Italian Long-Term Ecological Research for understanding ecosystem diversity and functioning. Case studies from aquatic, terrestrial and transitional domains

by

Maria Grazia Mazzocchi, Lucilla Capotondi, Michele Freppaz,
Antonella Lugliè, Alessandro Campanaro

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Italian Long-Term Ecological Research for understanding ecosystem diversity and functioning. Case studies from aquatic, terrestrial and transitional domains

Edited by Maria Grazia Mazzocchi, Lucilla Capotondi, Michele Freppaz, Antonella Lugliè, Alessandro Campanaro

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EDITORIAL

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Editorial

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The Long-Term time series of Ecological Research (LTER) allow us to follow the evolution of the ecosystems over decadal scales to understand the driving processes behind their temporal variability in relation to local and global stressors.

LTER-Italy (http://www.lteritalia.it) network includes terrestrial, freshwater, transitional and marine sites where ecological research is regularly carried out on decadal scale with the involvement of numerous universities, scientific and technical institutions, as well as local authorities. Since 2006, LTER-Italy is part of LTER-Europe (http://www.lter-europe.net) and LTER-International (https://www.ilter.network) networks. LTER-Italy consists of 79 research sites belonging to 25 different areas (parent sites) distributed throughout the Country and also including extra-national sites in the Ross Sea (Antarctica) and in the Himalayas (Nepal).

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Figure 1. Localisation of the LTER sites covered in this Special Issue. In the large panel, the Italian sites: terrestrial (brown), freshwater (light blue), transitional (green), marine (blue). In the inset: **A** Italy **B** Northern Europe **C** California (USA) **D** Ross Sea (Antarctica).

The present Special Issue entitled "*Italian Long-Term Ecological Research for understanding ecosystem diversity and functioning. Case studies from aquatic, terrestrial and transitional domains*" is the first published collection of studies performed at LTER-Italy sites which address the diversity and dynamics of ecosystems in different domains in response to natural and anthropogenic forcing. Eighteen papers in the volume present research carried out at 2 Antarctic and 16 Italian sites, one of which includes a comparative analysis with other International LTER sites in Northern Europe and USA (Figure 1 and Table 1). Two additional cross-domain papers address methodological and conceptual themes: Zilioli et al. (2019) tackle data management practices and interoperability for the monitoring of the Essential Biodiversity Variables in LTER-Italy; Ribera d'Alcalà (2019) compares terrestrial and marine ecosystems, highlighting their similarities and specific features in response to perturbations and climate change.

The studies focus on terrestrial (7 papers) and aquatic (11) domains, the latter including marine (7), transitional (3) and freshwater (1) ecosystems. The research sites span over a wide altitudinal range, from the mesopelagic ocean (-800 m) to the high-elevation mountain (2.800 m) and reflect the large diversity of the Italian territory and landscapes. The papers cover a broad range of topics, from biodiversity to biogeochemical fluxes, over time scales up to three decades. The different environments, organisms and processes are analysed with the common goal to discern the main modes

Table 1. List of the LTER sites covered in the papers of this Special Issue, with their names, DEIMS. ID (https://deims.org/docs/deimsid.html) and references, in the same order as they appear in the Index.

LTER site name	DEIMS-ID	Special Issue reference papers
Appennino centrale: Gran Sasso d'Italia, Italy	https://deims.org/c0738b00-854c-418f-8d4f-69b03486e9fd	Petriccione and Bricca 2019
Val Masino LOM1, Italy	https://deims.org/68a5673c-9172-48cc-88e5-b9408b203309	Balestrini et al. 2019
Istituto Scientifico Angelo Mosso (MOSSO), Italy	https://deims.org/17210eba-d832-4759-89fa-9ff127cbdf6e	Freppaz et al. 2019
Gran Paradiso National Park, Italy	https://deims.org/e33c983a-19ad-4f40-a6fd-1210ee0b3a4b	Cerrato et al. 2019
IT25 – Val Mazia/ Matschertal, Italy	https://deims.org/11696de6-0ab9-4c94-a06b-7ce40f56c964	Steinwandter et al. 2019
Foce Trigno-Marina di Petacciato (Campobasso), Italy	https://deims.org/1835cda2-b56d-400a-b413-ab5c74086dc5	Marzialetti et al. 2019
Foce Saccione-Bonifica Ramitelli (Campobasso), Italy	https://deims.org/088fe3af-c5bb-4cc8-b479-fe1ea6d5be80	Marzialetti et al. 2019
Montagna di Torricchio, Italy	https://deims.org/6b62feb2-61bf-47e1-b97f-0e909c408db8	Chelli et al. 2019
Lago Bidighinzu, Italy	https://deims.org/3707cf71-7e04-41e3-8afc-518b293f6c07	Pulina et al. 2019
Laguna di Venezia, Italy	https://deims.org/f7d94927-17be-4d3d-9810-e3c9bc91829c	Sfriso et al. 2019
Mar Piccolo of Taranto, Italy	https://deims.org/ede24c6e-9cf2-4cf8-8bf7-36ba327403b4	Petrocelli et al. 2019
Sacca di Goro, Italy	https://deims.org/b7869194-b220-473a-b035-feeadfa21aba	Pitacco et al. 2019
Valli di Comacchio, Italy	https://deims.org/70e1bc05-a03d-40fc-993d-0c61e524b177	Pitacco et al. 2019
LTER Marechiara, Italy	https://deims.org/0b87459a-da3c-45af-a3e1-cb1508519411	Zingone et al. 2019
Santa Barbara Coastal LTER, United States of America	https://deims.org/dbd399ed-9c26-4621-b479-7ab505c8aa35	Kröncke et al. 2019
Archipelago Research Institute, Finland (Seili LTER)	https://deims.org/9d4222a2-c50f-4fac-8b1d-3b685072b34d	Kröncke et al. 2019
North Sea Benthos Observatory, Germany	https://deims.org/50946250-c0fa-41b0-a917-17d2a3992eee	Kröncke et al. 2019
Golfo di Olbia, Italy	https://deims.org/3178d0fb-0789-4992-9c51-1ddb50b7e871	Kröncke et al. 2019
LTER Observatory HAUSGARTEN, Germany	https://deims.org/f6d9ed12-6bc1-47fb-8e81-ef24e9579596	Kröncke et al. 2019
Golfo di Trieste, Italy	https://deims.org/96969205-cfdf-41d8-979f-ff881ea8dc8b	Cerino et al. 2019; Monti-Birkenmeier et al. 2019; Franzo et al. 2019
Mooring A: Southwestern Ross Sea, Ross Island, Antarctica	https://deims.org/86b6465c-b604-4efa-9145-0805f62216f4	Chiarini et al. 2019; Azzaro et al. 2019
Mooring B: North Central Ross Sea, Joides Basin, Antarctica	https://deims.org/1fb62b9c-4d5c-4f1f-8882-807032337de7	Azzaro et al. 2019

of temporal variability and to understand the causes of the observed modifications. The heterogeneity of the presented research activities mirrors the variety of ecological issues that need to be addressed to achieve proper environmental conservation and management practices.

For the terrestrial environment, papers in this Special Issue investigate the effects of climate variability and habitat use on the biological and biogeochemical components of ecosystems from the sea-level coasts to the peaks of the Alps in a variety of habitat types, such as forests, grasslands and dunes. Amongst the "sentinels" of environmental changes, the readers will find the high-elevation plants reported by Petriccione and Bricca (2019), which varied significantly in the species coverage, and the butterflies whose variations in species distribution and community composition was investigated by Cerrato et al. (2019). Carbon and nitrogen cycles are influenced by climatic drivers, as well as by the atmospheric inputs and the study of these elements in the soil and soil solution provides important data on the ecosystem functioning, as outlined by Freppaz et al. (2019) in the alpine tundra and by Balestrini et al. (2019) in a montane forest. Alien species invasions are another effect of habitat and climate alteration that can be monitored and modelled in the LTER network. An example is the study of Marzialetti et al. (2019), in which the authors provide a first exploratory analysis of the environmental characteristics promoting the rapid growth and development of *Acacia saligna* in Italian dune ecosystems and identify coastal areas as the main habitats that are affected by the invasive process. From a methodological point of view, Chelli et al. (2019) designed a monitoring system for the study of species richness and assemblage patterns over time, based on a probabilistic sampling scheme. LTER sites are also important for their contribution to biodiversity and biogeography knowledge, as demonstrated by the finding in South Tyrol of *Opetiopalpus sabulosus*, a beetle inhabiting steppe-like grasslands, which represents the first record of the species for the European Alps and Central Europe (Steinwandter et al. 2019).

Multiannual research in aquatic domains has revealed a remarkable interannual variability in different ecosystems, from temperate to polar latitudes. Different kinds of natural factors seem to have affected microbial processes (Franzo et al. 2019) and phytoplankton patterns (Cerino et al. 2019) in the north Adriatic Sea, as well as vertical particle fluxes (Chiarini et al. 2019) and microbial metabolism (Azzaro et al. 2019) in the Ross Sea (Antarctica). Conversely, decreased anthropogenic forcing was found to be responsible for the significant changes of phytoplankton in a eutrophic reservoir (Pulina et al. 2019) and of benthic macrophytes in a coastal embayment (Petrocelli et al. 2019). The long-term analysis of various ecosystem components has also proved to be useful to identify key variables that may allow environmental dynamics to be followed in view of possible future limitations of sampling or analytical efforts. This is the case of tintinnids for marine microzooplankton (Monti-Birkenmeier et al. 2019), taxonomic surrogates for benthic assemblages in transitional ecosystems (Pitacco et al. 2019) and nutrients, such as nitrites and reactive phosphorus for the trophic status of the Venice Lagoon (Sfriso et al. 2019). The value of LTER investigations, generally conducted at a single site, is further enhanced by expanding the spatial scale of the

observations in a comparative effort, which is especially useful for the prediction of future changes in marine systems in response to climate variability and ocean warming (Kröncke et al. 2019). Finally, besides the record of fluctuations and trends, long-term ecological research plays an invaluable role as an inspiration for other research, while the LTER sites can also be used as natural laboratories to test hypotheses and answer specific scientific questions. They can therefore have a primary role in the development of the knowledge of ecosystems much beyond the local scale (Zingone et al. 2019).

The content of this volume underlines and confirms how observations and analyses of ecological variables and processes at multiple scales are keys to understand important ecological relationships. Similar ecosystems can respond differently to identical changes in system drivers, depending on their inherent ecological structure and functioning, their history and spatial location within the territory and connections with adjacent ecosystems. The insights gained by this kind of research contribute to multiple aspects: from the formulation of general laws of ecology to the development of policies to face the Grand Challenges under Global Change at local, regional and planetary levels.

Before leaving the readers to enter the core of this Special Issue, we wish to express our gratitude to all colleagues who submitted manuscripts to this Special Issue and to the 51 reviewers for their time, attentive criticisms and constructive comments and suggestions. Our special thanks to Boriana Ovcharova, Managing Editor and the entire Editorial Office of *Nature Conservation* for their kind assistance and technical support.

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RESEARCH ARTICLE

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Thirty years of ecological research at the Gran Sasso d'Italia LTER site: climate change in action

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Abstract

Since 1986, vegetation monitoring of alpine plant communities has been performed at the Gran Sasso d'Italia LTER site (https://deims.org/c0738b00-854c-418f-8d4f-69b03486e9fd) in the Central Apennines, through phytosociological relevés and abundance and coverage estimation of the vascular flora at fine scale. The monitoring activities for abiotic parameters regard air and soil temperatures, rainfall, snowfall and snow cover persistence.

A comparative analysis of changes in species composition, life forms, life strategies and morpho-functional types allowed recognition of dynamical processes (fluctuation and degeneration) and an increase in stress- and drought-tolerant and ruderal species, probably linked to a general process of climate change.

A trend of variation forced by increasing drought was recorded in high-mountain plant communities, normally within a dynamic fluctuation process. There has been a 50–80% change in species composition with respect to the total number of species observed over the years. Whereas the total number of species has increased in all communities, in high-mountain mesic grassland 20% of sensitive species have completely disappeared. Early signs of a degeneration process were already discernible after seven years: such signs are more evident in snow-dependent communities, with a quantitative increase in more thermophilic and drought-tolerant species and a parallel decrease in more mesic, cryophilic and competitive species. In particular, the following phenomena have been recorded in high-mountain mesic grassland, in agreement with predicted or observed phenomena in other Alpine or Arctic areas: (a) coverage increase (or appearance) of ruderal and stress- and drought-tolerant species; (b) coverage decrease (or disappearance) of cryophilic, mesic and competitive species.

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These short-term changes could lead, in the medium- or long-term, to a disgregation process affecting the high elevation plant communities of the Apennines (including the local extinction of most of the cold-adapted species), due to their very low resilience. The phenomena described may be linked to the observed climate change which occurred during the last century (in particular in the last 50 years) in the Apennines, consisting mainly, in the mountains, of a strong reduction in the duration of snow-cover and an increase in mean and minimum annual temperatures.

Keywords
LTER, climate change, alpine plant communities, Apennines, Braun-Blanquet approach

Introduction

According to the latest IPCC reports (2007, 2014), the current climate change is expected to cause, by the end of this century, a global average temperature increase between 1.8 and 4 °C. This warming is constantly accelerating, especially at middle and high latitudes which are experiencing a much higher rate. Climate change will become more marked in Southern Europe, in particular in the central-southern part of the Italian Peninsula (Mathez 2009), where a steep reduction in precipitation (20–30% ca.) and a strong increase in temperature (3.0–4.0 °C ca.) are expected in the next 80 years. High-resolution climate simulations (based on IPCC scenario RCP4.5, stabilization without overshoot pathway) predict for Italy in 2100 a marked reduction in total summer precipitation (June-August) of 24% and a decrease in snow coverage (h>1 cm) of 21 days/year and in frost days (t<0 °C) of 20 days/year (Bucchignani et al. 2016, Zollo et al. 2016). Recorded data for this part of Italy in the last ca. 100 years (Buffoni et al. 1999, Brunetti et al. 2000a, b) confirm this trend, with 15% less rainfall and temperatures 1 °C above average values, to the extent that the present Mediterranean-mountain climate may be described as shifting to a climate with definite Mediterranean characteristics, with precipitation peaks only in winter and snowfall becoming rarer. In addition, remote sensing data (IPCC 2001, 2007, 2014) show that the extension of yearly snow coverage has decreased by 10% in the last 50 years in all regions of the Northern Hemisphere. Data collected for the Italian Alps on the ground (meteorological stations) confirm a 30-year trend toward a decrease of snowfall, snow depth and coverage (Cannone et al. 2007).

Observed global climate warming in the 20th century (+0.7 °C in the last 60 years, +0.1 °C per decade in the same period, IPCC 2014) has already altered the vascular plant diversity at many high-elevation sites in the Alps (Braun-Blanquet 1955, 1957, Hofer 1992, Grabherr et al. 1994, 1995, 2001, Gottfried et al. 2002, Camenisch 2002, Pauli et al. 2003a, b, Walther et al. 2005, Pauli et al. 2007, Holzinger et al. 2008, Vittoz et al. 2008, Parolo and Rossi 2008, Erschbamer et al. 2009, 2011) and in other European mountain ranges (Klanderud and Birks 2003, Moiseev and Shiyatov 2003, Virtanen et al. 2003, Petriccione 2005, Steinbauer et al. 2018). Several modelling approaches focusing at large scale (Thuiller et al. 2005) and local scale within the Alps

and the Apennines have suggested remarkable warming-induced threats for reducing alpine plant diversity (Gottfried et al. 1999, Guisan and Theurillat 2000, Theurillat and Guisan 2001, Dirnböck et al. 2003, Stanisci et al. 2006, Frate et al. 2018). A significant decrease of vegetation coverage and an increase in species richness, with the associated lack of half of the original species and coverage decline of the most dominant species, have been found in the Central Alps in subalpine, alpine and nival plant communities in the last 50 years (Cannone et al. 2007, Cannone and Pignatti 2014). A general increase in the plant cover of most species (although not significant) has been recognized in some alpine and subalpine LTER sites in Austria, Switzerland and Italy in the montane, subalpine and alpine belts in the last 20 years (Rogora et al. 2018).

As far as the Central Apennines (Italy) is concerned, data collected at some key meteorological stations show a considerable recent decrease in precipitation (especially in spring and autumn) and a small increase in average temperature (Petriccione 2005). Snowfall data (Baldoni et al. 1999, Romeo and Scarpelli 2001) confirm the general trend, with a clear decrease in the last 80 years. The same trends are also recognizable in other localities, confirming the observed general trend towards drought conditions in Central and Southern Italy. These recorded and predicted changes are already producing noticeable effects of drought in most of the biocenoses distributed in the Mediterranean bio-climatic zone. On Mediterranean high mountains, the most endangered plants are the cryophilic species with a distribution range centred over the timberline in the alpine belt, showing morphological and functional adaptations to the severe physical environment of high elevation mountains (Körner 1999). Accordingly, alpine plant communities in this zone can be regarded as sensitive indicators of climate change (Körner 1994). Nevertheless, only a small number of papers have been published on this topic and those based on specific field observations are even less abundant. A first attempt to analyse possible shifts in the vegetation belts of Italy caused by climate change (Blasi 1996) considers only theoretical models based on small-scale bio-climatic maps. An extensive review of the possible impacts of climate change on sensitive land ecosystems in Italy predicts a short- and medium-term process of degeneration for most of the analysed biocenoses, with a gradual long-term transformation towards more drought-tolerant biocenoses (Petriccione 1995). Other studies start from the same approach, but the major emphasis is placed on especially sensitive biocenoses in Italy (Petriccione and Claroni 1996, Petriccione et al. 1998): the plant communities analysed are soil-controlled (flood-plain forests and marsh communities) or high-altitude biocenoses (sub-alpine shrubland and alpine tundra). For most of these communities, a medium-term process of degeneration is predicted, followed by a long-term process of regression. Since 1993, a trend of variation forced by increasing drought has been recorded in all plant communities above the timberline, normally within a dynamic fluctuation process. There has been a 10–20% change in species composition with respect to the total number of species observed over nine years (Petriccione 2005). Early signs of a degeneration process were already discernible after seven years: such signs are more evident in snow-dependent communities, with a quantitative increase in more thermophilic, drought- and stress-tolerant species and a parallel decrease in

more mesic, cryophilic and competitive species. In particular, the following phenomena have been recorded in alpine tundra, high-mountain mesic and snow-bed grassland, in agreement with predicted or observed phenomena in other Alpine or Arctic areas (Chapin et al. 1996, Theurillat and Guisan 2001, Welker et al. 2001): (a) coverage increase (or appearance) of chamaephytic, drought- and stress-tolerant species; (b) coverage decrease (or disappearance) of hemicryptophytic, cryophilic, mesic and competitive species. Another clearly-recorded effect of global warming on high elevation plant diversity is also the shift of some species from lower to higher altitudinal ranges. In the alpine belt of the Majella massif in the Central Apennines, the warmer eastern slopes are the first to be affected by the colonization of thermophilic species, whereas the north-facing exposures, with a shorter frost-free period, are the most conservative, showing greater inertia to the invasion process (Stanisci et al. 2005). Several species typical of the subalpine belt at their upper altitude limit, not recorded in previous studies, were mainly found on east-facing exposures. The species invasion process is able to modify the ecosystem functions, increasing fragmentation of habitats suitable for the survival of cryophilic species. Prediction models (GLM) in the Central Apennines (Stanisci et al. 2006) indicate a decline of alpine tundra affecting 75% of its current area, in relation to an increase in mean temperature of 1 °C. The predicted decline of the alpine belt in the Apennines will change the diversity pattern of plant species and communities in high elevation habitats with a strong extinction risk for local endemic cryophilic species. Over the last four decades, a significant increase in the frequencies of thermophilic, mesonitrophilic, caespitose hemicryptophytes and suffruticose chamaephytes has been detected in the alpine belt at an LTER site in the Central Apennines (Evangelista et al. 2016, Stanisci et al. 2016). Moreover, in the Northern Apennines a degeneration process was observed in chionophilic species (Rossi et al. 2004).

This paper is based on continuous ecological observations performed at an alpine research site ("Gran Sasso d'Italia", https://deims.org/c0738b00-854c-418f-8d4f-69b03486e9fd) established in 1986 and joining the LTER Italy network in 2007. The longest data series of vegetation data in the Apennines is available at this site, including up to 33 years of observations at community level. At European level, only data at species level are available for so long a time, except for an area in the Italian Alps (Stelvio National Park) where a 50-year data series is available (Cannone and Pignatti 2014). The site is part of the "Apennines: high elevation ecosystems" LTER site, which consists of "orographic islands" of alpine biocenoses in the central Mediterranean basin, along the Apennine chain, where many endemic and rare taxa occur. This species pool is critically endangered by climate warming, as reported in several studies (Petriccione 1995, Petriccione and Claroni 1996, Petriccione et al. 1998, Grabherr et al. 2001, Pauli et al. 2001, Petriccione 2005, Rossi et al. 2009, Stanisci et al. 2006, Gottfried et al. 2012). The first 18–25 years of observations at this site show a clear tendency of the high altitude plant communities to adapt to aridity (Petriccione 2012). A current process of gradual degeneration has been recognised, with a marked reduction in rare species adapted to colder climates and the invasion of more thermophilic species. Only 44–50% of the total number of species has survived with no significant changes,

whereas 40% are invaders. The remaining species were no longer found in the research plots (Petriccione 2012). The main purpose of the research site is to investigate and monitor structure and composition changes in plant communities in relation to climate change. The aim of this paper is to attempt to answer four questions: a) Are plant communities changing over time? b) Toward a new equilibrium? c) Are species responding in different ways? d) Is there a relationship between the changes in the features of the communities and the predicted and/or observed changes in the temperature and precipitation regimes?

Materials and methods

Study area

The LTER site EU IT 01-003-T "Appennino Centrale: Gran Sasso d'Italia", established in 1985, covers an area of ca. 0.5 km^2, with an elevation range from 2130 to 2385 m a.s.l. (approx. latitude 42°26N and longitude 13°33E, Suppl. material 1, Figure S1). The Gran Sasso d'Italia massif is located in the Central Apennines, which reach their highest elevation with the Gran Sasso peak (2914 m a.s.l.). The research site, one of the highest in Italy, has been a protected area both at National (as a National Park) and European level (as part of the EU Natura 2000 Network) since 1995. Land use in the site has remained unchanged over the last 50–100 years (personal observations, Falcucci et al. 2007): the only relevant activities are based on winter sports (a small ski resort is located at the southern boundary of the site), mountaineering and, more recently, nature tourism, all with very low impact on plant communities. In the past, land use was based on transhumant sheep farming, in progressive and rapid abandonment after the major political and economic change affecting Italy at the end of the 19th century (Clementi 1995). As a result, land management has not changed significantly, at least not since 1986, the year of ecological research at the site began.

The climate is Mediterranean-mountain (Pignatti 1969, Petriccione 2005), with an average annual temperature of 3.7 °C, average annual precipitation of 1170 mm, maximum rainfall in spring and autumn, no drought period in summer, but an extreme and prolonged frost period in winter, for 5–6 months, with prolonged snow cover for more than 6 months a year. The average monthly maximum temperature is not very high (17.4 °C), whereas the average monthly minimum temperature is very low (- 8.9 °C).

Since 1986, the same ecologists (first from the Universities of Rome and L'Aquila, from 1999 the Corpo Forestale dello Stato (National Forest Service) and since 2017 the Biodiversity and Park Protection Department of the Carabinieri) have been continuously studying the state of the high elevation vegetation, analysing all the plant species present in permanent plots where surveys are carried out once a year. Since 2013, the microclimate has also been studied directly using automatic temperature measurement devices installed in the soil.

The site consists of high elevation plant communities above the timberline, in the central Mediterranean basin, along the Apennine mountain range, included in the alpine and Mediterranean high-mountain altitudinal belts (Petriccione and Persia 1995). They belong to two habitat types protected by EU Habitat Directive no. 92/43/EEC and listed in its Annex I (attribution to EU habitat types according to Biondi et al. 2009 and personal observations). Two biocenoses are studied in detail, both described by Petriccione and Persia (1995) and characterized by perennial species, particularly well adapted to cold and drought conditions, with high resistance, but very low resilience: (a) high-mountain primary dry grassland (*Pediculari elegantis-Seslerietum tenuifoliae*, corresponding to habitat "6170 – Alpine and subalpine calcareous grasslands"), with non-continuous plant coverage, occurring between 2000 and 2300 m a.s.l. in the Central and Southern Apennines, in wind-swept peak and ridge zones with spatially limited and temporally discontinuous snow-cover, below zero night temperatures for ca. eight months a year and intensive cryo-nival phenomena (ice needles) in all seasons (except for the summer); soil is shallow (ca. 20 cm) and pH is basic (7.20–7.50, Furrer and Furnari 1960, Suppl. material 1, Figure S2); (b) high-mountain primary mesic grassland (*Luzulo italicae-Festucetum macratherae*, corresponding to the priority habitat "6230* – Species-rich *Nardus* grasslands, on siliceous substrates in mountain areas and submountain areas in Continental Europe"), with continuous plant coverage, occurring between 2000 and 2400 m a.s.l. throughout the Central Apennines, in wind-free zones with snow-cover for ca. six months a year, below zero night temperatures for ca. eight months a year and the absence of crio-nival phenomena (due to the prolonged snow-cover); soil is deep (cm 35–55 ca.) and pH is acid (4.50–5.90, Furrer and Furnari 1960, Suppl. material 1, Figure S3). The two plant communities are sampled on the basis of six permanent plots (three plots for each community), each measuring 100 m^2, grouped in two three-plot clusters, representative of a larger area of ca. 0.5 km^2. The site parameters observed include primary producers (species frequency and abundance, yearly) and microclimate (soil temperature, hourly, throughout the year). Some information on the microclimate characteristics of the *Pediculari-Seslerietum* community are already available for that specific site, although only for one summer season (Brucculeri and Petriccione 1994): the maximum recorded temperature is 27.5 °C.

Climate data

Climate data were collected at the official Campo Imperatore meteorological station (2135 m a.s.l.), located in the Gran Sasso d'Italia mountains, with over 70 years of standard precipitation and temperature observations (since 1942). Data on snow precipitation and the height of snow cover in winter were collected at the non-automatic METEOMONT station (managed by the Carabinieri, formerly the National Forest Service) at the same location, from 1988 to 2016. Total winter snow precipitation has been calculated on the basis of METEOMONT data (daily visual observations of height of new snow cover, in cm, assuming 1 cm of snow equal to 1 mm of liquid precipitation).

Continuous soil microclimate observations have been made at the site since 2013, by means of two data loggers in the soil (-10 cm depth), one for each community, according to the GLORIA methodology (Pauli et al. 2004). Near-surface ground temperature is a good proxy for the presence/absence of snow cover and therefore for tracking the related no-frost winter days (Lundquist and Lott 2008, Schmid et al. 2012). Unfortunately, several failure events affected one of the loggers and therefore data for the *Luzulo-Festucetum* is only available for 3 years (2013–2015, including two winters); in contrast, a valuable data series is available for the *Pediculari-Seslerietum* (5 years, from 2013 to 2018, including 5 winters). Climate data were analysed by linear regression model, using mean annual temperature, total annual precipitation, mean winter snow depth and total winter snow precipitation as response variables and "year" as explanatory variable. When data did not meet the model assumptions, non-parametric linear regression was used. The significance of the regressions was assessed using the F-test and the Theil-Sen test for simple linear regression and non-parametric linear regression, respectively.

Analysis at community level

For each of the six plots, 100 m^2 in area, a phytosociological relevé was performed (according to the Braun-Blanquet approach) in 1993 and repeated 6, 8 and 15 years later, in 1999, 2001 and 2008, on the same fixed surface area of the entire plot. Since 2008, data collection has been yearly, including three plots per community. In one case (*Pediculari-Seslerietum*), a relevé had also been performed seven years earlier (in 1986), even if in only one plot. All data are reported in Suppl. material 1, Table S1 (*Pediculari-Seslerietum* 1986–2017) and 2 (*Luzulo-Festucetum* 1993–2017). The coverage scale is according to Braun-Blanquet (1932, 1964), as modified by Pignatti (1952). Species nomenclature is according to Pignatti (2017–2019).

The ecology and distribution range of each plant community are described according to Petriccione (1994) and Petriccione and Persia (1995). The dynamical current tendencies (according to Falinski 1986, 1989) in each community were identified by indicator species (as reported by Petriccione and Claroni 1996).

Species composition changes were calculated by simply comparing year by year, each year with the original species composition (at 1986 or 1993) and with all species occurring during the 25–33 years of observation.

A temporal trend analysis of species composition, life forms (Raunkiaer 1934), life strategies (Grime 1977, 1984, 1996) and morpho-functional types (defined ad hoc, according to an inductive approach, Woodward and Cramer 1996) was performed for each community, on the basis of the relative number of species per group. On the basis of an ad hoc quali-quantitative checklist of easily recognizable morphological characteristics (occurring on leaves and stems), a multivariate analysis helped define four morpho-functional groups, named after their key characteristics: reptant (Rp), graminoid hemicryptophytes (Hgr), scapose and rosulate hemicryptophytes (Hsr) and

crassulent (Cr). Plant functional traits are considered as good predictors of community changes (Lavorel and Garnier 2002). All traits (directly measured in the field), are reported in Suppl. material 1, Table S1 *(Pediculari-Seslerietum* 1986–2017) and 2 *(Luzulo-Festucetum* 1993–2017).

To analyse changes over time at community level, the species cover value for each plot (Pignatti 1952) and for each year was transformed into percentage values, according to the following criteria (+: 1%, 1: 10%, 2: 30%, 3: 50%, 4: 70% and 5: 90%). Analysis of the communities was based on taxonomic diversity and functional structure, including life strategies (Grime 2006) and ecological features. In the first case, three taxonomic diversity indices were calculated: species richness, evenness and Simpson index. For functional and ecological analysis, we calculated the community weighted mean (hereafter CWMs), following the equation of Garnier et al. (2004):

$$CWM = \sum_{i=l}^{S} p_i x_i$$

where x_i is the mean trait values and pi is the relative abundance of the *i*-th species. To calculate the CWM with trait (hereafter CWM_{trait}) the matrixes "relevés x species (cover %)" and "species x trait values" were used. This index can be adequately used to quantify the relationship between a given trait and certain environmental conditions. The variation in life strategies was also approached using the above equation, multiplying the matrix "relevés x species (cover %)" with the matrix "species x Grime's strategy" using the same equation of Garnier et al. (2004) to weight life strategies at community level (hereafter CWM_{Grime}). For categorical traits, the relative frequency (%) of each trait state was obtained, while for continuous traits (such as plant height and leaf width), a single trait value was referred to the community mean value of that trait. In the case of species with multiple Grime's strategy, all strategies were computed separately, considering the contribution of each species to the community features according to its strategies.

Finally, an analysis of the change over time of the community's ecological features was performed using the ecological bioindicator values (hereafter BV) for the Italian flora (Pignatti 2005) to analyse changes in environmental conditions separately for both communities. The mean ecological indicator at community level was calculated in the same way as for CWM, by multiplying the matrix "relevés x species (cover %)" with "species xBV", to obtain a matrix "relevés x BV" (hereafter CWM_{BV}). Ecograms for the two plant communities were generated, as suggested by Pignatti (2005). Taxonomic diversity indices (Simpson, Species richness, Evenness), CWM_{trait}, CWM_{Grime} and CWM_{BV}were calculated for each observation year and for each community to assess the effects of temporal changes on different community facets (i.e. taxonomic, functional, Grime's live strategy and ecological) separately for the *Pediculari-Seslerietum* and *Luzulo-Festucetum*. A single linear regression model was developed, using as response variable each index separately and "year" as explanatory variable. The same approach was followed for the climatic analysis: a linear regression model was developed and the model assumption was verified. If the data did not meet the assumptions, a non-parametric linear regression model was used. The test results were corrected for multiple comparisons using Holm's correction to avoid Type I error.

Analysis at species level

At species level, changes in the cover of dominant and other selected species were analysed for both communities along a continuous temporal gradient (as performed by Evangelista et al. 2016, although along a non-continuous temporal gradient). For the *Pediculari-Seslerietum,* the analysis was performed on *Sesleria juncifolia*, *Carex kitaibeliana*, *Globularia meridionalis* (dominant), *Juniperus sibirica* (becoming dominant) and *Gymnadenia conopsea* (invader); for the *Luzulo-Festucetum*, on *Festuca violacea*, *Poa alpina* (dominant), *Carex caryophyllea*, *Trifolium pratense* (becoming dominant), *Trifolium thalii* (former dominant), *Silene ciliata* (mesophytic) and *Anthyllis montana* (xerophytic). In this case, species cover values were used as response variable and "year" as explanatory variable.

The same statistical method as described above was employed and a non-parametric linear regression model was used when the assumptions for the parametric model were not satisfied.

All the analyses were computed using R software (version 3.4.4, R Foundation for Statistical Computing, R Core Team 2015). The *stats* package (lm function) was used for the parametric linear regressions and the *mblm* packages version 0.12 (mblm function) for the non-parametric regression. CWMs were computed with the functcomp function in the FD package version 1.0–12 in R, otherwise taxonomic indices were computed with diversity function in the vegan package. For the Simpson index, the "Simpson" argument returning 1-D was selected. Finally, the *gvlma* package version 1.0.0.2 (gvlma function) and *stats* package (shapiro.test function) were used to validate the linear model assumptions. Temporal autocorrelation was checked with acf() function in stats package.

Results

Climate

Analysis of precipitation and temperature in the period 1950–2014 (65 years) shows (Fig. 1) an important and significant increase in mean annual temperature (+1.7 °C) and a small and not significant decrease in annual precipitation amount (-30 mm), especially in spring and autumn. Although the temperature trend seems very clear, the precipitation regime, on the other hand, is very variable year by year (with an average of 1170 mm), ranging from a minimum of 568 mm (year 1977) to a maximum of 1612 mm (year 1969), increasing the drought stress in very dry years: in recent decades, this has occurred in 2002, 2010 and 2011, with very low values of 700–800 mm.

For snow data, a significant variation (decrease) was found for snow precipitation only (Fig. 1, -147 mm), but not for the snow depth, which is not directly correlated. As for total precipitation, the snowfall regime is also very variable (Suppl. material 1, Figure S4) year by year (with an average of 163 mm), ranging from a minimum of only 40 mm (winter 2011/2) to a maximum of 309 mm (winter 2002/3), includ-

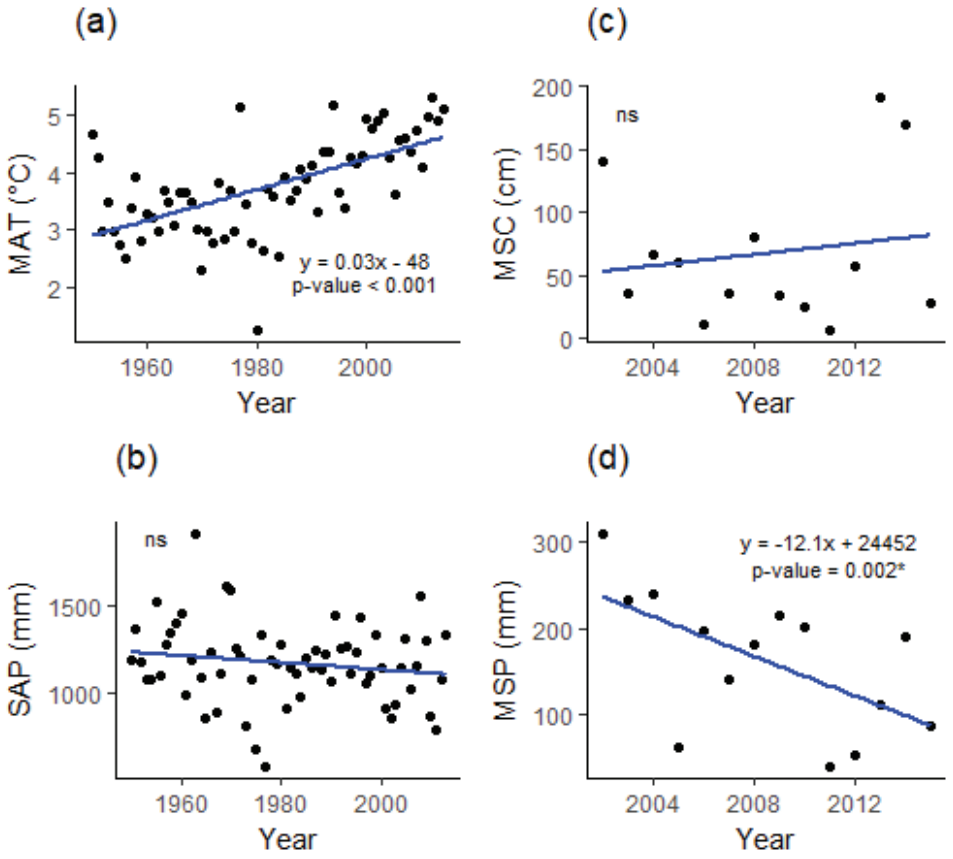

Figure 1. Changes in climate features during the period 1950–2014 at the Gran Sasso d'Italia LTER site (**a** mean annual air temperature (MAT) **b** mean annual total precipitation (SAP) **c** mean winter snow depth (MSC) for the period 1988–2016 **d** total winter snow precipitation (MSP) for the period 1988-2016 (SWE, mm); *: regression performed using non-parametric linear regression analysis; ns: non-significant).

ing frequent winters with mean height of snow cover lower than 20 cm (5 years). In winters with a very low snowfall amount, the height of snow cover (with an average value of 61 cm) reaches very low values (only 6 cm in winter 2011/2), whereas when snowfall is abundant the snow depth can reach very high values (192 and 170 cm in winters 2013/4 and 2014/5). But the snow depth can go down to very low values even in winters with a high snowfall amount, due to rapid and frequent high-temperature peaks, as in winter 2006/7, with 192 mm of snowfall and only 11 cm of snow cover.

The continuous soil microclimate observations performed at the site (Figs 2, 3), in accordance with data provided for only one year by Brucculeri and Petriccione (1994), show marked differences between the two communities. Whereas in winter 2013/4 the

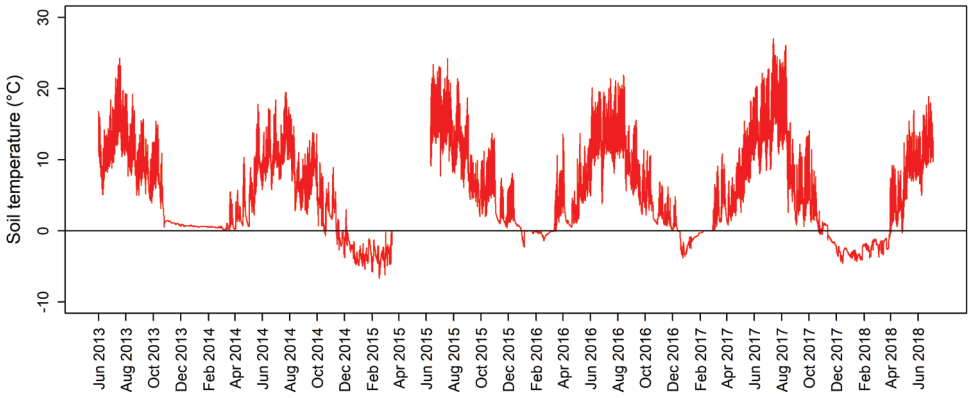

Figure 2. Daily Seasonal and annual changes in soil temperature (°C, recorded on a hourly bases -10 cm depth), at the dry grassland plots (Pediculari-Seslerietum) in the period May 2013-July 2018.

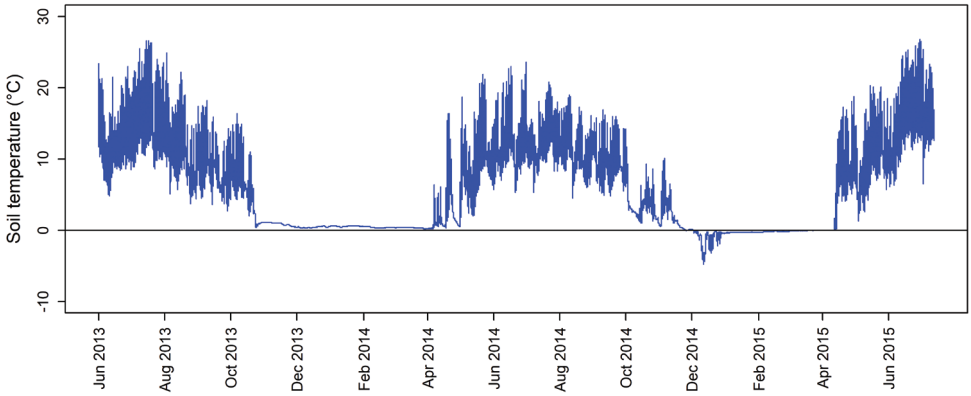

Figure 3. Seasonal and annual changes in soil temperature (°C, recorded on a hourly bases -10 cm depth) at the mesic grassland plots (Luzulo-Festucetum), in the period May 2013-July 2015.

abundant and persistent snowpack preserved the biocenoses from frost episodes (with temperature below 0 °C), in winter 2014/5 a very similar snowfall amount was so discontinuous that the dry grasslands were exposed to frost for 152 days (in other words, all the winter season, with min. absolute temperature up to -6.7 °C) and the sensitive mesic grasslands for 26 days (with min. absolute temperature up to -4.8 °C). The same frost episodes were recorded in the following winters: 8 and 14 days in 2015/6, 24 days in 2016/7 and the full winter season (99 days) in 2017/8, exposing the biocenoses to very dangerous frost stress, with a corresponding increase of mortality of roots and microbial fauna (Edwards et al. 2007)

Community level

Species composition changed by 10–15% per year in the period 1993–2017 (25 years) in both communities; the rate of change is also quite similar if the observation period is extended to a total of 32 years (possible only in the case of the *Pediculari-Seslerietum).* Long-term analysis, however, shows different trends: the dry grasslands (*Pediculari-Seslerietum*) have not lost any species, but have gained 53% of the total number of species occurring during the 32 years of observation; the mesic grasslands (*Luzulo-Festucetum*), on the other hand, have lost 20% of the original species (at 1993) and gained a very high number (81%) of the total number of species occurring during the 25 years of observation. Despite these very relevant changes in species composition, both communities maintain the same total plant coverage values over time (60–70% for *Pediculari-Seslerietum* and 90–95% for *Luzulo-Festucetum).*

The results show different biodiversity features of the two communities (taxonomic and functional) changing over time (Figs 4, 5). The *Pediculari-Seslerietum* community shows an increase in species richness only (even if not significant), while for the *Luzulo-Festucetum* there was an increase in patterns of species richness (significant), evenness and Simpson index (even if not significant), as the result of a clear downward trend in the coverage values of the former dominant species *Festuca violacea* and the invasion or expansion of many opportunistic species, such as *Trifolium pratense*.

As regards morpho-functional features (Figs 6–13), the *Pediculari-Seslerietum* community shows a significant decrease in the leaf width and a significant increase in dense leaf hairs. There are also many significant results associated with the *Luzulo-Festucetum* community: an increase pattern for leaves with sparse hairs and leaf width; a parallel decrease pattern for reclined stems is also discernible. Also traits like graminoid leaves and leaves without hairs show a significant decrease, probably due to the decrease in coverage of *Festuca violacea* (with graminoid leaves) and *Trifolium thalii* (with no hairs on the leaves) and parallel increase of *Trifolium pratense* (with hairs on the leaves).

As far as the analysis of Grime's life strategies (Figs 14–15) is concerned, both the *Pediculari-Seslerietum* and the *Luzulo-Festucetum* communities show an increase in species sharing the stress-tolerant strategy, even if non-significant in the first case.

As regards ecological indicators, generating ecograms of the two plant communities (Suppl. material 1, Figures S5, S6) makes it possible to obtain a graphic image of the ecological niche of each community, narrower in the dry grassland (stress adapted) and larger in the mesic grassland, which however shows a progressive narrowing over time. Indeed, no significant variations over time have been detected for the *Pediculari-Seslerietum* grasslands. On the other hand, the ecological features of the *Luzulo-Festucetum* mesic grasslands show many significant variations over time (Figs 16–17). In detail, our results highlighted a significant increase in the values of the ecological indicators of thermophile behaviour (T), with soil moisture (U) and nitrogen availability (N) decreasing.

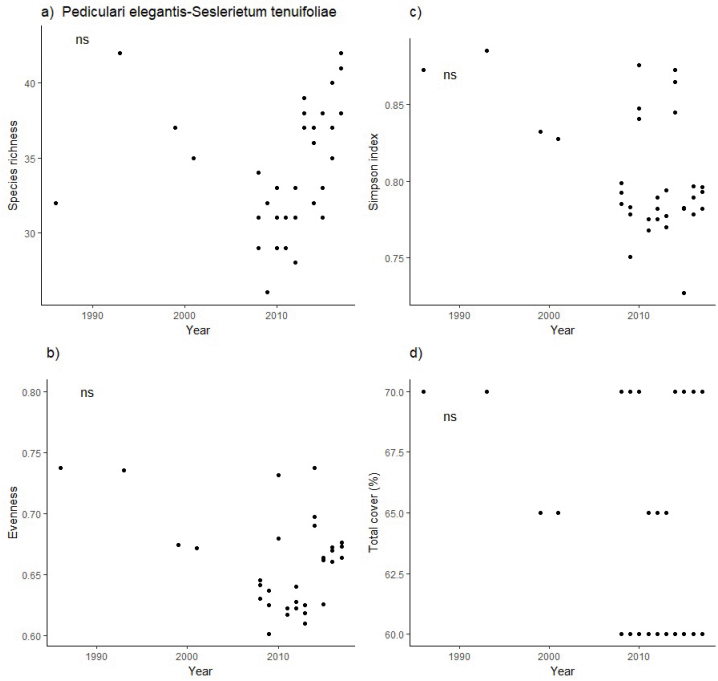

Figure 4. Changes in species diversity traits during the period 1986–2017 (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

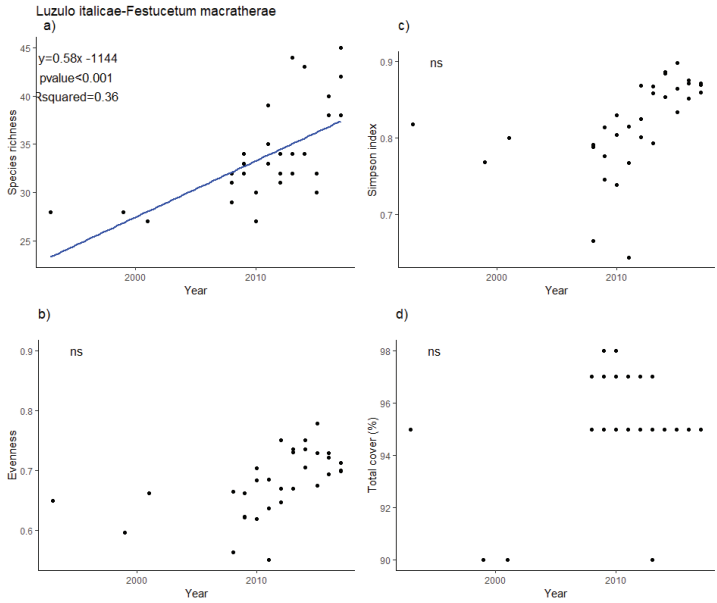

Figure 5. Changes in species diversity traits during the period 1993–2017 (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

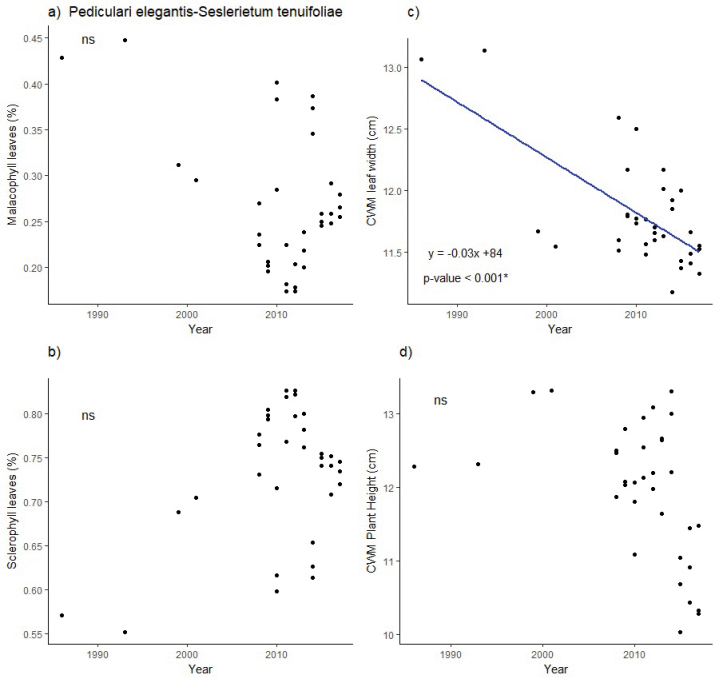

Figure 6. Changes in morpho-functional traits during the period 1986–2017 (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

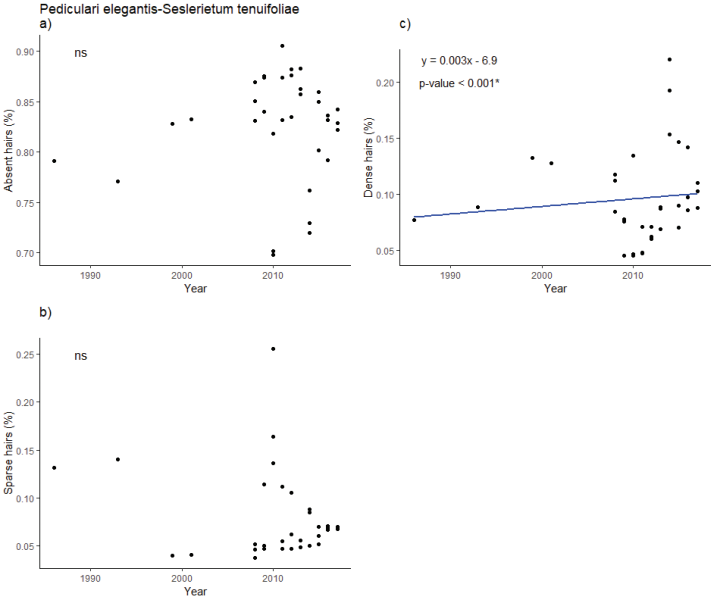

Figure 7. Changes in morpho-functional traits during the period 1986–2017 (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

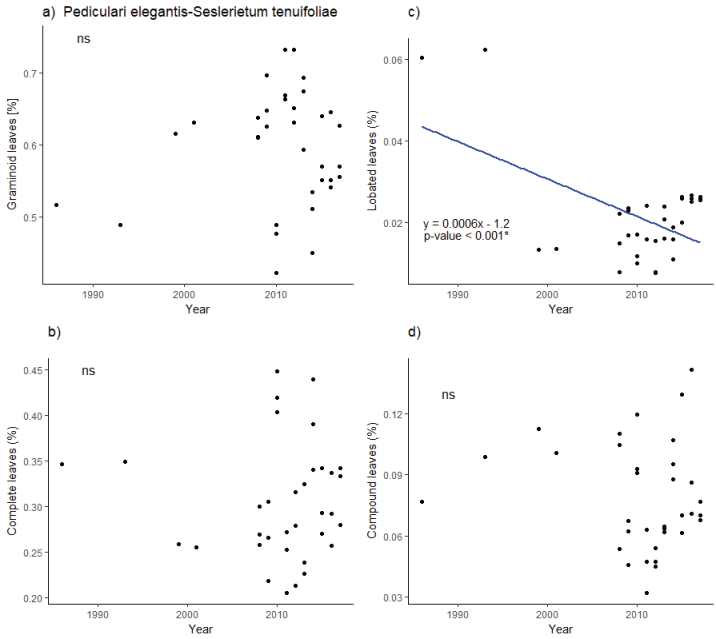

Figure 8. Changes in morpho-functional traits during the period 1986–2017 (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

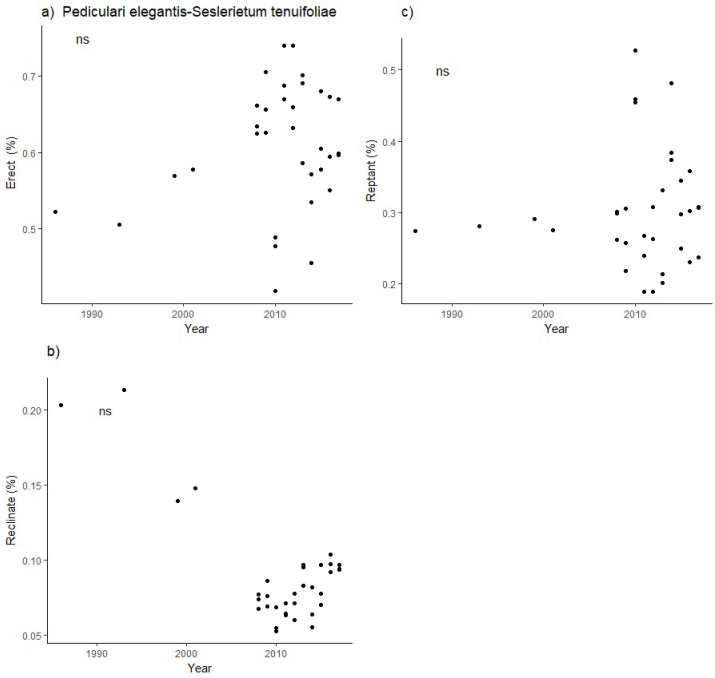

Figure 9. Changes in morpho-functional traits during the period 1986–2017 (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

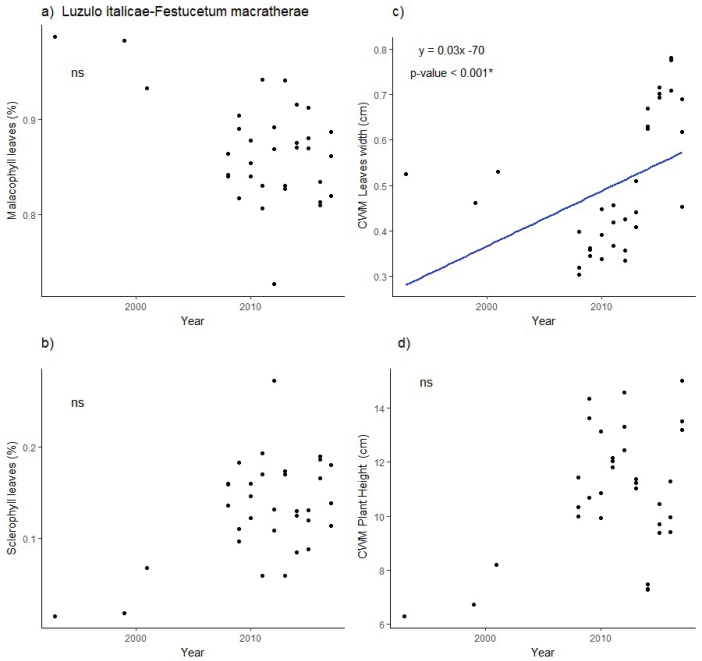

Figure 10. Changes in morpho-functional traits during the period 1993–2017 (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

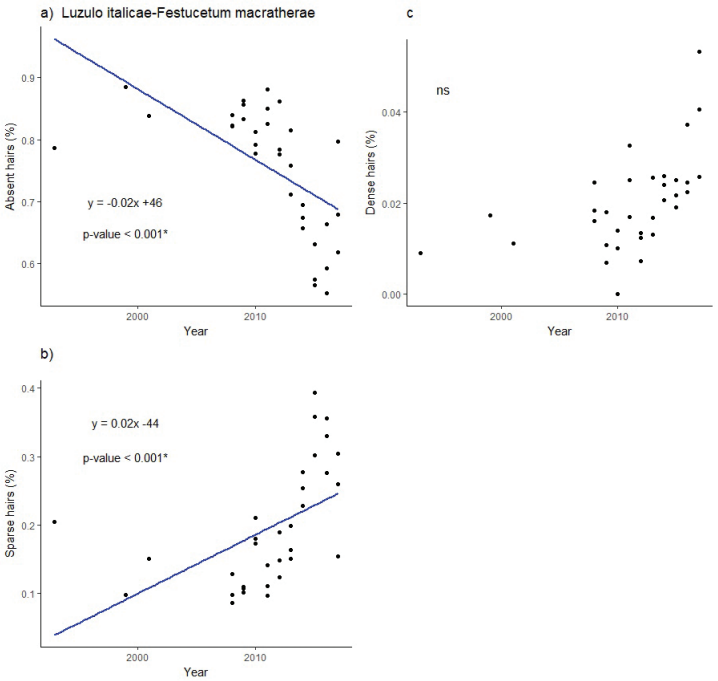

Figure 11. Changes in morpho-functional traits during the period 1993–2017 (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

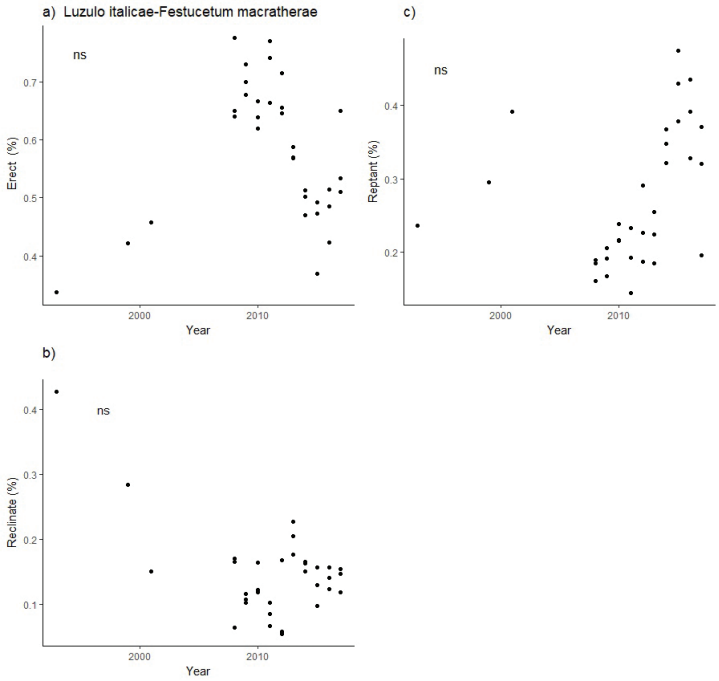

Figure 12. Changes in morpho-functional traits during the period 1993–2017 (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

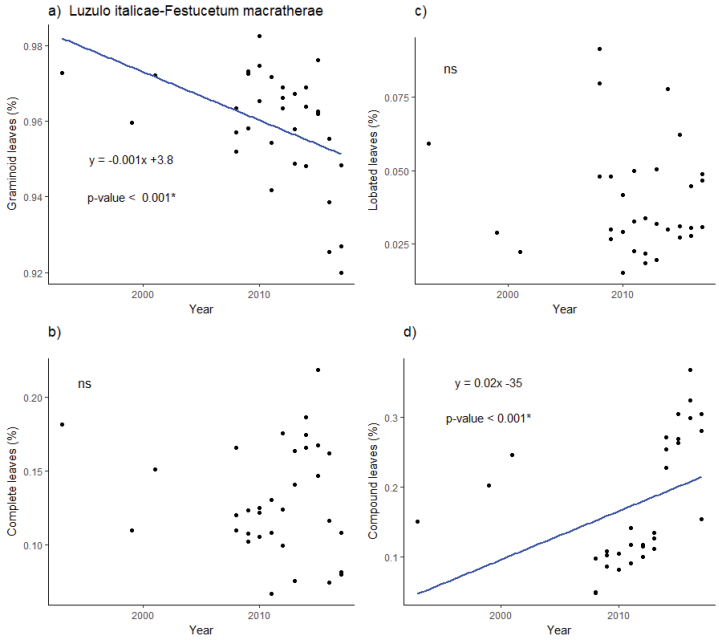

Figure 13. Changes in morpho-functional traits during the period 1993–2017 (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

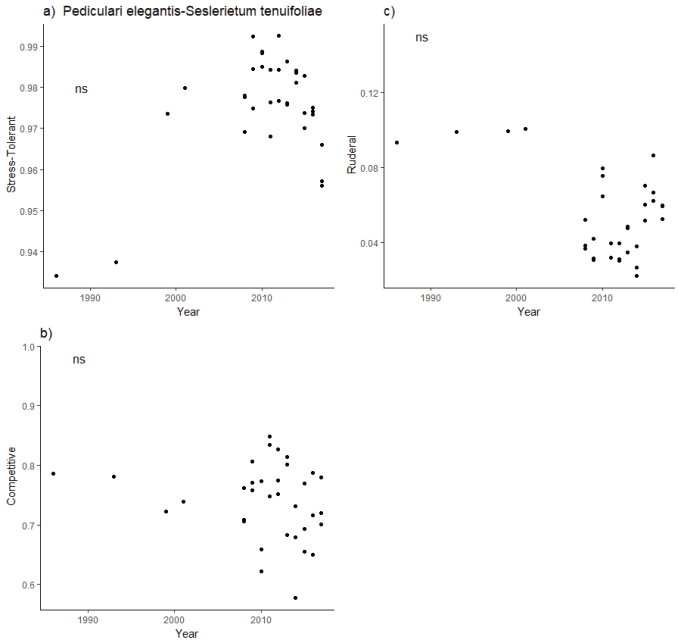

Figure 14. Changes in life strategies during the period 1986–2017 (Pediculari-Seslerietum plots) (S: stress-tolerant; R: ruderal; C: competitive) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

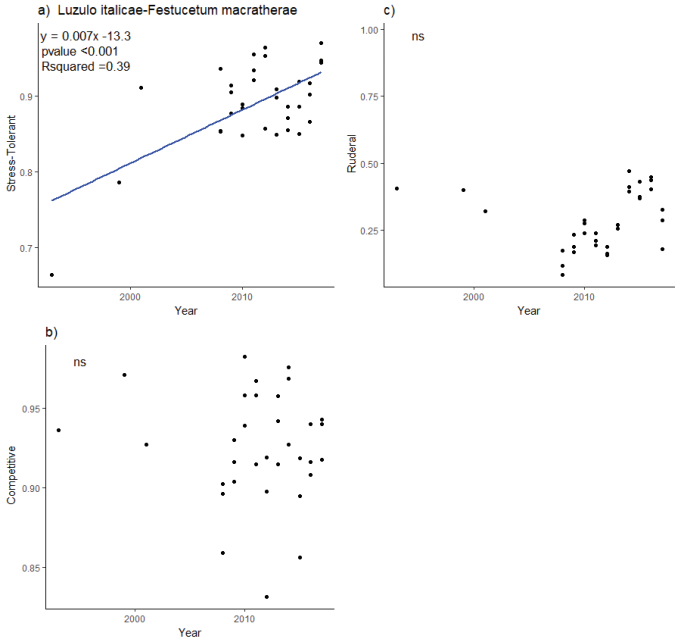

Figure 15. Changes in life strategies during the period 1993)-2017 (Luzulo-Festucetum plots) (S: stress-tolerant; R: ruderal; C: competitive) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

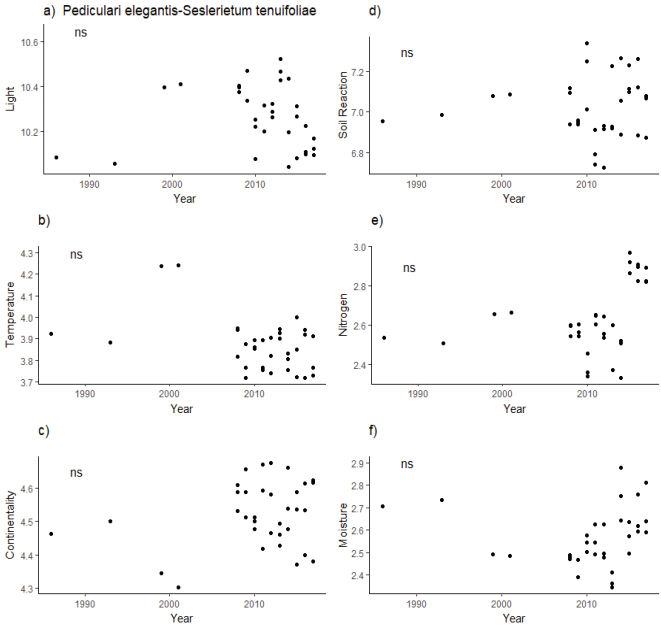

Figure 16. Changes in ecological bioindicator values during the period 1986–2017 (Pediculari-Seslerietum plots) (a): light availability; b): thermophile behaviour; c): continentality gradient; d): pH gradient; e): nitrogen availability; f): soil moisture) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

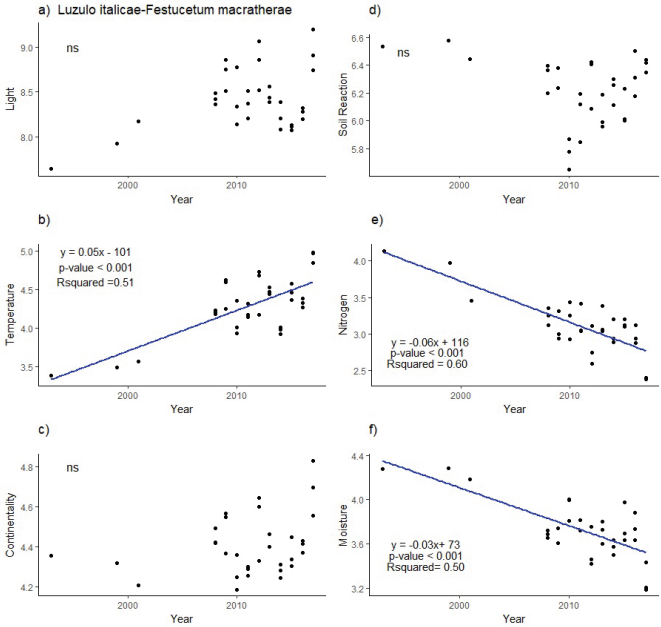

Figure 17. Changes in ecological bioindicator values during the period 1993–2017 at the Gran Sasso d'Italia LTER site (Luzulo-Festucetum plots) (a): light availability; b): thermophile behaviour; c): continentality gradient; d): pH gradient; e): nitrogen availability; f): soil moisture) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

Species level

The results of the analysis at species level highlighted a preferential response for the species belonging to the *Luzulo-Festucetum*, with a lack of response for the selected species of the *Pediculari-Seslerietum*. In detail, a significant decreasing trend has been found for the former dominant species *Festuca violacea* and a significative increasing trend for the new dominant species *Trifolium pratense* and the invaders *Carex caryophyllea* (even if not significant) which have only colonised the community since 2008 (Figs 18–19). On the contrary, *Trifolium thalii* is becoming ever rarer, although no significant trend has been detected.

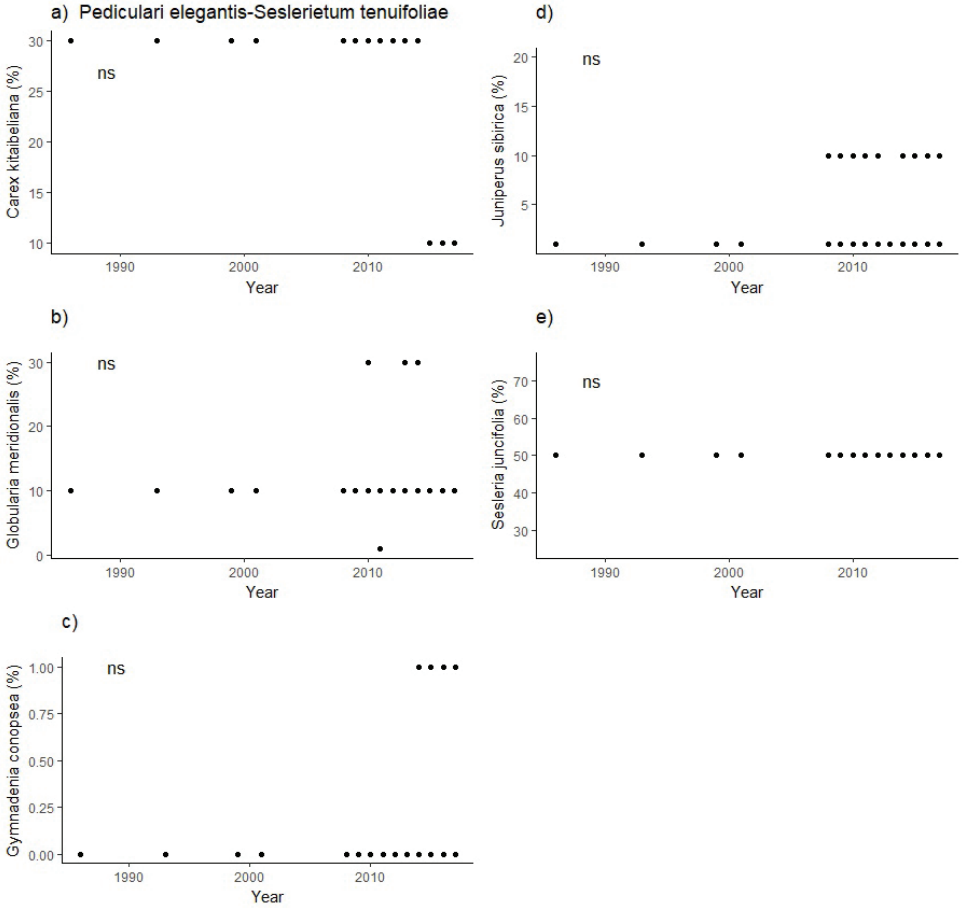

Figure 18. Changes in the coverage values of some selected species (Pediculari-Seslerietum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

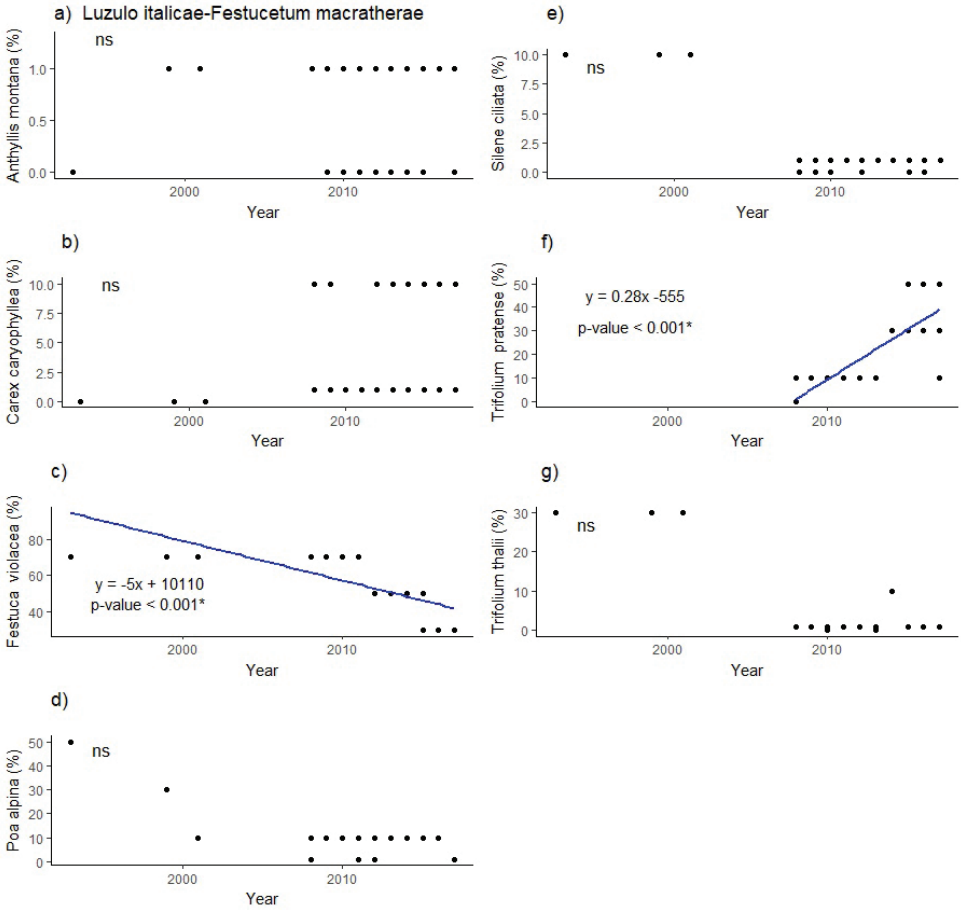

Figure 19. Changes in the coverage values of some selected species (Luzulo-Festucetum plots) (*: regression performed using non-parametric linear regression analysis; ns: non-significant).

Discussion

Climate data are concordant with those presented in Evangelista et al. (2016) for the Central Apennines. The increase in mean annual temperature and very high inter-annual variability in annual precipitation amount are able to produce a strong drought stress in the biocenoses, in particular in very dry years, more and more frequent in the last decades.

Decreased snowfall and snow persistence (and increased variability), together with the variability of total precipitation, increase the ecological stress at the research site, as confirmed by interpretation of the soil microclimate data collected during the last five years: the recorded frequent frost episodes, connected with the absence of protec-

tive snow cover, will expose the biocenoses to very dangerous frost stress. The official meteorological station at the same location (with sensors well above the soil surface) confirms these data, with 167 frost days in winter 2013–2014: only 6 days more than data related to the *Pediculari-Seslerietum* (which therefore remains exposed to frost for a few days only), but 10 days less than data for the *Luzulo-Festucetum* (which conserves the protective snow cover for longer). Decreased snowfall is in agreement with previous observations in the Apennines (Baldoni et al. 1999, Romeo and Scarpelli 2001) and in the Alps (Cannone et al. 2007).

The low yearly rate of species turnover observed at the site (confirming the preliminary results from Petriccione 2005) can be considered physiological in communities in a dynamical stage of fluctuation, such as these, but the long-term analysis shows very significant changes in species composition, with a high increase in the total number of species and a small but relevant lack of sensitive species (in the mesic grassland only). This trend can be seen as an emergent dynamical tendency: early signs of a degeneration process (also highlighted by an increase in invader species) were discernible after just seven observation years and were confirmed in subsequent years. Signs like these are more evident in snow-dependent communities *(Luzulo-Festucetum*), more sensitive to drought increase and snow cover shortage.

Although both communities preserve the same values of total plant coverage over time, in the case of the *Luzulo-Festucetum* the former dominant species (*Festuca violacea*) lost its past role, due to the invasion and expansion of an opportunistic species (*Trifolium pratense*). These results are in good agreement with those found in the Central Italian Alps in the subalpine, alpine and nival belts over the last 50 years (Cannone and Pignatti 2014), but they contrast with the vegetation coverage increase noted for many species in other Alpine ranges and for other LTER sites in the Central Apennines during the last 20 years (Rogora et al. 2018).

The significant changes of morpho-functional traits, noted in both communities, are interpretable as the effects of a decrease of snow cover and an increase in temperature and drought stress (Körner 1994) Small leaves (decrease in leaf width was found in the *Pediculari-Seslerietum* dry grassland) in fact reduce boundary layer resistance and help maintain favourable leaf temperatures and higher photosynthetic water-use efficiency-with high solar radiation and low water availability (Givnish 1987; Knight and Ackerly 2003). An increase in species with dense hairs could also be the result of drought stress adaptation over time. Hairs, in fact, protect plant species from solar intensity, but also retain air moisture and avoid water loss from the plant (Wagner et al. 2004).

Regarding the *Luzulo-Festucetum* mesic grassland, on the other hand, we found a more complex response to the variation over time. In line with the trend of increasing drought, we noted a significant decrease in species without hairs and a significant increase in species with sparse hairs. The observed increase of leaf width is apparently in contrast with increasing drought stress. However, this pattern may be the result of an increase in compound leaves, given that for these species the leaf, and not the leaflet, was measured. An increase in compound leaves along the aridity gradient has been noted (Givnish 1978) and leaves along a deciduous rachis are probably advantageous in dry environments.

The significant changes in life strategies, with an increase in species with a stress-tolerant strategy for the mesic community, are characteristic of biocenoses with increased ecological stress, undergoing changes towards adaptation to an increased drought. These results are in agreement with observations made in the Alps (Chapin et al. 1996, Theurillat and Guisan 2001, Welker et al. 2001, Cannone et al. 2007).

The progressive narrowing of the *Luzulo-Festucetum* ecogram over time confirms the correlation between the observed warmth and drought trends and the related changes in the sensitive mesic plant community. Our results exclude a possible effect on changes in vegetation caused by modified land use or nitrogen accumulation in the soil (as shown by the related ecogram, with a clear decrease in nitrogen availability indicators over time), unlike the assumptions of Evangelista et al. (2016).

Dry grassland shows higher resistance to an increased drought than mesic grassland. To improve the reliability of the findings, a regression model (non-parametric where assumptions were not met) removing data collected before 2008 (with no replicates) was also performed (Suppl. material 1, Table S3). The ecological meaning of the results is very similar, showing the preferential response of mesic grassland towards drought adaptation and the lower variation of dry grassland, again towards aridity.

Conclusions

The warming trend at global level is confirmed and reinforced by data related to the LTER site "Gran Sasso d'Italia": the mean annual temperature has increased by 1.7 °C over the last 65 years, corresponding to an average increase per decade of +0.26 °C. This is more than double the same values at global level (+0.7 °C in the last 60 years and +0.1 °C per decade, IPCC 2014), and very near the forecasted increase of +2.0° C by the year 2100 (IPCC 2014).

This exceptional warming in alpine areas, together with a decrease in total precipitation (as recognized for the Central Apennines as a whole, even if not significant at the site) and snowfall (significant at the site), an increase in climate inter-annual variability and extreme events, and a frequent lack of snow cover, are the combined drivers of the intense species turnover observed, occurring over the last 30 years in all the biocenoses studied, although more marked in snow-dependent communities. A quantitative increase in more thermophilic and stress- and drought-tolerant species and a parallel decrease in more mesic, cold adapted and competitive species have been clearly detected. These results confirm the preliminary assumptions provided in Petriccione (2012) for the first 18–25 years of observation at the same LTER site.

Ecological indicators demonstrate that the key factor in the ecological changes of the alpine biocenoses studied is drought, associated with the combined action of temperature increase, precipitation decrease and lack of snow cover and precipitation.

The two communities studied react in different ways to these abiotic drivers: (1) the *Pediculari-Seslerietum* dry grassland, highly resistant and well adapted to drought, frost and drastic temperature ranges, shows very slow or no changes over time (in accordance with the results of Frate et al. 2018); (2) the *Luzulo-Festucetum* mesic grass-

land, with low resistance (increase in species richness and invaders) and not adapted to drought and soil frost, shows important and rapid changes, increasing cover values for species with ruderal and stress-tolerant strategies, and a parallel decline in the former dominant species, towards first signs of drought stress.

The fluctuation stage typical of these primary alpine plant communities seems to be changing toward a dynamical tendency of degeneration, with an important disgregation of the community due to deterioration of the ecological connections: as in the Central Alps, this process can lead to an ecological vacuum or a disequilibrium state in the biocenoses (Cannone and Pignatti 2014).

In conclusion, our results enable us to answer the four questions listed in the introduction: a) plant communities are significantly changing over time, more for mesic grassland and less for dry grassland; b) toward a disequilibrium state; c) species are responding in different ways, altering the intra-community ecological connections; d) there is a relationship between the changes in the features of the communities and the predicted and the observed changes in the temperature and precipitation regimes.

Additional long-term observations over the next decades are, in any case, required to confirm the hypothesis of a cause-effect relationship between climate change and changes in plant communities and to exclude natural and unknown fluctuations.

The combined monitoring of vegetation (composition and structure) and temperature at high elevation will provide updated data on the processes currently underway on the high summits of the Apennines and will guide the local in-situ policies to conserve the associated plant communities and threatened species.

Acknowledgments

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Supplementary material 1

Supplementary tables and figures

Authors: Bruno Petriccione, Alessandro Bricca

Data type: measurements

Explanation note: **Table S1.** Pediculari-Seslerietum 1986–2017 (phytosociological releves). **Table S2.** Luzulo-Festucetum 1993–2017 (phytosociological releves). **Figure S1.** Geographical location of the LTER site "Gran Sasso d'Italia" (site area in pink; permanent plots indicated by red squares). **Figure S2.** Pedological profile of the Gran Sasso d'Italia Pediculari-Seslerietum community (soil depth in cm; from Furrer and Furnari 1960). **Figure S3.** Pedological profile of the Gran Sasso d'Italia Luzulo-Festucetum community (soil depth in cm; from Furrer and Furnari 1960). **Figure S4.** Changes in snow abundance during the period 1988-2016 (mean winter snow depth, in cm; total winter snow precipitation, SWE in mm*. 1cm of snowfall was assumed equal to 1mm snow water equivalent). **Figure S5.** Changes in the ecogram during the period 1986-2017 for the Pediculari-Seslerietum dry grassland plots (L: light availability, T: thermophile behaviour, C: continental gradient, U: soil moisture, R: pH gradient, N: nitrogen availability). **Figure S6.** Changes in the ecogram during the period 1993-2017 for the Luzulo-Festucetum mesic grassland plots (L: light availability, T: thermophile behaviour, C: continental gradient, U: soil moisture, R: pH gradient, N: nitrogen availability). **Table S3.** Results of statistical analysis based on data only from 2008 with replicates. Positive or negative slope coefficient refers to increase or decrease of given traits or indices. Parametric model refers to linear model (lm function in package stats), while non-parametric model refers to median based linear model (mblm function in package mblm). Only parametric model gives R2.

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RESEARCH ARTICLE

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Dynamic of nitrogen and dissolved organic carbon in an alpine forested catchment: atmospheric deposition and soil solution trends

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Abstract

A number of studies have reported decreasing trends of acidifying and N deposition inputs to forest areas throughout Europe and the USA in recent decades. There is a need to assess the responses of the ecosystem to declining atmospheric pollution by monitoring the variations of chemical species in the various compartments of the forest ecosystem on a long temporal scale. In this study, we report on patterns and trends in throughfall deposition concentrations of inorganic N, dissolved organic N (DON) and C (DOC) over a 20-year (1995–2015) period in the LTER site -Val Masino (1190 m a.s.l.), a spruce forest, in the Central Italian Alps. The same chemical species were studied in the litter floor leachates and mineral soil solution, at three different depths (15, 40 and 70 cm), over a 10-year period (2005–2015). Inorganic N concentration was drastically reduced as throughfall and litter floor leachates percolated through the topsoil, where the measured mean values (2 μeq L^{-1}) were much lower than the critical limits established for coniferous stands (14 μeq L^{-1}). The seasonal temperature dependence of throughfall DOC and DON concentration suggests that the microbial community living on the needles was the main source of dissolved organic matter. Most of DOC and DON infiltrating from the litter floor were retained in the mineral soil. The rainfall amount was the only climatic factor exerting a control on DOC and N compounds in throughfall and forest floor leachates over a decadal period. Concentration of SO_4 and NO_3 declined by 50% and 26% respectively in throughfall deposition. Trends of NO_3 and SO_4 in forest floor leachates and mineral soil solution mirrored

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declining depositions. No trends in both DON and DOC concentration and in DOC/DON ratio in soil solutions were observed. These outcomes suggest that the declining NO_3 and SO_4 atmospheric inputs did not influence the dynamic of DON and DOC in the Val Masino forest. The results of this study are particularly relevant, as they are based on a comprehensive survey of all the main compartments of the forest ecosystem. Moreover, this kind of long-term research has rarely been carried out in the Alpine region.

Keywords
Nitrate, N-saturation, throughfall, litter floor, DON, LTER

Introduction

Since the Industrial Revolution, forests have been exposed to elevated atmospheric fluxes of nitrogen (N) and sulphur (S), if compared to natural background. Throughout the second half of the 20th century, this has caused phenomena of N saturation and soil acidification in large areas of Europe and the United States (Aber et al. 1989, 1998; Gundersen and Rasmussen 1990). Over time, chronic inputs of N in temperate forests, i.e. through atmospheric deposition, can determine a shift from a N-limited growth state to a condition of N saturation in which the availability of inorganic N exceeds the N assimilation capacity of biological processes (Aber et al. 1989). The symptoms of an excess of N include (i) an increase of N mineralization and nitrification in soils, (ii) an imbalance of nutrients in plant tissues, (iii) an augmented acidification of soils, with the mobilization of toxic species e.g. aluminum (Al), and the leaching of acidifying compounds (Gundersen and Rasmussen 1990; Gilliam et al. 1996; Stoddard et al. 1999), and (iv) a diminished biodiversity in both aquatic and terrestrial ecosystems (Vitousek et al. 1997; Bobbink et al. 1998). The incidence of these detrimental effects on the environment led to the development of abatements strategies under the Convention on Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economic Commission for Europe (UNECE) (www.unece.org) aimed at reducing the emissions of acidifying pollutants all over Europe and the USA since the late 1970's. Rules for lessening emissions have proven successful and rates of acid and N deposition have declined in the past 20–30 years, in both Europe and North America (Shannon 1999; Waldner et al. 2014). In particular, the analysis of long-term trends of atmospheric deposition in the Southern Alps (Italy and Switzerland) revealed decreasing trends in both sulphate (SO_4) and nitrate (NO_3) concentration across a 30-year period (1984–2014). The decline of NO_3 started later (2006) compared to SO_4 and the inorganic N loads were still high (8.4–9.8 kg ha^{-1} y^{-1}) with respect to critical loads (Rogora et al. 2016). The reduction of S and N atmospheric loads enabled the recovery from acidification of a number of water bodies in Europe and the USA (Skjelkvåle et al. 2003, Wright et al. 2005, Garmo et al. 2014, Kopáček et al. 2016). However, especially for N, the relationship between the atmospheric deposition and the declining trends in surface waters is complex. Indeed, among others, climatic variations and climate-related factors (e.g. changes in terrestrial organic matter dynamic, increased drought events and forest dieback) are some of the elements that can "confound" the

trends in water chemistry of rivers and lakes (Webster and Brezonik 1995, Hejzlar et al. 2003, Mikkelson et al. 2013, Kopáček et al. 2016).

In order to assess responses of the ecosystem to the decline of N atmospheric loads it is important to investigate the transformation of N and C forms in the various compartments of the alpine forest ecosystem. Indeed, substantial changes of chemical species occur as rainfall passes through the tree canopies, reaches the forest floor and then percolates into the deeper layers of soil. Although long- term monitoring of soil solution chemistry is a valuable tool to evaluate abatement strategies and to verify the effective recovery of forest ecosystem from long-term inputs, this kind of study is rarely carried out in the Alps (Pannatier et al. 2011). Furthermore, the published long-term data series on atmospheric deposition and soil solution in forest ecosystems rarely include the organic form of N and C. Recently, however, scientific interest in the role of DON and DOC in the biogeochemical cycles of N and C has largely increased. The transfer of dissolved organic matter (DOM) from soil – the C storage compartment – to surface waters occurs mainly via soil solution, which therefore represents an important interface between the terrestrial and the aquatic ecosystems. The migration of DOC and DON from soil is regulated by a number of processes both biotic – such as the mineralization and the production of exudates by roots –, and physical ones – such as the adsorption into the soil matrix and the hydrological control over drainage and lateral export to surface waters (Camino-Serrano et al. 2014). Differently to inorganic NO_3 that is considered a valuable indicator of N saturation, many observations indicated that DON leaching from soils is likely not dependent on N deposition loadings (Perakis and Hedin 2002, Neff et al. 2003, Williams et al. 2001, Balestrini et al. 2013). Aber et al. (1998) described the DON behavior as the "leaky faucet hypothesis" according to which there is a persistent "leak" of DON from terrestrial catchments due to the DON decoupling from biological demand for N. Nevertheless, the results of long-term forest fertilization experiments included also great and persistent increases in soil solution DON flux (McDowell et al. 2004; Pregitzer et al. 2004). It is evident that to date the knowledge of the factors that regulate the production and retention of DON is too scarce to make predictions on its fate in the forest environment (Scott and Rothstein 2017).

The overall objective of the present study is to evaluate the biogeochemistry of N species and DOC at Val Masino forest, a remote site in the Italian central Alps, belonging to the Italian LTER network (LTER-Italy: www.lteritalia.it) and to the ICP Forests programme (www.icp-forests.org), launched in 1985 under the Convention on Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economic Commission for Europe (UNECE, www.unece.org). Since 1994, several studies have been carried out in this area in order to investigate the effects of S and N atmospheric deposition on the biological component of the ecosystems, first of all in the forest community (Balestrini et al. 2000, 2006, 2013; Balestrini and Tagliaferri 2001). The atmospheric depositions, in open field and under the forest canopy (throughfall), the mineral soil solutions at three depths, and the forest floor leachates have been monitored over 20, 13 and 10 years respectively, in order to provide a more in-depth comprehension of the connection among N deposition, terrestrial N processes, and

streamwater export. Specific aims of the present work are i) to investigate the chemistry of the environmental compartments that compose the forest ecosystem, ii) to identify the main controlling factors of NO_3, NH_4, DOC, DON concentrations over a decadal time scale, iii) to detect trends in inorganic N species and in C and N organic compounds in throughfall deposition, litter floor leachates and mineral soil solution, and iv) to investigate the effects of atmospheric depositions on soil solution chemistry.

Material and methods

Study area

The study area Val Masino (46°14'31.44"N, 9°35'51.51"E) is located at 1190 m a.s.l. in the upper watershed of the Masino River in the Central Alps (Northern Italy) (Figure 1). Around 85% of the upper basin is above 1850 m and is delimited by mountains rising to 3500 m. The overall soil cover percentage is 66% and only 13% of the catchment is forested. A more detailed description of the catchment is provided in Balestrini et al. (2013). The site is at a distance of approximately 140 km from the highly urbanised and populated Metropolitan City of Milan (Lombardy Region). Since 1995, Val Masino site has been a part of the national Integrated Programme for the Control of the Forest Ecosystems (CON.ECO.FOR) directly linked to the ICP Forests network (International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forest, http://icp-forests.net). Since 2006, the site has belonged to the Italian LTER network (LTER-Italy: www.lteritalia.it). The experimental site consists of two plots, one in open field and the other in a forested area located at the edge of conifer forest. The latter is a 900 m^2 fenced plot set up for the monitoring of throughfall deposition, soil solution and forest floor leachate. The dominant tree species is *Picea abies* followed by *Abies alba*. The geological substratum is volcanic granodiorite and the soil was classified as Cambic Umbrisol (Humic, Alumic, Skeletic) (Smart4Action project, http://smart4action.ise.cnr.it/). The main soil properties are shown in Table 1. The climate is continental, with the maximum amount of precipitation in summer and the minimum in winter. Snowfall typically occurs from November to April. Meteorological data have been provided by a multi-parameter weather station, with temperature sensor and precipitation gauge placed in the open field plot adjoining the forest. The 20-year mean amount of precipitation was 1598 mm y^{-1} (1995–2015) of which 30% occurred as snowfall. The annual mean air temperature from 1997 to 2015 was 7 °C.

Sampling and chemical analysis

All sampling activities and chemical analyses were conducted according to the international procedures adopted within the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forest (ICP Forests) (UN-ECE 1998).

Table 1. Soil properties of soil collected at Val Masino study area.

Depth cm	Clay %	Silt %	Sand %	pH ($CaCl_2$)	Exchangeable acidity cM kg^{-1}	Corg g kg^{-1}	TN g kg^{-1}	C/N
-4–0				4.3	3.6	381.1	15.0	25.4
0–10	1.4	9.5	89.1	3.9	6.6	80.1	5.6	14.3
10–20	1.5	15.2	83.3	4.3	3.9	45.7	3.7	12.5
20–40	1.2	16.3	82.5	4.3	3.3	37.9	3.1	12.1
40–80	1.2	16.7	82.1	4.5	2.6	33.7	2.4	14.2

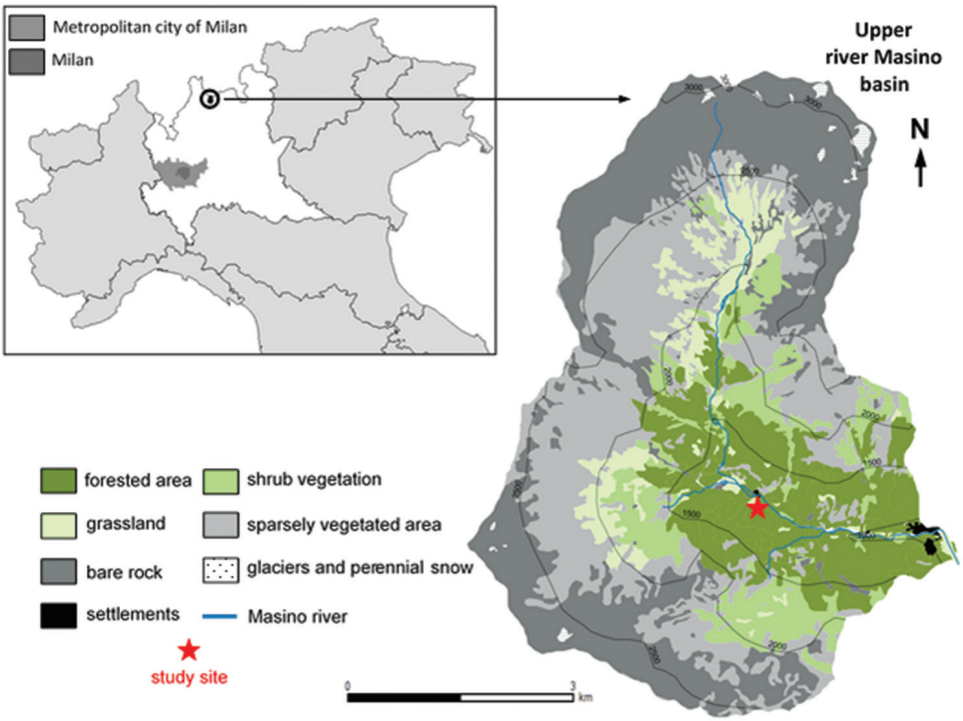

Figure 1. Location of the study site within the river Masino basin.

Since 1994 the experimental area has been equipped with 9 bulk rainwater samplers - polyethylene buckets (5L volume) with an 18 cm ID funnel - uniquely identified by a number and placed one meter above the ground. In the winter season 5 snow samplers (56 cm ID) replaced those for rain. Samplings and analyses of atmospheric deposition have been conducted on a weekly basis from 1995 to 2015, except for the 2007–2008 two-year period during which chemical analyses were conducted on bi-weekly combined samples. At the end of each 7-day sampling period, depositions collected by the throughfall samplers were combined to form one homogeneous sample and stored at 4 °C until shipping to laboratory. In 2007 and 2008, prior to chemical analyses, weekly samples were combined proportionally to obtain a 500 ml bi-weekly sample.

The monitoring of soil solution chemistry, instead, was carried out in two different periods. A first survey, from 1999 to 2001, was followed by a second longer investigation starting in 2005 and ending in 2015. The sampling system consisted of tension lysimeters made of 1 μm porosity ceramic cup connected via a plastic tube, to a 2L glass bottle to which the vacuum was applied. In 1998, six sampling points were equipped with lysimeters collecting water at 30 and 50 cm depth. During autumn 2005, changes were made to the experimental plan. Fifteen lysimeters collecting soil solutions at 15, 40, and 60–70 cm depth were placed at five sampling points. In order to monitor forest floor leachate, 3 zero-tension lysimeters, concave-shaped steel plates, were placed below the litterfall at 3 sampling points representative for different plant species and slope. These lysimeters worked by gravity and collected the precipitation passing through the canopy and the forest floor. Since soil disturbance due to lysimeter installation may affect water chemistry, samples collected in a first period (6–12 months) were discarded and regular sampling of soil solution began in 1999 and again in May 2006. Due to snow cover and the possibility of water freezing inside the tubes and the bottles, soil solution and forest floor leachates were mostly sampled from April to November. Analyses on soil solution and forest floor leachates were performed on bi-weekly samples.

The analysis was performed on filtered samples (0.40 μm), except for measurements of electrical conductivity (EC), pH, and total nitrogen (TN), for which unfiltered samples were used. Total organic nitrogen (TON) was obtained from TN minus total inorganic nitrogen (TIN). During 2005 we determined both total and dissolved N (TDN) and from 2006 to 2013 only TDN was assayed. Dissolved organic carbon (DOC) and total aluminum analyses were performed by high temperature catalytic oxidation (IR detection) and ICP-OES respectively.

Major anions (Cl, SO_4, NO_3) and cations (Ca, Mg, Na, K) were determined using suppressed ion chromatography, whereas NH_4 and total N were assessed by UV/VIS spectrophotometry.

For alkalinity we used a two end-points potentiometric titration with HCl 0.01M. The whole references for analytical methods and instrumentation used are presented in Suppl. material 1: Table S1.

The quality assurance controls included the participation in the ICP Forest ring tests, ICP Waters (organized by NIVA http://www.icp-waters.no/publications/), EMEP (https://www.nilu.no/projects/ccc/intercomparison/index.html) and WMO-GAW (http://www.qasac-americas.org/lis/) interlaboratory comparison programme. We used routine quality control charts based on certified or internal standards.

Data elaboration and statistics

We used volume-weighted means (VWM) for monthly and annual throughfall concentration and arithmetic mean for forest floor and soil solution monthly concentration. In order to obtain an annual estimate of the amount of soil water at each depth we

applied the mass balance approach for sodium (Na) (Bailey et al. 2003) assuming negligible weathering of Na from the mineral soil. From the soil solution annual fluxes we derived the VWM useful to make comparisons with throughfall VWM. For the statistical elaboration we processed the chemical data of soil solution collected at 30 cm depth (from the first and the 2005 surveys) together with those of soil solution collected at 15 cm depth during 2006–2015 decade. In the same way, we combined the dataset available for soil solution collected at 50 cm depth with the 40 cm depth dataset.

Statistical significance of trends and the slope of the derived relationships were assessed by means of Seasonal Kendall Test and Theil-Sen's slope estimator, respectively (Hirsch and Slack 1984; Ohlson and Kim 2015). Calculations were performed with the package 'rkt' (Marchetto 2017) in the R v.3.4.2 software environment (R Core Development Team 2016).

Results

Changes in throughfall and soil solution chemistry

Figure 2 illustrates the mean annual concentration of C and N chemical species measured in the throughfall deposition (THR), in the forest floor leachates (FF) and in the soil solution from various depths in the mineral soil (TS = soil solution at 15 cm depth; SS = soil solution at 40 cm depth; DS = soil solution at 70 cm depth).

The highest mean annual concentration of NO_3 occurs in throughfall (27.0 ±9.7 μeq L^{-1}, n = 21) and forest floor leachates (25.3 ±12.8 μeq L^{-1}, n = 10). A drastic decline of NO_3 is evident through the mineral soil where the concentrations ranged from 1.5 – 2.4 μeq L^{-1}. The reduced nitrogen form, NH_4, showed an analogous pattern with higher values in the throughfall (17.7±5.0 μeq L^{-1}, n = 21) and forest floor leachate (22.8 ±9.8 μeq L^{-1}, n = 10) and concentrations close to the detection limit in the soil solutions. A more homogenous pattern characterised SO_4, which showed a restricted range of values, between a minimum mean value of 18.4±7.1 μeq L^{-1} (n =10) measured in the forest floor and a maximum of 35.7±38.9 μeq L^{-1} (n = 14) at 40 cm depth in the mineral horizon (Suppl. material 1: Figure S1)

The forest floor was the principal source of base cations (BC), DON and DOC, which reached concentrations much more elevated in this leachate than in the other matrices. BC and DON concentrations followed the order FF>THR>TS>SS>DS, while for DOC the order was FF>TS>THR>SS>DS.

DON was the main form of N in all the analyzed matrices constituting the 45%, 70%, 85%, 83% and 68% in the throughfall, forest floor leachate, soil solution at 15, 40 and 70 cm depths, respectively.

Al concentrations measured in the last decade showed the highest mean concentration in the topsoil (468±94.1 μg L^{-1}, n = 11) to then gradually decrease with depth; the mean value was 247±51.1 μg L^{-1} (n = 9) (Suppl. material 1: Figure S1).

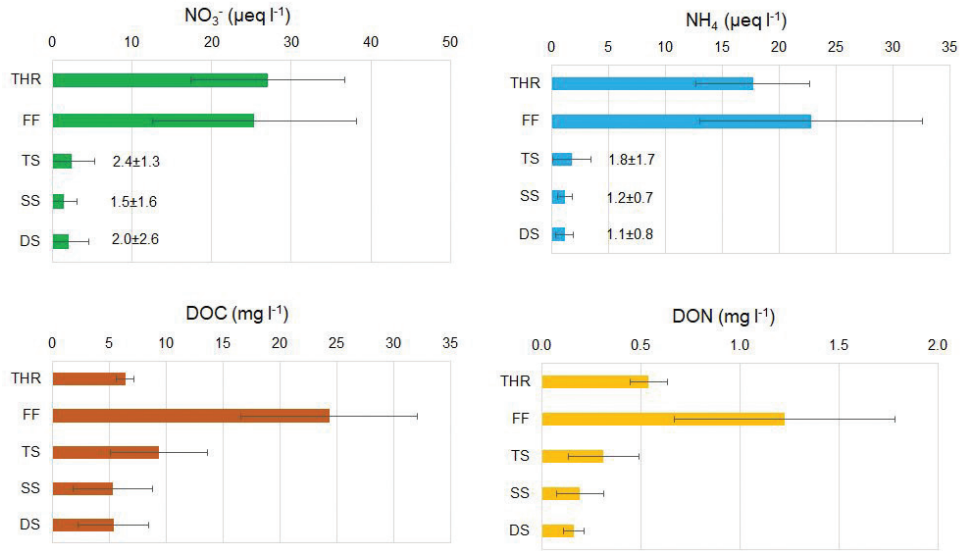

Figure 2. VWM concentrations of nitrate, ammonia, DOC and DON in water samples collected in different compartments at Val Masino site. Error bars represent the standard deviation. THR = throughfall depositions; FF = forest floor leachate; TS (topsoil) = soil solution at 15 cm depth; SS (subsoil) = soil solution at 40 cm depth; DS (deep soil) = soil solution at 70 cm depth.

Relationships between chemical species and influence of climatic factors

The Pearson product-moment correlation coefficients between monthly concentrations of the main chemical species within each environmental matrix are shown in Table 2. We found a strong significant positive correlation between NO_3 and SO_4 both in the throughfall deposition and forest floor leachates. Both NO_3 and SO_4 were well correlated with NH_4 in the same compartments.

DOC and DON were significantly correlated in throughfall, forest floor leachate and topsoil solution (15 cm depth), but not in the deeper soil layers. In all soil compartments, both DOC and DON concentrations were positively related to BC and Al except for DON in SS layer (40 cm depth).

Lastly, we examined the relationships of monthly concentrations between the environmental matrices (Table 3). NO_3 and SO_4 concentrations were significantly correlated between all matrices and depicted the hydrological pathway of solutes from throughfall to the deep soil solution. As far as DON concentrations are concerned, the relationship between throughfall and floor leachates was significant.

The influence of air temperature (minimum, maximum and mean), the amount of throughfall precipitation and snow (spring and total winter snow) on the concentration of the main chemical species was investigated at interannual scale.

The annual throughfall concentrations of DOC, DON, NO_3 were negatively linearly related to the amount of rain (Figure 3).

Table 2. Pearson correlation between monthly average concentration of throughfall depositions (THR), forest floor leachate (FF), topsoil (TS = topsoil solution at 15 cm depth), subsoil (SS = subsoil solution at 40 cm depth) and deep soil (DS = deep soil solution at 70 cm depth). Significance: * = p ≤ 0.05; ** =p ≤ 0.01; *** = p ≤ 0.001; ns: not significant.

	THR	FF	TS	SS	DS
NO_3:SO_4	0.771***	0.775***	ns	ns	ns
NO_3:NH_4	0.850***	0.668***	ns	ns	ns
NH_4:SO_4	0.696***	0.400***	ns	ns	ns
DON:DOC	0.533***	0.603***	0.461***	ns	ns
DOC:BC	0.760***	0.529***	0.453***	0.462***	0.257*
DON:BC	0.533***	0.444**	0.437***	ns	0.257*
DOC:H^+	0.259***	0.361*	0.443***	ns	ns
DON:H^+	0.196*	0.377**	0.586***	ns	ns
DOC:Al		0.715***	0.593***	0.614***	0.382**
DON:Al		0.355*	0.610***	ns	0.324*
Al:H^+		0.478***	0.745***	0.614***	0.424**

Table 3. Pearson correlation between monthly concentration of NO_3, SO_4 and DON in throughfall depositions (THR), forest floor leachate (FF), topsoil (TS = topsoil solution at 15 cm depth), subsoil (SS = subsoil solution at 40 cm depth) and deep soil (DS = deep soil solution at 70 cm depth). Significance: * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001; ns: not significant.

	NO_3	SO_4	DON
THR : FF	0.603***	0.577***	0.480***
THR : TS	0.710***	0.599***	ns
THR : SS	0.404***	0.447***	0.360**
THR : DS	0.387***	0.335**	0.282
FF : TS	0.397***	0.249*	ns
FF : SS	0.242*	ns	ns
FF : DS	0.477***	0.380**	ns
TS : SS	0.603***	0.768***	ns
TS : DS	0.534***	0.438***	ns
SS : DS	0.508***	0.525***	ns

Likely, DOC concentration in the forest floor leachates significantly decreased at the increasing of the throughfall precipitation (Suppl. material 1: Figure S2). For DON, even if a fairly similar trend can be visually perceived, the relationship with the amount of precipitation was not significant (p=0.19) (Suppl. material 1: Figure S2a). In the mineral soil, the precipitation amount had no influence on the concentrations of the analyzed species, whereas the amount of snow affected the chemical composition of the topsoil solution. In fact, negative linear relationships were found between snow amount and both H^+ and DON (Suppl. material 1: Figure S2). At deeper soil solution, only Al concentration was significantly affected by the snow.

The air temperature descriptors had no influence on the chemistry of the investigated environmental compartments.

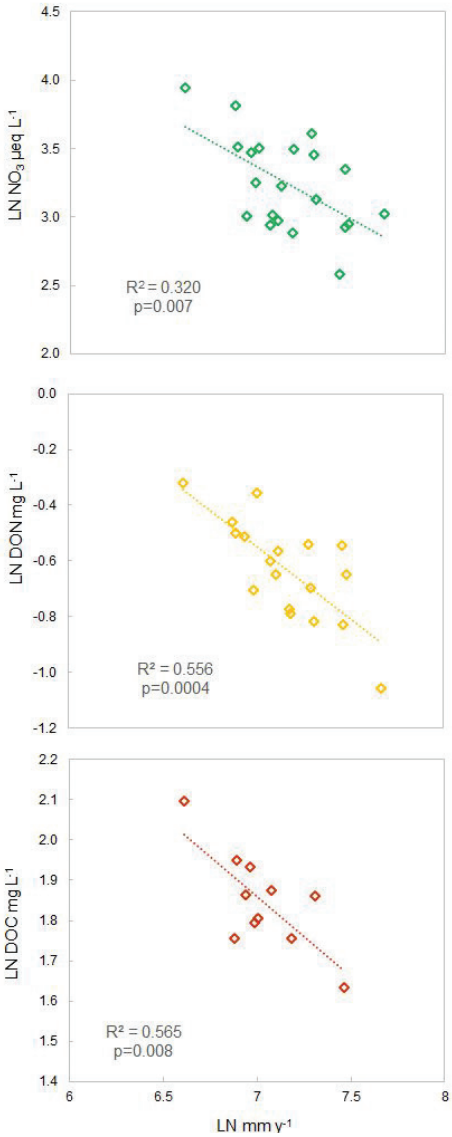

Figure 3. Relationship between the amount of precipitation (mm yr^{-1}) and the concentrations of NO_3, DON and DOC in the throughfall (mg L^{-1}).

Seasonality and annual trends

The monthly volume-weighted average concentrations of both inorganic and organic species in throughfall deposition show their maximum from March to the end of summer and minimum values in autumn and winter (Figure 4).

Despite high variability, a seasonal variation was quite evident for NH_4 and NO_3 in forest floor leachate with higher concentrations in warmer months (Figure 5). The

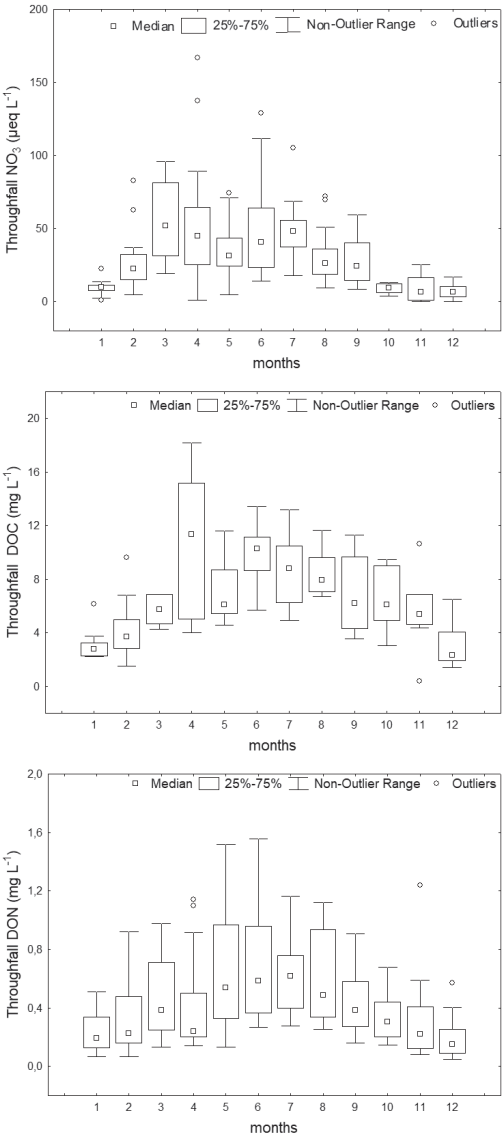

Figure 4. Box&Wisker showing the monthly variations of the NO_3, DON and DOC concentrations in the throughfall deposition.

concentration of all analyzed species in the mineral soil solutions did not exhibit seasonal variations during the study period. It is worth noting that it was generally not possible to sample soil solutions from January to March as the water in the external tubes of lysimeters was frozen. During 2007 and 2008, years characterized by warmer winter temperatures, we obtained samples for all months but we did not observe any important variation, except for slightly higher NO_3 concentrations in the topsoil solutions, from January to March 2007.

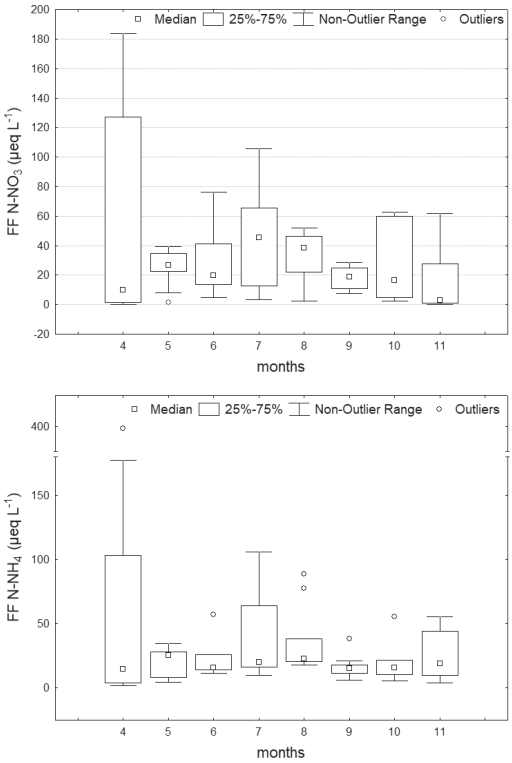

Figure 5. Box & Wisker showing the monthly variations of NO_3 and NH_4 concentrations in the forest floor leachates (FF).

Throughfall concentrations of NO_3 and SO_4 significantly declined during the last two decades (1995–2015) unlike NH_4 concentration that remained stable (Table 4). The declining annual rate of SO_4 was 3.6 fold higher than the one calculated for NO_3 trend. The concentration of H^+ significantly decreased in the throughfall deposition, and so did BC and DON concentrations. Due to the critical gaps in the data series relating to the DOC throughfall concentration, we could not test its trend. Nevertheless, the available data (Suppl. material 1: Figure S3) seem to indicate that a significant trend is not likely.

The annual trends of forest floor and mineral soil layers have been estimated over a period ranged from 9 to 13 years, thus shorter than that of throughfall (Table 4). Both SO_4 and NO_3 exhibited a decreasing trend in all matrices, with the exclusions of topsoil and subsoil respectively. NH_4 diminished significantly in the litterfall leachate and in the mineral soil at 40 cm depth. At 15 cm depth, the negative trend was not significant.

Unlike throughfall, in litter floor leachate H^+ increased while BC declined. Trends of DOC and DON concentration in the mineral soil solution resulted statistically significant in the deep soil layer only, and showed slightly declining and increasing values, respectively.

Table 4. Mann – Kendall trends for chemical species concentration analyzed in throughfall deposition (THR), forest floor leachate (FF), topsoil (TS = soil solution at 15 cm depth), subsoil (SS = soil solution at 40 cm depth) and deep soil (DS = soil solution at 70 cm depth). Significance of Sen's slope: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns: not significant.

	Period	H^+		NH_4		NO_3		SO_4		DOC		DON	
		Slope	%	Slope	%	Slope	%	Slope	%	Slope	%	Slope	%
THR	1995–2015	-0.10***	-3.19	ns		-0.51***	-1.44	-1.37***	-5.42	-0.09*	-1.32	-0.02***	-3.28
THR	2005–2015	-0.05**	-3.08	ns		ns		-0.80***	-4.95	ns		-0.02**	-5.22
FF	2006–2015	0.30***	13.4	-2.50***	-8.53	-2.03**	-6.86	-1.94***	-9.21	ns		ns	
TS 15 cm	2005–2015	ns	-0.61	ns		-0.23**	-4.87	ns		ns		ns	
SS 40 cm	2005–2015	ns	1.12	-0.10*	-3.78	ns		-1.67***	-6.60	ns		ns	
DS 70 cm	2006–2015	ns	-1.92	ns		-0.04*	-2.20	-1.64***	-6.45	-0.13*	-2.47	0.01*	4.04

Discussion

Patterns of inorganic species

The obtained results highlighted important modifications of the aqueous solution chemistry along the hydrological pathway through different environmental compartments from atmospheric deposition to the forest deep soil.

Particularly, nitrogen species exhibited the greatest quantitative and qualitative variations. Throughfall deposition and forest floor leachates were characterized by similar and comparatively high concentrations of NO_3 and NH_4, thus being a crucial source of inorganic N for the forest soil. Next, the passage to mineral layers led, for both NO_3 and NH_4, to a depletion greater than 90%, already in the topsoil (15 cm depth). The analysis of the relationships between chemical species has provided insights on the origin and fate of chemical compounds in the forest ecosystem. The strong positive correlation between NO_3 and SO_4 in the throughfall deposition suggests common precursors, i.e. SO_2 and NO_x emitted from anthropogenic sources derived from fossil fuel use. Furthermore, the linear relationship between NH_4 and both SO_4 and NO_3 could be explained considering the formation of ammonium sulfate and nitrate aerosols by a gas-phase reaction of ammonia with sulfuric and nitric acids in the atmosphere (Dentener and Crutzen 1994). The tree canopy acts as a natural surface able to intercept also dry deposition. Consequently, the throughfall deposition is considered a good estimate of the total (wet plus dry) deposition of SO_4 and NO_3. Nevertheless, a contribution from foliar leaching and/or uptake cannot be excluded in the cases of NO_3 and NH_4 (Balestrini et al. 2000). The same relationships (NO_3 vs SO_4, NO_3 vs NH_4, SO_4 vs NH_4) observed in the forest floor leachate, but not in the mineral soil solution, is rather unexpected as it could suggest that both NO_3 and NH_4 had a quite conservative behavior in this organic soil layer. The correlation found between the throughfall and the litter leachate NO_3 concentrations supports this hypothesis. Apparently, biotic processes consuming (i.e. through assimilation) and/or releasing inorganic N (with nitrification and mineralization) did not substantially change the concentration of NO_3 and NH_4 in this soil compartment. In order to investi-

gate further, we performed the above mentioned correlations on data grouped by season (Suppl. material 1: Table S2). During the warmer and snow-free period (July–October) the NO_3 concentration in THR was not correlated to the one in FF. Furthermore, the correlation between NO_3 and SO_4 was less significant compared to the November-June period. These outcomes suggest that during the growing season the biological processes are more effective and contribute to modify the FF nitrogen composition. Schwarz et al. (2011) measured $\delta^{15}N$ depleted values and higher NO_3 concentration in the litter leachate relative to throughfall in a tropical montane forest in Ecuador. The larger N stocks characterizing those soils compared to those of our site (Table 1) and the more favorable climatic conditions of Central America, likely explain the release of a large amount of NO_3 via ammonification and nitrification. In the Val Masino forest floor, the relatively constrained growing season and the short water residence time probably do not favor the biological retention processes of mobile species like NO_3. Thus, the resulting N-enriched overland flow may rapidly infiltrate the mineral soil and/or directly flow to streams as surface runoff during abundant rain events and wherever steep slopes are present.

Contrarily to the organic layer, the mineral soil represents an effective sink of inorganic N at our site, as expected in an N-limited ecosystem like a temperate forest. The potential sink processes of NO_3 include assimilation by plant, fungal and bacterial communities (e.g. Pena et al. 2013; Wang et al. 2018), denitrification and lateral leaching. In the case of NH_4, the adsorption to the soil matrix has to be considered a sink process in addition to the biological processes (uptake and nitrification).

The chemical composition of soil solution was used as an indicator of the effects of atmospheric pollution on forest ecosystem as well as to evaluate the efficacy of the flux deposition abatement policies (Iost et al. 2012). In order to calculate critical loads, thresholds for possible negative effects on ecosystems compartments were defined (UNECE 2007). For conifers, the acceptable N leachate concentrations to avoid nutrient imbalances was set at 0.2 mg N L^{-1}, while concentrations > 1 mg N L^{-1} suggest elevated leaching and risk of N saturation (Fischer and Lorenz 2011). The multiyear mean concentration of inorganic N recorded in the three mineral soil layers at the study site were much lower than these critical limits, even if during 2006 the median value was 0.17 mg L^{-1} i.e. very close to the critical limit. A recent approach used to assess the potential risk for the forest ecosystem consists of recording the number of individual exceedances of the critical limit (CLimE) in a long run (Iost et al. 2012). The analysis of inorganic N concentration data from 300 plots of the ICP Forest from 1990s to 2006 revealed that the safety limits were frequently exceeded in many European forests, especially in Central Europe (Iost et al. 2012). The annual mean of CLimE recorded in our study site showed the higher value in the topsoil compared to the deeper layers (12, 5 and 5% respectively at topsoil, subsoil and deep soil).

Pattern of organic constituents

The mixture of plant residues from the surrounding canopy and belowground that constitutes the forest floor is the principal source of organic compounds for the forest

soil. The concentrations of DOC and DON in the forest floor leachate were 3.5 and 2 folds those measured in the throughfall, which, however, substantially contributes to the soil enrichment. The mean annual concentration of throughfall DOC in the Val Masino plot (6.4±0.8 mg C L^{-1}) was very close to the values reported for spruce forests in the Italian Alps (Arisci et al, 2012), while DON concentration fell in the lower range reported by Michalzik et al. (2001) from 42 case studies in forest ecosystems of the temperate zone. The mean value of DOC in forest floor leachates, 21.8±4.4 mg C L^{-1}, was about half of that measured in spruce stands in Bavaria (Borken et al. 2011) and in coniferous forests of the temperate zone (Michalzik et al. 2001). Compared to DOC, few data are available for DON in percolating solution from organic soils. The values we observed are comparable to those reported in Michalzik et al. (2001) and higher than those measured in high-elevation spruce and fir forest at the Hubbard Brook Experimental Forest in the White Mountains, USA (Dittman et al. 2007). Organic matter in throughfall derives mainly from the leaching of plant tissues and consists principally of carbohydrates, and to a lesser extent aminoacids, pectic and phenolic substances, vitamins, and hormones (Tukey 1970). Analyzing the composition of dissolved organic matter (DOM) in spruce stands (Bavaria, Germany), Guggenberger et al. (1994) found that most fractions of carbohydrates in throughfall DOM occurred in an easily decomposable form and originated from the microbial community living on the needles. In contrast, the DOM in forest floor leachates was characterized by carbohydrates mainly derived from the degradation of lignocellulose. At our study site, the abundance of lichens on the trees and the seasonal evolution of DOC and DON concentration in throughfall, with maximum in the warmer months, suggest the importance of the temperature-dependent microbial activity for the DOM mobilization towards the litter level. A seasonal trend of DOC and DON in soil solution was not discernible, and therefore there was no evidence regarding the temperature control on the leaching of organic substances from the litter floor.

Most of infiltrating DOC and DON from the litter floor were retained in the mineral soil. Specifically, DOC concentration decreased to 39% in the topsoil and 31% in the deep soil, while DON reduced correspondingly to 29% and 15%. It is generally assumed that the principal processes responsible for the removal of carbon from the soil solution are abiotic, like precipitation as organo-metal complexes and/or by adsorption to solid Fe and Al phases (Guggenberger et al. 1994; Michalzik et al. 2001; Jansen et al. 2005; Fuss et al. 2011). We found strict linear relationships between both DON and DOC and Al in the top and subsoil solution (15 and 40 cm depth) but not in the deep soil (70 cm depth). These results suggest that the process of podzolisation is effective in the Val Masino soil and also that Al in the soil solution was probably in a complexed form, and therefore non-toxic. The capacity of mineral soil to retain DOM depends on soil features (e.g. texture, clay content) and the surface charge properties of DOM that change during adsorption (Scott and Rothstein 2017). Generally, the complex hydrophobic compounds are preferentially sorbed in soil layers, causing an enrichment of hydrophilic molecules in solution with increasing soil depth (Kaiser and Zech 1998; Scott and Rothstein 2014). These hydrophilic molecules are likely to have a relatively high N content (Lajtha et al. 2005) and are easier decomposable (Guggen-

berger et al. 1994). Even if some studies reported an effective reduction of DOC/DON ratio from surface to deep soil (Scott and Rothstein 2017), other studies did not confirm this tendency at regional scale and the ratios remained relatively constant with soil depth (Michalzik et al. 2001). At the studied spruce forest, we observed a declining C/N ratio in the soil matrix, particularly evident from the organic layer (C/N=25) to the topsoil at 15 cm depth (C/N=13). On the contrary, the average DOC/DON ratios of throughfall, forest floor leachate and mineral soil solution at three depths (13, 28.5, 35, 39, 38, respectively) indicated a slight N impoverishment of DOM moving through the soil profile. We hypothesize that a biodegradation of N-rich organic fractions, in addition to physical process, occurred within the first centimeters of soil resulting in a higher DOC/DON ratio of topsoil solution compared to litter leachate.

Effect of climatic factors on DOC and N species

Among the analyzed climatic parameters, the amount of rainfall seems to exert a primary control on DOC and N compounds in throughfall and forest floor leachates. In throughfall, DOC, DON, NO_3 and potassium (K) decreased as the amount of rain increased. This suggests a dilution effect occurring especially for chemical species strictly connected with the canopy, species that are washed off during the precipitation events. The decrease of DOC and DON concentrations in forest floor leachates and to a lesser extent also in the topsoil solution indicates that infiltrating water diluted DOC and DON concentrations. This is consistent with the results of Borken et al. (2011) that analyzed the site-to-site variability of DOC from organic layers and mineral soils of 22 forests in Germany. Wu et al. (2010) found a similar relationship between DOC concentration and precipitation in coniferous forests in Norway. Furthermore, we found an interesting effect in the role of snow amount in decreasing the concentration of DON and Al in the mineral soil. The presence of snow on forest floor for a relatively long period (November-April) likely supported a slow water infiltration during the snowmelt, which in turn influenced the translocation of DOM fractions richer in N. Some recent studies demonstrated the importance of snow cover duration as a key factor in regulating the N and C forms in high elevation alpine soils (Magnani et al. 2017).

A seasonal effect was evident for NO_3, NH_4, DON and DOC in the throughfall and, to a lesser extent, for inorganic nitrogen species in litter floor leachates. These findings, at least partly related to temperature, seem to suggest that biological processes occurring in the canopy and the organic soil layer somewhat controlled the N and C concentrations. In the case of throughfall, seasonal changes in the photochemical activity and in the atmospheric stability are to be taken into consideration, too. In the summer, contaminants reach the Val Masino from far away. On the contrary, in the winter the atmospheric deposition contains mostly ions of local origin (Balestrini et al. 2000). The seasonal influence of temperature on throughfall DOC and DON concentration has been observed in several studies (e.g. Borken et al. 2011; Solinger et al. 2001; Dawson et al. 2008).

We could not observe an effect of temperature on concentrations of N and C that could explain the yearly variations in all studied ecosystem compartments. The only exception was the positive relationship that linked NH_4 in the forest floor leachates to the annual minimum temperature. A regional scale study revealed that fluxes and concentration of DOC and DON were not connected to temperature changes (Michalzik 2001), whereas Borken et al. 2011 reported a positive correlation between DOC concentration and temperature in the organic layer leachates comparing forest stands located in different climatic regions. The lack of a temperature influence on DOC and DON in the organic soil layer could be explained by the occurrence of competing temperature-dependent processes that have an opposite effect, for example the mineralization and the mobilization of DOC (Michailzik et al. 2001). In our case, the relatively short annual data series is also to be taken into consideration.

Effects of trends in throughfall deposition on forest floor and mineral soil solution

The trends rates observed for NO_3 and SO_4 concentrations in throughfall deposition (-1.4% yr^{-1} and - 5.4% yr^{-1}, respectively) are in line with those recorded in European forests within the ICP-Forest network (Waldner et al. 2014). Differences between the two trends are evident in the studied area. SO_4 declined by 63% in a steady way from the first years of monitoring to 2015 and does not yet seem to have stabilised. NO_3 declined by 26% and only after 2006 a regular decline was recognizable. The inorganic N atmospheric flux (annual mean 8.01± 2.05 kg ha^{-1} yr^{-1}), making up 52.5% of the total N flux, also showed declining trends. Nonetheless, the TN loads measured in most recent years have continued to exceed the critical load of N as nutrient (5.8 kg N ha^{-1} yr^{-1}) by several kg ha^{-1} yr^{-1}.

Trends of NO_3 and SO_4 in forest floor leachates and mineral soil solution mirrored declining throughfall depositions. NO_3 decreased significantly at all depths (with the exception of 40 cm). Especially in the forest floor and topsoil solution the trend rates were higher than those calculated for throughfall deposition. The NO_3 trends in mineral soil solution were characterized by elevated concentrations in 2006 and 2007 followed by a drastic decline in the subsequent years (Figure 6). The concentrations measured in the topsoil (30 cm depth) from 1999 to 2001, not included in the trend analysis, were in line with the values recorded in the first years of the analyzed series, with the exception of 2006. In order to interpret this trend we have to consider that NO_3 yearly median concentrations in the mineral soil solution were most influenced by the higher concentrations generally recorded in spring months, just before summer, when NO_3 drastically declined to undetectable values. During the 2006 and 2007 spring seasons, we recorded some throughfall precipitation events characterized by NO_3 values that were among the highest measured in all data series, and which likely generated a NO_3-rich flux percolating through litter floor and mineral soil. Analyzing the weekly NO_3 data in the topsoil solution of the entire period under study (1999–2015), we observed a strong decline of NO_3 concentrations during a variable

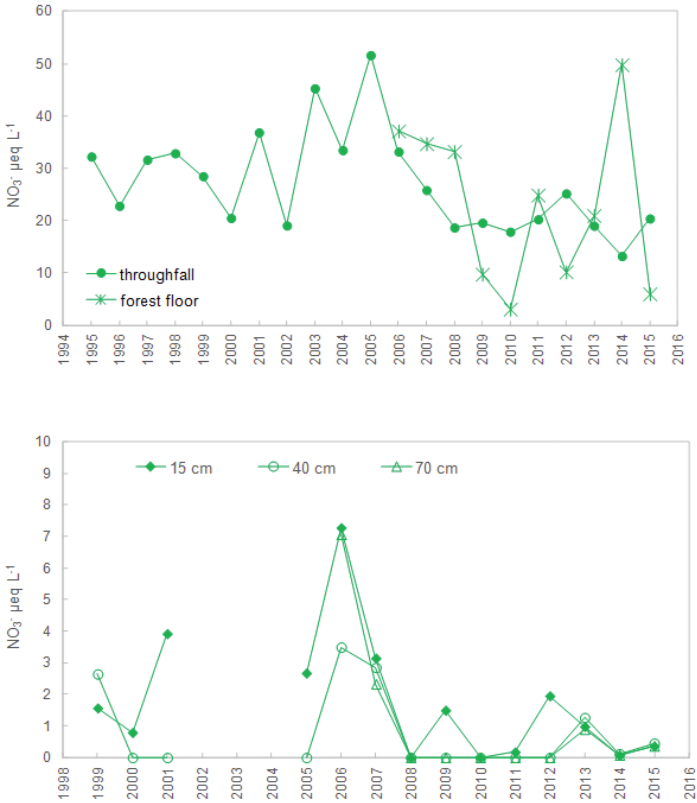

Figure 6. Annual NO_3 concentrations measured in throughfall and forest floor leachates (a) and in the topsoil solution (TS), subsoil solution (SS) and in deep soil solution (DS) (b) during the study period.

interannual period between March and June. In the last three years, NO_3 monthly concentrations never exceeded 2 µeq L^{-1}. These findings suggest that the overall ability of the forest ecosystem to consume NO_3 inputs from throughfall and litter has not changed, from 1999 to 2015, independently of the N loadings. What appears to have increased is the efficiency with which NO_3 is consumed in the topsoil, which is indeed related to the N inputs. We found a declining tendency of the number of individual exceedances of the critical limit (CLimE) per year in the mineral soil solution from 2005 to 2015 (Figure 7). A study carried out from 2006 to 2009 on 244 forest plots belonging to two European monitoring networks (ICP Forest and Swedish Throughfall Monitoring Network) showed a more frequent exceedance of the critical limit in soil solution samples in the forest plots where critical loads of N as nutrient had been exceeded, if compared to other plots in the same network (Waldner et al. 2015).

A currently debated topic is the influence of atmospheric N deposition loads on the DON losses in forest ecosystems. Some authors recorded an increase of DON and/or a decreasing DOC/DON ratio in soil solution when N loading rates were relatively elevated (McDowell et al 2004; Pregitzer et al. 2004, Brookshire et al. 2007). Brookshire

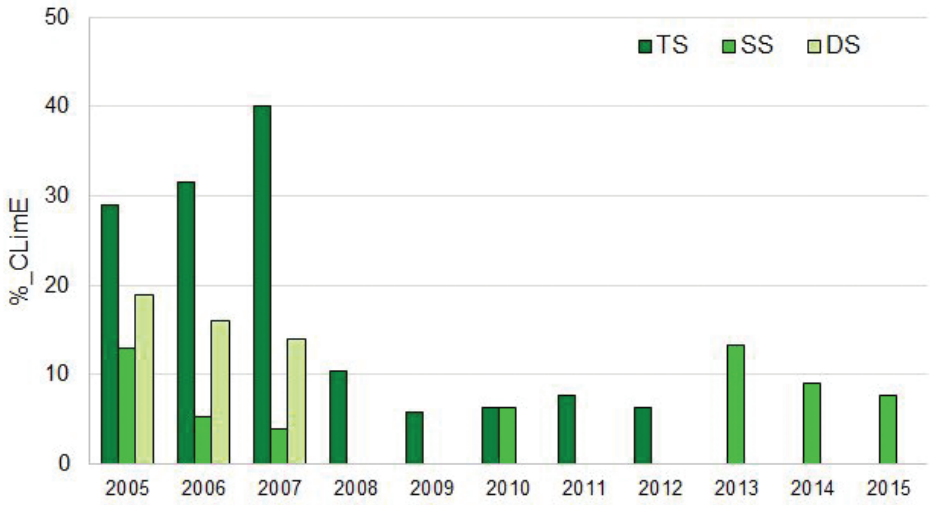

Figure 7. Percentage of individual exceedances of the critical limit (CLimE) per year in the topsoil solution (TS), subsoil solution (SS) and deep soil solution (DS) from 2005 to 2015.

et al. (2007) analyzed patterns in soil and stream C and N in forest watersheds spanning a broad gradient in atmospheric N loading (5–45 kg N ha^{-1} y^{-1}). These authors found that unpolluted forest sites lost DON in fixed proportion with DOC ("Passive Carbon Vehicle" hypothesis), while polluted sites exported disproportionately more DON compared to DOC, creating a shift in DOM stoichiometry ("Stoichiometric Enrichment" hypotheses). Because we observed a declining trend of N atmospheric loading, we could expect a concomitant decrease of DON in soil solution, coupled to that recorded for NO_3 at Val Masino forest. Instead, no trends in both DON and DOC concentration in soil solutions as well as in DOC/DON ratio were observed. Although our data series is relatively short for distinguishing clear trends in DOM (Waldner et al. 2014), these first outcomes suggest that the dynamic of DON was not dependent on the N atmospheric inputs. It is worth noting that a relationship between N atmospheric inputs and DON export was in most cases reported in the frame of long-term experiments of N fertilisation with 30–150 kg N ha^{-1} y^{-1}. As for now, there are very few long-term data on DON concentrations and vertical fluxes in temperate forests that exhibited declining N deposition trends (Verstraeten et al. 2016).

In recent years, the scientific community has been giving greater attention to the effects of reducing atmospheric loads of SO_4 on the increasing DOC concentration in surface waters and soil solution in regions that have previously experienced high loads of sulfate (e.g. Clark et al. 2010; Borken et al. 2011; Verstraeten et al. 2016). The increase in DOC concentrations in surface waters was explained by an increase of DOM solubility due to a lowering of soil solution ionic strength (Monteith et al. 2007) and an enhanced dissociation of organic acids (Clark et al. 2010). In our study area, the significant declining SO_4 trends observed in the litter floor leachates and soil solution were not accompanied by a simultaneous lowering of H^+ concentrations, which was,

conversely, very well recognisable in the throughfall deposition. Furthermore, while the SO_4 trends in the forest floor leachates and throughfall were rather synchronous, the soil solution concentrations steeply declined only until 2011. From that year on, they slightly increased to then show a decrease again during the last year of monitoring. The response of forest soils to the reduction of SO_4 deposition is complex and dependent on many site features (e.g. history of deposition, water fluxes, level of DOC concentrations and soil sulfur stocks). The desorption of inorganic SO_4 and the mineralization of organic sulfur in the soils can lead to a release of SO_4 that decouples the trends of sulfate deposition and sulfate concentrations in soil solution (Borken et al. 2011; Prechtel et al. 2001). In addition to the SO_4 temporal evolution, the fact that there are persistent higher concentrations of SO_4 in soil solution than there are in throughfall, indicates that the recovery process from acidification is not stable yet in the Val Masino forest.

Conclusions

The results of the present study confirm the utility of long-term monitoring of additional ecosystem compartments such as soil solution in providing a deeper knowledge of the fate of N and C in the forest ecosystem. The soil solution is a crucial interface connecting the N atmospheric deposition, the terrestrial processes and the streamwater N export. Particularly, the overland flow originating from the forest floor leachate in Val Masino forest represents an important source of inorganic N, which, in certain hydrological conditions, can directly flow to surface waters by-passing the soil layers where the N retention processes take place. The extremely low NO_3 concentrations measured in mineral soil solution indicated that a condition of N-limitation occurred independently from the range of N loadings recorded in the study period. The decreasing trend of NO_3 in atmospheric deposition observed at our site is in line with observations carried out in other alpine areas. The added value and the uniqueness of this research mainly lies in the evaluation of the response of an alpine forest ecosystem to the reduction of atmospheric pollution, both in temporal and quantitative terms. Contrary to ion species, DOC and DON concentrations do not seem to have been affected by changes in S and N inputs. More in-depth analysis and, possibly, a longer data series will provide a better identification of the most effective factors controlling the dynamic of DOM during the migration through different environmental compartments in the forest ecosystem.

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Supplementary material 1

Analytical methods, correlation tables and graphs showing additional results

Authors: Raffaella Balestrini, Carlo Andrea Delconte, Andrea Buffagni, Alessio Fumagalli, Michele Freppaz, Italo Buzzetti, Enrico Calvo

Data type: statistical data

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Climatic and pedoclimatic factors driving C and N dynamics in soil and surface water in the alpine tundra (NW-Italian Alps)

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Abstract

In alpine tundra the interannual and seasonal variability of C and N forms in soil and lake water during the short snow-free season could be significant and related to climatic and pedoclimatic variables. The hypothesis that not only the climatic and pedoclimatic parameters recorded during the summer season but also the ones measured during the previous snow-covered season could contribute to explaining the C and N dynamics in soil and surface water was tested along 10 snow-free seasons in 3 sites in the alpine tundra in the north-western Italian Alps (LTER site Istituto Mosso). Among the considered parameters, the snow cover duration (SCD) exerted a primary control on soil N-NH_4^+, DOC, C_{micr}, N_{micr} and DOC:DON ratio, with an inverse relationship. A long SCD might cause the consumption of all the subnival substrata by the soil microorganisms, determining a C starvation during the subsequent snow-free season. An opposite trend was observed for the lake water, where a longer SCD corresponded to a higher content of inorganic N forms. Among the pedoclimatic indices, the number of soil freeze/thaw cycles (FTC) recorded during the snow-covered season had a positive relation with most of soil C and N forms and N-NO_3^- in lake

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water. Only the soil DON showed an inverse pattern, and this result is consistent with the hypothesis that FTC released soil DON, subsequently decomposed and mineralized. Only N-NO_3^- had a significant intraseasonal variability, reaching the highest values in September both in soil and water, revealing a significant slowdown of the contribution of soil N immobilization processes.

Keywords
LTER, snow cover duration, soil temperature, freeze/thaw cycles, leaching, N-NO_3^-

Introduction

The alpine tundra is a high-mountain environment located above the tree-line, which occurs across a wide range of latitudes and landscapes with common properties such as: a short growing season, extended periods with air temperature below freezing, and long periods with snow-covered soils (Edwards et al. 2007, Knowles et al. 2015). Since snow controls soil organic matter decomposition and nitrogen (N) release (Walker et al. 1999, Groffman et al. 2001), variations in the depth and duration of snow cover result in large differences in pedoclimatic conditions and nutrient cycling, as well as in plant community composition (Walker et al. 1993, Fisk et al. 1998). Direct control involves the effects of snow cover on winter soil temperature by insulating soil from air temperature and/or on summer soil moisture. Indirect control involves the effects of snow cover variations on growing-season length and soil nutrients dynamics (Edwards et al. 2007).

Several studies performed in other ecotones, such as the boreal forests, demonstrated that the climatic conditions of the preceding winter are important driving factors of the C cycling during the growing season (e.g. Öquist and Laudon 2008, Haei et al. 2013). Consequently, not only the climatic and pedoclimatic conditions recorded during the growing season (e.g. soil temperature and moisture) can strongly affect the soil nutrient cycling, but also those of the previous winter season (e.g. snow cover duration, number of soil freeze/thaw cycles).

Although organic matter decomposition may be slow, the decomposition below the snowpack may still constitute a significant proportion of the total decomposition because the snow-covered season is long (Stark 2007). A thick snow cover usually maintains the soil temperature close to 0 °C throughout the snow-covered season, with significant microbial processes which could be influenced by the occurrence of freeze/thaw cycles, especially during the spring and autumn. The effects of freeze-thaw cycles may strongly depend on the number of cycles, the duration of the freezing period, and the minimum temperature (Grogan et al. 2004, Freppaz et al. 2007). After freeze/thaw events there is a significant flush of microbial respiration, most probably because soil microorganisms that die during the event represent an easily decomposable and nutrient-rich substrate for the surviving microorganisms (Schimel and Clein 1996, Herrmann and Witter 2002). Freeze/thaw events may release nutrients from the soil microbial biomass and/or through aggregates breakdown (Freppaz et al. 2007), being major drivers of nutrient mobilization in tundra systems. Research outcomes from a variety of forest and alpine ecosystems have shown increased mobilization of nitrate

associated with soil freezing, including sites in U.S. (Mitchell et al. 1996, Brooks et al. 1998), Germany (Callesen et al. 2007), and Japan (Christopher et al. 2008).

Snowmelt- and rainfall-driven leachate of nitrate is a key hydrochemical feature in montane catchments and has been studied extensively in order to understand its underlying mechanisms and ecological consequences (Stottlemyer 1992, Campbell et al. 2000, Sickman et al. 2003, Harms and Jones 2012). Soil characteristics and water pathways within soils are decisive for the nutrient transport from terrestrial to aquatic ecosystems. Water can be transported laterally through subsurface soil layers to streams or lakes (Khalili et al. 2010) and a variety of studies have found stream dissolved organic carbon (DOC) to be correlated with soil organic C and N (e.g. Houser et al. 2006) and allochthonous DOC (produced in the catchment and not in the lake itself) to represent the larger fraction of the total DOC in lakes (Sobek et al. 2007). Other studies have described a negative relationship between the export of nitrate and soil C:N ratios (e.g. Lovett et al. 2002).

In subarctic streams and rivers, higher inorganic N concentrations have been measured during the late growing season and fall (Petrone et al. 2006), suggesting a greater soil nutrient leaching during these periods potentially playing an important role in annual nutrient export. Alpine basins should not be considered as "teflon basins" since surface-groundwater interactions are a fundamental component of water quantity and quality even in areas with bedrock or talus deposits (Williams et al. 1997, Campbell et al. 2000).

Although much is known in the alpine tundra about litter decomposition, soil C and N cycling and microbial communities under the snowpack and during the period of snow melting (e.g. Brooks and Williams 1999, Baptist et al. 2010), less is known about the contribution of the pedoclimatic factors recorded during the snow-covered season on the soil N and C forms of the subsequent snow-free season. Our time series of soil N and C forms collected in the alpine tundra during the growing season under naturally changing snow cover characteristics (e.g. snow cover duration, cumulative snowfall), climatic (e.g. liquid precipitation) and pedoclimatic conditions (e.g. soil temperature) can provide information about the influence of these environmental factors on soil processes and water lake characteristics and the interactions between them. Our main goal was to quantify the influence of climate and pedoclimate factors on soil and lake water C and N cycling, examining the contribution of snow-covered conditions on the subsequent growing season. Given the general sensitivity of soil nutrient cycling in this ecosystem we expected that even small changes in these factors could significantly affect the soil C and N dynamics both on an interannual and seasonal basis.

Methods

Study area

The research area (Long Term Ecological Research [LTER] site Angelo Mosso Scientific Institute) is located in NW Italy (Piemonte Region), close to the Monte Rosa

Massif (4634 m a.s.l.), along the border with the Valle d'Aosta Region (Fig. 1). The study was conducted at three high-elevation research sites 1, 3, and 5, at elevations equal to 2840, 2770 and 2525 m a.s.l., respectively. The sites are located in the upper part of a glacial valley. The bedrock mineralogy is primarily micaschists, with some inclusions of ophiolites and calcschists. Soils are classified as Skeletic Dystric Regosol (site 1), Skeletic Umbrisol (Arenic) (site 3), and Skeletic Dystric Cambisol (site 5) (IUSS Working Group WRB, 2015). Soil total organic C (TOC) and N (TN) ranged from 6.5 to 75.0 g kg^{-1} and from 0.5 to 5.1 g kg^{-1}, respectively; soil pH ranged from 4.4 to 5.4 (Freppaz et al. 2010, Magnani et al. 2017a,b). In the years 2009, 2010, 2014, 2016, and 2017, the mean snow density before snowmelt onset was equal to 316 kg m^{-3}, while the N-NH_4^+ and N-NO_3^- stocks in the snowpack ranged between 0.24–0.82 and 0.53–2.92 kg ha^{-1}, respectively.

All the three study sites are ascribable to the 'Siliceous alpine and boreal grasslands' (habitat 6150, according to the EU Habitat Directive), but a large between-site difference in plant species composition was observed according to contrasting extremes of exposure and snow cover duration. Site 1 was a typical snow-bed community belonging to the *Salicion herbaceae* phytosociological alliance. Site 3 was an alpine microthermal *Carex curvula*-dominated grassland, ascribable to the *Caricion curvulae* alliance. Site 5, located at the lowest altitude, was dominated by *Agrostis schraderiana* Bech. (*Festucion variae* alliance).

Among the research sites, site 1 was located within the basin of the alpine lake Cimalegna, as shown in Figure 1. The Cimalegna Lake basin was delineated using a digital terrain model-DTM (cell size: 10×10m) produced by Regione Piemonte. The Cimalegna Lake basin covers an area of approximately 4.4 ha and is characterized by 35% rocks and 65% vegetated soil (Magnani et al. 2017b). The lake area was equal to 2700 m^2 and the max water depth was about 3.4 m. Further physical and chemical features of Cimalegna Lake are reported in Magnani et al. (2017b).

Although the Alpine Permafrost Index Map (APIM) (Boeckli et al. 2012) indicates high probability of permafrost presence in the basin (mean permafrost index > 0.8), the basin does not have permafrost because of the large presence of relatively well evolved soil with vegetation and massive bedrock outcrops, which are generally considered as indicating factors of warm conditions and/or permafrost absence.

Climatic and pedoclimatic measurements

Air temperature, liquid precipitation (during the snow-free season), and snowfall have been continuously recorded since 2005 by an Automatic Weather Station (AWS) located at 2901 m a.s.l. and belonging to the Italian Army (Comando Truppe Alpine – Ufficio Meteomont) (Fig. 1). At each research site, thermistors combined with data loggers (GEOTEST UTL-1, instrument sensitivity: ± 0.1 °C) were placed at a soil depth of 10 cm from fall 2007 until fall 2017 for the measurement of hourly soil temperature.

To assess the climatic conditions in the area, several indices were extracted from the AWS data (listed and described in Table 1): (i) cumulative snowfall (CS); (ii)

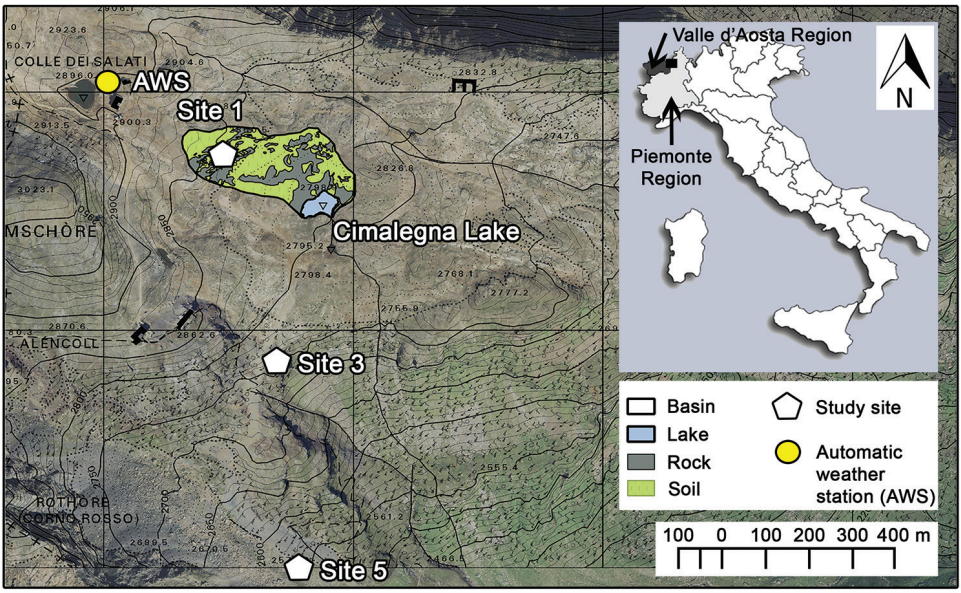

Figure 1. Localization of the study area in Italy, Cimalegna Lake basin and lake, soil sampling sites (1, 3 and 5), and the automatic weather station (AWS). Rock and soil land cover categories refer to the selected basin area of Cimalegna Lake.

heavy precipitation days (HPD); (iii) very heavy precipitation days (VHPD); (iv) consecutive wet days (CWD); (v) consecutive dry days (CDD). Moreover, to assess the site-specific pedoclimatic conditions, further indices were calculated (listed and described in Table 1): (i) snow cover duration (SCD), calculated from 1 October to 30 September (hydrological year) on the basis of the daily soil temperature data. When the daily soil temperature amplitude remained within a range of 1 °C, the day was defined as a "snow-covered day" (Danby and Hik 2007); (ii) melt-out day of snow (MOD); (iii) duration of soil freezing (DSF); (iv) soil freeze/thaw cycles (FTC), considered when the daily mean soil temperature dropped below and rose above 0 °C (Phillips and Newlands 2011); (v) mean soil temperature during the freezing period (MTF); (vi) mean soil temperature during the snow-covered season (MTSC); (vii) mean soil temperature during the snow-free season (MTSF); (viii) intensity of soil freezing (ISF). As suggested by Tierney et al. (2001), ISF was classified as "mild freezing", "mild/hard freezing" or "hard freezing" when the daily mean soil temperature ranged between 0 and -5 °C, -5 and -13 °C, or lower than -13 °C, respectively.

Soil and lake water sampling and analysis

Each soil study site consisted of three plots, each 9 m^2. From 2008 until 2010, once a year at the end of the snow-free season (September), and from to 2011 to 2017, monthly during the snow-free season, three topsoil samples (A horizon, 0–10 cm depth) were

Table 1. Indices used to assess the influence of climatic and pedoclimatic conditions on C and N forms in soil and water (adapted from Tiwari et al. 2018). * Melt-out day of snow (MOD) and snow cover duration (SCD) are considered pedoclimatic indices since they were calculated from soil temperature data.

Index	Term	Definition	Unit
Climatic index			
Cumulative snowfall	CS	Cumulative daily fresh snow calculated for each hydrological year (1 October to 30 September)	cm
Heavy precipitation days	HPD	Number of days, between samplings, when daily liquid precipitation >10 mm. For the first sampling the considered period is between melt-out day and sampling day	days
Very heavy precipitation days	VHPD	Number of days, between samplings, when daily liquid precipitation >20 mm. For the first sampling the considered period is between melt-out day and sampling day	days
Consecutive wet days	CWD	Maximum number of consecutive days, between samplings, when precipitation >1mm. For the first sampling the considered period is between melt-out day and sampling day	days
Consecutive dry days	CDD	Maximum number of consecutive days, between samplings, when precipitation <1mm. For the first sampling the considered period is between melt-out day and sampling day	days
Pedoclimatic index			
Snow cover duration*	SCD	Sum of "snow-covered days" in each hydrological year	days
Melt-out day of snow*	MOD	Date of complete snowmelt (indicated as day of the year - DOY)	DOY
Duration of soil freezing	DSF	Cumulative number of days, from October 1 to the melt-out day, when mean daily soil temperature <0 °C	days
Soil freeze/thaw cycles	FTC	Number of soil freeze/thaw cycles in each hydrological year	number
Mean soil temperature during soil freezing	MTF	Mean daily soil temperature when the soil is frozen, from October 1 to the melt-out day	°C
Mean soil temperature during the snow-covered season	MTSC	Mean daily soil temperature when the soil is snow-covered	°C
Mean soil temperature during the snow-free season	MTSF	Mean daily soil temperature between samplings. For the first sampling the considered period is between melt-out day and sampling day	°C
Intensity of soil freezing	ISF	Minimum soil temperature when soil is frozen	°C

collected, which in turn consisted of three subsamples in each plot. Soil samples were homogenized by sieving at 2 mm within 24 h of collection. At each sampling time, subsamples were dried at 100 °C overnight in order to obtain the gravimetric water content. An aliquot of 20 g of fresh soil was extracted with 100 mL K_2SO_4 0.5 M, whereas a 10 g aliquot was subjected to chloroform fumigation for 18 h before extraction with 50 mL K_2SO_4 0.5 M. Dissolved organic carbon (DOC) was determined with 0.45 µm membrane, which filtered K_2SO_4 extracts (extractable DOC) with a TOC analyzer (Elementar, Vario TOC, Hanau, Germany). Microbial carbon (Cmicr) was calculated from the difference in DOC between fumigated and non-fumigated samples corrected by a recovery factor of 0.45 (Brookes et al. 1985). Ammonium (extractable N-NH_4^+) concentrations in soil extracts were determined spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) by a modified Berthelot method involving reaction with salicylate in the presence of alkaline sodium dichloroisocyanurate (Crooke and Simpson 1971). Nitrate (extractable N-NO_3^-) concentrations in soil extracts were de-

termined spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) by the Greiss reaction as described by Mulvaney (1996) and modified by Cucu et al. (2014). Total dissolved nitrogen (TDN) in the extracts was determined as reported for DOC. Dissolved organic nitrogen (extractable DON) was determined as the difference between TDN and inorganic nitrogen ($N\text{-}NH_4^+ + N\text{-}NO_3^-$) in the extracts. Microbial nitrogen (Nmicr) was calculated from the difference in TDN between fumigated and non-fumigated samples corrected by a recovery factor of 0.54 (Brookes et al. 1985). Total nitrogen and total carbon were determined by elemental analysis (Carlo-Erba, Milano, Italy). A total of 267 soil samples were analyzed in the time-span 2008–2017.

Lake water was sampled approximately at the same time that soil was sampled, with a total of 79 samples analyzed in the time-span 2008–2017. The lake was sampled from 3 points on the shore with no vegetation, at ca. 10-cm depth. Shore samples were assumed not to be significantly different from mid-lake samples because the investigated lake was small and shallow (Mast et al. 2011). The analyses were performed in the laboratory on filtered samples (0.45 μm) within 48 h from the sampling. Ammonium ($N\text{-}NH_4^+$) concentrations in lakes were determined spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) by a modified Berthelot method involving reaction with salicylate in the presence of alkaline sodium dichloroisocyanurate (Crooke and Simpson 1971). Nitrate ($N\text{-}NO_3^-$) concentrations in the water samples were determined spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) by the Greiss reaction as described by Mulvaney (1996) and modified by Cucu et al. (2014). Since 2011, DOC was determined after filtering the water samples with a 0.45 μm membrane by a TOC analyzer (Elementar, Vario TOC, Hanau, Germany). Total dissolved nitrogen (TDN) was determined as reported for DOC. Dissolved organic nitrogen (DON) was determined as the difference between TDN and inorganic nitrogen ($N\text{-}NH_4^+ + N\text{-}NO_3^-$).

Statistical analyses

In order to assess the interannual variability in C and N forms in soils and lake water in the time-span 2008–2017, only the samples collected at the end of the snow-free season (September) were considered (during the first 3 years the sampling was carried out only during September), while for the evaluation of the intraseasonal variability, the monthly samples (July, August, September, October) were considered. We tested significant differences among years and months by one-way ANOVA and Bonferroni post hoc test ($p < 0.05$). Data were previously tested for homoscedasticity (Levene's test) and for normality (Kolmogorov–Smirnov test), and transformed when necessary.

In order to describe the variation of climatic and pedoclimatic indices over the 10 studied years, a Principal Component Analysis (PCA) was performed using all the variables described in Table 1 and calculated from the AWS (climatic indices) and from the data loggers in each site (pedoclimate indices). Climatic and pedoclimatic variables recorded during the winter seasons were referred to the measurement periods as follows: e.g.winter 2007–2008 was year 2008, winter 2008–2009 was year 2009, etc.

The influence of climatic and pedoclimatic variables on C and N forms in soil was evaluated by fitting generalized linear models (GLMs). Soil C and N forms were used as dependent variables and a gamma distribution was used because normality (tested with Kolmogorov-Smirnoff test) was not met (Zuur et al. 2009). Climatic and pedoclimatic variables (Tab. 1) were used in the models as explanatory variables. Highly collinear predictors ($r > |0.70|$) were excluded after a Pearson correlation analysis. Explanatory variables were standardized (Z-scores) to allow for analysis of effect size by scrutinizing model parameters (β coefficients). In order to evaluate the influence of climatic, pedoclimatic and soil properties on C and N forms in lake water, a similar GLM procedure was performed, using water properties as dependent variables and adding soil variables (from Site 1) as predictors.

All the statistical analyses were performed using SPSS v.19 (SPSS 2010).

Results

Climatic and site-specific pedoclimatic conditions

The mean annual air temperature measured at the AWS in the time-span 2008–2017 was -2.3 °C, with mean daily values ranging from a minimum of -21.1 °C (16 January 2017) to a maximum of +12.4 °C (24 August 2016) (Fig. 2a). The cumulative liquid precipitation during the snow-free season ranged between 285 mm in 2010 and 374 mm in 2015, with a maximum accumulated daily liquid precipitation of 103.8 mm (10 August 2015). The maximum snow depth was equal to 560 cm (28 April 2009). CS ranged from 605 cm in winter 2008 to 1099 cm in winter 2009 (Tab. 2). HPD ranged from 2 in 2014 to 12 in 2008, while VHPD comprised between 0 in 2014 and 7 in 2015 and 2016. The minimum number of CWD was 3 in 2009, while the maximum was 9 in 2016. CDD ranged between 5 in 2010 and 23 in 2014.

Soil temperature measured at all study sites during the snow-covered season was generally close to 0 °C (Fig. 2b). During the snow-covered season, the daily topsoil temperature ranged between a minimum of -8.3 °C in 2013 at site 3, while during the snow-free season it reached the maximum of 17.2 °C at site 5 in 2016. SCD was comprised between 210 (site 3, 2015) and 286 (site 1, 2014) (Tab. 3). The SCD resulted strongly positively correlated with the MOD ($r=0.88$, $p < 0.001$). Earliest MOD occurred at site 3 and 5 on DOY 139 (2011) while latest MOD occurred at site 1 on DOY 221 (2009). DSF ranged between 0 (site 5, 2015) and 269 (site 1, 2010). The maximum number of FTC (4) was measured at sites 3 and 5 in 2008. Lowest MTF (-1.6 °C) occurred at site 3 in 2013. Lowest MTSC (-1.6 °C) was measured at site 3 in 2012. Lowest MTSF was 4.5 °C at site 1 (2014). Conversely, the highest MTSF was 11.3 °C at site 5 (2009). ISF was always classified as mild freezing, except for 2 events of mild/hard freezing recorded at site 3 in 2013 (-8.3 °C) and in 2016 (-7.4 °C) (cf. Tierney at al. 2001).

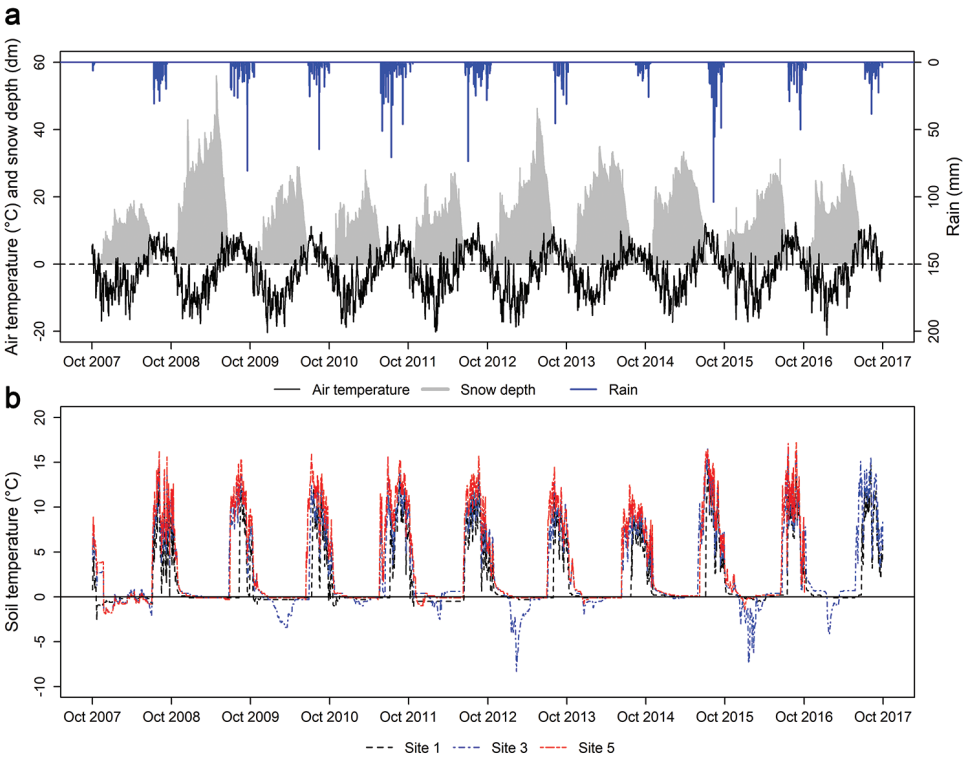

Figure 2. a Air temperature, snow depth and rain recorded at the Automatic Weather Station (AWS) from 1 October 2007 to 30 September 2017 (daily mean values) **b** soil temperature recorded in the topsoil (A horizon – 10 cm depth) at study sites 1, 3, and 5 (daily mean values).

Table 2. Climatic indices derived from the AWS data. CS (cumulative snowfall), HPD (heavy precipitation days), VHPD (very heavy precipitation days), CWD (consecutive wet days), and CDD (consecutive dry days). All indices are listed and described in Table 1.

Index	Unit	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Min	Max	Mean	St.dev
CS	cm	605	1099	879	752	890	947	820	820	756	608	605	1099	817	150
HPD	days	12	5	5	3	8	5	2	5	6	6	2	12.0	5.7	2.8
VHPD	days	4	2	2	4	4	3	0	7	7	3	0	7	4	2
CWD	days	7	3	6	4	4	7	5	7	9	6	3	9	6	2
CDD	days	18	9	5	9	6	5	23	12	9	16	5	23	11	6

The PCA revealed the distribution of the years in three different groups (Fig. 3). The year 2008 was characterized by a high number of FTC and very low MTF, ISF and MTSC. The years 2011, 2012, 2015 and 2017 were characterized by the earliest MOD and the shortest SCD, while the years 2009, 2010, 2013, 2014 and 2016 were characterized by the longest SCD.

Table 3. Site-specific pedoclimate indices measured at sites 1, 3, and 5 between 2008 and 2017. SCD (snow cover duration*), MOD (melt-out day of snow*), DSF (duration of soil freezing), FTC (soil freeze/thaw cycles), MTF (mean soil temperature during soil freezing), MTSC (mean soil temperature during the snow-covered season), MTSF (mean soil temperature during the snow-free season), and ISF (intensity of soil freezing). All indices are listed and described in Table 1. * SCD and MOD are considered a pedoclimatic index since they were calculated on the basis of the soil temperature data.

Index	Unit	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Min	Max	Mean	St.dev
Site 1															
SCD	days	249	272	271	255	241	284	286	250	283	262	241	286	265	16
MOD	DOY	181	221	192	187	163	211	205	186	193	175	163	221	191	17
DSF	days	205	246	269	208	235	217	223	15	102	124	15	269	184	79
FTC	number	3	1	3	1	2	1	1	1	1	1	1	3	2	1
MTF	°C	-0.3	-0.1	-0.1	-0.4	-0.5	-0.1	0.0	0.0	-0.3	-0.1	-0.5	0.0	-0.2	0.2
MTSC	°C	-0.3	0.0	-0.3	-0.2	-0.5	-0.1	0.0	0.1	0.0	0.1	-0.5	0.1	-0.1	0.2
MTSF	°C	5.8	8.5	7.6	7.2	6.8	5.1	4.5	9.0	7.7	6.4	4.5	9.0	6.9	1.4
ISF	°C	-2.6	-0.1	-0.8	-0.3	-1.1	-0.3	-0.1	0.0	-0.5	-0.1	-2.6	0.0	-0.6	0.8
Site 3															
SCD	days	222	246	222	219	223	247	231	210	274	228	210	274	232	19
MOD	DOY	182	174	179	139	162	184	162	148	188	146	139	188	166	18
DSF	days	167	125	211	154	58	184	125	59	122	57	57	211	126	55
FTC	number	4	1	2	2	2	1	1	1	1	1	1	4	2	1
MTF	°C	-1.0	-0.1	-1.1	-0.3	-0.9	-1.6	-0.5	-0.1	-0.9	-1.6	-1.6	-0.1	-0.8	0.6
MTSC	°C	-0.1	0.1	-0.8	-0.1	0.1	-1.1	-0.3	0.2	-0.6	0.2	-1.1	0.2	-0.3	0.4
MTSF	°C	8.1	10.0	9.1	8.5	8.3	6.6	7.2	10.0	6.5	9.1	6.5	10.0	8.3	1.3
ISF	°C	-2.1	-0.1	-3.4	-1.0	-2.5	-8.3	-1.9	-0.2	-7.4	-4.1	-8.3	-0.1	-3.1	2.8
Site 5															
SCD	days	219	244	235	219	216	253	235	222	248		216	253	232	14
MOD	DOY	183	178	164	139	160	182	161	157	165		139	183	165	14
DSF	days	190	156	176	147	163	160	181	0	68		0	190	138	63
FTC	number	4	1	1	1	1	1	1	0	1		0	4.0	1.2	1.1
MTF	°C	-0.7	-0.1	-0.1	-0.1	-0.5	-0.1	-0.1		-0.3		-0.7	-0.1	-0.2	0.2
MTSC	°C	-0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.3	0.2		-0.1	0.3	0.1	0.1
MTSF	°C	9.2	11.3	10.8	9.3	9.7	7.8	8.4	10.8	6.5		6.5	11.3	9.3	1.6
ISF	°C	-1.9	-0.3	-0.3	-0.3	-0.1	-0.1	-0.8		-1.4		-1.9	-0.1	-0.7	0.7

Influence of climatic and pedoclimatic variables on C and N forms in soil

On the interannual basis, the N-NO_3^- concentration was significantly higher in years 2008, 2009 and 2010, while the lowest values were recorded in years 2014, 2016 and 2017 (Fig. 4a). Conversely, the mean soil N-NH_4^+ concentration was rather constant among the years, with the exception of year 2010 (Tab. 4). The lowest soil extractable DON concentration was recorded between 2008 and 2011, while the highest value was measured in 2013. The DOC concentration was rather stable through years, with the exception of 2010. The highest DOC:DON ratio was measured in the years 2008, 2009, 2010 and 2011. The lowest microbial C concentration was recorded in the years 2013, 2014, 2015, 2016 and 2017, while the maximum was recorded in 2012. The highest microbial N concentration was measured in 2008. The highest C: Nmicr was measured

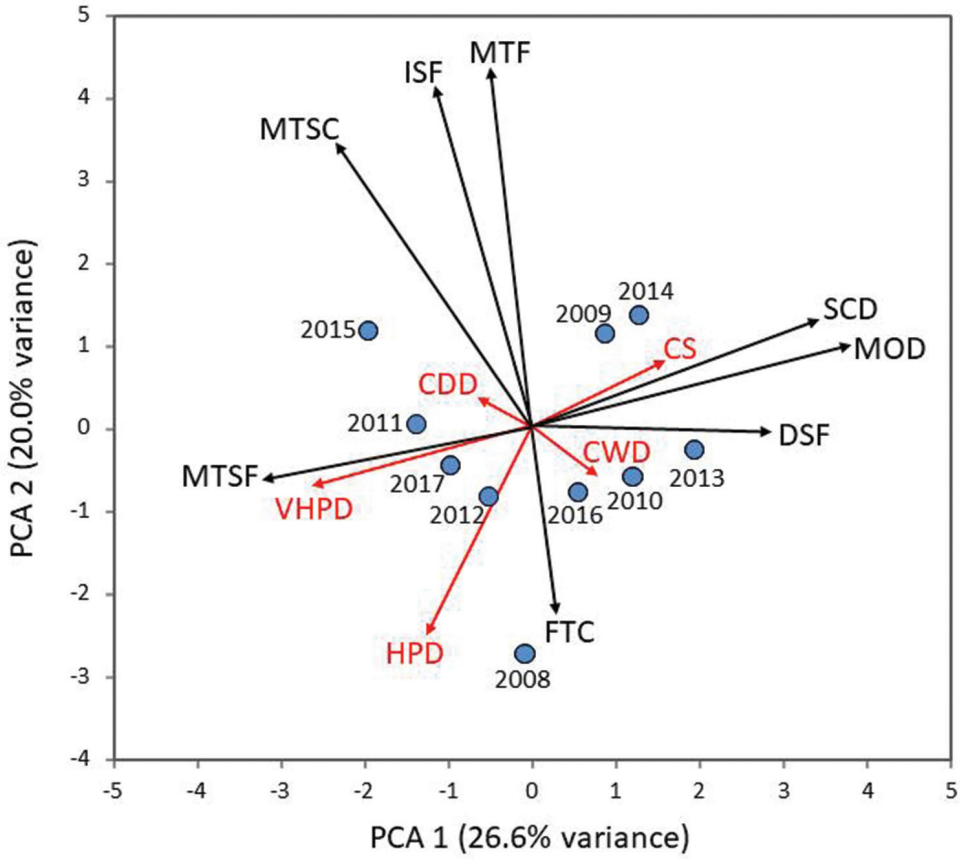

Figure 3. Principal Component Analysis showing the variation of climatic (red arrows) and pedoclimatic (black arrows) indices over the 10 studied years. Blue points represent the average coordinates of different sampling date and replicates within the same year. Descriptions for each climate and pedoclimate index can be found in Table 1.

in years 2011 and 2012, while the lowest was in 2014. On a seasonal basis, N-NO_3^- concentration significantly increased in September (Fig. 4b), while there were not significant differences between months in all the C and N forms considered in the study (Tab. 4).

All soil C and N forms were positively correlated with CS, with the exception of the microbial C:N ratio that was inversely correlated (Tab. 5). HPD was positively correlated with N-NO_3^- concentration, while VHPD was inversely correlated with DON. CWD was inversely correlated with N-NH_4^+, Cmicr and Nmicr while CDD was inversely correlated with the microbial C:N ratio (Tab. 5). Considering the site-specific indices, SCD was the main driving factor for the concentration of N-NH_4^+, DOC, Cmicr and Nmicr, which were all inversely correlated with SCD, as well as N-NO_3^-, TDN and Cmicr:Nmicr. DSF was positively correlated with N-NH_4^+, Cmicr and the Cmicr:Nmicr,

Table 4. Mean interannual and seasonal concentrations of N-NH_4^+, N-NO_3^-, DOC, TDN, Cmicr, Nmicr, DON (mg kg^{-1}), and values of C:Nmicr and DOC:DON at the 3 study sites in years 2008–2017. Letters represent significant differences between years and months ($p < 0.05$), letters are not reported when differences are not significant ($p > 0.05$).

Parameter	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Min	Max	Mean	St.dev
Interannual														
N-NH_4^+	9.9 (B)	9.7 (B)	21.2 (A)	6.9 (B)	6.9 (B)	5.4 (B)	4.5 (B)	5.1 (B)	2.2 (B)	4.4 (B)	2.2	21.2	7.6	5.3
N-NO_3^-	2.6 (B)	7.3 (A)	5.9 (A)	0.4 (BC)	1.0 (BC)	0.9 (BC)	0.2 (C)	1.2 (B)	0.2 (C)	0.2 (C)	0.2	7.3	2.0	2.5
DOC	324.2 (B)	308.2 (B)	576.5 (A)	251.7 (B)	335.7 (B)	302.6 (B)	212.0 (B)	365.6 (AB)	164.9 (B)	180.7 (B)	164.9	576.5	302.2	117.9
TDN	30.8 (AB)	32.7 (AB)	24.0 (B)	8.2 (C)	32.5 (AB)	61.9 (A)	39.7 (AB)	44.5 (AB)	34.8 (AB)	36.4 (AB)	8.2	61.9	34.5	13.8
Cmicr	2178.4 (B)	1150.4 (BC)	1769.1 (BC)	2106.5 (AB)	3029.8 (A)	872.3 (C)	1001.1 (C)	944.7 (C)	729.2 (C)	760.1 (C)	729.2	3029.8	1454.2	777.1
Nmicr	228.4 (A)	110.6 (AB)	102.7 (B)	67.3 (B)	98.5 (B)	64.4 (B)	135.7 (AB)	78.2 (B)	44.6 (B)	35.4 (B)	35.4	228.4	96.6	55.6
DON	18.3 (B)	16.1 (B)	6.7 (B)	4.4 (B)	32.4 (AB)	55.5 (A)	35.0 (AB)	38.8 (AB)	32.3 (AB)	31.7 (AB)	4.4	55.5	27.1	15.7
C:Nmicr	12.2 (BC)	11.0 (C)	19.4 (BC)	55.5 (B)	42.9 (A)	25.2 (BC)	8.1 (C)	12.2 (BC)	16.7 (BC)	21.1 (BC)	8.1	55.5	22.4	15.3
DOC:DON	17.6 (B)	24.5 (A)	41.5 (A)	33.7 (A)	11.0 (BC)	5.2 (C)	6.5 (BC)	11.7 (BC)	5.5 (C)	7.7 (BC)	5.2	41.5	16.5	12.8

	Jul	Aug	Sep	Oct	Min	Max	Mean	St.dev
Seasonal								
N-NH_4^+	6.0	6.8	7.8	6.2	6.0	7.8	6.7	0.8
N-NO_3^-	0.6 (A)	0.6 (A)	1.9 (B)	1.0 (A)	0.6	1.9	1.0	0.7
DOC	299.9	271.6	303.7	258.2	258.2	303.7	283.4	22.0
TDN	30.8	32.7	24.0	8.2	8.2	32.7	23.9	11.1
Cmicr	1476.9	1066.6	1482.7	1276.7	1066.6	1482.7	1325.7	197.5
Nmicr	104.3	107.7	100.8	90.5	90.5	107.7	100.8	7.4
DON	30.6	39.5	29.2	31.3	29.2	39.5	32.7	4.6
C:Nmicr	17.4	21.5	21.7	29.9	17.4	29.9	22.6	5.3
DOC:DON	17.7	11.0	12.5	12.7	11.0	17.7	13.5	2.9

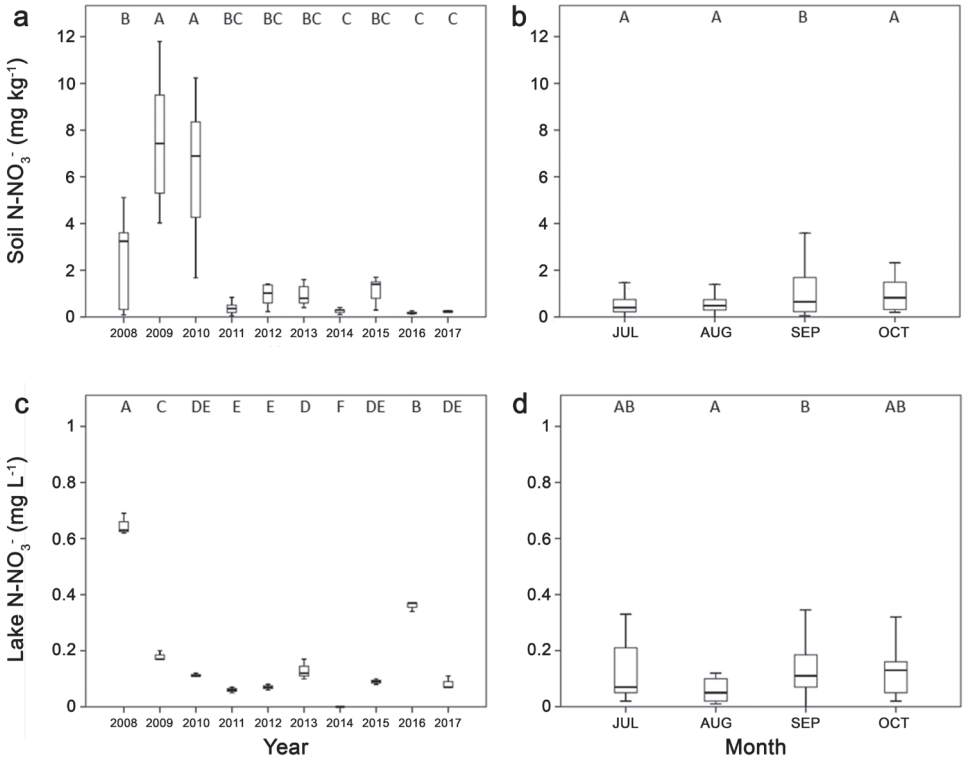

Figure 4. a Mean extractable soil nitrate ($N\text{-}NO_3^-$) concentrations (mg kg^{-1}) recorded in September at the three study sites in the time-span 2008–2017 (n = 87, in 2017 the site 5 was not sampled) **b** mean seasonal concentration $N\text{-}NO_3^-$ (mg kg^{-1}) in the 3 study sites in years 2008–2017 **c** mean water nitrate ($N\text{-}NO_3^-$) (mg L^{-1}) concentration recorded in September at the Cimalegna Lake during the years 2008–2017 (n = 30) **d** Mean seasonal concentration (mg L^{-1}) of $N\text{-}NO_3^-$ at the Cimalegna Lake in years 2008–2017 (n = 30). Upper-case letters represent significant differences between years and months ($p < 0.05$).

while it was negatively correlated with DON. In the latter case this parameter was the most influential among the considered variables. Also FTC had a significant influence on $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, DOC, Cmicr, Nmicr and DON. All these C and N forms were positively correlated with the exception of DON. MTF was negatively correlated with TDN, DON and the Cmicr:Nmicr ratio, while it was positively correlated with Nmicr. MTSC was positively correlated only with the Cmicr:Nmicr ratio (Tab. 5).

Influence of climatic, pedoclimatic and soil properties on C and N forms in lake water

On an interannual basis, the maximum $N\text{-}NO_3^-$ concentration in water was recorded in 2008, while the lowest was measured in 2011 and 2012 (Fig. 4c). The highest $N\text{-}NH_4^+$ values were recorded in 2008 and 2010 (Tab. 6). The highest DOC and

Table 5. Results of generalized linear models (GLMs) showing the effects of climatic and pedoclimatic variables on soil C and N forms. Explanatory variables were standardized (Z-scores) to allow for analysis of effect size by scrutinizing model parameters (β coefficients). *p*-values are also shown.

Predictor	N-NH_4^+		N-NO_3^-		DOC		TDN		Cmicr		Nmicr		DON		C:Nmicr		DOC:DON	
	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Climatic index																		
CS	**0.19**	**0.000**	**0.43**	**0.000**	**0.25**	**0.000**	**0.33**	**0.000**	**0.11**	**0.004**	**0.26**	**0.000**	**0.31**	**0.000**	**-0.13**	**0.014**	**-0.25**	**0.001**
HPD	-0.02	0.772	**0.37**	**0.000**	-0.02	0.575	**0.13**	**0.029**	0.08	0.134	0.11	0.155	0.14	0.083	0.04	0.566	**-0.35**	**0.002**
VHPD	0.07	0.225	0.10	0.340	0.06	0.141	**-0.14**	**0.019**	0.10	0.077	-0.06	0.477	**-0.18**	**0.018**	0.08	0.306	-0.02	0.835
CWD	**-0.14**	**0.018**	-0.20	0.053	-0.05	0.226	-0.02	0.783	**-0.13**	**0.037**	**-0.21**	**0.016**	-0.03	0.722	0.13	0.159	-0.05	0.711
CDD	0.03	0.473	-0.04	0.516	0.01	0.621	0.01	0.106	-0.07	0.100	0.08	0.111	0.04	0.486	**-0.22**	**0.000**	**-0.32**	**0.000**
Pedoclimatic index																		
SCD	**-0.38**	**0.000**	**-0.32**	**0.000**	**-0.39**	**0.000**	**-0.09**	**0.042**	**-0.45**	**0.000**	**-0.33**	**0.000**	-0.06	0.307	**-0.21**	**0.001**	**-0.62**	**0.000**
DSF	**0.14**	**0.010**	0.00	0.646	0.02	0.656	**-0.28**	**0.000**	**0.15**	**0.009**	-0.13	0.117	**-0.31**	**0.000**	**0.52**	**0.000**	**0.45**	**0.000**
FTC	**0.13**	**0.015**	**0.20**	**0.014**	**0.12**	**0.001**	-0.07	0.164	**0.12**	**0.007**	**0.23**	**0.002**	**-0.17**	**0.011**	-0.09	0.238	0.05	0.614
MTF	-0.02	0.685	0.06	0.481	-0.03	0.481	**-0.21**	**0.001**	-0.04	0.523	**0.18**	**0.032**	**-0.22**	**0.004**	**-0.31**	**0.000**	**0.37**	**0.002**
MTSC	0.00	0.991	-0.16	0.082	-0.03	0.509	0.04	0.484	0.01	0.210	-0.13	0.116	0.00	0.693	**0.33**	**0.000**	0.09	0.445
MTSF	0.05	0.277	-0.07	0.351	-0.01	0.753	-0.05	0.285	0.02	0.620	-0.03	0.606	-0.01	0.902	0.00	0.968	-0.15	0.177

Table 6. Mean interannual and seasonal concentrations of N-NH_4^+, N-NO_3^-, DOC, TDN, DON (mg L^{-1}), and values of DOC:DON at the Cimalegna Lake. Letters represent significant differences between years and months ($p < 0.05$), letters are not reported when differences are not significant ($p > 0.05$).

Parameter	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Min	Max	Mean	St.dev
Interannual														
N-NH_4^+	0.2 (B)	0.0 (C)	0.3 (A)	0.0 (C)	0.0 (C)	0.0 (C)	0.0 (C)	0.0 (C)	0.0 (C)	0.0 (C)	0.0	0.3	0.1	0.1
N-NO_3^-	0.6 (A)	0.2 (C)	0.1 (DE)	0.1 (E)	0.1 (E)	0.1 (D)	0.0 (F)	0.1 (DE)	0.4 (B)	0.1 (DE)	0.0	0.6	0.2	0.2
DOC				2.5 (A)	1.6 (B)	0.7 (C)	1.2 (BC)	1.5 (B)	1.6 (B)	1.1 (BC)	0.7	2.5	1.5	0.6
TDN				0.7 (A)	0.4 (B)	0.2 (CD)	0.2 (CD)	0.3 (C)	0.5 (B)	0.2 (D)	0.2	0.7	0.4	0.2
DON				0.6 (A)	0.3 (B)	0.1 (CD)	0.2 (C)	0.3 (B)	0.1 (D)	0.1 (D)	0.1	0.6	0.2	0.2
DOC:DON				4.3 (B)	5.6 (B)	6.8 (B)	6.0 (B)	6.1 (B)	19.7 (A)	18.3 (A)	4.3	19.7	9.5	6.5

	Jul	Aug	Sep	Oct	Min	Max	Mean	St.dev
Seasonal								
N-NH_4^+	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0
N-NO_3^-	0.1 (AB)	0.1 (A)	0.2 (B)	0.1 (AB)	0.1	0.2	0.1	0.1
DOC	1.7	1.8	1.4	1.5	1.4	1.8	1.6	0.2
TDN	0.5	0.4	0.4	0.4	0.4	0.5	0.4	0.1
DON	0.3	0.3	0.2	0.2	0.2	0.3	0.3	0.1
DOC:DON	6.3	6.6	10.5	6.7	6.3	10.5	7.5	2.0

DON concentrations were recorded in 2011, while the highest DOC:DON ratio was observed in 2016 and 2017. On a seasonal basis, the lowest N-NO_3^- concentration was recorded during August, while the maximum was in September (Fig. 4d). The N-NH_4^+ concentration did not show any significant difference between months, as reported for DOC and DON (Tab. 6).

Among the selected climatic indices, CS was inversely correlated with N-NH_4^+ (Tab. 7). VHPD was inversely correlated with the concentration of N-NH_4^+ and DON, while CWD was positively correlated with N-NH_4^+ and DOC. CDD was inversely correlated with N-NO_3^-. Considering the site-specific indices (site 1), SCD was positively correlated with N-NH_4^+, as FTC. DSF was inversely correlated with the DOC:DON ratio, while MTF was inversely correlated with DOC and DON. MTSF was positively correlated with the DOC:DON ratio. Among the soil C and N forms, the concentration of N-NH_4^+ in soil was inversely correlated with the N-NH_4^+ concentration in lake, as TDN and DON, while it was positively correlated with the DOC:DON ratio. The concentration of N-NO_3^- in soil was positively correlated with the N-NO_3^- concentration in lake, while it was inversely correlated with the DOC:DON ratio. The concentration of DOC in soil was positively correlated with N-NH_4^+, DOC, TDN and DON in water. The Cmicr was positively correlated with N-NH_4^+ and the DOC:DON ratio. The soil microbial N was positively correlated

Table 7. Results of generalized linear models (GLMs) showing the effects of climatic, pedoclimatic and soil variables on water C and N forms. Explanatory variables were standardized (Z-scores) to allow for analysis of effect size by scrutinizing model parameters (β coefficients). *p*-values are also shown.

Predictor	N-NH_4^+		N-NO_3^-		DOC		TDN		DON		DOC:DON	
	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Climatic index												
CS	**-0.32**	**0.023**	0.15	0.342	-0.02	0.760	-0.02	0.756	-0.09	0.460	0.07	0.536
HPD	0.07	0.684	0.24	0.201	0.03	0.744	-0.07	0.352	0.12	0.419	-0.11	0.359
VHPD	**-0.72**	**0.000**	0.01	0.950	-0.01	0.831	0.00	0.966	**-0.19**	**0.008**	0.11	0.115
CWD	**0.46**	**0.003**	0.28	0.060	**0.13**	**0.038**	0.12	0.061	0.09	0.434	-0.01	0.883
CDD	-0.19	0.161	**-0.38**	**0.000**	0.08	0.168	-0.01	0.924	0.07	0.466	-0.11	0.173
Pedoclimatic index												
SCD	**1.07**	**0.000**	**0.59**	**0.029**	-0.06	0.689	-0.02	0.922	-0.25	0.379	0.35	0.160
DSF	-0.24	0.120	-0.37	0.106	-0.02	0.878	0.11	0.307	0.26	0.154	**-0.38**	**0.029**
FTC	0.27	0.372	**0.59**	**0.046**	-0.25	0.313	**-0.54**	**0.028**	-0.69	0.134	0.31	0.447
MTF	0.21	0.156	**-0.38**	**0.023**	**-0.21**	**0.022**	**-0.51**	**0.000**	**-0.44**	**0.004**	0.13	0.359
MTSF	0.12	0.424	-0.24	0.071	0.03	0.675	-0.06	0.392	-0.15	0.193	**0.30**	**0.003**
Soil parameter												
S_N-NH_4^+	0.16	0.404	**0.47**	**0.023**	-0.12	0.228	-0.06	0.528	-0.19	0.211	0.23	0.124
S_N-NO_3^-	**-0.25**	**0.047**	**0.43**	**0.000**	-0.13	0.210	0.04	0.712	0.21	0.223	**-0.48**	**0.001**
S_DOC	**0.49**	**0.006**	0.25	0.078	**0.17**	**0.007**	**0.22**	**0.001**	**0.27**	**0.010**	-0.12	0.229
S_Cmicr	**0.43**	**0.009**	0.28	0.253	0.13	0.288	-0.02	0.886	-0.35	0.111	**0.64**	**0.001**
S_Nmicr	-0.38	0.082	**-0.49**	**0.046**	0.04	0.727	0.00	0.984	**0.43**	**0.041**	**-0.60**	**0.001**
S_DON	-0.24	0.075	-0.15	0.356	**-0.17**	**0.026**	-0.09	0.232	-0.08	0.560	-0.17	0.129
S_C:Nmicr	0.00	0.982	**-0.30**	**0.021**	0.00	0.966	-0.09	0.158	0.18	0.116	-0.23	0.049
S_DOC:DON	-0.26	0.053	**-0.42**	**0.009**	**0.08**	**0.299**	-0.05	0.478	0.03	0.831	-0.08	0.477

with DON, while it was inversely correlated with the DOC:DON ratio. The soil DON was inversely correlated with DOC in water, while the soil Cmicr:Nmicr ratio was inversely correlated with N-NO_3^- and DOC:DON ratio in soil.

Discussion

Influence of climatic and pedoclimatic variables on C and N forms in soil

Along the study period, the climate and pedoclimate conditions showed a great interannual variability, with some extreme meteorological events. For example, in 2008, the little and delayed snowpack accumulation and consequently the low insulation exerted by the snowpack caused a high number of soil FTC in all sites. In high-elevation ecosystems, the most frequent periods for FTC are spring and fall, when the soil cannot be covered by a consistent snowpack. Sometimes they can also occur throughout the winter, due to little snowpack accumulation and/or the wind action that causes a snow removal exposing the soil to cold air temperature (Hiemstra et al. 2002), as observed in winters 2013 and 2016, when soil mild/hard-freezing was recorded in site 3. FTC could damage the biological tissue of the microorganisms, resulting in the death of the soil microbial biomass, with the release of nutrients that are potentially immobilized by the surviving microorganisms (Brooks et al. 1995, Larsen et al. 2002). FTC may also have a disruptive effect on soil aggregation due to ice formation, which can result in "fresh" reactive surfaces becoming exposed, causing an increase in nutrient availability (Freppaz et al. 2007).

In our research sites, the resulting FTC number recorded during the snow-covered season significantly and positively correlated with the concentrations of most of the soil C and N forms in the subsequent growing season (cf. Haei et al. 2010). The only exception was DON, which was inversely correlated to FTC. FTC can damage or kill microbes, returning microbial protein and cell walls to the organic matter pool, while releasing amminoacids and other organic monomers into the DON pool (Schimel and Bennett 2004). This pool could be mineralized during the subsequent growing season, with a significant release of inorganic N forms (Grogan et al. 2004). As reported by Haei et al. (2010), in our study the increase in soil DOC pool was related to the number of FTC, revealing how changes in the insulation properties of the snow cover (e.g. little and delayed snowpack accumulation in late fall) may significantly affect the soil thermal status and consequently the biogeochemical processes during the winter, with consequence for DOC formation and export. As reported by Fuss et al. (2016), our results are consistent with soil frost causing a physical disruption of the soil matrix that resulted in the prolonged release of DOC for several months after the melting of snow rather than as a single pronounced pulse. The higher the number of FTC, the higher also the release of soil nitrate. This pattern is not consistent with the hypothesis that when soil freezing mobilizes DOC, the increased DOC availability can enhance microbial immobilization of NO_3^- and reduces losses (e.g. Groffman et al. 2001, Fuss et al. 2016).

The duration and intensity of soil freezing during the snow-covered season was positively related to an increase in the soil and microbial C:N ratio, suggesting the prevalence of fungi, characterized by a higher C:N ratio in comparison to bacteria. Lipson et al. (2002) reported that in an alpine dry grassland fungi are dominant in winter and more adapted to cold temperatures than bacteria, with a shift in structure and function between winter and summer.

In the time-span considered in this study, the interannual variability of the SCD and melt-out day was marked and it was possible to discriminate years with short and long snow-cover duration, which corresponded to early and late melt-out days, respectively. According to the conceptual model of Brooks and Williams (1999), our research area could be included in a transition zone between Zone II and Zone III, where small changes in SCD could have significant effects on the number of soil FTC and on the soil N and C dynamics (Magnani et al. 2017a). In particular, based on the weight of the variables in the GLM models, we showed that SCD had a first order control on the interannual variability of soil N-NH_4^+, N-NO_3^-, DOC, Cmicr, Nmicr and the DOC:DON ratio. Due to a deep snow cover soils seldom froze in the study area during fall, winter and spring (cf. Sickman et al. 2001). The soil temperature generally remained close to 0 °C, favoring the subnival decomposition processes and therefore the gradual consumption of organic substrate due to microorganism respiration (Lipson et al. 2000). A similar effect was reported for forest sites at a lower elevation (Schindlbacher et al. 2014). A number of studies reported a substantial decrease in subnival soil substrate availability from early to late winter (e.g. Zimov et al. 1996, Brooks et al. 2004) when Cmicr limitation increased, as inputs from plant litter were depleted. The winter-adapted microbial community ultimately succumbed to warmer temperatures and C starvation during spring thaw (Lipson et al. 2000).

Among the climatic indices, the cumulative snowfall was positively related to all the soil C and N forms. We assume that a higher cumulative snowfall in the study area corresponded to a higher soil water content and nutrient inputs into the soil during the spring melting of snow, enhancing the microbial activity and the C and N transformations. In the same area, Magnani et al. (2017a) reported a positive correlation between the soil water content and the microbial biomass. Soil moisture is commonly considered one of the main factors regulating the microbial activity during the growing season. In an alpine ecosystem, Lipson et al. (1999) reported the minimum level of soil microorganisms in correspondence to the lowest level of soil moisture during the growing season, underlying the controlling action of the soil moisture on soil microorganisms through a direct osmotic effect or through a diffusive effect on the availability of the substrate.

In contrast to what was found by Magnani et al. (2017a), the mean soil temperature recorded during the snow-free season (~7 °C) was not related to any of the soil C and N forms, and this could be the cause of their reduced seasonal changes. This is consistent with other studies in alpine meadows which found little evidence of impact of the pedoclimatic conditions on the DOC dynamics, in favor of biotic factors (e.g. quality of the ground and belowground biomass) (e.g. Luo et al. 2009).

Influence of climatic and pedoclimatic variables, and soil on C and N forms in water

A number of studies have demonstrated that some physical features of the catchment strongly influence the chemical composition of surface water, and may control ecosystem responses to global perturbations, such as changes in climate (Clow and Sueker 2000, Sickman et al. 2002, Lewis 2002, Kopácek et al. 2005, Helliwell et al. 2007, Balestrini et al. 2013). In particular, Balestrini et al. (2013) reported that in some North America and European high-elevation catchments, the areal extensions of developed soils are inversely related to nitrate concentrations in surface waters. Analogously, Helliwell et al. (2007) indicated the fundamental role that the soil biological community plays in the retention and loss of N and therefore the strict connection between soil and waters in mountain remote ecosystems.

In our study the resultant N-NO_3^- content in lake water positively correlated with the soil inorganic N forms. This concurs with the findings of Magnani et al. (2017b) that investigated the relationships between soil and water C and N forms in the same area but for a shorter time-span (2013–2015). These authors found a significant positive correlation between the nitrate concentration in soil and the nitrate concentration in lake water, revealing how the temporal variation of N-NO_3^- observed in the lake strictly reflected the temporal changes occurring in the soils (Campbell et al. 2002). We did not find any correlation between the rainfall patterns and the N-NO_3^- content in lake water. The reason could be that although in our study area we may expect that large summer storms can have a notable influence on surface water chemistry, in our GLM analysis we considered the sum of the rainfall events between the water samplings. Therefore we could not assess the potential contribution of the closest rainfall episodes to the water sampling time. This is in accordance with the fact that chemical responses to rain events during the summer are generally transient (Clow et al. 2003). A general increase in solute concentrations in lake was generally found during the fall as the relative importance of subsurface soils inputs to lakes increase and uptake of nitrogen by vegetation declines. During the warmer months, biological uptake and denitrification processes in the soils likely prevailed and led to a decrease in N-NO_3^- export. This is in accordance with the inverse relationship that we found between soil microbial N and N-NO_3^- content in water, revealing the importance of soil microbial N immobilization in limiting the leaching processes. During the fall, biological mediated immobilization processes slowed down with the result of N-NO_3^- rise in lake water and soil, a pattern in our study area especially evident in September, and reported also in the western United States mountains (NWT LTER site) where nitrate, dissolved organic carbon and nitrogen flushed from soils to streams (Williams et al. 2015). In our study area we found an increase in N-NO_3^- concentration both in soils and water especially during September, but in lake water also in July, revealing in this case a potential contribution of the snow melting through the ionic pulse phenomenon (e.g. Sickman et al. 2003).

As reported for the soil matrix, the number of FTC had an important control on the N-NO_3^- content in lake water and, as reported by Fitzhugh et al. (2001), it is

possible to hypothesize a strong $N\text{-}NO_3^-$ leaching response to soil frost. The soil temperature experienced during soil freezing events is usually not cold enough to directly kill roots, therefore a physical disruption of the soil matrix (e.g. frost heaving) may contribute to the fine root mortality and consequently to a reduced N uptake by plants (Fuss et al. 2016).

The SCD had a first order control on $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentration in water, but an opposite pattern was observed in comparison to soil C and N forms. A longer SCD caused an increase of both $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations in the lake water, which could be related to the reduction of the microbial nutrient immobilization processes in soil. In our study DOC concentration in water was positively related to the DOC concentration in soil, revealing how allochthonous DOC could represent a large fraction of the total DOC in lakes (Sobek et al. 2007), leached from terrestrial soils. The amount of DOC released from soils is determined by the production of leachable organic carbon in soils and by the water yield. Indeed, the CWD was found to influence the C forms both in soil and water, causing a decrease in the soil microbial biomass and a corresponding increase in DOC in water.

Conclusion

In the LTER site Istituto Mosso the C and N forms analyzed in soil and water for a decade showed a significant interannual variability, while a seasonal change was observed only for $N\text{-}NO_3^-$ both in soil and water lake, with the greatest values recorded in early fall, probably due to the slowdown of biological-mediated processes of N immobilization.

Both the climatic and pedoclimatic conditions recorded during the snow-free and snow-covered season significantly influenced the C and N forms in soil and water. A little and delayed snowpack accumulation caused a high number of soil freeze/thaw cycles, which resulted in a high nitrate content both in soil and water. The longer the snow cover duration, the lower are all the soil C and N forms measured during the subsequent snow-free season, with the exception of DON. An opposite trend was observed for the lake water, where a longer snow-cover duration caused a higher content of inorganic N forms, probably due to a reduction in soil N immobilization potential.

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RESEARCH ARTICLE

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Butterfly distribution along altitudinal gradients: temporal changes over a short time period

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Abstract

Mountain ecosystems are particularly sensitive to changes in climate and land cover, but at the same time, they can offer important refuges for species on the opposite of the more altered lowlands. To explore the potential role of mountain ecosystems in butterfly conservation and to assess the vulnerability of the alpine species, we analyzed the short-term changes (2006–2008 vs. 2012–2013) of butterflies' distribution along altitudinal gradients in the NW Italian Alps. We sampled butterfly communities once a month (62 sampling stations, 3 seasonal replicates per year, from June to August) by semi-quantitative sampling techniques. The monitored gradient ranges from the montane to the alpine belt (600–2700 m a.s.l.) within three protected areas: Gran Paradiso National Park (LTER, Sitecode: LTER_EU_IT_109), Orsiera Rocciavrè Natural Park and Veglia Devero Natural Park. We investigated butterflies' temporal changes in accordance with a hierarchical approach to assess potential relationships between species and community level. As a first step, we characterized each species in terms of habitat requirements, elevational range and temperature preferences and we compared plot occupancy and altitudinal range changes between time periods (2006–2008 vs. 2012–2013). Secondly, we focused on community level, analyzing species richness and community composition temporal changes. The species level analysis highlighted a general increase in mean occupancy level and significant changes at both altitudinal boundaries. Looking at the ecological groups, we observed an increase of generalist and highly mobile species at the expense of the specialist

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and less mobile ones. For the community level, we noticed a significant increase in species richness, in the community temperature index and a tendency towards homogenization within communities. Besides the short time period considered, butterflies species distribution and communities changed considerably. In light of these results, it is fundamental to continue monitoring activities to understand if we are facing transient changes or first signals of an imminent trend.

Keywords

Butterfly, community composition, mountain ecosystem, LTER, protected area

Introduction

Global warming and land use changes are considered among the main threats to biodiversity (Sala et al. 2000, Lemoine et al. 2007). Other driving factors may also interact with climate and land use changes to impact biodiversity (Brook et al. 2008); however, substantial alterations in the population and distribution of terrestrial species have already been detected worldwide, mainly in response to both of these effects (Mantyka-Pringle et al. 2012).

Mountain ranges are very sensitive to environmental changes and global warming (Huber et al. 2005, Beniston 2006). It is well established that the Alps have experienced a temperature increase over the last century (Beniston 2003, Brunetti et al. 2009) especially at higher altitudes (Acquaotta et al. 2014). In addition to global warming, the alpine chain has suffered (and is still suffering) loss of open habitats as a consequence of forest expansion (Hunziker 1995, Gellrich et al. 2007) due to traditional land use practices being abandoned (Hinojosa et al. 2016). Such a loss of open habitats is concomitant with a noticeable loss of species (Pauli et al. 2007), which are under threat because climatic and land use changes will probably continue into the future (Nogués-Bravo et al. 2007, Chamberlain et al. 2013, Pellissier et al. 2013).

The alpine biodiversity has already responded to these factors. Upward shifts of alpine plants (Walther et al. 2005, Pauli et al. 2012, Vittoz et al. 2013), invertebrates (butterflies, Wilson et al. 2007, Wilson and Gutierrez 2012; carabid beetles, Pizzolotto et al. 2014) and mountain birds (Scridel et al. 2018, Rocchia et al. 2018) have been documented. In addition, community composition has changed at high alpine sites (Keller and Körner 2003), with an accelerating increase of species richness, in many cases due to an expansion of generalist species (Walther et al. 2005, Pauli et al. 2007).

Although some responses are evident, few investigations have focused on alpine biodiversity temporal changes. As mentioned above, alpine environments are under threat, therefore more information is needed to understand how the main adverse factors (global warming and loss of open habitats) have affected, or are affecting, alpine biodiversity over time. Exploring temporal patterns of biodiversity is of great significance because future warming and related environmental changes are expected to cause substantial changes, not only in spatial distribution of species, but also in species turnover over time (Korhonen et al. 2010).

Long-term monitoring programs are fundamental tools to assess and monitor temporal changes of biodiversity (Morecroft et al. 2009; Magurran et al. 2010). However, long-term series are rarely available (Gaston et al. 2008). Therefore, it seems useful in the meantime to focus on short time-scales and investigate short-term biodiversity responses, establishing the basis for disentangling in the future if we are facing directional changes or just short-term fluctuations.

Protected areas are a key part of conservation strategies to mitigate and monitor losses of biological diversity due to changes in climate and land use (Kharouba and Kerr 2010). In those areas, the reduced direct anthropic pressure allows for more easily focusing on and detecting the effect of climate modifications, and the natural evolution of vegetation, on biodiversity. Moreover, thanks to their staff and logistic resources, protected areas have a primary role in carrying out long-term monitoring programs, with the aim of acquiring and sharing knowledge through research networks, such as the LTER network.

In 2006, a multi-taxa monitoring project in the NW Italian Alps was started (Biodiversity Monitoring Project), with the goal of analyzing long-term biodiversity changes in space and time along altitudinal transects in three protected areas (Viterbi et al. 2013).

Following the protocol developed and tested by the LTER site Gran Paradiso National Park (GPNP), seven taxa were monitored (Lepidoptera Rhopalocera, Orthoptera, Aves, Coleoptera Carabidae, Coleoptera Staphylinidae, Araneae, Hymenoptera Formicidae), using standardized, easy-to-apply and cheap sampling techniques. In 2007, the protocol was adopted by two other protected areas (Orsiera Rocciavré Natural Park, ORNP and Veglia Devero Natural Park, VDNP). The protocol has been repeated every 5 years (2 yr monitoring – 4 yr stop), and as a result, data from two sampling periods are now available (1st season, 2006–2008; 2nd season, 2012–2013).

In this framework, we focused on butterfly data deriving from the two sampling periods (1st, 2006–2008; 2nd, 2012–2013) of the Biodiversity Monitoring Project. Butterflies are an ideal indicator taxon (Dennis 1993; Hellmann 2002; Parmesan and Yohe 2003), strongly influenced by the abiotic environment and climatically sensitive (Hellmann 2002; Parmesan and Yohe 2003). Their ecological sensitivity and ability to disperse (Devictor et al. 2012) make butterflies a good model system to study species responses to climate and habitat changes (Stefanescu et al. 2011, Roth et al. 2014).

The main aim of our research consisted in assessing changes of butterflies' species distribution and community composition along altitudinal gradients over a short time period. We investigated butterflies' responses across multiple levels of biological organization following a hierarchical approach, in order to assess heterogeneity or consistency of changes among different levels. As a first step, we analyzed temporal changes of distribution for single species and for species classified into ecological groups (species level). Then, we explored temporal variation of species richness and of community composition (community level). We finally discussed the results in the light of the possible drivers of change within our study areas.

Materials and methods

Study areas, sampling design and data collection

Our study was carried out in three protected areas in the NW Italian Alps: Gran Paradiso National Park (GPNP; 720 km^2; 45°31'N, 7°19'E), Orsiera Rocciavrè Natural Park (ORNP; 110 km^2; 45°3'N, 7°19'E) and Veglia Devero Natural Park (VDNP; 86.2 km^2; 46°12'N, 8°14'E). GPNP is part of the Long-Term Ecological Research network (LTER, Sitecode: LTER_EU_IT_109).

All areas are characterized by mountain (alpine) conditions with vegetation ranging from mixed forest to alpine prairies and glaciers. The three parks have similar mean elevations and vegetation characteristics, but differ slightly in terms of climatic regime (the highest monthly precipitation and lowest annual mean temperature are found in VDNP).

A total of 11 altitudinal transects were set (one for each valley for each of the three parks) ranging from a minimum of 600 to a maximum of 2700 m a.s.l. The transects cover different altitudinal ranges and, overall, they involve three vegetation belts (montane, subalpine, alpine; Suppl. material 1: S1). Each altitudinal transect was composed of 4–7 sampling units (plots) separated by an altitude range of ca. 200 m, to allow independence of sampled data, for a total of 62 plots. Sampling units were circular plots with a radius of 100 m, where monitoring activities were carried out to obtain semi-quantitative data.

We sampled butterflies using linear transects along the diameter of the plot (200 m in length), walked along at uniform speed. According to the protocol by Pollard (1977), we recorded all butterflies observed within an imaginary 5×5 m square. Sampling was limited to sunny conditions, under calm-to-light wind. Each plot was visited once a month from June to August. Individuals were captured and released after specific identification, except for specimens that were difficult to identify, which were retained for further determination. Sampling efforts were characterized by 2 consecutive years of sampling and we collected data during two separate time periods: 1st sampling period (2006–2007 in GPNP; 2007–2008 in ORNP and VDNP) and 2nd sampling period (2012–2013 for all the three parks, GPNP, ORNP, VDNP).

In each plot, we also recorded the microclimatic conditions placing data-loggers (Thermochron iButton, DS1922L, Maxim, Sunnyvale, CA, U.S.) which recorded the air temperature every hour throughout the field season. Loggers were located in the center of each plot, at least 1 m above ground and were covered with a white shield.

By using land cover data derived from aerial photos and validated in the field (Agroselviter 2009; Meloni et al. 2009), we classified each plot according to four categories of main habitat cover type: woodland, ecotone (corresponding to transition habitats), grassland (including all grassland types jointly grouped, even if above or below the tree line) and rocks.

Moreover, during each sampling session, we noted, for each plot, land management data (dominant land use) considering three categorical variables: grazing, mowing and no activities.

Statistical analysis

Species level

The first step of our analysis focused on distributional changes of butterflies' species and groups of species. Following ecological requirements, species could change distribution along altitudinal gradient, either vertically or horizontally with the colonization or the loss of sites at similar altitudes.

To obtain a comprehensive framework on butterflies species distribution changes, we firstly assessed temporal changes in plot occupancy to detect an overall signal of expansion or retraction without considering an altitudinal directionality, then, we investigated altitudinal range changes between sampling periods.

Changes in plot occupancy

We defined occupancy as the number of plots occupied by each species in each sampling session (1st vs 2nd) and compared it by using a t-test for paired samples (significance level assessed after 999 randomizations, following Legendre and Legendre 2012). The randomization process allowed to perform the t-test even with non-parametric data (Legendre and Legendre 2012).

To identify which group of species changed the most over time, we analyzed if the occupancy increased/decreased equally between functional groups. We compared the changes in the number of plots per species (Δ plot, 2nd sampling session *minus* 1st sampling session), between the ecological groups of conservation interest by using non-parametric tests (Kruskal-Wallis or Mann-Whitney tests, depending on the number of ecological categories). Following the classification proposed by Balletto and Kudrna (1985) and Balletto et al. (2015), we classified butterflies' species according to: feeding specialization (from polyphagous to monophagous); altitudinal range (generalist, medium altitude, specialized); high altitude species (species typically found only in the subalpine and alpine vegetation belts); light ("shade loving", "sun loving"); dispersal capacity; habitat preferences (woodland, ecotone, open areas, screes) (Suppl. material 1: S2). We selected the ecological groups according to their potential responses in the light of possible drivers of change. In particular, feeding specialization and habitat preferences groups could highlight responses in relation to micro habitat changes while altitudinal range and high altitude species groups could have changed the occupancy status because of temperature increase. Moreover, we considered dispersal capacity because we hypothesized that species with high vagility can colonize easily new areas following both drivers of change.

To assess if individual species changed their occupancy of plots over time (if the frequency of presence/absence was statistically different between the two sampling periods), we applied the McNemar's test for paired nominal data in a 2×2 table (Sokal and Rohlf 1995). We organized data about gains and losses in plots according to the four following combinations: plots with species presence (*no change*) or species absence in

both sampling periods (*no change*); plots only colonized during the first sampling season (*loss*) or only during the second one (*gains*). We tested the 133 species common to both sampling periods, and we considered the species with p-values still significant after Bonferroni correction as "winners" or "losers", depending on the direction of changes.

Altitudinal range changes

We described the altitudinal range of each species with the following parameters:

- altitudinal optimum (mean and median value);
- higher limit (absolute maximum, 90th percentile);
- lower limit (absolute minimum, 10th percentile).

To quantify the amount of change, we compared these parameters between sampling periods using the t-test for paired samples. To compare non-parametric data we assessed significance level after 999 randomizations (Legendre and Legendre 2012). As in the case of occupancy rates, we also compared the changes in altitudinal range among ecological groups.

To test for species responses over time along the altitudinal range, we used the indicator species analysis (IndVal method) proposed by Dufrêne and Legendre (1997). The IndVal method combines the "specificity" of a species (its uniqueness in a specific group of sites) and its "fidelity" (its frequency within the same group). For each species, IndVal can range from 0 (no indication) to 1 (maximum indication). Statistical significances of IndVal are tested by means of a Monte Carlo test, based on 999 randomizations and were performed using the function *multipatt* of the '*indicspecies*' package (De Cáceres and Legendre 2009).

For this purpose, a species matrix (150 species) was created using the average abundance data between years for each plot and sampling period. A logarithmic transformation was applied to reduce the influence of extreme abundance values (Legendre and Legendre 2012).

We grouped together sampling sites into four altitudinal bands (1: 550–1200 m; 2: 1250–1650 m; 3: 1700–2150 m; 4: 2200–2700 m) and two time periods (season I, 2006–2008; season II, 2012–2013).

This methodology allowed us to test the association of each species with the altitudinal bands over time, resulting in 255 possible groups, but we restricted our analyses to the 62 groups that could be interpreted ecologically in terms of altitudinal constancy or shift (Suppl. material 1: S3). Depending on the degree of species association (IndVal) to altitudinal bands between sampling periods, we identified the following altitudinal changes. We classified as *altitudinal expansion* the case in which a species showed, in the second sampling period, new significant associations for higher altitudinal bands, at the same time maintaining the association to the altitudinal bands of the first sampling period. An *altitudinal upward shift* occurred when the species lost

its association to the lower altitudinal bands and gained new associations to higher ones during the second sampling period. At the same time, we identified *downward retraction* when a species lost its association to the lower bands, without gaining new associations with new higher ones. Then, we pointed out when the species became (*spread*) IndVal species or lost (*disappear*) their indicator value for specific altitudinal bands in the second sampling period. When no changes of associations occurred, the species remained *stable* over time (Suppl. material 1: S3).

Community level

Species' responses may affect communities' structure and composition (Wilson and Gutierrez 2012). Therefore, after a species level analysis, it is interesting to assess changes at the community level in order to compare the pattern of changes and to detect potential relationships. In this section, we investigated temporal changes of butterflies' communities from three different perspectives: species richness, community composition and community temperature index.

Species richness

To evaluate if sampling efficiency remained comparable throughout sampling periods, and if we consequently can carry out comparisons at the assemblage level, we performed a preliminary analysis of species richness estimators and sample completeness (expressed as the proportion of the observed species richness to the averaged estimated species richness). We calculated the averaged estimated species richness, through the mean value of four abundance-based estimators (ACE Abundance-based Coverage Estimator; Chao1; Jacknife1; Jacknife2), and correlated it with the observed species richness. Correlation was high and significant in both sampling periods (Spearman test; 1st sampling period, $\varrho = 0.937$, $p < 0.001$; 2nd sampling period, $\varrho = 0.918$, $p < 0.001$). Sample completeness was also high in both seasons (mean sample completeness among plots ± standard error; 1st = 67.59 ± 1.56; 2nd = 72.17 ± 1.31). Consequently, we considered the sampling efficiency between sampling periods as comparable, allowing us to carry out direct data comparisons.

To analyze how species richness per plot changed over time, we compared species richness between sampling periods using the t-test for paired samples (significance level assessed after 999 randomizations, following Legendre and Legendre 2012).

To understand if changes in species richness were mainly related to characteristics of specific plots, we analyzed it as a function of the following variables: altitude, temperature, geographic location, dominant vegetation cover (habitat type), and dominant land use. We considered the rate-of-change as a dependent variable (hereinafter *ROC*), defined as the differences in species richness between sampling periods (1st, 2006–2008; 2nd, 2012–2013), divided by the species richness of the first sampling

period (1st, 2006–2008). We analyzed the *ROC* through linear regression, and we compared variables in a multi-model context, according to two criteria: (i) avoiding the simultaneous use in the same model of highly correlated predictors (Spearman's rs >0.5); (ii) choosing predictors to represent biologically meaningful combinations of predictive variables, and consequently avoiding data dredging. All models were compared with the null model (intercept only) and all continuous variables were standardized, to enable comparisons between variables. The multivariate model selection was performed using Akaike information criteria, in its form corrected for small samples (AICc). As a measure of goodness-of-fit, we calculated the adjusted R^2. These analyses were performed by the R package '*MuMIn*' (Barton 2018).

Community composition

We analyzed community compositions by testing for changes both in location (significant changes in community composition per site over time) and dispersion over the years (significant changes in observed differences in community composition between sites, over time). Changes in location were tested by applying non-parametric MANOVA to Bray-Curtis distance matrixes, to test if the multivariate centroids of species composition were similar in the two groups, or not (Anderson 2001; McArdle and Anderson 2001). Non-parametric MANOVA (permutational MANOVA) is an analysis of variance using distance matrixes, which partitions sums of squares of a multivariate dataset. It was performed by the function *adonis* of the '*vegan*' package (Oksanen et al. 2012). The significance of the test was assessed by using *F*-tests based on sequential sums of squares obtained from permutations of the raw data (999 permutations). Since we had to keep in mind the temporal structure and the spatial dependencies of our sampling design (62 sites at two points in time), we applied a restricted randomization, which did not allow for permutations across samples. Changes in dispersion were tested by the *betadisper* function of the package '*vegan*', a multivariate analogous of the Levene's test for comparing group variances (Anderson 2001). To test if one time period is more variable than the other, non-Euclidean distances between objects and group centroids were handled by reducing the original distances to principal coordinates. To test for significance, we applied a similar randomization approach, as previously explained.

Community Temperature Index

The term "Species Temperature Index" (STI), refers to a quantitative description of the realized climatic niche of a species (Tayleur et al. 2016). To obtain such quantification over the Italian territory, we used presence data given by the database CkMap (Ruffo and Stoch 2005). CkMap is an atlas of the Italian fauna, promoted by the Italian Ministry of the Environment, which summarizes data related to the distribution of the Italian fauna on a gridded map (10×10 km). In our case, because we required STI that

referred to Alpine populations, we only focused on North Italy, considering 1396 cells. Every cell had a value of "1" (if occupied by the species) or "0" (if species presence was uncertain). Temperature data were obtained by the maps of Metz et al. (2014), using annual mean temperature (BIO1). In this way, we calculated mean temperature values for each species (realized niche optimum).

We used STI to calculate the "Community Temperature Index" (CTI), quantified as the mean STI of all the species present in a given community. We calculated the CTI for each community (plot) and sampling period, and we analyzed the changes in CTI over time.

We compared CTI values between sampling periods by using a t-test for paired samples. As for the case of *ROC*, we analyzed the temporal changes in CTI (Δ CTI) by a linear regression in a multi-model context regression, as a function of the same environmental variables and models.

Temperature analysis

To quantify changes in weather conditions between sampling periods, identified as a possible driving factor of changes in biodiversity, we compared monthly temperatures (from June to September), as a function of year, altitude and additive or interactive effects. Models were compared with the null one (intercept only) with Linear Mixed Effect Models (using the plot as random effect) and all continuous variables were standardized, to allow comparisons between variables. The multivariate model selection was performed using Akaike information criterion, in its form corrected for small samples (AICc). The analysis has been carried out using the R packages '*lme4*' (Bates et al. 2015) and '*MuMIn*' (Barton 2018).

All the analyses were carried out with the R software, version 3.3.3 (R Core Team 2017).

Results

Considering all sampling sites and time periods, we recorded 150 butterfly species, of which 133 were common to both periods, 5 were exclusive to the first sampling session and 12 were exclusive to the second sampling session (Suppl. material 1: S2).

Species level

Changes in plot occupancy

We observed a general increase in mean occupancy levels ($n = 150$, $t = -8.15$, $p = 0.001$; plot/species $1^{st} = 8.85 \pm 0.74$, $2^{nd} = 12.50 \pm 0.96$, change $= 3.65 \pm 0.45$).

The occupancy did not change equally between ecological groups. Concerning feeding specialization, strictly monophagous species differed from the other feeding groups (KW test, χ^2 = 9.82, df = 3, p = 0.020), even showing a slight decrease in the number of plots per species (polyphagous = 3.26 ± 0.99, one family = 4.10 ± 0.59, one genus = 3.68 ± 1.05, monophagous = -1.17 ± 0.98). We also recorded significant differences regarding the relationship with altitude. Altitudinal specialists increased less than the generalists (KW test, χ^2 = 13.13, df = 2, p = 0.001; generalists = 6.12 ± 1.03, medium = 3.04 ± 0.48, specialists = 2.32 ± 1.55) and also high altitude species showed a significantly less marked increase (MW test, W = 1070, p = 0.013, high altitude = 1.76 ± 1.32, others = 4.02 ± 0.46). "*Shade loving*" species showed the highest increase in mean occupancy levels (MW test, W = 2269, p = 0.041, "*shade loving*" = 4.53 ± 0.67, "*sun loving*" = 2.71 ± 0.57).

No significant differences have been recorded considering habitat (KW test, χ^2 = 2.04, p = 0.564) preferences and dispersal capacity.

We identified 17 species which significantly increased their distribution within the study area, of which 7 were also significant after Bonferroni correction. No species significantly decreased its occupancy level (Table 1).

Altitudinal range changes

We observed significant changes both at the minimum and at the maximum boundary of altitudinal range of the species. At the lower altitudinal limit, we observed a significant decrease in the absolute minimum value (t-test, n = 133, t = 3.03, p = 0.004, change = -96.62 m ± 31.85 m). At the higher altitudinal limit, we observed an increase in both the absolute maximum (t-test, n = 133, t = -3.08, p = 0.006, change = 75.19 m ± 24.01 m) and in the 90th percentile (t-test, n = 133, t = -2.63, p = 0.014, change = 55.15 m ± 20.97 m).

We also observed significant differences in the changes of the altitudinal range between ecological groups. In particular, high altitude species, compared to the others, showed a significant increase in the mean (MW test, W = 1657.5, p = 0.041; high altitude = 59.21 ± 21.24, others = -11.07 ± 18.09), median (MW test, W = 1657, p = 0.040; high altitude = 59.37 ± 19.90, others = -8.72 ± 17.58) and 10th percentile values (MW test, W = 1737, p = 0.012; high altitude = 68.12 ± 50.24, others = -72.11 ± 28.05).

"*Shade loving*" species lowered their minimum (MW test, W = 2738.5, p = 0.014; shade = -164.18 ± 45.34, sun = -28.03 ± 43.47) and 10th percentile values (MW test, W = 2702, p = 0.027; shade = -74.10 ± 36.66, sun = -19.09 ± 34.14) compared to the "*sun loving*" species.

Strongly vagile species increased their minimum boundary, while weakly and medium vagile species lowered their minimum boundary (KW test, χ^2 = 8.34, df = 2, p = 0.015; high = 139.29 ± 110.22, medium = -103.49 ± 37.93, low = -178.79 ± 62.12).

We identified 87 species associated with one of the selected group combinations (Fig. 1; Suppl. material 1: S4). In most cases, IndVal species resulted as *stable* through-

Table 1. Species which significantly changed their area of occupancy, expressed as the number of plots of presence during the first (Plot1) and the second (Plot2) sampling period. The number of plots gained or lost (Δ), the p-value of the McNemar's test (p-value) and its significance level (* $p < 0.05$, ** $p < 0.001$) after Bonferroni correction (p-adj) are also expressed.

Species	Plot 1	Plot 2	Δ	p-value	p-adj
Cyaniris semiargus	8	36	28	<0.001	**
Pieris bryoniae	16	43	27	<0.001	**
Cupido minimus	22	43	21	<0.001	**
Aglais urticae	37	55	18	0.004	
Callophrys rubi	9	27	18	0.001	*
Pyrgus malvoides	20	38	18	<0.001	*
Argynnis niobe	20	36	16	<0.001	**
Maculinea arion	10	24	14	0.001	
Colias alfacariensis	2	14	12	0.001	*
Lasiommata petropolitana	6	18	12	0.003	
Pieris rapae	20	32	12	0.009	
Polyommatus coridon	31	43	12	0.001	
Eumedonia eumedon	3	14	11	0.001	
Pyrgus cacaliae	2	13	11	0.010	
Erebia euryale	34	44	10	0.009	
Pyrgus carthami	1	11	10	0.003	
Argynnis aglaja	33	42	9	0.009	

out the time period: there were 52 species belonging to 7 different combinations of altitudinal bands. A total of 12 species expanded their altitudinal range, spreading the associations through the higher altitudinal band and remaining stable at the lower ones (*altitudinal expansion*, 5 combinations of groups). Even if with a low indicator value (*Celastrina argiolus*, IndVal = 0.510, p-value = 0.018; Fig. 1, Suppl. material 1: S4), only one species showed an *altitudinal upward shift*, gaining the association to a higher altitudinal band and losing it into the lower one. The general increase in species occupancy also resulted in 19 species becoming indicator species during the second sampling period of specific altitudinal bands (*spread*). Only three species were indicators of the lowest altitudinal range only during the first time period, showing a decline during the second sampling season (*disappear*). For no species did we record a *downward retraction*.

Community level

Species richness

Species richness significantly increased from the first to the second sampling period (t-test, n = 62, t = -9.76, p = 0.001, change = 8.82 ± 0.90).

The analysis of the *ROC* showed a significant effect of both land cover and land use: wooded habitats and managed plots increased the most (Table 2).

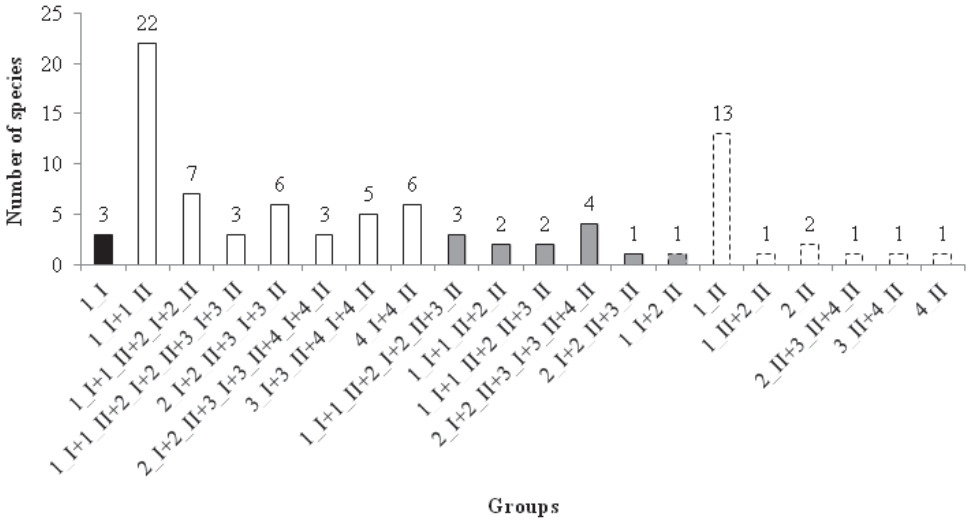

Figure 1. Changes along altitude. The number of significant IndVal species are shown for each combination of sampling periods (I = 1st sampling period, II = 2nd sampling period) and altitudinal bands (1, 550–1200 m; 2, 1250–1650 m; 3, 1700–2150 m; 4, 2200–2700 m). Different colours represent different statuses. Species that lost their indicator value with specific altitudinal bands during the second period (*disappear*; black, with continuous border); species stable over time (*stable*; white, with continuous border); species that showed, in the second sampling period, new significant associations for higher altitudinal bands, at the same time maintaining the association to the altitudinal bands of the first sampling period (*altitudinal expansion*; grey, with continuous border); species that lost their association to the lower altitudinal bands and gained new associations to higher ones during the second sampling period (*altitudinal upward shift*; grey with dotted border); species that became IndVal of specific altitudinal bands during the second sampling period (*spread*; white, with dotted border).

Community composition

Species composition significantly differed between time periods, but the obtained R^2 is extremely low, meaning a scarcely relevant change (non-parametric MANOVA, F-value = 5.87, r^2 = 1.91, p = 0.001). More interestingly, we observed a significant change in dispersion between sampling periods, with a lower dispersion around the median during the second sampling period (distance to centroids 1st sampling period = 0.584, 2nd sampling period = 0.528, p = 0.001; indicating a tendency towards homogenization; Fig. 2).

Community Temperature Index

We observed that the CTI significantly increased from the first to the second sampling period (t = -3.59; p = 0.001), indicating a common trend toward thermophily in all

Table 2. Best linear regression model for Rate Of Change (*ROC*). Coefficients (± standard error) of the selected variables are indicated in the cells. Adjusted r squared is indicated as a measure of goodness of fit. alt = altitude; alt^2 = altitude (second order); park = geographic location; rme = change in mean temperature; rmi = change in minimum temperature; vegetation = dominant cover type (land cover); use = land use; Tmin = mean seasonal minimum temperature during the first season. adj r^2 = adjusted R^2. ** $p < 0.01$; * $p < 0.05$; ° $p = 0.06$.

		ROC
alt		
alt^2		
park		
rme		
rmi		
vegetation		
	ecotone	-0.394 ± 0.141 **
	meadow	-0.277 ± 0.120 *
	rock	-0.349 ± 0.185 °
use	yes	0.257 ± 0.102 *
Tmin		
R^2 adj		20.64

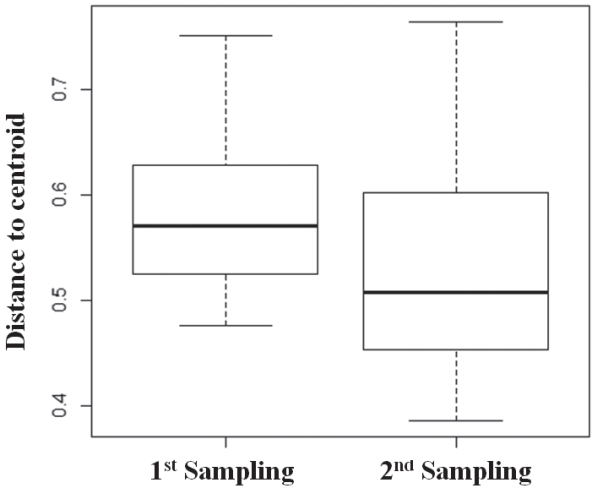

Figure 2. Homogenisation of community composition. Box-plot of the distances to the centroid of community composition, during each sampling period. A reduction in species heterogeneity at the community level from the first to the second period can be clearly seen.

the sampled areas. Moreover, interestingly, we observed that the change in CTI over time was mainly dependent on the geographic position of the sampling plots (Fig. 3; $R^2 = 14.17$, $p = 0.007$; geographic location, $p = 0.007$). In particular, we recorded the significantly higher increase in CTI in the colder areas, i.e. in the plots located in VDNP, which is characterized by the lowest temperature among our study areas.

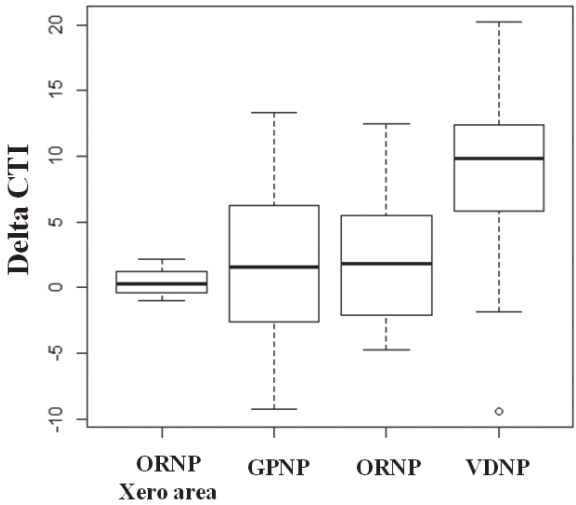

Figure 3. Changes in Community Temperature Index. We observed significant differences in ΔCTI between geographic locations (protected areas). The parks located in colder areas clearly show the highest increase in CTI.

Temperature

The analysis of field-recorded temperatures indicated significant differences between sampling periods, concerning the monthly mean and minimum temperatures observed during July, August and September (Table 3). In particular, we observed a second sampling period significantly warmer than the first one with a significant increase of minimum (+1.22 °C) and mean (+0.83 °C) temperatures. The values of increased temperatures reported refer to the seasonal average (from June to September).

Table 3. Results from the best linear model indicating the role of altitude and year (and their interaction) for determining differences between sampling seasons for each month and temperature parameters (Temp).

Temp	Month	Intercept	Altitude	Year	Altitude*Year	R^2
Mean	June	11.43	-2.46			80.2
	July	13.22	-2-72	0.11		91.9
	August	12.65	-2.62	0.2		87.8
	September	8.39	-2.45	0.28		89.2
Max	June	17.08	-1.76			46.8
	July	20.16	-2.03	-0.14		50.5
	August	19.35	-1.92			53.8
	September	15.38	-1.50			42.4
Min	June	7.33	-2.88			83.7
	July	8.3	-3.08	0.25		96
	August	8.29	-2.96	0.26		90.6
	September	4.22	--2.88	0.37		95.9

Discussion

Long-term monitoring, which is periodically and systematically repeated over time (usually decades), is crucial for correctly understanding the variables and mechanisms that determine species' distribution responses and patterns of community composition as a consequence of climate and land use changes (Magurran et al. 2010, Magurran and Henderson 2010, Legendre and Gauthier 2014). Interestingly, some recent studies in the Alps have shown how significant changes can also occur over short periods, highlighting the importance of verifying distributional and community changes at temporal intervals of less than 10 years (short-term changes).

Roth et al. (2014) described changes in community composition of birds, butterflies and plants in the Swiss Alps along an altitudinal gradient, over just 8 years. Erschbamer et al. (2009) showed how plant species richness in the Dolomites was significantly higher after only 5 years. In this framework, our study on butterflies composition and distribution along altitudinal gradients in the NW Italian Alps represents an important step towards a better comprehension of biodiversity patterns in mountain ecosystems, even if it was restricted to a short time-frame (2006–2007 vs 2012–2013). Indeed, understanding the spatial and temporal dynamics of species-rich communities is critical for understanding how environmental changes can affect biodiversity (McCann 2007).

Driving factors of change

Although our research is based on a short time frame, it is important to consider the role of potential driving factors of change, by analyzing climatic and land cover changes (whenever occurring) as explicative predictors.

Concerning variations in temperature between sampling periods (2006–2008 vs 2012–2013), we observed that the second sampling period was warmer than the first one. These observed differences are coherent with the trend recorded by Beniston (2006) for the Alps and are likely correlated to climate change. Indeed, Beniston (2006) observed an increase in minimum and mean temperatures over time, but no change in the maximum temperature.

Unfortunately, no punctual and detailed data on precipitations were available to introduce them into the analytical framework. However, analyzing data from the official weather stations of the Regional Meteorological Service located in the study areas, we observed an interesting pattern in snow cover, characterized by a reduction in the number of days with snow and by a seasonal shift in snow cover towards the spring, in particular since 2005. Such conditions are indicative of a warmer climate and they correspond to a common pattern for the entire Alpine chain (Scherrer et al. 2016).

Due to the short time frame of analysis, we considered the main habitat type (dominant land cover) as a constant variable because no huge structural changes have potentially occurred. However, because of the natural evolution of vegetation following climatic changes and land abandonment, minimal but effective land cover changes

in terms of microhabitat scale (e.g. shrubs encroachment) can be taken into account (Zurlo 2018).

Some sampling sites (plots) were characterized by low-intensity and sustainable grazing activities without any changes between sampling periods.

No other direct land use changes and habitat alterations occurred within our study sites because of the park status and LTER accredited site of our study area.

Species level

Plot occupancy

Despite the short time-frame under analysis, butterflies showed a general increase in mean occupancy levels but species-specific plot occupancy changes differed markedly among species. However, when grouping species according to homogeneous ecological traits, we observed consistent distributional responses, in accordance with several studies that have proved the effect of ecological and life-history traits in shaping species distribution (Forister et al. 2010, Chen et al. 2012, Auer and King 2014). Few studies to date have examined the underlying reasons for distribution changes and so far results have been equivocal (Angert et al. 2011; Buckley and Kingsolver 2012).

Monophagous, altitudinal specialists and high-altitude species appeared to be more limited than other species. These categories comprise species with a high level of specialization, consequently less prone to colonize new environments, even if climatic or environmental constraints are relaxed. In particular, monophagous species are strictly limited by the presence and the quality of their single larval host plant, and are already observed and predicted to be highly vulnerable to climatic/environmental changes (Romo et al. 2014). Our results concerning butterfly specialization are interesting and reflect what has been observed in central Europe concerning habitat specialization, where a decrease of specialized and low vagile species has been observed, along with an increase of generalist species and good dispersers (Habel et al. 2016).

A significantly higher increase in plot occupancy by "shade-loving" species compared to other species can be associated with a tendency towards a higher coverage of shrubs in open areas at low and medium altitude. In the European Alps, the effect of climate change is regionally affected by human activities. Cattle grazing in the alpine pastures has been decreasing throughout the past century, allowing rapid recolonization by trees and shrubs, where the treeline had been artificially lowered (e.g., Vittoz et al. 2008a; EEA 2010; Rocchia et al. 2018).

Interestingly, no species significantly decreased its area of occupancy, but 17 species increased their occupancy area, 7 of which in a particularly marked manner, still significant after Bonferroni correction (Table 1).

Aglais urticae and *Argynnis niobe* showed pronounced increase in plot occupancy. Our results are in contrast with other researches that reported a decline of these two species in other parts of Europe (Kulfan et al. 1997, Saarinen et al. 2003). Those spe-

cies are altitudinal generalists with a high dispersal capability like most of the other species which increased their area of occupancy within our study area (e.g. *Pieris rapae* and *Colias alfacariensis*). We therefore consider that our observations could be only transient, due to the high dispersal capacity of those species and their tendency to carry out vertical migrations. Particularly interesting was the increase in mean occupancy of *C. rubi.* Since its low feeding specialization (Balletto et al. 2015), this species could have increased its abundance or spread into new areas, without having limitations due to food availability. However, although we highlighted important plot occupancy changes both at ecological groups and species level, we should also consider that they could be influenced by many factors, such as species detectability, population dynamics and phenology, which could, in turn, affect plot occupancy detection. To disentangle such patterns, long-term monitoring and more detailed study on the population dynamics of target species will be essential.

Altitudinal range changes

As for plot occupancy analysis, we noticed a general process of altitudinal change, considering all butterflies species together.

Looking at the ecological groups, we observed that, even along altitude, generalist and highly mobile species were more prone than specialist and less mobile ones to change their distributional ranges. Particularly, high altitude species showed a significantly higher increase in their mean, median and 10th percentile altitudinal parameters, corresponding to a reduction in their lower altitudinal boundaries and in the surface available. Those species are already limited in their distribution. Their presence is, in many cases, limited by minimum temperatures (Pellissier et al. 2013) and, consequently, they cannot lower their altitudinal range nor, in many cases, raise it, due to the drastic decrease of vegetation cover at higher altitudes (a high occurrence of rock cover and a strong reduction of the availability of herbs and grasses). Moreover, the reduction in permafrost, which is a relatively new and rapid phenomenon, makes high altitude rock and screes unstable, preventing colonization by plant species (Cannone et al. 2007). However, we should also consider a limit of our sampling design, as we did not monitor plots over 2700 m a.s.l., consequently reducing our possibility to observe an expansion towards higher altitudes and a colonization of new plots by high altitude species.

Concerning altitudinal patterns, we used the IndVal test to identify species associated with specific altitudinal zones. Our analysis highlighted 52 species associated with one or more altitudinal zones at both time periods: these species are widely spread within their altitudinal zone, and stable over the investigated period, consequently representing species which – in the future – could be monitored to investigate important altitudinal changes. For 12 species, we observed new significant associations for higher altitudinal bands (*altitudinal expansion*). We recorded a change in association from a lower to a higher altitudinal band (*upward shift*) only for one species, *Celastrina argiolus*, which was associated with the first altitudinal zone (550–1000 m) during

the first sampling period, while during the second sampling period, it resulted characteristic for the second zone (1250–1650 m) only (see *statistical analyses – altitudinal range changes* for the description of the relationships between IndVal association and altitudinal changes). It is a species that favors woodland and shade, and probably an increase in minimum temperature with an increase in the shrub land coverage had an important effect in the shift of this species.

In contrast, other studies in European countries observed on the opposite a higher amount of species shifting their altitudinal distribution, disappearing from low elevation sites and colonizing higher altitudes (e.g., Hill et al. 2002; Konvička et al. 2003). The differences from our work, where most of the species changing their altitudinal association did not disappear from the lowest altitude sites, could be related both to the shorter temporal frame we analyzed and to the lower altitudinal gradient we monitored. Indeed, our low altitude sites are located in the montane belt, where the effects of climate and environmental changes could be less severe than in the lowland plains.

Finally for 19 species, the changes in their distribution determined new associations for specific altitudinal bands during the second sampling period (*spread*). These species represent important taxa to be followed over time, to understand if this pattern represents the first signal of an upward expansion.

Community level

Species richness

We observed significant changes in species richness per plot within the analyzed period. Butterfly communities are known to quickly change their arrangement due to environmental changes (Thomas 2005), and previous studies have indicated that butterflies could respond even faster than other taxa (Devictor et al. 2012).

We observed a clear and significant increase in species richness within our temporal frame, mainly related to land cover and land management. The highest rate of change was clearly evident in the wooded areas (woodland clearings), while ecotones (transitional areas dominated by shrubs, and mainly located inside the subalpine belts) showed the lowest rate of change. Other authors also observed similar results, although they were mainly related to individual species abundance. For example, Sgardeli et al. (2016) observed a higher increase in species abundance simultaneously with an increase of temperature within forest areas with respect to other areas. Indeed, on days with high temperatures and solar radiation (which are exacerbated in open areas), wooded areas can exert a buffering effect, protecting individuals from extreme temperatures and reducing temperature leaps (Oliver and Morecroft 2014). Even thermophilous and "sun-loving" butterfly species can be inhibited in their flight activity by high temperatures and solar radiation, with potentially dangerous consequences that have not yet been fully understood (Cerrato et al. 2016).

In grazed areas, we observed an increase in species richness that was twice as much as in the unmanaged area. This can be probably explained by the low-intensity, sustainable grazing of these sites. Although intense grazing activities can be detrimental for butterflies (Scalercio et al. 2014), it has been previously observed that a balanced grazing pressure can increase the presence of plant species belonging to Poaceae and Fabaceae (Fischer and Wipf 2002), which represent the most exploited plant families that host the larvae of many butterfly species. Moreover, grazing maintains woodland clearings and open herbaceous areas below the tree line, that would be rapidly colonized by shrubs and trees, without such management activities (Nagy and Grabherr 2009). Indeed, balanced grazing activities have the fundamental role of maintaining micro-habitat heterogeneity, which is, in turn, of high importance in reducing the potential detrimental effects of climate change to butterflies in mountain ecosystems (Klečková et al. 2014; Konvička et al. 2016).

Community composition

Although we observed major differences in species distribution between our sampling periods, we did not observe significant differences in community composition. These results were probably due to a consequence of the short time-frame of analysis.

Studies that have demonstrated variation in butterfly community composition clearly take into account longer time-frames (e.g., Habel et al. 2016) and, to our knowledge, no investigations have reported significant changes in community composition in such a short time-frame. Interestingly, however, we noticed a tendency towards biotic homogenization in butterfly community composition. Biotic homogenization refers to the increase in biological similarity between communities, a replacement process leading to a decrease in distinctiveness in community composition over time, as a result of the replacement of some specialist species with other generalists, which become more uniformly distributed across previously different assemblages (Olden and Rooney 2006). Indeed, species respond individually to changing environmental conditions, depending mainly on their physiological characteristics and habitat requirements (e.g., Wilson and Gutierrez 2012). This determines new species assemblages, which can only be appreciated by examining the entire communities throughout time (e.g., Wilson et al. 2007).

For example, a similar change in community composition over time, accompanied by an increase in community similarity, has been observed in the data analysis from the UK Butterfly Monitoring Scheme over a period of 20 years (Gonzàlez-Megìas et al. 2008). This tendency towards biotic homogenization has been observed in recent decades in different taxa, following land cover and climatic changes (e.g., Bühler and Roth 2011; Eskildsen et al. 2015). These phenomena often determine an increase of generalist and of highly vagile species, to the detriment of other taxa (e.g. Menéndez et al. 2006; Bonelli et al. 2011).

Community Temperature Index

The observed increase in CTI tended to derive from the increase in mean occupancy levels recorded by a large number of generalist, more termophilous species in the second sampling period compared to the first one. This phenomenon was particularly marked in the coldest areas, representing a potential threat for high altitude and microthermic species. Indeed, the generalist species successfully colonizing a higher altitude from a lower one can outcompete the more specialized and vulnerable species of the alpine belt, challenging the survival of their local populations in the long term. Our results are consistent with an increase in CTI already observed in other geographic areas (e.g., in Greece by Zografou et al. 2014 over 13 years; in the Swiss Alps by Roth et al. 2014 over 8 years; at European scale by Devictor et al. 2012 over two decades). In any case, our trend was observed over a shorter time-frame, and, if confirmed during the next monitoring sessions, could represent a warning signal for alpine butterfly fauna.

Conclusions

In recent decades, changes in species distribution and composition of communities have already been analyzed in some parts of the Alps. Nevertheless, data usually concern plants (e.g. Grabherr et al. 1994; Pauli et al. 2001; Vittoz et al. 2008a, 2008b), or birds (Popy et al. 2010; Scridel et al. 2017), with very little information regarding other taxonomic groups, and mainly concerning changes in the distribution of single species (e.g., Battisti et al. 2005).

Responses to climate and habitat changes vary widely between species of the same communities (Walther 2010). Consequently, it is of crucial importance to understand the heterogeneity of species responses and the implications of the changes in community composition that follow (Wilson and Gutierrez 2012). Moreover, many previous studies that aimed at understanding changes over time in community composition have relied on the comparisons of contemporary data with historical data (atlases, collection specimens), which were often collected in a non-standardized way and/or referred to a much coarser spatial grain (Wilson and Gutierrez 2012).

Sampling units placed in well-specified areas represent a more appropriate tool for investigative purposes (e.g., Archaux 2004; Viterbi et al. 2013; Brandmayr and Pizzolotto 2016), and our data from the Italian Alps, taken from exactly the same sites at 4-year intervals, represent a first attempt to fill these gaps, focusing on changes in mountain ecosystems and mainly considering community level responses. Moreover, thanks to the LTER network, such standardized and temporally replicated data are available for broader spatial scale analysis and comparison with data coming from other sites of the same network, greatly improving our capacity to understand biodiversity patterns.

Interestingly, and somewhat alarmingly, our results suggest that, even if the time-frame under analysis is relatively short, we already observed considerable changes, in

particular considering that the research was carried out in protected areas and LTER sites where habitat alteration by direct human effects is strongly reduced.

To summarize, in just 5 years, we observed:

- a *general increase* in *mean occupancy* level and in *species richness* of butterflies;
- *no significant changes* in the *mean altitudinal optimum*, but *significant changes* at *both altitudinal limits*.

Moreover, the observed changes differed across species, determining:

- an increase in shared species (a tendency to *homogenization*) within communities, even if the *overall community composition* did not change;
- a significant increase in *Community Temperature Index (CTI)*.

Considering these results, it is now even more important to continue our monitoring in order to understand in the near future if our observed patterns represent only transient changes or are the first signals of an imminent trend.

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Supplementary material 1

Supplementary data

Authors: Cristiana Cerrato, Emanuel Rocchia, Massimo Brunetti, Radames Bionda, Bruno Bassano, Antonello Provenzale, Simona Bonelli, Ramona Viterbi

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SHORT COMMUNICATION

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First records of *Opetiopalpus sabulosus* Motschulsky, 1840 (Coleoptera, Cleridae) for the European Alps

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Abstract

The genus *Opetiopalpus* from the family of checkered beetles (Coleoptera: Cleridae) is represented by 28 species worldwide, with 11 species found in the Palearctic and only four sparsely in Europe prior to 1998. One species, *Opetiopalpus sabulosus* Motschulsky, 1840, was recently found in Eastern Europe (i.e. Ukraine, Bulgaria, and Moldavia), with the most recent record in 2015 in Eastern Romania; no data are available for Central Europe. During a comprehensive sampling survey in 2016 in the dry inner-Alpine Vinschgau Valley (South Tyrol, Italy), one individual of *O. sabulosus* was recorded from soil core samples on an extensively managed steppe-like dry pasture at 2000 m a.s.l. This was the first record of *O. sabulosus* for the European Alps and Central Europe. Further intensive samplings were conducted in 2017 and 2018, in which one additional specimen at a dry pasture at 2500 m confirms the presence of this checkered beetle. *Opetiopalpus sabulosus* seems to have a cryptic lifestyle and therefore a low detection probability. The locations from where the species was recorded, all steppe-like dry grasslands that are part of the LTSER area "Val Mazia/Matschertal" within the LTER-Italia network, are characterized by low precipitation (730 mm at 2000 m a.s.l.) and traditional low input management (grazing cattle, sheep, and horses). Beside *O. sabulosus*, other rare and new species for South Tyrol and Italy were found at the sampling area. Therefore, our records underline the high biodiversity and the high nature conservational value of these steppe-like dry grasslands and the importance of long-term research to monitor such species.

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Keywords
new report, checkered beetles, Alpine grassland, dry pasture, LTER_EU_IT_097, LTER-Italia

1. Introduction

Cleridae are characterized as colorful, hairy beetles that are either predacious (especially on wood-boring or carrion-visiting insects) or flower visitors (Gerstmeier 2014). In the Palearctic, 215 Cleridae species (common name: checkered beetles) have been described to date (Gerstmeier 2014), of which the genus *Opetiopalpus* (subfamily Korynetinae) is represented by 11 species (28 worldwide); only four species have been sparsely found in Europe prior to 1998 (Gerstmeier 1998). A fifth species of the genus *Opetiopalpus*, *O. sabulosus* Motschulsky, 1840, was recently recorded from the peripheral regions of Europe. In general, *O. sabulosus* was recorded from North Africa, Armenia, Western Asia, Mongolia, and Siberia (Gerstmeier 1998) as well as from Eastern Europe (Ukraine, Bulgaria, and Moldavia; Löbl and Smetana 2007). To our knowledge, the most recent record, comprising six individuals, was from Eastern Romania (Kurzeluk 2015). However, as far as we are aware, there are no previous records for Central Europe for this species.

2. Material and methods

Site description

A comprehensive biodiversity study was conducted in 2016 at the "Muntatschinig" site in the Alpine Long-Term Socio-Economic Research (LTSER) platform "Val Mazia/Matschertal", a member of the national and international long-term ecological research networks LTER-Italy, LTER Europe, and ILTER (Vinschgau Valley, South Tyrol, Italy; site code LTER_EU_IT_097; 46°41.647'N; 10°37.029'E). For more details see Rief et al. (2017) and Hilpold et al. (2018). The "Muntatschinig" site ranges in elevation from approx. 1000 to 2500 m a.s.l. with slopes exposed to the south and south-west (Fig. 1).

Study design

Three soil core samples each (20 × 20 cm, 15 cm deep if possible) were taken randomly and evenly distributed at the extensively managed dry pastures P2000 (mean soil parameters: pH 4.47 ± 0.13, SOM 14.66 ± 2.27, C:N ratio 13.40 ± 0.98) and P2500 (pH 4.30 ± 0.30, SOM 15.99 ± 3.08, C:N ratio 12.81 ± 1.22) at four dates from April to October 2016 (Table 1; for details see Steinwandter and Seeber 2017). The

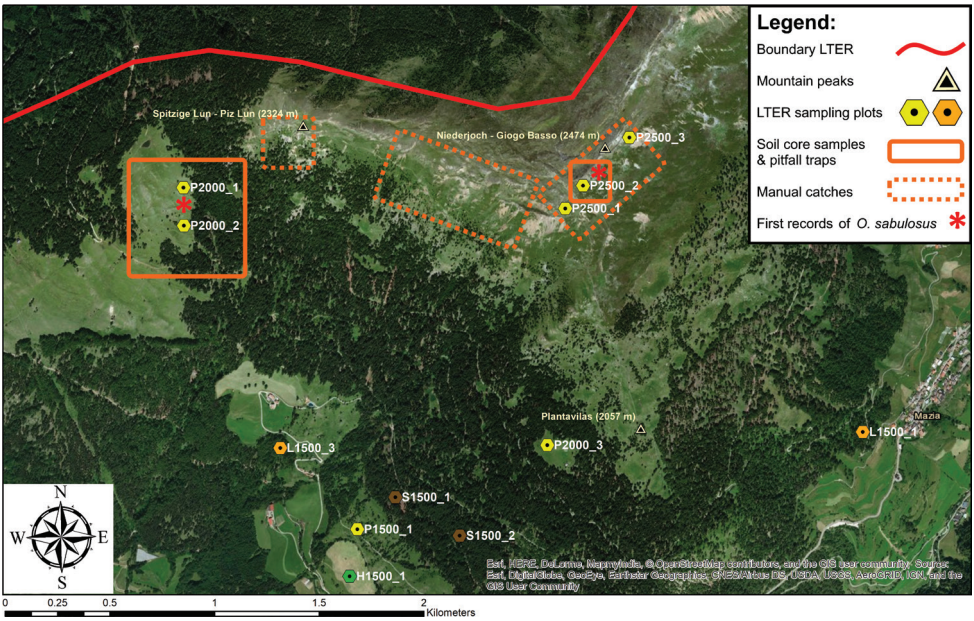

Figure 1. Aerial photograph of the upper "Muntatschinig" site in the LTSER area "Val Mazia/Matschertal" with the samplings from 2016 to 2018. The orange frames depict the *Opetiopalpus sabulosus* Motschulsky, 1840 (Coleoptera: Cleridae) sampling areas, hexagons the locations of permanent monitoring sites (e.g. L1500: Larch forests at 1500 m a.s.l., H1500: hay meadows, S1500: Spruce forests, P2000: dry pastures at 2000 m, etc.).

Table 1. List of samplings and methods at the "Muntatschinig" site in the LTSER area "Val Mazia/Matschertal" to find *Opetiopalpus sabulosus* Motschulsky, 1840 (Coleoptera: Cleridae). The sampling areas include steppe-like dry pastures at different elevations (e.g. P2000_1 represents plot number one of dry pastures P at approximately 2000 m a.s.l.) as well as summit areas. Details for each sampling date are given.

Date	Method	Sampling plots	Details
04.05.2016	soil cores	P2000_1-3, P2500_1-3	3 per plot
26.06.2016	pitfall traps	P2000_1-3, P2500_2	2 per plot, active for 14 days
30.06.2016	soil cores	P2000_1-3, P2500_1-3	3 per plot
31.08.2016	soil cores	P2000_1-3, P2500_1-3	3 per plot
31.10.2016	soil cores	P2000_1-3, P2500_1-3	3 per plot
25.05.2017	manual catches	P2000_1-2, P2500_2-3, Niederjoch, Spitzige Lun	hand-sampling, exhauster, sieving litter and lichens
21.05.2018	soil cores	P2500_2	3 per plot
19.06.2018	pitfall traps	P2500_2	3 per plot, active for 35 days
19.06.2018	soil cores	P2000_1-2	3 per plot
16.07.2018	pitfall traps	P2000_1-2	3 per plot, active for 27 days
16.07.2018	soil cores	P2000_1-2	3 per plot
06.08.2018	pitfall traps	P2000_1-2	3 per plot, active for 21 days
06.08.2018	soil cores	P2000_1-2	3 per plot
03.09.2018	pitfall traps	P2000_1-2	3 per plot, active for 28 days

samples were heat-extracted for 12 days in a modified Kempson apparatus (Kempson et al. 1963) in the laboratory of the Institute for Alpine Environment of Eurac Research, Bozen/Bolzano, Italy. All soil macro-invertebrates were stored in 75% ethanol solution until identification to family, and where possible, to species level, under a stereo microscope (Leica M205 C).

In late April 2017, the whole area around P2000_1, P2500_2, the "Spitzige Lun/Piz Lun" peak (2324 m), and the crest that ends in the "Niederjoch/Giogo Basso" peak (2474 m) was thoroughly investigated by hand collecting and litter sieving the shrub, grass, and lichen layers (Fig. 1). Furthermore, intensive sampling was undertaken in summer 2018 by taking randomly and evenly distributed three soil core samples and installing each three pitfall traps with propylene glycol as preservation fluid on each the P2000_1, P2000_2, and P2500_2 plots. They were emptied at the successive sampling date (2018 sampling, three to five weeks); in 2016 after two weeks in the field (Table 1).

Results and discussion

Two individuals of *O. sabulosus* Motschulsky, 1840 (Coleoptera: Cleridae) were found during all sampling attempts (Table 1). The sites (P2000_1 and P2500_2) are steppe-like dry pastures extensively grazed by cattle and sheep at 2000 m and 2500 m a.s.l., respectively. The whole area is exposed to the south (i.e SSW to SSE) and has a mean inclination of 10° (Fig. 2). Small herds (20–30 individuals) of cattle were grazing on the sites from June to October; at the "Niederjoch/Giogo Basso" peak also sheep were present. The P2000_1 dry pasture is surrounded in the north and east by a mixed Norway Spruce [*Picea abies* (L.) H. Karst.] and Swiss Stone Pine [*Pinus cembra* L.] forest (Fig. 2A). Small stones are dispersed over large parts of the area; in the northwest a small artificial reservoir can be found. The site is located close to a steep silicate rocky slope from the "Spitzige Lun/Piz Lun" peak (2324 m). The P2500_2 dry pasture is located close to the "Niederjoch/Giogo Basso" peak (2474 m) with stony and sandy areas at the foot of the small rocky peak (Fig. 2B).

The first specimen was extracted from one P2000_1 soil core sample taken on 31st August 2016 (1 male, leg. Steinwandter M, det. Kahlen M, 46°42.184'N; 10°34.203'E, Table 1). The specimen was 3 mm in size and had no physical damage (Fig. 3). The identification of the species followed Gerstmeier (1998) and was confirmed by both Manfred Kahlen, a Coleoptera specialist for the Central Alps (Kahlen 2018), and Roland Gerstmeier, a Cleridae specialist. The individual is stored in the Coleoptera collection of Manfred Kahlen at the "Tiroler Landesmuseen Betriebs G.m.b.H., Naturwissenschaftliche Sammlungen, Sammlungs- und Forschungszentrum in Hall in Tirol", Tyrol, Austria.

A second specimen was found two years after the first record, in a pitfall trap from the 2018 sampling collected on 19th June 2018 at the P2500_2 site (1 adult, leg. & det. Steinwandter M, 46°42.168'N; 10°35.700'E, Table 1). It is stored in the collection of the Museum of Nature South Tyrol, Bozen/Bolzano, Italy (Nr. INS 4709). Our investigations suggest that these are the first confirmed records of *O. sabulosus* for the European Alps and Central Europe.

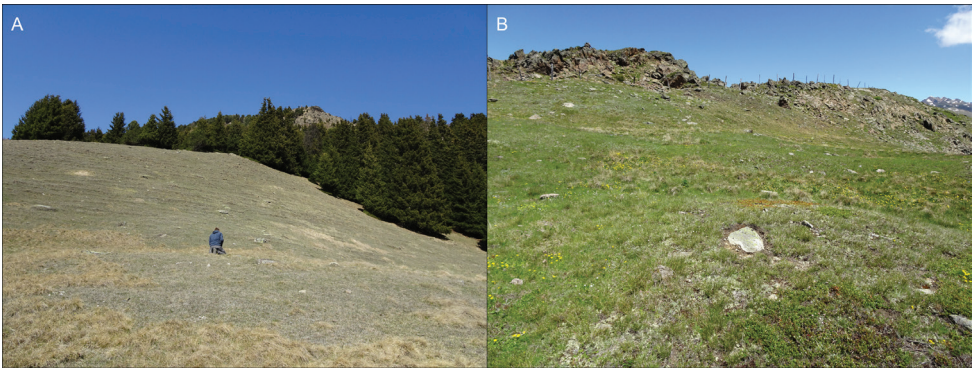

Figure 2. Photographs of the steppe-like dry pastures at the "Muntatschinig" site where *Opetiopalpus sabulosus* Motschulsky, 1840 was first recorded. The sites are part of the LTSER area "Val Mazia/Matschertal" at the research site "Muntatschinig" **A** P2000_1: photograph 2A was taken on 4th May 2016. In the background the "Spitzige Lun/Piz Lun" peak can be seen **B** P2500_2: photograph 2B was taken on 19th June 2018. The rocky "Niederjoch/Giogo Basso" peak can be seen in the left upper corner.

Figure 3. Habitus of the first record of *Opetiopalpus sabulosus* Motschulsky, 1840. Multiple photographs were taken using a stereo microscope (Olympus ZX 10) and a camera (Panasonic Lumix GH4), and rendered with a focus stacking software (Helicon Focus version 6.0) by Andreas Eckelt. The specimen is stored in the collection of Manfred Kahlen at the "Tiroler Landesmuseen Betriebs G.m.b.H., Naturwissenschaftliche Sammlungen, Sammlungs- und Forschungszentrum in Hall in Tirol", Tyrol, Austria.

The records on our P2000_1 and P2500_2 sites could indicate a small and cryptically living population adapted to inner-Alpine and steppe-like dry grasslands as well as rocky alpine landscapes. The fact that the specimens were found after two years underlines the presence of a small population of *O. sabulosus* in the LTSER area "Val Mazia/

Matschertal" and excludes a serendipitous finding. The Vinschgau Valley represents one of the driest regions of the whole European Alps and the only dry valley of the Eastern Alps (Braun-Blanquet 1961), with a mean annual precipitation of only 525 mm at the valley bottom, 730 mm at P2000_3; the mean annual temperature is 4.12 °C (Della Chiesa et al. 2014). All other recent findings of this checkered beetle were found in habitats that span from the lowland to the higher mountains up to 3200 m of the Northern Caucasus (Berlov and Berlov 2007), with preferences for dry grassland. Additionally, stony ground seems to be favorable, preferably providing insect cadavers as food source (Gerstmeier 1998).

We will continue to survey these steppe-like grassland areas in the inner-Alpine dry Vinschgau Valley to monitor the population and to gather more information about distribution, ecology and faunistics of *O. sabulosus*. The LTSER area "Val Mazia/Matschertal has already yielded several findings of new arthropod species for South Tyrol and Italy (e.g. Ballini et al. 2017 for Araneae, Arachnida; Schatz and Zanetti 2017 for Staphylinidae, Coleoptera), and provides the necessary means for biodiversity studies due to long-term sampling approaches.

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Modelling *Acacia saligna* invasion on the Adriatic coastal landscape: An integrative approach using LTER data

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Abstract

Invasive Alien Species (IAS) pose a major threat to biodiversity and ecosystem services worldwide. Even if preventing biological invasions should be the most cost-effective way to minimise the impact of IAS on biodiversity, new efforts are necessary to identify early signs of invasion and to assess invasion risk. In this context, the implementation of invasive Species Distribution Models (iSDMs) could represent a sound instrument that merits further research. *Acacia saligna* is an Australian vascular plant introduced into Europe during the last half century and is one of the most aggressive IAS in the Mediterranean basin.

In this work, we model the occurrence of *A. saligna* in the coastal landscapes of central Italy (Adriatic coast) while accounting for the simultaneous effect of multiple factors (propagule pressure, abiotic, biotic factors). The iSDM for *A. saligna* was implemented on a representative tract of the Adriatic coast in central Italy (Molise region), largely included in two Long-Term Ecological Research (LTER) sites which actively contribute to the description of the considered ecosystem status and possible future trends. By using a Generalised Linear Model (GLM) with a binomial distribution of errors based on field and cartographic geo-referenced data, we examined the statistical relationship between the occurrence of *A. saligna* and a comprehensive set of environmental factors. The iSDM effectively captured the role of the different vari-

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ables in determining the occurrence of *A. saligna* in the coastal dunes. Its occurrence is primarily related to Wooded dunes with *Pinus pinea* and/or *P. pinaster* (EU Habitat 2270) and distance from the sea and, to a lesser extent, with distance from roads and rivers. This research provides a first exploratory analysis of the environmental characteristics that promote the rapid growth and development of *A. saligna* in Italian dune ecosystems, identifying the habitats that are mainly affected by the invasive process in coastal areas and, by doing so, contributing to filling the gap between theory and practice in conservation decision-making. Finally, the LTER network benefitted from this research, confirming its relevance in providing useful information for modelling and monitoring invasion processes.

Keywords

Abiotic factors, Biotic factors, Invasive species distribution model, Propagule pressure, LTER

Introduction

Biological invasions are one of the major global drivers of biodiversity loss, often resulting in economic damage and public health care problems (Hulme et al. 2009, Simberloff et al. 2013).

The establishment, growth and expansion of invasive alien species depend on a combination of mechanisms related to both ecology of the species and the assembly of environmental factors (Lockwood et al. 2005, Richardson and Pyšek 2006, Malavasi et al. 2018). Indeed, biological invasions are promoted by a wide range of drivers that can be schematically grouped into three main components, the so called PAB factors: Propagule pressure (P), Abiotic characteristics of the invaded ecosystem (A) and Biotic interaction between invasive species and recipient community (B) (Catford et al. 2009). It is well known that invasions cannot occur without adequate propagule pressure (P), defined as the frequency of plant propagule introductions (Eppstein and Molofsky 2007). Although several authors agree that P represents the key driver in the invasion process (Lockwood et al. 2005, Colautti et al. 2006, Simberloff et al. 2013), abiotic drivers also play an essential role as the invasion will fail if the invading species cannot survive the environmental conditions of a site (Weiher and Keddy 1995, Gallien et al. 2014). Finally, an alien species entering into a new area can either gain or lose biotic interactions capable of facilitating or constraining the invasion (Mitchell et al. 2006).

In order to deal with biological invasions and for preventing their negative effects on ecosystem biodiversity and functioning (Hulme et al. 2009), the European Union adopted a new regulation (EU-No 1143/2014, hereinafter EU Alien regulation) which sets guidelines for the management of Invasive Alien Species (IAS). This regulation underlines the importance of invasion prevention, early warning and rapid response followed by eradication and control measures (Genovesi et al. 2015). As stated by the EU Alien regulation, preventing biological invasions should be the most cost-effective way to minimise the impact of IAS on biodiversity. Still, new methodologies aimed at identifying early signs of invasion are needed (Sitzia et al. 2016). In this context, the use of invasive Species Distribution Models (iSDMs), aimed at investigating the

relationship between alien occurrence and PAB factors, should offer an effective tool to better understand biological invasions (Guisan and Thuiller 2005, Tulloch et al. 2016). Specifically, iSDMs analyse the statistical relationship between the presence of alien species (dependent variable) and environmental predictors (independent variables) (Elith and Leathwick 2009); they also allow both identification of the strength of the relationship between species presence and environmental variables and project the probability of occurrence of the species in wide areas in which the species is not present. Over the last twenty years, iSDMs have been widely implemented for conservation and management purposes (Elith and Leathwick 2009, Franklin 2010). Part of the research efforts has been devoted to unravelling the influence of different invasion drivers on the occurrence of some invasive species (Bazzichetto et al. 2018a, Bellard et al. 2016, Thuiller et al. 2005) and predicting the probability of invasion of one taxon (e.g. Gutierres et al. 2011) or a group of taxa (e.g. Malavasi et al. 2018).

Despite all these efforts, further research is still necessary, orientated towards implementing invasive species distribution models for supporting IAS management. A taxon for which important research efforts have been undertaken, but still requiring multivariate analysis of the different invasion drivers, is the Australian genus *Acacia*. *Acacia* sp. is a highly aggressive genus and one of the major invaders in the world (Castro-Díez et al. 2011, Richardson et al. 2011, Richardson and Rejmánek 2011). Amongst them, *A. saligna* (Labill.) H. L. Wendl. is one of the most invasive taxa of the genus (Richardson and Rejmánek 2011) and its spread is particularly worrisome in Italy and the rest of Europe (Wilson et al. 2011). Although the high potential of invasion of *A. saligna* is acknowledged and the role of several environmental factors in driving its invasion has been separately investigated (Hadjikyriakou and Hadjisterkotis 2002, Nsikani et al. 2017, Yelenik et al. 2004), studies exploring the simultaneous influence of these factors are needed.

In light of this, the present study sets out to model the occurrence of *A. saligna* in coastal landscapes of central Italy (Adriatic coast) while accounting for the simultaneous effect of propagule pressure, abiotic and biotic factors. By implementing an iSDM, based on field and cartographic geo-referenced data, we explored the relationship between the presence of *A. saligna* and PAB factors. We assumed that the invasion by *A. saligna* across the dune mosaic is not homogeneous, but varies through space, according to the distribution of the main PAB factors.

By identifying the factors related to higher occurrence values of the alien taxa and by mapping the areas with different probabilities of occurrence, we can identify new tools able to contribute to the prioritisation of conservation actions in coastal ecosystems, as required by the EU Alien regulation.

It is also worth mentioning that such iSDM implementation benefitted from the presence within the study area of two Long-Term Ecological Research sites (LTER) (http://www.lter-europe.net/), which actively contributed to the description of the considered ecosystem status and its possible future trends. Indeed, the LTER network in which ecosystem experts monitor a wide range of environmental variables may offer a rich overview of alien species distribution and invasion drivers across different ecosystems and geographical areas.

Materials and methods

Target species

A. saligna is one of the most invasive taxa of the genus *Acacia* (Richardson and Rejmánek 2011). The species was introduced into the coastal areas of South Africa and of the Mediterranean basin for reforestation, dune stabilisation and ornamental purposes (Bar Kutiel et al. 2004, Gutierres et al. 2011,Wilson et al. 2011). It expanded in an uncontrolled manner in coastal areas of Algeria, Cyprus, Israel, Italy, Kenya, Morocco, Portugal, South Africa and Spain (Wilson et al. 2011, Yelenik et al. 2004). It successfully colonised arid environments of the Mediterranean region with poor and periodically burnt soils (Bell et al. 1993). In Italy, *A. saligna* was introduced in the 1950s for the stabilisation of inner dunes (Izzi et al. 2007; Tulloch et al. 2016). Nowadays, the species is present in many coastal areas of Italy (Celesti-Grapow et al. 2010; Del Vecchio et al. 2013) and it tends to colonise a narrow coastal strip between the Mediterranean scrubs and the *Pinus* sp. woodlands of the fixed dunes (Del Vecchio et al. 2013).

A. saligna has several ecological features that favour its expansion in non-native environments. The clonal and sexual reproduction, high rate of growth, short juvenile period and high tolerance to environmental stress (Del Vecchio et al. 2013, Milton and Hall 1981, Witkowski 1994) are all traits that allow its expansion in a wide variety of ecosystems. Furthermore, the production of a huge number of long-lived seeds and the secretion of allelopathic substances ensure the persistence of the species in the soil seed bank and its chance to sprout over long periods (Mehta 2000, Abd El-Gawad and El-Amier, 2015, Strydom et al. 2012). *A. saligna* forms dense monospecific stands in which several native species are excluded (Yelenik et al. 2004), leading to a simplification of the structure and diversity of native plant communities (Calabrese et al. 2017, Cohen and Bar Kutiel 2017, Del Vecchio et al. 2013, Hadjikyriakou and Hadjisterkotis 2002). It also alters the soil properties as its invasion promotes changes in microclimatic conditions (Mehta 2000, Richardson et al. 2011, Calabrese et al. 2017) and to hydrological and nutrient cycles (Witkowski 1991, Yelenik et al. 2004), in particular the N-cycle (Yelenik et al. 2004; Le Maitre et al. 2011)

Study area

The study was carried out on a representative tract of the Adriatic coast of central Italy (Molise region). The coast is mainly composed of recent sandy dunes (Holocene), which occupy a narrow strip along the seashore. The dune system has a simple structure, being usually characterised by a single dune ridge with low elevation (less than 10 metres) (Acosta et al. 2009, Carranza et al. 2008). Here, the psammophilous vegetation distributes following a well-defined coastal zonation due to the presence of a sea-inland environmental gradient (Acosta et al. 2003, Prisco et al. 2012).

The intense and rapid land use change (Malavasi et al. 2013, 2016) and the introduction of exotic species are amongst the main threats affecting native communities of Adriatic coastal ecosystems (Romano and Zullo 2014, Calabrese et al. 2017, Malavasi et al. 2018). Nonetheless, the coastal dunes of Molise still host many ecosystems of conservation concern in Europe (the so-called EU habitat types according to the European Directive 92/43/EEC; Stanisci et al. 2014). Most of the analysed coastal sectors are included in Sites of European Conservation Concern (Foce Trigno – Marina di Petacciato: IT7228221; Foce Biferno -Litorale di Campomarino: IT7222216; Foce Saccione-Bonifica Ramitelli: IT7222217) and belong to the European LTER network (Bertoni 2012, Drius et al. 2013) (Fig. 1).

Acacia saligna occurrence records

To implement the analysis, we used presence data of *A. saligna* mostly collected inside LTER sites during the years 2013-15 (Calabrese et al. 2017, Del Vecchio et al. 2013). We used 30 presence points and 95 random-absences collected at a minimum distance of 100 m between each other and distributed along the whole coastal dunes in the area (Hijmans 2012). The number of absences was chosen in order to guarantee model adequacy and provide an accurate prediction (Franklin 2010).

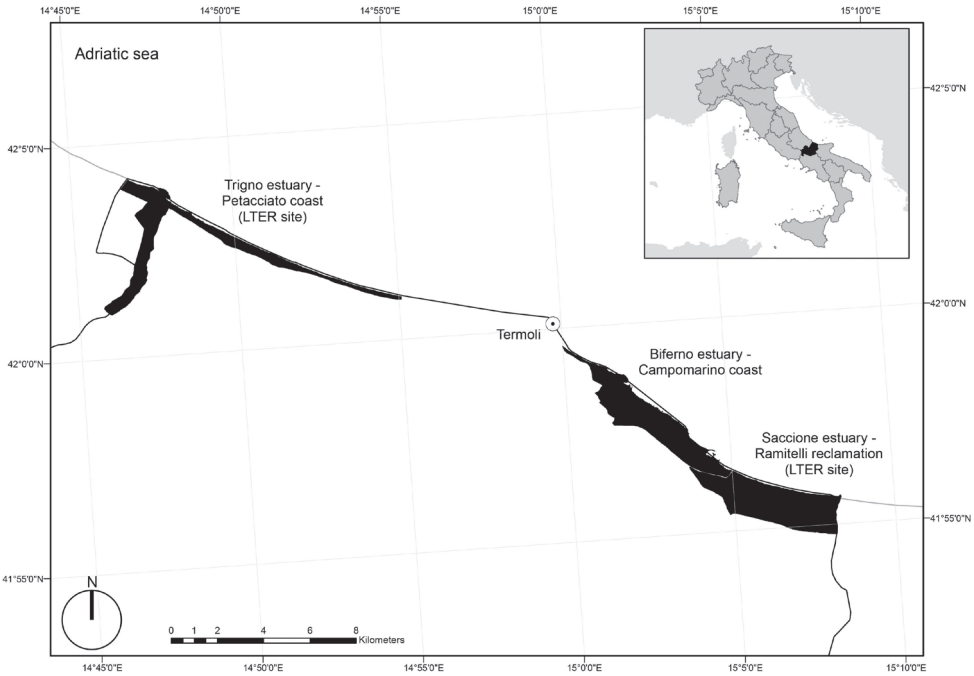

Figure. 1 Study area. Sites of European Conservation concern and LTER sites are shown in black. Coordinates are given in Datum: WGS 84.

Environmental data

In an ArcGIS environment (ArcGIS 10.2.2), a set of environmental variables was computed related to propagule pressure (P), abiotic (A) and biotic factors (B) and which were expected to influence the expansion of *A. saligna*. We selected road distance as a proxy of propagule pressure (Malavasi et al. 2014, Bazzichetto et al. 2018a), while sea distance and river distance were used as surrogates of abiotic conditions (Gutierres et al. 2011, Bazzichetto et al. 2018a). Finally, *Pinus* sp. wooded dune land cover and herbaceous dune vegetation land cover were used as a proxy of biotic conditions (Del Vecchio et al. 2013) (Table 1). Concerning propagule pressure, the distance from roads was considered because of its acknowledged role in favouring alien species dispersal (Le Maitre et al. 2004, Jørgensen and Kollmann 2009). Road distance was computed as the Euclidean distance between each point of occurrence of the invasive species and the closest road, including paved roads and secondary pathways (road data retrieved from https://planet.openstreetmap.org/; OpenStreetMap contributors 2017) that cross the dune systems. For abiotic factors, sea distance was considered because, in coastal dunes, it properly depicts the ecological conditions along the sea-inland stress gradient (Acosta et al. 2003, Drius et al. 2013, Bazzichetto et al. 2016). This sea distance was measured as the Euclidean distance of each point of occurrence from the shore line (obtained through photo-interpretation by Malavasi et al. 2013). Considering that *A. saligna* tends to prefer mesic conditions on arid landscapes, we used river/stream distance as a proxy of water supply (Le Maitre 2004, Gutierres et al. 2011). The distance from rivers was derived using the hydrography map and measured as the Euclidean distance of each point from principal rivers and streams of the Molise region (river data retrieved from https://planet.openstreetmap.org/; OpenStreetMap contributors 2017). Finally, as biotic factors, we considered the relationship between *A. saligna* occurrence with two natural dune vegetation types widely spread along the coast and characterised by different spatial structures and plant strategies: closed formations of *Pinus* woods and the herbaceous dune vegetation growing on fore dunes. We derived the percentage (%) of *Pinus* sp. woodlands and of herbaceous dune vegetation by ap-

Table 1. Predictors analysed. Propagule pressure (**P**), abiotic (**A**) and biotic (**B**) factors along with the corresponding proxy variables (predictors) used for implementing the iSDM.

Factor	Proxy variables (predictors)	Description
P	Road distance (m)	Euclidean nearest distance (m) from paved roads and secondary pathways
A	Sea distance (m)	Euclidean distance (m) from shoreline
	River distance (m)	Euclidean distance (m) from nearest river (main streams, river courses)
B	% *Pinus* sp. wooded dunes	Percentage of *Pinus* sp. wooded dunes within a 30 m radius window. Includes EU Habitat: 2270 – Wooded dunes with *Pinus pinea* and/or *Pinus pinaster*
	% Herbaceous dune vegetation	Percentage of herbaceous vegetation within a 30 m radius window. Includes embryonic shifting dunes (EC-2110), shifting dunes along the shoreline with *Ammophila arenaria* (EC – 2120) and *Malcolmietalia* dune grasslands (EC –2230).

plying a moving window procedure (buffer 30 m radius) FRAGSTATS; (McGarigal et al. 2012) across a fine scale (1:5000) land-cover map (Malavasi et al. 2013). The land-cover map conforms to the CORINE land-cover mapping procedure extended to a fourth level of detail for the forested and semi-natural categories (see Acosta et al. 2005 for details). The considered cover categories in the study area are linked with habitats of European conservation concern: wooded dunes with *Pinus pinea* and/or *Pinus pinaster* (EC-2270) and herbaceous dune vegetation, that includes the embryonic shifting dunes (EC-2110), shifting dunes along the shoreline with *Ammophila arenaria* (EC – 2120) and *Malcolmietalia* dune grasslands (EC – 2230). All the variables were reported into raster layers with 5 m resolution, which is an adequate spatial resolution for analysing the coastal dune environments in the Mediterranean basin (Bazzichetto et al. 2018a, Malavasi et al. 2018).

Species distribution models

The iSDM was based on a binomial Generalised Linear Model (GLM) aimed at analysing the relationship between the occurrence of *A. saligna* and the PAB variables (Hosmer and Lemeshow 2000). First, we performed collinearity analysis between these variables (see Appendix 1 for details) in order to exclude multi-collinearity (Brauner and Shacham 1998). Collinearity was assessed by means of Spearman's rank correlation (Ps) and by computing the Variance Inflation factor (VIF) (Brauner and Shacham 1998, Guisan and Thuiller 2005). A predictor was excluded by the model in case of high correlation, i.e. whenever the Spearman's correlation coefficient (Ps) was higher than 0.7 or lower than -0.7 and when VIF was higher than 3 (see Appendix 1 for collinearity analysis and variables selection).

Model performance was evaluated using two measures: the McFadden's R squared (McFadden 1973) and the area under the receiver operator curve (AUC) (Pearce and Ferrier 2000). The McFadden's R squared index, as the classical R squared, can assume continuous values from 0 to 1, with 0 indicating minimum and 1 maximum variability "explained" by the model (McFadden 1973). AUC has been widely used as a standard measure for assessing the predictive accuracy of SDMs (Lobo et al. 2008, Pearce and Ferrier 2000). In particular, the AUC was used to estimate the capacity of the model in predicting the presence of the species in location where the species was actually present and its absence where it was not recorded. AUC values between 0.5 and 0.7 indicate a poor discrimination capacity of the model, values between 0.7 and 0.9 represent a reasonable discrimination and an AUC greater than 0.9 indicates a very good discrimination capacity of the model (Pearce and Ferrier 2000). A robust estimation of the AUC can be gathered by cross-validating the model and averaging the single AUC values obtained from each cross-validation run. With this aim, the dataset was randomly partitioned into five subsets, using a 75% for model training and the remaining 25% to test prediction accuracy. Then, a final AUC value was computed by averaging between a single AUC obtained in each cross-validation run (Le Dell et al. 2015, Millar et al. 2011).

Results

According to the GLM model, the different PAB factors showed specific and significant relationships with the occurrence of *A. saligna*. (Table 2).

The variables, considered for modelling species occurrence, were independent with no significant Spearman's rank correlation coefficient and VIF values (see Appendix 1 for details).

The occurrences of *A. saligna* were associated with propagule pressure (P) (Table 2), with higher probabilities of finding the species in proximity to roads (Fig. 2). Concerning the abiotic variables (A), sea distance showed a significant and negative relationship with the presence of *A. saligna*, indicating that the species occurred in a specific sector of the dune system (50 – 100 metres to sea) (Table 2, Fig. 2). Similarly, *A. saligna* showed a significant negative relationship with river distance, indicating a link to humid conditions (Table 2, Fig. 2). Amongst the biotic factors (B), *A. saligna* was significantly associated with *Pinus* sp. dune woods. In particular, the probability of occurrence of *A. saligna* increased with higher percentages of *Pinus* dune woods (Fig. 2).

The fitted GLM explained 0.70 of the variability (McFadden's R squared= 0.70) and good predictive power (AUC mean = 0.96).

Discussion

In this study, we modelled the occurrence of *A. saligna* along the Adriatic coast in central Italy and identified the specific role of propagule pressure, abiotic and biotic factors in determining the presence of the species. The model showed a good power of prediction, as highlighted by the explained variability of the McFadden's R squared and predictive accuracy of the mean AUC, obtained through cross-validation.

Table 2. GLM model outcome. Response variable: *Acacia saligna* presence/absence; predictors: Propagule pressure, abiotic and biotic factors. For a detailed description of the predictors and the land cover types see Table 1.

Predictors	Estimate	Std. Error	Z value	p-value
Intercept	2.08	1.24	1.68	p>0.05
P (Propagule pressure)				
Road distance	-0.004	0.002	-2.059	*
A (Abiotic)				
Sea distance	-0.025	0.010	-2.639	**
River distance	-0.001	0.000	-2.289	*
B (Biotic)				
Pinus sp. dune wood	0.073	0.019	3.884	***
Herbaceous dune vegetation	0.003	0.016	0.202	p>0.05

*** p< 0.001;
** p < 0.01;
* p<0.05)

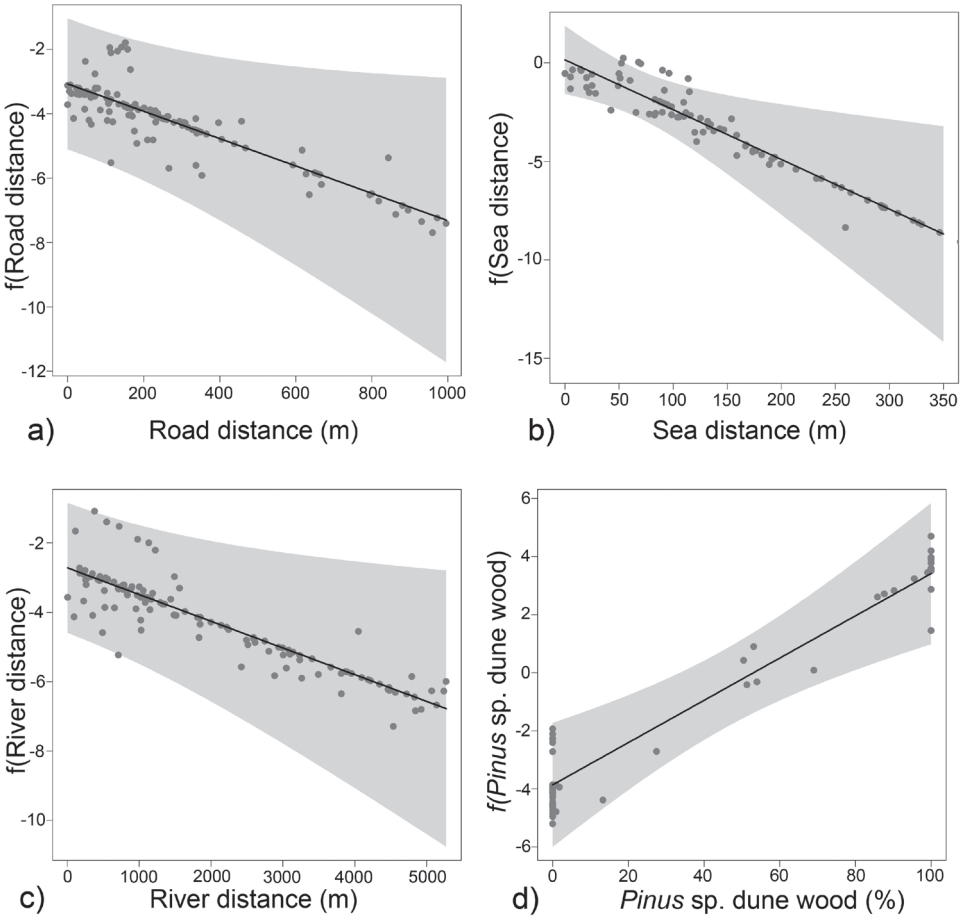

Figure 2. Regression curves. Relationship between *A. saligna* occurrence and the PAB predictors **a** Road distance **b** Sea distance **c** River distance **d** % of *Pinus* sp. dune wood). On the x-axis: predictors with the corresponding unit of measurement. On the y-axis: the residual values for each predictor.

Our results highlighted that the invasion by *A. saligna* was not spatially homogeneous, but varied across the coastal landscape, following the spatial distribution of the different PAB factors. *A. saligna* preferentially occurred close to the coastal pine forest, at an intermediate distance from the coastline, preferably 50-100 metres from sea and its presence was also related to distance from roads and rivers. Specifically, *A. saligna* occurrence is promoted by propagule pressure, which along the Mediterranean coasts can be related to distance from roads (Arévalo et al. 2005, Malavasi et al. 2014, Bazzichetto et al. 2018b). In addition to the acknowledged role of roads in supporting alien species dispersal (Jørgensen and Kollmann 2009, Le Maitre et al. 2004), roads can also fragment forested areas, thus altering the undergrowth light conditions (Gutierres et

al. 2011) and creating gaps of favourable habitat for *A. saligna* (Flory and Clay 2006, Gutierres et al. 2011, Parendes and Jones 2000). Indeed, forest edges are characterised by microclimatic conditions of temperature and soil moisture (Brothers and Spingarn 1992, Gehlhausen et al. 2000) that promote the growth of edge species (Carranza et al. 2012), most of them being weeds and aliens (Spellerberg 1998).

Moreover, the model also highlighted that abiotic factors regulated *A. saligna* invasion. Coastal dune ecosystems are characterised by a mosaic of habitats in which the gradual change in abiotic conditions shapes the growth of the species, thus determining the typical sea-inland ecological gradient (Acosta et al. 2003, Drius et al. 2013). Sea distance is a good proxy of such gradient (Bazzichetto et al. 2016) and the observed correlation of *A. saligna* occurrence with sea distance underlined the influence of this complex environmental gradient on the invasion process. Indeed, *A. saligna* tends to invade specific sectors of the dune system (Cohen et al. 2008, Midgley and Turnbull 2003) and, according to our results, this species preferentially occurred on sparsely vegetated fixed dunes. Probably this trend is also related with soil characteristics that, in the inner dune sectors, are more compact and with lower salt concentration (Santoro et al. 2011).

Similar behaviour was observed in other ecosystems characterised by dry sandy soils, (e.g. South-African fynbos, coastal sand dunes of Israel) in which *A. saligna* invades areas with open or patchy vegetation (Mehta 2000, Bar Kutiel et al. 2004) and it was attributed to its good competitive strategy for using water resources (Witkowski 1991, Yelenik et al. 2004).

In confirmation of this, river distance (a proxy of soil moisture) seems to favour *A. saligna* growth in this Adriatic sector. The observed correlation highlighted the tendency of the species to grow and develop in the most humid areas of arid coasts of the Mediterranean climatic region (Bar Kutiel et al. 2004; Gutierres et al. 2011). Due to its preference for mesic conditions on arid landscapes, *A. saligna* is commonly associated with areas close to the river courses on both inland sectors and dunes (Gutierres et al. 2011). Furthermore, the proximity of rivers, besides indicating the availability of moisture, should imply the presence of disturbance that removes competing plants, making such landscapes particularly sensitive to invasion (Mehta 2000, Gutierres et al. 2011).

Biotic conditions also affected the distribution of *A. saligna*, which preferentially invaded areas close to pine forests. The preference of the invader for pine forests on Mediterranean coasts is particularly worrisome because this formation, with high historical and social value for the territory (Del Vecchio et al. 2013), is a priority habitat of European conservation concern (Habitat 2270* Wooded dunes with *Pinus pinea* and/or *P. pinaster*) (Bonari et al. 2017, 2018). Thus, our results pinpointed the need for defining adequate management actions to counteract the invasion risk. Most of the areas covered by Wooded dunes with *Pinus pinea* and/or *P. pinaster* in the Italian peninsula derive from old afforestations (Falcucci et al. 2007) carried out to protect the inner coastal plains and for land reclamation purposes (Malavasi et al. 2013). The structure and floristic composition of these old pine formations has evolved over time and now is characterised by the presence of a good cohort of Mediterranean scrub species that forms the understorey corresponding to canopy gaps. The presence of canopy gaps that

allow the settlement of fast-growing and light-demanding species would make old pine forests particularly vulnerable to alien invasions (Burnham and Lee 2010, Del Vecchio et al. 2013, Gray 2005, Selmants and Knight 2003) and be threatened specifically by *A. saligna*. The results obtained using LTER data, should offer the basis for prioritising monitoring efforts on areas more susceptible to invasion, thus optimising the resources and time devoted to managing alien species expansion.

Similarly to that observed by Malavasi et al. (2018) using the LifeWatch biological database, the utilisation of the LTER network constitutes a sound tool for modelling biological invasions, promoting the sharing of unprecedented amounts of data amongst ecologists. Furthermore, the multi-temporal nature of LTER data offers a unique opportunity to monitor variations and environmental conditions over time and also to identify, through multi-temporal iSDMs (e.g. Carone et al. 2014), the factors and phenomena that underlie the changes occurring in invasive alien species distribution over time.

Conclusion

This integrative analysis of the occurrence of the non-native species *A. saligna* in coastal landscapes, including a single model using the simultaneous effect of propagule pressure, abiotic and biotic factors, allowed us to effectively depict those critical drivers for determining the presence of this highly invasive plant in the Mediterranean dunes and to define areas with different probabilities of invasion. Invasion by *A. saligna* was not homogeneous but varied across the coastal landscape, following the spatial distribution of different factors. Indeed, invasion preferentially occurred on coastal fixed dunes close to pine forests. The implemented iSDM provided valuable insights into the invasion process and it supplied an efficient prediction of the invasion processes in this stretch of the Adriatic coast, providing the necessary instruments for the assessment of invasion risks claimed by the EU Alien regulation (EEC 2014).

Finally, as the presence of a valuable amount of data collected across a network of LTER sites supported the implementation of a more effective iSDM, it is also true that the LTER network benefitted from such research, confirming its relevance in providing useful information for modelling and monitoring invasion processes. Furthermore, by monitoring variations in environmental conditions, it is possible to identify, through multi-temporal iSDMs, the factors and phenomena that allow alien species expansion over time. With this in mind, we hope that other LTER network-based case studies could be further carried out to provide integrated information across a wide range of monitored ecosystems and for increasingly larger areas.

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Appendix 1

The variables considered for modelling species occurrence are lower than the threshold values of both Spearman's rank correlation coefficient and VIF values (Table A1). Sea distance and herbaceous dune vegetation are the variables with greater correlation score (-0.6); instead the lowest correlation was found between herbaceous dune vegetation and road distance (0.00). Pinus dune wood in the multi-collinearity test has the higher VIF (2.56), while road distance has the lowest (1.12).

Table A1. Correlation analysis. Spearman's rank correlation coefficient and Variance Inflation factor (VIF) for all predictors.

Predictors	Spearman's rank correlation					VIF
	***Pinus* sp. dune wood**	**Road distance**	**Herbaceous dune vegetation**	**Sea distance**	**River distance**	
Pinus dune wood						2.56
Road distance	0.15					1.12
Herbaceous dune vegetation	0.01	0.00				1.22
Sea distance	-0.35	-0.05	-0.60			1.43
River distance	-0.07	0.17	-0.04	0.06		1.19

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RESEARCH ARTICLE

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Plant diversity changes in a nature reserve: a probabilistic sampling method for quantitative assessments

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Abstract

Species pool conservation is critical for the stability of ecosystem processes. However, climate and land use changes will likely affect biodiversity, and managers of protected areas are under increasing pressure to monitor native species diversity changes by approaches that are scientifically sound and comparable over time. Here we describe a plant diversity monitoring system in use since 2002 in the "Montagna di Torricchio" Nature Reserve (LTER_EU_IT_033), a Central Apennines representative area of 317 ha, most of which is under strict protection. The aim of this paper was to assess changes in plant species richness over time and to deduce the patterns of species assemblage. The monitoring system was based on a probabilistic sampling design representative of the different physiognomic vegetation types occurring in the Reserve. A total of 34 plots (10×10m) were sampled in 2002, 2003 and 2015, and their species presence/absence and relative coverage were estimated. Repeated measure ANOVA was used to test for plot-level and ecosystem-based changes in species richness along the study period. Temporal nestedness and temporal turnover metrics were used to assess patterns of species' compositional changes. The results

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showed significantly different levels of species richness depending on the year, with the lowest value in 2003, probably linked to extreme drought events. Forest systems were comparatively stable, demonstrating the capacity to buffer interannual climate variability. Regarding compositional changes along the entire period (2002–2015), we found random patterns of both temporal nestedness and turnover, indicating stability in species composition. However, we also showed the contemporary occurrence of species loss and species replacement processes, considering the dry year 2003, a finding which should be further explored through fine-scale studies to unravel mechanisms of community assembly under drought. The use of a probabilistic sampling design representative of the different physiognomic vegetation types proved to be advantageous in monitoring the Nature Reserve vegetation and collecting reliable quantitative information. This data, in turn, provides the basis for improvements in management practices and proposed adaptation measures.

Keywords

compositional changes, monitoring, LTER, species richness, temporal nestedness, temporal turnover

Introduction

Species pool conservation is critical to the stability of ecosystem processes (Loreau et al. 2001). However, climate and land use changes will likely affect biodiversity at local to landscape scale (Sala et al. 2000; Thomas et al. 2004). Despite commitments by the international community to halt biodiversity decline, conservation efforts targeting plant diversity are often hampered by the lack of suitable data for prioritising conservation action (Darbyshire et al. 2017). In particular, one of the main debates in the field of conservation biology is the assessment of results obtained in efforts to meet conservation goals fixed by international treaties, evaluating the efficiency of existing protected areas (Vellak et al. 2009; Capotorti et al. 2012; Pringle 2017). In Europe, the implementation of the EU Directives 09/147/CE (Birds Directive) and 92/43/CE (Habitats Directive) in the Natura 2000 network has been the main pillar for biodiversity conservation. Here, biodiversity monitoring is legally binding (i.e. articles 11 and 17 of the Habitats Directive, Bock et al. 2005). In this context, the assessment of species diversity is crucial, since this fundamental property of ecological communities can serve as a parameter for comparing assemblages in time and space, independently from species identities (Chiarucci et al. 2008 and references therein). Managers of protected areas are thus under increasing pressure to monitor species diversity changes by approaches that are scientifically sound and comparable over time. Many monitoring approaches are highly subjective in the choice of where to position observational units, thus violating the assumptions of randomness, independence and known probability (Chiarucci 2007). On the contrary, the selection of observational units driven by a probabilistic approach permits sound generalisation (Lájer 2007).

Global change trends are undoubtedly producing effects on species diversity in different ecosystems (e.g. van Vuuren et al. 2006; Chelli et al. 2017; Rogora et al. 2018). In addition, there is growing consensus on the major impact of interannual climate variability (Grime et al. 2008; Cleland et al. 2013) and extreme weather events on plant communities (Jentsch et al. 2007), including seasonal effects (Chelli et al. 2016) e.g., extreme drought (Wellstein et al. 2017). In order to assess these changes in plant

communities, species inventories and long-term monitoring of permanent plots are essential. Among different climatic parameters, interannual rainfall variation seems to have the most significant effect on plant species richness, composition and functioning (Adler and Levine 2007), especially in ecosystems where water is a primary limiting resource (Chelli et al. 2017). Regarding the direction of changes, resource supply influences species richness via its effects on the competitive interactions and growth rates of species already present in the local community (Adler and Levine 2007). Traditional explanations foresee that decreasing resources may increase species richness due to the reduced competitive strength of the dominant species (Grime 1979), or instead may reduce species richness by allowing a lower number of species to tolerate the environment (Preston 1962, Wright 1983). In Mediterranean-type ecosystems Chiarucci et al. (2003) found a negative effect of drought on species richness. However, it has been demonstrated that at relatively small geographic or local scales, different factors provide a spatial and functional buffer against the uncertainty of interannual climate variability and short-term extremes, as well as soil properties and heterogeneity (Jentsch and Beierkuhnlein 2008; Fridley et al. 2011), complex topography (Loarie et al. 2009), ecosystem structure (e.g. canopy cover in forests, Chen et al. 1999) and plant phenotypic plasticity (Wellstein et al. 2013).

In addition to climate, dynamic processes deriving from land use changes can produce significant changes in species richness and composition both in grasslands (Catorci et al. 2013; Giarrizzo et al. 2017) and forests (Bartha et al. 2008), due to competitive exclusion of subordinate species (Wellstein et al. 2014; Tardella et al. 2017) or to shifts in abiotic conditions (Campetella et al. 2011).

In addition to species richness, patterns of species assemblages over time are also particularly significant for understanding ecological processes (Kopecký et al. 2013). In this context, analysis of temporal beta diversity (variation of the species composition of plant assemblages over time) may reflect two different phenomena: species turnover and nestedness of assemblages. They result from two antithetic processes (Gaston and Blackburn 2000), namely, species replacement resulting in species turnover and species loss resulting in nestedness (Baselga 2010). Nestedness occurs when species-poor assemblages represent a subset of more species-rich communities (Patterson and Atmar 1986), and thus difference in species richness is a necessary condition for nestedness (Ulrich and Almeida-Neto 2012). According to Elmendorf and Harrison (2009), nestedness can occur when most species respond similarly to interannual variation in conditions. On the contrary, turnover can occur when different sets of species are present in different years, either because of different resource requirements (due to different environmental conditions) or as a result of competitive exclusion.

Despite their potential, such metrics have been rarely used in diachronic datasets (but see Elmendorf and Harrison 2009 in grasslands; Kopecký et al. 2013; Canullo et al. 2017, in forest ecosystems) and never used in monitoring programmes encompassing different ecosystems.

Temporal analyses of this kind could be affected by imperfect detection of species, such as when species are overlooked, misidentified, or when assessments contain errors, and this can lead to 'pseudoturnover' (Morrison 2016). Therefore, a stand-

ard, replicable and statistically representative method is suggested to avoid one of the most common causes of unreliability in the comparison of diversity assessments. To assess and compare biodiversity over time, a random sampling design is recommended (Magurran 2004).

The aim of this paper was to assess changes in vascular plant species richness over time and to deduce the patterns of species assemblage through a monitoring system based on a standard probabilistic sampling design. The "Montagna di Torricchio" LTER site (LTER_EU_IT_033), which includes typical Mediterranean-montane systems, was used as the study area. The study site is part of the Italian "Important Plant Areas" identified by Blasi et al. (2011) and is included in the Natura 2000 network (IT5330022 - Montagna di Torricchio).

In particular, we hypothesised that:

(a) species richness at plot level significantly changes over time according to inter-annual climate variability (including extreme climatic events, e.g. drought) and/or dynamic processes deriving from land-use change;
(b) forest ecosystems buffer climate variability and show a certain stability in terms of species richness over time, compared to grasslands. Finally, we explored the patterns of species compositional changes over time (in terms of both temporal nestedness and temporal turnover) along the entire monitoring period and for each couple of years of relevés. Given the scarcity of approaches exploring patterns of compositional changes in different ecosystems, our findings can be useful to support future hypotheses for monitoring programmes in complex landscapes.

Methods

Study area

The "Montagna di Torricchio" Nature Reserve is located in the Central Apennines, Italy (+130050E, +425740N, WGS84; Fig. 1a). It covers an area of 317 ha along two north- and south-facing slopes of a SW-NE oriented valley, spanning an elevation from 820 to 1490 m a.s.l. Mean annual precipitation reaches 1250 mm and mean annual temperature is around 11 °C. Jurassic-Cretaceous limestone prevails in the area (Kwiatkowski and Venanzoni 1994). The Reserve hosts different ecosystems, including xeric and mesic grasslands, grasslands with scattered shrubs, sub-Mediterranean hop-hornbeam (*Ostrya carpinifolia*) and ash (*Fraxinus ornus*) open woods, and beech (*Fagus sylvatica*) forests. The area has been under strict protection since 1970 with legacies for nature conservation, research and education. The management plan is devoted to protecting the natural processes that have been underway since the 1960s, when traditional management began to be abandoned, leading to natural succession. Previously, the grasslands had been managed as sheep-pastures, and forests had been coppiced with a rotation cycle of about 25 years (Campetella et al. 2016).

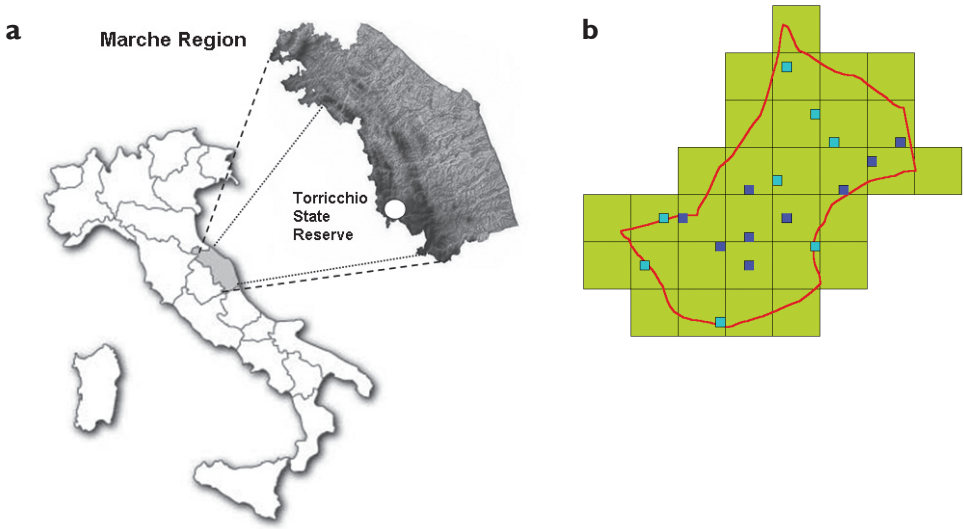

Figure 1. a Location of the "Montagna di Torricchio" Nature Reserve (LTER_EU_IT_033) in the Central Apennines, Marche Region, Italy (From: Wellstein et al. 2014) **b** Representation of the sampling system: the 25 ha grid was superimposed on the reference area, with the 17 macroplots randomly selected (each containing a cluster of four 10×10 m plots); the dark blue plots contain the clusters lying within the effective area of the Reserve, which is the subject of the present paper.

Sampling design

Based on a multi-scalar sampling protocol adopted to assess plant biodiversity (originally including three grain-levels: 1 m^2, 100 m^2 and 10,000 m^2, see Baffetta et al. 2007, Santi et al. 2010), the information on vascular plant diversity was collected at the 100 m^2 scale, 10×10 m (hereafter referred to as plot). The probabilistic sampling design was constructed on the Regional Technical Maps 325010 (1:10,000) georeferenced through the UTM (ED50) coordinates. The map was divided into a 500×500 m grid covering a reference surface for the Nature Reserve, resulting in 17 cells, and one macroplot of 100×100 m was randomly selected on each cell. A cluster of four random points, identified as the SW vertex of the corresponding 10×10 m plots, was generated within each single macroplot. Only the nine clusters fully included in the Nature Reserve were considered (Fig. 1b), located between 1,000 and 1480 m a.s.l. at both S- and N-facing slopes, encompassing xeric grasslands with *Bromus erectus*, *Sesleria nitida*, *Asperula purpurea*, *Brachypodium rupestre*, different successional stages (with shrubs as *Cytisus sessilifolius*, *Juniperus communis*, *Rosa* sp.), sparse thickets and forest communities dominated by *Fagus sylvatica*. Each plot was identified in the field, georeferenced (error ± 10m) and materialised with poles at the corners. Surveys were performed in the summer of 2002, with resampling in 2003 and 2015. Overall, 36 plots occurred in the Reserve; however, two of them were not considered because of problems with plot identification in the field, resulting in a total of 34 plots sampled. Field relevés

were performed to record the species composition and the relative structure of the vascular plant communities, by visual assessment of the abundance (Braun-Blanquet 1964). Further factors that helped us avoid overlooking or misidentifying species were our reference to a standard protocol and a common taxonomic source (Pignatti 1982), continuously updated knowledge of the species pool of the "Montagna di Torricchio" Nature Reserve, the availability of a dedicated section of the Herbarium CAME at the University of Camerino, and the assistance of specialists (as recommended by common international references; cf. Canullo et al 2013). Sintetic climatic data (yearly and seasonally aggregated) were obtained by yearly reports from the Agency for Agro-food Sector Services of the Marche Region (ASSAM).

Data analysis

Changes in species richness over time

The changes in the mean number of species in the study period, considering the entire dataset and the two main vegetation types separately (grasslands, including grasslands with shrubs, n = 22; forest habitats, n = 12), were tested using repeated-measures ANOVA. The Bonferroni post-hoc test was used to assess differences in species richness among the three study years.

Temporal nestedness analysis

In order to assess the species nestedness that occurred among the three survey periods (2002, 2003, 2015), we used temporal nestedness analysis (TNA). We compared the observed temporal nestedness (TN) between different survey periods (2002 vs 2003, 2003 vs 2015 and 2002 vs 2015) with the distribution of 999 TN values generated by random reshuffling of the survey period between temporally paired samples. More specifically, TNA is based on a comparison of the observed temporal nestedness between subsequent surveys from the same sample and the nestedness of the same subsequent surveys whose species configuration was randomly reshuffled according to species presence/absence collected in the field. In detail, (a) we calculated the Nestedness measure based on Overlap and Decreasing Fill for sites (NODFsites; Almeida-Neto et al. 2008), between each pair of the temporally sorted old and recent samples, then (b) we averaged these pairwise NODFsites values into a TN value of the whole data set and, finally (c) we compared the observed TN between the two selected survey periods to the distribution of 999 TN values generated by the randomisation procedure. For further details related to this methodology, please refer to Kopecký et al. (2013).

Temporal turnover analysis

To quantify the species temporal turnover (TT) that occurred among the three survey periods (2002, 2003, 2015), we used the Simpson dissimilarity index (βsim) based on

pairwise comparison (Baselga 2010; Canullo et al. 2017). Baselga (2010) described βsim as one of the components of the total beta diversity. Therefore the turnover analysis was calculated as follows:

$$\beta sim = \min(b,c)/[a+\min(b,c)]$$

where *a* is the number of species common to both plots, *b* is the number of species that occur in the first plot but not in the second and *c* is the number of species that occur in the second plot but not in the first (Baselga 2010). We used the same randomisation method as adopted for the TNA.

All statistical analyses were performed with R, version 2.14.1. In particular, the following R packages were used: the *stats* package for repeated measures ANOVA; the *betapart* package (function beta.pair) for the Simpson dissimilarity index calculation; the *vegan* package (function nestednodf) for temporal nestedness analysis.

Results

Species richness

Overall, 345 species were sampled during the monitoring period. The lowest number of species was recorded during 2003 (210 species), while 2015 showed the highest number of species (271 species). At plot level, species richness significantly differed among years (Sum of squares = 2811.78, df = 2, F = 24.69, $p < 0.001$, Fig. 2a). Considering the two vegetation macro-types separately, grasslands showed significantly different levels of species richness among the three years (Sum of squares = 2366.20, df = 2, F = 28.43, $p = 0.001$, Fig. 2b), while in forests we did not note significant changes in species richness at the plot level (Sum of squares = 555.38, df = 2, F = 3.21, $p = 0.060$, Fig. 2b).

Species compositional changes

The observed TN value along the entire period (2002–2015) did not differ significantly from the randomly generated assemblages (TN = 11.07, SES = -0.95, $p > 0.05$, Fig. 3a). On the contrary, the species assemblages in the year 2003 were temporally nested subsets of those from the year 2002 (TN = 31.28, SES = 4.36, $p = 0.001$, Fig. 3b), while in the comparison 2003–2015 the observed TN values were significantly lower than those of the randomly generated assemblages (TN = 4.10, SES = -2.95, $p = 0.001$, Fig. 3c), probably due to an increase in species richness over time.

Similarly, the observed TT value along the entire period (2002–2015) did not significantly differ from the randomly generated assemblages (TT = 0.37, SES = -1.56, $p > 0.05$, Fig. 4a). However, in the comparisons 2002–2003 and 2003–2015, the observed TT values were significantly higher than those of the randomly generated assemblages (TT = 0.65, SES = 3.57, $p = 0.001$, Fig. 4b; TT = 0.60, SES = 2.58, $p = 0.007$, Fig. 4c, respectively).

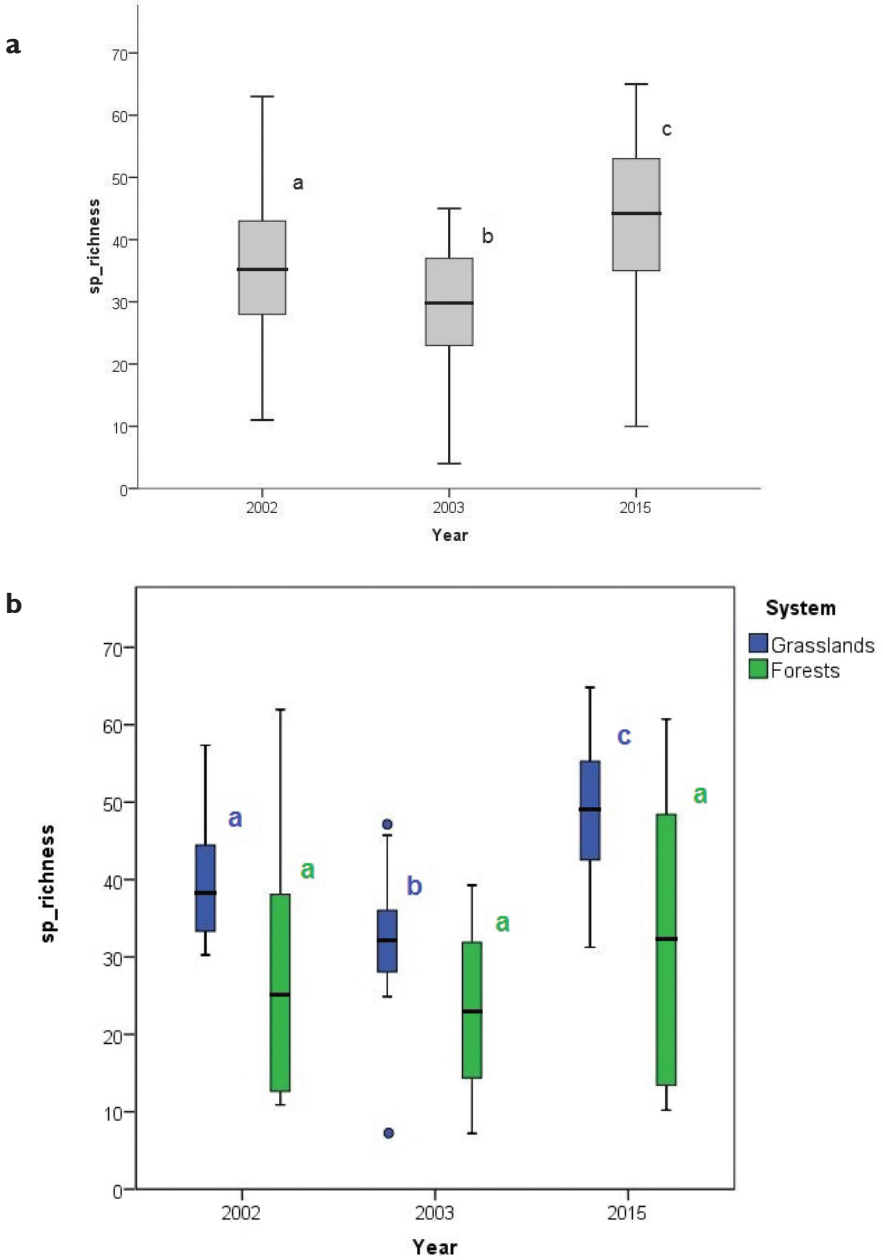

Figure 2. Mean values and variability of species richness per plot **a** for all the ecosystems, and **b** according to the two main ecosystems, i.e. grasslands (blue colour) and forests (green colour). Different letters indicate significant differences in mean values across time following post-hoc test results.

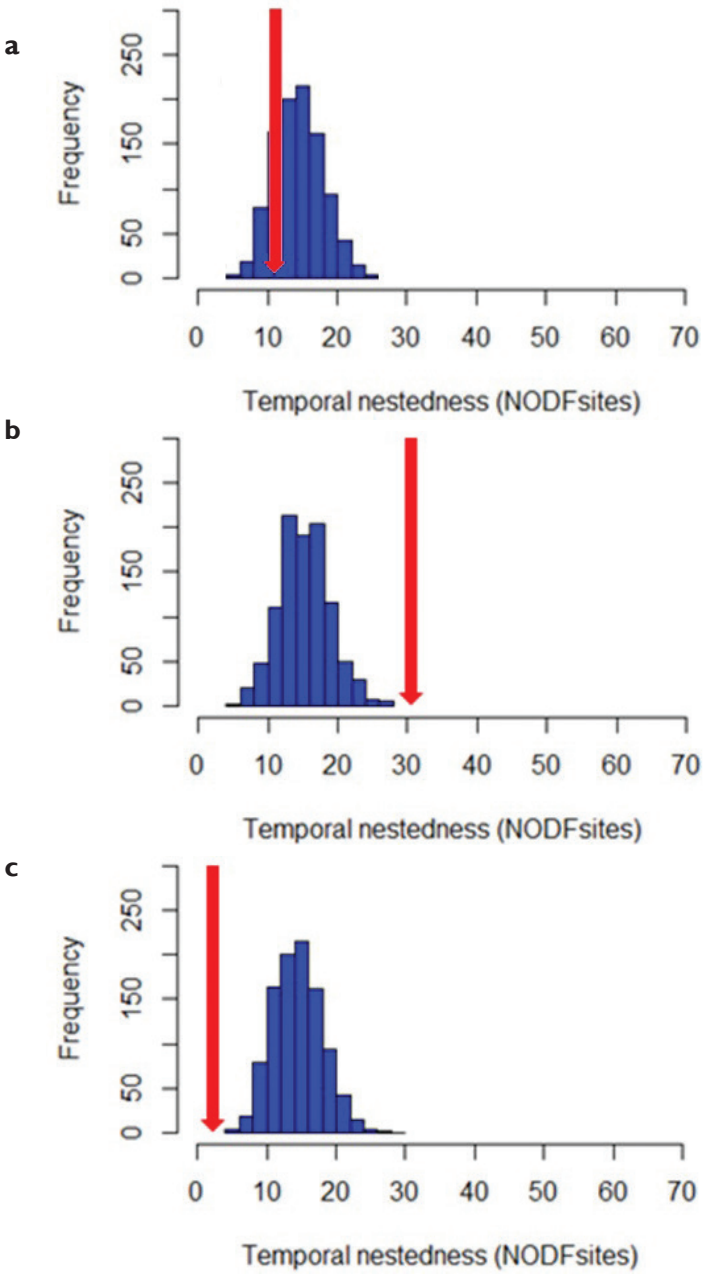

Figure 3. Temporal nestedness observed for **a** 2002 vs 2015 surveys **b** 2002 vs 2003 surveys and **c** 2003 vs 2015 surveys. The arrow indicates the position of the observed temporal nestedness value (NODFsites) with respect to the scores of the random loop (999 random nestedness values).

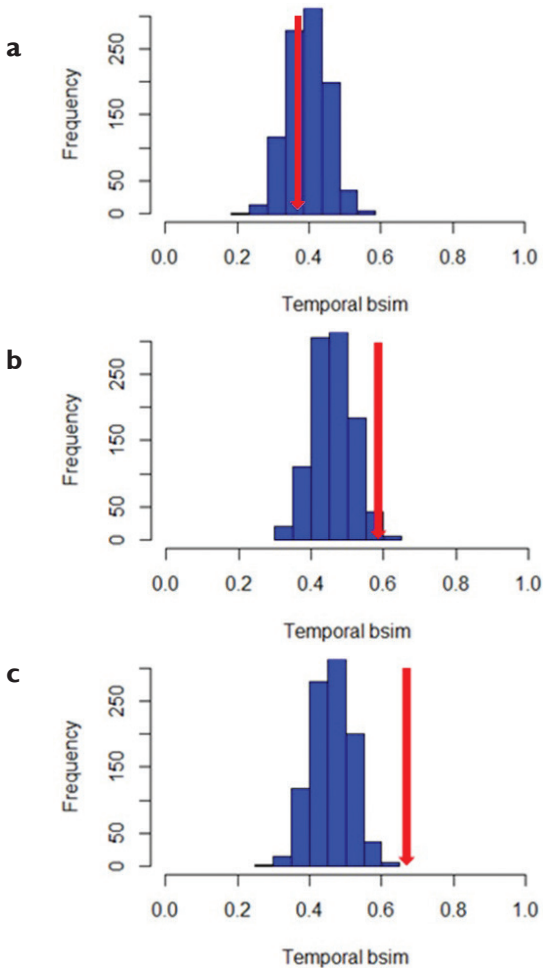

Figure 4. Temporal turnover observed for **a** 2002 vs 2015 surveys **b** 2002 vs 2003 surveys and **c** 2003 vs 2015 surveys. The arrow indicates the position of the observed temporal turnover value (bsim) with respect to the scores of the random loop (999 random turnover values).

Discussion

Our results showed significantly different values of species richness at the plot level, confirming our prediction, with the year 2003 having the lowest species richness value. The three study years showed different climatic conditions: the vegetative season of 2003 had 47% less precipitation than that of 2002, and 57% less than that of 2015. Focusing on spring, the year 2003 was particularly dry, registering 42% and 73% less precipitation than that measured for the spring seasons of 2002 and 2015, respectively (Source: ASSAM, Marche Region). Seasonal distribution of precipitation is known to influence processes triggering plant survival and growth, such as tiller production,

root-shoot biomass, root depth, canopy leaf area, stomatal conductance and photosynthesis (Zeppel et al. 2014). In particular, studies demonstrated the effect of spring climatic conditions on species growth rate (Battipaglia et al. 2009), plant functional traits (Wellstein et al. 2017), ecosystem productivity (Chelli et al. 2016), species cover and richness (Chiarucci et al. 2003) in sub-Mediterranean systems where water availability represents a strong limiting factor (Chelli et al. 2017). Our results, using a probabilistic sampling design and a diachronic dataset, confirm a significant reduction of species richness at plot level as a quick response to drought. Unfortunately, we did not repeat the sampling in the years immediately after the dry spell of 2003, thus we cannot infer information about species richness related to the timing of recovery.

Exploring in detail the pattern of species richness variation across the two main systems of the Nature Reserve (grasslands and forests), our results showed that forest ecosystems maintain a certain stability in terms of species richness at plot level over time, according to our predictions. The structure of ecosystems is known to influence local microclimate which in turn can buffer macroclimate variability. This is the case with forest ecosystems, where the canopy cover regulates temperature, light availability, wind speed and soil moisture, affecting ecological processes of the understory layer (Cervellini et al. 2017), including plant growth and survival, soil respiration and nutrient cycling (Chen et al. 1999). In addition, forest understories are mainly composed of perennial species, which are less sensitive to interannual climate variability. On the contrary, in line with our results, several studies reported yearly fluctuation of species richness in parallel with seasonal climatic conditions in Mediterranean and sub-Mediterranean grasslands (Chiarucci et al. 2003; Elmendorf and Harrison 2009). However, the relationship between these patterns and weather conditions is still not well understood. These systems are characterised by the occurrence of several annual species that can complete their life cycle rapidly during periods when conditions are favourable and survive unfavourable conditions (including drought) as seeds. In the studied grasslands we recorded during the dry 2003 the absence of many annual species, for example *Poa annua*, *Linum catharticum*, *Bromus hordeaceus*, *Alyssum minus*, *Myosotis* sp., *Bupleurum baldense*, and *Polygonum arenastrum*, all of which were present in the 2002 and 2015 relevés. On the contrary, perennial species occurring in grassland systems of the study area were more stable over time. They are mainly clonal and equipped with different types of belowground storage organs (e.g. rhizomes and tap roots, Halassy et al. 2005), and thus can deal with climate variability by using their stored energy and adjusting their growth rate (Wellstein et al. 2017). Instead, many perennial non clonal forbs and shrubs have deep rooting systems (e.g. *Astragalus sempervirens*, Wellstein et al. 2013), which are widely thought to buffer the plants from drought because they allow access to deeper water (West et al. 2012).

Along the entire monitoring period (2002–2015), the species' compositional changes were random, i.e. not related to significant patterns of species impoverishment or species turnover, indicating a certain stability in terms of species composition without tendencies due to the legacy of the previous land use. This result highlights that in the study area, the time interval of 13 years was not wide enough to detect successional effects in terms of compositional changes. However, comparisons including the dry year

2003 showed significant patterns of both species' impoverishment and turnover, with important implications in terms of community assembly. Our data probably illustrates how interannual environmental variation can generate communities that are nested across time: common species are always present, while rare species disappear in bad years such as the dry 2003, largely as a function of their relative abundances (Elmendorf and Harrison 2009). In parallel, we also observed a significant species turnover with respect to 2003, in line with Ulrich et al. (2017) who found that species turnover was expected to be important only in the case of high environmental heterogeneity (both spatially and temporally). This result probably depends on two mechanisms. First, the occurrence of species that can benefit from dry conditions (bad-year specialists, Elmendorf and Harrison 2009). This is in line with the findings of Wellstein et al. (2017) who showed how some grass species in sub-Mediterranean ecosystems (e.g. *Bromus erectus* and the endemic *Sesleria nitida*) surprisingly benefited from extreme drought. Second, the reduced competitive strength of dominant species due to drought (e.g. clonal grasses forming high-density tussocks or dense carpets, Wellstein et al. 2014), thus enabling more subordinate species to coexist. However, the contemporary occurrence of both species replacement and species loss processes resulted from this research in the dry year calls for more detailed diachronic studies. Here, experimental approaches manipulating water availability (see Jentsch et al. 2007; Jentsch and Beierkuhnlein 2008; Chelli et al. 2017) could be key to assessing fine-scale mechanisms of community assembly.

Conclusion

Thanks to the long-term approaches, LTER sites can provide unique insights potentially useful for managers of protected areas where there is the urgent need of monitoring species diversity over time. Our approach demonstrated the usefulness of long-term plant diversity monitoring programmes based on probabilistic sampling designs for the study of species richness and patterns of species assemblages over time in a protected area. Although we did not study environmental drivers directly, we showed how interannual climate variability could play a key role in shaping plant species richness and assemblages over time. Forest systems seem to buffer the impact of short-term variations, at least in terms of species richness. Nevertheless, the effects of interannual climate variability on species interactions and community structure are only beginning to be evaluated empirically, and more studies are needed. Accordingly, we suggest several considerations for future studies. First, spatial scale should be taken into account, since changes in species richness across vegetation types varies with scale of observation (Peet et al. 2014); moreover, a characteristic scale of spatial assemblages can emerge along the dynamics (e.g., Bartha et al. 2004). Second, plant cover values should be used as an indicator of species abundance, together with information on species presence/absence, to better assess community changes at multiple scales (Chiarucci et al. 2003). Third, approaches based on plant traits or functional groups can likely provide novel insights about the drivers of species diversity changes and ecosystem functioning (Suding et al. 2008).

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RESEARCH ARTICLE

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Multiannual decrement of nutrient concentrations and phytoplankton cell size in a Mediterranean reservoir

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Abstract

Reservoirs are primary water resources for many uses in the Mediterreanean region and need dedicated studies for understanding the complexity of their dynamics particularly vulnerable to local and global stressors. This study focused on phytoplankton variations in relation to seasonal environmental changes on a multiannual time scale (2006–2015) at a Mediterranean eutrophic reservoir (Bidighinzu Lake, Italy) belonging to the Italian, European and International Long Term Ecological Research networks. Phytoplankton cell density, volume and biomass and chlorophyll *a* concentrations were analysed together with meteo-climatic, hydrological, physical and chemical variables to detect trends and correlations. The period under study was also compared with previous years to assess the presence of significant differences in the environmental and planktonic compartments. Multiannual changes were more pronounced in summer than in the other seasons during the decade under study. The most conspicuous environmental changes were a significant decrease in summer nutrient concentrations in the reservoir and a simultaneous anthropic pressure reduction in the watershed. In addition, the mixing zone and euphotic zone ratio also increased. Multiannual changes in summer phytoplankton composition consisted of an increased density of smaller Bacillariophyceae and Cyanophyceae, which replaced larger species of the same phytoplankton classes. This resulted in opposite trends of total phytoplankton cell density (increasing) and mean phytoplankton cell volume (decreasing) over the study years. The nutrient decrement was statistically the strongest environmental driver of the phytoplankton changes observed in the reservoir. However, the

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mixing zone and the euphotic zone ratio and water temperature also significantly affected the multiannual phytoplankton variations. Therefore, we conclude that the success of small cell-sized phytoplankton in Bidighinzu Lake was most probably due to the synergic interactions of more environmental forces related to changing anthropic pressures and climate variability. Our results highlight the importance of long-term monitoring of reservoirs in the Mediterranean basin, especially in semi-arid regions where the need and scarcity of high quality water will be further exacerbated due to the global climate change.

Keywords
Phytoplankton, cell size, nutrients, climate change, reservoir, LTER

Introduction

In all aquatic ecosystems, phytoplankton growth depends on water temperature and on light and nutrient availability (Winder and Sommer 2012). These growth-limiting factors are in turn regulated by physical processes, such as water circulation, mixed-layer dynamics and upwelling, which are strongly linked to climate (Jovanović et al. 2017). Being the basis of pelagic trophic web as the primary producers, phytoplankton is a fundamental element for understanding the dynamics of the ecosystem and for predicting and managing the ecosystem responses to local and global changes (Guinder et al. 2010).

Different selective pressures, such as light availability, nutrient limitation, fluctuating nutrient supply or grazers, can influence the size structure in natural phytoplankton communities (Litchman et al. 2010). A shift to smaller cell sized phytoplankton has been predicted as a consequence of ocean warming, with profound consequences for the ecosystem's food web composition and efficiency, from the very bottom to the upper trophic levels (Polovina and Woodworth 2012, Boyce et al. 2015, Sommer et al. 2017a). Large losses of energy, nutrients and carbon throughout the food web can be expected when small phytoplankton dominates (Legendre and Rassoulzadegan 1996, Falkowski et al. 1998), with consequences on the ecosystem functioning (Kamenir and Morabito 2009).

Many studies have highlighted drastic modifications in phytoplankton cell size composition and abundance in lakes related to changes in nutrient concentrations (Salmaso 2010 and references therein). On the other hand, some physical variables, such as water mixing and underwater light availability, resulted in being the most important drivers in determining phytoplankton size and shape structure in several reservoirs (Naselli-Flores and Barone 2007).

This work examines phytoplankton variations in relation to seasonal environmental changes on a multiannual time scale in a warm monomitic eutrophic Mediterranean reservoir (Bidighinzu Lake, Sardinia, Italy), mainly used for drinking water for 20 urban areas and about 100,000 inhabitants. In the Mediterranean area, climate conditions (such as long lasting periodic droughts) seriously restrict the water availability, especially during summer and cause strong variations in the water level of reservoirs, which in turn affect the phytoplankton abundance and composition (Naselli-Flores 2003). In addition, in semi-arid areas, such as the Mediterranean region, reservoirs are the basic sources of freshwater for human uses (García-Ruiz et al. 2011). The Mediter-

ranean region is one of the climate change hotspots in the word (Giorgi and Lionello 2008), where a substantial decrease in precipitation (around 10%) is predicted for the period 2040–2070 by most of the current forecast models (García-Ruiz et al. 2011). Consequently, an increasing pressure on stored water resources is expected which will likely also affect the overall state of the reservoir ecosystem.

Multiannual observations allow detecting meaningful ecological shifts, distinguishing significant changes from the normal patterns and the background noise and the assessment of whether ecological changes are due to human or natural causes (Pugnetti et al. 2013). Investigating changes in phytoplankton cell size on a multiannual time scale may be useful for forecasting phytoplankton variations in relation to global change and other environmental impacts, such as those of local human activities (Weithoff and Gaedke 2017).

Phytoplankton and environmental variables have been monitored since 1978 (Marchetti et al. 1992) in Bidighinzu Lake, a site that belongs to the Italian (LTER-Italy), European (LTER-Europe) and International (ILTER) Long Term Ecological Research networks (www.ilter.network). A long term decreasing trend in nutrient concentrations in this reservoir has been recently reported for the period 1988–2012 (Mariani et al. 2015), accompanied by significant increasing trends in the density and biomass of Chlorophyceae, Chrysophyceae and Dinophyceae and significant decreasing trends in the density of Cyanophyceae Nostocales. In addition, the density and biomass of Bacillariophyceae increased in summer and decreased in winter, while those of Cyanophyceae Chroococcales increased in winter and decreased in summer. The present work focused on the structure and temporal dynamics of phytoplankton in the most recent decade (2006–2015) of this monitoring programme. We hypothesised that the reduction in nutrient concentrations in Bidighinzu Lake 1) may have been accompanied by a reduction in phytoplankton cell size, a descriptor not yet investigated in the Bidighinzu Lake and in other Mediterranean reservoirs and 2) may have been linked to variation in the anthropic pressures in the watershed and to climate variability.

Material and methods

Study site

The Bidighinzu Lake is located in the northern part of Sardinia (Italy, 40°33'22"N 8°39'41"E) at an altitude of 334 m a.s.l. (Figure 1). The reservoir was constructed in 1956, it has a surface area of 1.7×10^6 m^2, a theoretical maximum volume of 12.2×10^6 m^3, a maximum depth of 39.5 m and a mean depth of 7.3 m. The watershed has a surface area of 52.18 km^2. The main use of Bidighinzu Lake is to store drinking water. Problems affecting the potability of Bidighinzu Lake's water have occurred since the lake was built, particularly hypolimnetic deoxygenation and the excessive presence of algae in the epilimnion (Lugliè et al. 2001). To address these problems, an aeration system was installed adjacent to the water intake tower in 1966. A by-pass was built in 1987 to divert urban and

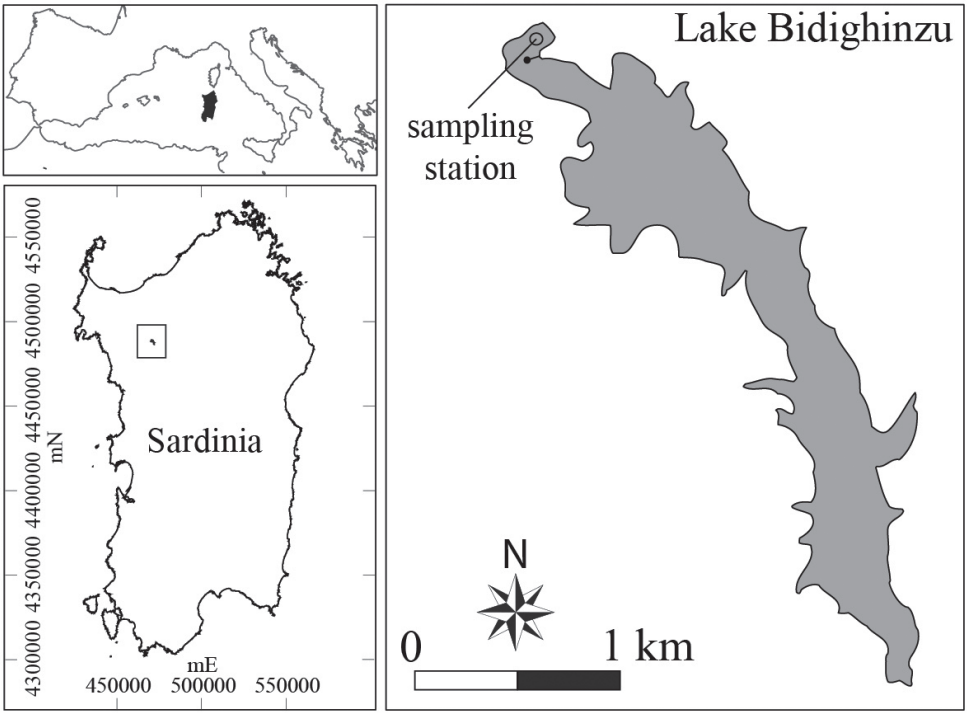

Figure 1. Location of the study site and sampling station.

industrial wastes from the town of Thiesi to downstream of the reservoir (Marchetti et al. 1992, Lugliè et al. 2001). An experimental water aeration system with micro-air bubbles was activated in the reservoir from spring 2014 to autumn 2015 (Mariani et al. 2016).

Multiannual sampling

Water sampling was conducted in the Bidighinzu Lake at monthly frequency from March 1988 to April 1989, from March 1994 to November 1997, from July to October 2003 and from March 2006 to October 2015 at a single station close to the deepest part of the reservoir (Figure 1). As part of the results of the WISER project (Water bodies in Europe: Integrative Systems to assess Ecological status and Recovery), the data analyses of phytoplankton and environmental variables collected at different stations within the reservoir demonstrated that the single sampling station, routinely considered, is representative of the entire Bidighinzu Lake (Thackeray et al. 2013). The sampling was more frequent in the summers of 2006, 2009, 2014 and 2015 (4 samples in each summer of these years), during the present study. Samples were collected at fixed depths using 2.5 l Niskin bottles at 0, 1, 2.5, 5, 7.5, 10, 15 m and subsequently at intervals of 10 m until 1 m from the bottom.

Analyses of environmental and meteo-climatic variables

The water transparency was measured with a Secchi disc and the water temperature was recorded using a multi-parametric probe (Hydrolab Datasonde 5 and YSI 6600V2). Water samples (1.5 l) from the Niskin bottles were stored in cold (4 °C) and dark conditions prior to laboratory analysis for ammonium ($N\text{-}NH_4$), nitrite ($N\text{-}NO_2$), nitrate ($N\text{-}NO_3$), total nitrogen (TN), reactive silica ($Si\text{-}SiO_4$), orthophosphate ($P\text{-}PO_4$) and total phosphorus (TP) according to Strickland and Parsons (1972). Total dissolved inorganic nitrogen (DIN) was calculated as the sum of ammonium, nitrate and nitrite. Total organic nitrogen (TON) was calculated by subtracting DIN from TN.

The Consiglio per la Ricerca in Agricoltura (CREA) provided daily meteorological data of rainfall and air temperature from a meteorological station nearby the Lake (about 15 km) from January 2006 to December 2015. Daily temperature values were monthly averaged and daily rainfall values were monthly cumulated. Monthly data on the climatic index WEMO (Western Mediterranean Oscillation) were provided by the Climatology Group of the University of Barcelona (Spain). This index measures the difference between the standardised atmospheric pressure recorded at Padua in northern Italy and San Fernando in south-western Spain (Martín-Vide and Lopez-Bustins 2006). Compared to the Mediterranean Oscillation index (Conte et al. 1989), which covers the atmospheric dynamics of the whole Mediterranean basin, WEMO is defined only within the synoptic framework of the western Mediterranean basin and its vicinities and shows a greater independence from the external dynamics of the Mediterranean basin (Martín-Vide and Lopez-Bustins 2006). WEMO is able to explain the pluviometric variability in the eastern fringe of the Iberian Peninsula, an area weakly or not related to the NAO (North Atlantic Oscillation index) pattern (Rodó et al. 1997, Martín-Vide and Lopez-Bustins 2006). Monthly data of the WEMO index were available continuously (i.e. without gaps) from 1988 to 2015.

Phytoplankton analyses

The phytoplankton samples (100 ml), taken from the Niskin bottles, were immediately fixed after collection in 2% acid Lugol's solution. Sample aliquots of 5 to 10 ml (depending on cell density) were analysed to estimate the cell density using the Utermöhl method (1958) with an inverted microscope (Zeiss, Axiovert 10). Cell counts were made at magnifications of 200× and 400× on at least 10% of the total bottom area of the sedimentation chamber. Additional non-fixed samples were observed immediately after collection to facilitate the identification of certain species. The species were identified following the taxonomic guides listed in Mariani et al. (2015). Results are shown at class level (and at order level only for the most abundant classes) in the present work.

Mean cell volume of each species was obtained by geometrical approximations from measurement of at least 30 cells in each sample according to Sun and Liu (2003). The mean volumes of all the species observed were added to obtain the total

cell volume in each sample. The total cell volume was divided by the total cell number to obtain the mean cell volume of the whole phytoplankton community in each sample (abundance-weighted mean cell volume according to Sommer et al. 2017a). The biovolume in each sample was converted to biomass based on the assumption that 1 mm^3 = 1 mg of fresh-weight biomass (Weyhenmeyer et al. 2013). The cell biomass of each species was determined in each sample by multiplying its mean cell volume by its cell density.

Water samples (1.5 l) for measuring chlorophyll *a* concentrations were stored in cold (4 °C) and dark conditions before the laboratory analysis (within 24 hours), which were conducted with a spectrophotometer (50 SCAM, Varian), according to Goltermann et al. (1978).

Data and statistical analysis (2006–2015)

Environmental data were depth-averaged for the entire water column, whereas phytoplankton data were depth-averaged only for the euphotic zone assuming that these organisms live and grow mainly in this water layer.

The depth of the euphotic layer was calculated as Z_{eu} = 2.5 times the Secchi disc depth (Poikane 2009). The depth of the mixed layer (Z_{mix}) was calculated as the zone from the surface to the depth where the temperature gradient exceeded 1 °C x m^{-1} (Ruttner 1963), except in the case of absence of stratification, when mixing depth was taken equal to the maximum depth of the reservoir. The mixing depth and euphotic depth ratio (Z_{mix}/Z_{eu}) was used as a good proxy for underwater light climate since it indicates the time spent by phytoplankton in good or poor light conditions when they are in the mixed water column: the highest is the Z_{mix}/Z_{eu} value in the lake, the lowest is the lifetime spent in suitable light conditions (Naselli Flores and Barone 2007).

Seasons were considered as: summer = July–September, autumn = October–December, winter = January–March and spring = April–June.

For each separate season, the non-parametric Mann-Kendall test (Gilbert 1987) was applied to detect significant monotonic trends in environmental and phytoplankton variables. Since the Mann-Kendall test results indicated the most significant trends for the summer season and for the phytoplankton cell density and cell volume datasets, all the subsequent statistical analyses were performed only on these phytoplankton descriptors in summer. Redundancy analysis (RDA, Ter Braak and Šmilauer 1998) was used to assess which environmental variable (amongst WEMO index, air temperature, rainfall, Z_{mix}/Z_{eu}, water temperature, Si-SiO_4, P-PO_4, TP, N-NO_3, N-NO_2, N-NH_4, DIN, TN and TON) significantly affected the multiannual patterns of the cell density and cell volume of all the phytoplankton classes observed consistently throughout the analysed decade (Bacillariophyceae, Chlorophyceae, Chrysophyceae, Conjugatophyceae, Cryptophyceae, Cyanophyceae, Dinophyceae, Euglenophyceae). Before analysis, phytoplankton data were $\log_{10}(x+1)$ transformed to stabilise variance and reduce the influence of dominant taxa on the ordination.

All statistical analyses were performed using R 3.4.3 software (R Core Team 2017). An *a priori* level of significance was established at $p < 0.05$, i.e. 95% confidence interval.

Statistical analyses of the long-term dataset (1988–2015)

The non-parametric Mann-Kendall test was applied to detect significant monotonic trends in the summer WEMO index over all the historical datasets.

One-way analysis of variance (ANOVA) was performed to assess significant differences in environmental (WEMO index, air temperature, rainfall, Z_{mix}/Z_{eu}, water temperature, Si-SiO_4, P-PO_4, TP, N-NO_3, N-NO_2, N-NH_4, DIN, TN and TON) and phytoplankton (cell density and cell volume of each phytoplankton class observed) variables amongst the four continuous time cycles of samplings available (1988–1989, 1994–1997, 2003, 2006–2015). Prior to ANOVA, all data were $\log_{10}(x+1)$ transformed to meet ANOVA assumptions: normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett test). When significant differences in the dependent variables based on these factors were observed, the post hoc Tukey's pairwise comparisons test was performed.

All the analyses were performed using R 3.4.3 software (R Core Team 2017). An *a priori* level of significance was established at $p < 0.05$, i.e. 95% confidence interval.

Watershed land cover and land use analysis

To explore the land cover and land use changes of the Bidighinzu Lake watershed (52.18 km^2) over time, a spatial data processing analysis with the support of a geographic information system (GIS) was performed (Padedda et al. 2015). The analysis was focused on the evaluation of land cover by using techniques of classification generating polygons by clipping the shape of the three time-series CORINE land cover maps: 1990 (EEA 2018a), 2000 (EEA 2018b) and 2012 (EEA 2018c). The automatic segmentation was carried out on the whole shape layer of the watershed of the reservoir. Each segmentation analysis was performed in order to obtain objects in accordance with the CLC system 2000 nomenclature (EEA 2000). For the purposes of this paper, the identified objects were aggregated into three homogeneous macro-classes (Urban Areas, Agricultural Areas and Natural and semi-Natural Areas) in accordance with the CLC classification at the 4th hierarchical level.

Results

The seasonal values of environmental and phytoplankton parameters considered during the decade 2006–2015 are reported in Tables 1 and 2, respectively.

Table 1. Minimum (Min), maximum (Max) and mean values ± standard deviation (SD) of environmental variables in Bidighinzu Lake considering the decade 2006–2015 (WEMOi, Western Mediterranean Oscillation index; Z_{mix}/Z_{eu}, mixing depth-euphotic depth ratio; Si-SiO_4, silicate; P-PO_4, orthophosphate; TP, total phosphorous; N-NO_3, nitrate; N-NO_2, nitrite; N-NH_4, ammonia; DIN, dissolved inorganic nitrogen; TN, total nitrogen; TON, total organic nitrogen). Number of observations are reported in Table 3.

	Winter			Spring			Summer			Autumn		
	Min	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**
WEMOi	-2.6	1.6	-0.2±1.0	-4.3	1.2	-1.4±1.4	-2.6	1.1	-1.0±0.9	-2.7	1.6	-0.6±0.9
Air temperature (°C)	4.8	10.8	8.3±1.5	12.4	22.8	17.1±3.5	19.2	26.0	23.1±2.0	7.1	18.9	12.5±3.7
Rainfall (mm)	4.0	104.6	47.8±26.8	0.8	111.0	47.6±31.0	0.0	101.2	16.4±23.4	2.6	191.6	59.5±41.9
Water temperature (°C)	6.6	11.5	9.3±1.4	12.0	26.4	16.8±3.3	17.7	24.8	20.4±1.8	8.6	19.1	15.0±2.2
Water transparency (m)	0.3	11.1	0.6±0.2	0.3	1.6	0.9±0.3	0.3	2.5	1.1±0.5	0.3	1.3	0.8±0.1
Z_{mix}/Z_{eu}	1.8	26.7	14.3±6.4	0.3	22.8	5.1±5.7	0.2	20	4.1±4.0	3.3	26.7	10.4±5.1
Si-SiO_4 (mg l^{-1})	0.7	9.5	6.1±2.0	0.0	9.2	4.8±1.8	0.2	62.0	5.1±10.0	0.1	7.1	2.6±0.8
P-PO_4 (mg m^{-3})	13.0	164.2	84.1±33.5	5.0	159.7	70.7±35.1	24.8	343.0	130.1±62.2	31.9	243.0	96.2±75.3
TP (mg m^{-3})	83.5	355.1	183.7±70.2	61.0	399.4	170.1±75.0	79.8	457.9	246.9±79.6	68.9	396.3	190.9±89.1
N-NO_3 (mg m^{-3})	328.8	1295.6	878.5±361.0	16.2	684.4	231.3±199.1	6.7	126.7	38.3±21.3	24.0	1435.3	344.3±97.5
N-NO_2 (mg m^{-3})	11.7	60.4	22.1±5.4	29.0	720.6	13.0±7.3	2.6	37.4	666.4±402.8	3.3	47.6	16.7±8.1
N-NH_4 (mg m^{-3})	25.4	648.0	111.1±180.0	2.9	37.0	171.9±171.0	40.0	1736.4	620.4±404.1	22.3	939.9	427.4±250.5
DIN (mg m^{-3})	504.9	1386.6	1011.6±295.5	63.0	803.6	416.3±182.4	73.2	1778.3	7.6±8.2	53.1	1977.0	788.4±229.0
TN (mg m^{-3})	1839.2	4049.2	2703.9±653.5	1171.7	3519.1	2114.2±596.6	1212.0	4658.7	2431.0±925.8	442.0	4131.8	2347.4±848.6
TON (mg m^{-3})	1004.0	3238.8	1692.3±584.1	858.1	3086.0	1697.9±588.6	344.5	4284.1	1766.1±985.6	146.2	3411.4	1572.2±872.8

Table 2. Minimum (Min), maximum (Max) and mean values ± standard deviation (SD) of phytoplankton variables in Bidighinzu Lake considering the decade 2006–2015. Number of observations are reported in Table 4.

	Winter			Spring			Summer			Autumn		
	Min	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**	**Min**	**Max**	**Mean±SD**
Total cell density (10^3 cells l^{-1})	448	52970	10298±13641	3068	515428	86784±133223	2633	1570361	203256±308627	1542	803909	149885±217932
Bacillariophyceae	56	2613	720±757	14	6300	594±1259	41	15128	3224±3493	216	6926	2530±2171
Chlorophyceae	78	4026	1123±1169	759	68065	9957±12968	324	36047	5657±7989	267	32226	9736±9146
Chrysophyceae	0	383	55±94	0	70	13±20	0	705	55±137	0	1344	127±298
Conjugatophyceae	0	35	7±10	0	62	8±15	0	336	42±75	2	1429	213±422
Cryptophyceae	0	24693	3706±6394	5	4143	972±1120	9	2299	498±571	39	9884	1465±2179
Cyanophyceae	0	27783	4618±8417	0	513989	75209±133927	1020	1562687	193740±308718	696	780464	135650±216116
Dinophyceae	0	81	11±24	0	58	4±11	0	148	15±27	0	56	12±16
Euglenophyceae	0	276	57±77	0	158	27±36	0	130	23±28	0	1528	153±325
Mean cell volume (μm^3)	0.52	25.68	6.95±6.64	0.06	18.44	1.97±3.57	0.03	34.67	3.12±6.58	0.09	14.80	2.40±3.87
Bacillariophyceae	3.12	256.49	66.59±91.78	1.54	941.19	169.59±228.36	0.13	120.96	20.08±29.79	1.48	70.09	1.48±70.09
Chlorophyceae	0.14	29.04	7.43±8.48	0.04	24.06	2.12±4.79	0.11	14.98	3.05±3.33	0.22	26.35	2.87±5.58
Chrysophyceae	0.11	2590.58	219.07±659.78	3.89	1469.34	270.71±407.10	4.10	1012.90	165.82±211.08	1.14	769.60	146.41±213.10
Conjugatophyceae	4.37	3306.90	542.12±826.55	73.73	12708.87	1367.56±3076.65	4.35	4772.39	475.39±824.45	0.56	3164.18	477.99±760.72
Cryptophyceae	0.12	178.12	22.07±47.11	0.04	61.57	9.02±15.50	0.70	209.54	15.39±36.20	0.26	44.28	9.61±12.64
Cyanophyceae	0.00	5.22	0.43±1.33	0.00	0.14	0.02±0.03	0.00	0.27	0.02±0.05	0.00	0.08	0.02±0.02
Dinophyceae	9.80	3834.09	637.89±1299.79	0.68	103248.12	14703.87±28210.65	73.333	172092.97	17474.80±35443.87	31.92	32951.37	5757.98±9131.45
Euglenophyceae	28.88	2283.72	414.01±574.54	43.14	4971.75	557.40±1035.71	1.89	2870.58	471.52±658.84	3.79	338.55	117.14±11.23
Total cell biomass (mg l^{-1})	0.20	9.19	3.46±2.51	0.28	29.86	3.87±5.57	2.14	68.86	13.12±13.43	0.65	21.60	9.18±5.90
Bacillariophyceae	0.08	7.54	2.39±2.57	0.03	24.95	1.52±4.58	0.05	65.43	6.78±11.70	0.49	16.99	3.42±3.47
Chlorophyceae	0.01	0.69	0.18±0.19	0.12	10.89	1.60±2.11	0.04	3.67	1.11±1.05	0.06	9.88	2.47±2.73
Chrysophyceae	0.00	0.04	0.01±0.01	0.00	0.04	0.01±0.01	0.00	2.39	0.13±0.47	0.00	1.39	0.10±0.30
Conjugatophyceae	0.00	0.09	0.01±0.02	0.00	0.11	0.01±0.02	0.00	0.45	0.06±0.10	0.00	1.51	0.20±0.38
Cryptophyceae	0.00	2.58	0.61±0.82	0.00	0.76	0.19±0.19	0.00	2.12	0.16±0.36	0.00	4.33	0.59±0.96
Cyanophyceae	0.00	0.16	0.02±0.04	0.00	5.55	0.46±1.15	0.01	21.22	3.99±5.85	0.00	13.22	1.32±3.43
Dinophyceae	0.00	0.23	0.02±0.06	0.00	0.25	0.02±0.06	0.00	7.34	0.68±1.40	0.00	4.81	0.70±1.27
Euglenophyceae	0.00	0.14	0.20±0.31	0.00	0.57	0.07±0.11	0.00	0.20	0.04±0.04	0.00	4.79	0.38±1.02
Chlorophyll *a* (mg m^{-3})	2.16	33.90	13.86±8.85	2.15	40.70	10.52±8.64	3.40	150.54	20.50±28.88	2.81	58.61	22.04±14.13

Multiannual trends of meteo-climatic and environmental parameters (2006–2015)

The Mann-Kendall test revealed no significant multiannual trends in the meteo-climatic variables considered nor in the water temperature and transparency during the study decade (Table 3). Instead, a very significant increasing trend of Z_{mix}/Z_{eu} ratio was observed in summer (Table 3). This increment was confirmed, although not significant, when the year 2015 (breaking of stratification due to the activation of the aeration system) was excluded from the analysis (Mann Kendall test: S = 12, p = 0.828, n = 35). The multiannual patterns of Z_{mix}/Z_{eu} summer mean values showed fluctuations over the years, from 0.80 in 2012 up to 11.0 in 2015 (Figure 2). Strong decreasing trends of TP, N-NH_4 and DIN were detected in summer (Table 3). TP values decreased from a summer mean value of 390.8 mg m^{-3} in 2006 to 160.3 mg m^{-3} in 2015 (Figure 2). DIN values decreased strongly from a summer mean value of 1351.4 mg m^{-3} in 2006 to 391.6 mg m^{-3} in 2015, with N-NH_4 always representing more than 80% of total DIN (Figure 3). Si-SiO_4 decreased in summer too, whereas TON increased, although their trends were close to the limit of statistical significance (Table 3). N-NH_4 decreased significantly in winter and DIN even in spring. TON and N-NO_2 increased significantly in autumn and TON even in spring (Table 3).

Table 3. Results of the Mann-Kendall test for detection of long-term trends (2006–2015) in the environmental parameters (WEMOi, Western Mediterranean Oscillation index; Z_{mix}/Z_{eu}, mixing zone and euphotic zone ratio; Si-SiO_4, silicate; P-PO_4, orthophosphate; TP, total phosphorous; N-NO_3, nitrate; N-NO_2, nitrite; N-NH_4, ammonia; DIN, dissolved inorganic nitrogen; TN, total nitrogen; TON, total organic nitrogen) in Bidighinzu Lake. Significant trends are in bold (S = Kendall score, it indicates the trend direction; p = significance; n = number of observations).

	Winter			Spring			Summer			Autumn		
	S	**p**	**n**	**S**	**p**	**n**	**S**	**p**	**n**	**S**	**p**	**n**
WEMOi	33	0.568	30	40	0.486	30	-34	0.556	30	15	0.802	30
Air Temperature	27	0.566	26	24	0.649	28	-6	0.912	26	20	0.657	25
Rainfall	38	0.440	27	20	0.721	29	53	0.303	28	20	0.707	28
Water Temperature	-17	0.544	24	2	0.986	30	52	0.469	35	21	0.620	24
Water Transparency	-21	0.444	24	62	0.274	30	-19	0.797	35	45	0.271	24
Z_{mix}/Z_{eu}	9	0.715	24	2	0.985	30	**181**	**0.007**	**35**	16	0.671	24
Si-SiO_4	-50	0.063	24	100	0.077	30	**-141**	**0.047**	**35**	-65	0.112	24
P-PO_4	-10	0.773	24	17	0.775	30	-62	0.386	35	-16	0.710	24
TP	-14	0.621	24	-27	0.643	30	**-175**	**0.013**	**35**	-39	0.346	24
N-NO_2	-23	0.404	24	-50	0.382	30	132	0.063	35	**83**	**0.042**	**24**
N-NO_3	12	0.677	24	-53	0.353	30	73	0.306	35	27	0.519	24
N-NH_4	**-56**	**0.037**	**24**	-67	0.239	30	**-159**	**0.025**	**35**	-33	0.427	24
DIN	10	0.733	24	**-157**	**0.005**	**30**	**-161**	**0.023**	**35**	35	0.399	24
TN	-6	0.850	24	103	0.069	30	113	0.112	35	63	0.124	24
TON	32	0.163	24	**128**	**0.027**	**30**	**221**	**0.040**	**35**	**113**	**0.031**	**24**

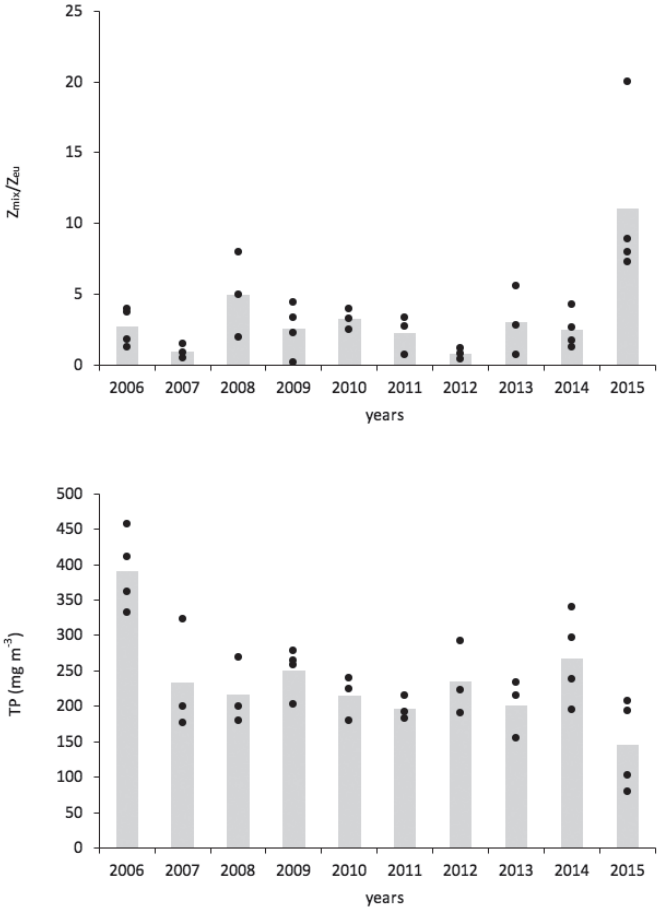

Figure 2. Multiannual (2006–2015) variation of mixing zone and euphotic zone ratio (Z_{mix}/Z_{eu}) (upper panel) and of total phosphorous (TP) (lower panel) in Bidighinzu Lake in summer. Each black dot represents a single sample and each column represents the mean value.

Multiannual phytoplankton trends (2006–2015)

A significant increasing trend resulted for total phytoplankton cell density in summer during the study decade (Table 4). This increment was confirmed also when the year 2010 (exceptional peak of the total phytoplankton density) was excluded from the analysis (Mann Kendall test: S = 190, p = 0.002, n = 35). Summer total phytoplankton cell density varied from about 20 × 10^6 cells l^{-1} in 2006 to about 126 × 10^6 cells l^{-1} in 2015, with an exceptional event (about 1 × 10^9 cells l^{-1}) in 2010, when a bloom of Cyanophyceae was observed (Figure 4). Cyanophyceae contributed most to the total phytoplankton cell density (> 60%), followed by Chlorophyceae (1–25%) and

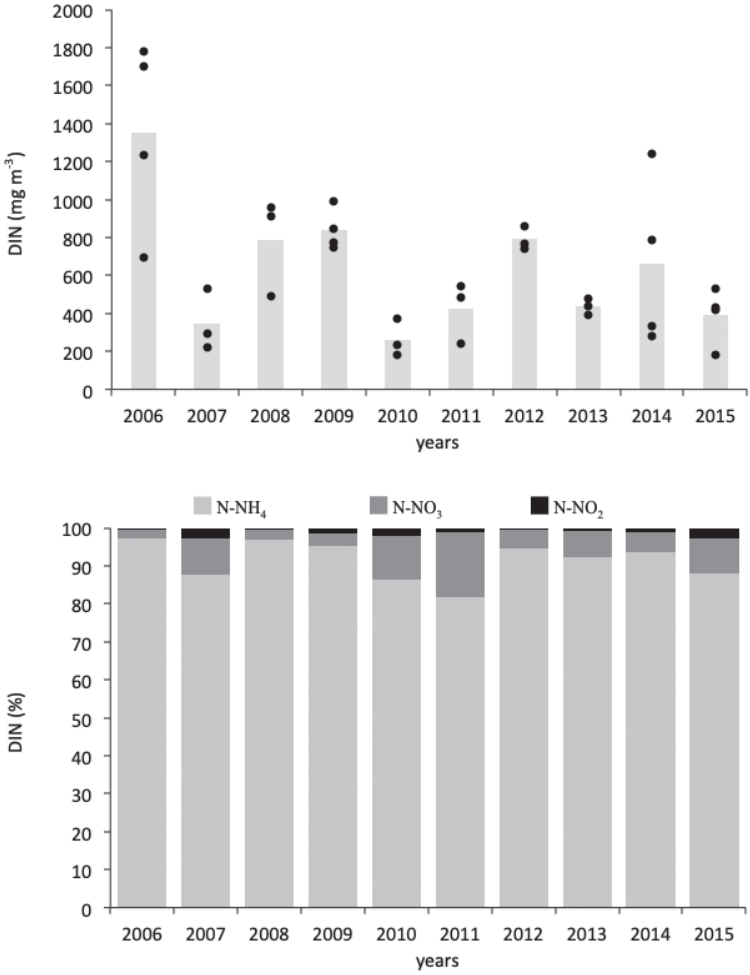

Figure 3. Multiannual (2006–2015) variation of dissolved inorganic nitrogen (DIN) (upper panel) and of the contribution of nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$) and ammonium ($N\text{-}NH_4$) to DIN (lower panel) in Bidighinzu Lake in summer. Each black dot represents a single sample and each column represents the mean value.

Bacillariophyceae (1–16%) (Figure 5). Significant increasing trends were observed for Bacillariophyceae and Cyanophyceae cell density in summer over the study period (Table 4). Bacillariophyceae cell density increased from a summer mean value of about 1×10^6 cells l^{-1} in 2006 to about 7×10^6 cells l^{-1} in 2015 (Figure 6A). Cyanophyceae cell density increased from a summer mean value of about 13×10^6 cells l^{-1} in 2006 to about 1×10^9 cells l^{-1} in 2010, decreasing to about 107×10^6 cells l^{-1} in 2015 (Figure 6C). Instead, no significant multiannual trend was observed for Chlorophyceae cell density (Table 4).

Table 4. Results of the Mann-Kendall test for detection of long-term trends (2006–2015) in the phytoplankton variables in Bidighinzu Lake. Significant trends are in bold (S = Kendall score, it indicates the trend direction; p = significance; n = number of observations).

	Winter			Spring			Summer			Autumn		
	S	p	n	S	p	n	S	p	n	S	p	n
Total cell density	24	0.300	24	106	0.065	30	**175**	**0.009**	**34**	43	0.236	24
Bacillariophyceae	30	0.192	24	**-142**	**0.008**	**30**	**249**	**0.000**	**34**	**81**	**0.024**	**24**
Chlorophyceae	4	0.892	24	12	0.836	30	-85	0.213	34	-65	0.071	24
Chrysophyceae	22	0.344	24	23	0.677	30	**201**	**0.003**	**34**	51	0.058	24
Cryptophyceae	-2	0.964	24	62	0.252	30	47	0.495	34	-49	0.176	24
Conjugatophyceae	-1	1	24	**-140**	**0.007**	**30**	**-212**	**0.002**	**34**	**-99**	**0.006**	**24**
Cyanophyceae	**60**	**0.008**	**24**	**130**	**0.015**	**30**	**167**	**0.014**	**34**	65	0.071	24
Dinophyceae	38	0.074	24	76	0.139	30	-68	0.318	34	12	0.744	24
Euglenophyceae	24	0.300	24	39	0.476	30	-10	0.894	34	-29	0.430	24
Mean cell volume	-18	0.444	24	-105	0.063	30	**-129**	**0.030**	**34**	-9	0.821	24
Bacillariophyceae	-18	0.444	24	**168**	**0.002**	**30**	**-197**	**0.004**	**34**	-29	0.430	24
Chlorophyceae	8	0.753	24	14	0.807	30	51	0.458	34	43	0.236	24
Chrysophyceae	7	0.766	24	5	0.910	30	**-128**	**0.049**	**34**	22	0.526	24
Cryptophyceae	17	0.428	24	34	0.536	30	-47	0.406	34	**87**	**0.015**	**24**
Conjugatophyceae	25	0.235	24	46	0.064	30	112	0.072	34	**129**	**0.000**	**24**
Cyanophyceae	-29	0.166	24	**-142**	**0.005**	**30**	**-195**	**0.004**	**34**	**-71**	**0.048**	**24**
Dinophyceae	-8	0.465	24	26	0.260	30	37	0.453	34	18	0.244	24
Euglenophyceae	-3	0.921	24	-41	0.378	30	-7	0.915	34	22	0.526	24
Total cell biomass	16	0.599	24	23	0.695	30	89	0.192	34	17	0.691	24
Bacillariophyceae	24	0.420	24	**-141**	**0.012**	**30**	**141**	**0.038**	**34**	**89**	**0.029**	**24**
Chlorophyceae	-6	0.861	24	21	0.721	30	-85	0.213	34	-53	0.197	24
Chrysophyceae	6	0.861	24	30	0.602	30	121	0.075	34	**111**	**0.006**	**24**
Cryptophyceae	-18	0.551	24	107	0.059	30	49	0.477	34	-25	0.551	24
Conjugatophyceae	2	0.972	24	**-143**	**0.009**	**30**	**-166**	**0.014**	**34**	-53	0.197	24
Cyanophyceae	20	0.505	24	67	0.239	30	13	0.859	34	25	0.551	24
Dinophyceae	28	0.323	24	89	0.101	30	-64	0.348	34	28	0.486	24
Euglenophyceae	16	0.599	24	-8	0.900	30	-19	0.790	34	-7	0.882	24
Chlorophyll *a*	4	0.892	24	15	0.803	30	9	0.909	34	-139	0.100	24

Considering the mean cell volume of the whole summer phytoplankton community, a multiannual decreasing trend was detected in the total assemblage, with a strong decrement in Bacillariophyceae and Cyanophyceae (Table 4). Summer Chrysophyceae cell volume also decreased, but to a lesser extent. The mean cell volume of the whole phytoplankton community decreased from a summer mean value of about 11 μm^3 in 2006 to about 1 μm^3 in 2015, apart from a high summer mean value of 18 μm^3 in 2012 (Figure 4). Summer Bacillariophyceae cell volume increased from a mean value of 45 μm^3 in 2006 to 58 μm^3 in 2009, then decreased to 4 μm^3 in 2015 (Figure 6B). Summer Cyanophyceae cell volume decreased from a mean value of 0.081 μm^3 in 2006 to 0.005 μm^3 in 2015 (Figure 6D). Dinophyceae was the most important class contributing to the mean cell volume of the whole phytoplankton community (19–97%), followed by Conjugatophyceae (1–67%) and Euglenophyceae (1–38%) (Figure 5).

No significant multiannual trends were observed for total phytoplankton biomass and chlorophyll *a* (Table 4).

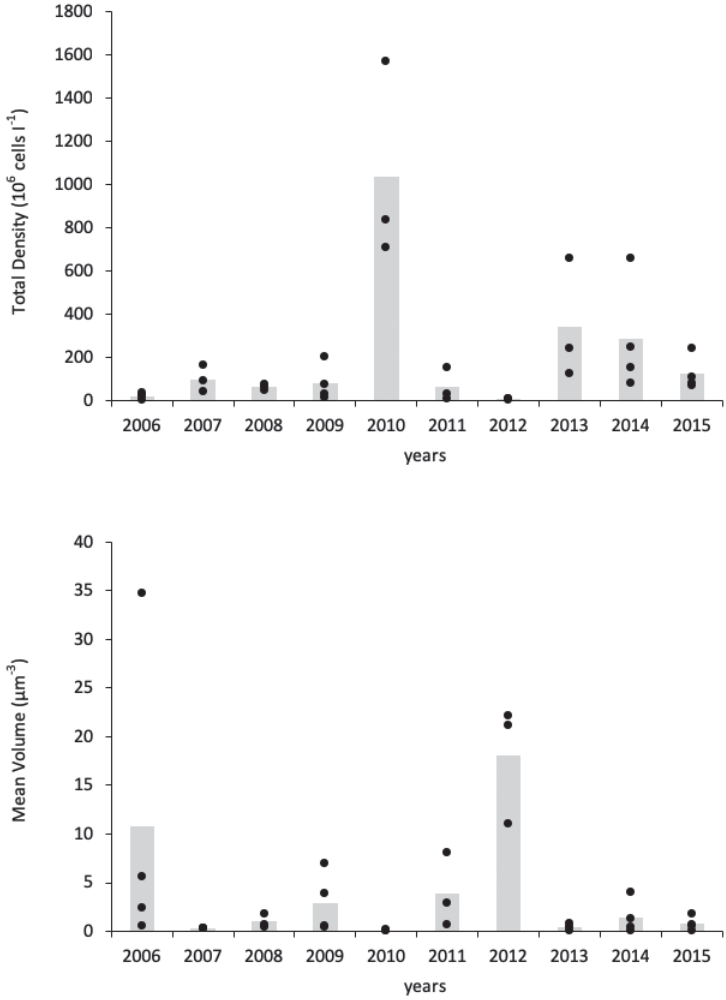

Figure 4. Multiannual (2006–2015) variation of total cell density (upper panel) and mean cell volume (lower panel) of the whole phytoplankton community in Bidighinzu Lake in summer. Each black dot represents a single sample and each column represents the mean value.

Multiannual changes in phytoplankton composition (2006–2015)

Opposite significant trends were observed for the Cyanophyceae Chroococcales and Bacillariophyceae Centrales: cell density increased (Mann-Kendall test: S = 197, p = 0.004, n = 34 and S = 253, p < 0.001, n = 34, respectively) while their cell volume decreased (Mann-Kendall test: S = -179, p = 0.008, n = 34 and S = -205, p = 0.002, n = 34, respectively). In addition, a multiannual decrement in summer was detected for the Cyanophyceae Nostocales only in cell density (Mann-Kendall test: S = -102, p = 0.040, n = 34; Suppl. materials 1, 2).

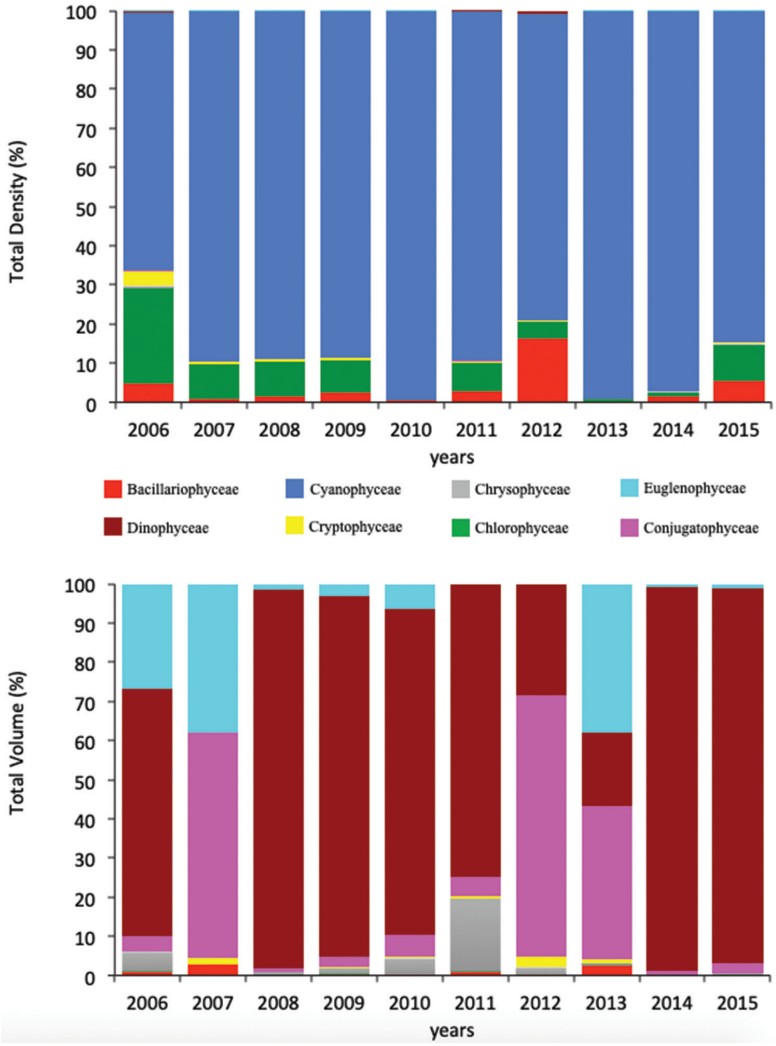

Figure 5. Multiannual (2006–2015) variation of the percentage contribution of all phytoplankton classes to the total cell density (upper panel) and mean cell volume (lower panel) of the whole phytoplankton community in Bidighinzu Lake in summer. BAC = Bacillariophyceae, CHL = Chlorophyceae, CHR = Chrysophyceae, CON = Conjugatophyceae, CRY = Cryptophyceae, CYA = Cyanophyceae, DIN = Dinophyceae, EUG = Euglenophyceae.

Chroococcales were mainly represented by *Aphanocapsa* sp., *Merismopedia* sp. and *Chroococcus* sp. (mean cell volume of 0.76 μm^3, 1.12 μm^3 and 77.15 μm^3, respectively) at the beginning of the study decade and by *Aphanothece* sp., *Aphanocapsa* spp. and *Merismopedia tenuissima* Lemmermann (mean cell volume of 1.30 μm^3, 0.44 μm^3 and 0.54 μm^3, respectively) at the end. Amongst Nostocales, *Dolichospermum flos-aquae* (Brébisson ex Bornet & Flahault) P. Wacklin, L. Hoffmann & J. Komárek (mean cell

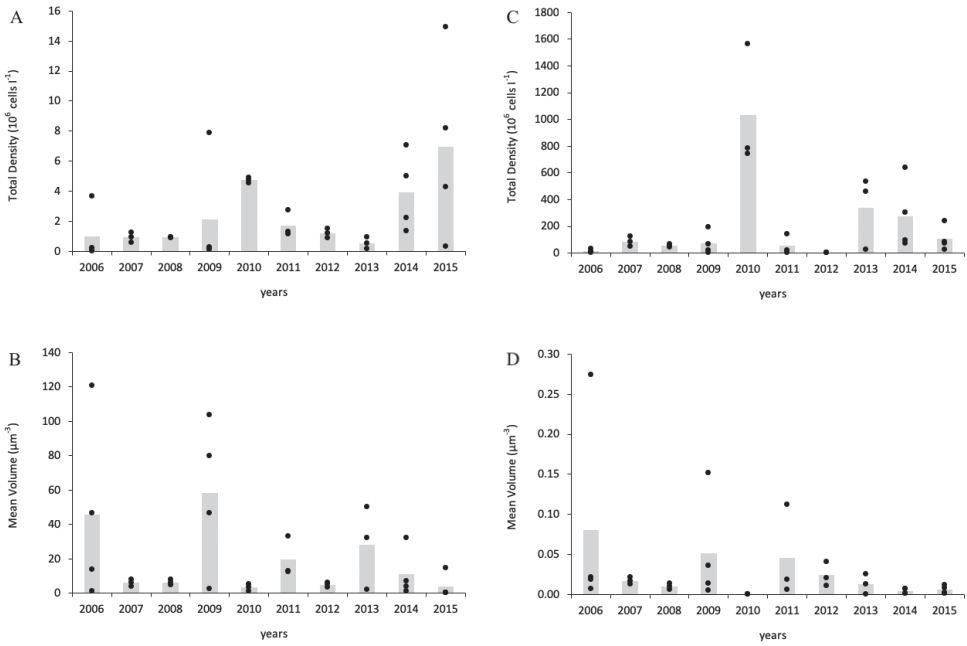

Figure 6. Multiannual (2006–2015) variation of total cell density (upper panels) and mean cell volume (lower panels) of Bacillariophyceae (A and B) and Cyanophyceae (C and D) in Bidighinzu Lake in summer. Each black dot represents a sample and each column represents the mean value.

volume of 73 μm^3) and *Dolichospermum spiroides* (Klebhan) Wacklin, L. Hoffmann & Komárek (mean cell volume of 519 μm^3) determined the highest cell density values at the beginning and at the end of the considered period, respectively. Centrales were mainly represented by *Cyclotella* spp. and *Aulacoseira granulata* (Ehrenberg) Simonsen (mean cell volume of 1035 μm^3 and 982 μm^3, respectively) at the beginning of the study and by *A. granulata* and *Aulacoseira granulata* var. *angustissima* (Otto Müller) Simonsen (mean cell volume of 608 μm^3 and 180 μm^3, respectively) during the last years.

Relationships between phytoplankton and environmental variables (2006–2015)

The results of the RDAs showed that, in summer, all environmental variables accounted for 57.8% and 53.8% of the variation in the 2006–2015 phytoplankton total cell density and volume, respectively. These results were confirmed when the years 2010 (exceptional peak of the total phytoplankton density) and 2015 (breaking of stratification due to the activation of the aeration system) were excluded from the analysis.

DIN ($F = 4.21$, $p = 0.004$), N-NH_4 ($F = 3.97$, $p = 0.002$) and TP ($F = 3.18$, $p = 0.007$) were significant environmental variables, which provided a greater explanation for the variability in summer total phytoplankton cell densitiy, followed by Z_{mix}/Z_{eu} ($F = 2.62$, $p = 0.029$) (Figure 7A). Total phytoplankton cell density and Cyanophyceae, Bacillariophyceae, Chrysophyceae and Euglenophyceae cell densities were negatively correlated

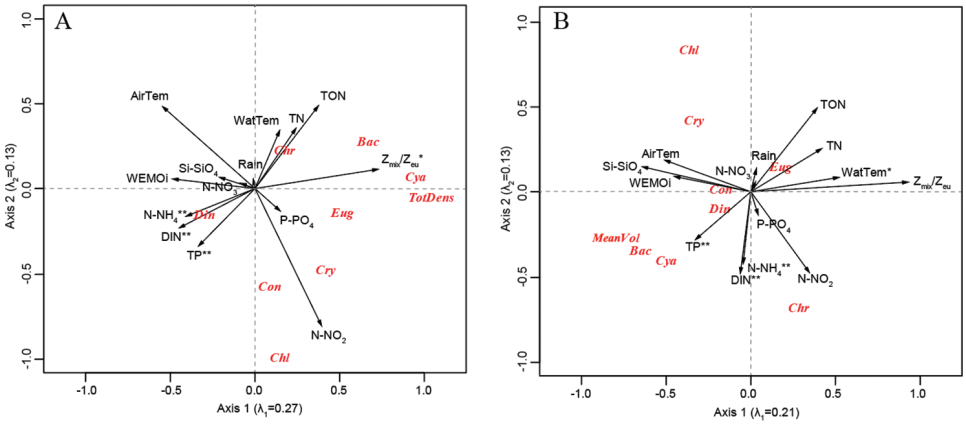

Figure 7. Results of Redundancy Analysis on the relationships between environmental explanatory variables (vectors) and phytoplankton variables (responses), considering (A) the total phytoplankton cell density and (B) the mean phytoplankton cell volume data during the decade 2006–2015 in Bidighinzu Lake in summer. Eigenvalues of the first two axes are indicated by λ_1 and λ_2. Asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) of environmental variables. WEMOi, Western Mediterranean Oscillation index; AirTem, air temperature; Rain, rainfall; Z_{mix}/Z_{eu}, mixing zone and euphotic zone ratio; WatTem, water temperature; Si-SiO_4, silicate; P-PO_4, orthophosphate; TP, total phosphorous; N-NO_3, nitrate; N-NO_2, nitrite; N-NH_4, ammonia; DIN, dissolved inorganic nitrogen; TN, total nitrogen; TON, total organic nitrogen; Bac, Bacillariophyceae; Chl, Chlorophyceae; Chr, Chrysophyceae; Con, Conjugatophyceae; Cry, Cryptophyceae; Cya, Cyanophyceae; Dino, Dinophyceae; Eug, Euglenophyceae; TotDens, total phytoplankton density; MeanVol, mean cell volume of the whole phytoplankton community.

with these nutrients, but Dinophyceae was positively correlated (Figure 7A). Instead, the cell density of total phytoplankton and of the phytoplankton classes mentioned above, with the exception of Dinophyceae, were positively correlated with Z_{mix}/Z_{eu}.

TP ($F = 3.34$, $p = 0.004$), DIN ($F = 2.82$, $p = 0.007$) and N-NH_4 ($F = 3.18$, $p = 0.009$), followed by water temperature ($F = 3.52$, $p = 0.027$), provided a greater explanation for the variability in summer mean phytoplankton cell volume (Figure 7B). Mean cell volumes of the whole phytoplankton community and of Bacillariophyceae, Cyanophyceae, Dinophyceae and Conjugatophyceae were positively correlated with TP and negatively correlated with water temperature (Figure 7B). Euglenophyceae cell volume was negatively correlated with TP Chrysophyceae and Chlorophyceae cell volumes were related positively and negatively to DIN and N-NH_4, respectively.

Long-term dynamics of meteo-climatic, environmental and phytoplankton parameters (historical dataset 1988–2015)

A significant decreasing trend (Mann-Kendall test: $S = -949$, $p < 0.001$, $n = 84$) was observed for the WEMO index in summer during the period 1988–2015. One-way ANOVA highlighted significant differences in the WEMO index, TP and P–PO_4

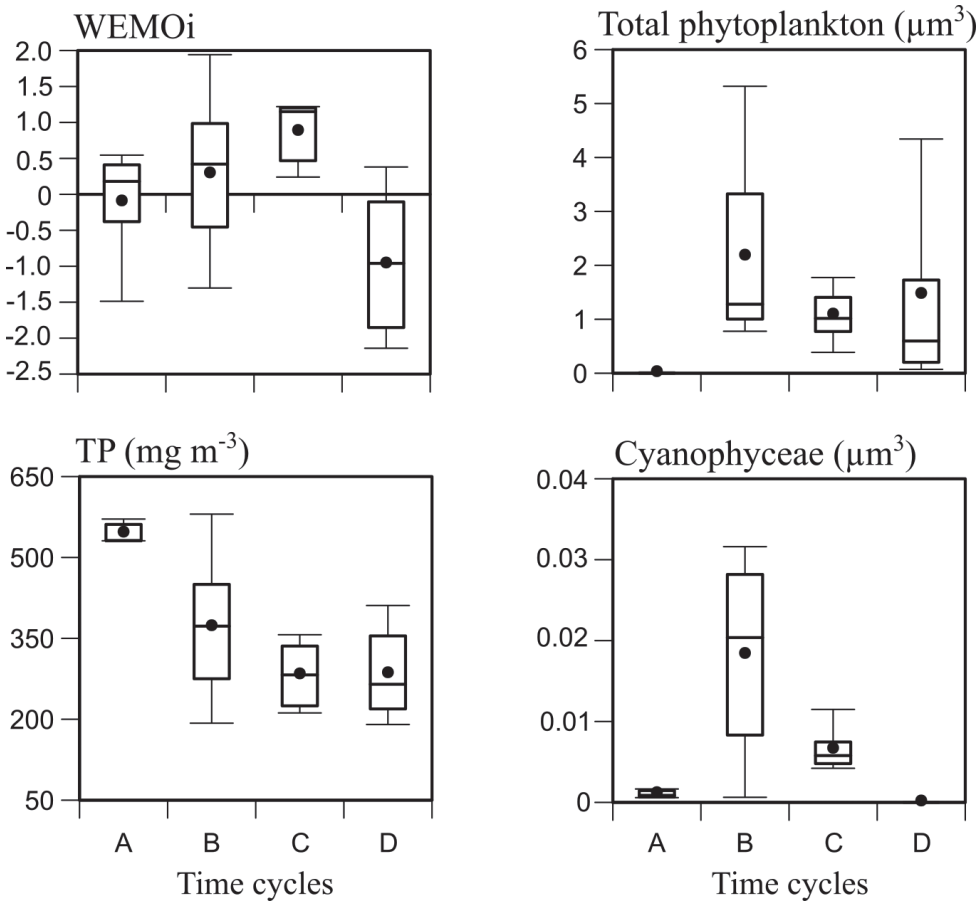

Figure 8. Variation of the Western Mediterranean Oscillation index (WEMOi), total phosphorous (TP), mean cell volume of the whole phytoplankton community (Total phytoplankton) and mean Cyanophyceae volume during the four time cycles of samplings (A = 1988–1989, B = 1994–1997, C = 2003, D = 2006–2015) in summer in Bidighinzu Lake. The lowest, second lowest, middle, second highest and highest lines in the box plots represent the 10th percentile, 25th percentile, median, 75th percentile and 90th percentile, respectively. Means are represented by black dots.

amongst the four time-cycles of samplings (i.e. 1988–1989, 1994–1997, 2003, 2006–2015; Table 5). Mean values of the summer WEMO index increased from -0.1 in the first cycle (1988–1989, A) to 0.8 in the third cycle (2003, C), then decreased strongly to -1.0 in the last period (2006–2015, D) (Tukey's test: B-D, $p = 0.011$; C-D, $p < 0.001$) (Figure 8). Mean values of summer TP decreased from 540.5 mg m^{-3} at the beginning of the time series (1988–1989, A) to 246.9 mg m^{-3} at the end (2006–2015, D) (Tukey's test: A-D, $p < 0.001$) (Figure 8). The analysis did not reveal significant differences in total phytoplankton cell density amongst the different time cycles, even if significant differences were detected in total cell density of Bacillariophyceae, Chlorophyceae, Chrysophyceae, Cryptophyceae, Conjugatophyceae, Dinophyceae and

Table 5. Results of the one-way ANOVA (F-test and p-value) to assess significant differences in selected environmental and phytoplankton variables amongst sampling year cycles (1988–1989, 1994–1997, 2003, 2006–2015) in summer in Bidighinzu Lake (WEMOi, Western Mediterranean Oscillation index; Z_{mix}/Z_{eu}, mixing zone-euphotic zone ratio; Si-SiO_4, silicate; P-PO_4, orthophosphate; TP, total phosphorous; N-NO_3, nitrate; N-NO_2, nitrite; N-NH_4, ammonia; DIN, dissolved inorganic nitrogen; TN, total nitrogen; TON, total organic nitrogen, ns = not significant).

	F	p		F	p		F	p
WEMOi	7.83	***	Total cell density	1.76	ns	Mean cell volume	3.60	*
Water Temperature	1.03	ns	Bacillariophyceae	10.92	***	Bacillariophyceae	77.56	***
Water Transparency	1.32	ns	Chlorophyceae	11.27	***	Chlorophyceae	406	***
Z_{mix}/Z_{eu}	1.24	ns	Chrysophyceae	13.35	***	Chrysophyceae	0.15	ns
Si-SiO_4	2.26	ns	Cryptophyceae	20.45	***	Cryptophyceae	46.96	***
P-PO_4	9.44	***	Conjugatophyceae	9.44	***	Conjugatophyceae	0.89	ns
TP	7.33	***	Cyanophyceae	2.01	ns	Cyanophyceae	123.3	***
N-NO_2	0.55	ns	Dinophyceae	9.47	***	Dinophyceae	9.43	***
N-NO_3	1.45	ns	Euglenophyceae	6.02	**	Euglenophyceae	3.91	*
N-NH_4	0.25	ns						
DIN	0.33	ns						
TN	2.21	ns						
TON	1.17	ns						

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Euglenophyceae, but not Cyanophyceae (Table 5). Instead, significant differences in mean cell volume of the whole phytoplankton community and in Bacillariophyceae, Chlorophyceae, Cryptophyceae, Cyanophyceae, Dinophyceae and Euglenophyceae cell volumes were assessed (Table 5). Summer mean cell volumes of total phytoplankton and Cyanophyceae increased at the beginning of the time series, from 0.004 μm^3 and 0.001 μm^3 to 2.48 μm^3 and 0.02 μm^3, respectively, from the first (1988–1989, A) to the second sampling cycle (1994–1997, B). Subsequently, summer mean values decreased strongly to 1.45 μm^3 and to a value very close to 0 μm^3 in the last sampling cycle (2006–2015, D) (Tukey's test: B-D, $p < 0.001$ for both) (Figure 8).

Long-term variation of watershed land cover and uses (1990–2000–2008)

Significant variations in land cover and land use have been assessed in the Bidighinzu Lake's watershed during the last 20 years. The GIS analyses of CORINE maps for the time series 1990, 2000 and 2012, highlighted a decrement in Agricultural Area of about 7.2% from 1990 (32.59 km^2) to 2000 (30.25 km^2) and of a further 16.8% from 2000 to 2012 (25.17 km^2), with a total decrement of about 23% from 1990 to 2012. On the other hand, Natural and semi-Natural Areas increased by about 14.6% from 1990 (16.63 km^2) to 2000 (19.06 km^2) and by a further 25.3% from 2000 to 2012 (23.89 km^2), with a total increment of about 44% from 1990 to 2012. A noteworthy variation in Urban Area was also assessed with an increase of about 2.5% from 1990 (1.61 km^2) to 2000 (1.65 km^2) and of a further 4.8% from 2000 to 2012 (1.73 km^2), with a total increment of about 7.5% from 1990 to 2012.

Discussion

This work showed that, during the decade 2006–2015, significant multiannual changes occurred in both environmental and phytoplankton variables in Bidighizu Lake, more in summer than during the other seasons. Our results confirmed those reported from other LTER studies in Sardinian reservoirs (Mariani et al. 2015, Pulina et al. 2016, Padedda et al. 2017). In the Mediterranean climate, winters are mild and wet and summers are warm-to-hot dry, whereas spring and autumn are more variable (Lionello 2012). Winter and summer are also the most critical seasons for freshwater ecosystems and predictions of most climate models (García-Ruiz et al. 2011) indicate that major environmental changes are expected during these periods (García-Ruiz et al. 2011, Lionello 2012).

Multiannual summer decrement in nutrient concentrations

We observed strong decreases in summer concentrations of various nutrients, i.e. Si-SiO_4, TP, DIN and N-NH_4 and a significant but weaker increment of TON values during the decade 2006–2015 in Bidighizu Lake. It has been previously documented that the phosphorous reduction in summer has been on-going since 1978, immediately after the application of recovery actions for the reservoir and it was most likely favoured by the diversion of urban and industrial wastes being initiated in 1987 (Marchetti et al. 1992, Lugliè et al. 2001, Mariani et al. 2015). The ANOVA results of the present study suggest that the decrease in summer nitrogen and silicate and the increase in TON instead may have been more recent and they had not been reported so far (Mariani et al. 2015).

Explaining how the dissolved nitrogen forms vary in lakes is not straightforward as they depend upon variations in natural and pollutant sources and a variety of physical, biological and metabolic features of the lake (Quirós 2003). The strong decrement of DIN values in Bidighinzu Lake could be attributed to the concomitant decrease of N-NO_3 and N-NH_4 in the reservoir. In eutrophic lakes, such as the Bidighinzu Lake, nitrification may stop in conjunction with summer thermal stratification associated with hypolimnetic relatively low dissolved oxygen and low pH. As a consequence, denitrification may increase and lead to a complete natural depletion of nitrate. In hypoxic and anoxic conditions, N-NH_4 is mainly generated as a product of decomposition of the organic matter instead of N-NO_3 (Wetzel 2001). Consequently, N-NH_4 concentrations also depend on the quantities of settled organic matter and oxygen concentrations in the waters. Further, in hypoxic and anoxic conditions, denitrification causes the formation of volatile nitrogen compounds that are released from aquatic ecosystems, contributing to reduced concentrations of inorganic nitrogen compounds (Wetzel 2001). Unfortunately, the rate of denitrification was not measured in Bidighinzu Lake; however, based on oxygen concentrations over time (data not shown), it was likely to be relevant in summer in the majority of the considered annual cycles. N_2-fixation due to Cyanobacteria was also not measured. Finally, as summer is

also the dry season in the Mediterranean climate, the reduction of external loadings in that season causes the loss of an important input of inorganic nitrogen carried by the drainage from river basin waters (Coppens et al. 2016, Padedda et al. 2010, 2017). In the present work, the GIS analysis of the Bidighinzu watershed land cover and land use variations over the last 20 years highlighted the significant decrease in agricultural areas and the opposing relevant increase in natural and semi-natural areas accompanied by a small increase in urban areas. These changes may have contributed significantly to a shift in material inputs, supporting the significant trends observed. In particular, considering the different forms of nitrogen, the significant increase of TON in the reservoir may be linked to the partially re-naturalisation of the watershed (Volungevicius et al. 2015). This latter, in turn, may also be responsible, at least in part, for the significant detriment of inorganic nitrogen compounds (DIN and $N\text{-}NH_4$) due to reduction in the use of fertilisers (Jomaa et al. 2016). This is a relevant outcome compared to other reservoirs in Sardinia, where dissolved inorganic nitrogen is generally the main component of TN. For example, Padedda et al. (2015) found a contribution of DIN around 60% of TN in Lake Cedrino, an ecosystem similar to Lake Bidighinzu.

Multiannual trends of summer phytoplankton

Relevant changes in summer multiannual phytoplankton dynamics were observed simultaneously to the summer nutrient reduction in Bidighinze Lake during the decade 2006–2015. The total phytoplankton cell density increased significantly, whereas the mean cell volume of the whole phytoplankton community decreased significantly. Consequently, it is not surprising that the total phytoplankton biomass and chlorophyll *a* concentration did not show any significant trend in the same period. The phytoplankton classes that contributed more to total density in the analysed decade remained mainly Cyanophyceae, followed by Chlorophyceae and Bacillariophyceae as observed in the previous years (Mariani et al. 2015). While significant multiannual trends were not observed for Chlorophyceae, the Cyanophyceae and Bacillariohyceae cell density increased significantly from 2006 to 2015 and their mean cell volume decreased strongly, determining the same multiannual trends for total cell density and mean cell volume of the whole phytoplankton community. According to the ANOVA results, the increment in total phytoplankton cell density was recent (i.e. concerning the period 2006–2015), whereas the reduction in mean cell volume of the whole phytoplankton community and of Cyanophyceae was found to have begun earlier, from the late 1990s. The strong decrease in mean phytoplankton cell volume can be explained by changes in the taxa composition of phytoplankton, not observed at classes' level, but present at order level and consisting of an increasing density of smaller taxa during the decade under study. In fact, the density of Cyanophyceae Chroococcales increased strongly, replacing the larger Cyanophyceae Nostocales at the end of the considered period. In addition, changes in Chroococcales species composition, with an increasing importance of smaller Chroococcales species (e.g. *Aphanothece* sp. and

Merismopedia tenuissima instead of *Aphanocapsa* sp. and *Chroococcus* sp.) caused a strong decrement of the Chrooccocales cell volume during the years. Similarly, the density of Bacillariophyceae Centrales, smaller than the Bacillariophyceae Pennales, increased significantly in the reservoir. Amongst Centrales, smaller species (e.g. *Aulacoseira granulata* var. *angustissima*) replaced the larger ones (e.g. *Cyclotella* spp.) in the last years of the study, inducing the reduction of mean Centrales cell volume during the decade. The increasing density of smaller Cyanophyceae Chroococcales and Bacillariophyceae Centrales during the long-term decrement in nutrient concentrations was also observed in Temo Lake, a reservoir located in the same geographical area of Bidighinzu Lake in Sardinia (Pulina et al. 2016), as well as in the natural subalpine Maggiore Lake (Kamenir and Morabito 2009).

Environmental drivers of multiannual phytoplankton variations

Statistical analysis revealed a strong relationship between the interannual patterns of algal nutrients and of total cell density and mean cell volume of the whole phytoplankton community at the Bidighinzu Lake in summer. Specifically, the summer TP and DIN (mainly N-NH_4) reduction affected, significantly and negatively, the summer total cell density. On the other hand, summer TP and DIN reduction significantly influenced the summer mean cell volume in a positive way. The decrement in phytoplankton size at lower nutrient concentrations was observed elsewhere, such as in another eutrophic Sardinian reservoir (Temo Lake, Pulina et al. 2016) and in different lake typology worldwide (e.g. Lehman 1991, Masson et al. 2000, Kamenir and Morabito 2009). The positive relationship between phytoplankton cell volume and nutrient concentrations was also detected experimentally in a highly eutrophic urban lake in Austria (Dokulil et al. 2007). Small-cell sized phytoplankton are expected to be favoured under lower nutrient concentration because of the high surface area to volume ratio and smaller diffusion boundary layer that enables rapid nutrient exchange through the cell surface (Harris 1994). In addition, the different strategies of nutrient utilisation can explain the distribution patterns of phytoplankton cell size classes along nutrient concentration gradients (Litchman and Klausmeier 2008). According to recent sudies on phytoplankton's nutrient uptake-related traits, low nutrient environments should favour smaller-celled species while high nutrient environments should favour larger-celled species since maximum nutrient uptake velocity (V_{max}) and half-saturation constant for nutrient uptake (K) are positively correlated with cell size (Litchman et al. 2007).

In the hypertrophic Lake Arancio (Sicily, Italy), as well as in many other Sicilian reservoirs, nutrients have never been observed to play an important role in determining the structure of the phytoplankton community (Naselli-Flores and Barone 2007). The morphological changes observed in phytoplankton of this lake were instead strongly related to water mixing and underwater light availability (Naselli-Flores and Barone 2007). Specifically, Z_{mix}/Z_{eu} was detected as one of the most important factors in determining phytoplankton structure in a study of 21 Sicilian reservoirs (Naselli-Flores

2000). In our study case, a significant increasing trend was detected for Z_{mix}/Z_{eu} values during the present study decade 2006–2015. This change might have been partially linked to the activation of the water aeration system in the reservoir only in the period 2014 and 2015. This experimental activity prevented the summer thermal stratification of Bidighinzu Lake in 2015 but not in 2014 (Mariani et al. 2016). Our data analysis showed that the summer Z_{mix}/Z_{eu} value was a significant positive driver of summer dynamics of total phytoplankton density in Bidighinzu Lake, although its role was statistically weaker than that of nutrients. Higher Z_{mix}/Z_{eu} values in Bidighinzu Lake enhanced an increment in total phytoplankton density and especially in smaller species density, though significant relationships with total phytoplankton cell volume were not observed. These findings agreed with the evidence that smaller cells have a lower sinking rate compared to the larger ones (Litchman and Klausmeier 2008) and may be more efficient at utilising low light because of the smaller packaging effect, with self-shading of light-capturing pigments (Kirk 1994, Litchman et al. 2010).

The strong size reduction of marine phytoplankton under increasing ocean warming has also been well documented, although without univocal evidence (Gardner et al. 2011). It remains controversial whether the cell size reduction under increasing temperatures is i) a direct temperature effect or ii) an indirect effect mediated by changes in size selective grazing or by enhanced nutrient limitation that alter the selection on phytoplankton's nutrient uptake-related traits, favouring smaller species over larger cells (Peter and Sommer 2013, Lewandowska et al. 2014). In the present work, no significant multiannual trends of meteo-climatic parameters were detected at the Bidighinzu Lake during the decade 2006–2015. However, summer water temperature significantly affected summer phytoplankton cell volume in the reservoir, though in a weaker way compared to the nutrients. The opposite trends observed for summer phytoplankton cell volume (decreasing) and summer water temperature (increasing), though not significant, seem to agree with the Atkinson's ecological rule, according to which the average size of individuals is inversely related to temperature (Atkinson et al. 2003), also supporting similar findings proposed by several study cases in literature (Sommer et al. 2017b and reference therein).

Conclusions

A significant multiannual decreasing trend in nutrient concentrations was detected in summer in the Bidighinzu Lake simultaneously with reduced anthropic pressure in its watershed. These environmental changes significantly affected the phytoplankton community of this Mediterranean reservoir, favouring a strong increment in the density of smaller Bacillariophyceae and Cyanophyceae taxa. This increment led to a significant multiannual increasing trend in total phytoplankton cell density and a strong multiannual decrease in mean phytoplankton cell volume. In addition, the variations of Z_{mix}/Z_{eu} and of water temperature in summer favoured smaller phytoplankton taxa, although their role in structuring phytoplankton communities was statistically weaker

compared to that of nutrients. We can not rule out that the consumer pressure may also have acted on the phytoplankton community of Bidighinzu Lake, as observed in other environments (Peter and Sommer 2012, Lewandowska et al. 2014, Boyce et al. 2015), but data on grazers are not available for our study area.

This work confirms the importance of acquiring long-term ecological data in studies on phytoplankton to understand the temporal evolution of aquatic ecosystems in relation to natural and anthropogenic forces. The affirmation of smaller phytoplankton cells in Bidighinzu Lake suggests a shift of the system towards a less energy-efficient trophic web based on smaller and lower-quality prey for grazing zooplankton and planktivorous fish. The ecological changes we observed in the reservoir were most probably due to the complex and synergic interactions between the investigated environmental variables related to changing anthropic pressures and climate variability. Considering the need and scarcity of high quality water in semi-arid regions and the paucity of studies on Mediterranean reservoirs, our findings provide useful information for our understanding of these crucial ecosystems and for their management and conservation, thus adding greater value to the LTER-Italy network.

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Supplementary material 1

Figure S1

Authors: Silvia Pulina, Antonella Lugliè, Maria Antonietta Mariani, Marco Sarria, Nicola Sechi, Bachisio Mario Padedda

Data type: multimedia

Explanation note: Multiannual (2006–2015) variation of the contribution of the orders Centrales and Pennales to the Bacillariophyceae total cell density (upper panel) and mean cell volume (lower panel) in Bidighinzu Lake in summer.

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Link: https://doi.org/10.3897/natureconservation.34.30116.suppl1

Supplementary material 2

Figure S2

Authors: Silvia Pulina, Antonella Lugliè, Maria Antonietta Mariani, Marco Sarria, Nicola Sechi, Bachisio Mario Padedda

Data type: multimedia

Explanation note: Multiannual (2006–2015) variation of the contribution of Cyanophyceae orders to the Cyanophyceae total cell density (upper panel) and mean cell volume (lower panel) in Bidighinzu Lake in summer (CHR = Chroococcales; NOS = Nostocales; OSC = Oscillatoriales).

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REVIEW ARTICLE

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Long-term changes of the trophic status in transitional ecosystems of the northern Adriatic Sea, key parameters and future expectations: The lagoon of Venice as a study case

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Abstract

The determination of the trophic status of transitional ecosystems from the physico-chemical and biological point of view is one of the requirements of the European Water Framework Directive (WFD 2000/60/EC). In Italy, its determination is implemented by the Regional Agencies for Environmental Protection (ARPAs) that have activated multi-annual monitoring programs. However, as the availability of funds is increasingly scarce, the number of environmental parameters to detect environmental changes should be conveniently managed.

The high number of environmental parameters, nutrient and macrophyte datasets available for the LTER-Italia site "Venice lagoon" can be an useful tool to analyze the trophic changes over recent years and to foresee environmental evolutions. Nutrient data on a spatial basis have been available since 1948, whereas macroalgal maps date back to 1980. The aim of this paper is to highlight the changes of the trophic status of the lagoon since the middle of the 20th century by considering the concentrations of nutrients in the surface sediments and in the water column, the variation of some physico-chemical parameters and the biomass of macroalgae and also to foresee the way it will possibly evolve. In fact, after many anthropogenic impacts that in the second half of the 20th century affected the lagoon, starting from

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the year 2010, the ecological status is progressively improving. Nutrients show a significant reduction both in the water column and in surface sediments, and the macrophytes are represented by species of higher ecological value while the opportunistic species such as the Ulvaceae are in strong regression.

Keywords
LTER-Italy network, trophic status, nutrient concentrations, waters, sediments, key parameters

Introduction

Almost half of Europe's population lives less than 50 kilometers from the sea and the resources of the coastal areas and transitional water systems (TWS) produce much of the economic wealth of the European Community (EU) (http://ec.europa.eu/environment/iczm/pdf/2000brochure_en.pdf). Urban settlements, industrial and agricultural activities, fishing, commercial traffic and tourism reduce vital space and introduce high quantities of nutrients and pollutants along the 89,000 kilometers of the European coasts, increasing eutrophication and pollution. TWS are mainly affected by anthropogenic impacts because of their shallow bottoms and closed morphology that rarely allows suitable hydrological renewal. These environments, which host habitats and species of conservation interest, are often severely degraded and require special attention. For this reason, they are monitored and have become the subject of numerous studies to understand and try to reverse the causes of their degradation.

The north-western Adriatic Sea is a closed shallow basin where various rivers flow, draining the Po plain and forming large TWS. From North to South three main lagoon systems are present: the lagoons of Marano-Grado, the lagoon of Venice and the lagoons and ponds of the Po Delta. Among them, the lagoon of Venice is the largest and most studied TWS in the Mediterranean Sea. The first biological studies of the lagoon date back to the end of the eighteenth century (Olivi 1794, Naccari 1828, Zanardini 1841 and subsequent papers of the same author, Meneghini 1842 and subsequent papers, De Toni 1889 and subsequent papers, Sighel 1938, Schiffner and Vatova 1938). They mainly dealt with the presence of macroalgae (Sfriso and Curiel 2007, Sfriso et al. 2009), showing only taxonomic data but without mentioning either the quantitative distribution of the species or any environmental parameter. The first studies of the species distribution and the monitoring of environmental parameters throughout the Venetian lagoon were made by Giordani-Soika and Perin (1970, 1974a, b), Perin (1975) and Perin et al.(1983) and refer to the distribution of some benthic taxa (some bivalves and polychaetes) and some nutrients (ammonium, orthophosphate, total phosphorus and total nitrogen) in the water column and surface sediments of the lagoon in 1948 and 1968. These data are the reference parameters to study the changes which have been recorded in the lagoon since the middle of the twentieth century. Later, studies began to address all the aspects concerning the lagoon. Regarding the macrophytes, nutrients and the main physico-chemical parameters of water and sediments, dozens of works are available. Maps of the macroalgal distribution and density have been drawn since 1980 (Sfriso and Facca 2007) whereas maps of nutrients in the water column and environmental parameters in the surface sedi-

ments date back to 1983 (Battaglia et al. 1983, Cossu and De Fraja-Frangipane 1985, Sfriso et al. 1995, 2003) for the central lagoon and to 1985 for the whole lagoon (Cossu and De Fraja-Frangipane 1985, Sfriso et al. 1988, Sfriso et al. 2003, Facca et al. 2014).

The present work investigates the way nutrient concentrations, macroalgal biomass and the environmental parameters of water column and surface sediments have changed since the middle of the last century. The aim is to explore the large dataset and highlight the most relevant parameters necessary to monitor trophic changes, in order to contribute to the institutional monitoring and environmental agencies' achievement in obtaining significant results with reduced efforts and lower costs.

Materials and methods

Sampling sites

The lagoon of Venice (https://deims.org/f7d94927-17be-4d3d-9810-e3c9bc91829c) is a polyhedric shallow water body located in the northern Adriatic Sea which has a water surface of ca. 432 km^2 and a mean depth of ca. 1.2 m (Fig. 1).The lagoon is connected to the sea through three large (400–900 m) and deep (15–50 m) mouths which divide it into three hydrological basins separated by watersheds which shift according to tides and winds. The present study refers to the three morphological basins: northern, central and southern lagoon. Burano and Torcello tidal marshes mark the separation between the central and the northern basin and the deep Malamocco-Marghera artificial canal the separation between the central and the southern one. The central basin (ca. 132 km^2) has been the most studied because it suffered the impact of industrial waste, urban sewage and other anthropogenic pressures such as commercial and touristic activities and the illegal fishing of Manila clam (*Tapes philippinarum* Adams & Reeve) by disruptive fishing gears (Sfriso et al. 2003).

Physico-chemical parameters, nutrient concentration and macroalgal biomass

Information on data collected before 1980 is reported in the cited papers whereas the surveys carried out by our research team, sampling procedures and analytical methods are summarized in the following pages.

Data on nutrients, macroalgal biomass and environmental parameters of the water column have been collected in the whole central lagoon since 1987 (34 sites). Monitoring surveys were carried out in 1993 (34 sites), 1998 (52 sites), 2003 (65 sites), 2011 (45 sites) and 2014 (34 sites). Data of ammonium concentration were also reported for the whole lagoon in 2011 (118 sites).

Thirty-four sites in early summer 1987, 1993, 1998 and 2003, and 31 sites in 2011 were monitored by collecting the 5 cm surface sediment top layer for phosphorus, total nitrogen analyses and determination of the sediment density.

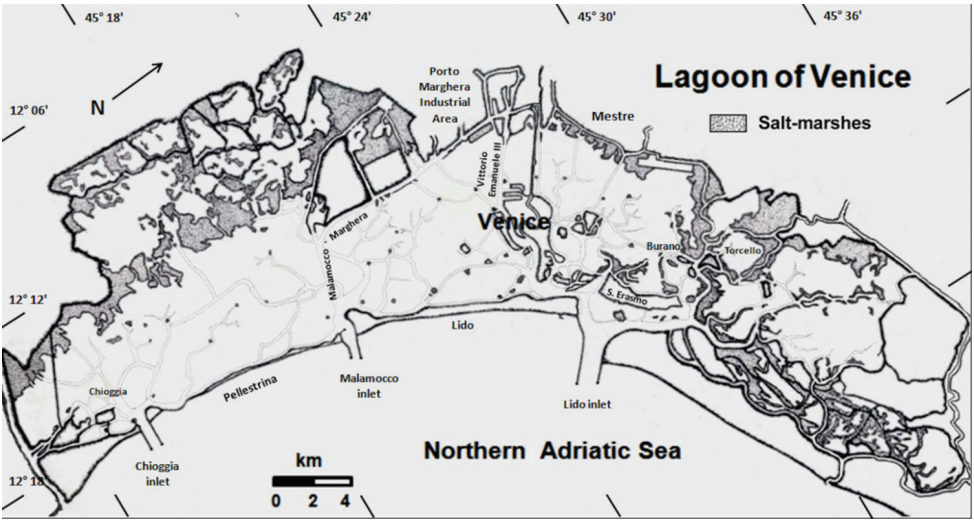

Figure 1. Map of the Venice Lagoon.

At each site, dissolved oxygen was measured with a portable instrument (Oxi 196 oximeter, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Oxygen concentrations were reported as percentage of saturation (%DO) taking into account water temperature and salinity. Water transparency was measured with the Secchi disk. Transparency measurements were reported as a percentage of water column visibility because the waters are shallow (0.5–2.5 m) and the tidal excursion is relatively high (0.5–1 m). A value corresponding to 100% means that the bottom was visible, 50% means that the disk disappeared half way to the bottom. pH and Eh were measured with a portable pH-meter (pH 25) of the CRISON Instruments (Barcelona, Spain).

Six water column samples were collected with a home-made cylindrical sampler (length: 1.50 cm, diameter: 4 cm) which was repeatedly plunged into the water and poured to a tank. Sub-samples of 0.1–1.0 L were filtered through GF/F Whatman glass fiber filters (porosity: 0.7 mm). Filters and water samples were stored frozen at -18 °C for chlorophyll-*a* (Chl-*a*) analyses (Lorenzen 1967) and nutrient (reactive phosphorus (RP) and dissolved inorganic nitrogen (DIN) as the sum of ammonium, nitrite and nitrate) determination according to Strickland and Parsons (1984). Other water sub-samples were collected for salinity, which was determined in the laboratory by chlorine titration according to Oxner (1962).

At each site three sediment cores (first 5 cm top layer) were collected with a Plexiglas corer (i.d. 10 cm) and carefully mixed together. Sub-samples were retained to determine density and nutrient (total nitrogen and total, inorganic and organic phosphorus) concentrations. Density was obtained as g cm^{-3} of wet and dry sediment according to Sfriso et al. (2003).

Total nitrogen (TN) concentrations were obtained by a Flash 2000 CHNS Analyser (Thermo Fisher Scientific spa), sediment freezing, lyophilization and pulverization. Inorganic (P_{inorg}) and total phosphorus (P_{tot}) were, respectively, determined before and

after combustion at 550 °C for two hrs, with dissolution in 1N HCl and spectrophotometric measurements according to Aspila et al. (1976). Organic phosphorus (P_{org}) was obtained by difference. All the analyses were carried out in duplicate on two different days and values were retained when the difference was <5%. To compare the nutrient concentrations of the surface sediments of each site, values were normalized with reference to the amount of dry sediment per volume unit (dry density).

The macroalgal biomass was sampled between late May and July, which is the period when it is most abundant. At each site, biomass samples were collected with a 71×71 cm square frame or with a rake when the biomass was low (3–6 replicates) according to Sfriso et al. (1991, 2014a). Samples were weighted with a mechanical/electronic balance according to the biomass amount. The number of sampling sites ranged from 65 (1987), when macroalgae were very abundant and widespread, to 34 (2014).

Statistical analyses

Data of different datasets of the whole central lagoon have been compared and means, standard deviations, maximum and minimum values have been determined. The Shapiro-Wilk test differentiated non-normal data, and then Friedman one-way ANOVA values ($p < 0.05$) and Spearman's non-parametric coefficients have also been calculated. The basic statistics and correlation analyses of the data collected in the 5 periods were carried out both separately (1987, 1993, 1998, 2003, 2011) and by considering the whole data set (1987–2011). The total information has been summarized in a table reporting the number of significant ($p<0.05$) correlations per single parameter and period. The principal component analysis (PCA) has been applied to log-transformed data to visualize the variance and the association between parameters of both single and total periods, and to explore the scores with a loading >0.7, which is the generalized standard used for this statistical analysis. Total data are visualized in a bi-plot, whereas the loadings >0.7 of the single variables of each period are reported in Table 5 showing the parameters with the highest variance in decreasing order.

The similarities and differences between the stations during the 5 sampling periods have been investigated by analyzing the same data in a transposed matrix and bi-plotting the results. The stations are grouped according to their ecological characteristics and comparisons between the separated or overlapped periods highlight their differences.

Data were processed by Statistica software, Release 10 (StatSoft Inc. Tulsa, USA) provided by an academic license.

Results

Macroalgal biomass and environmental parameters

The first detailed distribution of macroalgae over the whole lagoon took place in 1980 and was replicated in 2003 (Sfriso and Facca 2007). During this period the biomass

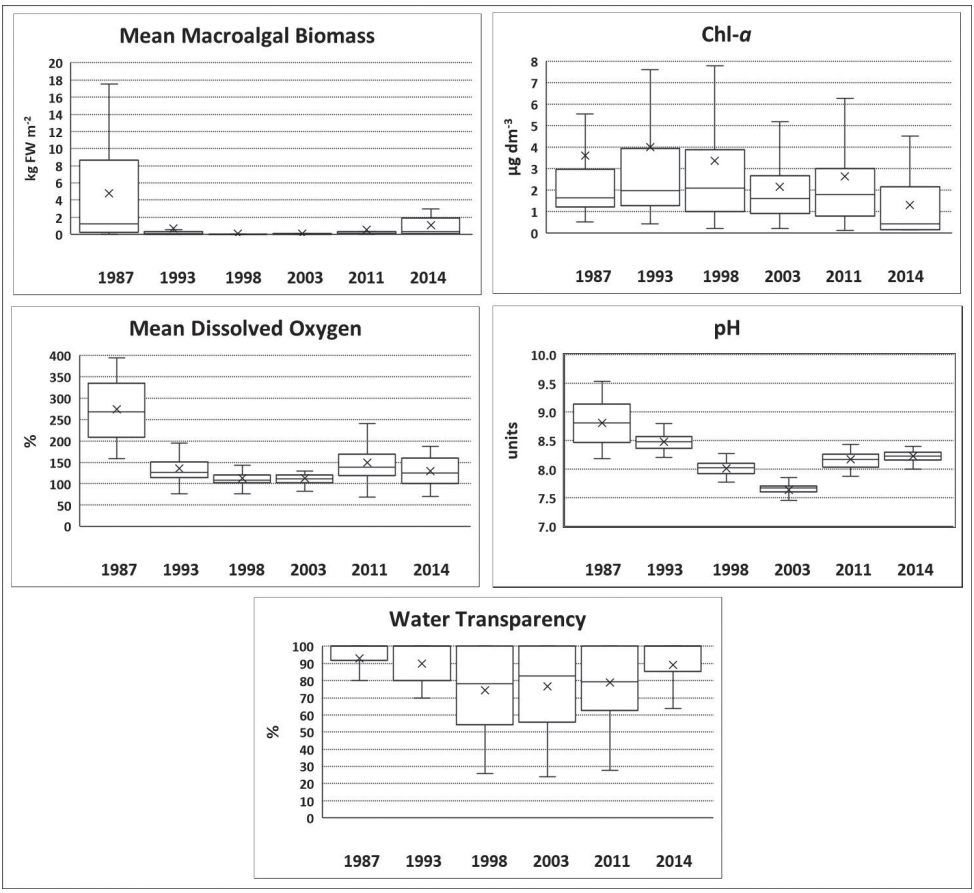

Figure 2. Trends of the mean, standard deviation, maximum, minimum and median values of the macroalgal biomass, Chl-*a*, dissolved oxygen, pH and water transparency in the central lagoon of Venice.

decreased by ca. 90% in the whole lagoon and by over 96% in the central lagoon. However, additional information is available for the central lagoon (ca. 132 km^2) because the biomass was mapped in 1987, 1993, 1998, 2003, 2011 and 2014, together with some physico-chemical parameters and the concentrations of nutrients in the water column and surface sediments. The highest mean biomass, mainly represented by *Ulva rigida* C. Agardh and *Ulva laetevirens* Areschoug, was recorded in late spring in 1987. It showed a mean value of 4.78 kg FW m^{-2} (65 sites, Fig. 2) and a density that in many sites ranged between 10 and 20 kg FW m^{-2}, reaching even 25 kg FW m^{-2} in one site. The mean biomass markedly decreased to 0.69 kg FW m^{-2} in 1993 and to 0.11 kg FW m^{-2} in 1998 (one-way ANOVA: $p<0.001$). In the following years, the biomass slowly increased, reaching a mean value of 1.05 kg FW m^{-2} in 2014 (one-way ANOVA: $p<0.001$). In the same periods, phytoplankton, recorded as Chl-*a,* was expected to increase. On the contrary, the mean concentration of Chl-*a* showed similar values in 1987 (3.61±5.62 µg dm^{-3}), 1993 (4.01±5.43 µg dm^{-3}) and 1998 (3.36±4.45 µg dm^{-3}) and decreased significantly (1.32±1.58 µg dm^{-3}) in 2014 (one-way ANOVA: $p <0.05$) (Fig. 2).

The environmental parameters mainly affected by the presence of macroalgae (i.e. the concentration of dissolved oxygen, pH and water transparency) showed a strongly related trend to the biomass variation (Fig. 2). In 1987, due to the high presence of macroalgal biomass, the mean %DO saturation was 274%, ranging between 159 and 394%. In 1993, when the biomass decreased significantly, the mean %DO halved to 135% (one-way ANOVA: $p<0.001$) and in the following periods fluctuated between 113 and 150% (one-way ANOVA: $p<0.001$). In 1987, the lowest %DO was higher than the mean values of the following periods. Moreover, since 1993 the lowest values have always been undersaturated. The pH values showed a similar decreasing trend (Fig. 2) with the highest mean value in 1987 (8.81) and the lowest one in 2003 (7.64) (one-way ANOVA: $p<0.001$). In contrast, the presence of high biomass, especially represented by laminar Ulvaceae that hamper sediment resuspension, enhanced water transparency which decreased significantly in 1998 and 2003 when the effort of clam harvesting was at its highest.

Nutrient concentrations

Changes of nutrient concentrations in the surface sediments of the Venice lagoon are reported in Table 1. The first data of total phosphorus (P_{tot}) and total nitrogen (N_{tot}) in the surface sediments date back to 1948–49 (Perin 1975, Perin et al. 1983), before the 2nd post-war industrial development. The highest changes were recorded for P_{tot} concentrations. During the period 1948–49, P_{tot} presented very low values: 24±16 μg g^{-1} (mean±SD). Twenty years later, in the period 1968–73, this value increased remarkably (164±79 μg g^{-1}) and even more in 1983 (454±126 μg g^{-1}), when it reached the highest mean concentration, i.e. 18.9 times the concentration measured in 1948–1949. In the following years, P_{tot} mean concentration decreased slightly, showing average values between 339 and 375 μg g^{-1} (Cossu and De Fraja-Frangipane 1985; Sfriso et al. 2003, Facca et al. 2014). The maximum value was recorded by Cossu and De Fraja-Frangipane (1985): 1102 μg g^{-1}. No record for the organic and inorganic fractions is available.

Total Nitrogen (N_{tot}) exhibited a mean value of 1.00±0.86 mg g^{-1} in 1948–49, increasing up to 1.86±2.20 mg g^{-1} in the period 1968–73, followed by a progressive decrease to 0.69 μg g^{-1} in 2011. The maximum value was recorded in the period 1968–73 by Perin (1975) with 3.56 mg g^{-1}.

Some more detailed information is available for the central basin of the Venice lagoon where the same operators monitored the surface sediment top layer (5 cm) of 31–34 stations in successive late spring-early summer periods, characterized by different scenarios (1987: presence of high algal biomass; 1993: sharp reduction of macroalgal biomass; 1998: intense Manila clam (*Tapes philippinarum* Adams and Reeve) harvesting; 2003: the highest Manila clam harvesting; 2011: sharp reduction of clam stocks (from ca. 40,000 tonnes in 2010 to ca. 2000 tonnes in 2012) and decrease of clam fishing activities).

During the period between 1987 and 2011 the mean concentrations of P_{tot} per volume unit (μg cm^{-3} of sediment) did not change significantly, except for the maximum value of 720 μg cm^{-3} in 1987, then progressively decreased to 473 μg cm^{-3} (-34%) in

Table 1. Nitrogen and phosphorus changes in surface sediment top layer (5 cm) in the whole lagoon.

		1948–2011								
		Sediment thickness	Phosphorus				Nitrogen			
		cm	Mean		SD	Max	Mean		SD	Max
			μg g^{-1}				mg g^{-1}			
Perin 1974	1948–49	30	24	±	16	50	1.00	±	0.86	1.96
Perin 1974	1968–73	30	164	±	79	250	1.86	±	2.20	3.56
Perin et al. 1983	1983	20	454	±	126	682	1.33	±	0.59	2.74
Cossu and De Fraja-Frangipane 1995	1987–88	20	339	±	215	1102	1.33	±	0.89	4.80
Sfriso et al. 2003	1987	5	386	±	96	720	1.21	±	0.60	3.00
Sfriso et al. 2003	1993	5	361	±	80	682	1.14	±	0.48	2.62
Sfriso et al. 2003	1998	5	375	±	65	541	0.93	±	0.48	1.37
Facca et al. 2009	2003	5	358	±	99	635	0.71	±	0.36	1.48
Facca et al. 2014	2011	5	367	±	114	896	0.69	±	0.75	2.89

Table 2. Changes of Total Phosphorus, Organic Phosphorus and Total Nitrogen in the central lagoon.

	Total Phosphorus					
	1987	1993	1998	2003	2011	changes
	μg/cm³					
site N°	**34**	**34**	**34**	**34**	**31**	**%**
Mean	386	361	375	358	383	≈
SD	96	80	65	99	50	
Min	227	184	257	201	281	
Max	720	682	541	635	473	-34
	Organic Phosphorus					
	1987	1993	1998	2003	2011	changes
	μg/cm³					
site N°	**34**	**34**	**34**	**34**	**31**	**%**
Mean	104	67	59	53	62	-40
SD	42	28	31	53	24	
Min	49	27	16	2	13	
Max	246	210	167	150	113	-54
	Total Nitrogen					
	1987	1993	1998	2003	2011	changes
	mg/cm³					
site N°	**34**	**34**	**34**	**34**	**31**	**%**
Mean	1.21	1.14	0.93	0.71	0.35	-71
SD	0.60	0.48	0.48	0.36	0.48	
Min	0.22	0.33	0.10	0.09	0.04	
Max	3.00	2.62	1.37	1.48	0.48	-84

2011 (Table 2). In contrast, the mean value of the organic fraction (P_{org}) lowered by ca. 40% (from 104 to 62 μg cm^{-3}, one-way ANOVA: $p<0.001$). On the whole, the ratio P_{org}/P_{tot} decreased from 26.9% to 16.2% and in 2011 the maximum P_{org} concentration was more than halved (from 246 to 113 μg cm^{-1}).

In the same period (1987–2011), the mean N_{tot} concentration decreased from 1.21 to 0.35 mg cm^{-3} (ca. -71.1%, one-way ANOVA: $p<0.001$) whereas the peak concentration lowered by 32%, from 3.00 to 2.05 mg cm^{-3}, although the lowest value was recorded in 1998 with 1.37 mg cm^{-3}.

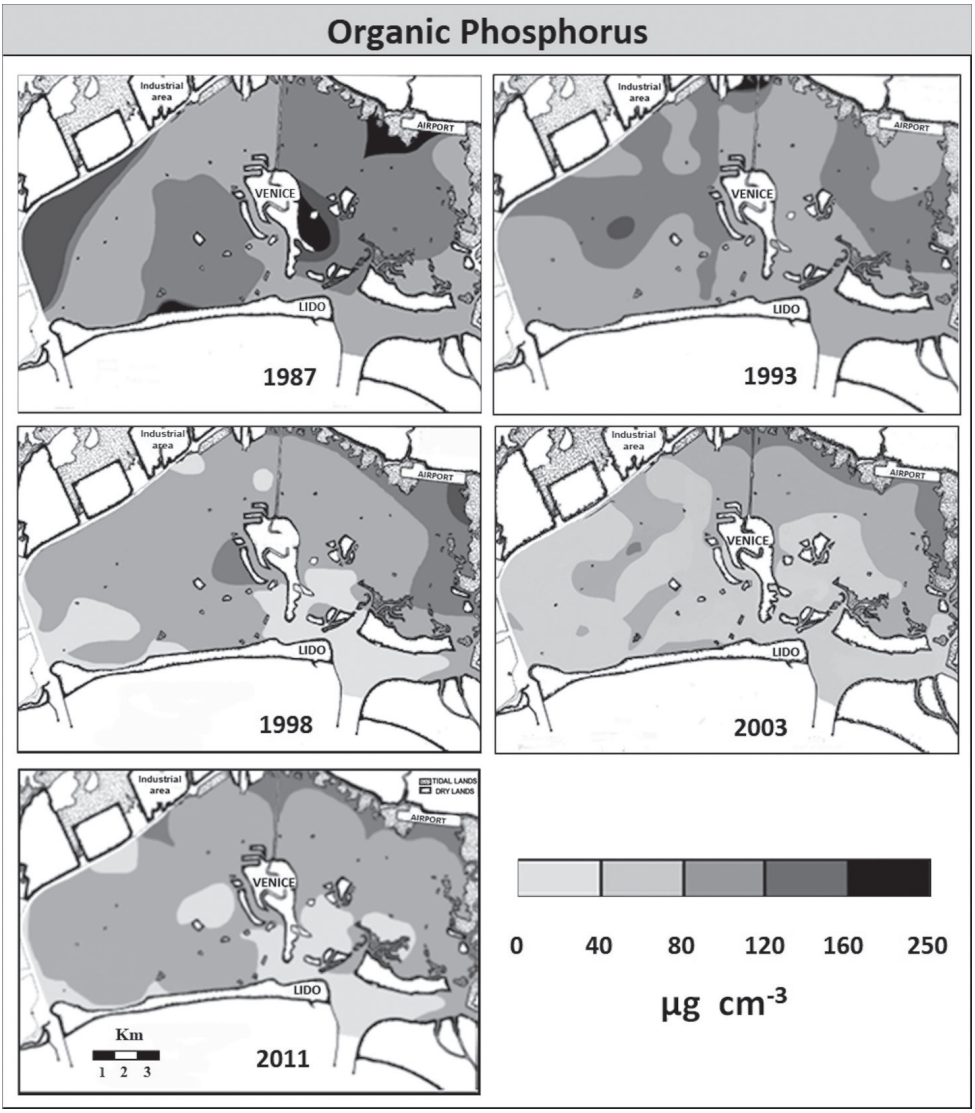

Figure 3. Maps of the organic phosphorus distributions in the 5 cm surface top layer of the central lagoon basin in 1987, 1993, 1998, 2003 and 2011.

Figs 3, 4 highlight the variation of P_{org} and N_{tot} loads in surface sediments of the central lagoon. In the first survey (1987) organic phosphorus showed high concentrations in 4 areas, one of which was affected by the industrial waste of Porto Marghera and the others by the urban discharges of Venice, Lido, Mestre and its hinterland (Fig. 3). In the following years, P_{org} concentrations decreased progressively and in 2011 the highest values were recorded along the mainland coast. Total nitrogen showed a temporal decrease even greater. Except for a station located at the north of the island of S. Erasmo, it generally decreased below 1 mg cm^{-3} (Fig. 4).

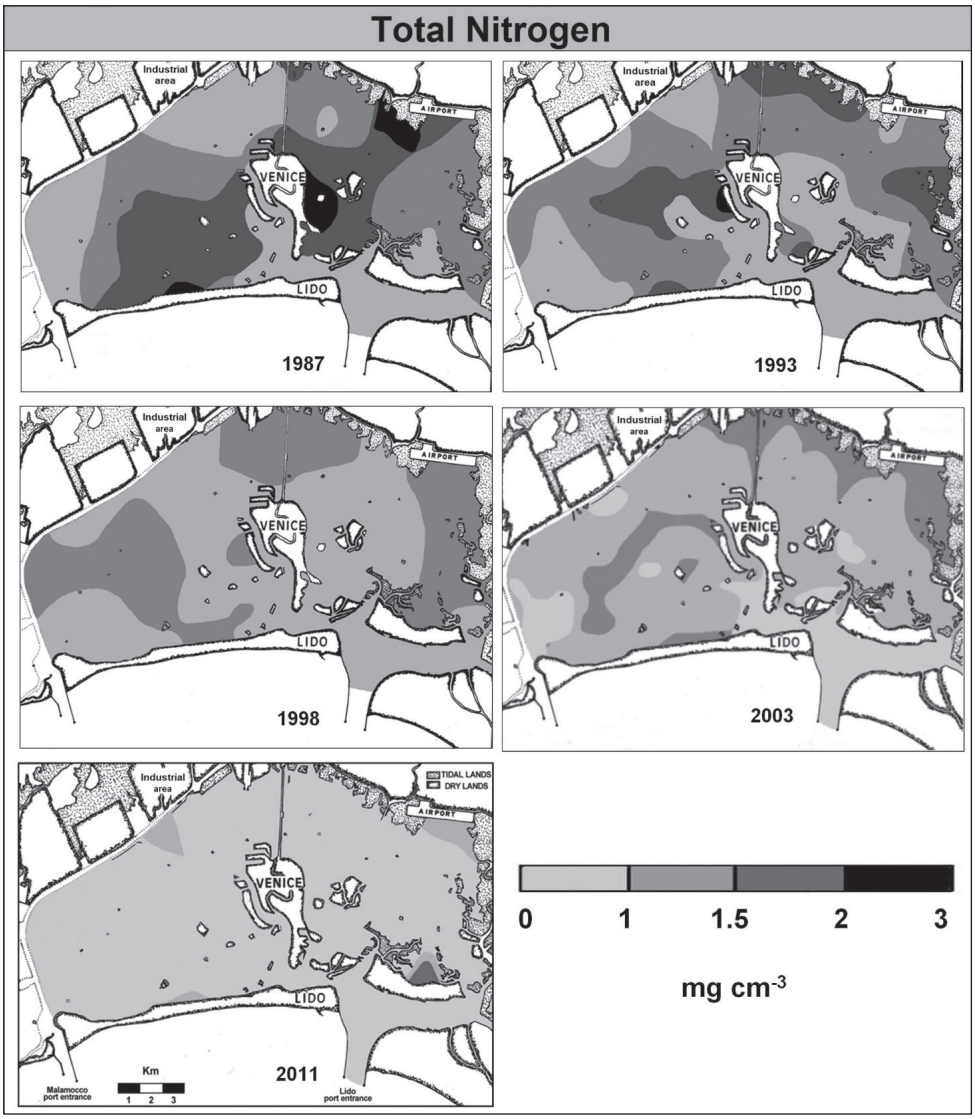

Figure 4. Maps of the total nitrogen distributions in the 5 cm surface top layer of the central lagoon basin in 1987, 1993, 1998, 2003 and 2011.

A significant decrease was also observed for the concentrations of nutrients in the water column Fig. 5). The first records date back to the period 1962–64 and deal with the concentration of ammonium in the canals of Porto Marghera industrial area and the lagoon surface in the vicinity. These values were very high (1000–2500 μM), reaching even 3800 μM in the industrial canals (Fig. 5). Ammonium concentration decreased to 500–1000 μM in the period 1970–72, lowering to 50–100 μM in 1985–8 (Fig. 5). In 2011 (Fig. 6) and 2014 the highest value recorded in that area was <10 μM.

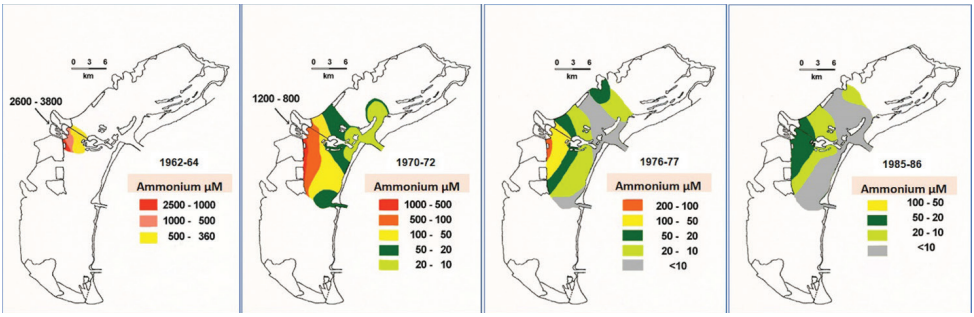

Figure 5. Distribution of ammonium in the water column in the central lagoon from 1962 to 1986 (from Cossu and De Fraja-Frangipane 1985).

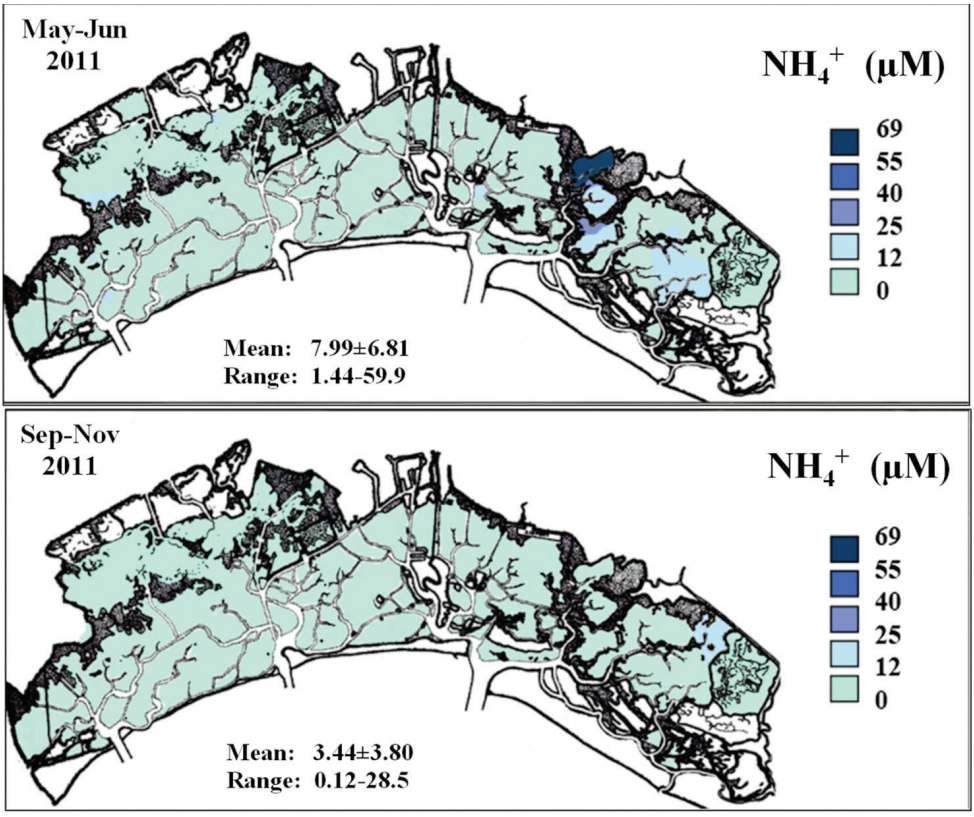

Figure 6. Map of ammonium distribution obtained for the whole lagoon by sampling 118 sites in spring and in autumn 2011.

By considering the whole central lagoon, information on the nutrient concentrations in the water column has been available since 1987. The mean concentration of reactive phosphorus (RP) decreased from 0.76 µM in 1987 (34 sites) to 0.19 µM in 2011 (45 sites), slowly increasing to 0.24 µM in 2014 (34 sites, Fig. 7) (one-way

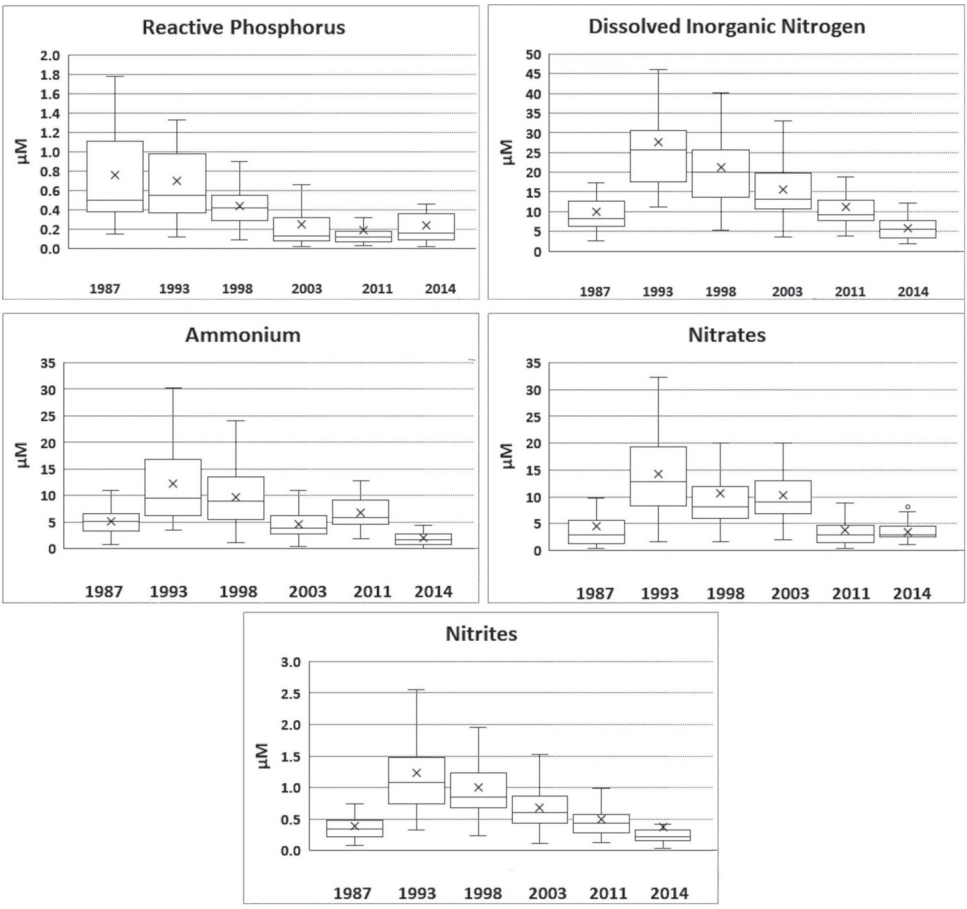

Figure 7. Trends of the mean, standard deviation, maximum, minimum and median values of the reactive phosphorus, dissolved inorganic nitrogen, ammonium, nitrate and nitrite concentrations in the central lagoon of Venice.

ANOVA: $p<0.001$). Conversely, the concentration of the dissolved inorganic nitrogen (DIN) was relatively low in 1987 (10.1 µM), in the presence of a very high macroalgal biomass, but it remarkably increased up to 27.6 µM in 1993 (32 sites) when macroalgae were negligible. Afterwards, DIN concentration progressively decreased to 5.9 µM in 2014 (one-way ANOVA: $p<0.001$). The mean concentrations of ammonium and nitrates showed the same pattern, increasing from 1987 (5.15 and 4.54 µM, respectively) to 1993 (12.2 and 14.2 µM, respectively) and decreasing in the following years to 2.01 µM (ammonium) and 3.51 µM (nitrates) (one-way ANOVA: $p<0.001$). Similarly, nitrites showed a mean concentration of 0.38 µM in 1987, increasing to 1.23 µM in 1993 and decreasing again to 0.37 µM in 2014 (one-way ANOVA: $p<0.001$).

Statistical analyses

The Spearman non-parametric correlation matrices of data collected in the central lagoon were determined by considering both the whole period: 1987–2011 and the years 1987, 1993, 1998, 2003, 2011, separately. On the whole, the highest number of significant ($p<0.05$) direct or inverse correlations was shown by RP (13 out of 18), salinity, Chl-*a* and nitrite (12 out of 18) and pH (11 out of 18) Tables 3, 4). Results depended on the different scenarios. In 1987, the highest number of correlations was found with temperature; in succession, other parameters were nitrite, macroalgal biomass and some parameters associated with the presence of biomass such as %DO, P_{tot} and P_{org} accumulated in the surface sediments and DIN in the water column. In 1993, macroalgal biomass started to decrease significantly and in 1998, when the biomass was negligible and clam fishing activities were affecting a wide part of the central lagoon, the parameters with a high number of significant correlations were salinity (12), water transparency (8) and $P_{inorg,}$ P_{tot} (10 and 8, respectively). In 2003, when clam fishing showed the highest efforts, water transparency (13) dropped down and high amounts of ammonium (15) and P_{org} (12) were released by the surface sediments. RP continued to decrease (12) in the water column whereas pH (13) showed the lowest values (Fig. 7). In 2011, the scenario changed and the number of correlations decreased (Table 4).

The principal component analysis highlighted the parameters with the highest variance (Table 5, Fig. 8). Their spatial distribution and association is displayed by bi-plotting the values of the first two components. The analysis of the whole dataset (1987–2011) shows the parameters with a loading >0.7 (Table 5). Among them, RP and nitrite (NO_2^-) played a key role in the changes of the environment as well as %DO (O_2) and P_{org} (Fig. 8). Moreover, the macroalgal biomass was associated with %DO, pH, water transparency, salinity and sediment density, and opposed to nutrient concentrations, Chl-*a* and water temperature. The analysis of the parameters of each monitoring period is reported in Table 5. On the whole, salinity displayed a loading >0.7 each single year but the value changes when the whole period is considered. Similarly, nitrate and water transparency showed significant loadings (4 out of 5) in the single periods, but not when they referred to the whole period. Nitrite, pH, P_{org} loadings were significant in three periods and only P_{org} did not display any significant value. These trends are also highlighted when the significant values of each parameter are summed.

The bi-plot of the first two PCA components of the transposed matrix highlights the similarities/dissimilarities between the stations of the different sampling periods (Fig. 9). All the stations sampled in 1987 and 1993 are opposite to the ones sampled in 2011. In contrast, results of sampling carried out in 1998 and 2003 partly overlap the ones occurred in other periods highlighting that the environmental conditions in 1998 and 2003 were intermediate.

Table 3. Spearman non-parametric coefficients for the total period 1987–2011 in the central lagoon.

Parameters	Temperature	pH	Eh	O_2	Salinity	Transparency	Chl-*a*	Macroalgae	RP	Ammonium	Nitrite	Nitrate	DIN	Density	P tot	P inorg	P org	TN
Temperature	1.00																	
pH	**-0.20**	1.00																
Eh	0.10	**0.26**	1.00															
O_2	-0.14	**0.63**	**0.41**	1.00														
Salinity	-0.15	0.12	**-0.19**	-0.07	1.00													
Transparency	-0.09	**0.37**	0.08	**0.33**	**0.40**	1.00												
Chl-*a*	0.15	0.07	**0.39**	**0.23**	**-0.56**	**-0.27**	1.00											
Macroalgae	-0.05	**0.34**	0.12	**0.35**	0.15	**0.29**	-0.14	1.00										
RP	0.05	**0.36**	**0.37**	**0.22**	**-0.45**	-0.06	**0.43**	-0.11	1.00									
Ammonium	-0.08	**0.16**	-0.06	-0.11	-0.15	-0.03	0.15	**-0.25**	**0.32**	1.00								
Nitrite	0.14	**-0.23**	0.05	**-0.30**	**-0.42**	**-0.23**	**0.31**	**-0.41**	**0.40**	**0.45**	1.00							
Nitrate	**0.25**	**-0.25**	**0.24**	-0.15	**-0.39**	-0.11	**0.35**	**-0.27**	**0.45**	**0.22**	**0.75**	1.00						
DIN	**0.16**	-0.12	0.15	**-0.19**	**-0.37**	-0.11	**0.35**	**-0.34**	**0.47**	**0.69**	**0.80**	**0.84**	1.00					
Density	-0.02	-0.06	-0.12	-0.07	**0.44**	**0.27**	**-0.27**	0.04	**-0.25**	-0.07	**-0.18**	-0.04	-0.07	1.00				
P tot	0.02	0.09	0.03	0.03	**-0.42**	**-0.34**	**0.21**	0.00	**0.22**	0.09	0.06	-0.07	-0.04	**-0.44**	1.00			
P inorg	0.03	-0.11	-0.08	-0.15	**-0.29**	**-0.40**	0.08	**-0.18**	0.11	0.09	0.14	-0.04	-0.01	**-0.28**	**0.87**	1.00		
P org	0.00	**0.32**	0.14	**0.27**	**-0.41**	-0.15	**0.32**	**0.23**	**0.32**	0.13	0.01	-0.05	0.00	**-0.56**	**0.66**	**0.28**	1.00	
TN	0.03	**0.27**	**0.37**	**0.34**	**-0.27**	0.05	**0.41**	0.06	**0.32**	0.11	**0.17**	0.06	0.11	**-0.44**	**0.32**	0.12	**0.52**	1.00

In bold $p<0.05$ per $r\geq0.16$

Table 4. Number of correlations among parameters.

	1997–2011	1987	1993	1998	2003	2011
RP	13	6	8	1	12	5
Salinity	12	7	9	12	9	6
Nitrite	12	8	4	6	11	6
Chl-*a*	12	2	8	6	10	1
pH	11	4	7	5	13	7
Porg (Sed)	10	7	9	7	12	4
O_2	10	7	9	4	11	2
Nitrate	10	4	5	4	9	5
TN	10	2	5	7	6	2
DIN	9	7	6	7	9	7
Transparency	9	3	6	8	13	4
Density (Sed)	9	4	8	7	7	4
Macroalgae	9	7	1	0	6	2
Ptot (Sed)	8	7	8	8	9	2
Eh	7	1	2	5	0	1
Ammonium	6	3	2	8	15	1
Pinorg (Sed)	6	5	5	10	8	1
Temperature	3	9	0	5	0	0

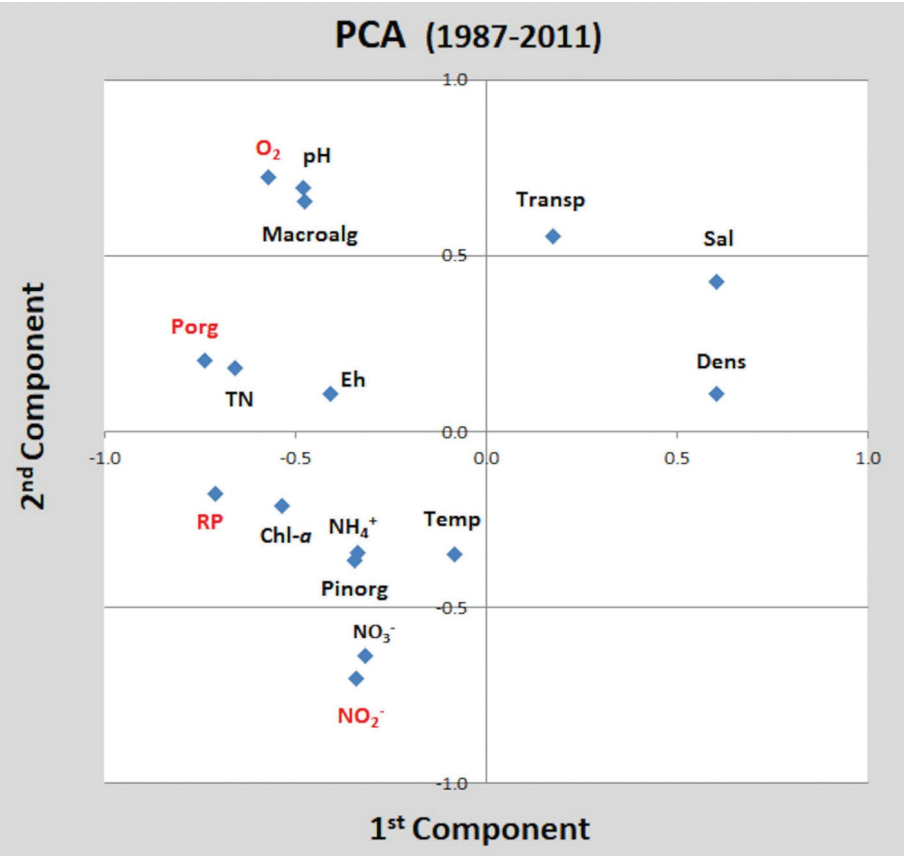

Figure 8. PCA bi-plot of the first two components during the whole 1987–2011 period.

Table 5. PCA loadings of the whole period and the single years.

Periods	Salinity	Nitrate	Transparency	Nitrite	pH	P org	RP	temperature	Chl-*a*	TN	O_2	Ammonium	Eh	Density	Macroalgae	P_{inorg}
1987–2011	0.61	0.64	0.35	0.70	0.69	**0.74**	**0.71**	0.35	0.54	0.66	**0.72**	0.34	0.41	0.60	0.65	0.37
1987	**0.75**	0.38	0.63	0.62	**0.76**	**0.73**	0.65	**0.85**	0.46	0.31	**0.74**	0.46	0.43	0.59	0.62	0.54
1993	**0.90**	**0.71**	**0.74**	**0.78**	0.64	0.61	**0.74**	0.22	**0.85**	0.57	**0.75**	0.52	0.45	0.55	0.22	0.60
1998	**0.70**	**0.83**	**0.83**	0.63	0.67	**0.76**	0.34	**0.77**	**0.74**	0.69	0.55	0.64	**0.84**	**0.81**	0.55	0.60
2003	**0.77**	**0.76**	**0.77**	**0.88**	**0.87**	**0.84**	**0.90**	0.35	0.66	**0.75**	0.65	**0.94**	0.20	0.63	0.30	0.62
2011	**0.76**	**0.78**	**0.72**	**0.86**	**0.73**	0.62	0.60	0.20	0.51	**0.74**	0.56	0.49	0.56	0.65	**0.76**	0.55
Total single years	**3.88**	**3.46**	**3.69**	**3.77**	**3.67**	**3.56**	**3.23**	**2.39**	**3.22**	**3.06**	**3.25**	**3.05**	**2.48**	**3.23**	**2.45**	**2.91**

in bold = loading >70

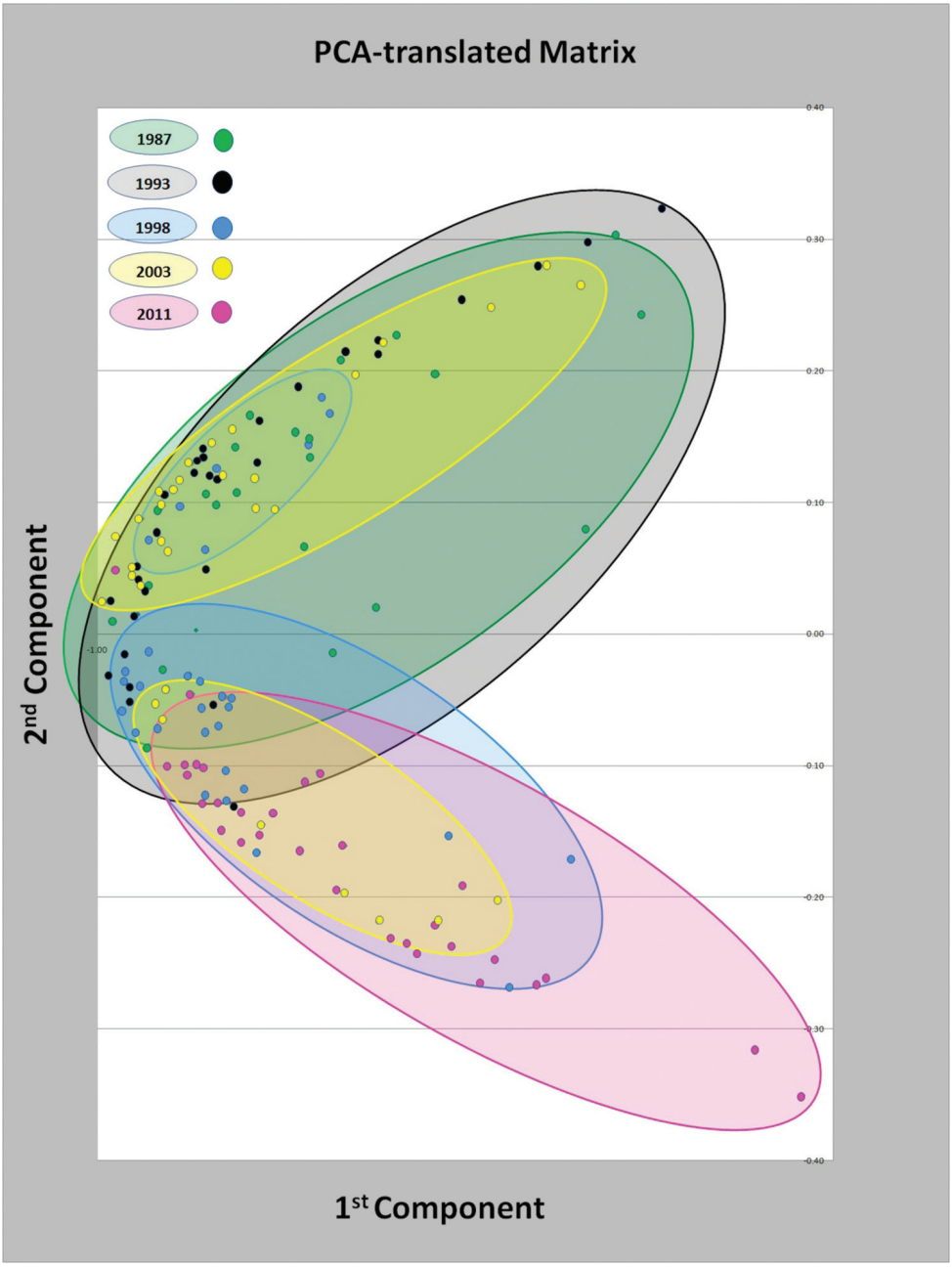

Figure 9. Transposed PCA bi-plot of the first two components during the whole 1987–2011 period. The separation or overlapping of the different years are highlighted by different colours.

Discussion

The lagoon of Venice is one of the most studied transitional environments of the Mediterranean Sea and the high number of available datasets enables us to understand environmental changes over the years and foresee its evolution. The trophic parameters and primary producers linked to their change have been studied assiduously since the early '80s, although for some of them, data have been available since the late '40s. Actually, since the 2nd post-war industrial development frequent changes of the environmental scenarios have witnessed the effect of different anthropogenic activities. Between the '60s and '80s, a significant increase of eutrophication was recorded (Wolf 1986, Dunnette and O'Brien 1992, Schramm and Nienhuis 1996). In Italy, it affected firstly freshwater environments, especially shallow lakes and ponds (Chiaudani et al. 1980), then transitional (Cossu et al. 1983, 1984, Facco et al. 1986, Sfriso et al. 1988) and coastal (Chiaudani et al. 1980, 1983) environments. The Venice lagoon, because of its historical and economic role, was the object of a very high number of environmental studies. In fact, hydrological interventions like the digging of wide and deep commercial canals (i.e. Vittorio Emanuele III, Malamocco-Marghera, namely "Canale dei Petroli"), the development of the industrial area of Porto Marghera, the increase of agricultural monoculture and population living in urban centers dramatically increased the flow of nutrients and pollutants into the lagoon (Marcomini et al. 1993). The first effect was a sharp increase in their concentration both in the water column and surface sediments which led to a change of primary producers and the spreading of nuisance macroalgae (Sfriso et al. 1988, Morand and Briand 1996, Ménesguen 2018). Since the early '90s, some synergic factors, mainly driven by climatic changes, have affected macroalgal growth (Sfriso and Marcomini 1996) that rapidly declined. In the meantime, the clam *Tapes philippinarum,* which had been introduced in the lagoon for economic purposes (Cesari and Pellizzato 1985), spread everywhere and fishing activities strongly affected the sediment texture by changing its grain-size and benthic communities (Pranovi et al. 2004, Sfriso et al. 2005). The main effect was the resuspension of high amounts of sediments, nutrients and pollutants (Sfriso et al. 2003, 2005). This scenario lasted until clam stocks were depleted and the lagoon began a progressive recovery of its ecological conditions which is still underway. In addition, in the past twenty years, many decrees and directives have contributed to reduce the trophic status of the lagoon (Ronchi-Costa Decree April 23th 1998, Water Framework Directive (2000/60/EC, etc.) which showed a remarkable resilience (Regione Veneto et al. 2012, 2015, Facca et al. 2014). Nowadays, the anthropogenic pressures that in the past used to affect the lagoon have decreased, especially nutrient enrichments and clam fishing, and the trophic conditions of the basin are regulated by the low concentrations of phosphorus. This element was banned from the detergent formulations (4–5% of total weight) in 1989 (Solidoro et al. 2010) and is presently the main factor limiting the primary production in the lagoon. Therefore, the Venice lagoon differs from many transitional and coastal areas that are affected by increasing

or steady high trophic conditions, such as the lagoons and ponds of the Po delta and the Valli di Comacchio (Munari and Mistri 2012, Sfriso et al. 2014b), the costs of Chine (Hu et al. 2010, Ye et al. 2011), the coasts of Brittany (Diaz et al. 2013, Perrot et al. 2014, Ménesguen 2018) the gulf of Mexico (Rabalais et al. 2009) and many others, and showed a return to good/high environmental conditions. The improvement of the environment is also highlighted by the increase of seagrass cover and the spreading of sensitive macroalgal taxa which in the Venice lagoon occurred both naturally and thanks to special projects. Recently, the project SeResto (Seagrass Restoration) developed a new strategic approach to meet HD & WFD objectives" (Bonometto et al. 2018, Sfriso et al. 2018) by transplanting aquatic angiosperms. The project funded by the European Community in the framework of LIFE12 NAT/IT/000331"Habitat 1150* (Coastal lagoon) recovery by SEagrass RESTOration" contributed to colonizing ca. 10 km^2 of the lagoon at different levels of coverage, ca. 4 km^2 of total plant cover, thus recovering fish and benthic communities. As the success of the project was due to the general recovery of the lagoon it is believable that in the absence of additional anthropogenic pressures, the environment should continue to improve its ecological conditions as evidenced by the monitoring of biological elements in accordance with WFD requirements (Regione Veneto et al. 2015).

Conclusions

This paper was prepared in the framework of the LTER-Italy network in order to analyze a part of the great amount of data collected in the Venice lagoon. The long-term analysis of the trophic status since the middle of the 20th century made it possible to highlight both the evolution of this environment and the parameters related to the observed changes. The lagoon eutrophication increased markedly from the 2nd post-war period until the end of the '80s, when high nutrient amounts were released into the environment (dissolved in the water column and accumulated in the surface sediments), triggering macroalgal blooms and favoring hyper-dystrophic conditions. The parameters which are related to the primary production (%DO, pH, water transparency, RP, P_{org}) showed the highest changes. In the following years, different environmental scenarios have occurred but the latest data show that the lagoon environment is improving and, without other additional anthropogenic pressures, it should keep a positive trend for at least 10 years or longer. Results highlight that the trophic status of a transitional environment, from the physico-chemical point of view, can be easily detected by measuring some driver parameters such as RP and nitrites. They are the most sensitive nutrients to the environmental changes, easy to analyze and low-cost. The analysis of these two parameters together with the measurement of pH and the oxygen concentration can support macrophyte assemblages for the assessment of the ecological status according to the WFD (2000/60/EC) and provide an exhaustive method for the determination of the trophic status of a transitional water system.

Acknowledgments

The authors thank the ARPA Veneto that funded the most recent monitoring of the whole lagoon (Regione Veneto et al. 2012, 2015) and the European Community that allowed the transplantation of aquatic angiosperms in the northern lagoon in the framework of the project LIFE12 NAT/IT/000331 SERESTO. A special thank you also goes to Dr. Orietta Zucchetta and the anonymous referee for the English editing.

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Successions of phytobenthos species in a Mediterranean transitional water system: the importance of long term observations

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Abstract

The availability of quantitative long term datasets on the phytobenthic assemblages of the Mar Piccolo of Taranto (southern Italy, Mediterranean Sea), a lagoon like semi-enclosed coastal basin included in the Italian LTER network, enabled careful analysis of changes occurring in the structure of the community over about thirty years. The total number of taxa differed over the years. Thirteen non-indigenous species in total were found, their number varied over the years, reaching its highest value in 2017. The dominant taxa differed over the years. The number of species in each taxonomic division also varied. In addition to the centuries-old exploitation of its biotic resources, mainly molluscs, the basin has been subject for a long time to a range of anthropogenic driving forces linked to urbanisation, shipbuilding, agriculture and military activities, which have caused chemical and biological pollution, eutrophication and habitat destruction. It may therefore be assumed that these changes were closely related to human activities. Indeed, it was observed that the reduction of only one of these forces, i.e. urban sewage discharge, triggered the recovery of phytobenthos. Therefore, it may be assumed that if the anthropogenic pressure on the Mar Piccolo was eased, it could once again become the paradisiacal place it was held to be in ancient times.

Keywords

LTER, Mar Piccolo, Mediterranean Sea, phytobenthos, transitional water systems

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Introduction

Human beings play a fundamental role in the ongoing degradation of coastal marine ecosystems (Bianchi et al. 2014). Coastal lagoons and transitional water systems (TWSs) generally are frequently chosen for human activities, due to their physicochemical and biocoenotic features. The main anthropogenic forcing factors are aquaculture, fishing and shipping, as well as tourism and recreation (Newton et al. 2014), with eutrophication and chemical pollution the most important effects on the environment (Pérez-Ruzafa et al. 2011a). Recently, biological pollution, caused by the introduction of non-indigenous species (NISs), has also become a significant issue in TWSs (Lotze et al. 2006). Heavy anthropogenic use severely damages such environments, which in contrast should be preserved by sustainable management. Extensive knowledge of the biotic and abiotic components of these systems is thus necessary, as well as an understanding of the cause-effect relationship between anthropogenic processes and environmental feedback (Pérez-Ruzafa et al. 2011a).

The availability of historic data on benthic populations could allow better measurement of the changes (Bianchi et al. 2014; Husa et al. 2014). Historical datasets could also make it possible to evaluate the effects of environmental recovery measures and to predict possible reactions to future changes (Gosz et al. 2010; Mirtl 2010; Bertoni et al. 2012). To this end, long-term ecological research (LTER), i.e. studies embracing long time-scales, could enhance our understanding of interactions amongst biotic components and the environment and help to distinguish between natural ecological processes and changes due to human actions (Bertoni et al. 2012).

The Mar Piccolo of Taranto (southern Italy) has belonged to the Italian LTER network since 2011 (LTER_EU_IT_095; https://deims.org/ac3f674d-2922-47f6-b1d8-2c91daa81ce1) (Cecere and Petrocelli 2012). It is a semi-enclosed coastal basin with lagoon features, which falls into the category of transitional waters (Viaroli and Basset 2009). Its phytobenthos has been studied since the second decade of the 20th century, albeit not continuously (Cecere and Petrocelli 2009). Comparison of historical floristic data dating back to the 1920s (Pierpaoli 1923; Cecere and Saracino 1999) with data collected in 1986, enabled initial assessment of the changes affecting phytobenthic assemblages in the Mar Piccolo over a period of sixty-four years (Cecere et al. 1991a). Subsequently, some other floristic lists, arising from studies carried out in the basin in 2001 and 2008 with a range of methods, became available (Sassanelli 2011; E. Cecere and A. Petrocelli, unpublished data). In addition, in 1989, a one-year quantitative study was performed, which gave the first ever information on the standing crop of seaweed in the Mar Piccolo and detected the dominant species (Cecere et al. 1992).

The aim of the present study was to increase our knowledge of the structure of phytobenthic assemblages in the Mar Piccolo of Taranto and to reconstruct the history of macrophytic communities in terms of both species composition and dominance changes during the last 30 years. For this purpose, seasonal quantitative data, collected at various sites in the Mar Piccolo in 2008 (Project VECTOR http://vector.conismamibi.it) and from spring 2011 to winter 2018 (I-LTER network), were carefully

analysed and compared with the floristic data collected in 1989. At the same time, again on the basis of already published data, speculative parallels were drawn with the demographic and socio-economic development of the town of Taranto (https://tiny-url.com/yacl8t6t; Nistri 1988).

Methods

Study site

The Mar Piccolo of Taranto is a lagoon-like basin located north of Taranto. It has a surface area of 20.72 km^2 and is divided into two sub-basins known as the First Inlet, to the west and the Second Inlet, to the east (Fig. 1). Hard substrata account for a limited area and are mostly artificial, consisting of concrete wharves and facilities for mussel farming. A small reef and a ruined stone pier, together with a few scattered stones and mussel shells, are the only natural hard substrata. Recent observations both *in situ* and by remote sensing showed that the anthropogenic impact on the Mar Piccolo is very high in the First Inlet but less severe in the Second Inlet and arises mainly from lines, poles and frames used for mussel farming (Bracchi et al. 2016). Hydrodynamism is limited, as is exchange with the adjacent Mar Grande basin (i.e. Mediterranean Sea), which occurs mainly through the Canale Navigabile (Fig. 1). The mean annual seawater surface temperature ranges from 7.5 °C to 32.3 °C and seawater surface salinity from 33.0 to 37.7 ‰ (Cecere et al. 2016a). Those values are strongly affected by inputs of freshwater from submarine springs and a few small rivers (Cecere and Petrocelli 2009).

Field activities

In 2008, sampling exercises were carried out at monthly intervals at three sites, two in the First Inlet (Station A and Station B) and one in the Second Inlet (Station C).

Station A (40°29'35"N, 17°14'17"E) was characterised by soft bottoms and artificial hard substrata, mainly concrete blocks. Station B (40°30'01"N, 17°15'10"E) was characterised by a soft muddy bottom. Station C (40°29'40"N, 17°19'18"E) had a soft muddy bottom with scattered concrete blocks.

In the period spring 2011 - winter 2018, four sites were seasonally sampled, two in the First Inlet (Station 1 and Station 2) and two in the Second Inlet (Station 3 and Station 4) (Fig. 1).

Station 1 (40°28'46"N, 17°13'41"E) was the only urban station, located in the old town. Artificial hard substrata prevailed, composed mainly of discarded plastic used in mussel farming activities but also concrete quays. Station 2 (40°30'03"N, 17°15'30"E) was located next to the mouth of a small river, with a soft muddy bottom and a few artificial substrates, mainly unlawfully built concrete quays used

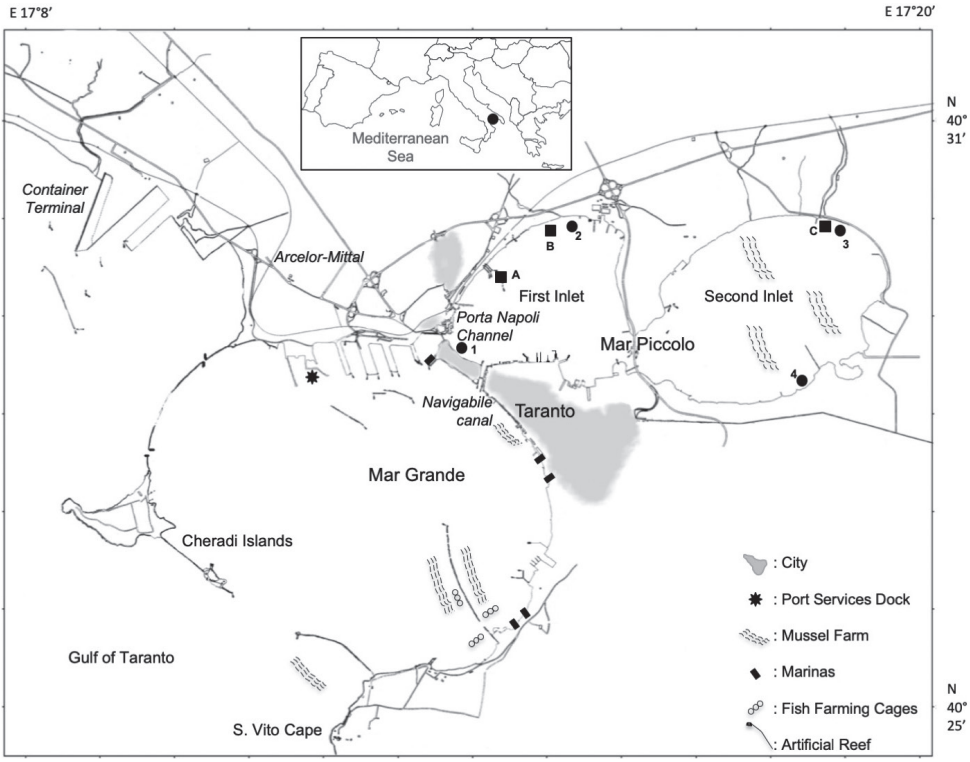

Figure 1. Location of Mar Piccolo in the Gulf of Taranto (Mediterranean Sea). ■ 2008 stations; ● 2011–2018 stations.

by mussel farmers. Station 3 (40°29'39"N, 17°19'22"E) was the only station characterised by natural hard substrata, rare in the Mar Piccolo, i.e. small rocks and the remains of a stone dock. It was close to the mouth of a small river. Station 4 (40°28'20"N, 17°18'25"E) was located in a zone where mussels and other seafood are manually cleaned, bagged and sold. A soft muddy bottom and concrete platforms were the main substrata.

The samples were handpicked from within a 50×50 cm square, randomly placed on the bottom. Three replicates were collected at each station. Seaweed thalli were stored in plastic bags and transported to the laboratory within a few hours. There, the macrophytes were sorted and each species was identified and weighed on a triple-beam balance. Data were expressed as kg wet weight m^{-2} (hereafter kg m^{-2}). A list of species was compiled for each dataset, including the unpublished floristic data from 1989, with nomenclature as in Guiry and Guiry (2018). In order to make the 1989 and 2008 datasets comparable, only data relating to the months that were also sampled in 2011–2018 (i.e. March for winter, May for spring, September for summer, December for autumn) were considered.

Data analysis

The quantitative data recorded in 2008 were not processed, since the sampling sites were not the same as those of 2011–2018. Therefore, only the total number of species, taxonomic divisions, biogeographical elements and dominant species were compared.

Since seasonal samplings started in spring 2011, to allow statistical tests to be performed on all four seasons of the year, the analysis was carried out during a period embracing the spring, summer and autumn of one year together with the winter of the following year (spring 2011 - winter 2012).

All univariate and multivariate analyses were performed using PRIMER v.6 (Primer-E Ltd., Plymouth, UK).

Two different matrices of the means were constructed from the three replicates collected at each station. The first matrix considered all the identified taxa and the second matrix only the NIS taxa. From the absolute abundance matrices (taxa vs. samples), the Bray-Curtis similarity index was calculated after log(x+1) transformation, in order to remove the effects of orders-of-magnitude differences between samples, to normalise the data and to increase the importance of smaller values, such as the mid-range species (Frontalini et al. 2009).

The PRIMER 'DIVERSE' routine was used to calculate the taxonomic richness (S), taxon abundance (n°taxa), Pielou's evenness index (J') and Shannon-Weaver diversity index (H') for each sample.

The statistical significance of spatial and temporal variations in the community structure across the defined factors, "year", "season", "basin" and "station", were tested by one-way analysis of similarities (ANOSIM). A two-way crossed analysis (year x station) was performed to highlight possible differences from year to year. In addition, bi-dimensional representations of the statistical comparisons amongst the samples collected during the seven years at the four stations were performed by means of non-parametric multidimensional scaling (nMDS) with superimposed hierarchical clustering and a cut-off at 80–90% similarity.

The SIMPER routine was used to identify the species that contributed most to dissimilarities amongst sampling sites (one-way procedure) and to explain the changes in terms of biomass or species composition (two-way procedure, years vs. sites).

All the multivariate tests were performed on the Bray-Curtis similarity matrix including all the identified taxa.

Results

The floristic lists of species collected in 1989, 2008 and 2011–2018 are reported in Table 1. The total number of taxa (Fig. 2, Table 1) differed over the years. Only nine taxa were found in all the considered periods (i.e. *Alsidium corallinum, Chaetomorpha linum, Dictyota dichotoma* var. *dichotoma, D. dichotoma* var. *intricata, Gracilaria bursa-pastoris, G. gracilis,*

Table 1. Floristic list (presence/absence data) of seaweeds and phanerogams recorded in the Mar Piccolo in the different years. B=biogeographic element, A=Atlantic, C=Cosmopolitan, CT=CircumTropical, IP=IndoPacific, M=Mediterranean.

	B	1989	2008	2011	2012	2013	2014	2015	2016	2017	2018
Unidentified Bacillariophyta		+			+	+	+				
Unidentified Cyanophyta		+	+								
Rhodophyta											
Acanthophora nayadiformis (Delile) Papenfuss	IP	+	+								
Agardhiella subulata (C. Agardh) Kraft *et* M.J. Wynne	A	+							+	+	
Aglaothamnion tenuissimum (Bonnemaison) Feldmann-Mazoyer	A									+	+
Aglaothamnion tripinnatum (C. Agardh) Feldmann-Mazoyer	A									+	
Alsidium corallinum C. Agardh	A	+	+	+	+	+	+	+	+	+	+
Alsidium helminthochorton (Schwendimann) Kützing	M	+									
Amphiroa beauvoisii J.V. Lamouroux	A			+	+	+	+	+	+	+	+
Antithamnion cruciatum (C. Agardh) Nägeli	A		+		+	+	+		+	+	
Antithamnion tenuissimum (Hauck) Schiffner	M					+					
Callithamnion corymbosum (J.E. Smith) Lyngbye	A	+	+		+			+	+	+	+
Caulacanthus cf. *ustulatus* (Mertens *ex* Turner) Kützing	C				+	+	+	+	+	+	+
Ceramium cimbricum H.E. Petersen f. *flaccidum* (H.E. Petersen) G. Furnari *et* Serio	C		+	+				+		+	
Ceramium comptum Børgesen	A								+	+	
Ceramium diaphanum (Lightfoot) Roth	C								+		
Ceramium incospicuum Zanardini	M		+					+			+
Ceramium siliquosum (Kützing) Maggs *et* Hommersand	C	+		+	+	+	+		+		
Chondracanthus acicularis (Roth) Fredericq	C		+	+	+	+	+	+	+	+	+
Chondracanthus teedei (Mertens *ex* Roth) Kützing	C		+		+	+	+	+	+	+	
Chondria capillaris (Hudson) M.J. Wynne	C	+	+								
Chondria dasyphylla (Woodward) C. Agardh	C		+								
Chylocladia verticillata (Lightfoot) Bliding	A		+	+	+	+	+	+			
Corallina officinalis Linnaeus	C				+	+	+	+		+	
Dasya baillouviana (S.G. Gmelin) Montagne	CT		+								
Dasya ocellata (Grateloup) Harvey	A		+				+		+		
Dasya punicea (Zanardini) Meneghini	A									+	
Dasya rigidula (Kützing) Ardissone	A				+	+					
Dasysiphonia sp							+	+	+		
Ellisolandia elongata (J. Ellis *et* Solander) K.R. Hind *et* G.W. Saunders	A			+	+	+	+	+	+	+	+
Erythrocladia irregularis Rosenvinge	C				+		+				
Gayliella flaccida (Harvey *ex* Kützing) T.O. Cho *et* L.J. McIvor	C		+	+						+	
Gelidiella lubrica (Kützing) Feldmann *et* Hamel	IP		+		+				+		
Gelidium crinale (Hare *ex* Turner) Gaillon	C				+	+	+	+	+	+	+
Gelidium pusillum (Stackhouse) Le Jolis	C		+		+			+			+
Gigartina cf. *pistillata* (S.G. Gmelin) Stackhouse	A								+	+	
Gracilaria bursa-pastoris (S.G. Gmelin) P.C. Silva	C	+	+	+	+	+	+	+	+	+	+
Gracilaria dura (C. Agardh) J. Agardh	A	+		+							
Gracilaria gracilis (Stackhouse) Steentoft, L.M. Irvine *et* Farnham	A	+	+	+	+	+	+	+	+	+	
Gracilaria longa Gargiulo, De Masi *et* Tripodi	M	+	+								
Gracilariopsis longissima (S.G. Gmelin) Steentoft, L.M. Irvine *et* Farnham	C	+	+	+	+	+	+	+	+	+	
Grateloupia cf. *filicina* (J.V. Lamouroux) C. Agardh	C	+		+	+	+	+		+	+	+
Grateloupia minima P.L. Crouan *et* H.M. Crouan	A							+		+	
Grateloupia turuturu Yamada	IP			+	+	+	+	+	+	+	+

	B	1989	2008	2011	2012	2013	2014	2015	2016	2017	2018
Griffithsia schousboei Montagne	A	+									
Herposiphonia secunda (C. Agardh) Ambronn	CT					+		+			
Herposiphonia tenella (C. Agardh) Ambronn	CT	+	+	+		+	+		+		
Heterosiphonia crispella (C. Agardh) M.J. Wynne	A							+			
Huismaniella nigrescens (Feldmann) G. Furnari, Cormaci, Alongi *et* Perrone	M				+						
Huismaniella ramellosa (Kützing) G.H. Boo *et* S.M. Boo	CT		+								
Hydrolithon cruciatum (Bressan) Y.M. Chamberlain	A		+						+		
Hydrolithon farinosum (J.V. Lamouroux) Penrose *et* Y.M. Chamberlain	C			+		+	+		+		
Hypnea cornuta (Kützing) J. Agardh	IP		+	+	+	+	+	+	+	+	
Hypnea musciformis (Wulfen) J.V. Lamouroux	CT		+				+	+	+	+	
Hypnea spinella (C. Agardh) Kützing	CT	+	+				+				
Jania rubens (Linnaeus) J.V. Lamouroux	C						+	+	+	+	
Jania virgata (Zanardini) Montagne	A							+			
Laurencia intricata J.V. Lamouroux	A		+								
Lomentaria clavellosa (Lightfoot *ex* Turner) Gaillon	A				+						
Lomentaria compressa (Kützing) Kylin	M	+									
Lophosiphonia obscura (C. Agardh) Falkenberg	C				+						
Nitophyllum albidum Ardissone	M				+						
Osmundea oederi (Gunnerus) G. Furnari	A		+							+	+
Osmundea pelagosae (Schiffner) K.W. Nam	M									+	
Peyssonnelia bornetii Boudouresque *et* Denizot	M			+							
Phymatolithon calcareum (Pallas) W.H. Adey *et* D.L. McKibbin *ex* Woelkerling *et* L.M. Irvine	A										+
Polysiphonia denudata (Dillwyn) Greville *ex* Harvey	C	+						+			
Polysiphonia elongata (Hudson) Sprengel	A		+						+		
Polysiphonia morrowii Harvey	IP				+		+	+	+	+	+
Polysiphonia subulata (Ducluzeau) Kützing	A	+					+	+		+	
Porphyra linearis Greville	A				+					+	
Porphyra umbilicalis Kützing	C	+				+		+			
Pterocladiella capillacea (S.G. Gmelin) Santelices *et* Hommersand	C			+						+	
Pterocladiella melanoidea (Schousboe *ex* Bornet) Santelices *et* Hommersand	A		+				+		+		
Pyropia leucosticta (Thuret) Neefus *et* J. Brodie	A						+				
Radicilingua reptans (Kylin) Papenfuss	A						+				
Radicilingua thysanorhizans (Holmes) Papenfuss	A	+	+			+	+	+	+	+	+
Rhodymenia ardissonei (Kuntze) Feldmann	A							+	+		
Rhodymenia pseudopalmata (J.V. Lamouroux) P.C. Silva	A	+	+							+	
Rytiphlaea tinctoria (Clemente) C. Agardh	A							+			
Solieria filiformis (Kützing) P.W. Gabrielson	A	+								+	
Spyridia filamentosa (Wulfen) Harvey	C	+	+	+	+	+	+	+	+	+	+
Stylonema alsidii (Zanardini) K.M. Drew	C		+								
Stylonema cornu-cervi Reinsch	A			+							
Wrangelia penicillata (C. Agardh) C. Agardh	CT			+							
Unidentified non geniculate Corallinaceae					+	+					
83		24	34	22	29	25	32	33	34	37	18
Ochrophyta											
Colpomenia peregrina Sauvageau	C				+	+	+	+	+	+	+
Colpomenia sinuosa (Mertens *ex* Roth) Derbès *et* Solier	C							+	+	+	
Cutlera chilosa (Falkenberg) P.C. Silva	M						+				
Cutleria multifida (Turner) Greville	C									+	+
Cystoseira barbata (Stackhouse) C. Agardh	IP	+		+	+	+	+				
Cystoseira compressa (Esper) Gerloff *et* Nizamuddin	A					+	+	+	+	+	

	B	1989	2008	2011	2012	2013	2014	2015	2016	2017	2018
Dictyota dichotoma (Hudson) J.V. Lamouroux v. *dichotoma*	C	+	+	+	+	+	+	+	+	+	
Dictyota dichotoma (Hudson) J.V. Lamouroux *var. intricata* (C. Agardh) Greville	C	+	+	+	+	+	+	+	+	+	+
Ectocarpus siliculosus (Dillwyn) Lyngbye	C	+			+	+	+		+	+	
Feldmannia mitchelliae (Harvey) H.-S. Kim	C								+		
Halopteris filicina (Grateloup) Kützing	C				+					+	
Hincksia dalmatica (Ercegović) Cormaci *et* G. Furnari	M							+		+	+
Nemacystus flexuosus (C. Agardh) Kylin var. *giraudyi* (J. Agardh) De Jong	M						+				
Padina pavonica (Linnaeus) Thivy	CT				+	+	+	+	+	+	+
Petalonia fascia (O.F. Müller) Kuntze	C							+		+	
Scytosiphon lomentaria (Lyngbye) Link	C				+			+	+	+	+
Sphacelaria cirrosa (Roth) C. Agardh	C							+	+	+	
Sphacelaria fusca (Hudson) S.F. Gray	C								+	+	+
Sphacelaria rigidula Kützing	C				+	+					
Undaria pinnatifida (Harvey) Suringar	IP		+								
20		4	3	3	9	8	9	10	11	14	7
Chlorophyta											
Bryopsis corymbosa J. Agardh	A									+	
Bryopsis cupressina J.V. Lamouroux	M								+		+
Bryopsis pennata J.V. Lamouroux	A				+	+	+				
Bryopsis plumosa (Hudson) C. Agardh	C					+	+	+			
Caulerpa cylindracea Sonder	CT		+			+					
Chaetomorpha linum (O.F. Müller) Kützing	C	+	+	+	+	+	+	+	+	+	+
Cladophora dalmatica Kützing	A		+	+							
Cladophora glomerata (Linnaeus) Kützing	C				+	+		+	+		
Cladophora hutchinsiae (Dillwyn) Kützing	C									+	+
Cladophora laetevirens (Dillwyn) Kützing	C		+	+	+	+		+	+	+	
Cladophora lehmanniana (Lindenberg) Kützing	A					+			+		
Cladophora prolifera (Roth) Kützing	A								+		
Cladophora ruchingeri (C. Agardh) Kützing	A	+									
Cladophora rupestris (Linnaeus) Kützing	A		+			+	+				
Cladophora sericea (Hudson) Kützing	C		+						+		
Codium fragile (Suringar) Hariot subsp. *fragile*	IP			+		+	+	+	+		
Ulva compressa Linnaeus	C	+		+				+	+	+	+
Ulva curvata (Kützing) De Toni	A	+	+		+	+	+				
Ulva flexuosa Wulfen	C	+			+	+				+	
Ulva intestinalis Linnaeus	C			+	+	+	+		+	+	+
Ulva laetevirens Areschoug	C	+	+	+	+	+	+	+	+	+	+
Ulva prolifera O.F. Müller	C	+	+		+	+		+	+	+	
Ulva pseudorotundata Cormaci, G. Furnari *et* Alongi	A	+	+	+	+	+	+	+	+		+
Ulva rigida C. Agardh	A	+					+		+	+	+
Umbraulva dangeardii M.J. Wynne *et* G. Furnari	A			+	+	+	+	+	+	+	
Valonia macrophysa Kützing	CT				+						
26		9	10	9	12	16	11	10	15	11	8
Spermatophyta											
Cymodocea nodosa (Ucria) Ascherson	A			+	+	+	+	+	+	+	+
Ruppia cirrhosa (Petagna) Grande	C				+						
Zostera noltei Hornemann	A								+		
3		0	0	1	2	1	1	1	2	1	1
TOTAL											
132		37	47	35	52	50	53	54	62	63	34

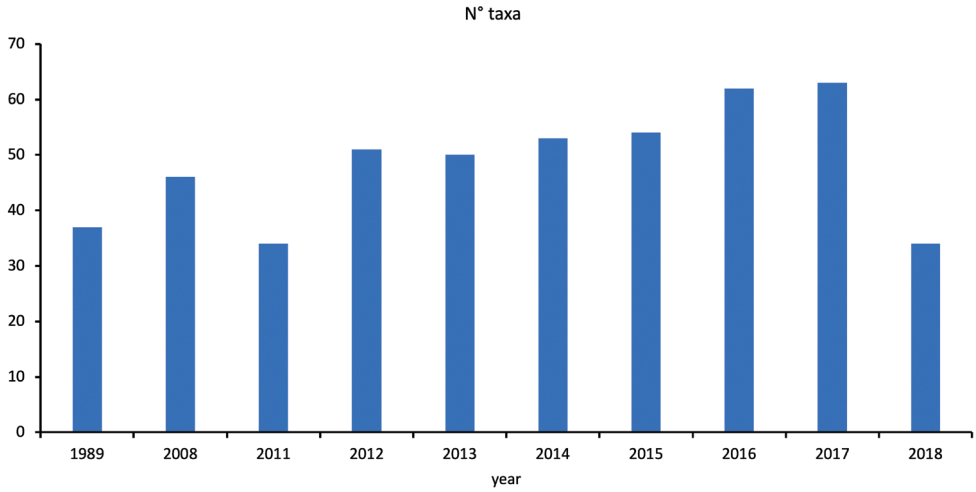

Figure 2. Trend in the total number of species. 2018 data for winter only. In addition to the analysed years 2008 and 2011–2018, unpublished floristic data from 1989 have been included.

Gracilariopsis longissima, Spyridia filamentosa and *Ulva laetevirens*). Seventy-six taxa recorded in 2011–2018 were not found in 1989 and 2008 (Table 1). Three taxa (i.e. *Acanthophora nayadiformis*, *Chondria capillaris* and *Gracilaria longa*) and unidentified Cyanophyta were recorded in 1989 and 2008 but not found in 2011–2018 (Table 1). Thirteen NISs in total were found. Their number varied over the years, reaching its highest value in 2017 (Table 2).

The number of species in each taxonomic division (i.e. Rhodophyta, Ochrophyta, Chlorophyta and Spermatophyta) also varied (Fig. 3). Rhodophyta were the most numerous in all years. Chlorophyta exceeded Ochrophyta until 2016. Spermatophyta were absent in 1989 and 2008 (Fig. 3).

In biogeographical terms, Cosmopolitan taxa ranked first each year, followed by Atlantic. Other interesting features were the low number of Mediterranean taxa and the increase in Indo-Pacific taxa from 2011 onwards (Fig. 4).

The dominant taxa, reaching a maximum yield of at least 5 kg m^{-2}, differed over the years. Table 3 shows the biomass values of these taxa each year, with the values shown in brackets when lower than 5 kg m^{-2}.

Considering the study period as a whole, the yearly biomass values (mean ± SD) for the whole basin ranged from 1.4±0.9 kg m^{-2} in 2014–2015 to 1.8±1.4 kg m^{-2} in 2017–2018 (Fig. 5a), see Suppl. materials 1–7: "Biomass data" for the original data used to perform this analysis. The site with the highest abundance by far was Station 1, with a pluriannual mean biomass of 2.5±1.2 kg m^{-2} (Fig. 5b). Concerning the season, there were no marked differences (R_{ANOSIM} = 0.017; p = 0.13), even though lower values were recorded in autumn than in the other seasons (Fig. 5c).

Species richness (S) and ecological diversity (H') increased over the years (Fig. 5d, g). The highest S was reached in 2016–17 when a total of 63 taxa (12.1±4.4 taxa per sample on average) were present and H' reached 1.2±0.4. Station 4 reached the high-

Table 2. Non-indigenous taxa recorded each year in the Mar Piccolo.

Species	1989	2008	2011	2012	2013	2014	2015	2016	2017	2018
Agardhiella subulata (C. Agardh) Kraft *et* M.J. Wynne	+							+	+	
Caulerpa cylindracea Sonder		+			+					
Codium fragile (Suringar) Hariot subsp. *fragile*			+		+	+	+	+	+	
Colpomenia peregrina Sauvageau				+	+	+	+	+	+	+
Dasysiphonia sp						+	+	+		
Grateloupia minima P.L. Crouan *et* H.M. Crouan							+		+	
Grateloupia turuturu Yamada			+	+	+	+	+	+	+	+
Hypnea cornuta (Kützing) J. Agardh		+	+	+	+	+	+	+	+	
Hypnea spinella (C. Agardh) Kützing	+	+				+				
Osmundea oederi (Gunnerus) G. Furnari		+							+	+
Polysiphonia morrowii Harvey				+		+	+	+	+	+
Solieria filiformis (Kützing) P.W. Gabrielson	+								+	
Undaria pinnatifida (Harvey) Suringar		+								
TOTAL 13	3	5	3	4	5	7	7	7	9	4

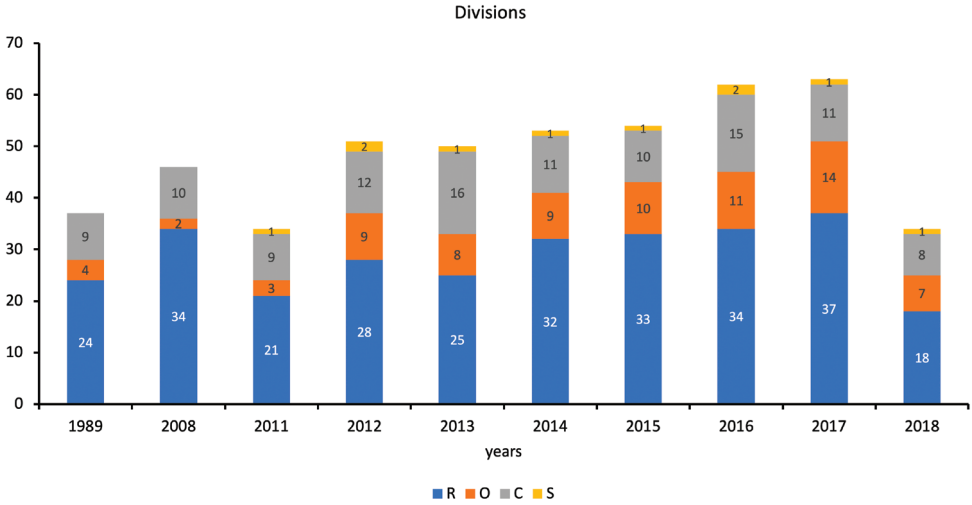

Figure 3. Trend in taxonomic divisions. 2018 data for winter only. In addition to the analysed years 2008 and 2011–2018, unpublished floristic data from 1989 have been included. R=Rhodophyta, O=Ochrophyta, C=Chlorophyta, S=Spermatophyta.

est S (12.4±4.5) and H'(1.2±0.4) (Fig. 5e, h). Higher S and H' values were observed in spring and winter than in autumn and, especially, in summer (Fig. 5f, i). J' values varied from 0.02 in autumn 2014 at Station 2 to 1 in autumn 2017 at Station 2.

Only small, marginally significant differences in the macrophyte community structure were apparent over the years (R_{ANOSIM} = 0.038; p = 0.03). The two-way crossed ANOSIM showed that there were no differences in community structure from one

Table 3. Maximum yearly yelds of dominant taxa (kg m^{-2}) in the Mar Piccolo of Taranto. In brackets values lower than 5 kg m^{-2}.

	2008	2011	2012	2013	2014	2015	2016	2017	2018 (winter)
Amphiroa beauvoisii	–	8.7	17.2	10.9	10.8	8.5	7.3	6.5	(0.93)
Chaetomorpha linum	29.9	10.8	11.3	(3.8)	5.4	(0.4)	6.1	(3.6)	(3.1)
Chondracanthus acicularis	(2.0)	(0.1)	(4.8)	6.8	(4.6)	(3.4)	(2.0)	11.6	(2.4)
Dictyota dichotoma var. *dichotoma*	(0.1)	(0.2)	(0.8)	(0.1)	(2.2)	6.5	10.3	11.4	–
Dictyota dichotoma var. *intricata*	(0.4)	6.3	(1.9)	9.5	6.9	(1.6)	(2.5)	9.3	10.0
Ellisolandia elongata	–	7.5	(1.4)	5.6	(2.6)	9.7	(1.4)	5.2	(0,1)
Hypnea cornuta	(2.3)	7.7	7.3	10.1	16.1	8.4	5.0	(4.1)	–
Spyridia filamentosa	(0.9)	(0.1)	(0.2)	(2.6)	(0.5)	(0.2)	(1.8)	7.1	(0.1)
Ulva intestinalis	–	–	(0.9)	–	(0.1)	–	5.1	(0.1)	(0.1)
Ulva laetevirens	(1.2)	9.7	(3.4)	(3.8)	(1.1)	(2.8)	(4.6)	(2.9)	(1.1)

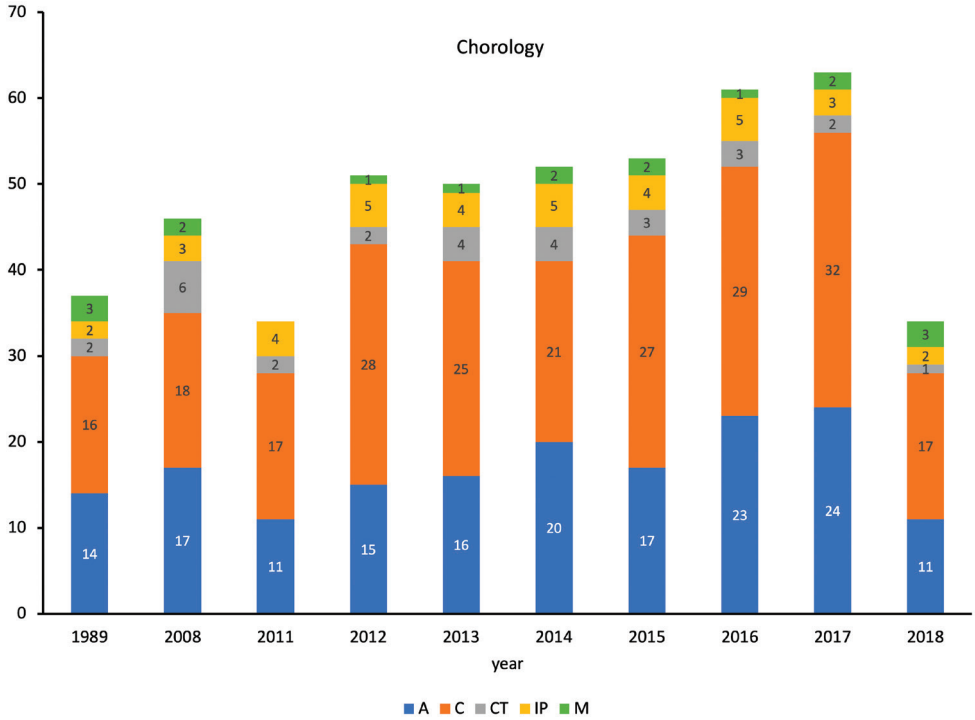

Figure 4. Chorological spectrum of taxa collected in the Mar Piccolo over the years. 2018 data for winter only. In addition to the analysed years 2008 and 2011–2018, unpublished floristic data from 1989 have been included. A=Atlantic, C=Cosmopolitan, CT=CircumTropical, IP=Indo-Pacific, M=Mediterranean.

year to the next, while there was variation between years distant from each other. For example, the pairwise test for 2011–2012 and 2017–2018 revealed significant variation in community structure (R = 0.363; p = 0.001), while the difference between

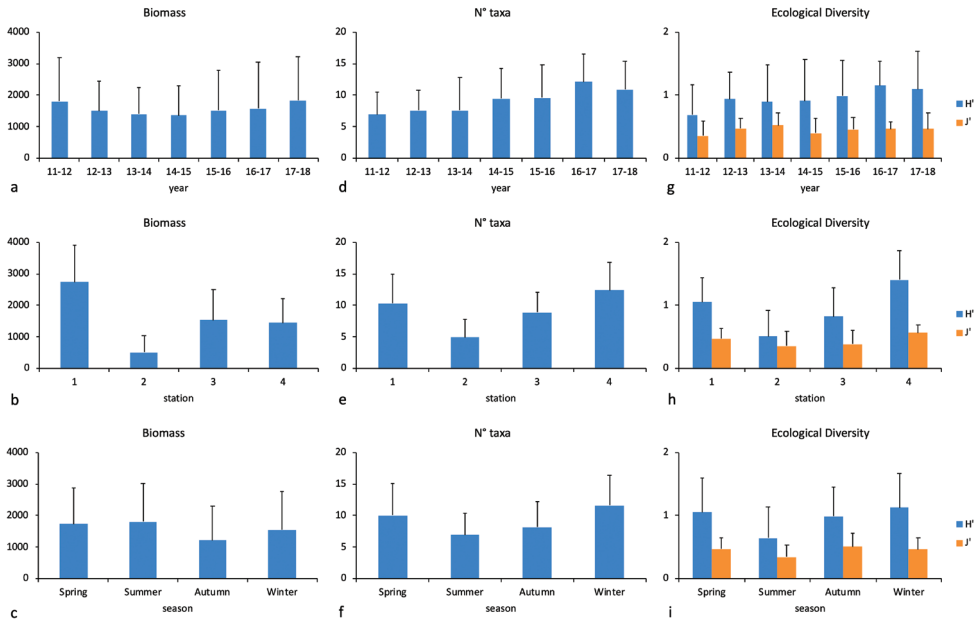

Figure 5. Mean values (±SD) calculated for the years of the study, sampling sites and seasons. **a, b, c** biomass (g m^{-2}) **d, e, f** species richness **g, h, i** ecological diversity.

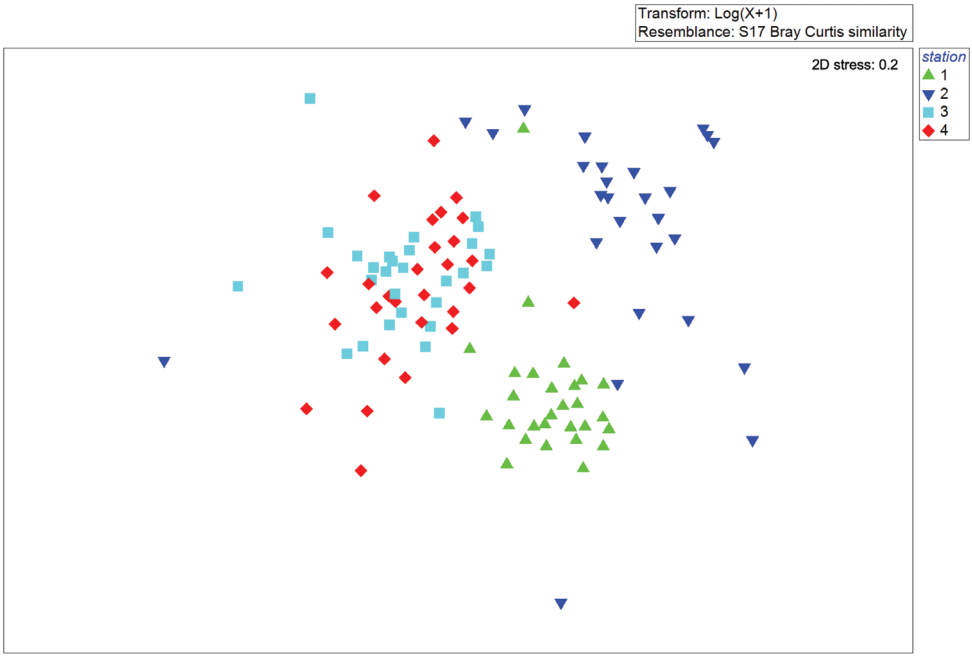

Figure 6. nMDS representation of the mean total biomass values reported for each sampling site in the period 2011–2018.

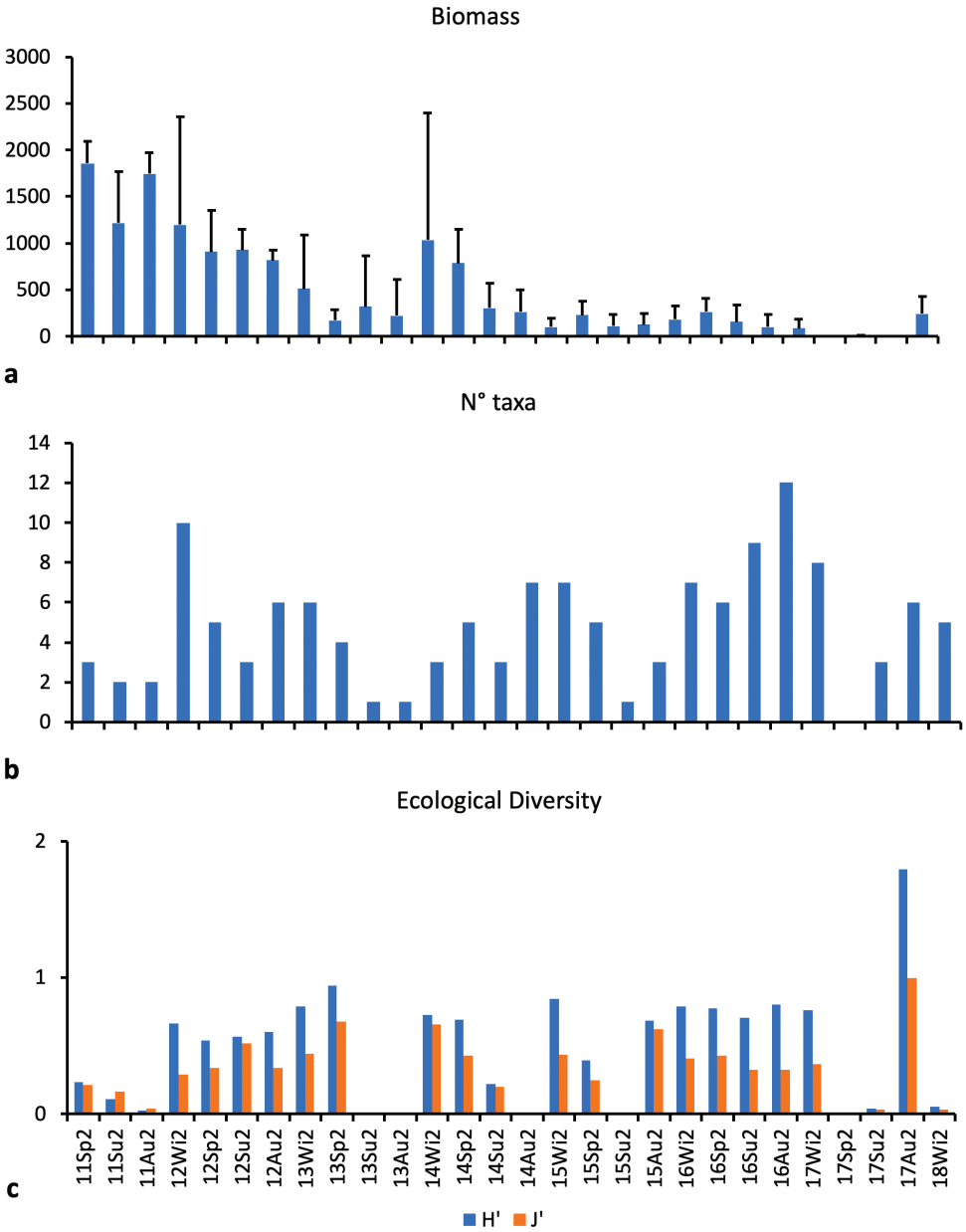

Figure 7. Mean values reported for Station 2 in the period 2011–2018. **a** biomass (g m^{-2}) **b** species richness **c** ecological diversity.

2016–2017 and 2017–2018 was not significant (R = 0.363; p = 0.3). Indeed, for immediately successive years, the R statistic was almost always negative.

The nMDS representation of the total biomass means per Station shows that the two stations in the Second Inlet were very close to each other and different from those

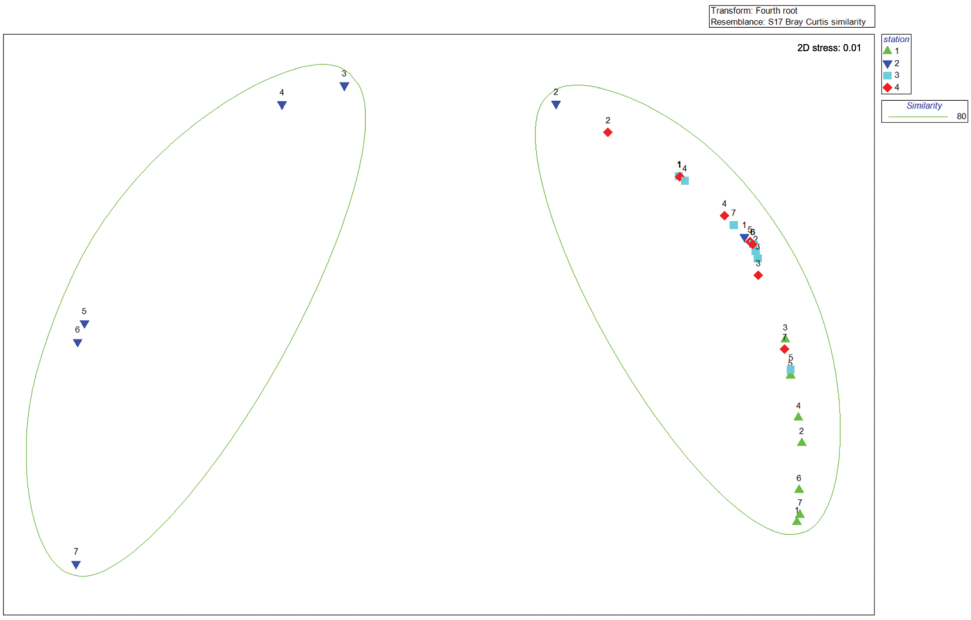

Figure 8. nMDS representation of the biomass means calculated over the period 2011–2018 at the four sampling sites. In the plot, the symbols indicate the station and the numbers refer to the sampling year (**1** 2011–2012 **2** 2012–2013 **3** 2013–2014 **4** 2014–2015 **5** 2015–2016 **6** 2016–2017 **7** 2017–2018).

in the First Inlet (Fig. 6). In addition, the analysis performed at the basin scale revealed a significant difference between the two inlets (R_{ANOSIM} = 0.47; p = 0.001).

Considering the analysis of each site, Station 2 was found to differ sharply from all the other stations (Fig. 7). Station 2 was found by the two-way SIMPER to have the highest dissimilarity in the pairwise comparisons with the other sites, higher than 97% in all cases. The ANOSIM confirmed this pattern (R_{ANOSIM} = 0.57; p = 0.001), all the pairwise tests showing high dissimilarity between stations, although Stations 3 and 4 in the Second Inlet were more similar to each other (R_{ANOSIM} = 0.169; p = 0.001). Station 2 had the lowest biomass and biodiversity (Fig. 5b, e, h). Regarding species richness and ecological diversity (Fig. 7b, c), Station 2 saw ups and downs over the years and a small recovery after 2016. Considering all the study years and sites, only 8.2% of the total biomass was recorded at Station 2, which saw a continuous fall in biomass values from 2011–2012 to 2017, when no macrophytes were collected in spring and negligible quantities were recorded in summer (4.9 ±8.4 g m^{-2}) and autumn (0.1 ±0.1 g m^{-2}). In winter 2018, higher values were measured again (Fig. 7a) when *Aglaothamnion tenuissimum* appeared, with relatively high biomass values (241 g m^{-2}).

Within the nMDS plot of the biomass means calculated over the years versus the sampling sites, in the first two years (2011–2012 and 2012–2013), Station 2 grouped with all the other sites, while in the other five years, sharp segregation was evident (Fig. 8).

Station 3 showed the highest abundance of NISs, both for the study period as a whole and year by year (Fig. 9b; Fig. 10). Starting from 2014–2015, Station 4 became more important (Fig. 10), reaching high biomass values, the highest NIS richness and ecological diversity (Fig. 9e, h). The biomass change was mainly accounted for by *Hypnea cornuta*, which reached 87% of total abundance in 2014–2015.

Discussion

LTER observations make it possible to detect the natural variability of ecological systems and the interaction between abiotic and biotic variables, as well as the effects on the environment of human activities (Kratz et al. 2003). Targeted studies have shown that seaweed species are strongly influenced by seawater temperature, salinity and other abiotic factors (Sousa-Dias and Melo 2008; Gallon et al. 2014).

In the Mar Piccolo, comparison of the situation dating back about thirty years and the results of the recent seven-year study show that qualitative and quantitative changes have occurred in the phytobenthic community and are ongoing. The statistical analysis showed that for the period 2011–2018, the structure of the phytobenthic community in the two basins of the Mar Piccolo differed and that a significant difference was also apparent between the sites investigated in the First Inlet, mainly due to the peculiar features of Station 2. At the same time, the results from the two stations of the Second Inlet were more similar even though with a significant difference in their community structure. Temporal variation was also observed, which was more evident when comparing distant years, while no significant differences were observed between successive years. Considering that the basin has been subject to a significant human pressure for centuries (especially from urban pollution and mussel farming) (Petrocelli et al. 2009) and that the last few years have seen the implementation of measures that have led to a change from eutrophic to oligotrophic conditions (Caroppo et al. 2011; Kralj et al. 2016), it may be assumed that most of these changes were of anthropogenic origin, beyond the natural alternation of macrophyte species (Ballesteros 1991).

Similar developments occurred over the 20 years from 1983 to 2003 in the Venice Lagoon, where a marked change in species composition was recorded (Sfriso and Curiel 2007). The disappearance of some species, generally considered as indicators of good environmental status, was attributed to the increase in pollution and eutrophication (Sfriso and Curiel 2007).

In the Orbetello Lagoon, a clear and progressive switchover of species occurred in the period 1983–2011, reflecting the worsening of ecological conditions in the lagoon, with the rise of *Chaetomorpha linum* (Lenzi et al. 2013). Over about 50 years (1953–2010), in the Nha Trang Bay (Vietnam, South China Sea), an increase in Chlorophyta, mainly Caulerpaceae, Cladophoraceae and Ulvaceae, was recorded (Titlyanov et al. 2015). Concurrently, Rhodomelaceae and Corallinaceae (Rhodophyta) and Sargassaceae and Dictyotaceae (Ochrophyta) decreased considerably. Chemical pollution

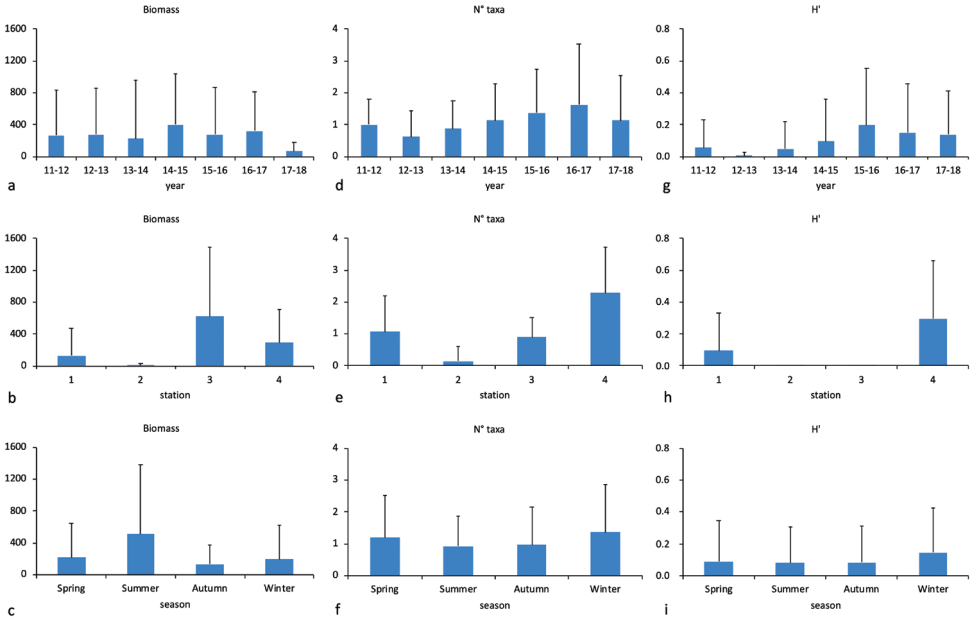

Figure 9. Mean numbers (±SD) of NISs in the period 2011–2018, sampling sites and seasons. **a, b, c** biomass (g m^{-2}) **d, e, f** species richness **g, h, i** ecological diversity.

of seawater due to the presence of inorganic and organic nitrogen and phosphorus compounds was held responsible (Titlyanov et al. 2015). Along the coast of the Gullmar Fjord (W Sweden), data on macroalgal vegetation, dating back to 1941, allowed a comparison with the situation over 40 years later, in 1998. Changes in species composition and depth distribution were observed, including a decrease in coarse filamentous and perennial species and an increase in small filamentous and ephemeral species, which were ascribed to the increased nutrient load (Eriksson et al. 2002). Since the 1950s, macroalgal communities in the Hardangerfjord (Norway) have seen a considerable increase in the number of species, mainly southern species with warm-water affinity and NISs, most probably due to the increase in seawater temperature (Husa et al. 2014). The increase in Rhodophyta was noteworthy, attributable to the installation of a hydro-electrical power plant which caused a change in salinity in the fjord (Husa et al. 2014). In a ten-year study carried out in Tancada Lagoon (NE Spain), the reduction in rooted macrophyte coverage was accompanied by an increase in unattached seaweeds. Amongst the latter, *Chaetomorpha linum* was the dominant species in autumn and winter, when it flourished with high concentrations of dissolved ammonium salts (Menéndez and Comín 2000).

Considering the floristic aspect, most of the species detected in the Mar Piccolo have also been reported in similar Mediterranean environments such as Greek and Cypriot TWSs (Christia et al. 2011) and French lagoons (Le Fur et al. 2018). Twenty-nine of them are also frequently found in euhaline Atlanto-Mediterranean coastal la-

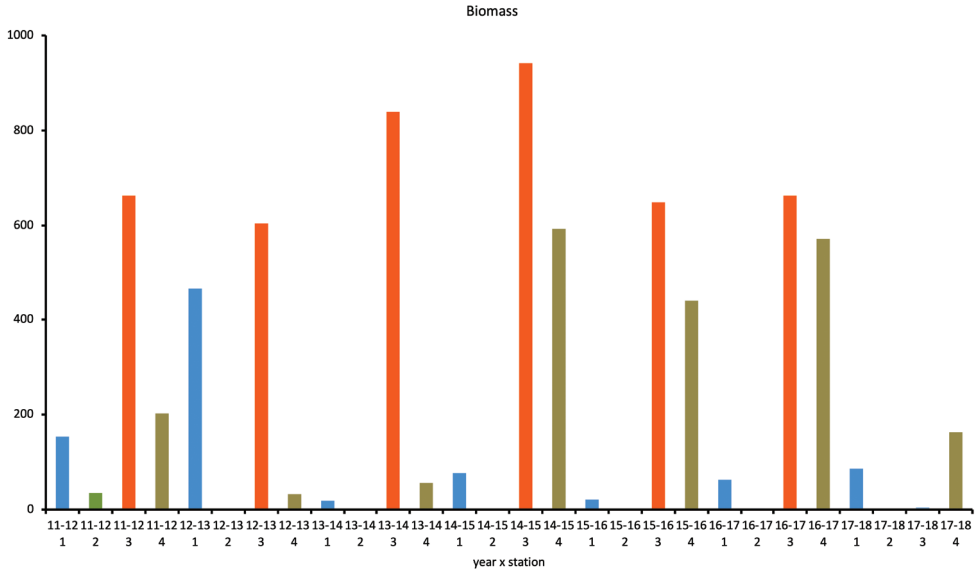

Figure 10. NIS biomass values (g m^{-2}) calculated for each year of the study at the four sampling sites.

goons (Pérez-Ruzafa et al. 2011b). These are mainly euriecious species, less sensitive to the continuous changes in the chemical and physical features of these environments (e.g. *Gracilaria bursa-pastoris* and *Ulva intestinalis*), but also NISs, most likely introduced through the importation of molluscs (e.g. *Colpomenia peregrina* and *Grateloupia turuturu*) (Cecere et al. 2011; Petrocelli et al. 2013). The predominance of Rhodophyta is seen to be a typical pattern in most TWSs (Falace et al. 2009; Ramdani et al. 2015; Le Fur et al. 2018), except for environments characterised by noticeable surges in nutrient concentrations and salinity, where Chlorophyta are dominant (Piñón-Gimate et al. 2008; Pellizzari et al. 2009). However, the most evident development in the last few years in the Mar Piccolo is the increase in Ochrophyta. This was probably due to the improvement of seawater quality following the closure of sewage pipes in the First Inlet (Alabiso et al. 2005; Kralj et al. 2016). Indeed, Ochrophyta are acknowledged to be the most sensitive seaweed species to urban pollution, which strongly affects their reproduction and physiology (Scherner et al. 2012).

Likewise, the chorological spectrum of Mar Piccolo phytobenthos has seen considerable fluctuation. The present situation is quite different from that of Italian marine flora in general, where the Atlantic and Mediterranean elements are prevalent, while the Indo-Pacific and Circum-Tropical elements are practically negligible (Furnari et al. 2010). Specifically, the increase in the Indo-Pacific element in the Mar Piccolo is most probably due to the introduction of NISs. In the Thau Lagoon (France), about 30% of seaweed taxa (i.e. 58 out of 179 taxa) are NISs native to the western Pacific. They are so numerous that the lagoon is considered "a Japanese biological island in the Mediterranean Sea" (Boudouresque et al. 2011). Their presence is considered a result

of the intensive importation of Japanese oysters for aquaculture purposes in the early 1970s (Boudouresque et al. 2011). In the Venice Lagoon, 20 NISs are of Pacific origin (Marchini et al. 2015). Here, the rise in NISs is attributed to the development of commercial activities such as ship traffic and mollusc importation (Sfriso and Curiel 2007). Similarly, the continuous increase in NISs in the Mar Piccolo could be ascribed to the massive importation of shellfish as a result of the serious crisis affecting Taranto mussel farming since 2011 (Cecere et al 2016b).

The total of 129 taxa recorded in the Mar Piccolo in the 2011–2018 period is probably an underestimate, since only four coastal stations were seasonally sampled from a total surface area of about 21 km^2. However, it was still comparable with that of other Mediterranean TWSs, considering that some of these environments have larger surface areas and probably offer a higher range of environmental conditions (Table 4).

Quantitative dominance showed the same fluctuations amongst both years and seasons. In 1989, Gracilariaceae and Solieriaceae had the highest standing crop throughout the basin (Cecere et al. 1992). The period 2011–2018 saw continuous changes in dominance amongst species (Table 3), unlike 2008, when only *Chaetomorpha linum* reached noticeable biomass values. The increase in geniculate Corallinaceae (e.g. *Amphiroa beauvoisii* and *Ellisolandia elongata*) was most probably linked to the decrease in nutrient load, as already observed in Spanish waters (Pinedo et al. 2013). A decrease of about one order of magnitude in the standing crop of submerged macrophytes was observed over about twenty years in the Lagoon of Venice, which also saw a marked dominance of seagrasses over seaweeds, with higher standing crops for *Cymodocea nodosa* (Sfriso and Facca 2007).

Considering differences in spatial patterns, it is well known that coastal lagoons are "a mosaic of assemblages", mainly depending on abiotic factors (Pérez-Ruzafa et al. 2008). In the Mar Piccolo, this was already highlighted in 1989, when noticeable spatial differences were recorded mainly due to a marked variability of nutrient concentrations. Specifically, Zone B, the nearest to Station 2, had the lowest biomass and biodiversity (Cecere et al. 1992). Therefore, the present study not only confirms previous observations, but, via multivariate analysis, sheds light on the remarkable change that has happened at this site since 2013. Specifically, the two-way SIMPER (years vs. sites) indicates that, at Station 2, the change was due to a huge decrease in biomass and not an apparent change in community composition. As already indicated by the univariate analysis, in 2016, an increase in species richness and diversity was registered here. However, *Chaetomorpha linum* and *Cymodocea nodosa,* which are considered to be structuring taxa, alone represented 80% of the total biomass, the others being mainly epiphytic species on *C. nodosa*. Of the latter, *Aglaothamnion tenuissimum,* commonly reported in epiphytic habits, developed a considerable quantity of pleustophytic biomass – uncommon in this species – in winter 2018. The area surrounding Station 2 is characterised by the presence of a small river discharging fresh water drained from surrounding farmland. The massive biomass development of *A. tenuissimum* is thus most probably due to an abnormal increase in nutrients, as already observed for epiphytes of *Posidonia oceanica* (Linnaeus) Delile (Prado et al. 2008), but it deserves to

Table 4. Number of seaweed and phanerogams taxa in some Mediterranean TWS. S=number of seaweeds; P=number of phanerogams.

TWS	S	P	Surface	Reference
Mar Piccolo	126	2	21 km^2	this study
Venice Lagoon	296	5	432 km^2	Sfriso et al. 2009
Thau Lagoon (France)	179	2	75 km^2	Boudouresque et al. 2010
Nadoor Lagoon (Morocco)	110	2	114 km^2	Ramdani et al. 2015
Stagnone of Marsala	108	4	20 km^2	Calvo et al. 2009
Mar Menor (Spain)	69	2	135 km^2	Pérez-Ruzafa et al 2008
Orbetello Lagoon	68	3	25 km^2	Lenzi et al. 2009
Marano and Grado Lagoon	41	4	160 km^2	Falace et al. 2009
Acquatina Lake	38	2	0.45 km^2	Petrocelli and Cecere 2010
Ganzirri Lake	32	3	0.34 km^2	Serio et al. 2009
Lesina Lagoon	36	2	51.36 km^2	D'Adamo et al 2009, E. Cecere and A. Petrocelli unpublished data
Caprolace Lagoon	28	3	2.26 km^2	Signorini et al. 2009a
Faro Lake	28	3	0.26 km^2	Serio et al. 2009
Fogliano Lagoon	10	2	3.95 km^2	Signorini et al. 2009b

be investigated further. The highest abundance of the NIS *Hypnea cornuta* at Station 3 may, in contrast, be ascribable to the presence of natural hard substrata, unique in the basin. Indeed, this species is characterised by organs of vegetative propagation (i.e. stellate propagules, thickened unattached fragments, basal parts) which also serve as over-wintering organs. In late autumn-early winter, the thalli decay but those organs survive the adverse season (Cecere et al. 2004, 2016a). The natural cavities in the rock at Station 3 probably represent a shelter supporting the survival of a higher number of propagules.

Considering a longer time period of about one century, complemented with information on changes in both the demography of Taranto, based on decade-long census data (https://tinyurl.com/yacl8t6t) and the forcing factors affecting the Mar Piccolo, changes in phytobenthos can speculatively be related to human pressure.

In the 1920s, Taranto had fewer than 100,000 inhabitants (https://tinyurl.com/yacl8t6t) and the only anthropogenic factors affecting the Mar Piccolo were mussel breeding and the shipyard of the Italian Royal Navy, established in 1889 (Nistri 1988). In that period, the waters of the Mar Piccolo seemed to be in good condition, hosting seaweed species commonly recorded in open and clean seawaters (Pierpaoli 1923), together with luxuriant meadows of the phanerogam *Cymodocea nodosa* (Pierpaoli 1923).

In the 1940s, a change was already observed, linked to the presence of the First Squadron of the Italian Royal Navy fleet during the Second World War (Pierpaoli 1959). Considerable quantities of both fuel and the residues of smoke bombs were observable in the areas where the ships were moored and where the anti-aircraft defences were placed. This probably adversely affected seawater quality and seaweed assemblages, with Ulvales growing on the docks and a noticeably lower number of Ochrophyta and Rhodophyta species (Pierpaoli 1959).

The period from the 1950s to the 1970s saw a considerable increase in the population of Taranto (https://tinyurl.com/yacl8t6t), linked to the development of new economic activities, chief amongst which was the ITALSIDER steelworks (now Arcelor-Mittal Italy). New districts were built and the town expanded (https://tinyurl.com/ycm2y68w, Nistri 1988). Along the shores of the Mar Piccolo, shipyard activities decreased (https://tinyurl.com/ya885vun) and the Italian Air Force training school was built (https://tinyurl.com/y8wpbgpu). Unfortunately, no information on the phytobenthos of the basin in that period is available, except for some notes in publications of general interest (Parenzan 1984).

At the beginning of the 1980s, the population of Taranto peaked along with the industrial activities (https://tinyurl.com/yacl8t6t). The demographic and economic boom resulted in the creation of 14 sewage outlets, both urban and military, which discharged untreated effluent into the Mar Piccolo until the late 1990s. The most immediate consequences of these factors were the eutrophication of the basin's waters and an increase in turbidity due to suspended particulate matter (Pastore 1993). Subsequent phytobenthos studies highlighted considerable changes with respect to Pierpaoli's data (Cecere et al. 1991a), with the following becoming more evident: excessive growth of nitrophilous species; the unattached status of most of the seaweeds; the loss of sexual reproduction and the onset of vegetative reproduction; the disappearance of the less tolerant species, amongst which brown seaweeds and in particular those of the *Cystoseira* genus; and the thinning out of the *Cymodocea nodosa* meadow (Cecere et al. 1988, 1989, 1991b; Saracino and Cecere 1991; Cecere et al. 1992). This situation was in line with observations in other coastal environments, where urban, agricultural and industrial sewage caused eutrophication, with a high level of inorganic nutrients and turbidity (De Casabianca et al. 1997). It is well known that untreated sewage discharged into coastal basins is the cause of severe modifications in benthic communities, due to increases in nutrients, toxic substances, turbidity and silt. Generally, in these conditions, filamentous and sheet-like algae prevail over thick algae, due to their higher uptake efficiency and faster growth (Díez et al. 2013). Specifically, in these environments, macrophytes represent an important structuring component. When nutrient levels rise, one of the first feedbacks is the shift of phytobenthic communities from rooted macrophytes and perennial seaweed species towards fast-growing and ephemeral macroalgae (De Casabianca et al. 1997; Obrador and Pretus 2010,; Scherner et al. 2013). In the Thau Lagoon, it was observed that the increase in eutrophication, mainly due to mussel farming activities, caused profound changes in macrophytobenthic communities. Pure eelgrass meadows (i.e. *Zostera marina* and *Nanozostera noltii*) were progressively replaced by nitrophilous communities of *Ulva rigida* and *Gracilaria bursa-pastoris* (De Casabianca et al. 1997). A quantitative study carried out along the Atlantic coast of Brazil proved that, in highly urbanised areas, calcareous algae coverage fell, most probably due to high concentrations of nutrients, which inhibited calcium carbonate synthesis. Another effect was the reduction in biodiversity and changes in macroalgal population structure. Indeed, Ochrophyta considerably decreased and Chlorophyta increased (Scherner et al. 2013).

The beginning of the new century saw the start of a new era; the population in Taranto began to decrease (https://tinyurl.com/yacl8t6t) and nine urban sewage outlets were closed (Caroppo et al. 2011). The physico-chemical features of the seawaters changed again, becoming less eutrophic and in some areas recovering their transparency (Alabiso et al. 2005; Kralj et al. 2016). A "re-oligotrophication trajectory" was thus triggered, with relatively rapid results (Le Fur et al. 2019). In addition, unlike other studies, the availability of historical data confirmed that a real recovery was in progress (Le Fur et al. 2019). Indeed, seaweed species, typical of clear seawater recorded by Pierpaoli (1923), reappeared (Petrocelli et al. 2014). In addition, many species recovered their capacity for sexual reproduction (Petrocelli et al. 2009), the *Cymodocea nodosa* meadow increased in density and plants began to produce flowers and fruits. A luxuriant meadow of another phanerogam, *Ruppia cirrhosa*, never previously recorded, was observed, with flowers and fruits (Petrocelli et al. 2009; Cecere et al. 2010). Similar occurrences have been observed in other transitional systems. A 22-year study of the recovery of seaweed populations after the installation of a sewerage system was carried out in the Abra de Bilbao (North Spain), a semi-enclosed bay, which for a long time had received urban and industrial effluent through a river (Díez et al. 2009, 2013). The increased algal cover at once degraded sites, the rise in the number of species concomitant with the improvement in seawater quality and the increase in similarity amongst stations were the most noteworthy results (Díez et al. 2009). An evaluation of long-term changes in macroalgal populations present around the coast of Catalonia (Spain, Mediterranean Sea) was performed over 17 years, in order to assess the effects of a sewerage system. An evident switch towards a decrease in both the number and abundance of nitrophilous Chlorophyta (e.g. *Cladophora* spp. and *Ulva* spp.) in favour of *Ellisolandia elongata* (as *Corallina elongata*), a stress-tolerant species, was recorded (Pinedo et al. 2013). The Saronikos Gulf (Greece, Aegean Sea) underwent a similar process, the heavily polluted conditions caused by rapid urbanisation from the mid-1950s to the mid-1990s giving way to oligotrophic seawaters by 1998, thanks to the installation of a sewerage system (Tsiamis et al. 2013). In the initial period, macroalgal communities were characterised by the dominance of nitrophilous Chlorophyta species such as *Cladophora* spp. and *Ulva* spp. Following the installation of the wastewater treatment system, seaweed communities became more biodiverse and Rhodophyta species, mainly Ceramiales, increased (Tsiamis et al. 2013). In Australia, near two decommissioned sewage outfalls, a decrease in species generally flourishing in eutrophic waters (e.g. *Ulva lactuca* and *Corallina officinalis*) was recorded, while Ochrophyta (e.g. *Colpomenia sinuosa* and *Petalonia fascia*) and Rhodophyta species (e.g. *Pterocladiella capillacea,* encrusting Corallinaceae) increased in abundance (Archambault et al. 2001).

Conclusions

The Italian LTER network combines a number of marine, terrestrial and freshwater ecosystems where ecological data have been collected for several decades. By means of interdisciplinary activities and cross-ecosystem research, it aims to make historic

datasets, in series of up to a century long, available for ecological research and preserve them for future generations (Bertoni et al. 2012).

In the Mar Piccolo, LTER studies enabled the analysis of historic qualitative and quantitative data and made it possible to draw up a history of its phytobenthos over about one century. On the basis of multidisciplinary observations of the whole basin conducted over many years (Petrocelli et al. unpublished data), it may be assumed that human activities, directly or indirectly, were the main cause of the changes. However, it is important to highlight that the basin showed high resilience. Indeed, following the removal of most of the urban wastewater discharges, which were the main cause of organic pollution, the basin was able to return to better conditions in just a decade. Therefore, it is realistic to hope that the Mar Piccolo could once again become the paradisiacal place described in ancient times, if only individual users adopt good habits. Indeed, strong pressures on the basin also arise from the careless dropping of litter of all types, such as mollusc shells, plastics and engine oil from fishing boats. In any case, long-term studies are ongoing, so it will be possible to monitor the changes year by year.

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Supplementary material 1

Biomass data in 2011–2012

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2011–2012.

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Supplementary material 2

Biomass data in 2012–2013

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2012–2013.

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Supplementary material 3

Biomass data in 2013–2014

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2013–2014.

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Supplementary material 4

Biomass data in 2014–2015

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2014–2015.

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Supplementary material 5

Biomass data in 2015–2016

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2015–2016.

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Supplementary material 6

Biomass data in 2016–2017

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2016–2017.

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Supplementary material 7

Biomass data in 2017–2018

Authors: Antonella Petrocelli, Ester Cecere, Fernando Rubino

Data type: measurement

Explanation note: Mean biomass values (g m-2), standard deviation and total number of taxa measured in each station and in each season in 2017–2018.

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RESEARCH ARTICLE

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Benthic studies in LTER sites: the use of taxonomy surrogates in the detection of long-term changes in lagoonal benthic assemblages

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Abstract

In benthic studies, the identification of organisms at the species level is known to be the best source for ecological and biological information even if time-consuming and expensive. However, taxonomic sufficiency (TS) has been proposed as a short-cut method for quantifying changes in biological assemblages in environmental monitoring. In this paper, we set out to determine whether and how the taxonomic complexity of a benthic assemblage influences the results of TS at two different long-term ecological research (LTER) sites in the Po delta region (north-eastern Italy). Specifically, we investigated whether TS can be used to detect natural and human-driven patterns of variation in benthic assemblages from lagoonal soft bottoms. The first benthic dataset was collected from 1996 to 2015 in a "choked" lagoon, the Valli di Comacchio, a lagoon characterised by long water residence times and heavy eutrophication, while the second was collected from 2004 to 2010 in a "leaky" lagoon, the Sacca di Goro, a coastal area with human pressure limited to aquaculture. Univariate and multivariate statistical analyses were used to assess differences in the taxonomic structure of benthic assemblages and to test TS on the two different datasets. TS seemed to work from species to family level at both sites, despite a higher natural variability of environmental conditions combined with multiple anthropogenic stressors. Therefore, TS at the family level may represent effective taxonomic surrogates across a range of environmental contexts in lagoon

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environments. Since the structure of the community and the magnitude of changes could influence the efficiency of taxonomic surrogates and data transformations in long-term monitoring, we also suggest periodic analyses at finer taxonomic levels in order to check the efficiency of the application of taxonomic substitutes in routine monitoring programmes in lagoon systems.

Keywords
LTER sites, Taxonomic sufficiency, Mediterranean lagoons, Benthic community

Introduction

Taxonomic sufficiency (TS) is an analysis technique developed in light of the current need for rapid and reliable procedures in marine impact assessment and monitoring. The basic concept behind TS (Ellis 1985) is that the identification of taxa at taxonomic levels, higher than the species level, enables the detection of changes in assemblages exposed to environmental stress without significant loss of information. In marine systems, TS is supported by a number of studies suggesting that using higher-level taxa (e.g. genera or families) is an affordable approach to depicting changes in the structure of macrobenthic assemblages. Such studies have been performed worldwide and have proven the efficiency of TS in different habitat types, from soft bottom (e.g. Vanderklift et al. 1996, Włodarska-Kowalczuk and Kędra 2007) to hard bottom (e.g. Mistri and Rossi 2000, Terlizzi et al. 2002), from high latitudes (e.g. Gray et al. 1990, Wlodarska-Kowalczuk et al. 2005) to tropical areas (e.g. Guzmán and García 1996, Warwick et al. 1990). Indeed, TS saves both time and costs, potentially obviating the need for arduous precise taxonomic identification and has, therefore, received increasing attention in recent years (Olsgard et al. 1998).

In particular, TS is mandatory when non-destructive sampling techniques have to be used and taxonomic resolution is low (Roberts et al. 1994, Terlizzi et al. 2003). It has been applied in the assessment of both environmental impacts – focusing on large pollution gradients, mostly including oil and heavy metal pollution (Dauvin et al. 2003) – and assemblage variation along natural gradients (Terlizzi et al. 2009). It is also useful for comparing data from different habitats or geographical regions (Warwick and Clarke 1993) when data at the species level could introduce noise into the analysis, given their higher dependence on natural variations and biogeographic areas (Warwick 1988b). In addition, TS has been tested as a tool for selecting Marine Protected Areas (Vanderklift et al. 1998).

That being said, the applications of TS, particularly in conservation studies, have been criticised (Terlizzi et al. 2003). First and foremost, the proliferation of TS studies has led to a fragmented knowledge base, since most attempts have been focused on finding a general taxonomic-sufficiency level, rather than on building a general theory of TS. Hence, factors influencing the effectiveness of TS still require a great deal of clarification (Dethier and Schoch 2006). Indeed, TS has been mainly used to describe the spatial patterns of macrobenthic communities – usually in response to heavy disturbances, such as oil spills (see Dauvin et al. 2003) – or to compare areas with different levels of anthropogenic disturbance (Arvanitidis et al. 2009). In contrast, data on

issues, such as using taxonomic surrogates to investigate long-term temporal patterns of marine assemblages, is still scant (Fraser et al. 2006, Musco et al. 2011).

This is mainly due to the lack of long-term monitoring programmes based on the description of variables at the species level (Musco et al. 2011), an issue we set out to address in this study. Specifically, we tested the efficiency of TS at two long-term ecological research (LTER) sites in the Po delta region of north-eastern Italy (Mediterranean Sea). The first benthic dataset was collected from 1996 to 2015 from a "choked" lagoon, the Valli di Comacchio – a lagoon characterised by long water residence times, while the second was collected over a period from 2004 to 2010 from a "leaky" lagoon (defined here as a lagoon which receives water and discharges it from one or more points), the Sacca di Goro. According to the Ramsar Convention, both LTER sites are wetlands of international importance and they constitute part of the Regional Park of the Po River Delta – one of the largest Mediterranean deltaic systems. They are also classified as Special Protection Areas (SPA) under the Birds Directive (2009/147/EC) in the Natura 2000 Italian network of protected areas.

At the same time, both sites are heavily affected by human pressures, mainly related to agricultural and aquacultural activities. The Valli di Comacchio is mostly affected by eel aquaculture and Sacca di Goro is subjected to intense bivalve fishing. Moreover, both are sites of eutrophication caused by excessive nutrient loads (Mistri et al. 2001) and contamination by pollutants of mainly agricultural origin (Pitacco et al. in press a, b). Given the ecological and economic importance of these sites, long-term monitoring is fundamental for understanding the effect of anthropogenic stress on the macrobenthic community and, therefore, planning efficient management plans for conservation of their ecosystems. Since such projects usually benefit from limited financial support, TS could be a useful tool for improving the cost/benefit ratio of environmental monitoring and enabling more efficient use of available resources (Chapman 1998).

The aim of the present study was, therefore, to test the efficiency of TS in long-term monitoring at each of the two LTER sites. Information loss was calculated for (i) different levels of taxonomic aggregation (from species to phylum) and (ii) different data transformations (row data, square root, logarithm, presence/absence), in order to understand whether and how the structure and taxonomic complexity of benthic assemblages influence TS results and whether TS can be used to detect natural and human-driven patterns of variation in long-term environmental monitoring.

Methods

Study area

The Valli di Comacchio (Figure 1) is the largest lagoonal system in the Po River delta and consists of choked lagoons with an average depth of 1 m. The Valli is characterised by limited water renewal (Table 1) and high nutrient load (Mistri et al. 2000), being surrounded by earthen dykes and separated from the Adriatic Sea by the 2.5 km wide

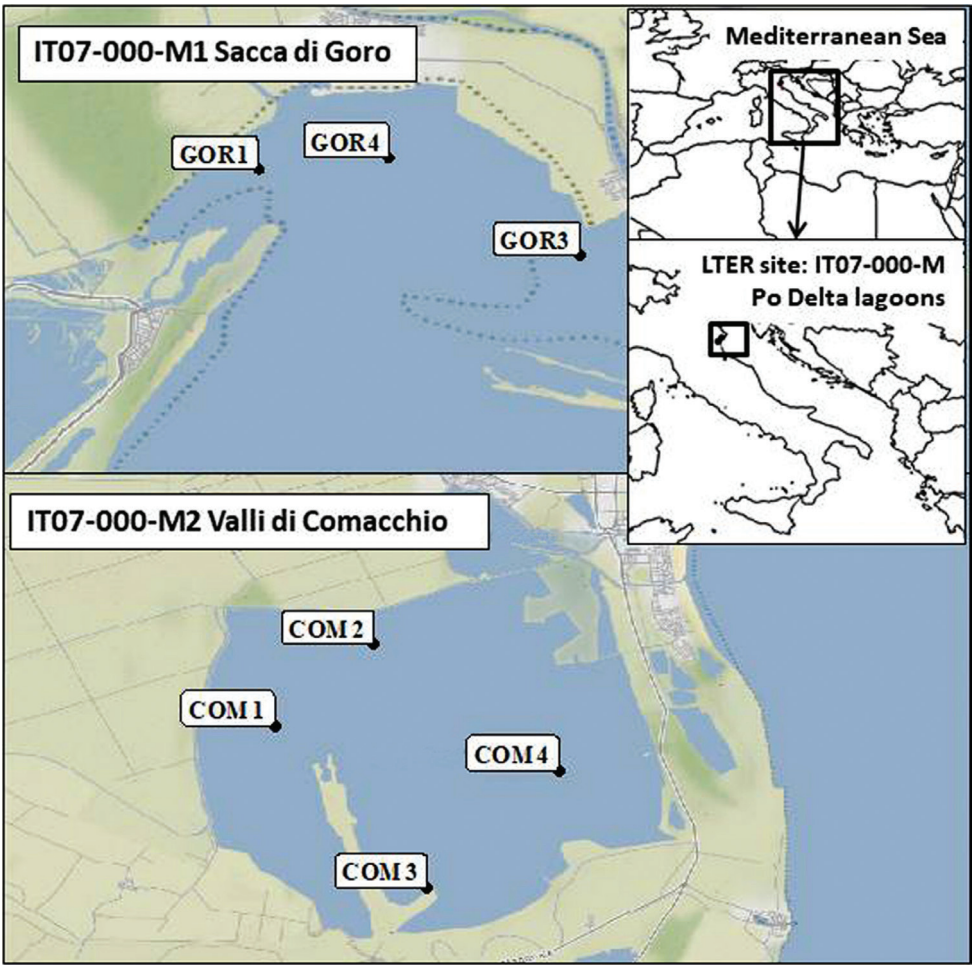

Figure 1. Map of the studied sites.

Spina spit. There are, however, two marine channels directly connected to the lagoons and freshwater input comes from the Reno River and a few drainage canals. Both marine and freshwater inflows are regulated by sluice gates and dams.

Sacca di Goro (Figure 1), on the other hand, is a leaky coastal lagoon, located in the southern part of the Po River delta, with an average depth of about 1.2–1.5 m. Salinity varies in relation to river and seawater inflows (Simeoni et al. 2007). The majority of its freshwater input is from the Po di Volano canal (about 3.5×10^8 m^3/y), which flows directly into the lagoon and the artificially regulated deltaic branch of the Po di Goro. Secondary freshwater sources are three irrigation canals with similar flow rates ($2.0–5.5\times10^7$ m^3/y) called Giralda, Romanina and Canal Bianco. Marine inflows – which vary according to tidal dynamics – originate from two estuarial mouths connecting the lagoon to the northern Adriatic Sea (Natali et al. 2016). Hence, the

Table 1. Physicochemical composition of the two LTER sites.

	Valli di Comacchio	Sacca di Goro
Extension (km²)	100	26
Average depth (m)	0.5–1.5	1.2–1.5
Residence time (d)	115	5
Salinity (psu)	10.9–40.1	6–30
Temperature (°C)	2.0–30.5	2.0–33.0
OM ‡	37.5 ± 5.5 †	44 ± 4.6 †
Sand (%)	44.3 ± 6 †	24.7 ± 2.6 †
Silt (%)	37.9 ± 5.9 †	59.5 ± 0.2 †
Clay (%)	17.8 ± 3 †	17 ± 3 †
References	Mistri et al. (2000); Munari et al. (2005); Pitacco et al. (*submitted-b*)	Corbau et al. (2016); Mistri et al. (2000); Pitacco et al. (*submitted-a*)

† Yearly averaged values, ‡ estimated by loss on ignition.

Sacca di Goro is characterised by large daily variations, depending on the height of the tides as well as seasonal fluctuations in the physicochemical composition of its waters (Table 1) (Corbau et al. 2016). Most of its lagoon bed consists of silty-clay sediments (Table 1), carried to the sea by the rivers, but there are also areas of mainly sandy sediments, in particular near the lagoon mouths and behind the spit (Simeoni et al. 2000).

Sampling

In the Valli di Comacchio lagoons (COM), sampling was performed from 1996 to 2015 at four sampling stations, COM1 to COM4 (Figure 1). Samples were taken seasonally throughout most of the study period. Sites COM1 (44°36.95'N, 12°07.38'E) and COM2 (44°38.47'N, 12°09.25'E), located in the northern part of the lagoon system, were subjected to both marine inflows and continental input and site COM3 (44°33.88'N, 12°10.25'E), in the southern part, to seasonal freshwater inflows from the Reno River. Site COM4 (44°36.10'N; 12°12.73'E), on the other hand, was located in the central, most confined area, which was only occasionally influenced by marine inflow when the Bellocchio drain was opened.

In the Sacca di Goro lagoon (GOR), sampling was performed from 2004 to 2010 at three sampling sites, GOR1, GOR3 and GOR4 (Figure 1). Samples were taken seasonally throughout most of the study period. Site GOR1 (44°49.65'N, 12°16.91'E), located in the western part of the lagoon, was influenced mainly by freshwater discharged from the Po di Volano and Giralda and is, therefore, characterised by variable salinity. Site GOR3 (44°48.73'N, 12°20.33'E) was located in the eastern part of the lagoon and site GOR4 (44°49.78'N, 12°18.31'E) in the central area and was influenced more by tidal exchange.

Three replicates were collected seasonally for macrofaunal community analysis using a 4l Van Veen grab. For the scope of the present work, averages of the replicates

were considered and sampling stations were used as replicates in order to obtain a global picture of the general status of each lagoon. These samples were sieved at 0.5 mm and preserved in 8% formalin. Animals were carefully sorted, identification was performed up to the species level in most cases (exceptions were due to the poor condition of the animals) and all specimens were counted.

Data analysis

At each of the LTER sites, the annual averages of the following structural indices were calculated: species richness (S), Shannon diversity index on $\log_e$ basis (H') and Pielou index (J'). A chi-square test, applied to Kruskal-Wallis (KW) ranks (Kruskal and Wallis 1952), was run to check for significant differences between years. Those calculations were performed using R version 2.4.0 (R Development Core Team 2008). For each of the two LTER sites, 'loss of information α' (α) was determined for each year according to Bacci et al. (2009). Specifically, the difference NTx – NTx+1 was calculated, in which NT expresses the number of taxa identified at the taxonomic level x and x+1 expresses the taxonomic level above level x. 'α' values give general information about the taxonomic heterogeneity-complexity within each level considered (higher values correspond to a greater information loss and vice versa). In order to describe the information loss along the taxonomic structure, the 'α' value was calculated stepwise from the lowest to the highest taxonomic level considered (species-genus, genus-family, family-order, order-class, class-phylum). The percentage was then calculated as follows: (NTx – NTx+1)/NTx * 100.

For each of the two LTER sites, abundance matrices were produced for each of the six taxonomic levels (species, genus, family, order, class and phylum) and for each of four different transformations (none, square-root, logarithm and presence/absence). Affinities between years were established using the Bray-Curtis similarity. For each dataset, a second-stage non-metric multi-dimensional scaling (MDS) ordination was plotted to visualise differences between similarity matrices at different levels of taxonomic aggregation and data transformation. Spearman's rank correlation coefficient (r_s) was calculated between matrices at the species and higher taxonomic levels. The stress of the two-dimensional plot was calculated using Kruskal's stress Formula 1 (Clarke and Green 1988). Stress is a measure of the reliability of the representation, a value < 0.1 being considered a good result (Clarke and Warwick 2001).

To test the effect of different data transformations on the effectiveness of taxonomic sufficiency, a third-stage resemblance matrix was built. This third-stage resemblance matrix, defined as a second second-stage resemblance matrix, constructed using rank correlations between corresponding elements in the set of second-stage matrices following Arvanitidis et al. (2009), was constructed for each dataset using second-stage matrices constructed for each data transformation. Spearman's rank correlations (r_s) between resemblance matrices were tested using RELATE, a non-parametric analogue to the Mantel test, using 9999 permutations. All these calculations were performed us-

ing the PRIMER v6 + PERMANOVA software package (Anderson et al. 2008, Clarke and Gorley 2006).

Identifying temporal changes in macrobenthic communities is fundamental for the efficiency of monitoring programmes. Therefore in order to identify breakpoints in each multivariate dataset, "Constrained Clustering Analysis" was performed on each of the following six matrices: species, genus, family, order, class and phylum. This technique, originally developed for stratigraphic analysis, is more suitable for time-series analysis than for ordinary unconstrained cluster analysis, since only adjacent clusters, according to sample order, are considered for merging. The Bray-Curtis similarity was calculated on the square-root transformed data and the CONISS algorithm, which relies on the incremental sum of squares (Grimm 1987), was used as an agglomeration method. The Broken Stick model (Bennett 1996) was applied to determine the number of significant groups identified by the cluster analysis. Calculations were performed using the 'vegan' and 'rioja' packages of R version 2.4.0 software (R Development Core Team 2008).

In order to test the significance of variations in taxa-abundance matrices between identified clusters, permutational multivariate analysis of variance, PERMANOVA (Anderson et al. 2008), was carried out on the six matrices corresponding to the different taxonomical levels. A single-factor design (with the number of levels corresponding to the number of clusters) and the "unrestricted permutation of row data" with 9999 permutations were applied, as recommended by Anderson et al. (2008). A multivariate t-statistic analogue was used for subsequent pairwise comparisons (Anderson et al. 2008) and, to test whether the between-group variation identified by cluster analysis was also due to a dispersion component, a dispersion homogeneity test (PERMDISP) was performed on the same matrices. The same procedure was followed for each type of data transformation and for each of the two LTER datasets. For all analyses, $p < 0.05$ was chosen as the significance threshold.

Results

Macrobenthic community structure at the two LTER sites

The Valli di Comacchio (COM) dataset comprised a total of 122 taxa at the lowest taxonomic level; these belonged to 9 phyla: Annelida, Arthropoda, Mollusca, Nemertea, Cnidaria, Platyhelminthes, Sipuncula, Echinodermata and Chordata. The annual average species richness (S), Shannon diversity (H') and Pielou equitability index (J') varied significantly throughout the study period (KW, $p < 0.05$), but displayed a general decreasing trend (Figure 2). Specifically, S fell from 19 ± 6 SD in 1999, to 6 ± 4 SD in 2011; H' from 1.72 ± 0.39 SD in 2001 to 0.77 ± 0.37 SD in 2012 and J' from 0.80 ± 0.20 SD in 1996 to 0.40 ± 0.22 SD in 2012.

In contrast, the Sacca di Goro (GOR) dataset comprised a total of 88 taxa at the lowest taxonomic level, in this case belonging to 7 phyla: Annelida, Arthropoda, Mol-

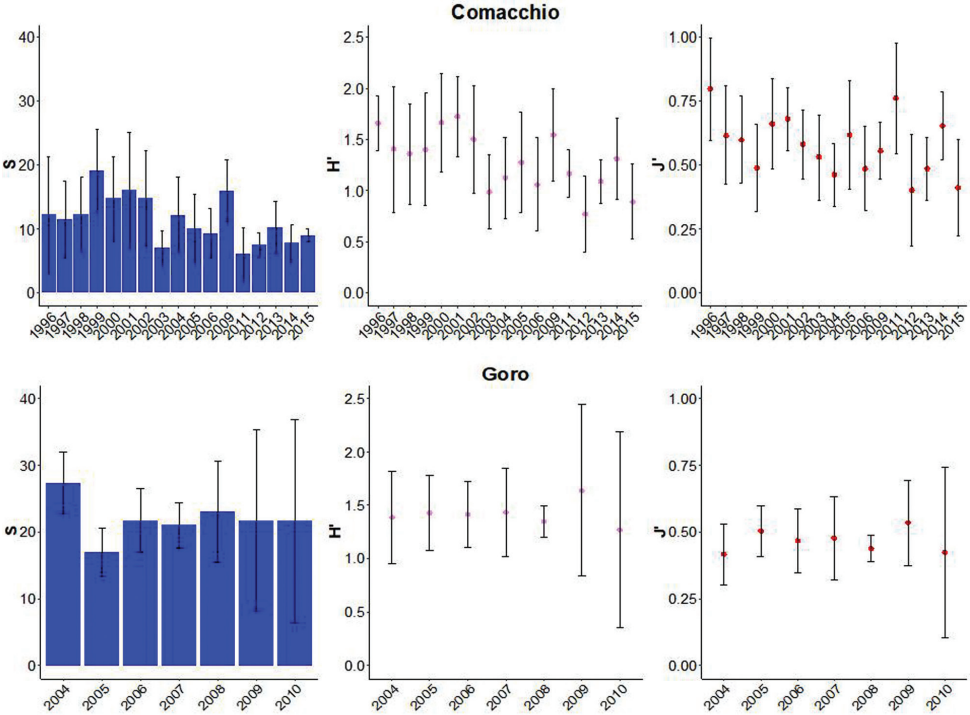

Figure 2. Variation in diversity indices across the study period at the two LTER sites. S: species richness, H': Shannon diversity index, J': Pielou diversity index.

lusca, Cnidaria, Nemertea, Sipuncula and Platyhelminthes. The annual averages for S, H' and J' (Figure 2) displayed no significant differences throughout the study period (KW, $p > 0.05$), although S fell from 27 ± 5 SD in 2004 to 17 ± 4 SD in 2005; H' from 1.64 ± 0.81 SD in 2009 to 1.27 ± 0.92 SD in 2010; and J' from 0.54 ± 0.16 SD in 2009 to 0.42 ± 0.32 SD in 2010.

Taxonomic complexity and information loss

At the COM site, the taxonomic complexity was highly variable throughout the study period, with total 'loss of information α', from species to phylum level, showing the highest values (45) in 2001 and 2002 and the lowest (8) in 2011 (Figure 3A). Only one genus contained eight species, two genera contained three species and nine genera contained two species; the remainder contained only one species. At the higher level, two families contained six genera, two contained five genera, three contained three genera and six contained two genera; the remaining families contained only one genus. Each family contained from 1 to 9 species. The most specious families were Spionidae and Phyllodocidae with 9 species, followed by Serpulidae with 7 species. Information

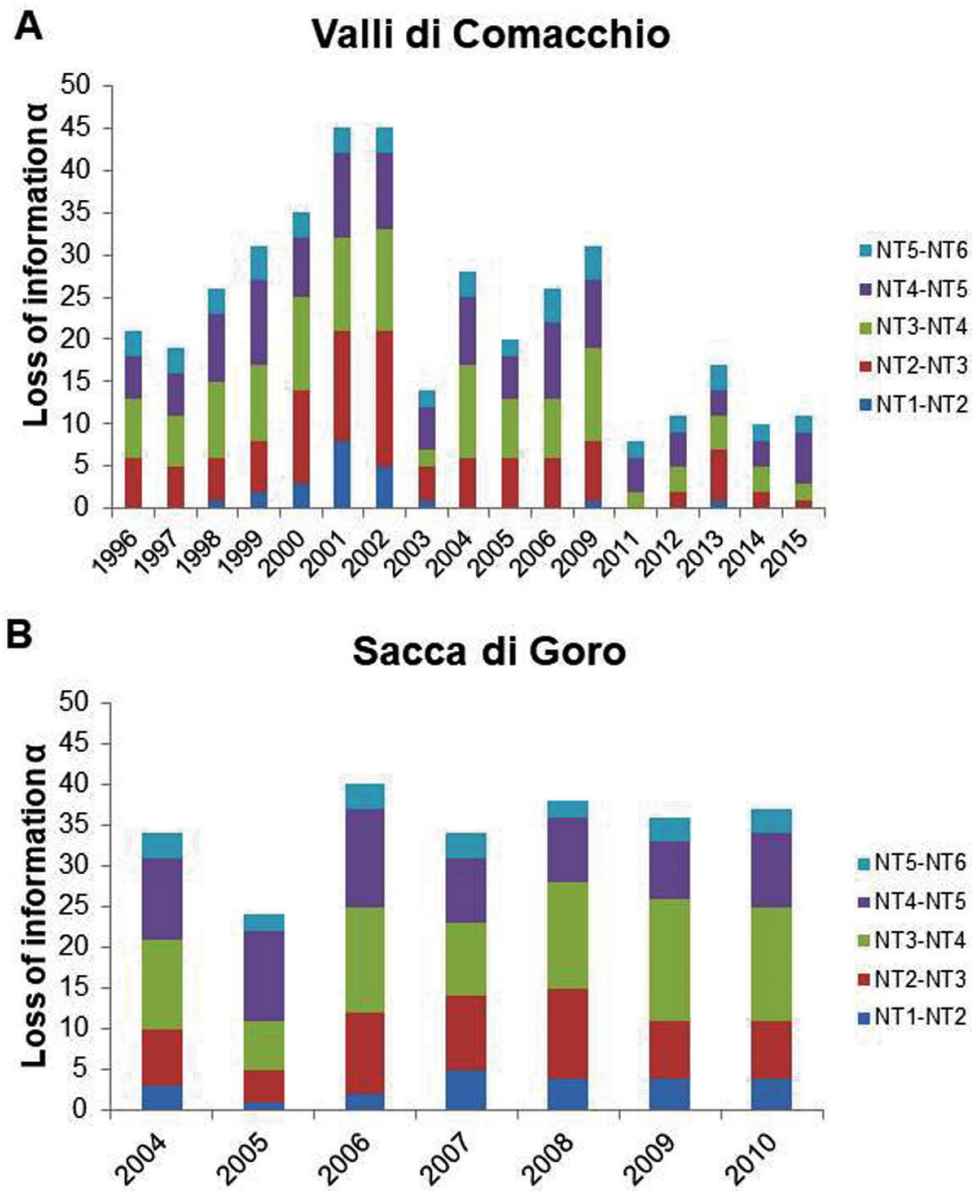

Figure 3. 'Loss of information α' from lower to higher taxonomic levels (NT1-NT6) at the two LTER sites: Valli di Comacchio lagoon (A) and Sacca di Goro lagoon (B).

loss (α) from the species to genus level was observed in 1998, 1999, 2000, 2001, 2002, 2003, 2009 and 2013. The highest loss (16%) was observed in 2001. Information loss (α) between genus and family levels was observed every year, with the exception of 2011, at percentages ranging from 7% in 2015 to 32% in 2002, indicating the presence of families with more than one genus. Information loss (α) was also observed

every year at the family-order level (from 11% in 2003 to 31% in 2009), order-class level (from 14% 2013 to 40% in 2011) and class-phylum level (from 6% in 2001 and 2002 to 20% in 2011).

At the GOR site too, the taxonomic complexity was more or less constant throughout the study period, with information loss (α) ranging from 24 to 40% from the lowest to the highest level (Figure 3B). A simple taxonomic structure was recorded for this site: only two genera contained three species, ten genera contained two species and the rest contained only one species. At the highest level, only two families contained four genera, three families contained three genera, seven contained two genera and the rest contained only one genus. Each family contained from 1 to 7 species. The Spionidae family was the most specious with 7 species represented. Information loss (α) was recorded every year in the steps between all levels considered, descending from species to phylum. The greatest information loss between species and genus levels (13%) was observed in 2007 and the lowest (6%) in both 2005 and 2006. The information loss between genus and family levels was highest (26%) in 2008 and lowest (14%) in 2005; at the family-order level, it was highest (35%) in 2009 and lowest (21%) in 2005 and at the order-class level, it was highest (39%) in 2005 and lowest (16%) in 2009; while at the class-phylum level, it varied from 5% in 2008 to 8% in 2004 and 2007.

Information loss in multivariate data structure

The ordination of similarity matrices in second-stage MDS plots (Figure 4) showed a typical 'fan' pattern for each investigated dataset, with a vertical and horizontal spread of points at increasing taxonomic aggregation and data transformation, respectively. Low stress values were obtained (<0.1) in both cases. The effects of aggregation and transformation, however, varied between the two datasets.

For the COM dataset, there were good correlations between ordination plots at the species and genus levels (always $r_s > 0.96$; $p < 0.05$) and between the species and family levels (always $r_s > 0.88$; $p < 0.05$), whatever the type of transformation considered (Figure 4A). Data points, derived from species and genus abundance from matrices with the same data transformation, tended to overlap, indicating that the similarity matrices were very closely related. Conversely, at higher levels, the similarity progressively decreased (order: $r_s > 0.78$; $p < 0.05$, class: $r_s > 0.49$; $p < 0.05$, phylum: $r_s > 0.29$; $p < 0.05$), in particular for untransformed and square-root transformed matrices (Figure 4A). The pattern of similarity between matrices with different levels of taxonomic aggregation was consistent between different data transformations, as indicated by the third-stage correlation matrix (Table 2); this shows high correlations, in particular between untransformed, square-root and log-transformed second-stage ordination matrices (always $r_s > 0.9$; $p < 0.05$). Information loss increased with increasing taxonomic aggregation, but the distances between ordination plots at different taxonomic levels varied with the strength of the data transformation. Correlations between ordination plots at species and genus levels ranged from $r_s = 1$ for untransformed data to $r_s = 0.96$

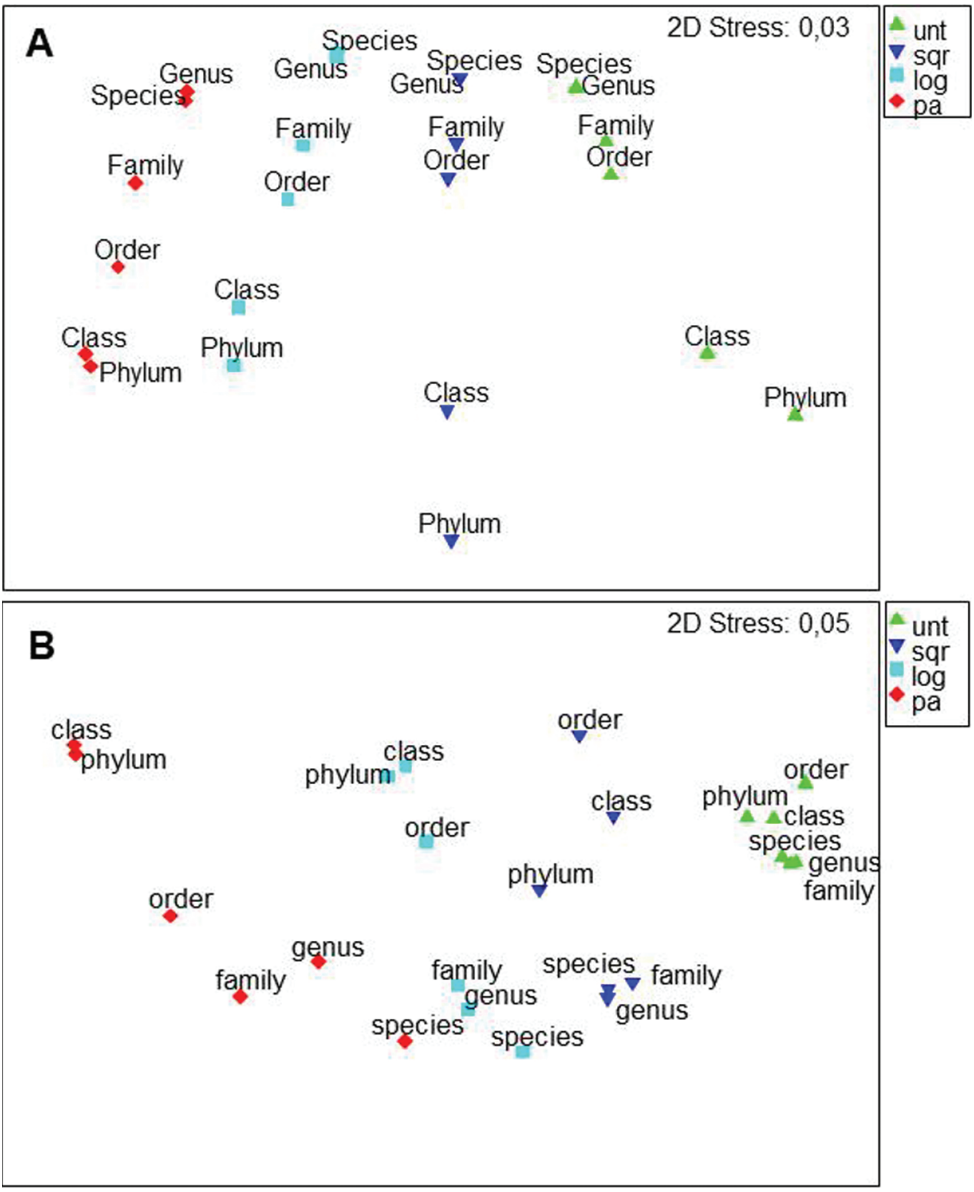

Figure 4. Second-stage MDS ordination of resemblance matrices derived from species, genus, family, order and phylum abundance data at the two LTER sites: Valli di Comacchio lagoon (**A**) and Sacca di Goro lagoon (**B**). unt: untransformed data, sqr: square-root transformed, log: log-transfomed, pa: presence/absence data.

for species-abundance transformation; between species and family levels, these varied from $r_s = 0.94$ for untransformed data to $r_s = 0.89$ for species-abundance transformation, indicating a greater information loss as the transformation strength increased. Such a variation was more marked between order-, class- and phylum-level ordination

plots, but did not follow a general trend. Correlation between similarity matrices at species and order aggregation varied from $r_s = 0.92$ (untransformed) to $r_s = 0.78$ (presence/absence); between species and class aggregation matrices, it varied from $r_s = 0.65$ (presence/absence) to $r_s = 0.49$ (square root); and between species and phylum aggregation, it varied from $r_s = 0.63$ (presence/absence) to $r_s = 0.29$ (square root). This suggests that, at higher levels of aggregation, information loss was lower at stronger transformations, in particular between class and phylum levels.

For the GOR dataset, the ordination plot showed a clear clustering pattern amongst untransformed similarity matrices at all different taxonomic levels (Figure 4B). The significantly high values of Spearman's correlation between the species similarity matrix and matrices at the higher taxonomic levels (always $r_s > 0.95$, $p < 0.05$) indicated that very little information about the general structure of the community was lost going from the species to higher taxonomic levels. However, for this dataset, this pattern was not consistent amongst matrices with different data transformations (Figure 4B), as shown by the low correlation values of the third-stage matrix (always $r_s < 0.9$; Table 2). The distance between matrices at different taxonomic aggregations increased with increasing data transformation strength. Furthermore, similarity matrices at the genus and family levels displayed a significantly high correlation with matrices at the species level ($r_s > 0.90$ and $r_s > 0.80$, respectively; p-value < 0.05), irrespective of the data transformation. This indicates that reasonably little information was lost between species and genus and family levels, even at the strongest transformation. Conversely, in similarity matrices aggregated at higher taxonomic levels (order, class and phylum), information about the structure of the benthic assemblages markedly decreased, as indicated by the low correlation values with species similarity matrices (order: $r_s > 0.63$; class: $r_s > 0.47$; phylum: $r_s > 0.50$; p-value < 0.05). Interestingly, for those higher taxonomic levels, the distances from similarity matrices at species levels did not always increase with decreasing taxonomic resolution. In fact, the phylum-level similarity matrices were closer to those at the species- rather than class-level matrices, especially with the square-root transformation (Figure 4B).

In the Valli di Comacchio, constrained cluster analysis, based on square-root transformation (Figure 5) at the lowest taxonomic level, yielded 7 main groups, separated by 5 breakpoints between the years: 1997–1998, 1998–1999, 1999–2000, 2002–2003, 2009–2011 and 2013–2014. In the Sacca di Goro LTER site, on the other hand, constrained cluster analysis (Figure 6) on square-root transformed data at the lowest taxonomic level showed 4 main groups, separated by 3 breakpoints between the years: 2005–2006, 2006–2007 and 2008–2009.

The results of the PERMANOVAs and PERMDISPs applied at different levels of taxonomic resolution and data transformation on all datasets are summarised in Table 3. For the Valli di Comacchio dataset, PERMANOVA highlighted significant differences ($p < 0.05$) in macrobenthic assemblages between the groups identified by cluster analysis at the species level. Those differences were significant across all taxonomic levels and data transformations, with only one exception: such differences were not retained at the phylum level with presence/absence transformation. Nevertheless,

Table 2. Spearman correlations (r_s) resulting from the third-stage correlation matrix, showing the effect of data transformation on differences between aggregation matrices.

	COMACCHIO		
	None	Square root	Logarithm
Square-root	0.975		
Logarithm	0.932	0.907	
Presence/absence	0.686	0.632	0.854
	GORO		
	None	Square root	Logarithm
Square-root	0.568		
Logarithm	0.254	0.436	
Presence/absence	0.333	0.361	0.861

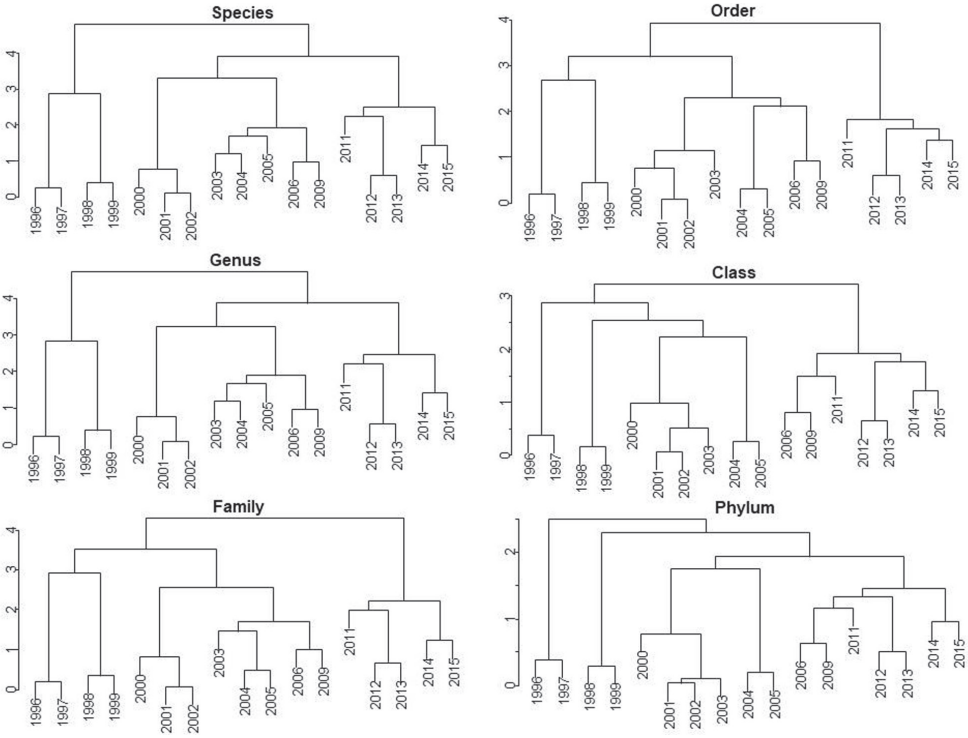

Figure 5. Constrained cluster analysis of macrobenthic data on Valli di Comacchio lagoon, aggregated to different taxonomic levels.

pairwise comparisons revealed that not all possible combinations of clusters showed significant differences. The number of significant pairwise differences decreased from the species to higher taxonomic levels (Table 3), thereby indicating that the ability of PERMANOVA to discriminate amongst groups decreased. PERMDISP analyses

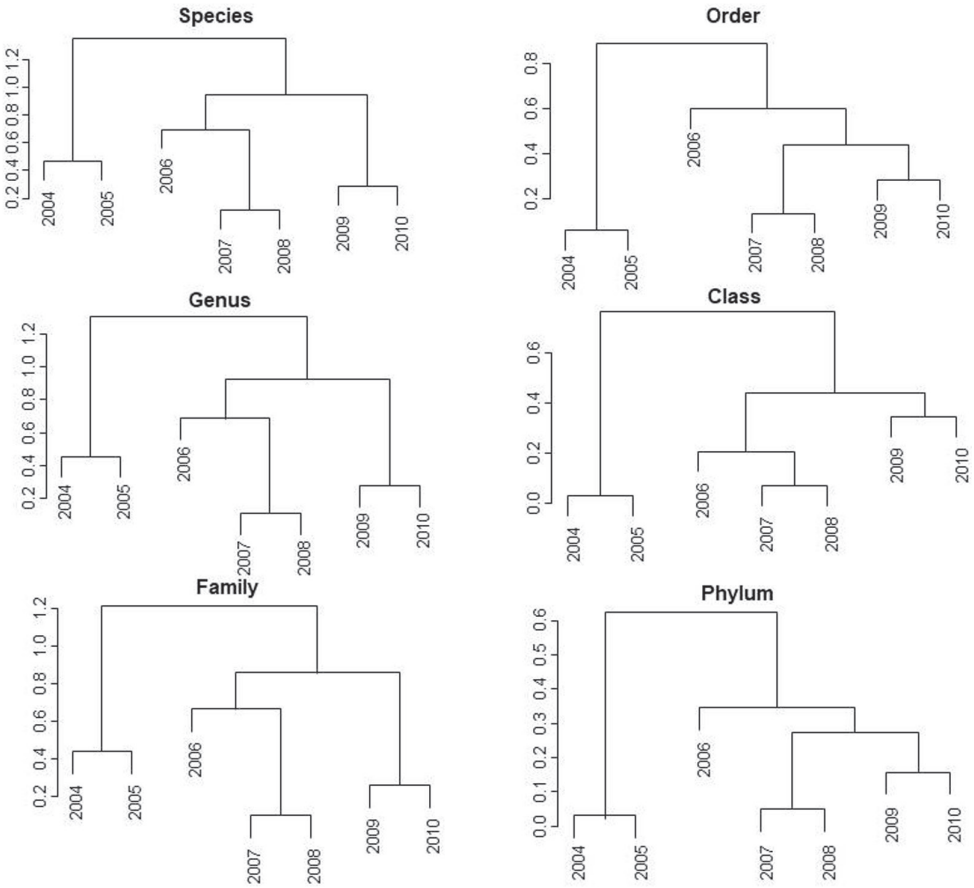

Figure 6. Constrained cluster analysis of macrobenthic data on Sacca di Goro lagoon, aggregated to different taxonomic levels.

showed that these differences between clusters were due to a combined effect of sample location and dispersion ($p < 0.05$), with the only exception being presence/absence data, in which no significant differences in dispersion between cluster groups was observed ($p > 0.05$). Significant differences in dispersion were retained across taxonomic levels up to the phylum level for untransformed data, the order level for square-root transformed data and the family level for log-transformed data.

Regarding the Sacca di Goro dataset, PERMANOVA highlighted significant differences ($p < 0.05$) in macrobenthic assemblages between groups identified by cluster analysis at the species level when each of the three transformations were used, but not when data remained untransformed. For each of the transformations (square-root, logarithm and presence/absence data), the significance of the differences decreased with increasing taxonomic level, with no significance being detected at the phylum level. Additionally,

Table 3. Significance of cluster groups (PERMANOVA), percentage of significant pairwise combinations between those groups and the significance of differences in dispersion between cluster groups (PERMDISP).

LTER dataset	Data transformation	Cluster groups		Taxonomic resolution					
				Species	Genus	Family	Order	Class	Phylum
COM	None	9	PERMANOVA p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0003
			Significant pairwise combinations	81%	81%	81%	78%	56%	36%
			PERMDISP p-value	0.001	0.001	0.001	0.001	0.016	0.004
	Square root	7	PERMANOVA p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0005
			Significant pairwise combinations	81%	86%	86%	71%	57%	38%
			PERMDISP p-value	0.001	0.002	0.006	0.015	0.064	0.005
	Logarithm	8	PERMANOVA p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0004
			Significant pairwise combinations	96%	96%	93%	82%	86%	54%
			PERMDISP p-value	0.005	0.019	0.017	0.113	0.189	0.011
	Presence-absence	6	PERMANOVA p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0697
			Significant pairwise combinations	93%	93%	80%	67%	80%	27%
			PERMDISP p-value	0.220	0.253	0.191	0.233	0.022	0.002
GOR	None	4	PERMANOVA p-value	0.08	0.08	0.11	0.14	0.08	0.13
			Significant pairwise combinations	50%	50%	50%	33%	33%	33%
			PERMDISP p-value	0.299	0.291	0.372	0.153	0.401	0.463
	Square root	4	PERMANOVA p-value	0.01	0.01	0.02	0.02	*0.05*	0.10
			Significant pairwise combinations	50%	50%	50%	33%	50%	33%
			PERMDISP p-value	0.004	0.001	0.008	0.002	0.044	0.061
	Logarithm	5	PERMANOVA p-value	0.001	0.003	0.004	0.03	0.04	0.11
			Significant pairwise combinations	50%	50%	50%	20%	0%	0%
			PERMDISP p-value	0.001	0.001	0.002	0.011	0.011	0.001
	Presence-absence	5	PERMANOVA p-value	0.003	0.01	0.01	0.02	0.04	0.28
			Significant pairwise combinations	30%	20%	20%	10%	10%	0%
			PERMDISP p-value	0.001	0.002	0.001	0.308	0.090	0.025

in this case too, pairwise comparisons revealed that not all possible pairwise combinations of clusters differed significantly and the number of significant pairwise differences decreased from the species to higher taxonomic levels (Table 3). Once again, therefore,

the ability of PERMANOVA to discriminate between groups appeared to decrease. PERMDISP analyses, on the other hand, revealed no differences between clusters, either in untransformed data or in terms of dispersion ($p < 0.05$). For other transformations, significant differences between clusters ($p < 0.05$) were due to a combined effect of location and dispersion. Significant differences in dispersion were retained across taxonomic levels, up to the phylum level, for log-transformed data, the class level for square-root transformed data and the family level for presence/absence data.

Discussion

Macrobenthic community characteristics at the two LTER sites

The macrobenthic communities at both LTER sites were characterised by reduced richness and diversity (low S and H') and were badly structured (low J'), as is typical in transitional environments, in particular those of the Po River delta (e.g. Marchini et al. 2008, Munari et al. 2010, Pitacco et al. 2018). Nevertheless, the two LTER sites differed in terms of their hydromorphological features and anthropogenic impact, differences that were reflected in their respective macrobenthic communities. Specifically, at the Val di Comacchio site, the community showed high variability in annual richness, diversity, equitability and taxonomic complexity, whereas at the Sacca di Goro site, the community, characterised by lower interannual variations, showed higher annual species richness and diversity (S and H'), but at the same time, reduced equi-distribution of taxa (low J') and low taxonomic complexity. From the COM dataset (Valli di Comacchio), a clear decreasing trend of structural indices (S, H' and J') was observed; changes at the community level at this site, in addition to the major factors driving those changes (mainly eel aquaculture and climate changes), have been described thoroughly in previous papers (e.g. Mistri et al. 2000, Munari and Mistri 2014b, Munari et al. 2005, Pitacco et al. 2018). As for the GOR dataset (Sacca di Goro), structural indices failed to depict a temporal trend in the macrobenthic community, but changes were revealed through multivariate analyses. Sacca di Goro, as a leaky lagoon, is characterised by a high spatial variability in physicochemical parameters, related to the distance from the rivers and the sea and by large daily fluctuations linked to the height of the tides (Corbau et al. 2016). Furthermore, in recent years, Sacca di Goro has been subjected to frequent sediment dredging related to fishery activities, as well as restoration and maintenance projects (such as macroalgal removal in the event of blooms) fundamental for the local clam fishery productivity (Corbau et al. 2016). Nonetheless, the macrobenthic population at this site showed high resilience, recovering rapidly (within months) after these types of disturbance (Munari and Mistri 2014a), which could explain the high infra-annual variability of structural indices. The effects of such stressors and restoration actions on the macrobenthic community have been exhaustively described in previous papers (e.g. Corbau et al. 2016, Munari et al. 2006, Munari and Mistri 2014a).

Information loss along taxonomic groups

Information loss, in terms of the percentage of 'α', was reasonably low for both datasets from both species-to-genus (< 20%) and genus-to-family levels (< 40%), despite the higher variability of the taxonomic complexity at the COM site. Indeed, the suitability of TS for taxonomically complex communities, as well for simple, species-poor ones, has also been observed in different habitat types (Bacci et al. 2009, Mistri and Rossi 2001). In fact, the efficiency of TS is not dependent on the number of species belonging to the same genus or family, but instead on their response to disturbance (Dauvin et al. 2003). At the GOR site, the most specious family, Spionidae, was the only one that could represent a limitation for TS. Although the family itself was classified as tolerant according to the AMBI library (EGIII), it comprised a number of species, not only tolerant (EGIII) but also opportunistic (EGIV and EGV). At the COM site, not only the family Spionidae, but also the family Serpulidae represented limitations for TS, comprising sensitive (EGI), indifferent (EGII) and tolerant species (according to the AMBI library; www.ambi.azti.es). Instead, the family Phyllodocidae, although responsible for high α values at the COM site, comprised only two genera, with almost exclusively similar species and did not, therefore, present a limitation for TS. In all cases, no variation in sensitivity (in terms of AMBI groups) was observed at the genus level, suggesting that the genus level provided a good representation of the response to disturbance.

Variations in the general structure of the macrobenthic community (multivariate analyses) were maintained with reasonably low information loss, considering both location and dispersion components, from species to genus and from species to family levels, almost irrespective of the data transformation, in both datasets (as shown by MDS, Spearman's correlation, hierarchical clustering, PERMANOVA and PERMDISP analyses). However, the response to TS differed between the two datasets for higher levels of taxonomic aggregation. In fact, information loss, due to both location and dispersion components, at taxonomic levels higher than the family level was quite high with respect to the species level at both sites. Our results are consistent with investigations performed in a western Mediterranean lagoon, where the ordination models derived from species and family abundances were very similar both in terms of location and dispersion effects, while further aggregation to the class level altered the observed spatial patterns (Tataranni et al 2009).

Although loss of information about the structure of the benthic assemblages increased with decreasing taxonomic accuracy at the COM site, this was not the case for the GOR dataset. In fact, at the GOR site, aggregation at the phylum level yielded better results than aggregation at the class level. Hence, TS, using family as surrogate, could be a good compromise between time/costs and efficiency, while genus remains the best surrogate for the identification of temporal variations in the macrobenthic community. That being said, the sufficiency level of taxonomic resolution could be strongly context-dependent (Terlizzi et al. 2003) and could change according to the bio-geographic background (Roy et al. 1996) or habitat type (Chapman 1998). More-

over, it could vary according to different relationships of abundance and redundancy between species. For instance, Vanderklift et al. (1998) found that, in an Australian marine bay, genus richness highly reflected species richness, but families only well described species richness for fish assemblages and not for plants. In addition, Warwick et al. (1990) found little information loss related to taxonomic aggregation at the family level in a macrobenthic community from Bermuda, but significant information loss at the level of the meiobenthic community, in particular regarding nematodes.

A review of the current literature on taxonomic sufficiency (Table 3), performed considering only papers testing the efficiency of the use of higher taxa as surrogates for species in marine macrobenthic invertebrates, showed that, when the entire community was analysed, the family level is recognised as the best surrogate by many authors worldwide. This review revealed that analyses performed at the family level are considered suitable for assessing the response of macrobenthic invertebrates to both anthropogenic impact and natural gradients of environmental variation in most of the published papers. The suitability of family levels has acquired a consensus worldwide, from polar to tropical regions and from transitional (both estuaries and coastal lagoons) to marine habitats.

Information loss with data transformations

The choice of data transformation required particular attention. It is well known that data transformation can influence the results of consequent analyses to a similar extent to the choice of taxonomic resolution (Olsgard et al. 1997), but only a small percentage of published papers on TS have analysed this aspect in detail (Table 4). As suggested by the 'fan' shape of the second-stage MDS plots, reported also in other habitat types (e.g. Olsgard et al. 1998; Olsgard and Somerfield 2000), the effects of transformation and taxonomic resolution operate largely independently. In particular, data transformation can critically affect data dispersion and is considered a common remedy for heterogeneity of variance in univariate analyses (Anderson et al. 2008).

In our case, irrespective of data transformation, differences in dispersion amongst clusters remained significant, at least to the family level, in accordance with Tataranni et al. (2009). At higher taxonomic levels, the effect was more evident and changed according to the strength of the data transformation and the dataset considered. The decreasing similarity of ordination matrices with increasing strength of data transformation observed at the two LTER sites clearly indicates that abundance was a key factor in characterising the macrobenthic community. The importance of abundance was further confirmed by the greater information loss with the use of presence/absence transformation at both LTER sites. In particular, at the COM site, the high correlation between second-stage similarity matrices (representing different transformations) indicated that transformation had little effect on the efficiency of TS, although loss of information regarding the general pattern of the community from one taxonomic level to the one above slightly increased with the strength of transformation. The only

Table 4. Taxonomic sufficiency documented for natural and anthropogenically induced benthic patterns. Atl= Atlantic Ocean, NS = North Sea, Pac = Pacific Ocean, Med = Mediterranean Sea, CS= Caribbean Sea, Arc = Arctic ocean, Aus = Australia, SA= South Africa.

Habitat	Area	Taxonomic group	Sufficient taxonomic level	Effect of data transformation	Reference
Soft bottom – pollution gradients					
Marine water, intertidal to subtidal	Atl	Macro and meiobenthos	Family for meio, phylum for macrobenthos	Not considered	Warwick 1988a
Marine waters, 0–3000 m	NS	Macrobenthic invertebrates	Family or phylum	Not considered	Gray et al. 1990
Transitional waters, estuarine	Pac	Macrobenthic invertebrates	Family (1 mm mesh), species (0.5 mm mesh)	Not considered	Ferraro and Cole 1992
Coastal waters, 14–380 m	NS	Macrobenthic invertebrates	Family	Increased from order to higher levels, no effect for family	Olsgard et al. 1998
Coastal waters	Med	Macrobenthic invertebrates	Family	Not strong till family level	Karakassis and Hatziyanni 2000
Coastal waters, 65-380 m	NS	Macrobenthic invertebrates	Family	Evident complex effect	Olsgard and Somerfield 2000
Soft bottom – natural gradients					
Coastal water, fjord	Atl	Macrobenthic invertebrates	Family	Not strong	Warwick 1988b
Coastal water	Atl	Macro and meiobenthos	Family (except nematodes)	Not considered	Warwick et al. 1990
Coastal water, fine sand	CS	Polychaetes, crustaceans, molluscs	Phyla (from genera)	Effect of fourth root only at phylum level	Guzmán and García 1996
Transitional waters, lagoons	Med	Macrobenthic invertebrates	Family	Effect only for levels above order, with P/A data	Mistri and Rossi 2001
Transitional waters, estuarine	Med	Macrobenthic invertebrates	Family	Not considered	De Biasi et al. 2003
Transitional waters, estuarine	Atl	Macrobenthic invertebrates	Order	Not considered	Chainho et al. 2007
Coastal waters, 38–380 m	Arc	Macrobenthic invertebrates	Order	Not considered	Włodarska-Kowalczuk and Kędra 2007
Coastal waters, 1–120 m depth	Med	Macrobenthic invertebrates	Family	Not considered	Bacci et al. 2009
Transitional waters, coastal lagoons	Med	Macrobenthic invertebrates	Family	Not considered	Munari et al. 2009
Transitional waters, coastal lagoons	Med	Macrobenthic invertebrates	Family	Important	Tataranni et al. 2009
Coastal waters, silty-sandy, 31–37 m depth	Med	Polychaetes	Family	Not considered	Musco et al. 2011
Soft bottom – natural and pollution gradients					
Coastal water, Intertidal to subtidal	Aus	Macrobenthic invertebrates	Family and order	Not considered	Vanderklift et al. 1996
Hard bottom – pollution and natural gradients					
Coastal area, rocky intertidal	SA	Macrobenthic invertebrates	Phylum for regional differences, family for pollution differences	P/A data regional differences significant only at the species level	Lasiak 2003

Habitat	Area	Taxonomic group	Sufficient taxonomic level	Effect of data transformation	Reference
Hard bottom – natural gradients					
Intertidal area, mangrove forest	Aus	Macrobenthic invertebrates	9 major groups (mix of orders, classes and phyla)	Fourth root retained differences between sites, but altered patterns within sites	Chapman 1998
Coastal waters	Med	Macrobenthic invertebrates	Family	Evident complex effect	Mistri and Rossi 2000
Intertidal and subtidal, ≤ 5 m	Atl	Macrobenthic invertebrates	Phylum	Not considered	Pagola-Carte and Saiz-Salinas 2000
Coastal area, kelp forest	Pac	Macrobenthic invertebrates	Family	Mild effect	Anderson et al. 2005
Hard and soft bottom – pollution gradients					
Coastal waters, 5–90 m depth	Med	Molluscs and polychaetes	Order for molluscs, genus for polychaetes	Absent or mild, stronger on hard bottom	Bevilacqua et al. 2009
Hard and soft bottom – natural gradients					
Transitional waters, estuarine	Pac	Macrobenthic invertebrates	Family	Not considered	Dethier and Schoch 2006
Coastal water, till 200 m	Pac	Molluscs	Family for tropical–polar latitudinal diversity, not for regional climatic gradients	Not considered	Roy et al. 1996
Marine and estuarine	Pac	Fish, invertebrates and plants	Genus for plants, family for fishes, class for invertebrates	Not considered	Vanderklift et al. 1998
Coastal waters, 11–380 m	Med, Atl, Pac, Arc	Molluscs	Family	Not considered	Terlizzi et al. 2009

exception was the strongest transformation (presence/absence), in which the contribution of taxa abundance was null.

Conversely, at the GOR site, the correlation between second-stage matrices (representing different transformations) was, in general, very low, indicating a stronger effect of transformation on the efficiency of TS. Interestingly, for the GOR dataset, weak data transformation (i.e. raw data) was sufficient to allow good correspondence amongst similarity matrices at the species level and all taxonomic surrogates up to the phylum level. This suggests that abundance was fundamental in determining temporal changes in the macrobenthic community at the GOR site. Nevertheless, both PERMANOVA and PERMDISP analyses on untransformed data failed to discriminate between groups identified by cluster analysis. For the GOR dataset, therefore, the choice of data transformation was more important to the correct evaluation of the efficiency of TS. Indeed, the populations within transitional waters typically show dramatic seasonal, annual and interannual variations, ranging from disappearance to complete dominance during periods of dystrophic crisis (Arvanitidis et al. 2009). Hence, changes in annual abundance could be biased by seasonal or occasional local variations, with changes in the abundance of the dominant species obscuring all other changes in the macrobenthic community.

Untransformed data is the most commonly used for TS in environmental monitoring (Musco et al. 2011), but in our case, the best results were obtained with square-root data transformation. Our results suggested caution in the use of both untransformed data and strong transformations, at least for transitional environments, particularly in the absence of strong pressures causing marked effects on all aspects of the macrobenthic community, as was the case with the GOR dataset. Indeed, recent publications (Table 4) have shown that the effect of transformations could vary across different environmental conditions, spatial scales and habitat types. For instance, data transformation affected differences between similarity matrices at varying taxonomic resolutions in hard-bottom natural gradients, while this effect was less pronounced in soft-bottom gradients (Bevilacqua et al. 2009). As a consequence, the choice of data transformation should not rely exclusively on the best statistical match between similarity matrices, but should be more a biological question, as already suggested by previous investigations. A weak transformation gives a narrow view of the community, deeply influenced by the most abundant taxa, whereas a strong transformation yields a wider view of the community, in which all taxa have the same weight, regardless of their relative abundance (Karakassis and Hatziyanni 2000; Mistri and Rossi 2001). Therefore, if changes in the dominant species are considered important, using raw abundance data will provide just such a narrow view of community structure, whereas if all species are considered to be equally important, then a strong transformation is more appropriate (Olsgard and Somerfield 2000). In short, investigators should bear in mind that the choice of data transformation may be affected by the structure of the analysed community, the scale of the changes therein, the taxonomic level considered and the specific objective of each individual investigation.

Conclusions

Our results showed that TS could be an efficient tool for long-term monitoring programmes. Our results also showed how LTER observations are critical for detecting meaningful ecological shifts and assessing whether ecological changes are due to human or natural causes. LTER data are particularly important for the identification of temporal trends, as many ecological processes develop at temporal scales that are longer than have typically been considered in traditional short-term ecological research studies. The higher natural variability of environmental conditions, combined with multiple stressors of anthropogenic origins at the two LTER sites analysed, did not represent an impediment for TS in detecting temporal changes in the macrobenthic community. For the two LTER sites analysed, the solution providing the best compromise between time/cost and information loss was a square-root transformation using family data as the taxonomic surrogate. Given the rising importance of long-term data series for detecting trends and changes at community levels, in particular in greatly fluctuating environments such as transitional waters, TS could be a useful method of enabling an increase in sampling frequency, together with a higher spatial resolution, while still reducing costs. Increasing the available information on a temporal scale could also help

reduce the bias of seasonal and local variations and, therefore, increase the efficiency of environmental management actions and biodiversity conservation measures.

At the same time, our results showed that the structure of the community and the magnitude of changes influence the efficiency of taxonomic surrogates and data transformations. Therefore, great care in the choice of those aspects of TS is necessary, in particular at sites where the effect of disturbance on community structure is not marked, as was the case for the GOR dataset. The best choice could be a function of environmental conditions, habitat types and biogeographic area. Therefore, care is needed when generalising outcomes in the field of TS and pilot studies are required to distinguish the most suitable procedure on a case-by-case basis (Chapman 1998, Pagola-Carte and Saiz-Salinas 2000). Knowledge about the species present and their biology and ecology is still an indispensable prerogative for defining the most suitable taxonomic level for TS (Terlizzi et al. 2003). Indeed, taxa including many abundant species might contain rare species with key roles in the structure of communities that only fine taxonomic analyses and manipulative experiments may reveal (Mistri et al. 2001). We therefore suggest that analyses at a finer taxonomic level should be performed periodically in routine monitoring programmes in order to provide, case by case, baseline knowledge for interpreting changes in communities and to check the efficiency of taxonomic substitutes and data transformation in monitoring programmes. In particular, baseline knowledge seems particularly important for long-term monitoring in lagoon systems affected by several natural and anthropogenic factors.

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REVIEW ARTICLE

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Time series and beyond: multifaceted plankton research at a marine Mediterranean LTER site

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Abstract

Plankton are a pivotal component of the diversity and functioning of coastal marine ecosystems. A long time-series of observations is the best tool to trace their patterns and variability over multiple scales, ultimately providing a sound foundation for assessing, modelling and predicting the effects of anthropogenic and natural environmental changes on pelagic communities. At the same time, a long time-series constitutes a formidable asset for different kinds of research on specific questions that emerge from the observations, whereby the results of these complementary studies provide precious interpretative tools that augment the informative value of the data collected. In this paper, we review more than 140 studies that have been developed around a Mediterranean plankton time series gathered in the Gulf of Naples at the station LTER-MC since 1984. These studies have addressed different topics concerning marine plankton, which have included: i) seasonal patterns and trends; ii) taxonomic diversity, with a focus on key or harmful algal species and the discovery of many new taxa; iii) molecular diversity of selected species, groups of species or the whole planktonic community; iv) life cycles of several phyto- and zooplankton species; and v) interactions among species through trophic relationships, parasites and viruses. Overall, the products of this research demonstrate the great value of time series besides the record of fluctuations and trends, and highlight their primary role in the development of the scientific knowledge of plankton much beyond the local scale.

Keywords

phytoplankton, zooplankton, time series, long-term research, LTER, Mediterranean Sea

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…time-series programs act as intellectual flywheels that create and sustain ever larger, complementary programs where the scientific outcome of the integrated effort is much larger than the sum of its parts

David Karl (2010)

Introduction

Long-term ecological research is recognized to contribute prominently to scientific advances as well as to inform environmental policy, which makes investments in continuous observations highly cost-effective (Edwards et al. 2010, Hughes et al. 2017). Yet, long-term studies have often been derogatorily tagged as 'routine monitoring', only aimed at recording natural events, whereby the sampling scheme that repeats over time with the same sustainable modality would hardly deem to deserve consideration alongside scholarly scientific activities. In terms of publications, observational activities are also considered scarcely rewarding, or likely to produce only descriptive papers. As a matter of fact, long-term time series are less than what would be needed and their spatial coverage is extremely limited (Koslow and Couture 2013). In the marine environment, few programs among the twentieth-century time-series have lasted more than a few decades (Duarte et al. 1992) probably because of their higher inherent costs and lower accessibility compared to the terrestrial environment. Especially in the case of plankton, the dominance of microbial life, which is characterized by small temporal and spatial scales in life cycles and distributions (Ribera d'Alcalà 2019) imposes a sampling frequency that is hardly compatible with the logistic and economic constraints of marine research. In fact, long-term ecological studies are less numerous in the world seas than in freshwater and terrestrial ecosystems (e.g., Mirtl et al. 2018), and they are mostly coastal and only in a few cases include the study of plankton.

Nonetheless, remarkable examples exist of the crucial role of time series as a baseline for the definition of the marine ecosystem status change and the understanding of the impact of climatic and anthropic forces on the structure and function of oceanic ecosystems (McGowan 1990, Karl 2010, Koslow and Couture 2013, Cloern et al. 2016). Time-series analysis has highlighted long-term changes and regime shifts that are at the basis of the fluctuations for plankton and fish populations in relation to climate oscillations in the north Atlantic (Beaugrand 2004, Edwards et al. 2010, Harris 2010) and in the Pacific (Venrick et al. 1987, Karl et al. 2001), which has been made possible by such regular and persistent surveys as the Continuous Plankton Recorder program (Reid et al. 2003), the long-term observations in San Francisco Bay (Cloern and Jassby 2012) and at the CALCOFI and station ALOHA programs in the North Pacific Ocean (Karl et al. 2001, Koslow et al. 2011).

In the studies mentioned above, the data collected over many years have mainly been used to highlight interannual patterns, episodic events and long-term changes in the system. On the other hand, it is rarely taken into account that, while recording changes over time, a sustained sampling plan can help to gather fundamental information on the biology of the species and illuminate the mechanisms of their succession and the relationships among the components of the system. In addition, the precious infrastructural asset of a time series provides a backbone for complementary studies that are stimulated by questions stemming from the results of continuous observations (Karl 2010, Zingone et al. 2010b).

This paper is an overview of a series of studies that have been produced in relation to plankton observations conducted at the Long Term Ecological Research site "Mare-Chiara" (LTER-MC) in the Gulf of Naples (Mediterranean Sea) since its establishment in 1984. These studies include both ecological investigations aimed at tracing the time variability of the pelagic system and complementary research based on experiments or additional observations flanking the routine monitoring for shorter periods of time. The latter complementary studies were prompted by the idea that the interpretation of observational data must be grounded in a deep understanding of the diversity, biological assets and ecological interactions of plankton populations, which is the key to the prediction of the response of natural populations to changing scenarios. Our aim is to highlight the fundamental contribution of time series to the development of plankton knowledge that can be of general interest much beyond the local scale.

The research site: general characteristics

The Gulf of Naples (GoN) extends between 40°50'N, 40°32'N, 13°52'E, 14°28'E in the Mid Tyrrhenian Sea, with an area of ca 870 km^2 and an average depth of 170 m (Fig. 1). A population of about 4 million people lives in the coastal area, which has been inhabited since pre-Roman times. Large-scale industrial activity is today reduced, while small and medium enterprise activities are significant and growing. For several years, urban and industrial discharges have largely been submitted to different types of treatments. However, some highly impacted areas are still present, sometimes located close to relatively pristine localities that are part of Marine Protected Areas (http://www.parks.it/indice/RM/map.php?reg=15) (Tornero and Ribera d'Alcalà 2014). Besides scattered municipal effluents, modest freshwater inputs are provided by the Sarno River from the southern coast and the Volturno River from the adjacent Gulf of Gaeta to the North, which results in high salinity values (> 38) often also close to shore.

First studies on the plankton of the GoN predate the foundation of the Stazione Zoologica Anton Dohrn of Naples (SZN) in 1872 (e.g., Costa 1858). In the following decades studies on plankton biodiversity were occasionally conducted with different objectives, and included taxonomic studies on copepods (Giesbrecht 1892) and ecological studies focused on the phenology of microplankton species (Issel 1934, Indelli 1944, De Angelis 1958) and on zooplankton distribution (Hure and Scotto di Carlo 1968).

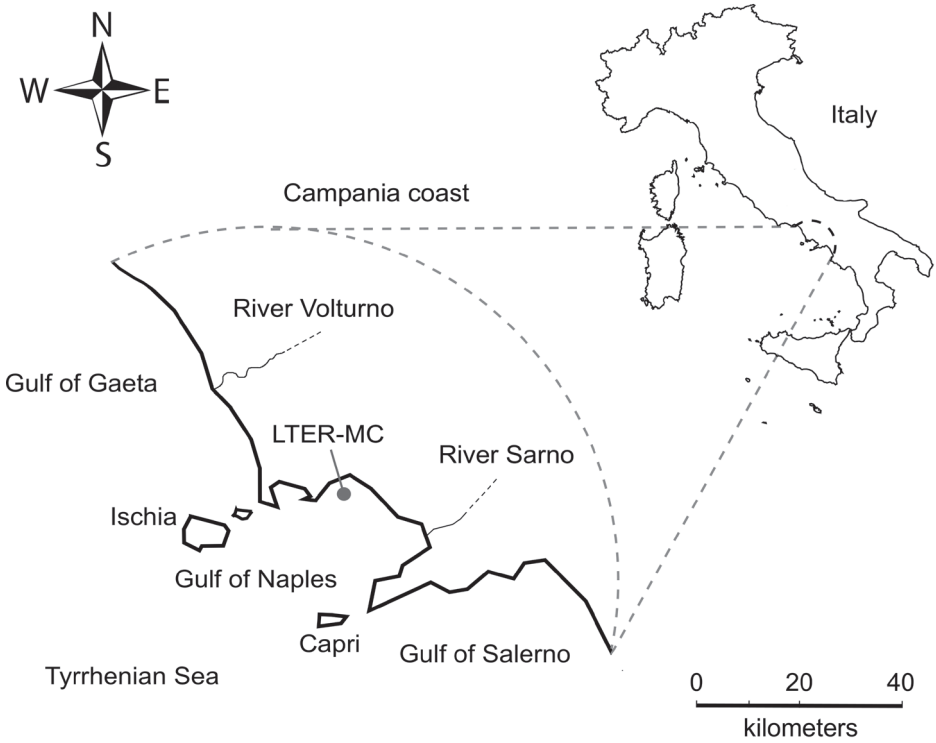

Figure 1. Map of the Gulf of Naples with the location of the station LTER-MC.

Yet regular ecological researches into the planktonic system of the GoN did not start until the 1970's (Carrada et al. 1981, 1982). Since 1984 these researches have mainly been based on data collected fortnightly (until 1991) or weekly (1995 to date) at the site MareChiara (Scotto Di Carlo et al. 1985, Ribera d'Alcalà et al. 2004), which in 2006 became part of the Italian, European and international LTER networks as LTER-MareChiara (LTER-MC). Research at this single point has been complemented over the years by several sampling campaigns and studies expanding over the whole area of the GoN and at times in the adjacent Gulfs (Marino et al. 1984, Ribera d'Alcalà et al. 1989, Zingone et al. 1990, 1995a, Ragosta et al. 1995, Iermano et al. 2012, Cianelli et al. 2017).

The station LTER-MC is located 2 nautical miles off the coast of the city of Naples in an area that can be alternatively influenced by the eutrophic coastal zone and the oligotrophic waters of the mid Tyrrhenian Sea (Fig. 1). Based on the data from a series of weekly sampling cruises in the inner Bay of Naples in summer 1983 (Zingone et al. 1990), the site was selected so as to avoid the conditions more directly reflecting the most intensive nutrient inputs from the southeastern coast of the inner Bay, and to capture the variability deriving from the exchange with the offshore waters. In fact, estimates of primary production conducted over the first part of the time series range from

Table 1. Environmental and biological variables at station LTER-MC (1984–2014). All values refer to surface waters, except zooplankton abundance which refers to 0–50 m layer of the water column.

	Temperature (°C)	Salinity	Chlorophyll *a* (µg L^{-1})	Phytoplankton (Cells mL^{-1})	Zooplankton (Ind. m^{-3})
Minimum	13.2	36.2	0.1	7.5×10	1.1×10^2
Maximum	28.9	38.3	26.8	2.2×10^5	2.3×10^4
Median	19.6	37.7	1.1	5.7×10^3	1.3×10^3

260 g C m^{-2} yr^{-1} in the period 1984–1991 to 151 g C m^{-2} yr^{-1} in the years 1997–2000 (Pugnetti et al. 2006), which ranks the study area as oligo-mesotrophic (Nixon 1995). Research on the spatial representativeness of station LTER-MC has shown that the site is more affected by coastal than offshore waters and can occasionally be influenced by the nearby Gulf of Gaeta (Carrada et al. 1981, Iermano et al. 2012), whereas it does not seem significantly affected by the Sarno River (Cianelli et al. 2017). On the other hand, complementary research conducted on a wider area (e.g. Marino et al. 1984, Ragosta et al. 1995, Zingone et al. 1995a) indicates that environmental conditions in the inner Gulf of Naples are quite distinct from the nearby mid-Tyrrhenian coasts.

The dataset collected at the LTER-MC site since 1984 includes physical (temperature and salinity), chemical (O_2, NO_2, NO_3, NH_4, PO_4, SiO_4) and biological (chlorophyll *a*, phytoplankton and mesozooplankton) data, all concerning different depths of the water column except phytoplankton which are analyzed in surface waters and mesozooplankton which are collected in the 0–50 m depth layer (Table 1). A number of other variables have been added over the years, including pigment spectrum by High Performance Liquid Chromatography (HPLC) since 1996 and, since 2007, particulate Organic Carbon (POC), Total Nitrogen and Phosphorous (TN e TP), Dissolved Organic Carbon (DOC), bacteria and picoplankton by flow-cytometry and mesozooplankton carbon and nitrogen content. Data over relatively long periods were also collected for microzooplankton (March 1984–March 1985, September 1996–December 2009), ^{14}C primary production (1984–1989 and 2007) and egg production and viability in the copepod *Centropages typicus* Krøyer, 1849 (1995–2015).

Temporal variability at station LTER-MC

Tracing the ecosystem variability over decadal scales is the distinctive essence of long-term research and the only approach that allows for discerning regular patterns, trends and shifts occurring in the environment. In long-term studies, one of the primary questions is whether significant changes occur in the overall system or in some of its components, which at LTER-MC has so far been addressed over the years 1984–2006. In that period, a pronounced interannual variability was evident in all environmental variables, with only a few significant trends, e.g., increase in summer temperature and decrease in chlorophyll *a* concentrations (Mazzocchi et al. 2011, 2012). Chlorophyll

markedly declined until 2002 (Zingone et al. 2010a), followed by a weak positive trend from 2003 to 2006 (Mazzocchi et al. 2012). Since 1995, phytoplankton increased in cell numbers but decreased in average cell size (Ribera d'Alcalà et al. 2004) mainly because of a decrease, across the years, in the proportion of larger vs. smaller diatom species (Fig. 2). The decrease for average cell size was evident until the early 2000s but a trend reversal has been observed in recent years (Sarno and Zingone unpubl. data in Morabito et al. 2018).

Copepods are the most numerous among the zooplankton groups and shape the patterns of the entire community. They showed higher abundances in the 1980s than in the two successive decades; this trend reversed in the years 2004–2006, mainly due to the increase of the calanoids *Paracalanus parvus* (Claus, 1863), *Acartia clausi* Giesbrecht, 1889 and *Centropages typicus* (Mazzocchi et al. 2012). The latter two species showed changes in their phenology with a significant anticipation of their population cycle in relation to positive temperature anomalies, a typical "earlier when warmer" response observed at different latitudes (Mackas et al. 2012). The decrease in chlorophyll concentrations suggests that station LTER-MC has acquired less coastal characteristics over the years, a signal that is also seen in the disappearance of a few copepod species typical of confined areas and increased abundance of species that thrive in offshore waters (Mazzocchi et al. 2012).

Over a longer time scale, the comparison with previous investigations in the GoN of the early 1930s showed essential changes in the tintinnid (ciliates) community in terms of dominant species (Modigh and Castaldo 2002) and in the occurrence of dinoflagellates species of the genus *Tripos* (as *Ceratium*) as a response to local warming (Tunin-Ley et al. 2009).

The interannual variability at station LTER-MC is remarkable, but it is the seasonal forcing that plays the main role in shaping the local pelagic system by deeply affecting the environmental features (Ribera d'Alcalà et al. 2004) and the plankton assemblages, from phytoplankton (Zingone et al. 1990, 1995a, 2003, 2010b) to microzooplankton (Modigh 2001, Modigh and Castaldo 2002) and mesozooplankton (Mazzocchi and Ribera d'Alcalà 1995, Mazzocchi et al. 2011, 2012). The only exception seems to be represented by the quite constant abundance of picoplankton, which, however, was studied over a single annual cycle (Modigh et al. 1996). The depiction of the average annual cycle of plankton at the sampling site is supported by long-term records which highlight the recurrence of regular patterns during most of the time series (Fig. 3). The comparison of the average seasonal cycle of zooplankton (1984–2006) with that of other five Mediterranean time-series at stations encompassing a wide range of trophic status has revealed that local drivers are dominant over large-scale climate influence for these coastal stations (Berline et al. 2012).

In thoroughly mixed water column conditions, minimum annual concentrations of chlorophyll in December-January, with a dominance of nanoflagellate species, are generally followed by late winter increase in February-March, mainly driven by large colonial diatoms which include several *Chaetoceros* species, *Pseudo-nitzschia delicatissima* (Cleve) Heiden and *Thalassionema bacillaris* (Heiden) Kolbe. These winter blooms

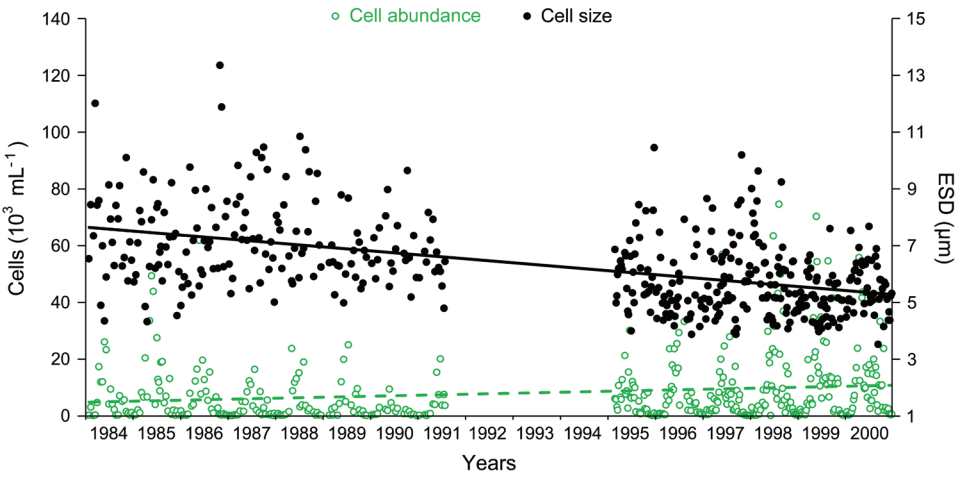

Figure 2. Trends in cell number and size of phytoplankton species at station LTER-MC. Redrawn from Ribera d'Alcalà et al. (2004). The marked decrease in cell size mainly reflected the decrease of larger diatom species and the increase of small-sized ones. ESD: Equivalent Spherical Diameter.

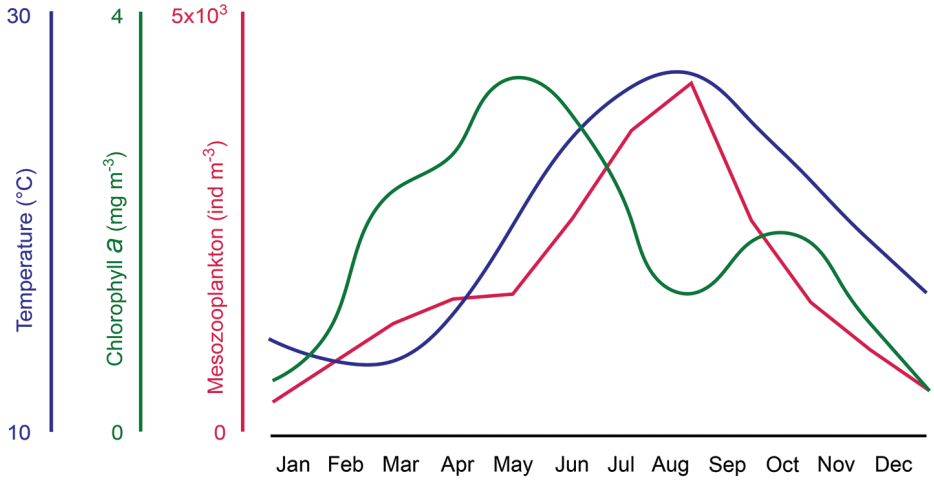

Figure 3. A schematic, averaged seasonal cycle of temperature, chlorophyll *a* and mesozooplankton abundance at station LTER-MC as recorded in the period 1984–2014.

are allowed by the non-limiting light amount especially under stable meteorological conditions and are reinforced by freshwater inputs contrasting intense vertical mixing (Zingone et al. 2010b). The reduced grazing due to the annual minima for micro- and mesozooplankton also contributes to the build-up of the winter biomass. In the winter ciliate assemblage, autotrophic species (*Mesodinium*) show the highest contribution,

while among metazoans copepods occur with numerous small-sized species (e.g., *Clausocalanus*, *Calocalanus*, *Ctenocalanus vanus* Giesbrecht, 1888, *Oithona*, oncaeids and corycaeids) and a few larger-sized (>1.5 mm) genera which thrive in deep offshore waters and are brought to the coastal site by the winter water mass circulation. The winter zooplankton are also characterized by a high percentage contribution of appendicularians and meroplankton, the latter group likely reflecting the intense reproductive activity of benthic organisms, which indicates that phytoplankton blooms in this season fuel the zoobenthos rather than being exploited by the scarcely abundant holoplankton.

Spring is the period of growth of the whole plankton compartment in the GoN, with a conspicuous phytoplankton peak confined to surface waters generally occurring in May, mainly caused by diatoms (e.g. *Skeletonema pseudocostatum* Medlin and *Leptocylindrus aporus* (F.W. French & Hargraves) Nanjappa & Zingone and phytoflagellates. Spring also sees the highest biomass of ciliates, which are dominated by the mixotrophic choreotrichs, and a copepod peak dominated by *Acartia clausi* in early spring followed by *Centropages typicus* in late spring-early summer.

In summer, with the annual peak of surface temperature (26 °C ± 1.5 °C in August), phytoplankton are mainly characterized by intense blooms of small-sized, often non-colonial diatom species (e.g., *Chaetoceros tenuissimus* Meunier) and of phytoflagellates, along with an increase of dinoflagellate biomass. Ciliates are represented by a variety of mixotrophic *Strombidium*, while mesozooplankton show the highest abundance and the lowest diversity of the entire year, along with the dominance of cladocerans (*Penilia avirostris* Dana, 1842) and copepods (mainly *Paracalanus parvus*).

The water column stratification disrupts in autumn, when depth-integrated temperature and salinity reach their maximum annual values. Phytoplankton show a less regular third annual peak in October-early November which is driven by recurrently stable weather conditions (Saint Martin's summer) that allow the exploitation of nutrients of terrestrial origin and mainly contributed by colonial diatoms (*Leptocylindrus* spp., *Chaetoceros socialis* Lauder, *Thalassiosira rotula* Meunier, etc.) (Zingone et al. 1995a). Among ciliates, the autotrophic *Mesodinium rubrum* (Lohmann, 1908) and the mixotrophic *Strombidium* spp. and *Tontonia* spp. occur with high concentrations. The copepods *Temora stylifera* (Dana, 1849), *Clausocalanus furcatus* (Brady, 1883) and *Oithona plumifera* Baird, 1843, together with chaetognaths, appendicularians and doliolids, account for most mesozooplankton abundance in this period of the year.

Overall, in spite of the high interannual variability observed in environmental variables, the different phases of the annual cycle are remarkably regular for the whole community structure and for the most common species of all planktonic compartments (Mazzocchi and Ribera d'Alcalà 1995, Modigh 2001, Modigh and Castaldo 2002, Ribera d'Alcalà et al. 2004, Mazzocchi et al. 2011). This feature highlights the resilience of coastal communities, which may favor their persistence over time (Mazzocchi et al. 2012, Cianelli et al. 2017). Recurrent species-specific seasonal patterns are observed not only in species of the copepod genera *Clausocalanus* and *Oithona* (Mazzocchi and Ribera d'Alcalà 1995, Castellani et al. 2015), but also in several phytoplankton taxa, such as the dinoflagellate *Tripos* (as *Ceratium*, Tunin-Ley et al., 2009), the diatom *Pseudo-nitzschia*

(Ruggiero et al. 2015), cryptomonads (Cerino and Zingone 2006) and numerous other species (Zingone and Sarno 2001, Ribera d'Alcalà et al. 2004), which suggests that the seasonal succession is mainly regulated by biological factors, such as life-cycle processes, species-specific physiological performances and inter-specific interactions.

Plankton diversity at LTER-MC

LTER-MC is one of the few sites where marine plankton diversity is regularly monitored at the species level, with routine sample observations complemented by detailed taxonomic studies based on microscopy and molecular analyses. This peculiar approach stems from the conviction that a sound taxonomic knowledge and a clear definition of the ecological units of interest (i.e., species identification) are fundamental to the study of seasonal and long-term variability of plankton communities, to assess the conditions in which phyto- and zooplanktonic species occur and succeed, and, ultimately, understand the pelagic ecosystem functioning. These principles also explain the attention paid to quality control procedures of both diversity and chemical physical data (Zingone et al. 2015, Sabia et al. 2019). Indeed, the relevance of precise identification is fully supported by the results so far obtained on the taxonomic and molecular diversity of the GoN plankton, as detailed in the following.

Studies on plankton diversity in the GoN boast an ancient tradition dating back to the beginning of the XIX century (Delle Chiaje 1823, Costa 1858) and have intensified following the SZN foundation (Daday 1888, Giesbrecht 1892, Schröder 1900). Much higher attention has been paid to taxonomy of the GoN plankton since the start of the LTER-MC project, which has led to the clear demonstration of the high diversity of these assemblages, with more than 750 microalgal and 212 mesozooplankton taxa recorded over the years. These numbers are certainly underestimated, particularly for protists, because many groups are still in need of detailed taxonomic investigation, but also for planktonic metazoans (Fig. 4), which also need deep investigation at the molecular level (Di Capua et al. 2017) and are recently revealing a high level of cryptic diversity (Hirai et al. 2017, Kasapidis et al. 2018). A current view is that hotspots of diversity correspond to hotspots of expert taxonomists, a joke that points to the lack of objective criteria for the comparison of plankton diversity among different sites. Nonetheless, it is tempting to hypothesize that the GoN presents suitable characteristics to host a high biodiversity for plankton organisms. In fact, the Mediterranean Sea confers the area subtropical characteristics despite its temperate latitude, while the fertility of coastal waters may act as an incubator of diversity for a high number of species, including those from offshore waters that are frequently driven into the Gulf. The interplay of hydrography, climate and trophic conditions likely creates optimal conditions for a high number of species to coexist in the area, a hypothesis that comparative studies with objective molecular methods will allow to test over coming years.

The taxonomic insights on the LTER-MC plankton have concerned particularly microalgae, which remain the least known plankton compartment – the smaller the

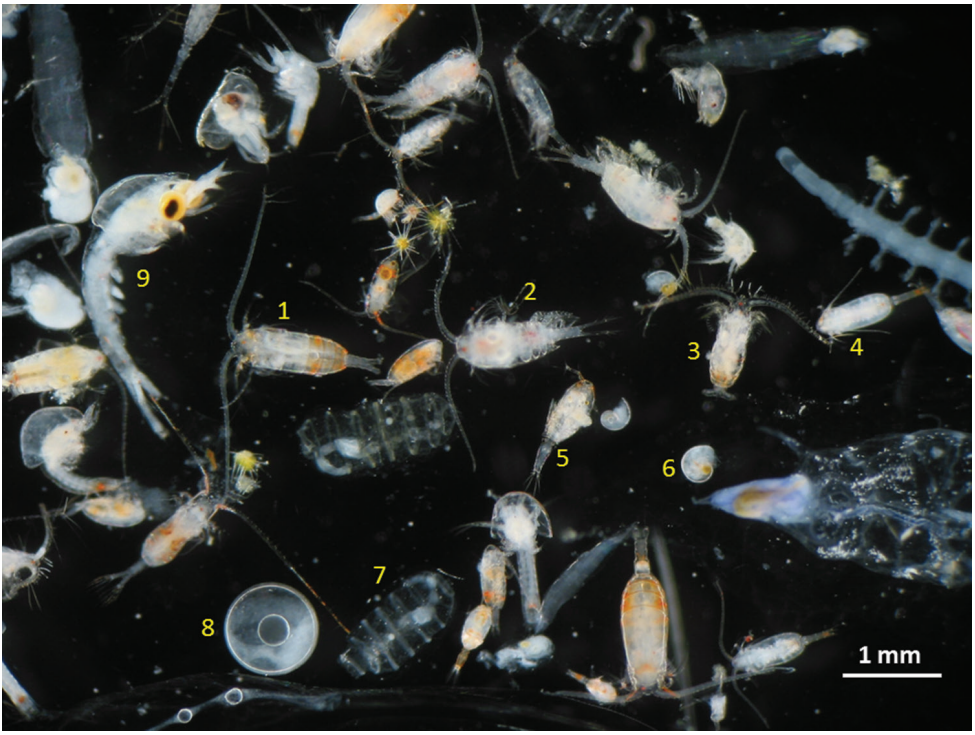

Figure 4. A mixed zooplankton sample from station LTER-MC with indicated some common taxa. Copepods: **1** Calanidae **2** *Temora stylifera* **3** *Calocalanus* **4** *Clausocalanus* **5** Oncaeidae **6** gastropod larva **7** doliolid **8** fish egg **9** decapod larva.

organism, the larger the taxonomic deficit. Cultivation of microalgal strains obtained from natural samples under controlled laboratory conditions, introduced at SZN in the 1980's, has been a powerful tool for the characterization of poorly known species. Flagellates hardly identified in fixed material were investigated at LTER-MC over their seasonal cycle using the Serial Dilution Culturing method (Throndsen 1978, Cerino and Zingone 2006), which eventually turned out to be a valuable source of material for deeper taxonomical and ecophysiological investigations. In addition, electron microscopy frequently coupled with cultivation allowed taxonomic insights of interest much beyond the GoN, like the rediscovery of Mediterranean flagellate species never found since their early description (Throndsen and Zingone 1988, 1994, Zingone et al. 1995b), and the description of calcareous resting stages of dinoflagellates and their assemblages in sediments from the GoN (Montresor et al. 1993, 1994, D'Onofrio et al. 1999).

Over the years, the regular observations of plankton samples from LTER-MC and the development of taxonomic expertise at SZN have allowed for spotting organisms not readily classifiable, paving the way for the re-description of ill-defined taxa and the discovery and formal description of more than 20 microalgal species new to science. These latter studies, often integrating the resting stage features as a further taxonomic

Table 2. Phytoplankton species originally described with different methods from LTER-MC and surrounding waters. C: cultivation, M: Light and Electron Microscopy, MB: Molecular Biology, LC: Life Cycle studies, RS: Resting Stage description.

Species	Methods	References
Diatoms		
Bacteriastrum parallelum Sarno, Zingone & Marino	C, M	Sarno et al. 1997
Chaetoceros throndsenii (Marino, Montresor & Zingone) Marino, Montresor & Zingone	C, M, RS	Marino et al. 1987, Marino et al. 1991
Leptocylindrus aporus (F.W. French & Hargraves) Nanjappa & Zingone	C, M, MB	French and Hargraves 1986, Nanjappa et al. 2013
Leptocylindrus convexus Nanjappa & Zingone	C, M, MB, RS	Nanjappa et al. 2013
Leptocylindrus hargravesii Nanjappa & Zingone	C, M, MB, RS	Nanjappa et al. 2013
Pseudo-nitzschia mannii Amato & Montresor	C, M, MB, LC	Amato and Montresor 2008
Skeletonema dohrnii Sarno & Kooistra	C, M, MB	Sarno et al. 2005
*Tenuicylindrus belgicus** (Meunier) Nanjappa & Zingone	C, M, MB	Meunier 1915, Nanjappa et al. 2013
Dinoflagellates		
Alexandrium mediterraneum U. John	C, M, MB	John et al. 2014
Alexandrium tamutum Montresor, Beran & U. John	C, M, MB	Montresor et al. 2004
Azadinium dexteroporum Percopo & Zingone	C, M, MB	Percopo et al. 2013
Biecheleria cincta (Siano, Montresor & Zingone) Siano	C, M, MB	Siano et al. 2009, Balzano et al. 2012
Prorocentrum nux Puigserver & Zingone	C, M	Puigserver and Zingone 2002
Protoperidinium parthenopes Zingone & Montresor	M	Zingone and Montresor 1988
Protoperidinium vorax Siano & Montresor	C, M	Siano and Montresor 2005
Scrippsiella precaria Montresor & Zingone	C, M, RS	Montresor and Zingone 1988
Scrippsiella ramonii Montresor	C, M, RS	Montresor 1995
Prasinophytes		
Crustomastix stigmatica Zingone	C, M, MB	Zingone et al. 2002
Dolichomastix tenuilepis Throndsen & Zingone	C, M, MB	Throndsen and Zingone 1997
Prymnesiophytes		
Phaeocystis cordata Zingone & Chrétiennot-Dinet	C, M, MB	Zingone et al. 1999a
Phaeocystis jahnii Zingone	C, M, MB	Zingone et al. 1999a

*new genus

character, were initially based on morphology and ultrastructure (Table 2 and Fig. 5). Subsequently, the introduction of molecular methods has provided additional information that has robustly supported the establishment of new species and their correct attribution in cases of poor morphological characters, i.e. in flagellates. For example, without the support from molecular data, the tiny single cells flagellate *Phaeocystis cordata* Zingone & Chrétiennot-Dinet (Fig. 5d) would have hardly been attributed to a genus that is mainly identified by its conspicuous colonial stages (Zingone et al. 1999a).

Successfully, taxonomic research at LTER-MC has retained a traditional morphological approach at the same time as embracing different aspects of the species identity, such as phylogeny (Kooistra et al. 2010 , Gaonkar et al. 2018), life cycles (Levialdi Ghiron et al. 2008, Amato et al. 2005, D'Alelio et al. 2009b), mating compatibility with closely related species (Amato et al. 2007, D'Alelio et al. 2009a), biochemistry (Lamari et al. 2013, Nanjappa et al. 2014b), physiology and metabolomics (Degerlund et al. 2012, Huseby et al. 2012), feeding strategies (Modigh and Franzè 2009, Mahadik 2014), behavior (Bianco et al. 2013, Mahadik et al. 2017) as well as ecological aspects like species

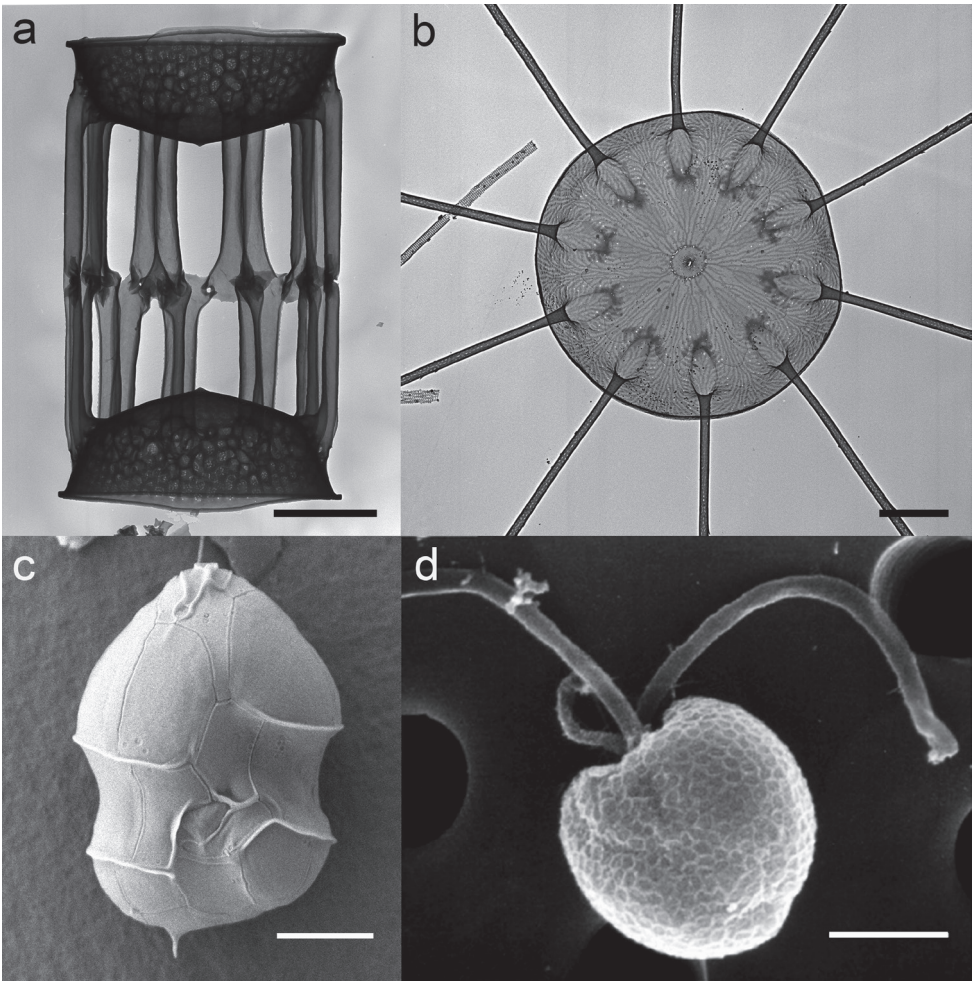

Figure 5. Some of the microalgal species discovered in the Gulf of Naples. **a** *Skeletonema dohrnii* Sarno & Kooistra, a species very similar to *S. marinoi* Sarno & Zingone, discovered instead in the Adriatic Sea. Both species bloom in late winter-early spring, whereas the most abundant species in the GoN, *S. pseudocostatum*, blooms in late spring **b** *Bacteriastrum parallelum* Sarno, Zingone & Marino, a solitary diatom species in a genus entirely consisting of colonial species **c** *Azadinium dexteroporum* Percopo & Zingone, a dinoflagellate producing several toxins of the group azaspiracids, and the first in this genus discovered in the Mediterranean Sea **d** *Phaeocystis cordata* Zingone & Chrétiennot-Dinet, a prymensiophyte that differs from all the congeneric species because it apparently lacks a colonial stage.

diversity during blooms (Orsini et al. 2004) and phenology (Cerino et al. 2005, D'Alelio et al. 2010). It was precisely the latter trait, which can only be inferred from long-term observations, that draw attention to certain taxa, e.g., '*Skeletonema costatum* (Greville) Cleve' showing different seasonality between the Tyrrhenian and Adriatic seas, or '*Leptocylindrus danicus* Cleve' occurring all year round in the GoN. These phenological peculiarities inspired detailed studies on these previously ill-defined taxa, with relevant insights

into their actual diversity and the seasonal occurrence of the redefined species, which demonstrate the reciprocal advantages of taxonomic and ecological studies conducted at the same place. Over the years, this cutting edge approach to taxonomy has resulted in pioneering researches that have soundly demonstrated cryptic and pseudo-cryptic diversity as common features in microbes. Results obtained on iconic phytoplankton species such as the dinoflagellate *Scrippsiella* (D'Onofrio et al. 1999, Montresor et al. 2003) and the diatoms *Pseudo-nitzschia* (Amato et al. 2007, Amato and Montresor 2008), *Skeletonema* (Zingone et al. 2005, Sarno et al. 2005, 2007) and *Leptocylindrus* (Nanjappa et al. 2013) have definitely challenged some common paradigms on marine microbes. For example, the idea that they are ubiquitous and opportunistic, being able to thrive in any place (e.g., Finlay 2002), has been contrasted by the first evidences of clearly distinct temporal and phylogeographic patterns (Kooistra et al. 2008, Nanjappa et al. 2013, Ruggiero et al. 2015), subsequently confirmed also for cryptic species with HTS-metabarcoding investigations (Nanjappa et al. 2014a, Gaonkar 2016, Pargana 2017).

The description of diversity has been further deepened at the population level in the case of the diatom *Pseudo-nitzschia multistriata* (Takano) Takano, selected as a model, which was found to consist of genetically distinct populations (Fig. 6) in samples collected at LTER-MC over four consecutive years (Tesson et al. 2014). This result, further confirmed in two subsequent years (Ruggiero et al. 2018), has been interpreted as the outcome of genetic recombination regularly occurring in this species (D'Alelio et al. 2009a, 2010) coupled with the rapid diversification during vegetative growth (Tesson et al. 2013). Interestingly, during the autumn bloom in 2013, a 'clonal expansion' event took place, with the sudden increase of diversification by mutation and the subsequent dominance of a single genotype. The availability of genomic resources for *P. multistriata* will allow deeper investigations into the dynamics of evolutionary adaptation to changing environmental conditions in the GoN.

Accurate data on species diversity gathered at the LTER-MC site have also enabled the discovery of a number of potential Invasive Alien Species (IAS). The definition and detection of IAS in plankton organisms are particularly tricky and biased by several factors, such as difficult identification, spatial patchiness and ephemeral occurrence. All these problems can be partially overcome in places where plankton species are properly identified over a long-term period. At the LTER-MC station, at least two diatom species, *Pseudo-nitzschia multistriata* and *Skeletonema tropicum* Cleve, were never recorded until 1995 and 2002, respectively, despite their relatively easy identification (Zingone et al. 2003, Zenetos et al. 2010, Corriero et al. 2016,) and the non-indigenous calanoid copepod *Pseudodiaptomus marinus* Sato, 1913 (Sabia et al. 2015) has suddenly appeared in recent years. Interestingly, at least one case of local extinction has also occurred with *Pseudo-nitzschia subpacifica* (Hasle) Hasle, never retrieved in the area as of 1988 (Zingone et al. 2003).

Considering the possible impact of potentially toxic and harmful species in such a densely populated area as the GoN, the high attention paid to the taxonomy and distribution of these species in the area is not surprising. A more detailed description and a sound taxonomic assessment have been provided for several harmful diatom

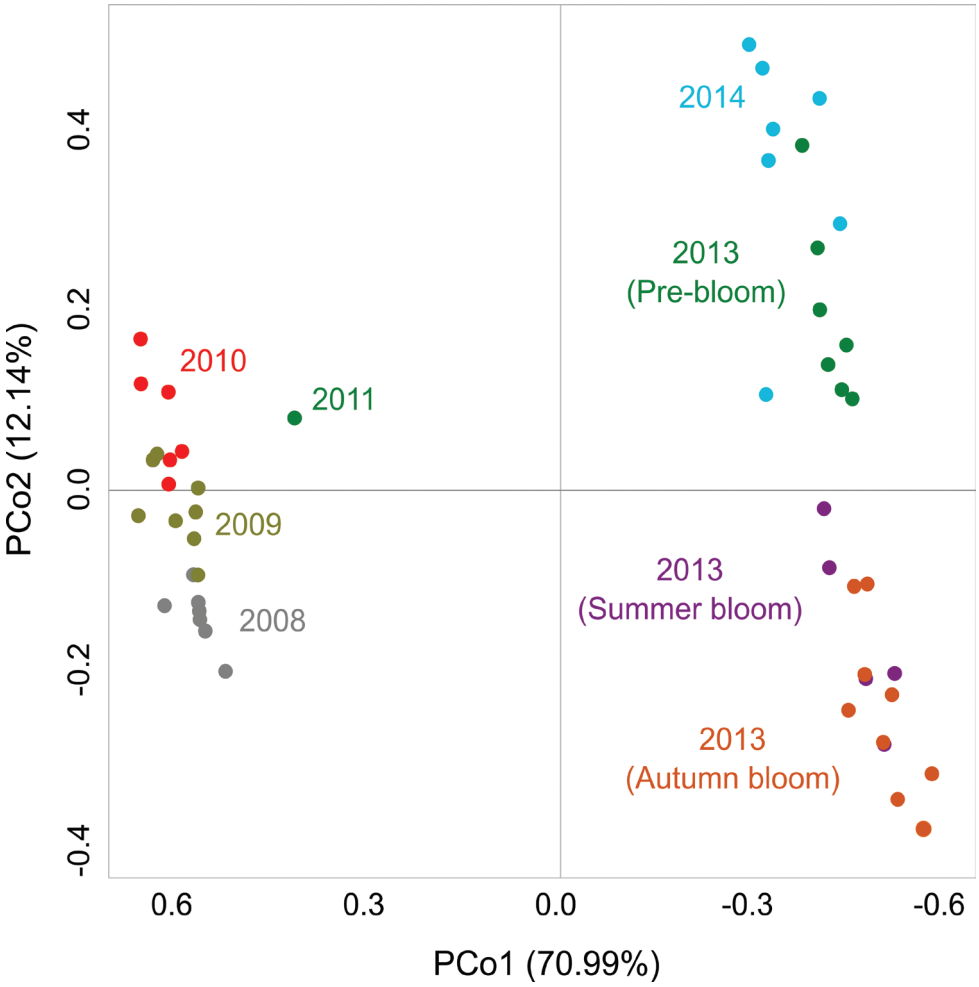

Figure 6. Different *Pseudo-nitzschia multistriata* populations, identified by microsatellite marker analysis, succeeding one to the other at station LTER-MC from 2008 to 2014. Redrawn from Ruggiero et al. (2018).

(e.g., Orsini et al. 2002, Cerino et al. 2005) and dinoflagellate species (e.g., Zingone et al. 1998, Escalera et al. 2014, 2018). In many cases, non-toxic species have been discovered in the GoN which only show subtle morphological differences from the toxic congeneric ones, as in the case of the dinoflagellates *Alexandrium tamutum* Montresor, Beran & U. John, similar to the toxic *A. minutum* Halim (Montresor et al. 2004), and *A. mediterraneum* U. John (group II), similar to the toxic *A. catenella* (Whedon & Kofoid) Balech (group I) and *A. pacificum* Litaker (group IV) (John et al. 2014), and the diatom *Pseudo-nitzschia mannii* Amato & Montresor, hardly distinguishable from the toxic *P. calliantha* Lundholm, Moestrup & Hasle (Amato and Montresor 2008). Recently, the first Mediterranean species of the dinoflagellate genus *Azadinium*, *A. dexteroporum* Percopo & Zingone (Fig. 5c), has been discovered in the GoN (Percopo et al. 2013),

along with a suite of new, probably toxic azaspiracids that it produces (Rossi et al. 2017). Overall, about 50 toxic and/or potentially harmful species have been detected over the years in the area, where the time series and additional sampling have contributed relevant data on their distribution and seasonality (Zingone et al. 2006b and unpublished data). This precious information can help with identifying periods of the year of higher risk of toxic species occurrence, providing guidance to informed monitoring and contributing to improve the management of toxicity outburst and the protection of food security.

Molecular diversity: the metabarcoding approach

The detection and quantification of species that are difficult to identify with morphology-based methods have been a goal for many years at LTER-MC, where a number of different attempts have been made to introduce adequate methods (McDonald et al. 2007a, b, Barra et al. 2013, Santamaria et al. 2015, McNamee et al. 2016). The direct access to molecules that are diagnostic for the individual taxa has opened a whole new range of possibilities for the study of microbial life, which has been scarcely known hitherto because of incomplete sampling, difficult cultivation and inadequate identification tools. To obtain the whole list of species from a single sample has been a long held dream that has now come true with the introduction of metabarcoding. The first and most comprehensive experiments using this approach have concerned sampling cruises at a local and worldwide scale (e.g. Moon-Van der Staay et al. 2001, de Vargas et al. 2015). Yet it has soon been acknowledged that the use of this new powerful tool is maximized at places with a sound background knowledge of the system, which allows for taking full advantage of the new information by integrating it with that deriving from classical methods (Davies et al. 2014, Stern et al. 2018).

Metabarcoding studies at the LTER-MC site started soon when the new molecular technologies became available to marine research. First tests demonstrated the potential of metabarcoding to overcome the two most arduous obstacles in diversity studies, i.e., the bad identification of species hardly seen in fixed material (e.g., tiny flagellates) and the difficulty to trace cryptic species in the environment. A great diversity and abundance of Prymnesiophyceae, until that time uncovered, was highlighted for the first time at LTER-MC in a study using dot blots and clone libraries (McDonald et al. 2007a), which also confirmed results on the seasonality of several cryptophyte species obtained with culturing methods (Cerino and Zingone 2006). In addition, hardly distinguishable, toxic and non-toxic species of the diatom genus *Pseudo-nitzschia* were first identified using PCR on environmental DNA samples with genus-specific LSU rDNA primers, followed by cloning (McDonald et al. 2007b). The latter experiment confirmed the idea, initially proposed based on strain isolation and culturing, that during seasonal blooms several almost identical congeneric species may coexist (Orsini et al. 2004). Subsequently the whole annual cycle of *Pseudo-nitzschia* species was reconstructed with the same approach, highlighting the high diversity of this genus and the different seasonality of the apparently alike species that belong to it (Ruggiero et al.

2015). In all these cases, the samples to submit to molecular analysis were selected based on the knowledge of the seasonal occurrence of flagellates and *Pseudo-nitzschia* acquired in years of morphology-based investigations, while the interpretation of the results was robustly supported by the comparison between molecular and light-microscopy results from the same samples, thus definitely proving that genomic observatories do benefit from background and contextual knowledge obtained with classical methods.

The huge diversity of prymnesiophytes in the plankton was subsequently confirmed by the first metabarcoding study in the GoN that used High Throughput Sequencing (HTS) on protist amplicons obtained with specific haptophyte primers, in the frame of the EU project BioMarKs (Bittner et al. 2013). Within the same project, the LTER-MC station was compared with five other European sites addressing the distribution of protists in coastal waters (Massana et al. 2015), rare species (Logares et al. 2014), the sediment assemblages (Forster et al. 2016) and the diatom community (Piredda et al. 2018).

Temporal changes in planktonic protist compositions at LTER-MC were investigated in a dedicated metabarcoding study carried out on eight sampling events over one year (Fig. 7, Piredda et al. 2017b). The presence of about 6,000 OTUs, many of which not attributable to any known protist genus, in such a limited number of samples, pointed to the high and largely unexplored diversity of the marine microbial world, at the same time revealing high variations of their diversity across the seasons, with a maximum in winter. Also in this case a correspondence was highlighted with samples analyzed with classical methods focusing on diatoms, for which the most complete datasets exist for both reference sequences and morphological identification. Metabarcoding analyses were also successfully applied to diatom resting stages, obtaining a quite complete picture of the diversity of the seed bank in the LTER-MC sediments (Piredda et al. 2017a).

The implementation of molecular studies in long term plankton observatories is nowadays occurring at several places, with slightly different approaches and methods (Stern et al. 2018) that need to be harmonized to allow for full intercomparability of the results. In this respect, the experience and knowledge gained at the LTER-MC site represent a useful contribution to the development of appropriate methodologies as well as a convincing example of the value of introducing such molecular approaches in classic, morphology-based ecological research on plankton organisms.

Life cycles

The LTER-MC time series has offered the precious opportunity not only to deepen the knowledge on plankton diversity but also to shed light on different phases in the life cycles of individual species, such as the many developmental stages of copepods or the benthic stages of many planktonic protists. Understanding the structure of species life cycles, along with the external (environmental) or internal (biological) cues that determine life-stage shifts and impact their viability, provides a framework to interpret the success and occurrence of the species across the seasons and the way they interact with the environment and with other organisms.

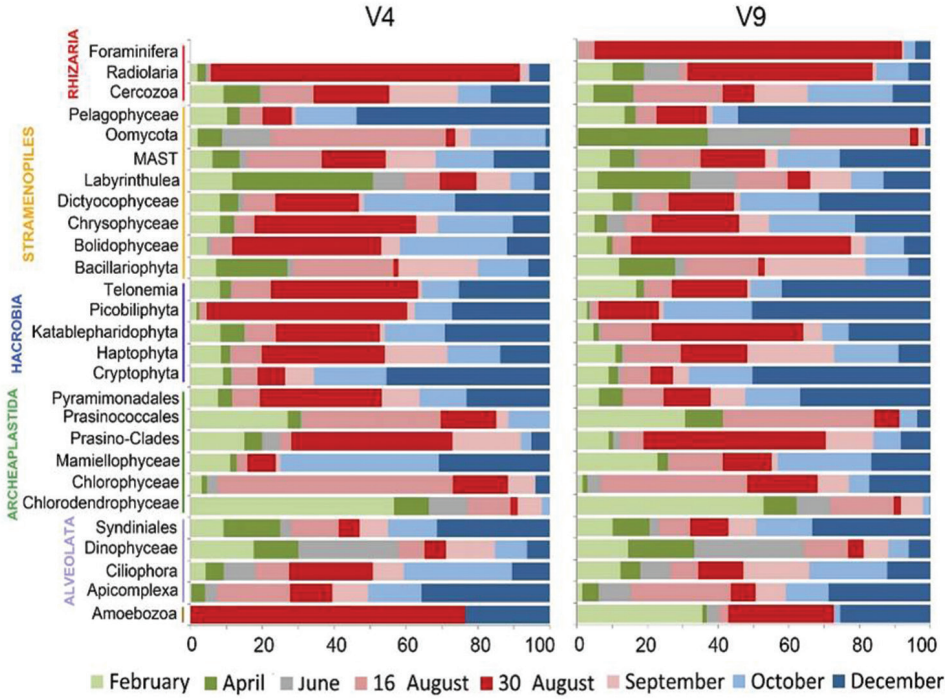

Figure 7. Protist seasonality at station LTER-MC, as revealed by the relative abundance of reads obtained by High Throughput Sequencing (HTS)-metabarcoding using two different 18S rDNA sequence fragment, V4 and V9. For each protist group, read abundance on different sampling dates was normalized over the total abundance of that group, in order to show the marked differences in seasonal patterns among groups. With the exception of a few cases, V4 and V9 gave similar results. From Piredda et al. (2017b), by permission of Oxford University Press.

For plants and metazoans, life cycle is defined as a series of changes and developmental stages that an organism passes through from the beginning of its life until its death. In protists, different stages correspond to distinct forms in which cells of a species exist in the environment, which may exhibit different morphologies, perform in different ways and follow a different destiny (von Dassow and Montresor 2011). The capability to enter a resting phase is probably the aspect of the life cycle of planktonic unicellular organisms that has attracted the most attention. Resting stages have a different morphology and/or physiology as compared to the vegetative cells that grow in the water column. Resting stages can survive in the sediments for many years, thus constituting a sort of 'seed bank' of diversity which, upon germination, inoculates the water column with vegetative cells. In the GoN, 59 dinoflagellate cyst morphotypes (less than 20% of the dinoflagellate species found in the plankton) were recorded in surface sediments and sediment traps (Montresor et al. 1998), with high production rates (up to 1.7×10^5 cysts m^{-2} day^{-1}) and rather similar species composition over two years investigated (Fig. 8a). The cyst assemblage was dominated by calcareous cysts of

Scrippsiella species and small organic-walled cysts produced by unarmoured dinoflagellates. The abundance of viable diatom resting stages in sediments from LTER-MC was instead estimated with the Most Probable Number method applied to Serial Dilution Cultures (MPN-SDC), because their small size prevents their direct observation in the sediments. On eight sampling dates spanning over a year-and-a-half, 30 diatom species (ca 1/3 of those recorded in the plankton) were obtained from sediment germination (Montresor et al. 2013), of which the most abundant were *Skeletonema pseudocostatum*, *Leptocylindrus danicus*, *Chaetoceros socialis* (Fig. 8b) and other Thalassiosirales. A pilot experiment comparing the results of the MPN-SDC and the HTS-metabarcoding approaches demonstrated a good match between the two methods (Piredda et al. 2017a).

Another crucial phase of protist life cycle is sexual reproduction which, besides its importance for genetic recombination (for the *Pseudo-nitzschia* genus, see D'Alelio and Ruggiero 2015 and Ruggiero et al. 2018), in diatoms produces large sized cells that counteract the progressive cell miniaturization that features their vegetative phase (Montresor et al. 2016). In the laboratory, basic information was obtained on sexual reproduction of selected *Pseudo-nitzschia* species from LTER-MC (Amato et al. 2005, Amato and Montresor 2008, D'Alelio et al. 2009b). These potentially toxic species have become models for several studies partly because they are heterothallic, i.e., sex can only occur when cells of opposite mating type are co-cultured, which has also allowed for testing the biological species concept and elucidating the actual diversity among cryptic or pseudo-cryptic species (Amato et al. 2007).

At LTER-MC, one of the few massive sex events ever recorded for diatoms in the natural environment has taken place, whereby two different species, *P.* cf. *delicatissima* and *P.* cf. *calliantha,* were found to undergo sex at the same time (Sarno et al. 2010). The ephemeral nature of these events may explain why such reports are so scanty, but cell-size variations and the presence of large-sized cells formed following sexual reproduction can provide an indirect way to infer the occurrence of diatom sex in the natural environment. Indeed, records of *P. multistriata* cell-size in plankton samples from LTER-MC over ten years, coupled with a modelling approach, showed that this species undergoes sex every two years (D'Alelio et al. 2010), which to date represents the only indication of an internal clock that rules the occurrence of sex and the shift of growth phase across the seasons in diatoms. More sophisticated tools are offered today by genes expressed during sexual reproduction, first identified in some diatom species from LTER-MC (Patil et al. 2015, Basu et al. 2017, Nanjappa et al. 2017), which can be searched in protist meta-transcriptomes, with a great potential to shed light on the actual occurrence of sexual events in the natural environment.

Life history traits in zooplankton at station LTER-MC have been analysed in conspicuous copepod species, such as *Acartia clausi*, *Centropages typicus* and *Temora stylifera*, with particular focus on reproduction and development. Results of experimental and *in situ* studies on egg production, hatching success, survival and temporal distribution of naupliar and copepodite stages showed remarkable differences among species that highlight the characteristic strategies of species co-occurring in temperate areas (e.g., Ianora and Buttino 1990, Ianora 1998, Di Capua and Mazzocchi 2004, Carotenuto et al. 2006). In *C. typicus* and *A. clausi*, the highest egg production rates were

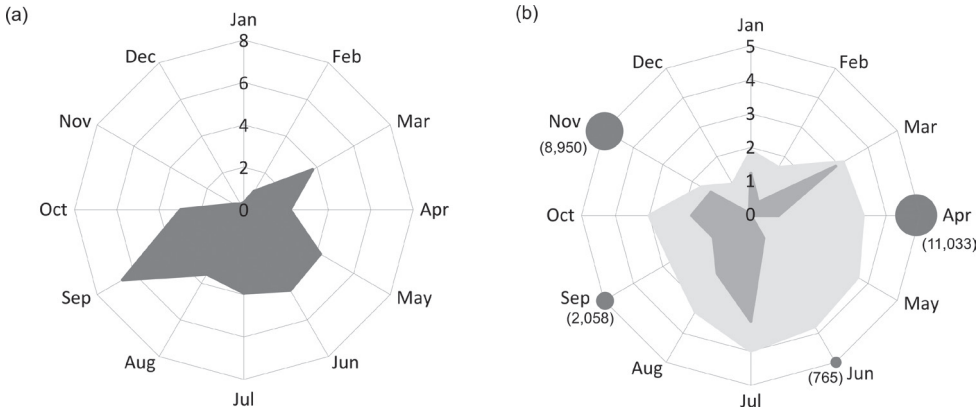

Figure 8. Phytoplankton resting stages at station LTER-MC. **a** Dinoflagellate cyst fluxes (cysts × 10^5 m^{-2} d^{-1}); average monthly values over two years (1994 and 1995) (data from Montresor et al. 1998) **b** abundance of spores of *Chaetoceros socialis* (circles, viable spores × 10^3 g^{-1} of wet sediment) in April, June, September and November, and average monthly cell abundance (log10(n+1) ml^{-1}) of spore-forming diatoms (light gray polygon) and of *C. socialis* in the plankton (dark gray polygon) (data from Montresor et al. 2013).

recorded at relatively low female abundance and *viceversa* suggesting that copepods invest resources towards reproduction only in specific periods of their annual cycle, independently from the number of females in the population (Ianora and Buttino 1990, Ianora et al. 1992). However, Ianora and Poulet (1993) provided the first evidence that egg hatching success is also linked to food quality, with egg production and viability benefiting from a dinoflagellate diet and being impaired by a diatom diet. These results set the ground for a series of studies showing that some diatom species produce secondary metabolites (PUA and various oxylipins) that affect hatching success and naupliar viability (Ianora and Miralto 2010). The crucial role of food quality but also of different mortality rates of the various developmental stages in shaping population dynamics was proven by a Lagrangian individual-based model run with data of *T. stylifera* populations at LTER-MC (Mazzocchi et al. 2006). Indeed mortality, by both natural causes and predation, is a fundamental process that affects zooplankton population dynamics. As an example, the average percentage of carcasses at LTER-MC has been estimated to be around 10% of the total copepod population, with juveniles always more vulnerable as compared to adults (Di Capua and Mazzocchi 2017).

Overall these studies, along with similar ones from other areas (e.g., Johnson et al. 2008, Renz and Hirche 2006), highlight the importance of knowing population demography for different species in order to better understand the variability in structure and temporal course of zooplankton at sea. Significant long-term trends of zooplankton populations have been observed around the world (Harris et al. 2014) and some evidence of declines is appearing also in the GoN (M.G. Mazzocchi, I. Di Capua and I. Carotenuto unpublished data), which should be elucidated by tracing the environmental changes that may have occurred in critical periods of the year, thus affecting specific life cycle phases of individual species. This is very relevant also for the possible repercussions on the trophic web structure and the productivity of the sea.

Biological interactions

In addition to endogenous rhythms dictated by life cycles and exogenous environmental forcing, the occurrence and seasonality of plankton species can be determined or modulated by positive or negative interactions with other co-occurring organisms. One such obvious case is represented by trophic relationships, which have received much attention since the first studies on plankton ecology and have started to be investigated at LTER-MC as well.

Plankton communities are complex ensembles of unicellular organisms with different metabolism types, from strictly photoautotrophic to phagotrophic and mixotrophic (i.e., microzooplankton or protozooplankton), which can shift between heterotrophy and autotrophy, and multicellular organisms with distinct diets, from predominant herbivory to omnivory or strict carnivory: plankton organisms therefore can display multiple trophic interactions, which result in complex food-webs (e.g., D'Alelio et al. 2016b). Grazing experiments in the GoN involving the lower levels of the plankton food-web, namely phyto- and microzooplankton, have revealed: i) a seasonality in the consumption of phytoplankton by microzooplankton, with higher rates during winter compared to spring and summer (> ~30%) (Modigh and Franzè 2009); and ii) a strong 'cannibalism' among heterotrophic protists, with up to 78% of microzooplankton production not exported to higher trophic levels but consumed within microbial trophic-loops (Franzè and Modigh 2013). Furthermore, grazing experiments with diatoms suggested that copepods from LTER-MC, despite size- selectivity, display a predominantly generalist and plastic trophic behavior (Mahadik 2014, Mahadik et al. 2017).

The multiple and flexible trophic interactions occurring within plankton have recently been explored taking advantage of the detailed information on species that occupy different trophic levels and using LTER-MC as a model system for developing conceptual and computational models of the plankton food web. A first model (D'Alelio et al. 2015) has targeted the whole plankton community during the summer season in the years 2002–2009, from nanoflagellates to predatory mesozooplankton (individual size spanning from 5 μm to 2×10^3 μm). Co-variations of species trends were used as evidence of the different trophic links, which were assembled into networks that were investigated in order to identify the response of the system as a whole to different trophic conditions (e.g., Loreau 2010). Besides a well-resolved reconstruction of the web of trophic interactions, it has been possible to test the response of this web to short-term physical-chemical variability typical of the coastal GoN (Ribera d'Alcalà et al. 2004, Cianelli et al. 2017). The trophic network (Fig. 9) was dominated by links connecting phytoplankton and mesozooplankton during coastal, eutrophic states and by microbial loops during offshore, oligotrophic states (D'Alelio et al. 2015). Other interesting characteristics of the GoN plankton food web were that: i) the resource intermittency may propagate from individuals to individuals' interactions; ii) the community may respond 'adaptively' to oscillations between eu- and oligotrophy; and iii) biological diversity seems to be the main driver of this latter system response.

Building on this conceptual model, a food-web computational model was subsequently developed and fed with data of carbon biomass fluxes derived from LTER-MC

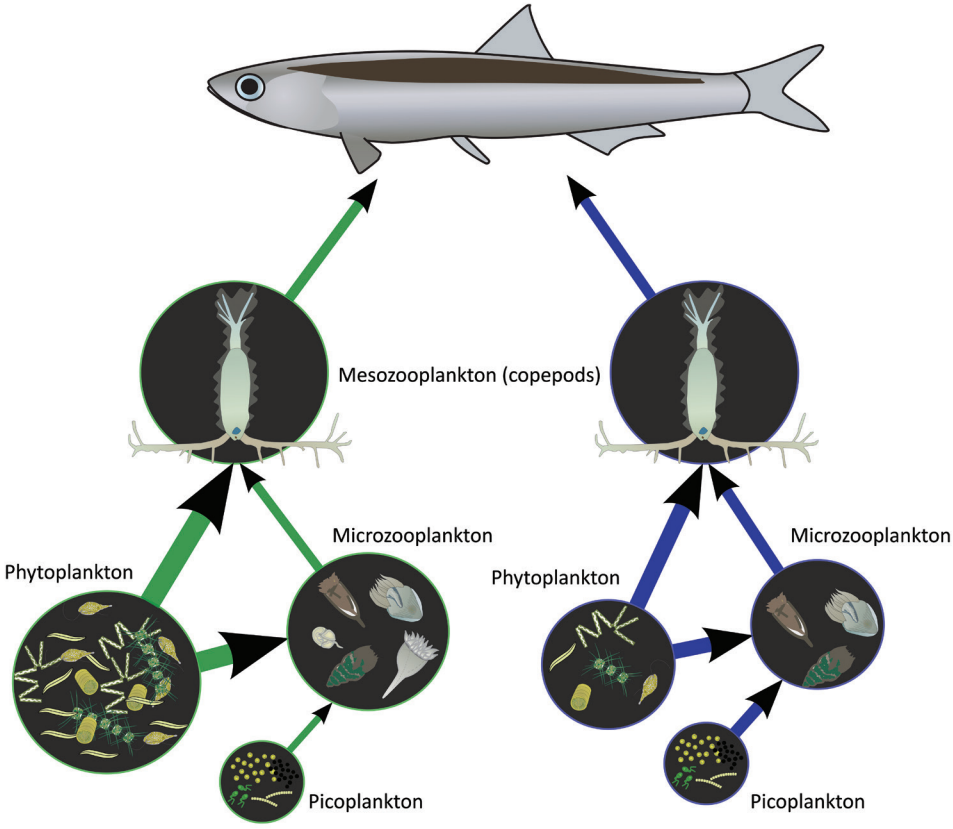

Figure 9. A simplified version of a food web in the Gulf of Naples in eutrophic (Green) and oligotrophic (Blue) summer conditions, modified from D'Alelio et al. (2016a). Briefly, in the Green system state, both copepods and microzooplankton exert a strong grazing pressure on phytoplankton, while in the Blue state, copepods increase their predation over microzooplankton, which in turn shifts its predation from phytoplankton to bacterial plankton or picoplankton. These trophic mechanisms stabilize the delivery of organic matter from copepods to fish.

studies, complemented with available knowledge concerning the biological characteristics of plankton organisms from the GoN (D'Alelio et al. 2016a, b). The model built by exploiting the Ecopath methodology allowed for reconciling, within a single network framework, three important ecosystem properties: how many species are there (community biodiversity), how these relate reciprocally (community structure) and what they do collectively (community functioning) (Thompson et al. 2012). The Ecopath model indicates that almost all consumers in the plankton food-web are capable of switching trophic preferences based on available resources, i.e., when the system shifts between eu- and oligotrophic states, and it also suggests the ecological importance of neglected biological processes, such as mixotrophy and differentially selective feeding by micro- and mesozooplankton. These characteristics expand the hyperspace of potential-

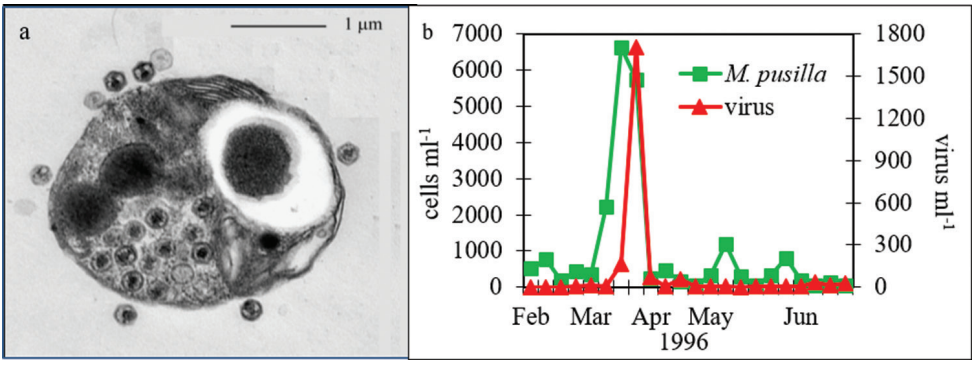

Figure 10. Viral infection in the prasinophyte *Micromonas pusilla*. **a** Transmission electron micrograph of an infected cell **b** abundance of the virus and its host at station LTER-MC in spring 1996 (data from Zingone et al. 1999b). In the following two years the seasonal trend of host and virus appeared to be less coupled, which pointed to complex relationships between different viral and host strains.

ly available trophic pathways, thus conferring the protozoan-metazoan links the highest trophic efficiency (i.e., the highest ratio between the biomass taken up by a trophic level and that delivered by the same trophic level to the subsequent one). This high efficiency allows dampening the negative effect of the decrease in primary production on planktivorous fish production at the oligotrophic system state. Specifically, when phytoplankton biomass was seven-fold lower than at the eutrophic state, calanoid copepods significantly increased their grazing pressure on protozooplankton, which, in turn, increased their pressure on picophytoplankton, less consumed by calanoids because of their small cell size. Instead the food-web was highly dissipative at the lowest trophic levels, i.e., at phytoplankton-protozooplankton trophic step: the matter and energy not delivered at the higher level of the web was apparently used by protozooplankton as a resource to maintain species survival and, thus, the diversity of unicellular heterotrophs.

Unlike trophic relationships, other forms of interactions among organisms in the plankton have received less attention until recently. For example, the impact of viruses on the dynamics of planktonic populations is still hardly known and few are the species for which viruses have been identified. One such case is *Micromonas pusilla* (Butcher) Manton et Parke, a small prasinophycean flagellate abundant at LTER-MC from autumn to early spring. Viruses specific for this species (Fig. 10a) were recorded all over the period in which the host was present at sea, along with a great intraspecific variability in host susceptibility to the viruses (Zingone et al. 1999b). In some cases, peaks of the host were accompanied by an increase in viral concentrations, showing that viruses were responsible for algal mortality (Fig. 10b). However, based on results of three consecutive years, there was no evidence that viruses were able to terminate the host bloom. In fact, viral strains isolated from the GoN showed differences in infection capability when tested on strains of *M. pusilla* from the same area and from different geographic sites (Zingone et al. 2006a). In addition, host cultures generally recovered after the infection and became resistant to infection from the same virus. These results highlight

the complex dynamics of algal-virus interactions, where diversity in viral infection capability, coupled with differences in intraspecific susceptibility of the host and differences in acquired resistance to the viruses, interplay to generate a sort of equilibrium.

Parasites also represent a poorly known loss factor in planktonic population dynamics. The limited information available at LTER-MC was obtained for the copepod *Paracalanus parvus* where females and juveniles were parasitized by dinoflagellates (Ianora et al. 1987, 1990). Infections by *Syndinium* were greatest in juveniles (up to 30%) than in adult females (8%) and completely absent in males. The identification of parasites in unicellular organisms is challenging, but HTS-based metabarcoding approaches are now available and showed that Syndiniales can be relatively abundant components of the protist community (Piredda et al. 2017b).

Conclusions and future perspectives

More than three decades of studies at station LTER-MC in the Gulf of Naples have definitely proved the relevance and potentiality of this research site as a precious asset not only to trace plankton changes at different scales and under different environmental conditions but also as a natural laboratory and a source of inspiration for complementary scientific research that has widened substantially our knowledge of the planktonic organisms and of the system as a whole.

As typical for coastal areas, the temporal course of the water column environment has shown to be remarkably variable throughout the years. Nevertheless, a notable resilience has characterized the plankton assemblages both in their bulk properties and at the level of individual species, with repeated seasonal patterns pointing to some biological and functional adaptability. These properties are also featured in the flexible organization of the food web under different hydrographic conditions, which points to a behavioral plasticity of individual species, as also disclosed by targeted experimental studies. Still the trends that have been recorded for temperature, chlorophyll and phytoplankton size, and the significant changes recorded in the abundance and phenology of some species need to be investigated in depth in their role of possible sentinel of environmental changes.

A fraction of the hidden marine diversity has been uncovered with the description of a high number of phytoplankton species and the elucidation of crypticity, which have greatly improved the capability to interpret seasonal and biogeographical patterns so far blurred by the misidentification of the significant taxonomic units. These results support the consideration that precision in taxonomic identification is a requisite of ecological studies, and in many cases higher taxa (e.g., genera or classes) include too much diversity to be ecologically meaningful. Further, placing specific, intraspecific and population variability in the frame of natural environmental variability has proven to be a good opportunity to shed light on both the ecological meaning and the evolutionary potential of diversity. High diversity in planktonic elements has also emerged from the analysis of small-scale behavior and life-history traits such as development, reproduction and dormancy, as well as natural and pathological mortality. Overall,

the results of these studies highlight the fundamental role of biological processes and individual performances in the coexistence and/or succession and phenological characterization of the species, beyond the influence of environmental conditions.

In spite of the quite wide-ranging results obtained so far, much remains to be done in terms of exploring and filling the knowledge gaps emerging from them and gaining further comprehension of the planktonic system. In addition to in-depth studies on the data set and complementary research in line with what has been done so far, the complexity of plankton diversity and dynamics prompts us to further extend and intensify our efforts using novel approaches. To this end, an augmented marine observatory is being established which couples traditional and -omics approaches applied at the fixed LTER-MC and periodically over a larger spatial grid. In addition, sustained recording by means of fixed mooring bearing optical and acoustic sensors and biomolecular samplers is planned to complement the traditional data gathering procedure. The augmented observatory is aimed at a complete characterization of plankton communities (meta-barcoding and meta-genomics) and of their functions (meta-transcriptomics) through the analysis of barcodes, complete gene sets and their expression patterns. This empowerment of the LTER-MC research activities will also allow investigating other planktonic taxa neglected so far (e.g., jellyfish and fish larvae) and including further trophic links into pre-existing ecological networks and will likely shed further light on the processes underlying the extraordinary plankton world.

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RESEARCH ARTICLE

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Comparison of biological and ecological long-term trends related to northern hemisphere climate in different marine ecosystems

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Abstract

Data from five sites of the International Long Term Ecological Research (ILTER) network in the North-Eastern Pacific, Western Arctic Ocean, Northern Baltic Sea, South-Eastern North Sea and in the Western Mediterranean Sea were analyzed by dynamic factor analysis (DFA) to trace common multi-year trends in abundance and composition of phytoplankton, benthic fauna and temperate reef fish. Multiannual trends were related to climate and environmental variables to study interactions. Two common trends in biological responses were detected, with temperature and climate indices as explanatory variables in four of the five LTER sites considered. Only one trend was observed at the fifth site, the Northern Baltic Sea, where

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no explanatory variables were identified. Our findings revealed quasi-synchronous biological shifts in the different marine ecosystems coincident with the 2000 climatic regime shift and provided evidence on a possible further biological shift around 2010. The observed biological modifications were coupled with abrupt or continuous increase in sea water and air temperature confirming the key-role of temperature in structuring marine communities.

Keywords

ILTER network, marine communities, multiannual trends, biological shifts, climate changes, climate indices, northern hemisphere

Introduction

Long-term ecological research aiming at understanding sources of natural variability in species composition, dominant species and functional diversity of marine communities is essential for disentangling the effects of natural environmental drivers and climate variation from the direct effects of local human activities (Giron-Nava et al. 2017; Mirtl et al. 2018). Many marine ecosystems around the world have undergone dramatic changes in species composition or a reduction in species diversity (Perry et al. 2005; Worm et al. 2006; Steffen et al. 2007; Suikkanen et al. 2007, 2013; Rockström et al. 2009; Kröncke et al. 2011) due to human activities and climate-driven ocean warming in the northern Pacific and Atlantic Oceans during recent decades (Deser et al. 2000; IPCC 2014).

An analysis of changes across different biological components and geographic regions in relation to climate indices has the potential to provide a novel insight into the role of climate in influencing trends in marine biodiversity (Dornelas et al. 2014; Bowler et al. 2017a, b). Changes and shifts in biodiversity of single or different biological components can be part of biological regime shifts (BRS). BRS are defined mainly on the basis of changes in the ecosystem as a whole (Scheffer et al. 2001; Collie et al. 2004; Conversi et al. 2015) and are typically characterized by infrequent and abrupt changes in ecosystem structure and function, occurring at multiple trophic levels and on large geographic scales (Collie et al. 2004; Cury and Shannon 2004; deYoung et al. 2008; Bakun 2005; Lees et al. 2006). Marine biological regime shifts have been explained mainly as a result of multiple causes, e.g. climate regime shifts (CRS), overexploitation of resources or a combination of climate and volcanic eruptions (Scheffer and Carpenter 2003; Fisher et al. 2015; Gardmark et al. 2015; Levin and Möllmann 2015; Reid et al. 2016). Of particular note was the 1988 BRS, which was detected in marine ecosystems globally (e.g., Reid et al. 2016; Conversi et al. 2010), while the 2000 CRS/BRS has been subject to only a few long-term studies in specific marine systems (Kröncke et al. 2013a; Beaugrand et al. 2014; Dippner and Kröncke 2015; Perretti et al. 2017). All these studies have shown that signals of climate variability can be detected by an increase in warm-temperate species, a decrease in cold-temperate species at various trophic levels of the marine ecosystem, and subsequently in changes in species composition and species richness.

The trends in biodiversity described above were strongly correlated with increasing sea surface temperatures (SST) and regional climate variability (Dornelas et al. 2014). These variables have been proven useful for characterizing basin-scale variation in the world's oceans and offer great potential for investigating inter-annual and inter-decadal climate oscillations, biological and ecological regime shifts, and climate teleconnections. Various climatic indices have been developed for these purposes and their modes typically involve different mechanisms over different regions. For example, the North Atlantic Oscillation (NAO) (Hurrell 1995) and the North Pacific Index (NPI) (Trenberth and Hurrell 1994) are the leading climate modes of sea level pressure variability over the northern Atlantic and Pacific Oceans, respectively. These indices along with the Pacific Decadal Oscillation (PDO) (Mantua 1999) and the El Niño/Southern Oscillation (ENSO) (Philander 1990) are the four dominant modes of climate variability in the Northern Hemisphere (Tsonis et al. 2007). NAO has been widely used to characterize the role of climate variability in the responses of terrestrial (Mysterud et al. 2003), freshwater (Straile et al. 2003) and marine ecosystems (Drinkwater et al. 2003; Dippner 2006). Hameed and Piontkovski (2004) developed an indicator alternative approach to the NAO that uses the pressures at the atmospheric centers of action (Azores High and Icelandic Low, IL) as independent variables and showed that the standardized time series of the pressure, zonal and meridional position of the IL is a good predictor of various ecosystem attributes such as the north wall position of the Gulf Stream and the zooplankton abundance in the Gulf of Maine. In the southern North Sea, the NAO was a good predictor of the structure of macrofauna communities in the following spring (Kröncke et al. 1998), which allowed the development of forecast equations (Dippner and Kröncke 2003). However, the NAO lost its persistency around the year 2000, when a climate regime shift (CRS) occurred (Dippner et al. 2014). Thus, the North Sea Environmental index (NSE), a multivariate predictor constructed from a mixture of global and regional climatic time series, was developed to better predict inter-annual and inter-decadal climate variability in the North Sea (Dippner and Kröncke 2015). Similar climate predictors have been developed for other regions, such as the Arctic Oscillation (AO) (Thompson and Wallace 1998), the Pacific North American pattern (PNA) (Barnston and Livezey 1987), the Indian Ocean Dipole (IOD) (Saji et al. 1999, Webster et al. 1999), and the Western Mediterranean Oscillation (WeMO) (Martin-Vide and Lopez-Bustins 2006).

Combining different climate modes with vertical propagating Rossby waves to and from the stratosphere (Ineson and Scaife 2009) results in a coupled "super-loop" NAO → PDO → ENSO → PNA → stratosphere → NAO, which contains the major signals of low-frequency variability in Northern Hemisphere (Wang et al. 2009). The analyses of climate modes are used for the identification of climate phenomena such as teleconnections and regime shifts. For example, high spectral phase coherence between two climate modes indicates the existence of a teleconnection. Such teleconnections have been identified between the NAO and ENSO (Huang et al. 1998), ENSO and IOD (Saji and Yamagata 2003; Tozuka et al. 2007), and ENSO and AO (Jevrejeva et al. 2003).

CRS are often considered as changes in the trend of global mean air temperature. Yasunaka and Hanawa (2002) used empirical orthogonal functions' analysis to evaluate the SST and sea level pressure (SLP) fields in the northern hemisphere and identified different CRS in the 20th century. Swanson and Tsonis (2009) proposed the following mechanism for CRS. When different modes of climate variability are synchronized (defined as the root-mean-square correlation coefficient between all pair of climate modes) and the coupling strength (defined as the phase of an individual climate mode relative to the phases of other modes) between the modes increase simultaneously, the climate system becomes unstable and moves into a new state (Tsonis et al. 2007; Swanson and Tsonis 2009; Wang et al. 2009). Such synchronization of CRS occurred four times during the 20th century (1910–1920, 1938–1945, 1956–1960, and 1976–1981) and an increase in coupling strength occurred three times (1910–1920, 1938–1945, and 1976–1981). The consequence was a change in the trend of global mean air temperature (Swanson and Tsonis 2009). The last identified synchronization and increase in coupling strength causing a climate shift was identified in 2001/2002 (Swanson and Tsonis 2009). These identified periods were related to the turning points of the Atlantic Multidecadal Oscillation (AMO; Sutton and Hodson 2005). The AMO has a periodicity of ~60–80 years (Schlesinger and Ramankutty 1994). Thus, a relatively cold period at the beginning of the Twentieth Century was followed by a warm period in the 1940s and 1950s, another cold period in the 1970s and 1980s, and a warm period in the 1990s (Ting et al. 2013).

Comparative ecosystem analysis has been effectively used to improve our understanding of the processes controlling the biodiversity, productivity, and resilience of marine ecosystems (Murawski et al. 2009), and develops general principles of biological shifts (Conversi et al. 2015; Beaugrand et al. 2015). In this paper, we analyzed long-term trends in biological and climate data from five sites belonging to the European (LTER-Europe) and International (ILTER) Long Term Ecological Research networks (www.ilter.network), located in different marine basins along a latitudinal gradient in the northern hemisphere (i.e., the Northeastern Pacific, Western Arctic Ocean, Northern Baltic Sea, Southern North Sea and Western Mediterranean Sea; Fig. 1). The main objectives of the study were to investigate: (1) whether common trends in abundance or biomass across different biological groups or ecological parameters of pelagic and benthic components of marine communities occurred in the last two decades, and (2) whether biological shifts corresponded to shifts in ocean or air temperature and regional climate variability in the different marine regions. We also sought (3) to determine, whether any such changes could be explained by species-specific temperature affinities during cold and warm phases.

Material and methods

The International Long-term Ecological Research Network (ILTER), founded in 1993, comprises 44 active member LTER networks representing 700 LTER Sites and ~80 LTSER Platforms across all continents, active in the fields of ecosystem, criti-

cal zone and socio-ecological research (Mirtl et al. 2018). ILTER maintains global environmental observation and ecological research networks and infrastructures for excellent science. The role of ILTER is to investigate ecosystem structure, function, and services in response to a wide range of environmental strengths using long-term, place-based research.

Long-term ecological research sites

The **Santa Barbara Coastal LTER** (https://deims.org/dbd399ed-9c26-4621-b479-7ab505c8aa35) in southern California (USA, Fig. 1) was established in 2000 to investigate the roles of a wide range of marine and terrestrial processes in structuring kelp forest ecosystems in the face of changing climate and human use. The study site extends 120 km and is bounded by the continental coastline and an archipelago of four islands located 30 – 40 km offshore. Its east-west orientation contributes to complex circulation patterns that are influenced by the intersection of the cold southward-flowing California Current and the warm, northward-flowing southern California Counter Current (Hendershott and Winant 1996; Harms and Winant 1998). This circulation and resulting strong temperature gradients create a major biogeographic boundary at the western border of the Channel for a wide variety of marine taxa (Valentine 1966; Briggs 1974), and make the Santa Barbara Channel an ideal location to examine shifts in species composition driven by climate variability. Details in sampling and methods are given by Reed et al. (2016).

The arctic **LTER Observatory HAUSGARTEN** (https://deims.org/f6d9ed12-6bc1-47fb-8e81-ef24e9579596) in the Fram Strait between NE Greenland and the Svalbard archipelago (Fig. 1) was established in 1999 to detect and track the impact of large-scale environmental changes on the marine ecosystem in the transition zone between the northern North Atlantic and the central Arctic Ocean (Soltwedel et al. 2005, 2016). Today, HAUSGARTEN observatory constitutes a network of 21 permanent sampling sites, the majority of which are located along a bathymetric transect between ~250 and ~5,500 m water depth at about 79N from the Kongsfjorden (Svalbard) in the east, along the Vestnesa Ridge towards the Molloy Hole (i.e., one of the deepest known depressions in the Arctic Ocean), and across the Greenland continental margin. Three sampling sites close to the ice edge between 79°30'N and 80°00'N in the north-eastern Fram Strait and a supplementary site in a permanently ice-free area at 78°30'N in the eastern part of the strait complete the network. The Fram Strait is the only deep-water connection between the Nordic Seas and the central Arctic Ocean, with a sill depth of about 2600 m. The hydrography in the eastern part of the strait is characterized by the inflow of relatively warm and nutrient-rich Atlantic Water into the central Arctic Ocean (Beszczynska-Möller et al. 2012). Cooler and less-saline Polar Water exits the central Arctic Ocean as the Eastern Greenland Current in the western part of the Fram Strait (de Steur et al. 2009). Hydrographic patterns in the strait result in a variable sea-ice cover, with predominantly ice-covered areas in the west, permanently ice-free areas in

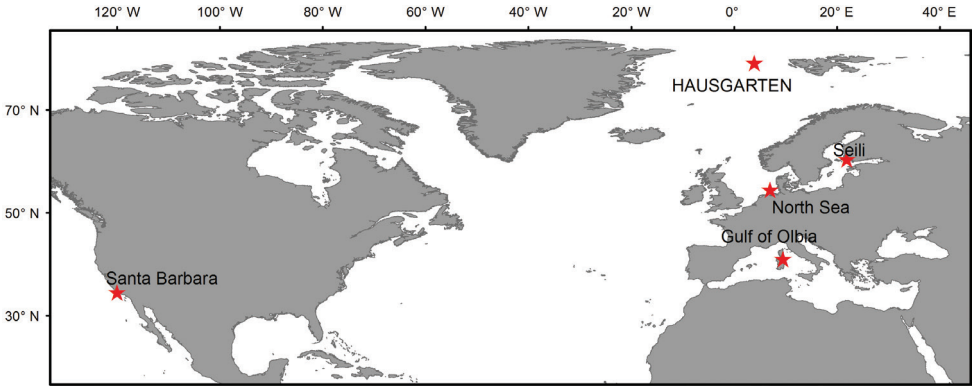

Figure 1. Locations of the LTER study sites.

the southeast, and seasonally varying ice conditions in the central and north-eastern parts. Details in sampling and methods are given by Soltwedel et al. (2005, 2016).

The **Baltic Sea LTER site Seili** (https://deims.org/9d4222a2-c50f-4fac-8b1d-3b685072b34d) is located in the Archipelago Sea in Finland (60°15'33"N; 21°57'39"E) (Fig. 1), which is part of the northern Baltic Sea, and hence a brackish water marine area. According to the Water Framework Directive of the European Union, this coastal area belongs to the Southwestern middle archipelago of the Finnish coastal waters. The area is characterized by a large number of skerries and islands; the median depth of this part of the Archipelago Sea is 40 m. The depth of the sampling site is 50.6 m. The salinity ranges between 5.1 and 7.0 (median value 5.9). The sampling area is described in detail by Kauppila (2007 and references therein). Samples for phytoplankton have been taken since 1991. Details in sampling and methods are given by HELCOM (2017).

The **LTER North Sea Benthos site** (https://deims.org/50946250-c0fa-41b0-a917-17d2a3992eee) constitutes a network of seven sampling areas in the North Sea. Box A, the area used for this study, is situated in the German Bight about 25 nautical miles northwest of the Island of Helgoland, in close proximity to the 30 m depth contour near the former glacial valley of the River Elbe (Fig. 1). The mean depth of this area is 40 m and the water column is generally well mixed due to tidal mixing throughout the year. The freshwater run-off from continental rivers results in low salinities and regionally high nutrient input in the area, which can cause enhanced primary production and high food supply for benthic fauna (Kröncke et al. 2004). The area is also characterized by strong seasonal variability in temperature and salinity as well as by high bottom stress due to strong tidal currents (Neumann et al. 2017). The sediments consist of more than 20% (<63 µm fraction) mud in the south-west corner gradually decreasing towards the north-east corner (0–5%). The time series started in 1998 and has since been sampled annually with the exception of 2013, when it was not sampled. Details in sampling and methods are given by Neumann et al. (2008).

The **Mediterranean Sea LTER site Gulf of Olbia** (IT14-002-M) (https://deims.org/3178d0fb-0789-4992-9c51-1ddb50b7e871) has belonged to the LTER-Italy network since 2006. It is situated on the eastern coast of Sardinia (Italy, Fig. 1). Morphologically, it is a ria, 5 km long, 2 km wide, with a total area of 6.5 km^2, a mean depth of 5 m, and a maximum depth of 10 m. The Gulf receives an inflow from the Padrongianus River in its southern part. The town of Olbia, located in the inner part of the Gulf, represents one of the most important passenger harbors in the Mediterranean in addition to a commercial and an industrial harbor (www.olbiagolfoaranci.it). It is also the largest mussel- and clam-farming area in Sardinia (Sannio et al. 1996; Bazzoni et al. 2015) and one of the most important in Italy. Since the early 1990s, the Gulf has been monitored at three stations for phytoplankton abundance and composition as well as environmental variables. In particular, the Gulf of Olbia has been monitored with respect to its trophic state, phytoplankton abundance and composition, focusing on the presence of harmful algae, since the early 1990s (Sannio et al. 1996, 1997; Lugliè et al. 2003a, b; Garcés et al. 2007; Satta et al. 2010; Bazzoni et al. 2015; Pulina et al. 2016). The first data were collected in 1987 in connection with an event of water discoloration due to a phytoplankton bloom (Sechi et al. 1987).

The collection of LTER data started in 1992 for phytoplankton. Details in sampling and methods are given by Pulina et al. (2016).

Sampling, data sets and data analysis

Table 1 provides an overview on the LTER study sites, including the sampling period, and the climate and biological data sets considered in the dynamic factor analysis (DFA).

Dynamic Factor Analysis (DFA)

DFA is a smoothing and dimension reduction technique to identify common trends in multivariate time-series and to determine the effect of explanatory variables. The "common trends" represent the underlying dynamic pattern over time in the considered system (Zuur et al. 2003a). Unlike other dimension-reduction techniques such as principal component analysis (PCA) or multidimensional scaling (MDS), DFA is especially designed for sequential time-series data. Another advantage of this method is that it can deal with missing data in time series, as they also occur in our time series (e.g. Olbia, Hausgarten, North Sea).

The time series were modeled as a function of a linear combination of common trends, a constant level parameter, two or more explanatory variables, and noise (Zuur et al. 2003a, b). A detailed mathematical description of the DFA algorithm is given in Zuur et al. (2003a) and Zuur et al. (2007).

Table 1. Location, depth, sampling periods and designs, climate and biological data sets used for the DFA at the five LTER sites.

	Santa Barbara Coastal LTER	LTER Observatory HAUSGARTEN	Baltic Sea LTER Seili	LTER North Sea Benthos Observatory	Western Mediterranean Sea LTER Gulf of Olbia
Location	USA, Santa Barbara Channel	Germany, Arctic Ocean Fram Strait	Finland, Archipelago Sea	Germany, North Sea, German Bight	Italy, Tyrrhenian Sea (Sardinia), Gulf of Olbia
Geographical coordinates	~34°25`N, ~119°57`W	~79°N, ~4°20`E	~60°15`N, ~21°58`E	~54°22`N, ~7°10`E	~40°55`N, ~9°33`E
Sampling depth	7-11 m	250-5500 m	Surface to twice the Secchi depth	36-43 m	Surface water layer (-30 cm)
Sampling period	July to August	Autonomously year round sampling; field study June	July to September	July to August	July to August
Sampling design	7 transects	Satellite remote sensing, annual sampling in summer months with water samplers, plankton nets, multicorers	1-2 times per month sampling using a water sampler	Nine random replicates per year with a 2 m beam trawl	Fortnightly or monthly samplings at one station collecting water samples with a Niskin bottle
Climate data	PDO	IL	NAO	NSE	WeMO
	Summer bottom temperature	Water temperature (0–100 m)	SST	SST	Air temperature
Period	2002-2016	2000-2016	1991-2014	1998–2017	1996-2014
Biological data	Abundance of 12 fish species	Chlorophyll *a*, phytoplankton composition (6 groups), ash-free dry weight, biogenic sediment compounds, bacterial numbers and biomasses, meiofauna abundance	Phytoplankton biomass (9 groups)	Abundance of 16 epifauna species	Phytoplankton cell density as percentage composition on the base of contribution of 9 major groups

DFA was used to analyze time series of phytoplankton (Baltic Sea, Western Mediterranean, Arctic Ocean), benthic fauna (North Sea, Arctic Ocean), benthic bacteria (Arctic Ocean) and temperate reef fish (Santa Barbara Channel). Water or air temperature and various climate indices (PDO, IL, NAO, NSE, WeMO) were used as explanatory variables. Several DFA models were tested for each time-series, ranging from the simplest (one common trend plus noise) to the most complex (two common trends, up to three explanatory variables plus noise). Models were fitted with both a diagonal covariance matrix and a symmetric non-diagonal covariance matrix and compared using the Akaike's information criterion (AIC) (Akaike 1974), which depends on the maximum likelihood estimates and is a measure of goodness-of-fit. Depending on the number of parameters, the model with the lowest AIC value was selected as the most appropriate one. Factor loadings were used to assess the relationship between response variables and the common trends (cut-off point of 0.2). The diagonal elements of er-

ror covariance matrix obtained by the model were used as a measure of "misfits" of the fitted model. Large diagonal elements mean that the corresponding time series are not fitted well (Zuur and Pierce 2004). Significant ($p < 0.05$) relationships between the response and explanatory variables were assessed by using the estimated regression coefficients and their associated t-value, whereby t-values > 2 (in absolute sense) indicate strong relationships. All time series data (response variables) were square root transformed and both response and explanatory variables were standardized by subtracting their time-averaged mean and dividing by their temporal standard derivation (normalization). Computations were carried out using Brodgar computer software, developed by Highland Statistics LTD., UK (www.brodgar.com).

Results

The transformed and standardized time-series of the different biological components of marine communities and environmental parameters are presented in Fig. 2. The time series showed considerable variability with no obvious trends. To estimate the underlying common trends of the various biological components, different sets of DFA models, depending on the length of the time-series in relation to different climatic indices, were used for each of the LTER study sites (Fig. 3). The AIC values (Table 2) indicate the best model fit in each study case.

LTER Santa Barbara Channel: Sixteen dynamic factor models were calculated to estimate the underlying common trend of the fish time-series differing in the covariance matrix employed (diagonal versus non-diagonal), the number of trends (one or two) and the included explanatory variables (mean summer bottom temperature, PDO index or both). The AIC values indicated that the best model fit was obtained for a non-diagonal matrix with two common trends explained by bottom temperature and the PDO index (AIC = 415) (Table 2).

Both trends showed overall increases from 2001–2017 (Fig. 3). The first common trend was characterized by a sharp increase from 2001 to 2008 and a decrease until 2012 followed by a leveling out and slight increase. The second common trend showed a modest decrease from 2001 to 2006, followed by an increase afterwards (Fig. 3). Factor loadings showed that the first trend was related to the blue rockfish *Sebastes mystinus* (0.30). The second trend was correlated with the señorita *Oxyjulis californica* (0.41), the blacksmith *Chromis punctipinnis* (0.32), the pile surfperch *Phanerodon furcatus* (0.2) and the blackeye goby *Rhinogobiops nicholsii* (-0.23). Relatively low diagonal elements of the error covariance matrix (< 0.50) were obtained for all of these species except the pile surfperch (0.58) highlighting a good model fit of the individual series for these species (Table 3).

The estimated t-values for the explanatory variables bottom temperature and PDO are given in Table 4. Bottom temperature had a significant influence on the painted greenling *Oxylebius pictus* (-3.36), the señorita *Oxyjulis californica* (-2.48), and the kelp bass *Paralabrax claturatus* (2.44). Furthermore, the abundances of the blacksmith

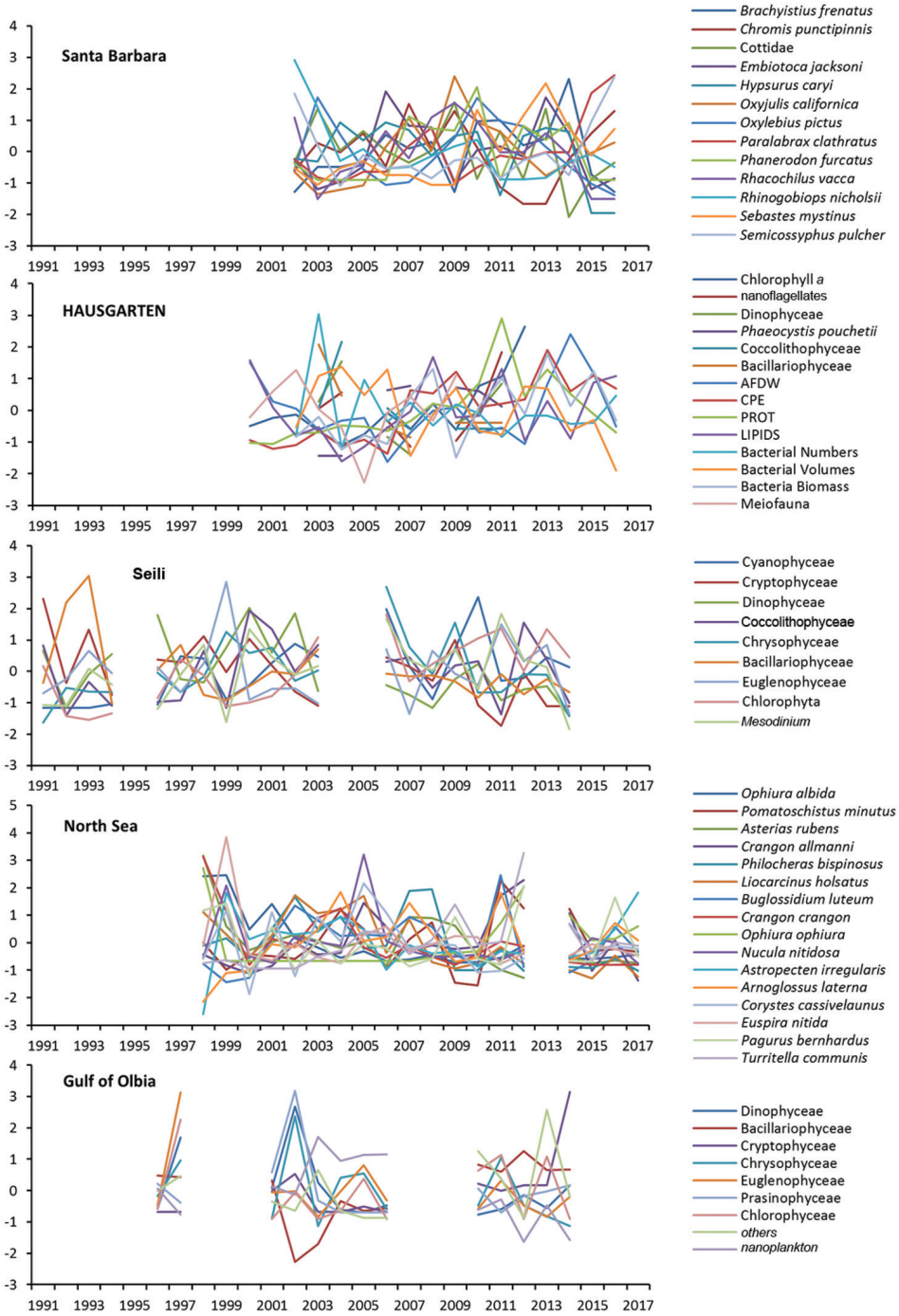

Figure 2. Transformed and standardized time-series at the various LTER sites. AFDW = organic matter in the sediments as ash-free dry weight, CPE = sediment-bound chloroplastic pigments, PROT = particulate proteins in the sediments.

Table 2. Model selection based on values of Akaike's information criterion (AIC). The optimal Dynamic Factor Analysis model with one or two trends is given in bold. TEMP = summer bottom temperature, PDO = Pacific Decadal Oscillation index, SST = sea surface temperature, PRES = pressure, LAT = latitude, LONG = longitude, NAO = North Atlantic Oscillation index, NSE = North Sea Environmental index, AirTemp = air temperature, WeMO = Western Mediterranean Oscillation index.

	Diagonal		Non diagonal	
Number of trends	**1**	**2**	**1**	**2**
SANTA BARBARA				
M common trend + noise	530	518	477	480
M common trend + noise + TEMP	525	515	457	462
M common trend + noise + PDO	525	521	436	444
M common trend + noise + TEMP + PDO	517	506	416	**415**
HAUSGARTEN				
M common trend + noise	500	489	x	x
M common trend + noise + SST	506	479	x	x
M common trend + noise + PRES	510	493	x	x
M common trend + noise + LAT	515	501	x	x
M common trend + noise + LONG	518	475	x	x
M common trend + noise + SST + PRES	503	478	x	x
M common trend + noise + SST + LAT	520	485	x	x
M common trend + noise + SST + LONG	515	467	x	x
M common trend + noise + PRES + LAT	518	468	x	x
M common trend + noise + PRES + LONG	473	480	x	x
M common trend + noise + LAT + LONG	521	460	x	x
M common trend + noise + SST + PRES + LAT	499	**424**	x	x
M common trend + noise + SST + PRES + LONG	491	473	x	x
M common trend + noise + SST + LAT + LONG	508	428	x	x
M common trend + noise + PRES + LAT + LONG	478	439	x	x
M common trend + noise + PRES + LAT + LONG + SST	506	479	x	x
SEILI				
M common trend + noise	**549**	554	569	582
M common trend + noise + SST	555	557	574	583
M common trend + noise + NAO	552	555	569	582
M common trend + noise + SST + NAO	559	560	572	581
NORTH SEA				
M common trend + noise	874	855	790	784
M common trend + noise + SST	871	843	768	761
M common trend + noise + NSE	883	857	728	721
M common trend + noise + SST + NSE	876	845	626	**620**
GULF OF OLBIA				
M common trend + noise	379	369	317	329
M common trend + noise + AirTemp	381	374	314	325
M common trend + noise + WeMO	387	376	313	313
M common trend + noise + AirTemp + WeMO	394	379	309	**308**

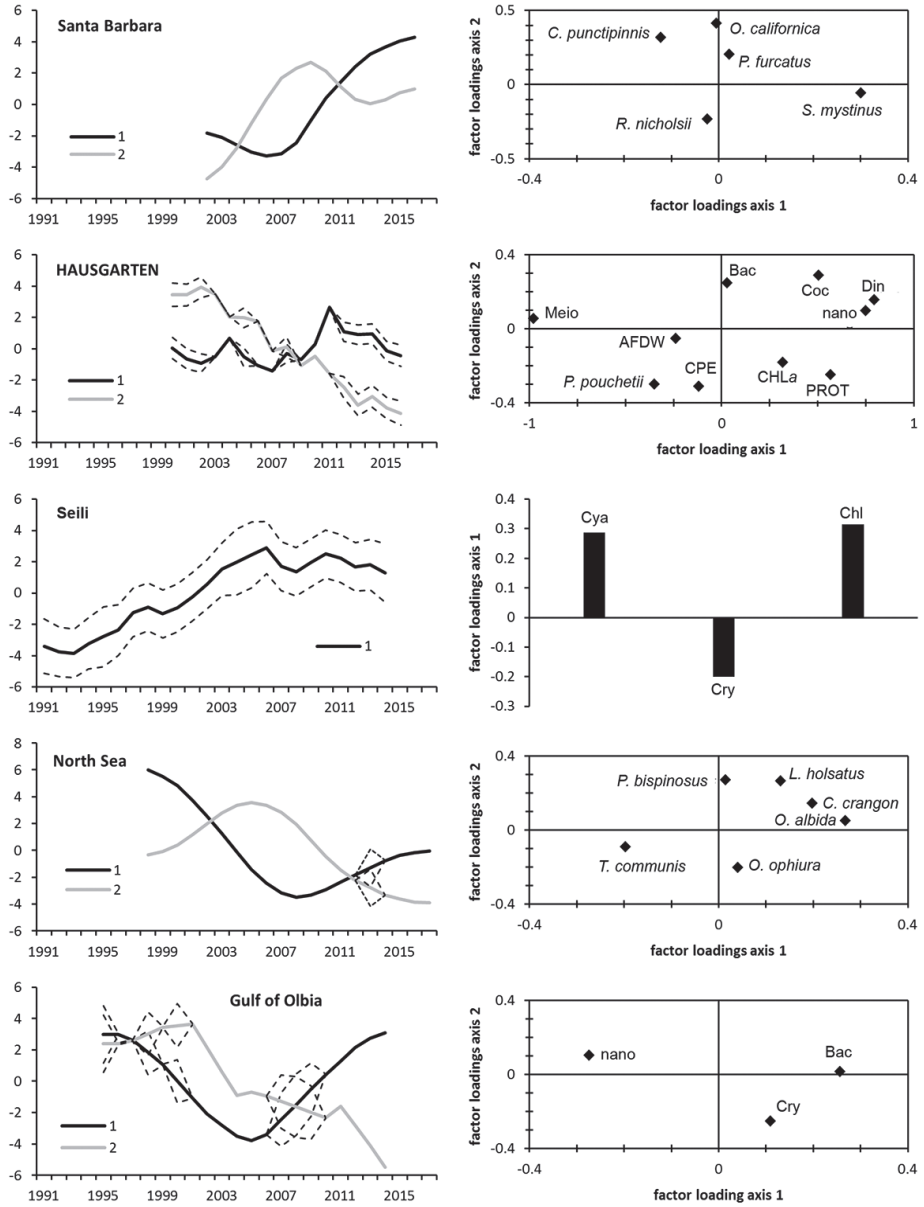

Figure 3. Common trends (left) and corresponding factor loadings (right) for the five LTER sites obtained by means of DFA. Only factor loadings above the cut-off of 0.2 in absolute value are shown. Common trends and factor loadings are untitled. Dashed line in graphs indicates the confidence interval of the DFA model. Bac = Bacillariophyceae, Chl = Chlorophyceae/Chlorophyta, Coc = Coccolithophyceae, Cry = Cryptophyceae, Cya = Cyanophyceae, Din = Dinophyceae, nano = nanoplankton/nanoflagellates, Ppou = *Phaecystis pouchetii*, CHL*a* = chlorophyll *a*, AFDW = organic matter in the sediments as ash-free dry weight, CPE = sediment-bound chloroplastic pigments, PROT = particulate proteins in the sediments, Meio = meiofauna.

Table 3. Measures of fit (diagonal elements of error covariance matrix) for the different time-series. Relatively low diagonal elements of the error covariance matrix (< 0.50) indicate good fit. AFDW = organic matter in the sediments as ash-free dry weight, CPE = sediment-bound chloroplastic pigments, PROT = particulate proteins in the sediments.

SANTA BARBARA		HAUSGARTEN		SEILI		NORTH SEA		GULF OF OLBIA	
B. frenatus	0.68	Chlorophyll *a*	0.47	Cyanophyceae	0.51	*O. albida*	0.18	Dinophyceae	0.64
C. punctipinnis	0.18	nanoflagellates	0.18	Cryptophyceae	0.76	*P. minutus*	0.77	Bacillariophyceae	0.34
E. jacksoni	0.58	Dinophyceae	0.27	Dinophyceae	0.85	*A. rubens*	0.65	Cryptophyceae	0.49
H. caryi	0.64	*P. pouchetii*	0.20	Coccolithophyceae	0.89	*C. allmanni*	0.40	Chrysophyceae	0.41
O. californica	0.23	Coccolithophyceae	0.20	Chrysophyceae	0.83	*P. bispinosus*	0.49	Euglenophyceae	0.68
O. pictus	0.42	Bacillariophyceae	0.14	Bacillariophyceae	0.80	*L. holsatus*	0.32	Prasinophyceae	0.76
P. clathratus	0.35	AFDW	0.49	Euglenophyceae	0.95	*B. luteum*	0.69	Chlorophyceae	0.52
P. furcatus	0.58	CPE	0.12	Chlorophyta	0.41	*C. crangon*	0.43	others	0.63
R. vacca	0.42	PROT	0.13	*Mesodinium*	0.79	*O. ophiura*	0.51	nanoplankton	0.25
R. nicholsii	0.43	LIPIDS	0.65			*A. irregularis*	0.82		
S. mystinus	0.29	Bact_Numb	0.67			*A. laterna*	0.61		
S. pulcher	0.57	Bact_Vol	0.58			*C. cassivelaunus*	0.78		
		Bact_Biom	0.38			*E. nitida*	0.65		
		Meio	0.35			*P. bernhardus*	0.62		
						T. communis	0.50		

Chromis punctipinnis (6.14), black surfperch *Embiotica jacksoni* (-2.90) and the kelp perch *Brachyistius frenatus* (-2.27) were significantly related to the PDO.

LTER Observatory HAUSGARTEN: Thirty-two dynamic factor models, using SST as well as the intensity and positions of the IL as explanatory variables, revealed two trends in the planktonic community and underlying sediments. The AIC values (Table 2) indicated that the best model fit was obtained for a diagonal matrix with two common trends and bottom temperature, air pressure and latitude as explanatory variables (AIC = 424). The first common trend showed two major peaks in 2004/2005 and 2011/2012. The second common trend showed an overall decrease between the years 2000 and 2016 (Fig. 3). Factor loadings showed that the first trend was related to chlorophyll *a* (0.32), nanoflagellates (0.75), Dinophyceae (0.79), *Phaeocystis pouchetii* (-0.35), Coccolithophyceae (0.50), organic matter in the sediments (AFDW; 0.24), particulate proteins in the sediments (PROT; 0.57) and meiofauna (-0.98). The second trend was related with Bacillariophyceae (0.25) and the sediment-bound chloroplastic pigments (CPE; -0.31). Among the considered biological groups, the best model fits were found for the proportion of Bacillariophyceae, nanoflagellates, Coccolithophyceae and *Phaeocystis pouchetii* (Table 3).

t-values indicated a significant influence of SST on the proportion of Bacillariophyceae (-4.64) and *Phaeocystis pouchetii* (3.65) (Table 4). The air pressure anomaly of the IL strongly covaried with the proportion of Bacillariophyceae (4.08), Coccolithophyceae (-3.51), and protein concentrations (4.24) in the sediments (Table 4). The proportions of Coccolithophyceae (4.79) and Bacillariophyceae (-4.47) also showed a strong relationship to the latitudinal position of the IL (Table 4).

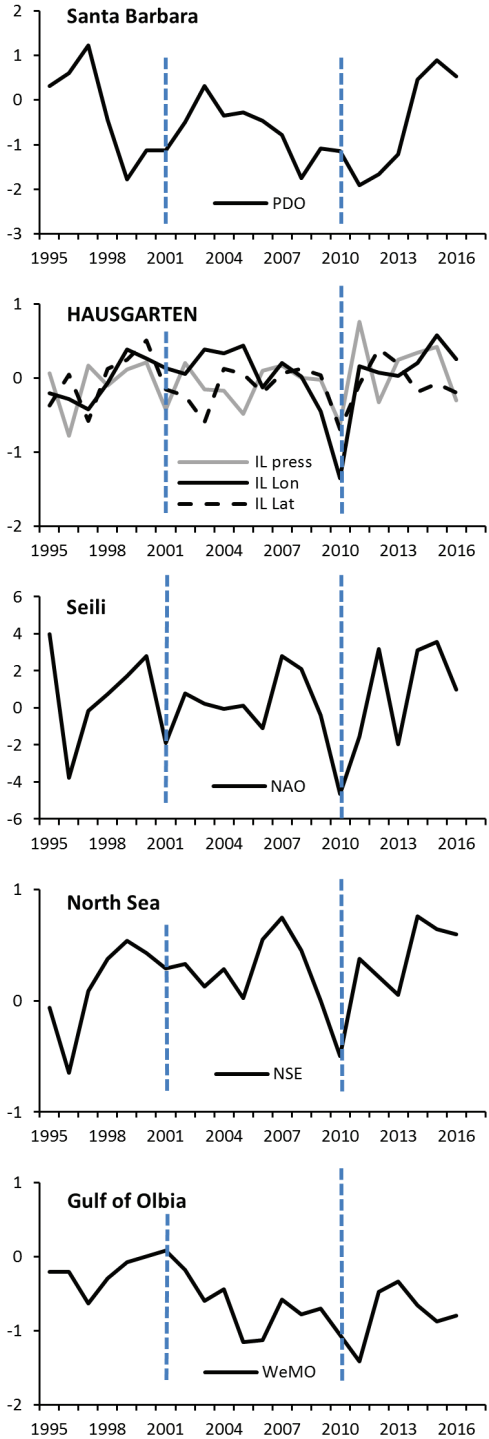

Figure 4. Multiannual yearly mean variability of the different climate indices (PDO, IL, NAO, NSE, WeMO) considered in the study. The blue dotted lines indicated the Climate Regime Shifts (CRS).

Table 4. Estimated t-values for the explanatory variables. Only values above 0.2 are shown. TEMP = summer bottom temperature, PDO = Pacific Decadal Oscillation index, SST = sea surface temperature, PRES = pressure, LAT = latitude, NSE = North Sea Environmental index, AirTemp = air temperature, WeMO = Western Mediterranean Oscillation index, PROT = particulate proteins in the sediments.

		t- values	
SANTA BARBARA		**TEMP**	**PDO**
O. californica		-2.48	
O. pictus		-3.36	
P. clathratus		2.44	
B. frenatus			-2.27
C. punctipinnis			6.14
E. jacksoni			-2.90
HAUSGARTEN	**PRES**	**LAT**	**SST**
Bacillariophyceae	4.08	-4.47	-4.64
Coccolithophyceae	-3.51	4.79	
PROT	4.24		
P. pouchetii			3.65
NORTH SEA		**SST**	**NSE**
C. allmanni		-2.37	-3.22
E. nitida		-2.48	
P. bernhardus		-2.71	
C. crangon			-2.03
GULF OF OLBIA		**AirTemp**	**WeMO**
Chrysophyceae		-3.45	
Dinophyceae			2.38
Chlorophyceae			-2.87

Meiofauna density at HAUSGARTEN observatory was the only faunal parameter included in the DFA. Unfortunately, meiofauna data had to be restricted to results provided by Hoste et al. (2007) and Grzelak (2015) for the central HAUSGARTEN site (2500 m water depth), covering the years 2000 till 2009. Results from the DFA revealed no strong relationships with the explanatory variables chosen for the analysis; however, the model exhibited a fairly good fit with the observed values (Table 4).

LTER Baltic Sea Seili: Sixteen dynamic factor models were calculated to estimate the underlying common trend of the phytoplankton time-series differing in the covariance matrix employed (diagonal vs. non-diagonal), the number of trends (one or two) and the included explanatory variables, i.e. SST and NAO (Table 2). The AIC values indicated that the best model fit was obtained for a diagonal matrix with one common trend and no explanatory variables (AIC = 549). The common trend showed an increase from 1991 to 2005, and leveling out afterwards (Fig. 3). Based on the factor loadings, the trend was positively related to biomasses of Chlorophyta (0.32) and Cyanophyceae (0.29) and negatively to Cryptophyceae (-0.20). Observed and fitted series for the best correlated taxa indicated that the first two groups were fitted reasonably well, with measures of 0.41 and 0.51, respectively (Table 3). Because the best model

fit did not contain any explanatory variables, relations of phytoplankton time series to explanatory variables could not be examined.

LTER North Sea Benthos Observatory: Sixteen dynamic factor models were calculated to estimate the underlying common trend of the epibenthic time-series differing in the used covariance matrix (diagonal and non-diagonal), and the number of trends (one or two) and the included explanatory variable (SST or NSE or both). The AIC values indicated that the best model fit was obtained for a non-diagonal matrix with two common trends and SST and NSE as explanatory variables (AIC = 620) (Table 2).

The first common trend showed a sharp decrease from 1998 to 2007, followed by an increase afterwards (Fig. 3). The second common trend was characterized by an increase from 1998 to 2005 and a steady decrease from 2005 onwards. Factor loadings showed that the first trend was positively related to the brittle star *Ophiura albida* (0.27) and the brown shrimp *Crangon crangon* (0.20), and negatively related to the Auger shell *Turritella communis* (-0.20). The second trend was positively correlated with the shrimp *Philocheras bispinosus bispinosus* (0.27) and the swimming crab *Liocarcinus holsatus* (0.27) and negatively with the brittle star *Ophiura ophiura* (-0.20). Observed and fitted series for the best correlated species (Table 3) indicated a good model fit for the above species. In contrast, the individual model fit was low for the sea star *Astropecten irregularis* (0.82), the masked crab *Corystes cassivelaunus* (0.78) and the goby *Pomatoschistus minutus* (0.77).

The estimated t-values for the explanatory variables SST and NSE are given in Table 4. SST covaried significantly with hermit crab *Pagurus bernhardus* (-2.71), the common necklace shell *Euspira nitida* (-2.48) and the shrimp *Crangon allmanni* (-2.37). Furthermore, the NSE was significantly related to the shrimp *C. allmanni* (-3.22) and *Crangon crangon* (-2.03). The shrimp *C. allmanni* was related to both explanatory variables.

Mediterranean Sea LTER Gulf of Olbia: The best fit of the sixteen model outputs for phytoplankton was one with a non-diagonal error covariance matrix, containing two common trends (Fig. 3), and with air temperature and WeMO as explanatory variables (the lowest AIC = 308) (Table 2).

The first common trend showed higher and similar values at the beginning (1996–1997) and at the end of the considered time series (2013–2015), with a central part of lower values (1998–2012, minimum value in 2005) (Fig. 3). The second common trend was slightly increasing at the beginning (1995–2001), with a subsequent prominent decrease till the end of the study period, except for a small rise in 2011.

According to the largest factor loadings (Fig. 3), percentage contribution of Bacillariophyceae to total phytoplankton cell density followed strongly the first common trend (0.26) in a positive way. On the contrary, percentage contribution of nanoplankton to total phytoplankton cell density resulted in a negative factor loading (-0.27). The second common trend strongly correlated with the percentage contribution of Cryptophyceae to total phytoplankton cell density, with a negative sign (-0.25). Best fits were found for the time-series of nanoplankton (0.25), Bacillariophyceae (0.34), Chrysophyceae (0.41) and Cryptophyceae (0.49) (Table 3).

Considering the estimated t-values (Table 4), air temperature had a significant influence on percentage contribution to total phytoplankton cell density of Chrysophyceae (t value = -3.45), whereas WeMO influenced the percentage contribution of Chlorophyceae (t value = -2.87) and Dinophyceae (2.38).

Trends in ocean temperature and climate modes

Data from the five LTER sites considered in this study revealed an overall increase in temperature (SST, bottom and air temperature; data not shown). SST increased 0.8–3 °C at the northeastern Pacific, North Sea and Baltic Sea sites between 1995 and 2016. Air temperature at the LTER site in the Western Mediterranean Sea increased by 0.8–1.1 °C, while at the Arctic LTER site HAUSGARTEN SST increased by 0.06 °C y^{-1} for the time-period 1997–2010 (Beszczynska-Möller et al. 2012), but also revealed distinct interannual variability (Walczowski et al. 2017). The time-series of summer observations exhibited 5–6 year cycles with distinct warm-water anomalies (WWA). Recent WWAs in Fram Strait occurred between 2005 and 2007, in the years 2011/2012, and towards the year 2016.

Figure 4 shows the long-term variability of climate indices. Temperature increases in the HAUSGARTEN, in the North Sea and the Baltic Sea were related to increasing IL/NAO/NSE indices. The increase in air temperature at the LTER site in the Western Mediterranean Sea coincided with extreme negative WeMO values. There was a strong shift at the Santa Barbara Channel LTER site in the north-eastern Pacific from decreasing bottom temperature and negative PDO indices towards increasing SST of about 3 °C and positive PDO indices around 2014. The 2000 CRS is obvious as well, a similar pattern in all indices around 2010, which might be a hint of another CRS, as yet undetected.

Discussion

The comparison of climatic and long-term ecological data at the five LTER sites located in the north-eastern Pacific, the western Arctic Ocean, the northern Baltic Sea, the southern North Sea and the western Mediterranean Sea revealed a great similarity in common trends in marine systems in the northern hemisphere.

Common multiannual trends and biological shifts

At four of the five LTER sites considered in this study, the DFA revealed the presence of two common trends with temperature and climate indices as explanatory variables. Despite the different biological components and marine ecosystems analyzed, multiannual common trends within a site were quasi-synchronous, but usually in opposite directions. Common trends first crossed near the end of 1990s/early 2000s and then again around

2010, which coincided with the 2000 CRS and probably a new, yet to be described CRS around 2010. We found one inversion of tendencies of biological components at the Arctic LTER HAUSGARTEN and two inversions at sites in the north-eastern Pacific Ocean (Santa Barbara), the Western Mediterranean Sea (Gulf of Olbia) and the North Sea. A similar signal was also observed at the Baltic Sea site (Seili), where the single common trend reached its maximum in 2005, for which no explanatory variable was identified, similar to other long-term plankton studies (e.g. in the North Sea, van Beusekom et al. 2009). Three LTER data sets (Western Mediterranean Sea, North Sea, Baltic Sea) analyzed in this study started before 2000. The observed changes in the common trends and composition of the marine communities at these sites coincided with the 2000 CRS (Tsonis et al. 2007; Swanson and Tsonis 2009; Wang et al. 2009) and could provide further evidence for the presence of BRS, as already reported for other marine systems worldwide (Bond et al. 2003; Peterson and Schwing 2003; Collos et al. 2009; Pulina et al. 2011; Kröncke et al. 2013a; Beaugrand et al. 2014; Dippner and Kröncke 2015; Reid et al. 2016; Perretti et al. 2017). Beaugrand et al. (2015) reported quasi-synchronous regime shifts in the late 1980s and the mid- to late 1970s related to temperature and the Arctic circulation in multiple marine systems from two oceans and three regional seas in the Northern Hemisphere. Because changes monitored in the north-eastern Pacific Ocean since 2002 are similar to those from three other LTER sites considered in our study, they can probably also be related to the 2000 CRS. Turning points of the common trends at four of the sites in the mid to late 2000s might be explained as biological shifts between two CRS. Four of the five LTER sites revealed also biological shifts around 2010, which coincided with shifts in the climate indices and might be evidence for a new CRS.

The congruent trends in climate indices and CRS at the five LTER sites suggest that decadal fluctuations in atmospheric and ocean circulation are teleconnected between the Atlantic and Pacific Ocean regions as also found for previously reported CRS (Schwing et al. 2003). The congruent trends of climate indices and of common trends in biological variability provide evidence that a major part of inter-annual and inter-decadal biological variability can be attributed to physical forcing.

Biological responses to CRS and the role of temperature

We found consistent biological modifications to abrupt or continuous increases in sea water and air temperature and associated climatic indices at all five of the LTER sites analyzed in this study.

Temperature modulates a multitude of processes at the cellular, organismic and ecosystem levels of organization, and it can act as a mediator between organisms and climate (Kröncke et al. 2013a; Dippner and Kröncke 2015). Recent changes observed in the northern Atlantic communities caused by the 2000 CRS were similar to those caused by the CRSs in the 1920s and 1930s (Drinkwater 2006; Deser 2000). Ecosystem changes associated with warmer than normal SST and positive values of the NAO in the 1920s and 1930s included a general northward movement of cold-temperate species of

fish and benthic invertebrates. These changes reversed during the CRS of 1938–1945 (Tsonis et al. 2007; Swanson and Tsonis 2009; Wang et al. 2009), when temperature dropped again. Additional support for the important role of temperature in structuring marine communities comes from other studies that examined the relationship between population trends and species latitudinal distributions, which may, to some extent, act as a proxy for temperature niche of fish (Holbrook et al. 1997; Brooks et al. 2002; Heath 2005; Reed et al. 2016) and marine invertebrates (Neumann and Kröncke 2011; Birchenough et al. 2015; Hiddink et al. 2015; Gardmark et al. 2015: Bowler et al. 2017a, b).

In our study, at the south-eastern North Sea LTER site, the first occurrence of the epifaunal angular crab *Goneplax rhomboides* coincided with the 2000 CSR. The angular crab extended its distribution range from the north-eastern Atlantic to the North Sea, which was facilitated by an increase in water temperature (Neumann et al. 2013). The link between temperature and the occurrence of the angular crab is most likely given by effects on the survival of its larvae as it was the case for the Pacific Oyster *Crassostrea gigas*, when rising temperatures facilitated the successful survival of larvae and promoted the colonization of the entire German Wadden Sea (Brandt et al. 2008). Further, a shift in the functional composition of epifauna occurred in 2002, largely coinciding with the 2000 CSR (Neumann and Kröncke 2011). These results suggest that climate-induced variability of SST primarily affects the reproduction of epifaunal species rather than other functional traits. Drivers of this functional shift were the shrimps *Crangon* and *C. allmanni* as well as the hermit crab *Pagurus bernhardus*, whose abundances were strongly related to SST and NSE in this study. This corresponds to the results of Henderson et al. (2006), who also found that the recruitment of *C. crangon* is correlated to SST and the winter NAO index. This could be explained by the positive link between temperature and the duration of the larval stage as well as the frequency of breeding of shrimp species (Bergström 2000; Wear 1974). In contrast, Temming and Damm (2002) found higher recruitment of *C. crangon* after cold winters and postulated that predators get over-saturated in years with a pronounced peak of recruits and consequently more recruits would survive. Direct effects of temperature on key stages of reproduction are also known for *P. bernhardus* where timing and frequency of breeding is favored by cold water temperatures (Lancaster 1990). Unfortunately, the study by Neumann and Kröncke (2011) ended in 2008, but if the results of our analyses are generalizable, then we would expect that the 2010 CRS had similar effects on the functional composition of epifauna in the southern North Sea since these three species were positively related to the DFA trends. The epibenthic community of the North Sea LTER site was also severely affected by the cold winter of 1995/1996, resulting in the outbreak of the opportunistic brittlestar *Ophiura albida*. This outbreak was followed by characteristic post-disturbance succession stages from 1998 to 2000 (Neumann et al. 2008, 2009), which is clearly reflected in trend 1 of the DFA analysis. The period from 2000 onwards coincided with the 2000 CRS and was characterized by a continuous decrease of *O. albida* and by an increase in diversity and secondary production due to an increase in SST.

The biomass and diversity of reef fish at the Santa Barbara Coastal LTER site also shifted towards warm-temperate species with warm water affinities (Reed et al. 2016).

The DFA results for fish from this site demonstrated that species composition responded strongly to temperature and climate mode, the PDO. Changes in fish communities were driven by common species that tend to have warm-water or cold-water affinities such as the blue rockfish *Sebastes mystinus,* the señorita *Oxyjulis californica*, the blacksmith *Chromis punctipinnis* and the blackeye goby *Rhinogobiops nicholsii* that, interestingly, are all partially or wholly planktivorous and depend on zooplankton including copepods and salps for their diet (Love and Ebeling 1978). This trophic dependency may make these species more responsive to climatic forcing.

It is widely recognized that temperature has also a strong influence on the physiology of planktonic organisms because it controls basic metabolic processes (Vidussi et al. 2011). The role of temperature in structuring phytoplankton is still strongly debated, in part due to the covariation of temperature, physical structure of water masses, nutrients and grazing pressure in marine ecosystems (Gardner et al. 2011; Boyce et al. 2015; Marañón et al. 2015; Sommer et al. 2017). Instead, there is growing evidence that the decline in cell size together with the shift of species' ranges toward higher latitudes and changes in phenology, could be considered as one of the major universal biotic responses to global warming, even if with no contradictory evidence (Daufresne et al. 2009; Sommer et al. 2017). Cascading consequences of these changes on marine pelagic ecosystem functioning are still to be evaluated and should be considered in the context of a revised paradigm of pelagic ecosystem structure and function (Verity et al. 2002). Previous studies determined a shift from larger to smaller species at Western Mediterranean, Baltic and Artic LTER sites around the mid-2000s (Suikkanen et al. 2013; Nöthig et al. 2015; Pulina et al. 2016). The results obtained in the present study showed that these common trends in phytoplankton composition found at the above mentioned sites could be connected to climate variability and especially to the temperature variation. For example, at the LTER Arctic HAUSGARTEN observatory, the WWA during the years 2005–2007 caused a major shift in the composition of unicellular plankton organisms with the affirmation of the coccolithophores *Phaeocystis pouchetii*, a significantly increased proportion of nanoflagellates between 2009 and 2011 and a reduction of diatoms (Nöthig et al. 2015). A similar change was observed at the LTER Mediterranean site, but slightly earlier during 2002–2004, in correspondence with the strongest variation of temperature. An intense decrement in summer phytoplankton abundance was observed due to an abrupt Bacillariophyceae decrease and an anomalous increase of Prasinophyceae and Chrysophyceae in 2002. There was also the beginning of a more lasting predominance of nanoplankton replacing larger species of Bacillariophyceae, Dinophyceae and Euglenophyceae (Pulina et al. 2016). The LTER Baltic site experienced a significant increase in Cyanobacteria biomass and, similarly to the Arctic site, in small Coccolithophyceae of the genus *Chrysochromulina* coupled to the increase in SST (Suikkanen et al. 2013). In the present study, results from the DFA confirmed these trends on the considered longer dataset, especially for the northernmost sites. Instead, at the Mediterranean site, further relevant modifications were detected in the phytoplankton composition from 2011, when further strong temperature instability occurred, bringing to a turnaround for Bacillariophyceae and to an increase for Cryptophyceae.

Quantitative and qualitative changes in phytoplankton have unavoidable consequences at the other trophic levels. Several studies of benthic communities revealed that their diversity and function depended on the amount and quality of organic matter produced in the water column, even at extreme depths (Rowe and Pariente 1992; Clare et al. 2017). The DFA's results of the Arctic HAUSGARTEN time-series (2000–2016) revealed strong relationships between the phytoplankton composition and concentrations of biogenic sediment compounds, indicating organic matter in surface sediments with the explanatory variables chosen for our analysis. Higher amounts of sediment-bound chloroplastic pigments seemed to provide additional food supply for meiofauna and megafauna, which resulted in increases in abundance with a time lag of 1–2 years in larger organism size classes, as also found in other deep-sea areas (Sibuet et al. 1989; Rowe and Pariente 1992; Kröncke et al. 2013b).

Conclusions

Time series data collected at the five widely distributed marine ILTER sites in the Northern Hemisphere revealed relatively synchronous changes in the abundance and species composition of marine biota across the study regions. The community changes coincided with the 2000 CRS and provide the first data on a possible additional CRS in 2010, highlighting the existence of teleconnections among the climate modes in the Northern Hemisphere. Although recent climate models predict further increases in global temperature, future CRS might cause unexpected changes in marine communities. The significant relationship described in this study between climate modes and marine communities mediated by sea water or air temperature in the Northern Hemisphere can aid in predicting future changes in these marine systems in response to climate variability and ocean warming. Such improvements in ecological forecasting should prove useful to environmental managers responsible for implementing measures aimed at mitigating adverse ecological effects of future changes in climate. Our findings highlight the importance of spatially distributed quantitative Long-Term Ecological Research in developing a predictive understanding of ecological responses to climate change in the world's oceans and they underpin the continued need for long-term research within the global and the national LTER networks, as already suggested in similar studies (Pugnetti et al. 2013; Mirtl et al. 2018; Morabito et al. 2018). Last but not least, ILTER facilitate collaborations among scientific groups with different scientific topics and this study, catching the opportunity of the Special Issue proposed by the LTER-Italy network, demonstrates it.

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RESEARCH ARTICLE

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Phytoplankton temporal dynamics in the coastal waters of the north-eastern Adriatic Sea (Mediterranean Sea) from 2010 to 2017

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Abstract

Phytoplankton community structure was analysed from 2010 to 2017 at C1-LTER, the coastal Long-Term Ecological Research station located in the Gulf of Trieste, which is the northernmost part of the Mediterranean Sea. Phytoplankton abundance and relevant oceanographic parameters were measured monthly in order to describe the seasonal cycle and interannual variability of the main phytoplankton taxa (diatoms, dinoflagellates, coccolithophores and flagellates) and to analyse their relationship with environmental conditions. Overall, phytoplankton abundances showed a marked seasonal cycle characterised by a bloom in spring, with the peak in May. During the summer, phytoplankton abundances gradually decreased until September, then slightly increased again in October and reached their minima in winter. In general, the phytoplankton community was dominated by flagellates (generally <10 µm) and diatoms co-occurring in the spring bloom. In this period, diatoms were also represented by nano-sized species, gradually replaced by larger species in summer and autumn. Phytoplankton assemblages differed significantly between seasons (*Pseudo-F* = 9.59; $p < 0.01$) and temperature and salinity were the best predictor variables explaining the distribution of the multivariate data cloud. At the interannual scale, a strong decrease of the late-winter bloom was observed in recent years with the spring bloom being the main phytoplankton increase of the year.

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Keywords
Phytoplankton diversity, interannual variability, seasonality, Long-Term Ecological Research, Adriatic Sea, nutrients

Introduction

Marine ecosystems are experiencing many different changes in response to natural processes, human activities and climate change. These changes are rapidly altering nearly every chemical, physical and biological property affecting the growth of marine microorganisms (Hutchins and Fu 2017). Phytoplankton are a key component of marine ecosystem dynamics, contributing about half the global net primary production (Field et al. 1998). They represent the base of the food web and play a pivotal role in global nutrient cycles and particle export to the bottom. As primary producers, they are directly dependent on abiotic variables and are very sensitive to environmental changes of which they are actually important indicators (Hays et al. 2005). Therefore, tracking changes in the phytoplankton community structure can help to forecast ecosystem changes and plan sustainable management of the seas and oceans. For this reason, for instance, phytoplankton community diversity and temporal modification have been included as useful indicators in the Marine Strategy Framework Directive (MSFD) to determine the achievement of Good Environmental Status for the protection and conservation of the marine ecosystem. However, to disentangle climate and anthropogenic pressures from natural variability, many years of observations are needed (Henson et al. 2010) and, with this in mind, long-term data offer a useful instrument for achieving this aim and improving decision-making in ocean and coastal management (Edwards et al. 2010). Several marine research institutes maintain time series of physical, chemical and biological parameters for over a decade and are referred to as Long-Term Ecological Research sites (LTER). In Italy, the Italian Long-Term Ecological Research Network (LTER-Italy) includes terrestrial, freshwater and marine sites where observations are carried out at multidecadal scale. This study is based on data collected at the station C1-LTER that, since 2006, has been formally included in the LTER-Italy as part of the northern Adriatic LTER site.

The analyses of time series available for the northern Adriatic LTER site have highlighted that the northern Adriatic has experienced significant modifications of environmental conditions and trophic structure (Giani et al. 2012). The authors suggested that these changes were probably indicative of gradual eutrophication that occurred in the period from 1970 to mid-1980, followed by an oligotrophication process in the period 2000–2007, and drew attention to the possible effects on fish productivity. Phytoplankton interannual and inter-decadal variability, as well as temporal trends, have been reported for the northern Adriatic (Ninčević Gladan et al. 2010; Mozetić et al. 2010; Bernardi Aubry et al. 2012; Cabrini et al. 2012; Marić et al. 2012; Mozetić et al. 2012). An analysis encompassing 38 years (1970–2007) showed a strong decrease of chlorophyll *a* concentration in the whole northern basin probably due to a reduction of freshwater discharges and phosphorus used in agriculture (Mozetić et al. 2010). A recent analysis

carried out in the Mediterranean Sea, encompassing more recent years (1998–2014) and based on satellite-derived chlorophyll concentration, found a positive trend of chlorophyll concentration in the northern Adriatic Sea (Salgado-Hernanz et al. 2019). Different regime shifts have also been reported for phytoplankton abundances, community composition and seasonal cycle (Cabrini et al. 2012; Marić et al. 2012; Mozetić et al. 2012). At the C1-LTER station in the Gulf of Trieste, Cabrini et al. (2012) analysed a long-term dataset (1986–2010) and reported a regime shift of phytoplankton abundances in 1994, but no relationship with environmental forcing was presented.

The aim of this study is to analyse the temporal dynamics of the phytoplankton community at a seasonal and interannual scale during the past eight years (2010–2017) in the coastal waters and infer the possible environmental drivers shaping the variability of phytoplankton assemblages.

Methods

Study area

The Gulf of Trieste is a semi-enclosed basin located in the north-western part of the Adriatic Sea, characterised by shallow depths (maximum 25 m) and a strong influence of freshwater inputs. Two main rivers, the Isonzo and Timavo Rivers, enter the gulf along the shallower north-west coastline, whereas several submarine freshwater springs flow along the eastern karstic coast. The Isonzo River is the major source of freshwater and nutrients in the gulf and deeply modulates the hydrology, biogeochemistry and productivity of this coastal area (Cozzi et al. 2012). The oceanographic properties of the gulf are highly variable due to a marked seasonal cycle of seawater temperature (from winter minima of 5 °C to summer maxima > 26 °C) and strong salinity gradients (25–38) (Malačić and Petelin 2001). The overall circulation is mostly cyclonic and mainly influenced by the Eastern Adriatic Current, flowing northwards along the eastern coast and advecting warmer, saltier and more oligotrophic waters coming from the Ionian Sea (Poulain and Cushman-Roisin 2001), and by winds typical of the area, the cold Bora wind from the east-north and mild Scirocco wind from the south (Querin et al. 2007).

Sampling and environmental parameters

Data considered in this paper were collected at the C1-LTER station (45°42'2.99"N and 13°42'36.00"E, bottom depth: 17 m) located in the Gulf of Trieste. C1-LTER, 270 m far from the coast, north of the town of Trieste (Fig. 1), is part of the LTER network (http://www.lteritalia.it/) since 2006 and is sampled monthly since 1986 (http://nettuno.ogs.trieste.it/ilter/BIO/history.html). Data presented here cover a period of eight years for a total of 384 samples collected monthly from January 2010 to December 2017 at four depths (0.5, 5, 10 and 15 m) by 5 L Niskin bottles.

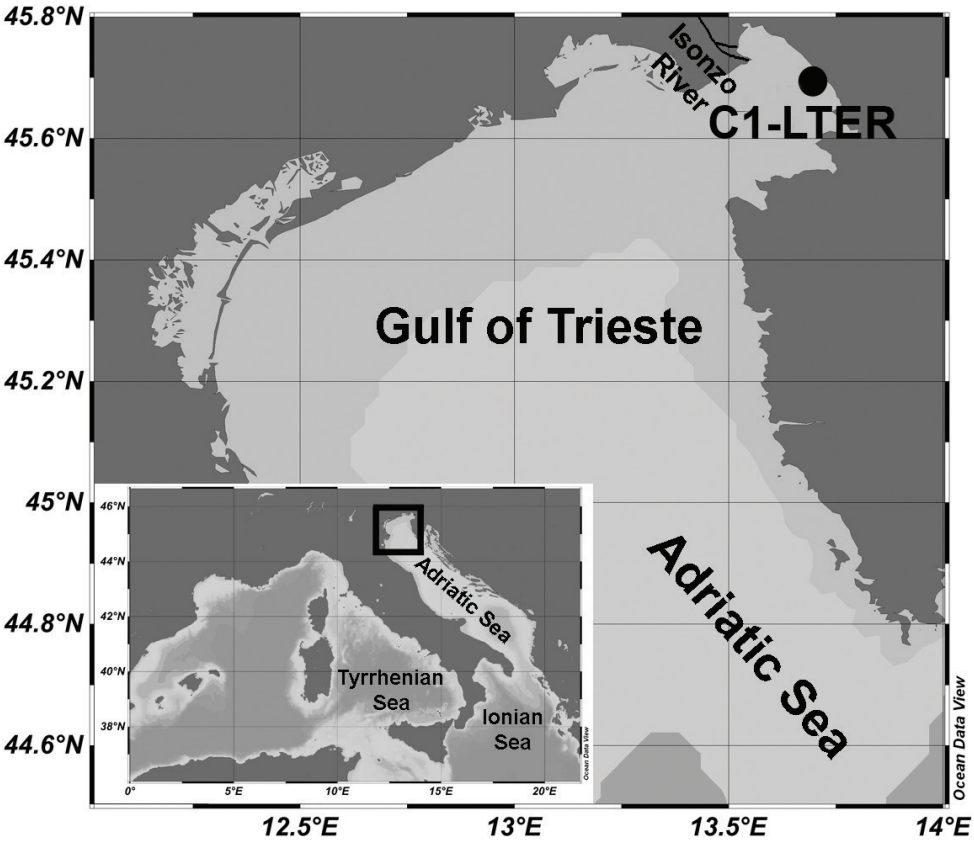

Figure 1. Map of the study area in the northern Adriatic Sea (Mediterranean Sea) showing the location of the sampling station (C1-LTER).

CTD profiles of temperature and salinity were obtained with an Idronaut Ocean Seven (models 401 and 316) or SBE 19plus SEACAT multiparametric probe, calibrated every 6/12 months.

Total precipitations data, provided by ARPA FVG – OSMER e GRN, Trieste (http://www.meteo.fvg.it/), were registered at the station situated at Molo Fratelli Bandiera (45°38'59.99"N, 13°45'8.07"E).

The Isonzo River discharge data, provided by Regione Autonoma Friuli Venezia Giulia, were calculated by a rating curve from the hydrometric level registered at Turriaco station (13 km from the Isonzo River mouth).

Samples for the determination of dissolved inorganic nutrient (nitrite, $N\text{-}NO_2$, nitrate, $N\text{-}NO_3$, ammonia, $N\text{-}NH_4$, phosphate, $P\text{-}PO_4$, and silicate, $Si\text{-}Si(OH)_4$) concentrations were pre-filtered through precombusted size glass-fibre filters (Whatmann GF/F), stored at -20 °C and then analysed colorimetrically with a Bran + Luebbe Autoanalyzer 3, up to December 2013, and afterwards with a QuAAtro (Seal Analytical), according to Hansen and Koroleff (1999). The concentration of dissolved inorganic

nitrogen (DIN) was calculated as the sum of the concentrations of nitrite, nitrate and ammonia. The detection limits for DIN, phosphates and silicates were 0.02, 0.01 and 0.01 μmol L^{-1}, respectively.

Temperature and salinity profiles and nutrient concentrations were plotted using Ocean Data View ver. 4.7.10 (Schlitzer 2015).

Phytoplankton abundance

For phytoplankton analysis, samples were fixed with pre-filtered and neutralised formaldehyde (1.6% final concentration) (Throndsen 1978). A variable volume of seawater (10–50 mL) was settled in an Utermöhl chamber depending on cell abundance (Utermöhl 1958; Zingone et al. 2010). Cell counts were performed using an inverted light microscope (LM) (Olympus IX71 and LEICA BMI3000B) equipped with phase contrast. Cells (minimum 200) were counted along transects (1–2) at a magnification of 400×. Additionally, half of the sedimentation chamber was also examined at a magnification of 200× for a more precise identification of less abundant microphytoplankton (>20 μm) taxa. Phytoplankton specimens were identified to the lowest possible taxonomic level and species/genus names were checked for validity against AlgaeBase (Guiry and Guiry 2018), and relevant recent publications. Heterotrophic species of some dinoflagellate and nanoflagellate genera, and of the protozoan class Cercozoa, were also included since they are usually considered in phytoplankton studies. Identified taxa were reported per major groups such as diatoms, dinoflagellates, coccolithophores and flagellates, the latter including several phytoplankton classes (mostly smaller than 10 μm forms of uncertain taxonomic identification under LM afterwards named nanoflagellates, chryso-, chloro-, crypto-, dictyocho-, eugleno-, prasino-, primnesiophytes, ebridea, choanoflagellates and incertae sedis). With 'total phytoplankton', through the manuscript, it is intended all species/taxa detectable in light microscopy; therefore, prokaryotic phytoplankton and the majority of picoeukaryotes (< 1 μm) were not considered.

Statistical analyses

The distributions of nutrient concentrations and main phytoplankton group abundances were checked for significant differences among seasons, years, months and depths through analysis of variance (Kruskal-Wallis ANOVA). When significant differences were observed ($p < 0.05$), post hoc comparisons of mean ranks of all pairs of groups (Siegel and Castellan 1998) were also performed to further assess these statistically significant differences.

A non-parametric Spearman rank order correlation was used to assess the relationship in surface waters among oceanographic parameters (temperature, salinity, DIN, $P\text{-}PO_4$ and $Si\text{-}Si(OH)_4$), total precipitations on the three days preceding sampling, Isonzo River discharge on the day preceding sampling, considering the dataset grouped

per season. Additionally, the influence of environmental variables (temperature, salinity, DIN, P-PO_4 and Si-$Si(OH)_4$) on the phytoplankton groups and taxa was considered using the water column integrated values. These analyses were performed using the Statistica 7.0 software package (StatSoft).

A reduced taxa dataset (85 taxa) was used to calculate the Indicator Value Index (IndVal) and to perform multivariate statistical analyses: from the whole dataset comprising 122 taxa, any taxa with a lower than 10% frequency percentage were eliminated and species abundances were integrated on four depths using the trapezoidal method. The trapezoidal rule works by approximating the region under the graph of the function as a trapezoid and calculating its area.

Before performing the multivariate analyses, the species abundance values were log(X+1) transformed to diminish the effect of the most abundant species, and the dissimilarity matrix was computed based on the Bray-Curtis index. The environmental variables (temperature, salinity, total precipitations, Isonzo River discharge and nutrient concentrations) were first tested for multi-collinearity and symmetric distribution using PRIMER's Draftsman Plot tool and then normalised. The dissimilarity matrix was calculated based on the Euclidean distance.

To assess differences in species composition among seasons, a PERMANOVA test was applied considering the 'season' as a fixed factor. Unrestricted permutations of row data and 999 permutations were performed.

The effect of abiotic variables (temperature, salinity, total precipitations, Isonzo River discharge, dissolved inorganic nitrogen, phosphates and silicates) on the phytoplankton community was assessed by distance-based redundancy analysis (dbRDA, Legendre and Anderson 1999) that produced a better model of the relationship between the multivariate data cloud and environmental variables.

All these analyses were performed using the PRIMER software package (v. 7), including the add-on PERMANOVA+ package.

To assess species characterising seasons (winter: January, February and March; spring: April, May and June; summer: July, August and September; autumn: October, November and December), the IndVal (Dufrêne and Legendre 1997) was applied, grouping all samples per season. The indicator considers both the specificity and the fidelity, namely whether a species is abundant in a specific type of habitat and predominantly found in this type of habitat, respectively. The analysis was performed using the labdsv package in the R program (v. 3.3.0).

Results

Seasonal cycle and interannual variability of oceanographic parameters and freshwater inputs

The seasonal cycle and interannual variability of oceanographic parameters are showed in Figures 2, 3. Water temperature showed a marked seasonal cycle (Fig. 2A) with winter minima and summer maxima. In general, late autumn and winter were character-

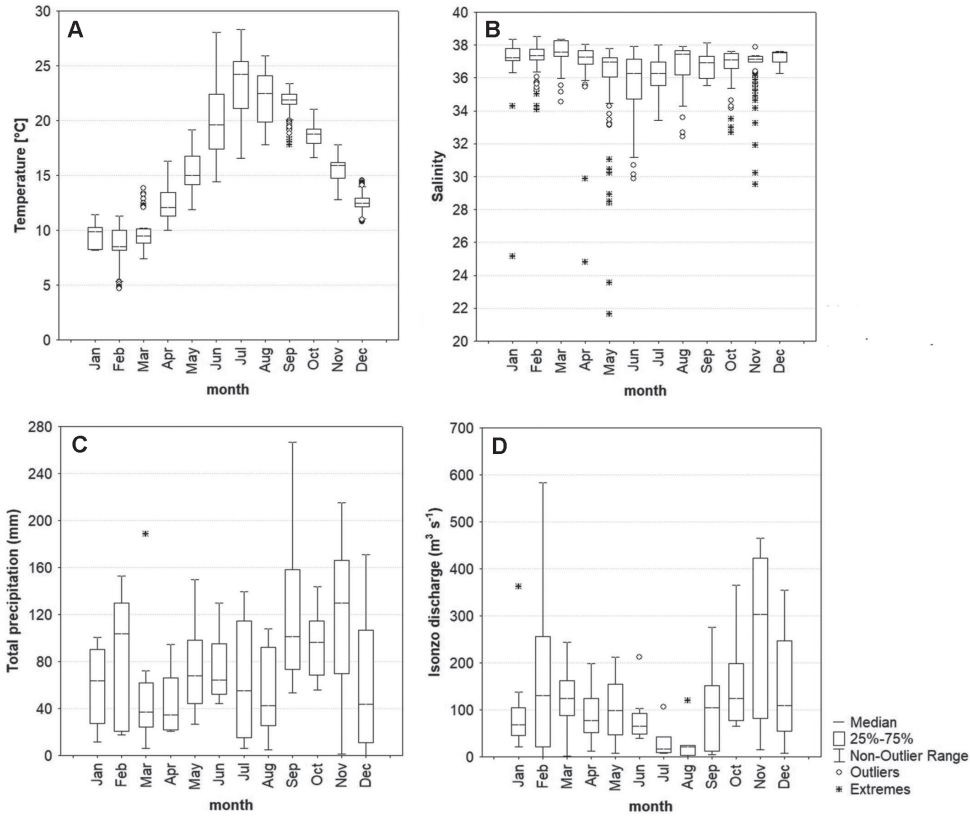

Figure 2. Seasonal cycles of temperature profiles (**A**), salinity profiles (**B**), precipitations (**C**) and Isonzo River discharge (**D**). In the box plot, the bold line represents the median, the box the 25th and 75th percentiles of the distribution, the whisker the non-outlier range, the circle the outliers and the star the extremes.

ised by vertical mixing of the water column, while the thermal stratification started in April-May and became more pronounced in June-August (Fig. 3A). After winter cooling, water temperature increased until July, which was on average the hottest month, with the highest value of 28.34 °C recorded in 2015 at the surface, and then decreased through the autumn months (Fig. 2A). During the first three years, seawater temperature was particularly low in winter (medians along the water column, 9.01, 8.61 and 7.57 °C in 2010, 2011 and 2012, respectively), with the lowest value of 4.71 °C in February 2012 at the bottom. In the successive winters, 2013–2016, seawater temperature was higher (medians along the water column ranged from 9.44 to 11.34 °C). The winter of 2017 was colder again (median 8.51 °C).

The salinity also showed a clear seasonality with the minimum recorded in spring and the maximum in winter (Fig. 2B). Generally, salinity values ranged from 34.00 to 38.50; however, low values (< 30) were occasionally detected at the surface: in May, June and November 2010 (21.66, 29.86 and 29.52, respectively), April 2013 (24.83), January 2014 (25.14), and May 2016 (28.51).

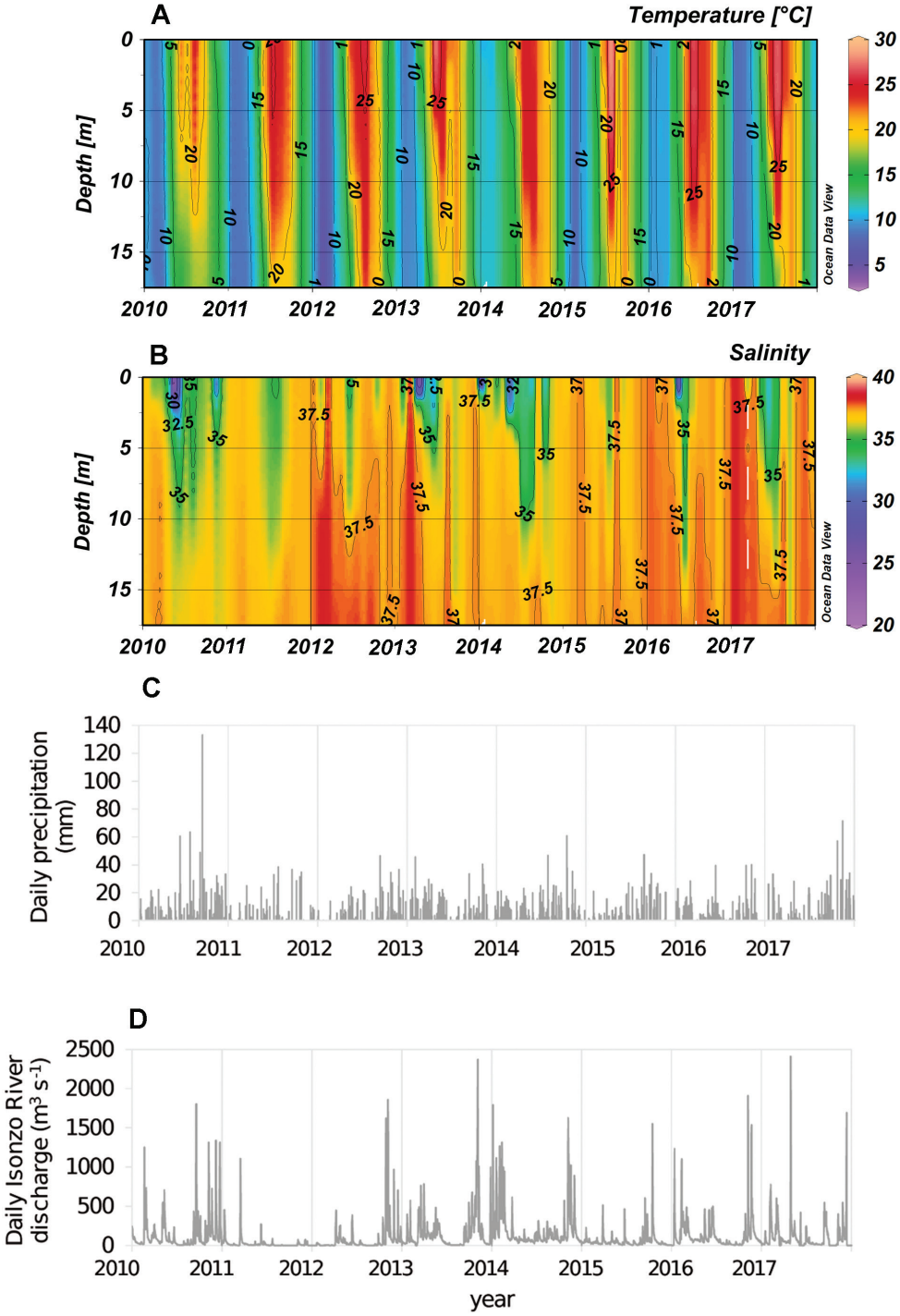

Figure 3. Interannual distributions of temperature profiles (**A**), salinity profiles (**B**), precipitations (**C**) and Isonzo River discharge (**D**).

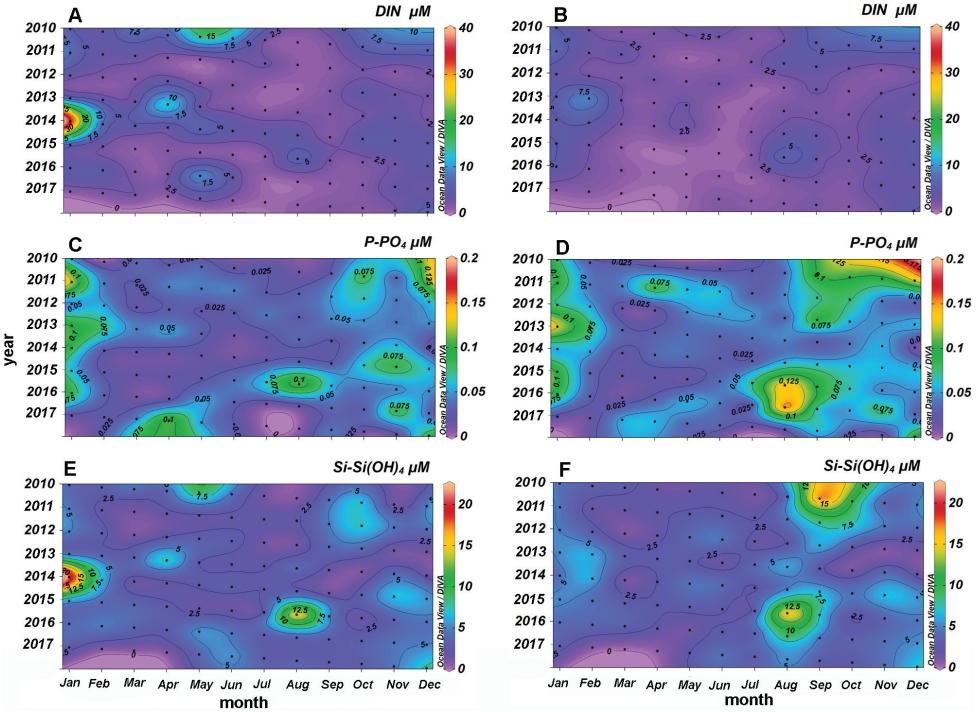

Figure 4. Seasonal and interannual distribution of dissolved inorganic nitrogen (DIN) (**A–B**), phosphate (P-PO_4) (**C–D**) and silicate (Si-Si$(OH)_4$) (**E–F**) concentrations in the two layers 0.5–5 m (**A, C, E**) and 10–15 m (**B, D, F**).

The precipitation regime was characterised by a rainy period in late summer-autumn (September to November) and two drier periods in March-April and July-August (Figs 2C, 3C).

The annual cycle of the Isonzo River discharge displayed minima in summer and two maxima, the biggest one in autumn and a lower one in winter (Figs 2D, 3D). River flows in 2014 were particularly high (annual median, 95.97 m^3 s^{-1}), whereas scarce loads were recorded in 2011, 2012 and 2015 (annual medians, 8.51, 13.59 and 31.59 m^3 s^{-1}, respectively) (Fig. 3D).

Dissolved inorganic nitrogen (DIN) concentration ranged from undetectable values to 71.27 µmol L^{-1}, measured in January 2014 at the surface (Fig. 4A, B). Concentrations were significantly higher (H = 64.86, p < 0.001) in autumn-winter than in spring-summer (mean ± SD, 3.79 ± 5.34 and 2.41 ± 3.82 µmol L^{-1}, respectively). Deviations from this cycle, with particularly high concentrations in the spring-summer period, occurred mainly in the surface layer. In 2010, 2013 and 2014, higher nitrogen concentrations (annual means ± SD, 4.87 ± 2.32, 3.26 ± 2.54 and 5.05 ± 5.26 µmol L^{-1}) than those recorded in other years (about 2.0 µmol L^{-1}) were observed. The statistically significant differences were 2010 vs 2011, 2012, 2015, 2016 and 2017, 2014 vs 2017 (H = 44.58, p < 0.001).

Phosphate concentrations ranged from undetectable values to 0.28 μmol L^{-1} in June 2015 at the surface, and higher values were observed from late summer throughout autumn and winter, mainly in bottom waters (Fig. 4C, D).

Silicate concentrations ranged from 0.07 μmol L^{-1} in November 2010, at 5 m depth, to 40.73 μmol L^{-1} in January 2014 at the surface, with a mean value (± SD) of 3.88 ± 3.67 μmol L^{-1}. Silicate generally showed an increase in summer in the deeper waters (Fig. 4F). Occasionally, higher concentrations were observed at the surface (Fig. 4E).

The correlations among oceanographic variables and freshwater inputs, considering the seasons separately, are reported in Table 1. In the surface waters, in winter, salinity was negatively correlated with the Isonzo River discharge ($p < 0.05$), as well as DIN ($p < 0.001$) and P-PO_4 concentrations ($p < 0.01$). Temperature ($p < 0.05$), DIN ($p < 0.05$) and Si-$Si(OH)_4$ ($p < 0.01$) concentrations were positively correlated with the riverine discharge. In spring, salinity was negatively correlated with temperature ($p < 0.001$), Isonzo River discharge ($p < 0.01$), DIN and Si-$Si(OH)_4$ concentrations ($p < 0.001$). DIN ($p < 0.05$) and Si-$Si(OH)_4$ ($p < 0.01$) concentrations were positively correlated with the riverine discharge. In summer, salinity was inversely correlated with seawater temperature ($p < 0.05$). The riverine discharge was significantly correlated with precipitations ($p < 0.05$). Seawater temperature ($p < 0.05$) and Si-$Si(OH)_4$ concentrations ($p < 0.05$) correlated, respectively, inversely and directly with precipitations. Finally, in autumn, salinity was negatively correlated with precipitations ($p < 0.001$), riverine discharge ($p < 0.01$), DIN ($p < 0.001$) and P-PO_4 ($p < 0.05$). Riverine discharge was positively correlated with precipitations ($p < 0.01$).

Phytoplankton seasonal cycle

Phytoplankton displayed a seasonal cycle characterised by minima in late autumn-winter (from December to February, with the monthly median always lower than 8.0 × 10^5 cells L^{-1} at all depths) (Fig. 5).

In March, abundances started to increase, reaching the main peak in spring (May), with a median value of about 4 × 10^6 cells L^{-1} in the 0.5–5 m layer and about 2.3 × 10^6 cells L^{-1} at 10–15 m, although these differences among depths were not statistically significant. From June, phytoplankton abundance gradually decreased and was low throughout the summer. Slightly higher abundances were observed at 10 m during this period. A further slight increase was observed in October (Fig. 5).

Considering the whole dataset, the phytoplankton community was dominated by flagellates (66%) and diatoms (29%), followed by coccolithophores and dinoflagellates (3 and 2%, respectively), with all groups showing a marked seasonal cycle (Fig. 6).

On average, flagellates showed the highest abundances in spring-summer (H = 127.46, $p < 0.001$), from April to July, and minima in winter (Fig. 6A–B). The maximum abundance value (5.6 × 10^6 cells L^{-1}) was recorded in July 2010, at 5 m depth. Abundances were slightly higher in surface waters than in deeper ones, particularly in June

Table 1. Spearman rank correlations among oceanographic variables and freshwater inputs at surface in different seasons (WIN: winter; SPR: spring; SUM: summer; AUT: autumn). Significant values (< 5%) are marked in bold. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

WIN	Temperature	Salinity	Precipitation	Isonzo discharge	DIN	P-PO_4	Si-$Si(OH)_4$
Temperature	1						
Salinity	-0.241	1					
Precipitation	-0.036	-0.289	1				
Isonzo discharge	**0.497***	**-0.439***	0.336	1			
DIN	**0.456***	**-0.690*****	0.199	**0.528***	1		
P-PO_4	0.062	**-0.529****	-0.017	0.220	**0.549****	1	
Si-$Si(OH)_4$	0.378	-0.332	0.204	**0.547****	**0.619****	**0.510***	1
SPR	**Temperature**	**Salinity**	**Precipitation**	**Isonzo discharge**	**DIN**	**P-PO_4**	**Si-$Si(OH)_4$**
Temperature	1						
Salinity	**-0.648*****	1					
Precipitation	-0.299	0.265	1				
Isonzo discharge	0.253	**-0.533****	0.191	1			
DIN	0.339	**-0.805*****	-0.183	**0.493***	1		
P-PO_4	-0.334	0.073	-0.071	-0.006	-0.030	1	
Si-$Si(OH)_4$	**0.471***	**-0.734*****	-0.203	**0.580****	**0.773*****	0.112	1
SUM	**Temperature**	**Salinity**	**Precipitation**	**Isonzo discharge**	**DIN**	**P-PO_4**	**Si-$Si(OH)_4$**
Temperature	1						
Salinity	**-0.464***	1					
Precipitation	**-0.456***	0.113	1				
Isonzo discharge	-0.289	-0.108	**0.448***	1			
DIN	-0.403	-0.176	0.392	0.176	1		
P-PO_4	-0.358	0.221	0.182	-0.026	0.259	1	
Si-$Si(OH)_4$	**-0.421***	0.234	**0.476***	0.187	**0.497***	**0.430***	1
AUT	**Temperature**	**Salinity**	**Precipitation**	**Isonzo discharge**	**DIN**	**P-PO_4**	**Si-$Si(OH)_4$**
Temperature	1						
Salinity	-0.240	1					
Precipitation	0.275	**-0.683*****	1				
Isonzo discharge	0.344	**-0.609****	**0.630****	1			
DIN	-0.117	**-0.660*****	**0.504***	0.329	1		
P-PO_4	-0.051	**-0.422***	0.331	0.233	**0.605****	1	
Si-$Si(OH)_4$	-0.003	-0.238	0.004	-0.056	**0.471***	**0.773*****	1

(Fig. 6A–B), but the differences were not statistically significant. The flagellate group was mainly represented by small (< 10 µm) forms of uncertain taxonomic identification (80%), cryptophytes (11%), prasinophytes (4%) and prymnesiophytes (2%), whereas chryso-, eugleno-, dictyochophytes and heterotrophic taxa accounted for only 3%.

Diatoms showed minima from December to February (monthly median of about 10^4 cells L^{-1}), then increased in late winter and peaked in spring ($H = 40.39$, $p < 0.001$), in May (monthly median 1.6×10^6 cells L^{-1}) (Fig. 6C–D), with a maximum of 5.2×10^6 cells L^{-1} in May 2013 at the surface. In June, a remarkable decrease down to 2.1×10^5 cells L^{-1} was observed and from July to September they attained very low abun-

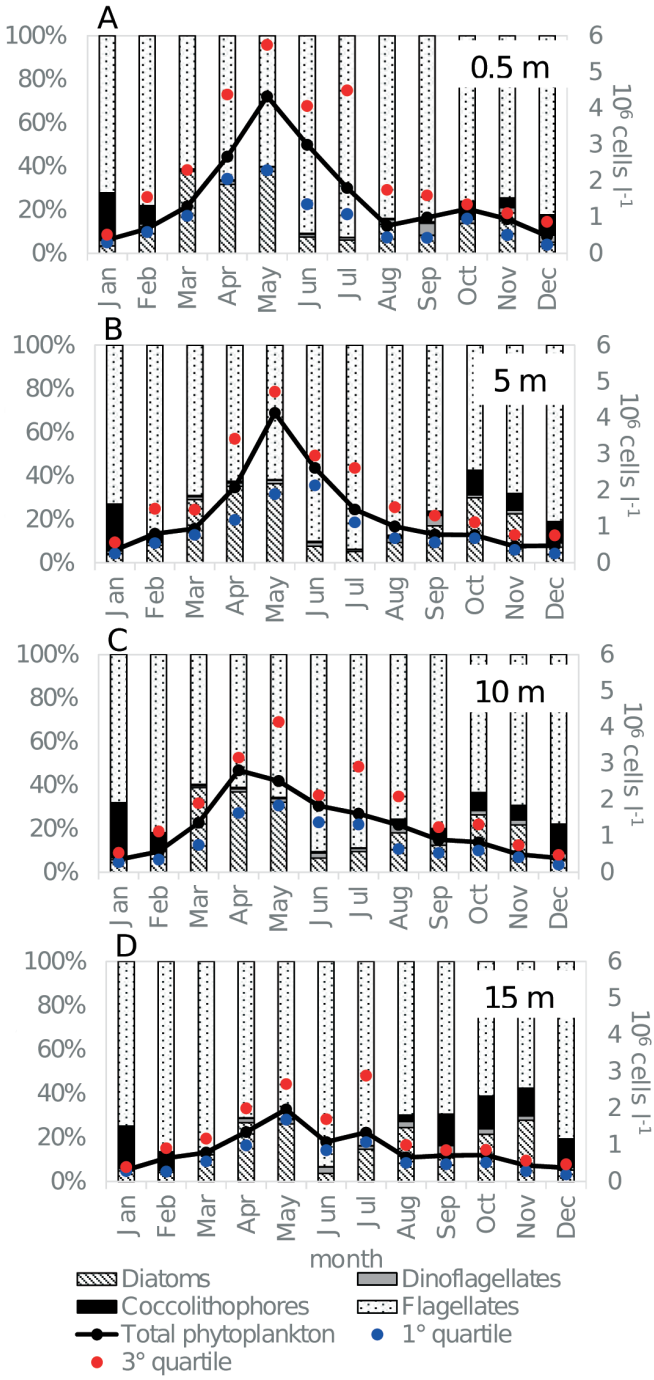

Figure 5. Monthly medians (black lines) and first and third quartiles (blue and red circles, respectively) of phytoplankton abundance and the relative contribution of the main phytoplankton groups (flagellates, diatoms, dinoflagellates, and coccolithophores) (bars) from 2010 to 2017 at the four sampled depths (0.5, 5, 10 and 15 m) at the C1-LTER station.

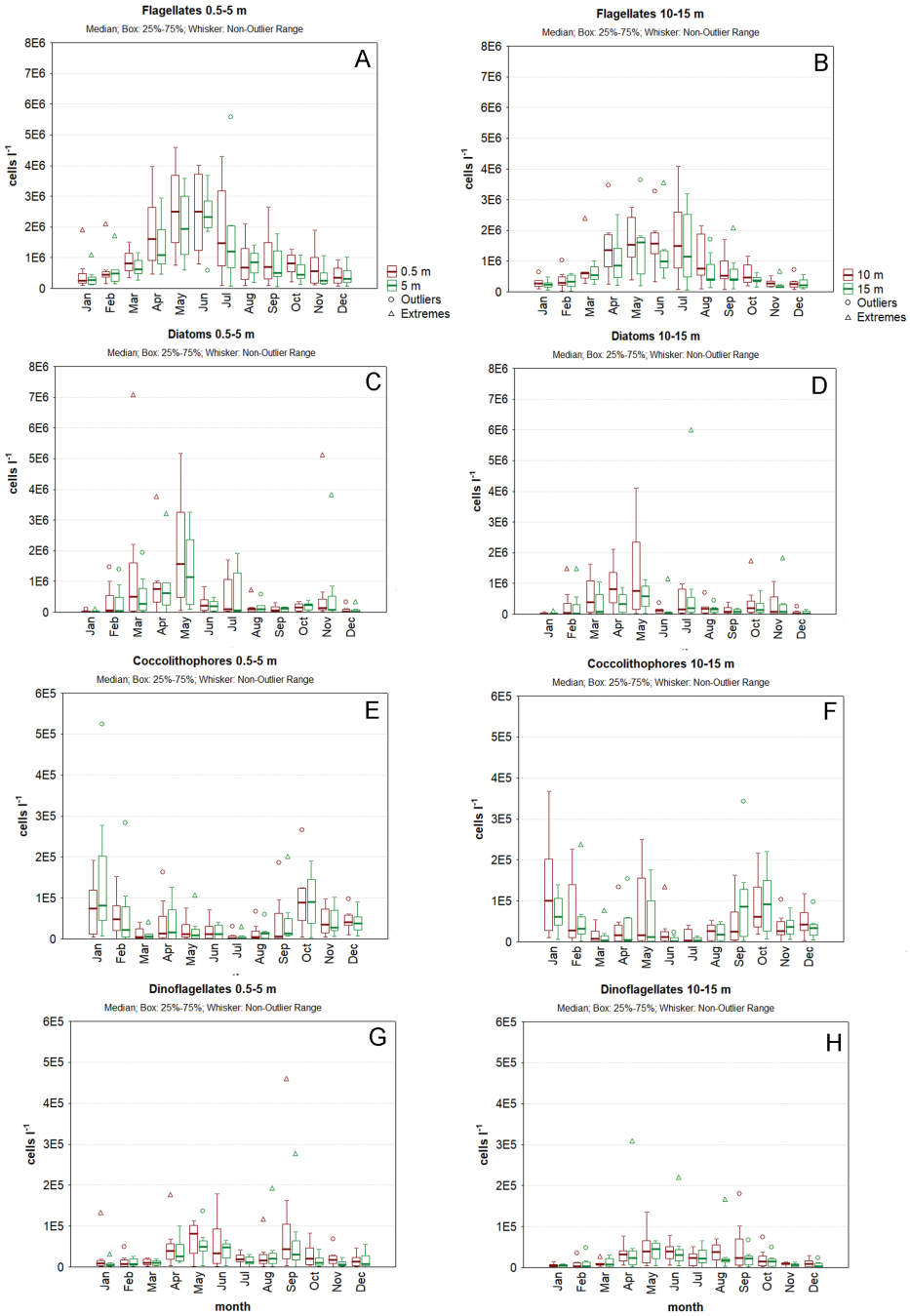

Figure 6. Seasonal cycle of flagellates (**A–B**), diatoms (**C–D**), coccolithophores (**E–F**) and dinoflagellates (**G–H**) at 0.5 and 5 m depth (**A, C, E, G**) and at 10 and 15 m depth (**B, D, F, H**). In the box plot, the bold line represents the median, the box the 25th and 75th percentiles of the distribution, the whisker the non-outlier range, the circle the outliers and the triangle the extremes. Note: y-scales are different for diatoms/flagellates and dinoflagellates/coccolithophores.

dances (monthly median < 1.0 × 10^5 cells L^{-1}), with occasional increases in July. A further, very low, increase was observed in October-November (monthly median 1.5 and 1.4 × 10^5 cells L^{-1}, respectively). In winter, the most present taxa were *Skeletonema* spp., *Pseudo-nitzschia* spp. and *Chaetoceros* spp. The spring bloom was generally dominated by different small (< 20 µm) species of the genera *Chaetoceros*, *Cyclotella* and *Bacteriastrum*, replaced by larger species (e.g. *Cerataulina pelagica* (Cleve) Hendey, *Pseudo-nitzschia* spp. *delicatissima* complex, *Leptocylindrus* sp.) in summer and autumn.

Coccolithophores were most abundant in autumn-winter (H = 53.42, p < 0.001) (Fig. 6E–F) with additional episodes of high abundance in May at 10 and 15 m (Fig. 6F). *Emiliania huxleyi* (Lohmann) W.W.Hay & H.P.Mohler was the most abundant coccolithophore species (on average, 63% of the total coccolithophores), followed by undetermined forms (20%), *Ophiaster* sp. (4%), *Acanthoica quattrospina* Lohmann (4%), *Calciosolenia brasiliensis* (Lohmann) J.R.Young (3%), *Rhabdolithes* (formerly *Rhabdosphaera*) *claviger* (G.Murray & Blackman) Voeltzkow (2%) and *Syracosphaera pulchra* Lohmann (2%).

Dinoflagellates showed higher abundances in spring and late summer (H = 94.18, p < 0.001) (Fig. 6G–H), with undetermined naked and thecate forms being the most abundant taxa (on average, 44.1 and 35.2% of the total dinoflagellates, respectively).

Diatoms were negatively correlated with DIN and silicates (p < 0.001), while dinoflagellates positively with temperature (p < 0.001) and negatively with DIN (p < 0.05) (Table 2). Coccolithophores showed a positive correlation with phosphates (p < 0.001) and silicates (p < 0.01), while flagellates were positively correlated with temperature (p < 0.01) and negatively with salinity (p < 0.01), DIN (p < 0.001), phosphates (p < 0.01) and silicates (p < 0.05) (Table 2).

Phytoplankton assemblages were significantly diverse among seasons (*Pseudo-F* = 9.59, p < 0.01) and the IndVal calculation gave an indication of which species characterised different seasons (Table 3). Considering the species with an IndVal higher than 0.4, the winter season was characterised by only three taxa, the diatoms *Skeletonema* spp., the dictyochophyte *Octactis octonaria* (Ehrenberg) Hovasse and the heterotrophic taxon of choanoflagellates. In spring, a higher number of species was typical for the season: the small diatoms *Cyclotella* spp., *Chaetoceros throndsenii* (Marino, Montresor, & Zingone) Marino, Montresor & Zingone and *Chaetoceros* spp., a mixed assemblage of dinoflagellates (*Torodinium robustum* Kofoid & Swezy, *Prorocentrum cordatum* (Ostenfeld) J.D.Dodge, *Lessardia elongata* Saldarriaga & F.J.R.Taylor and undetermined naked forms), flagellates (undetermined cryptophytes, *Leucocryptos marina* (Braarud) Butcher, *Commation* sp., *Ollicola vangoorii* (W.Conrad) Vørs and *Meringosphaera mediterranea* Lohmann) and undetermined coccolithophores. The summer seasons were characterised by a mix of large diatoms (*Proboscia alata* (Brightwell) Sundström , *Guinardia flaccida* (Castracane) H.Peragallo, *Rhizosolenia* spp., *Thalassionema* spp., *Cerataulina pelagica* (Cleve) Hendey), dinoflagellates (*Ceratoperidinium falcatum* (Kofoid & Swezy) Reñé & Salas, *Dinophysis fortii* Pavillard, undetermined thecate dinoflagellates), the coccolithophore *Rhabdolithes claviger* (G.Murray & Blackman) Voeltzkow and the prasinophyte *Pseudoscourfieldia marina* (J.Throndsen) Manton. Finally, in

Table 2. Spearman rank correlations among oceanographic variables with water column integrated phytoplankton groups and taxa. Significant values (< 5%) are marked in bold. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

	Temperature	Salinity	DIN	P-PO_4	Si-$Si(OH)_4$	Diatoms	Dinoflagellates	Coccolithophores	Flagellates	Total phytoplankton	*Chaetoceros* spp.	*Skeletonema* spp.
Temperature	1											
Salinity	**-0.455*** **	1										
DIN	**-0.332*** **	-0.161	1									
P-PO_4	-0.063	-0.022	**0.404*** **	1								
Si-$Si(OH)_4$	-0.069	0.084	**0.469*** **	**0.416*** **	1							
Diatoms	0.130	-0.151	**-0.308** **	-0.122	**-0.516*** **	1						
Dinoflagellates	**0.380*** **	-0.199	**-0.248* **	-0.187	0.004	**0.336*** **	1					
Coccolithophores	-0.165	0.081	0.126	**0.347*** **	**0.286** **	-0.105	-0.006	1				
Flagellates	**0.290** **	**-0.306** **	**-0.373*** **	**-0.317** **	**-0.214* **	**0.497*** **	**0.765*** **	-0.084	1			
Total phytoplankton	0.200	**-0.292** **	**-0.373*** **	**-0.246* **	**-0.373*** **	**0.752*** **	**0.640*** **	-0.046	**0.906*** **	1		
***Chaetoceros* spp.**	0.095	-0.085	**-0.384*** **	-0.164	**-0.406*** **	**0.712*** **	**0.311** **	-0.162	**0.377*** **	**0.531*** **	1	
***Skeletonema* spp.**	**-0.451*** **	0.156	**0.216* **	-0.071	-0.003	0.125	**-0.238* **	-0.122	-0.149	-0.070	0.098	1

Table 3. List of phytoplankton taxa characterised by the highest and significant IndVal for each season (win: winter; spr: spring; sum: summer; aut: autumn).

	Group	Indval	*p*-value
Skeletonema spp.	win	0.592	0.004
Und. choanoflagellates	win	0.491	0.014
Octactis octonaria	win	0.409	0.001
Protoperidinium bipes	win	0.292	0.04
Cyclotella spp.	spr	0.800	0.001
Prorocentrum micans	spr	0.734	0.001
Chaetoceros throndsenii	spr	0.724	0.001
Chaetoceros spp.	spr	0.608	0.004
Torodinium sp.	spr	0.590	0.001
Und. Cryptophyceae	spr	0.576	0.001
Prorocentrum cordatum	spr	0.517	0.006
Leucocryptos marina	spr	0.516	0.002
Commation sp.	spr	0.514	0.001
Ollicola vangoorii	spr	0.507	0.001
Lessardia elongata	spr	0.500	0.001
Meringosphaera mediterranea	spr	0.484	0.001
Und. coccolithophores	spr	0.427	0.001
Und. naked dinoflagellates	spr	0.423	0.005
Diplopsalis group	spr	0.414	0.001
Protoperidinium steinii	spr	0.391	0.001
Alexandrium spp.	spr	0.322	0.037
Und. Prymnesiophyceae	spr	0.279	0.047
Dinobryon faculiferum	spr	0.227	0.034
Proboscia alata	sum	0.888	0.001
Hermesinum adriaticum	sum	0.847	0.001
Ceratoperidinium falcatum	sum	0.824	0.001
Guinardia flaccida	sum	0.710	0.001
Und. pennate diatoms	sum	0.668	0.001
Rhizosolenia spp.	sum	0.611	0.001
Asteromphalus spp.	sum	0.586	0.001
Pseudoscourfieldia marina	sum	0.571	0.002
Rhabdolithes claviger	sum	0.508	0.001
Thalassionema spp.	sum	0.498	0.013
Und. thecate dinoflagellates	sum	0.453	0.016
Dinophysis fortii	sum	0.451	0.001
Cerataulina pelagica	sum	0.434	0.050
Tripos furca	sum	0.395	0.005
Gyrodinium spp.	sum	0.387	0.026
Hemiaulus hauckii	sum	0.364	0.004
Gonyaulax polygramma	sum	0.340	0.005
Chaetoceros lorenzianus	sum	0.320	0.008
Prorocentrum dactylus	sum	0.312	0.010
Dinophysis caudata	sum	0.300	0.009
Leptocylindrus mediterraneus	sum	0.280	0.021
Bacteriastrum jadranum	sum	0.269	0.016
Phalacroma rotundatum	sum	0.268	0.025
Syracosphaera pulchra	aut	0.776	0.001
Calciosolenia murrayi	aut	0.700	0.001
Diploneis spp.	aut	0.638	0.001
Dactyliosolen blavyanus	aut	0.594	0.001
Lioloma pacificum	aut	0.546	0.001
Ophiaster spp.	aut	0.505	0.001
Guinardia striata	aut	0.447	0.002
Asterionellopsis glacialis	aut	0.435	0.001
Dictyocha fibula	aut	0.369	0.011
Chaetoceros socialis	aut	0.363	0.001
Und. Euglenophyceae	aut	0.352	0.027
Paralia sulcata	aut	0.260	0.029

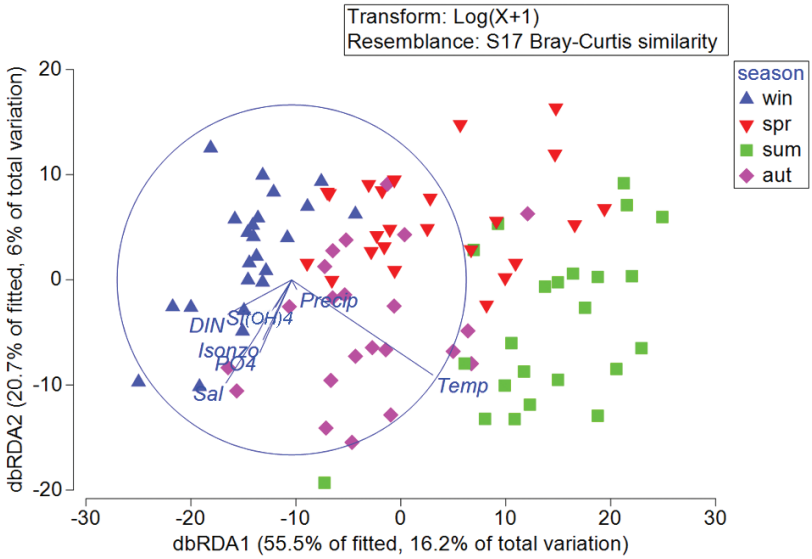

Figure 7. Distance-based redundancy analysis (dbRDA) plot of phytoplankton assemblages. Vectors of abiotic variables (temp: temperature; sal: salinity; precip: total precipitations in the three days preceding the sampling; Isonzo: Isonzo River discharge in the seven days preceding the sampling; DIN: dissolved inorganic nitrogen concentration; PO4: phosphate concentration; Si(OH)4: silicate concentration) affect the construction of the constrained ordination picture; the longer the vector, the bigger the effect of the variable.

autumn, very few species were typical, some coccolithophores (*Syracosphaera pulchra* Lohmann, *Calciosolenia murrayi* Gran, *Ophiaster* sp.) and some large diatoms (*Dactyliosolen blavyanus* (H.Peragallo) Hasle, *Lioloma pacificum* (Cupp) Hasle, *Guinardia striata* (Stolterfoth) Hasle, *Asterionellopsis glacialis* (Castracane) Round).

The dbRDA analysis also revealed temporal differences of phytoplankton assemblages (Fig. 7), with summer and winter samples well-separated, whereas autumn and spring samples were slightly overlapping. Temperature and salinity proved to be the best predictor variables, explaining the greatest variations in the data cloud: dbRDA1 was strongly correlated with temperature (r = 0.81), while dbRDA2 was related to salinity (r = -0.59). Phosphate showed a moderate correlation with dbRDA2. Temperature was associated with summer assemblages, salinity and Isonzo River discharge with autumn samples, and nutrients with winter samples.

Phytoplankton interannual variability

The temporal distribution from January 2010 to December 2017 of water column integrated abundance values of the main phytoplankton groups showed interannual variability for maximum values and occurrence of these maxima (Fig. 8).

The two main taxonomic groups were flagellates and diatoms during the whole analysed period, with a predominance of flagellates (annual medians > 6×10^5 cells

L^{-1} for flagellates, < 2 × 10^5 cells L^{-1} for diatoms). Two exceptions were observed in 2011 and 2012, when flagellates showed particularly low abundances (annual median 1.7 and 4.5 × 10^5 cells L^{-1}, respectively) (Fig. 8B). Particularly high flagellate abundances were recorded in July 2010 (up to 5.6 × 10^6 cells L^{-1} at 5 m), even though the highest annual median (1.2 × 10^6 cells L^{-1}) was observed in 2016. Diatoms showed low interannual variability in their annual median values (between 4.5 × 10^4 and 1.1 × 10^5 cells L^{-1} from 2011 to 2016 and about 2.0 × 10^5 cells L^{-1} in 2010 and 2017). However, annual peaks varied in abundance and occurrence (Fig. 8C). During the first years (2010–2012) of the analysed period, diatom increases were observed in late winter-early spring (March-April): in 2010, due to *C. simplex* Ostenfeld (mean March-April along the water column 3.5 × 10^5 cells L^{-1}), *Pseudo-nitzschia* spp. *delicatissima* complex (1.1 × 10^5 cells L^{-1}) and *Skeletonema* spp (1.0 × 10^5 cells L^{-1}); in 2011, due to *Chaetoceros* spp. (1.2 × 10^5 cells L^{-1}), *Pseudo-nitzschia* spp. *seriata* complex (7.3 × 10^5 cells L^{-1}) and *Skeletonema* spp. (1.7 × 10^6 cells L^{-1}); in 2012, due to *Pseudo-nitzschia* spp. *delicatissima* complex (2.7 × 10^5 cells L^{-1}) and *Skeletonema* spp. (3.5 × 10^4 cells L^{-1}). From 2013 onwards, *Skeletonema* spp. abundances decreased significantly (never higher than 9.2 × 10^3 cells L^{-1}) in winter and blooms were recorded in spring (May) (with highly variable maximum abundance values), due to different species belonging to the *Chaetoceros*, *Bacteriatrum* and *Cyclotella* genera. In 2010, additional high diatom increases were detected in July (0.5–15 m-integrated abundance 2.0 × 10^6 cells L^{-1}, maximum 6.0 × 10^6 cells L^{-1} at 15 m) and November (0.5–15 m-integrated abundance 2.3 × 10^6 cells L^{-1}, maximum 5.1 × 10^6 cells L^{-1} at the surface). The main species present in July were *Pseudo-nitzschia* spp. *delicatissima* complex (5.2 × 10^6 cells L^{-1} at 15 m), *Chaetoceros* cf. *simplex* (3.3 × 10^5 cells L^{-1} at 15 m) and *C. socialis* H.S. Lauder (1.0 × 10^5 cells L^{-1} at 15 m), whereas in November it was *Pseudo-nitzschia* spp. *delicatissima* complex (5.0 × 10^6 cells L^{-1} at 15 m). Finally, the lowest diatom abundances were recorded in 2012.

Coccolithophores displayed the typical seasonal cycle, with the highest abundances in autumn-winter, in all investigated years (Fig. 8D); however, additional increases were observed in 2010, 2011, 2014 and 2017. In August 2010, a very unusual peak (1.5 × 10^6 cells L^{-1}) due to *Emiliania huxleyi* was recorded at 15 m depth, whereas abundance in the upper layer (0.5–10 m) was very low. In May 2011, high *E. huxleyi* abundance (2.5 × 10^5 cells L^{-1}) was recorded only at 10 m depth, whereas in 2014 and 2017 the increase in April-May was noted at all depths and was due to more species, *E. huxleyi*, *Acanthoica quattrospina*, *Ophiaster* sp. and undetermined coccolithophores. In 2012, 2013 and 2016, coccolithophore abundances were lower (annual medians lower than 2.0 × 10^4 cells L^{-1}) than those observed in 2010, 2011, 2014 and 2015 (annual medians between 2.9 and 3.7 × 10^4 cells L^{-1}), whereas in 2017 higher values (annual median 5.5 × 10^4 cells L^{-1}) were recorded.

Dinoflagellates were always present in spring-summer (Fig. 8E) with the lowest annual median in 2011 (2.9 × 10^3 cells L^{-1}) and the highest in 2013 (3.3 × 10^4 cells L^{-1}). Sometimes, higher abundances were recorded at specific depths, generally due to undetermined naked and thecate forms. For instance, in September 2015, the highest density was observed at 0.5–5 m (4.6 and 2.8 × 10^5 cells L^{-1}).

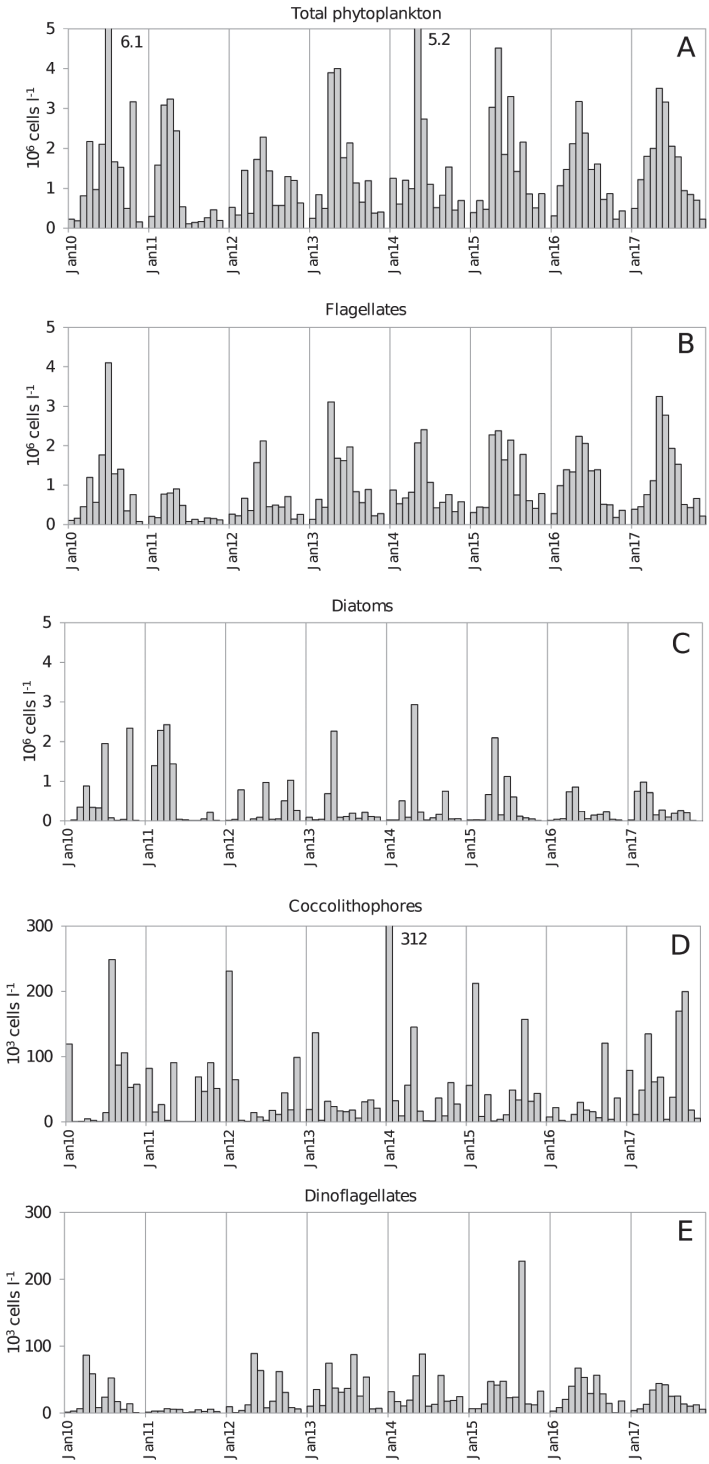

Figure 8. Temporal variations from January 2010 to December 2017 of depth integrated abundances of total phytoplankton (**A**), flagellates (**B**), diatoms (**C**), coccolithophores (**D**) and dinoflagellates (**E**).

Discussion

Seasonal cycle

This study presents the temporal dynamics, over eight years, of the phytoplankton community at a coastal station located in the north-eastern Adriatic, a highly variable environment. A marked seasonality, with warm summers and cool winters, was observed, which is typical of the area (Cossarini et al. 2012). Phytoplankton also showed a marked seasonal cycle with a maximum in spring (May), a gradual decrease during the summer, a further small increase in October and the lowest values in winter.

Phytoplankton attained minimum values in winter, in contrast to a previous study (Cabrini et al. 2012), covering 25 years (1986–2010), which described a phytoplankton annual cycle characterised by a late winter-early spring peak dominated by flagellates and the diatoms *Pseudo-nitzschia* spp., *Skeletonema marinoi* Sarno & Zingone, *Thalassiosira* spp., and *Chaetoceros* spp.. Instead, in the period 2010–2017, a spring bloom characterised the median seasonal cycle as previously observed in different area of the Adriatic Sea such as the north-western (Bernardi Aubry et al. 2012), north-eastern (Mozetić et al. 2012) and middle basin (Totti et al. 2000). Diatoms and flagellates (forms generally <10 µm in size) generally co-occurred during this period, as reported by Ribera d'Alcalà et al. (2004) in the Tyrrhenian Sea, but in contrast to the general findings in other Mediterranean areas where phytoplankton maxima are often dominated by diatoms (e.g. Nunes et al. 2018). In the north-eastern Adriatic, a dominance of nanoflagellates was often observed in spring (Marić et al. 2012; Mozetić et al. 2012; Godrijan et al. 2013; Talaber et al. 2014). Mozetić et al. (2012) explained that this could be due to the lack of control by their grazers, the microzooplankton, that indeed showed a strong reduction of their spring peak in the period 1998–2010 compared to the period 1986–1990 (Monti et al. 2012). Diatoms peaking in spring were also nano-sized, generally small (often <15 µm) species belonging to genera *Cyclotella*, *Bacteriastrum* and *Chaetoceros* (solitary or colonial species). This contradicts the general view of the typical spring diatom assemblages often being dominated by large species, such as *Pseudo-nitzschia* and large *Chaetoceros*, (e.g. Ribera d'Alcalà et al. 2004; Daniels et al. 2015). However, Daniels et al. (2015), who investigated the dynamics of the phytoplankton community structure at two contrasting sites in the Icelandic and Norwegian basins, found that the typical large diatoms dominated the spring bloom in the Icelandic basin, while very small (even <5 µm) diatoms dominated in the Norwegian basin. Similarly, Leblanc et al. (2018) described a massive spring bloom of the smallest known diatoms (*Minidiscus*) in the northwest Mediterranean Sea and, using a metabarcoding approach, showed the importance of this diatom at global scale. The dominance of nano-sized species in a spring bloom could have noteworthy implications for carbon export because larger phytoplankton cells more easily sink and, eaten by zooplankton, are exported to the bottom as fecal pellets, whereas smaller organisms are shifted towards the microbial loop, thereby reducing the efficiency of the carbon pump. On the contrary, Leblanc et al. (2018) evidenced high sinking rates for *Minidis-*

cus, thus demonstrating its important role in the export of carbon to deep oceans. The spring bloom could be triggered by higher nutrient availability in the period March-May, especially in surface waters due to riverine discharges, as shown by the significant correlations with salinity and with DIN and Si-Si$(OH)_4$. After May, phytoplankton abundances progressively decreased and attained low values in the summer. This differs from other northern Adriatic areas where summer blooms were reported, often due to diatoms (Bernardi Aubry et al. 2004, 2012; Kraus and Supić 2011; Marić et al. 2012; Mozetić et al. 2012; Godrijan et al. 2013; Talaber et al. 2014). In the northern Adriatic, these blooms were related to the spreading of the Po River plume towards the Istrian coast, carrying low salinity water with high inorganic nitrogen concentration (Godrijan et al. 2013; Viličić et al. 2013) or to episodic events of precipitations in the southern part of the Gulf of Trieste (Mozetić et al. 2012). In the northern part of the Gulf of Trieste, low Isonzo River discharge and low precipitations characterised the summer months, with the exception of some episodic storms in August that actually can cause episodic diatom increases. Seawater temperature reached maximum values and the water column was stratified with low inorganic nitrogen concentration. A mix of large diatoms and dinoflagellates, along with the coccolithophore *Rhabdolithes claviger* and the flagellate *Pseudoscourfieldia marina*, were typical of the period. The increase in the number of dinoflagellates is a typical feature of the summer period throughout the northern Adriatic (Bernardi Aubry et al. 2004, 2006; Ninčević Gladan et al. 2010; Cabrini et al. 2012; Marić et al. 2012) as shown by the positive correlation with temperature, also found by Ninčević Gladan et al. (2010). Generally, non-red-tide dinoflagellates are reported to be well-adapted to thermal stratification conditions with low nutrient availability (Latasa et al. 2010). The coccolithophore *Rhabdolithes claviger* was also identified as a distinctive summer coccolithophore in the Adriatic Sea (Bernardi Aubry et al. 2006; Godrijan et al. 2013; Cerino et al. 2017; Godrijan et al. 2018) as well as in other Mediterranean areas (Cros and Fortuño 2002; Dimiza et al. 2015; Karatsolis et al. 2017) and in thermal stratified waters worldwide (Okada and McIntyre 1977; Hagino et al. 2000). It is reported to be related to high temperature and low nitrate concentrations (Haidar and Thierstein 2001; Bernardi Aubry et al. 2006; Godrijan et al. 2013).

In late summer, the increase of silicates in deeper waters, under the pycnocline, as also observed in previous works (Cossarini et al. 2012), could be related to the degradation of diatoms.

In autumn, the high nutrient availability due to the riverine discharges and mixing of the water column and the still favourable light conditions triggered a second phytoplankton increase, although much smaller. Autumn blooms have been reported in the Gulf of Trieste (Cabrini et al. 2012; Mozetić et al. 2012) and in other areas of the north-eastern Adriatic Sea (Kraus and Supić 2011; Marić et al. 2012; Godrijan et al. 2013; Talaber et al. 2014). However, Mozetić et al. (2012) and Marić et al. (2012) observed a strong reduction in amplitude of this bloom from the period 1989–2002 to 2003–2009 and from 1972–1999 to 2000–2009, respectively. Coccolithophores (*Syracosphaera pulchra*, *Calciosolenia murrayi*, *Ophiaster* sp.) and some large diatoms

(*Dactyliosolen blavyanus*, *Lioloma pacificum*, *Guinardia striata*, *Asterionellopsis glacialis*) were typical of this period. The presence of coccolithophores in autumn assemblages has already been reported in the eastern Adriatic Sea (Šupraha et al. 2011; Cabrini et al. 2012; Cerino et al. 2017; Godrijan et al. 2018; Skejić et al. 2018). In general, autumn coccolithophores are related to high nutrient concentrations (Godrijan et al. 2013), as indicated by the positive correlation between coccolithophores and phosphate concentration. Moreover, the positive correlation between coccolithophore abundance and silicates could be explained by the role of silicates in the calcification process of some coccolithophores (Durak et al. 2016).

Interannual variability

In recent decades, the Gulf of Trieste has experienced considerable changes in its oceanographic, biogeochemical and biological features (Giani et al. 2012). Some of these changes are still ongoing and can affect phytoplankton abundance and dynamics. Obviously, eight years are not sufficient to support the existence of trends; however, the analysis of multi-annual periods can help to distinguish between interannual variability and possible ongoing long-term changes in phytoplankton dynamics.

Changes in phytoplankton abundances have been extensively reported at both global (e.g. Rousseaux and Gregg 2015) and local scale. For instance, in the Adriatic Sea, in the past years, different studies, using datasets of varying time spans, have reported regime shifts of chlorophyll *a* concentrations (Ninčević Gladan et al. 2010; Mozetić et al. 2010) and phytoplankton abundances (Cabrini et al. 2012; Marić et al. 2012; Mozetić et al. 2012; Ljubimir et al. 2017). Cabrini et al. (2012), analysing a long-term dataset (1986–2010) of phytoplankton abundances in the same area in the Gulf of Trieste, described two shifts, one in 1995 with a decrease of phytoplankton abundances and another in 2009 with an increase, but concluded that the shortness of the second period (2009–2010) did not allow for stating the beginning of a new regime. Analysing the years immediately after (2010–2017), the upward trend observed by Cabrini et al. (2012) was confirmed.

The most striking change recorded during this study was the strong decrease of the phytoplankton annual maximum in late winter-early spring, typical of the study area (Cabrini et al. 2012; Cibic et al. 2018). Changes in phytoplankton maxima over the seasonal cycle have already been reported in the Adriatic Sea (Viličić et al. 2009; Mozetić et al. 2010; Marić et al. 2012). Nanoflagellates and diatoms dominated this maximum, and among diatoms, *Skeletonema* spp., *Chaetoceros* spp. and *Pseudo-nitzschia* spp. *delicatissima* complex were the most representative taxa. The bloom decrease was due mainly to a strong reduction of *Skeletonema* spp. abundance compared to previous years, during the 1986–2000 period in particular, when this genus was the main taxon of late winter-early spring blooms (Cabrini et al. 2012). The taxon we reported as *Skeletonema* spp. was never characterised via electron microscopy or molecular markers; therefore, we prefer to refer to *Skeletonema* spp.. However, it is most probably *S. marinoi* (former *S. costatum*), which is extensively reported as being

responsible for the winter-early spring blooms in the northern Adriatic Sea (Sarno et al. 2005; Marić Pfannkuchen et al. 2018). The decrease of this species had already been observed in the area by Cabrini et al. (2012) who suggested the nutrient reduction as a possible cause. However, the temperature increase could have also played a role in this decline. Indeed, this species was reported as typical of cold waters (Bernardi Aubry et al. 2004) and laboratory experiments also confirmed the preference of this species for temperature below 10 °C (Kaeriyama et al. 2011). Accordingly, a negative correlation between *Skeletonema* spp. and temperature was observed and in the period 2010–2012, when high *Skeletonema* spp. abundances were recorded, mean annual seawater temperatures in winter were very low compared to the period 2013–2017. The increase of surface water temperature in the northern Adriatic Sea (Degobbis et al. 2000; Solidoro et al. 2009; Giani et al. 2012) and worldwide in coastal environments (Lima and Wethey 2016) is generally recognised. A warming trend, with an increase in surface water temperature, particularly intensified since 2008, of about 1.1 °C in the period 1979–2015, has been observed along the eastern Adriatic coast, although the authors pointed out the existence of multidecadal sea temperature variability (Grbec et al. 2018). A decrease in *Skeletonema* abundances has also been reported for Narragansett Bay in the period 1980–1997 with the decline being greatest during the winter-spring bloom (Borkman and Smayda 2009). The authors proposed an influence of the North Atlantic Oscillation (NAO); therefore, the years with low NAO tended to have colder winters and the winter-spring bloom dominated the *Skeletonema* annual cycle. A decline in *Skeletonema* dominance replaced by *Chaetoceros* was also observed in the eastern Seto Inland Sea (Nishikawa et al. 2010). However, longer observations are needed to definitively support the view that temperature is possibly responsible for the *Skeletonema* bloom decrease, and other factors should be considered. The diversity and dynamics of phytoplankton assemblages are complex processes driven by several interacting abiotic and biotic factors and, in addition to other physical-chemical drivers, biological interactions, such as grazing pressure and/or viral infections can have a role in shaping phytoplankton assemblages. The implications of a decreased bloom can be significant if one considers the importance of the synchronisation of the phenological cycles of primary producers and their consumers for matter and energy transfer through the food web (e.g., Edwards and Richardson 2004; Thackeray et al. 2016; Kodama et al. 2018). In the same way, changes in the composition of these blooms can also affect consumers because the food quality of various species can differ both in terms of stoichiometric composition and morphological characteristics, such as the presence of setae or cell projections (e.g., Finkel et al. 2010).

Conclusions

Long-term sampling offers a unique opportunity to analyse multiannual datasets and describe complete seasonal cycles, thus unveiling possible changes occurring in phytoplankton community structure in highly variable environments such as coastal ecosys-

tems where the distinction between natural variability and temporal trends is more difficult due to local disturbances. In the north-eastern part of the Gulf of Trieste, phytoplankton displayed a marked seasonal cycle strongly influenced by temperature and salinity, as revealed by multivariate analysis. This cycle was characterised by a spring peak dominated by nanoflagellates and small diatoms, triggered by high nutrient availability due to riverine discharges, and a second small increase in autumn dominated by nanoflagellates, larger diatoms and coccolithophores, possibly favoured by higher nutrient availability deriving from the mixing of the water column. In summer, stormy events could cause episodic diatom increases. At interannual scale, a strong decrease of the late winter-early spring bloom was observed in recent years, with the spring bloom becoming the main peak during the year. If the role of temperature will be confirmed with further analyses, it may have significant implications in the view of climate changes as drivers of long-term changes in phytoplankton dynamics. However, because long-term data series are considered necessary to determine whether the changes are actual ongoing trends, or fall within the interannual variability of phytoplankton communities, continuous monitoring of these alterations is very important. Therefore, LTER sites offer ideal study fields for this purpose and provide data for defining the environmental status, as required by the Marine Strategy. Additionally, the LTER-Italy network allows for sharing methodologies, ecological data and knowledge which would provide the opportunity to establish collaborations at the national (with other LTER-Italy sites), regional (LTER- Europe) and international (LTER-International) levels.

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RESEARCH ARTICLE

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Long-term changes in abundance and diversity of tintinnids in the Gulf of Trieste (Northern Adriatic Sea)

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Abstract

Abundance and composition of the planktonic tintinnid ciliates were studied in the Gulf of Trieste (Northern Adriatic Sea, NE Mediterranean Sea) from July 1998 to July 2016. Tintinnids were collected biweekly-monthly from the LTER station C1 (200 m offshore, 17.5 m depth) at four depths (surface, 5 m, 10 m and 15 m). The maximum tintinnid abundance reached 4476 ind. L^{-1} at surface in February 2016. The tintinnid community comprised a maximum of 35 species and was dominated by the genera *Stenosemella*, *Tintinnopsis*, *Codonellopsis*, *Salpingella* and *Eutintinnus*. The most abundant species were *Stenosemella nivalis*, *Tintinnopsis nana*, *Codonellopsis schabi*, *Salpingella rotundata* and *Eutintinnus apertus*. We found a species-specific correlation with the abiotic factors considered, i.e., temperature and salinity. Temperature was positively correlated with *S. rotundata* and *E. apertus* and negatively with *S. nivalis*. Salinity was negatively correlated with the majority of the detected species. Agglutinated species presented winter maxima while hyaline species showed higher abundance in summer-autumn. Some key species were present over the whole period studied. Significant differences within the water column were not seen in the species composition, but were seen in the relative abundances of the same species at different depths. *Stenosemella nivalis*, *S. ventricosa* and *Tintinnopsis beroidea* can be considered as keystone species in the area and their possible loss can be seen as a signal of changes in the structure of the entire planktonic system.

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Keywords
Tintinnids, Adriatic Sea, LTER

Introduction

Tintinnid ciliates represent a fraction of microzooplankton (broadly heterotrophic planktonic organisms spanning 20 µm to 200 µm). They have a key position in the plankton food web as major consumers of picoplankton and nanoplankton and serve as prey for metazoans (Capriulo et al. 1991). Although they represent only a small part of the planktonic ciliate community (generally less than 10% of biomass), they can be very important in a variety of ecosystems (Dolan 2000). In coastal waters, they are occasionally abundant (Kršinić 1987, Modigh and Castaldo 2002) and are reported to ingest up to 27% of the annual primary production (Capriulo and Carpenter 1983, Verity 1987). Tintinnids are ideal for studying changes in the structure or composition of the plankton communities (Thompson et al. 1999). In fact, they are recognised from the lorica morphology, a quite simple feature that ensures homogeneity in the taxonomic identification with a low risk of misclassification when different researchers are involved over the years in long-term studies.

Tintinnids are the most investigated component of microzooplankton communities in the Mediterranean Sea where the west-to-east increase in species richness and in taxonomic diversity has been well documented (Dolan 2000, Dolan et al. 2002, Modigh and Castaldo 2002, Gómez and Gorsky 2003, Sitran et al. 2007, 2009). Previous studies in the Adriatic Sea have shown that tintinnids are an important plankton fraction, characterised by variable abundances along the basin (Lipej 1992, Fonda Umani et al. 2010, Moscatello et al. 2004, Bojanić et al. 2006, 2012, Kršinić and Grbec 2006). For this area, studies on tintinnids have focused both on their seasonal distribution (Fonda Umani et al. 2005a, Milani et al. 1991, Monti et al. 2012) and trophic role (Fonda Umani and Beran 2003, Fonda Umani et al. 2005b).

The Gulf of Trieste is the most northern part of the Adriatic Sea, with a surface area of about 600 km^2 and a maximum depth around 23 m (Malej and Malačič 1995). The main freshwater input in the Gulf is through the Isonzo River and the hydrodynamics is driven by a wind regime characterised by strong wind events, by the interaction with the Adriatic circulation and by seasonal shifts from stratification to mixing (Cossarini and Solidoro 2007). The circulation pattern is variable; however, the prevailing East-North-East wind is the most efficient driver for the water exchange (Solidoro et al. 2009). Over the last decade, an increase in seawater temperature and salinity was detected for the area, particularly at the surface, and a reduction in nitrate and silicate was recorded (Malačič et al. 2006, Mozetič et al. 2012). The area remains phosphorus-limited with P concentrations always low, ranging from 0.05 to > 3 µM (Fonda Umani et al. 2007, Lipizer et al. 2011). The satellite-based chlorophyll concentrations indicated an oligotrophication trend from 1998 to 2007 (Mozetič et al. 2010). More recently, Giani et al. (2012) reported an increase in phytoplankton biomass since 2007.

Biological factors, such as primarily prey abundance and predator presence, influence tintinnid behavior and ecophysiology (Montagnes 2013). Nevertheless, species diversity is not always dependent on food availability and quality or predator abundance (Dolan and Gallegos 2001), and abiotic factors can also be important in shaping tintinnid communities. The influence of salinity on tintinnids has been generally overlooked because of their euryhaline distribution and their ability to survive over a large range of salinity values (Montagnes 2013). Temperature may influence the tintinnid ingestion rate, growth and cell size (Montagnes 2013). Very few data exist on tintinnid diversity and their relationship with abiotic factors such as salinity and temperature, especially for the Adriatic Sea.

In this paper, we analysed the long-term development of tintinnids collected in the Gulf of Trieste over a period of 18 years. The tintinnid assemblages were studied in terms of abundance and diversity along the water column and in relation of temperature and salinity. The main goal of this study was to identify the main patterns of temporal variability of the whole tintinnid community and its main components, and the possible key species.

Materials and Methods

Sampling method

Sampling was carried out in the Gulf of Trieste aboard different boats from July 1998 to July 2016 at the LTER station C1 (https://deims.org/96969205-cfdf-41d8-979f-ff881ea8dc8b). The sampling station is located 200 m offshore at 45°42'3"N, 13° 2'36"E (bottom depth 17.5 m) (Figure 1). Water samples were collected early in the morning, biweekly (from June 2002 to August 2005) or monthly (the rest of the period) at four depths (0 m, 5 m, 10 m, and 15 m), using Niskin bottles. Two litre samples were transferred into polycarbonate bottles and immediately preserved with formaldehyde buffered with $CaCO_3$ (4% final concentration). Temperature and salinity data were recorded by a CTD probe (Idronaut Ocean Seven 316 and SeaBird 19 Plus) not connected with the Niskin bottles.

Sample processing and analysis

In the laboratory, the samples were allowed to settle for 3 days, after which the initial 2 L volume was reduced to 200 mL by siphoning through a glass tube made specifically for this purpose, under a fume hood. The reduced samples were stored in dark glass bottles and subsamples (50 mL) were analysed using inverted microscopes (Leitz and Leica DMI 3000B) at x 200 magnification following the Utermhöhl method (Utermöhl 1958). The entire sedimentation chamber was analysed and tintinnid abundance was expressed as number of individuals per liter (ind. L^{-1}). We counted a variable num-

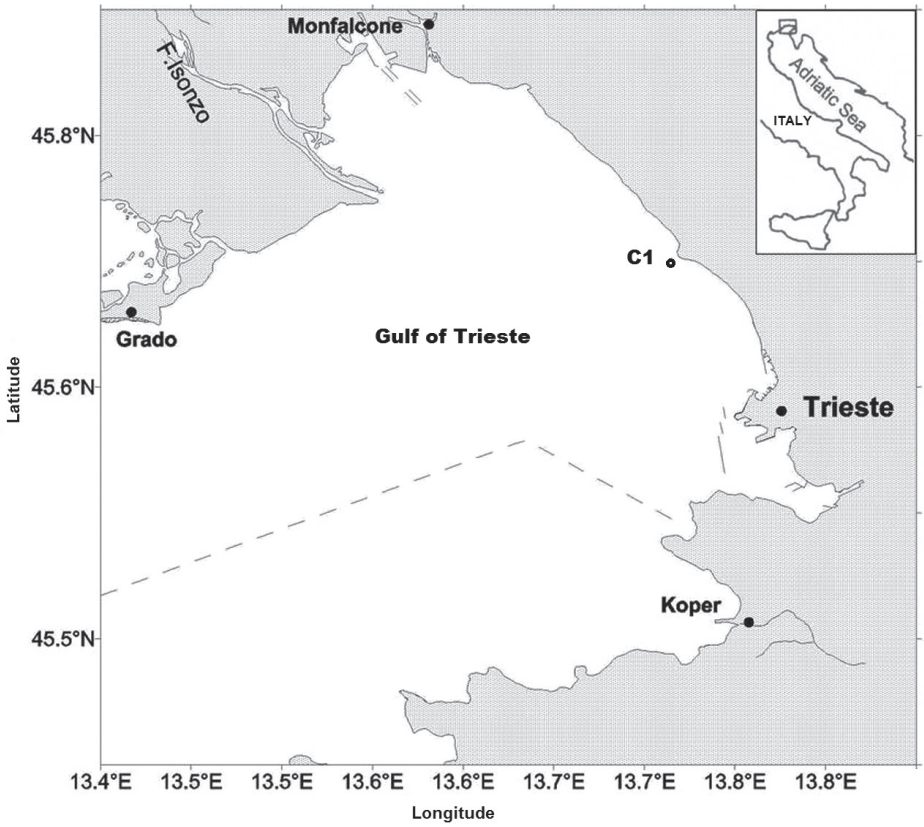

Figure 1. Map of the Gulf of Trieste showing the location of the LTER sampling station C1.

ber of tintinnids in each sample, from the minimum of 0 to the maximum of 2238 tintinnids (median value 16 tintinnids), without any replication. The samples were analysed within one month of the collection date. A total of 1000 samples were analysed over 18 years. For estimating the abundance within the water column (0–15 m), the integrated values were calculated following the trapezoidal rule (Walsby 1997).

Tintinnids were determined according to the classifications by Kofoid and Campbell (1929), Rampi and Zattera (1982) and Kršinić (2010a). Taxonomic criteria were maintained homogeneous over the whole period by a series of inter-calibration exercises among the different researchers (six), under the supervision of the responsible taxonomist (M. Monti-Birkenmeier). The final classification followed Kršinić (2010a) and species names were updated according to the most recent nomenclature. In particular, *Amphorella quadrilineata* was changed in *Amphorides quadrilineata*, *Amphorella quadrilineata* var. *minor* in *Amphorides quadrilineata* var. *minor*, *Dictyocysta elegans* var. *speciosa* in *Dictyocysta elegans*, *Favella campanula* in *Favella adriatica*, *Tintinnopsis levigata* in *Tintinnopsis cylindrica*, *Tintinnopsis minuta* in *Tintinnopsis nana*. Furthermore,

species detected less than twice over the period were grouped to the genus or order level (*Amphorellopsis tetragona* to *Amphorellopsis* sp.; *Codonella aspera* to *Codonella* sp.; *Tintinnopsis baltica* and *Tintinnopsis bütschlii* to *Tintinnopsis* spp.; *Craterella* sp. to Choreotrichida unid.). Empty loricae of tintinnids were not differentiated from filled ones because tintinnid protoplast is attached to the lorica by a fragile strand that detaches easily during collection and fixation of the samples. Furthermore, it is not possible to detect the presence/absence of the cell inside for the agglutinated loricae.

Statistical analysis

Statistical analysis was carried out using the PRIMER-7 software package (Anderson et al. 2008) to find out about similarity among years and seasons and to estimate tintinnid species richness and diversity in each sample. ANOSIM and pair-wise tests were performed to analyse the similarity pattern in the dataset considering the factors depth, month and year. In order to test the influence of season, salinity and temperature on the community, we performed Permutational Multivariate Analysis of Variance (hereafter called PERMANOVA) (Anderson and Walsh 2013) on square-rooted Bray-Curtis dissimilarity matrix for the abundance of each taxon. On the same matrix, a distance based Redundancy Analysis (dbRDA) was performed considering temperature and salinity as environmental variables. The significance of the two RDA axes and of the environmental variables considered in the analysis was tested using "vegan" package (Oksanen et al. 2016) in R environment while for the Indicator Value (IndVal), which measured species-season association, considering the relative abundances and the relative frequency in a seasonal sub set of samples, the "labdsv" package was used (Dufrêne and Legendre 1997). The IndVal test was used to test which species were representative for each season and to test the significance of this relationship over the considered period. The box plots and Spearman's correlation coefficients between species and abiotic factors were calculated using the STATISTICA 8.0 software.

Results

Tintinnid abundance

The total tintinnid abundance ranged from 0 to 4476 ind. L^{-1} (February 2016) and the median values ranged from 22 ind. L^{-1} (surface) to 26 ind. L^{-1} (bottom) from July 1998 to July 2016. Tintinnid abundance integrated along the water column showed a high peak at the beginning of the series (775 ind. L^{-1} in October 1998) and decreased afterwards remaining < 250 ind. L^{-1} from March 2000 to April 2007 (Figure 2). Since May 2007, a slow but continuous recovery of tintinnid abundance was registered up to the highest peak recorded in February 2016 (856 ind. L^{-1}).

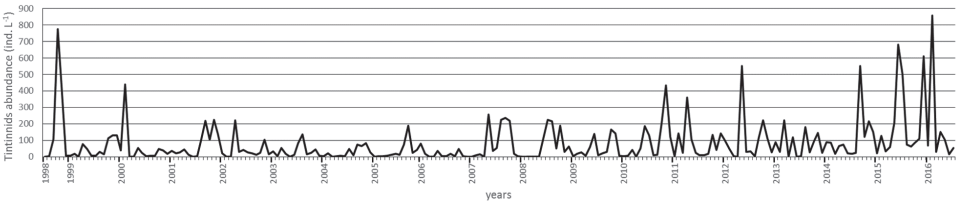

Figure 2. Long-term fluctuations of total tintinnid abundance (ind. L^{-1}) integrated within the water column (0–15 m) from July 1998 to July 2016 in the Gulf of Trieste.

The average seasonal pattern of tintinnid abundance in the integrated water column showed the lowest median values from January to April, and the highest ones from September to November (Figure 3). The pattern was different considering the average abundance for agglutinated and hyaline species. Agglutinated species presented winter maxima whereas hyaline species showed the highest abundance during summer-autumn (Figure 4).

The pair-wise test of PERMANOVA revealed significant differences (p-perm < 0.05) among the different sampled layers, with the exception of 5 m and 10 m depths that showed the highest similarity (Table 1). The highest values of tintinnid abundance were registered at 0 m and 15 m depths (Figure 5). At intermediate depths, the abundances were higher than 800 ind. L^{-1} only in October 2011, May 2012 and July 2015 (Figure 5).

Temporal and vertical patterns of the main genera and species of tintinnids

During the present study, tintinnids belonging to 19 genera and 35 species were recorded. Twenty of these were open sea species and 15 were estuarine-neritic species. The species list shows the frequency of occurrence and maximum abundance of each taxon (Table 2). Tintinnids were dominated by the agglutinated genera *Stenosemella* (*S. ventricosa* and *S. nivalis*) and *Tintinnopsis* (e.g., *T. nana*, *T. beroidea* and *T. radix*) and by *Codonellopsis schabi*. These species present a lorica covered by particles, are more abundant in coastal areas and are considered neritic. The most frequently found hyaline species belonged to *Eutintinnus* and *Salpingella* genera, with open sea species as *E. apertus*, *E. fraknoi*, *S. decurtata* and *S. rotundata* (Table 2). The pair-wise test showed a significant difference (p-perm < 0.05) among most of the years and we did not detect any clear trend in the similarity matrix for the studied period. The highest similarity (17.60) was registered between 2015 and 2016 (Suppl. material 1).

The number of tintinnid species detected in each year from 1998 to 2016 varied between 13 (2006) and 24 (2008) (Table 3). In most of the years, the number of species was very similar with an average species number of 18 ± 3 stdev. Only three species were present in all the years: *S. nivalis*, *S. ventricosa* and *T. beroidea*. A group of six species (*S. rotundata*, *E. fraknoi*, *E. tubulosus*, *T. nana*, *T. campanula* and *S. decurtata*) was

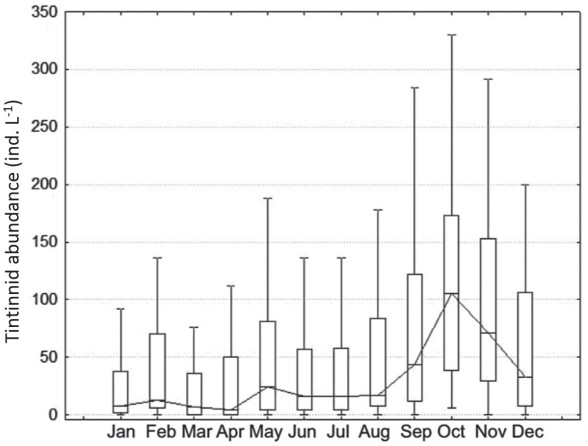

Figure 3. Box plots of monthly averaged abundance of tintinnids (ind. L^{-1}) integrated within the 0–15 m water column. The black lines represent the median, the white rectangles represent the dispersion of the data (25–75%), and the black bars show the non-outlier range.

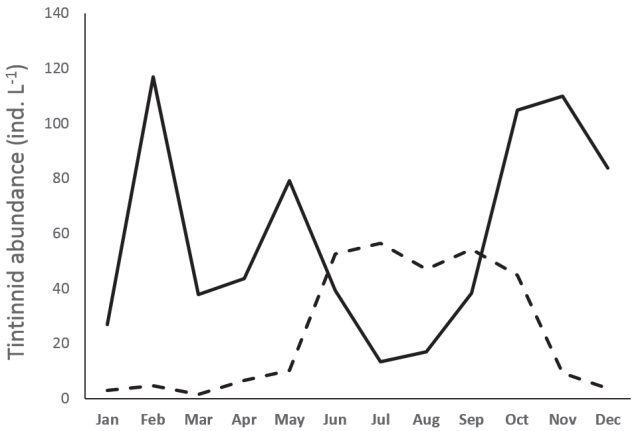

Figure 4. Monthly averaged abundance (ind. L^{-1}) of agglutinated (black) and hyaline (dashed) tintinnids integrated within the 0–15 m water column from July 1998 to July 2016.

Table 1. Results of pair-wise tests of PERMANOVA for the factor depth, built on similarity (Bray Curtis) matrix of tintinnid composition.

Groups	*t*	*p* (perm)
0–5 m	1.61	0.004
0–10 m	1.80	0.002
0–15 m	2.10	0.001
5–10 m	1.15	0.192
5–15 m	2.02	0.002
10–15m	1.45	0.013

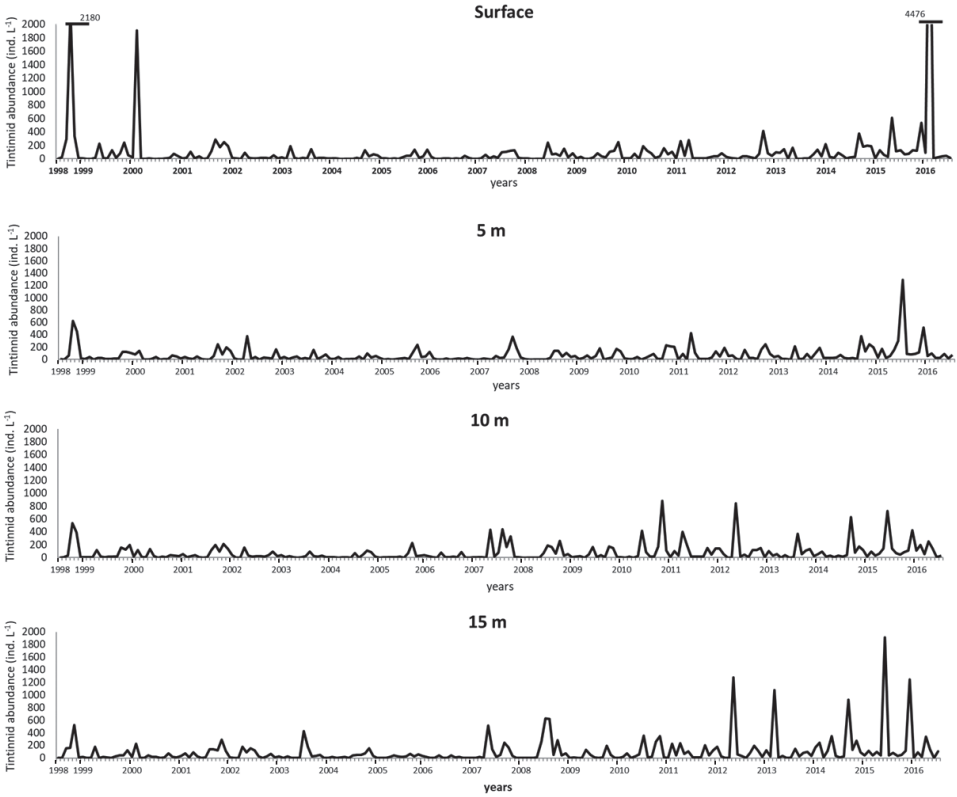

Figure 5. Long-term fluctuations of total tintinnid abundance (ind. L^{-1}) at different depths (surface, 5 m, 10 m and 15 m) from July 1998 to July 2016 in the Gulf of Trieste.

absent only in few years (1–3 y). Five species were detected only in two years or less and should be considered rare taxa (*Dictyocysta lepida*, *Rhabdonella spiralis*, *Protorhabdonella curta*, *Favella adriatica* and *Amphorides* cfr. *amphora*). In particular, *D. lepida* was detected only at the end of the considered period (2015). Conversely, the presence of *Tintinnopsis parvula* and *T. radix* was not registered after 2010.

Considering the vertical distribution of the key species, the light differences detected among layers were due to the different abundances of the most representative species (*S. nivalis*, *S. rotundata*, *S. ventricosa*, *T. nana*, *C. schabi* and *Salpingella decurtata*) regardless the stratification of the water column. *Stenosemella nivalis* was absent at surface only in 1998 while *S. ventricosa* and *T. beroidea* were absent in many occasions at different depths (Suppl. material 2). Among the rarer species, *D. lepida* was detected at all depths only in 2015. *Amphorides* cfr. *amphora*, *P. curta* and *R. spiralis* were never collected at the surface. *Rhabdonella spiralis* was never collected at 0 m and 5 m, *P. curta* at 10 m and *A.* cfr. *amphora* at 15 m. *Favella adriatica* was collected only in 2008 and 2013 at different depths (Suppl. material 2).

The number of tintinnid species was lower during winter time, started to increase in spring and reached the maximum in summer-autumn. The specie richness was al-

Table 2. Occurrence (number of samples in which the species was present), frequency, maximum abundance (ind. L^{-1}) and distribution of each tintinnid taxon recorded in the Gulf of Trieste between 1998 and 2016 (total number of samples = 1000). C, common (20–100%); QC, quite common (8–20%); QR, quite rare (3–8%); R, rare (1–3%); RR, very rare (<1%), following the classification of Travers (1971). NES, neritic-estuarine species; OSS, oceanic or open sea species, following Kršinić (2010a, b).

	Occurences	Frequencies	Max abundance	Neritic or Open sea
Acanthostomella conicoides	39	QR	60	OSS
Amphorides cfr. *amphora*	3	RR	4	OSS
Amphorides laackmanni	9	RR	28	OSS
Amphorides quadrilineata	19	R	20	OSS
Amphorides quadrilineata var. *minor*	4	RR	20	OSS
Amphorellopsis acuta	66	QR	66	OSS
Amphorellopsis sp.	2	RR	2	OSS
Codonella sp.	19	R	160	OSS
Codonellopsis schabi	126	QC	444	NES
Codonellopsis sp.	34	QR	35	NES
Dadayiella ganymedes	4	RR	8	OSS
Dadayiella sp.	1	RR	2	OSS
Dictyocysta elegans	50	QR	28	OSS
Dictyocysta lepida	9	RR	12	OSS
Eutintinnus apertus	95	QC	604	OSS
Eutintinnus fraknoi	100	QC	208	OSS
Eutintinnus lusus-undae	68	QR	380	OSS
Eutintinnus stramentus	16	R	28	OSS
Eutintinnus tubulosus	96	QC	650	OSS
Eutintinnus sp.	17	R	208	OSS
Favella adriatica	4	RR	4	NES
Favella sp.	6	RR	24	NES
Helicostomella subulata	67	QR	972	NES
Leprotintinnus nordqvisti	11	R	10	NES
Metacylis joergenseni	39	QR	48	NES
Metacylis sp.	79	QR	512	NES
Protorhabdonella curta	2	RR	10	OSS
Protorhabdonella sp.	7	RR	4	OSS
Rhabdonella spiralis	2	RR	6	OSS
Salpingella decurtata	149	QC	156	OSS
Salpingella rotundata	194	QC	1280	OSS
Salpingella sp.	98	QC	120	OSS
Steenstrupiella steenstrupii	16	R	24	OSS
Steenstrupiella sp.	1	RR	2	OSS
Stenosemella nivalis	429	C	4476	NES
Stenosemella ventricosa	212	C	360	NES
Tintinnopsis angulata	5	RR	38	NES
Tintinnopsis beroidea	122	QC	208	NES
Tintinnopsis campanula	63	QR	32	NES
Tintinnopsis compressa	42	QR	324	NES
Tintinnopsis cylindrica	46	QR	46	NES
Tintinnopsis nana	196	QC	254	NES
Tintinnopsis parvula	71	QR	72	NES
Tintinnopsis radix	88	QC	48	NES
Tintinnopsis spp.	115	QC	900	NES
Undella subcaudata var. *acuta*	8	RR	32	OSS
Choreotrichida unid.	180	QC	670	

Table 3. Occurrence of each tintinnid species recorded in the studied period (1998-2016) in the Gulf of Trieste. Black squares indicate that the species was present at least once in the corresponding year. White squares indicate the absence of the species in those years.

	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Stenosemella nivalis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Stenosemella ventricosa	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Tintinnopsis beroidea	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Salpingella rotundata	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■
Eutintinnus tubulosus	■	■	■	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■
Tintinnopsis campanula	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	□
Tintinnopsis nana	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	■	■	■	■
Eutintinnus fraknoi	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	□	■	■
Salpingella decurtata	■	■	□	■	□	■	■	■	■	■	■	■	■	■	□	□	■	■	■
Codonellopsis schabi	■	□	□	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	□
Eutintinnus apertus	□	□	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■
Eutintinnus lusus-undae	■	■	■	■	■	■	□	■	■	■	■	□	■	■	■	■	■	■	□
Helicostomella subulata	■	□	□	■	■	■	■	□	□	■	■	■	■	■	■	■	■	■	■
Tintinnopsis cylindrica	■	■	□	■	□	□	■	□	□	■	■	■	■	■	■	■	■	■	□
Dictyocysta elegans	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■
Tintinnopsis compressa	■	■	□	□	■	■	■	■	□	■	■	■	■	■	□	□	□	■	■
Tintinnopsis parvula	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	□	□	□	□
Tintinnopsis radix	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	□	□	□	□
Metacylis joergenseni	■	■	■	■	□	■	■	■	■	■	■	■	□	□	■	□	■	□	□
Amphorellopsis acuta	□	□	□	□	■	■	□	■	□	■	■	□	□	■	■	■	■	■	□
Steenstrupiella steenstrupii	□	□	□	□	■	□	□	□	■	□	■	□	□	□	■	■	■	■	■
Acanthostomella conicoides	□	□	□	■	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■
Amphorides laackmanni	□	□	□	□	□	□	□	□	□	□	■	□	□	■	■	■	■	■	□
Undella subcaudata var. *acuta*	□	□	□	□	□	□	■	□	□	■	■	□	□	□	□	■	□	■	□
Amphorides quadrilineata	■	□	□	□	□	□	■	□	□	□	□	□	□	□	□	□	□	■	■
Dadayiella ganymedes	□	□	□	□	■	□	□	□	□	□	□	□	■	■	□	□	■	□	□
Eutintinnus stramentus	□	□	□	□	□	□	□	□	□	■	■	□	■	□	□	□	□	■	□
Leprotintinnus nordqvisti	□	□	□	■	□	□	□	□	□	■	■	■	□	□	□	□	□	□	□
Tintinnopsis angulata	□	■	□	□	□	□	□	□	□	□	□	■	■	□	□	□	□	□	□
Amphorides cfr. *amphora*	□	□	□	□	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□
Favella adriatica	□	□	□	□	□	□	□	□	□	□	■	□	□	□	□	■	□	□	□
Protorhabdonella curta	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Rhabdonella spiralis	□	□	□	□	□	□	■	□	□	□	□	□	□	□	□	□	□	□	■
Dictyocysta lepida	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	□
Number of detected species	20	18	14	19	18	18	19	17	13	22	24	18	21	18	17	19	19	22	16

ways < 12 from January to April, while it was > 25 from August to November (Figure 6). The Shannon Index (H') exhibited the same trend with values between 0.18 and 1.24 (Figure 6).

The pair-wise test showed a significant difference (*p*-perm < 0.05) in the community composition during the year. A significant pattern in composition similarity was detected and the highest similarity values were detected between contiguous months (Suppl. material 3).

The most representative species showed different abundance trends. *Stenosemella nivalis* reached the maximum average abundance (109 ± 537 ind. L^{-1}) in February while *S. ventricosa* in November (22 ± 65 ind. L^{-1}); both species presented the lowest values in August (< 2 ind. L^{-1}). The most common *Tintinnopsis* species showed the maxima in

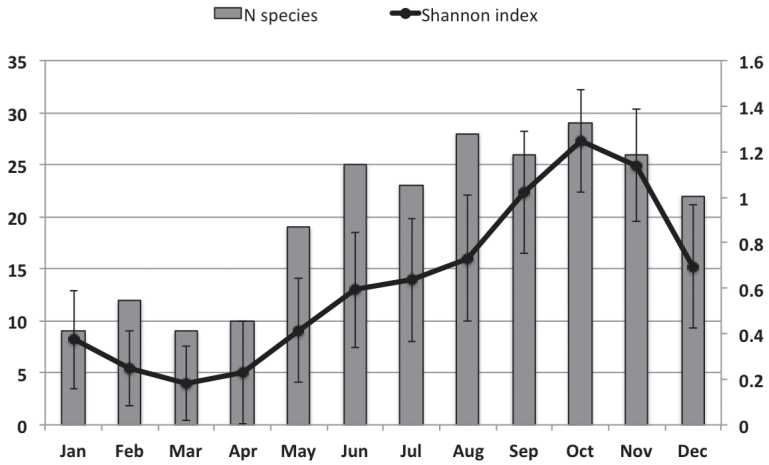

Figure 6. Seasonal patterns of tintinnid species richness (N species, left axis) and diversity index (Shannon Index + stdev, right axis) in the period from July 1998 to July 2016.

autumn. In particular, the data of the abundances integrated in the water column for *T. nana*, reached the average maximum value of 23 ± 43 ind. L^{-1} in October, followed by 11 ± 25 ind. L^{-1} in November. *Codonellopsis schabi* was present only from September to January reaching the maximum in November (16 ± 51 ind. L^{-1}) (Suppl. material 4). The hyaline genera presented the maximum average abundance from June to October. In this period, the two most common *Salpingella* species (*S. decurtata* and *S. rotundata*) showed a different trend. *Salpingella rotundata* reached the maximum average abundance in July (27 ± 143 ind. L^{-1}) while *S. decurtata* in October (8 ± 19 ind. L^{-1}). The genus *Eutintinnus* presented maxima in June or July. In particular, the most frequent species (*E. apertus*, *E. fraknoi* and *E. tubulosus*) showed maximum average abundance with values around 10 ind. L^{-1} in spring and summer (Suppl. material 4).

The results of IndVal test are synthesized in Table 4 where only the species which correlated significantly ($p < 0.05$) with each season are reported. These results confirm the seasonal pattern registered for the abundance. In particular, *S. nivalis* is the only species which significantly correlated to winter. All the species presenting high and significant IndVal values in summer are hyaline species. The majority of the species with high IndVal values in autumn are agglutinated species.

The tintinnid seasonal trend was strictly related to temperature and salinity values (PERMANOVA: pseudo-f = 1.1158 $p < 0.05$ for temperature and pseudo-f = 1.1531 $p < 0.01$ for salinity). The dbRDA plot highlighted the distribution of the samples on the base of the similarity in tintinnid composition and the two abiotic variables taken into consideration (Figure 7). The samples collected during winter and those collected during summer were antipodal distributed on the dbRDA plot, while the samples collected during spring and autumn showed a higher variability with respect to both salinity and temperature (Figure 7A).The total variation explained by dbRDA axes showed values of 6.8% for axis 1 and 0.3% for axis 2; the significance of axes

Table 4. List of tintinnid taxa and significant IndVal for each season; taxa are ordered by season and decreasing IndVal values. aut=autumn; spr=spring; sum=summer; win=winter.

	Season	IndVal	*p*-value
Codonellopsis schabi	aut	0.4307	0.001
Tintinnopsis nana	aut	0.3280	0.001
Stenosemella ventricosa	aut	0.2200	0.001
Tintinnopsis spp.	aut	0.2008	0.001
Tintinnopsis radix	aut	0.1933	0.001
Tintinnopsis beroidea	aut	0.1572	0.001
Dictyocysta elegans	aut	0.1410	0.001
Codonellopsis sp.	aut	0.1092	0.001
Tintinnopsis campanula	aut	0.1024	0.001
Salpingella decurtata	aut	0.0839	0.01
Tintinnopsis parvula	aut	0.0796	0.001
Amphorides quadrilineata	aut	0.0410	0.002
Amphorides laackmanni	aut	0.0319	0.001
Leprotintinnus nordqvisti	aut	0.0262	0.002
Eutintinnus tubulosus	spr	0.0912	0.008
Helicostomella subulata	spr	0.0642	0.024
Tintinnopsis compressa	spr	0.0623	0.002
Tintinnopsis cylindrica	spr	0.0413	0.003
Acanthostomella conicoides	spr	0.0361	0.022
Codonella sp.	spr	0.0273	0.012
Tintinnopsis angulata	spr	0.0150	0.029
Salpingella rotundata	sum	0.3105	0.001
Eutintinnus apertus	sum	0.1823	0.001
Metacylis sp.	sum	0.1076	0.001
Metacylis joergenseni	sum	0.1065	0.001
Eutintinnus fraknoi	sum	0.1020	0.001
Eutintinnus lusus-undae	sum	0.1006	0.001
Amphorellopsis acuta	sum	0.1002	0.001
Salpingella sp.	sum	0.0860	0.001
Undella subcaudata var. *acuta*	sum	0.0162	0.04
Favella sp.	sum	0.0132	0.044
Stenosemella nivalis	win	0.2882	0.001

and considered variables were tested via ANOVA.CCA ($p < 0.01$). The influence of temperature and salinity was also tested for each species. In Table S5 (Suppl. material 5), the Spearman's correlation values are presented. *S. nivalis* occurred with high abundance in the winter samples highlighting a clear negative correlation with temperature (Spearman value = -0.439) (Figure 7B). A strong positive correlation with temperature (0.34) was found for *S. rotundata* (Figure 7C) that reached the highest abundance in the summer samples. *Eutintinnus tubulosus* showed a weak but significant correlation with temperature (0.17) (Figure 7D) while *C. schabi* did not have any correlation with abiotic variables (Figure 7E). The Spearman's correlation values highlighted that salinity was negatively correlated with the majority of species and positively correlated only with *S. nivalis*.

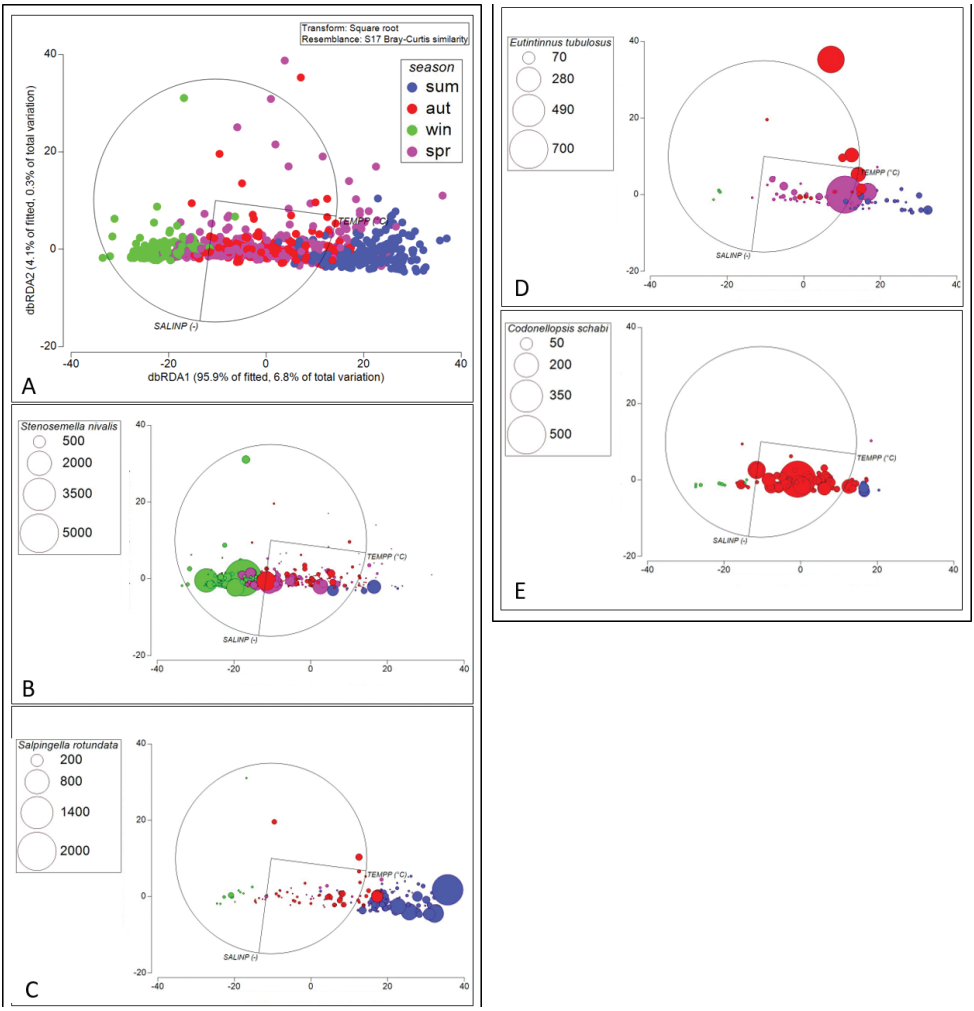

Figure 7. dbRDA (distance bases Redundancy Analysis) plot on the base of similarity in tintinnid composition constrained by temperature and salinity with axis significances (**A**). The colours represent samples collected in different seasons (blue=summer, red=autumn, green=winter, violet=spring). In the bubble plots (**B–E**), the bubble scale reports the relationship between bubble diameters and abundance measured for the most representative species: **B** *Stenosemella nivalis* **C** *Salpingella rotundata* **D** *Eutintinnus tubulosus* **E** *Codonellopsis schabi*.

Discussion

This study revealed that, in the Gulf of Trieste, tintinnid abundance integrated within the water column decreased remarkably after the beginning of the year 2000, followed by a continuous but slow recovery starting from the year 2007. This pattern matched that of phytoplankton biomass observed in the same area during the period 2001–2007, characterised by the reduction of river runoff, increase of surface salinity and decreasing concentration of nitrate and silicate (Giani et al. 2012, Mozetič et al.

2010, 2012). The reduction of river supply was due to a long period of drought, which can be foreseen as frequent in the near future due to the ongoing climatic changes.

The total tintinnid abundances were similar to those recorded in the same area by previous studies but higher to the rest of the Adriatic Sea. In the period 1986–1990, at the same station, but only for the surface, the tintinnid abundance reached a maximum value of about 1000 ind. L^{-1} (Monti et al. 2012). Fonda Umani et al. (2005a) registered values always below 200 ind. L^{-1} at 9 stations in the northern Adriatic Sea. In the middle Adriatic Sea, Bojanić et al. (2012) found values of about 100 cells L^{-1} at a station in the Split Channel, and Vidjak et al. (2009) reported a maximum of 3.3 ind. L^{-1} at a station along the Krka River estuary. In the open waters of the south Adriatic Sea, Kršinić and Grbec (2006) found values with a maximum of 33 ind. L^{-1}. The higher abundance values found in the Gulf of Trieste, compared to the middle and south Adriatic, reflect the patterns of the other plankton community compartments in the area, which are characterised by high standing stock but low diversity (Fonda Umani 1996).

The total tintinnid abundance, integrated within the water column, showed relevant inter-annual fluctuations characterised by autumn maxima and, only in few cases, summer peaks. All the maxima, both in summer and in autumn, were due to blooms of *S. nivalis* and other few species. The isolated abundance peaks of some neritic species, such as *S. nivalis*, might be explained by the seasonal recruitment from cysts, as consequence of particular environmental conditions (Kamiyama 2013). The dense lorica of *Stenosemella* genus, agglutinated with mineral particles, may in fact allow the species belonging to this genus to rapidly sink to the bottom, where they can lie as cysts (Capriulo et al. 1982). The excystment of these resting stages can play an important role in recruiting new tintinnid populations in the water column (Paranjape 1987). Very unfortunately, this hypothesis could not be tested in our study because cysts were not considered in the microscopic analyses. In June 2015 (15 m) and July 2015 (5 m), the high abundances were due to *Helicostomella subulata* and *S. rotundata*, respectively. The abundance peaks of some oceanic species, such as *S. rotundata*, can be explained by the advection of water masses from the middle and south to the northern Adriatic (Kršinić 2010b). In fact, *S. rotundata* is primarily distributed in the surface layer of the Otranto Strait and in the open waters of the south Adriatic (Kršinić 2010b). Conversely, *H. subulata* is considered an estuarine species very rare or absent in the open waters of south and middle Adriatic (Kršinić 2010b). This species was quite rare in our samples and only occasionally bloomed in spring. *Helicostomella subulata* was reported as abundant during the warmer months in the south-east part of the Gulf of Trieste by Lipej (1992), and along the Adriatic coast in relation to river inputs (Kršinić 1987, Bojanić et al. 2012) or in proximity to harbors (Moscatello et al. 2004).

The seasonal pattern registered in this study differed between agglutinated and hyaline species. Tintinnids build the lorica according to the material available in the water column and the two lorica types, in general, correspond to different habitats: agglutinated lorica to coastal environments and hyaline lorica to open waters. In our time series, the open sea species were more numerous (27) than the neritic ones (20). The former were more abundant in summer, in agreement with the worldwide pattern

of hyaline loricae dominating the communities in summer and agglutinated loricae in winter (Dolan and Pierce 2013). Species with agglutinated loricae may be better adapted to turbulent conditions, which are more frequent in the winter season (Dolan and Pierce 2013). In the Adriatic Sea, agglutinated species dominate along the coastal areas while hyaline species characterise the open sea areas of middle and southern Adriatic (Kršinić and Precali 1997). The presence of oceanic species in the Northern Adriatic Sea can therefore be connected to the advection of higher salinity waters from the middle Adriatic.

At station C1, we found the four most widely distributed and reported species in the world: *A. quadrilineata*, *D. ganymedes*, *S. steenstrupii* and *E. apertus* (Dolan and Pierce 2013). However, with the exception of *E. apertus*, they were rare or very rare in the Gulf of Trieste. On the contrary, *S. nivalis*, *S. ventricosa* and *T. beroidea* were recorded in all the years considered. These species are common in the whole Mediterranean Sea (Dolan 2000) and they are often dominant in coastal tintinnid communities (Fonda Umani et al. 2010). In the Gulf of Trieste, they can be considered as keystone species and their presence and fluctuation in abundance must be considered an important signal of possible changes in the whole plankton community. In a study conducted from June 1999 to July 2002, *S. nivalis* and *T. nana* were present at 9 stations in 3 transects across the northern and central Adriatic Sea (Fonda Umani et al. 2005a). The southern transect was characterised by *D. ganymedes*, *E. lusus-undae*, *X. longicaudata* and *R. spiralis*, typical of warmer and salty waters. In our study, *R. spiralis* and *D. ganymedes* were recorded only in few years and can be considered rare species. Their presence in the northern part of the Gulf of Trieste can suggest a possible previous water intrusion from southern areas.

In our study, tintinnids were represented by 19 genera and 35 species. This number of taxa agreed with other studies within the same area. Lipej (1992) found 30 species in the southeast part of the Gulf of Trieste, along the Slovenian coast, from 1989 to 1991. Kršinić (2010b) found 50 species for all the North Adriatic Sea, with a large number of oceanic species (30). In the period 1998–2010, Monti et al. (2012) recorded 40 species in the surface samples of the same site. The lower number of species in the present study in comparison to Monti et al. (2012) can be justified by the revision of taxonomy that we applied more recently to our dataset following Kršinić (2010a). As indicated in Methods, we also shifted to the genus level some species that were very rare and/or with identification problems, which caused a reduction of species number. In comparison to other time series of tintinnids in coastal areas of the Mediterranean Sea, the number of species we found in this study was generally lower. For example, in the Northern Ionian Sea, Sitran et al. (2007) examined the tintinnid community at a fixed station for two years (2003–2004) and found 79 species belonging to 23 genera. At a station in the Southern Tyrrhenian Sea, the same authors (Sitran et al. 2009) found 67 species belonging to 22 genera. At a coastal site in the Gulf of Naples, 57 species were identified in 4 years (Modigh and Castaldo 2002), but only seven accounted for 81% of total numbers (*T. minuta*, *T. beroidea*, *Metacylis annulifera*, *E. tubulosus*, *H. subulata*, *S. curta* and *S. decurtata*). The lower number of tintinnids species found in this study, in comparison to other areas of the Mediterranean Sea, reflects the reduction in the

number of species for all planktonic compartments observed in the Gulf of Trieste due to the extreme variability of the environmental conditions of the area (Ingrosso et al. 2016). The hydrodynamic features of the Gulf of Trieste are mostly controlled by pulses of external inputs (e.g., terrigenous material, fresh waters, middle Adriatic water advection), that may alter the phytoplankton successions (Cataletto et al. 1995, Fonda Umani 1996). In particular, the high and unpredictable variability of the river inputs cause remarkable variability in salinity values (from 33 to 38) (Malačič et al. 2006) and nutrient concentrations (e.g., from 2 to 84.2 μmol L^{-1} for N/NO_3, and from 0.05 to > 3 μmol L^{-1} for P/PO_4) (Cataletto et al. 1995). Ample is also the range of sea surface temperature that shows a seasonal cycle from winter minima of 6°C to summer maxima of >29°C (Celio et al. 2006).

Generally, the lowest species richness and diversity in the Mediterranean tintinnid assemblages is recorded in summer months, reflecting the seasonal minimum of primary production (Dolan and Pierce 2013). In contrast, in our study, the lowest species richness occurred in winter (8–12 species) probably due to the extreme cold temperature and high salinity in this season (Ingrosso et al. 2016). The highest species richness values were detected in the autumn, in accordance with other studies in costal and confined areas. Bojanić et al. (2012) detected higher numbers of tintinnid species and high diversity (H' = 0.42–2.71) at a costal Adriatic station, between September and November. In the southern Adriatic Sea (from January 1980 to May 1990), Kršinić and Grbec (2006) detected the highest number in October. For the Ionian Sea the highest value of diversity index (H' = 1.3–2.7) for tintinnids was found in September and November (Sitran et al. 2007).

In our study, the differences in the vertical distribution of tintinnid composition were not significant, as reported for similar depths by Sitran et al. (2007). However, we observed significant differences in the relative abundance of the most common species among the different depths. The water column in the Gulf of Trieste, as already stated, is subject to strong variations in temperature, salinity, and vertical stratification during the year (Malačič and Petelin 2001). During spring, the freshwater input and surface heating cause the thermohaline stratification, which increases in strength during summer. In autumn and winter, convective and mechanical mixing, induced by water cooling and wind, disrupts the vertical stratification leading to a mostly homogeneous water column. Contrary to our expectation, the increase of vertical stability in summer did not enhance the vertical differences in abundance of the key species. This result may suggest that tintinnids abundances are more linked to the food availability, i.e. phytoplankton at the surface and re-suspended organic matter at the bottom.

The seasonal pattern of tintinnid species can be strictly related to temperature and salinity. Tintinnid diversity appeared to be positively linked to salinity at a coastal station in the Ionian Sea (Sitran et al. 2007) and along the Krka river estuary (Vidjak et al. 2009), whilst Dolan and Gallegos (2001) highlighted how tintinnid diversity in the estuarine environment was high and increased with decreasing salinity. The difference among the results of these authors may depend on the environmental factors that characterize the different studied areas. Temperature dependent distribution of different tintinnid species has been reported in the middle Adriatic (Bojanić et al. 2012) as

well as in other areas (Abboud-Abi Saab 1989, Koray and Őzel 1983, Posta 1963). In our work, we found that temperature showed direct or inverse correlation according to species, while salinity was negatively correlated with the majority of species. These results suggest that temperature may be more relevant than salinity in shaping the tintinnid community. The abundance of most tintinnids was related to temperature, but the particular response of each tintinnid to the environmental factors was species specific, as found by Kamiyama and Tsujino (1996) in Hiroshima Bay. In our work, the variation in temperature correlation was strictly related to the species seasonality and consequently to their presence and abundance. Therefore, the expected global increase in temperature could have, in the future, an important effect on the tintinnid community in the Gulf of Trieste.

Conclusions

This paper presented the characteristics of tintinnid assemblages along an 18-year period at a coastal site of the Gulf of Trieste that belongs to the Italian LTER (Long Term Ecological Research) network. Our long-term study has highlighted clear seasonal patterns and large interannual fluctuations of diversity and abundance. Three species appeared to be as keystone species for their persistent occurrence and relevant abundance and can be used to monitor the long-term evolution of the whole microzooplankton community in the Gulf of Trieste. The lack of significant differences among the community composition and relative abundance between 5 and 10 meter depths may suggest to reduce the sampling effort to the surface and bottom depths to monitor the tintinnid assemblages in this shallow marine area.

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Supplementary material 1

Table S1. Result of pair-wise test on factor year

Authors: Marina Monti-Birkenmeier, Tommaso Diociaiuti, Serena Fonda Umani

Data type: statistical data

Explanation note: The years: 1999–2000; 1999–2001; 2003–2005; 2005–2006; 2008–2010; 2012–2014, did not show significant difference

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Link: https://doi.org/10.3897/natureconservation.34.29841.suppl1

Supplementary material 2

Table S2. Occurrence for each tintinnid species recorded in the studied period (1998–2016) in the Gulf of Trieste at each sampling depth (A, surface; B, 5 m; C, 10 m; D, 15 m)

Authors: Marina Monti-Birkenmeier, Tommaso Diociaiuti, Serena Fonda Umani

Data type: occurence

Explanation note: Black squares indicate that the species was present at least once in the corresponding year. White squares indicate the absence of the species in those years.

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Link: https://doi.org/10.3897/natureconservation.34.29841.suppl2

Supplementary material 3

Table S3. Result of pair-wise test on factor month

Authors: Marina Monti-Birkenmeier, Tommaso Diociaiuti, Serena Fonda Umani

Data type: statistical data

Explanation note: The months: Dec-Jan; Dec-Feb; Jan-Feb; Jan-Mar; Feb-Mar, did not show significant differences.

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Link: https://doi.org/10.3897/natureconservation.34.29841.suppl3

Supplementary material 4

Figure S4. Abundance trend of the most representative agglutinated species: *S. nivalis, S. ventricosa*, *T. nana*, *C. schabi* and hyaline species: *S. rotundata*, *S. decurtata*, *E. fraknoi*, *E. apertus*

Authors: Marina Monti-Birkenmeier, Tommaso Diociaiuti, Serena Fonda Umani

Data type: abundance

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Link: https://doi.org/10.3897/natureconservation.34.29841.suppl4

Supplementary material 5

Table S5. Sperman's correlation values with temperature and salinity for each tintinnid taxon detected in the studied period

Authors: Marina Monti-Birkenmeier, Tommaso Diociaiuti, Serena Fonda Umani

Data type: statistical data

Explanation note: in red the significative correlation $p < 0.05$

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RESEARCH ARTICLE

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Microbial processing of sedimentary organic matter at a shallow LTER site in the northern Adriatic Sea: an 8-year case study

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Abstract

Benthic prokaryotes are the key-players in C-cycling at the sediment-seawater interface, one of the largest biologically active interfaces on Earth. Here, microbial-mediated processes, such as the degradation of organic matter and the incorporation of mobilized C into microbial biomass, depend on several factors such as environmental temperature and substrate availability, especially in shallow sediments at mid-high latitudes where seasonal fluctuations of these variables occur. In the present study, four degradative activities (β-glucosidase, lipase, chitinase and aminopeptidase), Heterotrophic C Production (HCP), Total Organic C (TOC), Total Nitrogen (TN) and Biopolymeric C (BPC) were investigated seasonally from April 2010 to April 2018 in the surface sediments of a shallow Long-Term Ecological Research (LTER) station of the northern Adriatic Sea. Significant temperature-dependences were described by Arrhenius-type equations for HCP and each of the degradative activities tested with the exception of aminopeptidase. The relatively low apparent Activation Energies suggested that these microbial-mediated processes were enhanced by the availability of palatable substrates over the study period. Nevertheless, a clear and tight dependence from such substrates was detected only for aminopeptidase, the most pronounced degradative activity observed.

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TN was identified by the stepwise multiple regression analysis as the environmental variable that mainly drove this exoenzymatic activity. Enhanced aminopeptidase rates mirrored peaks of TN that seemed, in turn, linked to the seasonal proliferation of benthic microalgae. By supplying prokaryotes with promptly available substrates, these autotrophs, represented mainly by diatoms, seemed to play an important role in the C-cycling regulation at the studied LTER station.

Keywords

Benthic prokaryotes; exoenzymatic activities; Heterotrophic C Production; temperature; Total Nitrogen; Long-Term Ecological Research; LTER

Introduction

Collectively termed prokaryotes, bacteria and archaea play a pivotal role in C-cycling and geochemical processes as the main elements responsible for the degradation of organic matter and the incorporation of mobilized C into microbial biomass. In the marine environment, the majority of organic matter is, in fact, in a polymerized form that cannot be assimilated directly by the prokaryotic cells since only small molecules (<600 Da), such as aminoacids and monosaccharides, can pass through the membrane due to the activity of permeases. In order to make the organic matter available, prokaryotes induce the production of non-cytoplasmatic enzymes that hydrolyze polymers and oligomers into assimilable monomers (Chróst 1992). Although these active molecules can be cell-bound or cell-free (Baltar 2018), in the present study both kinds are inclusively referred to as exoenzymes. Their targets are specific chemical bonds that link the subunits that form organic polymers (i.e. sugars, peptides, nucleotides, structural and storage polysaccharides). The end-up products of exoenzymatic reactions are low molecular weight compounds that are ultimately incorporated into prokaryotic cells and used for growth.

Although both exoenzymatic activities and organic matter uptake (Heterotrophic C Production – HCP) are known for being influenced by several environmental variables (e.g. temperature, pH, salinity, availability of substrates, etc.), it is difficult to discriminate between the influence exerted by each of them and to evaluate any synergistic or antagonistic effect. Among the several variables that may influence microbial dynamics, the role of temperature and substrate availability aroused the interest of scientists for years (Pomeroy and Wiebe 2001, Calvo-Díaz et al. 2014, Lønborg et al. 2016). While some studies investigated only the effect of temperature on microbial C uptake (e.g. Lønborg et al. 2016), others tried to relate the observed microbial metabolic changes to both of them. For instance, Calvo-Díaz and co-authors (2014) measured bacterial production over two annual cycles in temperate coastal waters (southern Gulf of Biscay) and detected an overall switch from a more pronounced control exerted by resource availability in summer-early autumn samplings to a more prominent effect of temperature during winter and spring. Overall, these kinds of studies highlighted a variable susceptibility of bacterioplankton to both temperature and substrate availability, making quite difficult the extrapolation of any general description about their interaction (Pomeroy and Wiebe 2001).

To date, the majority of investigations pertaining to this issue have been carried out on bacterioplankton while organic matter processing in the sediments remained

almost neglected, notwithstanding some exceptions in freshwater (Zoppini and Marxsen 2010, Pohlon et al. 2013, Hill et al. 2017) and estuarine environments (Middelburg et al. 1996; Danovaro et al. 2002). The general lack of studies focusing on marine sediments represents an important knowledge gap because the sediment-water interface not only represents one of the largest interfaces on Earth (Middelburg 2018), but it is also the site where all physicochemical and biological processes become more intensive (Frankowski and Bolałek 1999). It functions, in fact, as a key interface in the Earth system since the processes that take place there determine whether organisms' remains are recycled within the biosphere (short-term cycle) or transferred to the geosphere (long-term cycle) (Middelburg 2018). Microbial-mediated processes in the sediments acquire, therefore, a very important role from a biogeochemical point of view. This highlights the need to study the potential influence of temperature and substrate availability/composition on benthic microbial degradative activities and C uptake. The issue is particularly pressing nowadays because understanding microbial dynamics dependence from temperature and substrates may help to detect any biogeochemical alteration due to large-scale, Climate Change-related events.

In the present study, the rates of HCP and of four benthic exoenzymatic activities (β-glucosidase, lipase, chitinase and leucine aminopeptidase) were measured over a time span of eight years (2010–2018) in order to untangle whether the bottom temperature and organic matter amount and composition acted as forcing factors on microbial metabolism and to what extent. The study was carried out at the sediment-water interface of the shallow coastal station C1 (northern Adriatic Sea), which is part of the Long Term Ecological Research-LTER network. The investigation of the same station over time allowed us to discard any attribution of any observed metabolic variation to environmental variables different from temperature and organic matter, as variations of the bathymetry and of the sediment grain size.

Materials and methods

Study site

The Gulf of Trieste is the northernmost part of the Adriatic Sea with a surface area of about 600 km^2 (Olivotti et al. 1986). Almost completely landlocked, this shallow basin (average depth of 17 m, Celio et al. 2002) is isolated from the rest of the Adriatic by a sill (~22 m depth) between Grado and the Salvore peninsula (Ogorelec et al. 1991). Freshwater inputs, which come mainly from the Isonzo River, show a high interannual variability (Comici and Bussani 2007) which affects salinity whose values range from 29 to 38 at the surface (Malačič et al. 2006). The Gulf experiences annual fluctuations of temperature (from 5 °C to ≥ 24 °C at the surface and from 6 °C to ≥ 20 °C at the bottom) and the water column is usually stratified during summer. A high variability of the water column profile, due to both riverine outflow and water temperature, is enhanced by an alternation between strong cold winds from the east-north east (i.e. Bora)

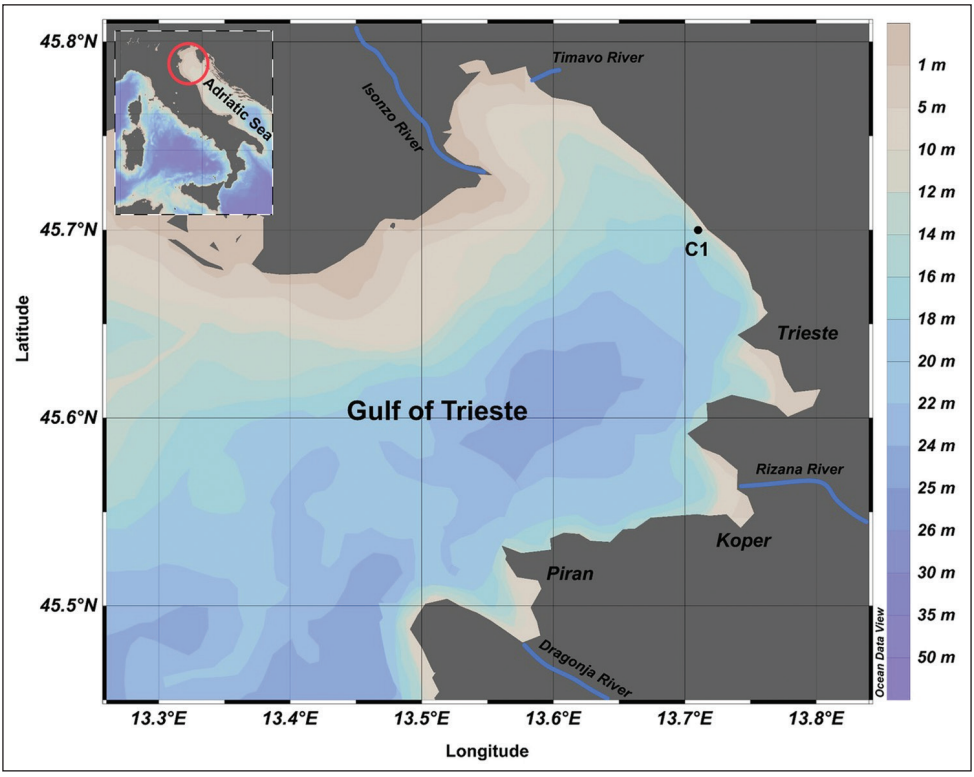

Figure 1. Location of the St. C1 (45°42'03"N, 13°42'36"E). This LTER site is nearby the outer border of the Marine Protected Area of Miramare, along the Italian coastline of the Gulf of Trieste (northern Adriatic Sea).

and mild winds from the south (i.e. Scirocco) (Lipizer et al. 2011). Sedimentation is mainly controlled by river inputs rather than by marine currents (Brambati and Catani 1988). The annual average sedimentation rate is about 1 mm yr^{-1} in the middle of the gulf and increases to 2.5 mm yr^{-1} in front of the Isonzo mouth (Covelli et al. 1999 and references therein). The sediments are mainly sandy-mud although soft bottoms can vary from sands with patches of rocks to detrital mud (Brambati and Catani 1988).

The study was carried out at the station LTER-C1, located ca. 200 m offshore (45°42'03"N, 13°42'36"E) at a depth of around 18 m, nearby the outer border of the Marine Protected Area (MPA) of Miramare (Figure 1). This small MPA is divided into two distinct zones: the inner part (30 ha), subjected to a regime of integral protection (i.e. all human activities are banned with the exception of a little corridor for diving) and a surrounding buffer zone (90 ha), sheltered by boats and professional fishing.

Sampling

From April 2010 to April 2018, sampling campaigns were performed every 3–4 months. During each sampling, the bottom sea temperature was measured using a Seabird 19 Plus-

Seacat probe. The bottom water was sampled by means of a 5-L Niskin bottle. Three virtually undisturbed sediment cores were taken by a KC Haps bottom corer (KC-Denmark, Silkeborg, Denmark) using polycarbonate sample tubes (13.3 cm I.D. resulting in a sampling surface of 127 cm^2). The sediments were collected within an experimental area of max 40 m^2. Once in the laboratory, the cores were partially extruded and the oxic sediment layer (0–1 cm ca) of each core was collected, homogenised and subsampled for all the analyses.

Total Organic C (TOC), Total Nitrogen (TN) and Biopolymeric C (BPC)

For TOC and TN analyses, sediment was freeze-dried, grounded in a ceramic mortar and then sieved on a 250 μm iron steel sieve (Endecotts LTD, UK). Triplicate subsamples of about 8–12 mg were weighed on a microultrabalance with an accuracy of 0.1 μg, directly into silver and tin capsules for TOC and TN, respectively. Before TOC determination, subsamples were treated directly into capsules with increasing concentrations of HCl (0.1 N and 1 N) to remove the carbonate fraction (Nieuwenhuize et al. 1994). Carbon and nitrogen contents were determined using a CHNO-S elemental analyzer (mod. ECS 4010, Costech, Italy) according to Pella and Colombo (1973). Standard acetanilide (Costech, purity ≥ 99.5%) were used to calibrate the instrument and empty capsules were also analyzed in order to correct for blank. Quality control of measurements was performed using internal standards and it was also verified for carbon against the certified marine sediment reference material PACS-2 (National Research Council Canada). The relative standard deviations for three replicates determination were lower than 3%. TOC and TN concentrations are expressed as mg C g^{-1} d.w. and mg N g^{-1} d.w., respectively.

For BPC analyses, subsamples of homogenised sediment were freeze-dried and processed in triplicates for the determination of carbohydrates, lipids and proteins. Colloidal and EDTA extractable carbohydrates (CHO-H_2O and CHO-EDTA, respectively) were analyzed following the method described by Blasutto et al. (2005). Lipids (LIP) were analyzed according to Bligh and Dyer (1959) while proteins (PRT) were determined following Hartree (1972). The concentrations of CHO, PRT and LIP were expressed as glucose, bovine serum albumin and tripalmitine equivalents, respectively. Data were converted to carbon equivalents using the conversion factors proposed by Fichez (1991): 0.45 for CHO, 0.50 for PRT and 0.75 g C g^{-1} for LIP.

Heterotrophic C Production (HCP)

Heterotrophic C Production (HCP) rates were carried out following the method of ^{3}H-leucine uptake for sediment samples (van Duyl and Kop 1994, as detailed by Franzo et al. 2016). Each sediment sample (0.2 mL of 1:1 v/v slurry) was added to 6 μCi of ^{3}H-leucine (Perkin Elmer) and incubated in the dark for 1 h at *in situ* temperature. After incubation, radiotracer incorporation was stopped by adding 80% ethanol (1.7 mL). After two washes of the samples with ethanol (80%) by mixing, centrifuging and supernatant removal, the sediment was transferred with ethanol (80%) onto

a polycarbonate filter (0.2 µm mesh size). Subsequently, the filters were washed twice with 5% trichloroacetic acid. Samples were heated in 2M NaOH for 2 h in a water bath at 100 °C, cooled on ice and centrifuged at 425 g for 3 minutes. One mL of supernatant was transferred to scintillation vials and 10 mL of Hionic Fluor (Perkin Elmer) scintillation fluid was added. For each sample, three replicates and two ethanol-treated blanks were analyzed. Activity in the samples was determined by a β-counter (Packard TRI-CARB 2900TR Liquid Scintillation Analyzer).

Exoenzymatic activities

Extracellular enzymatic activities were assayed using fluorogenic substrate analogues (Hoppe 1993) derived from 7-amino-4-methyl-coumarin (AMC) and 4-methyl-umbelliferone (MUF). Leucine aminopeptidase activity was assayed as the hydrolysis rate of leucine-AMC. β-glucosidase, lipase and chitinase were assayed using MUF-β-D-glucoside, MUF-oleate and MUF-*N*-acetyl-β-D-glucosaminide, respectively. Enzyme activities were expressed in terms of the rate of MUF or AMC production. Sediment slurries were prepared by adding 6 mL of 0.2 µm-filtered bottom water to 0.5 g of wet sediment. After the evaluation of saturating concentrations performed during the first sampling, hydrolysis rates were measured by incubating slurries with (final concentrations) 800-µM MUF-β-D-glucoside, 400-µM leucine-AMC and 200-µM MUF-oleate and MUF-*N*-acetyl-β-D-glucosaminide (Sigma) for 1 h in the dark and at *in situ* temperature. Before spectrofluorometric measurement, each sample was centrifuged 2 min at 1008 *g*. Fluorescence increase due to MUF and AMC hydrolysed from the model substrates was measured using a Shimadtzu RF-1501 spectrofluorometer (MUF = 365-nm excitation and 455-nm emission; AMC = 380-nm excitation and 440-nm emission). Standard solutions of MUF and AMC were used to produce calibration curves with 0.2 µm-filtered bottom water. Triplicate blanks without fluorogenic substrate were used to determine the natural fluorescence increase in the samples not attributable to the tested enzymes.

Arrhenius plots

The Arrhenius-type relationship (linearity of a natural logarithm vs inverse absolute temperature) between temperature and microbial activities was tested assuming that chemical kinetics controls the observed rates. According to the Arrhenius law, the temperature sensitivity of each exoenzymatic activity and of HCP is defined by:

$$\text{Microbial Activity} = A \cdot e^{-Ea/R*T}$$

where A is the theoretical microbial activity in the absence of the Ea; Ea is the Activation Energy, i.e. the energy barrier to be surpassed in order for the reaction to take place (in J mol^{-1}); *R* is the universal gas constant (8.314 J mol^{-1} K^{-1}); *T* is the temperature in Kelvin (K). The factor $e^{-Ea}/R \cdot T$ is proportional to the fraction of substrate molecules

with kinetic energies in excess of Ea (Arrhenius 1889). An estimate of Ea can be derived from the slope of an Arrhenius plot, which is built on the natural logarithm of each microbial activity against the inverse absolute temperature (1/T). The Ea can thereafter be calculated by multiplying the regression slope by *R*. Strictly, the Arrhenius law should be applied to a well-defined enzymatic reaction with a constant Ea and with temperature as the only factor affecting the rate. Since the organic matter consists of myriads of compounds degraded by a wide variety of microbial populations, the temperature response measured with this approach is actually the sum of all processes involved. Therefore, the calculated Ea should be seen as an apparent Ea (Middelburg et al. 1996).

Linear regression analyses were used for the Arrhenius plots. Prior to regressions, normality was checked and the confidence level was set at 95% using STATISTICA7.

Statistical analyses

A stepwise multiple regression analysis was performed in order to establish the relationship between microbial activities (HCP, β-glucosidase, lipase, chitinase and leucine-aminopeptidase) and environmental variables (temperature, TOC, TN, CHO-H_2O, CHO-EDTA, PRT and LIP). All non-normally distributed data series were log(10)-transformed before running the analysis.

In order to highlight any overall temporal pattern of microbial activities at the station LTER-C1, a cluster analysis was performed on HCP, β-glucosidase, lipase, chitinase and leucine aminopeptidase rates. The Euclidean distance and the complete linkage were applied and data were log10(x+1) transformed and normalized prior to analysis. Afterwards, the Mann-Whitney test was applied in order to test for significant differences of all microbial and environmental variables among the cluster groups. Only statistically significant results are presented.

The Spearman-Rank correlation analysis was performed to test the linear relationship between TOC and TN. Correlation coefficients (r) were considered significant at *p*-values less than 0.05.

All statistical analyses were performed using STATISTICA7 with the exception of the cluster analysis that was conducted with PRIMER software v5 (PRIMER-E Ldt, Plymouth, UK).

Results

Ranging between 7.5 °C in Mar12 and 22 °C in Jul16 (Table 1), the bottom temperature followed a seasonal pattern throughout the 8-year study, with higher values during summer samplings (June-September) and colder conditions during winter-early spring (December-March). TN concentrations varied between 1.11 ± 0.04 and 1.76 ± 0.06 mg N g^{-1} (Dec16 and Feb11, respectively) while TOC ranged from 9.85 ± 0.23 to 18.09 ± 0.14 mg C g^{-1} (in Dec16 and Jul10, respectively). Although both variables showed a certain temporal variability, overall higher amounts characterized sum-

Table 1. Environmental variables measured at the sea bottom (Temp = temperature) or in sediment surface layer. CHO-H2O =colloidal carbohydrates; CHO-EDTA = Carbohydrates extracted in EDTA; PRT = proteins; LIP = lipids; n.a. = not available. With the exception of Temp, values are averages of 3 replicates.

	Temp	CHO-H2O			CHO-EDTA			PRT			LIP		
	°C	μg C g^{-1}			μg C g^{-1}			μg C g^{-1}			μg C g^{-1}		
Apr10	11.18	70.10	±	1.56	149.92	±	6.93	557.49	±	29.55	626.36	±	30.02
Jul10	16.53	27.48	±	1.10	170.18	±	2.77	627.84	±	26.79	1069.42	±	43.39
Nov10	15.50	71.27	±	2.19	141.35	±	4.26	590.21	±	24.23	937.49	±	51.83
Feb11	8.71	43.04	±	0.13	184.81	±	5.85	426.90	±	15.75	859.93	±	33.14
Jun11	15.12	95.04	±	3.52	285.17	±	17.05	957.12	±	13.33	1192.15	±	8.77
Sep11	19.42	85.24	±	3.67	219.45	±	5.95	888.92	±	38.09	1328.65	±	2.08
Dec11	12.72	60.08	±	1.49	206.73	±	6.51	881.94	±	11.34	976.34	±	23.38
Mar12	7.47	85.14	±	5.31	97.01	±	5.07	1109.63	±	26.85	835.08	±	6.09
Jul12	19.13	85.45	±	5.23	347.14	±	3.90	1123.10	±	27.13	1134.46	±	54.83
Nov12	15.00	n.a.			n.a.			n.a.			n.a.		
Feb13	9.00	108.25	±	4.67	140.14	±	1.96	512.84	±	20.86	769.26	±	31.29
Apr13	9.50	164.02	±	2.36	267.04	±	16.11	639.92	±	29.61	959.88	±	44.41
Nov13	16.60	110.82	±	4.75	312.71	±	15.07	637.75	±	17.55	956.62	±	26.33
Apr14	13.50	112.60	±	8.82	177.39	±	8.13	574.66	±	13.30	861.99	±	19.96
Jul14	18.20	115.73	±	3.12	93.83	±	4.45	573.84	±	28.98	860.75	±	43.47
Mar15	10.00	87.49	±	2.19	225.99	±	12.70	691.42	±	13.71	823.81	±	50.11
Jun15	19.50	144.25	±	0.35	274.63	±	10.11	444.93	±	19.48	954.55	±	55.66
Oct15	17.40	76.13	±	2.14	253.43	±	1.35	613.61	±	10.57	748.53	±	26.94
Jan16	9.45	93.06	±	3.36	267.29	±	6.12	322.10	±	18.81	1067.77	±	30.71
Apr16	13.10	102.60	±	1.16	308.67	±	19.61	316.30	±	14.38	662.53	±	30.14
Jul16	22.00	129.87	±	2.10	325.18	±	8.49	462.49	±	30.21	921.30	±	34.09
Oct16	18.70	119.41	±	6.30	94.38	±	2.53	419.14	±	23.58	705.92	±	37.81
Dec16	13.00	47.67	±	2.08	274.07	±	4.33	231.85	±	7.90	447.87	±	78.07
Apr17	11.40	139.45	±	4.51	437.44	±	21.64	321.30	±	16.38	686.25	±	39.41
Jul17	17.70	147.92	±	1.59	420.28	±	1.10	470.87	±	24.20	479.54	±	30.24
Oct17	19.20	96.40	±	2.49	411.50	±	6.10	385.77	±	0.90	581.62	±	22.67
Jan18	10.90	80.76	±	0.59	386.35	±	11.10	295.40	±	10.21	759.16	±	55.64
Apr18	10.20	133.16	±	2.41	482.11	±	20.69	511.04	±	40.92	822.06	±	22.47

mer samplings while lower ones were observed mainly during winter (Figure 2). With concentrations between 27.48 ± 1.10 (Jul10) and 164.02 ± 2.35 μg C g^{-1} (Apr13), colloidal carbohydrates (CHO-H_2O) represented the minor fraction of the BPC pool throughout the entire study (Table 1). While minima of carbohydrates extracted in EDTA were comparable to CHO-H_2O (~100 μg C g^{-1}), overall this biopolymeric fraction showed more elevated concentrations, especially from April 2017. During the last five samplings (2017–2018), in fact, CHO-EDTA accounted for values >386.35 ± 11.10 μg C g^{-1} reaching the highest concentrations of the whole study period (Table 1). Representing on average 31% of the BPC pool, proteins were the second dominant fraction after lipids. Notwithstanding, proteinaceus material was clearly more abundant in 2011–2012 samplings while overall lower amounts were observed from January 2016 (Table 1). Lipids accounted on average for 48% of BPC and the values ranged from 447.87 ± 78.08 to 1328.65 ± 2.08 μg C g^{-1} in Dec16 and Sep12, respectively (Table 1). A clear seasonal pattern, e.g. higher values during summer and lower ones during winter, was observed for none of the four biopolymeric fractions considered.

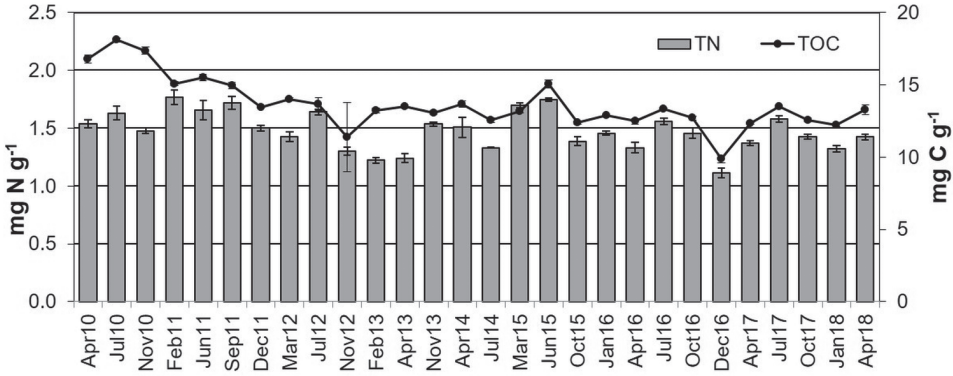

Figure 2. Mean concentrations of TN and TOC (±SD) over the 8-year study.

Minima of HCP rates characterized winter samplings (Nov12 and Dec16) while maxima were measured in spring and early-summer (Apr16 and June15) (Figure 3a). The degradation of polysaccharides was more pronounced in summer and early-autumn samplings. This was more evident in Jun11 and Sep11 when the fastest hydrolytic rates were measured (~17 nmol cm^{-3} h^{-1}) while, throughout the entire study, velocities > 5 nmol cm^{-3} h^{-1} were observed in June, July or October with the exception of Apr13 (Figure 3b). With an average rate of ~4 nmol cm^{-3} h^{-1}, the degradation of lipids was the less pronounced microbial activity in the sediments of the station LTER-C1 during the 8-year study. Notwithstanding, similarly to β-glucosidase, the rates were >5 nmol cm^{-3} h^{-1} only in June, July, September and October and reached peaks equal to 16.94 ± 1.43 and 12.18 ± 0.85 nmol cm^{-3} h^{-1} in Jul12 and Jun15. On the contrary, values lower than 1 nmol cm^{-3} h^{-1} were measured in Mar15, Apr16 and Jan18 (Figure 3c). Focusing on the degradation of chitin, two evident maxima were observed in Jun11 (32.99 ± 0.81 nmol cm^{-3} h^{-1}) and in Jul17 (16.95 ± 0.33 nmol cm^{-3} h^{-1}). Similarly to the hydrolytic activities described above, chitinase rates resulted >5 nmol cm^{-3} h^{-1} in June, July, September and October while lower values characterized the remaining samplings (Figure 3d). In the present study, the highest hydrolitic activity was addressed to the degradation of polypeptides since the measured rates resulted in at least 1 order of magnitude faster than those ascribed to the other exoenzymes considered. With the exclusion of the highest value measured in Jun11 (568.59 ± 24.14 nmol cm^{-3} h^{-1}), summer and early-autumn samplings showed velocities comparable with those observed during early-spring (March and April) (Figure 3e).

Significant temperature-dependences were described by Arrhenius-type equations for HCP and all the degradative activities tested with the exception of leucine-aminopeptidase (Figure 4). The calculated Ea was lower for HCP rates (29.27 kJ mol^{-1}) and overall higher for the degradation of the main macromolecules according to the following order: chitin (55.61 kJ mol^{-1}), polysaccharides (63.99 kJ mol^{-1}) and lipids (75.21 kJ mol^{-1}).

The stepwise multiple regression analysis confirmed the influence of the bottom temperature on both the microbial C uptake and the majority of the tested exoenzymatic activities. The only exception was represented by the aminopeptidase since it was

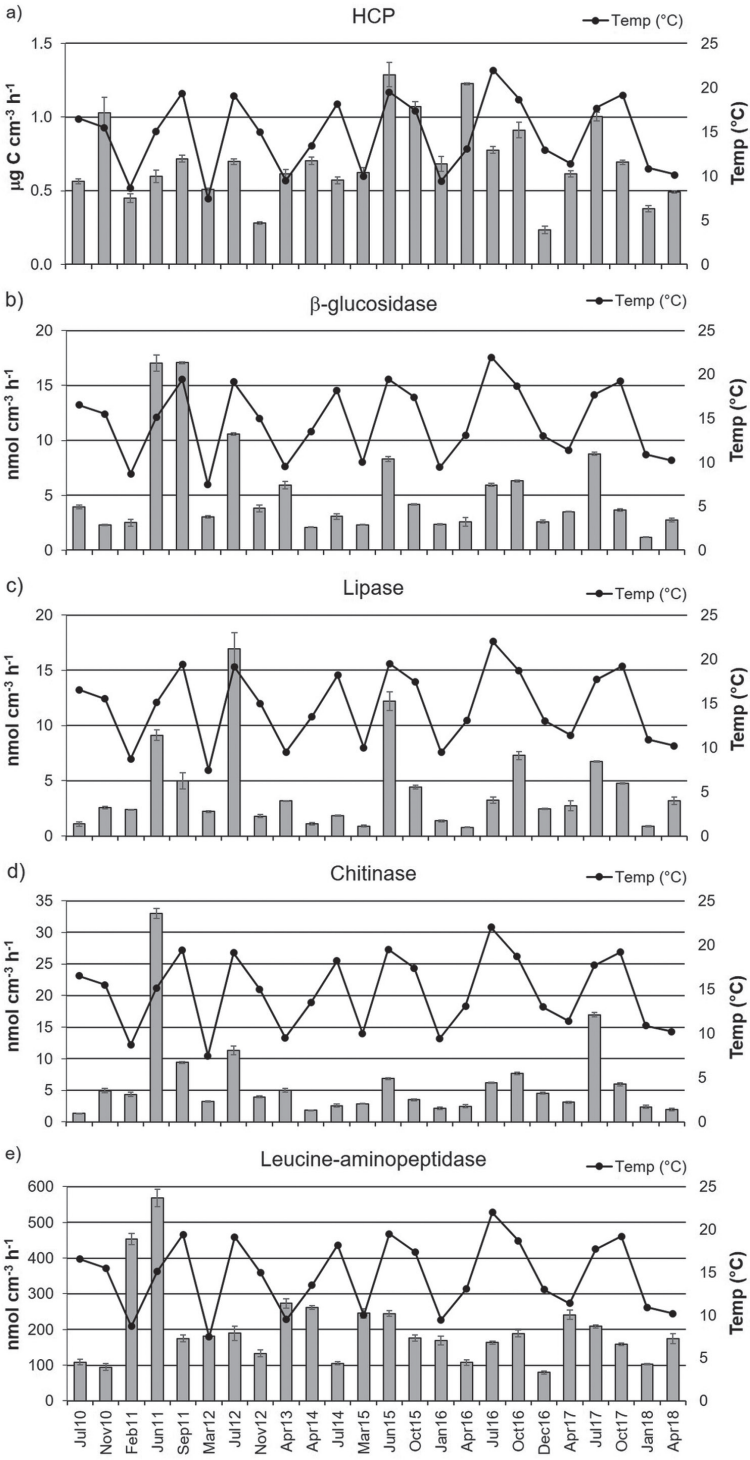

Figure 3. Rates of the tested microbial activities from July 2010 to April 2018.

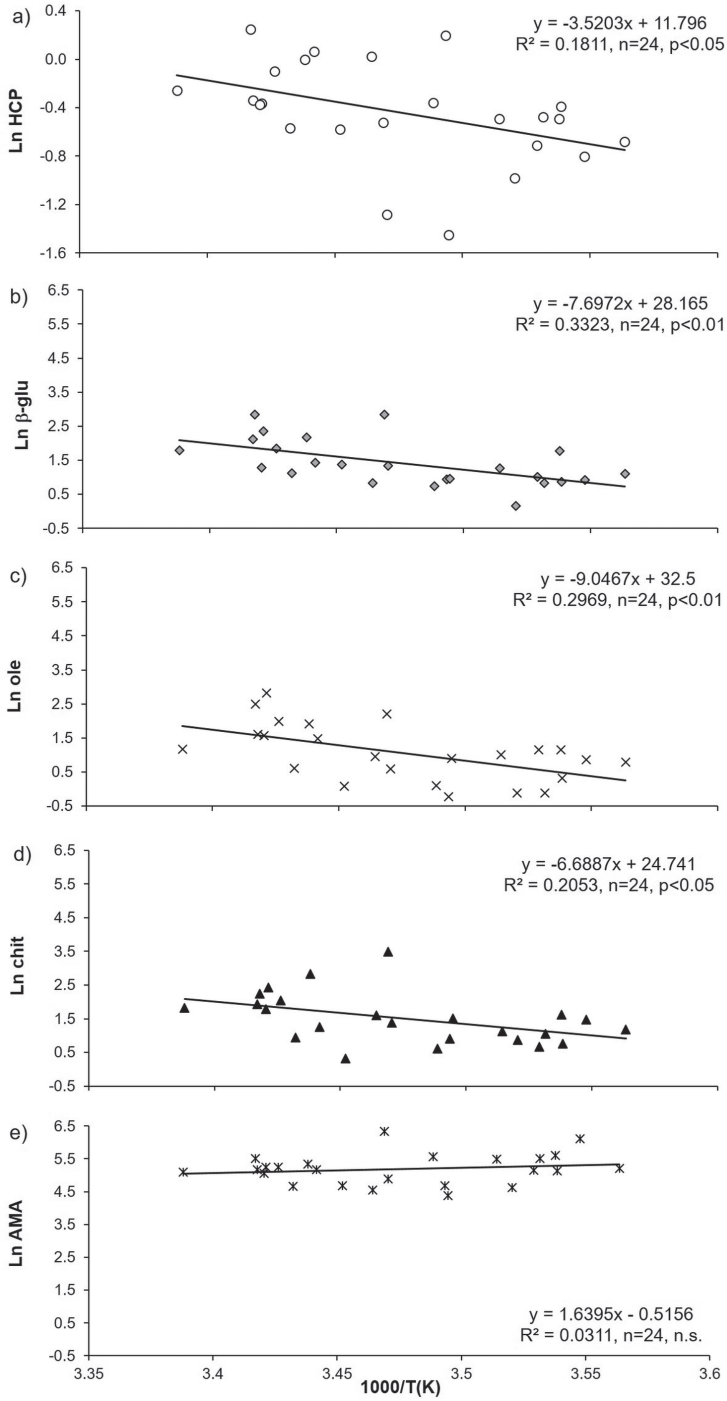

Figure 4. Arrhenius plots of the natural logarithm of each microbial activity against the inverse absolute temperature (1/T). HCP = Heterotrophic C Production (**a**); β-glu = β-glucosidase (**b**); ole = lipase (**c**); chit = chitinase (**d**); AMA = leucine aminopeptidase (**e**); n.s.= not significant.

Table 2. Stepwise multiple regression analysis between microbial activities and environmental variables (*p<0.05; **p<0.01; ***p<0.001). HCP = Heterotrophic C Production; AMA = Leucine aminopeptidase activity; Temp = bottom temperature; TOC = Total Organic C; TN = Total Nitrogen; CHO-H2O =colloidal carbohydrates; CHO-EDTA = carbohydrates extracted in EDTA; LIP = Lipids; PRT = proteins.

	F	Adj r^2	n	Variables
HCP	3.410*	0.30	23	Temp* CHO-H2O TOC LIP
β-glucosidase	6.115**	0.54	23	Temp** PRT CHO-H2O LIP CHO-EDTA
Lipase	5.584**	0.38	23	Temp** PRT* CHO-EDTA
Chitinase	3.275*	0.24	23	Temp* PRT CHO-EDTA
AMA	8.359***	0.50	23	TN*** CHO-H2O* Temp*

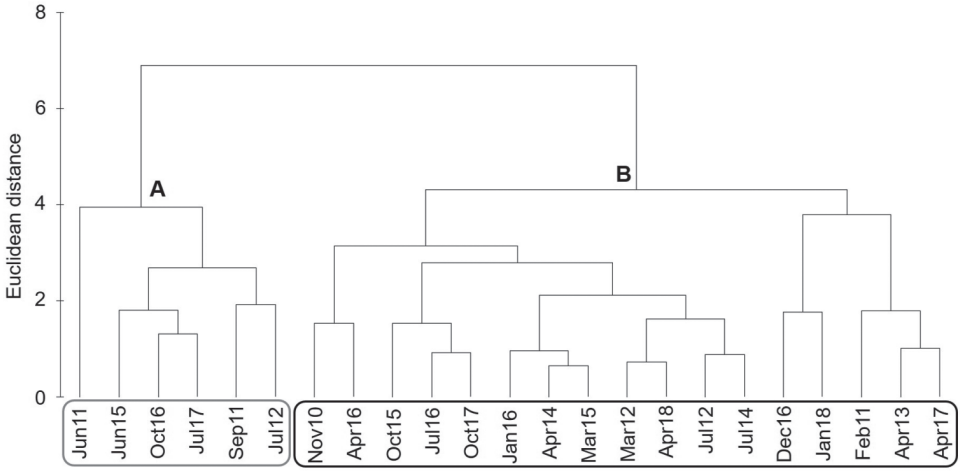

Figure 5. Cluster analysis performed on the tested microbial activities. Euclidean distance and the complete linkage were applied.

explained for 50% by N-containing material, expressed in terms of TN, and, to a lesser extent, by colloidal carbohydrates and bottom temperature (Table 2).

The cluster analysis performed on the tested microbial activities clearly separated the majority of summer and early-autumn samplings (Group A) from the remaining observations (Group B; Figure 5). The Mann-Whitney test pointed out that group A

gathered samplings with significantly faster rates of β-glucosidase, lipase and chitinase ($Z=3.57$, $p<0.001$) than those belonging to group B. On the contrary, HCP and protease velocities were not statistically different between the two groups identified. Focusing on the environmental variables, the Mann-Whitney test indicated that only the bottom temperature ($Z=2.52$, $p<0.05$) and TN ($Z=2.45$, $p<0.05$) significantly differed between A and B, i.e. higher values for the former group and lower ones for the latter.

Discussion

Over a time span of eight years (2010–2018), five benthic microbial processes - i.e. HCP, β-glucosidase, lipase, chitinase and aminopeptidase - were related to the temporal fluctuations of the bottom temperature and to the variable amount and composition of sedimentary organic matter. Overall, the fastest rates of both organic matter degradation and microbial growth were observed in warmer conditions while a limited C reworking was detected at lower temperatures. This is a general pattern already observed in the water column (Arnosti et al. 2011, Calvo-Díaz et al. 2014) and at the sediment-water interface (Perliński and Mudryk 2018). Arnosti et al. (2011) suggested the existence of a latitudinal gradient for the degradation of marine dissolved organic carbon: the warm superficial waters of tropical and subtropical areas showed faster degradative rates than their cold counterpart at higher latitudes. Focusing on the effects of temperature temporal fluctuations on microbial metabolism, Perliński and Mudryk (2018) measured the highest hydrolytic activities during the vegetation period (spring, summer and autumn) at the sediment-water interface along a coastal river estuary. Similarly, a pronounced seasonal variability was reported in the water column also at St. C1, with winter minima and maxima from April to October for six exoenzymatic activities measured on a monthly basis between 2000 and 2005 (Celussi and Del Negro 2012). As shown by our data, analogous empirical evidences characterized also the sediments of this site and were statistically validated by the Mann-Whitney outputs and by the cluster analysis. Throughout the study period, in fact, summer and early-autumn samplings, characterized by overall faster microbial activities (group A), gave results that were significantly separated from those in group B, which gathered samplings with overall low (e.g. Nov12 and Dec16) and intermediate velocities (i.e. Apr13, Marc15 and Apr17). Furthermore, the significant correlations between the bottom temperature and the majority of the tested processes pointed out in the Arrhenius plots supported the dependence of the microbial metabolism from this environmental variable.

The Arrhenius plots describe a single microbial-mediated reaction that is performed on a homogeneous substrate in terms of composition and bioavailability. Undoubtedly this is an ideal situation that does not occur in natural environments since the bulk of organic material is composed by a wide plethora of molecules, each characterized by a dissimilar composition and, therefore, by a different degree of complexity (Burdige 2006). Notwithstanding, in the present study the outputs of the stepwise correlation analysis confirmed the dependence of the microbial metabolism from the bottom tem-

perature for all the tested activities. While very little is known about marine sediments, temperature sensitivity of organic matter degradation by microbes has been studied extensively in soils (Davison and Janssens 2006; Davison et al. 2012; Sierra 2012). The carbon quality temperature hypothesis (CQT) is based on the principles of enzyme kinetics combined with the Arrhenius law. According to CQT, substrate quality can be defined by changes in Ea, i.e. the more recalcitrant the substrate, the higher its corresponding Ea (Sierra 2012). In the present study, Ea values (29.27- 75.21 kJ mol^{-1}) were similar to those calculated for organic matter degradation in marine sediments (Middelburg et al. 1996) and for heterotrophic prokaryotic production in the epipelagic and the mesopelagic (Lønborg et al. 2016). Although the highest Ea measured during this 8-year study implies a somewhat more recalcitrant nature of sedimentary lipids, this value was lower than that proposed for the degradation of actual recalcitrant organic matter in marine sediments (~ 200 kJ mol^{-1}; Burdige 2011), suggesting that at St. C1 microbial-accessible substrates were available to benthic prokaryotes over the entire study period. The Ea calculated for HCP further corroborated this hypothesis. Its value was, in fact, clearly lower than the theoretical Ea of 62.7 kJ mol^{-1}, which indicates the energy for the overall heterotrophic metabolism if the temperature were the only factor involved. The consequent implication is that environmental factors other than temperature likely influenced prokaryotic C uptake at St. C1 (e.g. the organic matter pool) over the entire study period (Brown et al. 2004; López-Urrutia and Morán 2007). During this 8-year time span, benthic prokaryotes likely benefited from favorable conditions of substrate availability that may have supported the observed HCP rates by lowering the energy required for activating this process.

In the present study, the degradation of proteins was the only exoenzymatic activity that resulted weakly temperature-dependent, as indicated by the lack of significance in the corresponding Arrhenius plot. On the other hand, this hydrolytic activity resulted mainly related to the substrate availability, expressed in terms of TN. In coastal sediments, this pool comprises a wide spectrum of inorganic and organic compounds, with the former fraction mainly represented by fixed ammonium ions entrapped in illite crystal structure (Schubert and Calvert 2001). At St. C1, this inorganic fraction cannot be excluded since its amount was reported to not exceed ~20% of the whole pool (Faganeli et al. 1991). Nevertheless, TOC (wt %) and TN (wt %) resulted tightly correlated (n=28, r=0.67; $p<1\times10^{-5}$) as reported also by Rumolo et al. (2011) in the harbour of Naples (Southern Tyrrhenian Sea). As inferred by these authors, this strong correlation allows us to reasonably assume that TN concentrations measured at St. C1 were mainly organic (Rumolo et al. 2011).

In the Gulf of Trieste, a preferential decomposition of organic nitrogen and total phosphorous with respect to organic carbon was suggested by Faganeli et al. (1991) as a result of a stable isotope study on marine sediments. The stepwise correlation analysis in the present study further confirmed this overall pattern since TN was indicated as the environmental driver that mainly influenced the most pronounced hydrolytic activity observed, i.e. the degradation of polypeptides. Despite the lack of significant correlation between aminopeptidase and bottom temperature, also this exoenzymatic

activity showed an overall temporal pattern with faster rates in summer samplings. This pattern mirrored mainly seasonal TN fluctuations rather than temperature, posing the question about the possible environmental drivers responsible for TN temporal variability. In coastal areas where the euphotic zone extends to the sediment-water interface, benthic microalgae are known for producing readily-usable organic carbon (Welker et al. 2002 and references therein). Accordingly, Hardison et al. (2013) demonstrated in a mesocosm study that microphytobenthos, and in particular benthic diatoms, determined a sensible increase of both TN and sediment organic matter (SOM) not only in terms of amount but also of lability. The latter was investigated by means of a suite of Specific Total Hydrolysable Amino Acids (THAA) and Phospholipids Linked Fatty Acids (PLFA) for which it was possible to ascribe the respective major organic matter sources. During the experiment time-course, the authors observed that the increase of TN and SOM was mainly due to the PLFA attributable to diatoms, demonstrating therefore the role of these microalgae in contributing to the lability of the organic carbon pool. Furthermore, the observed increase of PLFA concentrations of bacterial origin indicated that diatom proliferation supported the heterotrophic bacterial community, highlighting an important link between these two assemblages. The shallow depth of St. C1 allows the solar radiation to reach the seafloor and, consequently, the growth of an active and abundant community of microalgae is promoted (Welker et al. 2002, Cibic et al. 2007). Seasonal peaks of abundance during summer have been already reported and ascribed to favorable conditions of irradiance and temperature (Welker et al. 2002, Cibic et al. 2012). Considering the period 2010–2018, such peaks of abundance have been documented during the first two years (Franzo et al. 2016) and were observed also afterwards (Cibic, personal communication). The proliferation of benthic microalgae may have contributed not only to the observed TN peaks but also to an increased lability of the substrates fueling, therefore, microbial-mediated processes and in particular those oriented to the degradation of polypeptides. The future implementation of the study of microbial metabolism with microphytobenthic data would allow the exploration of the interactions between these two benthic assemblages mediated by palatable organic material of photosynthetic origin.

Conclusions

Over a time span relatively long for data of microbial-mediated processes, this study allowed us to understand how two environmental factors – i.e. temperature and substrate availability – determine the microbial C-cycling in temperate shallow marine sediments. Similar to the soils and the water column, a clear dependence of prokaryotic activities from temperature was confirmed at the sediment-water interface, a biologically active interface largely neglected despite its ecological importance.

Overall, relatively low Ea values were measured, suggesting that the availability of microbial-accessible substrates to prokaryotes was not negligible throughout the study period. In particular, the presence of palatable substrates likely lowered the Ea required

for HCP, resulting, in fact, in a value clearly lower than that theoretically required if the temperature were the only factor involved.

Nevertheless, a clear and tight dependence from the substrate availability was detected only for aminopeptidase, the most pronounced degradative activity observed. Identified as the substrate that mainly drove the degradation of polypeptides, TN varied temporally, posing therefore the question on what caused such fluctuations. Known for increasing TN concentration and bioavailability, benthic microalgae are suggested here as the best candidates. Future implementations with detailed macromolecular fingerprinting of sedimentary organic matter would allow the identification of the substrates preferentially used by benthic prokaryotes and to link them definitely to their probable microalgal origin.

Our 8-year dataset demonstrated to be sufficiently robust to obtain a glimpse about the effects of temperature and substrates on shallow microbial metabolism. According to the LTER philosophy, its continuation over the years deserves to be promoted because only in a long-term time span the functional-microbial perspective would contribute in detecting possible biogeochemical deviations, especially in consideration of current global challenges as Climate Change-related events. The membership of St. C1 to LTER-Italy represents an added value for our current and future results because the network will magnify their sharing and dissemination, in accordance with the perspective of Open Science.

Acknowledgements

Being St. C1 part of the LTER national and international networks (LTER-Italy, LTER-Europe, ILTER), this study has been carried out in the context of LTER initiatives. The authors would like to thank C. Comici, C. De Vittor and M. Kralj for analytical support and the three reviewers for their constructive comments.

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Interannual variability of vertical particle fluxes in the Ross Sea (Antarctica)

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Abstract

This study presents new data on biogenic and terrigenous particle fluxes collected by an oceanographic mooring (Mooring A) deployed in the south-western Ross Sea (Antarctica) in the frame of the Italian Long-Term Ecological Research network (LTER-Italy). Results from the years 2005 and 2008 document high mass fluxes during the summer and early autumn seasons, not coincident with the algal bloom. Downward particle fluxes exhibit a high inter-annual variability of both particulate fluxes and composition that seem related to the different factors as the phytoplankton increases, occurring between the beginning of February and the end of March, to the variations in the sea ice extent and to the resuspension and/or lateral advection processes. The flux variability may have been influenced by Iceberg B-15 that resided in the investigated area between 2000 and 2005. The decoupling of biogenic silica and organic carbon cycles is documented by differences in the rates of their respective key processes: biogenic silica dissolution and organic carbon degradation.

Keywords

Particle flux, sediment trap, mooring, Antarctica, Ross Sea, LTER

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Introduction

The Ross Sea is the region with the highest values of primary production and carbon sequestration through the biological pump in the Southern Ocean (Arrigo et al. 2008a; Hoppe et al. 2017). It represents a key area for investigating changes in primary productivity and their relationship with sea ice cover, physical and chemical parameters of the water column and the availability of nutrients as the iron supply. All these parameters affect the phytoplankton blooms, the zooplankton grazing and, consequently, both retention and export of carbon within the water column and at the seafloor (Frignani et al. 2000). The export of carbon from the surface to the deep ocean via the sinking of particulate organic matter is a major process of the ocean carbon cycle and a key factor in the regulation of the atmospheric CO_2 level (Siegenthaler et al. 2005).

The study of particles collected by automated sediment traps provides an important tool for establishing how and when environmental and biological factors modulate export of the organic matter produced at the surface. The export fluxes change within and between ocean regions as a function of primary productivity and temperature (Laws et al. 2000) and vary inter-annually (Ducklow et al. 2008; Church et al. 2013). However, the processes governing the export from the surface to the deep sea and its efficiency are still poorly known, especially in the Southern Ocean (Takahashi et al. 2009). Previous sediment trap data from Antarctic and Arctic seas suggested a close temporal coupling between the spring-summer phytoplankton blooms and high particle flux (Collier et al. 2000; Anadon and Estrada 2002; Fischer et al. 2002; Arrigo et al. 2008b; Ducklow et al. 2008, 2015; Honjo et al. 2010; Forest et al. 2011; Sampei et al. 2012; Weston et al. 2013).

The aim of this research was to increase the current knowledge of the seasonal and inter-annual variability of vertical particle fluxes in the Ross Sea and to understand the factors influencing the quantity and the quality of the trapped material. For this purpose, we analysed the sediment trap samples collected at two different depths (360 and 770 m) from the Mooring site A during 2005 and 2008. This site is part of the Italian Long-Term Ecological Research network (LTER-Italy) and provides a suite of data essential for investigating the biogeochemical cycles in the south-western Ross Sea.

The present study also allowed us to record the Ross Sea particle flux variability during and after the residence of Iceberg B-15, which occurred near the investigated area between 2000 and 2005.

This large (230 × 80 km^2) iceberg with a draft of 300 m calved from the Ross Ice Shelf near Ross Island on 21 March 2000 and broke into two parts (B-15A and B-15B) on 10 May 2000. B-15B exited the Ross Sea in 2000, while B-15A was transported by currents and was pinned between Ross and Franklin Islands for a period of five years (Arrigo and van Dijken 2004). Icebergs may disturb regional ocean processes for several years with important impacts on physical oceanography (Robinson and Williams 2012) and the local ecosystem (Arrigo et al. 2002; Seibel and Dierssen 2003). Recording and understanding the spatial and temporal variability of downward particle fluxes in relation to the iceberg presence is of great interest, particularly when considering the present climate warming scenarios.

Study area

The Ross Sea is located in the Pacific sector of the Southern Ocean, between Cape Colbeck at 158°W and Cape Adare at 170°E. On the southern side, it is bounded by the Ross Ice Shelf, at around 78.5°S, which extends over nearly half the continental shelf and is about 250 m thick on its northernmost side (Budillon et al. 2002). The Ross Sea is characterised by a wide and deep continental shelf with a mean depth of 500 m. The circulation on the Ross Sea shelf is rather complex and variations in physical properties along the seawater column determine a vertical structure from the surface to the bottom.

Mooring A is located in a quite flat area characterised by high biogenic fluxes through the water column (Langone et al. 2003) and by weak currents at the bottom flowing from Terra Nova Bay (TNB) polynya (Mangoni et al. 2017). In this region, the long-lasting seasonal ice cover inhibits fine sediment and organic matter accumulation on the sea-floor (Langone et al. 1998). Here currents are quite weak, allowing large particles to sink in close proximity (< 20 km) to the production area (Jaeger et al. 1996; Frignani et al. 2000). As a consequence, high primary productivity typically leads to high accumulation of biogenic silica in nearby sediments (Jaeger et al. 1996).

Diatom blooms occur abundantly in the Ross Sea (Smith and Nelson 1985; Leventer and Dunbar 1996) and, when ice melts, these blooms border the marginal ice zone for most of the austral summer (Ravaioli et al. 1999). Investigations performed in this area report that diatoms are the dominant primary producers and their fluxes are characterised by both seasonal and interannual variability (Leventer and Dunbar 1996) principally linked to changes in the water current regime (Frignani et al. 2000).

The area has been affected by the presence of Iceberg B-15 between 2000 and 2005 and reopened in the year 2005 when the Iceberg moved (Manzoni 2006).

Materials and methods

Data collection

Mooring A (https://deims.org/86b6465c-b604-4efa-9145-0805f62216f4) was deployed in 1991 in the south-western region of the Ross Sea polynya, between Franklin Island and Ross Island (Fig. 1), at 76°41'S latitude and 169°02'E longitude at 832 m water depth, to monitor the oceanographic regime and the accumulation rate in this region. It was active until year 2010 and was managed through collaboration between Italian and American scientific institutions.

Mooring A was equipped with two time-series McLane Labs PARFLUX sediment traps, coupled with an Aanderaa RCM9 single point current meter and an SBE 16 SeaCat conductivity and temperature recorder at about 360 and 770 m water depths, respectively. The upper level trap allowed the measurement of the export from the pro-

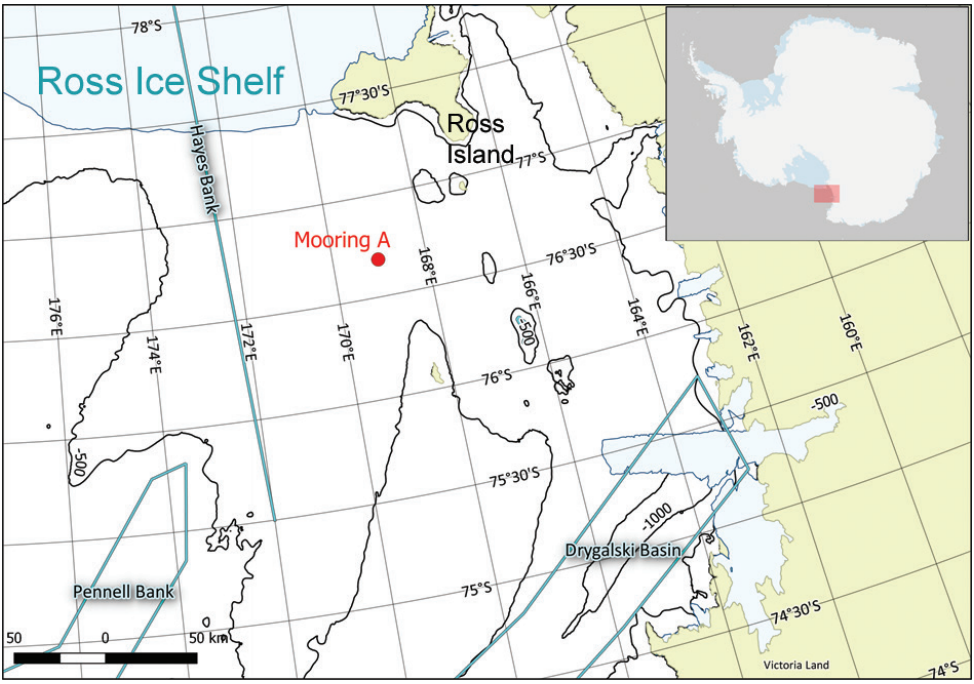

Figure 1. Position of Mooring A in the south-western Ross Sea. The bathymetric contour levels were produced using the dataset provided by the International Bathymetric Chart of the Southern Ocean, IBCSO (Arndt et al. 2013) and the QGIS 2.14 software package.

ductive layer of the water column while the near-sea bottom trap provided data about the water-sediment interface fluxes. The depth of the top trap was selected in order to avoid possible damages caused by the passage of Iceberg B-15 (2000–2005), which caused the loss of an instrument array during previous studies.

In 2005, both sediment traps were equipped with 21 collection cups, while in 2008, each trap contained 13 cups. The sediment traps were cone-shaped with a collecting area of 0.5 m^2 and they collected material between 8 February 2005 and 16 January 2006, and between 1 February 2008 and 1 February 2009. To prevent organic degradation during deployment, trap sample cups were filled with filtered seawater containing a pH-buffered (1 g l^{-1} of sodium borate) solution of 5% formalin.

Unfortunately, the 2005 dataset was not complete as some bottles broke during transport to the laboratory and the little amount of material collected in some trap cups (< 20 mg) precluded geochemical analysis. However, 11 samples from the top trap and 12 from the bottom trap were analysed and 8 of them, both from the top and bottom traps, were related to the same sampling period. A total of 22 and 26 samples were analysed from the 2005 and the 2008 deployments, respectively. Trap samples were kept constantly refrigerated and later stored in a cold room at 4 °C at the CNR-ISMAR laboratory in Bologna.

Sample treatment and analytical methods

Samples collected during 2005 were treated and analysed at the CNR-ISMAR of Bologna following a modified Heussner's method proposed by Chiarini and co-workers (Heussner et al. 1990; Chiarini et al. 2013), while the samples collected during 2008 were split and analysed at the Stanford University following the Dunbar and Mucciarone (2003) method. These two methods differ in the sample preparation, as in the modified Heussner's method the splitting procedure is carried out with a peristaltic pump, whereas in the Dunbar and Mucciarone (2003) protocol, it is done with a Folsom Plankton Splitter. As a consequence, the protocol of Dunbar and Mucciarone (2003) is faster than the modified Heussner's method. Despite the differences, the results obtained with the two adopted methods were comparable (Chiarini et al. 2013).

First, the supernatant was removed and part of it was preserved for further analyses, then the sample was sieved through a 580 µm filter mesh to separate the coarse sediment from the finest sediment. The larger-sized swimmers that might block the peristaltic pump were removed before the sample was split to obtain three different fractions of material depending on its initial abundance. Of these material fractions, one was kept as an archive, one was kept wet and the last one was freeze-dried. An accurate picking was carried out under a stereomicroscope in order to remove organisms which entered the trap actively (active flux) and to obtain only the passive flux for biogenic silica, carbon and nitrogen analyses.

Biogenic silica (BioSi, considered as opal $SiO_2 \cdot 1.5H_2O$, with about 10% of water) content was obtained using the DeMaster (1981) dissolution method. Total nitrogen and organic carbon contents were determined using a Carlo-Erba CHN analyser (Stanford: Carlo-Erba NA 1500). The composition of the particulate matter was calculated as:

$$\%CaCO_3 = (\%C_{tot} - \%C_{org}) \cdot 8.33$$
$$\%\text{lithogenic} = 100 - (\%C_{org} \cdot 2) - \%CaCO_3 - \%\text{BioSi} - \%N_{tot}.$$

The flux estimations were subject to uncertainties (about 6%) related to the efficiency of material collection and to chemical (material solubilisation), physical (hydrodynamic) and biological (swimmers/active migration) factors (Sanchez-Vidal et al. 2015). BioSi, organic carbon, $CaCO_3$ and total mass flux determinations present errors of about 4.1%, 2%, 1–5% and 4.5%, respectively (Collier et al. 2000; Sanchez-Vidal et al. 2015). Chemical analyses were conducted on grounded freeze-dried samples both at CNR-ISMAR and Stanford University.

Sea ice concentrations were derived from daily data from the National Snow and Ice Data Center, obtained with the DMSP-F11, F13 and F17 Special Sensor Microwave Imager/Sounder (SSMIS), using the NASA Team algorithm for calculation of the sea ice concentration (Cavalieri et al. 1996). The sensor resolution is 25 × 25 km^2 and the sea ice concentration data represented the daily values averaged on the grid

cell containing the location of Mooring A. Sea ice concentration data were processed starting from November 2004 to December 2005 and from November 2007 to December 2008. Chlorophyll-*a* (chl-*a*) concentrations (available from 1997) were derived from the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor on the Aqua satellite (4 km resolution). The data can be considered cloud free, since they are averaged over 8 days in order to reduce the bias due to cloud cover. The accuracy for chl-*a* data from MODIS is ± 35%. Data were processed using the Giovanni online data system, developed and maintained by the NASA GES DISC (http://disc.sci.gsfc.nasa.gov/giovanni/overview/index.html). Chl-*a* concentration data were processed from 1 October 2004 to 6 March 2005 and from 1 October 2007 to 6 March 2008, averaged over an area of about 45 km of radius centred on the Mooring A site. The chl-*a* concentrations were averaged over the region 76.60S–76.89S latitude and 167.87E–169.10E longitude for comparison with the sea ice data and water column particle fluxes.

Mass balance of particle fluxes

The mass balance is based on the assumptions that lithogenic fluxes are conservative and that the material laterally advected has the same composition of vertical flux (Frignani et al. 2000). Consequently, the values obtained are considered rough estimates.

As the lithogenic material is the only material that does not undergo degradation when sinking to the seafloor, it is used to determine the magnitude of resuspension and lateral advection. The amount collected by the top trap was considered as 100% and the difference between the top and the bottom trap was then determined by the amount of material removed or resuspended. The fraction of material that did not result from vertical sinking was thus determined.

Assuming the material that arrives into the bottom trap due to external inputs has the same composition as the material related to the vertical flux, the amount of BioSi and organic carbon (OC) gathered or removed can then be determined. To do this, we considered the BioSi and the OC removed or added with the same percentage of lithogenic material with respect to the top level. The relationship between the BioSi and the OC amount collected in the bottom trap and the expected amount indicated the percentage of dissolution or degradation of the various elements along the water column.

Results

Particle fluxes and composition

Annual integrated mass fluxes during 2005 were 42.2 g m^{-2} yr^{-1} at the top trap and 102.7 g m^{-2} yr^{-1} at the bottom trap, while during 2008, the values were 47.8 g m^{-2} yr^{-1} and 36.1 g m^{-2} yr^{-1}, respectively (Table 1).

During 2005 year, total mass fluxes were lower in the top trap (from 1.9 mg m^{-2} d^{-1} to 557.3 mg m^{-2} d^{-1}) than in the bottom trap (from 32.8 mg m^{-2} d^{-1} to 712.5 mg m^{-2} d^{-1}). In contrast, during 2008, mass fluxes were higher in the top trap (from 5.0 mg m^{-2} d^{-1} to 409.6 mg m^{-2} d^{-1}) and lower in the bottom trap (from 6.0 mg m^{-2} d^{-1} to 226.1 mg m^{-2} d^{-1}) (Table 2 and Fig. 2).

In the top trap, the highest mass fluxes were observed in February and in March 2005, while in the bottom trap, they increased in the second part of January and in April 2005. In general, BioSi fluxes were about one order of magnitude higher than

Table 1. Annual integrated fluxes of total mass, biogenic silica (BioSi), organic carbon (OC), nitrogen (N), calcium carbonate and lithogenic (g m^{-2} yr^{-1}), and SiO_2/OC molar ratio during 2005 and 2008 at Mooring A. The sampling days represent the integrated period of the fluxes at the two levels (top trap and bottom trap). In brackets, the estimated values of annual mass fluxes in 2005 (see text for details).

Years	Level	Sampling days	Mass	BioSi	OC	N_{tot}	$CaCO_3$	Lithogenic	molar SiO_2/OC	Molar C/N
2005	top	257	42.2 (54.9)	15.0	2.3	0.4	7.6	15.6	1.0	7.0
	bottom	229	102.7 (113.2)	33.1	3.9	0.7	6.8	54.3	1.8	7.2
2008	top	365	47.8	10.8	3.8	0.6	20.1	11.8	0.4	7.5
	bottom	365	36.1	9.1	2.5	0.4	12.1	9.7	0.8	7.6
1994*	top	355	59.3	26.3	10.6	–	–	–	0.5	9.2
	bottom	355	86.9	52.4	4.1	–	–	–	2.5	9.6

*Data from Langone et al. (2003)

Table 2. Temporal series of total mass, relative content (%) of biogenic silica, organic carbon, nitrogen and $CaCO_3$ and absolute values of organic carbon (OC) and biogenic silica (BioSi) fluxes at Mooring A in 2005. Days indicate the sampling time. Anomalous values are marked with an asterisk (*).

Bottle	Start	Stop	Days	Total flux (mg m^{-2} d^{-1})	%OC	%BioSi	%N	%$CaCO_3$	OC (mg m^{-2} d^{-1})	BioSi (mg m^{-2} d^{-1})
Top 1	08/02/2005	15/02/2005	7	395.1	6.3	39.5	1.2	14.6	25.0	156.1
Top 2	15/02/2005	25/02/2005	10	557.3	6.4	44.8	1.2	13.1	35.9	249.6
Top 5	15/03/2005	31/03/2005	16	514.2	4.6	49.5	0.8	10.1	23.7	254.5
Top 7	15/04/2005	30/04/2005	15	342.8	2.3	22.5	0.9	42.7	19.4	77.0
Top 9	31/05/2005	15/06/2005	15	30.2	4.7	0.3*	0.8	85.3	1.4	0.1
Top 10	15/06/2005	30/09/2005	107	39.6	2.0	18.7	0.9	17.3	2.2	7.4
Top 11	30/09/2005	01/11/2005	32	1.9	2.4	23.8	0.4	1.1	0.1	0.5
Top 13	15/11/2005	01/12/2005	16	25.7	4.1	25.5	0.7	0.6	1.0	6.6
Top 14	01/12/2005	16/12/2005	15	40.3	5.8	27.7	0.9	3.4	2.4	11.2
Top 15	16/12/2005	01/01/2006	16	107.0	5.2	22.6	0.8	1.3	5.5	24.2
Top 17	08/01/2006	16/01/2006	8	65.7	1.0	46.5*	1.6	3.8	6.3	30.5
Bot 1	08/02/2005	15/02/2005	7	397.7	6.5	44.7	1.2	4.2	25.6	177.8
Bot 2	15/02/2005	25/02/2005	10	466.9	5.7	46.6*	1.1	11.5	26.4	217.6
Bot 4	15/03/2005	31/03/2005	15	569.7	3.7	50.9*	0.7	10.6	20.8	290.2
Bot 7	15/04/2005	30/04/2005	15	550.9	5.4	39.4	1.0	16.0	29.9	217.1
Bot 9	31/05/2005	15/06/2005	15	32.8	13.5	9.2	2.2	42.0	4.4	3.0
Bot 10	15/06/2005	30/09/2005	107	216.7	3.0	17.6	0.5	4.5	6.6	38.1
Bot 12	30/09/2005	01/11/2005	14	230.2	1.3	27.0	0.2	1.1	3.0	62.2
Bot 14	15/11/2005	01/12/2005	15	83.1	1.6	26.4	0.3	0.4	1.3	21.9
Bot 15	01/12/2005	16/12/2005	16	233.5	1.9	28.5	0.3	0.6	4.4	66.5
Bot 16	16/12/2005	01/01/2006	7	372.9	3.0	43.0	0.5	0.1	11.2	160.2
Bot 17	08/01/2006	16/01/2006	8	712.5	5.0	39.7	0.8	0.8	35.6	282.5

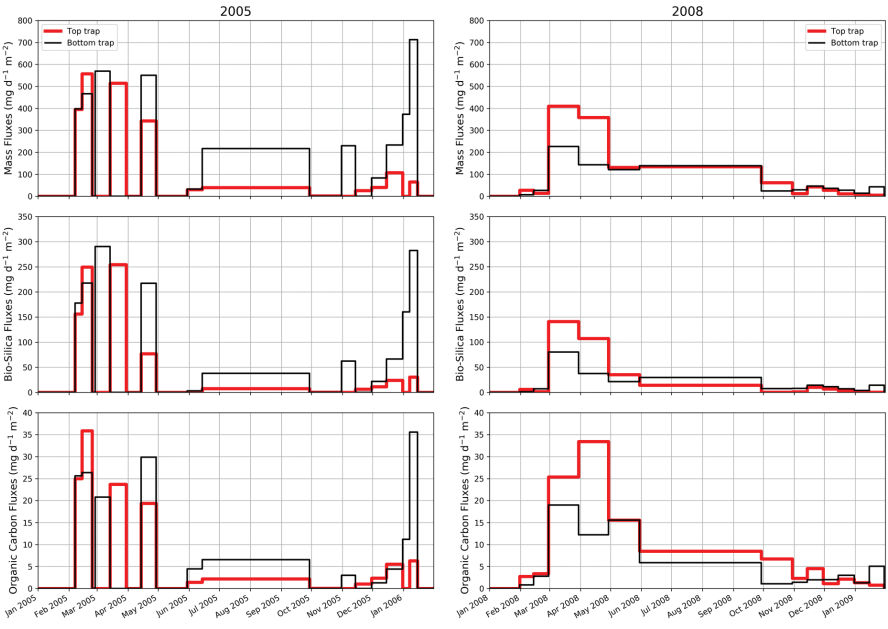

Figure 2. Mass, biogenic silica and organic carbon fluxes at the top and bottom traps measured in 2005 (left) and in 2008 (right) at Mooring A.

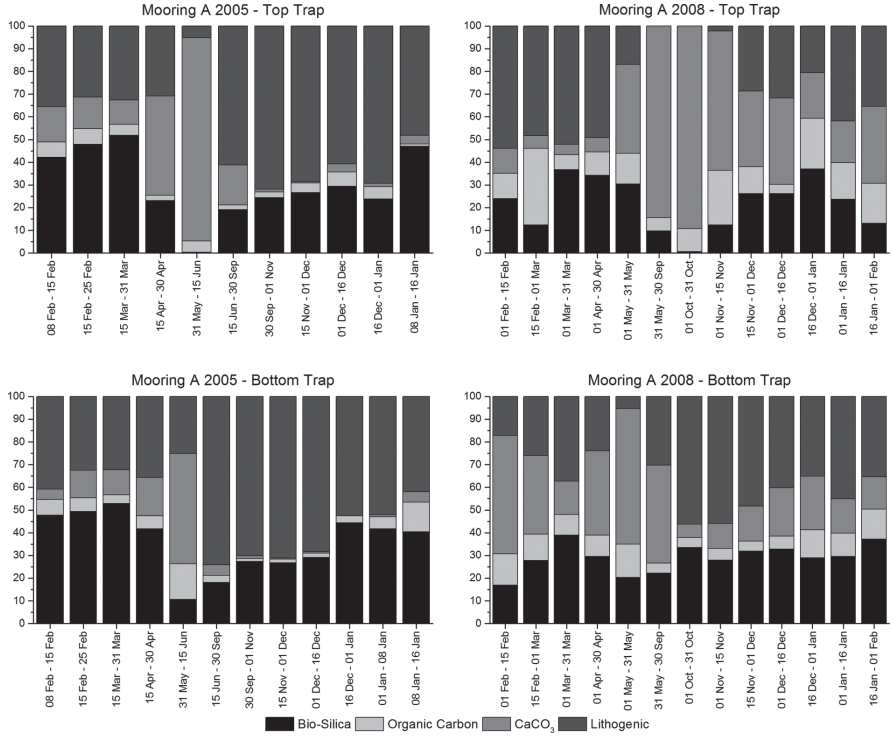

Figure 3. Percentage sample composition at the two levels of Mooring A during 2005 and 2008.

organic carbon fluxes and reached their maximum values during March 2005 and March 2008 at both levels (Fig. 2). The percentages of the major components of the total mass flux are listed in Table 2 (2005) and Table 3 (2008) while their temporal variations are shown in Figure 3. The amount of biogenic material, i.e., OC and BioSi, reached about 50% of the total flux during the austral summer of both years. In 2005, we observed higher concentrations of lithogenic material in both traps, with values of about 70% from September to January (Fig. 3). During 2008, the particle composition in the top trap samples showed high seasonal variability. In the first part of the year, lithogenic material comprised about 50% of each sample. From May to November, samples were predominantly composed of $CaCO_3$, with concentrations ranging from 70% to 90%.

In order to compare our results with previous ones obtained from Mooring A in 1994 by Langone et al. (2003), we tentatively estimated the missing data from 2005 using the fluxes from the periods immediately before and after the data gaps as well as the typical mass flux pattern related to these periods (Table 1). Estimated mass fluxes were around 500 mg m^{-2} d^{-1} during the first 15 days of March and about 350 mg m^{-2} d^{-1} during the first 15 days of April and we obtained an integrated mass flux of

Table 3. Temporal series of total mass, relative content (%) of biogenic silica, organic carbon, nitrogen and $CaCO_3$ and absolute values of organic carbon (OC) and biogenic silica (Bio-Si) fluxes at Mooring A in 2008. Days indicate the sampling time.

Bottle	Start	Stop	Days	Total flux (mg m^{-2} d^{-1})	%OC	%BioSi	%N	%$CaCO_3$	OC (mg m^{-2} d^{-1})	BioSi (g m^{-2} d^{-1})
Top 1	01/02/2008	15/02/2008	15	27.4	10.0	21.5	1.3	9.9	2.8	5.9
Top 2	15/02/2008	01/03/2008	14	14.5	25.2	9.3	4.5	4.2	3.4	1.3
Top 3	01/03/2008	31/03/2008	30	409.6	6.2	34.5	1.0	4.2	25.4	141.2
Top 4	31/03/2008	30/04/2008	31	346.8	9.3	31.0	1.5	5.7	33.4	107.4
Top 5	30/04/2008	31/05/2008	31	131.4	11.9	26.8	2.5	34.5	15.6	35.2
Top 6	31/05/2008	30/09/2008	122	135.1	6.3	10.6	0.8	82.4	8.5	14.3
Top 7	30/09/2008	01/11/2008	32	60.2	10.8	0.7	3.1	85.4	6.7	0.4
Top 8	01/11/2008	15/11/2008	14	11.8	19.4	10.0	4.4	49.5	2.3	1.2
Top 9	15/11/2008	01/12/2008	16	42.7	10.6	23.4	1.9	29.8	4.5	10.0
Top 10	01/12/2008	16/12/2008	15	27.7	4.0	25.1	0.7	36.5	1.1	7.0
Top 11	16/12/2008	01/01/2009	16	11.7	18.3	30.2	3.1	16.4	2.1	3.5
Top 12	01/01/2009	16/01/2009	15	9.6	13.9	20.4	2.3	15.7	1.3	2.0
Top 13	16/01/2009	01/02/2009	16	5.0	15.0	11.1	2.1	28.8	0.8	0.6
Bot 1	01/02/2008	15/02/2008	15	6.8	12.1	14.9	1.9	45.85	0.8	1.0
Bot 2	15/02/2008	01/03/2008	14	28.6	10.4	24.9	1.6	31.03	2.8	7.1
Bot 3	01/03/2008	31/03/2008	30	226.1	8.4	35.6	1.5	13.45	19.0	80.4
Bot 4	31/03/2008	30/04/2008	31	138.3	8.5	27.0	1.9	34.04	12.2	37.4
Bot 5	30/04/2008	31/05/2008	31	121.3	12.8	17.7	2.5	52.00	15.6	21.5
Bot 6	31/05/2008	30/09/2008	122	139.3	4.2	21.2	0.5	41.43	5.9	29.5
Bot 7	30/09/2008	01/11/2008	32	23.8	4.3	32.1	0.8	5.51	1.1	7.6
Bot 8	01/11/2008	15/11/2008	14	29.3	4.8	26.6	0.8	10.45	1.4	7.8
Bot 9	15/11/2008	01/12/2008	16	46.3	4.2	30.5	0.8	14.79	2.0	14.2
Bot 10	01/12/2008	16/12/2008	15	36.6	5.5	30.9	1.0	20.04	2.0	11.3
Bot 11	16/12/2008	01/01/2009	16	27.3	11.0	25.8	1.9	20.98	3.0	7.1
Bot 12	01/01/2009	16/01/2009	15	13.9	9.3	26.8	1.5	13.76	1.3	3.7
Bot 13	16/01/2009	01/02/2009	16	43.3	11.7	32.8	1.8	12.63	5.1	14.2

54.9 g m^{-2} yr^{-1}. The fluxes for the periods 15 February – 25 February and 15 March – 31 March were 557.30 and 514.16 mg m^{-2} d^{-1}, respectively, so we estimated a flux of 500 mg m^{-2} d^{-1} for the period in-between. Following this procedure, the annual flux at the bottom was 113.2 g m^{-2} yr^{-1}.

Sea ice

The sea ice coverage in mooring area, analysed from November 2004 to December 2005 and from November 2007 to December 2008, exhibited the highest values from April to November and the lowest values in January and February, in both years. In the timeframe of the present study, the sea ice concentration decreased from November to January with a minimum value at the end of February in both 2005 and 2008. From the end of February, the sea ice concentrations increased, reaching values greater than 90% by the end of March. The increase in the ice cover was quite fast, occurring in less than 5 weeks, in some cases. From April to November, sea ice concentrations oscillated around an average of 90.5%, sometimes reaching 100% (Fig. 4).

Physical properties of sea water

In 2005, the temperature recorded at the top trap (360 m) showed little variability from January to June (mean -1.89 °C, max -1.84 °C, min -1.91 °C). The salinity ranged from 34.61 to 34.70, with constant values of about 34.64 until June, increasing to 34.74 and exhibiting more variability through January 2006 (Fig. 5a). Temperatures at the bottom trap (770 m) showed quite constant values near -1.89 °C. Salinity values were quite variable from January to June 2005, averaging around 34.74, then they began to decrease until August. From August through the end of the year, salinity values were constant with a mean value of 34.72 (Fig. 5b). During 2008, at the top level (360 m), the temperatures showed nearly constant values (-1.90 °C) from mid-June through to December 2008. The highest salinity (34.69) was recorded in early February, then it decreased from March to June (34.60). From June, salinity increased until November and then decreased again (Fig. 5c).

Swimmers

In the year 2005, the top sediment trap collected very few intact organisms that could be considered active swimmers and they were mainly represented by crustaceans and polychaetes. They were removed before calculating the vertical fluxes. Abundant empty or broken shells of the pteropod *Limacina helicina*, faecal pellets of different shapes and sizes, and degraded organisms (considered as part of the passive flux) were found in late summer and early autumn samples. Mucilaginous material was abundant from February to mid-March. It is worth mentioning that half of the top trap bottles con-

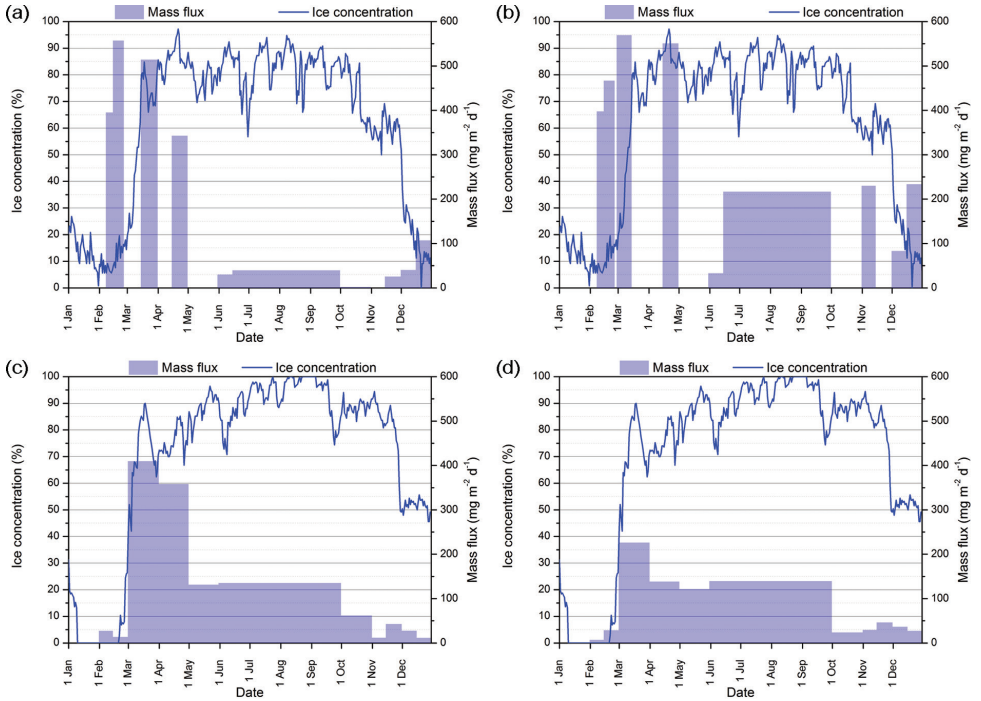

Figure 4. Sea ice concentration and mass fluxes during 2005 and 2008 **a, c** at the top and **b, d** at the bottom sediment traps, respectively.

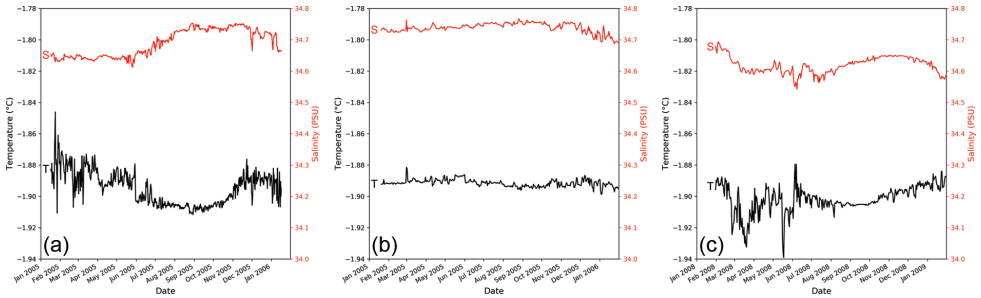

Figure 5. Temperature (T) and salinity (S) values **a** at the top and **b** at the bottom levels in 2005 and **c** the surface level measure in 2008.

tained only passive flux. The swimmers were few also in the bottom trap samples. From February to the end of September 2005, many empty and broken shells of *L. helicina* were found. The period of maximum abundance of swimmers did not coincide with that of maximum particulate fluxes.

During 2008, in the top trap, the period of maximum abundance of swimmers was from March to April. During the same period, many specimens of *L. helicina* were found with the maximum flux in March (700 specimens m^{-2} d^{-1}).

Chlorophyll-*a*

Chl-*a* concentrations are reported in Figure 6. During 2004–2005, chl-*a* concentrations gradually increased up to the highest value reached from 18 December 2004 to 25 December 2004 (1.4 mg m^{-3}), then they decreased to ~0.2 mg m^{-3} from January to March 2005 (Fig. 6a). During 2007–2008 (Fig. 6b), the data series showed a maximum chl-*a* concentration of 4.5 mg m^{-3} (between 1 January and 8 January 2007) and values generally higher than those of the 2004–2005 period. It should be noted that, while the presence of chl-*a* in the area was constant from November 2004 to January 2005, the satellite did not record the presence of chl-*a* from 18 December 2007 until 3 January 2008 (value marked with a star in Fig. 6b).

Discussion

Variability in particles fluxes

During the present study, the maximum mass fluxes at the top and at the bottom sampling levels were detected in the year 2005 just before the onset of maximum sea ice concentration and during sea ice formation (between the beginning of February and the end of March). During winter, a period of high and stable sea ice cover, the

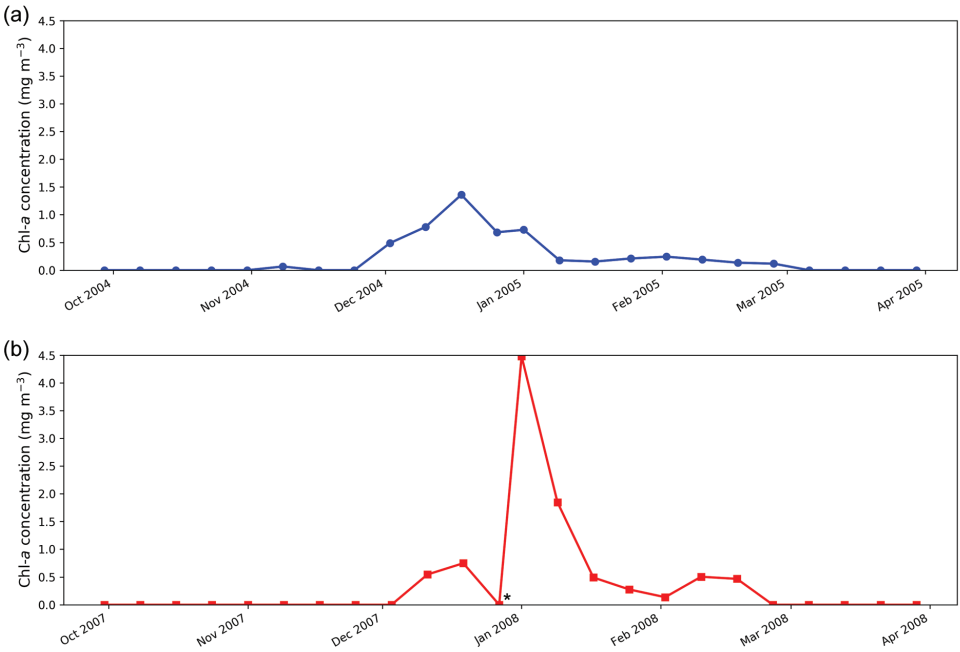

Figure 6. Chl-*a* concentrations in the periods **a** 01 November 2004 – 15 March 2005 and **b** 01 November 2007 – 15 March 2008.

trap at 360 m collected very few materials, whereas abundant material was detected near the seafloor. We interpreted these results with the possible presence of resuspension and/or lateral advection processes occurring during cold periods. As previously mentioned, in 2005 Iceberg B-15 was present near Mooring A area (less than 20 km distant). The satellite images in 2005 showed an anomalous, wide stripe of thick sea ice present between the iceberg and the shoreline in the austral summer (Fig. 7a), whereas in 2008, a more typical ice-free area was present (Fig. 7b). We hypothesise that this iceberg provided a great amount of both lithogenic and organic materials, according to previous studies that documented an increase in the export fluxes near icebergs (Shaw et al. 2011; Sherman et al. 2011; Smith et al. 2011). In addition, the material increase in the bottom sediment trap may have also been due to lateral advection induced by the modified oceanographic pattern in this area. In fact, Iceberg B-15A influenced the oceanographic pattern by blocking the inflow of Antarctic Surface Water (AASW) (Robinson and Williams 2012).

The maximum mass flux registered in 2008, from both the top and bottom traps, was detected during January – March, when the sea ice concentration begun to rise. During winter, both traps collected about the same amount of material, which may suggest that the collected material could have originated from gravitational sinking along the water column and no or very low post-depositional processes were active. It is also worth noting the presence of significant oscillations in the sea ice cover (minimum and maximum values of 56.8% and 97.2%, respectively) in 2005 and the higher concentration (minimum and maximum values of 66.8% and 100.0%, respectively) with less intense changes in 2008. This may be explained with the recent mass balance of the ice sheets observations by Zwally et al. (2015) who reported that, during the years 2003 – 2008, the Antarctic mass gain from snow accumulation exceeded the mass loss from ice discharge.

The interpretation of the BioSi/OC and OC/N molar ratios may help in understanding the processes which occurred in the sampling years, particularly at the bottom trap. Generally, SiO_2/OC values were higher at the bottom than at the top trap in both

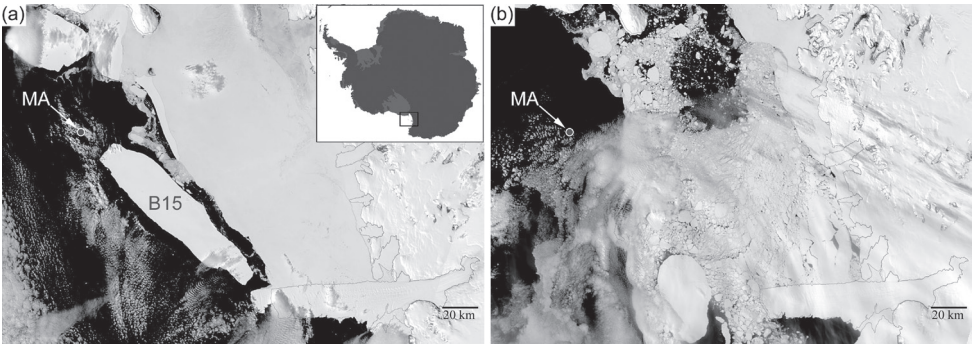

Figure 7. True-colour imagery of the area near Mooring A on 02 January 2005 **a** and 02 January 2008 **b** The location of Iceberg B-15 is also shown (**a**).

years. This indicated the decoupling of Si and C cycles during both years, probably due to a different preservation of biogenic silica compared to organic carbon along the water column. Consequently, the hypothesis of rapid sinking phenomena during 2008 is weakened. On the other hand, the average of the C/N molar ratio was about the same at both depths and in 2005 (with an average of 7.0 at the top and 7.2 at the bottom) and in 2008 (7.5 and 7.6, respectively, at the top and bottom). As introduced in the Materials and Methods section, diatoms are the dominant primary producers in the Ross sea area, and the observed C/N ratios suggest that diatom assemblages varied more than carbonates in the later years. This ratio is slightly lower than the Redfield ratio (an empirical stoichiometric ratio calculated for carbon, nitrogen and phosphorous for phytoplankton and deep-ocean sediments) estimated at about 8 for Antarctic diatoms (DeMaster et al. 1996). In fact, the C/N molar ratio commonly increases along the water column, because nitrogen degrades more quickly than carbon. In addition, according to Collier et al. (2000), if the area is subjected to resuspension processes, the material from the seafloor (with high carbon and low nitrogen contents) may drive this ratio even higher. Our BioSi/OC and OC/N data showed almost constant values at both depths. This might suggests that either: i) the resuspension processes were negligible during both years and the collection of particles in the seafloor trap was then driven only by lateral advection processes or ii) resuspension processes occurred (raising BioSi content) together with an increase in both carbon and nitrogen due to the exports from Iceberg B-15A (2005) and high productivity and preservation along the water column (2008).

Our data document that mass flux peaks occurred in mid-February and in mid-March during 2005, whereas only one peak was detectable during 2008. However, in the Ross Sea, the peak of primary productivity is usually in December or early January (Nelson et al. 1996; Smith et al. 2000; Arrigo and van Dijken 2004) as evidenced by the chl-*a* concentrations, suggesting a time lag of about two - three months between the productivity peak and the higher mass fluxes measured. This observation has already been reported by studies performed in this area (Dunbar et al. 1998; Collier et al. 2000; Langone et al. 2003), but it does not have a unique explanation at the moment. As hypothesised in previous studies, the main driving force(s) responsible for the observed time lag could be: i) the time lag between the growth of phytoplankton and zooplankton community development (Dunbar et al. 1998; Smith and Dunbar 1998; Boyd and Newton 1999); ii) a late diatom bloom associated with winds or a pulse of iron (Collier et al. 2000; Peloquin and Smith 2007), and iii) small or low density aggregates or particles that sink at a slower rate in the water column (Smith and Dunbar 1998; Becquevort and Smith 2001). However, the available data do not seem to support unequivocally one or more of these hypotheses. For example, the chl-*a* concentrations in 2008 seemed to support hypothesis ii), because they showed the presence of some productivity in late summer (between February and March).

It is well known that iron plays a fundamental role in the development of the phytoplankton community, as it is one of the micro-nutrients needed for the synthesis of chl-*a*. In the Ross Sea, this element is mainly supplied by two sources (Collier et al. 2000): dust released into the ocean, with different origins (melting of sea ice,

transported by winds from local and/or distant sources) and resuspension processes due to currents (e.g., Modified Circumpolar DeepWater current, MCDW). However, very recent studies of Winton et al. (2016) suggest that only dust as a source is not enough to regulate phytoplankton growth in the SW Ross Sea. As reported by a recent work (Castagno et al. 2017), also the intrusion of CDW could be pointed as one of the drivers for the benthic development, in addition to being a source of heat and salt. According to the data available, we may suggest that, in the sampling years, the CDW intrusion did not occur because the temperature values were constantly below -1.0 °C.

In 2005, the delay in vertical mass flux that followed the peak of production was likely related to zooplankton grazing, since this area was dominated by diatoms that are important component of the diet of many zooplankters, while *Phaeocystis antarctica* is less grazed (Smith et al. 2003). Indeed, we observed abundant faecal pellets during the period of high flux of particulate material. In 2008, the shift of the mass flux peak of about a month later may be explained by a late reduction in sea ice cover. Consequently, the major algal growing period was also delayed.

In 2008, the extent of the area with high chl-*a* concentration was very limited, probably due to the presence of the sea ice. Chl-*a* concentrations were high only during 20 days in January and the algal bloom did not have a wide extension also in the neighbouring areas. Additionally, in comparison with 2005, the extension and concentration of chl-*a* were significant by the end of November until the first week of January, when the extension remained the same, even if the concentration was much lower. It can therefore be inferred that the phytoplankton bloom in 2005 started at the end of November and that it lasted more than a month, while in 2008 its extension was greatly reduced, although the time length of the bloom was about the same when considering the whole area.

The area of the Ross Sea considered in the present study has a substantial component of diatoms and Nelson et al. (1996) calculated an average SiO_2/OC production ratio of about 0.60–0.65 in the ice edge diatom bloom. The 2005 SiO_2/OC values measured at 360 m were about twice this production ratio, while in 2008, the average ratio was only 0.4. This could be due, in addition to low productivity, to a very limited diatom bloom and to the predominance of non-siliceous algae.

Quantification of particles fluxes

The availability of particle fluxes from two levels of sampling during 2005 and 2008 allowed us to carry out an approximated mass balance to investigate the processes along the water column (Figures 8 and 9). As previously discussed, the fluxes of 2005 could have been influenced by the contribution of different processes (resuspension, lateral advection, presence of Iceberg B-15A). Indeed, we obtained a lithogenic contribution from these processes of 38.7 g m^{-2} yr^{-1}, which is 2.4 times the vertical flux. To evaluate the expected values of biogenic silica and organic carbon fluxes at the bottom, we consider an external input 2.4 times the top flux. This means an input of 36.28 g Bio-Si m^{-2} yr^{-1} and 5.56 g OC m^{-2} yr^{-1}, respectively. Balancing the BioSi and the OC fluxes, we

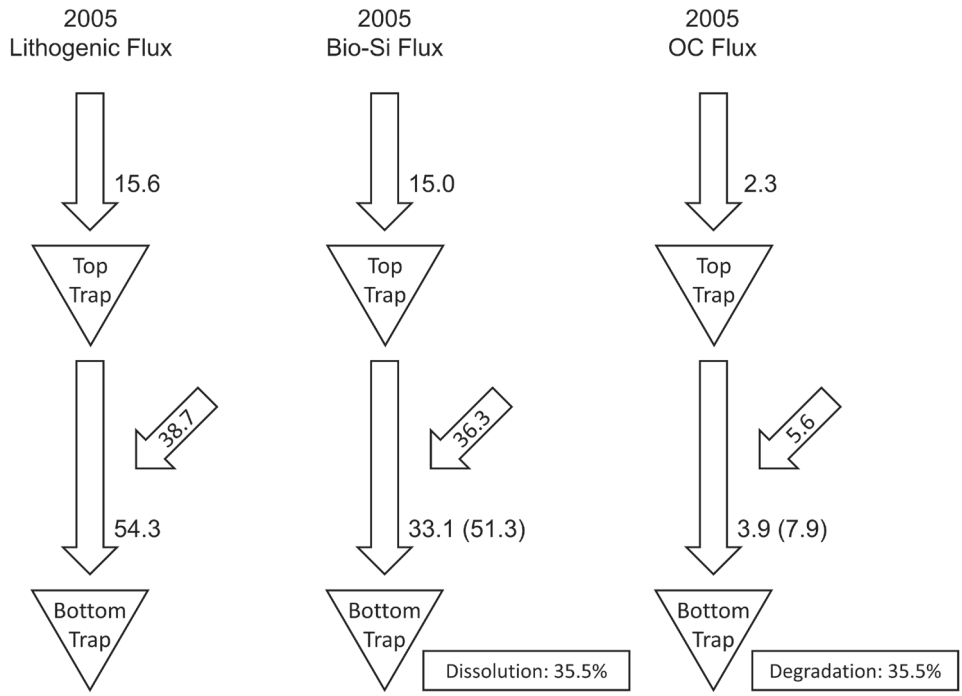

Figure 8. Mass balance and processes of lateral advection in 2005. The numbers near the vertical arrows represent lithogenic, biogenic silica and organic carbon annual integrated fluxes (g m^{-2} yr^{-1}) at the top and bottom trap during 2005. The values near the sideways arrows represent the amount from external inputs and numbers in brackets are the expected fluxes.

get an expected value of silica of 51.28 g m^{-2} yr^{-1} against a measured value of 33.1 g m^{-2} yr^{-1}, which would denote there was a dissolution of about 36% of the biogenic silica in transit from the upper to the bottom trap (DeMaster et al. 1992; Nelson et al. 1996; Collier et al. 2000; Langone et al. 2000; Greenwood et al. 2001). Considering organic carbon, the expected value is 7.86 g m^{-2} yr^{-1} against a measured value of 3.9 g m^{-2} yr^{-1} and thus a degradation of about 50% (Figure 8). These results underline the decoupling of carbon and silica cycles with a higher rate of carbon degradation compared to silica dissolution in this area in line with the results provided by literature (Nelson et al. 1996; Dunbar et al. 1998).

Since the time series of samples was not complete in 2005, the balance was recalculated considering only the periods in which samples at the top and at the bottom levels were both available. The results showed dissolution of biogenic silica of 32% and a degradation of organic carbon of 67%, thus confirming, also in this case, the different cycling rates of OC and BioSi.

In 2008, as previously mentioned, fluxes into the bottom trap were lower than those at the upper level. Considering the measurement errors, this trend was maintained for about 293 days, during periods of high ice coverage. During the remaining 75 days of the year, when the sea was free from ice, other processes, i.e., focusing (ac-

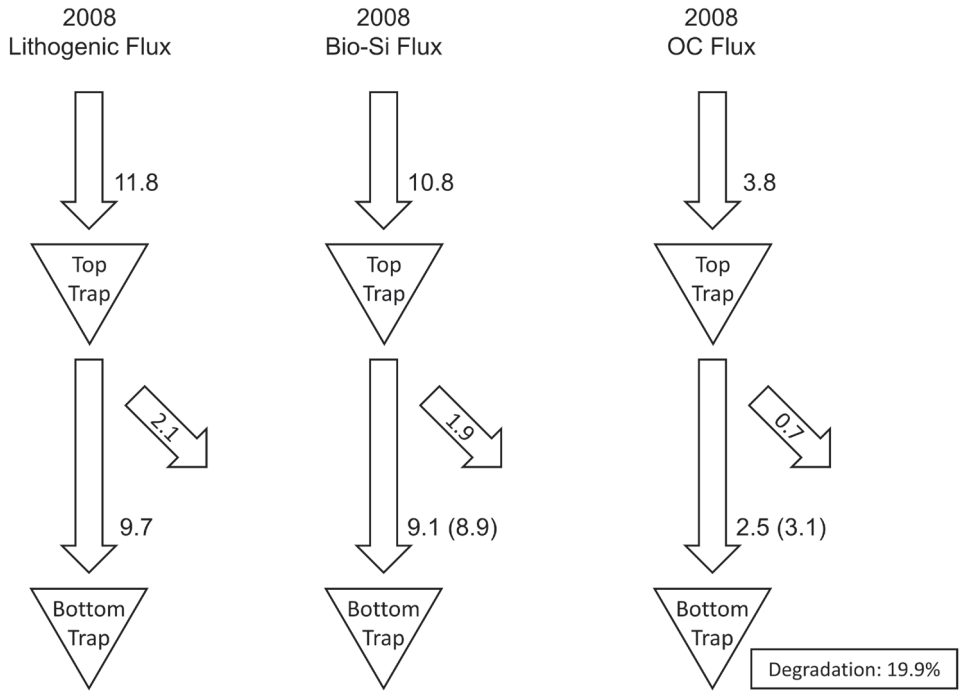

Figure 9. Mass balance and processes of lateral advection in 2008. The numbers near the vertical arrows represent lithogenic, biogenic silica and organic carbon annual integrated fluxes (g m^{-2} yr^{-1}) at the top and bottom trap during 2008. The values near the sideways arrows represent the removed amount and numbers in brackets are the expected fluxes.

cumulation of material), may have taken place. In this case, the balance showed no or negligible dissolution for the silica; in fact, the expected value was 0.2 g m^{-2} yr^{-1} less than the actual value and these two values could be considered equal, taking into account the measurement errors. Conversely, organic carbon had an expected value of 3.12 g m^{-2} yr^{-1} with an actual value of 2.5 g m^{-2} yr^{-1}, meaning that OC degradation was 19.9% (Fig. 9). Additionally, this value was much lower than the value estimated for 2005.

At the top level, particle fluxes during 2005 and 2008 did not differ much. In 2005, they were slightly higher, likely due to a higher primary productivity, as indicated from the chl-*a* values. In contrast, the comparison of the bottom levels showed that the particle fluxes values in 2005 were significantly higher than in 2008.

In 2008, external inputs from lateral advection and/or resuspension were probably negligible, as suggested by the balance of biogenic silica and organic carbon fluxes. This may be due to the low primary productivity that characterised the entire area of the Ross Sea where low chl-*a* concentrations scattered in small areas were observed. During 2008, the ice concentration was greater than in 2005 and the Ross Sea was never completely ice-free. The greater amount of sea ice that surrounded the Ross Sea during summer certainly influenced surface and bottom waters, causing reduced hydrodynamics.

Conclusions

The analysis and correlation of different variables, i.e. particle fluxes, sea ice cover and chl-*a* concentrations, collected from Mooring A, located in the Ross Sea during the years 2005 and 2008, allowed us to provide, for the first time along an extended period of time, *in situ* data on biogeochemical processes in the Ross Sea, which are still poorly understood.

The peaks of mass and biogenic fluxes were recorded in February and March, delayed by one/two months from the algal blooms that usually occur in December/January, related with the processes of ice formation and melting. This can be due to the interaction of different factors, such as the time lag between the growth of phytoplankton and zooplankton community development, the late diatom bloom associated with winds or a pulse of iron, and small or low density aggregates or particles that sink at a slower rate in the water column. The highest percentages of biogenic silica were detected from February to April in both investigated years (2005 and 2008). The mass balance between the top and bottom traps was related to different processes, such as resuspension and/or lateral advection. Also, the presence of Iceberg B-15A could have influenced the amount of sinking material.

Our results highlight the presence of high interannual variability in the biogeochemical processes in the Ross Sea and support the importance of time-series observations. Long-term data sets as in the present research can provide, indeed, sound grounds to identify the type and role of the driving forces that may explain the observed variability.

As a final remark, this study underlines the high importance of interdisciplinary long-term surveys, such as those proposed by the LTER network, in obtaining representative time-series of physical, geochemical and biological data to investigate ecosystem dynamics. In fact, since the climate change and anthropic pressure are expected to intensify, long-term research constitutes a baseline reference for the future management and conservation of the ocean and other ecosystems.

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RESEARCH ARTICLE

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Microbial metabolic rates in the Ross Sea: the ABIOCLEAR Project

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Abstract

The Ross Sea is one of the most productive areas of the Southern Ocean and includes several functionally different marine ecosystems. With the aim of identifying signs and patterns of microbial response to current climate change, seawater microbial populations were sampled at different depths, from surface to the bottom, at two Ross Sea mooring areas southeast of Victoria Land in Antarctica. This oceanographic experiment, the XX Italian Antarctic Expedition, 2004-05, was carried out in the framework of the ABIOCLEAR project as part of LTER-Italy. Here, microbial biogeochemical rates of respiration, carbon dioxide production, total community heterotrophic energy production, prokaryotic heterotrophic activity, production (by ^{3}H-leucine uptake) and prokaryotic biomass (by image analysis) were determined throughout the water column. As ancillary parameters, chlorophyll *a,* adenosine-triphosphate concentrations, temperature and salinity were measured and reported. Microbial metabolism was highly variable amongst stations and depths. In epi- and mesopelagic zones, respiratory rates varied between 52.4–437.0 and 6.3–271.5 nanol O_2 l^{-1} h^{-1}; prokaryotic heterotrophic production varied between 0.46–29.5 and 0.3–6.11 nanog C l^{-1} h^{-1}; and prokaryotic biomass varied between 0.8–24.5 and 1.1–9.0 µg C l^{-1}, respectively. The average heterotrophic energy production ranged between 570 and 103 mJ l^{-1} h^{-1} in upper

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and deeper layers, respectively. In the epipelagic layer, the Prokaryotic Carbon Demand and Prokaryotic Growth Efficiency averaged 9 times higher and 2 times lower, respectively, than in the mesopelagic one. The distribution of plankton metabolism and organic matter degradation was mainly related to the different hydrological and trophic conditions. In comparison with previous research, the Ross Sea results, here, evidenced a relatively impoverished oligotrophic microbial community, throughout the water column.

Keywords

Microbial respiration, heterotrophic production, heterotrophic energy production, Ross Sea, Antarctica, LTER

Introduction

The present work aims to explore the carbon fate through microbes in an area of the Ross Sea (RS) and to identify signs and patterns of microbial responses to current climate change.

The Southern Ocean (SO) plays an important role in world climate since it is considered the engine of the worldwide oceanic currents. The dramatic seasonal variability in environmental factors in SO generates a significant stress on the biota which must endure constant sunlight, oscillating temperatures and melting ice phenomena in spring-summer time. The annual pelagic primary production is largely confined to this period, when light and nutrient conditions are favourable for the phytoplankton growth. Studies on global change have revealed that the SO is becoming a larger carbon sink with over 30–40% of total carbon uptake occurring there (Lancelot 2007, Khatiwala et al. 2009). Recently, an early spring retreat of sea ice, along with a decrease in seasonal sea-ice cover, an increase of light availability and a presumed constraint on primary production, has also been reported (Dinasquet et al. 2018, Constable et al. 2014). Furthermore, the low iron-limited primary productivity ensures that SO falls into a high-nutrient low-chlorophyll (HNLC) type of environment (Minas et al. 1986, Dinasquet et al. 2018, Deppler and Davidson 2017).

One of the most productive and peculiar area of the SO continental shelf zone is the RS (Nelson et al. 1996). This region (1.55 million km^2 of ocean bordering Antarctica from ice edge to deep ocean) was established as a Marine Protected Area (MPA) in December 2017. Here, a peculiar and relatively simply trophic web exists. The phytoplankton, dominated by either diatoms or *Phaeocystis* sp., depending on whether the euphotic zone is stratified or deeply mixed, is grazed either by krill or silver fish that, in turn, sustain the higher trophic levels (Deppler and Davidson 2017). The RS includes a mosaic of functionally different marine ecosystems mainly linked to sea ice distribution whose variability induces unpredicted cascade effects on trophic dynamics and carbon and nutrient drawdown (Catalano et al. 2006, Smith et al. 2007, Vichi et al. 2009). Accordingly, different fates involve the organic matter transfer, carbon export and depth sequestration. The relevance of high latitude oceans, such as the RS, to models and budgets of global carbon cycling has stimulated recent studies of organic matter degradation in the RS water column (Nelson et al. 1996, Carlson and Hansell 2003, Langone et al. 2003, Azzaro et al. 2006, Misic et al. 2017).

Catalano et al. (2006) confirmed that, in spring-summer periods, about 90% of the total carbon derived from new production was exported *via* higher trophic levels. Comparing the oxidation and sinking of organic matter through the deep water found that, according to sediment-traps estimates, 63% of organic carbon, remineralized to CO_2 by microbial respiration, originated in the particulate organic matter (*POC*) pool (Azzaro et al. 2006). Such evidence highlighted *POC* as the main organic fuel in the RS biological pump. The oxidation rate, fuelling the dissolved organic fraction, was not measured by sediment traps (Jiao et al. 2010, Legendre et al. 2015), but it was a smaller fraction, here in the RS, than it was in other oceans (Azzaro et al. 2006).

Although microbes constitute the sentinel of ecosystem evolution (Dutta and Dutta 2016), to date, relatively little is known in the RS about the microbial contribution to the degradation of the carbon pool. Amongst the biological processes and metabolic activities, respiration has particularly been neglected despite its great impact on environmental ecology (Packard 2017). Respiration is controlled by the respiratory electron transport system activity (*ETS*) in all organisms on the planet (Packard 1969, Packard et al. 1971, Lane 2006, Packard et al. 2015). It functions in aerobic and anaerobic conditions, as well as in extreme or deep marine environments (Koppelmann et al. 2004, Azzaro et al. 2006, Packard and Codispoti 2007, Baltar et al. 2010). The *ETS* assay was originally designed by Packard (1971). The biochemical relationship amongst the biological energy currency, adenosine triphosphate (*ATP*) and *ETS* was completely unknown before the 1940s. In few words, in all living organisms, during cellular respiration, the electron transport system produces the bulk of the cell energy in the form of *ATP* molecules. However, earlier, the idea of capturing biologically usable energy from respiration was appreciated by the biophysicist, Alfred Lotka (1925). He felt that Darwinian natural selection was the result of competition between organisms for energy. Those individuals that extracted, stored and used energy most efficiently survived and reproduced more often than their competitors. Building on this concept, Howard Odum described energy flow in freshwater streams (Odum 1956). David Karl recently argued that biological energy production in the ocean should be assessed to understand ocean productivity better (Karl 2014).

Still, none of the earlier work calculated energy production from the biochemical processes that produce this energy. They used distantly related proxies such as biomass or, the more related one, heat production (Pamatmat et al. 1981). Here, we calculate the heterotrophic side of biological energy production (*HEP*) in the RS. *HEP* is the *ATP* generation that results from protons being pumped across the prokaryote plasmalemma or the eukaryote mitochondrial inner membrane by the respiratory *ETS*. This pumping stores energy in the form of a proton gradient across these membranes. It is manifest as both a hydrogen-ion gradient, with a pH change across the membrane as high as 1 pH unit (Procopio and Fornés 1997) and a voltage gradient with an electromotive force (EMF), equal to 170–225 mV. Both cross-membrane gradients, when there is no electron or proton leakage, generate a force across the membrane that is equal to the EMF in millivolts. These gradients, above about 225 mV, force protons back across the membrane, through the molecular motor, *ATP* synthetase (EC 3.6.3.14), to catalyse

the phosphorylation of *ADP* to *ATP* (*ADP* + Pi + H^+_{out} ⇌ *ATP* + H_2O + H^+_{in}) (Berg et al. 2002). This is the main way by which heterotrophic organisms produce their energy. The biochemistry of the connection between *ATP* and the *ETS* was poorly known until the Nobelist, Severo Ochoa, determined how three *ATP* molecule were produced for every oxygen atom (P/O ratio) used in respiratory oxygen consumption (Ochoa 1943). A decade later, Odum (1956) described energy flow in fresh water streams. Much later, working with the copepod *Acartia tonsa*, Dana 1849, Kiørboe et al. (1985) used modern biochemical understanding of the relationship between respiration and oxidative phosphorylation to calculate *ATP* production during growth and egg production. In this millenium, Karl (2014), cognisant of Odum's thinking, as well as the ocean research of Holm-Hansen and Booth (1966) and Packard et al. (1971), advocated assessing biological energy production in the ocean to help understand regional differences in primary productivity. Here and in Packard et al. (2015), we build on this reasoning to calculate *HEP* and the *ATP* turnover rate for the seawater of the RS. This *HEP* is *ATP* produced by respiratory O_2 consumption (RO_2) both in the epipelagic microplankton community of phytoplankton, prokaryotes and protozoans and in the mesopelagic microplankton communities of prokaryotes and protozoans.

In order to analyse the role of prokaryotic metabolism as a regulator of the organic carbon budget, seawater samples were taken in a quadrilateral area of the RS where four mooring sites were located. Fifteen stations were sampled in the epipelagic layer (from surface to 100 m) and in the mesopelagic one (from > 100 m to 800 m) to broaden the evaluation of the whole area.

The prokaryotic biomass (*PB*) was detected by Image Analysis cell counts and volume measurements. The *ETS* assay was adopted to calculate respiration rates of microplankton (< 200 µm) in terms of oxygen utilisation (*OUR*), carbon dioxide production (*CDPR*) rates and heterotrophic energy production (*HEP*). In some stations, the prokaryotic heterotrophic activity and the heterotrophic carbon production by ^{3}H-leucine uptake (*PHA* and *PHP*, respectively) were measured. Ancillary parameters were chlorophyll *a* (*CHLa*), adenosine triphosphate (*ATP*) concentrations and the hydrological parameters. We tried to simultaneously analyse the bulk C metabolism of prokaryotic assemblage, by direct measurement of different independent parameters (respiration and heterotrophic production) and their interconnections (*HEP, PCD* and *PGE*).

The aims of the paper were 1) to monitor the role of microbes as regulators of the organic carbon transfer in the biogeochemical processes, 2) to compare the obtained data with other surveys in RS and 3) to use microbial respiratory and metabolic activity patterns as proxies to describe microbial ecosystem trends.

Methods

During the XX Italian PNRA (National Programme of Antarctic Research, year 2004/05) expedition, in the framework of the ABIOCLEAR project (Antartic BIOgeochemical cycles-CLimatic and palEoclimAtic Reconstructions), an oceanographic cruise was carried out from 4 January to 14 February 2005 aboard the Italian R/V Italica. In a quadrilateral

area between four mooring sites (mooring A and B, monitored in the framework of LTER-Italy activity in Antarctica and moorings H and D), a total of fifteen stations were sampled throughout the water column, from surface to 800 m depth, using a Rosette sampler with 24, 12 l, Niskin bottles. The Rosette was mounted on a CTD equipped with a Sea-Bird 9/11 plus multiparametric probe (SeaBird Electronics) that sensed temperature (*T*), conductivity (salinity, *S*) and dissolved oxygen (*DO* [SBE 43]). In Figure 1, the map of the sampling area and in Table 1, the names of stations, their coordinates, maximum depths and studied parameters are reported. In Suppl. material 2: Table S1, the acronyms of the studied parameters and the link amongst some of them are reported.

Trophic measurements (ATP and CHLa)

For *ATP* measurements, 1 l of seawater was prefiltered through a 250 µm net and then filtered through a 0.22 µm membrane filter. The filter was immediately plunged into 3 ml boiling TRIS–EDTA phosphate buffer (pH 7.75) and the *ATP* was extracted at 10 °C for 3 min and kept frozen (-20 °C) until laboratory analysis in Italy (Holm-Hansen and Paerl 1972). The filtrate, from the *ATP* sample, was stored in sterile polycarbonate bottles. Extracts for *ATP* determination were prepared according to Holm-Hansen and Paerl (1972) and analysed by measuring the peak height of the firefly bioluminescence with a Lumat LB9507 luminometer by EG&G Berthold. The conversion factor, *C/ATP* = 250, was adopted to convert *ATP* values into carbon biomass units (*C-ATP*) according to Karl (1980). The accuracy of this conversion is ± 20% (Skjoldal and Båmstedt 1977). According to *ATP* concentrations, Karl (1980) classified the trophic status of marine systems as follows: oligotrophy when *ATP* is < 100 ng l^{-1}; moderate trophism *ATP* > 100 < 500 ng l^{-1} and eutrophy *ATP* < 500 ng l^{-1}.

CHLa concentrations, as an index of phytoplankton biomass, were determined in the water column from surface to a maximum of 160 m depth. The water samples (1 l) were filtered on Whatman GF/F glass-fibre filters, according to Lazzara et al. (1990). After filtration, the filters were immediately stored at -20 °C. *CHLa* was extracted in 90% acetone and read before and after acidification. Determinations were carried out with a Varian Eclipse spectrofluorometer. Maximum excitation and emission wavelengths (431 and 667 nm, respectively) were selected on a pre-scan with a solution of *CHLa* from *Anacystis nidulans* (Sigma-Aldrich Co). The conversion factor of *C-CHL* = 100 was adopted to convert *CHLa* values into carbon biomass units (Smith et al. 1998).

The Trophic State Index (*TSI*), applied to classify the stations according to their algal biomass, was calculated from the chlorophyll measurements (Carlson 1983: $TSI(CHLa) = 9.81 \ln(CHLa) + 30.6$).

Prokaryotic determinations (abundance and biomass)

Samples for prokaryotic abundance (*PA*; including bacteria, archaea and cyanobacteria) were collected into sterile Falcon vials (50 ml). Each sample was immediately fixed

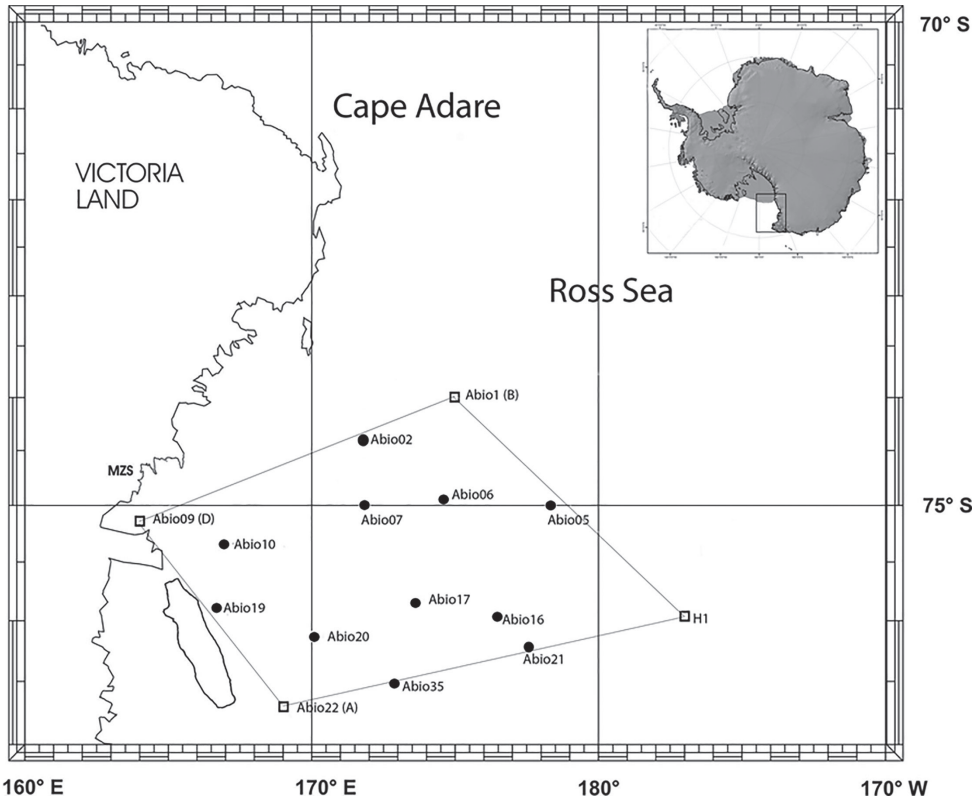

Figure 1. Map of the study area within the Ross Sea in the framework of the ABIOCLEAR Project in Summer 2005. The sampling stations are included within the polygon delimited by four mooring stations.

Table 1. Station names, sampling dates, geographical coordinates, maximum depths and basic parameters. *PA*= prokaryotic abundance; *PB*= prokaryotic biomass; *CHLa*= chorophyll a; *ATP*= adenosine triphosphate; *ETS*= electron transport system activity; *PHA*= prokaryotic heterotrophic activity.

Station	Date	Latitude	Longitude	Depth	Studied basic parameters
Abio09-D	1/10/2005	75°06.77'S	164°25.55'E	1002	*PA, PB, CHLa, ATP, ETS*
Abio10	1/11/2005	75°20.96'S	166°54.77'E	461	*PA, PB, CHLa, ATP, ETS*
Abio19	1/16/2005	75°50.81'S	167°14.62'E	590	*PA, PB, CHLa, ATP, ETS*
Abio22-A	1/17/2005	76°41.49'S	169°04.74'E	789	*PA, PB, CHLa, ATP, ETS, PHA*
Abio01-B	1/30/2005	74°00.53'S	175°05.67'E	590	*PA, PB, CHLa, ATP, ETS, PHA*
H1	2/1/2005	75°58.20'S	177°17.64'E	616	*PA, PB, CHLa, ATP, ETS, PHA*
Abio05	2/2/2005	75°00.00'S	178°19.92'E	390	*PA, PB, CHLa, ATP, ETS*
Abio02	2/3/2005	74°17.85'S	171°35.14'E	458	*PA, PB, CHLa, ATP, ETS*
Abio06	2/4/2005	74°57.31'S	174°35.19'E	400	*PA, PB, CHLa, ATP, ETS, PHA*
Abio07	2/5/2005	75°04.92'S	171°44.70'E	548	*PA, PB, CHLa, ATP, ETS*
Abio20	2/6/2005	76°06.97'S	170°09.72'E	605	*PA, PB, CHLa, ATP, ETS, PHA*
Abio35	2/8/2005	76°30.67'S	172°17.60'E	639	*PA, PB, CHLa, ATP, ETS*
Abio21	2/8/2005	76°14.06'S	179°06.10'E	350	*PA, PB, CHLa, ATP, ETS*
Abio16	2/9/2005	75°58.39'S	176°29.49'E	454	*PA, PB, CHLa, ATP, ETS*
Abio17	2/9/2005	75°50.94'S	173°35.09'E	374	*PA, PB, CHLa, ATP, ETS*

in pre-filtered formaldehyde (0.2 µm porosity; 2% final concentration) and stored at 4 °C until analysis. Within three months, two replicates of each sample were filtered through polycarbonate black membrane filters (0.2 µm porosity; GE Water & Process Technologies) and stained for 10 min with 4',6-diamidino-2-phenylindole (DAPI, Sigma, final concentration 10 µg ml^{-1}) according to Porter and Feig (1980). Stained cells were counted under a Zeiss AXIOPLAN 2 imaging epifluorescence microscope (magnification: Plan-Neofluar 100× objective and 10× ocular; HBO 100 W lamp; filter sets: G365 excitation filter, FT395 chromatic beam splitter, LP420 barrier filter) equipped with the digital camera AXIOCAM-HR. Images were captured and digitised on a personal computer using the AXIOVISION 3.1 software. Linear dimensions of cells were measured and their shapes equated to standard geometric figures according to Lee and Fuhrman (1987), Fry (1990) and Massana et al. (1997). Volume of single cells (*VOL*, as μm^3) was calculated according to Bratbak (1985). Cell carbon content (*CCC*, as fg C $cell^{-1}$) was derived from cell volumes according to Loferer-Krößbacher et al. (1998). Total prokaryotic biomass (*PB*, as µg C l^{-1}) was calculated by multiplying *PA* by *CCC,* locally derived from single cell *VOL* (La Ferla et al. 2015).

Metabolic rates

ETS measurements and relative rates

Respiratory rates were quantified according to the tetrazolium reduction technique (Packard 1971, 1985) as modified for the microplankton community (Kenner and Ahmed 1975). The *ETS* assay allowed an estimation of the maximum velocity (*V*max) of the dehydrogenases transferring electrons from their physiological substrates (NADH, NADPH and succinate) to a terminal electron acceptor (O_2) through their associated electron transfer system. Details on our *ETS* measurements in Antarctic and peri-Antarctic areas have been described previously (Azzaro et al. 2006, Crisafi et al. 2010, Misic et al. 2017). Briefly, subsamples (from 2 to 20 l) were pre-filtered through a 250-µm mesh-size net and concentrated on GF/F-glass-fibre filters (nominal pore diameter 0.7 µm) at reduced pressure (< 1/3 atm). Although the filter porosity was specific for microplankton, GF/F filters retain also particles colonised by very small heterotrophs. The filters were immediately folded into cryovials and stored in liquid nitrogen to prevent the enzymatic decay (Ahmed et al. 1976) until they were analysed in the laboratory (< 3 months). The assays were performed in duplicate and homogenates were incubated for 30 min in the dark at the *in situ* temperature (± 0.5 °C) of the sample. The *ETS* was corrected for *in situ* temperature with the Arrhenius equation using a value for the activation energy of 11.0 kcal mol^{-1} (Packard et al. 1975, Arístegui and Montero 1995).

The specific standard deviation (i.e. the percentage of the standard deviation of the replicates on the average value of the same replicates), due to the analytical procedures and sample handling, was about 35%.

ETS (µl O_2 l^{-1} h^{-1}) was considered equal to the respiration rate in the epipelagic zone and converted to respiratory Carbon Dioxide Production Rates (*CDPR*) (µg C l^{-1} d^{-1}) by using the following (Eq.1):

$$CDPR = (ETS*12/22.4) * (122/172) \text{ (Eq.1)}$$

where 12 is the C atomic weight, 22.4 the O_2 molar volume and 172/122 the Takahashi oxygen/carbon molar ratio (Takahashi et al. 1985). Real respiratory rates have been calculated using the conversion factors from *ETS V*max to *CDPR* as referred by La Ferla and Azzaro (2001b) and Azzaro et al. (2006), in the epi- and mesopelagic layers, respectively.

Cell specific respiratory rates (*CSRR*) were calculated by dividing normalised *CDPR* values to the normalised cell abundance values in each station by adopting a prokaryotic contribution of 50% and 80% at epipelagic and mesopelagic layers, respectively, assuming that the activities we measured were mainly due to the prokaryotic fraction and that all the cells have similar activity levels.

Heterotrophic energy production (*HEP*) *determination*

Today, the P/O ratio is thought to be closer to 2.5 rather than 3.0 (Ferguson 2010, Moran et al. 2012). With this ratio and using *ETS* activities to compute respiration, one can derive *ATP* production rates in a particular oceanic region through the determination of the *HEP* (Packard et al. 2015). *HEP,* in micro Joules (µJ), can be calculated from seawater respiration (*R*) in µmol O_2 h^{-1} l^1 using the following equation (Eq.2):

$$HEP \text{ (µJ } h^{-1} l^{-1}) = R \text{ (µmol } O_2 h^{-1} l^{-1}) * 2 * 2.5 * 0.048 * 10^{-6} \text{ (Eq.2)}$$

where 2 is the number of electron pairs that participate in reduction of one molecule of O_2 to two molecules of H_2O; 2.5 is the modified P/O of Ochoa (1943), the *ATP* produced by the flow of one electron pair; 0.048 is the Gibbs Free Energy (ΔG) per micro mol *ATP* in J·(µmol *ATP*)$^{-1}$ (Hinkle 2005, Ferguson 2010, Moran et al. 2012, Packard et al. 2015); and 10^{-6} converts Joules to micro Joules. If, HEP in milli Joules (mJ) is desired, simply replace the factor, 10^{-6}, in Eq. 2, with the factor, 10^{-3}. The turnover time (τ) of *ATP* in the microplankton community, from which came the sample, is the molar ratio of the *ATP* concentration to the *ATP* production rate, in the microplankton. The calculation steps are shown in Table 2.

Prokaryotic heterotrophic activity (*PHA*) *and production* (*PHP*)

PHA was evaluated by ^{3}H-leucine incorporation rate assay using the microtubes method described by Smith and Azam (1992). Briefly, triplicate 1.7 ml subsamples and duplicate zero-time killed (trichloroacetic acid-TCA, 5% final concentration) blanks were

Table 2. Heterotrophic Energy production (*HEP*) and adenosine triphosphate (*ATP*) turnover time in microplankton in the Ross Sea water column.

Pelagic Zone	Depth Interval (m)	Potential Respiration (Φ) (μmol O_2 l^{-1} h^{-1})	*ATP* (ng l^{-1})	Respiration (μmol O_2 l^{-1} h^{-1})	*HEP* (*ATP* Production) (μmol l^{-1} h^{-1})	*HEP* (Energy Production) (mJ l^{-1} h^{-1})	*ATP* Turnover Time (τ) (min)
Euphotic	2–100	9.13 ± 5.93	123 ± 72.2	2.38 ± 1.54	11.88 ± 7.71	570 ± 370	1.25 ± 0.76
Epipelagic	2–160	8.97 ± 3.27	110 ± 71.0	2.33 ± 0.85	11.67 ± 4.25	560 ± 204	1.15 ± 0.77
Mesopelagic Upper (A)	100–500	4.02 ± 3.49	30.35 ± 31.68	1.05 ± 0.91	5.23 ± 4.54	251 ± 218	0.74 ± 0.49
Mesopelagic Upper (B)	160–500	3.03 ± 3.19	27.49 ± 32.58	0.86 ± 0.83	4.31 ± 4.15	207 ± 199	0.78 ± 0.53
Mesopelagic lower	500–800	1.65 ± 0.88	13.48 ± 6.76	0.43 ± 0.23	2.15 ± 1.15	103 ± 55	0.76 ± 0.53

incubated in 2 ml polypropylene microcentrifuge tubes (Safe-lock, Eppendorf) with L-[4,5-^{3}H] leucine (Amersham, GE Healthcare, SA 61Ci/mmol) giving final concentration of 20 nM. Microtubes were distributed in floating racks into darkened containers with seawater disposed in a refrigerated box. Samples were incubated for 170–190 min at a temperature between -0.5 and -0.8 °C. Incubation was stopped with TCA (5% final concentration). Pellets were washed twice with 5% TCA and 80% ethanol and finally supplemented with 1 ml of liquid scintillation cocktail (Ultima Gold MV Perkin Elmer Life and Analytical Sciences). The liquid-scintillation analysis was effected in a Perkin Elmer Model Wallac 1414 WIN Spectral counter using the internal quenching control. Saturation curves analysis and time-course experiments were carried out at four different stations (from 2 to 300 m depth) with hourly controls for up to six hours of incubation. This procedure was established in a previous cruise in the RS, carried out within the framework of the Victoria Land Transect Project – VLTP-2004 (PNRA XIX Expedition). During *PHA* time-course experiments, linearity in the ^{3}H-leucine uptake was observed in one sample (25 m depth) between 1 and 6 hours of incubation (r = 0.96), while in the other three samples (2, 270 and 310 m depths), linearity was observed only between 1 and 3 hours of incubation (r = 0.83 and 0.88, respectively).

Prokaryotic heterotrophic production (*PHP*) was calculated from the ^{3}H-leucine incorporation rate (*PHA*) expressed in moles incorporated per unit time and volume (Kirchman et al. 1985) according to the equation $PHP = PHA*CF$ where CF is a conversion factor expressed in kg C mol^{-1}. CF were determined following a "semi-theoretical" approach using the molecular weight of leucine, the leucine content of cellular protein and the cellular carbon equivalent of the protein according to Simon and Azam (1989) and the isotope dilution (*ID*) *in situ* experimentally determined according the rectangular hyperbola fitting method of van Looij and Riemann (1993), as described by Pedrós-Alió et al. (2002). In particular, *ID* was determined from two samples collected at 20 and 45 m depth, respectively and two at 300 m depth. It varied between 1.04 and 1.37. The mean value of 1.25 was used to calculate the *CF* that was equivalent to 1.94 kg C (mol leucine)$^{-1}$ (Table 3).

PHP was expressed as production of biomass (as C) per time unit and volume.

Table 3. Isotopic Dilution (*ID*) detected from leucine incorporation rates (*V*max) in samples Abio19 (45 and 300 m depth) and Abio05 (20 and 300 m depth), respectively. *ID* mean value 1.25 ± 0.14.

Station	depth (m)	*V* max ± SD p mol l^{-1} h^{-1}	*ID*	confidence interval 95%
Abio19	45	18.058 ± 0.870	1.06	0.95–1.18
	300	0.357 ± 0.069	1.24	1.00–1.48
Abio05	20	9.785 ± 4.813	1.37	0.69–2.04
	300	0.932 ± 0.225	1.34	1.01–1.66

Derived parameters as Specific Growth Rate d^{-1} [*SGR* (μ) = *PHP/PB* (Prokaryotic biomass)] and Biomass Turnover Time (days) [*BTT*(g) = ln(2)/μ] were calculated according to Kirchman (2001).

In comparison, PHP_D, PB_D, SGR_D and BTT_D were calculated using 107 fg C μm^{-3} cell and an *ID* = 1 according to Ducklow et al. (2001). Cell specific incorporation rates (*CSIR*) were calculated by dividing normalised *PHP* values to the normalised cell abundance values in each station.

The prokaryotic C requirement was computed as Prokaryotic Carbon Demand (*PCD*), i.e. *PHP*+*CDPR* by using normalised data, assuming the contribution of prokaryotes to total microbial community respiration as 50% in the epipelagic layer and a contribution of 80% in the mesopelagic ones (La Ferla et al. 2005, del Giorgio et al. 2011, Baltar et al. 2015). The Prokaryotic Growth Efficiency (*PGE*) was calculated as *PHP/PCD* and the isotope dilution (*ID*) *in situ* was experimentally determined according the rectangular hyperbola fitting method of van Looij and Riemann (1993), as described by Pedrós-Alió et al. (2002) using HYPER 32 fitting software.

Data processing

In order to detect possible influences between environmental factors and microbial variables, Spearman Rank correlation coefficients were calculated for the microbiological data and environmental parameters using the SigmaStat software V3.0 and the Mann and Whitney test using the PAST.exe (Hammer et al. 2001). The analyses of variance (one-way ANOVA and Kruskal-Wallis) were applied to some parameters to assess the significance of the differences between depth layers and stations.

Data were integrated with depth according to the trapezoidal method and normalised to the depth: from 2 to 100 m for the epipelagic layer; from 100 to 800 m for the mesopelagic layer.

The depth-integrated rate ($\int R$ dz in mg C m^2 d^{-1} l^{-1}) for the water column was calculated within the depth interval between Z_1 and Z_2 using the following equation (Eq. 3):

$$\int R\, dz = y\, (Z_2^{(x+1)} - Z_1^{(x+1)} / (x+1) \quad \text{(Eq.3)}$$

Results

Temperature ranged between -2.01 (H1, 500 m) and 1.48 °C (Abio09-D, 5 m) and salinity between 34.11 (Abio10, 25 m) and 34.79 (Abio09-D, 600 m). In Suppl. material 1: Figure S1, the temperature and salinity at surface and 200 m depth layers are shown. In the surface layer, a frontal structure was evidenced by temperature (*T*) and salinity gradients. Temperature clearly decreased eastwards from 1.5 to -0.6 °C, corresponding to mooring stations Abio09-D and H1, respectively. Salinity was similarly distributed along a non-linear front that cut eastwards and southwards through the RS with a salinity of 34.3. At the 200 m depth-horizon, temperature showed a wedge of relatively warm water, with temperatures ranging from -1.3 to -0.7 °C, that had crept between two cooler water masses, with temperatures ranging from -1.9 to -1.3 °C. Salinity, in Suppl. material 1: Figure S1, showed an eastwards decreasing trend (from 34.66 to 34.46).

In Table 4, the ranges, mean values and standard deviations of the trophic analyses, prokaryotic determinations and metabolic rates detected in the epipelagic and mesopelagic layers are reported. *CHLa* showed very low concentrations throughout the study area (mean values 0.14 and 0.058 mg m^{-3} in the epi- and mesopelagic layers, respectively). In the epipelagic layer, the minimum and maximum values were detected at stations Abio20 (5 m) and Abio09-D (5 m), respectively; in the mesopelagic layer, they occurred at stations Abio10 (150 m) and Abio02 (110 m), respectively.

The average *CHLa* value in the epipelagic layer was 2.4 times higher than in the mesopelagic one. In Figure 2a, the *C-CHLa* vertical distribution is reported. TSI were always lower than 30, categorising the RS as oligotrophic for Summer 2005 (Suppl. material 3: Table S2).

ATP sharply decreased with depth (Figure 2b) by a factor of 4 (Table 4). On average, *ATP* ranged between 124 and 29 ng l^{-1} in the epi- and mesopelagic layers. The minimum and maximum concentrations in the epipelagic layer were observed in stations H1 (100 m) and Abio09-D (5 m), respectively; in the mesopelagic layer, at stations H1 (500 m) and Abio02 (110 m), respectively. The biological carbon, calculated from the *ATP* (*C-ATP*), decreased with depth from a mean of 31 micro g C l^{-1} in the epipelagic euphotic zone to a mean of 7 micro g C l^{-1} in the mesopelagic zone between 100 and 800 m (Table 4 and Figure 2b). According to Karl's classification, stations H1, Abio09-D, Abio06, Abio05 and Abio02 manifested moderate trophism (> 100 < 500 ng l^{-1}) whereas Abio07, Abio20, Abio35 manifested oligotrophy (*ATP* < 100 ng l^{-1}).

The calculation of the average *TSI*, chosen to establish the station trophic state, ranged from a high of 24 at H1, the most offshore station, to a low of 16 at Abio20, a station much closer to shore (Suppl. material 3: Table S2, Figure 1). Stations H1, Abio09-D, Abio21, and Abio05 were richer than the other stations. Their averaged *TSI* surpassed 23. Stations H1, Abio21 and Abio05 grouped together as the three most offshore stations, but station Abio09-D was the most inshore one. At the other end of the scale, station Abio20, as mentioned above, had the lowest *TSI*. A one-way ANOVA analysis confirmed this variability amongst the stations ($P < 0.000314$, $n = 80$).

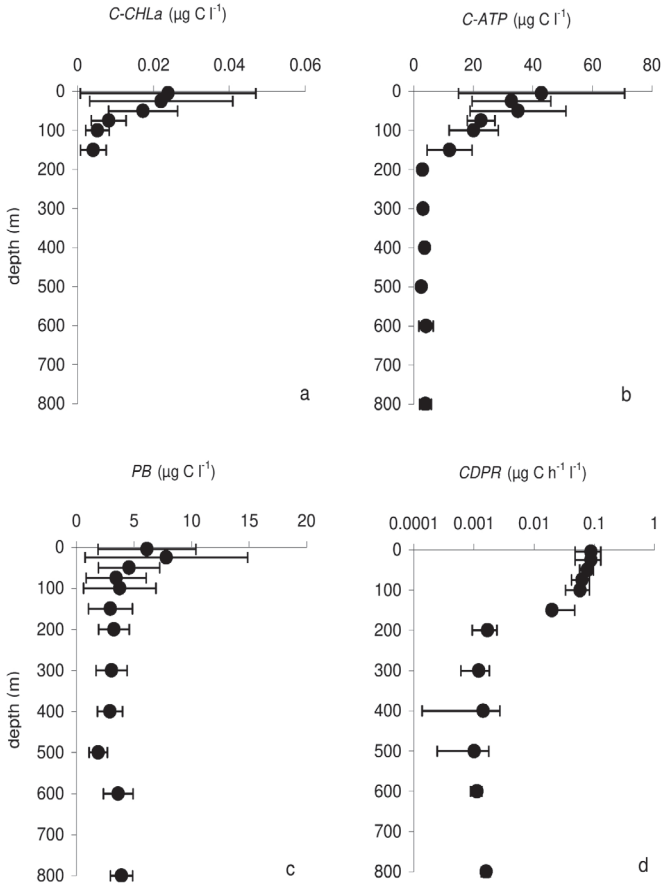

Figure 2. Depth profiles of mean values and standard deviations of the carbon content derived from chlorophyll *a* (*C-CHLa,* **a**) and adenosine triphosphate (*C-ATP,* **b**), the prokaryotic biomass (*PB,* **c**) and the carbon dioxide production rates derived from ETS activity (*CDPR,* **d**) in the Ross Sea water column.

Prokaryote Abundance (*PA*) was in the order of 10^4–10^6 cell ml^{-1} in the epipelagic layer and 10^4–10^5 cell ml^{-1} in the mesopelagic layer (Table 4). The Cell Carbon Content (*CCC*) showed a discrete variability in both the studied layers and ranged between 11 and 36 and between 13 and 45 fg C $cell^{-1}$ in epi- and mesopelagic layers, respectively (Suppl. material 4: Table S3). *PB* modulated by both *PA* and *CCC*, showed a decreasing trend and high variability in the upper layers, particularly at 25 m (Figure 2c). The minimum value was detected at 500 m and an increase below occurred. The values in the epipelagic layer were 1.7 times higher than in the mesopelagic one. In the near-surface layer, *PB* min and max values were detected at stations Abio05 (100 m) and Abio09-D (25 m), respectively. In the deep layer, *PB* min and max values were obtained at stations Abio05 (100 m) and Abio22-A (100 m), respectively.

Table 4. Range, mean and standard deviations, sampling numbers (n) of trophic parameters (*CHLa, C-CHLa*, *ATP* and *C-ATP*), prokaryotic abundance and biomass (*PA* and *PB*) and metabolic rates (*ETS, CDPR* and *PHP*); detected in the epipelagic (0–100 m) and mesopelagic (>100<800 m) depth layers. *CHLa*= cholrophyll *a*; *C-CHLa*= cholrophyll *a* in carbon units; *ATP*= adenosine triphosphate; *C-ATP*= adenosine triphosphate in carbon units; *PA*= prokaryotic abundance; *PB*= prokaryotic biomass; *ETS*= electron transport system activity; *CDPR*= carbon dioxide production rate; *PHP*= carbon prokaryotic heterotrophic production.

	CHLa	***C-CHLa***	***ATP***	***C-ATP***	***PA***	***PB***	***ETS***	***CDPR***	***PHP***
	mg m^{-3}	**mg C m^{-3}**	**ng l^{-1}**	**ng C l^{-1}**	**cells ml^{-1}**	**µg C l^{-1}**	**µl O_2 l^{-1} h^{-1}**	**µg C l^{-1} h^{-1}**	**µg C l^{-1} h^{-1}**
0–100 m depth									
min	0.019	1.89	42.76	10689	5.70E+4	0.8	0.052	0.0043	0.0005
max	0.539	53.90	380.49	95123	1.44E+6	24.5	0.437	0.1660	0.0295
mean	0.141	14.11	123.78	30944	2.87E+5	5.2	0.201	0.0740	0.0100
SD	0.116	11.56	70.26	17564	2.55E+5	4.5	0.076	0.0323	0.0077
n	67	67	39	39	67	67	66	66	25
100–800 m depth									
min	0.007	0.67	4.77	1193	5.66E+4	0.8	0.006	0.0002	0.0003
max	0.244	24.44	130.69	32673	6.76E+5	15.1	0.271	0.0965	0.0061
mean	0.058	5.77	29.04	7260	1.51E+5	3.1	0.086	0.0122	0.0019
SD	0.051	5.14	30.66	7664	8.45E+4	1.9	0.076	0.0244	0.0015
n	25	25	47	47	78	78	81	81	36

ETS showed a decreasing trend with depth and the values in the epipelagic layer were 3 times higher than in the mesopelagic one (Table 4). In the upper layer, *ETS* minimum and maximum values were detected at stations Abio 22-A (80 m) and Abio10 (25 m), respectively. In the deeper layer, they were observed at stations Abio16 (400 m) and Abio10 (150 m), respectively.

CDPR showed a decreasing trend with depth with a discrete variability in the deep layers (Figure 2d). It sharply decreased with depth by a factor of 6 (Table 4). The minimum value in the epipelagic layer was achieved at station Abio16 (2 m) and the maximum value at Abio10 (25 m). In the mesopelagic layer, minimum and maximum values were observed at Abio16 (400 m) and Abio17 (120 m), respectively.

The cell specific respiratory rate (*CSRR*) calculated on depth-integrated and normalised data (Figure 3), in the epipelagic layer varied between 10.1×10^{-3} (Abio16) and 390×10^{-3} (at station Abio02) fg C cell^{-1}; in the mesopelagic waters, they varied between 1.46×10^{-3} (Abio16) and 50.1×10^{-3} (Abio17) fg C cell^{-1}.

The *HEP* calculations for the epipelagic and mesopelagic waters of RS are given in Table 2. The average *HEP* (and standard deviation) in epipelagic zone down to 160 m was 560 ± 20 µJ h^{-1} l^{-1}. Over this depth range, *HEP* ranged from as high of 108 µJ h^{-1} l^{-1}, at station Abio06 (2 m depth), to 15 µJ h^{-1} l^{-1}, at station Abio22-A (80 m). The *HEP* calculations for the euphotic zone (upper 100 m) were the same (Line 1, Table 2). The range was 108 to 15 µJ h^{-1} l^{-1} and the average and standard deviation was 570 ± 370 µJ h^{-1} l^{-1}. In mesopelagic waters below 160 m down to 500 m, *HEP* was 80% lower, averaging 118 ± 90 µJ h^{-1} l^{-1}. If we make the calculation from and including 100, the *HEP* down to 500 m was larger. It averaged 251 ± 218 µJ h^{-1} l^{-1}. The database for

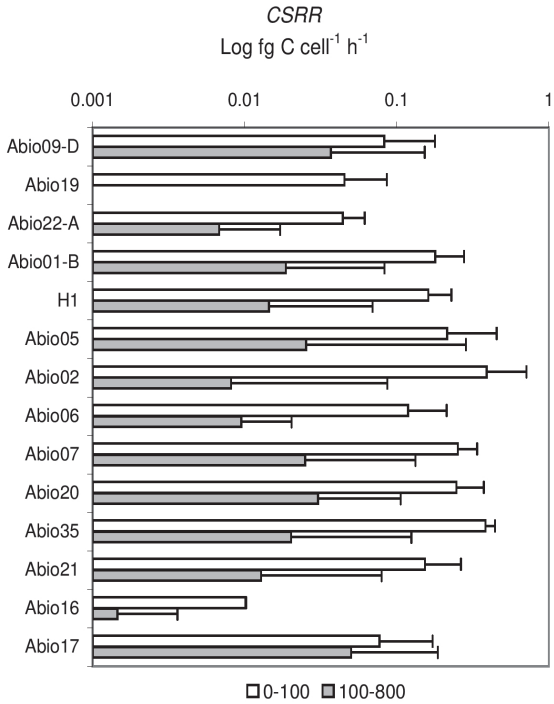

Figure 3. Cell Specific Respiratory Rates (*CSRR*) and SD for each station in the epipelagic and mesopelagic layers (0–100 m, 100–800 m). In the upper and deeper layers, the prokaryotic contributions to total respiration were considered to be the 50% and 80%, respectively, of the total carbon dioxide production rates (*CDPR*).

the mesopelagic waters between 500 and 800 m was smaller than the one between 100 and 500 m, in only 15 compared to 74 measurements. These measurements yielded an average deep-mesopelagic *HEP* of 103 ± 55 μJ h^{-1} l^{-1}. The *ATP* turnover rate in the cells of the microplankton was remarkably constant with depth, in the order of magnitude of a minute. It only decreased 39% from the euphotic zone to the lower mesopelagic zone, from 1.25 ± 0.76 to 0.76 ± 0.53 minutes.

The prokaryotic heterotrophic activity (*PHA*) – in term of leucine incorporation rates – varied between 0.213 and 19.035 pmol l^{-1} h^{-1} (Figure 4). Peaks of activity were detected in surface (Abio01-B, Abio20 and Abio06 stations) or at 20–25 m depth (Abio22-A and H1 stations). Decreasing *PHA* was detected with depth followed by an increase of activity in the bottom samples (Figure 4). In station Abio22-A, normalised *PHA* in the epipelagic layer (12.4 nmol m^{-3} h^{-1}) was one order of magnitude higher than that observed in the other stations (Table 5). *PHA* observed in 0–50 m layers was on average 8.05 ± 4.52 pmol l^{-1} h^{-1} (n = 15). The cell specific incorporation rate (*CSIR*) varied between 1.992 and 70.844 z mol leucine $cell^{-1}$ h^{-1} with the highest values in the upper 50 m depth (28.20 ± 15.97 z mol leucine $cell^{-1}$ h^{-1}) while, in mesopelagic waters, they turned out to be one order of magnitude less (6.48 ± 3.63 z mol leucine $cell^{-1}$ h^{-1}, n = 26). Throughout the different water columns, *CSIR* strictly reflected what has already been observed in *PHA* (see Figure 4).

Table 5. Leucine incorporation rates (*PHA* expressed in nmol m^{-3} h^{-1}) integrated in the depth intervals 1–100 m (epipelagic layer) and 100 m-bottom (mesopelagic layer) and normalized.

Station	depth (m)	*PHA*	
		1–100 m	100 m – botton (m)
Abio22-A	789	12.420	2.093 (600)
Abio01-B	590	2.651	0.463 (585)
H1	616	5.398	0.799 (600)
Abio06	400	3.805	2.589 (390)
Abio20	605	3.926	1.353 (500)

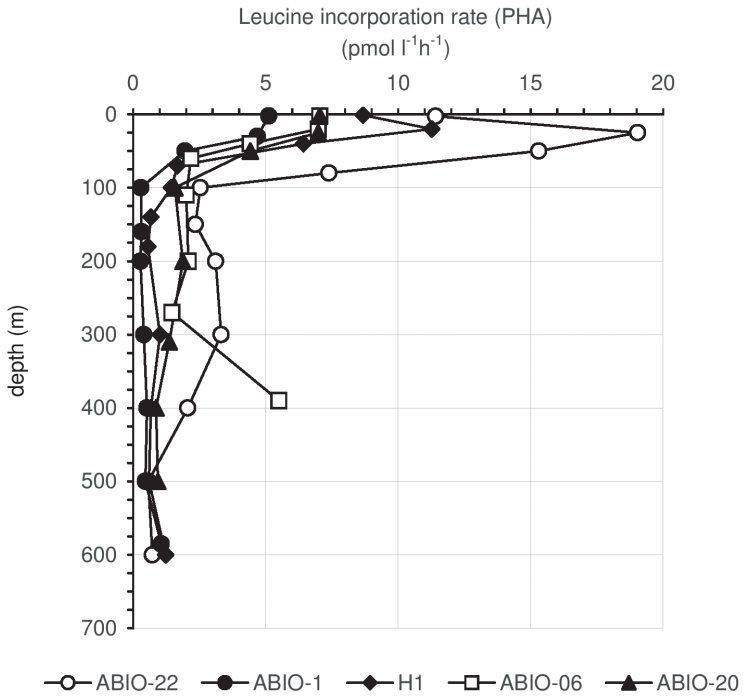

Figure 4. Leucine incorporation rates (*PHA*) at the five stations in the Ross Sea, Summer 2005.

PHP showed the same distribution of *PHA* throughout the water column. In the epipelagic layer, *PHP* varied between 0.46 and 29.51 ng C l^{-1} h^{-1} with the highest value at station Abio22-A at 25 m depth. In mesopelagic waters, *PHP* varied 0.33 and 6.11 ng C l^{-1} h^{-1} and it was, on average, 5 times lower than in upper layer (Table 4). In Table 6, the *PHP, PB, SGR* and *BTT* calculated at the ABIO22, ABIO01, H1, ABIO06 and ABIO20 stations are reported. In the euphotic layer, *SGR* day^{-1} ranged from 0.005 to 0.18 with a mean of 0.053. With the conversion factors used by Ducklow et al. (2001), SGR_D day^{-1} ranged from 0.013 to 0.44, with a mean of 0.128. Contrary to the *SGR,* the *BTT* (days) ranged from 3.79 to 128.31 (mean = 28.1) and BTT_D ranged from 1.55 to 52.04 (mean = 11.6). In mesopelagic waters, *SGR* and *BTT* were lower and higher, respectively, than those observed in the upper 100 m of the epipelagic zone.

Table 6. Prokaryotic heterotrophic production (*PHP*), Prokaryotic biomass (*PB*), Prokaryotic specific growth rate (*SGR*) and Biomass turnover time (*BTT*) calculated in the five indagated stations (ABIO22, ABIO01, H1, ABIO06 and ABIO20). PHP_D, PB_D, SGR_D and BTT_D were calculated using a different factor (107 fg C μm^{-3} cell^{-1} and *ID* = 1) according to Ducklow et al. (2001).

	Depth	n	Mean	SD	Range
PHP (ng C l^{-1} h^{-1})	0–100 m	22	12.182	9.492	0.575–36.883
	100–800 m	26	2.027	1.629	0.413–6.441
PHP_D (ng C l^{-1} h^{-1})	0–100 m	22	9.746	7.593	0.460–29.506
	100–800 m	26	1.622	1.303	0.330–5.153
PB (μg C l^{-1})	0–100 m	20	5.179	3.594	1.921–15.127
	100–800 m	25	3.222	1.939	1.074–9.028
PB_D (μg C l^{-1})	0–100 m	20	1.735	1.242	0.608–5.125
	100–800 m	25	1.077	0.676	0.338–3.101
SGR day^{-1}	0–100 m	20	0.053	0.047	0.005–0.183
	100–800 m	25	0.016	0.011	0.003–0.038
SGR_D *day*$^{-1}$	0–100 m	20	0.128	0.116	0.013–0.446
	100–800 m	25	0.040	0.027	0.006–0.095
BTT (*days*)	0–100 m	20	28.12	30.43	3.79–128.31
	100–800 m	25	65.29	50.44	18.24–254.05
BTT_D (*days*)	0–100 m	20	11.63	12.50	1.55–52.04
	100–800 m	25	27.458	23.152	7.26–118.26

Table 7. Prokaryotic carbon demand (*PCD*) and prokaryptic growth efficiency (*PGE*) depth-integrated and normalized values in the epi- and mesopelagic depth layers.

Station	0–100 m depth		100–800 m depth	
	PCD mg C m^{-3} h^{-1}	*PGE* %	*PCD* mg C m^{-3} h^{-1}	*PGE* %
Abio09-D	–	–	0.0069	26.23
Abio22-A	0.051	37.82	0.0052	60.21
Abio01-B	0.0354	11.62	0.0052	22.28
H1	0.0417	19.87	0.0045	27.58
Abio06	0.0551	18.99	0.0029	26.21
Abio20	0.0457	13.31	0.0079	26.71
Abio16	–	–	0.0037	88.49

The normalised prokaryotic C demand (*PCD*) ranged between 0.035 and 0.055 mg C h^{-1} m^{-3} in the epipelagic layer with high values at Abio22-A and Abio06. In the mesopelagic layer, it ranged between 0.003 and 0.008 mg C h^{-1} m^{-3} with the highest value at Abio20 (Table 7). Comparing the two layers, the averaged *PCD* was about 9 times higher in the epipelagic layer than in mesopelagic one (Kruskal-Wallis One-Way ANOVA: $P < 0.003$).

The normalised *PGE* ranged between 12 and 37% in epipelagic layer, with the lowest value at Abio01-B and the highest at Abio22-A. In the mesopelagic layer, *PGE* ranged between 22 and 88% with the minimum value at station Abio01-B and the maximum at Abio16. In contrast to the *PCD*, the averaged *PGE* was twice as high in the mesopelagic zone as in the epipelagic one (Kruskal-Wallis One-Way ANOVA: $P < 0.048$).

Table 8. Spearman–Rank correlations among microbial and environmental parameters in the whole data set. O_2= dissolved oxygen; *S*= salinity; *DEN*= density; *PA*= prokaryotic abundance; *PB*= prokaryotic biomass; *CCC*= cell carbon content; *ATP*= adenosine triphosphate; *CHLa*= chorophyll *a*; *PHP*= prokaryotic heterotrophic production; *CDPR*= carbon dioxide production rates.

CHLa vs.	***r***	**P**	**n**	***ATP vs.***	***r***	**P**	**n**
Depth	-0.545	0.0000	80	Depth	-0.836	0.0000	79
T °C	0.5	0.0000	80	*T °C*	0.776	0.0000	78
O_2	0.626	0.0000	73	O_2	0.543	0.0000	67
S	-0.505	0.0000	73	*S*	-0.762	0.0000	67
DEN	-0.522	0.0000	73	*DEN*	-0.747	0.0000	67
PA	0.347	0.0024	75	*PA*	0.399	0.0000	78
PB	0.318	0.0056	75	*CCC*	-0.255	0.0243	78
ATP	0.899	0.0000	42	*PB*	0.229	0.0437	78
PHP	0.46	0.0139	28	*CHLa*	0.899	0.0000	42
CDPR	0.346	0.0028	73	*CDPR*	0.775	0.0000	76

PB vs.	***r***	**P**	**n**	***CDPR vs.***	***r***	**P**	**n**	***PHP vs.***	***r***	**P**	**n**
Depth	-0.285	0.0000	134	Depth	-0.851	0.0000	136	Depth	-0.683	0.0000	56
T °C	0.197	0.023	134	*T °C*	0.714	0.0000	136	*T °C*	0.47	0.0000	56
O_2	0.459	0.0000	122	O_2	0.607	0.0000	123	O_2	0.784	0.0000	47
S	-0.215	0.0174	122	*S*	-0.731	0.0000	123	*S*	-0.545	0.0000	47
DEN	-0.205	0.0237	122	*DEN*	-0.726	0.0000	123	*DEN*	-0.554	0.0000	47
PA	0.898	0.0000	134	*PA*	0.304	0.0000	125	*PA*	0.662	0.0000	56
CCC	0.258	0.0027	134	*CCC*	-0.256	0.0040	125	*PB*	0.618	0.0000	56
ATP	0.229	0.0437	78	*PB*	0.216	0.0157	125	*CHLa*	0.46	0.0139	28
CHLa	0.318	0.0056	75	*CHLa*	0.346	0.0028	73	*ETS*	0.615	0.0000	54
PHP	0.618	0.0000	56	*ATP*	0.775	0.0000	76	*CDPR*	0.657	0.0000	54
CDPR	0.216	0.0157	125	*PHP*	0.657	0.0000	54				

The Spearman-Rank correlation analysis of the whole dataset yielded the outputs shown in Table 8; only the significant correlation coefficients (*r*) are reported, together with their significance level (P) and data number (n). Numerous significant correlations were computed amongst the hydrological, trophic and microbial parameters. *PB, CDPR* and *PHP* showed the largest number of highly significant correlations with most of the hydrological, trophic and microbiological parameters. No significant relations were detected between *CCC* vs. *PHP* and *CHL* or between *PHP* and *ATP*. In Table 9, the stations and depths, which showed the minimum and maximum values of each parameter in the epi- and mesopelagic layers, are summarised. Finally, the depth integrated data of standing stock - in terms of prokaryotic and autotrophic biomass (*PB* and *C-CHLa*), total biomass (*C-ATP*) and remaining heterotrophic biomass (*HB*) – and rates – in terms of respiratory and prokaryotic heterotrophic production rates (*CDPR* and *PHP*) – are reported in Suppl material 5: Table S4, *C-CHLa*, *PB* and *C-ATP,* amounted to 1545, 1681 and 4605 mg C m^{-2}, respectively. The remaining heterotrophic component (*HB*) presumably accounted for a biomass of 1379 mg C m^{-2}. *CDPR* remineralised 8.279 mg C m^{-2} h^{-1} with higher rates in the upper layers while *PHP* accounted for 1.697 mg C m^{-2} h^{-1} with a similar weight in the epi- and mesopelagic layers.

Table 9. Stations and depth with minimum and maximum values of each parameters in the epi- and mesopelagic layers. *CHLa*= chorophyll *a*; *ATP*= adenosine triphosphate; *PA*= prokaryotic abundance; *CCC*= cell carbon content; *PB*= prokaryotic biomass; *ETS*= electron transport system activity; *CDPR*= carbon dioxide production rates; *PHA*= prokaryotic heterotrophic activity.

	0–100 m		100–800 m	
	min	**max**	**min**	**max**
CHLa	Abio20 – 5m	Abio09(D) – 5m	Abio10 – 150m	Abio02 – 110m
ATP	H1 – 100m	Abio09(D) – 5m	H1 – 500m	Abio02 – 110m
PA	Abio05 – 100m	Abio09(D) – 25m	Abio22(A) – 100m	Abio07 – 500m
CCC	Abio20 – 2m	H1 – 2m	Abio05 – 200m	Abio09(D) – 800m
PB	Abio05 – 100m	Abio09(D) – 25m	Abio05 – 100m	Abio22(A) – 100m
ETS	Abio22(A) – 80m	Abio10 – 25m	Abio16 – 400m	Abio10 – 150m
CDPR	Abio16 – 2m	Abio10 – 25m	Abio16 – 400m	Abio17 – 120m
HEP	Abio22(A) – 80m	Abio06 – 2m	Abio16 – 400m	Abio10 – 150m
PHA	Abio01(B) – 100m	Abio22(A) – 25m	Abio06 – 270m	Abio16 – 250m

Discussion

Trophic conditions and prokaryotic biomass

The environmental assessment revealed a general picture of low trophism on a spatial scale as exemplified by the *CHLa* concentration. Analysis of the *ATP* concentrations also indicated modest or poor trophism. Considering *ATP* as a quantitative proxy for total living biomass (Holm-Hansen and Paerl 1972, Karl 1980), a relatively homogeneous biomass occurred throughout the RS. However, according to Karl's classification, most stations manifested moderate trophism or oligotrophy. In a previous study carried out in January-February 2001 in the RS, an extensive algal biomass and variable *ATP* estimates corresponding to different trophic statuses were observed (Azzaro et al. 2006, La Ferla et al. 2015). In particular, marked peaks of *ATP* were found (up to 1752 ng l^{-1}), revealing strong eutrophy according to Karl (1980). In our study, *ATP* determinations confirmed the trophic evaluation derived by *TSI*. In the 0–100 m depth layer, the autotrophic biomass (in terms of *C-CHLa*) accounted for 20–59% (mean value 44 ± 19%) of the total biomass (in terms of *C-ATP*). The highest and the lowest ratios of autotrophic biomass occurred at stations Abio09-D and Abio20, respectively, thus corroborating the previous statements about *TSI* and *ATP* estimates. In the epipelagic layer, the integrated *CHLa* values revealed a low phytoplankton standing stock varying from 4.76 to 23.68 mg m^{-2}. In contrast, in austral summer 2014, Mangoni et al. (2017) detected high autotrophic biomass (integrated *CHLa* up to 371 mg m^{-2}) in the epipelagic layer of the RS.

PA was in a range comparable to similar measurements made in several Antarctic marine environments (see table S1 in La Ferla et al. 2015). Comparison to previous studies in the RS confirmed the results of Monticelli et al. (2003) in Terra Nova Bay and Celussi et al. (2009) in Cape Adare.

PB decreased with depth by a factor of 1.7. However, it was higher than other prokaryotic measurements made previously in the RS (Buitenhius et al. 2012, Carlson et al. 1999, Steward and Fritsen 2004). The utilisation of a variable cell carbon content (from 11 to 45) to calculate the biomass in each sample could partially explain this pattern. Generally, two standard PA to biomass conversion factors are utilised in ecological marine studies: 20 fg C $cell^{-1}$ (Lee and Fuhrman 1987) or 9.1 fg C $cell^{-1}$ (Buitenhuis et al. 2012). Since cell carbon content is directly linked to cell-size variability (Young 2006), the utilisation of unvarying factors to convert abundance to biomass might distort the evaluations by overestimating or, in this case, underestimating the actual carbon amounts (La Ferla et al. 2015).

Respiration and CO_2 production

The *ETS* assay, originally designed by Packard (1971), continues to be successfully adopted in oceanic regions because of its high sensitivity and resolution levels that are not attainable with other methods based on sample incubation (del Giorgio and Williams 2005). Moreover, the response to the bias derived by the utilisation of empirical conversion factors from the *ETS V*max into actual rates of O_2 consumption and metabolic CO_2 production has largely been discussed and countered (del Giorgio and Williams 2005, La Ferla et al. 2010, Packard et al. 2015, Filella et al. 2018). Independently, good correlations between *ETS* and *in vivo* respiration rates were obtained in surface seawater samples in the framework of the ABIOCLEAR cruise. In the aphotic zone between 100 and 600 m depth of the RS, Azzaro et al. (2006) reported decreasing *ETS* activity throughout the water column and, despite the algal bloom in 2001, the ETS activity fell in a narrower range (0.017–0.170 µl O_2 l^{-1} h^{-1} than our data in the mesopelagic layer. In Summer 2014, Misic et al. (2017) observed different *ETS - POM* relationships, but ones consistent with the characteristics of a phytoplanktonic bloom of the *Phaeocystis* type. Moreover, their results featured averaged *ETS* values twice ours in the epipelagic and mesopelagic layers. *CDPR* was also twice ours in the upper layers and 4.6 times higher than ours in the mesopelagic one. The comparison between these findings evidenced great differences in the metabolic rates on an inter-annual scale and corroborated the importance of heterotrophic signals in understanding climate trends.

Heterotrophic energy production

HEP calculations in the microplankton quantify the energy generation due to the decomposition of *ATP* by a group of enzymes (*ATP*ases) in plasmalemma membranes of constituent bacteria and archaea as well as in constituent eukaryote mitochondrion. It represents a new metric in oceanographic analysis. The only other oceanic region, for which *HEP* has been calculated, is the Peru Current Upwelling at 15° S (Pisco, Peru).

The *HEP* calculations for the epipelagic and mesopelagic waters of the Peru Upwelling at 15° S are given in Packard et al. (2015). The average *HEP* (and standard deviation) in the epipelagic zone down to 150 m was 24 ± 30 × 10^3 μJ h^{-1} l^{-1}. In a transect (C-Line) for 185 km across the Peru Upwelling, epipelagic *HEP* ranged from a high of 108 × 10^3 μJ h^{-1} l^{-1}, at station C10, over the Peru Trench, 71 km from the coast, to a low of 2 × 10^3 μJ h^{-1} l^{-1}, 22 km further offshore at station C12. In mesopelagic waters below 150 m down to 1000 m, *HEP* was 96% lower than it was in the epipelagic zone, averaging only 84 ± 59 μJ h^{-1} l^{-1}. These values, from the Peru Upwelling system (Packard et al. 2015), are more than an order of magnitude higher than the values from the RS.

Prokaryotic heterotrophic activity and production

Time course experiments on *PHA* showed results in agreement with those detected in the VLTP-2004 project (Monticelli, personal communication). Linearity occurred within 1 and 6 hours for samples collected at 25 m depth and in a smaller time lapse for the others. Along the water column, higher *PHA* was always observed in the photic layers while reduced activity in the aphotic waters occurred. An increase in activity was always observed in the bottom samples, i.e. those taken a few metres from the sea floor. The increase of heterotrophic bacteria metabolisms in benthic boundary layers is a known phenomenon observed in other water columns (Packard and Christensen 2004).

At station Abio22-A, *PHA* was particularly high at all depths, with the highest normalised rates (12.442 nmol m^{-3} h^{-1}) in the photic layer. This was one order of magnitude higher than equivalent normalised *PHA* calculations observed at the other four stations. The mean leucine incorporation rate observed in the 0 - 50 m layer was 8.05 pmol l^{-1} h^{-1} (sd = 4.52, n = 15), the same order of magnitude as observed by Ducklow et al. (2001) in the RS during late spring period. The *PHA* observed in our cruise was also in accordance with that observed by Pedrós-Alió et al. (2002) in the Gerlache strait (Antarctic Peninsula) in late spring and summer cruises. The cell specific incorporation rate (*CSIR*) strictly reflected the distribution of *PHA* throughout the water column with the highest values in the surface to 50 m depth layer.

In our experiment, an isotope dilution of 1.25 was used to calculate the *CF*. It was equivalent to 1.94 kg C mol leu^{-1}. Considering the variability observed in *ID* determinations and the coefficient of variation (CV%) detected in the triplicate samples for leucine incorporation-rate analysis (mean = 14.3%, sd = 11.3%, n = 84), the *ID* should be not too far from 1 (assuming no isotope dilution). That value corresponds to a theoretical *CF* = 1.55 kg C mol leu^{-1} (Simon and Azam 1989). Similar *ID* values (mean = 1.27 corresponding to *CF* = 1.96 kg C mol leu^{-1}) were detected in the Antarctic Peninsula area in late spring-summer where the empirical method carried out simultaneously produced on average *CF* = 0.81 kg C mol leu^{-1} (Pedrós-Alió et al. 2002). In the framework of experiments conducted in subtropical northeast Atlantic Ocean, Baltar et al. (2010) discussed the incongruence often observed between empirical and theoretical *CFs* estimates as well as their variability in the mesopelagic water column (range 0.13–

0.85 kg C mol^{-1} leu). They argued that, in the deep domain, the carbon limitation and the slower cell growth take place, further reducing the deep water *CFs* as compared to the theoretical ones. The choice of theoretical, semi-theoretical or empirical *CF* can markedly affect the *PHP* estimation and, consequently, the derived parameters. In the case of a significantly different *CF*, it would be appropriate to use both values to furnish the best information about the carbon flux in the prokaryotic compartment. *PHP* followed the same distribution of *PHA* throughout the water column with a maximum value corresponding to a production peak at 25 m depth at station Abio22-A.

In the euphotic layer, *SGR* d^{-1} calculated with our *CFs*, resulted 2.4 times lower than SGR_D d^{-1} calculated using the conversion factors used by Ducklow et al. (2001). Contrary to the *SGR,* the *BTT* (days) was 2.4 times higher than that calculated with Ducklow's *CFs*. In mesopelagic waters, *SGR* and *BTT* were the opposite of those observed in the upper 100 m of the epipelagic zone.

From mean hourly values, the *CDPR/PHP* ratios in the epi- and mesopelagic layers were 11.75 and 0.80 μg C l^{-1} h^{-1}, respectively.

Prokaryotic metabolic patterns

Prokaryotic (bacterial and archaeal) activity is often measured using the *PGE* that defines the balance between catabolic and anabolic prokaryotic processes (Baltar et al. 2015). It corresponds to the proportion of dissolved organic carbon (*DOC*) that is converted by microorganisms into biomass and might be consumed by higher trophic levels (Eichinger et al. 2010). *PGE* is considered the most important factor affecting the C budget (Van Wambeke et al. 2002) and it indicates the efficiency of organic substrates recycling by prokaryotes (Mazuecos et al. 2015). The determination of *PGE* depended on the choice of the methodological procedures and approach, i.e. mostly respiration estimates and leucine to carbon *CF*. Indirect respiration estimates were often obtained from the sinking biogenic particles (Sweeney et al. 2000), from the bacterial production experimental data and from an empirical contribution of respiration (Ducklow et al. 2001; Ducklow 2003) or by sediment traps (DiTullio et al. 2000, Langone et al. 2000, Langone et al. 2003, Nelson et al. 1996). Sometimes, *PGE* is arbitrarily considered to be 30% or 36% (Manganelli et al. 2009) and a few papers have simultaneously identified the actual respiration and heterotrophic production rates (La Ferla et al. 2005, Zaccone et al. 2003, del Giorgio et al. 2011, Baltar et al. 2009, 2015 and references herein) in pelagic waters. In deep waters in the Atlantic Ocean, Baltar et al. (2010) determined *PGE* variations in the range < 1–34%. This high variability resulted in being highly correlated to the empirical conversion factors determined and adopted to calculate *PHP* (Baltar et al. 2010). High variability of *PGE,* on both space and time scales in ocean samples, has often been assessed (Lemée et al. 2002, Reinthaler and Herndl 2005) and low values of *PGE* (< 15%) have been associated with oligotrophic conditions (Biddanda et al. 2001, del Giorgio et al. 2011). In short, at low growth efficiency rates, more dissolved organic matter is remineralised,

keeping the nutrients cycling within the microbial cycle; conversely, at high growth efficiency rates, the dissolved organic matter is more efficiently transferred into the particulate phase thus strengthening the carbon distribution throughout the trophic food web (Cajal-Medrano and Maske 2005). In our study, the highest *PGE* values were determined at stations where oligotrophic conditions at the sea-surface were evidenced by *TSI*. Typically, most of the primary production in low-productivity environments is respired by bacteria (Biddanda et al. 2001). In epipelagic layers, the prokaryotic respiratory processes exceeded heterotrophic production, whilst high *PGE* was surprisingly calculated in the mesopelagic layer showing consistent differences with the upper layer. In addition, excluding the Abio16 and Abio22-A stations, where high *PHPs* were determined, the averaged *PGE* value in the mesopelagic layer surpassed the value in the epipelagic one. These findings were consistent with the high values detected in the deep layers of the Mediterranean Sea (La Ferla et al. 2005 and 2010) where *PGE* data did not correlate with primary production, but rather confirmed that the *PCD* could not be sustained solely by the *DOC* of autochtonous origin and/or by phytoplankton exudation. In the RS, Azzaro et al. (2006) compared estimates of carbon flux by sediment traps and found that about 63% of organic carbon, remineralised by respiration, was derived from the *POC* pool, confirming the decomposition rates of Ducklow et al. (2001) in the vertical *POC* flux. Weak bacteria-primary production coupling has also been ascribed to temperature restriction of metabolic utilisation (Pomeroy and Wiebe 2001) or to grazing together with the lack of bio-available dissolved organic matter (Bird and Karl 1999). The relatively high *PGE* probably also reflected the capability of prokaryotic cells to individually divide (fast growing communities) or to increase in size. We found that, in our samples, volumetric determinations in the mesopelagic layer were higher than in the epipelagic layer. They fell into the range of 0.038 to 0.196 μm^3 (data not shown). Furthermore, the circulatory dynamics of the water masses could also explain the high *PGE* at depth. When vertical convective processes occur with the sinking of surface water masses, a consequent enrichment of fresh organic matter in the deep layers happens (La Ferla and Azzaro 2001a, Azzaro et al. 2012). In temperate seas, the lateral advection of newly-formed water masses (both intermediate and deep) from convective regions as well as the lateral injection in the winter, of organic matter from the canyons and shelves, enhanced C respiration in deep layers (Packard et al. 2008, La Ferla et al. 2010). The horizontal *PGE* variability in the water layers suggested that, in mesopelagic waters, prokaryotes are able to use the available organic matter and convert it into biomass more efficiently than in epipelagic ones. This finding could be interpreted as an adaptive physiological response. It reflects the ability of deep-water microbes to efficiently exploit the available *DOC* at great depths. Placenti et al. (2018) suggested this mechanism for the deep Mediterranean Sea. Another aspect, that was unfortunately not considered, concerns the maintenance of the prokaryotic biomass and metabolism in terms of energetics when assessing the role of microbes in oceanic carbon cycles (Eichinger et al. 2010). According to Baltar et al. (2015), a combination of environmental stressors could enhance the proportion of the energy flux devoted to cell maintenance, inducing increases in cell specific respiration and decreases in *PGE*.

In incubation experiments, slow-growing bacterial communities tended to have low *PGE* and to respire a high portion of the secondary production in terms of leucine uptake (del Giorgio et al. 2011). Moreover, in oligotrophic systems, low *PGE* may result from the maintenance of active transport and from the production of exoenzymatic hydrolysis with high bacterial energy demand (del Giorgio and Cole 1998).

However, using mean hourly normalised values, the ratio *CDPR/PHP* (μg C l^{-1} h^{-1}) in the epipelagic layer was 3 times higher than in the mesopelagic one, presumably due to the occurrence of autotrophic respiration (Marra and Barber 2004). The prokaryotic carbon requirement (*PCD*) was particularly low in the mesopelagic layer where autotrophic production was lacking.

The significant relationship between *CDPR* and other physical and chemical parameters measured, suggests that respiration is strictly interconnected with environmental forces. Respiration varied in response to changes in hydrology according to Rivkin and Legendre (2001). In addition, it co-varied with different microbial parameters showing the consistent patterns of diverse aspects of microbial metabolism, as previously postulated by del Giorgio et al. (2011). The close link between the diverse aspects of prokaryotic patterns would imply that changes in the metabolic variables synergistically mediate the fate of organic matter by influencing the composition of organic material reaching the sediments (Catalano et al. 2006), the distribution of particulate and dissolved matter with depth (Carlson et al. 2000, Fabiano et al. 2000, Misic et al. 2017) and the remineralisation through the water column (Azzaro et al. 2006, 2012).

In all the stations, *CSRR* was surprisingly higher in the epipelagic layer than in the mesopelagic one. This suggests that a valuable contribution of organic matter of phytoplanktonic origin might sustain the heterotrophic metabolism in the upper layer. When we calculate *CSRR* by *ETS V*max, i.e. without utilisation of *ETS* to carbon conversion factors, almost all the mesopelagic values would be lower than surface ones with averaged *CSRR* value of 0.34 and 0.29 fg C $cell^{-1}$ in the epi- and mesopelagic layers, respectively (data not shown). Although different from other reports from temperate seas (Placenti et al. 2018, Baltar et al. 2009), the *CSRR* in epipelagic water was higher than in the mesopelagic one, suggesting more actively respiring cells in the upper layers. Nevertheless, high *CSRR* values were found at stations where *TSI* was low, suggesting the importance of cell-specific respiration in oligotrophic conditions, in agreement with the findings of Baltar et al. (2015) for subantarctic waters. Conversely, *CSRR* in Summer 2014 was higher in the deeper layers than in the surface ones (Misic et al. 2017). Nevertheless, high *CSRR* values were found at stations where *TSI* was low, which also corroborates the importance of prokaryotic respiration in the surface layer.

The cell-specific incorporation rate (*CSIR*) strictly reflected the distribution of *PHA* throughout the water column with the highest values in the surface to 50 m depth layer. The average *CSIR* was similar to that detected by Ducklow et al. (2001) and Pedrós-Alió et al. (2002) in the upper 50 m layer of the RS during Summer 1997 as well as in the Antarctic Peninsula, respectively. A decreased availability of organic carbon for synthesising new biomass could explain this finding (Baltar et al. 2015). The *CSIR*-temperature Spearman-rank relationships were positive in the photic layer and negative in the

aphotic one (data not shown). The positive correlation observed in the epipelagic layer amongst heterotrophic rates, *CHLa* and respiration, allowed us to consider a direct or indirect (previous exoenzymatic hydrolysis) flux of labile *DOC* from phytoplankton biomass and detritus towards the new prokaryotic biomass. In the XIX PNRA expedition (VLTP-2004 project), a mean of 2.35 μg C l^{-1} h^{-1} potentially mobilised by leucine aminopeptidase + ß-glucosidase activities, was detected in the photic layer off Victoria Land (Monticelli, personal communication). In the photic zone of Terra Nova Bay (RS), during summer 2000, the daily flow of C towards new prokaryotic biomass was equivalent to 0.2% of C, potentially mobilised by exoenzymatic activities (Monticelli et al. 2003).

Overall, in Summer 2005, the investigated area of the RS contributed in different ways to the epi- and mesopelagic layer carbon metabolism. *Per* sea-surface area, the autotrophic (by *C-CHLa*), prokaryotic (*PB*) and total standing stocks (C-*ATP*) amounted to 1545, 1681 and 4605 mg C m^{-2}, respectively. The remaining heterotrophic component (*HB*) presumably accounted for a biomass of 1379 mg C m^{-2}. The prokaryotic biomass appeared to be predominant in the mesopelagic layer with respect to the epipelagic one (depth integrated *PB* ratio epi/meso: 0.4). The entire heterotrophic production accounted for 1.697 mg C m^{-2} h^{-1} with a similar weight in the epi- and mesopelagic layers (depth integrated *PHP* ratio epi/meso was 1.03). Respiration remineralised 8.279 mg C m^{-2} h^{-1} with higher rates in the upper layers (depth integrated *CDPR* ratio epi/meso was 2.7).

Conclusions

This study was carried out within a time series of research conducted since the nineties in the Ross Sea. Through their metabolic rates, microorganisms worked as regulators of the organic carbon transfer in the Ross Sea and impacted Antarctic biogeochemical cycles. In this experiment, highly variable microbial metabolism was detected at all stations and depth layers. At the same time, coherent metabolic patterns were detected using different, independent, methodological approaches. The distribution of plankton metabolism and organic matter degradation was mainly related to the general oligotrophic conditions occurring during Summer 2005. The processes of heterotrophic production, respiration and growth efficiency revealed relatively low levels of carbon remineralisation. Compared with other cruises carried out in the Ross Sea, dramatic changes were found on an inter-annual scale. Monitoring the heterotrophic microbial patterns in long term series is proving to be an interesting approach in furthering understanding of biogeochemical trends. In contexts such as the mooring sites of LTER-Italy, it needs to be better known due to the climate-change implication of Antarctic Ocean on the global scale.

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Supplementary material 1

Figure S1

Authors: Maurizio Azzaro, Theodore T. Packard, Luis Salvador Monticelli, Giovanna Maimone, Alessandro Ciro Rappazzo, Filippo Azzaro, Federica Grilli, Ermanno Crisafi, Rosabruna La Ferla

Data type: measurement

Explanation note: Isopleths of temperature and salinity at the sea-surface and at 200m depth in the Ross Sea during the ABIOCLEAR Project. The CTD stations are indicated by black dots.

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Link: https://doi.org/10.3897/natureconservation.34.30631.suppl1

Supplementary material 2

Table S1

Authors: Maurizio Azzaro, Theodore T. Packard, Luis Salvador Monticelli, Giovanna Maimone, Alessandro Ciro Rappazzo, Filippo Azzaro, Federica Grilli, Ermanno Crisafi, Rosabruna La Ferla

Data type: parameters data

Explanation note: Acronyms of the studied parameters and link among some of them. The specific steps are described in the text.

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Supplementary material 3

Table S2

Authors: Maurizio Azzaro, Theodore T. Packard, Luis Salvador Monticelli, Giovanna Maimone, Alessandro Ciro Rappazzo, Filippo Azzaro, Federica Grilli, Ermanno Crisafi, Rosabruna La Ferla

Data type: measurements

Explanation note: Trophic State Index (TSI) calculated from Chlorophyll a concentrations (CHLa in mg m^{-3}) in the epipelagic layer and Adenosine Triphosphate (ATP in ng l^{-1}) concentrations detected in the epi- and mesopelagic layers of each station.

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Supplementary material 4

Table S3

Authors:Maurizio Azzaro, Theodore T. Packard, Luis Salvador Monticelli, Giovanna Maimone, Alessandro Ciro Rappazzo, Filippo Azzaro, Federica Grilli, Ermanno Crisafi, Rosabruna La Ferla

Data type: statistical data

Explanation note: Ranges, mean values and standard deviations of Cell Carbon Content (CCC as fg C $cell^{-1}$) determined in each station in the epi- and mesopelagic layers.

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Link: https://doi.org/10.3897/natureconservation.34.30631.suppl4

Supplementary material 5

Table S4

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Data type: measurement

Explanation note: Depth integrated data of prokaryotic (PB) and autotrophic (by C-CHLa) biomass, total standing stocks (C-ATP) and remaining heterotropic biomass (HB); respiratory (CDPR) and prokaryotic heterotrophic production (PHP) rates in the epi-, mesopelagic layers and total water column.

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RESEARCH ARTICLE

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Feeding Essential Biodiversity Variables (EBVs): actual and potential contributions from LTER-Italy

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Abstract

The conceptual framework of Essential Biodiversity Variables (EBVs) aims to capture the major dimensions of biodiversity change by structuring biodiversity monitoring and by ruling data collection amongst different providers. Amongst the research infrastructures adopting and implementing the EBV framework, LTER-Europe - the European node of ILTER (International Long-Term Ecological Research) - follows the approach to compare site-based biodiversity observations within and across its networks. However, a synoptic overview of their contributions with EBVs-relevant data is still missing, since data are not made available for several reasons. In this paper, we assess the capacity of LTER-Italy, one of the richest and heterogeneous networks of LTER sites in Europe, to provide data to "Species Distribution" and "Species Abundance" EBVs without inspecting and downloading their contents. To this aim, we mine the EBVs information which is publicly structured and shared by LTER site managers through DEIMS-SDR, the LTER-Europe online metadata repository. We classify the sites according to two types of contributions: (i) the *actual* contribution, based on metadata of datasets and (ii) the *potential* contribution, based on metadata of sites. Through these assessments, we investigate if LTER-Italy monitoring activities can provide EBVs measures and which sites currently provide datasets. By comparing the two contributions,

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we pinpoint the factors hampering the accessibility of LTER-Italy data and suggest solutions to increase the discoverability and reusability of LTER-Italy EBVs measurements. The research provides the first overview of EBVs monitored in LTER-Italy and the corresponding data management practices, as well as an evaluation of the interoperability of this network with respect to other research organisations for legal and technical aspects.

Keywords

Essential Biodiversity Variables, LTER-Italy, DEIMS-SDR, metadata analysis, research infrastructure assessment, EML, EMF

Introduction

Despite its indisputable role for human well-being and for ecosystem functioning (Díaz et al. 2006), biodiversity is threatened by anthropogenic stressors (Barnosky et al. 2011; Dirzo et al. 2014). The United Nations Convention on Biological Diversity (CBD) was organised to encourage countries to reduce pressures on biodiversity. In 2010, the CBD identified the "Aichi Targets" as specific goals for 2011–2020, to steer subscribing parties in developing national plans for assessing biodiversity loss and for providing solutions through policy regulations. Despite the increasing data volume in the last decades (Kelling et al. 2009), the fact that two-thirds of reports previously submitted to CBD lacked evidence-based information on biodiversity change (Bubb and Chenery 2011; UNEP CBD 2010) revealed the need for better managing this knowledge to actually enable meaningful estimation for policy practices.

Proliferation of studies is not always accompanied by integration of data in decision-making (Sutherland et al. 2012) as the exchange of knowledge between science and policy requires brokering and it is slowed by the lack of information effectively asked by policy-makers (Sutherland et al. 2012) or by the lack of access to reliable datasets through adequate tools.

The conceptual framework of Essential Biodiversity Variables (EBVs) was endorsed by the CBD (UNEP CBD 2010, 2016) to address this issue, wherein it defines a minimum set of essential measurements to facilitate the reporting of data amongst practitioners (Pereira et al. 2013) and to quantify the major dimensions of biodiversity change. This is supported by different works (Pereira et al. 2013; Weltzin et al. 2014; Geijzendorffer et al. 2016), where the EBVs framework is used as an abstraction layer of measurements by means of which the primary observations captured from any biodiversity initiative can be related to Aichi Targets. The Group on Earth Observations Biodiversity Observation Network (GEO BON) developed the framework and grouped 22 EBVs in six main classes (i.e. Genetic Composition, GC; Species Populations, SP; Species Traits, ST; Community Composition, CC; Ecosystem Structure, ES; Ecosystem Function, EF), each representing a level of biodiversity organisation, which requires appropriate datasets. The comprehensive nature of this conceptual framework enables providers at any scale of study to help cover the six levels and allows prioritising data mobilisation if essential measurements are lacking in monitoring

programmes (Geijzendorffer et al. 2016). Both scholarly and citizens science projects (eBird; TEAM), as well as online publishers (Pangaea; GenBank; LPI) can provide EBVs-relevant data, while projects (e.g. GLOBIS-B) or worldwide observation systems such as GEO BON and the Global Ocean Observing System (Muller-Karger et al. 2018) currently work to make the framework operational at research level. Amongst these, the European node of the International Long Term Ecological Research network (LTER-Europe) is embracing the EBVs framework to compare biodiversity observations across its networks and sites (Haase et al. 2018; Mollenhauer et al. 2018). Within LTER-Europe, monitoring activities on multiple biotic and abiotic ecosystem parameters are carried out by research sites grouped in national scale observing systems, i.e. networks. According to the INSPIRE Thematic Working Group "Environmental Monitoring Facilities" (2012) (Wohner et al. 2018), the research sites are the environmental monitoring facilities in which the LTER networks are organised and those focusing on biodiversity are unique data sources for three reasons: (i) they provide biotic in-situ data "enriched" by complementary abiotic measures, (ii) they provide data with high temporal coverage as their activities are planned with long-term view and (iii) as they belong to a network system and are distributed in different places of a specific country, they provide data with high geographic coverage. As previously highlighted by other authors (Kissling et al. 2018a; Haase et al. 2018), LTER networks are good candidates to supply values for almost all EBVs classes by monitoring the three LTER realms (marine, terrestrial and freshwater) (Geijzendorffer et al. 2016). Additionally, long-term datasets are recommended data sources for CBD indicators (UNEP CBD 2010) and the LTER geographical organisation makes it possible to set-up a distributed environmental facility to provide measurements with specific country coverage.

Although ongoing efforts are undertaken to align monitoring programmes to the EBV concept, the capacity of LTER networks to deliver relevant data has not been described yet, even if reported by authors of EBVs studies (Geijzendorffer et al. 2016) and by scientific products of networks activities. The presence of such description would instead be very useful for governments and advisory bodies to be aware of the LTER role in collecting updated measurements and for biodiversity practitioners to assemble its data resources within a worldwide extent (Peterson et al. 2018; Schmeller et al. 2017; Hardisty et al. 2019a).

Integration of biodiversity datasets from multiple sources is one of the current challenges faced by ecological informatics. It requires the use of standardised measurement protocols, the adoption of common data standards, ontologies, the creation of controlled vocabularies (Rosati et al. 2017), the use of virtual laboratories (Hardisty et al. 2013) and tools for EBVs data visualisation as well (e.g. European Biodiversity Portal, EBP; Global Biodiversity Information Facility, GBIF). Recently, Kissling et al. (2018a) proposed the concept of a global EBV data product built by integrating existing heterogeneous primary data, appropriately harmonised. As proposed for "Species traits" (Kissling et al. 2018b), "Species abundance" and "Species distribution" (Kissling et al. 2018a), a standard data processing workflow is necessary for the aggregation of primary data into global data products. According to the number of data management and

analysis procedures which are accomplished in the pipeline, three types of harmonised datasets are generated (i.e. EBV-usable, EBV-ready, derived and modelled EBV data).

At the same time, metadata compiled in standardised forms are fundamental for aggregation of biodiversity datasets. Metadata support different processes of data integration, by facilitating the discovery and the reuse of generated data to other scientists (Michener 2006). In order to assess the fitness for purpose of primary datasets, the EBVs metadata should provide indications about the three dimensions of the variable (space, time and taxonomy) and related attributes (extent, resolution and measurement units) (Kissling et al. 2018a). Particularly, this information can be shared through different standards (Wilkinson et al. 2016), developed to allow machine-to-machine interaction and to provide comprehensive information to understand and reuse datasets, including that related to content, context, quality, structure and accessibility (Michener et al. 1997). Even if two Biodiversity Research Infrastructures (BRIs) are successfully tested to build EBV data products, limits to aggregation still remain (Hardisty et al. 2019b).

The EBVs framework is a theory-driven and academic approach to biodiversity monitoring. On the one hand, it helps to attain consensus on what is essential to monitor and where to focus the limited financial resources to assure the assessment of biodiversity change (Vihervaara et al. 2017). On the other hand, it does not establish methods and instrumentations to allow integration of measures (Haase et al. 2018; Hardisty et al. 2019b), scarcely fostering the combination of data from multiple sources or the attitude to share them through public repositories so that many potential data resources remain hidden to end-users.

To be reliable, the above-mentioned description of LTER EBVs-relevant data has to pinpoint how the data can be integrated without missing the identification of all the potential sources of the research infrastructure considered. In fact, although facilities such as global IT aggregators (e.g. GBIF) or e-Science infrastructures (e.g. LifeWatch) increase the access to different users, scientists apply restrictions to data (e.g. commercial use) by limiting access and confidential sharing practices hamper the review of their contents. Moreover, the lack of funding for data curation and publishing activities limit their sharing through digital archives.

The objective of our study is to demonstrate the capacity of LTER-Italy to provide EBVs data through the analysis of its metadata resources, by considering that: (I) data (e.g. the dataset itself) has not always been published for several reasons; (II) not all LTER sites measure the biodiversity components, but monitoring occurs according to the ecological research focus of the programme.

Materials and methods

To free the analysis of the LTER network from data inspection and to identify the specific causes of restricted access to datasets, we examined EBVs information structured in metadata of LTER sites and datasets published by site managers in the Dynamic Eco-

logical Information Management System – Site and Data Set Registry (DEIMS-SDR), that is the most comprehensive catalogue of field observations sites in environmental research networks (Mirtl et al. 2018; Wohner et al. 2019). We focused on LTER-Italy, which we introduce in the "Case study" subsection. In the "Mapping EBV information" subsection, we provide the first mapping of the EBV information in two DEIMS Metadata Models and in "Collection of EBV information from metadata" and the following subsections, we propose a method to collect and analyse the metadata compiled for LTER-Italy sites. As meaningful metrics, we define two types of contributions to "Species Abundance" (SA) and "Species Distribution" (SD) EBVs which belong to the "Species Populations" class: the potential contribution of the network, assessed by processing its Sites Metadata and the actual contribution of the network, based on its Data Set Metadata. While the former reveals the rate of sites that can provide EBV primary data, the latter reveals the rate of sites that are currently sharing metadata for SA and SD datasets. An overview of the workflow for obtaining the two metrics from site metadata and from datasets metadata, respectively, is depicted in Figure 1.

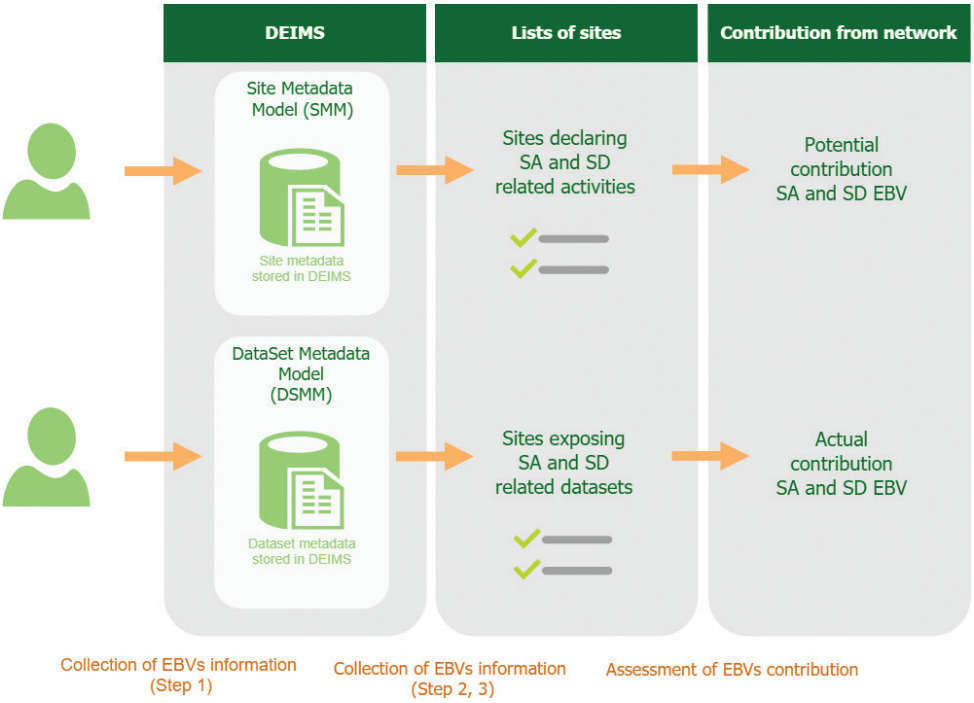

Figure 1. Metadata analysis overview. The diagram illustrates the activities required to perform the metadata analysis. The collection of EBV information from metadata is articulated in three steps which are followed by EBVs actual and potential contribution assessment for Species Abundance (abridged as "SA") and Species Distribution (abridged as "SD").

Case study

The LTER-Italy network is the Italian node of LTER-Europe and consists of 104 sites registered on DEIMS-SDR. It is the richest, amongst the European national LTER networks, with respect to the number of sites and it is one of the more heterogeneous for monitored ecosystems (Mollenhauer et al. 2018). In our analysis, we considered only those sites that are distributed inside the boundaries of the country, since our ultimate goal is to evaluate how this national monitoring programme can contribute to EBVs measurement or provide evidence-based data for local, regional and national governments in reporting to CBD; therefore, we excluded the 9 extraterritorial sites from the metadata analysis.

To avoid redundancy, we also excluded from our analysis the metadata from 23 Italian macrosites, as every macrosite aggregates the metadata of the sites it groups, which are individually analysed.

Hence, we analysed the metadata related to 72 sites and, in particular, we selected only those which compiled the metadata element "eLTER Parameter" (illustrated in the following subsection) which constitutes an informative tagging of the research activities of a site. The resulting set of sites is our statistical data sample and amounts to 43 sites.

Mapping EBV information

For the purpose of the present study, metadata of datasets and sites in LTER-Italy, stored in DEIMS-SDR, are queried: The two metadata models (DEIMS-SDR Metadata Models), in which these metadata are structured, are the Site Metadata Model (SMM) and the Data Set Metadata Model (DSMM). Both models contain elements referable to EBVs that allow us to assess whether a site can be an EBVs data provider and if a dataset can be reused (e.g. for its aggregation with other EBVs measurements). While the first model contains explicit references to EBVs, for the second we had to establish which elements should have been taken into consideration in our analysis. To this aim, we followed the metadata requirements described by Kissling et al. (2018a) and we checked which elements were actually compiled by site managers with this information.

EBVs information can be explicitly found from SMM in the "eLTER Parameters" element, whose content is a list of keywords from a hierarchically structured controlled vocabulary. The vocabulary is related to the LTER framework for standard observations (Haase et al. 2018; Mollenhauer et al. 2018) that combines the Ecosystem Integrity (EI) and the EBVs conceptual frameworks. Figure 2 illustrates the organisation of keywords relative to "Species Abundance" and "Species Distribution" observations.

As shown in Figure 2, the information relative to the EBVs monitoring programme is under the "Biodiversity" node of the tree structure of the vocabulary, further subdivided into three branches corresponding to the realms in which the EBVs are monitored: Terrestrial, Rivers and Lakes, Marine. These realms correspond to the biomes that

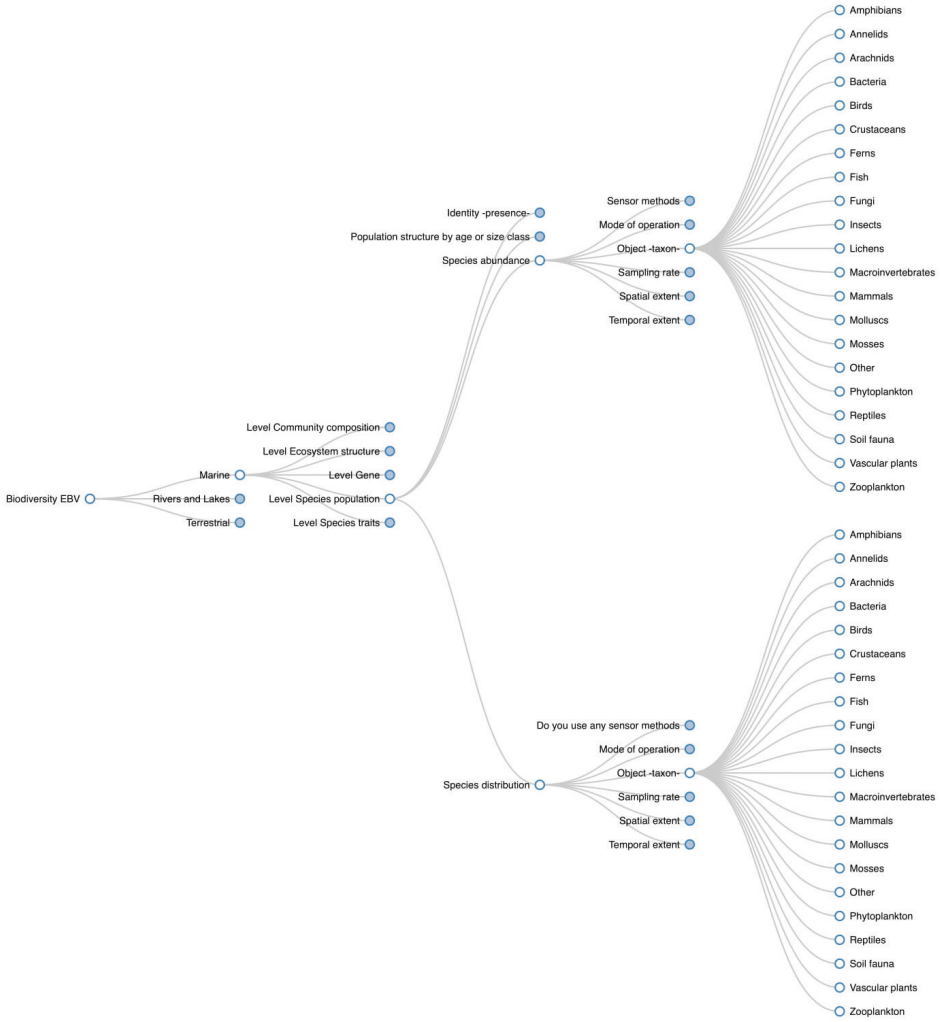

Figure 2. Organisation of EBVs-related keywords for the metadata element "eLTER Parameter". As an example, the Figure illustrates the tree structure for the Marine realm. The Figure shows the metadata field "Object (taxon)", associated with the eLTER Parameters element and analysed for LTER-Italy metadata. According to the realm selected, specific taxonomic terms are exposed. Empty circles provide the branches illustrated. Light blue circles are not expanded in the Figure.

are used for the classification of LTER-Italy sites in the following analysis. Each branch contains the following six fields related to aspects of the monitoring programme:

1. *Object (taxon)*, which represents information on the taxonomic extension of the measurements (in a site metadata document, the presence of a keyword from this branch indicates a taxonomic group of interest for the monitoring campaigns of the site);

2. *Spatial extent*, which corresponds to the spatial dimension of measurements, e.g. single sampling point, sampling surface;
3. *Temporal extent (sampling history)*, which corresponds to the temporal range of measurements;
4. *Mode of operation*, which describes the measurements process: either a continuous or campaign-based sampling;
5. *Sampling rate (or sampling rate per campaign)*, which corresponds to the frequency of measurements;
6. *Sensor methods*, which corresponds to the automatic tools possibly used to collect measurements.

DSMM does not instead contain information explicitly referred to EBVs.

For this reason, we analysed the DSMM to identify elements suitable for the provision of the information suggested by Kissling et al. (2018a) to identify and aggregate data provided by any research infrastructure to build global data products, i.e.:

a. the EBVs measured, that can be found in the elements "Abstract", "Title" and "Keywords" of DSMM;
b. the EBVs dimensions (taxonomy, time and space);
c. the EBVs attributes for each dimension (extent, resolution and measurement units);
d. the EBVs uncertainties related to the measurement of each dimension;
e. the workflow steps accomplished to provide a usable-EBV dataset with reference to the standard data processing pipeline described in the above-mentioned work. The first three steps are: (1) identify and import raw data and associated metadata; (2) check data-sharing agreements and licences; (3) check data completeness and consistency. We considered the accessibility and completeness of metadata with respect to this information a requirement itself.

Table 1 reports the mapping of b. c. and d. to DSMM elements, while Table 2 details the mapping of the workflow steps described in e.

The mapping was obtained by analysing the model and selecting suitable elements to provide the information considered and by checking them with compiled metadata.

Collection of EBV information from metadata

The EBV information, described in the previous section, was collected for every site of LTER-Italy and structured in a database. The steps that we followed to collect the EBV information from the metadata elements, both of the sites and of the datasets, are presented below:

1. The investigator accesses the metadata through the public web interface of DEIMS-SDR and reads the content of the selected metadata elements exposed in a human-readable format. Through DEIMS-SDR, it is possible to read sites and datasets meta-

data shared by the network and, in particular, the values for the elements synthesised in Table 3. As reported in Kliment and Oggioni (2011), dataset metadata can be automatically exported according to standard XML schema Ecological Metadata Language (EML) and site metadata can be exported according to the standard schema Environmental Monitoring Facilities (EF). However, some information actually stored following DEIMS schemas, has no counterpart in these machine-readable standard schemas exposed by the platform. For instance, the "eLTER parameter" element of site metadata, which plays a key role in our analysis, is missing. For this reason, the process cannot be entirely automated and manual retrieval of values is required.

2. The investigator records the values of the variables under consideration for every site in a database. This database constituted the groundwork from which we derived the descriptive statistics presented in this study: it is publicly available in the form of a spreadsheet (Zilioli and Oggioni 2018).
3. The investigator uses the database to identify two lists of sites:
   a) the list of sites declaring SA and SD activities, obtained from site metadata;
   b) the list of sites exposing SA and SD related datasets, obtained from dataset metadata;

Table 1. Information suitable for building EBV data products mapped to DEIMS-SDR DSMM elements. The table illustrates elements which report information on EBV dimensions, attributes and uncertainties. The name of the related fields appears between parentheses while "ND" is used when elements to report the information are missing in the model.

EBV Dimensions ► **EBV Attributes and Uncertainties ▼**	**Taxonomy**	**Space**	**Time**
Extent (e.g. how many and which species are documented; sampling locations, satellites; length of time series, continuous recording, time period of collection of records)	Taxonomic coverage - Biological classification (field_bio_classification) OR Keywords (field_keywords_envthes) OR Title (field_title)	Geographic (field_related_sites)	Temporal extent (field_date_range)
Resolution (e.g. species, genus, higher taxonomic level; volume, resolution of satellite sensors; time window of sampling; sampling frequency)	Keywords (field_keywords_envthes) OR Title (field_title)	Abstract (field_abstract)	Sampling time span (field_sampling_time_span)
Measurement units (e.g. taxonomic entity for which species distribution and abundance data are sampled; metres, cubic metres, degrees; hours, days, months, years, decades)	Title (field_title) OR Abstract (field_abstract)	Method (field_related_links)	Minimum sampling unit (field_minimum_sampling_unit)
Uncertainties (e.g. wrongly recorded coordinates; precision of time of collection; identification and observation uncertainty differences in taxon concepts)	ND	ND	ND

Table 2. Information suitable for building EBV data products mapped to DEIMS-SDR DSMM elements. The table associates workflow steps required to build EBV-usable datasets to DSMM elements carrying the appropriate information. The name of the related field appears between parentheses.

Workflow Step ▼	DSMM elements ▼
Identify and import raw data and associated metadata (1)	Data set Title (field_title); DOI (field_doi), Online location (field_online_locator)
Check data-sharing agreements and licences (2)	Principal and granted permission (field_access_use_termref) Intellectual rights (field_dataset_rights)
Check data completeness and consistency (3)	Quality assurance (field_quality_assurance)

The total number of LTER-Italy sites of list a) are used to measure the EBVs Potential Contribution (PC) of the network; the total number of LTER-Italy sites of list b) are used to measure the Actual Contribution (AC) of the network.

Assessment of EBVs Potential Contribution (PC)

We measure the potential capacity of LTER-Italy to provide SA (or SD) data as the number of sites monitoring the selected variable against the total number of sites in our sample, as formalised in the following formula:

$$PC_v(LTER - Italy) = \frac{SM_v(LTER - Italy)}{S_{Total}},\ v \in \{SA,\ SD\} \subset EBV$$

where PC_v (LTER-Italy) is the Potential Contribution of LTER-Italy to EBV variable v. EBV in the formula represents the set of all EBVs: In our study, we are limiting v only to SA (Species Abundance) or SD (Species Distribution). SM_v is the number of Sites with the site metadata compiled for variable v. S_{total} is the number of sites taken into consideration as described in the "Case study" subsection.

Assessment of EBVs Actual Contribution (AC)

We measure the actual capacity of LTER-Italy to provide SA (or SD) usable data as the number of sites providing at least one dataset metadata compiled for the selected variable against the total number of sites in our sample (S_{total}):

$$AC_v(LTER - Italy) = \frac{SDM_v(LTER - Italy)}{S_{Total}},\ v \in \{SA,\ SD\} \subset EBV$$

where AC_v (LTER-Italy) is the Actual Contribution of LTER-Italy to EBV variable v. In our study, v is limited to SA (Species Abundance) or SD (Species Distribution) amongst

Table 3. Selection of Site Metadata and Data Set Metadata elements for analysis.

Site Metadata	Data Set Metadata
Site name	Dataset title
eLTER parameters (Biodiversity (EBV) – Object (taxon))	Related site
	Abstract
	Keywords
	Access and use constraints
	Intellectual rights
	Online distribution
	Geographic
	Temporal extent
	Taxonomic coverage

other possible EBVs. SDM_v is the number of Sites with at least one dataset metadata in which one of the elements reported in Table 3 is compiled for variable v. Again, S_{total} is the number of sites taken into consideration, as described in the "Case Study" subsection.

Collection and Assessment of Data management practices

For the considered LTER-Italy sites, we also imported the values of metadata elements belonging to the "Data management" and "Data sharing policies" sections, which contain additional information about data handling and sharing practices. We decided to enrich the EBV information retrieved through eLTER parameters with that describing the data management practices exposed in Site Metadata, so as to identify the researcher's attitude towards sharing data with external users. Although these data management practices are declared by the site managers in relation to their whole activity and not specifically referred to EBVs, we consider this information suitable for describing technological characteristics of the site (e.g. storage media and formats used, web services created and general policies applied to ecological data) and helpful to explain discrepancies between PC and AC.

For this assessment, we selected the following elements of SMM:

- *Data Storage Location*: this element describes the general design of data storage and the number of storage locations for data. By compiling this element, the site manager provides information on central or distributed data storage, the number of storage locations within an organisation as well as the storage locations situated by other organisations;
- *Storage format*: this element describes the different formats in which data are managed or are available to end users;
- *Data services*: this element describes which services are provided to end users (external or internal) to connect to data;
- *General data policy*: this element describes which rewarding actions and restrictions to user or activities are applied to data;
- *Data Request Format*: this element describes how the datasets can be requested from the site.

Results

The application of the methodology to the LTER-Italy case study resulted in the outcomes presented in this section.

EBVs Potential Contribution from LTER-Italy

The Potential Contributions from LTER-Italy are:

PC_{SA} (LTER – Italy) = 53%
PC_{SD} (LTER – Italy) = 42%

It is possible to group the sites in accordance to the biome they declare to monitor as explained in subsection "Mapping EBV information". Figures 3, 4 and 5 represent these groups of sites, the "Terrestrial", "Marine" and "River and Lakes", respectively. In these figures, we present not only the number of sites measuring SA and SD, but also the number of sites measuring other EBVs, to contextualise the contribution of the network to EBVs framework in a wider perspective.

The figures profile each biome-specific group with respect to the whole set of EBVs and each bar counts the number of sites declaring activities related to the corresponding EBV. Hence, through this analysis of metadata, we can compare our main analysed potential contributions (for SA and SD) with other EBVs monitored by LTER-Italy sites.

For marine biome, LTER-Italy accounts for 10 sites as potential providers for SA and SD EBV measures, which represent 23% of the sample in both cases. For terrestrial biome, LTER-Italy accounts for 8 and 7 sites for SA and SD EBV, respectively, which represent 19% and 16%; for the River and Lakes biome, the network accounts 5 (SA) and 1 (SD) sites, i.e. 12% and 2%, respectively.

SA and SD are the most measured EBVs. We can distinctly consider the total number of sites for every biome and restrict the analysis to them. In this case, the evaluation of the potential contribution to each biome is: 100% for SA, 88% for SD in Terrestrial biome; 77% for SA, 77% for SD in Marine biome; 50% for SA, 10% for SD in Rivers and Lakes biome.

Although there is a high number of biodiversity monitoring sites for both EBVs in each biome, the analysis suggests the presence of bias in long-term monitoring of biodiversity for EBVs, different from SA and SD. In fact, "Genetic composition" is an under-represented EBV class as only one site in LTER-Italy provides measures for "Allelic diversity" EBV; moreover, with respect to the six GEO BON classes that groups EBV (see "Introduction" section), while the Marine and Terrestrial biomes of Italy can be potentially described with 4 of the 5 class of EBVs, the Rivers and Lakes biome can be potentially described by data which cover only 2 out of 5 classes. By considering together SA and SD, monitoring sites which are potentially able to provide useful data are 72% of our sample.

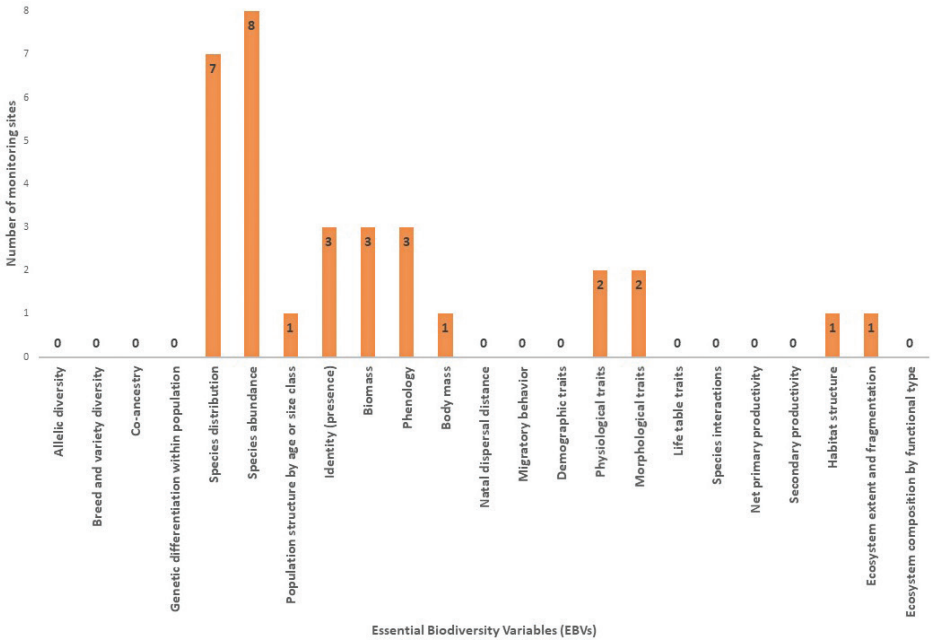

Figure 3. EBVs coverage for Terrestrial Biome sites of LTER-Italy (total number 8). The height of the bars represents the number of Terrestrial sites that are claimed to measure the selected variable.

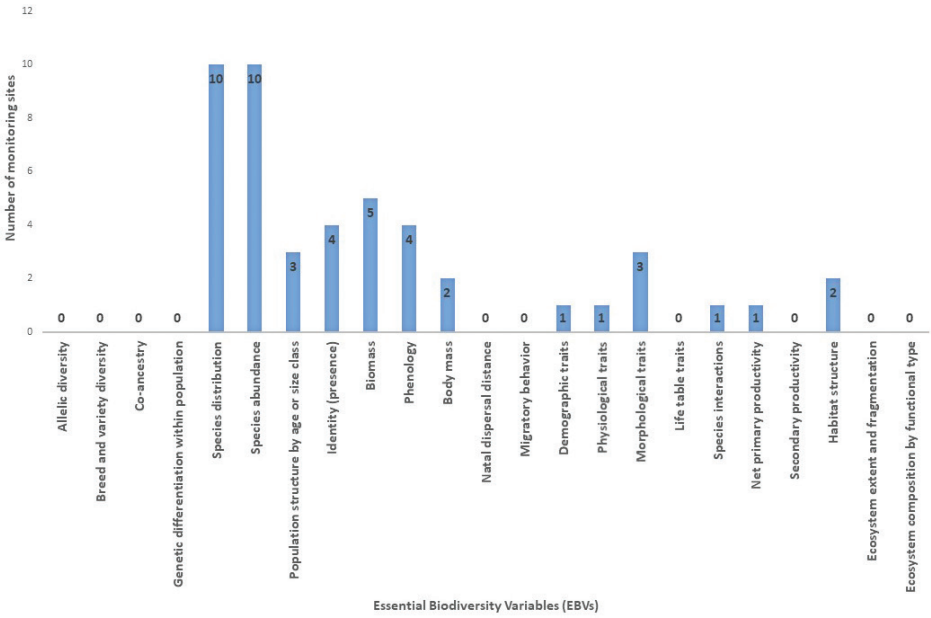

Figure 4. EBVs coverage for Marine Biome sites of LTER-Italy (total number 13). The height of the bars represents the number of Marine sites that are claimed to measure the selected variable.

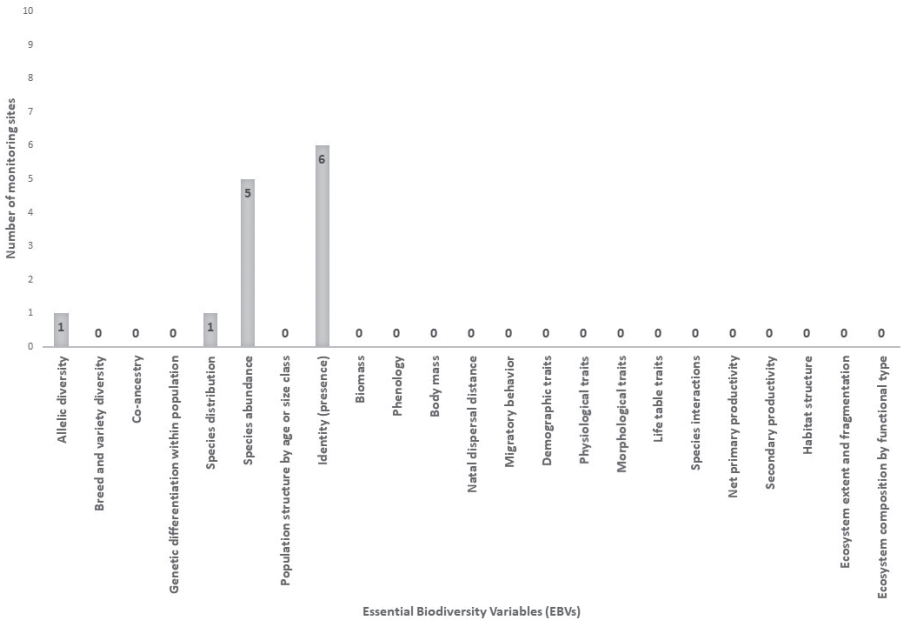

Figure 5. EBVs coverage for Rivers and Lakes Biome sites of LTER-Italy (total number 10). The height of the bars represents the number of Freshwaters sites that are claimed to measure the selected variable.

EBVs Actual Contribution from LTER-Italy

The Actual Contributions from LTER-Italy are:

AC_{SA} (LTER – Italy) = 14%
AC_{SD} (LTER – Italy) = 14%

The two contributions are the same because all the dataset metadata are referred to as "Species Abundance", thus providing measures of presence and absence of species which are useful also for "Species Distribution".

We can expand this numeric result with some further consideration on EBV dimensions (taxonomy, space, time) and attributes (extent, resolution, measurement unit, uncertainties), trying to evince the adequacy of metadata with respect to those discussed in section "Materials and Methods".

Figure 6 presents the percentage of completeness of dataset metadata with respect to these information requirements, by considering the full (100%) metadata completeness when information is given in each dimension of EBVs (taxonomy, space and time) for the three attributes (extent, resolution and measurement units).

Regarding the observed taxonomic groups, metadata are accessible for plankton (phytoplankton, zooplankton) and vascular plants. The orientation of the network to focus on these taxonomic groups is confirmed by the analysis of site metadata by

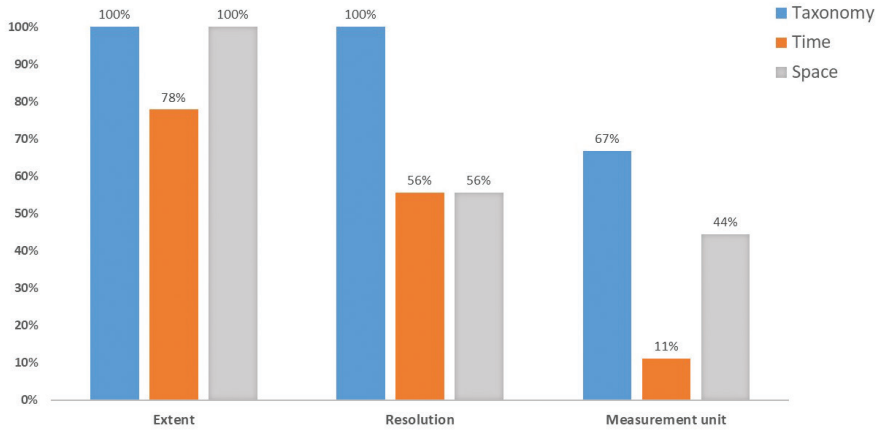

Figure 6. Metadata completeness with respect to EBV dimensions (taxonomy, space and time) and attributes (extent, resolution and measurement units) as mapped in Table 1.

which we observe that 29 sites can provide abundance measures for Phytoplankton and 13 sites for Zooplankton. Even if, in 100% of cases, the metadata element providing information for the taxonomy extent is compiled, the terms used do not belong to the ecological controlled vocabulary: Identification of organisms is given through free texts defining heterogeneous groups of taxonomic categories. Traditional methods (e.g. vegetation surveys, cells counting) are used to provide data along different spatial and temporal extents as described in "Materials and Methods".

Metadata are provided for long time-series datasets covering about 25–30 years or shorter periods. The 78% of metadata illustrated a sampling frequency time of five months, but resolution is provided by 56% and measurement units are not provided for 90% of metadata. In 100% of cases, sampling areas are carefully georeferenced through the metadata element "Geographic", reporting information about the spatial extent with altitudes and bounding coordinates provided by geotagging devices. However, also in this case, resolution and measurement units are provided in 56% and in 44% of metadata, respectively.

Figure 7 shows the completeness of metadata elements presented in Table 2, required by the workflow to integrate primary data in an EBV data product. A total of 100% of metadata and datasets is identified by elements about online distribution or by a title. In particular, the "Resource Locator" element accounts for the available ways to access the dataset resource: it is the navigation section of a metadata record, pointing users to the location where a dataset can be retrieved or where information about how to acquire a dataset can be obtained (e.g. the Uniform Resource Locator, URL; the E-mail to request a dataset). The "Resource Locator" element can also consist of a Digital Object Identifier (DOI) pointing directly and persistently to the dataset. This element covers two cases: The first is when an interested person must write asking for the data and, in this case, an e-mail address is provided; the second is when data are published

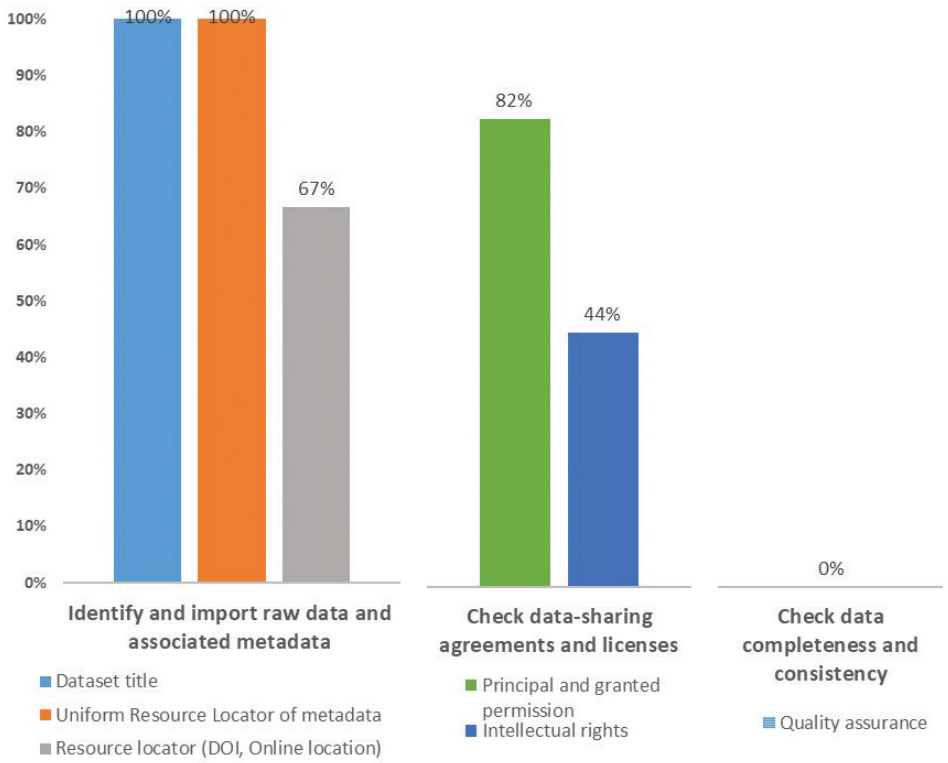

Figure 7. Metadata completeness with respect to the information mapped in Table 2. The three stepsrequired to obtaining EBV-usable datasets (i.e. datasets with measurements and observation protocols in thecorrect formats) are reported. Bars of different colours correspond to percentages of information provisionneeded for checking the workflow steps through different metadata elements. In particular, bar 1 (lightblue) corresponds to the completeness percentage for "Dataset Title"; bar 1 (orange) for "Uniform ResourceLocator of metadata"; bar 1 (grey) for "Resource Locator" of dataset; bar 2 (green) for "Principal and granted permissions"; bar 2 (blue) for "Intellectual rights"; bar 3 for "Quality assurance".

online and are retrievable through the provided URL or DOI. We highlighted in the figure the percentage of dataset URLs, 67%. The presence of URLs should indicate the online availability of a dataset stored within remote servers, project or institutional online repository, but unfortunately, none of the datasets proved to be publicly available, neither to be downloaded nor to be viewed, thus limiting access to the structure, the format and the observation protocols used to create data.

Licences and data-sharing agreements are applied to 82% of datasets through the metadata element "Principal and granted permission". In particular, there are distinctions in licensing based on intended use of datasets (for research, for public). For research uses, the actual granted permissions are "Free for access and use upon request" and "Free for access", while for generic public uses, the "Other restrictions according to rules defined in in-

tellectual rights" are applied by the providers and more finely defined by the metadata field "Intellectual rights". The field "Intellectual rights" is specified for 44% of datasets and, in the case of generic public uses, it almost always asks for "co-authorship on publications resulting from the use of dataset". We found just one dataset with "No access" granted.

Data quality information is not provided for any dataset; hence no dataset appears to be EBV-usable at metadata analysis level.

Data management practices

Figure 8 describes data management practices of potential EBVs contributors with respect to characteristics defined in subsection "Collection and Assessment of Data Management practices" of "Materials and Methods".

Data storage location is "central" (i.e. in the server of an institution) for 79% of sites, while in 10% of sites, data are distributed amongst repositories of different institutions and, in 11% of sites, data are distributed within the same institution (i.e. multiple places for data within the organisation that maintains and manages data).

With respect to *storage format*, 62% of the sites organise their data in structured files or spreadsheets, while 21% of sites declare their management of spatial datasets. Finally, dataset's proprietary formats are chosen by only 7% of sites.

Services for data access are not specified by 72% of sites while 14% exploit standard web services and 7% declare sharing its datasets through a generic "data portal".

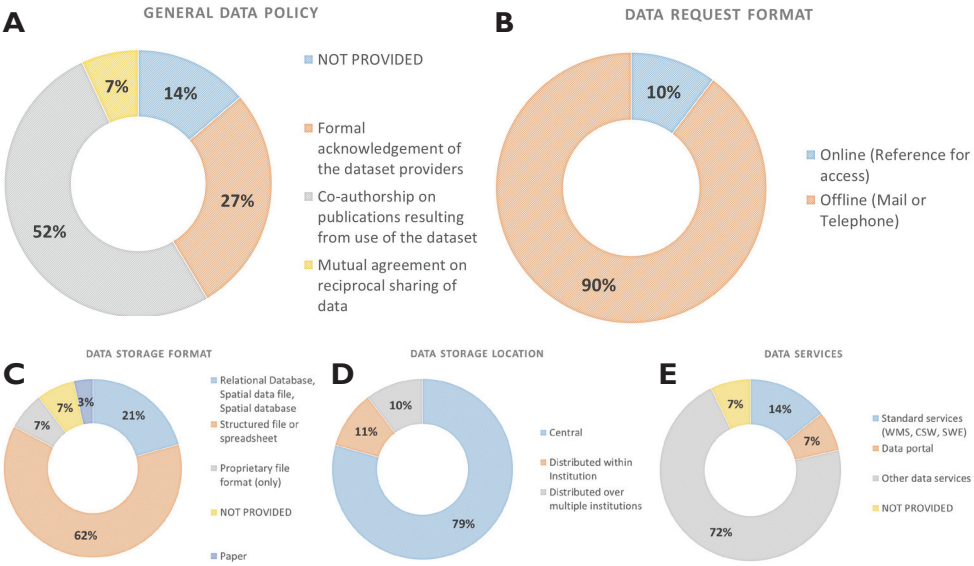

Figure 8. Data management practices associated with EBVs potential contributor sites. The Figure separately illustrates the relative percentages of sites for **A** policies applied to data **B** request formats for release data to external users **C** storage formats **D** storage location **E** web services used to make access to data.

A general preference for offline release of data, that explains the Actual Contribution results, is evident in the analysis of the *data request format*: only 10% of sites give online access to data, while 90% of sites prefer to be contacted by telephone or mail for giving access to data.

Finally, focussing on the *general data policy,* the data usage must be acknowledged by 52% of sites through demand for co-authorship on publications resulting from the use of datasets; mutual agreement on reciprocal data sharing are required to data users in 7% of cases only, while information is not provided at all by 14% of sites.

Discussion

Researchers and policy-makers are called to take joint actions to face biodiversity emergencies, as highlighted by the growing demand for readily accessible data that can be integrated and analysed in support of political decisions (Hardisty et al. 2013; Hoffmann et al. 2014). Even if biodiversity management literature reports advances with works relating EBVs to governmental policy (Turak et al. 2017a), the information pertinent to these essential measurements can be hidden to public users in the Web for different reasons, spanning from technical obstacles (e.g. limits to data discovery) to legal constraints (e.g. restrictions applied to sensitive data). In this paper, we investigate whether LTER-Italy provides measures for SA and SD by freeing the analysis from the need to directly access data. It is of interest to examine which specific motivations possibly hamper the public accessibility to data.

For the discussion of results, it is important to consider the following. First, LTER research is driven by specific scientific questions, posed by individual scientists or groups. These programmes are typically decentralised, rarely harmonised at global level and unevenly distributed geographically (Haase et al. 2018). Moreover, the selection of biotic and abiotic variables to monitor is at the sites' discretion, according to the available instrumentation and ecological focus. To monitor biodiversity is not a mandate of any central funding body or any coordinating scientific committee, hence not all the sites are expected to provide these measures. Second, communicating biodiversity change to wider audiences remains challenging, even if necessary to make biodiversity measurements into effective management actions (Turak et al. 2017b). Thus, a comprehensive, trustworthy and synoptic overview of monitoring and research capacity of scientific networks is needed.

Through the analysis of EBV information derived from metadata, we described the potential and actual contribution of LTER-Italy to provide EBV related datasets for collection and mobilisation of SA and SD measures (Hardisty et al. 2019a). We demonstrate LTER-Italy's good potential in providing EBVs, but also the discrepancy in data provision for SA and SD, which is graphically represented in Figure 9.

In fact, while 53% of sites potentially provide SA and 42% of sites SD data (Figure 9a), the number of sites which actually collect datasets metadata are 14% for both (Figure 9b); moreover, no dataset is accessible due to web resource localisation problems (e.g. URL pointing to no resource, dead link, broken link, location shifting), thus limiting web users in accessing the primary data.

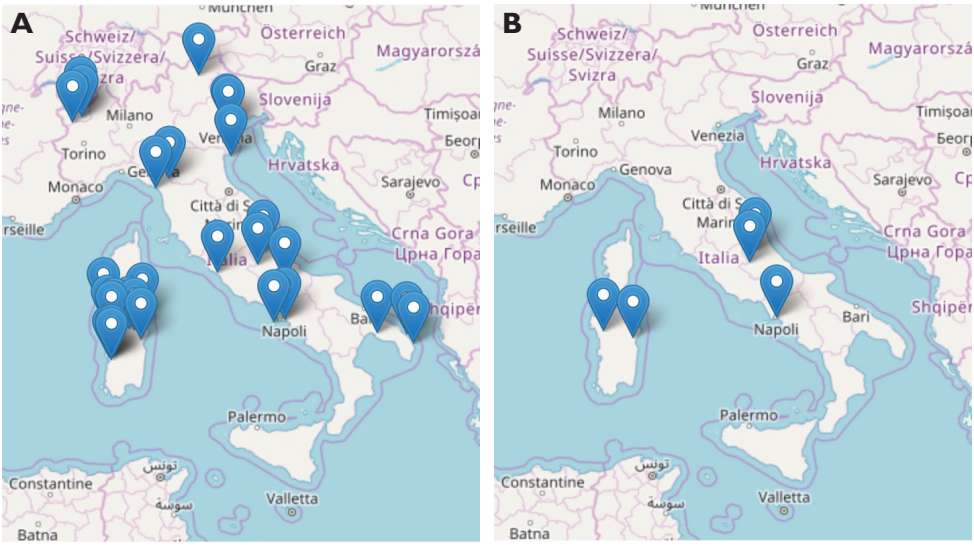

Figure 9. LTER-Italy potential and actual contribution sites. LTER-Italy sites which potentially supply SA and SD site-based, long-term measures are represented with a placeholder in **A** while sites which currently provide SA and SD metadata for primary datasets are represented in **B**.

Our metadata analysis suggests that community-related reasons are the factors which can explain the gap between the network's potential and actual capacity, thus providing clues to making data more accessible. Although several studies highlight that scientists often do not make their data available in digital form, for reasons including insufficient time and lack of funding (Tenopir et al. 2011), the analysis of data management practices rather suggests that the community is open to release its data, but preferably through offline media, instead of doing it by applying additional restriction to online distribution tools: this is consistent with our results that only a small part of the community (21% of sites) uses data-sharing services (standard Web services, data portals) and the greater part (79% of sites) centrally archives datasets, rather than distributing them through different storage media. However, these results are not conflicting with the more general attitude of the community to share data, but they pinpoint the need for tailored solutions to improve discoverability and reusability of data in this scenario. In fact, 52% of the general policies and 60% of licences, applied directly to biodiversity data, indicate that scientists approve data sharing for: (i) research purposes insofar as the collaboration is rewarded with citations or co-authorship (e.g. licences chosen are "Free for access and use upon request"; "Co-authorship on publications resulting from use of the dataset"); (ii) public purposes, insofar a formalised recognition, coming from the use of data, is given.

In such a context of limited online access to data, well-compiled metadata are even more necessary. Different types of metadata can compensate for the choice to regulate access to data, by supplying information for discovering and mining EBV information.

Different from the data management workflow described in Kissling et al. (2018a, 2018b), we found that published metadata of sites play an essential role in provid-

ing sound information about which EBVs are monitored. First, being compiled by site managers, they offer trustworthy indications about the observed properties of biodiversity. This means that the metadata contained in DEIMS-SDR are suitable wherever an authoritative assessment of measured parameters is needed, for example, when semi-quantitative or qualitative analysis are required for Ecosystem Integrity and Ecosystem Services assessments or for biodiversity change assessment (Turak et al. 2017a; Stoll et al. 2015), which are currently carried out with time-consuming surveys of key stakeholders and researchers. However, by providing the first mapping between EBVs metadata requirements and elements of DEIMS-SDR metadata models, we underline that technical improvements, facilitating the retrieval of EBVs information, have also to be addressed, particularly to assure its thorough exportation in standard formats (EML, EMF).

Second, metadata can be useful to identify thematic focus of any network (not only LTER) exposing metadata in DEIMS-SDR. In fact, through metadata analysis, we assess that LTER-Italy conducts biodiversity measures through different numbers of sites in every realm. Marine and terrestrial biomes are described with a higher number of EBV classes (5 and 4, respectively) with respect to freshwaters biome (2 classes) and with different frequencies for each EBV. SA and SD are the most measured EBVs, but the analysis shows that not all the sites provide these measures. The result can direct financial resources to activate monitoring activities, at least by volunteers of local communities through citizen science projects which present several advantages over traditional *in situ* field surveys for the collection of SA and SD data (Chandler et al. 2017, Kissling et al. 2018a).

The analysis of site metadata can provide spatial and temporal coverage, sampling frequency and monitored taxa, without the need for exploring related data, thus facilitating the planning of harmonised research activities at network scale. The method highlights, in fact, the capacity of the network in supplying data for taxa groups which are less monitored than invertebrates or vascular plants, towards which there is a bias described in the EBV-related literature (Proença et al. 2017). However, since metadata are compiled by site managers, they can be incomplete for elements that are not mandatory, as in the case of the "eLTER Parameter". For this specific reason, our analysis was limited to a sample of the community which is not representative for the comprehensive capacity of LTER-Italy to monitor EBVs. For example, ten sites are excluded from the analysis, as their metadata reported information on biodiversity solely in the element "Parameter". However, even if this element reports information related to generic species abundance and distribution measures, it provides information neither with respect to other measures referable to the EBV concept, nor at the level of detail required to obtain a comprehensive representation of the investigated objects and scales. To expand the statistics to the whole network, all sites have to describe research activities through specific metadata elements and the site metadata analysis needs to be completed with that related to datasets metadata. Through datasets metadata, we attempted to evaluate more deeply whether their information enables the reuse of datasets and whether datasets are accessible to other investigators: for example, to provide *in situ* data for Calibration and Validation activities of remote sensing analysis, as described in Mirtl et al. (2018). A dataset is deemed EBV-usable if (1) primary data and associated metadata are identified and imported, (2) data-sharing agreements and licences are checked and

(3) data completeness and consistency are described. Through the analysis of DEIMS-SDR, no dataset can be considered EBV-usable in the above sense. In fact, 67% of metadata provide an online location for data, partially satisfying (1); 82% of metadata satisfy (2), indicating data-sharing agreements associated to data and no metadata satisfy (3), offering information about quality check. This further enforces the need for metadata curation so as to assure visibility to EBVs monitoring activities of the network.

With respect to other worldwide providers, we conclude that LTER-Italy can contribute to SA and SD measures and that interoperability to integrate them with other data is partly achieved at two levels (Haslhofer and Klas 2010) as described below:

- Legal interoperability, which occurs at metadata level, where general data policies applied from sites, principal and granted permissions, as well as intellectual rights related to datasets, are specified.
- Technical interoperability, which occurs at metadata level and is assured by the DEIMS-SDR IT infrastructure, which allows the export of EBVs metadata in standard schema.

Nevertheless, these two levels are not fully achieved because (i) LTER-Italy dataset metadata just partially report how to allow the reuse of data without directly contacting owners and (ii) the implementation of mapping DEIMS-SDR metadata models to standard schemas needs to be completed. For these reasons, the next section is dedicated to suggestions for the improvement of both the IT infrastructure and the data provider support system, in order to expand the visibility of LTER sites with respect to SA and SD measures.

Conclusion and recommendations

The EBV concept should become the window into biodiversity observation systems upon which researchers, managers and decision-makers can better interact. Related web resources aid the streamlining of the EBV information exchange amongst different stakeholders insofar as its discovery and reuse are assured. The synoptic, comprehensive and harmonised overview of the set of local research which resulted by mining this information is of particular importance for LTER observational design purposes, as monitoring programmes need to be more coordinated and improved through sites' collaborations. This paper suggests a method, based on metadata analysis, to reveal capacities and gaps in these networks with disparate focuses on ecology to provide EBVs measurements. Since the present analysis exploits metadata of field observations, harmonised through the EBV concept and described in the DEIMS-SDR repository, it can be applied to every research organisation using this information system (e.g. Murgia Alta EcoPotential site does not belong to LTER-Italy, but its site managers can benefit from DEIMS-SDR metadata models to expose information), by offering an approach both to coordinate monitoring schemes for primary data collection and to evenly assess the role of Biodiversity Research Infrastructures (BRIs) (Hardisty et al. 2019a). LTER-Italy, the network to

which we apply this method, is a relevant case study, as it is deployed in a country that is extremely rich in biodiversity: it has the highest number and density of both animal and plant species within the European Union, as well as a high rate of endemisms (Convention on Biological Diversity, Country Profile, Italy). Thus, assessing the monitoring coverage of this system is essential for the conservation management of biological diversity and to centrally design its research activities, which actually represent a collection of individual monitoring studies that vary across time scales and research focuses.

Our results demonstrate a documented capacity to provide essential measures at two different levels of interoperability through the information system DEIMS-SDR, but underline the need to support the community and to optimise the EBV information retrieval to improve the assessment and hence the effectiveness of LTER as an observing system. The analysis behind this work also allows us to provide some recommendations regarding the tools proposed for the LTER network. As discussed, DEIMS-SDR can be exhaustively consulted only through the user interface and provides information on the attributes of each of the EBV dimension (see Table 1).

In order to provide the same analysis for different LTER networks or for a set of sites (e.g. those based on networks or projects), we would suggest:

1. to formally structure EBVs information both in SMM and in DSMM to (i) give visibility to those sites which choose to restrict the online data-sharing and (ii) to enable the automating of EBV information analysis through specific metadata elements. Particularly, we suggest:
   I. to complete the implementation of the mapping between DSMM and EML schema, following that which is described in Kliment and Oggioni (2011), where "Taxonomic coverage" within DMSS (field_bio_classification) was effectively mapped to the EML corresponding field (taxonomicClassification);
   II. to improve the description of datasets and their discovery: the DSMM should provide a field where the corresponding EBV or eLTER parameter could be inserted, as it currently happens for sites;
   III. to map the values of eLTER parameter field (field_elter_parameters) in the field observedProperty of the EF metadata exposed by DEIMS. Currently, through EF schema, only the contents of the "Parameter" field (field_parameters_taxonomy), i.e. description of the observed parameters and parameter groups at the site, are provided without the hierarchy of details for methods and instrumentations provided by eLTER parameters;
2. to ensure metadata completeness through curation staff to create a legacy of well-designed and documented long-term observations. In fact, the process of creating and publishing metadata is relatively new amongst scientists despite its value in domains like ecology, where metadata improve the reusability of data. For example, protocols and instruments information are needed to assure interpretation of data over time and to allow comparisons when different methods are adopted. Metadata compilation is error prone (Kervin et al. 2013) and also perceived as a burden from researchers which often results in incomplete metadata provision. Nevertheless, curation staff (e.g. data stewards, librarians, help desk) can support scientists by stimulating their willingness

to share (meta)data by identifying contextual causes which hamper the practice (Zilioli et al. 2019) or by lightening the compilation procedures with informatics facilities (Fugazza et al. 2016; Fugazza et al. 2018; Pavesi et al. 2016). Specifically addressing the issue of this paper, we suggest ensuring a careful recording of the EBVs information by employing dedicated personnel to assist scientists in creating reliable metadata.

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Similarities, differences and mechanisms of climate impact on terrestrial vs. marine ecosystems

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Abstract

Comparisons between terrestrial and marine ecosystems are generally not in the main stream of scientific literature even though Webb (2012) listed several points for which the transfer of knowledge and concepts related to one or to the other system would benefit our understanding of both. Even sharing this view, the leading hypothesis behind this contribution is that the pelagic system, where the dominant biotic component by number and biomass is microscopic, has specific features which strongly differentiate it from the above-the-surface terrestrial systems. Due to this, climate change, i.e. changes in temperature, precipitation and most importantly in the dynamics of the two fluid media, atmosphere and ocean, act with different mechanisms which prevents proceeding with analogies in many cases. In addition, the non-linearity of most of the processes and responses to perturbations requires, in order to obtain reliable forecasts or hindcasts, a detailed analysis of the path followed by the system which is normally overlooked in the step-change simulations or projections.

Keywords

terrestrial, marine, climate change impact, LTER

Background

Organisms, communities and ecosystems are continuously exposed to variations of weather components, namely, solar radiation, temperature, atmospheric pressure, humidity, cloud coverage, precipitation, wind speed and direction. These variations may

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be significant over the short natural cycles of daily and seasonal periods. However, their medians and the squared deviations around the median over a few decades are generally constrained within narrow intervals for each specific geographic location. Those characterise the climate of the specific site (IPCC 2007, IPCC 2013). Therefore climate is just the emergent pattern of weather variations in a specific site over a few decades. If the values, mentioned before, change systematically beyond the typical ranges then, by definition, a climate change is taking place. This raises the question of why climate change, which is a persistent change of the median values of weather components, generally lower than the short term variance of weather variability, has a significant impact on ecosystems.

The weather components listed above are all abiotic processes/variables of the atmosphere or the sun. The reciprocal feedback between abiotic atmospheric processes and biota has been well established in the last decades (IPCC 2013) but, by tradition, we still speak of climate forcing and ecosystem response. The reason may be that three of the key processes that affect the climate system, the radiation emission by the sun, directly and the earth rotation and tectonics, *sensu lato*, indirectly, are substantially out of the control of biota.

Considering the above definition of climate, another question arises: is a significant climate change presently going on? Recalling the previous statements, climate change means that the weather/climate variables display a persistent change in space and time at regional and global scales. This has always occurred in the past. Primarily because of the orbital coupling of sun-earth system whose main manifestation are the Milankovitch cycles (Berger 1988) or for climate system internal dynamics, such as pluridecadal oscillations (Mann 2007) and also for exceptional events causing dramatic changes in climate leading to drastic reshaping of soil occupation, mass extinctions etc (Whiteside and Grice 2016). Several indicators prove that we are going through a climate change at a global scale (IPCC 2013). The current debate on the issue is focused on whether the present climate change is faster than what could be expected because of 'natural' processes, whether this is causing unprecedented shifts on earth ecosystems and, more importantly, whether the anthropogenic activities are causing it. IPCC reports (IPCC 2007, IPCC 2013), as well as numerous studies (Scheffers et al. 2016, amongst others), provide convincing evidence that this is the case for the latter. Therefore, aside from designing prevention and mitigation policies, it becomes relevant to predict how earth ecosystems will respond to this accelerated climate change. This is not an easy task, because of the complexity of natural systems, having a vast number of interactions and non-linear responses that characterise their functioning. This is particularly true for marine ecosystems, which are definitely less known than terrestrial ones. This leads to a further question: do the trends observed in terrestrial systems, which are easier to observe, provide the needed conceptual schemes to predict the response of marine ecosystems to climate change?

Despite the strong interest for climate change impact on earth ecosystems and the fundamental effort carried out by the IPCC, the interaction between 'terrestrial' and 'marine' scientific communities is not frequent and the number of studies dealing

with differences between the two systems is small, if compared with the studies on individual systems.

Literature on climate change and its established or potential impacts, is incredibly vast and it is beyond the scope of this review to summarise it all. The focus of this contribution will be the differences and similarities between terrestrial and marine ecosystems that are relevant in the analysis of the response to climate change. This will aim at highlighting areas of research needing more exploration, hoping to stimulate further discussion on the topic.

Ecosystems responses follow trajectories in a multivariate space

While our immediate perception of climate is more related to temperature and rain, all other variables, mentioned in the previous section, contribute to shape the climate. These interact via several feedbacks, involving all of them varying in time. An instantaneous set of values for these or their means and variances, would only provide partial information on how climate might affect us. A typical example is the difference between a mild continuous rain and an intense storm producing a flood, with the same total amount of water having fallen. Therefore, a more appropriate characterisation of climate would be a representation of the contemporary change of all the state variables in a multidimensional space, i.e. a phase space, which would design a trajectory. This trajectory, even if purely phenomenological, would be a much better descriptor of the climate and would be more informative of its potential impact on biota. Likewise, changes in species abundance would not fully characterise the biotic response. Their trajectory mapped in the phase space, which reflects the rates associated with their activities and the consequent interactions, would instead be the best descriptor of how the system functions (Tett et al. 2013, Crise et al. 2015). A prediction of the shape of both trajectories in the time to come is the main scope of climate studies. Due to the non-linearity of the processes in complex systems, as atmosphere and ecosystems, each point on those trajectories depends on the previous path along that trajectory, meaning that simulating changes for a sudden step-variation of state variables might not properly provide information on how the final status of the analysed system will be. We are then confronted with two problems and correspondent knowledge gaps. The first problem is to develop methods for analysing the changes along the trajectories followed by all the state variables linked to the main climate drivers which result from their mutual interactions. A similar process should also be carried out for the biotic component with the added complexity of the reciprocal feedback between biotic and abiotic processes. This approach is adopted with the so-called transient simulations to distinguish them from the equilibrium simulations (e.g. Millar et al. 2015)

The second problem relates to the biotic response which has two components which we can briefly summarise in acclimatisation and adaptation. The first is related to the tolerance of the organism to changes in environmental conditions and may involve internal biochemical adjustments up to epigenetic modifications. The second is instead

related to modifications at genomic and genetic levels. While information that relate to the first component can be generated in experimental set-ups or gathered from regular *in situ* observations, the extent and the characteristics of the second process are unpredictable. What might be within reach in the near future is an estimate of the probability of possible genomic/genetic changes building on an increased knowledge of genome dynamics, the ability to evolve and speciation rates for different class of organisms.

To overcome our knowledge gaps on many mechanisms that drive the time-dependence of the trajectories, the present approaches rely on two strategies: i. they reconstruct the trajectory by numerically simulating a reduced set of processes (e.g. Sokolov et al. 2018); ii. they mine the data with more and more advanced statistical techniques to extrapolate from present information (e.g. Sarhadi et al. 2017). However, both approaches, with their intrinsic limits, focus on the final state of the systems. Even when a reliable prediction is generated, the underlying trajectory is seldom analysed and, therefore, the complex interactions that drive the system to the final state are overlooked. In addition, transient simulations are generally carried out for one or a reduced set of forcings, for example, the progressive increase of CO_2. This implicitly assumes that all changes are caused by a primary driving change, the increase of temperature, which is due to the increase of CO_2. As discussed below, the above limitations affect our predictions of marine ecosystems more than those of terrestrial ecosystem, because of their different functioning.

The role of the time

Those ecosystems trajectories, discussed above, develop in the phase space over time. Time is, therefore, the shared context within which all the interactions and changes take place. Time is not the driver of the changes, which depend on fluxes of matter, energy and information amongst the ecosystem components, but time is the main scaling factor which allows for characterising, quantifying and comparing the changes.

The fundamental question in ecology is how the ecosystems function or, which is substantially the same, which are their dynamics? Tracking the different states in the phase space of the ecosystems is the prerequisite for answering that question. The evolution of Man has been strongly coupled with his capability of exploiting natural resources which, even when Man was a gatherer or a hunter, had to rely on associations but also on predictions. The birth of agriculture is based on having acquired knowledge on the coupled cycles of plants and environmental forcing, i.e. on parts of ecosystem dynamics. All this leads to the essential role of ecological time series. All the present knowledge about Earth functioning is based on our reconstruction of its dynamics for the past and for the present, made possible by observations over time. Long Term Ecological Research (LTER), a very recent formalisation of a long implicitly known practice, is often perceived as a specific niche of ecological research. In fact, the only difference with any other ecological observations is the time scale, by definition longer than episodic observations focused on specific processes and, often, the sampling in a fixed, spatially limited area. Both traits often have originated criticisms, for the long term sustainability, the former and for

their representative nature, the latter. The former is more dependent on societal awareness and willing-to-support, therefore asking for an improved outreach activity. The latter, instead, can be overcome by promoting observational strategies that integrate the periodical observations with sampling efforts focused on the characterisation of the spatial context and/or on specific processes that could be revealed by periodic sampling. This can be better achieved by building networks of sites and is fundamental for directing ecological research (e.g. Vanderbilt and Gaiser 2017, Zingone et al. 2019). The discussion that follows is largely based on the information collected in observational efforts over time.

Solid earth vs. ocean

Besides the obvious visible differences between the terrestrial and marine environments, e.g. in the first animals walk and in the second they swim, it is still an object of discussion if all the other differences prevent the identification of general ecological rules, valid for both realms. The existence of general rules in ecology, which would allow prediction of the structure of any ecosystem, once assigned initial conditions and fluxes, is part of a long lasting debate. Lawton (1999) argued against the existence of such laws, even if he admitted that macroecological patterns may display regularities beyond the ensemble of contingent events that may involve the different organisms in the ecosystem. This would imply that the final state reached after a perturbation or a transition would not be strictly dependent on the trajectory followed by the system, at least at a coarse, macro scale. This, in turn, might imply that some general structural properties, e.g. architecture of food webs or mechanisms, e.g. trophic or mutualistic interactions, should be shared by all ecosystems, independently from the components, which would be more exposed to contingent events. From this general statement, one could then assume that marine and terrestrial systems should share several common traits and that the separation by the two scientific communities is more related to tradition or differences in observation tools, than to fundamental differences in the systems (e.g. Webb 2012). However, the analysis conducted by Chase (2000) on various characteristics of the food webs in the two environments, amongst which the existence of cascades, the number of trophic levels and the dominant size of primary producers, led him to conclude that some differences, at least in the functioning and structure, exist. This would suggest that their response to climate change might also follow different paths, a possibility also discussed by him (Chase 2000).

Indeed, there are several evident differences between the two systems, especially if one compares the pelagic systems with the above-surface terrestrial ecosystems which are the most distant in terms of characteristics and are the focus of this contribution.

First of all, let us consider the dimensionality. Pelagic marine systems are fully three-dimensional and the total volume is occupied by organisms. In terrestrial systems, the third dimension has a limited thickness, with the exception of the overlying atmosphere which, however, hosts only a very small fraction of biomass (Bar-On et al. 2018). For marine organisms, this requires a significant tolerance to an extended

pressure range which is another specific trait of the marine systems. On the other hand, on land, differences in altitude may determine drastic changes in environmental conditions on short linear distances and, therefore, much sharper gradients. Another remarkable difference is the reduced gravity in the marine systems with a parallel increase in friction during movement. Energy and signal transfers are also substantially different. Light is rapidly absorbed by water and modified in its spectral properties (Kirk 2011), implying less available radiant energy and potential constraints. Light is also attenuated in the terrestrial environments but, above the surface, mostly under canopies and with a different pattern of spectral modulation (Depauw et al. 2012) On the other hand, spectral changes with depth can be a source of detectable signals by organisms (Jaubert et al. 2017). This is partially compensated by acoustic signals which propagate faster and over longer distances in the water (Hovem 2010), a fact which explains why sound and mechanical signals are widespread in marine ecosystems (Dusenbery 1992). However, there are two key differences between the two systems. One is the water availability. Water is the fundamental element of life, at least the life that we know. Terrestrial organisms rely on water supplied by precipitation or humidity in the air and in the soil while marine organisms are embedded in water, even if this means that they have to handle the osmotic pressure of the medium. The other is the medium on which organisms rely to make their life. Terrestrial systems are organised on a fixed substrate and the mobile component, the atmosphere, is where the organisms just transit to move from one fixed site to another. In the marine environment, the medium is mobile with most of the biomass living in it (Bar-On et al. 2018) and in a size range such as to make it prevalently transported by the movement.

Those differences are significant, but not all of them may directly affect the response to climate change. Some of them are crucial, i.e. temperature, water availability and medium motion, others enter the game indirectly, e.g. dimensionality, while those remaining have relevance from an evolutionary point of view but do not modify the impact of climate change on the two systems.

Inferring and comparing potential impacts of climate change

In Table 1, the three climate components, mentioned above, are listed together with the direct dominant effect that a change in their amplitude would have on terrestrial and pelagic ecosystems. In the last row, there is an additional component, the movement of the fluid media (atmosphere and oceanic water) which is strongly intertwined with the others, in fact it depends on them, but exerts a complex, direct, very specific impact on biota.

Temperature

Temperature is by far the most studied climate variable in terms of its impact on organisms and ecosystems. Temperature increase, or global warming, framed in the context of a rotating planet and of an active terrestrial crust, is the primary driver of all the

Table 1. Climate components and their direct impact on terrestrial and marine ecosystem processes.

Change in	Impact on land	Impact in the ocean
Temperature	Organism metabolism	Organism metabolism
Precipitation	Drought	None direct
Cloudiness	Light availability	Light availability
Motion of the fluid media (atmospheric and ocean circulation)	Evapotranspiration, seeds spreading and immigration	Displacement and mixing

other changes in climate components. Temperature is also a fundamental modulator of biotic activity and, therefore, changes in temperature produce a direct impact on organisms and ecosystems.

The reason why temperature is so important has been long known and is rooted in basic physical and chemical mechanisms. Put in a simple way, it is related to the rates by which the chemical reactions, on which organisms base their metabolism, occur and on the rates of the processes that allow the reagents involved in those reactions to meet. The reactions occurring in an organism vary from billions to trillions per second and are of several kinds. This makes it very difficult to find a universal, simple relationship between temperature and the overall biochemical functioning of an organism. However the attempts to find general patterns are numerous and are all based on the exponential dependence of chemical reaction rates on temperature and the 'activation energy', which quantifies the energy barrier that reagents need to overcome in order to lead to the products. This 'law' is generally attributed to Arrhenius (1889). Building on this and on a previous analysis by West et al. (1997) on the allometric relationship between body mass and metabolic rates (Kleiber 1932, Gillooly et al. 2001), Gillooly et al. (2001) highlighted that most metabolic reactions strongly rely on the back and forth transformation of the ADP/ATP couple, which has an activation energy of 0.65 EV and that all the activation energies are within the range of 0.2–1.2 EV. With this mechanistic model, they showed that a wide set of empirical data could be fitted by a line in a plot of the log of allometrically biomass normalised metabolic rate vs. the inverse of temperature, refining previous more empirical, similar fits. This generalisation has been actively debated within the scientific community since its first proposition (Arrhenius 1889, Gillooly et al. 2001, Clarke 2004, Clarke and Fraser 2004, Gillooly et al. 2006, amongst others). However, the acceptable correspondence between the general 'law' and the data states two important points: 1. There is an exponential increase in metabolic rates of all organisms with temperature and 2. This increase is constrained within a range which is in the order of one order of magnitude. This implies that an increase of only two degrees may increase the metabolic rate by 20–30%. However, this general rule provides only the range of variation of whole metabolic rates with temperature. As a matter of fact, when zooming in on the different components of the metabolism, differences appear. For example Alcaraz et al. (2013) showed that, for arctic zooplankton, the relationship of metabolic rates with the temperature changes depending on the considered element, e.g. phosphorus vs. nitrogen. The exponential increase in rate with temperature proceeds until the decreased performance of enzymes, the higher demand to support the higher metabolic rate etc. limits or forces it to decrease. This produces the typical bell

shaped temperature dependence of optimal metabolic activity which is organism-specific (Clarke 2017). In other words, all organisms display a temperature range of existence which clearly results from their evolutionary history and context and whose optimal range is relatively narrow. Echtoderms are obviously more exposed to external temperature changes. Additionally, on the descending side of the curve, the relationship with temperature might be process dependent. For example, Alcaraz et al. (2014) showed that carbon ingestion by and respiration of the copepod *Calanus glacialis* scale differently with temperature, thus causing a sharp discontinuity in the temperature window in which they can survive. Are these changes due to global warming having a similar impact on land and ocean? This is not the case. The heat capacity coefficient of water makes the seawater more resilient to heating, meaning that it is more difficult to produce a sudden temperature increase unless this is coupled to the dynamics of the water. On the other hand, the same heat capacity of water makes it a better medium to transfer heat from one place to another. Poloczanska et al. (2013) showed that warmer isotherms display a faster latitudinal change in the ocean than on land. In addition, Sunday et al. (2012) showed that there is a sharp difference between land and sea in the redistribution patterns of organisms, associated with isotherm migrations. On land, the poleward migration is occurring both in the leading and in the trailing part of their distribution while, in the ocean, there is no displacement on the warmer, equator-wards side. This hints at additional roles played by other components of the climate systems, like moisture and soil characteristics (Sunday et al. 2012), which have certainly greater inertia than flowing water. The bottom line is that the fluidity of marine systems favours a faster spreading of thermal changes compared to the land environment. The recent analysis by Beaugrand and Kirby (2018) is based on the assumption of a bell shaped optimal curve for each organism and, therefore, its predictions built on the decoupling between presence in the fluid and change in its temperature because of changes in heat fluxes.

However, while the annual average increase may predict coarse structural community changes to some extent, the details of changes cannot be anticipated, thus, leading back to the need for characterising the path. As an example, Maffucci et al. (2016) showed that the annual temperature increase in the Tyrrhenian Sea occurs mostly in the summer season, with winter temperatures staying within the previous range of variations. This is having a strong impact on the reproduction success of the sea turtle *Caretta caretta.* The increase in summer temperature has favoured a northwards expansion of the species while the winter temperatures that are still too cold are hampering the survival of the juveniles, thus impacting on the species recruitment.

While the response of the two systems might be different in the tempo and in the mode, there are reported cases of a significant synchronism. In a comprehensive analysis of a global regime shift in the 1980s, Reid et al. (2016) provided robust evidence of an all-encompassing impact of a rebound in global temperature after a sudden decrease following the El Chichòn eruption. This alternation of decrease-increase in temperature produced a widespread change in phenology as well as in productivity of both terrestrial and marine organisms. What remains elusive is the reason why some organisms showed more prominent responses than others, whether the amplitude of response was comparable and, if so, with which metrics a comparison would be reliable. This relates,

in my view, to the physical dynamics of the two systems which is not described by a change in the value of state variables, such as temperature.

Before analysing in more detail this important aspect of earth ecosystems, we analyse another key difference between land and ocean, that being the water availability.

Precipitation

Water is provided to terrestrial areas by precipitations which also feed surface water networks that supply water to the more confined areas surrounding them. Roughly one quarter of the rainfall comes from the ocean but precipitation in the ocean is more than four times greater than on land (Gimeno et al. 2012). However, precipitation in the two systems has completely different impacts. On land, precipitation is a key term that rules humidity of the soil and of the atmosphere and, therefore, affects the water availability to plants and animals and impacts their physiology (Carnicer et al. 2011). By contrast and despite the fact that most of the precipitation occurs on the sea, the impact on marine organisms of fresh water added to the surface ocean is negligible, even in the case of large precipitation events. This does not mean that precipitation has no influence on marine ecosystems, but its main impact is not direct.

The increase in temperature increases the amount of water vapour in the atmosphere and, therefore, more intense precipitation events (Trenberth 2011). The poleward shift of storm track observed and predicted by existing models (Tamarin and Kaspi 2017; and references therein) will surely affect the terrestrial vegetation at mid latitudes. In general, a change in precipitation acts in quite complex ways, due also to a concurrent, anthropogenic increase of CO_2 in the air, which affects plant physiology. Dai et al. (2018) showed that the surface drying effect, due to Green House Gases-induced warming, dominates over the wetting effect of plants' physiology in response to increasing CO_2. The reduction of stomatal conductance and, therefore of evapotranspiration, caused by the increase in CO_2, does not mitigate surface drying. The link between warming, atmospheric moisture content, plant evapotranspiration and precipitations is one of the most important examples of feedbacks involving biota and climate and is an important term in climate simulations (Carnicer et al. 2011). This is specific for terrestrial ecosystems, even though the regulation of precipitation by marine plankton has been proposed many years ago by Charlson et al. (1987), though acting through a completely different mechanism.

Precipitation is not just liquid water. It can also fall as snow. Snow has a very important impact on the cycle of plants and animals in the terrestrial environment. In fact, the presence of snow allows the reduction of heat loss by the soil, while simultaneously providing the needed humidity to the soil. A careful analysis by Wang et al. (2018) showed how the trade-off between snow accumulation and its starting time (earlier start would increase snow accumulation) may be detrimental to the fate of plants. An example of this phenomenon can be found in the Italian Apennines, where Petriccione and Bricca (2019) showed that the reduction of snowfall, with a consequent increase in drought, had strongly impacted the community structure of plant coverage with the

disappearance of 20% of sensitive species; "a quantitative increase in more thermophilic and drought-tolerant species and a parallel decrease in more mesic, cryophilic and competitive species". Therefore, there had been an increase in diversity with a parallel extirpation of some species which did not suit the environment anymore.

This is another example of the relevance of the time course of changes. The presence of snow prevents the evaporation of water from soil whilst providing it at the time of snow melting. Its absence or even a change in the time of melting can go out of phase with the germination of some plants, thus hampering their growth. Global warming will increase the precipitation in the form of water with respect to snow. All this will impact the terrestrial ecosystems almost exclusively.

There is only one process in marine ecosystems which, even if being it is extremely weakly coupled with precipitation, shares some similarities with the effect of snow on land and this is the presence of ice. It is well known that, because of the particular properties of water, ice is colder but lighter than liquid water. This is also true when it contains a certain amount of salt. Therefore ice covers the sea surface layer as snow covers the soil. Ice cover prevents heat exchange between water and the atmosphere, thus allowing for water to remain liquid underneath. This isolating role is very similar to what the snow does for the soil.

Some species, e.g. ice diatoms or the antarctic krill *Euphausia superba,* have part of their life cycle linked to the presence of ice (Nicol 2006). If the ice does not form or melts before the time of the typical seasonal cycle, the *E. superba* life cycle is altered and, apparently, there is a higher probability that salps prevail (Atkinson et al. 2004).

It is worth mentioning that ice formation is driven only by heat fluxes, while snow presence is also driven by precipitation patterns.

Cloudiness

Cloudiness, besides its impact on precipitation which has been discussed before, is a modulator of radiant energy reaching the earth surface. While clouds may increase the total radiation impinging on earth surface by a small amount with respect to a cloud-free sky, because of its diffuse component, the most frequent effect is to significantly reduce it by a factor that can reach 70–80% (Pfister et al. 2003). A decreasing trend in cloud coverage, even if slight, has been reported (Karlsson et al. 2017). Even though there are pluriannual trends, on average, the oscillation between brightening and dimming is more related to aerosols, often of anthropogenic origin, than to cloudiness (Wild 2009). Decrease in light intensity has an impact on photosynthetic performance of autotrophs either via preventing photoinhibition, thus improving it, or limiting the available energy in shade environments, thus reducing it. The impact of reducing or increasing light flux, which will also depend on changes in the spatial distribution of cloudiness, might have a stronger influence on the marine ecosystems because a large part of the ocean hosts a maximum of phytoplankton biomass in the subsurface layer. The Deep Chlorophyll Maximum (DCM) does not always correspond to a maximum in biomass or production, because of the rapid attenuation of light in the water col-

umn (see Cullen 2015). The dynamics of DCM are associated with the total available light coming from above, the nutrient vertical fluxes coming from below, as well as with the dynamics of the mixed layer (Letelier et al. 2004, Lavigne et al. 2015). A reduction in the available light has an impact, not only on its total production, but also on the composition of the community, i.e. on its diversity (Huisman et al. 2006) and this occurs on fairly large parts of the ocean. Interestingly, several reconstructions of past climate are based on the ratio between surface and sub-surface organism distributions, used as a proxy for mostly the stratification and mixed layer dynamics, while the cloudiness is generally neglected (e.g. Incarbona et al. 2013).

Motion of fluid media

While the alteration of the heat budget of the Earth system is the primary driver of the climate change and also affects the elements we have briefly analysed above, the main modulators of the changes are the concurrent motions of the two fluids, the atmosphere and the oceanic water. The Earth rotation adds complexity to the non-linear interactions amongst thermal gradients, water vapour release by the ocean, soil and vegetation and, therefore, air moisture content, ultimately determining the trajectories of air transport. Likewise in the ocean, the rotation affects the paths of currents, with salinity being the additional variable instead of moisture content. The full description or a short synthesis of all the above processes is beyond the scope of this paper for its length and complexity on its own. It has to be said that ocean and atmosphere are strongly coupled and they affect each others' dynamics but the impact on biota displays significant differences in the two environments. Atmospheric transport is what determines the spatial distribution of cloud coverage, the precipitation and, partly, the horizontal heat fluxes, thus acting as a modulator of the three terms analysed above, with their different impact. It also contributes, via atmospheric deposition, to transport essential nutrients. However, the relevance of this process for the two systems is significant. As an example in the terrestrial environment, the transport of nitrogen and phosphorus has been estimated to increase the CO_2 removal by forests by 9% at most (Wang et al. 2017), while in the ocean, the transport of iron has been estimated to impact on CO_2 removal by more than 12%. The differential impact is, however, mostly on the access to the internal stocks in the two systems. Apart from the atmospheric transport, wind is a key term for vertical mixing in the ocean and therefore for the upward flux of new nutrients that drive large part of primary production in the ocean. There is no equivalent on land, where, instead, the wind is crucial for the evapotranspiration. In fact, one of the expected effects of global warming is the increase in stratification in the mid-latitudinal belt with a less effective vertical mixing even at quasi constant wind field (Doney et al. 2011). The impact is even more dramatic if possible changes in wind field are included because of their impact on the oceanic general circulation and the ice coverage (Moore et al. 2018). Growing effort is being placed on simulating all the possible effects of change in atmospheric and ocean dynamics on biogeochemical cycles, often producing contrasting scenarios. The point I want to

make here is that the 'motion of media' acts very differently in the two systems and through different mechanisms. The other difference is on the dispersal. Seed dispersal is the main mechanism for colonisation of new space by terrestrial plants. However, the timescale of this process may be long (months to years) and must cope with the previous occupation of soil. In the marine environment, dispersal not only involves the propagules of organisms, but it also transfers entire communities exposing them to a complex change in environmental conditions (Beaugrand et al. 2002) and with a large impact on the food web (Beaugrand et al. 2002, Beaugrand et al. 2003). This also has no equivalent in the terrestrial systems, especially on short timescales. This process reinforces the point that a prediction of the change must be based on the integration of the continuous change in space and time of the moving water, i.e. on an integral along the trajectory of the current.

Is there a coupling between the two systems?

So far, I have discussed the different mechanisms by which climate acts on the marine pelagic and above-the-surface terrestrial ecosystems. However, between the two, there are no rigid barriers and one may wonder which could be, or could have been, the reciprocal feedbacks within the two sub-systems under climate forcing and the impact on biota. Indeed, if we exclude the few organisms that divide their life between solid Earth and ocean, such as, for example, some birds, a few mammals etc., the reciprocal feedback between the two systems, even when driven by biota, acts via abiotic players, i.e. the atmosphere and the water. Though, it can act on a wide range of scales up to the macroevolutionary timescale, thus being coupled with, but not necessarily driven by, climate. The iconic example is the origin of oxygenic photosynthesis which, via the accumulation of oxygen produced essentially in the marine environment (Sánchez-Baracaldo 2015, Fischer et al. 2016), also caused a drastic change in terrestrial environment and biota. More recent events, on a geological time scale, are the sapropel depositions, which are accumulations of reduced carbon in the sediments of the Mediterranean Sea (Rohling et al. 2015). Those events are driven by co-occurring processes, such as Monsoon intensification, increase in precipitation and runoff. There are several lines of evidence that the consequent increase of stratification and, possibly, the increase in export production fuelled higher nutrient inputs from land, with a parallel weakening of the thermohaline circulation in the basin and caused a decrease in oxygenation rates of the deep layer, leading to euxinia or anoxia. Any change in the hydrological cycle on land which, as discussed above, is also affected by terrestrial plants and activity, impacts, via runoff, the marginal seas and coastal areas (Meybeck et al. 2006) and can track climate fluctuations via the paleobiological changes and their isotopic signatures (e.g. Sprovieri et al. 2012). A third example is the observed increase in iron transport from land during glacial times initially hypothesised by Martin (1990). The harsher climate on land during those periods eventually produced an increase in iron transport to the ocean with a parallel increase in carbon vertical export and, very likely, a change in community structure. The impact of the recorded increase in iron transport to the

ocean is still at the centre of an intense debate, but the evidence supporting the coupling between changes induced on terrestrial environment on land and those in the marine ecosystem is definitely robust. A very crude generalisation would be to separate the scales. At the interface between land and sea, i.e. on a small scale, climate change on land would act via runs-off and would be more frequent from the land to the sea, even though it can be hypothesised that habitat destruction on the near-shore due to storm intensification might impact on on-shore habitats. On a larger scale, the atmosphere would likely play a prominent role, also via global biogeochemistry.

What knowledge gaps do we have to fill and how could they be filled?

We discussed above that temperature changes play a role at multiple levels. Prediction of temperature future trends is still a great challenge for Earth system models (Bonan and Doney 2018) due also to the feedback of biota. This in turn depends on their physiological fitness, as well as on their capabilities for acclimatisation and, eventually, modification of their fitness via mutation. The importance of biota feedback can be generalised to many more processes and is the key challenge that the scientific community will face in the near future. This can only be obtained via a holistic approach that should merge classical observational methods with the recently developed new tools, such as bio-optics, bioacoustics and -omics (Karsenti et al. 2011, Coles et al. 2017, Crise et al. 2018, amongst others). It should consider in more detail the multiple interactions occurring within communities which are differently modulated in the marine and terrestrial environments and display plasticity and complex outcomes (Tylianakis et al. 2008, D'Alelio et al. 2016, amongst others). It should be continued in time as the LTER community is showing for many years. In fact, most of the issues discussed in this paper were stimulated by the observations conducted over the last decades at the LTER station MC in the bay of Napoli (Zingone et al. 2019). This information, especially if integrated with modern techniques and approaches, especially those based on bioptical and molecular methods, can feed a new generation of models (Coles et al. 2017, Stec et al. 2017, D'Alelio et al. 2019).

We are facing a faster change in the environment even with regards to climate because of anthropogenic actions. We know from the past that climate change induces ecosystem changes. However, our data result from a natural filtering process that transmits only the prominent changes. The importance of 'history' has been stressed before. In the background section, I raised the question on why biota respond to changes in environmental conditions that are often smaller than the variations they experience in one single year. I believe that this too is related to the time course of the change. Short term fluctuations are tolerated by organisms which, in addition, tune their life cycles to the most suited conditions for them, meaning that many of them do not experience the whole range of variations of the seasonal cycle or adopt different solutions to cope with it. Besides the cycle of illumination which is the same for the ocean and the land, terrestrial ecosystems experience a more fluctuating environment with wider ranges of variations for the key climatic components. While the heat capacity of seawater buffers the temperature, an equivalent buffering role is partially played by the soil with

respect to water availability. On the other hand, the real modulating player is the motion of the medium, on land because of the impact of wind in moisture transport and evaporation and evapotranspiration and in the ocean because of the currents. The main difference is that, on land, the inertia is much higher because the motion of medium changes the conditions but does not move the organisms, whereas in the ocean, organisms in many cases move with the water. Nectonic organisms are in-between because they may move independently from the medium, to some extent. This does not always mean that they can escape the impact of changes, because a moving environment also changes their environment.

In any case, the conceptual framework for the two systems has some relevant differences and even if one wants to build on unifying theories, the real impact must be based on the integral of the changes over time which depends on different processes on land and in ocean.

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