

Research Article

Morphological and genetic characteristics of garfish *Belone belone* (L., 1760) (Belonidae, Teleostei) population from the southern Bulgarian Black Sea coast

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Abstract

This study was conducted to investigate genetic and some morphometric and meristic characteristics of garfish *Belone belone* from Nesebar in the Bulgarian Black Sea coast. Twelve morphometric characters were measured, and six meristic characters were counted for each individual. Based on both sexes' morphological and meristic analyses, no statistically significant sexual differences were observed. Additionally, DNA barcoding was done. The fragment of the cytochrome oxidase subunit I (COI) gene of mitochondrial DNA was sequenced to supplement the species identification and population diversity study. Two haplotypes were found out of 39 sequences, indicating a low level of haplotype diversity (0.146 ± 0.072). Nucleotide diversity was also found to be low (0.00023 ± 0.00011). The Nesebar population of *B. belone* requires conservation efforts, due to the highly decreased mtDNA genetic diversity.

Key words: *Belone belone*, Bulgarian Black Sea coast, genetic, meristic analysis, morphometric

Introduction

The Belonidae family, represented by a total of 10 genera and 44 nominal species in marine and freshwater ecosystems, constitutes one of the important groups of the ichthyofauna of the Eastern Atlantic, the Mediterranean and the Black Sea (Fricke et al. 2022; Öztürk 2023). Three subspecies have been recognized (Collette and Parin 1986): *B. belone belone* (Linnaeus, 1760) – restricted to the north-eastern Atlantic, *B. belone gracilis* Lowe, 1839 - distributed from the south of France in the Mediterranean Sea to the Canary Islands in the Atlantic, and *B. belone euxini* Günther, 1866 - which is found in the Black Sea and the Sea of Azov. The garfish *Belone belone euxini* (Günther, 1866), distributed in the Black Sea and Azov Sea (Zaitsev and Mamaev 1997), is one of the most important pelagic fish species in the Black Sea artisanal fishery. A few studies concerning some aspects of biometric, biological, and electrophoretic analysis (Dobrovolov et al. 1980; Prodanov

1982; Dorman 1988), were carried out for populations that inhabited the Bulgarian Black Sea. In spite of its wide distribution in the Black Sea, the knowledge of biology and ecology of this species is still scarce and for genetics absent.

Morphometrics and meristics characters are the two types of morphological characters that have been most frequently used to describe populations of exploited marine fish species (Turan 2004; Uyan and Turan 2017). In addition, morphometric parameters of a fish species have a major role to ensure whether there is any disparity between the same species of different geographic regions (Naeem and Salam 2005).

Genetic diversity is the basis of species adaptability and evolution, and there is a positive linear relationship between intraspecific genetic diversity and the adaptability of the species to the environment (Cruz et al. 2013; Turan et al. 2016). Nucleotide diversity and haplotype diversity are important indicators in terms of revealing mtDNA genetic variation in populations (Liu and Zhang 2009; Jiang et al. 2019). Among the most common mitochondrial genes used in detecting genetic diversity and population structure, the mtDNA cytochrome c oxidase subunit I (COI) gene represents useful genetic marker to assess cryptic diversity and population genetic diversity and structure in many fish species (Ivanova et al. 2021; Wei et al. 2023). So far mtDNA COI is mainly used for species identification and phylogeny of *Belone belone* from North Atlantic and Mediterranean (Turkish waters) (Knebelsberger et al. 2014; Turan et al. 2023), and there have been no reports about genetic diversity and population structure of garfish populations based on COI gene. In addition, except for one study of allozymes (Dobrovolov et al. 1980), no other molecular markers have been used in the genetic diversity analysis of different Black Sea populations of *B. belone*. The studies of population genetic structure and genetic diversity could provide guidance for the establishment of fishing quotas to prevent overharvesting (Zhao et al. 2019).

The aim of this paper is to represent for the first time the morphological and genetic diversity of one *Belone belone* population along the Bulgarian Black Sea coast, which as a commercially important fish species could aid in its conservation.

Methods

Morphological study

B. belone specimens were collected in December 2022 from the Bulgarian Black Sea coast (Nesebar) (Fig. 1). A number of 40 garfish individuals were used for morphological study and 39 of them were additionally used for molecular analyses. Biometric measurements were performed using BioMorph (Kutlu and Turan 2018) on the fresh fish. Twelve morphometric and six meristic body characters were examined. The analyzed morphometric and meristic characters are presented in Fig. 2 and in Tables 1, 2, respectively. The total and standard lengths were measured to the nearest 0.1 cm (Fig. 3). The rest of the morphometric characters were measured to the nearest 0.01 mm. Measurements of the head were expressed as percentages of the head length whereas other body measurements were expressed as percentages of the standard length. Statistical analyses were performed with the SPSS 5.5 software package and level of significance of $\alpha=0.05$ was accepted. The determination of the sex was made by direct examination of the gonads after opening the abdominal cavity.

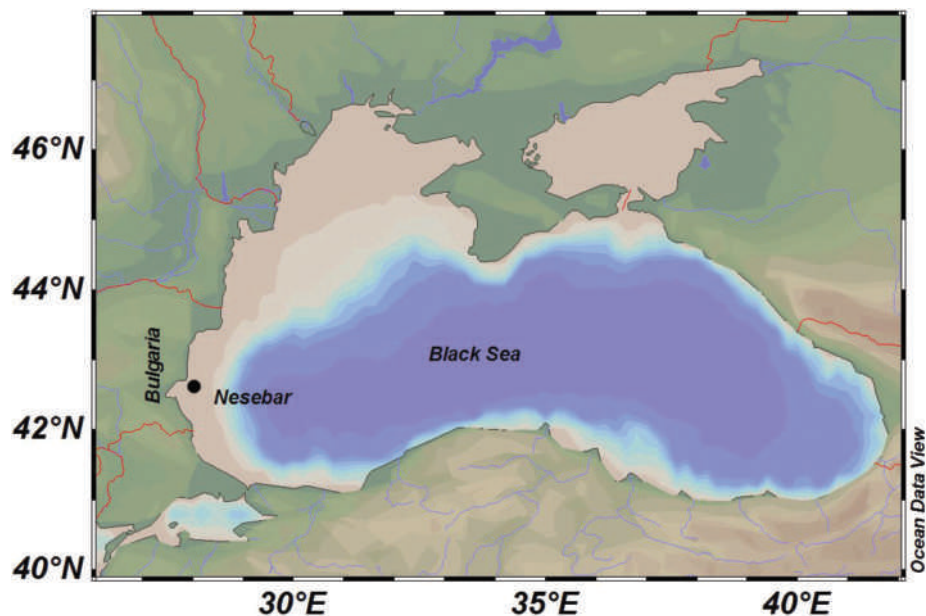


Figure 1. Location of the sampling station.

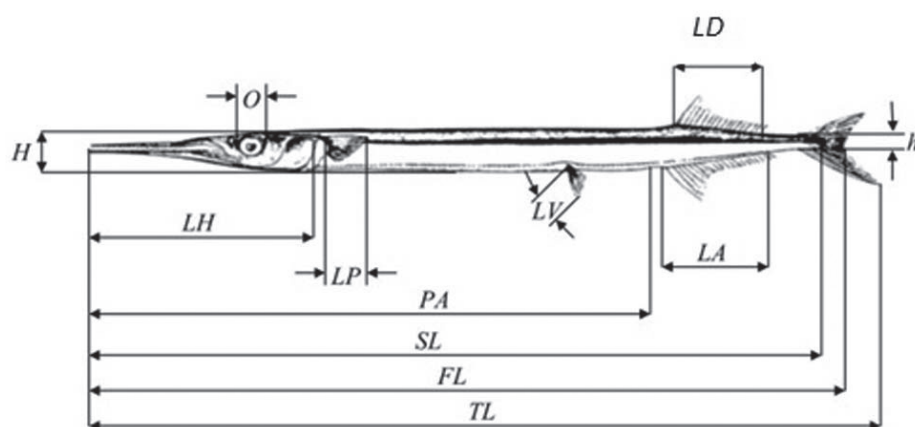


Figure 2. Morphometric measurements of garfish. Figure is from Collette (2016).

DNA extraction and PCR amplification

Tissue sample from the pectoral fin was cut and preserved in 96% ethanol at 4 °C. The genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN), and the target DNA was amplified with universal set of mitochondrial primers – cytochrome c oxidase subunit I (COI) FishF2: 5'TCGACTAATCATA-AAGATATCGGCAC3' and FishR2: 5'ACTTCAGGGTGACCGAAGAATCAGAA3' (Ward et al. 2005). The polymerase chain reaction (PCR) was carried out in a reaction volume of 50 µl containing 1 µl of each primer, 25 µl of the mastermix (MyTaq™ HS Mix) and 2 µl of the target DNA. The PCR conditions included the following parameters: 95 °C for 1 min, and 95 °C for 30 sec, 54 °C for 30 sec, 72 °C for 1 min (35 cycles), 72 °C for 10 min. A quality control of the PCR product was performed by electrophoresis on 1% agarose gel. The DNA sequencing was performed by Macrogen Europe B.V. The obtained haplotype sequences were submitted to GenBank under the accession number OR554252 and OR554253.

Table 1. Relative relationships of measured body proportions of *Belone belone* in the Bulgarian Black Sea coast. n-number of fish studied, \bar{X} -mean value, SD-standard deviation; SE-standard error of mean value; CV-coefficient of variation.

Relation	Sex	n	Range	$\bar{X} \pm SD$	$\bar{X} \pm SE$	CV (%)
FL/TL	♀	26	32.7±39.9	35.3±1.79	35.3±0.35	5.08
	♂	14	30.0±37.8	34.7±2.15	34.7±0.57	6.21
	Total	40	30.0±39.9	35.1±1.91	35.1±0.30	5.46
SL/TL	♀	26	31.1± 39.4	34.1±1.80	34.1±0.35	5.29
	♂	14	25.8± 36.6	33.2±2.88	33.2±1.80	8.69
	Total	40	25.8±39.4	33.8±2.25	33.8±0.35	6.65
PA/TL	♀	26	24.1±30.3	48.9±1.32	48.9±0.26	5.01
	♂	14	23.2±28.0	26.2±1.24	26.2±0.33	4.72
	Total	40	23.2±30.3	26.3±1.28	26.3±0.20	4.86
HL/TL	♀	26	10.1±11.5	19.5±0.39	19.5±0.07	3.73
	♂	14	9.5±10.9	10.3±0.33	10.3±0.09	3.25
	Total	40	9.5±11.5	10.4±0.37	10.4±0.06	3.60
O/LH	♀	26	0.8±1.0	0.9±0.06	0.9±0.01	7.03
	♂	14	0.7±0.9	0.9±0.06	0.9±0.02	7.01
	Total	40	0.7±1.0	0.9±0.06	0.9±0.01	7.19
H/TL	♀	26	1.5±1.9	1.7±0.12	1.7±0.02	7.02
	♂	14	1.5±1.9	1.7±0.12	1.7±0.03	7.18
	Total	40	1.5±1.9	1.7±0.12	1.7±0.02	6.99
h/H	♀	26	0.7±0.8	0.7±0.04	0.7±0.01	5.59
	♂	14	0.7±0.8	0.7±0.03	0.7±0.01	3.78
	Total	40	0.7±0.8	0.7±0.04	0.7±0.01	5.53
LD/TL	♀	26	4.3±5.3	4.8±0.03	4.8±0.06	6.93
	♂	14	4.3±5.1	4.2±0.03	4.7±0.08	6.44
	Total	40	4.3±5.3	4.7±0.32	4.7±0.05	6.94
LP/TL	♀	26	1.8±2.4	2.2±0.13	2.2±0.02	5.89
	♂	14	2.0±2.4	2.2±0.11	2.2±0.03	4.96
	Total	40	1.8±2.4	2.2±0.12	2.2±0.02	5.55
LA/TL	♀	26	5.2±6.3	6.1±0.36	6.1±0.07	5.84
	♂	14	5.1±6.2	6.4±0.37	6.4±0.10	5.78
	Total	40	5.1±6.3	6.2±0.36	6.2±0.06	5.82
LV/TL	♀	26	1.4±1.9	1.7±0.10	1.6±0.02	6.19
	♂	14	1.4±1.9	1.7±0.12	1.7±0.03	6.91
	Total	40	1.4±1.9	1.7±0.11	1.7±0.02	6.37



Figure 3. The captured specimen of garfish *Belone belone*.

Table 2. Meristic characteristic of garfish from Bulgarian Black Sea. n-number of fish studied, \bar{X} -mean value, SD-standard deviation; SE-standard error of mean value; CV-coefficient of variation.

Meristic characteristic	Sex	n	Range	$\bar{X} \pm SD$ (%)	$\bar{X} \pm SE$ (%)	CV (%)
No rays in dorsal fin (<i>D</i>)	♀	26	16–19	17.8±0.65	17.8±0.13	3.67
	♂	14	16–19	17.6±0.75	17.6±0.20	4.30
	Total	40	16–19	17.7±0.68	17.7±0.11	3.88
No rays in pectoral fin (<i>P</i>)	♀	26	12–19	13.0±0.20	13.0±0.04	1.51
	♂	14	13–19	13.0±0.01	13.0±0.01	0.01
	Total	40	12–19	13.0±0.16	13.0±0.03	1.22
No ray in anal fin (<i>A</i>)	♀	26	19–23	21.9±1.02	21.9±0.20	4.64
	♂	14	19–23	21.6±1.01	21.6±0.27	4.66
	Total	40	19–23	21.8±1.01	21.8±0.16	4.63
Upper teeth	♀	26	13–15	14.0±0.28	14.0±0.05	2.02
	♂	14	14–14	14.0±0.01	14.0±0.00	0.01
	Total	40	13–15	14.0±0.23	14.0±0.03	1.62
Gill	♀	26	31–34	33.1±0.88	33.2±0.17	2.65
	♂	14	31–34	32.9±0.83	32.9±0.22	2.52
	Total	40	31–34	33.1±0.86	33.1±0.14	2.60
Vert.	♀	26	80–81	80.0±0.20	80.0±0.04	0.24
	♂	14	80–81	80.2±0.36	80.2±0.10	0.45
	Total	40	80–81	80.1±0.27	80.1±0.04	0.33

Results

The total length of female varied between 30.0 cm±37.8 cm (TL_{min} - TL_{max}) and 32.7 cm± 39.9 cm for males. No statistically significant difference was noted between sexes (KOLMOGOROV-SMIRNOV Test, $P < 0.05$) for all morphometric characters: total length $P(T \leq t) = 0.54$, fork length $P(T \leq t) = 0.50$, standard length $P(T \leq t) = 0.29$, pre-anal distance $P(T \leq t) = 0.74$, head length $P(T \leq t) = 0.29$, eye diameter $P(T \leq t) = 0.18$, maximum body height $T \leq t) = 0.81$, minimum body height $P(T \leq t) = 0.24$, length of the dorsal fin basis base $P(T \leq t) = 0.63$, length of anal fin basis $P(T \leq t) = 0.75$, length of pectoral fin $P(T \leq t) = 0.44$, length of pelvic fin $P(T \leq t) = 0.82$. The fork length, standard length, pre-anal distance, length of anal fin, length of dorsal fin, maximum body height, length of pelvic fin, minimum body height was found as 87.5%, 84.4%, 65.8%, 14.5%, 11.9%, 4.3%, 4.1% and 1.8% of the total length of the fish respectively (Fig. 4A). Head length, length of pectoral fin, and eye diameter were found as 26.1%, 5.9% and 2.2% (Fig. 4B). Of all investigated specimens 65% were females and 35% were males.

The coefficient of variation CV was relatively low ($CV < 9\%$) for all morphometric measured body proportions of *Belone belone*. The lowest value was recorded for the relationship of length head and total length (LH/TL) in male ($CV = 3.25\%$), while the highest value was registered for relationship (SL/TL) of standard and total length in male ($CV = 8.69\%$) (Table 1).

The dorsal fin is long, unbranched with 16–19 spiny rays. The pectoral and anal fin comprised of 13–19 and 19–23 fin rays respectively. The coefficients of variation CV were relatively low for all meristic characteristics. The lowest value was recorded for the pectoral fin rays in total ($CV = 0.01\%$), while the highest value was registered for anal fin rays in male ($CV = 4.66\%$). No statistical difference was observed between sexes (Table 2).

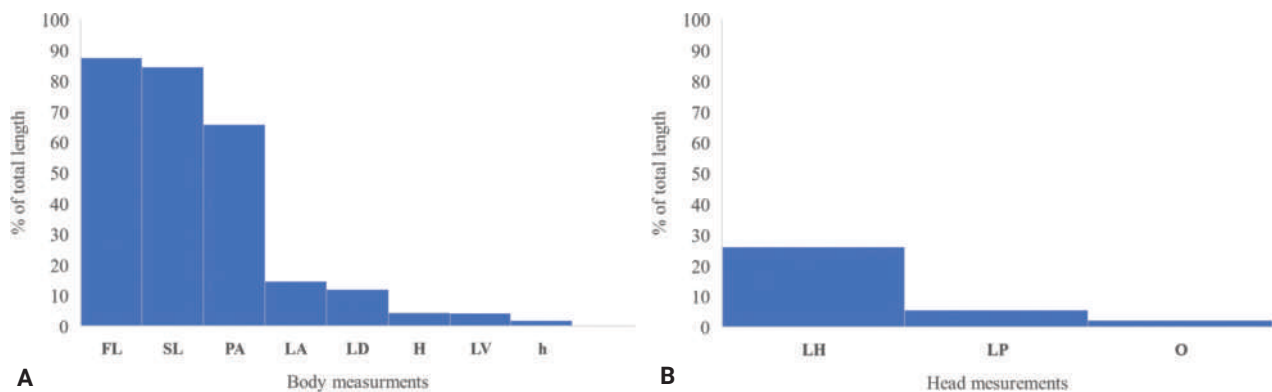


Figure 4. Morphometric measures of garfish in relation to the **A** total length and **B** head length of the fish in percentage.

Some length-length relationships (FL/TL, PA/TL, SL/FL, SL/TL, LH/TL) were fitted well by linear regression and show high determination (R^2) with the exception of the relationships:

(H/TL, LV/TL, LD/TL, h/H, LP/TL, LH/O), which displayed very small values of R^2 (Fig. 5A–K).

The obtained COI gene fragment (633 bp) showed high percent identity (100% for haplotype 1 and 99.84% for haplotype 2) with *B. belone* sequences deposited in GenBank (KJ204729 North Sea, KJ128428 Sweden), supporting the species identification. From the 39 garfish samples analyzed, only two haplotypes were found, indicating low level of haplotype (0.146 ± 0.072) diversity. The first haplotype (H1) was dominant, presented in 36 of the samples, whereas the second one (H2) was found only in 3 of them. Low nucleotide diversity (0.00023 ± 0.00011) was also found.

Discussion

The range of TL observed in the present study (31–39.90 cm) was generally congruent with results from Bosphorus area (31–59 cm, Yüce 1975; 29–58 cm, Samsun et al. 2006) and previous investigation from the Black Sea (32–57 cm, Samsun et al. 1995; Samsun 1996; 34–52 cm, Erkoyuncu et al. 1994), with the exception of the upper limit of total length, which is significantly higher than that given in this research (Table 3). For garfish in the Black Sea, Prodanov (1982) and Kaya and Saglam (2017) reported the total length 25.60–57.90 cm and 49.5 cm, respectively. Other authors have received slightly higher estimates of TL, between 50.5 and 60.3 cm (Samsun 1995; Bilgin et al. 2004; Samsun et al. 2006; Polat et al. 2009).

The observation of infrequent growth in total length could be a result of hereditary factors (Borges 2001) or may be associated with the variations in morphologic characteristics (Bauer 1961). Fish populations exposed to high fishing pressure react to this pressure by reproducing at smaller ages and sizes (Helfman et al. 2009).

The feminine rate of garfish population is dominant with 65% and was higher than that reported for southern Ireland (58.3%: Dorman 1989), for Swedish waters (38.6%: Dorman 1991) and also for the Black Sea (53.4%: Samsun et al. 1995; 51.5%: Samsun 1996). Hemida (1987) explained this dominance by high natural mortality in males.

It was noted that R^2 values from the trends (Fig. 5) were above 0.70. R^2 values of the total length against morphometric parameters increase in the order

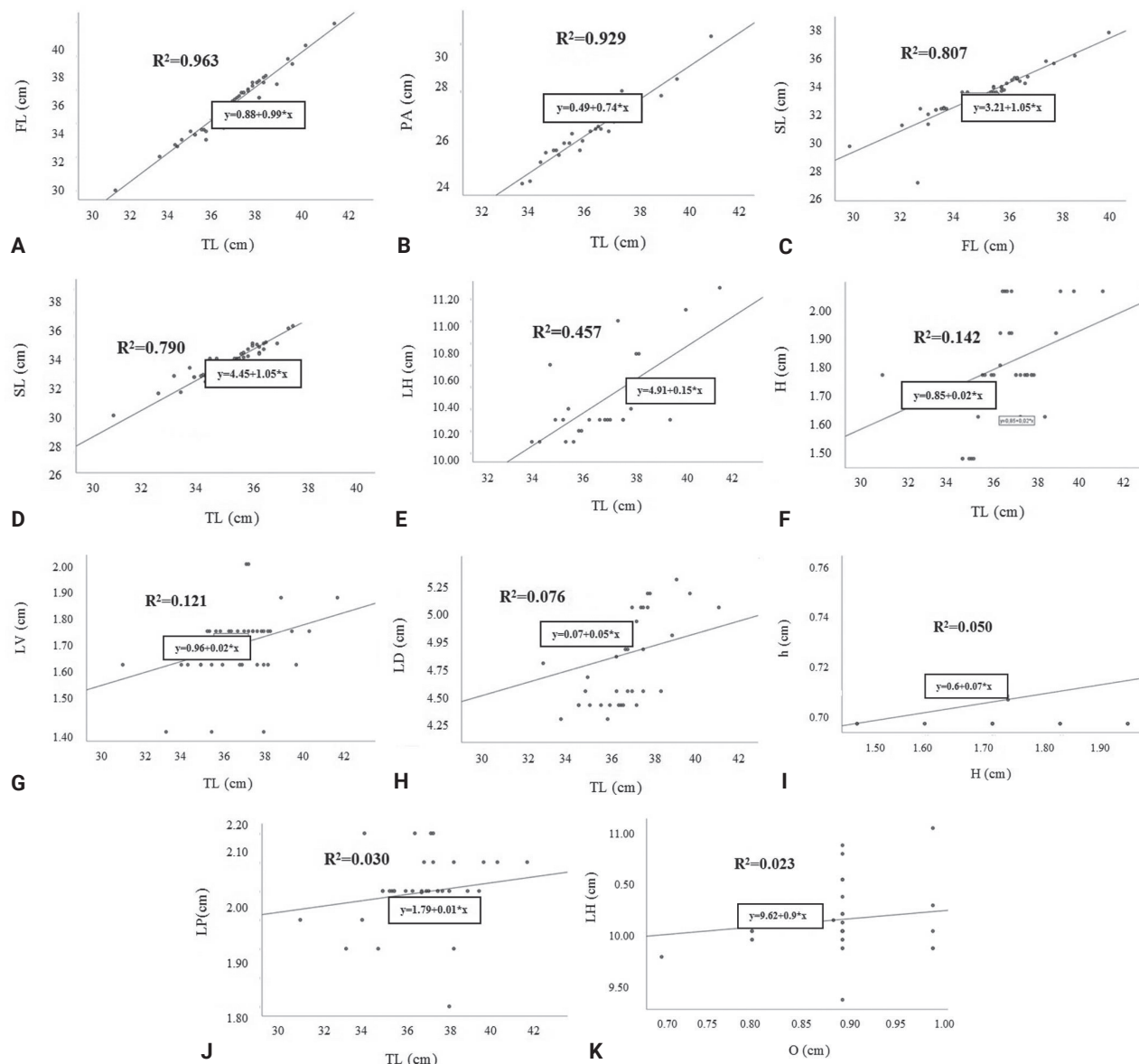


Figure 5. Length-length regression and coefficient of determination R^2 .

FL > SL > PA. This entails that below 80% of changes in the total length were predicted by the selected morphometric parameters selected for this study.

Data from the literatures on morphometric relations are comparable since they refer to total body length. However, a study carried out by Fehri-Bedoui and Gharbi (2004) showed lower value for morphometric relationships of fork and total and standard and total length (FL/TL= 70% SL/TL=78%). The present findings show that both relationships (FL/TL= 87.5%, SL/TL=84.4%) are close to data reported by Zorica and Čikeš Keč (2011) (FL/TL=96.2% SL/TL=93.8%) and Sinovčić et al. (2004) (SL/TL=82%). The differences between this study with previous and other studies in different regions may have been caused by the dissimilarities of habitats, sampling time, differences in population and fishing gears used in sampling (Kalayci and Yeşilçiçek 2012).

Zorica and Čikeš Keč (2011) reported that the head constitutes 22.6% of the total body length in *Belone* from the Adriatic Sea. We determined that the head makes up 26.05% of the standard body length in sample of Bulgarian

Table 3. Comparison of the maximum length recorded in different areas.

Area	Authors	Length (cm)	Length type	Fishing gear
Black Sea, Turkey	Bilgin et al. 2014	65.1	TL	Surrounding net
Black Sea, Turkey	Polat et al. 2009	60.3	TL	Surrounding net
Black Sea, Turkey	Bilgin et al. 2004	58.0	TL	Purse seine, Surrounding net
Black Sea, Turkey	Samsun et al. 2006	58.0	TL	Purse seine, Surrounding net
Black Sea, Turkey	Samsun 1995	52.2	TL	Surrounding net
Black Sea, Turkey	Kalayci and Yeşilcicek 2012	50.5	TL	Purse seine, Gillnet
Black Sea, Turkey	Kaya and Saglam 2017	49.5	TL	Trammel net
Bosporus, Turkey	Yuce 1975	57.5	FL	Unknown
Aegean Sea, Greece	Koutrakis and Tsikliras 2003	27.0	TL	Beach seine
Mediterranean Sea, Tunisia	Ben Smida et al. 2014	40.1	TL	Unknown
Black Sea, Bulgaria	Prodanov 1982	57.9	TL	Unknown
Black Sea, Bulgaria	Present Study	39.9	TL	Trammel net

Table 4. Comparison of the maximum length recorded in different areas.

Area	Authors	No rays in Dorsal fin (D)	No rays in Pectoral fin (P)	No rays in Anal fin (A)	Vert.
Black Sea	Bănărescu (1964)	14–16		II/18–20	
Black Sea	Svetovidov (1964)	II/14–16	9–11	II/18–20	
Black Sea	Prodanov (1982)	II/14–18	II/11–12	II/17–21	74–81
Mediterranean, Atlantic, Black Sea	Colette and Parin (1986)	16–20	11–14	19–23	75–84
Adriatic Sea	Jardas (1996)	16–19	11–14	19–23	80–81
Present study		II/16–19	I/13–19	II/19–23	80–81

Black Sea coast. According to the Tortonese (1970) and Jardas (1996) data about maximum body height H/TL , relationships have been calculated slightly lower as follows $H/TL=0.06\%$. According to the Tortonese (1970) and Jardas (1996) data about LH/TL relationships have been calculated as $LH/TL=28.6$. In this study values of the relative relationship between length head and total length ($LH/TL=26.05\%$) were in agreement with the observations given by Zorica and Čikeš Keč, (2011) $LH/TL=22.6\%$ and Tortonese (1970) and Jardas (1996). Several researchers reported the number of dorsal fin rays in Black Sea between 14–18 and 16–20 for the Mediterranean and Adriatic Sea (Bănărescu 1964; Svetovidov 1964; Prodanov 1982; Collette and Parin 1986; Jardas 1996). The meristic characters reported for species in the Adriatic Sea show the same result with respect to dorsal and anal fin rays (Table 4). The result of dorsal and pectoral fin rays showed low variability compared with results given by previous studies from other research areas. Similarly, to the Bănărescu (1964) and Svetovidov (1964), we found the presence of spines in anal and dorsal fins (Table 4). According to Svetovidov (1964), the presence or absence of the upper teeth on the hard palate, found in the present research, could be a reason for subspecies differentiation. Lindsey and Harrington (1972) found that the number of spines and pectoral fin rays in some fishes depended on the temperature of the water in which the fertilized egg develops, with increasing temperature and the number of indicated meristic characters improving. With regard to the latter, the number of vertebrae and rays in the pectoral fins is determined for 4 and 8 days, respectively. These data show that a number

of abiotic environmental factors, especially during the development of fertilized eggs, play a major role in the formation of plastic and meristic characters (Prodanov 1982).

Genetic diversity is an important part of biodiversity, and its level reflects the environmental adaptability, evolutionary potential, and viability of species. Haplotype and nucleotide diversity are two important indicators for evaluating population genetic diversity. Low haplotype (0.146) and nucleotide (0.00023) diversity of COI found in Nesebar population could be a result of population decline, probably caused by overfishing. The results indicated that some measures should be taken to protect the resources of *Belone belone* along the Bulgarian Black Sea coast. The information on genetic diversity and population structure of *B. belone* are critical for phylogenetic relationships, resource conservation and fisheries management. Suggesting future studies, the genetic diversity analysis of other genetic markers should be supplemented.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Data curation: YR. Formal analysis: PI, MY, ND. Investigation: ND, MY, PI, VR. Methodology: VR, PI, MY, ND. Software: MY. Visualization: MY. Writing - original draft: ND, PI, VR, MY. Writing - review and editing: MY, VR, PI, CT, ND.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Research Article

A baseline assessment of anthropogenic macrolitter on dunes along the Bulgarian Black Sea Coast using visual census and Unmanned Aerial Systems

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Abstract

Beach-dune systems are among the most dynamic and sensitive elements of coastal ecosystems in the world. They represent an intersection between human activities, flora, fauna and economic interests in tourism. The Bulgarian Black Sea shoreline spans 518.7 km and comprises 131 km (25%) of the depositional coast, including beaches and 46 dune systems. Over the past three decades, heavy anthropogenic impacts have been observed, significantly altering the cleanliness of the beach-dune systems along the Bulgarian Black Sea Coast (BBSC). The research initially began as an initial assessment of macrolitter on dunes (MLD) using Unmanned Aerial Systems (UAS). However, due to concerning data obtained in the first year, it transitioned into a mid-term monitoring program conducted between 2018 and 2022. The baseline assessment is based on a visual census, UAS mapping and manual image screening procedure in a GIS environment for litter mapping in 40 areas of litter monitoring (ALMs) along the Bulgarian Coast. Throughout the five-year monitoring period, the most abundant type of MLD was “Artificial polymer materials,” accounting for 83.4% of the total number, followed by “Paper/Cardboard” (6.2%), “Glass/Ceramics” (2.8%), “Metal” (2.8%), “Processed/Worked wood” (1.83%), “Rubber” (1.29%), and “Cloth/Textile” (1.17%). Generally, 95% of the total litter amount was assessed from Land-based sources and 5% from Sea-based sources. The COVID-19 pandemic indirectly affected the cleanliness of the Bulgarian dunes due to restrictions on foreign travel, which increased the domestic tourist pressure on the Bulgarian beaches, resulting in a more significant amount of waste accumulating on the beaches and dunes. The abundance experienced an increase of 39% between 2018 and 2021. A similar upward trend (+41%) was observed in the density of macrolitter on the dunes. Based on visual census data, the average density was estimated to be 0.54 ± 0.35 items/m². The spatial distribution of MLD is a complex combination of anthropogenic impact and wind processes that affect various eco-geomorphological elements of the beach-dune system. The embryonic dunes retained only 16% of the total items (Dav: 0.32 ± 0.12 items/m²). The highest litter density was registered on the foredunes (Dav: 0.71 ± 0.21 items/m²; 28% of total items). The backdunes contained the highest litter abundance, accounting for 55% in larger areas (Dav: 0.59 items/m²). Density litter maps established that dune vegetation acted as a natural trap, retaining 40% more macrolitter compared to areas without dune plants. A Clean Dune Index (CDI) was developed to evaluate the cleanliness of Bulgarian dunes. Based on aggregated CDI data for 2018–2022, the cleanliness of the dunes along the Bulgarian Coast was categorised as “moderate” (CDIav:10.89). Dune systems near the most visited resorts were

classified as “extremely dirty”, with the highest CDI values recorded at Kavatsite (27.22), Nessebar – South (25.01), Bolata (24.69), Asparuhovo - Varna (24.33) and Slanchev bryag (24.09). On the other hand, the dune systems at Ropotamo and Lipite were rated with the lowest CDI – 0.95 and 1.2. Dunes are sensitive habitats and require minimal anthropogenic impact, which requires the intensification of the use of high-resolution remote sensing methods for litter mapping. The quality of the presented data and the results obtained outline drones as a future primary tool for beach and dune surveys.

Key words: Abundance, baseline assessment, Bulgarian Black Sea Coast, dune pollution, macrolitter, monitoring, unmanned aerial systems (UAS)

Introduction

World Ocean and marine litter

Sandy beaches and coastal dunes are widespread landforms along coastlines worldwide (Martínez et al. 2008; Bird 2011; Luijendijk et al. 2018, etc.) and hold significant geological, biological, and ecological importance (Hesp 2002; Masselink et al. 2011; Jackson et al. 2019, etc.). Currently, the impact of anthropogenic marine litter (AML) on these landforms and the marine environment is recognised as a threat to marine wildlife under Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy, MSFD (European Commission 2008) and international provisions such as the United Nations Sustainable Development Goal 14 (UN RES/70/1 2015). Recent global efforts have led to the United Nations signing the first resolution specifically targeting plastic pollution. This resolution aims to guide the development of a legally binding treaty by 2024 (UNEP 2022).

In recent decades, there has been a noticeable increase in the accumulation of AML, especially plastic debris, in various environmental compartments spanning from the north to the south pole (Galgani 2015; Galgani et al. 2015; Monteiro et al. 2018, etc.). Marine litter (ML) consists of plastic, glass/ceramic, metal, wood/paper, textiles, and rubber (Galgani et al. 2013a), with plastic being the predominant type, comprising 60–80% of all litter (Barboza et al. 2019). Anthropogenic debris has become a significant pollutant in marine, coastal and terrestrial environments, posing risks to species’ survival and potential adverse effects on human health (Galgani et al. 2013b; Wright et al. 2013; Panti et al. 2019). The most significant threats to human health and implications for environmental and ecological well-being are associated with anthropogenic ML (Leal Filho et al. 2019). Negative impacts of microplastic pollution on the marine environment include entanglement (Gregory 2009; Thiel et al. 2018), ingestion (Savoca et al. 2021), bioaccumulation of persistent organic and inorganic pollutants (Teuten et al. 2009; Ranjani et al. 2022) and a transfer of non-native species through hitchhiking (Al-Khayat et al. 2021). Furthermore, ML has detrimental effects on the economies of coastal countries (McIlgorm et al. 2011; Brouwer et al. 2017; Beaumont et al. 2019), apart from its negative impact on marine biodiversity. Land-based sources contributed to nearly 80% of the anthropogenic microplastics entering the oceans (UNEP Regional Seas Programme 2005; Allsopp et al. 2006; Eunomia 2016). Borrelle et al. (2020) estimated that

in 2016, global plastic waste production ranged from 19 to 23 million tons, a substantial portion of which eventually found its way into aquatic ecosystems.

Growing global environmental concerns have led to increased efforts to monitor and quantify the accumulation of anthropogenic litter in marine environments. Consequently, numerous surveillance programs have been established worldwide in recent decades to assess the extent of litter found in oceans and coastal areas (e.g., Maes et al. 2019). Beach-dune ecosystems are particularly important to monitor in the context of ML or beach litter (BL) due to the various anthropogenic pressures (recreational activities, fisheries, urbanization, etc.) and environmental factors (wind, waves, tides, etc.) that can contribute to their contamination. However, many strategies proposed for collecting stranded ML in coastal areas primarily focus on the subaerial beach (e.g., OSPAR 2010; Gargani et al. 2013a; GESAMP 2019). A notable global initiative addressing the issue of plastic pollution in oceans and coastal areas is the Marine Beach Litter Strategy, which provides valuable data on the extent of the AML problem (Bhuyan et al. 2021; Zielinski et al. 2022; Cesarano et al. 2023; Diem et al. 2023; Mugilarasan et al. 2023, etc.).

Marine litter in the Black Sea

Regarding the marine environment of the Black Sea, land-based waste is identified as the primary contributor, accounting for over 70% of all marine pollution (World Bank 2020). Alongside issues such as nutrient runoff, eutrophication, wastewater discharges and heavy metal accumulation, ML is recognised as a significant and complex environmental concern in the Black Sea basin (BSC 2019; World Bank 2020). The enclosed nature of this sea, with limited water replenishment, restricted vertical mixing and dynamic surface circulation, makes it highly vulnerable to environmental degradation. Additionally, ML pollution is a relatively recent problem for the Black Sea, exacerbating its vulnerability to environmental harm. Consequently, the countries surrounding the Black Sea have undertaken numerous surveys in recent years to gain a comprehensive understanding of the scale of the issue and develop strategies to reduce the inflow of ML into the basin. For instance, the Project “Assessing the vulnerability of the Black Sea marine ecosystem to human pressures – ANEMONE” has played a significant role in these efforts (Paiu et al. 2020; ANEMONE 2021).

Over the past decade (after 2013), the countries surrounding the Black Sea have conducted numerous studies to comprehend the extent of the issue and develop strategies to reduce the inflow of ML into the basin. These studies have revealed concerning levels of anthropogenic litter at various sites along the coastlines of Romania (Muresan et al. 2017; Paiu et al. 2017, etc.), Bulgaria (Brouwer et al. 2017; Simeonova et al. 2017, 2020; Bobchev 2018; Simeonova and Chuturkova 2019, 2020; Toneva et al. 2019; Panayotova et al. 2020; Chuturkova and Simeonova 2021; Bekova 2023; Bekova and Prodanov 2023, etc.), Georgia (Machitadze et al. 2020) and Turkey (Topçu et al. 2013; Terzi and Seyhan 2017; Şahin et al. 2018; Aytan et al. 2020; Gülenç et al. 2020; Öztekin et al. 2020; Terzi et al. 2020; Bat et al. 2022; Erüz et al. 2023, etc.). Significant amounts of floating and seafloor ML, as well as ML sourced from rivers, were identified (BSC 2007; Ioakeimidis et al. 2014; Lechner et al. 2014; Suaria et al. 2015; Moncheva et al. 2016; Öztekin and Bat 2017; Slobodnik et al. 2018; Stanev

and Ricker 2019; Aytan et al. 2020; Berov and Klayn 2020; Doncheva et al. 2020; Miladinova et al. 2020; Raykov et al. 2020; Slabakova et al. 2020; Terzi et al. 2020; Panayotova et al. 2021; Erüz et al. 2022; González-Fernández et al. 2022; Pogojeva et al. 2023). ML was also reported in biota (Tonay et al. 2020; Aytan et al. 2021, 2022; Terzi et al. 2022; Mihova et al. 2023; Zlateva et al. 2023) and in archaeological contexts (Prahov et al. 2021) in the Black Sea area. Georgieva et al. (2023) also highlighted alarming data regarding the widespread distribution of microplastic pollution along the Bulgarian Black Sea Coast. In general, these studies emphasize the necessity of assessing the distribution of micro-, meso- and macrolitter in the Black Sea and their potential risks to humans and coastal ecosystems.

There is a lot of research on mapping anthropogenic debris on coastal dunes (Poeta et al. 2014; de Francesco et al. 2018; Rangel-Buitrago et al. 2018, 2021; Šilc et al. 2018; Menicagli et al. 2019, 2023; Andriolo et al. 2020a, 2020b, 2021a, 2021b; Gonçalves et al. 2020a, 2020b; Turner et al. 2021; Andriolo and Gonçalves 2022; Corbau et al. 2023, Gallitelli et al. 2023; Mancuso et al. 2023, etc.). Unfortunately, the litter on dunes along the Bulgarian Black Sea coast has not been studied until now. Given the results we obtained (mostly land-based), especially for the dunes studied, it is appropriate to avoid the commonly accepted term “Marine Litter” (ML) and use instead the term “MacroLitter on Dunes” (MLD) with the size of the items > 2.5 cm.

UAS approaches in litter mapping

The Unmanned Aerial Systems (UAS), also known as Unmanned Aerial vehicles (UAV) or drones, should be considered not only as an alternative to the conventional visual census but also as a new methodology to advance knowledge on the dynamics of litter, with the potential to play a significant role in providing data for the development of litter models on coasts over time (Gonçalves et al. 2022). A new survey strategy that is based on UAS has been used in recent years to map and detect (manual image screening and machine learning techniques) the abundance and distribution of accumulated macrolitter items on sandy beaches (e.g., Deidun et al. 2018; Fallati et al. 2019; Andriolo et al. 2020a, 2020b, 2021a, 2021b, 2023; Gonçalves et al. 2020a, 2020b; Lo et al. 2020; Escobar-Sánchez et al. 2021; Merlino et al. 2021; Papakonstantinou et al. 2021; Andriolo and Gonçalves 2022). This method has been demonstrated to be efficient in terms of both time and cost while enhancing the accuracy of measurements of coastal macrolitter pollution with RTK mode drones. Andriolo et al. (2020a, 2020b, 2021a) proposed a new framework based on the combined use of UAS and a mobile application to map and quantify marine litter accumulation on coastal dunes. Drones were introduced in Bulgaria for mapping anthropogenic waste and its influence on beach-dune systems in 2018, launching long-term monitoring studies of beach-dune systems (Prahov et al. 2021; Bekova 2023; Bekova and Prodanov 2023; Prodanov et al. 2023a).

Aim of the study

One of the significant gaps in scientific knowledge that requires attention is monitoring macrolitter on coastal dunes in the Black Sea region. This article

aims to fill a gap in the research on dune pollution with macrolitter as well as draw attention to this ignored issue. After worrisome results in the first year, our study switched to mid-term monitoring using a standard visual census aided by UAS photogrammetry. The work aims to obtain an answer to the question “How dirty are the dunes along the Bulgarian Black Sea coast?” and, on the other hand, to understand “How significant is the role of plants on mobile and stabilized dunes for trapping litter?”. The paper aims to present a baseline assessment of abundance, density, spatial distribution, litter sources and categories and an evaluation of cleanliness in 40 dune systems along the Bulgarian Black Sea Coast.

Materials and methods

Dune systems along the Bulgarian Black Sea Coast

According to the latest data from UAS of the Institute of Oceanology at the Bulgarian Academy of Sciences (IO-BAS), the Bulgarian Black Sea shoreline length is 518.7 km (Prodanov et al. 2020a, 2023a, 2023b). Between Cape Sivriburun and Rezovo, approximately 25% (131 km) of its length presents depositional landforms consisting of beaches and 46 beach-dune systems (Prodanov et al. 2023a). During the years, dunes along the Bulgarian Coast have been relatively understudied in geomorphological, morphodynamic (Peychev 2004; Peychev and Peev 2006; Prodanov et al. 2019a, 2019b, 2020b, 2021a, 2021b, 2023a; Prodanov 2023) and geological aspects (Petrov 2013; Valchev 2014, 2015; Sinnyovsky and Sinnyovska 2016, 2017), or as habitats (Gushev and Tzonev 2015; Tzonev 2015a, 2015b, 2015c, 2015d, 2015e, 2015f; Valcheva et al. 2019, 2020, 2021). Anthropogenic macrolitter on coastal dunes has not been studied. Our research focused on the largest dune systems that had been significantly impacted by anthropogenic activities in recent years (Fig. 1). The study sites are located on embryonic dunes, foredunes with dune crest to 7–8 m and back-dunes (blowouts and interdune slacks) covered by plants that trap the litter (Prodanov et al. 2023a).

Areas of litter monitoring

The first step was to pre-define the areas for litter monitoring (ALM), also called Sampling Units (SU) in the dune systems (DS) and create vector shape files with study areas as templates for subsequent campaigns (seasons). The MLD surveys were conducted in the spring and autumn seasons from 2018 to 2022 (Fig. 2A). The Bulgarian coastal dune systems have never been subject to monitoring for anthropogenic pollution. This study used the master list in the Guidelines for monitoring marine litter in European seas to classify macrolitter (Galgani et al. 2013a). An area of litter monitoring was planned in 87% of all dune systems along the Bulgarian Black Sea Coast. The 40 selected areas (with a minimum size of 100 m length x 50 m width) were defined according to the spatial distribution of dune landforms varying in height, often covered by dune vegetation that retains significantly more litter than the active beach (Fig. 2B). The area of monitored dunes was assessed at 242 359 m² (2.45% of the total area of dunes). They embrace 5 174 m along the coastline, which is equal to 1% of the current length

of the Bulgarian shoreline of 518.7 km (Prodanov et al. 2023a, 2023b). In order to facilitate the creation of a density litter correlation with previous data, the back-dunes of the beaches from the national monitoring program (BLKBG-D10-Marine litter 2016; Simeonova et al. 2020; Kalinov et al. 2021; Bekova 2023; Bekova and Prodanov 2023) were included in the present research.

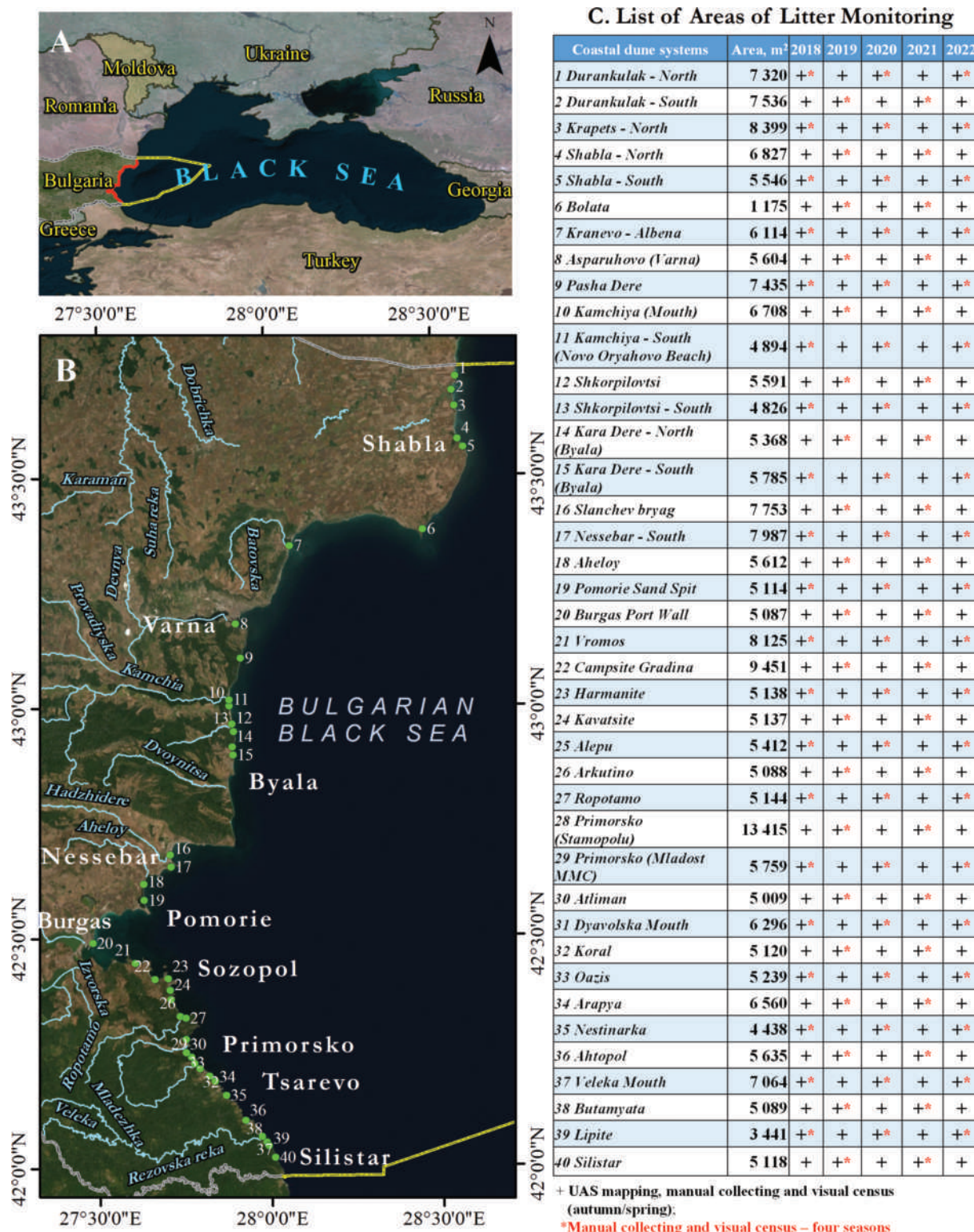


Figure 1. Dune areas for litter monitoring along the Bulgarian Coast in 2018–2022.

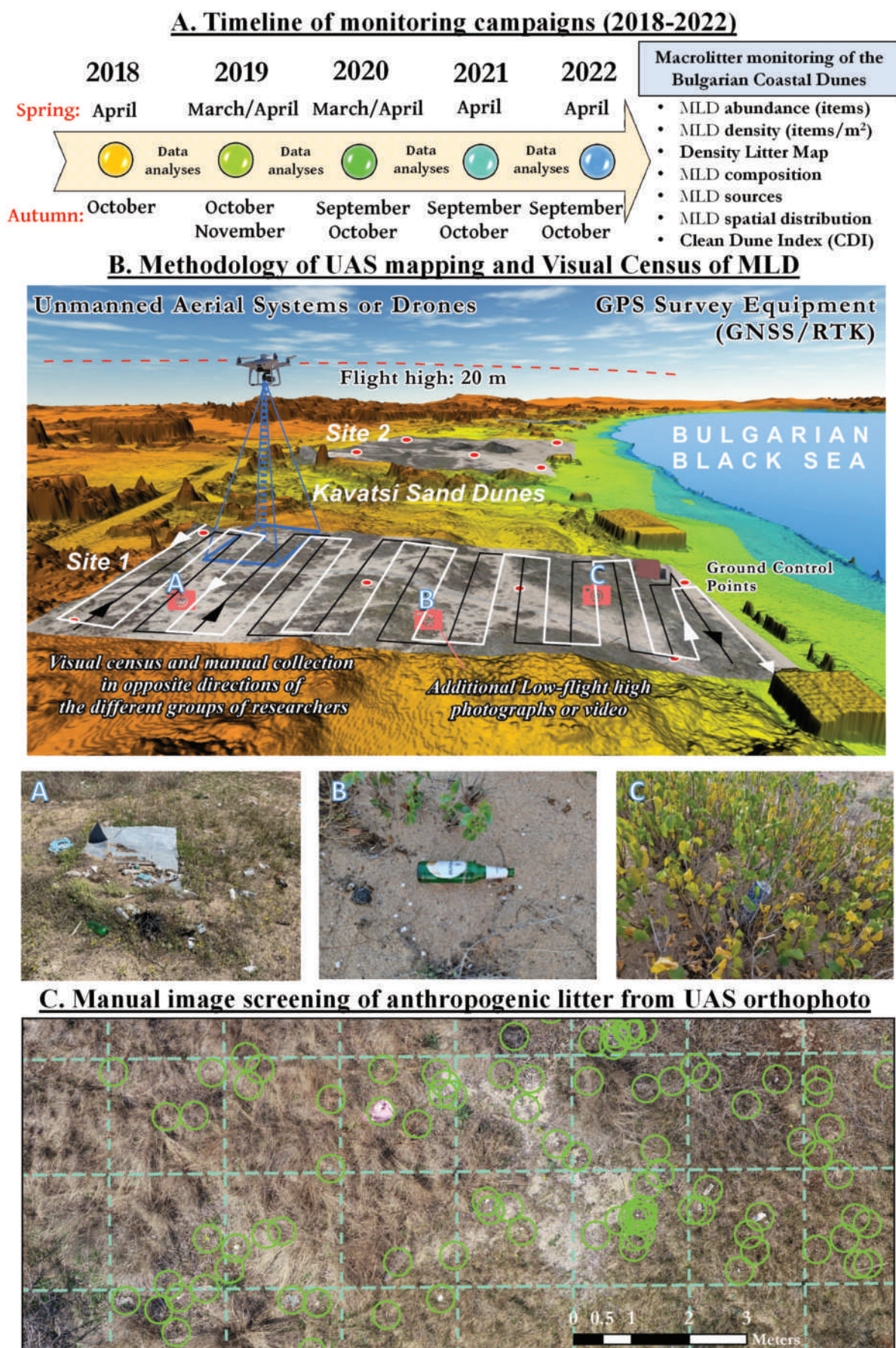


Figure 2. Timeline, methodology of field survey and example of MS procedure along the Bulgarian Coast.

Field surveys

UAS missions and acquired data

A multirotor quadcopter DJI Phantom 4 RTK (DJI-P4RTK), equipped with a 20M pixel camera, was used to collect high-resolution aerial images on dune systems during spring and autumn in the period 2018–2022 (Fig. 2A), following standard procedures (Prodanov et al. 2020a; Gonçalves et al. 2022). A drone-mounted multi-frequency on-board GNSS receiver provided reliable centimetre-level accuracy in positioning. Minimum five ground control points were collected within the study areas by a Hi-Target V90 GNSS RTK system (Fig. 2B). Flying at the height of 20 m with the camera lens placed perpendicular to the flight direction, the drone acquired images (4000 × 3000) with an overlap of 90% front and 90% side. The drone needed about 60 min to complete the study sector (3 flight missions). Following the double-grid photogrammetric survey, imagery in an oblique camera mode was taken from 5 meters above the dune surface to provide a high-resolution image that could be used to support the classification of litter during MS, if necessary.

Agisoft Metashape (v1.5.3-v.1.7.2) was used for the Structure from Motion Multi-View Stereo (SfM-MVS) post-processing stage to generate a Digital Surface Model (DSM) and raster RGB orthophotomosaic (OM) (Fig. 2B, 2C). The nominal spatial resolution of the final mosaic was GSD 0.3 cm raised by low-altitude flight settings. In the period 2018–2022, a large amount of photogrammetric data from different surveys was used, ultimately aiding in wrapping up the monitoring campaigns (Prodanov et al. 2019a, 2019b, 2020a, 2020b, 2021a, 2021b, 2023a, 2023b; Kotsev et al. 2020; Bekova 2023; Bekova and Prodanov 2023; Prodanov 2023).

Visual census, composition and source identification

A primary monitoring strategy consisting of a two-season visual census of MLD larger than 2.5 centimetres was implemented in the second phase. A critical component of the monitoring procedure involved assuring the quality and accuracy of MLD identification through observers. During the five years of the campaigns, the objective was to achieve full coverage of visual census and classification at least once in each area of litter monitoring (Fig. 1). Following the observers in the opposite direction methodology (Fig. 2B), a minimum of three IO-BAS researchers conducted visual census and classifications. The classification of macrolitter was performed using the Marine Litter (ML) categories outlined by Galgani et al. (2013a), ensuring consistency in the classification and characterization of the identified litter. Level 1 - Materials from the MSFD: Artificial polymer materials, Rubber, Cloth/textile, Paper/Cardboard, Processed/worked wood, Metal, Glass/Ceramics, Unidentified.

To investigate for correlation between litter sources on beaches and dunes, we used a bottom-up strategy (found litter types were attributed to possible sources), more specifically, the attribution-by-litter type method, which was in line with the approach provided by Veiga et al. (2016), as it was used for the Bulgarian beaches by Chuturkova and Simeonova (2021). The possible sources are given below:

- Public litter - Items dropped or left by the public on the coast or inland and carried by winds and rivers;

- Fishing litter - Includes commercial and recreational items - e.g., fishing line, nets, rope, weights and buoys;
- Sewage-related debris - Items flushed down the toilet, such as cotton bud sticks, tampons and panty liners;
- Shipping litter - Items dropped or lost from ships;
- Fly-tipped litter - Illegal disposal of waste, including furnishings, pottery and ceramics;
- Medical litter - Includes anything medical such as inhalers, plasters, syringes;
- Non-sourced litter - Items too small or damaged to identify or not obviously attributable to a given source.

Abundance and density of litter

As stated above, the dunes along the Bulgarian Black Sea coast have not been studied in the pollution context. The lack of data and increased anthropogenic pressure on the dunes, particularly over the past three years (Prodanov et al. 2023a), prompted us to ponder two primary aspects: the cleanliness of coastal dunes and the role of vegetation in trapping litter. To understand the cleanliness status of the Bulgarian dunes and how important vegetation is in litter trapping, we investigated the abundance (items) and density (items/m²) based on the visual census. The values were given as mean ± SD. The visual description of MLD density in the assessment areas is presented in Table 1. The MLD densities in each sampling unit were calculated by Formula (1):

$$D_{ALM,Year,Season} = \left(\frac{\text{Total number of litter}}{\text{Assessment area}} \right), \quad \left[\frac{\text{number of items}}{m^2} \right] \quad (1)$$

Manual image screening procedure and Litter Maps

Using MS data, Density Litter Maps were generated for each assessment area for analysis of the distribution of MLD (Fig. 3B). To survey the distribution and density of the macrolitter, the orthophotomosaics were subdivided into 2 m × 2 m plots in which the density was quantified in items/m² (Fig. 3C), following the workflow of Gonçalves et al. (2020b). The DLM results allowed for the identification of MLD hotspots (or fly-tipped areas) and a future correlation between the density of litter on beaches and dunes (Fig. 3C).

In line with the methodology employed by Gonçalves et al. (2020b), our approach involved the systematic division of each image into a 2-meter square

Table 1. MLD density and visual description adopted for the dune survey.

Density	Visual description (Alkalay et al. 2007)	Adopted visual description for dune surveys (Present study)
0–0.1 items/m ²	no litter is seen	no litter is seen
0.1–0.25 items/m ²	no litter is seen over a large area	no litter is seen over a large dune area
0.25–0.5 items/m ²	a few pieces of litter can be detected	a few pieces of litter can be detected
0.5–1 items/m ²	a lot of waste on the shore	a lot of waste in the dune area
More than 1 items/m ²	most of the shore is covered with plastic debris	most of the dune area is covered with debris

grid, establishing a structured and consistent framework for manual image screening (MS). The operator followed MS, encompassing several key steps:

- i. Visual Screening: The operator was tasked with visually examining the RGB orthophotomosaic uploaded in a GIS.
- ii. Identification: They were required to identify any litter items present.
- iii. Classification: When image quality permitted accurate recognition, the operators classified the litter items according to the categories outlined by Galgani et al. (2013a) and litter sources (Veiga et al. 2016). In this study, when assigning a specific category to an ML object during the image screening was impossible, the feature was characterised with the attribute “unidentified”.
- iv. Geospatial Marking: A critical component of the procedure involved placing a geospatial placemark at the approximate centre of each identified item within a Geographic Information System (GIS) environment (Fig. 2C).
- v. Shapefile Creation: Operators generated individual shapefiles for each designated area of litter monitoring.

This comprehensive procedure facilitated the precise mapping of MLD and its precise localization, enabling the creation of a Dune Litter Map (as illustrated in Fig. 3A). Furthermore, it allowed for the analysis of BL density, as depicted in Fig. 3B.

Results

Abundance and density of macrolitter on dunes

The launch of monitoring on dunes in 2018, aided by unmanned aerial systems, provided accurate data on the MLD abundance, density, composition and spatial distribution. The results provided a new perspective on the pollution mechanism existing between the shoreline and the backdunes. The presented results for abundance, litter density and Clean Coast Index are based on visual census data, as well as MS data for analysis of the spatial distribution of MLD.

It was determined that there was a long-term trend toward an increase in both key parameters: abundance (the number of litter items) and density of macrolitter (items/m²), shown in Fig. 4, Appendix 1 and 2. The abundance reached its maximum during 2021 with average values of $3\,710 \pm 2\,678$ items per assessment area. The average abundance experienced an increase of 39% between 2018 and 2021. It is pertinent to acknowledge that this period coincided with the occurrence of force majeure circumstances, notably the COVID-19 epidemic, which exerted significant pressure on the Bulgarian Black Sea coast due to the influx of local tourists.

Over 5% of the dune area has been under strong anthropogenic influence in recent years due to recreational activities and interventions, camping and inadequate management of dunes (Prodanov et al. 2023a). Along the Bulgarian coast, these factors have a negative effect on the density and concentration of hotspots. The map of average densities (Appendix 1) illustrates the significant imprint left by tourists in the most frequently visited beach-dune systems. In 15% of the assessment areas, the highest litter density ($Dav > 1$ items/m²) was recorded: (24)

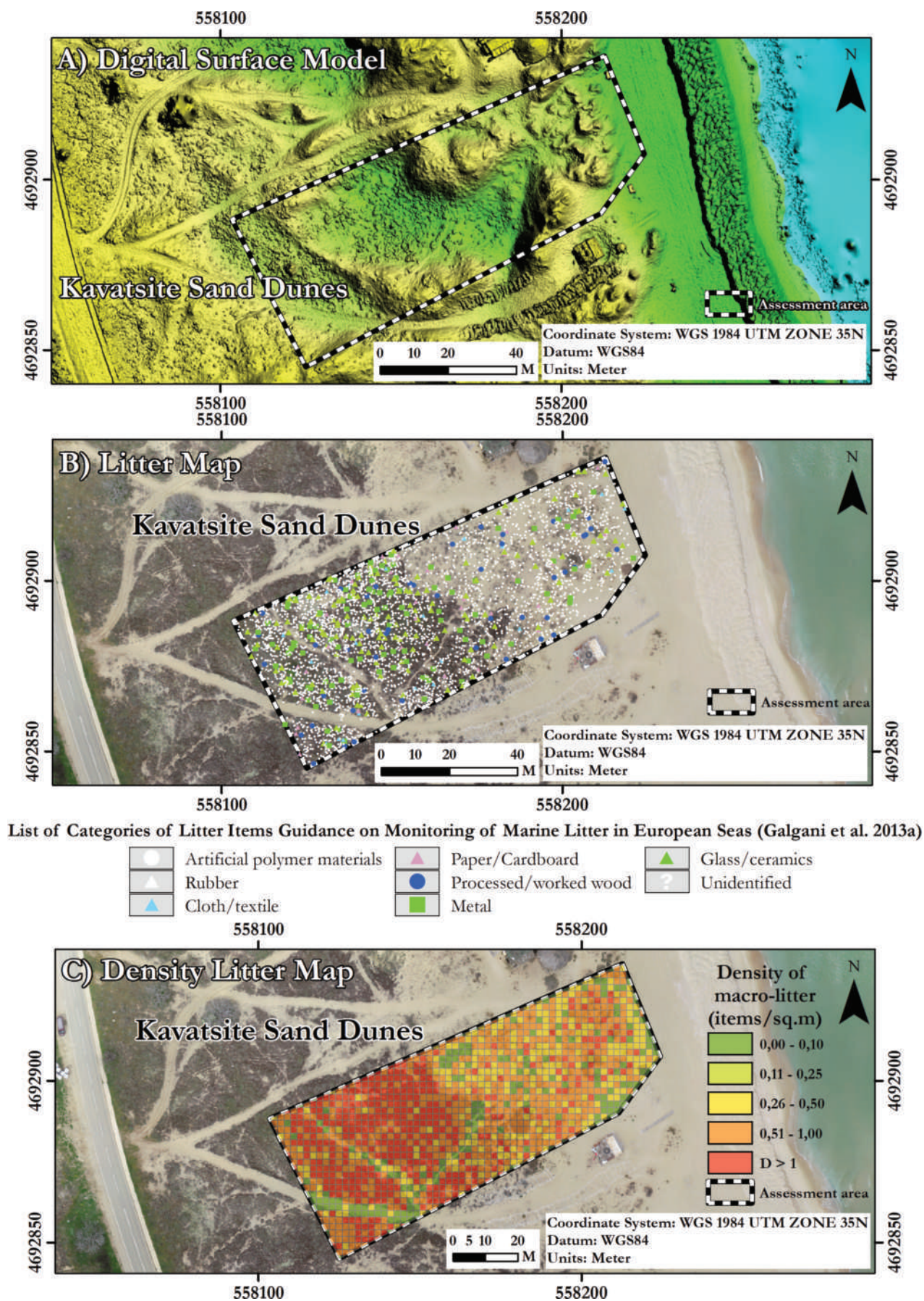


Figure 3. Example of manual screening and Density Litter Map on Kavatsite dunes in April 2021.

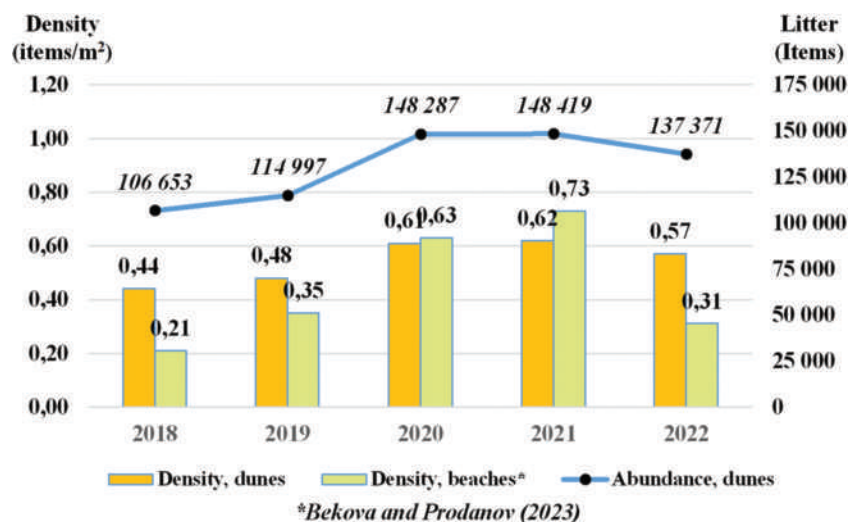


Figure 4. Mid-term variation (2018-2022) of total abundance (items) and average densities (items/m²) based on visual assessment of macrolitter on dunes along the Bulgarian Coast.

Kavatsite – 1.36; (17) Nessebar - South - 1.25; (6) Bolata – 1.23; (8) Asparuhovo (Varna) - 1.22; (16) Slanchev bryag - 1.25; and (20) Burgas Port Wall - 1.03.

Regarding density, most assessment areas were categorised as “a few pieces of litter can be detected” - 38% (0.26–0.5 items/m²) and “a lot of waste in the dune area” - 30% (0.5–1 items/m²). They were prevalent along the entire coastline, especially near campsites and resorts such as Krapets, Arkutino, etc. Still, there were relatively clean dunes with a low density of macrolitter, despite the strong anthropogenization, lack of maintenance and systematic cleaning. They were categorised as “no litter is seen over a large dune area” - 12% (0.1 to 0.25 items/m²) and “no litter is seen” - 5% (0 to 0.1 items/m²). A total of two assessment areas were categorised as “no litter” representing areas with low tourist impact: (39) Lipite and (27) Ropotamo (Appendix 1).

Seasonal variation of macrolitter on dunes

According to Simeonova et al. (2017), the amount of litter on the beach had the highest values in summer. Terzi and Seyhan (2017) and Aytan et al. (2020) reported an increase in pollution during the spring season in the southwestern part of the Black Sea. Panayotova et al. (2020) found that Asparuhovo Beach was the most polluted in spring. Our research indicated that the highest density (Dav,18–22,Autumn: 0.62 items/m²) occurred in autumn, after the end of the tourist season. In winter, strong north, northeast and east winds blew some of the litter off the dunes and spring monitoring campaigns registered lower densities (Dav,18–22, Spring: 0.46 items/m²). This study found a significant seasonal variation in MLD density of approximately 25%, with the greatest litter amount discovered in autumn, following the peak of the tourist season.

Composition of macrolitter on dunes

The identified MLD was classified according to the Master List of Categories of Litter Items - Level 1 by MSFD Technical Subgroup on Marine Litter (Galgani et al. 2013a): Artificial polymer materials, Rubber, Cloth/textile, Paper/Cardboard,

Processed/worked wood, Metal, Glass/ceramics, Unidentified. It was not surprising that the high dominance of plastic litter (artificial polymer materials) on the beach (Simeonova et al. 2017, 2020; Simeonova and Chuturkova 2019; Toneva et al. 2019; Panayotova et al. 2020; Chuturkova and Simeonova 2021; Kalinov et al. 2021) was also found on the dunes. Over the past five years, low variations in the amount of plastic polymer litter were observed, and in terms of percentage, it was the most prevalent waste along the Bulgarian Black Sea coast (Figs 5A, 6; Appendix 3). Special consideration should be given to the category "Paper/Cardboard" - 6.17%. That was the second most prevalent type of litter on our dunes. Due to inadequate waste-disposal facilities, a significant amount of that litter category was wind-transported from the active beach to the dunes. The unique conditions provided by the plants on the dunes aided in the trapping of paper waste. Rubber, Cloth/textile, Processed/worked wood, Metal, and Glass/ceramics were scarce on the dunes and did not exceed the 2% threshold (Fig. 5A; Appendix 3).

Sources of macrolitter on dunes

The results obtained during the monitoring campaigns are an initial assessment of the sources, composition and density of macrolitter on the Bulgarian Black Sea dunes. Understanding how increasing human pressure affects sensitive dune habitats is of the utmost importance when studying coastal dunes in the context of litter pollution. Sources of MLD were separated into two main classes: land-based sources (LS) and sea-based sources (SS). To a large extent, SS were dominated by waste discarded by fishing and shipping. Small-mass macrolitter, such as fishing nets and ropes, was successfully transported to the dunes. Our study found that the high frontal dunes covered with vegetation played an important trapping role in the retention of SS litter. SS did not exceed 5% of the total litter during any of the monitoring campaigns, with an average prevalence of 4.79%. At the same time, recreational areas, resorts and campsites were the main LS (95.21% of the total amount of macrolitter).

Even though the produced data were a pilot for the dune systems, it was essential to investigate the connection between the litter on the dunes and the litter on the beaches adjacent to them (if possible). Therefore, we went beyond the previous studies of beach litter (Panayotova et al. 2020; Chuturkova and Simeonova 2021) and examined the differences in the obtained results in an eco-geomorphological aspect. According to Veiga et al. (2016), the summary data for the period 2018–2022 is shown in Fig. 5B. The Public litter comprised the majority of MLD, accounting for 44.3% of the total number of items registered during that time. In contrast to the beach, where Fly tipped litter was 5.2% of the total amount (Chuturkova and Simeonova 2021), on dunes, that source generated 33.6% of litter items. The contribution of the other sources (Medical - 13.6%, Fishing - 2.8%, Shipping - 1.1, and Sewage Related Debris - 0.4%) was considered insignificant. Therefore, the estimated Non-sourced anthropogenic litter was 4.2%.

Spatial distribution of MLD

In contrast to active beaches, which are dominated by wave processes and tourism, dunes are dominated by the trapping function of dune vegetation. But are plants the most essential for waste retention?

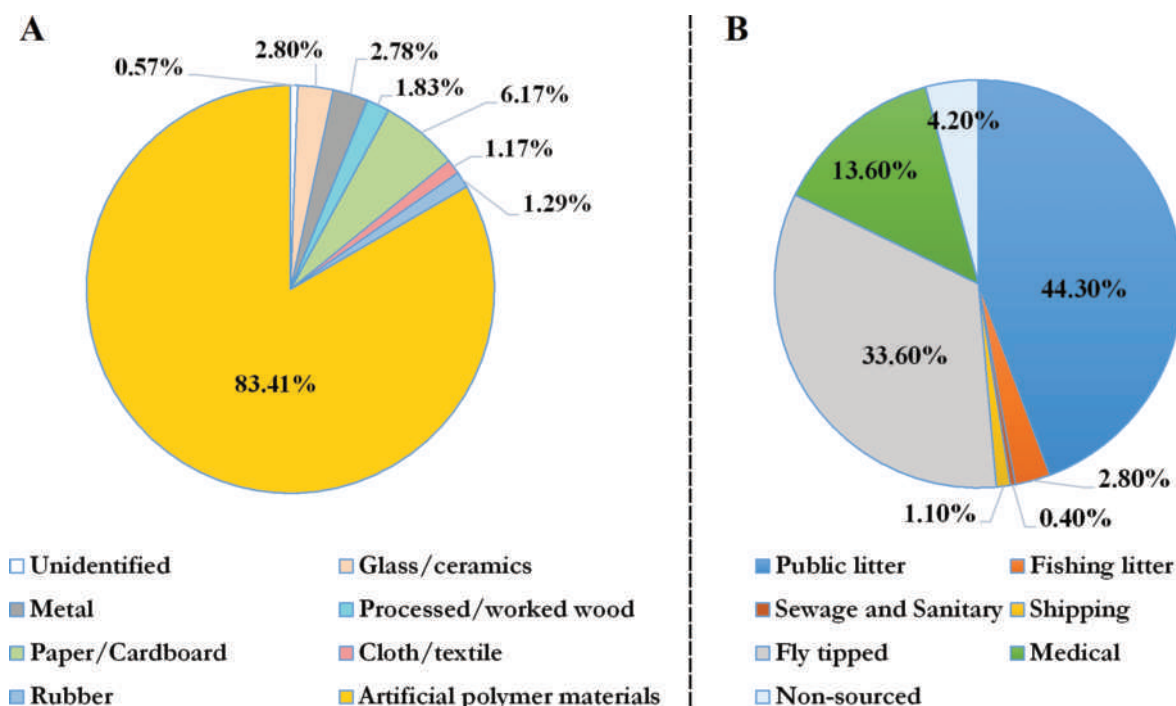


Figure 5. **A** average percentage distribution (%) of MLD materials according to Master List of Categories of Litter Items (Galgani et al. 2013a) for 2018–2022 **B** sources of MLD aggregated in 2018–2022.

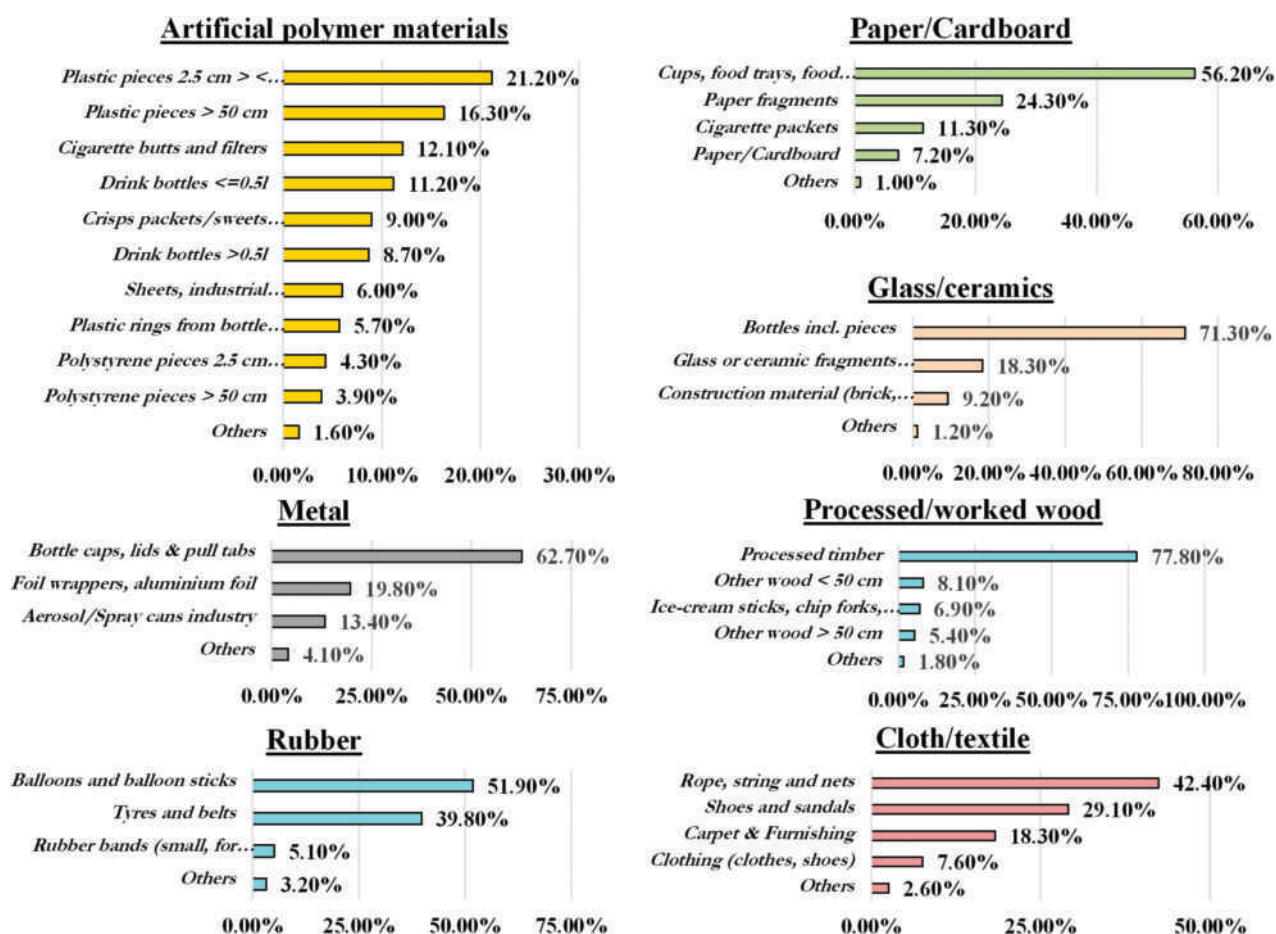


Figure 6. Average percentage distribution (%) of MLD subcategories according to Master List of Categories of Litter Items (Galgani et al. 2013a) for 2018–2022.

Geomorphologically, the main types of dunes that comprise the accumulative landscape are embryonic, foredunes (frontal) and secondary (backdunes), which are not currently subject to wave action. From an ecological point of view, dunes are habitats with unique vegetation (Gusseff and Tzonev 2015; Tzonev 2015a, 2015b, 2015c, 2015d; Valcheva et al. 2019, 2020, 2021). The embryonic dunes represent the initial barrier against marine debris. They “armour” the frontal dunes and retain only 16% of the total litter items (Fig. 7). The average

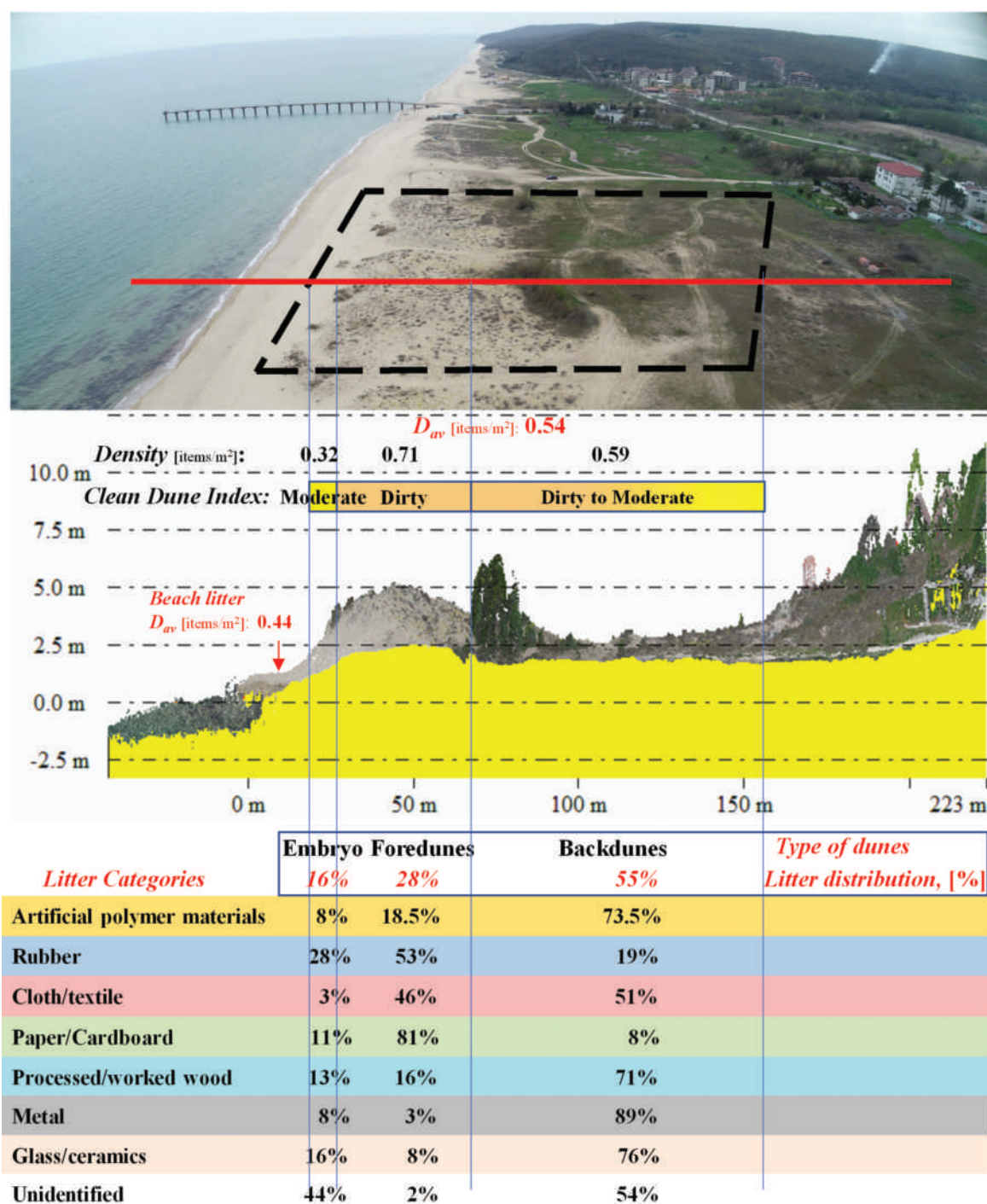


Figure 7. A general profile with CDI categorisation, average densities, composition and spatial distribution of litter on dunes along the Bulgarian Black Sea Coast for the monitoring period 2018–2022 (example: Shkorpilovtsi beach-dune system).

density was estimated to be 0.23 items/m² with Rubber, Paper/Cardboard, Processed/worked wood and Glass/ceramics dominating the litter composition.

The highest litter density was observed on the foredunes, with an average value of 0.80 items/m² (28% of total items). The backdunes contained the highest amount of litter items (55%), with an average density of 0.58 items/m². Land-based sources were the main cause of that great abundance of litter. A significant portion of litter was found in the dune vegetation, increasing the litter density in the backdunes by 20–25% compared to the beaches and foredunes (see example DLM in Fig. 3B). It should be noted that there were small areas where fly-tipped waste was observed in 38 out of 40 assessment areas, negatively impacting the beach (Fig. 2A, B).

Discussion

Effectiveness of drones in litter mapping on dunes

In our examination, drones are practical and convenient for operation. However, there is still uncertainty and subjectivity when it comes to classifying small-size litter, especially in stabilized vegetated dunes. The discussion highlights the advantages of using drones for pollution research. One more reason for the future application of drones for litter mapping on the dunes is their protective nature, and manual collecting should be avoided. An analysis of collected data reveals that the MS procedure was highly effective, identifying 92.1% of the BL items initially identified by a visual survey (Fig. 8). Notably, the MS procedure was supported with low-altitude video imaging at 5 m above each beach surface using an oblique camera, which an operator used to classify items more accurately. The identification of BL from the video contributed an additional 2%, as the final score increased to 94.1%. Furthermore, the “hidden” macro litter in the dune plants compelled us to reduce the flying height to 20 m (in some areas to 10 m), resulting in very high-resolution orthophotomosaics between

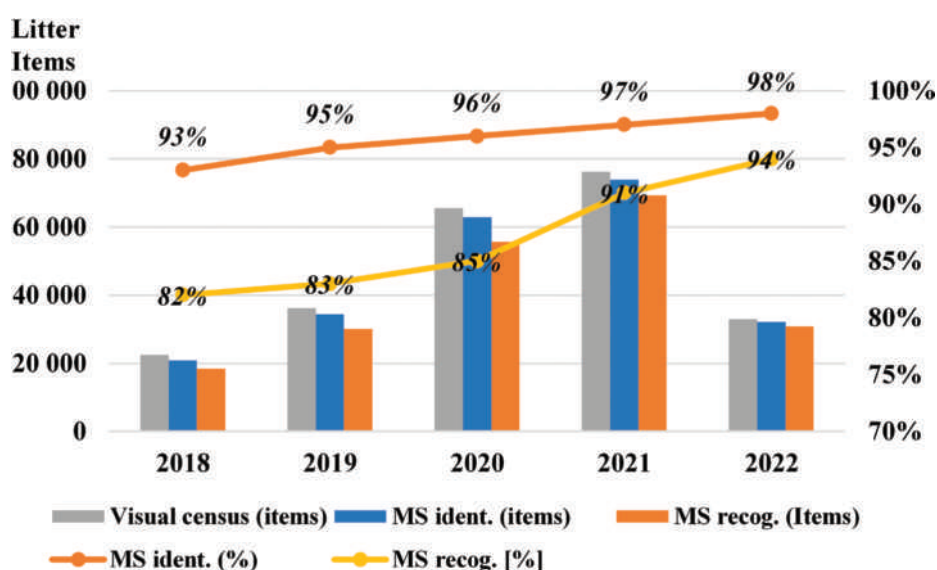


Figure 8. Comparison between the results from visual census and MS procedure of RGB orthophotomosaics along the Bulgarian Black Sea Coast.

0.3–0.5 cm/pix GSD. Of course, very low-altitude missions necessitated longer flight times, but, on the other hand, that expedited the macrolitter image screening process from orthophotomosaics. This recognition was successful on 84% of the items in our study. However, it is important to note that the operator was familiar with the categories of objects commonly found along Bulgarian coastlines and dunes (Bekova 2023; Prodanov and Bekova 2023) and actively participated in the field manual collecting and visual census.

From another point of view, the development of orthophoto and DSM also enables an objective definition of the various dune types (embryonic, foredunes and backdunes) and features (dune blowouts, trails, or dune pathways). Future research could combine litter mapping/monitoring with coastal geomorphological studies (Bastos et al. 2018; Duo et al. 2018; Gonçalves et al. 2018, 2020a, 2020b; Laporte-Fauret et al. 2019; Pagán et al. 2019; Andriolo et al. 2020a, 2020b; Bekova and Prodanov 2023; Corbau et al. 2023; Prodanov et al. 2023a, etc.) and coastal dune vegetation mapping (Meng et al. 2017; Suo et al. 2018) using drone data.

Still, there is no sustainable solution for automatically detecting and classifying litter on dunes. In contrast, for beaches, uniform standards of operation are being discussed to enhance the reliability of research (Gonçalves et al. 2022, etc.). Despite the successful application for litter mapping (Andriolo et al. 2020a, 2020b, 2021a; Gonçalves et al. 2020a, 2020b, etc.), we believe that the use of an automated object-based image segmentation technique and the service of a machine-learning classifier is not yet sufficiently improved to identify and recognize litter on/in high dune vegetation (white/grey/wooded dunes), which comprises more than 70% of the assessment areas along Bulgarian coast (Figs 2, 7).

Our experience shows that the opportunities of the UAS-based methodology presented in the study outweigh the disadvantages. The main line of controversy is between “time-consuming manual collecting with intrusive impact on dunes” versus “fast UAS mapping with minimal dune impact”. According to the results of our research, traditional field measurements could, under ideal conditions, survey two study sites per day, while drone surveys mapped four assessment areas per day, including beaches that were close to one another (Bekova and Prodanov 2023). Due to the sensitive coastal dune habitats, we need to continue to conduct our research using remotely non-destructive drone-based technology with minimal anthropogenic impact, following a trend away from minimizing manual sampling, which inevitably has a negative impact on dune habitats.

Development of a Clean Dune Index (CDI) for evaluation of the Bulgarian Black Sea coastal dunes

Data pertaining to the density, composition and sources of litter on the dunes have been presented as a means to assess the level of dune cleanliness. As Alkalay et al. (2007) proposed, a Clean Coast Index (CCI) was calculated based on the density of anthropogenic litter covering the beaches. But should CCI threshold values actually apply to dunes?

The configuration of the coastal system is influenced by various factors such as morphology, anthropogenic activities, climatic conditions and vegetation type. However, it is important to note that the impact of these factors

on beach and dune forms is not uniform. Thus, it can be concluded that the CCI proposed by Alkalay et al. (2007) may not be suitable due to its mapping of different eco-geomorphological components. The present study established significant differences in density values, exceeding 50%, between dunes and beaches in some locations. This finding is consistent with the results reported by Simeonova et al. (2020), Kalinov et al. (2021), Bekova and Prodanov (2023) in their respective studies. The beach-dune system located at Veleka Mouth is considered to be one of the emblematic cases. According to Simeonova et al. (2020), beach litter density was estimated to be 0.09 items/m² in 2019. Bekova and Prodanov (2023) conducted UAS orthophoto screening and identified a density of 0.55 items/m². At the same time, our study found a density value of 0.66 items/m² of macrolitter on the dunes.

A comparative analysis was performed using data from standardised UAS mapping of beach and dune assessment areas (Table 2) to determine the extent to which dunes are more polluted than beaches and whether that was valid for all beach-dune systems. The observed dissimilarities in density between BL and DL suggested that the threshold values of the widely accepted CCI index (Alkalay et al. 2007) were not fully applicable for evaluating the spatial distribution of MLD. In order to conduct a comprehensive evaluation of cleanliness, a Clean Dune Index (CDI) was developed using the CCI (Alkalay et al. 2007) as a basis. However, the threshold values were increased to account for the significant trapping role of dune vegetation that spans up to 200 m inland and reaches up to 0.5 m in height. The primary objective of the CDI, as introduced for the first time in this study, is to provide data consumers with a clear indication that the outcomes exclusively pertain to dune formations and do not encompass any statistical information concerning litter disposal on the beach. Formula (2) was used to determine the CDI of each area of litter monitoring during the monitoring campaigns.

$$CDI_{ALM\ Year\ Season} = \left(\frac{\text{Total number of litter items}}{\text{Area of assessment}} \right) \times K \quad (2)$$

where K is a coefficient and equals 20, the assessment area (m²) between the dune toe (foot line of the seaward dune slope) and the dune hee of the back-dunes (or minimum 50 m length inland) was estimated, as shown in Fig. 3A. The disparity between the suggested CDI and CCI (Alkalay et al. 2007) was solely attributed to the tolerance in threshold values and interpretation of the definition.

Table 2. Comparison of average litter density on dunes and beaches (Bekova and Prodanov 2023) in 2018–2022 (see the full table in Appendix 4).

Year	D _{beaches} ± SD	D _{dunes} ± SD
2018	0.21 ± 0.14	0.44 ± 0.28
2019	0.35 ± 0.23	0.48 ± 0.30
2020	0.63 ± 0.39	0.61 ± 0.40
2021	0.73 ± 0.43	0.62 ± 0.40
2022	0.31 ± 0.22	0.57 ± 0.40
Average density	0.44 ± 0.28	0.54 ± 0.35
Total Average Percentage difference, [%]	21.12% ~ 20%	

The thresholds of the CDI were increased by the total average percentage difference between the density of macrolitter found on beaches and dunes, which was established to be 20% for the Bulgarian coastal dunes (Appendix 4). The variability of the 20% difference may be subject to modification in accordance with the specific eco-geomorphological conditions of various countries, dune systems/dune habitats. It is recommended that litter surveys be conducted on the beach and back dunes whenever possible. It will provide insight into the general pattern of macrolitter distribution in beach-dune systems.

Are the dunes clean or dirty along the Bulgarian Black Sea coast?

Based on an in-depth evaluation of data obtained from the mid-term monitoring, the Bulgarian Black Sea dune systems were categorised as “moderate” with $CDI_{av,18-22}$: 10.89 (Fig. 9; Appendix 1). Throughout the monitoring campaigns spanning from 2018 to 2022, an equal distribution was observed among the primary categories of CDI, namely, very clean to clean (20%), moderate (45%) and dirty to extremely dirty (35%). Nevertheless, trends could be discerned among the sampling unit in relation to their proximity to leisure areas, such as Kavat-site and Gradina campsites, etc.

Moderately clean dunes at Dobrudzha Coast

The elongated dune systems were classified as “moderate” Dobrudzha dunes (Fig. 9; Appendix 1). They are located in the following assessment areas: (1) Durankulak - North, (2) Durankulak - South, (3) Krapets - North, (4) Shabla - North and (5) Shabla - South. The beach-dune systems are oriented in a meridional direction, extending from Durankulak to Shabla. The moderate pollution observed on the dunes at the area assessments (3) Krapets-North could be attributed to the unauthorised camping activities taking place in the backdunes. The beach-dune systems found in the northernmost regions were typically less affected by anthropogenic activities owing to the relatively lower influx of tourists compared to the southern coast. The units (6) Bolata stands in contrast to the aforemen-

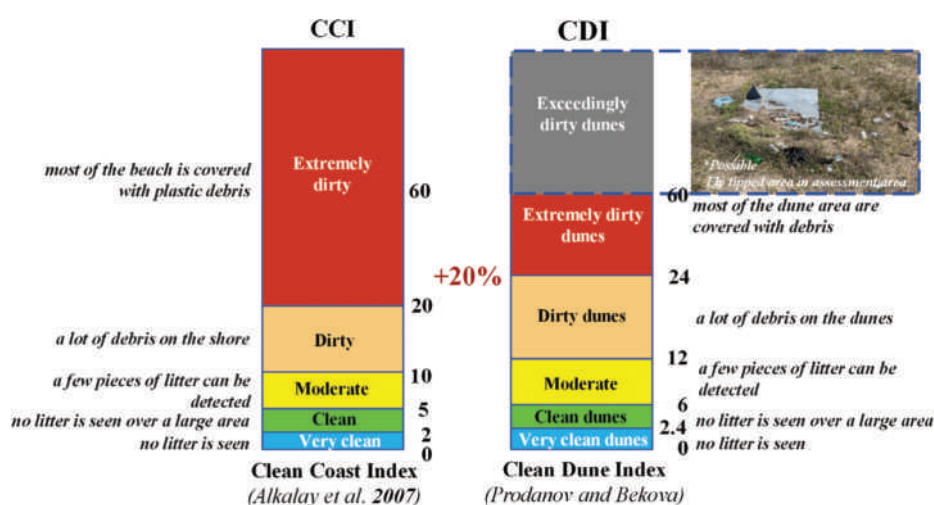


Figure 9. Clean Dune Index (CDI) for evaluation of the Bulgarian Black Sea coastal dunes with an additional class of Exceedingly dirty dunes.

tioned statements. Bolata dunes are well-shaped compact sand forms covered by dense vegetation. Recently, vehicle access has been allowed in the immediate vicinity. Consequently, there is an increase in tourist pressure. Bolata Cove is characterised by its scenic beauty and limited spatial extent. The dynamic of debris within the cove is constrained by the presence of high cliffs behind the dunes, designated as “extremely dirty” status (CDIav,Bolata,18–22: 24.69).

Dunes along the Central Coast

This group comprises the coastal dunes stretching from Cape Galata to Cape Emine (Fig. 9; Appendix 1). The preserved coastal dunes of the central coast section encompass the assessment areas (9) Pasha Dere, (10) Kamchiya (Mouth), (11) Kamchiya - South (Novo Oryahovo Beach), (12) Shkorpilovtsi, (13) Shkorpilovtsi - South, (14) Kara Dere - North (Byala), (15) Kara Dere - South (Byala). The study identified two distinct areas, namely (14) Kara Dere - North (Byala) and (15) Kara Dere - South (Byala), based on their geographical location. Due to the comparable CDI values, the scope of the research was broadened to encompass the clean dunes of Pasha Dere and Kara Dere, which had been protected from pollution owing to the limited number of tourists. The CDI values observed in these assessment areas varied between 2.77 and 7.42.

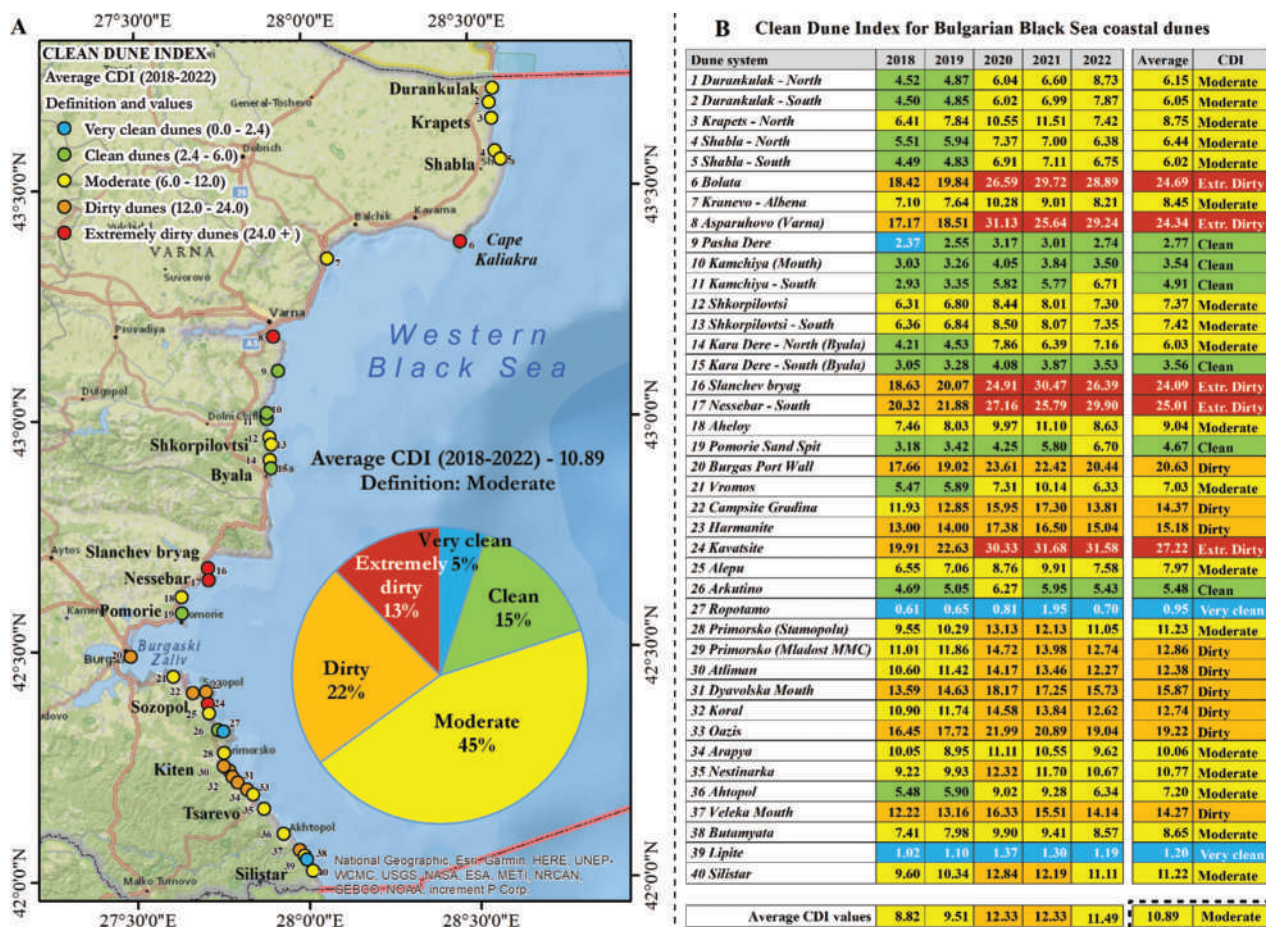
Extremely dirty dunes at Slanchev bryag and Nessebar Coast

A significant human footprint was detected on the assessment units (16) Slanchev Bryag and (17) Nessebar–South, located south of Cape Emine (Fig. 9; Appendix 1). The anthropogenic impact was reflected in “extremely dirty” dunes at the abovementioned assessment areas (CDIav,Slanchev bryag,18–22: 24.09 and CDIav,Nessebar,18–22: 25.01). The plastic litter most commonly found in the environment comprises cigarette butts, plastic bottles and medical masks. The insufficiency of waste disposal facilities for tourists is a pressing issue. In addition, the coastal region is subject to severe wind conditions that facilitate the transportation of substantial quantities of litter from the beach to the inland backdunes.

Dirty dunes along Burgas-Medni Rid Coast

The present group provides a concise overview of the dune systems located within the assessment areas: (20) Burgas Port Wall, (21) Vromos, (22) Campsite Gradina, (23) Harmanite, (24) Kavatsite, (25) Alepu, (26) Arkutino and (27) Ropotamo (Fig. 10). The distribution of MLD did not exhibit a discernible trend, as evidenced by the CDI values encompassing from 0.95 to 27.22. Notably, the Bulgarian coastal dunes showed high and low cleanliness levels.

“Extremely dirty” dunes at Kavatsite. The locality is emblematic, with the wide beach strip visited by thousands of tourists in summer. The well-developed, irregularly dune ridges have “sheltered” the vast amount of litter from the beach. The DLM (Fig. 3C) demonstrated the role of plants with a considerable supply of litter from the beach. The index reached alarming values, CDIav,Kavatsite,18–22: 27.22. It is also an important fact that in the dune systems (24) Kavatsite and (16) Slanchev Bryag, the highest values of plastic waste (92%) for the Bulgarian coast were recorded (Fig. 9).



Dirty dunes at Strandzha Coast

The southernmost coast of Bulgaria is characterised by small settlements and villages surrounded by areas intended for recreational activities/camping. In summer, many tourists visit the area extending from Primorsko to Silistar, significantly reflecting the local ecosystem. Most dune systems were classified as "dirty" or "moderate" according to CDI values (Fig. 9; Appendix 1). The "dirty" dunes included assessment areas (29) Primorsko, (30) Atliman, (31) Dyavolska Mouth, (32) Koral, (33) Oazis, (34) Arapya, (35) Nestinarka, (36) Ahtopol and (37) Veleka Mouth. As "Moderate" polluted dunes were categorised: (28) Primorsko, (34) Arapya, (35) Nestinarka, (36) Ahtopol, (38) Butamyata and (40) Silistar. Their CDI values ranged between 7 and 19. Many assessment areas experienced significant human-induced pressure on dune systems, which were often close to commercial establishments such as beach bars and pubs. Plastic litter included items such as bottles, cigarette butts, and filters.

Ropotamo and Lipite - the cleanest dunes along the Bulgarian Coast

The study sites were deliberately chosen to include dune systems that were hard to reach, isolated from recreational activities, with limited tourist presence and a minimal anthropogenic impact on the beach. Based on the established criteria,

two dune systems, (27) Ropotamo and (39) Lipite, were chosen. As expected, after a 5-year observation period, the assessment areas were categorised as “very clean”. In fact, these assessment areas exhibited the highest level of cleanliness among the study dune systems along the Bulgarian coast. The study determined that both assessment areas showed low abundance and density of litter, resulting in the lowest values of CDlav, Ropotamo, 18–22: 0.95 and CDlav, Lipite, 18–22: 1.20. Plastic litter, comprising items such as cigarette butts, bottles and fishing equipment, was prevalent on their territory. Despite the discovery of camping remnants during the field surveys, these dune systems remained relatively unaffected by human intervention. Due to their “pristine” eco-geomorphological settings, their exceptional cleanliness has earned them recognition as a reference for “very clean” coastal dunes on the Bulgarian Black Sea coast (Fig. 9; Appendix 1).

COVID-19 pandemic impact on dune cleanliness

The impact of the COVID-19 pandemic on plastic pollution in coastal and marine environments has been a subject of study in various parts of the world. For example, in neighbouring Greece, Kouvara et al. (2022) examined the impact of the ended COVID-19 pandemic on plastic pollution in the coastal/marine environment. The presence of personal protective equipment (PPE) such as face masks, gloves, and wet wipes significantly increased during the pandemic, contributing to plastic pollution. Face masks constituted the majority of PPE items found in the marine environment. COVID-19-related items accounted for a small percentage of the total litter, while wet wipes showed higher densities compared to the pre-pandemic period. Similar results of the negative impact of the COVID-19 pandemic on coastal environments have been documented in a wide range of studies worldwide (e.g., Okuku et al. 2021; Ormaza-Gonzalez et al. 2021; Hayati et al. 2022; Segal et al. 2022), especially the role of PPE associated with the epidemic (Akhbarizadeh et al. 2021; Benson et al. 2021; Chowdhury et al. 2021; De-la-Torre and Aragaw 2021; De-la-Torre et al. 2021; Haddad et al. 2021; Sajorne et al. 2022). From another point of view, Souza Filho et al. (2023) reported a decreasing abundance of beach litter. During the COVID-19 pandemic, beach closures and reduced public traffic led to a significant reduction of up to 83% in litter on the surveyed beaches, mostly related to recreational activities. But what was the situation during the COVID-19 epidemic on the Bulgarian beaches?

First, the 5-year timeline for monitoring dune litter remained unaffected by the COVID-19 pandemic. Bulgaria introduced a short lockdown in early March 2020, which coincided with the winter season and the beginning of spring when vacationers and campers were not expected due to low temperatures.

Second, in an effort to regulate international travel, Europe imposed restrictions during the summer seasons of 2020 and 2021. Consequently, the dunes in Bulgaria indirectly experienced the impact of the pandemic. The imposition of travel restrictions resulted in an immediate increase in domestic tourist activity on Bulgarian beaches. As a result, there was a notable 39% increase in litter abundance and a corresponding 41% rise in litter density (Figs 5, 10B). The final map of the Clean Dune Index (CDI) clearly outlines a significant jump in the amount of macrolitter, particularly single-use plastic packaging, plastic bottles, and discarded face masks. The index values revealed an increase in 38 out of the 40 areas of litter monitoring (Fig. 9B). In addition to the findings

mentioned earlier, it is worth noting that the period 2020–2022 presented a new challenge for dune ecosystems: the availability of face masks. With the advent of the COVID-19 pandemic, face masks became an essential protective measure, but unfortunately, their improper disposal resulted in an increased accumulation of litter in the dune vegetation (Fig. 11). This observation shed light on the weaknesses in waste management infrastructures and the need for improved systems to deal with the disposal of these pandemic-related items.

Conclusions

This research article presents various aspects, trends and results of mid-term 5-year monitoring of macrolitter on the Bulgarian Black Sea dunes. The 2018–2022 monitoring assessed macrolitter (size > 2.5 cm) abundance, density, composition, and sources on dunes within 40 assessment areas. According to the Master List of Categories of Litter Items in the Guidance on Monitoring of Marine Litter in European Seas (Galgani et al. 2013a), “Artificial polymer materials” accounted for 83.4% of MLD during the five-year monitoring, followed by “Paper/Cardboard” (6.2%), “Glass/ceramics” (2.8%), “Metal” (2.8%), “Processed/worked wood” (1.83%), “Rubber” (1.29%), and “Cloth/Textile” (1.17%). The COVID-19 pandemic indirectly affected Bulgarian dunes’ cleanliness by increasing domestic tourist pressure due to foreign travel restrictions. MLD abundance increased by 39%, peaking in 2021. The average density was assessed as $Dav,18-22: 0.54 \pm 0.35$ items/m² - “a lot of waste in the dune area” - with a maximum of $Dav,21: 0.62$ in summer 2021.

The spatial distribution of macrolitter on dunes is a complicated combination of anthropogenic impact and wind processes affecting the various eco-geomorphological elements of the beach-dune system. Only 16% of items were retained by embryonic dunes ($Dav,Embryo,18-22: 0.23$ items/m²). Foredunes had the most litter (28% of total items; $Dav,Foredunes,18-22: 0.80$ items/m²). In larger areas, backdunes had 55% litter ($Dav,Backdunes,18-22: 0.58$ items/m²). The Density Litter Maps showed that dune vegetation trapped 40% more macrolitter than sand forms without dune plants.

The Clean Dune Index was developed to assess the Bulgarian Black Sea coast dune systems’ cleanliness. Dune cleanliness along the Bulgarian coast was categorised as “moderate” by aggregated CDI data for assessment units ($CDIav,18-22: 10.89$). In 2018–2022, the dunes near the most popular resorts were classified as “extremely dirty”: (24) Kavatsite: 27.22, (17) Nessebar – South: 25.01, (6) Bolata: 24.69, (8) Asparuhovo (Varna): 24.33, and (16) Slanchev bryag: 24.09. The lowest CDI was for the Ropotamo and Lipite dune systems. Ropotamo 0.95 and Lipite 1.2 were “very clean”.

The manual screening procedure on the UAS orthophotos identified 93.1% of litter items initially identified by a visual survey. This recognition worked on 87% of registered litter items. UAS data is invaluable for litter location, but classifying it requires an orthophotomosaic with GSD between 0.3–0.5 cm/px. Due to the sensitive coastal dune habitats, we are obligated to continue to conduct our research using remotely non-destructive drone-based technology with minimal anthropogenic impact, following a trend away from minimizing manual sampling, which inevitably has a negative impact on dune habitats (Bekova 2023; Bekova and Prodanov 2023).



Figure 11. Example of macrolitter on dunes in assessment areas along the Bulgarian Coast.

The research on macrolitter pollution along Bulgarian dunes shows that poor management, tourist non-eco culture, and lack of clean-up activities after every summer season have resulted in a high number of dirty and extremely dirty dunes (35% of monitored dune systems).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

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No ethical statement was reported.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Appendix 1

Table A1. Monitoring data of abundance, densities, and Clean Dune Index in 2018-2022 at Bulgarian Black Sea coastal dunes.

Area of litter monitoring		2018			2019			2020			2021			2022			Average (2018-2022)	
Beach-dune system	Area m ²	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Density	CDI _{av}
1 Durankulak - North	7 320	1 655	0.23	4.52	1 782	0.24	4.87	2 212	0.30	6.04	2 416	0.33	6.60	3 197	0.44	8.73	0.31	8.73
2 Durankulak - South	7 536	1 695	0.22	4.50	1 826	0.24	4.85	2 267	0.30	6.02	2 635	0.35	6.99	2 967	0.39	7.87	0.30	7.87
3 Krapets - North	8 399	2 693	0.32	6.41	3 294	0.39	7.84	4 429	0.53	10.55	4 832	0.58	11.51	3 115	0.37	7.42	0.44	7.42
4 Shabla - North	6 827	1 882	0.28	5.51	2 027	0.30	5.94	2 516	0.37	7.37	2 390	0.35	7.00	2 178	0.32	6.38	0.32	6.38
5 Shabla - South	5 546	1 244	0.22	4.49	1 340	0.24	4.83	1 917	0.35	6.91	1 971	0.36	7.11	1 871	0.34	6.75	0.30	6.75
6 Bolata	1 175	1 082	0.92	18.42	1 166	0.99	19.84	1 562	1.33	26.59	1 746	1.49	29.72	1 697	1.44	28.89	1.23	28.89
7 Kranevo - Albena	6 114	2 170	0.35	7.10	2 337	0.38	7.64	3 142	0.51	10.28	2 755	0.45	9.01	2 511	0.41	8.21	0.42	8.21
8 Asparuhovo (Varna)	5 604	4 812	0.86	17.17	5 186	0.93	18.51	8 723	1.56	31.13	7 186	1.28	25.64	8 193	1.46	29.24	1.22	29.24
9 Pasha Dere	7 435	881	0.12	2.37	948	0.13	2.55	1 177	0.16	3.17	1 118	0.15	3.01	1 019	0.14	2.74	0.14	2.74
10 Kamchiya (Mouth)	6 708	1 015	0.15	3.03	1 093	0.16	3.26	1 357	0.20	4.05	1 289	0.19	3.84	1 175	0.18	3.50	0.18	3.50
11 Kamchiya - South (Novo Oryahovo Beach)	4 894	716	0.15	2.93	819	0.17	3.35	1 424	0.29	5.82	1 411	0.29	5.77	1 641	0.34	6.71	0.25	6.71
12 Shkorpilovtsi	5 591	1 764	0.32	6.31	1 900	0.34	6.80	2 358	0.42	8.44	2 240	0.40	8.01	2 041	0.37	7.30	0.37	7.30
13 Shkorpilovtsi - South	4 826	1 533	0.32	6.36	1 651	0.34	6.84	2 050	0.42	8.50	1 947	0.40	8.07	1 775	0.37	7.35	0.37	7.35
14 Kara Dere - North (Byala)	5 368	1 130	0.21	4.21	1 217	0.23	4.53	2 110	0.39	7.86	1 716	0.32	6.39	1 923	0.36	7.16	0.30	7.16
15 Kara Dere - South (Byala)	5 785	882	0.15	3.05	950	0.16	3.28	1 179	0.20	4.08	1 120	0.19	3.87	1 021	0.18	3.53	0.18	3.53
16 Slanchev bryag	7 753	7 223	0.93	18.63	7 779	1.00	20.07	9 656	1.25	24.91	11 813	1.52	30.47	10 231	1.32	26.39	1.20	26.39
17 Nessebar - South	7 987	8 113	1.02	20.32	8 738	1.09	21.88	10 846	1.36	27.16	10 301	1.29	25.79	11 941	1.50	29.90	1.25	29.90
18 Aheloy	5 612	2 092	0.37	7.46	2 253	0.40	8.03	2 797	0.50	9.97	3 115	0.56	11.10	2 422	0.43	8.63	0.45	8.63
19 Pomorie Sand Spit	5 114	813	0.16	3.18	875	0.17	3.42	1 086	0.21	4.25	1 482	0.29	5.80	1 713	0.33	6.70	0.23	6.70
20 Burgas Port Wall	5 087	4 492	0.88	17.66	4 838	0.95	19.02	6 005	1.18	23.61	5 704	1.12	22.42	5 199	1.02	20.44	1.03	20.44
21 Vromos	8 125	2 223	0.27	5.47	2 394	0.29	5.89	2 972	0.37	7.31	4 118	0.51	10.14	2 573	0.32	6.33	0.35	6.33
22 Campsite Gradina	9 451	5 639	0.60	11.93	6 073	0.64	12.85	7 538	0.80	15.95	8 176	0.87	17.30	6 526	0.69	13.81	0.72	13.81
23 Harmanite	5 138	3 339	0.65	13.00	3 596	0.70	14.00	4 464	0.87	17.38	4 240	0.83	16.50	3 865	0.75	15.04	0.76	15.04
24 Kavatsite	5 137	5 113	1.00	19.91	5 812	1.13	22.63	7 789	1.52	30.33	8 136	1.58	31.68	8 111	1.58	31.58	1.36	31.58

Area of litter monitoring		2018			2019			2020			2021			2022			Average (2018-2022)	
Beach-dune system	Area m ²	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Items _{av}	D _{av}	CDI _{av}	Density	CDI _{av}
25 Alepu	5 412	1 773	0.33	6.55	1 909	0.35	7.06	2 370	0.44	8.76	2 681	0.50	9.91	2 052	0.38	7.58	0.40	7.58
26 Arkutino	5 088	1 193	0.23	4.69	1 285	0.25	5.05	1 595	0.31	6.27	1 515	0.30	5.95	1 380	0.27	5.43	0.27	5.43
27 Ropotamo	5 144	156	0.03	0.61	168	0.03	0.65	209	0.04	0.81	502	0.10	1.95	181	0.04	0.70	0.05	0.70
28 Primorsko (Stamopolu)	13 415	6 407	0.48	9.55	6 900	0.51	10.29	8 809	0.66	13.13	8 135	0.61	12.13	7 415	0.55	11.05	0.56	11.05
29 Primorsko (Mladost MMC)	5 759	3 170	0.55	11.01	3 414	0.59	11.86	4 238	0.74	14.72	4 025	0.70	13.98	3 669	0.64	12.74	0.64	12.74
30 Atliman	5 009	2 655	0.53	10.60	2 859	0.57	11.42	3 549	0.71	14.17	3 371	0.67	13.46	3 072	0.61	12.27	0.62	12.27
31 Dyavolska Mouth	6 296	4 278	0.68	13.59	4 607	0.73	14.63	5 719	0.91	18.17	5 432	0.86	17.25	4 951	0.79	15.73	0.79	15.73
32 Koral	5 120	2 791	0.55	10.90	3 006	0.59	11.74	3 731	0.73	14.58	3 544	0.69	13.84	3 230	0.63	12.62	0.64	12.62
33 Oazis	5 239	4 309	0.82	16.45	4 641	0.89	17.72	5 761	1.10	21.99	5 472	1.04	20.89	4 987	0.95	19.04	0.96	19.04
34 Arapya	6 560	3 297	0.50	10.05	2 936	0.45	8.95	3 645	0.56	11.11	3 461	0.53	10.55	3 155	0.48	9.62	0.50	9.62
35 Nestinarka	4 438	2 045	0.46	9.22	2 203	0.50	9.93	2 734	0.62	12.32	2 597	0.59	11.70	2 367	0.53	10.67	0.54	10.67
36 Ahtopol	5 635	1 543	0.27	5.48	1 662	0.29	5.90	2 540	0.45	9.02	2 616	0.46	9.28	1 786	0.32	6.34	0.36	6.34
37 Veleka Mouth	7 064	4 314	0.61	12.22	4 647	0.66	13.16	5 768	0.82	16.33	5 478	0.78	15.51	4 993	0.71	14.14	0.71	14.14
38 Butamyata	5 089	1 885	0.37	7.41	2 030	0.40	7.98	2 520	0.50	9.90	2 393	0.47	9.41	2 182	0.43	8.57	0.43	8.57
39 Lipite	3 441	176	0.05	1.02	190	0.06	1.10	236	0.07	1.37	224	0.07	1.30	204	0.06	1.19	0.06	1.19
40 Silistar	5 118	2 457	0.48	9.60	2 646	0.52	10.34	3 285	0.64	12.84	3 120	0.61	12.19	2 844	0.56	11.11	0.56	11.11
Mean values:		2 666	0.44	8.82	2 875	0.48	9.51	3 707	0.62	12.33	3 710	0.62	12.33	3 434	0.57	11.49	0.54	11.49
Minimum:		156	0.03	0.61	168	0.03	0.65	209	0.04	0.81	224	0.07	1.30	181	0.04	0.70	0.05	0.70
Maximum:		8 113	1.02	20.32	8 738	1.13	22.63	10 846	1.56	31.13	11 813	1.58	31.68	11 941	1.58	31.58	1.36	31.58
Standard deviation:		1 913	0.28	5.52	2 068	0.30	5.99	2 657	0.40	7.93	2 679	0.40	7.91	2 641	0.40	8.03	0.35	8.03
Standard error:		48	0.01	0.14	52	0.01	0.15	66	0.01	0.20	67	0.01	0.20	66	0.01	0.20	0.01	0.20

Appendix 2

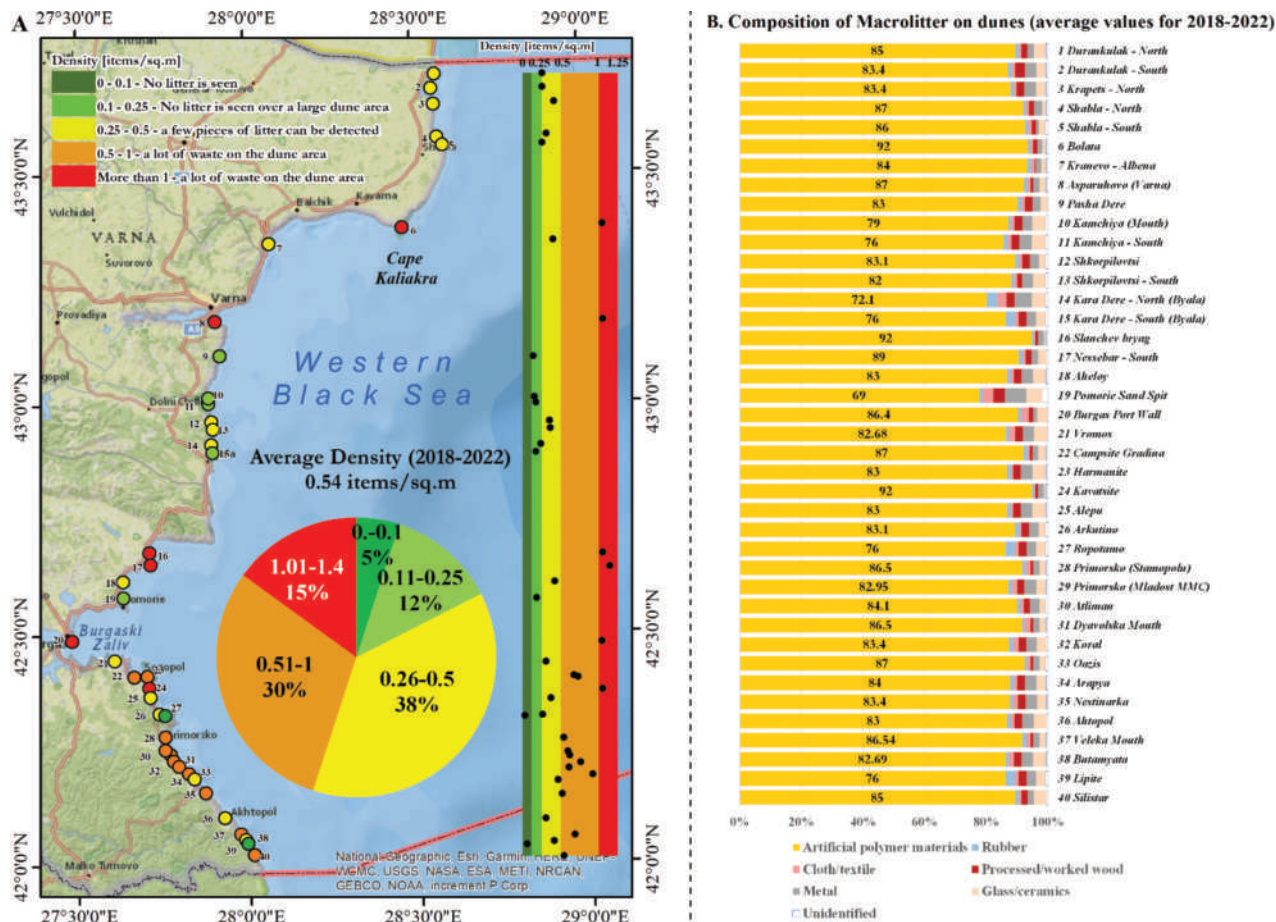


Figure A1. A Map of the average densities of MLD along the BBSC B the average values of litter composition on the ALMs in 2018–2022 (see Appendix 1).

Appendix 3

Table A2. Composition of litter on dunes along the Bulgarian Black Sea Coast in 2018–2022.

Area of litter monitoring	Artificial polymer materials	Rubber	Cloth/Textile	Paper/Cardboard	Processed/Worked wood	Metal	Glass/Ceramics	Unidentified
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
1 Durankulak - North	85.0	1.1	0.8	4.9	1.9	1.9	3.8	0.6
2 Durankulak - South	83.4	1.4	1.0	4.2	2.9	3.6	3.0	0.6
3 Krapets - North	83.4	1.1	1.0	5.1	2.3	3.6	3.0	0.6
4 Shabla - North	87.0	0.9	1.0	5.4	1.5	2.6	1.0	0.6
5 Shabla - South	86.0	1.1	1.0	7.1	1.2	1.0	2.0	0.6
6 Bolata	92.0	0.8	1.0	1.6	1.3	1.6	1.1	0.6
7 Kranevo - Albena	84.0	1.2	1.0	10.0	0.3	1.6	1.4	0.5
8 Asparuhovo (Varna)	87.0	1.2	1.0	5.7	0.8	1.8	2.0	0.6
9 Pasha Dere	83.0	1.2	1.0	8.0	2.3	2.3	2.0	0.2
10 Kamchiya (Mouth)	79.0	0.9	1.0	9.3	2.3	2.9	4.0	0.6
11 Kamchiya - South (Novo Oryahovo Beach)	76.0	1.2	1.0	11.3	2.3	3.6	4.0	0.6

Area of litter monitoring	Artificial polymer materials	Rubber	Cloth/ Textile	Paper/ Cardboard	Processed/ Worked wood	Metal	Glass/ Ceramics	Unidentified
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
12 Shkorpilovtsi	83.1	1.1	1.0	7.1	2.3	2.8	2.0	0.6
13 Shkorpilovtsi - South	82.0	0.9	1.0	6.9	1.4	3.3	3.9	0.6
14 Kara Dere - North (Byala)	72.1	3.1	2.8	10.0	2.3	5.1	4.0	0.6
15 Kara Dere - South (Byala)	76.0	2.7	1.0	12.0	2.3	2.6	2.8	0.6
16 Slanchev bryag	92.0	0.8	0.3	3.0	0.8	1.9	0.6	0.6
17 Nessebar - South	89.0	1.4	1.0	1.7	1.8	2.1	2.6	0.5
18 Aheloy	83.0	1.2	1.0	4.3	2.3	3.6	4.0	0.6
19 Pomorie Sand Spit	69.0	1.0	3.1	11.2	3.3	6.1	4.7	1.6
20 Burgas Port Wall	86.4	1.1	2.3	4.3	1.3	1.3	3.1	0.2
21 Vromos	82.7	1.1	1.8	4.3	2.3	3.6	4.0	0.2
22 Campsite Gradina	87.0	1.3	0.6	5.6	0.8	1.8	2.3	0.6
23 Harmanite	83.0	1.2	0.9	4.3	2.3	3.6	4.1	0.6
24 Kavatsite	92.0	0.8	0.3	3.0	0.8	1.9	0.6	0.6
25 Alepu	83.0	1.2	0.8	4.3	2.3	3.6	4.2	0.6
26 Arkutino	83.1	1.4	0.6	7.0	2.3	2.8	2.2	0.6
27 Ropotamo	76.0	2.7	1.1	11.9	2.3	2.6	2.8	0.6
28 Primorsko (Stamopolu)	86.5	1.2	1.5	5.6	0.8	1.8	2.0	0.6
29 Primorsko (Mladost MMC)	83.0	1.4	1.3	4.9	2.3	3.6	3.0	0.6
30 Atliman	84.1	1.1	1.0	6.6	1.8	2.8	2.0	0.6
31 Dyavolska Mouth	86.5	0.8	1.9	5.6	0.8	1.8	2.0	0.6
32 Koral	83.4	1.3	1.7	4.6	2.3	3.3	3.0	0.4
33 Oasis	87.0	1.0	0.9	5.9	0.8	1.8	2.0	0.6
34 Arapyra	84.0	1.2	1.0	4.3	2.3	3.6	3.0	0.6
35 Nestinarka	83.4	1.1	1.4	4.9	2.3	3.6	3.0	0.3
36 Ahtopol	83.0	1.2	1.2	4.3	2.3	3.6	4.0	0.4
37 Veleka Mouth	86.5	1.4	1.3	5.6	0.8	1.8	2.0	0.6
38 Butamyata	82.7	1.1	1.4	4.3	2.3	3.6	4.0	0.6
39 Lipite	76.0	2.7	1.0	12.0	2.3	2.6	2.8	0.6
40 Silistar	85.0	1.2	0.8	4.9	1.9	1.9	3.8	0.5
Average, [%]	83.4	1.3	1.2	6.2	1.8	2.8	2.8	0.6

Appendix 4






















Table A3. Comparison of average litter density on dunes and beaches (Bekova and Prodanov 2023) in 2018–2022.

Year	2018		2019		2020		2021		2022	
D-dunes; B-beaches	D	B	D	B	D	B	D	B	D	B
Areas of Litter Monitoring (Dune system)	Litter Density, [items/m ²]									
1 Durankulak - North	0.23	0.11	0.24	0.14	0.30	0.32	0.33	0.41	0.44	0.17
2 Durankulak - South	0.22	0.11	0.24	0.18	0.30	0.33	0.35	0.39	0.39	0.09
3 Krapets - North	0.32	0.12	0.39	0.18	0.53	0.35	0.58	0.41	0.37	0.11
4 Shabla - North	0.28	0.10	0.30	0.15	0.37	0.29	0.35	0.34	0.32	0.13
5 Shabla - South	0.22	0.12	0.24	0.19	0.35	0.36	0.36	0.44	0.34	0.10

Year	2018		2019		2020		2021		2022	
D-dunes; B-beaches	D	B	D	B	D	B	D	B	D	B
Areas of Litter Monitoring (Dune system)	Litter Density, [items/m²]									
6 Bolata	0.92	0.51	0.99	0.82	1.33	1.54	1.49	1.85	1.44	0.41
7 Kranevo - Albena	0.35	0.18	0.38	0.28	0.51	0.53	0.45	0.63	0.41	0.32
8 Asparuhovo (Varna)	0.86	0.49	0.93	0.78	1.56	1.10	1.28	1.22	1.46	0.26
9 Pasha Dere	0.12	0.06	0.13	0.09	0.16	0.17	0.15	0.21	0.14	0.05
10 Kamchiya (Mouth)	0.15	0.07	0.16	0.12	0.20	0.22	0.19	0.27	0.18	0.06
11 Kamchiya - South	0.15	0.10	0.17	0.16	0.29	0.31	0.29	0.37	0.34	0.08
12 Shkorpilovtsi	0.32	0.15	0.34	0.25	0.42	0.46	0.40	0.55	0.37	0.10
13 Shkorpilovtsi South	0.32	0.15	0.34	0.25	0.42	0.46	0.40	0.56	0.37	0.07
14 Kara Dere - North (Byala)	0.21	0.10	0.23	0.16	0.39	0.31	0.32	0.37	0.36	0.18
15 Kara Dere - South (Byala)	0.15	0.07	0.16	0.12	0.20	0.22	0.19	0.27	0.18	0.13
16 Slanchev bryag	0.93	0.48	1.00	0.77	1.25	1.45	1.52	1.55	1.32	0.82
17 Nessebar - South	1.02	0.49	1.09	0.79	1.36	1.48	1.29	1.44	1.50	0.86
18 Aheloy	0.37	0.16	0.40	0.25	0.50	0.47	0.56	0.56	0.43	0.27
19 Pomorie Sand Spit	0.16	0.10	0.17	0.16	0.21	0.29	0.29	0.35	0.33	0.17
20 Burgas Port Wall	0.88	0.43	0.95	0.69	1.18	1.08	1.12	1.31	1.02	0.76
21 Vromos	0.27	0.15	0.29	0.23	0.37	0.44	0.51	0.53	0.32	0.26
22 Campsite Gradina	0.60	0.30	0.64	0.48	0.80	0.90	0.87	1.08	0.69	0.53
23 Harmanite	0.65	0.32	0.70	0.51	0.87	0.95	0.83	1.14	0.75	0.56
24 Kavatsite	1.00	0.57	1.13	0.90	1.52	1.34	1.58	1.49	1.58	0.75
25 Alepu	0.33	0.17	0.35	0.27	0.44	0.50	0.50	0.60	0.38	0.25
26 Arkutino	0.23	0.09	0.25	0.14	0.31	0.27	0.30	0.32	0.27	0.16
27 Ropotamo	0.03	0.02	0.03	0.03	0.04	0.06	0.10	0.07	0.04	0.03
28 Primorsko (Stamopolu)	0.48	0.23	0.51	0.37	0.66	0.70	0.61	0.84	0.55	0.37
29 Primorsko (Mladost MMC)	0.55	0.27	0.59	0.43	0.74	0.80	0.70	0.96	0.64	0.36
30 Atliman	0.53	0.26	0.57	0.41	0.71	0.77	0.67	0.93	0.61	0.34
31 Dyavolska Mouth	0.68	0.29	0.73	0.46	0.91	0.87	0.86	1.04	0.79	0.51
32 Koral	0.55	0.27	0.59	0.42	0.73	0.80	0.69	0.96	0.63	0.35
33 Oasis	0.82	0.26	0.89	0.56	1.10	1.05	1.04	1.11	0.95	0.55
34 Arapya	0.50	0.21	0.45	0.34	0.56	0.63	0.53	0.75	0.48	0.27
35 Nestinarka	0.46	0.22	0.50	0.36	0.62	0.67	0.59	0.81	0.53	0.38
36 Ahtopol	0.27	0.15	0.29	0.24	0.45	0.45	0.46	0.54	0.32	0.26
37 Veleka Mouth	0.61	0.30	0.66	0.48	0.82	0.89	0.78	1.07	0.71	0.52
38 Butamyata	0.37	0.18	0.40	0.29	0.50	0.54	0.47	0.65	0.43	0.32
39 Lipite	0.05	0.02	0.06	0.04	0.07	0.07	0.07	0.09	0.06	0.04
40 Silistar	0.48	0.21	0.52	0.33	0.64	0.62	0.61	0.74	0.56	0.36
Average annual density, [items/m²]	0.44	0.21	0.48	0.35	0.61	0.63	0.64	0.73	0.53	0.31
Total Average density, Time period 2018-2022, [items/m²]	Dunes – 0.54					Beaches – 0.44				
Total Average Percentage difference, [%]	21.12% ~ 20%									

Research Article

Effects of anthropogenic and environmental stressors on the current status of red mullet (*Mullus barbatus* L., 1758) populations inhabiting the Bulgarian Black Sea waters

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Abstract

The red mullet (*Mullus barbatus* Linnaeus, 1758) is a keynote species for the Bulgarian Black Sea ecosystem and fisheries; nevertheless, existing knowledge on population status is very scarce. The present study was intended to assess the health status and adaptive potential of *M. barbatus* populations inhabiting the Bulgarian waters of the Black Sea. Our findings revealed that populations of *M. barbatus* are exposed to a variety of anthropogenic and environmental stressors. The species' status was assessed using representative genetic, morphological, biochemical and chemical biomarkers from specimens obtained in the research area's northern and southern regions. Based on mtDNA markers, genetic analysis revealed low haplotype and nucleotide diversity, typically observed in overexploited or “threatened” populations. Examining the morphology of the specimens revealed no discernible pattern of differentiation. Except for aluminium and chrome, metal and PAH concentrations in fish were below the regulatory thresholds. The specimens from the southern region ingested more microplastics than those from the northern region. The majority of specimens collected from the southern region also exhibited elevated levels of oxidative stress and decreased antioxidant defence, which can be interpreted as an early indication that they had reached the limits of their adaptive potential. Further research on the composite effects of the stressogenic environment on the Black Sea biota are critically needed, as well as the introduction of new indicators and thresholds at molecular and cellular levels for adequate monitoring of both the ecological state of the marine environment and its biota.

Key words: Black Sea, fish morphology, genetic diversity, metal accumulation, microplastics, oxidative stress, red mullet

Introduction

Progressively, marine ecosystems are exposed to multiple stressors of both natural and anthropogenic origin, which can have direct and indirect impacts with interdependent and complex effects on the marine environment and

biota. Human impacts on marine ecosystems include physical disturbance of the marine environment, biota and habitats; inputs of nutrients, litter and toxic substances; overexploitation of marine resources; introduction of waterborne pathogens and alien species; and increased use of coastal and seabed resources, which are overlaid on the effects of changing natural conditions (Bailly et al. 2015; Bernal et al. 2017).

As a result of environmental pressure, marine fish species are subjected to a wide variety of stressors that impair their optimal physiological functioning and adaptive capacity, which are heavily dependent on the ecological state and fluctuations of their immediate environment (Farag et al. 2009; Birnie-Gauvin et al. 2017). Multiple toxic contaminants can accumulate in the marine environment and biomagnify along the food chain (Streit 1998; Feng et al. 2020; Tenji et al. 2020; Butnariu 2022). The response of marine fish species to environmental stress can be polymorphic, depending on habitat, species sensitivity and vulnerability and other intrinsic variables, as well as the magnitude, duration and intensity of exposure to particular stressor/s. In addition, substantial evidence has recently accumulated suggesting that alterations at the molecular and cellular levels of biological organisation can have large effects at higher hierarchical levels by distressing the survival, growth and reproduction of organisms (Steinberg 2012; Petitjean et al. 2019; Fu et al. 2020).

The Black Sea has been documented as one of the seas heavily impacted by human activities, such as pollution and discharges from land-based sources on the territories of the central and eastern European countries along the river Danube (Zaitsev and Mamaev 2008). In the 1980s, the Black Sea ecosystem suffered substantial structural and functional changes as a result of multiple anthropogenic and natural influences (Ivanov and Beverton 1985; Daskalov 2002; Pauly and Watson 2005; Keskin et al. 2017). Eutrophication, overfishing and the introduction of alien species were referred to as the primary causes of the observed ecosystem disruption and severe loss of biodiversity (Prodanov et al. 1997; Zaitsev et al. 1997; Caddy 2008). Recently, the bioaccumulation of microplastics (MPs) has emerged as a new hazard, as it has been demonstrated to have harmful impacts on marine biota. In the Black Sea, MPs were registered in sediments (Săvucă et al. 2017; Cincinelli et al. 2021; Pojar et al. 2021a), seawater (Öztekin et al. 2017; Aytan et al. 2020a, 2020b; Berov and Klayn 2020; Pojar et al. 2021b; Bat and Öztekin 2022; Terzi et al. 2022) and marine organisms: zooplankton (Aytan et al. 2016, 2020a, 2020b), bivalves (Gedik and Gozler 2022) and fish (Atamanalp et al. 2021; Aytan et al. 2021; Eryaşar et al. 2022).

It has been proven that tracking explicit biomarkers in wild populations is an indispensable method for evaluating the harmful effects of an unstable environment. According to this perspective, changes in genetic diversity and genetic structure reflect the “health” of an ecosystem (Belfiore and Anderson 1998; Medina et al. 2007), which is defined as a comprehensive, multiscale, dynamic, hierarchical assessment of system resilience (Ehrenfeld 1992).

Various biomarkers, including oxidative stress’ (OS) ones, are currently being used to study and monitor the marine environment and marine ecosystems. (van der Oost et al. 1996; Samet and Wages 2018). The stress-response approach is currently gaining attention in studies of the environment’s multiple stressogenic effects on Black Sea biota and ecosystem functionality (Silkina et al. 2014; Vinagre et al. 2014; Yakimov et al. 2018; Bozcaarmutlu et al. 2020;

Alexandrova et al. 2021, 2022a, b). In addition, changes in cellular pro- and anti-oxidant balance can be utilised to assess the general response of all aerobic organisms to multiple stressors. Numerous studies have shown that, in fish, various factors of the aquatic environment can affect oxidative processes in cells (Lushchak and Bagnyukova 2006; Stoliar and Lushchak 2012; Vinagre et al. 2012; Chowdhury and Saikia 2020; Gopi et al. 2021). These effects, can intensify the production of reactive oxygen species (ROS) (pro-oxidant processes) and/or reduce the cellular antioxidant defence (antioxidant enzymes and non-enzymatic antioxidants). The imbalance in favour of oxidative processes is referred to as oxidative stress (OS). OS has been recognised as a promising molecular biomarker tool for determining the extent to which the environment has stressogenic effects on living organisms (Van Straalen 2003; Steinberg 2012; Hook et al. 2014; Samet and Wages 2018). Lower levels of biological organisation are more vulnerable to stressogenic environments; thus, alterations at these levels can be used as early warning indicators for changes that will eventually occur at the population, community and ecosystem levels.

Benthic and piscivorous marine species have been reported to be amongst the most vulnerable to the effects of pollutants (Tenji et al. 2020) and, hence, appropriate indicators of the state of the marine environment (Goksøyr et al. 1996; van der Oost R et al. 1996).

The red mullet is one of the most important species for the Black Sea's fisheries and marine ecosystem; nevertheless, little is known about their populations along the Bulgarian Black Sea coast. *M. barbatus*, as a benthivorous fish, has the potential to serve as a sentinel and bioindicator species for detecting the Black Sea ecosystem's stressogenic and genotoxic potential. Consequently, a multi-biomarker approach was employed to assess the current status of the *M. barbatus* populations as a multifaceted response to environmental stresses, with the incorporation of genetic and OS markers as an intelligible metric for ecological stress. Other physiological markers, such as morphological variation and physiological state, were found to be useful towards determining the health status of *M. barbatus* and, as a result, were also studied within the scope of this research.

The goal of this study was to provide an initial evaluation of the status of *M. barbatus* populations as a key species with significant economic value in the Bulgarian Black Sea waters, as well as its adaptive capacity, using representative biomarkers.

Material and Methods

Study area and sampling

Species sampling was carried out as part of a multispecies survey from 5 July to 15 July 2021. A random selection of 36 to 38 sampling sites was surveyed in the study area covering the coastal and shelf waters at depths 15 to 100 m in front of the Bulgarian Black Sea coast and the samples under consideration in this study were conditionally taken as representative for the "north region" – the area in front of Kavarna (sampling site 34) and the "south region" – the area in front of Sv. Vlas (sampling site 24) in coastal waters at depths 15–19 m (Fig. 1).



Figure 1. Sampling sites along the Bulgarian Black Sea coast.

The marine environment at both sites is known to be under substantial anthropogenic pressure. The eutrophication and organic content of the marine environment at the two sites were quantified using the pollution index PI (ranging from 0 to 1), in line with Guidance Document No. 23 on eutrophication of the overall strategy for WFD implementation (Guidance, WCE 2009). Furthermore, for the sampling regions, the time series of annual mean sea surface temperature (2003–2021) and salinity (1993–2022) were acquired using NASA OBPB (NASA OBPB 2020) and Copernicus Marine Service (Lima et al. 2020; Ciliberti et al. 2021). Breakpoints indicating trends in temperature and salinity regimes in the studied regions were detected using segmented regression analysis. The analyses and graphical representations were carried out using the statistics and programming software R 4.0.5 (R Core Team 2021), packages ‘ggplot2’ (Wickham et al. 2016) and ‘segmented’ (Muggeo 2008).

Assessment of heavy metals accumulation

A total of 25 fish specimens (15 sampled at the Sv. Vlas site and 10 sampled at the Kavarna site) were deep-frozen immediately after capture. The fish size range was consistent with Descriptor 8 “Pollutants in the Marine Environment” of the Monitoring Programme. The main elements were analysed on whole fish (pollutant priority substances: cadmium – Cd, mercury – Hg, lead – Pb, nickel – Ni, polycyclic aromatic hydrocarbon – benzo (a) pyrene and specific pollutants: arsenic – As, chromium – Cr, aluminium – Al, iron – Fe, copper – Cu, manganese – Mn and zinc – Zn) were carried out on whole fish. The water content and free fats were measured in each sample, with the aim of subsequent normalisation of the concentration of pollutants. The analysis of the samples was carried out by an accredited laboratory (SGS Bulgaria Ltd., <https://www.sgs.bg/en>) using standard methods (atomic absorption and gas chromatography). The assessment of the state of the biota in terms of pollutants was based on the methodology described in Guidance Document No. 32 on Biota Monitoring (Guidance Document No. 32 2013). As a first step in the matrices, results that were below the limit of determination were replaced by ½ limit of quantification (LOQ). Metal concentrations in fish were then standardised by weight, whereas organic contaminants were normalised by fat content. The values obtained after normalisation by dry weight were compared to the maximum permissible limits for seafood (Commission Regulation (EC) No 1881/2006 of 19 December 2006), as enacted in Bulgarian national law (Ordinance No 5/9.02.2015 2015).

Fish morphology and biometric analyses

The biometric analysis includes the measurement and evaluation of 22 morphometric and four meristic features on 77 specimens (33 from Sv. Vlas and 40 from Kavarna). A Vernier caliper was used to measure the features with a precision of 0.1 mm. The methodology used by Mahmoud et al. (2016) to differentiate the morphology of *M. surmuletus* was applied to the morphometric measurements and meristic counts of *M. barbatus* (see Suppl. material 1: fig. S1).

To test statistical significance and validate the results, several statistical methods were used: parametric tests to verify the normal distribution of length-frequency data (LFD), which was used in linear regression models and non-parametric tests to identify statistically significant similarities in and between the samples (Analysis of Similarities – ANOSIM). The length-weight relationship (LWR) and ratios, such as standard length (SL), total length (TL), head length (HL), body depth/height (BD/BH) and BD/BH – SL (Fakunmoju et al. 2014) were modelled and their parameters were analysed.

All parametric and non-parametric statistical tests, modelling and computations were carried out using the MATLAB programming environment (THE MATH WORKS, INC. MATLAB version 2020a), the vegan package (Oksanen et al. 2019) and R version 4.0.5 (R Core Team 2021).

Molecular genetic analyses

Tissue samples were obtained from the dorsal fins of 79 specimens (39 from Kavarna and 40 from Sv. Vlas) and preserved in 96% ethanol at 4 °C for DNA

analysis. The genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN) and the target DNA was amplified with mitochondrial primers – cytochrome c oxidase subunit I (COI) and cytochrome b (Cyt b). The polymerase chain reaction (PCR) using mitochondrial primers (COI) and (Cyt b) was carried out in a reaction volume of 50 µl containing 2 µl of each primer, 25 µl of the Mastermix (MyTaq™ HS Mix, Bioline Reagent Ltd.) and 2 µl of the target DNA. The mitochondrial cytochrome c oxidase subunit I (COI) was amplified using universal primers, according to Ivanova et al. (2007) and for Cyt b using universal primers, according to Keskin and Can (2009). The conditions, conducive for PCR amplification for COI and Cytb included the following parameters: for COI 95 °C for 1 min, 95 °C for 15 s, 52 °C for 25 s, 72 °C for 1 min (35 cycles) and 72 °C for 1 min; for Cyt b: 95 °C for 2 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (35 cycles) and 72 °C for 10 min. PCR product quality control was performed by electrophoresis on 2% agarose gel. The mtDNA sequencing was performed by Macrogen Europe B.V. The mtDNA sequence data was further analysed by applying MEGA7 (Kumar et al. 2018). The number of haplotypes, haplotype connectivity and TCS Networks were constructed by means of Popart (Clement et al. 2002). The haplotype (H) and nucleotide (π) diversity were estimated with DnaSP 5.10.01 (Librado and Rozas 2009).

Oxidative stress assessment

Tissue preparation

M. barbatus specimens of the same length were analysed (supposedly to have individuals of the same age). The 26 samples were immediately shock-frozen and transported to the laboratory for optimal preservation (12 from the Sv. Vlas region and 14 from the Kavarna region) (Secci and Parisi 2016). On the day of analysis, the samples were dissected and their livers and gills were extracted and homogenised in 0.1 M potassium phosphate buffer (pH 7.4). After centrifuging homogenates at 3000 g for 10 minutes, the post-nuclear fraction was used to measure lipid peroxidation (LPO) and glutathione (GSH) contents. Antioxidant enzyme activities were measured using the supernatant obtained from re-centrifuging a portion of the post-nuclear fraction at 12,000 g for 20 minutes at 4 °C.

Measurement of oxidative stress biomarkers

Oxidative stress biomarkers were assayed using kits, purchased by Sigma-Aldrich Co. LLC, USA: MDA assay kit (Cat. No: MAK085) for determination of lipid peroxidation; Glutathione Assay Kit (Cat. No: CS0260), Superoxide dismutase (SOD) determination kit (Catalogue No: 19160), Catalase Assay Kit (Catalogue No: CAT100), Glutathione Peroxidase Cellular Activity Assay Kit (Cat. No CGP1), Glutathione-S-transferase (GST) Assay Kit (Cat. No CS0410) and Acetylcholinesterase Activity Assay Kit (Cat. No MAK119). The assays were performed strictly following the manufacturer's instructions. Protein concentration was measured according to Lowry et al. (1951) and calculated from a standard curve, obtained using bovine serum albumin as a standard.

This study also employed a version of a previously introduced Specific Oxidative Stress (SOS) index (Yakimov et al. 2018). The SOS index was com-

puted as the difference between the pro-oxidative (PrO) score and the antioxidant (AO) score of the OS markers measured in *M. barbatus* specimens. The pro-oxidative (PrO) score (index) is the mean Z-score of the pro-oxidant markers (LPO) measured ($\text{PrO} = \text{mean}(\text{zLPO})$). The antioxidant (AO) score (index) is the average of the anti-oxidant Z-scores (in this case GSH, SOD, CAT, GPX and GST) measured ($\text{AO} = \text{average}(\text{zGSH} + \text{zSOD} + \text{zCAT} + \text{zGPX} + \text{zGST})$). In order to calculate the Specific Oxidative Stress (SOS) index, the AO score was subtracted from the PrO score ($\text{SOS} = \text{PrO} - \text{AO}$). Close to zero values for each of the three indices represent the mean of the difference (i.e. the balance). Negative and positive values indicate deviation from the mean (above or below).

Determination of ingested microplastics in *M. barbatus*

Each sample (40 collected at Sv. Vlas and 39 collected at Kavarna) was wrapped in aluminium foil and frozen at $-20\text{ }^{\circ}\text{C}$ immediately after identification on-board. Lusher, Dehaut and Karami's (Dehaut et al. 2016; Karami et al. 2017; Lusher et al. 2020) methodology was utilised to extract and evaluate microplastics in biota. A reduced-cross-contamination methodology was applied during the analysis and liquid and air exposure controls were taken into account in the result processing. The samples were defrosted at room temperature and carefully rinsed in the laboratory. The gastrointestinal tract (GIT) was separated and weighed. The sampled stomach and intestines were digested with filtered 10% potassium hydroxide at $40\text{ }^{\circ}\text{C}$ for 48–72 hours (until ready) in glass containers covered with metal foil. Subsequently, they were filtered with glass micro-fibre filters with a pore size of $1.2\text{ }\mu\text{m}$ and a diameter of 47 mm (Whatman glass micro-fibre filters, Grade GF/C) with a vacuum system, Rocker MultiVac 300, made of stainless steel. Using an Olympus SZ30 stereomicroscope with a 110AL2X WD 38 lens, the type, colour and size of the identified microplastics were determined directly from the glass Petri dishes, which were opened solely for the purpose of identifying the particle structure.

Results

Sampling sites' environmental characteristics and pressures

In general, the PI in the sampling locations was low to moderate and most pronounced in the south (Table 1, Fig. 2A). Both sampling sites are located in close proximity to or within regions with high physical disturbance pressure from fishing (swept area ratio (SAR) ≥ 2) (Todorova et al. 2021). During the sampling period, the fishing intensity was estimated to be 0.5 h/km^2 at the southern sampling site (Sv.Vlas) and $100+\text{ h/km}^2$ at the northern sampling site (Kavarna) (Fig. 2B), despite the fact that cargo traffic intensity (Fig. 2C) was found to be low at both sampling sites in 2021.

Segmented regression analyses identified two breakpoints for both variables (temperature and salinity) during the studied period, the most recent of which occurred in the southern region in 2010 for SST and 2014 for SSS and in the northern region in 2011 and 2014, respectively (Table 2 and Suppl. material 1: fig. S2A–D), indicating a nearly decade-long increase in annual mean surface temperature and salinity.

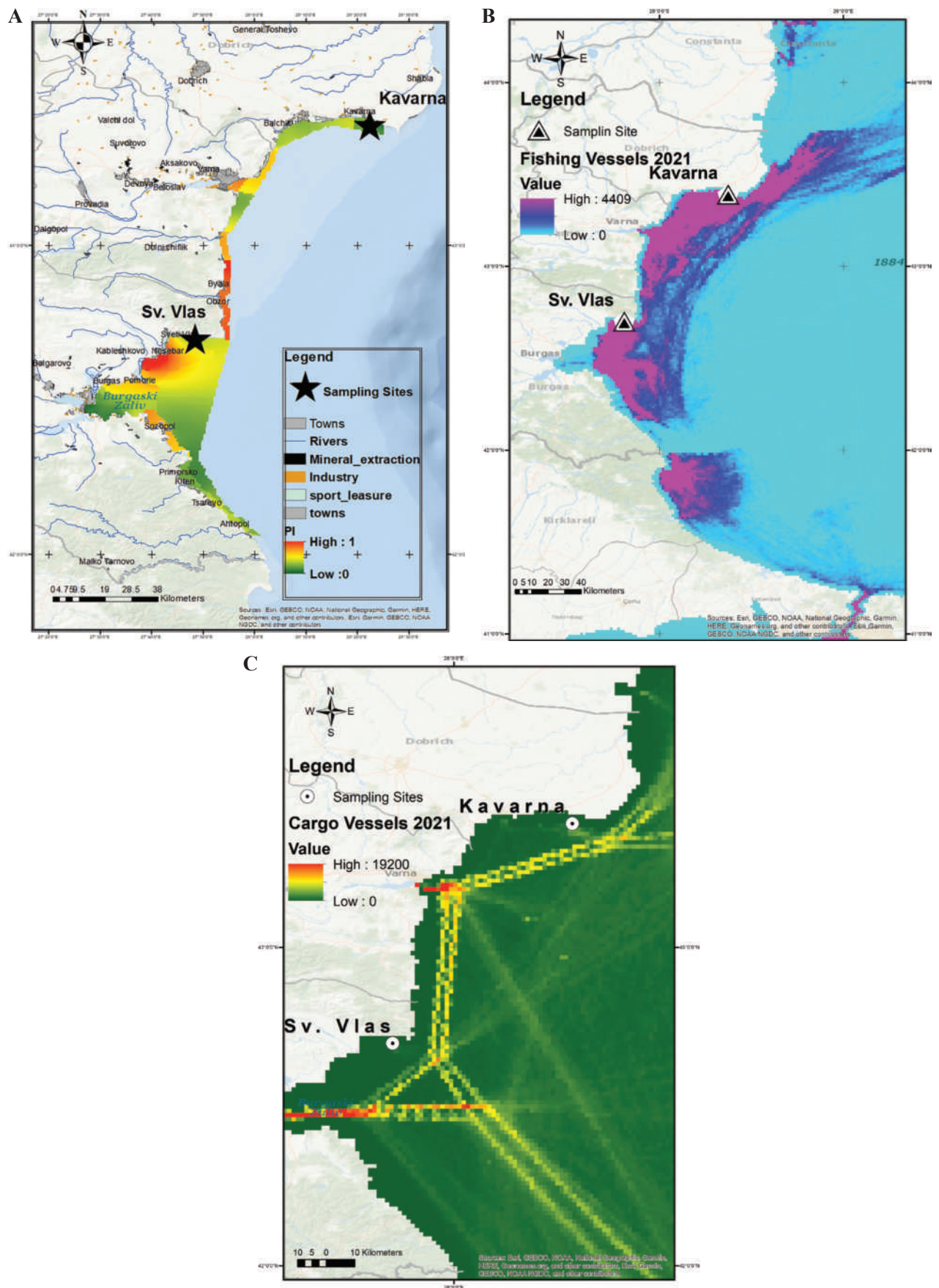


Figure 2. A sampling sites, overlain with PI (eutrophication + organic) B sampling sites overlain with fishing intensity layer (routes km²) in 2021 and C cargo ships traffic intensity layers in 2021 (EMODNet 2022).

Table 1. Sampling stations and anthropogenic impact data in the sampling period.

Date	Sampling station	Lon [DD]	Lat [DD]	Depth [m]	Region position	Pollution index (PI)*	Fishing density (h/km ² per month in July 2021**	Fishing intensity in 2021 (routes/km ²) **	Cargo traffic intensity in 2021 (routes/km ²) **
13.07.21	Kavarna	43.388	28.329	15	northern	0.213	> 100+	1155	13
9.07. 21	Sv. Vlas	42.698	27.775	19	southern	0.288	0.5	450	0

*Report analysis and interpretation of data on the ecological state of marine waters – 2021,

** (EMODNet 2021).

Table 2. Breakpoint analysis of annual mean surface sea temperature (SST) and salinity (SSS) time series (2003–2021 for SST and 1993–2022 for SSS) in the sampling regions.

Sampling station	Variable	Time series data period	Estimated breakpoints (year)	Std. error and R ²	Data source
Kavarna	T_{mean} [°C]	2003–2021	2007 ∨ and 2010 ↗	$R^2=0.59$; $std.err=0.48$	1*
Kavarna	S_{mean} [PSU]	1993–2022	2008 ∨ and 2014 ↗	$R^2=0.61$; $std.err=0.23$	2*
Sv. Vlas	T_{mean} [°C]	2003–2021	2007 ∨ and 2011 ↗	$R^2=0.49$; $std.err=0.53$	1*
Sv. Vlas	S_{mean} [PSU]	1993–2022	2007 ∨ and 2014 ↗	$R^2=0.55$; $std.err=0.26$	2*

*1 (NASA OBPG 2020),

*2 (Lima et al. 2020; Ciliberti et al. 2021).

Heavy metal accumulation

The majority of the normalised concentrations of the studied elements were below the maximum allowable concentrations for seafood specified in the relevant regulatory documents (Guidance Document No. 32 2013) (Table 3). The aluminium concentration in fish from the Kavarna site (higher than 30 mg kg⁻¹) and the chromium concentration in fish from the Sv. Vlas site (higher than 0.3 mg/kg) both exceeded the toxicologically acceptable level in biota. The results for mercury were below its limit of determination (0.05 mg kg⁻¹) and, therefore, not taken into account.

Fish morphology

Analysis of morphometric characteristics revealed that specimens captured in the southern region had a lower allometric coefficient than those sampled in the northern region (Table 4, Suppl. material 1: fig. S3A, B); however, specimens from both regions exhibited negative allometric growth. The estimated Fulton condition coefficient for Sv. Vlas specimens was slightly higher than the value calculated for Kavarna specimens, with the most likely cause being the larger size class range represented in the length frequency data (LFD) collected for Kavarna specimens (Suppl. material 1: table S1). The BD-HL ratio varied significantly by sampling site and all other ratios displayed very close values, with a difference of 0.01–0.02 in favour of the samples collected at the Kavarna site, indicating greater variability in the morphometric characteristics of specimens within each size class.

According to ANOSIM results, the morphometric and meristic characteristics of male and female *M. barbatius* specimens did not differ statistically and the specimens collected from the two sites appeared to have a relatively similar biometric structure (Suppl. material 1: table S2).

Table 3. Normalised concentrations of metal elements in *M. barbatus*.

Element	As	Cd	Pb	Al	Fe	Sn	Co	Mn	Cu	Ni	Cr	Zn	Benzo (a) pyrene
Sampling station	$\mu\text{g kg}^{-1}$												
Sv. Vlas	1.17	0.028*	0.028	21.1	263	0.028*	0.028*	1.34	1.05	0.07	0.55	9.54	0.266
Kavarna	0.93	0.024*	0.066	61	525	0.024*	0.024*	1.64	0.83	0.19	0.32	10.2	0.169

* – below the detectable limit.

Table 4. Length-weight relationship (LWR) modelling results and TL-SL, BD-HL/SL, HL-SL/TL ratios and Fulton's condition factor, calculated for specimens taken at Sv. Vlas and Kavarna.

LWR		
Sampling site:	Sv. Vlas	Kavarna
No of samples	n = 33	n = 40
LWR model: $W_{LWR}(i) = a * L(i)^b$	$W_{LWR} = 0.029 * L^{2.62}$	$W_{LWR} = 0.016 * L^{2.685}$
R^2 ($\alpha=0.05$)	$R^2=0.90$	$R^2=0.92$
Fulton condition factor: $K=W/L^3 * 100$	$K=1.11(\text{std. dev} \pm 0.129)$	$K=1.08(\text{std. dev} \pm 0.131)$
Ratios		
TL/SL ratio	TL/SL ratio=1.21(std. dev \pm 0.069)	TL/SL ratio=1.22(std. dev \pm 0.048)
BD-HL ratio	BD/HL ratio=0.55(std. dev \pm 0.09)	BD/HL ratio=0.61 (std. dev \pm 0.11)
BD-SL ratio	BD/SL ratio=0.16(std. dev \pm 0.04)	BD/SL ratio=0.18 (std. dev \pm 0.05)
HL-SL ratio	HL/SL ratio=0.29 (std. dev \pm 0.04)	HL/SL ratio=0.3 (std. dev \pm 0.04)
HL-TL ratio	HL/TL ratio=0.24(std. dev \pm 0.03)	HL/TL ratio=0.25 (std. dev \pm 0.04)

Molecular genetic analyses

The obtained mitochondrial DNA sequences were used to determine the number of different haplotypes. A total of 16 haplotypes for COI (626 bp) and 13 haplotypes for Cyt b (298 bp) were identified (Table 5). The majority of identified COI haplotypes originated from one prevalent set of haplotypes (Hap 3) following a single nucleotide substitution (Fig. 3A). Five haplotypes were unique for Sv. Vlas and three for Kavarna populations, while for Cyt b, the observed unique haplotypes were three in Sv. Vlas and four in Kavarna (Table 5, Fig. 3A, B).

The analyses of COI showed high values of haplotype diversity ($H_d > 0.5$) only in the population of Sv. Vlas (0.520) and lower diversity in the region of Kavarna (0.396), as well as low values of nucleotide diversity ($\pi < 0.5\%$), varying from 0.00077 (Kavarna) to 0.00245 (Sv. Vlas) (Table 5).

Analyses of Cyt b showed high values of haplotype diversity ($H_d > 0.5$), ranging from 0.533 (Sv. Vlas) to 0.613 (Kavarna) and low values of nucleotide diversity ($\pi < 0.5\%$), ranging from 0.00199 (Sv. Vlas) to 0.00245 (Kavarna) (Table 5).

Oxidative stress assessment

Assessing the level of OS biomarkers in individual *M. barbatus* fish can provide a direct indication of their condition. The biomarker values demonstrated significant differences between the liver and gills of *M. barbatus*, the two organs that actively respond to environmental stresses. There were differences in the levels

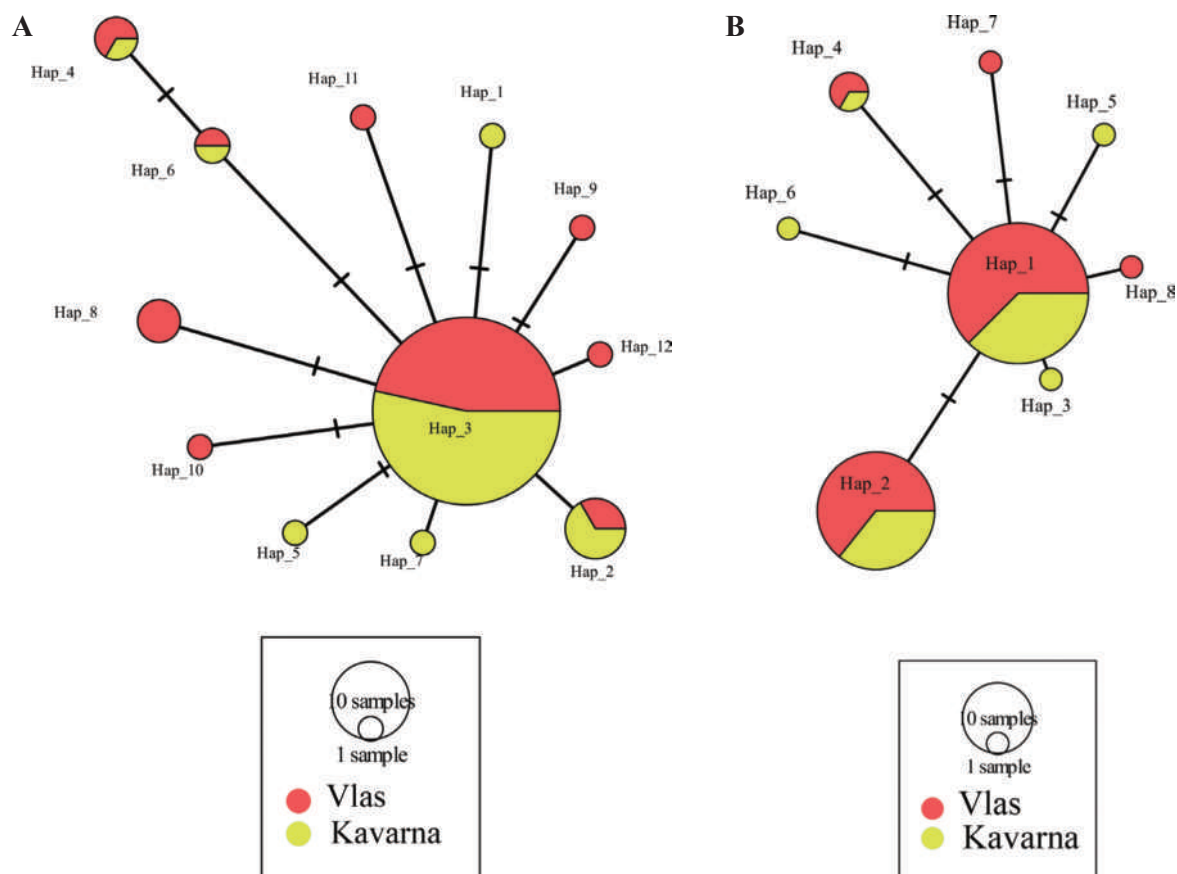


Figure 3. **A** haplotype network obtained from the TCS analysis, based on the distribution of COI haplotypes **B** haplotype network obtained from the TCS analysis, based on the distribution of Cyt b haplotypes*. *The size of the circles indicates the frequency of occurrence of each haplotype by the studied region. Small lines represent substitutions between haplotypes.

Table 5. Genetic diversity parameters of two sampling sites of *M. barbatus*, based on mtDNA sequence data.

	Sampling site	n	H	pHap	Hd	π	D	k	Fs
COI	Sv. Vlas	39	9	5	0.520 ± 0.096	0.00111 ± 0.00026	-1.81204*	0.696	-6.660
	Kavarna	40	7	3	0.396 ± 0.095	0.00077 ± 0.00022	-1.75657	0.482	-5.205
Cyt b	Sv. Vlas	40	6	3	0.556 ± 0.072	0.00212 ± 0.00036	-1.17640	0.63205	-2.732
	Kavarna	39	7	4	0.632 ± 0.048	0.00257 ± 0.00033	-1.23402	0.76653	-3.310

n – number of sequences; H – number of haplotypes; pHap – number of private haplotypes; Hd – haplotype diversity; PS – polymorphic sites; π – nucleotide diversity; D – Tajima's D value; *p < 0.05; Fs – Fu's Fs value.

of lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and acetylcholine esterase (AChE) in fish from both sampling sites, as well as superoxide dismutase (SOD) activity in fish from the Sv. Vlas sampling site (Table 6). Similar GSH concentrations were found in the liver and gills of fish from Kavarna and Sv. Vlas. In contrast, the LPO, CAT and GST activities in the liver and gills of fish from the Sv. Vlas sampling site were significantly greater than those of fish from the Kavarna sampling site (Table 6).

In general, the majority of fish samples from the Sv. Vlas sampling site had elevated OS, as indicated by the high levels of LPO in the liver and the low levels of GSH; nonetheless, they also had elevated CAT and GST activities in the liver and gills.

Table 6. Biomarkers of OS (mean \pm SD) in liver and gills and weight and length of *M. barbatus* from two different sampling sites (Kavarna and Sv.Vlas) off the Bulgarian Black Sea coast.

Weight [g]	Length [cm]	organ	Biomarker							Mean SOS
			LPO (lipid peroxidation)	GSH glutathione	SOD (superoxide dismutase)	CAT (catalase)	GPx (glutathione peroxidase)	GST (glutathione-S-transferase)	AChE (acetylcholine esterase)	
			nmoles/mg prot	ng/mg prot	U/mg prot					
Kavarna										
19.84 ± 2.15*	11.84 ± 0.41	Liver	0.57 ± 0.05 ^{†*}	413.22 ± 34.41*	25.69 ± 1.55*	4.84 ± 0.58 ^{†*}	3.45 ± 0.25	76.50 ± 7.64 ^{†*}	73.96 ± 5.45 [†]	-0.659
		Gills	13.26 ± 1.43 [†]	434.11 ± 33.63*	23.92 ± 2.16*	1.87 ± 0.32 ^{†*}	8.33 ± 0.83*	39.01 ± 3.00 ^{†*}	277.96 ± 24.59 [†]	
Sv. Vlas										
16.43 ± 0.96*	11.36 ± 0.19	Liver	0.91 ± 0.04 ^{†*}	245.03 ± 16.05*	17.94 ± 1.51*	9.37 ± 0.82 ^{†*}	3.30 ± 0.34 [†]	268.75 ± 24.63 ^{†*}	82.69 ± 7.72 [†]	-0.103
		Gills	8.81 ± 2.26 [†]	203.91 ± 13.49*	13.21 ± 0.69*	4.26 ± 0.24 ^{†*}	12.86 ± 0.85 ^{†*}	65.30 ± 3.04 ^{†*}	284.10 ± 26.09 [†]	

[†]- significance of difference ($p < 0.05$) between the liver and gills of the fish from the same region,

* – significance of difference ($p < 0.05$) between sampling sites.

In this study, the Specific Oxidative Stress (SOS) index was utilised for an integrated assessment of the cellular oxidative process balance. The results of the SOS measurements revealed that only a few *M. barbatus* specimens from Kavarna exhibited activation of pro-oxidative processes and activation of their antioxidant system (Fig. 4). In contrast, the majority of *M. barbatus* specimens from the Sv. Vlas sampling site exhibited a high level of oxidative stress and, in some cases, the antioxidant system was even suppressed (Fig. 4). This strongly suggested that the marine environment of the southern Black Sea region (Sv. Vlas sampling site) was ultimately more stressful for *M. barbatus* than the marine environment of the northern region (Kavarna sampling site). The mean values of the SOS index for the two sampling locations also reflected these results (Table 6).

In an effort to evaluate the potential effects of the OS on the body condition of *M. barbatus* individuals, we intended to measure the correlation between OS markers and Fulton's K or the length and weight of the fish. However, in this study, we utilised only *M. barbatus* specimens of equal length, indicating that they were likely of the same age (Table 6). This practically limited the analyses of the possible relationships of the OS markers and body condition indicators; consequently, it was relevant in this case to analyse only the relationships between the OS markers and fish body weight. The interdependence between individual fish weight and measured OS markers in fish from the two sampling sites was investigated using correlation analyses (Pearson's r). No significant correlations were observed between fish weight and OS markers in the liver and gills of *M. barbatus* individuals from the Kavarna sampling site. On the contrary, significant correlations were discovered between weight and LPO in red mullet from the Sv. Vlas sampling site ($r = -0.812$; $p < 0.05$) and CAT ($r = -0.786$; $p < 0.05$) for liver and between fish weight and LPO ($r = -0.763$; $p < 0.05$) and AChE ($r = -0.794$; $p < 0.05$) for gills.

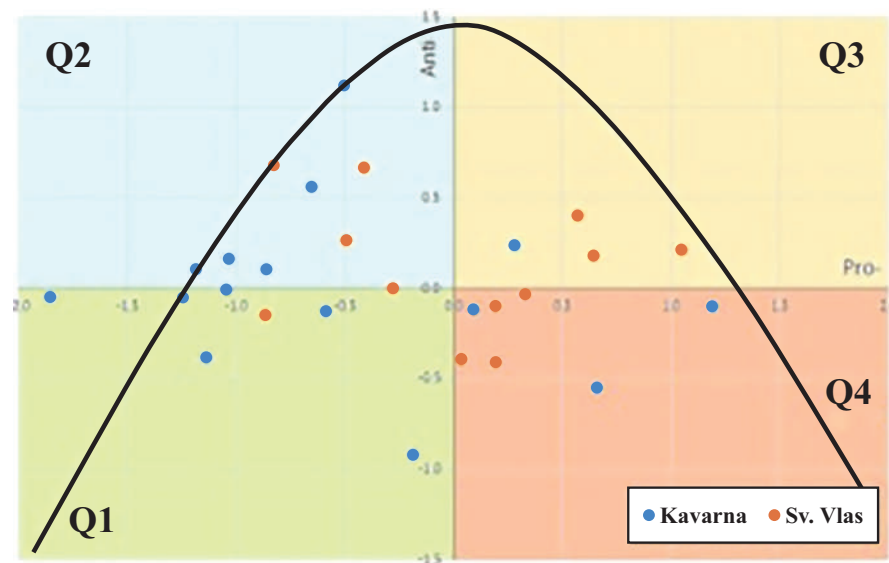


Figure 4. Distribution of the Specific Oxidative Stress (SOS) indices for *M. barbatus* specimens from the sampling sites Kavarna and Sv. Vlas in the space of the pro- and antioxidant indices axes (standardised data) divided into four quadrats (Q*) corresponding to the deviation of the balance of the pro- and antioxidant processes. * Q1 – low pro-oxidants, low antioxidants; Q2 – low pro-oxidants, high antioxidants; Q3 – high pro-oxidants, high antioxidants; Q4 – high pro-oxidants, suppressed antioxidants.

Analyses of microplastics in *M. barbatus* gastrointestinal tract

A total of 74 plastic particles were found in GIT of 26 specimens, 15 sampled at Sv. Vlas and 11 sampled at the Kavarna site (33% of all examined, 16 out of 44 females and 10 out of 35 males), pointing to the fact that 60.8% of MPs were found in female specimens. The average number of plastic particles per fish was estimated to 0.94 ± 1.81 particles individual⁻¹ for all individuals and respectively 2.85 ± 2.15 particles individual⁻¹ only for those who have ingested plastics. The number of particles documented varied from 0 to 6 per specimen in Sv. Vlas and 0 to 10 per specimen in Kavarna (Fig. 5). The types of plastics detected were predominantly fibres (75.7%) and fragments (24.3%) (Suppl. material 1: fig. S4). The most common plastic colour distinguished for fibres was grey (36.5%), followed by blue (29.7%) and for fragments green (21.6%) (Suppl. material 1: fig. S5).

The vast majority (98.4%) of the ingested particles were MPs in the size class < 5 mm. The small MPs size class (1 µm – 1mm) represented 73.4% of all plastics found, of which the share of the 1–500 µm size class was estimated to be 48.4% of the total number of small particles, with an average length of 255.7 µm and the 501–1000 µm size class was represented with a 25% share, with an estimated average length of 778.4 µm. Large microplastics (1–5 mm) accounted for 25% of all plastics reported, with an average length of 2034.2 µm. Mesoplastic (> 5 mm) were represented by only 1.6% of all plastics registered in the study. Particles with a length above 25 mm (macroplastics) were not detected.

A correlation between the total length and weight of the sampled fish, the weight of their GIT and the number of MPs was not established (verified by Spearman Rank Correlation, $\rho \sim 0.2$).

Additionally, the results showed that the specimens sampled at Sv. Vlas had a higher number of ingested MPs (1.125 ± 1.842 particles individual⁻¹),

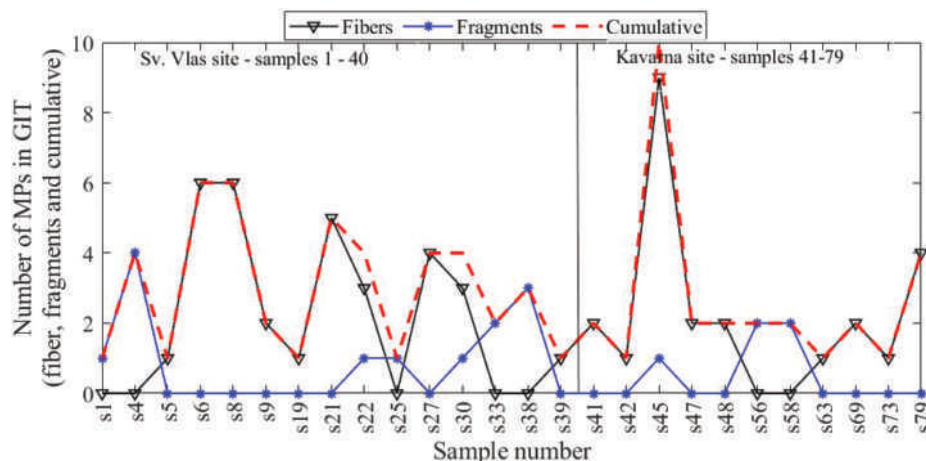


Figure 5. Number of microplastics found in GIT of *M. barbatus*, in the specimens sampled at Sv. Vlas and Kavarna sites.

compared to those sampled at Kavarna (0.743 ± 1.787 particles individual⁻¹), despite the fact that the LFD (Sv. Vlas $TL_{mean} = 11.767 \pm 0.798$; Kavarna $TL_{mean} = 12.377 \pm 1.859$) showed that only smaller size classes and age groups were represented in the Sv. Vlas sample.

Discussion and conclusions

Historically, physico-chemical analyses of environmental parameters formed the backbone of the risk assessment of pollution and alterations to the Black Sea ecosystem. Recently, multi-biomarker approaches have been developed (Beliaeff and Burgeot 2002; Yancheva et al. 2018) to assess the effects of exposure to contaminants and the responses of marine biota to environmental stress. Biochemical and physiological indicators are presently an essential tool for ecological research (Steinberg 2012). The general response of marine organisms to environmental pressures (abiotic, biotic and anthropogenic) is the activation of oxidative processes in their cells (Birnie-Gauvin et al. 2017) and the latter were also utilised in the assessment of the condition of the studied *M. barbatus* populations.

Depending on the spatial dispersal of the species, the findings of this study indicate that Black Sea marine environment pressures can have diverse and multidimensional effects on *M. barbatus* populations. The alterations and variations in the balance of oxidative processes in *M. barbatus* are a polymorphic response to the stressogenic effects of the environment. Specimens from the southern Bulgarian Black Sea coast had elevated OS levels, whereas those from the northern had low genetic diversity. Based on PI data and other stressors, such as the accumulation of heavy metals and MPs in biota, the southern region was evidently more polluted and its stressogenic effects from an OS perspective were more pronounced. In the northern region, fishing was clearly identified as a major threat and its effects on the genetic diversity of the *M. barbatus* population were more distinct.

Genetic diversity is one of the major determinants of the “health” and resilience of fish populations. Reduced genetic diversity may result in decreased population viability and small effective population size, despite the possibility of a high abundance or biomass and an increased probability of extinction (Hauser et al. 2002; Kenchington 2003; Martinez et al. 2018; Prunier et al.

2020). Our study revealed the absence of genetic structure amongst the analysed populations, indicating the existence of a single stock of the studied species. In addition, the low haplotype and nucleotide COI diversity of the *M. barbatus* population in the northern region (Kavarna) was correlated with the higher values of commercial catches reported in the northern Black Sea during the period 2018–2020 (Suppl. material 1: table S3). The effects of overfishing have been linked to a decline in genetic diversity, which may result in the loss of fish species' capacity to adapt to future environmental changes (Zhang et al. 2020; Petit-Marty et al. 2021). Moreover, fishing is typically a highly selective activity, so it has the potential to alter the population structure by favouring slow-growing, early-maturing specimens (Kenchington 2003). Practically, fishing pressure may have significant genetic effects on fish stocks without clearly pronounced contributions to the near-extinction of fish stocks. These findings indicate the need for further investigation of *M. barbatus* populations inhabiting Bulgarian Black Sea waters and implementation of conservation plans and measures.

The analysis of morphometric features revealed that specimens captured in the southern region had a lower allometric coefficient and specimens from both regions exhibited negative allometric growth. The condition of the fish specimens, as estimated by the Fulton condition coefficient (K), was comparable at both sites, although the mean value of K for the samples captured in the Sv. Vlas region was slightly higher than the value calculated for the samples captured at the Kavarna site. The larger size class range represented in the length frequency data (LFD) collected for the specimens sampled at the Kavarna site may account for this difference. The BD-HL ratio varied significantly between both sampling sites, whereas all other ratios exhibited very similar values. The absence of morphological variation between the sexes of *M. barbatus* was previously reported for Mullidae species (Mahe et al. 2014). Our data on OS biomarkers allowed us to study the interdependence only between individual fish weight and measured OS markers at the two sampling sites. In *M. barbatus* individuals from the Kavarna sampling site, no significant correlations were found between fish weight and OS markers both in the liver and in the gills. On the contrary, in the specimens sampled at Sv. Vlas, significant negative correlations were found between fish weight and LPO and CAT for liver and between fish weight and LPO and AChE for gills. Nevertheless, the observed BD-HL ratio variation between the specimens sampled in the two regions need further investigation to allow generalisation, as species occupying different substrate types are more likely to develop a diverse diet composition resulting in consequent morphological variation (Mahe et al. 2014). The latter may add valuable knowledge specifically in terms of the *M. barbatus* ability to adapt; nonetheless, given the outcomes of the genetic analyses, it is more likely that the observed differences are due to phenotypic plasticity than to genetic variation.

Our study provided the first data on the types and quantities of MPs ingested by *M. barbatus* in the Black Sea waters of Bulgaria. The particles found in the GIT of the sampled fish were more than twice as numerous as those found in the same species by Aytan et al. (2021) and Eryaşar et al. (2022) in the Turkish Black Sea waters and nearly comparable to Atamanalp's (Atamanalp et al. 2021) findings (Suppl. material 1: fig. S6). In all of the latter case studies, including our own, fibres were the most prevalent morphological form, followed by fragments (Eryaşar et al. 2022; our study) and microsheets/films (Aytan et

al. 2021). The MPs particles discovered in the GIT of *M. barbatus* in our study were more abundant in Sv.Vlas samples, which corresponded with the higher OS in fish specimens from this location. The possible effects of MPs bioaccumulation in marine biota are still being investigated. Nonetheless, some case studies have reported the induction of OS by MPs in the tissues of both invertebrate (Hu and Palić 2020; Costa et al. 2022) and vertebrate – primarily fish (Alomar et al. 2017; Espinosa et al. 2019; Kim et al. 2021). In this regard, our findings suggest that the higher number of accumulated MPs may have also contributed to the increased OS observed in *M. barbatus* specimens from Bulgaria's southern Black Sea coast (Sv. Vlas).

The toxic effects of various heavy metals and PAHs may be one of the numerous potential causes of OS induction in fish. Chromium concentrations were found to be higher in the tissues of Sv. Vlas specimens in our study. Particularly, trivalent and hexavalent chromium forms are involved in redox cycling (Stohs and Bagchi 1995). In a comparison of the effects of hexavalent and trivalent chromium ions on goldfish, it was discovered that both ions induce oxidative stress (Kubrak et al. 2010). The exposure of Chinook salmon *Oncorhynchus tshawytscha* (Farag et al. 2006) and *Channa punctatus* (Awasthi et al. 2018) to hexavalent chromium resulted in oxidative DNA damage and elevated LPO in the tissues; and that of *Labeo rohita* resulted in significantly elevated activity of SOD, CAT and GR in the liver, gills, muscles and brain (Kumari et al. 2014); that of *Oncorhynchus mykiss* led to increased brain SOD and GR (Li et al. 2011). As a conclusion, the specific effects of heavy metals accumulation in *M. barbatus* tissues in the Black Sea on their health status and physiological state need to be further studied.

Water temperature and salinity are proven to be basic abiotic factors that govern species' spatial distribution and developmental stages, having multiple effects on species' physiology and can consequently also affect OS induction in marine organisms. In our study, breakpoint analysis of mean annual surface sea temperature and salinity time series revealed shifts towards increase of the mean annual temperatures and salinity in the last decade in both of the studied regions. In general, the rise in seawater temperature can cause higher production of ROS in marine organisms. It has been recognised that higher water temperatures can trigger intracellular ROS production and metal-induced cell death to a greater extent (Park et al. 2020). A rise in the seawater temperature is also associated with elevated metabolic rates, higher oxygen consumption and respiratory chain flux and, thereby, increased ROS production (Halliwell and Gutteridge 2015). The OS response, on the other hand, is related to the optimal temperature for the species. It is at its lowest at the optimal temperatures and increases outside the upper and lower thermal optimum limits (Vinagre et al. 2014). Adaptation to temperature changes with a reduction in OS has also been previously reported (Vinagre et al. 2014). Thus, the anticipated increases in temperature due to climate change are very likely to induce higher OS and trigger adaptive mechanisms in marine biota. Moreover, as the various environmental stressors interact with one another, the strength of their effects on OS cannot be attributed to a single stressor (Min et al. 2014; Fadhlouli and Couture 2016). Consequently, further research is needed to explain the relative proportion of species' synergetic responses to multiple stressors and their interactive effects that remain unaccounted for.

In conclusion, the present study demonstrated evidently that *M. barbatus* populations along the Bulgarian Black Sea coast are exposed to a variety of

stressors that differ by region and habitat's ecological conditions. Our study displayed that the species can tolerate environmental and anthropogenic variations to some extent; however, some of the studied specimens from the southern region exhibited high oxidative stress and suppressed antioxidant defence, while those from the northern region exhibited low genetic diversity. The latter can serve as an early indicator that the studied population may be approaching its adaptive capacity limits. In order to effectively monitor the ecological condition of the marine environment, it is recommended that research into the multiple stressor effects of the Black Sea environment on the biota must be intensified. This includes the development of new indicators and the determination of thresholds, also at the molecular and cellular levels.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: IZ, AA, ND, KS, PI, NC. Data curation: VS, IP, NV, VD. Formal analysis: VS, ES, VD, AG, MY, ND, ET, AA, MA, BD, OH, IZ, PI, SM. Investigation: PI, NC, IZ, VR, YR, AA, DD, ES. Methodology: VR. Project administration: VR. Resources: DD, YR, KP, VR, MY. Validation: SM, AG, ET, IZ, NV, OH, BD, ND, MA, IP, KP, PI. Visualization: VD, VS, IP. Writing – original draft: IZ. Writing – review and editing: IZ, NC, AA, KS.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary data

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Data type: figures and tables (.docx file)



Explanation note: Schematics of measurements taken for biometric analysis on the body of *M. barbatus* (Mullidae species – Mahmoud et al., 2016). Breakpoint analyses (segmented regression models fit) of SST and in a) southern region in the area of Sv. Vlas – sampling site 24 and b) northern region in the area of Kavarna – sampling site 34 and SSs c) in the region of sampling site Sv. Vlas and d) in the region of sampling site Kavarna. Graphic representation of LWR models vs. measured weight-length values per sampling site (a) – Sv. Vlas, b) – Kavarna). First row: large fragments identified in the GIT of two female specimens sampled in Kavarna (first row, first image) and Sv. Vlas (first row, second image); Second row: fibers identified in the GIT of *M. barbatus*. Percentage share of fragments and fibers identified in *M. barbatus* GIT by color. Number of particles found in GIT of *M. Barbatus* per case study in the Black Sea region. Summary statistics of morphometric features measurements of sampled specimens per sampling site. Analysis of Similarities (ANOSIM) results of *M. barbatus* morphometric and meristic features in (by sex) and between the samples. EAFA Official statistics for *M. barbatus* landings reported in the period 2018-2020.

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Research Article

Spatial distribution of sand dunes along the Bulgarian Black Sea Coast: inventory, UAS mapping and new discoveries

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Abstract

Coastal sand dunes are amongst the world's most sensitive and dynamic landforms. Unfortunately, during the last thirty years, heavy anthropogenic alterations have been observed, encompassing the greater part of the Bulgarian Black Sea coast (BBSC), which has changed the land-sea interactions significantly. As a consequence, the depositional coast has shrunk to 131 km or 25% of the aggregate Bulgarian Black Sea shoreline length. Although our research reveals that 86% of BBSC dunes are included in the Natura 2000 network of protected sites established under the Habitats Directive (Council Directive 92/43/EEC 1992), they are often heavily modified, subjected to environmental vandalism and destroyed due to mismanagement or lack of accurate information and prevention. These facts were the main reason for carrying out an inventory of the Bulgarian Black Sea coastal dune systems in 2021–2022. Our research aimed to identify all dune systems/sand dunes, update their spatial distribution and classify the observed coastal sand dunes landforms along the BBSC. The article demonstrates a successful methodology for combining unmanned aerial systems (UAS), Structure-from-Motion (SfM) photogrammetry, in situ sediment sampling, video imaging and verification and GNSS-RTK ground control points for coastal mapping. As of June 2022, over 97% of the Bulgarian shoreline has been surveyed with this technique, excluding military areas and national security sites. Based on the acquired data, as of 2021, the shoreline length was estimated to be 518.7 km at a scale 1:5000. The integrated UAS approach includes using Digital surface models (DSM), raster orthophotomosaics (OM) and 3D models, based on SfM photogrammetry to analyse the coastal topography, detect dune forms and update their spatial distribution. Throughout the inventory, 46 beach-dune systems were identified along the BBSC, which were divided into 62 dune sectors. The area of coastal dune systems was estimated at 988.21 ha (0.0089% of Bulgaria) and a total length of 73 km (14% of the shoreline). A comprehensive geomorphological analysis of the relationships between landforms morphology, aeolian and morphodynamic processes, vegetation density and type was the basis for the coastal dune landforms (CDLs) or dune systems to be classified into primary (312 ha; 32%) and secondary (676 ha; 68%). Additionally, the CDLs were classified according to Natura 2000 habitats: fixed (grey) dunes (546.27 ha; 55.28%), wooded dunes (222.61 ha; 22.53%), shifting (white) dunes (150.30 ha; 15.21%), embryonic dunes (68.3 ha; 6.91%) and humid dune slacks (0.94 ha; 0.09%). The highest positioned CDLs on the Balkan Peninsula were registered at perched Sozopol Sand Dunes (61 m a.s.l.) and cliff-top dunes at Arkutino (50.2 m a.s.l.). The multi-temporal analysis of photogrammetric DSMs and raster OMs showed the permanent loss of five dune systems in the Pomorie-Burgas-Rosenets coastal sector. The accrued UAS data approach allowed us to identify and map eight dune systems for

the first time: Zlatni Pyasatsi (Panorama), Asparuhovo (Varna), Byala, Atanasovska Kosa, Central Beach (Burgas), Chernomorets, Kavatsite (partly) and Rezovo-Kastrich. A high anthropogenic footprint was registered on 50.7 ha (5.1%) of the entire dune surface. In the final stage of the study, human interventions that caused degradation and permanent loss of dunes (12 ha) over the last 15 years along the BBSC were shown. The main causes for dune degradation along BBSC have been documented, such as massive tourism development after the socialist period, road construction, recreational pressure exerted on the dunes, human trampling, lack of designated footpaths in areas with fixed and mobile dunes, off-road vehicles and parking lots (especially at camping sites), dumping of garbage and anthropogenic marine litter on the sand dunes etc.

Key words: anthropogenic pressures, Bulgarian Black Sea Coast, coastal dune landforms, drones, dune degradation, dune systems, shoreline length, unmanned aerial systems (UAS)

Introduction

Coastal dune landforms (CDLs) are common sand forms of depositional landscapes that exist along the shores of oceans, seas and other water bodies in the world, where geomorphological settings, waves and currents interact with the available sediment and psammophilous vegetation to create varieties of forms and habitats located behind the active beach (Psuty 2008; Huggett 2016, etc.). Geomorphologically, the coastal dunes were formed at various timescales, but most of them were shaped out in Holocene and modern times from sand supplied to beaches from the sea floor (notably during the Holocene marine transgression) and alongshore sources, such as cliffs or bluffs in soft sandstone or glacial drift deposits (Bird 2008).

Beach and dune definitions

Beaches, as the most dynamic component of coastal systems, offer an exposed sediment source. Luijendijk et al. (2018) assessed that 24% of the world's sandy beaches are persistently eroding at a rate exceeding 0.5 m/yr over the study period (1984–2016), while 27% are accreting. About 16% (18%) of sandy beaches are experiencing erosion (accretion) rates exceeding 1 m/yr. Hence sandy beaches are often enclosed by some dune forms, produced by sand transported by wind activity and deposited at the vegetated landward section of the beach (Davidson-Arnott 2009). CDLs range from small forms, less than a metre in height, a few metres in width and along shore extents on small rocky embayments to much greater geomorphological features that may be 100 m or more in height, extending for tens of km alongshore, on sandy barrier systems or in low coastal plains (Davidson-Arnott 2009). Most of the sediments transported from the backshore are initially trapped by vegetation colonising the beach area, just landwards from the limit of storm wave action, leading to the development of a foredune ridge parallel to the shoreline (Davidson-Arnott 2009). In addition, Davidson-Arnott (2009) generalises some facts in terms of coastal sediment budgets. Essentially, beaches are the source, while dunes represent the sink. As fine sediments are generally scarce on active sandy beaches, coastal dune deposits have very small amounts of sediment with < 0.15 mm grain size. Likewise,

because of the small air density compared to water, particles > 1 mm are selectively left on the beach during deflation and are also generally scarce. Bird (2008) summarises that dune sands have similar characteristics to the beach sands from which they derive and generally consist of quartz, feldspar and calcareous particles (including foraminifera, bryozoa, comminuted shells and corals), sometimes also containing heavy minerals, such as rutile and ilmenite.

Although the CDLs are unique aeolian landforms, their evolution is closely related to the vegetation's role in reducing wind speed and facilitating the deposition of sediments (Masselink et al. 2011) and represent habitats of important environmental and landscape values. Moreover, dunes form natural coastal defences because they act as a sand reserve and a physical barrier protecting landward territories (Fryberger et al. 1979; Jay 1998; Sabatier et al. 2009; Harley and Ciavola 2013). Geomorphologically, dunes are classified into primary (foredunes and embryonic dunes) and secondary, with different morphological and morphodynamic characteristics, each one modified by different processes (Psuty 1989; Davidson-Arnott 2009; Masselink et al. 2011 etc.). The morphodynamics of secondary dunes are similar to terrestrial (desert) dunes, but primary dunes are fundamentally different. Sherman and Bauer (1993) consider primary dunes to be essential coastal forms because they are strongly coupled to the nearshore processes on the fronting beach. By appearance and morphology, dunes can be differentiated into foredunes, parabolic, blowouts, transgressive sheets and dune fields (Hesp 1991, 1999, 2002, 2011; Bird 2008) and are associated with ridges, mounds, terraces and low-lying swales, formed by different geomorphological processes over the years (Bird 2008).

Brief reference on sand dunes inventories in Europe and Bulgaria

Early publications on sand dunes over the European Union include a Council of Europe Report and a wide-ranging study in a special Catena publication (Géhu 1985; Bakker et al. 1990). In Sand Dune Inventory of Europe (Doody 1991), the Coastal & Marine Union (EUCC) has promoted dune conservation on European coasts and nowadays (with a total area of more than 5300 km²), they have acquired high importance for the community and nature preservation. The inventory also provides a brief description of the dune formation types, size of the overall resource, vegetation, important dune sites, comments on conservation issues and a list of references (Delbaere 1998; Doody 2008). Doody (2013) makes a retrospective analysis of the significant steps in the scientific activity of dune inventories in Europe. Recently, Jackson et al. (2019) and Gao et al. (2020) have provided a comprehensive analysis of stabilisation trends of coastal dunes by utilising a larger temporal window and more dedicated dune sites. In the context of increasing coastal urbanisation, climate change and associated sea-level rise, shorelines have become more vulnerable to overdevelopment, dune erosion and dune vanishing, especially mobile sand dunes. They are suggested to have a much higher ecological diversity and species richness, while stabilised sand dunes could be more tolerant to future sea-level rise.

At the European level, coastal dunes have a conservation significance as natural habitats and are meant to be protected from negative human impacts (Council Directive 92/43/EEC 1992; Martinez and Psuty 2004; European Commission 2008; Doody 2013; Janssen et al. 2016). The coastal sand dune

habitats are considered conservationally important at the national level, pursuant to Appendix 1 of the Biological Diversity Act (BDA 2002). The coastal dunes are also included in Volume 3 (Natural Habitats) of the Red Data Book of Bulgaria (Tzonev 2015a, 2015b, 2015c, 2015d; Tzonev and Gushev 2015). In addition, Gushev and Tzonev (2015) summarise that a substantial part of the total dune area in Bulgaria is included in the Natura 2000 network: 58% of embryonic dunes (ED), 73% of white (shifting) dunes (WSD), 89% of grey (fixed) dunes (GD), 100% of wooded dunes (WD) and 67% of humid dune slacks (HDS).

Human activities and anthropogenic pressures on coastal dunes

Human intervention is critical when discussing dune mobility alterations, their stabilisation and re-mobilisation. Therefore, in the near future, it will be necessary to increase scientific and public awareness, as well as to take careful actions to preserve or possibly even restore the dune mobility and balance the ecosystem services that coastal dunes offer. Failure to address the degradation and loss of dunes could result in hazardous consequences (Gao et al. 2020). Naturally, the distribution and ecological state of the dunes was not always excellent. The degradation and loss of coastal dunes come as a consequence of different activities performed on the coast by humans. These actions can be categorised into six groups and, to a major or minor extent, all of them affect coastal dunes: housing and recreation, industrial and commercial use, waste disposal, marine litter, agriculture, aquaculture and fisheries, military activities etc. Gómez-Pina et al. (2002) define the main dune management problems in Spain, which are unfortunately valid for dune fields all over the world: massive tourist development, road and boulevard construction, dune mining, littoral drift interruption, dune recreational pressure, inadequate waterfront construction, human trampling, off-road vehicles and parking lots, agricultural practices and afforestation, garbage dumping, water extraction, civil engineering works and military use (Martínez et al. 2013; Vallés and Cambrollé 2013 etc.).

The second half of the 20th century saw the beginning of specific anthropogenic processes primarily represented by urbanisation, industrialisation and tourism. These processes have significantly altered the natural and environmental state of many coastal and river ecosystems, resulting in intense erosional processes and the destruction of numerous dune systems (Aguilera et al. 2020; Zhai et al. 2020 etc.). In reality, 30% of the world's coastlines are eroding, while dune systems have reduced by 70% in Europe alone (Luijendijk et al. 2018; Mentaschi et al. 2018; Vousdoukas et al. 2020 etc.). Furthermore, by causing sea level rise, climate change can expedite these processes (De Figueiredo et al. 2018; Forgiarini et al. 2019; Reguero et al. 2019; FitzGerald et al. 2020). As an integral part of coastal systems, threats affecting European dune systems, such as urbanisation, extraction of materials, recreational seashore activities (including mechanical impact caused by trampling, campsites etc.), pollution, invasive species and natural system modifications (Defeo et al. 2009; Malavasi et al. 2014).

Aim of the study

In accordance with the definition by numerous authors (Bird 2008; Psuty 2008; Davidson-Arnott 2009; Masselink et al. 2011; Huggett 2016), in this article,

the Bulgarian Black Sea coastal dunes are considered as sand landforms, a fundamental element of the coastal system. In Bulgaria, the spatial register of beaches, dunes and other coastal features is maintained as an integral thematic module of the Cadastral-Administrative Information System (CAIS) of the Republic of Bulgaria (<https://kais.cadastre.bg/en>) which should be up-to-date. This online-accessible webGIS-based information system is administered by the Geodesy, Cartography and Cadastre Agency (GCCA) to the Bulgarian Ministry of Regional Development and Public Works (MRDPW-Bulgaria). These duties of GCCA are in line with the Black Sea Coast Spatial Development Act and the related Ordinance No. 1/ 16 September 2008, that regulates the creation, maintenance and data update of the specialised maps and registers of the coastal features illustrated (i.e. beaches, dunes, coastal lakes, estuaries, lagoons, wetlands, hydro-technical structures etc.) (Ordinance 2020). Currently, however, CAIS fails to reflect all coastal dunes and sites in the country.

Due to the mobile nature of ED and WSD, they often shift their locations and boundaries. Hence, they change their size and position, which has not been systematically reflected in the specialised maps and dedicated thematic module on coastal features in CAIS over the years. Such geodata gaps lead to various issues concerning beach management and respective measures by local authorities, relevant ministries and executive agencies. Moreover, a large number of unidentified and unclassified dune areas and forms have been found during the dune inventory over the years and described herein.

Last but not least, throughout the last 15 years, the number of hydrotechnical facilities and ports along the BBSC has grown significantly, resulting in the formation of new beaches and, in some places, new dune areas (MSPRB 2021; UASME 2021). It was precisely the cited data discrepancies between the thematic coastal module of CAIS and the actually observed features in situ that, in 2020, led to the decision to launch an inventory of all beach-dune systems along the BBSC by a research team affiliated with the Institute of Oceanology – Bulgarian Academy of Sciences (IO-BAS).

The objectives of the presented study herein are: **(1)** a spatially explicit inventory of beach-dune systems (dune landforms) and preparation of an up-to-date list of dune systems locations along the BBSC; **(2)** detailed mapping of newly identified dune systems at a scale of 1:1000; **(3)** data update on the spatial distribution of coastal dune landforms; **(4)** assessment of the aggregate dune area affected by anthropogenic alterations; **(5)** assessment of the area and type of dunes lost over the past 15 years.

Materials and methods

Study area

Geomorphology of the Bulgarian Black Sea Coast

The geomorphological settings along the western Black Sea coast provide excellent conditions for the formation of coastal sand dune landforms. Tătui et al. (2019) report that 68% of the shoreline is morphodynamically stable (change between -1 m/yr and 1 m/yr) and 13% is dominated by accumulative processes (over 1 m/yr), which allows for the formation of local beach-dune systems

in some low-lying coastal sectors of Bulgaria, Romania, Ukraine, Russia and Turkey (Bird 2010).

The relief of the Bulgarian coastal region is a result of intricate interactions amongst tectonics, neotectonic movements and exogenous weathering and accumulation processes, such as erosion, abrasion, aeolian transport and deposition (Popov and Mishev 1974; Cheshitev et al. 1989; Keremedchiev 2001; Krastev 2002; Peychev 2004; Peychev and Peev 2006; Filipova-Marino-va 2007; Keremedchiev and Stancheva 2007; Aleksiev 2012; Filipova-Marino-va et al. 2013; Dimitrov et al. 2023). Over the years, zonation of the Bulgarian coastal area has been carried out on the basis of different criteria, for example, morphostratigraphic specifics (Lilienberg 1966), morphostructural properties (Popov and Mishev 1974), morphodynamic systems (Peychev and Andreeva 1998), morphometric parameters (Keremedchiev 2001), morphodynamic activity (Keremedchiev and Stancheva 2007), morphographic settings (Dimitrov et al. 2023) etc. In this regard, the authors of this paper consider that the dune systems analysis should correlate with the following six morphographic subdivisions on the Bulgarian Black Sea coastal zone (Fig. 1): Northern zone - Dobrudzha and Franga-Avren sectors; Central zone - Lower Kamchiya (Kamchiya) and Stara Planina (East Balkan Mts.) sectors; Southern zone - Burgas and Med-ni Rid-Strandzha sectors.

Bulgarian Black Sea beach-dune systems

In Bulgaria, the beach-dune systems are a valuable recreational resource and the most frequent landforms created in coastal depositional environments, making their preservation crucial. The accumulative forms comprise more than 70 beaches along the Bulgarian coast, including a total of 19 beach-dune systems (Stancheva 2010; Stanchev et al. 2013). They are prevalent along the southern Bulgarian Black Sea coast (Popov and Mishev 1974), but this information will be updated in the study presented herein. Most of the beach-forming substrates along the coast of Bulgaria are of terrigenous origin. Once eroded from the nearby coastal source areas, they are subsequently transported by rivers and surface streams and eventually deposited and re-distributed in the littoral zone. As a result, estuaries, lagoons, inlets, bays, gul-lies and ravines are essentially linked to the spatial distribution of beach-dune systems. Furthermore, due to overlapping characteristics of the coastal mor-phodynamics (sand strips) and the aeolian transport (dunes), beach-dune sys-tems are characterised by rather intense spatio-temporal variations (Popov and Mishev 1974; Krastev 2002; Keremedchiev and Cherneva 2003; Peychev 2004; Stancheva 2010; Stancheva et al. 2011; Kotsev et al. 2020; Prodanov et al. 2021b etc.).

Coastal landforms differ as to the degree of influence by morphodynamic processes that predominate in the littoral zone. They also exhibit notable dif-ferences in terms of their origin. However, in recent years, the anthropogenic activity or anthropogenic load has become yet another significant factor re-garding coastal change (Stancheva et al. 2011; Stanchev et al. 2013; MSPRB 2021; UASME 2021), which applies with full force to the dune fields: Nesse-bar (Stancheva et al. 2011) and campsites, for example, Kavatsi, Smokinya, Gradina etc. (Stancheva 2010; MSPRB 2021). Over the years, dunes along the

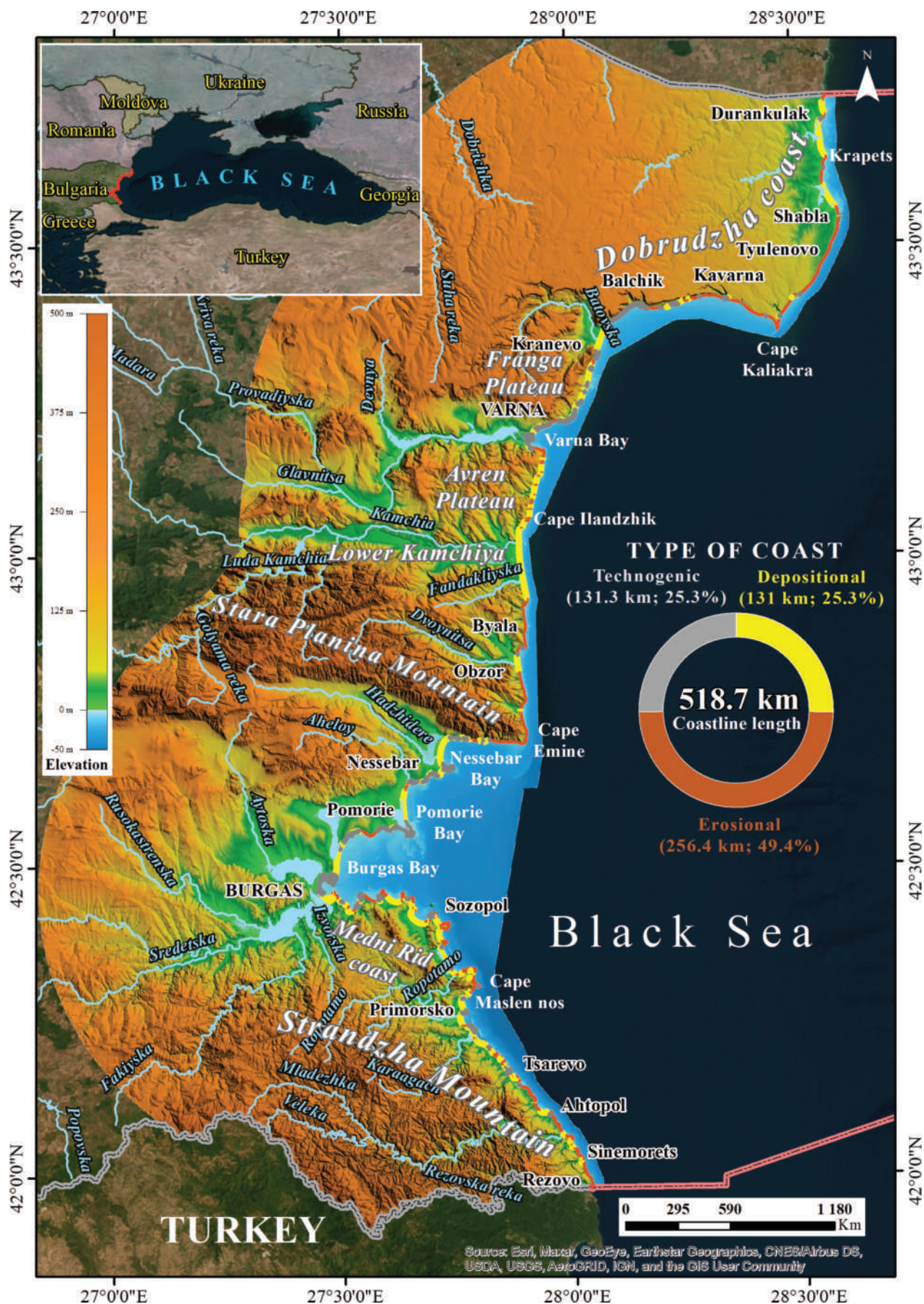


Figure 1. Bulgarian Black Sea shoreline and spatial distribution of depositional, erosional and technogenic coastal types.

Bulgarian coast have been relatively understudied in geomorphological, morphodynamic (Passports of the Bulgarian Beaches 1994; Kenderova et al. 1999; Prodanov et al. 2019a, 2020b, 2021a; Kotsev et. 2020; Prodanov 2023) and geological aspects (Petrov 2013; Valchev 2014, 2015; Sinnyovska and Sinnyovska 2016, 2017) or as Natura 2000 Habitats. Dunes of the first row at the back of a beach's active portion are termed foredunes (corresponding to the so-called embryonic and mobile/white dunes) (Tzonev 2015a, 2015b), while these located further landwards are known as stabilised (grey) dunes, which may also be covered by arboreal vegetation (vegetated dunes) (Gussev and Tzonev 2015; Tzonev 2015c, 2015d). The negative, often moist depressions located amongst dune ridges are termed dune slacks (Tzonev 2015d). Gussev and Tzonev (2015) summarize that a substantial part of the Bulgarian coastal dune areas is included in the Natura 2000 Network: 58% of embryonic dunes (ED), 73% of white (shifting) dunes (WSD), 89% of grey (fixed) dunes (GD), 100% of wooded dunes (WD) and 67% of humid dune slacks (HDS). In recent years, an increase in non-psammophytes, grass and shrubland species has been observed, probably due to tourist activity and technogenic pressure, which should be addressed as an issue in the future (Valcheva et al. 2019, 2020, 2021).

Climate of the Bulgarian Black Sea coast. Prevailing winds, aeolian transport and dune formation

Redistribution and accumulation of sand through aeolian transport represent the essence of dune formation. Dune locations are indivisibly related to the beach exposure towards the prevailing strong winds direction. For the BBSC, threshold values for aeolian transport are assumed to be 15 m/s (Popov and Mishev 1974). The prevailing eastern and north-eastern winds are of utmost significance, although such coming from the west quarter are also important concerning aeolian transport and subsequent dune formation. At beaches open towards the east and prevailing strong north-easterly winds, dunes form in the middle, especially in the southern section of the beaches. Accordingly, at sand strips exposed towards the north, dunes are formed in the middle and western beach sections (Popov and Mishev 1974).

Inventory data source

Archive of IO-BAS

At the end of the 1970s, IO-BAS carried out the first scientific studies on the BBSC for mapping the beach-dune systems. For a decade, in-situ campaigns were carried out to map all sand beaches and coastal dunes (as landforms) and determine their morphodynamic properties. Unfortunately, the results of these studies were kept confidential during Bulgaria's socialist period and, currently, only the beach and dune locations are available in the IO-BAS archives. After 1991, at the beginning of the democratic period, IO-BAS resumed systematic studies of the Bulgarian coast. The most detailed records of beach-dune systems on the BBSC date from that time (Passports of the Bulgarian Beaches 1994).

Cadastral-Administrative Information System of the Republic of Bulgaria (CAIS): Thematic module for the Bulgarian Black Sea coast

The webGIS-based information system for cadastral data and services - CAIS comprises several components containing and visualising thematic geospatial data, amongst which is a module dedicated to coastal features (i.e. beaches, dunes, estuaries, lagoons, wetlands, coastal protection infrastructure etc.), as listed in Article 6, paragraphs 4 and 5 of the Black Sea Coast Spatial Development Act (BSCSD 2008). However, despite being a highly valuable (and openly available) data source, the cited thematic module of CAIS fails to represent the up-to-date locations and boundaries of all the above-mentioned coastal features. Amongst the reasons for these drawbacks are certain peculiarities of data updates on behalf of the GCCA, cadastral parcel-based approach in delineating contours of the cited features, discrepancies in the coastal classifications used, delayed in-situ surveys on behalf of the authorities in charge, resulting property rights issues due to the legal status of the cited coastal features etc.

Methods

The study combines various techniques, such as spatio-temporal GIS-aided analysis of archival data, drone mapping, sedimentological analysis and visual observations, integrated into a GIS database. For the accuracy of our research, field geodetic GNSS measurements were performed at each beach-dune system (Fig. 2).

UAS mapping and field surveys

The use of drones for photogrammetric surveys is widespread in studies of beach-dune systems around the world (Papakonstantinou et al. 2016; Suo et al. 2017; Laporte-Fauret et al. 2020, 2022; Fabbri et al. 2021). In the last five years, drones have become the most efficient and inexpensive method for environmental monitoring, habitat and vegetation mapping and analysis of geomorphic processes/changes (Turner et al. 2016; Choi et al. 2017; Maduraperuma et al. 2018; Guisado-Pintado et al. 2019; Pagán et al. 2019; Prodanov et al. 2019a, 2019b; Suo et al. 2019; Kotsev et al. 2020; Prodanov 2023). Scientific UAS surveys in Bulgaria are focused on the SfM-derived georeferenced data for different purposes, such as geomorphological or landscape mapping (Dimitrov and Savova 2019; Prodanov et al. 2019a, 2019b, 2021a, 2021b; Kotsev et al. 2020; Tcherkezova et al. 2020; Dinkov et al. 2021; Tcherkezova 2021; Trendafilova and Dechev 2021; Prodanov 2023), landslide processes (Atanasova and Nikolov 2021; Pashova et al. 2021; Nankin et al. 2022), geoarchaeological mapping (Peev and Prodanov 2020; Prahov et al. 2020; Tzvetkov 2020; Vajsov et al. 2020) etc. The cited references prove the wide range of digital results obtained, making drones a suitable mapping tool for beach-dune systems along the BBSC.

Since 2018, IO-BAS has been using Phantom 4 Pro, Phantom 4 RTK and WingtraOne drones (Figs 2, 3). As of June 2022, over 97% of the Bulgari-

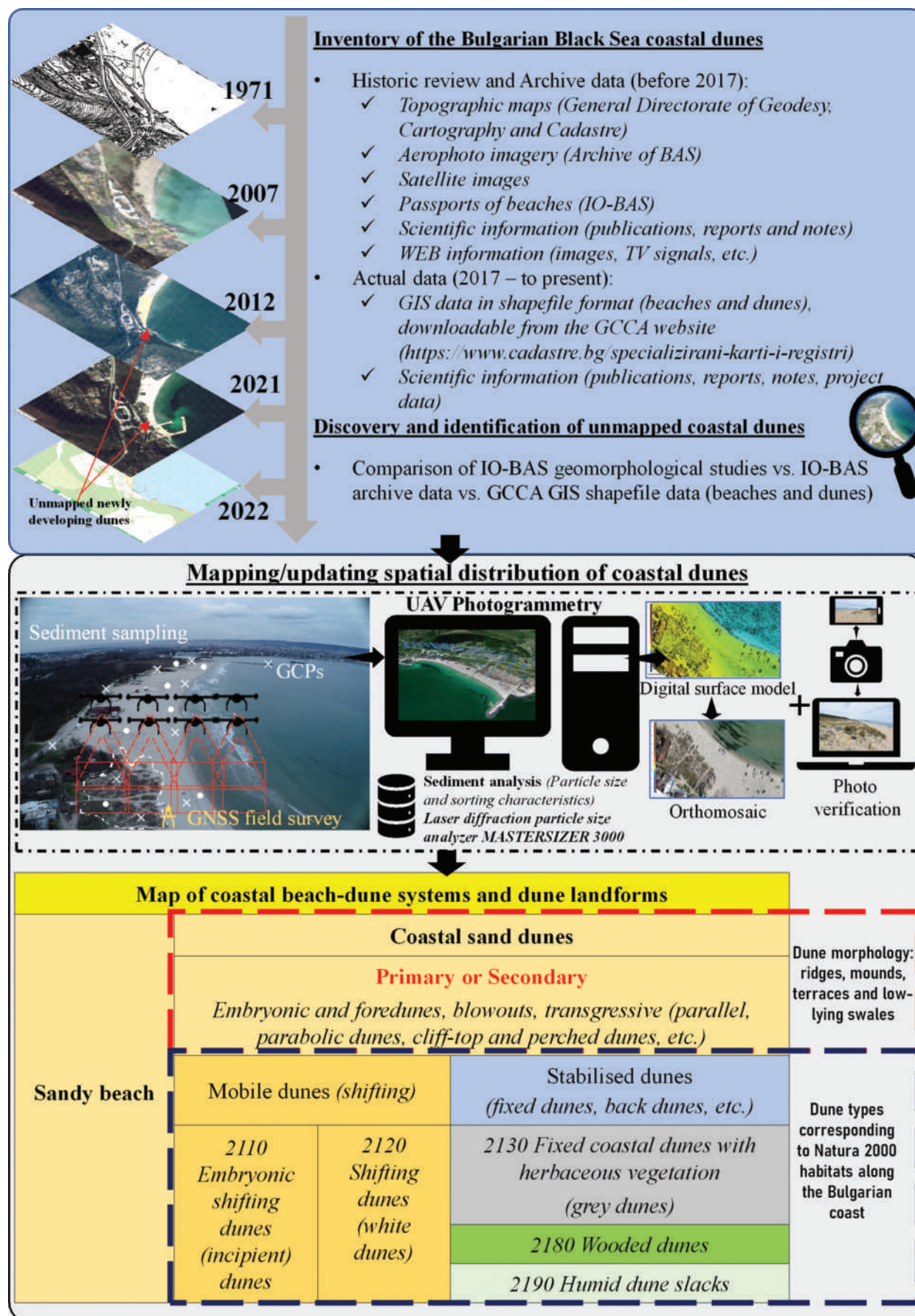


Figure 2. Methodology for inventory and mapping of coastal sand dune landforms along the Bulgarian Black Sea Coast.

an coast has been surveyed using these UAVs, excluding military areas and national security sites (Prodanov et al. 2020a etc.). Furthermore, geodetic GNSS surveys were performed to physically trace dune landforms in wooded areas (such as the Koral coastal area, town of Kiten) and to locate 2316 ground control points (GCPs) in order to increase the accuracy of the UAV photogrammetric measurements. In addition, photo verification of landforms and plant species within the range of beach-dune systems was performed at over a thousand locations (Figs 2, 3). Flight data were processed by specialised photogrammetric software, such as Agisoft Metashape, Pix4D and Global Mapper. The digital surface models (DSMs) were brought to the Baltic Height System. All vector and raster files representing survey results were combined into a coherent geodatabase, with WGS 1984/UTM zone 35N as a spatial reference.

Grain-size sampling and analysis of beach-dune sediments

Sediment sampling was performed by the IO-BAS team within each beach-dune system along the BBSC. Samples were taken at 1632 stations between February 2018 and May 2022 along morpho-lithologic transects and distinct locations on the CDLs (Fig. 3). Sands were collected from the beach face, beach face crest, summer berm, high beach winter berm, seaward dune slope, the crest and lee slope of the primary dune, a transition zone between primary and secondary dunes, middle sections of secondary dunes and the end of the secondary dunes. Sediment characteristics were determined using a Mastersizer 3000 Laser Diffraction Particle Size Analyzer in the Laboratory of Lithodinamics and Sediment Analysis at IO-BAS. The study used a grain-size distribution and statistics package for the analysis of unconsolidated sediments by sieving and laser granulometry (Blott and Pye 2001). The sediment stations were classified according to the Wentworth grain-size scale (Wentworth 1922) and Gradistat (Blott and Pye 2001).

Beach-dune systems and hierarchical subordination of sectors

For CDLs, two main levels of hierarchical subordination were used. Primarily, they correspond to **(I)** beach-dune systems - they represent unified beach and dunes in a geomorphological sense often named on their localities (Fig. 4, Suppl. material 1). The secondary level is **(II)** beach-dune sectors compliant with the coastal thematic module of CAIS (Suppl. material 1).

Identification and classification of coastal dune landforms

The geomorphological field surveys were conducted systematically outside the tourist season and low level of anthropogenic influence on dunes. In the present study, the spatial distribution of CDLs was based on morphometric analyses of the DSMs, visual analyses of the OM or 3D photorealistic models, as well as in situ verification. From a geomorphological point of view, the division of CDLs into primary (embryonic and foredunes) vs. secondary (blowouts, transgressive, parabolic, cliff-top, perched etc.) or fixed vs. mobile dunes is important for the study of land-sea interactions and coastal natural resources.



Figure 3. Example of field surveys and UAS mapping of coastal dune landforms using WingtraOne and DJI Phantom 4 RTK drones at Kavatsite-Smokini beach-dune system (Location on Fig. 4).

The vegetation field observation (verification) was conducted systematically in the summer (July -August) of 2021 and 2022. In the light of the Natura 2000 European ecological network and the EU Habitats Directive, the inventory of CDLs carried out and newly mapped dunes were classified according to their characteristics in the sense of the Red Data Book of Bulgaria (Tzonev 2015a, 2015b, 2015c, 2015d; Tzonev and Gushev 2015). Furthermore, field observation of the vegetation type and anthropogenic influence on the dunes was conducted within the active touristic period in the summer seasons of 2021 and 2022. As a result, the CDLs were divided into shifting/mobile dunes (embryonic and white dunes) and relatively stabilised dunes (fixed grey dunes, wooded dunes and humid dune slacks) - Suppl. material 1.

Results

Spatial distribution of dune systems along the Bulgarian Black Sea coast

The results presented herein summarise the field study of the IO-BAS team in the period between 2018 and 2022. The combination of UAS (drone) mapping, DSM-based morphometric analysis, sediment sampling, visual recognition (verification) and plant reconnaissance allowed the identification of 46 beach-dune systems along the BBSC (Fig. 4, Suppl. material 1). The coastal dune systems consist of 14% of the Bulgarian shoreline (total length of 73 km) with an aggregate area of 988 ha (0.0089% of Bulgaria). A comprehensive analysis of the relationships between local morphology, aeolian and morphodynamic processes and stabilization by density vegetation was the basis for dune classification into two broad types: primary and secondary.

Primary dunes are located closest to the shoreline. They are significantly affected by wave processes (e.g. overwashing, storm erosion) and are in a dynamic interrelationship with the beach. Primary dunes include embryonic dunes on the backshore and foredunes on the seaward edge of the dune system. Their area amounts to 311.9 ha (32% of the total dune area). As a result of coastal progradation, secondary dunes (dune systems) are found further inland, where they are no longer affected by wave processes. These dunes encompass various landforms, including blowouts, dune fields, parabolic dunes, transgressive dunes, mature foredune ridges and cliff-top (perched) dunes. These distinct types of coastal dune landforms collectively span an area of 676.3 ha, accounting for 68% of the total dune area.

According to Natura 2000 Habitats, the most widespread is the stabilized grey dunes (546.47 ha, 55.28% of the total dune area). They are typical for the entire BBSC. The white dunes are mainly confined to the foredune zone and have an area of 150.30 ha (15.21% of the total dune area). The shifting embryonic dunes are common in front of foredunes, with a total area of 68.30 ha (6.91%). Wooded dunes are characteristic of the backwardmost parts of the beach-dune systems. In nearly all cases, they mark the outer end of the dunes and their area is 222.61 ha (22.53% of the total dune area). The least widespread type is the dune slacks, with an aggregate area of barely 0.94 ha (0.09% of the total dune area).

Dunes along Dobrudzha coast (Cape Sivriburun - Batova River Mouth)

Four beach-dune systems have been identified and mapped along the northern Bulgarian coast: Durankulak, Krapets, Ezerets-Shabla and Bolata (Fig. 4, Suppl. material 1). Examples of well-formed primary and secondary dunes are observed in the Durankulak-North coastal sector. The first row represents foredunes of medium sand and the wide beach strip provides conditions for the formation of embryonic dunes. Secondary dunes in the section are flat-to-undulating sand sheets to sand sheets that rise in elevation downwind and end up in a slip face, for which we will use the term ramp dunes. After the construction of the Durankulak Fishing Port (Fig. 5), the geological conditions and the exposure of the coast to wind waves (Valchev et al. 2014) have contributed to the accumulation of a narrow beach and adjacent embryonic dunes have been formed (Fig. 4, Suppl. material 1).



Figure 4. Spatial distribution of sand dune systems along the Bulgarian Black Sea coast.



Figure 5. 3D model of newly identified coastal sand dune landforms north of Durankulak Fishing Port from 2018 (see location on Fig. 4).

Dunes accumulated over barrier beaches attached to the land at both ends are characteristic of the Dobrudzha coast. Mobile white and stabilised grey dunes separate Durankulak, Ezerets and Shabla lakes from the Black Sea. The average width of the dune systems is approximately 60 m. South of Cape Shabla, the erosional morphology dominates. The coast consists of karstified rock platforms or vertical cliffs without sand beaches. A small sand-dune system exists in the area of Bolata Cove. The dunes are small in size and close to the shoreline (Prodanov et al. 2019b). Although they are protected by an old dilapidated military port, they have been subjected to high anthropogenic pressure and are almost completely destroyed. Sediment profiles show the presence of coarse sand in the active beach area, passing into medium sand within the primary and secondary dune areas. The finest sands occur at the end of the secondary dunes located immediately next to lakes or backwoods at Durankulak - North.

Dunes along Franga-Avren coast (Batova River Mouth - Cape Paletsa)

The Franga-Avren coast is also known as the Varna coastal region (Fig. 4). Coastal dune landforms were identified and mapped in the following sectors: Batova River Estuary, Zlatni Pyasatsi Panorama Resort (Fig. 6), Asparuhovo quarter (Varna) – Fig. 7 and Pasha Dere Ravine. South of the Batova River Estuary, the specific geological and geomorphological conditions do not allow the formation of long beach-dune systems. Most beaches are narrow and heavily altered by the thriving tourism along the Varna coast. Beaches have been exploited and groomed for many years, which does not allow the formation of dunes on them. In some places, small embryonic dunes were found that did not exceed the minimum mapping unit and were, therefore, not reflected in Suppl. material 1.

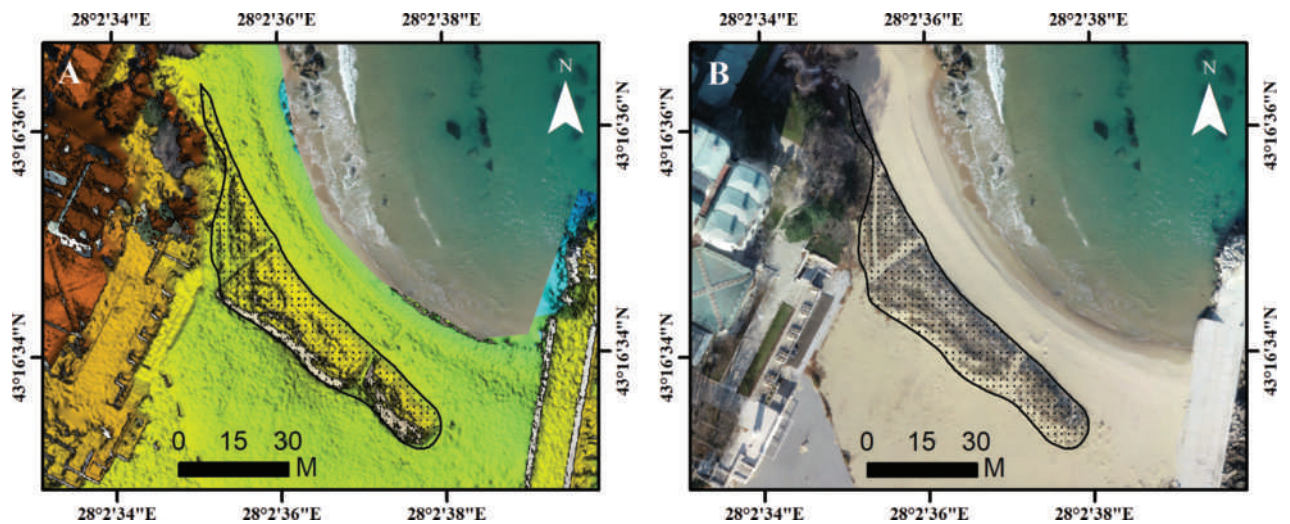


Figure 6. Newly identified coastal sand dune landforms at Riviera Beach, Zlatni Pyasatsi Resort (A digital surface model B orthophotomosaic from 2022) - (see location on Fig. 4).

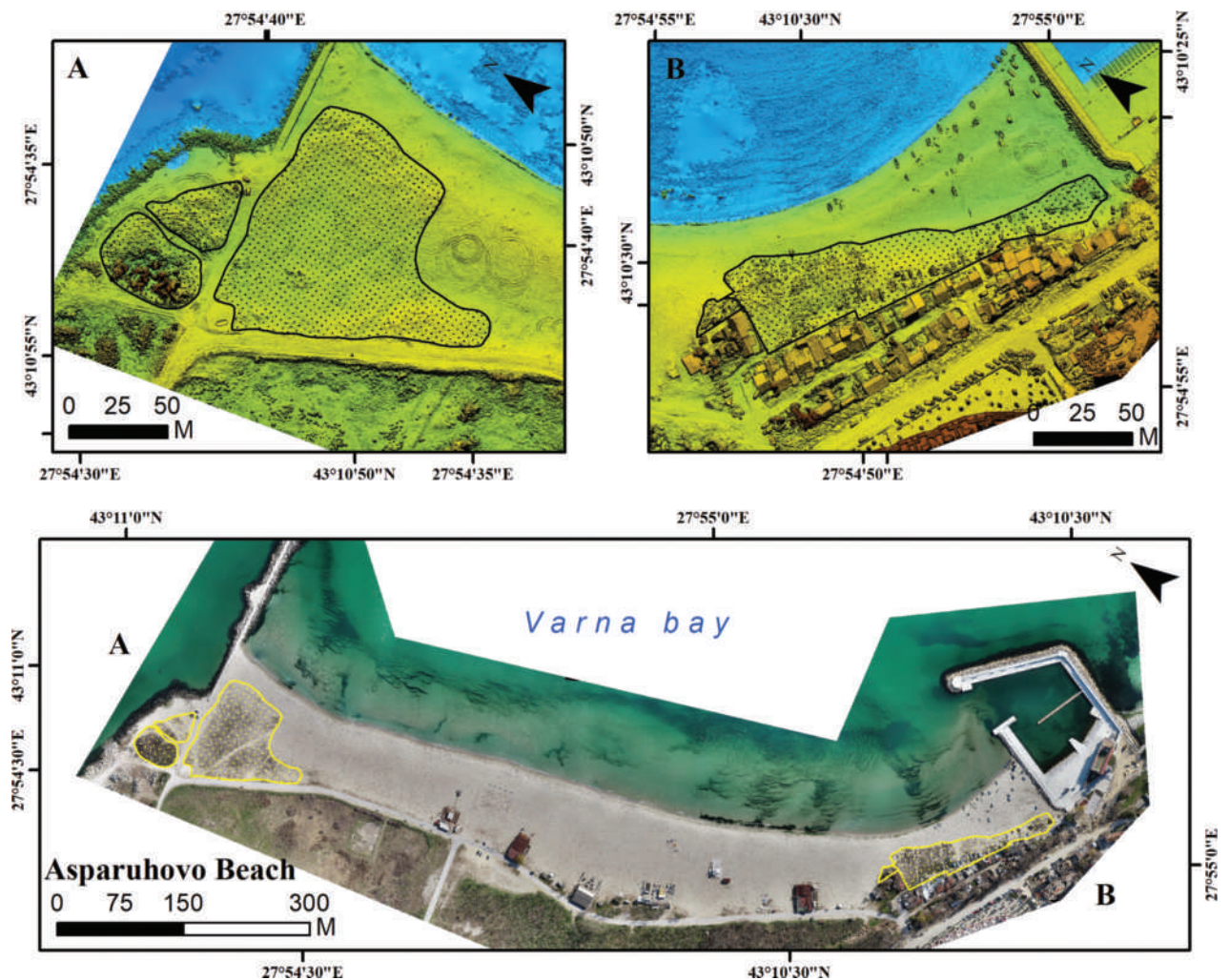


Figure 7. Newly identified coastal sand dune landforms (embryonic, grey and wooded dunes) at Asparuhovo Beach in the southern part of Varna Bay (A digital surface model B orthophotomosaic from 2022) - (see location on Fig. 4).

In the second half of the 20th century, a period of mass industrialisation, the Varna coast was heavily armoured by hydrotechnical facilities – groins, sea-walls etc. To a large extent, these constructions have changed land-sea interactions. These help for dune formation at some urban and resort sectors of the Varna coast by the deposition of sea sand after the construction of the T-groins (Fig. 6) and Karantinata Fishing Port (Fig. 7). A few remnants of the large dune field separating Varna Lake from the Black Sea remained under the landscaped part of Asparuhovo District. South of Cape Galata, the development of embryonic dunes positioned in front of the Pasha Dere Ravine was reported.

Dunes along Kamchiya coast (Cape Paletsa – Cape Karaburun)

Kamchiya coast is emblematic of the BBSC. The shoreline between Capes Paletsa and Karaburun is 12.9 km long, which makes Kamchiya-Shkorpilovtsi the longest beach on the Bulgarian coast. Over 90% of it is a well-formed beach-dune system with an area of 326 ha, comprising five different sectors: Kamchiya – North (Romantika Campsite), Kamchiya River Mouth, Kamchiya-South (Novo Oryahovo Village), Shkorpilovtsi and Shkorpilovtsi-South (Samotino Village). Dunes in the five sectors differ due to variations in the vegetation types, geological and geomorphological processes that affect them. North of the Kamchiya River mouth, low, up to 1–1.5 m high embryonic dunes were formed along the entire beachfront (Prodanov et al. 2021b). Immediately next to the river mouth, in a small area between the flood terrace and the road, stable wooded dunes are present (Fig. 4, Suppl. material 1). The low-lying relief is a precondition for the different nature of the dunes near the villages of Novo Oryahovo and Shkorpilovtsi. In some areas, the secondary (wooded) dunes reach up to 1 km inland, where the sand is still medium-grained. Along the flattened central dune fields, prevalence of grey dunes was established. Their stabilised sand ridges vary between 1.5 and 3 m in height. The foredunes are mainly white (mobile), at places armoured by embryonic mobile dunes of small size up to 25–40 cm high, formed by medium-grained sand.

Passing south from the mouth of the Fundakliyska River, the beach-dune system becomes narrower and the existing grey dunes are limited by a road that marks the northern border of the Balkan Mountains. This narrow strip is an example of misclassified dunes in CAIS. It is important to note that a wide strip of wooded dunes was recorded in the Shkorpilovtsi - South (Samotino) sector. During the field surveys in the Cape Karaburun direction, it was established that the narrow sand-dune system of embryonic and grey dunes passes over steep cliffs covered by cliff-top wooded dunes (Fig. 4, Suppl. material 1).

Dunes along the Balkan Mountains coastal area (Cape Karaburun – Aheloy)

In this sector, there are steep flysch cliffs all along the shoreline with narrow beach strips at the cliff bases, formed mainly by eroded and abraded material. In the southern part, the cliff descends almost to sea level at the Hadzhiyska River Mouth and the low cliff sections between the towns of Nessebar and Aheloy. The depositional relief is represented by stable beach-dune systems

at Kara Dere Ravine, the town of Byala, Irakli Protected Area, Slanchev Bryag (Sunny Beach) Resort and the town of Nessebar (Figs 4, 8, Suppl. material 1).

Small embryonic dunes (0.287 ha) were identified and mapped during the field surveys. These dunes have formed after the construction of the Byala fishing port that has created a sheltered environment from the eastern storm processes, facilitating aeolian and deposition processes. Over the past three years, the dunes have progressively transitioned into white (shifting) dunes. It is important to pay attention to their preservation in the future, as they are in an area with strong anthropogenic pressure, such as fishing activities, boat launching etc. In Kara Dere, Vaya River Mouth and Irakli sectors, an extension of the embryonic dunes and some newly formed ones have been discovered (Fig. 4, Suppl. material 1).

The most significant beach-dune systems along central parts of BBSC are observed in the coastal sectors of Slanchev Bryag Resort and the town of Nessebar (Fig. 4, Suppl. material 1). The low-lying relief and abundance of sand material at Slanchev Bryag Resort are the main reasons for the presence of coastal dune landforms inside the resort complex. Over the years, many secondary dunes have been formed. Embryonic dunes, white (shifting) dunes, grey (fixed) dunes (GD) and wooded dunes (WD) with a total area of 46.98 ha were identified. The comparative spatio-temporal analysis of coastal dune dynamics showed that 14.80% of their area was subjected to strong anthropogenic pressure as a consequence of expanding tourism (Fig. 4, Suppl. material 1).

The Nessebar dunes are distributed between the Nessebar Sand Dune Field and Nessebar – South coastal segments (Fig. 8). Their area is almost twice as large as those of Slanchev Bryag. Again, the local geomorphological conditions are the leading factor in shaping out their modern boundaries. At the Nessebar Sand Dune field, dunes are 100% secondary, formed under conditions of intense aeolian transport in the past. The sediment nourishment of the Nessebar grey and wooded dunes nowadays is rather limited due to increased distance from the source province (the beach of Nessebar - South) as well as the presence of primary foredunes and vegetation cover posing an effective barrier to aeolian transport. Another coastal issue at the Nessebar Sand Dune Field in terms of sand nourishment is the urbanisation and large-scale development of the Mladost residential area of Nessebar in the 1960s that altered the wind flow from the north side of the dunes at Slanchev Bryag - South and the associated aeolian sand transport (Fig. 4, Suppl. material 1).

Dunes along the Burgas coast (Aheloy – Chernomorets)

Pomoriyska Sand Spit, Pomorie, Sarafovo, Burgas (Central), Burgas (South), Kraymorie, Vromos and Chernomorets beach-dune systems have been identified and the dunes on Atanasovka Sand Spit were mapped for the first time (Fig. 4, Suppl. material 1). Over the years, numerous factors have had an impact on the coastal spatial pattern of Burgas and Pomorie Bays. Similar to Durankulak Lake, Pomoriysko and Atanasovsko Lakes are separated from the sea by sand strips known as Pomoriyska and Atanasovska sand spits. Geomorphologically, they also represent barrier beaches attached to land at both ends and covered with well-formed foredunes with a maximum height of 3.7 m (Suppl. material 1). Over the years of development, the sand spits have been colonised

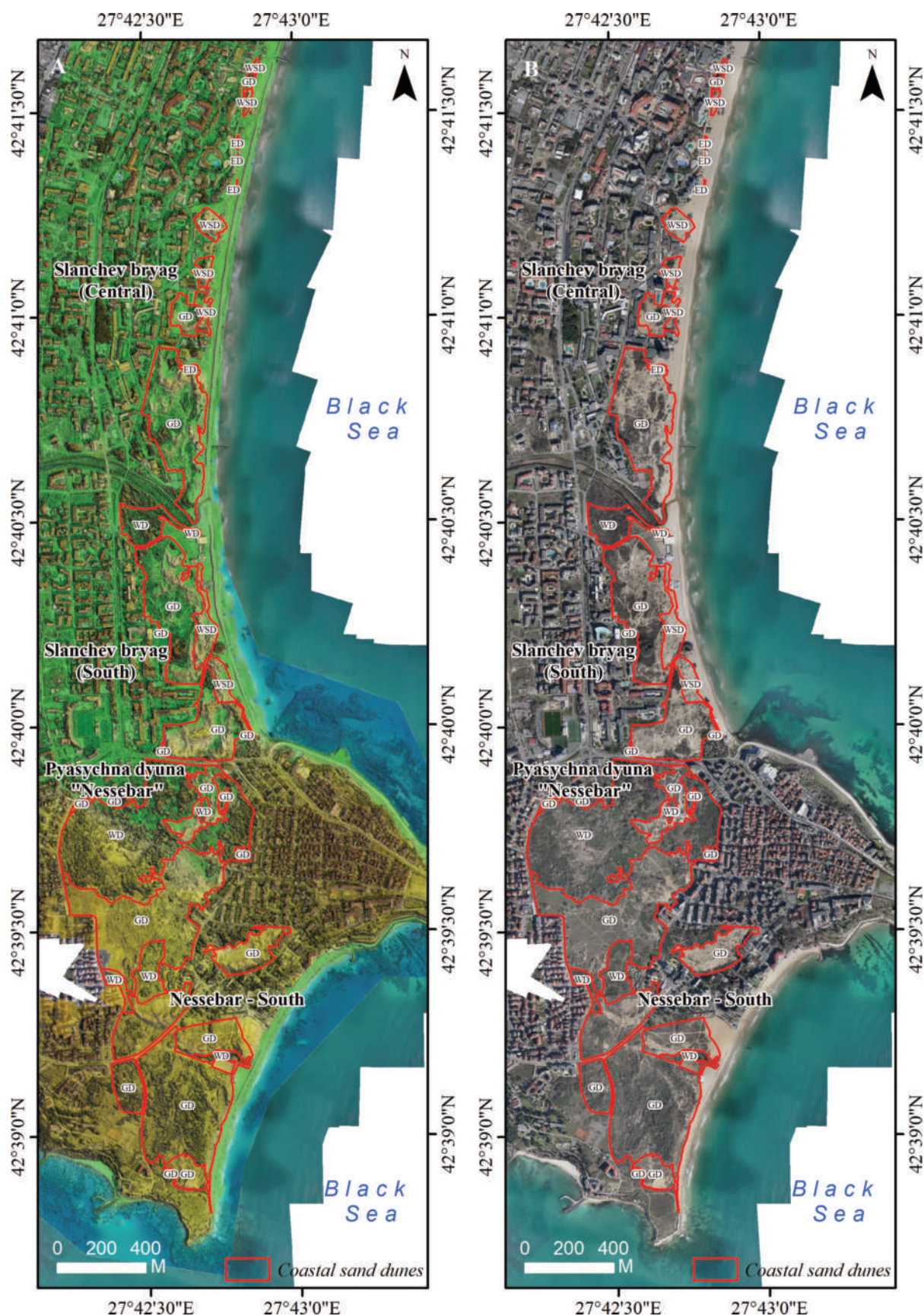


Figure 8. Coastal sand dune systems at Slanchev Bryag Resort and Nessebar coastal sectors - embryonic dunes (ED), white (shifting) dunes (WSD), grey (fixed) dunes (GD), wooded dunes (WD) **A** UAV photogrammetric DSM from 2022 **B** UAV orthomosaic from 2022 (see location on Fig. 4).

by specific psammophilous species characteristic of grey dunes, for example, sand cornflower (*Centaurea arenaria* M.Bieb. ex Willd.), white-stemmed musk (*Jurinea albicaulis* subsp. *kilaea* (Azn.) Kožuharov), Euxinian campion (*Silene euxina* (Rupr.) Hand.-Mazz.), milkweed (*Cionura erecta* (L.) Griseb.) etc. (Tzonev 2015c). In the Sarafovo beach-dune system, grey dunes also predominate over white ones. Dunes composed of medium-grained sand have been developing unhindered within the Burgas (Central) Beach for many years. Although moderately exposed to wave action (Valchev et al. 2014), positive sediment balance and weak, but constant accretion have contributed to the formation of mobile embryonic and white dunes (Fig. 9, Suppl. material 1). Our research team mapped these dune landforms for the first time at the beginning of 2022. Unfortunately, as of October 2022, they still do not appear in the thematic coastal module of CAIS, necessitating an immediate update of the relevant coastal geodatabase of GCCA.

Strong anthropogenic pressure on these dune systems was registered in the last years of field surveys. South of the City of Burgas, the dunes undergo even more significant human alterations, affecting up to 65–70% of their area in the Burgas (South) and Burgas (Pobeda quarter) depositional sectors (Fig. 4, Suppl. material 1). The permanently lost beach-dune systems were established along the Burgas coast: Pomorie (East), Europa Campsite, Lahana-West, Lahana-East and Rosenets (West) at Cape Chukalya (Fig. 4).

Dunes along Medni Rid - Strandzha coast

The largest number of beach-dune systems (25) have been identified along the Medni Rid - Strandzha coast. Out of 157 km of shoreline between the town of Chernomorets to the north and the Village of Rezovo to the south, approximately 32 km represent depositional relief comprising the following beach-dune systems: Gradina Campsite (Sozopol Bay), Sozopol, Harnani and Kavatsite-Smokini Beaches (Fig. 10), Alepu and Arkutino Lagoons (Fig. 11), Ropotamo River mouth, Primorsko (Stamopolu – Perla Beach), Primorsko – South Beach, Atliman Bay, Kiten, Koral Beach and Yug Campsite, Lozenets, Oasis, Arapya, Tsarevo (Popski plazh), Nestinarka and Ahtopol Beaches, Veleka and Butamyata River mouths (Fig. 12), Lipite and Listi Beaches, Silistar River mouth and Rezovo-Kastrich Beach (Suppl. material 1). Generally, the systems consist of well-formed primary dunes, including foredunes and incipient dunes. Usually, the first row is represented by 7–9 m in high white/grey dunes and armoured by embryonic dunes (Kavatsite, Smokini, Arkutino etc.). In rare cases, as at Butamyata Beach, the dune field is further shaped out by the small estuary. However, in the last ten years, dunes have evolved from small embryonic to grey ones and occupy a much larger portion of the beach. Unfortunately, this beach-dune system has been systematically destroyed every year by excavation, trampling and grooming as part of the beach preparation for the summer season because a significant portion of them is not reflected in the coastal thematic module of CAIS. In this sense, the Kavatsi Dune Area is an example of strong anthropogenic pressure on the dunes (Valcheva et al. 2022). Therefore, an urgent update is required of the relevant geodatabase of GCCA in order to protect them (Suppl. material 1).

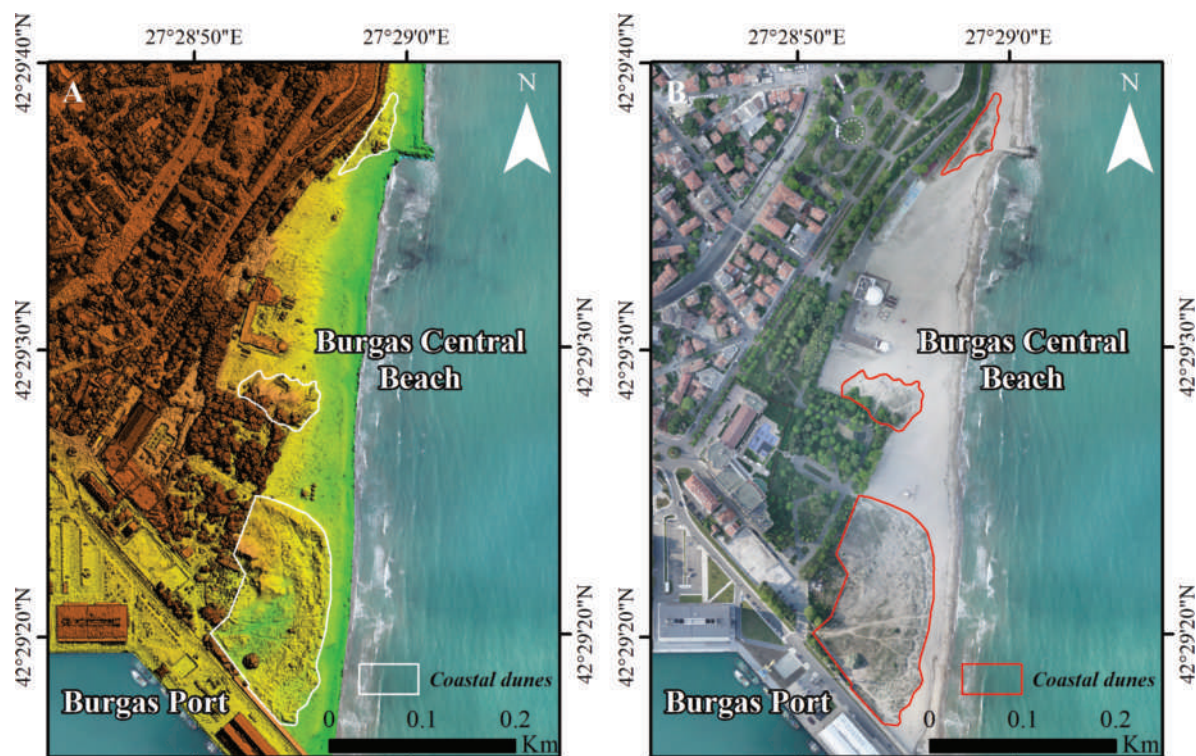


Figure 9. Newly identified coastal dune system at Burgas (Central) Beach - embryonic dunes (ED), grey (fixed) dunes (GD)
A UAV photogrammetric DSM from 2022 **B** UAV orthomosaic from 2022 (see location on Fig. 4).

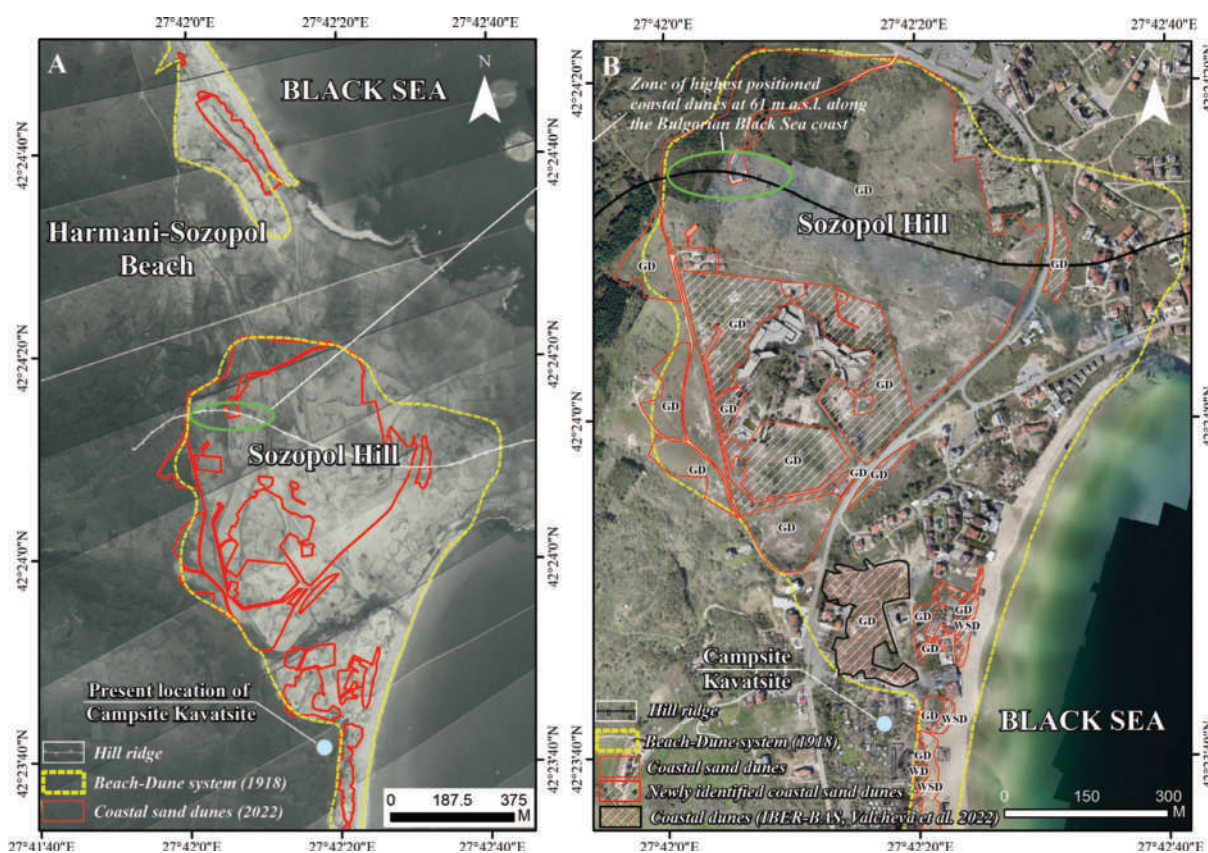


Figure 10. Coastal sand dune systems at Kavatsite Beach, Sozopol - embryonic dunes (ED), white (shifting) dunes (WSD), grey (fixed) dunes (GD) and wooden dunes (WD); **A** spatial distribution of beach-dune systems in 1918 (Aerial Photomosaics 1918) **B** UAS orthomosaic from 2022.



Figure 11. **A** an aerial view of the Arkutino beach-dune system from 2022 (photography of Dr. Bogdan Prodanov) **B** UAV digital surface model of the Arkutino sector with the second highest positioned coastal dunes (50.2 m a.s.l.) along the Bulgarian coast from 2022 (see location on Fig. 4).

The highest positioned coastal dunes along the Bulgarian coast were registered at the perched **Sozopol Sand Dunes** (grey dunes), located at Sozopol Hill at an impressive height of 61 m above sea level in coordinates: 42°24'16.5907"N, 27°42'00.0501"E (Fig. 10). It is noteworthy to mention that, in the past, prior to the high level of anthropogenic impact on the Sozopol Hill and Budzhaka Locality, these dunes have been nourished with sand through aeolian transport from two sources simultaneously - the Harmanite Beach (from the northern direction) and

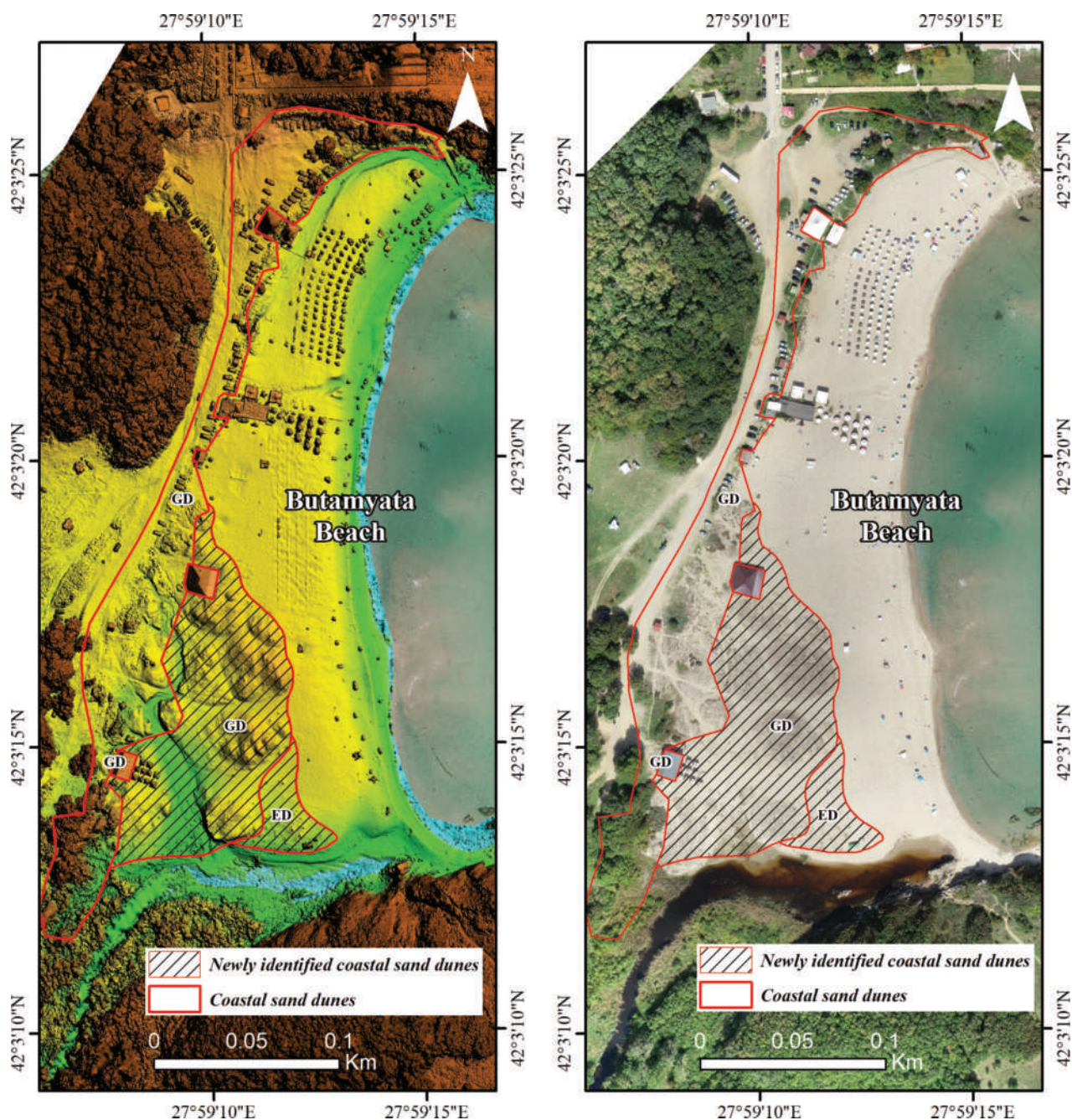


Figure 12. Coastal sand dune system at Butamyata Beach (Sinemorets) - embryonic dunes (ED) and grey (fixed) dunes (GD) **A** UAV photogrammetric digital surface model from 2022 **B** UAV orthomosaic from 2022 (see location on Fig. 4).

the Kavatsite Beach (from the southern direction) (Fig. 10, Suppl. material 1). The Sozopol cliff-top dunes have been formed by wind activity, which has transported medium to fine sand from the aforementioned beaches. However, over the last two decades, construction activities in the northern part of Kavatsi and the southern part of Harmani have discontinued the supply of sand to this secondary dune system (Fig. 10A). These dunes have had a chance to remain relatively undisturbed from human intervention, allowing for the colonisation and stabilisation of psammophyte vegetation on their sandy surfaces. The sediment analysis of seven stations taken within these dunes exhibits an extremely poor sorting, indicating a low intensity of morphodynamic processes of the dune sand at this hypsometric level.

The second highest positioned dunes were registered in Arkutino coastal sector. The system is composed of a long row of frontal dunes, discontinued by small-size blowouts, followed inland by mosaics of parabolic and cliff-top dunes, which reach a height of 50.2 m a.s.l at coordinates: 42°19'32.6673"N, 27°44'10.2816"E (Fig. 11). In many sections of the Medni Rid - Strandzha coast, the embryonic dunes have been colonised by psammophytes that are characteristic of white dunes, slowly increasing their area. As a result, 63% of the dunes are stabilised and, over the years, together with the wooded dunes, have preserved their area of 45 ha (12%). Most beach-dune systems consist of secondary dunes. They were classified as parabolic and cliff-top dunes, blowouts, sand sheets (dune fields) that rise in elevation downwind and end up as a slip face (Arkutino, the Sozopol Sand Dune Area etc.).

Discussion

General morpho-sedimentological profile of dune systems along the Bulgarian Black Sea coast

At this point, we have discussed some correlations and relationships in dune morphology and the spatial distribution of beach-dune sands. The analyses of available data show that 88% of the active beaches on the Bulgarian coast are mainly composed of medium-grained sand and the remaining 12% consist of coarse-grained sand. In rare cases, sediments from external sources were found during the surveys, but these were not included in the summary results. Sedimentological analyses from all sediment stations in the period 2020 – 2022 showed fine to coarse sands (from 0.131 mm to 0.78 mm diameter) deposited in the Bulgarian Black Sea beach-dune systems. Accumulative and aeolian processes have contributed to the formation of progradation beaches with alternating embryonic/white and stabilised/grey or wooded dunes (Fig. 13).

Kavatsite-Smokini beach-dune system is an excellent example, in which the general cross-shore profile shows a complex distribution of dunes subjected to intensive anthropogenic pressure due to tourist development and human trampling (Figs 14–17). A correlating cross-shore diagram of the grain-size distribution (D_{50}) and sorting of sediments is added to the general morpho-sedimentological profile and shows decreasing grain size diameter in the landward direction. Analyses of sand from the beach face, beach face ridge, summer berm and high beach winter berm at each beach identify that moderately well-sorted coarse sands (greater than 0.5 mm in diameter) dominate the seaward slope of the main dunes and the active beach that is influenced by wave processes. Foredune morphology continues to evolve as foredune ridges develop through a combination of reduced sediment supply and increased surface stabilisation by plant species. As a result, they are mostly composed of well-sorted to moderately well-sorted medium sands. An inverse relationship was established for the Bulgarian Black Sea dune systems - as the distance from the ridge of the primary front dune to the land increases, the diameter of the sediment decreases within the average size of the sand, while its sorting increases to well-sorted (Fig. 13).

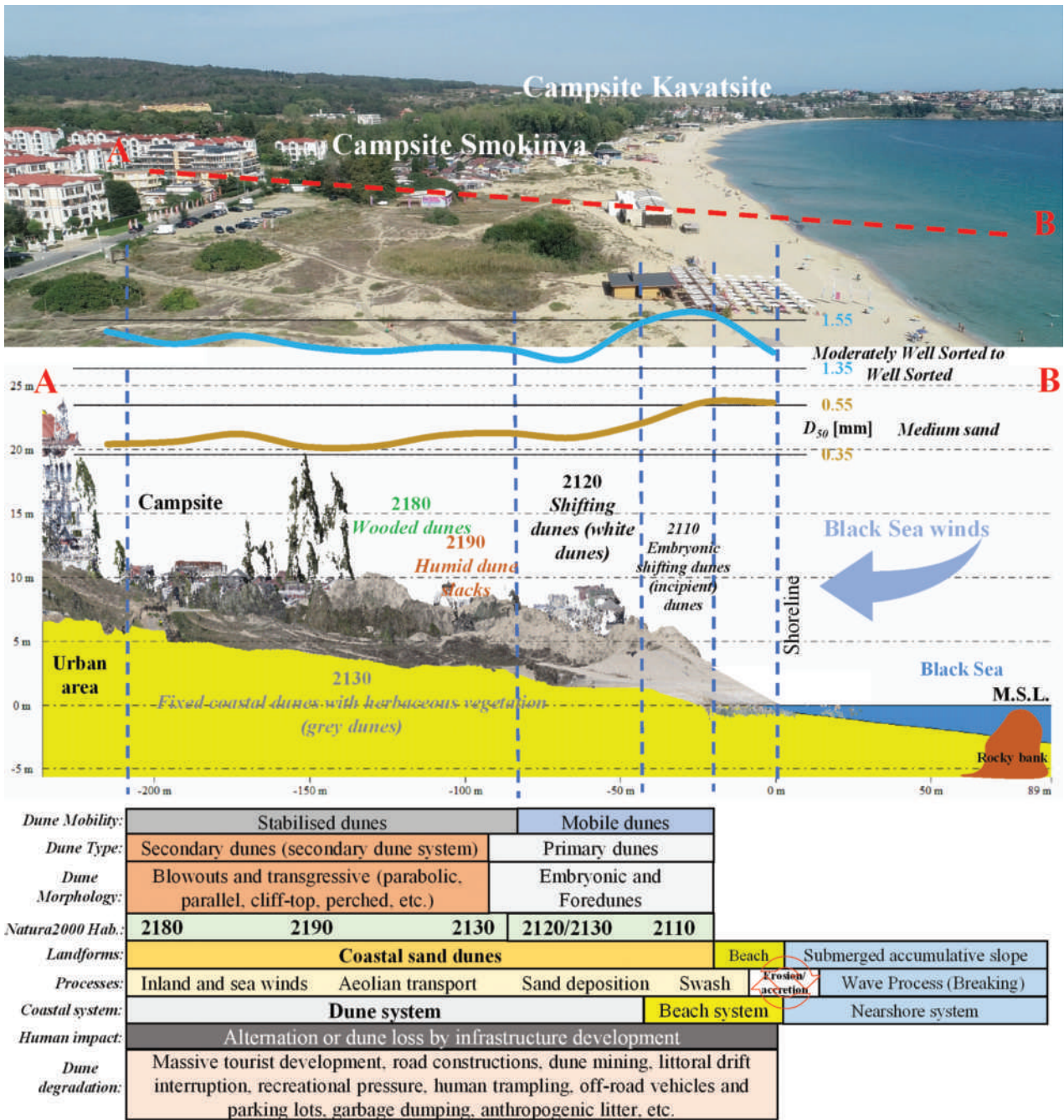


Figure 13. A general geomorphological profile of the beach-dune systems along the Bulgarian Black Sea coast (example of the Kavatsite-Smokinya beach-dune system).

Alteration, degradation and loss of coastal dunes due to human activities

The most important issue to be addressed is the increasing anthropogenic pressure on dune formations. The Bulgarian Black Sea coastal dune landforms have always been of interest to tourists and holidaymakers during the summer season. The development of tourism and the adjacent beach-dune infrastructure are the main reasons for the large altered area of 50.72 ha (5.1% of the total area of dunes). An extremely high degree of human intervention and dune degradation (over 50% of their area) was registered at seven dune systems: Byala, Burgas (South), Burgas (Pobeda), Asparuhovo (Varna),

Kamchiya River mouth and Bolata Cove, Kavatsite and Smokinya campsites (Fig. 4, Suppl. material 1).

Emblematic examples of dune mismanagement are within the resort towns of Sunny Beach, Nessebar and the campsites around Sozopol (Gradina, Kavatsite and Smokinya) - (Figs 14–17). In the last 15 years, the loss of coastal dunes along the Bulgarian Black Sea Coast due to various human activities has been estimated at 12.5 ha. The inventory found a permanent loss of 3.2 ha at five dune systems: Pomorie (East), Evropa Campsite, Lahana, Lahana (West) and Rosenets (West) near Cape Chukalya. The losses fall entirely within the Burgas coast. Shoreline exposure of the northern Burgas Bay coast and the impact of storm processes have resulted in necessary additional wave break construction and fortification to protect the Pomorie-Burgas Road. On the southern shore of Burgas Bay, during the construction of the port and the tourist complex at Cape Chukalya, 0.03 ha of white dunes have been wiped out (Fig. 4).

Human interventions that have caused the degradation of 50.72 ha (5.1% of dune areas) and 12 ha of permanent dune losses along the BBSC can be identified as a consequence of massive tourist development after the socialist period, road construction, dune recreational pressure, human trampling, lack of walkways over fixed and mobile dunes, off-road vehicles and parking lots (especially at campsites), garbage dumping and marine litter on CDLs. The above-mentioned losses of dune systems in the last 15 years are outside designated Natura 2000 sites, which directs the discussion towards their conservation beyond the boundaries of the European ecological network. Another issue remains the illegal effacement of dunes (mostly grey dunes) inside privately-owned land behind the beaches designated for real estate



Figure 14. Sand dunes breaching (degradation) from off-road vehicles and beach cleaning techniques at Veselie campsite, September 2022.



Figure 15. Beach bar on the white dunes at Smokinya Beach, South Bulgarian Black Sea coast, September 2022.



Figure 16. Destruction of the protective fence of “Pyasachna Liliya” Reserve by off-road, poor dune management and walkways, Southern Bulgarian coast, September 2022.

development. Coastal dunes within the limits of such cadastral parcels are often trampled and subsequently covered artificially with a soil layer in order to disguise the action, with the owners almost always stating that no dunes have ever existed on their property.



Figure 17. Example of recreational construction on the coastal sand dune landforms of Kavatsite Campsite, Southern Bulgarian Black Sea coast, September 2022.

Adequacy of protected areas boundaries in the light of CDL conservation: “Pyasachna liliya” Managed Reserve, Kavatsite Dune Area

A disturbing fact emblematic of coastal mismanagement concerning dune preservation is the current boundaries of “Pyasachnata liliya” (The Sea Daffodil) Managed Reserve at Kavatsite Area (Fig. 18). In fact, subject to preservation are three spatially separate dune polygons that are artificially united into a single protected area. Squeezed amongst adjacent campsites and recently established beach bars and nightclubs, the boundaries literally “slice” through the existing CDL ridge in the southern part of the Reserve. Such a “delineation approach” runs counter to fundamental principles of nature conservation when designing the extent of a given protected area, for example, spatial coherence, ecological connectivity and landscape-scale consistency of the boundaries.

Accordingly, ludicrous decisions of this kind towards an environmentally sensitive area raise questions regarding the adequacy of management decisions taken on behalf of the public environmental authorities, as well as towards the employees’ expertise who are in charge of coastal nature conservation. Additionally, Fig. 18 demonstrates an example of the negative anthropogenic influence of rake-up (cleaning) of beach sand, causing physical destruction/loss of embryonic dunes in front of the Managed Reserve.

Long-term legal preservation of coastal dune landforms in Bulgaria

As of today, the network of protected areas preserving coastal dune landforms in Bulgaria remains inadequate. Nevertheless, it is crucial to note that dunes are granted special legal protection according to the BSCSD (2008). Currently, a common approach in Bulgaria involves designating coastal dune areas as natural landmarks, compliant with the Protected Areas Act of the Republic of Bulgaria (PAA 1999).

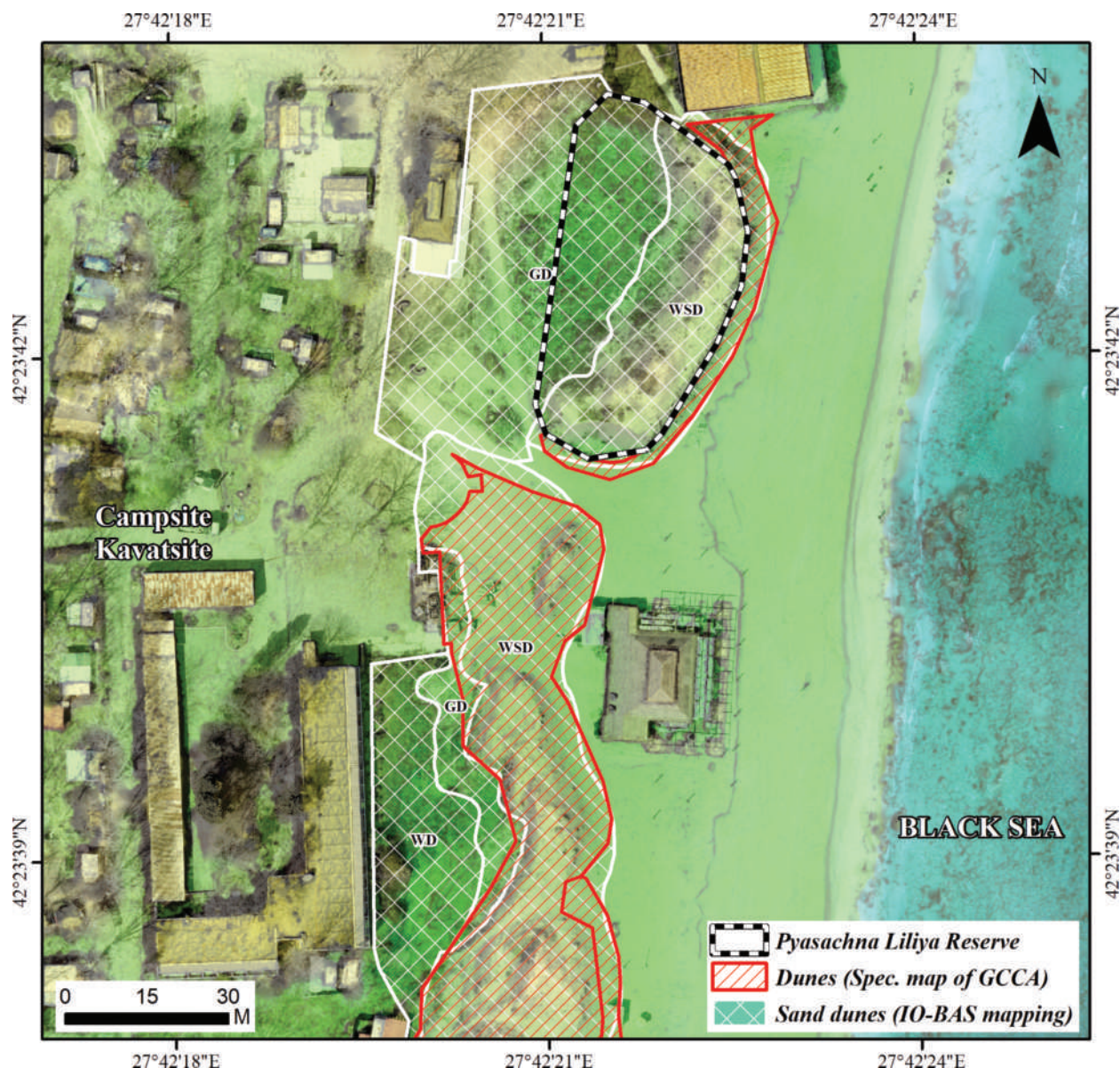


Figure 18. Coastal dune landforms at “Pyasachna liliya” Managed Reserve, Kavatsite Area: embryonic dunes (ED), white (shifting) dunes (WSD), grey (fixed) dunes (GD), wooded dunes (WD). An example of physical rake-up/cleaning of beach sand, causing degradation of embryonic dunes.

In light of the existing situation, the authors of the study herein strongly advocate for necessary legal actions to be initiated by the Ministry of Environment and Water. Their proposal is to designate all remaining dune areas along the Bulgarian coast as natural landmarks, aligning with the provisions stated in the above-cited act. This step, on behalf of the relevant authorities, would significantly enhance the protection of these ecologically valuable coastal landforms.

Data adequacy of CAIS, thematic module for the Bulgarian Black Sea Coast

Results of our surveys revealed several weaknesses and data discrepancies of the coastal thematic module (shown in Suppl. material 1) discussed herein, amongst which:

- Obsolete data, incorrect classification and erroneous cartographic representation (i.e. spatial and thematic) of coastal beaches and dunes and incorrectly illustrated spatial extents of beaches and dunes;
- Incorrectly depicted contours (boundaries) of geomorphological features (i.e. coastal beaches, dunes, estuaries, lagoons, wetlands etc.).

The above drawbacks dictate the necessity for rapid measures aimed at data accuracy improvement of the specialised maps maintained by GCCA and the related geospatial files of the coastal geomorphological features in CAIS. Amongst these, improvement of the coastal surveying approaches, including the use of UAVs as equivalent to the geodetic survey on the ground, mandatory participation of experts with proficiency in coastal geomorphology and dune phytocoenology in the mapping campaigns, as well as the willingness of the executive authorities in charge to collaborate with academia is highly recommendable and in line with the recently-amended legislation in the field of coastal spatial development (Ordinance No.1/Art.6 2020). Furthermore, designing a scientifically sound national programme for mapping and monitoring the geomorphological features and hydrotechnical facilities along the BBSC should be amongst the priorities of the MRDPW in Bulgaria. Finally, such a national programme should naturally include scientifically sound guidelines for mapping with clear (non-ambiguous) definitions of dunes of all types and dune fields/systems in morphological and habitat aspects and subsequent cartographic representation of the results, based on good practices from other littoral EU Member States.

Conclusions

The integration of remote sensing methods in geomorphological mapping and monitoring dune formations and anthropogenic changes is demonstrated in the study presented. The identification of a significant number of previously unknown dune landforms within a span of two years was made possible by the easy utilisation of unmanned aerial systems. Based on our UAS surveys, the Bulgarian Black Sea shoreline length was estimated to be 518.7 km at a scale 1:5000, stretching between Cape Sivriburun and Rezovo Village. The sand beaches encompass 131 km (25%) of the Bulgarian coastline. The coastal dune systems consist of 14% of the Bulgarian shoreline (total length of 73 km) with a total area of the Bulgarian coastal sand dune landforms at 988.21 ha (9.8 km²), 0.0089% of Bulgaria, differentiated into 46 beach-dune systems.

The primary dunes along the Bulgarian coast are significantly influenced by wave processes and have a dynamic interrelationship with the beach, but it is essential not to forget their coastal flood defence role. The area of primary dunes (foredunes and embryonic dunes) is 311.9 ha (31%) and their distance to the shoreline varies from 12 m to 80 m for wide beaches. Within the BBSC, secondary dunes (676.3 ha; 69%) prevail over the primary ones. These secondary dunes are positioned further inland, where the effect of the wave process was not observed. Given their mobile character, embryonic dunes rarely exceed 1.5 m in height, while the foredunes in places reach 7 m at Arkutino dunes, 9 m at Kavatsite-Smokini dunes and 4–5 m at Durankulak and Krapets, but in general, they are a long ridge with average heights of 3.5–4.2 m along the Bulgarian Black Sea Coast.

The different natures of dunes are determined by morphodynamics and, subsequently, stabilisation by vegetation. As we have already noted, most foredunes are presented by shifting (white) dunes – 150.3 ha (15.2%) and “armoured” by embryonic dunes – 68.3 ha (6.9%). Generally, CDLs are dominated by grey (fixed) dunes – 546 ha (55.3%) and wooded dunes – 222 ha (22.5%), which reach 1 km inland between Kamchiya River Mouth and Shkorpilovtsi Resort. Dune slacks are minimised and observed in the context of enclosing dunes – 0.94 ha (0.09%).

The largest mapped dune systems, such as Kamchiya-Shkorpilovtsi (326 ha), Nessebar (98 ha), Arkutino (94 ha), Kavatsi-Smokini (59 ha), Gradina (57 ha), Slanchev Bryag (47 ha), include mature foredune ridges, blowouts and transgressive dunes. In addition, they comprise cliff-top dunes, perched dunes, flat, vegetated plains and interdune slacks. The cliff-top or “perched” dunes are relatively uncommon, in general, but in some cases, such as Nessebar, Sozopol, Kavatsite, Krapets, Durankulak, Arkutino, Koral, Ahtopol etc., the geomorphological settings and dominant wind direction predispose the migration of sand inland over the bedrock or, in some cases, over loess surfaces as of Krapets and Durankulak. The highest positioned coastal dune landforms on the Balkan Peninsula were found at “perched” Sozopol Sand Dunes (61 m a.s.l.) and Arkutino dunes (50.2 m a.s.l.).

Massive tourist development and poor dune management (regulation, restriction, actual data about spatial distribution, lack of short-term monitoring of dune integrity) have caused destruction and dune surface changes on 50.7 ha in 5.1% of dune areas (see Table 1). For example, the inventory shows permanent losses of five beach-dune systems (12 ha) for the last 15 years along the Burgas coast, located outside Natura 2000 sites. Within this research, we identified and mapped eight dune systems for the first time: Zlatni Pyasatsi – Panaroma, Asparuhovo – Varna, Byala, Atanasovska kosa, Burgas – Central Beach, Chernomorets, Kavatsite (partly) and Rezovo-Kastrich. A stunning fact is that 16.8% of the dunes identified are outside the Natura 2000 network, mainly around the cities and resorts along the Bulgarian Black Sea coast.

Table 1. General information of coastal dune landforms, anthropogenic area and permanent loss of dune systems along the Bulgarian Black Sea Coast up to 2022.

CDL area [ha]	Anthropogenic area of CDLs		Morphological type of CDLs, [ha]		CDLs, corresponding to Natura 2000 habitats, [ha]					Permanent loss of CDLs [ha]
	[ha]	[%]	Primary	Secondary	ED 2110	WSD 2120	GD 2130	WD 2180	HDS 2190	
988.21	50.04	5.06	311.9	676.3	68.30	150.15	546.41	222.61	0.94	12
Percentage, %			32	68	6.91	15.19	55.29	22.53	0.09	five dune systems

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

B.P. conceptualised research, inventory, geomorphological and GIS analysis; B.P., R.B., T.L. designed and performed the UAS surveys, SfM processing, generating of DSMs and OMs; B.P., R.B. performed sedimentological sampling and analysis, maps, tables, graphics and appendix; B.P., I.K., L.D. analysed the coastal dune forms. I.K., R.B., L.D. assisted with the landscape analysis, vegetation observation and refinement of the overall text in English; I.K. analysed the relevant Bulgarian legislative acts on coastal planning and development; L.D. supervised the accuracy of ground geodetic measurements and dune characterisation and provided critical notes.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

List of identified beach-dune systems along the Bulgarian Black Sea coast

Authors: Bogdan Prodanov

Data type: word file



Explanation note: ED – embryonic dunes, WSD – shifting (white) dunes, GD – fixed (grey) dunes, WD – wooded dunes and HDS – humid dune slacks.

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Research Article

Exploring life-history traits of an endangered plant (*Vicia biennis* L.) to support the conservation of marginal populations

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Abstract

We aimed to investigate the reproduction-related traits of *Vicia biennis* L., an endangered and poorly known wetland species in its western marginal populations (in Hungary), and discuss the conservational and ecological implications. We measured the mass, viability, and physical dormancy of half-year-old seeds in five in-situ collected seed lots, while potential seed longevity (i.e., seed bank type) was estimated from repeatedly germinating subsamples from four ex-situ collected seed lots for 3–8 years. Plant survival, flowering, and seed production were studied in different light-, irrigation-, and competition conditions in a botanical garden experiment. We found that 1) half-year-old seeds have a high germination capacity (78–100%), 2) and high level of physical dormancy (72–100%) in all examined Hungarian populations, and 3) the seeds can preserve their germination capacity for more than five years, although their viability sharply decreases, probably falling below 10% within ten years, when they are stored at room temperature. The results of the botanical garden experiment suggested that 1) the species is annual, not biennial; 2) it shows strong sensitivity to precipitation and low competitiveness for water; and 3) it can produce hundreds of seeds even in suboptimal (dry or shady) conditions. Although the species was found to be well-adapted to a temporally heterogeneous environment, its future vulnerability can increase depending on the duration of dry seasons. Further investigation of genetic diversity and soil seed bank is needed to estimate the actual vulnerability of the species while strengthening the populations through seed sowing, and additional vegetation control in the habitats is suggested.

Key words: Fabaceae, germination, physical dormancy, seed longevity, seed production



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Introduction

Recently, several studies have shown that human-induced loss, fragmentation, and degradation of natural habitats, together with accelerating climate change, are among the most important drivers of current and future biodiversity crisis (Sala et al. 2000; Jaureguiberry et al. 2022). These processes may also

significantly affect species distribution patterns (Parmesan and Yohe 2003), leading to a significant level of species turnovers and species extinctions in several regions, such as the Pannonian region (Thuiller et al. 2005)).

An increasing number of models were developed to predict the responses of species and communities to future environmental changes in order to prioritize conservation actions (Thuiller et al. 2008). There is a growing demand for using trait-based approaches in plant ecology and related sciences (Violle et al. 2007) as well as in species distribution modelling (Violle and Jiang 2009; Violle et al. 2014). This approach needs high-quality functional trait data of the target species, which led to the development of large trait databases, e.g., TRY (Kattge et al. 2011), SID (Anon 2020) or LEDA (Kleyer et al. 2008). Although these databases have achieved unprecedented coverage (Kattge et al. 2020), there are still some critical knowledge gaps: seed-related traits except for seed mass (Jiménez-Alfaro et al. 2016; Saatkamp et al. 2019), and generally, the traits of rare species (Kattge et al. 2020) are surprisingly underrepresented in these databases and the literature.

Life history traits of rare plant species are often poorly known, and the relative importance of these species in maintaining ecosystem functioning has been underestimated for a long time (Grime 1998). However, several studies have shown that rare species often support important and vulnerable ecosystem functions (Lyons et al. 2005; Mouillot et al. 2013) as they are usually characterized by unique functional trait combinations in the given community (Jain et al. 2014). Furthermore, they potentially have an essential role in stabilizing food webs (Säterberg et al. 2019) and can even strengthen a community's resistance to invasions (Lyons and Schwartz 2001). Consequently, their role in maintaining ecosystem functioning and ecosystem services can be significant despite their small number in the community (Dee et al. 2019). However, rare species are considered more vulnerable to climate change, habitat loss, disturbance, or biological invasions; thus, their loss can contribute disproportionately to the current extinction crisis (Pimm et al. 1988; Enquist et al. 2019).

For the effective in-situ and ex-situ conservation of rare species, it is essential to have a basic knowledge of life-history traits related to germination and seedling establishment that can considerably affect population growth and stability (Saatkamp et al. 2019; Pence et al. 2022). This knowledge is also inevitable for planning effective reintroductions of rare species (Godefroid et al. 2011) and fundamental in the case of annual and biennial plants for which recruitment by seed is a crucial process due to their short life-cycle.

This study aimed to examine the life strategy of a poorly known, endangered plant species (*Vicia biennis* L.) and its reproduction-related traits in order to reveal potential sources of the species' vulnerability and understand its current demography and distribution.

Vicia biennis L. is a wild legume species with a 1–3m long, branching and climbing herbaceous stem system. Originally it was described as a biennial (hemitherophyte) plant, which is preserved in its Latin name, but recently, this was questioned in the literature. Nowadays, it is occasionally referred to as annual (Leht 2005; Király 2009), annual/biennial (Lesku and Molnár 2007; POWO 2023) or as a perennial liana with “periodic shoot reduction to a remnant shoot system that lies relatively flat on the ground surface” (Botta-Dukát et al. 2023).

Although the species has a wide distribution area (from Hungary to Kazakhstan), its known populations are often isolated and situated far from each oth-

er. They live near wetland habitats: edge of gallery forests, willow bushes, or reed-framed watersheds in the steppe zone. These habitat types are profoundly affected by land use, climatic changes, and invasive alien species (Stohlgren et al. 1998; Schindler et al. 2016). According to Soó (1980), its habitats are characterised by moist, compacted clay or alluvial soils.

The westernmost populations live in the Pannonian Biogeographic Region in Hungary, where the species is endangered and strictly protected by law (Király 2007). It was probably always rare in this country and was among the first plants proposed for protection in 1908 (Farkas 1999). Herbarium specimens from the 1900s are sporadic (Molnár et al. 2000), but new populations have been found continuously since the late 1990s. In the last 25 years, 13 populations have been recorded in the “Vascular Plants of Hungary online database” (Fig. 1) (Bartha et al. 2023). These populations were found along the River Tisza and its tributaries, but not necessarily in wetland habitats. For example, Somlyay and Bauer (2013) found larger populations in near-water fallows and even in dry, weedy hay meadows, suggesting that the plant may not be strongly associated with wetlands, or at least, it can appear intermittently on drier habitat types too. According to the different ecological indicators collected together in the Pannonian Database of Plant Traits (Sonkoly et al. 2022), *Vicia biennis* is a disturbance-tolerant species and prefers moist, sunny/semi-shaded and nitrogen-rich habitats (Soó 1980; Borhidi 1995).

The size of the found populations was highly variable, ranging from one specimen to more than 100 individuals. However, despite all conservational efforts, this shows a high within-population variation between years. Based on this and the vulnerability of wetland habitats, the species was suggested for ex-situ conservation by the Hungarian National Park Directorates (Házi and Lesku 2006), and an ex-situ population was established in 2009 in the MATE botanical garden, Gödöllő (Fig. 1).

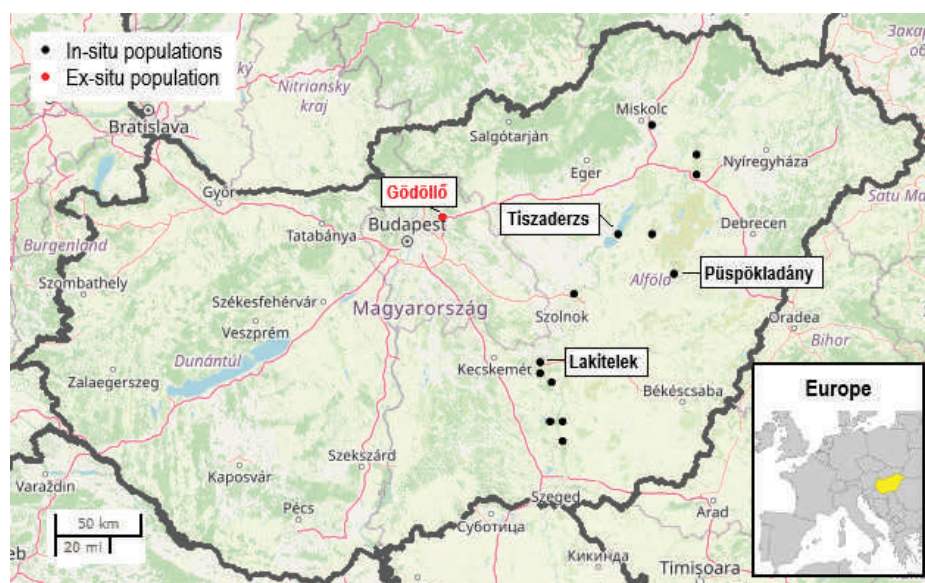


Figure 1. Location of the ex-situ and in-situ populations in Hungary validated after 1990. Examined populations (Tiszaderzs, Püspökladány and Lakitelek) are highlighted. The map is based on the data of the Vascular Plants of Hungary online database (Bartha et al. 2023).

Despite the species' endangered conservation status, vulnerability, and its well-known crop (e.g., *Vicia sativa*) and weed (e.g., *V. cracca*) relatives, only morphological data and no functional trait data are available about *V. biennis* in the literature or public databases (e.g., SID (Anon 2020), TRY (Kattge et al. 2020)), making predictions and conservation actions even more challenging.

In our research, we aimed to experimentally investigate some reproduction-related traits of the species in semi-natural conditions: Seed and germination-related traits (i.e., seed mass, potential seed longevity, dormancy, germination capability) were examined in greenhouse experiments, while plant survival, flowering, seed production and their reaction to different light exposure and precipitation were investigated in a botanical garden experiment. The main questions and hypothesis of the study were the followings:

1) What is the degree and variance of mass, physical dormancy, and germination capacity (viability) of seeds collected from different in-situ populations?

A vast majority of species in the Fabaceae family produce seeds with some kind of dormancy – mostly physical (PY) or combinational (i.e., physical + physiological, PY+PD) dormancy (Baskin and Baskin 1998; Van Assche and Vandeloos 2010; Jayasuriya and Wijetunga 2013). The primary role of physiological dormancy (PD) is to inhibit germination until environmental conditions are favourable for seedling establishment (e.g., waiting for the end of the summer period in the case of winter annuals (Van Assche and Vandeloos 2010)). In contrast, seeds with physical dormancy (PY) cannot germinate even in favourable conditions until their water-impermeable structures in the seed coat break due to mechanical or thermal effects (Baskin and Baskin 2004; Renzi et al. 2014). From a long-term conservational perspective, physical dormancy (PY) can be more important in the bet-hedging of annual or biennial species and greatly impact population dynamics, as it can positively correlate with the seeds' longevity as well as delay germination for many years. (Cohen 1966; Childs et al. 2010).

Due to the limited number of seeds available from the endangered populations of *V. biennis*, we decided not to test all potential dormancy types but rather focus on the more important physical dormancy (PY). Based on the expected life form of the species (i.e., summer annual) and the seed characteristic of other *Vicia* species, high between-population variances in seed dormancy, weight, and viability were expected. As the examined in-situ populations are small and variable (containing ~10–50 individuals), detecting signs of inbreeding depression (e.g., low viability of seeds) was also conceivable.

2) What is the potential seed longevity (seed bank type) of *Vicia biennis*? How long can the seeds potentially preserve their germination capacity?

The longevity of seeds (i.e., the time they can preserve their germination capacity) is a crucial seed trait affecting the survival of endangered, annual/biennial plant populations. Although this highly depends on the species' attributes, environmental conditions affect it too (Bekker et al. 2003). For the effective conservation of a species, maximum ex-situ seed longevity (i.e., the time the seeds can be stored in controlled conditions without losing their germination

capacity), potential in-situ seed longevity (i.e., the time the seeds can persist in the soil without losing their germination capacity) and realized in-situ seed longevity (i.e., the actual seed longevity on a given habitat) can be all important. Ex-situ seed longevity is usually measured by controlled seed ageing experiments (Newton et al. 2014), while long-term seed burial experiments can help to estimate in-situ seed longevity (e.g., Csontos et al. 2016). The former is more expensive and requires proper lab equipment, while the latter is time-consuming and highly affected by the soil and weather conditions. In addition, both approaches require hundreds/thousands of seeds, which can be problematic for an endangered species. In a simplified approach, seed longevity can also be estimated by ecological correlates (Probert et al. 2009): e.g., in-situ seed persistence is correlated with some seed characteristics (mass, shape, and density in the soil) (Thompson et al. 1993; Bekker et al. 1998). However, these simplified approaches are usually limited only to determine the potential seed bank type of the species, i.e., if a species probably has a transient seed bank (with seeds viable for less than one year), a short-term persistent seed bank (with seed persistence between 1–5 years) or a long-term persistent seed bank (with seeds preserving their viability for more than five years) (Thompson 1993).

Although we did not have the conditions (lab and a sufficient number of seeds) to conduct a proper longevity test, we wanted to estimate at least the potential seed bank type of the species by testing the viability of seeds with different ages in the short-term ex-situ seed collection. Based on the known ecological correlates, it is not clear what seed bank type to expect: E.g., species with similarly large seed sizes have a higher chance to have a short-lived, transient seed bank (Csontos 2001) but short life histories (e.g., annual life form) are usually associated with increased seed persistence in soils (Thompson et al. 1998).

3) How do light, precipitation, and root competition affect mortality, growth, flowering, and seed production?

V. biennis often grows close to wetland habitats in the half-shaded edge of taller vegetation (e.g., bushes or cattails); thus, we hypothesized that the species has a relatively low light requirement and high water demand for healthy development and seed production.

Materials and methods

Examined populations

Three in-situ and one ex-situ Hungarian population were selected as seed sources to study the seed traits of the species (Fig. 1).

The Tiszaderzs population was found in 1999, near a canal connected to an oxbow of the River Tisza (Cserőközi-Holt-Tisza). At that time, the known habitats of the species were threatened by invasive weeds (e.g., *Amorpha fruticosa*, *Vitis* spp., *Echinocystis lobata*, *Solidago gigantea*) (Molnár et al. 2000). In 2009, the individuals occurred 20–50 m from the canal, mainly in a young, semi-shaded poplar forest (*Populetum canadensis cultum*) in the neighbourhood of a holiday home zone. However, a few individuals had already appeared in the more open willow bushes (*Calamagrosti* – *Salicetum cinereae*) nearby. Regarding the

vegetation, although we found some typical softwood gallery forest species (e.g., *Salix alba*, *Rubus fruticosus*, *Galium mollugo*) in the forest habitat, most of the recorded plants were disturbance-tolerant weeds (e.g., *Amaranthus albus*, *Cirsium vulgare*, *Artemisia vulgaris*, *Tanacetum vulgare*, *Equisetum arvense*) and *V. biennis* had to cope with many invasive neophytes (e.g., *Conyza canadensis*, *Erigeron annuus*, *Amorpha fruticosa*) too (Endrédi 2010; Endrédi and Nagy 2012). Between 2009 and 2016, the estimated population size oscillated between 40 and 5 individuals (Endrédi 2012; Sőth 2017).

In the last decade, the forest habitat has become more shaded, and despite all conservational efforts (e.g., early spring weed control and some reintroduction attempts), the number of *V. biennis* individuals showed a significant decrease here. Meanwhile, the shift of the population towards the continuously disturbed (cut) edge of willow bushes was observed.

The Püspökladány population was found in 2009 in a ditch between fields. Here, the plant community was mainly composed of typical wetland species (e.g., *Typha angustifolia*, *T. latifolia*, *Bolboschoenus maritimus* s. l., *Lycopus exaltatus*, *Butomus umbellatus*) (Gulyás 2013). Two years later, in 2011, the population size was about a dozen (Gulyás 2013), and we found a similar number (10–15) of individuals in the year of the seed collection (2015), climbed up to cattail (*Typha* spp). We do not have information about the conservation of this population.

The Lakitelek population was recorded in 2012. A few dozen individuals occurred on a pond's steep shore, and a similar number of plants were found nearby, on the embankment between artificial ponds (Somlyay and Bauer 2013). In 2014, we found about 10–15 plants climbed up to the vegetation on the edge of a pond. In 2017 and 2018, we did not find the species here, while the cover of disturbance-tolerant weeds has increased dramatically. Although, on the edge of a nearby artificial pond, a few dozen individuals appeared in 2017 (Ércz 2018).

The ex-situ population was established in 2009 in the Botanical Garden of MATE (Gödöllő). For the establishment, seeds were collected from the Tiszaderzs population in 2007 and germinated in 2009. The species' preference for light and moist conditions was tested in the first year. The results and the conditions are presented in this paper. In the following years, the population was maintained partly by spontaneous germination and partly by additional seed sowing and seedling planting. All seeds/plants used for the sowing and plantation originated from the previous seed lots of the same ex-situ population. The number of seed-producing individuals in this population varied between 50 and 100, and about 50% of the mature seeds was collected each year.

Seed collection and storage

In the germination tests, we used both in-situ and ex-situ collected seeds. In this paper, we will use the term "seed lot" for seeds collected from the same population in the same year, while "seed sample" refers to subsamples of seed lots for germination experiments or weighing.

Five in-situ seed lots were collected in four years (2013, 2014, 2015, 2017) from the three different populations (Fig. 1): Tiszaderzs (13T and 14T), Lakitelek (14L and 17L) and Püspökladány (15P). In the years of seed collections, all populations contained only a relatively small number of individuals: The Tiszaderzs population contained approximately 40 individuals, the Lakitelek

population was formed by 10–15 (2014) and 20–30 (2017) individuals, while the Püspökladány population included only 10–15 individuals. Population size was estimated partly by counting the stems right above the ground and partly based on the more or less separated canopies, as in some cases, the vegetation density (dense willow bushes, cattail or the “carpet” formed by the target species) made it impossible to count each stem separately.

We performed only one seed collection in a vegetation season to minimize the disturbance of these endangered populations. The Tiszaderzs and Lakitelek populations were sampled twice in two different years, while in the Püspökladány population, only one seed collection was performed in 2015. Samplings were performed in August, in the middle of the yielding period. Following the ENSCONET (2009) seed-collecting manual’s suggestions for small populations, the aim was to collect less than 20% of the total seeds available. Depending on the size of the population, ~7–20 individuals (~50–100% of the population) were selected randomly, and 2–5 mature (brown and dried) pods were collected from them. The collected seed lots accounted for only a small proportion of the plants’ total seed production as the species has continuous flowering and yielding during summer/autumn (Endrédi 2010).

Pods were opened, and only mature (round, pigmented) and intact seeds were selected for storage. In total, ~46–150 seeds were collected per population during one collection. Seeds from the same lot (collected in the same year and population) were mixed. Whereas previous observations suggested that the species is a spring germinator and a preliminary study did not show a significant effect of cold winter stratification (1 month in 4 °C) on germination (Endrédi 2010), seeds were kept in the dark, in paper bags, at room temperature (23 °C), and in ambient moisture conditions for about six months before the experiment. This time interval should be sufficient to eliminate the after-ripening period the seeds potentially need (i.e., primary dormancy) before germination.

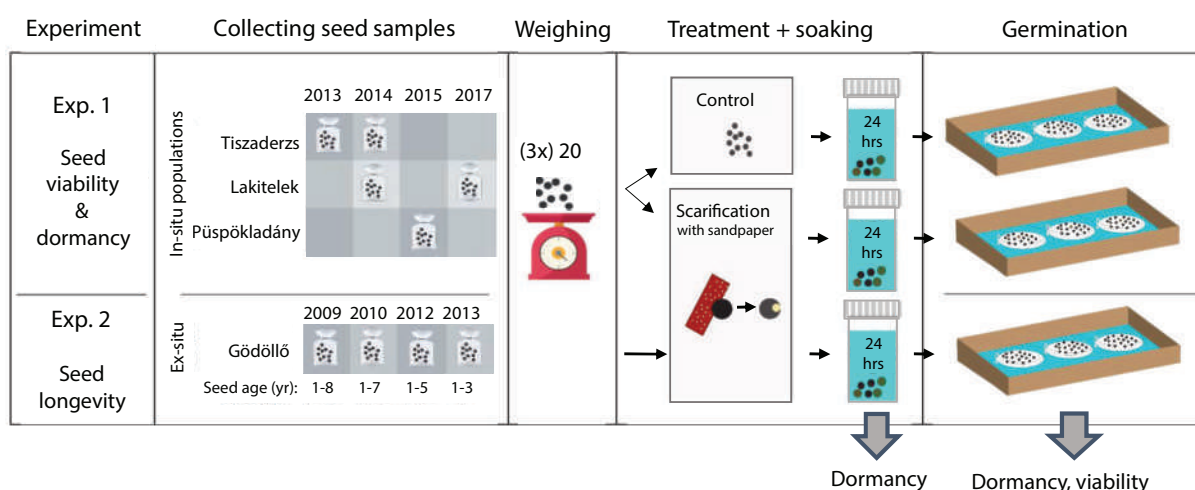
As the size of the in-situ collected seed samples was not appropriate for repeating germination tests for years, seeds from the ex-situ population were used for the longevity experiment. For the longevity experiment, we used seeds collected from the population in four different years: 2009, 2010, 2012, and 2013 (seed lots 09EX, 10EX, 12EX, and 13EX, respectively). These ex-situ seed lots were stored under the same conditions as the in-situ seeds: in the dark, within paper bags, at room temperature (23 °C), and in ambient moist conditions.

Germination tests

Two series of germination experiments were conducted in greenhouse conditions to determine 1) the viability (estimated via germination capability) and physical dormancy of half-year-old seeds and 2) the average seed longevity (i.e., the time until the seeds can preserve their germination capacity). Experimental setups are shown in Fig. 2a. and Table 1.

Before the experiments, the average seed mass of all seed lots was estimated by weighing three subsamples containing exactly 20–20 seeds. In the case of Lakitelek seed lots (14L and 17L) we did not have 3×20 seeds; thus, we weighed all the 46–50 seeds individually to get the average seed masses. Subsamples were measured in grams with an accuracy of 0.0001 g.

a) Germination experiments



b) Field experiment

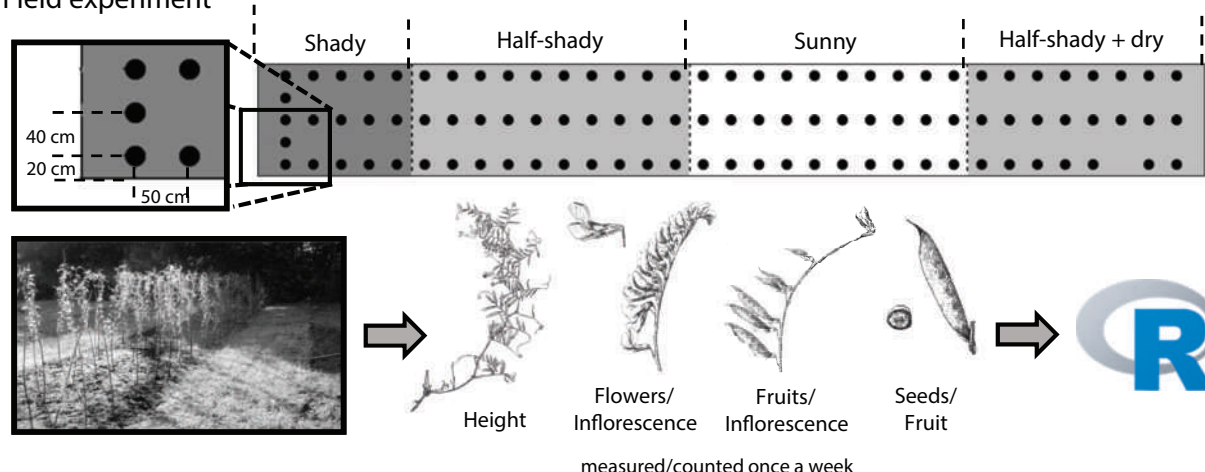


Figure 2. Materials and experimental setups of **a** the germination tests and **b** the botanical garden experiment.

Experiment 1 – Viability and dormancy tests

In the first experiment, we tested the viability and dormancy of the in-situ collected seeds, mainly focusing on physical dormancy (PY).

From the largest seed lot (15P), 120 seeds were randomly selected for the experiment, while only 80–80 seeds were available from the Tiszaderzs seed lots (13T and 14T), and all the 46–50 seeds of the Lakitelek seed lot (14L and 17L) were used in the experiment (Table 1). Before the germination test, half of the seeds in each sample were scarified individually (i.e., the seed coat was perforated) by sandpaper to break the possible physical dormancy (i.e., water-impermeable layer in the seed coat). The other half of the sample was treated as unscarified control. All (control and scarified) seeds were soaked for 24 hours in tap water. From each treatment group, at least 20 seeds (23 for 14L and 25 for 17L) were germinated in Petri dishes, with 1–3 replicates, depending on the number of available seeds (see details in Table 1).

We recorded the number of imbibed seeds daily to study the degree of physical dormancy. Physically dormant seeds could not intake water; thus, their appearance (size and colour) did not change, while seeds with broken hard

seed coats visibly swollen and their colour faded. Based on this, the physical dormancy of the seed lots was estimated as follows:

$$PY_i = 1 - ND_i = 1 - \frac{IMB_C}{IMB_C + UN_C}$$

PY_i= level of physical dormancy in population i

ND_i= ratio of non-dormant seeds in population i

IMB_C= number of imbibed seeds in the control group

UN_C= number of unchanged seeds in the control group

Table 1. Origin, treatments and number of seeds used in germination experiments. Note that seed age is rounded up as the seeds were collected in summer and germinated in spring.

Experiment	Year of collection	Source population	Seed sample	Seed age (yr)	Treatment	Sample size (n)
I. (viability & dormancy)	2013	Tiszaderzs	13T	1	Control	2*20
					Scarified	2*20
	2014	Tiszaderzs	14T	1	Control	2*20
					Scarified	2*20
		Lakitelek	14L	1	Control	23
					Scarified	23
	2015	Püspökladány	15P	1	Control	3*20
					Scarified	3*20
	2017	Lakitelek	17L	1	Control	25
					Scarified	25
II. (longevity)	2009	Gödöllő (EX)	09EX	1	Scarified	3*20
				2		3*22
				3		3*22
				7		3*20
				8		3*20
	2010	Gödöllő (EX)	10EX	1	Scarified	3*22
				2		3*22
				6		3*20
				7		3*20
	2012	Gödöllő (EX)	12EX	1	Scarified	3*20
				4		3*20
				5		3*20
	2013	Gödöllő (EX)	13EX	1	Scarified	3*20
				3		3*20
				4		3*20

Germination (root/shoot emergence) was monitored daily to investigate seed viability. As scarification was a reliable method to break physical dormancy (see in the Results section), the viability of the in-situ collected seed lots was estimated based on the germination of the scarified, imbibed seeds as physical dormancy did not prevent the germination of viable seeds in this group. However, the possible occurrence of viable seeds with combinational dormancy (PY+PD) “hiding” among the non-germinated, imbibed seeds was also considered in interpreting the results. Thus, seed viability of the in-situ collected seed lots was calculated as:

$$SV_i = \frac{GERM_{SC}}{GERM_{SC} + NG_{SC}}$$

SV_i = seed viability in population i

$GERM_{sc}$ = number of germinated seeds in the scarified group

NG_{sc} = number of non-germinated seeds in the scarified group

Experiment 2 – Longevity tests

In the second experiment series between 2009 and 2018, four ex-situ collected seed lots (09EX, 10EX, 12EX, and 13EX) were sampled and germinated repeatedly for 3–8 years to record the temporal dynamics of their germination capability (Table 1). Before the experiments, all seeds were scarified and soaked in tap water for 24 hours to break physical dormancy.

All germination experiments were performed in a greenhouse from the beginning of March when the temperature in the greenhouse varied between 10 °C and 20 °C. All seeds were placed onto wet filter papers in Petri dishes, and irrigation was automatic by diffusion with the help of 2cm × 20cm filter paper stripes. Imbibition and root/shoot emergence were monitored daily for 30 days.

Botanical garden experiment

Survival, development, and reproduction success were studied in 2009 in the newly established ex-situ population (Fig. 2b).

For establishing the ex-situ population, seeds of Tiszaderzs population were collected in 2007 and germinated in March 2009. In June, the well-developed (longer than 30 cm) specimens were planted on a 15 × 2m plot with sandy, fertilized, and homogeneously mixed soil and naturally diverse light conditions (Endrédi 2012; Fig. 2b). Ninety-eight individuals were planted in 3 rows and 33 columns. In the same column, individuals grew under the same light conditions, and the columns followed a natural light gradient regarding the time spent in direct sunlight (see Fig. 2b). To decrease root competition, plants were placed at least 40cm from each other: the distance between the rows was 80cm, while between the columns it was 50cm (see Fig. 2b). To estimate the plants' sensitivity to root competition, marginal specimens were planted only 20cm from the grassy edge of the seedbed.

Before planting, we classified the columns according to their light conditions (Fig. 2b): 1) shaded microsites (columns 1–5, called “Shady”), which were never exposed to direct sunlight due to a large shading tree, 2) sunny microsites (columns 16–26, called “Sunny”) which were exposed to direct sunlight the whole day, and 3–4) two half-shaded microsites (columns 6–15 & columns 26–33, called “HS” and “HS + dry, respectively) with at least some hours of direct sunlight and shade every day. As the Tiszaderzs population (i.e., the source population) grew in half-shady conditions (Endrédi 2010), we used the same condition to test the species' sensitivity to precipitation. For this purpose, the two half-shady microsites were irrigated differently: while the first three microsites (Shady, Sunny, and HS) were irrigated once a week for an hour, the last half-shady microsite (HS + dry) got irrigation only every second week. This experimental setup enabled us to study plant survival, development, and reproduction success in different microsites, reflecting a wide range of light conditions and two different precipitation scenarios occurring in the different in-situ populations. We randomly distributed the specimens that germinated on the same day between the four microsites and randomly selected a column for them within the microsites to minimize the effect of germination time.

Measurements: We measured the length of the stems weekly. The number of inflorescences per individual, flowers per inflorescence, green pods per inflorescence, seeds per green pods, and the number of mature legumes were counted weekly, too. Mature pods were collected, and seeds were counted as well.

As flowering and seed production is continuous during the summer, note that with monitoring once a week, particular flowers could be counted more than once, and we could not collect all of the mature legumes before the escape of some seeds. Consequently, we cannot estimate the exact number of produced flowers or seeds, but the standard sampling makes it possible to compare the seed production of the individuals grown under different conditions.

Data processing and software

Data visualization and statistics were performed using R statistical software (R Core Team, 2019). Figures were drawn by the 'ggpubr' (Kassambara 2019) package, except Fig. 1 and Fig. 2. Fig. 1 was made with the help of 'maps' and 'mapdata' (Brownrigg et al. 2018) packages, while the river shape file is from (Tapiquén 2015), and Fig. 2 was drawn in Inkscape (Bah 2010).

Germination capabilities and dormancy of seeds in the different seed samples, as well as the effect of seed weights on seed germination probability, were compared by logistic regressions (*glm* function; R Core Team 2019), while linear model (*lm* function; R Core Team 2019) was used to compare the average seed weights of in-situ seed samples.

Regarding the garden experiment, the probability of mortality after the plantation was analysed within 6-week periods (i.e., the probability of survival in the first six weeks, between 6–12 weeks, 12–18 weeks, and 18–24 weeks after plantation). The effect of microsites (i.e., light and precipitation conditions) and root competition (marginal/inner individuals) on this probability was analysed by logistic regression. The maximum heights of plants growing in different microsites and within different root competition conditions were compared with linear models. Post hoc pairwise comparisons were made using the *glht* function of the 'multcomp' package, which automatically gives adjusted p-values (Hothorn et al. 2008). The impact of microsites and root competition (i.e., marginal or inner position) on the number of legumes and seeds was examined using the microsites/root competition in the non-parametric Kruskal-Wallis test, and pairwise Wilcoxon-test was used for posthoc pairwise comparisons.

Results

Germination tests

Experiment 1 – Viability and dormancy tests

The results of the first experiment are summarized in Fig. 3.

Generally, the seeds of the species showed high viability (on average, 78–88% of the scarified seeds germinated), and there were no significant differences between the different in-situ seed lots (adjusted $p > 0.08$, logistic regression) (Fig. 3a). Furthermore, the level of physical dormancy was also high in every seed lot (adjusted $p > 0.88$, logistic regression): only 0–10% of the control seeds showed visually noticeable water uptake (Fig. 3b), indicating the absence of water-imperme-

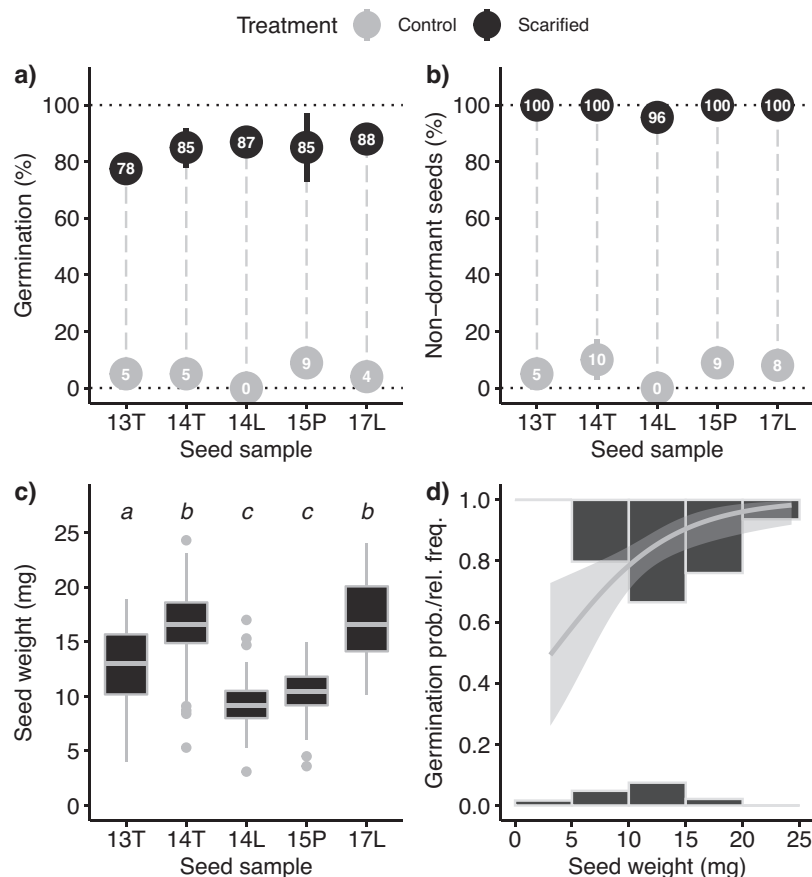


Figure 3. Results of the first germination experiment: seed viability (a), dormancy (a, b), seed weight (c) and their relationship (germination probability/relative frequency of scarified seeds with different weights) (d) in the case of half-year-old, in-situ collected seeds. Different characters in panel c indicate significantly different seed lots (linear model, Tukey's contrasts). In panel d, the heights of the bars represent the relative frequencies of germinated (hanging bars) and non-germinated (standing bars) seeds in the different seed weight classes. The sum of the heights of all bars is equal to one. E.g., 4.78% of the examined scarified seeds weighed between 5–10 mg and showed no germination (second standing bar), while another 20.21% of all tested seeds fell into the same weight category but did germinate in the experiment (second hanging bar). The fitted line shows the relationship between the seed weight and the germination capability, predicted by the logistic regression model.

able seed coat (i.e., the absence of physical dormancy). Individual scarification proved to be a suitable method for breaking this high level of physical dormancy – 96–100% of the seeds could take up water after the treatment (Fig. 3b), with a significant 72–87% increase in germination capability as well (Fig. 3a).

Almost all seed samples were different in average seed mass ($p < 0.001$, linear model) (Fig. 3c), even the ones that came from the same population but different years (13T/14T and 14L/17L pairs), and those collected in the same year but from different populations (14T/14L). However, heavier seeds had a higher germination probability ($p < 0.01$, logistic regression), germination probability of seeds with more than 10 mg mass was found to be high, exceeding 0.8 (Fig. 3d).

Experiment 2 – Longevity tests

The results of the second experiment series are shown in Fig. 4.

Despite the highly protective seed coat (i.e., the generally high initial physical dormancy) and high initial viability, all examined ex-situ collected seed samples

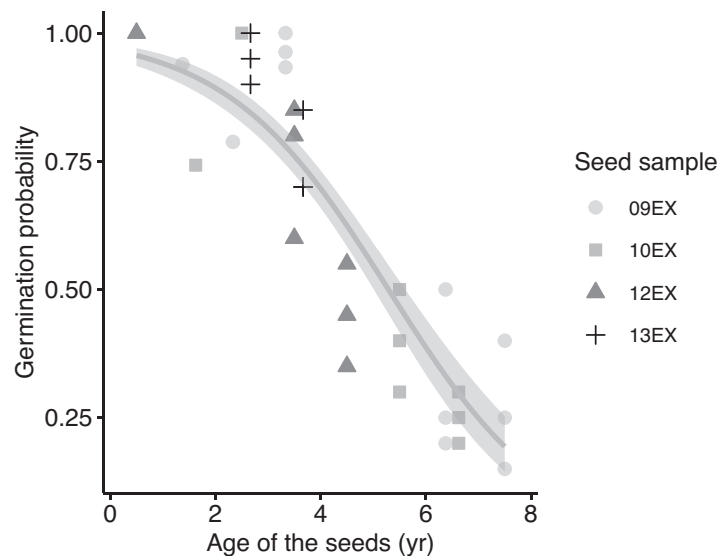


Figure 4. Temporal changes in seed viability of ex-situ collected seeds.

showed a sharp decrease in germination capability in the first 5–6 years ($p < 0.001$, logistic regression) (Fig. 4). The logistic regression model suggests that germination capability probably decreases below 10% within ten years when these seeds are stored at room temperature and ambient relative humidity.

Botanical garden experiment

Summary statistics (mean and SD) of traits (lifespan, height, pod, and seed production) measured on the different microsites are summarized in Table 2.

Table 2. Summary statistics (mean and sd) of the traits of plants grown in the botanical garden in different light and precipitation conditions (Shady= always shaded weekly irrigated microsite ($n=17$), HS= half-shaded, weekly irrigated microsite ($n=30$), Sunny= always sunny, weekly irrigated microsite ($n=30$), HS+dry= half-shaded, biweekly irrigated microsite ($n=23$)).

Microsite	Lifespan (weeks)		Max. height (cm)		Number of pods/individual		Number of seeds/individual		Number of seeds/pods	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Shady	21.00	3.78	201.37	41.42	80.43	39.79	264.43	111.56	3.39	0.63
HS	18.33	5.96	276.91	50.82	148.07	76.67	529.25	256.75	3.62	0.40
Sunny	15.30	8.82	209.00	54.43	184.50	130.95	679.91	507.77	3.69	0.36
HS + dry	14.39	5.98	161.47	49.52	53.45	51.44	183.05	152.20	3.67	0.49

Survival and development

All ex-situ planted individuals ($n=98$) germinated, established and finished their life cycle within one vegetation season (from spring to autumn) as summer annual plants. Within the 24-week-long field experiment, the highest average lifespan (21 weeks after plantation) was found in shady conditions, and a decreasing trend was observed along the light gradient (Half-shady (HS)=18.3 weeks, and Sunny=15.3 weeks, Fig. 5a). However, individuals growing on the drier half-shady part (HS + dry) of the plot showed the lowest average lifespan (14.4 weeks).

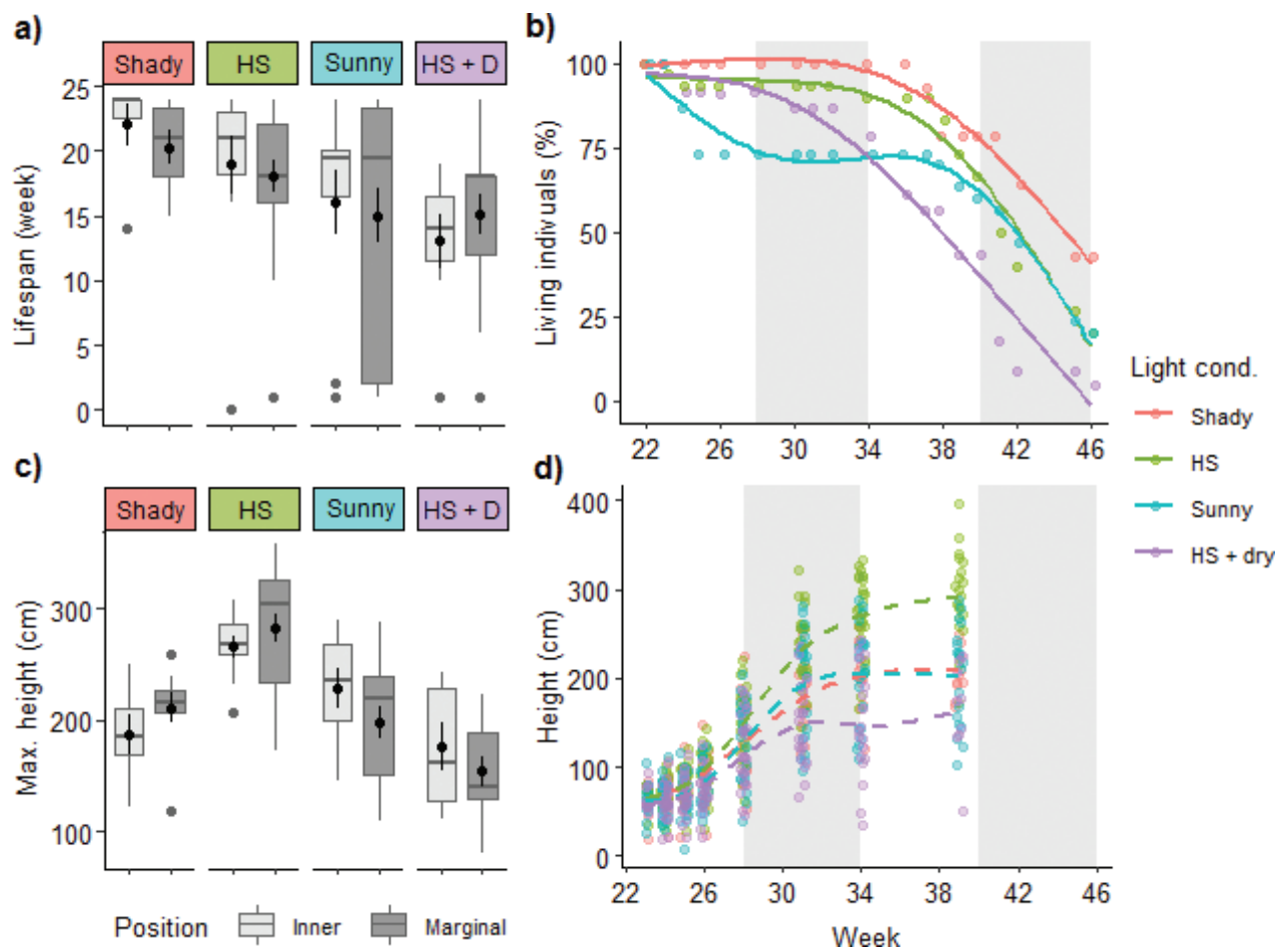


Figure 5. Survival and growing in different light conditions and competition: lifespan after plantation (mean + SE) (a), temporal patterns in survival (b), maximum height (mean + SE) (c) and temporal patterns in growing (d). Lighter boxplots show individuals with no competitors within 40cm, while darker boxplots indicate plants with neighbouring grass in 20cm distance. Dots and bars on the boxplots show mean and SE values. Grey and white stripes on panels b and d indicate 6-week-long periods.

Plants grown in the marginal position showed a slightly, but not significantly lower average lifespan than their neighbours in the middle of the plot. The temporal change in mortality is presented in Fig. 5b. Plants growing under direct sunlight were found to be more vulnerable to the stress caused by planting, as 26.7% of the individuals died in the first few weeks after planting. In contrast, in half-shady areas, the mortality rate was 18–20% less, while in the shade, none of the individuals died within the first six weeks. In general, the mortality rate started to increase 12 weeks after plantation (from week 34, middle of August, after the plants reached their maximum height (Fig. 5d)), but plants growing under different light conditions showed different patterns: on the drier half-shady part of the plot, the increase started some weeks earlier than on the shady or sunny parts.

The plants reached their maximum height 12–16 weeks after plantation (Fig. 5d), and it was significantly the highest (mean=276.9 cm, sd=50.8) in the half-shady, weekly irrigated microsite (HS), while the smallest plants (with an average of 161.5 cm and sd=49.5) were found on the half-shady, but rarely irrigated microsite (HS + dry) (Table 2). There was no difference between the maximum height of plants growing in shady (mean=201.4 cm, sd=41.4) and sunny (mean=209.0 cm, sd=54.4) conditions, but both groups differed significantly from the half-shady groups ($p < 0.03$, linear regression). There was no significant

difference in the maximum stem length of plants growing at the edge/middle of the plot as well (i.e., in different root competition conditions, Fig. 5c).

Reproduction

Flowering started around week 28, reached the peak around week 33, and ended around week 39 (Fig. 6b). However, the timing of flowering seems to be independent of the light and competition conditions (Fig. 6a, b), the number of flowers per individuals showed some differences (Fig. 6b): plants which were grown in the half-shady and weekly irrigated microsite (HS) had more flowers than those in the other groups.

The first pods appeared two weeks after the start of flowering. The number of pods showed a similar temporal pattern to the flower's (Fig. 6d), with the highest values in the half-shady, weekly irrigated area (HS), but the average total number of matured pods per individual (Fig. 6c) was similarly high in the sunny area too ($p=0.61$, pairwise Wilcoxon test). All the other group pairs differed significantly ($p<0.04$, pairwise Wilcoxon test). As the number of seeds in the pods was independent of the conditions (Table 2), the total number of seeds showed

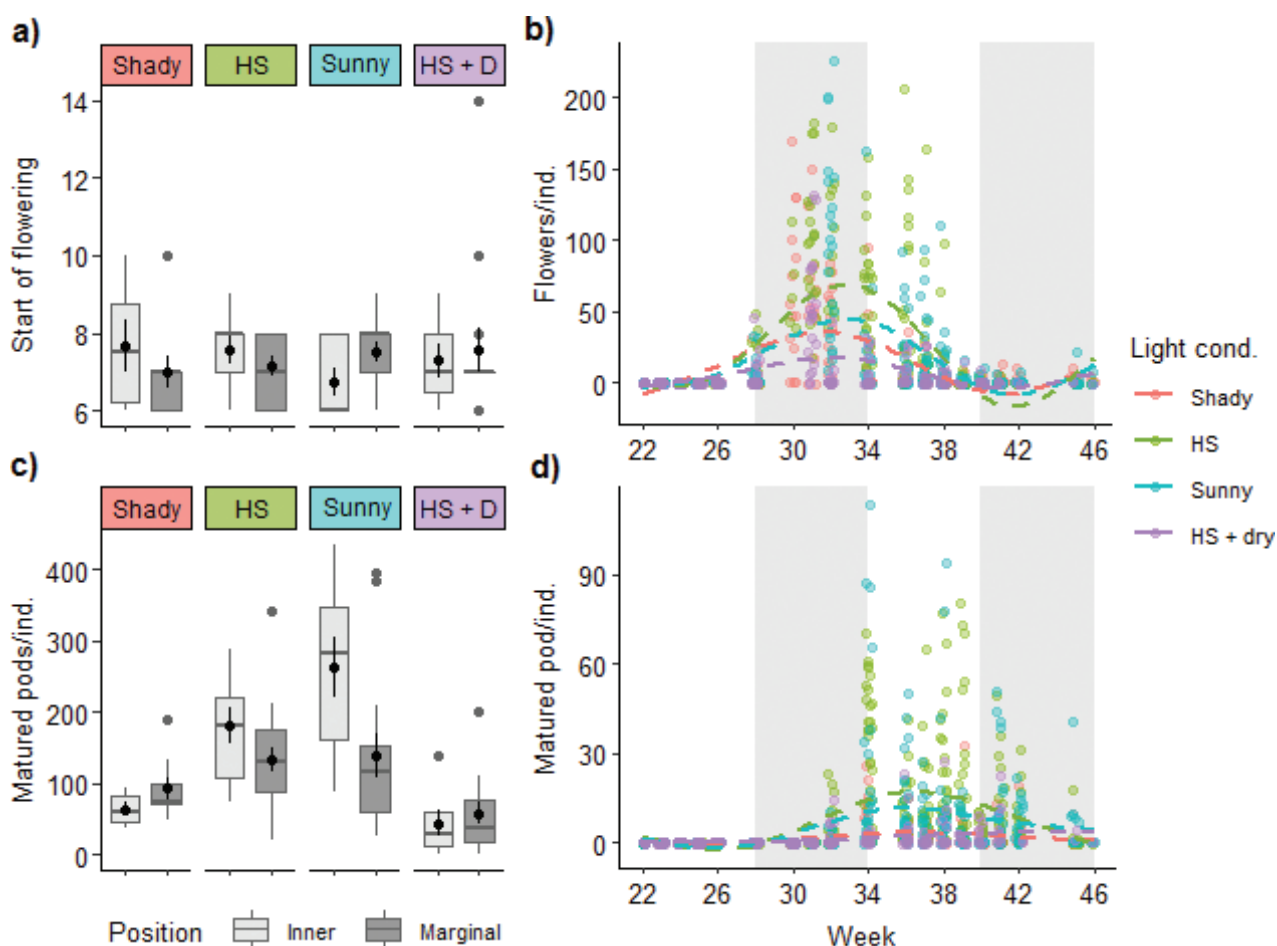


Figure 6. Flowering and yielding in different light conditions and competition: first week of flowering after plantation (a), temporal patterns in flowering (b), number of matured pods/individual (c) and temporal patterns in producing pods (d). Lighter boxplots show individuals with no competitors within 40cm, while darker boxplots indicate plants with neighbours within 20cm distance. Dots and bars on the boxplots show mean and SE values. Grey and white stripes on panels b) and d) indicate 6-week-long periods.

a similar pattern as the pods: the plants produced the lowest number of seeds in the half-shady, dry (HS + dry) area (mean=183.05, sd=152.2), slightly more in shady conditions (Shady, mean=264.4, sd=111.6), while the best performance was observed on the weekly irrigated half-shady (HS, mean=529.3, sd=256.8) and sunny (mean=679.9, sd=507.8) microsites (Table 2). Regarding the edge effect, only the sunny group showed a significant difference; plants grown in the middle of the plot, far from each other, produced more pods and seeds than their neighbours on the edge of the plot, where the root competition with grasses was more emphasized ($p=0.029$, Wilcoxon test).

Discussion

To summarize our results, *Vicia biennis* seeds showed high seed viability and physical dormancy in all the studied Hungarian populations, while seed mass was highly variable across years and source populations. The germination results suggest that the species has a long-term persistent seed bank, but seed viability sharply decreases below 10% within ten years in dry storage and room temperature.

All specimens in our experiment behaved as annual (not biennial), and their mortality, growth, and reproduction showed a strong sensitivity for irrigation/soil moisture content: on sandy but fertilized soil and in half-shady conditions, the plants performed significantly better (lived longer, grew higher and produced more flowers and seeds) when they were irrigated weekly and not only every second week. When getting the same amount of water, the plants had longer stems and more flowers in the half-shady area than in shady or sunny conditions, but the pod and seed production were similar or even higher (when competition for water was low) on the sunny part of the plot. However, higher competition for the limited water resulted in a significant decrease in seed production.

Annual plant species are typically associated with highly disturbed and temporally variable habitats, where producing seeds with different dormancy levels and high longevity can efficiently “spread the risk” caused by the unpredictable environment (Cohen 1966; Childs et al. 2010). Seed dormancy is a widespread, adaptive strategy for avoiding unfavourable conditions in a seasonally variable environment (Westoby 1981; Rubio de Casas et al. 2017). In theory, seed dormancy is generally more important for annuals as the vegetative phase of their life is short, and a massive part of their populations exist only in seed banks (Cohen 1966; Pake and Venable 1996; Childs et al. 2010). However, even in the case of annuals, high dormancy is rather expected in highly unpredictable, stochastic environments (Venable and Brown 1988; Philippi 1993; Volis and Bohrer 2013). Furthermore, environmental factors (i.e., mainly annual precipitation) in the year of seed production can also modify the ratio of dormant seeds produced, especially in habitats with poor water supply (Cseresnyés-Bózsing 2010).

V. biennis is mainly connected to the edge of temporal or persistent wetland habitats, and we found that the adult plants show a strong sensitivity to water availability. Based on this, a possible explanation for the generally observed high seed dormancy across populations and years can be the widespread insufficient precipitation or high inter-annual variation in precipitation in their habitats. The long-term climate data of Hungary support this: in the last decades, there was a significant decrease in spring precipitation and the number of wet days (with more than 1mm rainfall), and an increasing trend in the duration of dry seasons,

while a large inter-annual variation can be observed for all the three parameters (Lakatos et al. 2018). On the other hand, high dormancy can also result from the strong competition for water (Harel et al. 2011). Evidence suggests that in some cases, mostly in disturbed areas, *V. biennis* can appear further away from water bodies, while in the Tiszaderzs and Lakitelek populations, it has disappeared from areas where the vegetation cover had increased. In addition, our botanical garden experiment showed that in a sunny area, where the higher evaporation rate can lead to lower soil moisture, increased root competition significantly negatively affected seed production. Based on these, the adult plants seem sensitive to insufficient water supply and have low competitiveness for water. Thus, consistently high physical dormancy can indicate temporally suboptimal conditions in all examined populations (i.e., increasing aridity or competition), or it may be a sign that the species or its local populations are adapted to a highly unpredictable, stochastic environment (Cohen 1966; Childs et al. 2010).

Seed mass is also important in coping with environmental stress. At an individual level, larger seeds support a higher survival rate for seedlings (Westoby et al. 2002) and adult plants (Metz et al. 2010) and can increase the longevity of the seeds (by the greater storage capacity), especially when combined with a hard seed coat (which effectively protects the embryo) (Debeaujon et al. 2007). However, the larger predation pressure on these seeds can limit the seed mass in a population (Volis and Bohrer 2013), and as there is a trade-off between seed size and seed number, seed size depends more on the local environment of the mother plant too (Debeaujon et al. 2007). For example, many studies found that plants produce larger seeds in shaded habitats than in more open areas (e.g., Salisbury 1942; Hodkinson et al. 1998; Sonkoly et al. 2017).

In this study, we found significant differences in the seed weights between the different populations and years. In general, we found significantly larger seeds in the larger populations (Tiszaderzs and Lakitelek in 2017) containing more than 20 individuals, while the 10–15 individuals living in the smallest populations (Püspökladány and Lakitelek in 2014) produced the smallest seeds. Although seed mass usually correlates with seed viability (i.e., seeds developed in unfavourable conditions are often less viable), we found high seed viability in all populations. A possible explanation for this is that even these populations with small seeds have reached an average seed weight of 10 mg, which – according to our logistic model – can be enough to maintain high seed viability (i.e., an expected germination capacity higher than 0.8). These results suggest that the small numbers of individuals in the populations have not yet led to a significant deterioration in seed quality.

At the species level, seed longevity shows some correlation with other seed traits, like seed mass: species with smaller average seed mass are more likely to have long-term persistent seed banks (i.e., seeds that are still viable after five years in the seed bank) (Thompson et al. 1993; Hodkinson et al. 1998; Csontos 2001). According to the 8-grade seed weight category system of Csontos (2001), *V. biennis* has relatively large seeds belonging to the 7th group (i.e., species with average seed weight between 10.1 and 50 mg). Based on this, a transient seed bank (with seeds that are viable for less than a year after dispersal) would be expected, but according to Thompson et al. (1998), the majority of variance in seed persistence cannot be explained by seed mass, because seed longevity is more a species attribute, and more dependent of phylogeny and life history of the species than seed mass. Most *Vicia* species living in the same region have

similar average seed weights as *V. biennis*, and many have persistent seed banks (Csontos 2001; Kleyer et al. 2008). However, data about seed bank types of *Vicia* species are scarce and sometimes contradictory. On the other hand, shorter life histories correlate with increased seed longevity and habitat specialists of disturbed habitat types (often annuals) show higher persistence in soils than those of stable, undisturbed habitats (Thompson et al. 1998). These findings support our results that *V. biennis* is more likely to have a long-term persistent seed bank, and its seeds can preserve their germination capability for more than five years. Although we experienced a sharp decrease in germination after five years of dry storage at room temperature, our results do not necessarily reflect the true (ex-situ or in-situ) seed longevity. In their natural environment (i.e., in lower temperatures of the natural seed bank), seeds can remain viable for a longer time than in our experiment, and the realized persistence in the soil also depends on other factors (i.e., soil moisture, depth, temperature fluctuations, seed predation, microorganism). Based on the LEDA database, no *Vicia* species has yet been found with a seed life expectancy exceeding 25 years (Kleyer et al. 2008), so we do not expect *V. biennis* to have higher seed longevity, but for a more precise determination, appropriate experiments, either in-situ or ex-situ, are needed.

From a conservation perspective, the most crucial question is how threatened the species actually is by climate change or habitat change/loss. According to the climate models of Hungary (Lakatos et al. 2018), due to the decreasing annual precipitation and increasing mean annual temperature, the soil moisture content is expected to decrease, and the frequency and duration of droughts will increase in the future. The rainfall deficit will affect the summers the most when *V. biennis* is most sensitive to adequate soil moisture and lower competition for water during flowering. This makes it increasingly unlikely that stable populations will be established further away from wetlands. However, wetlands are not only becoming increasingly threatened globally but are also changing locally. With increasingly rare periods of extreme rainfall events, regulated shorelines and reduced grazing, the competition-poor environment required by the species is becoming less and less likely.

The species' long-term survival will depend on how it can adapt to these changes or 'escape' them and find more suitable areas.

Adaptive capacity is based on the adequate genetic diversity of populations. In small, highly fluctuating populations, genetic diversity is more likely to be reduced by inbreeding and genetic drift, and these populations may become more sensitive to environmental change (Bouzat 2010).

The probability and level of inbreeding depression depend on the species' mating system. Although self-compatibility of flowers is common in the Fabaceae family (e.g., Zhang and Mosjidis 1995), we do not have information about the possibility or frequency of self-pollination in the case of *V. biennis*. It also needs to be discovered how isolated the populations of the species actually are. Regarding pollinators, only common *Bombus* species have been observed as active pollinators to date (A. E. personal observation). The estimated foraging distance of these species does not exceed 1000m (Cresswell et al. 2000), so they are not suitable for maintaining gene flow between the natural populations of the plant. However, the contribution of other pollinator species cannot be excluded.

The primary mechanism to escape from the negative effects of climate and habitat change is dispersal. Similarly to other *Vicia* species (e.g., Oleas et al.

2018), in the case of *V. biennis*, short-distance dispersal (for a few meters) is achieved by the explosive opening of mature fruits and the ejection of seeds (called “ballochory”) (A. E., personal observation), but other mechanisms of dispersal are unknown. However, maintaining a large, persistent and highly dormant soil seed bank may be an appropriate strategy to avoid unfavourable conditions, not in space but in time. However, this strategy is only successful if conditions in the habitat become optimal at specific intervals.

Marini et al. (2012) suggest that the three most important traits that can help cope with the effects of habitat loss and isolation are annual life form (i.e., short generation time usually coupled with large persistent seed banks), high competitive ability and animal dispersal (i.e., non-random long-distance dispersal helping to reach suitable habitats). Although *V. biennis* is probably an annual plant with reproductive traits well adapted to unpredictable environments, its competitive ability – especially for water – is low, and there is no reliable data about animal dispersal. Based on this, the species’ sensitivity to further habitat loss or isolation can be high, and if the frequency of wet years or the disturbance events in suitable wetlands decreases under a threshold, seed banks can be depleted, and these small western marginal populations can disappear.

Based on the above, the following conservation priorities are suggested:

1. To assess the actual vulnerability of the species, it is essential to assess the populations’ current genetic diversity and isolation. This should be complemented by an investigation of the state of the soil seed bank and appropriate experimental studies of seed longevity.
2. In-situ conservation actions should primarily target the vegetation of the habitat and the seed bank of the species: continuous, controlled disturbance of the sites and reinforcement of the seed bank by seed sowing can help the species to persist in a given area. In the long-term, assisted migration may also be an option, where seed sowing is used to help the species spread to new areas likely to provide suitable conditions in the longer term.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

AE and JGYN designed the experiments and collected the seed material. AE, ÁS, and DÉ did the germination experiments, AE conducted the botanical garden experiment, analyzed the data, and wrote the first version of the manuscript. JGYN, BD, and OV contributed to the final version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Review Article

Does a flashing artificial light have more or conversely less impacts on animals than a continuous one? A systematic review

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Abstract

Background: Light pollution has been increasingly recognised as a threat to biodiversity, especially with the current expansion of public lighting. Although the impacts of light intensity, spectral composition and temporality are more often studied, another component of light, its flicker frequency, has been largely overlooked. However, flashing light could also have impacts on biodiversity, and especially on animal behaviour and physiology.

Objective: This systematic review aimed at identifying the reported physiological and behavioural impacts of flashing light on animals when compared to continuous light.

Methods: We followed the standards recommended by the Collaboration for Environmental Evidence (CEE) in order to achieve a comprehensive, transparent and replicable systematic review. Citations were primarily extracted from three literature databases and were then screened for relevance successively on their titles, abstracts and full-texts. Retained studies were finally critically appraised to assess their validity and all relevant data were extracted. Only studies which compared a flashing light to a continuous one were included.

Results: At first, we found 19,730 citations. Screening and critical appraisal resulted in 32 accepted articles corresponding to 54 accepted observations—one observation corresponding to one species and one outcome. We collated data on four main taxa: Aves (the most studied one), Actinopterygii, Insecta and Mammalia as well as on plankton.

Conclusions: The impacts of flashing light are currently critically understudied and varied between species and many light specificities (e.g. frequency, wavelength, intensity). Therefore, no definitive conclusions could be drawn for now. Thus, research on flashing light should be pressingly carried out in order to better mitigate the impacts of Artificial Light at Night (ALAN) on wildlife. In the meantime, we would recommend precautionary principles to be applied: flashing lighting should be limited when not deemed essential and flicker frequencies managed to prevent animals from experiencing any potential harm from flashing light.

Key words: Blinking light, critical flicker fusion frequencies, dark infrastructure, dynamic lighting, light emitting diodes, nature, sensory disturbance, vision

Introduction

Since the 1990s, species diversity has been decreasing at an accelerating pace (IPBES 2019; IUCN 2021). Among the main drivers affecting biodiversity, land degradation, overexploitation, climate change, chemical pollution and invasive species are now commonly acknowledged as the most impacting ones (IPBES 2019). However, during the last decades, other types of anthropogenic drivers have intensified as well, such as light pollution, which is now considered a serious cause of biodiversity erosion (Hölker et al. 2010, 2021). Indeed, satellite-detectable light has increased by at least 49% between 1992 and 2017, notably due to increased urbanisation and economic growth (Sánchez de Miguel et al. 2021). Simultaneously, Light Emitting Diode (LED) installations have increased, due to their reduced energetic consumption and cost compared to previous technologies such as gas discharge lamps (Zissis et al. 2021), and could lead to a potential ‘rebound effect’ where more anthropogenic light may end up being emitted (Kyba et al. 2017).

Artificial Light at Night (ALAN) have a wide range of impacts on biodiversity from alterations of an individual’s physiology, behaviour and reproduction to ecosystem-wide consequences through impacts on species mobility, relationships and habitat use, threatening community persistence at the landscape level (Falcón et al. 2020; Pérez Vega et al. 2022). These effects of ALAN may depend on several key components of the light source (e.g. intensity, spectral composition, temporality, spatial distribution). For instance, Simons et al. (2021) showed the importance of light intensity on the distribution of runs of the California grunion *Leuresthes tenuis* and roosts of the western snowy plover *Charadrius nivosus* and identified a threshold of 50 mlx to 100 mlx at which the behaviours of both species started to be impacted by ALAN. Increasing intensity levels of ALAN have also been shown to alter cane toads *Rhinella marina* activity patterns and to strongly decrease their corticosterone levels (Secondi et al. 2021). As for ALAN spectral composition, Deichmann et al. (2021) observed an overall significant decrease of insect attraction to filtered-amber LED lamps (deprived in blue spectrum) in a tropical forest environment. Considering the temporality of ALAN, Davies et al. (2017) observed that switching off lighting during periods of low demand while also dimming, was the most promising alternative in order to mitigate the impacts of ALAN on the composition of grassland spider and beetle assemblages. However, on higher levels of the food chain, part-night lighting schemes have been shown to poorly reduce the ecological impacts of ALAN on bats (Azam et al. 2015; Hooker et al. 2022).

Another component of anthropogenic light sources has been largely overlooked despite its potential effects on species: its flicker frequency. Flicker results from the alternating nature of power supply (i.e. 50 Hz in Europe and 60 Hz in the United States) and may usually reach a frequency of 100 Hz (or 120 Hz). All light technologies may be affected by flicker, in particular vapour discharge—such as high-pressure sodium lamps—and LED which are more commonly used as outdoor lighting. Additionally, the expansion of the LED market has also enabled new advanced dynamic lighting to flourish, as exemplified by flashing shop fronts and ad panels or new traffic-regulated street lamps (Falcón et al. 2020; ICNIRP 2020). For the sake of conciseness and clarity and because the difference between flashing and flickering is purely based on how humans perceive flashing light, we will solely use ‘flashing’ in the rest of the

article. Indeed, in the literature, a low flicker frequency (usually under 10 Hz) is more often called a flashing whereas a higher flicker frequency (usually superior to 100 Hz) is more often called a flicker.

The perception of a flashing light is variable according to the species and depends on a threshold frequency value, called the Critical Fusion Frequency (CFF) (Frank 1999; Boström et al. 2017). To this day, the knowledge of CFF distribution within the animal kingdom is still patchy (Inger et al. 2014). However, Lafitte et al. (2022) recently identified that some animal species, and more especially nocturnal and crepuscular ones, had exceptionally high CFF and could theoretically perceive ALAN as flashing. However, whether they are perceived or not, flashing lights have already been linked to alteration of behaviour and physiology in several species. For instance, Barroso et al. (2015) recorded fewer captured insects at traps lit with a flashing light compared to the ones lit with a continuous one. Greenwood et al. (2004) found that the starling *Sturnus vulgaris* was preferentially attracted to a continuous light source compared to a flashing one. Examining the ocular physiology of guinea pigs exposed to a 1 Hz flashing LED, Zhi et al. (2013) found that a significant myopia had been induced by flashing light after just three weeks of treatment. Flashing has also been linked to potential significant effects on human health such as headaches and eyestrains, as discovered by Wilkins et al. (1989).

Hence, we propose this systematic review which aimed at answering the following question: what are the known physiological and behavioural impacts of flashing artificial light on animals when compared to continuous light? We chose to only consider and report results comparing continuous and flashing lights because we felt they were the only ones to really evaluate the effect of the flashing characteristic of a light stimulus, as opposed to the effect of the light stimulus as a whole. We followed the method recommended by the Collaboration for Environmental Evidence (CEE) (CEE 2018). Adapted to the field of ecology, CEE systematic reviews are based on standardized protocols and provide a transparent, accurate and unbiased reporting of evidence to help practitioners make informed and efficient decisions (Haddaway et al. 2016; Berger-Tal et al. 2019; Pullin et al. 2022). We used a comprehensive search strategy based on several databases and performed a critical analysis of accepted observations in order to judge the level of confidence which could be granted to the reported impacts of flashing light.

Methods

This review followed the method for systemic reviews recommended by the Collaboration for Environmental Evidence (CEE) (CEE 2018) and conformed to ROSES RepOrting standards for Systematic Evidence Syntheses (Haddaway et al. 2017) (see Suppl. material 1). The procedure typically includes (i) a literature searching phase, (ii) a screening process related to several eligibility criteria, (iii) a critical appraisal phase during which the susceptibility to bias of each selected article is evaluated and (iv) the extraction of all relevant data in the form of a narrative synthesis. Deviations from CEE standards are listed in the section “Review limitations”.

Search for literature

We carried out a search for literature on three accessible databases from the Web of Science platform (Clarivate): Web of Science Core Collection, Biological

Abstracts, and Zoological Records. These databases were chosen for their functionalities, which enabled an advanced search strategy to be carried out, and because of their wide coverage on biological and ecological matters. For the Web of Science Core Collection search, SCI–EXPANDED, SSCI, A&HCI, CPCI–S, CPCI–SSH, BKCI–S, BKCI–SSH, ESCI and CCR–EXPANDED citation indexes were used. As for Biological Abstracts and Zoological Records, we had access to all indexed databases (respectively 1969–present and 1864–present). In order to achieve the best recovery of citations, several successive search strings were designed by both ecological scientists from the French National Museum of Natural History (MNHN) and physicists from the National Conservatoire of Arts and Crafts (CNAM). Each one was tested for comprehensiveness on a pre-established test list of articles—comprised of 35 articles identified as relevant while scoping the evidence on Web of Science Core Collection and Google scholar at the beginning of the project (see Suppl. material 2)—until the following search string was finally accepted:

("light* flash*" OR "flicker* light*" OR "blink* light*" OR "light* strob*" OR "strob* light*" OR "light* wink*" OR "light* puls*" OR "puls* light*" OR "intermittent* light*" OR "dynamic* light*" OR "light*dim*" OR "dim* light*" OR "discontinuous light" OR "dynamic illumination" OR "flash rate" OR "change\$ of light*") AND (ecolog* OR biodiversity OR ecosystem\$ OR species OR vertebrate\$ OR mammal\$ OR reptile\$ OR amphibian\$ OR bird\$ OR fish* OR invertebrate\$ OR arthropod\$ OR insect\$ OR arachnid\$ OR crustacean\$ OR centipede\$ OR animal\$ OR plant\$* OR bacteri* OR microorganism*)).

The search was then conducted on "Topic" (TS) on 1 February 2021 and reached a comprehensiveness of 86%, corresponding to the percentage of articles from the test list retrieved by the search string.

Screening process and eligibility criteria

Following CEE guidelines for systematic reviews (CEE 2018), a three-stage screening process was carried out on all citations to select only those relevant to our question, starting with titles, then abstracts and finally full-texts. Citation eligibility screening relied on Population–Exposure–Comparator–Outcome (PECO) criteria. At title and abstract screening stages, only restricted Population and Exposure criteria were considered due to the limited amount of available information (Table 1).

At the full-text screening stage, these Population and Exposure criteria were further refined (Table 2). Indeed, while our first aim was to assess the effects of flashing light on biodiversity as a whole, we had to limit the scope of this systematic review due to the high volume of literature retrieved by the search string

Table 1. List of eligibility criteria at title and abstract screening stages.

	Include	Exclude
Population	- All wild and domesticated species in all types of ecosystems (e.g. animals, fungi, plants, micro-organisms)	- Humans
		- Isolated organs (except those from the visual pathway, optical nerve and/or pineal gland)
Exposure	- Artificial flashing light sources at all wavelengths and correlated colour temperatures	- Natural (e.g. lightning) or unknown light sources

and thus, of accepted citations after title and abstract screening. As such, we decided to only keep alive and conscious wild animals while domesticated, dead (or animal parts) and anaesthetised animals were excluded—the generalisability of their results was considered too low for our review objectives. Plants, fungi and micro-organisms were discarded as well. In addition, Comparator–Outcome inclusion/exclusion criteria as well as language, document type and document content criteria were assessed as well. Only articles comparing a continuous light source to a flashing one were included as we considered that they were the only ones to really assess the sole effect of the flashing characteristic of a light stimulus as opposed to the effect of the light stimulus as a whole. Articles only comparing the obscurity (no light) to a flashing light source or comparing several flicker frequencies were thus excluded. Ideally, the flashing characteristic of light would be the only varying factor between the control and the exposed groups but the presence of confounding factors (e.g. type of light source, spectral composition, temporality) was not considered as an exclusion criteria and was further assessed during critical appraisal (see section ‘Critical appraisal’). Only studies published in English and/or French were retained in this systematic review in respect to the competences of the review team. Articles without an appended abstract were not screened due to their high number and because of time limitations.

Screening was carried out by at least two reviewers: ML and RS for titles, ML, RS and YR for abstracts, ML, RS and AL for full-texts. For title and abstract screening, a Randolph’s Kappa coefficient was computed on a random sample of 5% of all articles in order to assess the consistency of the inclusion/exclusion decisions between screeners. This process was repeated until reaching a Kappa coefficient value higher than 0.6, usually considered sufficient (Adams et al. 2019; Ghordouei Milan et al. 2022). All disagreements between reviewers were discussed before beginning the screening process to resolve any differences in the understanding of eligibility criteria. To prevent any conflicts of interest, special care was taken, at each stage of the screening process, to ensure that no reviewer would screen articles they co-authored.

Table 2. List of eligibility criteria at the full-text screening stage.

	Include	Exclude
Population	- All wild animal species in all types of ecosystems	- Domesticated animals
	- Alive specimens	- Humans, plants, fungi and micro-organisms
	- Conscious specimens	- Dead specimens and therefore isolated organs, tissues or cells
Exposure	- Artificial flashing light sources at all wavelengths and correlated colour temperatures	- Anaesthetised specimens
	- Short-lived flashing patterns	- Natural or unknown light sources
		- Very slow flashing light patterns spreading on possibly several hours (e.g. circadian patterns)
Comparator	- Studies comparing a continuous light source to a flashing one	- Studies only comparing the obscurity (no light) to a flashing light source
		- Studies comparing several flicker frequencies
Outcome	- Physiological and/or behavioural responses	
Language	- Articles written in English and/or French	
Document type	- Journal article, book chapter, technical report, Ph.D. or M.Sc. theses	
Document content	- Primary research articles	- Reviews and meta-analyses, modelling studies without experimental data

Other sources of literature

A call for literature—and in particular non peer-reviewed articles published in French and/or English—was also carried out by contacting a group of 40 experts on 12 February 2021. Indeed, as there exists a publication bias where only significant results may be accepted for publication, the CEE advocates for grey literature to be included in the literature search of systematic reviews to limit the risk of overestimating the effect of the exposure on the studied population (Haddaway and Bayliss 2015; CEE 2018).

Other sources of literature were added to improve the comprehensiveness of our search. First, we included references dealing with flashing light coming from Adams et al. (2019, 2021), who recently published a systematic map on the effects of artificial light on bird populations. Additionally, some other articles on the impacts of flashing light on animals identified by the review team but not directly extracted from the three considered databases were also included. All corresponding documents were screened on their full-texts according to the same inclusion/exclusion criteria as described above.

Critical appraisal

Critical appraisal is one of the defining stages of systematic reviews, albeit it remains rarely performed in environmental evidence syntheses (Stanhope and Weinstein 2022). Its aim is to assess the extent of systematic error that can be found in primary research articles included in the systematic review. Systematic error is usually estimated thanks to pre-built and objective risk of bias criteria (see below in the context of this review) and may lead to the exclusion of research considered as highly susceptible to bias.

Accepted articles after screening stages were split into observations, an observation corresponding to one species and one outcome, in order to carry out a critical appraisal and assess the validity of each single observation for a given article—e.g. an article analysing two responses of three different species would be split into six observations which would then be critically appraised individually. A test was conducted on a subsample of observations by two reviewers (RS and AL), then critical appraisal was performed by AL for all observations. To prevent any conflicts of interest, special care was taken to ensure that no reviewer would critically appraise articles they co-authored.

When hypothesizing a 'gold standard protocol', carried out in the context of an ideal and quasi-perfect study supposedly granted with unlimited financing, time and workforce (CEE 2018), we were able to identify six risks of bias criteria to evaluate the validity of each observation:

- the type of experimental design (Control criterion),
- the number of individuals (Replication criterion),
- the number of measures (Repetition criterion),
- the randomisation of individuals throughout experimental groups (Randomisation criterion),
- if individuals really perceived the exposure to flashing light (i.e. flicker frequency higher than their CFF) (Exposure criterion),

- if confounding factors have been accounted for (Confounding factors criterion),
- if any other risk of bias was detected (Other bias criterion).

Each of these criteria was assigned a 'high', 'medium' or 'low' risk of bias (see Suppl. material 7 for details). Finally, for each accepted observation, an overall risk of bias was attributed:

- 'high' for an observation which had a high risk of bias in the control or replication criteria or more than two high risks of bias criteria,
- 'medium' for an observation which had a medium risk of bias in the control or replication criteria or more than two medium risks of bias criteria,
- 'low' for remaining observations.

We considered an observation to be unreliable in the total absence of control or replication, therefore resulting in its exclusion. However, due to expected in-situ experimenting challenges and because we wanted to ensure the best comprehensiveness of study designs, in-situ observations with only one experimental site (but several replicates) were still kept but were given a high risk of bias in the Replication criterion.

Data extraction

Data on the influence of flashing light for a particular species or taxa were extracted by one reviewer (AL) although a test was first conducted on a subsample of observations by two reviewers (RS and AL) to assess agreement between reviewers. Metadata were also extracted for each observation, namely locations, specificities of population (age, sex) and light sources (type, wavelength, power, luminance, correlated colour temperatures and flicker frequency) as well as outcomes (e.g. behaviour, weight, mortality). Each species was associated with its taxonomic class and name updated with the latest taxonomy (GBIF 2021). Critical appraisal risks of bias were also appended to each observation included in the database.

Data synthesis and presentation

Accepted observations are described in an exhaustive narrative synthesis (see Suppl. material 9) and are arranged by subgroups based on taxa, outcomes and risks of bias. All statistical analyses were carried out on the R software (November 2021, version 4.1.2) and graphs were customized thanks to the 'ggplot2' package (Wickham 2016).

Results

Screening process and critical appraisal

A total number of 19,730 citations were extracted from the three databases from which 5,253 citations were kept after title screening. Among them, 2,145 citations had no indexed abstracts and were discarded. After abstract

screening, 2,594 citations were kept. With the addition of 68 citations identified through the call for grey literature and 63 identified by the review team, 2,130 PDFs were successfully retrieved and screened on full-texts. The screening process resulted in 32 accepted articles (see Suppl. material 3 for inclusions/exclusions on titles, abstracts and full-texts, Suppl. material 4 for full-text reasons for exclusion and Suppl. material 6 for unobtainable full-texts).

All 32 articles accepted after the screening process were then split into 62 observations—an observation corresponding to one species and one outcome—and subjected to critical appraisal. Among them, 54 observations were accepted on which 22.2% (12 observations) were rated with a high, 70.4% (38 observations) with a medium and 7.4% (4 observations) with a low risk of bias (see Suppl. material 7). Complete screening and critical appraisal processes are presented on Fig. 1.

Bibliometric results

Year of publication

The earliest accepted observations were published in 1972. However, this research subject boomed at the start of the 2000s and the vast majority of observations (51 observations) were investigated between 2000 and 2020, with a slight increase over time (see Suppl. material 9).

Literature sources

The majority of accepted observations came from our search on Web of Science Core Collection database (32 observations) while 10 were provided thanks to the work carried out during Lafitte et al. (2022)'s systematic review on CFF, 8 were extracted thanks to Adams et al.'s systematic map on the effect of ALAN on birds (Adams et al. 2019, 2021), three were identified by the review team and finally one was provided thanks to the call for grey literature. No observations from Zoological Records or Biological Abstracts databases were accepted in the end.

Observation location

The United States (US) is the primary research location with 22 observations, followed by the United Kingdom (11 observations), Canada (6 observations) and Germany (4 observations). The 11 remaining observations were conducted either in Egypt, Switzerland, Israel, Japan, Taiwan and Brazil, as well as one joint experimental observation carried out between the US and Israel (Fig. 2 and Suppl. material 9).

Type of light source exposures

Most of the 54 observations used LED (17 observations), 11 used gas discharge lamps and three used incandescent bulbs. Experiments were also carried out thanks to lasers (3 observations), a video projector (1 observation) or a monitor screen (1 observation). Sometimes, several light sources were used at the same time: for instance, LED and gas discharge (4 observations), LED

ROSES Flow Diagram for Systematic Maps. Version 1.0

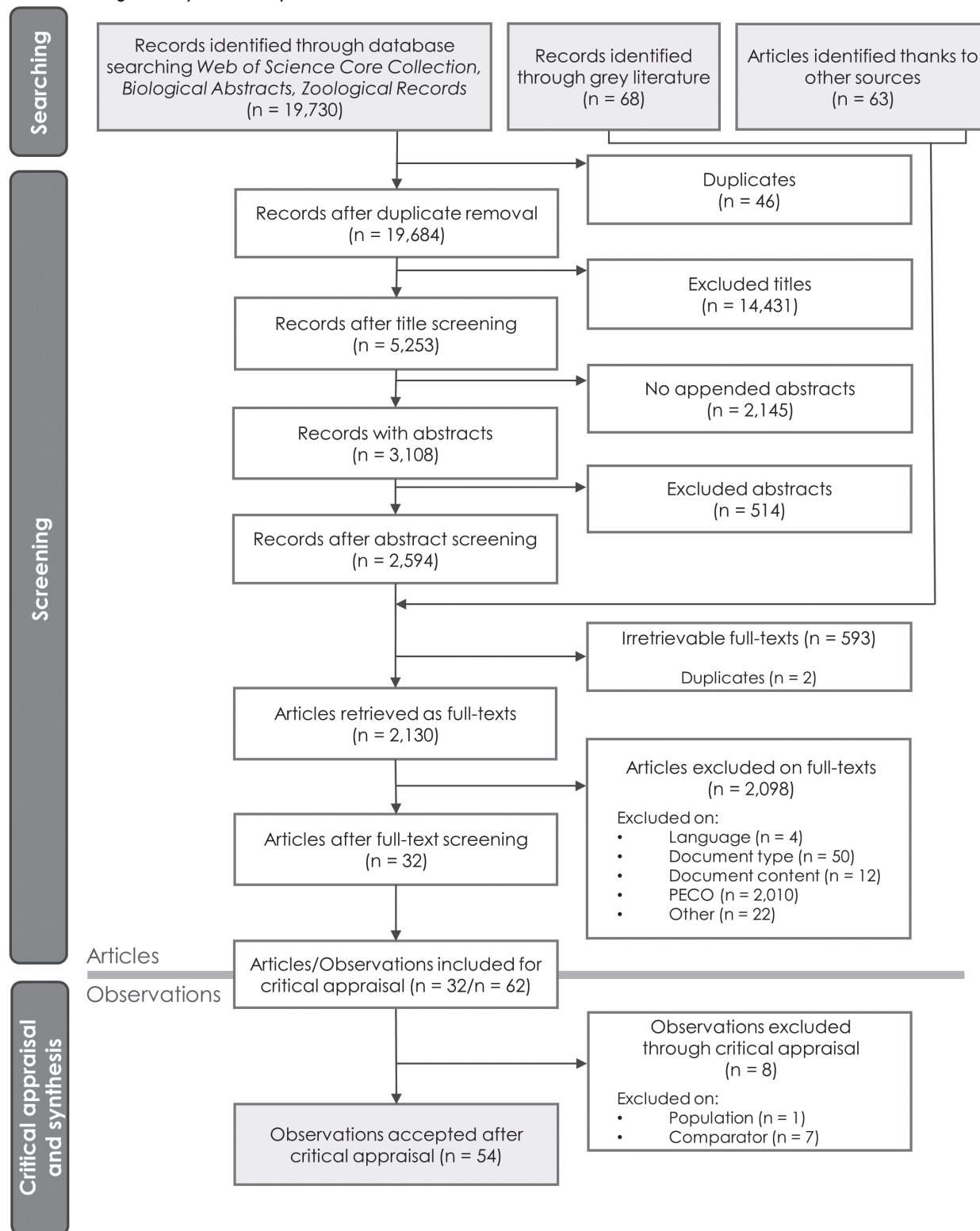


Figure 1. ROSES flow diagram reporting the screening process of articles and observations included in the review. (Haddaway et al. 2018). 'PECO' stands for Population–Exposure–Comparator–Outcome eligibility criteria.

and incandescent (3 observations). In some cases, the light source was not sufficiently reported which resulted in some observations having an unclear light source appended to them (see Suppl. material 9).

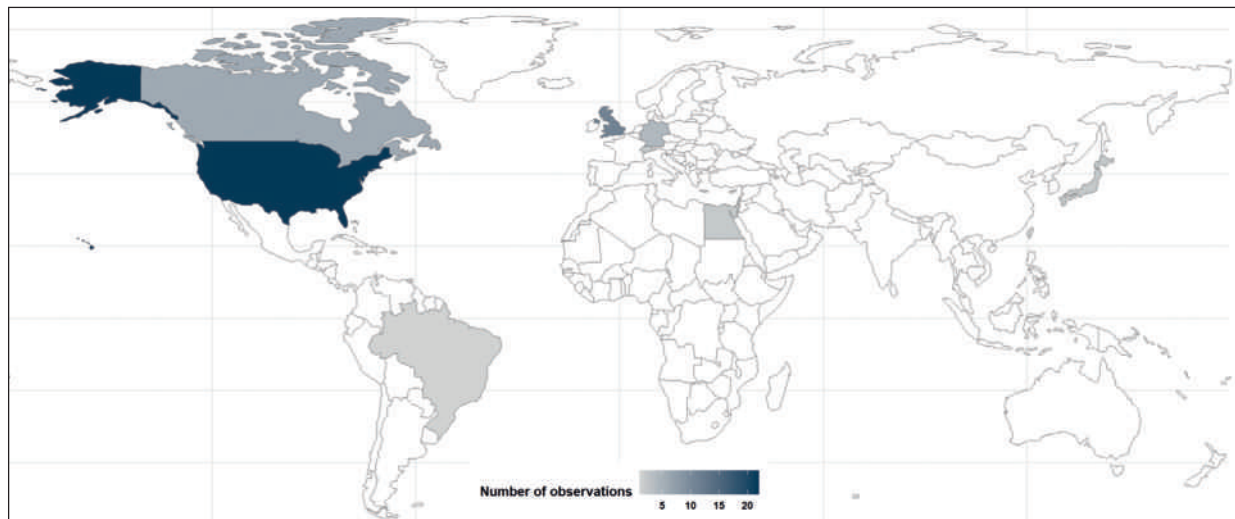


Figure 2. World map showing the number of included observations by country. The joint Israel/United States observation is not shown.

Studied taxa

Data on the four main taxonomic classes Aves (28 observations), Actinopterygii (10 observations), Insecta (8 observations) and Mammalia (6 observations) were collated (Fig. 3A). Additionally, two observations investigated the effects of flashing light on plankton (notably on Malacostraca and Polychaeta larvae). While a fraction of observations was conducted in-situ (18 observations), 70% of observations (36 observations) were carried out in laboratories. The starling *S. vulgaris* was the most investigated species with 13 observations (Fig. 3B), followed by the brown-headed cowbird *Molothrus ater* (4 observations) and the cat flea *Ctenocephalides felis* (3 observations). All other species or taxa were only studied once or twice.

Measured outcomes

In the vast majority of cases, observations measured the effects of flashing light on animals' behaviour (Fig. 4). Phototactic behaviour—i.e. the attraction of animals to flashing light when compared to a continuous one (or which should be perceived as continuous based on their CFF)—was mostly investigated (33 observations), but activity level (6 observations) and other types of behavioural responses such as disorientation, feeding, aggression (6 observations) were also assessed. Observations on the physiological responses of animals exposed to flashing light were also collected albeit more sparsely. Cortisol levels were studied four times while haematocrit, memory, neuronal activity, ocular physiology and weight were studied once each.

The impacts of artificial flashing light

Before reading the following results, the reader has to be reminded that only observations comparing a flashing light to a continuous one were included; all other comparisons were not reported in this review—e.g. obscurity compared to flashing light or comparing several flicker frequencies.

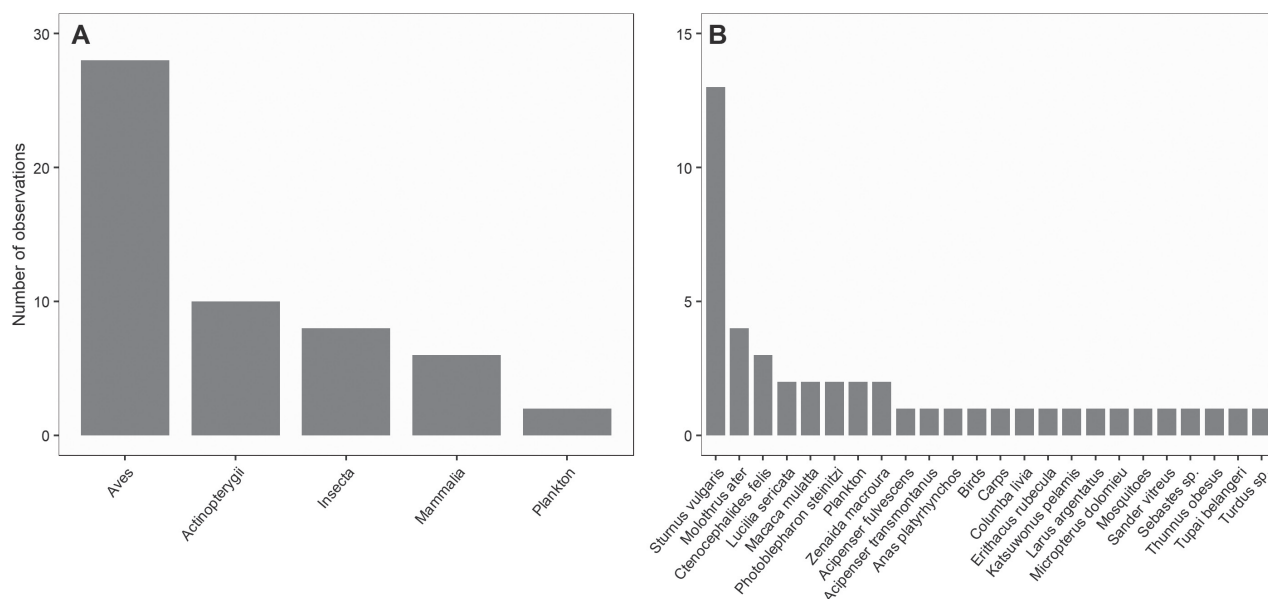


Figure 3. Proportion of included observations by taxa **A** total number of included observations by taxonomic classes and **B** number of included observations by detailed taxa.






	 Aves			 Actinopterygii			 Insecta		 Mammalia		 Plankton
Types of outcome	L	M	H	L	M	H	M	H	M	H	M
Phototactic behaviour	3	9	2	•	7	1	4	4	1	•	2
Activity level	•	•	3	1	•	•	•	•	2	•	•
Behaviour	•	4	•	•	1	•	•	•	•	1	•
Cortisol level	•	4	•	•	•	•	•	•	•	•	•
Haematocrit	•	1	•	•	•	•	•	•	•	•	•
Memory	•	1	•	•	•	•	•	•	•	•	•
Neuronal activity	•	•	•	•	•	•	•	•	•	1	•
Ocular physiology	•	•	•	•	•	•	•	•	1	•	•
Weight	•	1	•	•	•	•	•	•	•	•	•

Figure 4. Summary of the number of observations by outcomes and risks of bias for all taxa. 'L' low risk of bias, 'M' medium risk of bias, 'H' high risk of bias, '•' no data.

Taking the example of phototactic behaviour, the most studied outcome with 33 observations (60% of the corpus), a clear and definitive conclusion on the effects of flashing light remains hard to draw (Fig. 5)—even though one could argue that flashing light might be as attractive as continuous light, or even slightly less so. Overall, the impacts of flashing light are highly variable according to taxa (Fig. 6) as well as light parameters.

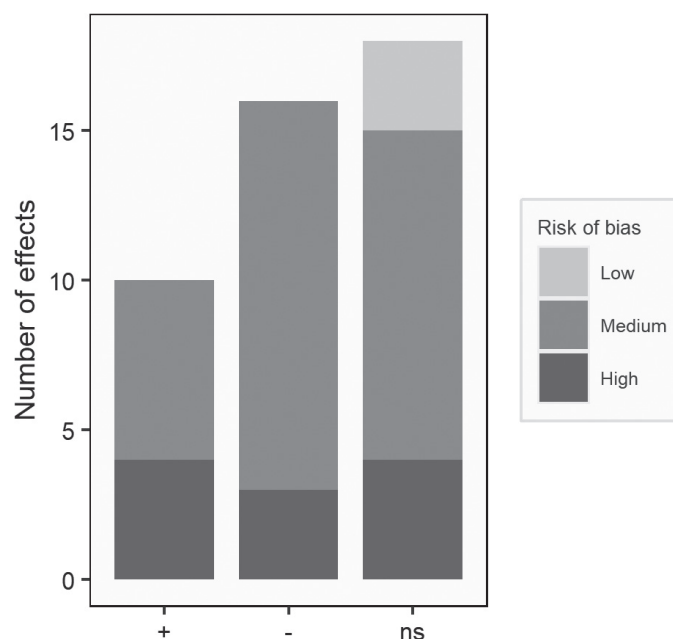


Figure 5. Number of reported effects for the outcome phototactic behaviour. ‘+’ animals are more attracted to a flashing light than a continuous one, ‘-’ animals are less attracted to a flashing light than a continuous one, ‘ns’ no significant effect. Sample size: Aves (n = 14 observations), Actinopterygii (n = 8), Insecta (n = 8), Mammalia (n = 1), Plankton (n = 2). As directions of effects are not homogeneous between the different types of reported outcomes, we decided to only show the number of effects for phototactic behaviour, the most studied outcome which accounts for 60% of the corpus with 33 observations.






	 Aves			 Actinopterygii			 Insecta		 Mammalia		 Plankton
Types of outcome	L	M	H	L	M	H	M	H	M	H	M
Phototactic behaviour	ns	+ - ns	- ns	•	+ - ns	ns	+ - ns	+ - ns	ns	•	ns
Activity level	•	•	+ ns	+ ns	•	•	•	•	ns	•	•
Behaviour	•	+ - ns	•	•	+ - ns	•	•	•	•	-	•
Cortisol level	•	+ - ns	•	•	•	•	•	•	•	•	•
Haematocrit	•	ns	•	•	•	•	•	•	•	•	•
Memory	•	+	•	•	•	•	•	•	•	•	•
Neuronal activity	•	•	•	•	•	•	•	•	•	+ -	•
Ocular physiology	•	•	•	•	•	•	•	•	ns	•	•
Weight	•	ns	•	•	•	•	•	•	•	•	•

Figure 6. Summary of flashing light effects by outcomes and risks of bias for all different taxa. ‘L’ low risk of bias, ‘M’ medium risk of bias, ‘H’ high risk of bias, ‘+’ flashing light increases the outcome compared to continuous light, ‘-’ flashing light decreases the outcome compared to continuous light, ‘ns’ no significant effect, ‘•’ no data.

Due to this strong heterogeneity of results, we chose to provide, in the following section, a brief summary of our main findings. For a full and exhaustive narrative synthesis of all observations and results included in this systematic review, we refer the reader to Suppl. materials 8, 9.

On birds, we collated 28 observations. First, in 5 observations, flashing light appeared to be less attractive than continuous light to night-migrating birds and might thus help lower the number of avian fatalities with communication towers or wind turbines, even though such results could be wavelength-dependent (Evans et al. 2007; Gehring et al. 2009; Gehring 2010; d'Entremont 2015; Rebke et al. 2019). The impact of flashing light on bird vehicle deterrence was inconsistent and seemed to be species-, frequency- and speed-dependent (Blackwell and Bernhardt 2004; Blackwell et al. 2009; Doppler et al. 2015). Avian preference for high-frequency lighting (i.e. lighting frequency superior to 30,000 Hz) over low-frequency (i.e. lighting frequency of 100 Hz) followed a more complex pattern than predicted and may depend on the spatial frequency of the surrounding environment (Greenwood et al. 2004; Smith et al. 2005)—for example, a black and white grating. Bird behavioural responses to flashing light stimuli seemed very variable but were often altered after the exposure (Greenwood et al. 2004; Smith et al. 2005; Evans et al. 2012; Wiltshko et al. 2016). Flashing light was hypothesised to induce greater stress levels in birds kept under low-frequency lighting but results proved to be highly inconsistent (Maddocks et al. 2001; Greenwood et al. 2004; Smith et al. 2005; Evans et al. 2012). Bird activity levels have been shown to be affected by flashing light but such results were dependent on the species and type of light source (Lustick 1972). In addition, one observation on the common pigeon *Columbia livia* also indicated that flashing stimuli may be easier to remember for birds (Fetterman 2000). Haematocrit and weight were also investigated once and were not shown to be impacted by flashing light when compared with a continuous one (Smith et al. 2005).

On fishes, we reported the results of 10 observations. Fish phototactic behaviour was found to be highly variable and seemed to be, in part, species-, frequency- and wavelength-dependent (Ruebush et al. 2012; Rooper et al. 2015; Ford et al. 2018, 2019; Elvidge et al. 2019; Oshima et al. 2019), and even more so when flashing stimuli can be used by some fish species to communicate with conspecifics (Hellinger et al. 2020). In contrast to continuous light, flashing light was also found to significantly alter the behaviour of the flashlight fish *Photoblepharon steinitzi* (Hellinger et al. 2020) as well as to influence daily fluctuations in activity levels of the nesting smallmouth bass *Micropterus dolomieu* (Foster et al. 2016).

Regarding insects, 8 observations were collected. Overall, flashing light was shown to produce an effect on insect phototactic behaviour, but results were highly species- and frequency-dependent (Müller et al. 2011; Barroso et al. 2015; Eichorn et al. 2017; Liu et al. 2017; Bolliger et al. 2020). In addition, flashing light may be particularly important for insects as some species seem to use flashing signals to identify conspecifics and sexually-mature partners (Eichorn et al. 2017).

Mammals were investigated in 6 observations. Bat activity level was reported in two observations and phototactic behaviour once. Both outcomes were not found to be significantly influenced by flashing light (Jain et al. 2011; Bolliger et al. 2020). One observation on the tree shrew *Tupaia belangeri* showed that flashing blue light could cause myopia when the continuous blue one did not (Gawne et al. 2017)—the result, however, depended on the spectral composition of the light source. Two observations also found that the rhesus monkey *Macaca mulatta* could be less efficient at

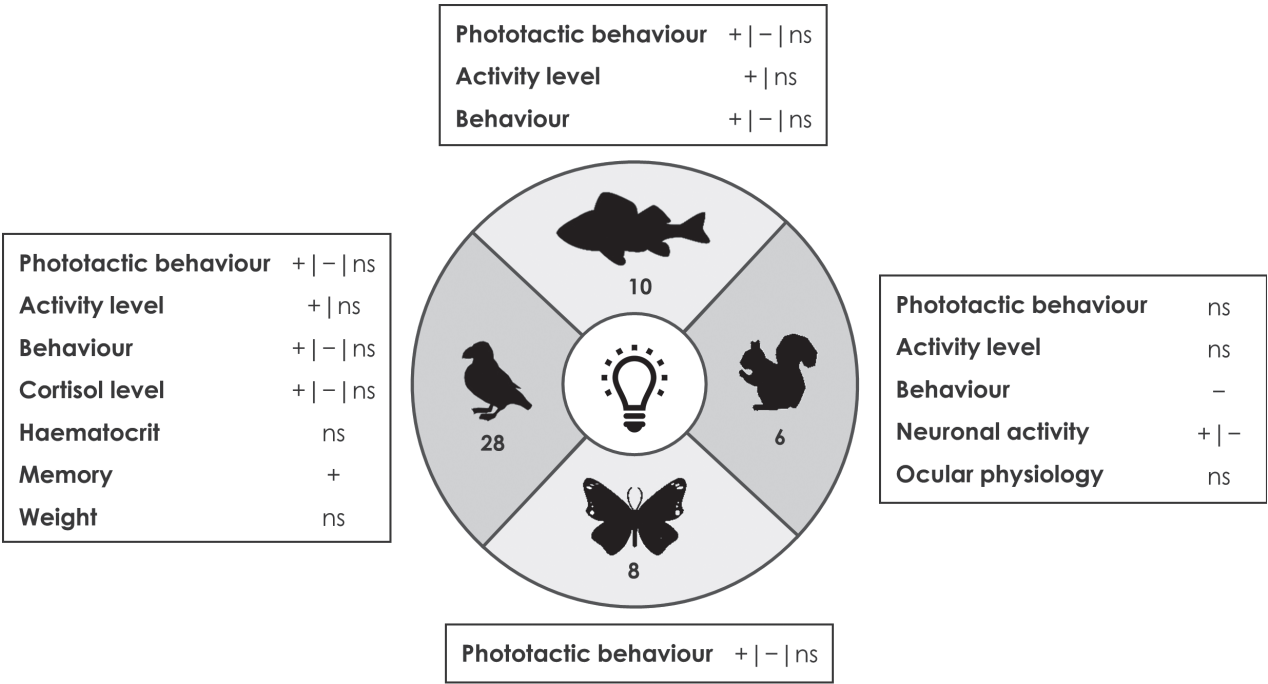


Figure 7. Summary of results for the four main studied taxonomic classes. ‘+’ flashing light increases the outcome compared to continuous light, ‘-’ flashing light decreases the outcome compared to continuous light, ‘ns’ no significant effect. For clarity, the two observations on plankton phototactic behaviour are not shown but were both found to be non-significant.

discriminating the direction of moving stimuli with a flashing background (Churan and Ilg 2002).

Finally, we also collated two studies on the phototactic behaviour of plankton, which did not find any significant impact of flashing light over continuous light (Dougherty et al. 2016).

Discussion

Within this systematic review, which aimed at summarising the physiological and behavioural impacts of flashing light on animals, 32 articles accounting for 54 observations were accepted. After carrying out screening and critical appraisal, 28 observations on birds, 10 on fishes, 8 on insects, 6 on mammals, as well as 2 on plankton were collected (Fig. 7). Overall, we found that: (i) the impacts of flashing light may vary according to the species and taxonomic classes; (ii) the various specificities of light sources (e.g. flicker frequency, light intensity, spectral composition, duration) may also influence the response of animals to flashing light; (iii) the available literature is scarce and more research should be carried out rapidly in order to give more definitive conclusions; (iv) therefore, in the meantime, precautionary principles should be applied to avoid adding potential negative impacts on sensitive animals.

The impacts of flashing light may vary between species and taxonomic classes

While the evidence still seems scarce, our results indicate that the effects of flashing light are highly variable between species and taxonomic classes. We

found that, in some animal species, a flashing light could be less harmful than a continuous one. For example, the brown-headed cowbird *M. ater* showed a lower attraction to a 2 Hz flashing light (Blackwell et al. 2009; Doppler et al. 2015). Similarly, flashing lights on communication towers have been shown to reduce the number of avian collisions (Evans et al. 2007; Gehring et al. 2009; Gehring 2010; Rebke et al. 2019). Bolliger et al. (2020) showed that, in a street with intermittent lighting, fewer insects, and more especially fewer heteropterans, may be trapped compared to the one that would be lit all night. Alternatively, species exposed to flashing light may sometimes experience the same kind of effects as for a continuous light. For instance, bats were studied thrice and the impact of a flashing light was not shown to differ from that of a continuous one (Jain et al. 2011; Bolliger et al. 2020). No differences in plankton phototaxis to flashing or continuous light was observed either (Dougherty et al. 2016). Lastly, in some cases, flashing light have been shown to be more impactful than continuous light. For instance, one observation showed that flashing light could lead to greater daily fluctuations in fish activity levels (Foster et al. 2016). Some fish species, like the lake sturgeon *Acipenser fulvescens*, may also be more attracted to flashing light (Elvidge et al. 2019), which could then disturb their overall behaviour and hamper their ability to feed or reproduce. Such variability in the responses of different taxa to light pollution has already been highlighted in vertebrates, for which the impacts of ALAN on melatonin and circadian rhythms may be highly dependent on the species and taxonomic class considered (Grubisic et al. 2019). For bats too, the effects of ALAN can greatly vary between different species and foraging guilds (Voigt et al. 2021). These differences of responses to ALAN between and within taxa preclude us from drawing general conclusions on the impact of light pollution and, in our particular case, flashing light.

The impacts of flashing light may also vary according to several parameters of the light source

In addition to variations between species and taxonomic classes, the response to flashing light may also differ according to the type of exposure to the light source—i.e. flicker frequency, light intensity, wavelength and/or duration.

First and foremost, the response to flashing light depends on the frequency at which the source flashes. For instance, Eichorn et al. (2017) found that male green bottle flies *Lucilia sericata* were greatly attracted to flicker frequencies of 178 Hz, 190 Hz and 250 Hz, while no differences in phototaxis between flashing and continuous lights were found for flicker frequencies of 110 Hz and 290 Hz. In particular, as there exists for each species a threshold frequency at which a flashing light begins to be perceived as continuous, defined as the CFF, the importance of a light source flicker frequency seems paramount. Indeed, a species perception of a flashing light source could theoretically be inferred thanks to the knowledge of its CFF and the flicker frequency of a light source. To that end, Inger et al. (2014) first reviewed the actual perception of flashing light by animals by collating one of the first databases on animal CFF. Lafitte et al. (2022) then updated this work by following the method of systematic reviews recommended by the CEE (CEE 2018) and were able to collate a comprehensive database of 200 CFF values. As they identified that animal CFF ranged

from 0.57 to a maximum of 500 Hz, they argued that outdoor lighting should exceed this upper threshold in order to limit the impacts of ALAN on wild animals. In addition, they reported that some nocturnal animals (e.g. moths and fishes) had CFF higher than the 100 Hz threshold sometimes found in some lighting technologies such as LED. Based on this analysis of both CFF and light source flicker frequencies, it can be assumed that some species could be subjected to the potential adverse effects of flashing light.

However, comparing a species CFF with the flicker frequency of a light source may prove insufficient in order to conclude on the existence or absence of impacts of flashing light on animals. Indeed, in real in-situ conditions, many factors can accentuate or limit the perception of a flashing light by an animal (Fig. 8). Indeed, the specificities of light sources, such as intensity, spectral composition, correlated colour temperatures, or timing and duration of exposure, have often been linked to the variability observed in the reported effects of ALAN on animals (Grubisic et al. 2019; Voigt et al. 2021). In the case of flashing light, we therefore advocate for a better regulation of outdoor lighting, as a precautionary measure. The light intensity and therefore the distance to the light source may influence the extent of potential impacts. Indeed, as a brighter continuous light source may be perceived from further away compared to a dimmer one, we therefore advocate for keeping light levels of flashing lights as low as possible. The orientation of the light source is also crucial as a horizontal, or worse, upward-oriented flashing light source may be detected from further away. In relation to the chromatic visual capabilities of each taxon, the spectral composition of the light source could also influence how flashing light is perceived and how it potentially impacts animals. Indeed, Evans et al. (2007) and

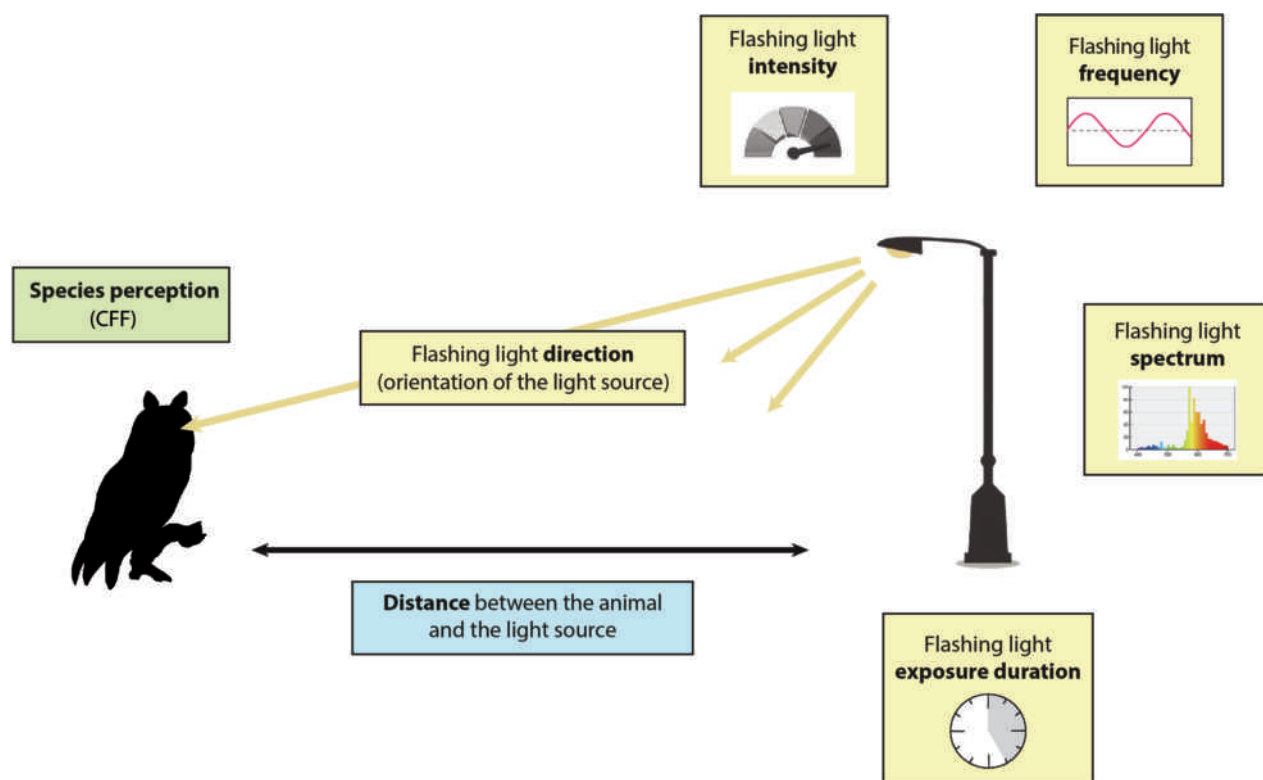


Figure 8. The in-situ perception of flashing light by animals depends on several parameters of the light source.

Rebke et al. (2019) showed that red-coloured lights, whether flashing or not, were less attractive for night-migrating birds, unlike other light colours such as green, blue or white which were less attractive only when flashing. Gawne et al. (2017) found that flashing light could induce myopia in the tree shrew *T. belangeri*, but the response seemed highly wavelength-dependent as well. Finally, the duration of the exposure to flashing light could also influence the extent of the recorded impacts. For instance, in several publications, we identified that similar exposures to flashing light, but with varying durations, produced very different behavioural responses in birds (Greenwood et al. 2004; Smith et al. 2005; Evans et al. 2012).

Thus, the impacts of flashing light on animals may be considered highly variable and may depend on the species, the taxonomic class, various parameters from the light source and on the surrounding environment (e.g. buildings, surfaces, vegetation).

A huge lack of knowledge for a timely subject

In the end, this systematic review highlights a dearth of knowledge on the effects of flashing light on animals. Although the research on this subject has gained momentum since the 2000s, the evidence remains scarce on this matter. While we were able to identify a relative knowledge cluster on birds' phototactic attraction to flashing light, many other taxa and outcomes were at least poorly studied or simply not investigated. These knowledge gaps on the effects of flashing light should be filled pressingly as lighting is expected to get more and more dynamic with on-demand or sensor lightings being currently rapidly scaled up. While these new technologies could help limit the duration of the exposure to ALAN, the new type of light pollution they may produce and its impacts on biodiversity are not fully understood for now. Likewise, LED, which may flash depending on their technology, are currently being deployed all over the world to reduce the energy consumption of lighting (Zissis et al. 2021) but without taking into account their potential adverse effects on animal populations.

Moreover, among the studies included in this systematic review, very few in-situ experiments were carried out. As such, the generalisability of these studies to real-world situations is low. Only one study dealt with sensor lighting (Bolliger et al. 2020) and some others investigated flashing lights on communication towers (Gehring et al. 2009, 2010) and wind turbines (d'Entremont 2015). We were not able to find any study on the effects of illuminated advertising, billboards or flashing signs which are very common sources of outdoor flashing light.

Another surprising point is that the majority of included studies involved diurnal species, with the starling *S. vulgaris* being the most investigated species. Indeed, diurnal species can be impacted by ALAN—for example, ALAN disturbs their sleep and can have repercussions on their immunology (Ouyang et al. 2017; Sun et al. 2017; Ulgezen et al. 2019). Nonetheless, nocturnal species are probably the most likely to discern whether night-time lighting is flashing or not.

Then, it appears from all previous points that more research on the subject of flashing light should be pressingly carried out in order to keep up with the fast-paced evolution of lighting practices.

Recommendations for further research

First, the studied species and taxonomic classes which were identified in this systematic review should be further investigated. Then, more research is pressingly needed on key taxa which have not yet been studied and could also be at risk of being impacted by flashing light—e.g. moths, amphibians, nocturnal raptors, glow worms. Further research on additional outcomes should also be contemplated such as fitness, foraging or reproductive behaviours as well as other key physiological or spatial outcomes—e.g. immunity, movement, spatial distribution. More in-situ studies should be carried out in order to take into account all light source parameters which may influence a species sensitivity to flashing light—i.e. distance from the light source, orientation, spectrum, intensity. In the case of these in-situ experiments, several locations should also be studied to account for local heterogeneity in species repartition. Based on our criteria for critical appraisal, we advocate for authors to use more robust experimental protocols (Fig. 9). For instance, few studies had protocols comparing two populations, one unexposed and one exposed, before and after the exposure to flashing light—i.e. BACE designs.

We also would like to stress the need for a better reporting of experimental designs specifications (Fig. 9). Light sources were rarely completely described and information on the flicker frequency of flashing or continuous light sources was rarely reported. This lack of reported data on the light sources used to expose specimens to light disturbances has already been noted in another review on the impacts of ALAN on melatonin and circadian rhythms on vertebrates (Grubisic et al. 2019). However, the actual perception of a light source as continuous by one species can only be proven by crossing the flicker frequency of the continuous light source with the CFF of this species. Therefore, if the flicker frequency of the continuous light source is not provided, it cannot be verified if the two light stimuli were perceived any differently by the specimens under scrutiny. In addition, other parameters of the light source like wavelength and light intensity could also influence the results of the experi-

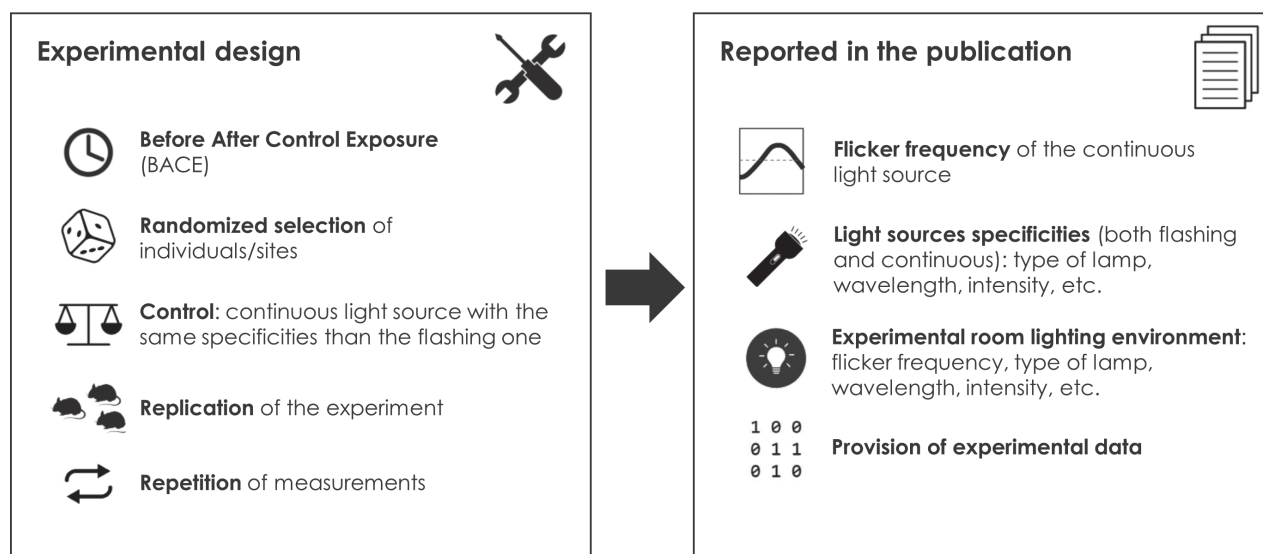


Figure 9. Selected recommendations for more robust and better reported experimental designs and results.

ment and should be reported thoroughly. As such, we recommend light sources to be strictly the same between the control and treatment groups in order to avoid adding any potential confounding factors. Finally, data on laboratories lighting conditions is rarely provided, which could also alter experimental results as some indoor light sources could flash and thus impact the specimens being investigated.

Review limitations

Our methodology comprised some biases which have to be pointed out. First, while the majority of articles found in this review came from our literature search, more than a third was provided by additional sources of literature, indicative that the scope of our search string might have been too limited.

In addition, we sometimes had to decide to reduce our requirements compared to CEE guidelines (CEE 2018) due to time limitations and financing constraints. First, we could not request supplementary databases (e.g. Scopus) or include search engines (e.g. Google Scholar) in our search strategy. However, doing so may have increased the number of test list articles indexed in the requested databases which would have probably increased the reliability of our search strategy. In addition, while consistency checks between reviewers were performed for the title and abstract screening stages, we could not do so for full-text screening. Likewise, CEE guidelines (CEE 2018) call for a double independent assessment by two reviewers during critical appraisal and data extraction. However, in this review, only one reviewer critically appraised and extracted data from all observations accepted after full-text screening. A test between two reviewers on a subsample of articles was still performed before starting critical appraisal and data extraction to check their agreement.

In addition, citations for which an appended abstract was not available were discarded during the screening process. Indeed, searching for these additional 2,145 full-texts was deemed to represent an unfeasible additional workload within the scope of our project. We nevertheless made sure to create an additional database which lists these citations without abstract (see Suppl. material 5). We hope that this database will prove useful for whomever would want to continue this work. Moreover, due to the high level of accepted citations after title and abstract screening and while the initial scope of this review included plants, microorganisms, domesticated animals as well as the impacts of ALAN timing on circadian rhythms, we had to downgrade our expectations and only carry out this systematic review on the sole behavioural and physiological impacts of flashing light on animals. However, if one wishes to disentangle the impacts of flashing on the other identified taxa, we made sure to create easily available categories in Suppl. material 3 to facilitate a potential future full-text screening on these citations. We chose to only consider and report results comparing continuous and flashing lights as we judged they were the only ones to really assess the effect of the flashing characteristic of a light stimulus alone, as opposed to the effect of the light stimulus as a whole. Any update of this systematic review could then also try to assess additional types of comparators which could be useful to draw a more complete picture. Indeed, some studies may also evaluate the effects of several different flicker

frequencies or compare the obscurity (no light) to a flashing light source, such as in this recent study by Krivek et al. (2022).

We are aware that these limitations may reduce this review's scope but we believe that this work remains one essential first step in order to better identify and mitigate the impacts of artificial light on biodiversity.

Conclusion

Within this systematic review, more than fifty observations on the behavioural and physiological impacts of flashing light on animals were collected. Birds were the primarily studied taxon while fishes, insects and mammals were less investigated. Phototaxis to flashing light was the most studied outcome but, overall, very few outcomes were investigated. We found little available evidence on nocturnal species: bats were found to be alarmingly understudied while nocturnal raptors as well as glow worms have not been the subject of any research so far. The impacts of flashing light seemed to vary greatly between studied species. On the one hand, flashing light can be more impactful on animals than continuous light. On the other hand, more surprisingly, in the case of night-migrating birds, it might also reduce animals' phototaxis to ALAN and therefore limit some effects of light pollution. In some other cases, responses to flashing and continuous lights were not found to differ.

As LED and dynamic lighting are currently being rapidly scaled up, this systematic review represents a relevant first step in order to better grasp the actual state of the evidence base regarding the effects of flashing light on biodiversity. However, our results highlighted a crucial lack of knowledge and we therefore advocate for further research to be pressingly carried out. Many more species and outcomes should be investigated and more in-situ experiments conducted in order to better understand real-world lighting situations—e.g. illuminated signs and advertisements, sensor lighting, wind turbines. Then, an update of this review should be contemplated as it will surely allow for more complete and definitive conclusions on the impacts of flashing light to be drawn.

In the meantime, based on these first provisional results, we argue that some precautionary measures should be taken to reduce the potential adverse effects of flashing light on animals. First, from the point of view of lamp engineers and manufacturers, flicker frequencies should be kept way beyond the currently known highest critical frequencies of the animal kingdom—i.e. 500 Hz. Secondly, from a lighting management perspective, new regulations should be implemented in order to better consider this understated flashing parameter of light pollution—as it is the case for more acknowledged characteristics of light such as direction, spectral composition and intensity.

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Additional information

Conflict of interest

The authors declare the following competing interests: Gaël Obein is the president of the AFE (French Association on Lighting) and Virginie Nicolas is the president of the ACE (French Association of Lighting Designers and Lighting Engineers).

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: YR, RS, GO, VN. Data curation: ML, AL. Formal analysis: AL. Investigation: RS, YR, AL, ML. Project administration: RS, YR. Writing – original draft: RS, AL. Writing – review and editing: ML, RS, AL, VN, YR, GO.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

ROSES form

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol

Data type: excel file

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Link: <https://doi.org/10.3897/natureconservation.54.102614.suppl1>

Supplementary material 2

Test list and comprehensiveness

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol

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Supplementary material 3

Citation screening

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Supplementary material 4

Citations excluded at full-text screening

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Supplementary material 5

Citations with irretrievable abstracts

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Supplementary material 6

Unobtainable articles at full-text screening

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol

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Link: <https://doi.org/10.3897/natureconservation.54.102614.suppl6>

Supplementary material 7

Observation critical appraisal

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol

Data type: excel file

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Link: <https://doi.org/10.3897/natureconservation.54.102614.suppl7>

Supplementary material 8

Systematic review observation database

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol

Data type: excel file

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Link: <https://doi.org/10.3897/natureconservation.54.102614.suppl8>

Supplementary material 9

Additional bibliometric results and narrative synthesis

Authors: Alix Lafitte, Romain Sordello, Marc Legrand, Virginie Nicolas, Gaël Obein, Yorick Reyjol





Data type: word file

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Research Article

Vegetation changes along an urbanisation and atmospheric pollution gradient in Mexico

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Abstract

Green areas are important places for biodiversity conservation within cities, but their vegetation is affected by various anthropogenic factors. This study used an exploratory approach to examine the influence of urbanisation and air pollution-related factors on the indicators for the composition and structure of vegetation in an urban area in northeast Mexico. Based on the spatial analysis of the major air pollutants, four sampling categories were delimited (rural, low, moderate and high urbanisation). The differences between categories, based on vegetation structure, were determined using non-parametric Kruskal-Wallis tests. The Importance Value was calculated for the species. The floristic similarity was compared using NMDS and PERMANOVA unidirectional. The relationship between environmental variables and abundance of species was evaluated using CCA. One hundred and ten plant species were collected, including ten alien species. The highest abundance and species richness were registered in the rural site. The general tendency of vegetation structure is to plants decreasing with respect to the increase in the levels of urbanisation and air pollution present in the study area. The association between the environmental variables and plant communities along the urbanisation gradient was significant, being the relative humidity, the particles lower than 2.5 µm, the dew point and the heat index as the most important variables. The understanding of the nature and variability of vegetation within green areas contributes to increasing our knowledge about the distribution of the environmental services they provide and the composition of the faunal communities that depend on them. For this reason, this study relates the plants of a specific area of northeast Mexico with the environmental quality present in an urban area.

Key words: air pollution, environmental variability, Monterrey Metropolitan Area, urbanisation, vegetation structure



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Introduction

The demographic growth dynamics faced by cities represent a serious threat to the environment, as well as to the health and quality of life of its inhabitants (Vlahov and Galea 2002). The unsustainable use of natural resources, intense land-use changes, increasing density of urban/industrial centres and the

growing emission of pollutants irreversibly damage the environment (García et al. 2013). These effects not only harm living beings, but also generate phenomena that affect the ecosystem (López et al. 2001). Likewise, the accelerated urbanisation changes the structure of cities and affects their climate and that of their surrounding area (Tang et al. 2008). This urbanisation process occurs more rapidly in countries located in regions classified as developing economies. Particularly in Latin America, where it is estimated that 75% of the population live in cities (UN-HABITAT 2010).

In Mexico, air pollution has deteriorated air quality in various cities, including the Metropolitan Area of the Valley of Mexico, the Metropolitan Area of Guadalajara and the Monterrey Metropolitan Area (MMA) (García et al. 2012; Cerón et al. 2014; Mancilla et al. 2015; Menchaca et al. 2015). It is appropriate to point out that there is a perception problem in society as there is no clear awareness of pollutant emissions, their concentrations and damage to health, urban infrastructure and ecosystems (Lezama and Graizbord 2010). The State of Nuevo León, in the northeast of Mexico, has an unregulated urban growth. Its main urban sprawl, the MMA presents serious environmental problems: geological and hydrological risks, water scarcity, loss of green areas, air pollution, amongst many others (Badillo et al. 2015; Orta et al. 2016; Sanchez-Castillo et al. 2016; Sisto et al. 2016; Ybáñez and Barboza 2017).

Studies of species diversity in urban ecosystems are needed to understand the effect of anthropogenic development on ecosystem integrity and sustenance (Mukherjee et al. 2015). To study the effects of urbanisation on ecosystem structure and function, researchers have used the urban-rural gradient methodology (Pennington et al. 2010). Urban-rural gradients are generally realised on large spatial scales and, in some cases, have been conceived as a linear transect radiating from the city centre towards less disturbed landscapes. Studies employing this method have documented declines in plant species diversity, basal area and density of native species as sites become more urbanised. These studies, which show a decrease in species richness as urbanisation increases, follow a general disturbance hypothesis (Porter et al. 2001; Moffatt et al. 2004; Burton et al. 2005; Duguay et al. 2007).

On the other hand, the intermediate disturbance hypothesis has been one of the main models used to interpret urban plant diversity patterns (Johnson and Swan 2014). The theory has been applied to explore the co-existence of native and non-native species along urban-rural gradients or within the urban environment between patches that vary in level of disturbance (e.g. Porter et al. (2001); ManSecak and Wein (2006); Catford et al. (2012)). The expectation is that species diversity will be maximised in intermediate locations, where native and invasive species are found in the same communities, in relatively uniform proportions.

Previous studies of large-scale urban-rural gradients have documented that those urban forests are more deteriorated than their “natural” or rural counterparts (Paul and Meyer 2001; Güler 2020). Consequently, they reduce the perceived ecological value of remnant vegetation within highly modified landscapes. However, it is important to understand the potential ecological and social value of remnant urban vegetation (Turner et al. 2004; Czaja et al. 2020). Given that more than 60% of the world’s population will reside in urban areas by 2050, these forest fragments in urban settings could provide critical ecosystem

services for both people and other species (Bernhardt and Palmer 2007; Zeg-eye et al. 2023).

For our study, we characterised the remnant vegetation of the MMA, north-east Mexico, along an urbanisation gradient, based on parameters of atmospheric pollution. The objectives of this study were: (1) Identify the plant species richness in the MMA, northeast Mexico; (2) Compare the variation in richness, abundance and diversity of plant species amongst urbanisation categories; (3) Quantify the value of importance of the species by urbanisation category; and (4) Analyse the influence of environmental variation (air pollutants, climatic factors and soil) on the abundance and richness of plant species. Our hypothesis is that the structure and composition of the vegetation decrease with respect to the increase in urbanisation levels in the MMA.

Methods

Study area

The MMA is the largest urban area in northeast Mexico and the third largest urban centre in the country, extending from 25°15' to 26°30' north latitude and from 99°40' to 101°10' west longitude (Fig. 1A, B). The area is bounded by the coastal plain of the Gulf of Mexico and the Sierra Madre Oriental Mountain Range. Several municipalities compose the geographical area of MMA: Apodaca, Cadereyta, García, General Escobedo, Guadalupe, Jiménez, Juárez, Monterrey, Salinas Victoria, San Nicolás de los Garza, San Pedro Garza García, Santa Catarina and Santiago (Alanís 2005; González et al. 2011; Mancilla et al. 2015). The main vegetation cover found at MMA is forest, scrubs and grasslands (Carpio et al. 2021). The MMA has a vehicle fleet of 2.5 million vehicles (Castillo-Nava et al. 2020) and 5.3 million inhabitants (INEGI 2021), which is probably even higher today. Likewise, there is a variety of industrial complexes that include the production of glass, steel, cement and paper, amongst others (Menchaca et al. 2015). The city centre has an average altitude of 540 m a.s.l., the characteristic climate is dry steppe, hot and extreme with temperatures above 35 °C during the summer and below 8 °C during the winter (Alanís 2005; González et al. 2011; Menchaca et al. 2015).

Delimitation of the urbanisation gradient

Since November 1992, the MMA has operated a network of air quality monitoring stations known as the Integral Environmental Monitoring System (SIMA). The SIMA network is currently made up of 14 recording stations distributed according to criteria from meteorological, land use and population density studies. The measurements recorded at these monitoring stations are: PM₁₀ (particulate matter less than 10 µm), PM_{2.5} (particulate matter less than 2.5 µm), carbon monoxide (CO), ozone (O₃), nitrogen oxides (NO_x) and sulphur dioxide (SO₂). In addition, some meteorological variables are reported, such as barometric pressure, rainfall, relative humidity, solar radiation, temperature and wind direction and magnitude (Arreola and González 1999; González et al. 2011; Mancilla et al. 2015). The data recorded by the SIMA stations for air quality and meteorological variables (2009–2018) were obtained from the National

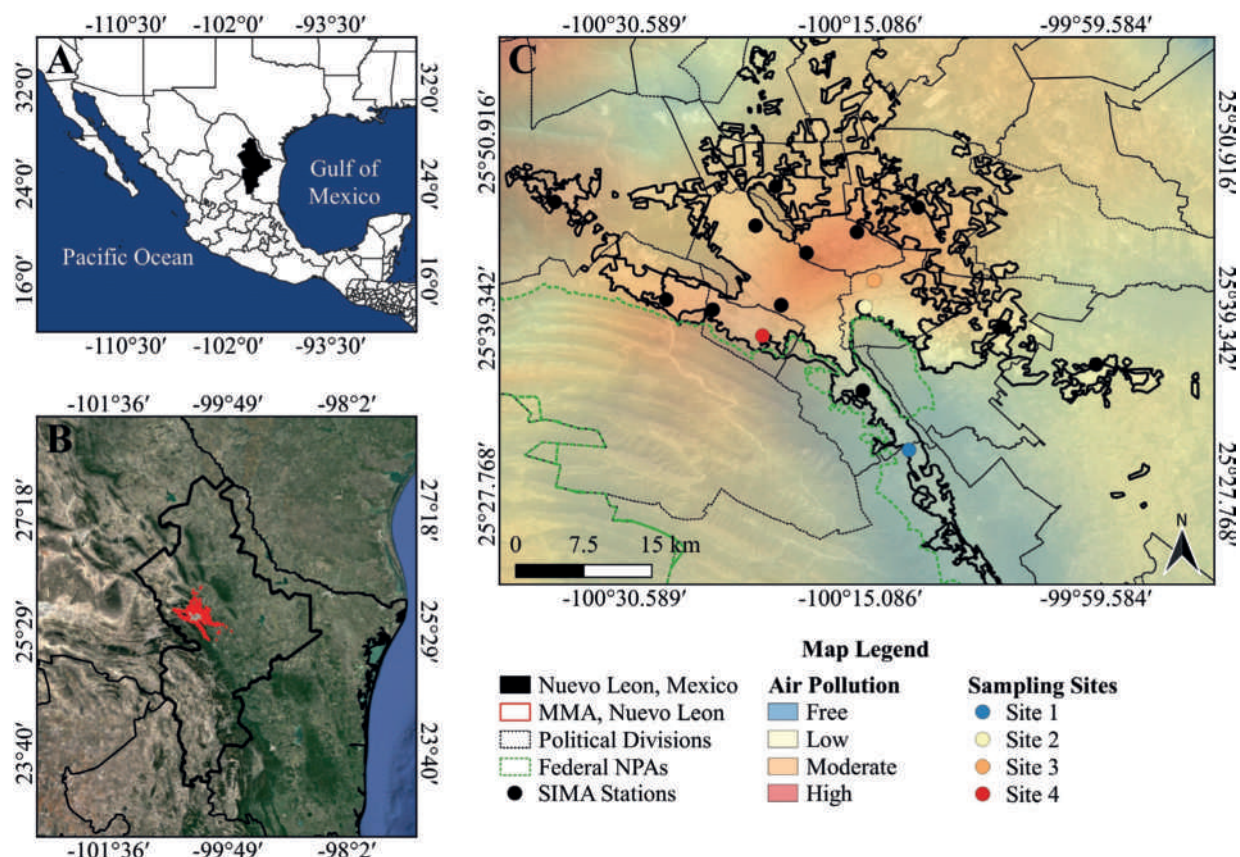


Figure 1. Study area and location of sampling sites **A** location of Nuevo Leon in Mexico **B** location of the MMA inside Nuevo Leon **C** location of sampling sites according with the air pollution levels.

Air Quality Information System (SINAICA). Obtaining descriptive measures for each year and for each of the recording stations was carried out in the Statistica 13.3 programme (TIBCO Software Inc. 2017).

To identify the main pollutants that describe air quality in the MMA during the 2009–2018 period, a Principal Component Analysis (PCA) was carried out. Subsequently, to differentiate the changes in the spatial distribution of pollutants that are indicators of air quality in the MMA, maps were created using the annual average information on each monitoring station. Mapping was done using Inverse Distance Weighting (IDW) interpolation, with a Distance Coefficient of 2 and the output raster pixel size reset to 15 metres. As a reference of the extension, the minimum and maximum distances of the vector sections corresponding to the urban areas that make up the MMA were taken; these sections were obtained from the national layer of Land Use and Vegetation Series 6 (INEGI 2016). The procedures described above were performed using Quantum GIS 3.2 software (Quantum GIS Development Team 2018). As a result, four categories of urbanisation by atmospheric pollution were generated: rural (lower than $3.22 \mu\text{g}/\text{m}^3$ of $\text{PM}_{2.5}$), low (3.22 to $10.56 \mu\text{g}/\text{m}^3$ of $\text{PM}_{2.5}$), moderate (10.56 to $17.92 \mu\text{g}/\text{m}^3$ of $\text{PM}_{2.5}$) and high (17.92 to $25.3 \mu\text{g}/\text{m}^3$ of $\text{PM}_{2.5}$) (Fig. 1C).

Selection of sampling sites

Four permanent sampling sites were delimited, based on the spatial superposition of three geographic elements: (1) the interpolation of the main air pollutants

was used for determination of the urbanisation gradient in the study area (Fig. 1C); (2) images obtained from the Google Earth Pro software were used to differentiate the spatial presence or absence of vegetation cover and (3) a mesh with a grid size of 150×150 m was delimited to select sampling areas with complete vegetation cover. Overlay and selection procedures were performed in Quantum GIS 3.2 software. The rural site is located in the Municipality of Santiago, a rural area without substantial urbanisation or air pollution and with secondary submontane scrub vegetation ($25^{\circ}30'41.184''\text{N}$, $100^{\circ}11'53.159''\text{W}$). The low urbanisation site is located in the central zone of the Municipality of Guadalupe with low values of air pollution and secondary vegetation of submontane scrub ($25^{\circ}40'4.944''\text{N}$, $100^{\circ}14'45.564''\text{W}$). The moderate urbanisation site is located in the northern zone of the Municipality of Guadalupe with moderate air pollution and secondary vegetation of submontane scrub ($25^{\circ}42'44.017''\text{N}$, $100^{\circ}13'58.825''\text{W}$). The high urbanisation site is in the Municipality of San Pedro Garza García with high air pollution and anthropogenic submontane scrub vegetation ($25^{\circ}38'11.112''\text{N}$, $100^{\circ}21'30.815''\text{W}$) (Fig. 1C).

Sample collection and processing

During April 2019, the analysis of preliminary samples obtained in the study area was carried out. The Clench model was used to calculate the minimum sample size to be used, based on the method and parameters indicated by Jiménez-Valverde and Hortal (2003). According to the analysis, between 5 to 8 sampling units are needed to register the 95% of the richness of each site. Vegetation was assessed using 20 quadrats of 10×10 m (0.01 hectares), which were evenly distributed amongst the four categories of urbanisation by air pollution (five quadrats per category). The quadrats were located from inside the sampling site (150×150 m) (2.25 hectares) randomly and they were placed using the tool random points inside a polygon in Quantum GIS 3.2 software. The quadrats are located in patches of natural and native vegetation. The evaluation was carried once per season: dry season (November to April) and rainy season (May to October), during the period from May 2019 to April 2020. The seasons were defined, based on the historical data of the monthly total values of temperature and rainfall (average from 2009 to 2018), which were obtained from the SIMA stations located within the study area (Fig. 2).

Measurements were carried out independently for each of the vegetation strata. For the herbaceous stratum, five sub-quadrats of 1×1 m (5 m^2 in total per quadrant) were delimited. In the shrub stratum, two 5×5 m sub-quadrats (50 m^2 in total per quadrant) were evaluated. Finally, the tree stratum was evaluated in the entire quadrat, 10×10 m (100 m^2 in total per quadrat). The dimension of the quadrat and sub-quadrats was established according to the criteria described by Brower et al. (1998).

In each quadrat/sub-quadrats, the following measurements were made: (1) height of the plant (from its base at ground level to the highest branch); (2) largest and (3) smallest diameter of the aerial projection of the plant. The number of individuals of each morphospecies assigned in the field was quantified and their identification in the laboratory was carried out using the works of Alanís and González (2003), Stubbendieck et al. (2003) and Zurita and Elizondo (2009); likewise, the botanical nomenclature was homogenised using

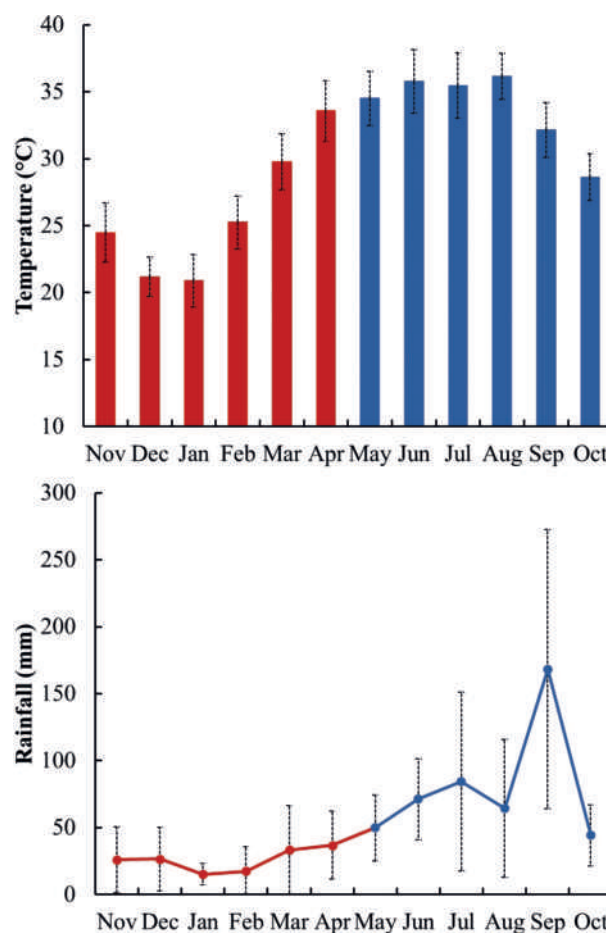


Figure 2. Monthly average variation of temperature and rainfall in the MMA. Dry season (red colour) and Rainy season (blue colour).

the International Plant Names Index base (IPNI 2022). Villaseñor and Espinosa-García (2004) were mainly followed to determine which plant species were not native to the MMA region.

Microenvironment measurement

The microenvironmental variables were measured in each of the quadrants using a Kestrel 5500 portable weather station, a CEM – DT1308 digital luxmeter, a CEM – DT9881 particle counter and a HB – 2 soil moisture and pH meter, simultaneously with the sampling of the vegetation, recording the following variables: maximum wind speed (MWS) and average wind speed (AWS) (obtained during five minutes of exposure), temperature (T), relative humidity (RH), heat index (HI), dew point (DP), evapotranspiration (E), solar radiation (SR), particles of 2.5 ($PM_{2.5}$) and 10 microns (PM_{10}), soil pH (SpH) and soil moisture (SM). Measurements were carried out in the centre of each quadrat during the early hours of the morning, noon and before sunset, avoiding direct solar radiation.

Data analysis

Species richness was measured as the total number of species observed in the study area, as well as in each of the sites. Significant differences in the

number of species between sites were determined using non-parametric Kruskal-Wallis tests, in Statistica 13.3 software. Sampling efficiency was calculated for the entire study area and for each site using the interpolation and extrapolation methodology proposed by Chao and Jost (2012), available in the iNEXT package (Hsieh et al. 2016) for version 3.5.3 of R (R Development Core Team 2019).

Differences in plant abundance between sites were calculated with a Kruskal-Wallis test. For the analysis of alpha diversity, we adopted the analytical method of Chao and Jost (2015) to obtain profiles in which diversity is evaluated in terms of “effective numbers of species” (qD), an approach that is equivalent to the numbers of Hill (Hill 1973). Hill numbers include three widely-used measures as special cases: species richness ($q = 0$), Shannon diversity (the exponential of Shannon entropy, $q = 1$) and Simpson diversity (the inverse of Simpson concentration, $q = 2$), all of which are expressed in units of “species equivalents”. The analysis was performed for the entire study area and for each site using the SpadeR package (Chao et al. 2016), in R 3.5.3.

Vegetation cover was calculated according to the criteria described by Ramírez (2006). Differences in vegetation cover between sites were determined with a Kruskal-Wallis test. To examine differences in species composition between sites, we performed non-metric multidimensional scaling (NMDS) analysis, using the Bray-Curtis Index as the similarity matrix. A PERMANOVA was also performed to test for differences in species composition between sites. Both analyses were performed using the Vegan package (Oksanen et al. 2019) in R 3.5.3.

For each species, its abundance was determined according to the number of individuals, its dominance based on cover and its frequency based on its presence in the sampling quadrats. These results were used to obtain a weighted value at the taxon level called Importance Value (IV), which acquires percentage values on a scale from 0 to 100 (Mueller-Dombois and Ellenberg 1974). The IV was calculated for each site separately.

Finally, a canonical correspondence analysis (CCA) was carried out to determine the relationship between the microenvironmental variables and the abundance of the recorded species in each plot, which also includes a Monte Carlo permutation test to evaluate the significance of the microenvironmental variables in the analysis. For the CCA, the average values of the microenvironmental variables of each season of the year were used (dry and rainy season). The CCA was done using the Vegan package in R 3.5.3.

Results

A total of 12,878 plants of 42 families, 104 genera and 110 species were quantified. From this total, 17 species (594 individuals) were trees, 34 (2,595 individuals) were shrubs and 59 (9,689 individuals) were herbaceous (Table 1). The greatest abundance and richness of tree species in the study area was found in the Fabaceae family with 35.0 and 23.5% of the total registered, respectively. Likewise, Fabaceae presented the highest abundance and richness of shrub species with 29.3 and 29.4% of the total registered, respectively. Asteraceae showed the highest abundance and richness of herbaceous species with 21.6 and 18.6% of the total recorded, respectively (Table 1).

Table 1. Taxonomic list, abundance and IV of the species found in an air pollution gradient in the MMA. Legend: Site 1 = Rural, Site 2 = Low urbanisation, Site 3 = Moderate urbanisation, Site 4 = High urbanisation.

Taxon	Key	Abundance				IV			
		Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
Tree									
Boraginaceae Juss.									
Ehretia anacua (Terán & Berland.) I.M. Johnst.	Eana	16	6	0	0	8.1	4.7	0.0	0.0
Cannabaceae Martinov									
Celtis laevigata Willd.	Clae	26	22	0	0	12.1	12.7	0.0	0.0
Ebenaceae Gürke									
Diospyros texana Scheele	Dtex	22	0	0	0	10.7	0.0	0.0	0.0
Fabaceae Lindl.									
Ebenopsis ebano (Berland.) Barneby & J.W. Grimes	Eeba	20	28	16	0	10.2	14.6	17.3	0.0
Havardia pallens (Benth.) Britton & Rose	Hpal	18	12	6	0	9.6	8.7	5.7	0.0
Leucaena leucocephala (Lam.) de Wit*	Lleu	0	26	16	0	0.0	12.6	18.9	0.0
Prosopis glandulosa Torr.	Pgla	26	18	22	0	12.4	9.6	21.2	0.0
Fagaceae Dumort.									
Quercus fusiformis Small	Qfus	0	0	0	24	0.0	0.0	0.0	22.4
Juglandaceae DC. ex Perleb									
Carya illinoensis (Wangenh.) K. Koch	Cill	0	0	0	6	0.0	0.0	0.0	5.1
Oleaceae Hoffmanns. & Link									
Fraxinus americana L.*	Fame	0	0	14	16	0.0	0.0	16.0	16.9
Ligustrum lucidum W.T. Aiton*	Lluc	0	0	0	22	0.0	0.0	0.0	18.0
Rutaceae Juss.									
Sargentia greggii S. Watson	Sgre	20	0	0	0	9.3	0.0	0.0	0.0
Salicaceae Mirb.									
Salix nigra Marshall	Snig	0	22	0	0	0.0	11.8	0.0	0.0
Sapindaceae Juss.									
Koelreuteria elegans (Seem.) A.C. Sm.*	Kele	0	30	22	16	0.0	14.1	21.0	14.1
Sapindus saponaria L.	Ssap	34	0	0	0	15.4	0.0	0.0	0.0
Ungnadia speciosa Endl.	Uspe	0	0	0	26	0.0	0.0	0.0	23.5
Sapotaceae Juss.									
Sideroxylon celastrinum (Kunth) T.D. Penn.	Scel	24	18	0	0	12.2	11.2	0.0	0.0
Shrub									
Asparagaceae Juss.									
Yucca treculeana Carrière	Ytre	33	0	0	0	2.9	0.0	0.0	0.0
Asteraceae Bercht. & J. Presl									
Gochnatia hypoleuca (DC.) A. Gray	Ghyp	34	0	0	0	3.0	0.0	0.0	0.0
Bignoniaceae Juss.									
Tecoma stans (L.) Juss. ex Kunth	Tsta	0	59	0	0	0.0	7.5	0.0	0.0
Boraginaceae Juss.									
Cordia boissieri A. DC.	Cboi	54	44	0	0	4.4	6.3	0.0	0.0
Cactaceae Juss.									
Opuntia engelmannii Salm-Dyck ex Engelm.	Oeng	40	0	0	0	3.6	0.0	0.0	0.0

Taxon	Key	Abundance				IV			
		Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
Cannabaceae Martinov									
Celtis pallida Torr.	Cpal	53	32	0	0	4.2	4.9	0.0	0.0
Capparaceae Juss.									
Capparis flexuosa Vell.	Cfle	39	0	0	0	3.4	0.0	0.0	0.0
Euphorbiaceae Juss.									
Adelia vaseyi (JM Coult.) Pax y K. Hoffm.	Avas	43	36	0	0	3.7	5.4	0.0	0.0
Fabaceae Lindl.									
Acacia berlandieri Benth.	Aber	59	40	0	0	4.7	5.9	0.0	0.0
Acacia farnesiana (L.) Willd.	Afar	0	0	53	0	0.0	0.0	12.8	0.0
Acacia rigidula Benth.	Arig	49	0	0	0	4.1	0.0	0.0	0.0
Bauhinia mexicana Vogel	Bmex	36	0	0	0	3.4	0.0	0.0	0.0
Caesalpinia mexicana A. Gray	Cmex	0	33	50	61	0.0	5.0	12.6	19.6
Dalea scandens (Mill.) R.T. Clausen	Dsca	45	0	0	0	3.9	0.0	0.0	0.0
Erythrina herbacea L.	Eher	43	0	0	0	3.8	0.0	0.0	0.0
Eysenhardtia texana Scheele	Etex	28	38	40	45	3.0	5.5	11.0	15.2
Mimosa monancistra Benth.	Mmon	0	40	43	0	0.0	5.8	11.7	0.0
Parkinsonia aculeata L.	Pacu	0	0	57	0	0.0	0.0	14.2	0.0
Lythraceae J. St.-Hil.									
Punica granatum L.*	Pgra	0	0	0	41	0.0	0.0	0.0	15.1
Malpighiaceae Juss.									
Malpighia glabra L.	Mgla	46	33	0	0	3.9	5.1	0.0	0.0
Mascagnia macroptera (Moc. & Sessé ex DC.) Nied.	Mmac	54	50	48	63	4.3	6.7	12.3	19.6
Myrtaceae Juss.									
Psidium guajava L.*	Pgua	0	0	0	41	0.0	0.0	0.0	14.6
Oleaceae Hoffmanns. & Link									
Forestiera angustifolia Torr.	Fang	29	45	0	0	2.8	6.2	0.0	0.0
Rhamnaceae Juss.									
Condalia hookeri M.C. Johnst.	Choo	49	56	0	0	4.0	7.3	0.0	0.0
Karwinskia humboldtiana (Schult.) Zucc.	Khum	51	0	0	0	4.3	0.0	0.0	0.0
Ziziphus obtusifolia (Hook. ex Torr. & A. Gray) A. Gray	Zobt	33	0	0	0	2.9	0.0	0.0	0.0
Rubiaceae Juss.									
Randia obcordata S. Watson	Robc	42	53	0	0	3.6	7.2	0.0	0.0
Rutaceae Juss.									
Helietta parvifolia (A. Gray ex Hemsl.) Benth.	Hpar	45	41	48	0	3.8	5.7	12.5	0.0
Zanthoxylum fagara (L.) Sarg.	Zfag	47	41	0	0	3.8	5.9	0.0	0.0
Salicaceae Mirb.									
Neopringlea integrifolia (Hemsl.) S. Watson	Nint	58	0	0	0	4.5	0.0	0.0	0.0
Scrophulariaceae Juss.									
Leucophyllum frutescens (Berland.) I.M. Johnst.	Lfru	35	36	0	0	2.9	5.4	0.0	0.0
Simaroubaceae DC.									
Castela erecta Turpin	Cere	49	0	0	0	3.5	0.0	0.0	0.0
Verbenaceae J. St.-Hil.									
Citharexylum berlandieri B.L. Rob.	Cber	43	0	0	0	3.2	0.0	0.0	0.0

Taxon	Key	Abundance				IV			
		Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
<i>Lantana camara</i> L.	Lcam	66	26	52	47	4.5	4.1	12.9	15.9
Herb									
Acanthaceae Juss.									
<i>Elytraria bromoides</i> Oerst.	Ebro	79	0	0	0	3.0	0.0	0.0	0.0
<i>Justicia pilosella</i> (Nees) Hilsenb.	Jpil	80	114	0	0	3.0	4.2	0.0	0.0
<i>Ruellia nudiflora</i> (Engelm. & A. Gray) Urb.	Rnud	0	122	0	0	0.0	4.3	0.0	0.0
<i>Tetramerium nervosum</i> Nees	Tner	101	0	0	0	3.5	0.0	0.0	0.0
Apocynaceae Juss.									
<i>Asclepias curassavica</i> L.	Acur	101	76	96	90	3.5	3.2	4.0	5.5
<i>Telosiphonia lanuginosa</i> (M. Martens & Galeotti) Henrickson	Tlan	0	0	105	104	0.0	0.0	4.3	5.9
Asteraceae Bercht. & J. Presl									
<i>Bidens odorata</i> Cav.	Bodo	114	94	119	114	3.8	3.7	4.5	6.3
<i>Calyptocarpus vialis</i> Less.	Cvia	0	101	103	93	0.0	3.8	4.1	5.7
<i>Chromolaena odorata</i> (L.) R.M. King & H. Rob.	Codo	111	0	0	0	3.8	0.0	0.0	0.0
<i>Helianthus annuus</i> L.	Hann	0	0	94	0	0.0	0.0	3.9	0.0
<i>Jefea lantanifolia</i> (S. Schauer) Strother	Jlan	0	93	0	0	0.0	3.6	0.0	0.0
<i>Sanvitalia ocymoides</i> DC.	Socy	64	97	0	0	2.1	3.8	0.0	0.0
<i>Simsia eurylepis</i> S.F. Blake	Seur	0	77	95	0	0.0	3.3	4.0	0.0
<i>Thymophylla pentachaeta</i> (DC.) Small	Tpen	0	0	0	132	0.0	0.0	0.0	7.1
<i>Tridax coronopifolia</i> (Kunth) Hemsl.*	Tcor	99	101	103	131	3.4	3.8	4.2	6.8
<i>Verbesina persicifolia</i> DC.	Vper	61	0	0	0	2.1	0.0	0.0	0.0
<i>Wedelia acapulcensis</i> Kunth	Waca	96	0	0	0	3.5	0.0	0.0	0.0
Commelinaceae Mirb.									
<i>Commelina erecta</i> L.	Cere	99	89	95	87	3.3	3.5	3.9	5.3
Convolvulaceae Juss.									
<i>Evolvulus alsinoides</i> (L.) L.	Eals	0	0	119	0	0.0	0.0	4.6	0.0
<i>Ipomoea hederacea</i> Jacq.	Ihed	91	84	69	0	3.3	3.3	3.1	0.0
<i>Merremia dissecta</i> (Jacq.) Hallier f.	Mdis	0	101	98	0	0.0	3.7	3.9	0.0
Euphorbiaceae Juss.									
<i>Acalypha monostachya</i> Cav.	Amon	0	0	0	82	0.0	0.0	0.0	5.1
<i>Cnidoscolus rotundifolius</i> (Müll. Arg.) McVaugh	Crot	0	103	0	0	0.0	3.9	0.0	0.0
<i>Croton cortesianus</i> Kunth	Ccor	103	114	0	0	3.6	4.3	0.0	0.0
<i>Euphorbia hirta</i> L.	Ehir	0	78	109	0	0.0	3.3	4.3	0.0
Fabaceae Lindl.									
<i>Canavalia villosa</i> Benth.	Cvil	101	0	0	0	3.5	0.0	0.0	0.0
<i>Desmanthus virgatus</i> (L.) Willd.	Dvir	95	0	0	0	3.4	0.0	0.0	0.0
<i>Mimosa malacophylla</i> A. Gray	Mmal	112	0	0	0	3.7	0.0	0.0	0.0
Lamiaceae Martinov									
<i>Ocimum micranthum</i> Willd.*	Omic	81	76	0	0	3.1	3.2	0.0	0.0
<i>Salvia coccinea</i> Buc'hoz ex Etl.	Scoc	93	102	106	105	3.4	3.9	4.1	5.9
Loasaceae Juss.									
<i>Cevallia sinuata</i> Lag.	Csin	0	0	0	89	0.0	0.0	0.0	5.3

Taxon	Key	Abundance				IV			
		Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
Malvaceae Juss.									
Abutilon trisulcatum (Jacq.) Urb.	Atri	0	105	113	0	0.0	3.9	4.3	0.0
Malvastrum americanum (L.) Torr.	Mame	99	0	0	0	3.4	0.0	0.0	0.0
Melochia pyramidata L.	Mpyr	78	95	99	96	2.9	3.7	4.0	5.5
Waltheria indica L.	Wind	0	0	97	0	0.0	0.0	4.0	0.0
Nyctaginaceae Juss.									
Cyphomeris crassifolia (Standl.) Standl.	Ccra	57	0	0	0	2.1	0.0	0.0	0.0
Oleaceae Hoffmanns. & Link									
Menodora heterophylla Moric. ex DC.	Mhet	0	0	63	0	0.0	0.0	2.5	0.0
Onagraceae Juss.									
Oenothera rosea L'Hér. ex Aiton	Oros	0	0	55	0	0.0	0.0	2.2	0.0
Papaveraceae Juss.									
Argemone grandiflora Sweet	Agra	0	0	63	0	0.0	0.0	2.4	0.0
Passifloraceae Juss. ex Roussel									
Passiflora foetida L.	Pfoe	69	64	0	0	2.3	2.3	0.0	0.0
Petiveriaceae C. Agardh									
Rivina humilis L.	Rhum	68	60	0	0	2.3	2.3	0.0	0.0
Poaceae Barnhart									
Aristida adscensionis L.	Aads	100	0	0	0	3.5	0.0	0.0	0.0
Bouteloua curtipendula (Michx.) Torr.	Bcur	0	0	0	104	0.0	0.0	0.0	5.9
Cenchrus spinifex Cav.	Cspi	90	114	0	0	3.3	4.2	0.0	0.0
Eragrostis barrelieri Daveau*	Ebar	0	0	84	0	0.0	0.0	3.7	0.0
Melinis repens (Willd.) Zizka*	Mrep	0	0	91	94	0.0	0.0	3.9	5.4
Panicum hallii Vasey	Phal	0	90	0	0	0.0	3.5	0.0	0.0
Paspalum unispicatum (Scribn. & Merr.) Nash	Puni	0	0	69	0	0.0	0.0	2.7	0.0
Setaria leucopila (Scribn. & Merr.) K. Schum.	Sleu	102	107	0	0	3.5	3.9	0.0	0.0
Pteridaceae E.D.M. Kirchn.									
Adiantum tricholepis Fée	Atri	92	0	0	0	3.4	0.0	0.0	0.0
Cheilanthes aemula Maxon	Caem	88	0	0	0	3.1	0.0	0.0	0.0
Ranunculaceae Juss.									
Clematis drummondii Torr. & A. Gray	Cdru	122	98	134	0	4.1	3.8	5.0	0.0
Rubiaceae Juss.									
Spermacoce glabra Michx.	Sgla	45	67	0	0	1.7	2.5	0.0	0.0
Sapindaceae Juss.									
Cardiospermum halicacabum L.	Chal	0	103	102	84	0.0	3.8	4.1	5.2
Solanaceae Juss.									
Solanum elaeagnifolium Cav.	Sela	0	0	95	0	0.0	0.0	4.0	0.0
Solanum triquetrum Cav.	Stri	54	0	0	0	1.9	0.0	0.0	0.0
Verbenaceae J. St.-Hil.									
Lantana canescens Kunth	Lcan	98	90	106	96	3.4	3.5	4.2	5.6
Phyla nodiflora (L.) Greene	Pnod	0	0	0	136	0.0	0.0	0.0	7.1
Verbena canescens Kunth	Vcan	0	0	0	112	0.0	0.0	0.0	6.4
*Introduced species.									

Koelreuteria elegans (Seem.) A.C. Sm. was the species with the highest abundance of trees (68 individuals), 11.4% of the total number of individuals recorded in the study area. On the other hand, *Mascagnia macroptera* (Moc. & Sessé ex DC.) Nied. presented the highest abundance of shrubs (215 individuals) (8.3%). *Bidens odorata* Cav. was the species with the highest abundance of herbaceous plants (441 individuals) (4.6%) (Table 1). From the total of the reported species, ten are alien species, amongst which, *Koelreuteria elegans* (Seem.) A.C. Sm. and *Tridax coronopifolia* (Kunth) Hemsl. are present in most of the sampling sites (Table 1).

Variation per urbanisation category

Sapindus saponaria L. was the tree species with the highest IV (15.4%) in the rural site. On the other hand, *Ebenopsis ebano* (Berland.) Barneby & J.W. Grimes was the most important tree species (14.61%) in the low urbanisation site. *Prosopis glandulosa* Torre. was the most important species (21.21%) in the moderate urbanisation site. Likewise, *Ungnadia speciosa* Endl. was the most important species (23.48%) in the high urbanisation site (Table 1).

Acacia berlandieri Benth. was the shrub species with the highest IV (4.68%) in the rural site. On the other hand, *Tecoma stans* (L.) Juss. ex Kunth was the most important shrub species (7.53%) in the low urbanisation site. *Parkinsonia aculeata* L. was the most important species (14.23%) in the moderate urbanisation site. Likewise, *Caesalpinia mexicana* A. Gray was the most important species (19.63%) in the high urbanisation site (Table 1).

Clematis drummondii Torr. & A. Gray was the herbaceous species with the highest IV (4.06%) in the rural site. On the other hand, *Ruellia nudiflora* (Engelm. & A. Gray) Urb. was the most important herbaceous species (4.31%) in the low urbanisation site. *Clematis drummondii* Torr. & A. Gray also turned out to be the most important herbaceous (5.02%) in the moderate urbanization site. Likewise, *Phyla nodiflora* (L.) Greene turned out to be the most important species (7.12%) in the high urbanisation site (Table 1).

Comparisons between sites showed significant differences ($P < 0.05$) for species richness, height and coverage between all sites (Table 2). Abundance was significantly different ($P < 0.05$) between all sites, except for the comparison between the sites with moderate and high urbanisation (Table 2). All the parameters (abundance, species richness, height and coverage) decreased with increasing levels of urbanisation or pollution. In the rural site, 425.2 ± 87.7 individuals and 61.8 ± 3.4 species were registered, representing a sampling coverage of 99.9%. In the low urbanisation site, the values were reduced to 350 ± 68.5 individuals and 50.9 ± 1.6 species (coverage of 99.9%). For the moderate urbanisation site, 296.9 ± 62.4 individuals and 36.2 ± 1.6 species were registered (coverage of 99.9%), while for the high urbanisation site, 215.7 ± 35.5 individuals and 27.2 ± 0.6 species (coverage of 100%).

For ^0D , ^1D and ^2D , the rural site had the highest diversity. All comparisons between sites were significantly different (with 95% confidence intervals) (Table 2). The one-way PERMANOVA test detected significant differences in species composition between all sites ($\text{SS}_{\text{total}} = 7.05$; $\text{SS}_{\text{within-group}} = 1.25$; $F = 55.81$, $P < 0.001$). Plant communities sampled formed separate groups in the NMDS diagram (Stress = 0.11) (Fig. 3).

Table 2. Richness, abundance, height, coverage and diversity profiles along the urbanisation gradient in the MMA. Legend: ⁰D = species richness expressed in units of species; ¹D = Shannon diversity expressed in units of species; ²D = Simpson diversity expressed in units of species.

Ecological parameter	Rural	Low urbanisation	Moderate urbanisation	High urbanisation
Richness *	61.8 ± 3.4a	50.9 ± 1.6b	36.2 ± 1.6c	27.2 ± 0.6d
Abundance*	425.2 ± 87.7a	350 ± 68.5b	296.9 ± 62.4c	215.7 ± 35.5c
Height *	1.2 ± 0.1a	0.9 ± 0b	0.7 ± 0c	0.8 ± 0.1d
Coverage *	558.3 ± 103.1a	205.7 ± 31.1b	117.4 ± 22.9c	118.5 ± 19.9d
⁰ D **	68 ± 0a	54 ± 0b	40 ± 0.2c	29 ± 0d
¹ D **	61.2 ± 0.8a	47.3 ± 0.8b	35.5 ± 0.5c	25.1 ± 0.5d
² D **	56.5 ± 1.2a	43.4 ± 1b	33.4 ± 0.7c	23.3 ± 0.7d

* Values with different letters between columns are significantly different using Kruskal-Wallis test: richness between sites, $K = 36.6$, $DF = 3$, $P = 0.0001$; abundance between sites, $K = 17.5$, $DF = 3$, $P = 0.0001$; height between sites, $K = 32.5$, $DF = 3$, $P = 0.0001$; coverage between sites, $K = 31.2$, $DF = 3$, $P = 0.0001$. ** Diversity values with different letters between columns are different, using 95% confidence intervals.

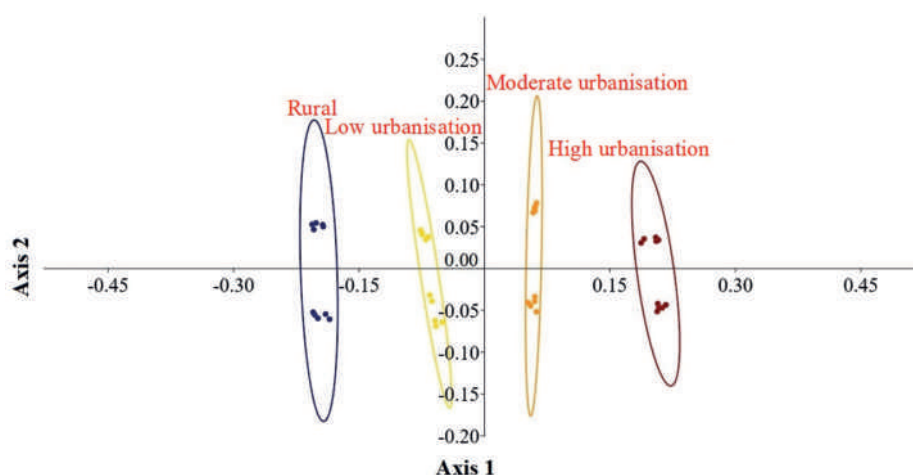


Figure 3. Non-Metric Multidimensional Scaling (NMDS) ordination of plant communities by urbanisation categories.

Plant responses to environmental variation

The MWS, T, RH, HI, DP, E, $PM_{2.5}$ and SpH were the significant environmental variables ($P < 0.05$) used in the CCA (Table 3). CCA showed significant association between the environmental variables and the plant communities along the urbanisation gradient (Total inertia = 81.3%; $P < 0.001$). The variables most related with the plant abundance in the gradient were: RH and $PM_{2.5}$ for Axis 1 (Eigenvalue = 0.441; Inertia = 56.6%). For Axis 2 (Eigenvalue = 0.193; Inertia = 24.7%), DP and HI were the most important variables. *Quercus fusiformis* Small, *Carya illinoensis* (Wangenh.) K. Koch, *Fraxinus americana* L., *Ligustrum lucidum* W.T. Aiton, *Koeleria elegans* (Seem.) A.C. Sm., *Caesalpinia mexicana* A. Gray, *Punica granatum* L., *Psidium guajava* L., *Lantana camara* L., *Thymophylla pentachaeta* (DC.) Small, *Tridax coronopifolia* (Kunth) Hemsl., *Eragrostis barrelieri* Daveau, *Melinis repens* (Willd.) Zizka and *Verbena canescens* Kunth are associated with conditions of high concentration of $PM_{2.5}$, higher RH and alkaline SpH. On the other hand, *Ehretia anacua* (Terán & Berland.) I.M. Johnst., *Diospyros texana* Scheele,

Table 3. Environmental values registered along the urbanisation gradient in the MMA. Environmental variables marked (*) are significant ($p < 0.05$) according to the Monte Carlo permutation test. MWS = maximum wind speed; AWS = average wind speed; T = temperature; RH = relative humidity; HI = heat index; DP = dew point; E = evapotranspiration; SR = solar radiation; $PM_{2.5}$ = 2.5 μm particles; PM_{10} = 10 μm particles; SpH = soil pH; SM = soil moisture.

Environment variable	Rural	Low urbanisation	Moderate urbanisation	High urbanisation
MWS (Km/h) *	5.2 \pm 1.5	4.2 \pm 0.7	4.9 \pm 2.3	15 \pm 9.7
AWS (Km/h)	3 \pm 1	1.9 \pm 0.4	2.2 \pm 0.8	1.7 \pm 0.8
T (°C) *	26.4 \pm 0.5	27.9 \pm 1.7	26.5 \pm 1.6	20.8 \pm 2.7
RH (%) *	49.1 \pm 6.1	62.2 \pm 3.7	65.6 \pm 3.8	74.6 \pm 3.4
HI (°C) *	26.2 \pm 1.7	32.2 \pm 4.3	26.8 \pm 1.2	22 \pm 2.8
DP (°C) *	14.1 \pm 1.9	20.9 \pm 2.1	18.8 \pm 1.7	16.6 \pm 2
E (°C) *	18.4 \pm 1.5	22.2 \pm 1.3	20.4 \pm 1.4	19.3 \pm 1.9
SR (Klux)	9.4 \pm 1.5	7.5 \pm 1.5	9.1 \pm 1.8	7.6 \pm 3.2
$PM_{2.5}$ *	256.1 \pm 101.1	410.4 \pm 94.6	396.7 \pm 33.4	1181.4 \pm 455.6
PM_{10}	47 \pm 20	71.5 \pm 22.4	41.4 \pm 8	69.2 \pm 23.5
SpH *	7.1 \pm 0.4	7.2 \pm 0.6	7.3 \pm 0.5	8 \pm 0.7
SM (%)	11.6 \pm 4.8	16.6 \pm 7.2	11.7 \pm 4.3	14 \pm 5

Ebenopsis ebano (Berland.) Barneby & J.W. Grimes, *Prosopis glandulosa* Torr., *Sapindus saponaria* L., *Gochnatia hypoleuca* (DC.) A. Gray, *Tecoma stans* (L.) Juss. ex Kunth, *Forestiera angustifolia* Torr., *Randia obcordata* S. Watson, *Zanthoxylum fagara* (L.) Sarg., *Citharexylum berlandieri* B.L. Rob., *Verbesina persicifolia* DC., *Croton cortesianus* Kunth, *Cenchrus spinifex* Cav., *Clematis drummondii* Torr. & A. Gray, *Solanum triquetrum* Cav. and *Lantana canescens* Kunth are related to the low $PM_{2.5}$ concentration, RH and neutral SpH (Fig. 4).

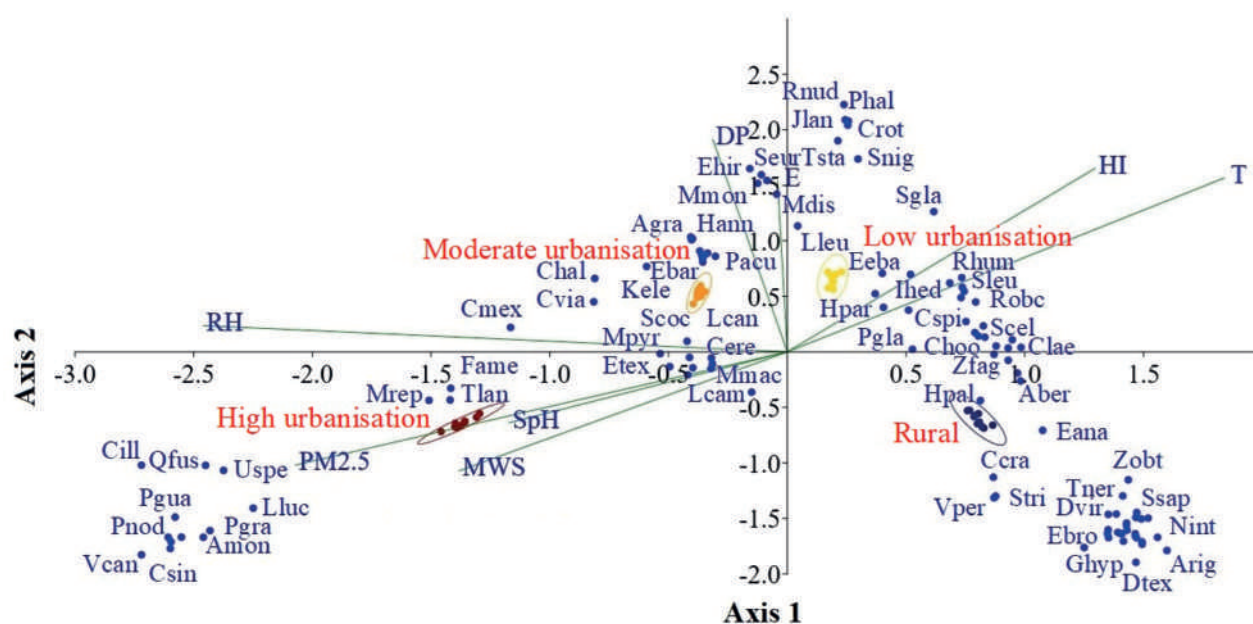


Figure 4. Canonic Correspondence Analysis (CCA) of the plant communities and significant environmental variables corresponding to the urbanisation gradient. MWS = maximum wind speed; T = temperature; RH = relative humidity; HI = heat index; DP = dew point; E = evapotranspiration; $PM_{2.5}$ = 2.5 μm particles; SpH = soil pH.

Discussion

We use the air quality records to define an urbanisation gradient in the MMA, where the height, cover, abundance, species richness and diversity were the parameters recorded in sites with different levels of urbanisation. It was found that all the parameters decreased with increasing urbanisation levels; thus, in accordance with the general disturbance hypothesis, the general tendency of plant distribution at the levels of urbanisation and pollution present in the MMA is to decrease.

It is important to note that urban gradient studies are clearly a simplification of the complex patterns produced by urbanisation, such as air pollution (Alberti et al. 2001; Hahs and McDonnell 2006; McKinney 2008). The negative effect of urbanisation on plant species richness has been related to a variety of factors ranging from the pollution and habitat degradation to introduction of alien species and others societal impacts (McKinney 2002, 2006; Hope et al. 2003; Grimm et al. 2008; Vilà et al. 2011). While the impact of urbanisation on plant richness depends upon the size of an urban area, the overall loss of habitable areas in urban zones normally results in a lower richness of plants. On the other hand, the expansion of urban areas is associated with an influx of non-native plant species that tend to counterbalance this urban effect by increasing overall plant richness (Duguay et al. 2007; Gavier-Pizarro et al. 2010; Cameron et al. 2015). These patterns were observed in the MMA region, notably, we recorded a decrease in the number of species as urbanisation levels increase and an increase in the abundance of introduced species in sites with higher urbanisation level.

The integrity of plant communities is vulnerable to intense land-use modification associated with urbanisation (Richardson et al. 2007). Significant changes in species composition along urban-rural gradients have been reported in Baltimore, Maryland (Groffman et al. 2003), Winnipeg, Manitoba (Moffatt et al. 2004) and Columbus, Georgia (Burton et al. 2005; Styers et al. 2010). Species richness and density of native plants were shown to decrease near urban areas (Porter et al. 2001; Moffatt et al. 2004), whereas invasive richness and density increased with urban development in the south-eastern United States. (Burton et al. 2005). These studies applied an urban-to-rural gradient approach to study sites located over a large geographic region from a densely populated urban landscape to a relatively unpopulated rural landscape. Our results corroborate similar studies of declining plant populations in urban-rural gradients, suggesting that habitat degradation may be a devastating threat to the persistence of certain sensitive taxa, such as plants present only in rural sites.

The replacement of local native species by alien species causes the floras of cities in different biogeographic regions to be increasingly homogeneous (i.e. beta diversity is reduced) (Kühn and Klotz 2006; Schwartz et al. 2006). However, the introduction of non-native species in urban areas can make them relatively biologically diverse at smaller scales. Our results show a clear differentiation in species composition (beta diversity) between sites on the urban-rural gradient. Hope et al. (2003) and Turner et al. (2005) demonstrate that certain anthropogenic habitats may have similar or greater alpha diversity than the more natural habitats of the region. However, our results show a greater diversity for sites without apparent urbanisation, but it decreases as urbanisation levels increase. The low diversity in such habitats may reflect a high degree of land change, thus causing significant stress to the plant community in urban areas (Pennington et al. 2010).

Certain native and alien species represent ecological indicators of different levels of urbanisation (LaPaix and Freedman 2010). *Sapindus Saponaria*, *Acacia berlandieri* and *Clematis drummondii* were the species with the highest IV value in the rural site. On the other hand, *Ungnadia speciosa*, *Caesalpinia mexicana* and *Phyla nodiflora* were the most important species in the high urbanisation site. Our species with the highest IV differ from those mentioned by Alanís-Rodríguez et al. (2015) for areas contiguous to the MMA. In contrast, Estrada-Castillón et al. (2012) report plant associations made up of species mentioned in our study, clarifying that the plant communities with the highest deterioration are associated with the areas adjacent to the metropolitan zone.

From the CCA, we identified plant species associated with urbanisation (Kremen 1992). Amongst which, invasive alien species, such as *Ligustrum lucidum*, *Koeleria elegans*, *Tridax coronopifolia*, *Eragrostis barrelieri* and *Melinis repens*, were found in the more urbanised sites. These species are highly tolerant to urban growth conditions and appear capable of exploiting environmental conditions associated with urbanisation (McKinney 2002). Native species, such as *Quercus fusiformis*, *Carya illinoensis*, *Caesalpinia Mexicana*, *Lantana camara*, *Thymophylla pentachaeta* and *Verbena canescens*, were amongst the most common species to observe in urbanised sites and likely present adaptations capable of tolerating disturbance associated with urbanisation. In contrast, native species, such as *Diospyros texana*, *Sapindus saponaria*, *Gochnatia hypoleuca*, *Zanthoxylum fagara*, *Citharexylum berlandieri*, *Verbesina persicifolia*, *Solanum triquetrum* and *Lantana canescens*, were found only in the less urbanised sites. Consequently, these species are highly intolerant to process associated with urbanisation, highlighting the importance of green areas as refuges for these species. These results are consistent with the large-scale studies by Moffatt et al. (2004) and Burton and Samuelson (2008), who reported a predominance of exotic and pioneer species in more urbanised areas compared to rural areas.

The composition and structure of vegetation in peri-urban and urban areas can vary due to climate, soil conditions, ecological disturbances and human influences (Jim and Liu 2001; Jim 2002; Pedlowski et al. 2002; Escobedo et al. 2006). For this study, the conditions of RH, DP, HI and $PM_{2.5}$ were the variables that best describe the vegetation structure in the MMA. Other studies have documented these characteristics. For example, Stewart et al. (2009) in New Zealand and Godefroid and Koedam (2003) in Belgium studied different plant assemblages in urban and peri-urban temperate forests. In Latin America, Grau et al. (2008) in Tucumán, Argentina and Baumgardner et al. (2012) in Mexico City, analysed the role of the structure and composition of peri-urban forests as a function of the watershed and regional air quality, respectively. In addition, Puric-Mladenovic et al. (2000) in Canada and Christopoulou et al. (2007) in Greece, discussed the loss of peri-urban natural areas due to urbanisation.

Other anthropogenic factors of vegetation structure and composition have been found in other urban and subtropical areas of the world (Jim 2002; Grau et al. 2008). For example, people in southern China prefer green areas characterised by high tree cover and large trees (Jim and Chen 2006). Furthermore, socioeconomic and educational levels may play a role in the structure and composition of forests in Brazilian urban areas (Pedlowski et al. 2002). In Kenya, peri-urban mangroves have been affected by industrial pollution and sewage (Mohamed et al. 2009).

The approach used in this research implies a relationship between microclimatic variations and plant species at the plot level. This analysis assumes the influence of environmental variables (independent variables) on the species (Dolédéc et al. 2000). However, the relationship between both factors is interdependent. That is, the structure of the vegetation and the characteristics of the plants influence the abiotic variation (Guariguata and Ostertag 2001; Renaud et al. 2010; Lienard et al. 2015; Hardwick et al. 2015) and, at the same time, the presence of certain microclimatic conditions allows the development of each plant species (Arroyo-Rodríguez et al. 2017). Therefore, the microclimate is one of the first factors to change after disturbance (Norris et al. 2012; Parr 2012; Hardwick et al. 2015).

Overall, our study analysed the effects of urbanisation on vegetation and changes in vegetation structure were detected as levels of urbanisation increased. However, studies in subtropical regions of North America show how, in addition to urbanisation, demography also affects the structure of the vegetation, mainly tree structure in built-up areas (Zhao et al. 2010; Flocks et al. 2011). Additionally, other studies in South America document the effect of socioeconomics in vegetation structure (De la Maza et al. 2002; Pedlowski et al. 2002; Escobedo et al. 2006). The ability of parks and areas of remaining native vegetation to promote biodiversity depends largely on their design and the types of management activities to which they are subjected. For example, while regionally rare native species can be found within cities, they are often associated with habitats that have not been greatly altered (Godefroid 2001; Godefroid and Koedam 2003). Such partnerships strengthen the call to protect plant communities within the urban landscape and emphasise the need for ecological knowledge to guide park design and management.

Conclusions

For the first time in north-eastern Mexico, the vegetation structure was monitored on a rural-urban gradient, where the height, cover, abundance, species richness and diversity were the parameters recorded in sites with different levels of urbanisation. It was found that all the parameters decreased with increasing urbanisation levels; thus, in accordance with the general disturbance hypothesis, the general tendency of plant distribution at the levels of urbanisation and pollution present in the MMA is to decrease. The association between environmental variables and the plant community along the urbanisation gradient was significant, the conditions of RH, DP, HI and $PM_{2.5}$ being the variables that best describe the vegetation structure in the MMA. Understanding the nature and variability of vegetation within green spaces contributes to increasing our knowledge about the distribution of the environmental services it provides and the composition of the faunal communities that depend on it. Likewise, it provides valuable information to prioritise the strategic management of the vegetation of urban green spaces so that it provides the greatest benefit for humans and biodiversity.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Edmar Meléndez-Jaramillo, sampling sites selection, fieldwork, plants identification, data analysis and document writing; Laura Sánchez-Castillo, fieldwork, database compilation and document writing; Ma. Teresa de Jesús Segura Martínez, sampling sites selection, plants identification and completed document review; Uriel Jeshua Sánchez-Reyes, data analysis, results interpretation and completed document review.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Short Communication

Lost in hostile lands: moths of conservation concern in cultivated and suburban areas of south Italy

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Abstract

Cultivated and suburban areas are usually considered unfavourable to the vulnerable components of biodiversity and regarded as not interesting from a conservation point of view. However, remnants of semi-natural vegetation can be embedded in such areas, becoming possible refuges for wild biodiversity despite the high anthropogenic pressures. With the present study, we raise awareness that, in some cases, these areas can be regarded as biodiversity treasure chests, even when apparently poor and with low appeal for conservationists. We demonstrate the importance of urbanised and cultivated landscapes by providing new records of two lepidopteran species rare for the Italian Peninsula, namely *Amphipyra* (*Pyrois*) *cinnamomea* and *Boudinotiana notha*. The European range of *A. cinnamomea* has become strongly reduced, seeming extinct in some Central European regions and the few Italian records mostly date back several decades. *B. notha* has very few relict populations in Mediterranean Europe, some of which are in peninsular Italy, where it is threatened by urbanisation and reduced precipitations expected in the next decades. Our findings confirm the importance of small and highly fragmented patches of semi-natural vegetation for biodiversity conservation, as they can allow species of conservation interest to persist in hostile lands.

Key words: *Amphipyra cinnamomea*, biodiversity, *Boudinotiana notha*, habitat fragmentation, Lepidoptera



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Introduction

Unveiling the role of non-protected areas in sustaining wild diversity is crucial for biodiversity conservation, as, in most cases, natural parks are surrounded by cultivated lands that represent a barrier to wild diversity (Forman 1995; Conrad et al. 2004) and can rarely lead to a genetic drift in populations of vulnerable species (Britten and Baker 2002). Many countries in Europe and North America have addressed this problem by introducing Agri-Environmental Schemes (AES) as an attempt to reverse declines in farmland biodiversity by providing financial incentives to farmers for adopting less intensive, environmentally sensitive agricultural practices (Fuentes-Montemayor et al. 2011). In addition, a key point of the European Community Agricultural Policy (https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/cap-glance_en) is to maintain the integrity of the ecosystem service of pollination by funding the

monitoring of abundance and diversity of Hymenoptera Apoidea, Diptera Syrphidae and Lepidoptera (Ollerton 2017). Thanks to this policy, the number of studies on species distribution, community composition and abundance of wild pollinators in non-protected areas is rising across Europe (Millard et al. 2021).

During the last decades, knowledge on lepidopteran diversity has increased in Calabria, the southernmost region of peninsular Italy, thanks to several monitoring programmes of forested habitats carried out mostly within natural and protected areas (Ienco et al. 2020; Scalercio et al. 2022). However, the largest part of the regional territory is represented by non-protected urbanised and cultivated areas where only a few studies have been conducted. To fill this gap, some monitoring programmes of nocturnal Lepidoptera were launched in olive groves (Scalercio et al. 2007; Sabatino et al. 2021) and in suburban areas (Zucco and Scalercio 2023).

The complexity of the Calabria territory favours the frequent records of Lepidoptera every time that a new type of habitat is surveyed through monitoring activities (Infusino et al. 2016; Greco et al. 2018). This happens even in suburban and cultivated areas where semi-natural habitats are strongly fragmented in very small patches (Scalercio and Catania 2020), which seem to be large enough to enable wild diversity to permeate hostile environment and maintain populations.

The aim of the present study is to demonstrate that suburban environments that are cultivated and/or subject to anthropogenic interventions can host species richness similar to natural habitats and are, therefore, worthy of attention and protection. The important role of semi-natural vegetation patches in sustaining wild pollinators is confirmed in this paper by new findings of lepidopteran species in a hilly olive grove surrounded by semi-natural vegetation and in a suburban area. During 2022, we found the night-active Noctuidae *Amphipyra* (*Pyrois*) *cinnamomea* (Goeze, 1781), whose populations are declining across Europe (SwissLepTeam 2010; Wachlin and Bolz 2012), and the day-active Geometridae moth *Boudinotiana notha* (Hübner, 1803). For this latter species, the habitat is being strongly reduced by human activities and vulnerable to the expected climatic scenarios (Zaimes 2020), but very little data of its presence in peninsular Italy are available so far. Here we provide new records of these two lepidopteran species in south Italy, the critically revised literature data and the records publicly available on web platforms for Italian fauna, with notes concerning their conservation status at local, national and continental levels.

Material and methods

The sampling sites are located within the Crati Valley, one of the largest plains in the Calabria Region (Fig. 1). Nocturnal samplings of *A. cinnamomea* were carried out in the Coppone locality (Altomonte Municipality, Province of Cosenza) during a monitoring activity of the moth diversity inhabiting an olive orchard and its surroundings. Within and around the selected olive orchard, which extends for 3.1 hectares, we installed a network of ten UV LED light traps (Infusino et al. 2016), weekly activated from July to September 2022 during nights favourable to moth activity, with low wind and little or no rain and temperatures near the mean of the period and not lower than 15 °C. Sampled specimens were sorted and identified in the Laboratory of Faunistic Management and Forest Biodiversity of the Research Centre for Forestry and Wood (Rende, Province of Cosenza) and preserved in the scientific Collection of Lepidoptera of that laboratory.

Diurnal sampling of *B. notha* was done by net in a suburban area near the Research Centre for Forestry and Wood, where urbanised and cultivated lands are dominant and the semi-natural vegetation is mostly represented by remnants of riparian forests. The findings occurred during a monitoring activity of butterflies near an Italian Butterfly Monitoring Scheme transect (<https://butterfly-monitoring.net/ebms>). One was spotted on the ground, captured and brought to the laboratory for identification. After this first occasional finding on 10 March 2022, daily surveys were organised to find new specimens. However, no other individuals of this species were found despite the occurrence of sunny days optimal for the flight activity of adults (Hausmann 2001). The search during the same period of 2023 in the same area and in other suitable areas along the Crati River near the cities of Rende and Cosenza was unsuccessful.



Figure 1. Location of study area. Collecting sites of *Amphipyra cinnamomea* (red and white squares) and *Boudinotiana notha* (blue and white squares) are indicated. Aerial photographs (Google Earth) illustrate the landscape composition around the collecting sites at two different spatial scales.

Results and discussion

Amphipyra (Pyrois) cinnamomea (Goeze, 1781)

Fig. 2

New records. Calabria: Coppone (Altomonte Municipality, Cosenza Province) 39.689°N, 16.115°E, 5.VII.2022 (1 male), 19.VII.2022 (1 male).

Two adult male specimens, newly emerged, (Fig. 2) were found in July in two out of ten sites monitored in the study area, at the margin of semi-natural patches occasionally burned and near to human artefacts (Fig. 3). No specimens

were collected within the cultivated land and within the best-preserved habitats represented by riparian forests with *Populus*, *Ulmus*, *Lonicera* and others deciduous trees on which the larvae are known to feed (Rákosy 1996).

Other studies report adults from the beginning of spring-time (Fiori 1880) to October (Prola et al. 1978), likely as the result of adult aestivation. In peninsular Italy, the mimetic and elusive *A. cinnamomea* was found in Mediterranean habitats with *Quercus ilex* (Parenzan 1979), in Mediterranean maquis (Nappini and Dapporto 2009) and in areas with sparse *Quercus virgiliana* trees and olive groves (this study). This species has long been considered rare (Bertoloni 1849; Fiori 1880) and only five specimens have been found in Italy during the last century (Fig. 4). Records in Sardinia should be confirmed, as the only available record is the generic citation in the Italian checklist (Raineri and Zilli 1995). Moreover, the citation in Parenzan and Porcelli (2006) is wrong as the mention in Rocci and Turati (1925) refers to *Sideridis cinnamomea* Turati, 1913 that is a form of *Mythimna sicula* (Treitschke, 1835) described from Sardinia (Seitz 1938).

Süssenbach and Fiedler (1999) found that the abundance of species belonging to the genus *Amphipyra* can be underestimated by using light traps only, as they appear to be more abundant when bait traps were used. However, *A. cinnamomea* seems to be very rare in any case as it has never been detected with this method in Italy and it is also absent in the species list gathered from a survey of Lepidoptera carried out with bait traps in an area 20 km around the collecting locality (Scalercio 2006).

A. cinnamomea is considered a Mediterranean species extinct north of the Alps since 2007 (Fibiger et al. 2007). In Germany, it seems to be extinct and last records from Rhineland-Palatinate, Hessen and Baden-Württemberg date back to 1881 (Wachlin and Bolz 2012). It was common near Wiesbaden around the 1880s, becoming rare and lastly recorded in 1898 or 1899 (Steiner 1997). In Switzerland, its range was strongly reduced as it was only recorded in the south-westernmost part of the Central Highlands (SwissLepTeam 2010) as all records after the 1960s were from the vicinity of Valaisan Rhône between Briga and Martigny (Wymann et al. 2015).

General distribution. *Amphipyra* (*Pyrois*) *cinnamomea* is recorded from Andorra, Austria, Bosnia and Herzegovina, Bulgaria, Corsica, Croatia, French mainland, Germany, Greek mainland, Hungary, Italian mainland, North Macedonia, Romania, Slovenia, Spanish mainland, Switzerland, Serbia, Montenegro and doubtfully from Sardinia and Slovakia (Karsholt and Nieuken 2013). Outside Europe, it is only known for a few specimens collected in Turkey and Iran (Fibiger and Hacker 2007).



Figure 2. *Amphipyra* (*Pyrois*) *cinnamomea*. specimens collected during the present study (Coppone, Altomonte, Cosenza) **a** male, wingspan: 45 mm (19.VII.2022) **b** male, wingspan: 48 mm (5.VII.2022).



Figure 3. Calabrian collecting sites of *Amphipyra (Pyrois) cinnamomea*.

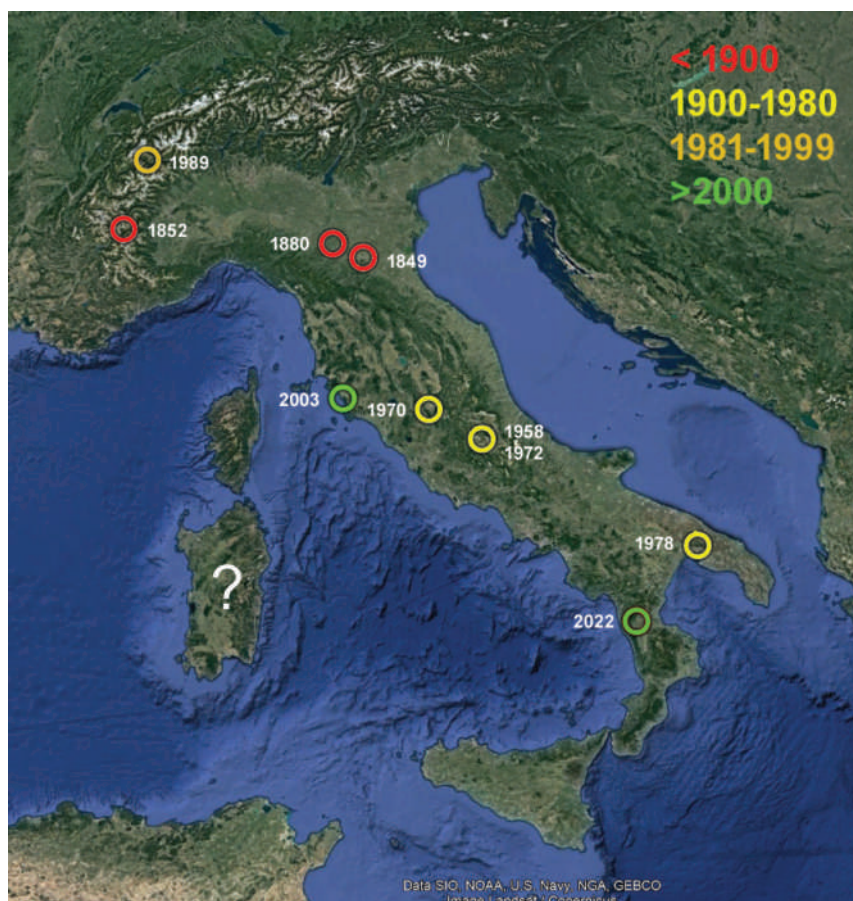


Figure 4. Italian records of *Amphipyra (Pyrois) cinnamomea*.

Italian distribution. Valle d'Aosta: Vens, Aosta, 1850 m elev., 16.IX.1989 (1 ex.) (Faquaet 1991).

Piemonte: Alpi, Northern Savoia, luglio-agosto (Ghiliani 1852).

Emilia: Bologna Botanic Garden, summer, 1 larva (Bertoloni 1849); San Faustino, Modena, 2 specimens at the beginning of spring-time (Fiori 1880).

Toscana: Collelungo, Parco Regionale della Maremma, Grosseto, VIII.2003 (1 ex.) (Nappini and Dapporto 2009).

Umbria: San Faustino, Terni, 4.X.1970 (1 ex.) (Prola et al. 1978).

Abruzzo: San Potito, L'Aquila, 10.VIII.1958 (1 ex.), 9.X.1972 (1 ex.) (Prola et al. 1978).

Puglia: Pianelle, Taranto, 350–450 m elev., 2.VI (1 male) (Parenzan 1979).

Sardegna: (Raineri and Zilli 1995).

***Boudinotiana notha* (Hübner, 1803)**

Fig. 5

New record. Calabria: Contrada Rocchi (Rende, Cosenza Province), 39.3675°N, 16.2282°E, 10.III.2022 (1 male).

In Calabria, *Boudinotiana notha* was found in a suburban area demonstrating that it can survive also where only remnants of riparian forest grow (Fig. 6), which is the habitat type where the larvae can be found. Preferred habitats are characterised by wind-protected areas, such as glades, valleys, forest-rides usually on wet, peaty or sandy soils near groundwater (Hausmann 2001). It occurs often near pioneer trees of *Populus tremula*, but also old trees. Adults can be attracted by *Salix* and rap runs. It flies exclusively during the day on sunny mornings and, during the afternoon, flies around *P. tremula* or rests on their twigs. It can be found from 0 m up to 1000 m above sea level (Hausmann 2001). For pupation, the *B. notha* caterpillar makes a hole in rotten wood and the pupation tubes are closed by a small cover made of gnawed wood (<https://lepiforum.org>). The species is univoltine and flies from late February to mid-April in Italy, from early April to mid-May in northern Europe. Larval stages can be found from early May to early July in Italy, from June to July in northern Europe. It overwinters as pupa, sometimes for two or three years (Hausmann 2001).

Several records are available from web platforms of citizen science, whereas published data are very scarce in Italy. It was commonly found from the Po Valley to the Alps, in Piemonte and Lombardia and scattered records are reported from Alpine areas in other regions, with the exclusion of Valle d'Aosta. It is very rare in peninsular Italy where it was recorded in a small area of Tuscany, in one locality of the Circeo National Park in Lazio and in a lowland forest in Puglia (Fig. 7). It was recorded also on other species of *Populus*, for example, *P. nigra* or exceptionally on *Salix* in Europe (Hausmann 2001). Adults were repeatedly observed also in *P. alba* stands (Zdeněk Laštůvka, pers. comm). Despite the field efforts carried out after the first finding in the same location and its neighbouring areas, but also in well-preserved riparian habitats of the Crati Valley during 2022 and 2023, no additional individuals were observed. Adults are very active in sunny days and easy to be detected as demonstrated by the large number of observations available for this species in citizen-science platforms. Then, the lack of additional observations leads us to hypothesise that the presence of very small populations are likely in need of conservation actions. Beside the habitat reduction due to the increasing urbanisation at low altitudes, its habitat is strongly threatened by the reduced precipitations expected in the next decades, more pronounced in the Mediterranean Basin (Brunetti et al. 2012).

General distribution. *Boudinotiana notha* can be found mostly in Europe and in part of Asia, in particular Russia and Japan (GBIF.org). In Europe, it occurs in the central-eastern part of the continent, specifically Austria, Belarus, Belgium, United Kingdom, Bulgaria, Croatia, Czechia, Danish mainland, European Russia, Estonia, European Turkey, Finland, French mainland, Germany, Hungary, Kaliningrad Region, Latvia, Lithuania, Luxembourg, Norwegian mainland, Poland, Romania, Slovakia, Slovenia, Spanish mainland, Sweden, Switzerland, The Netherlands and Ukraine (Karsholt and Nieuken 2013).

Italian distribution. Piemonte: Eremo, Torino (Giorna 1791–1793); Località Madonnina, Brignano Frascata, Alessandria, 3.III.1997 (Cabella and Fiori 2006);

Ponte Stura Ghiaia Grande, Alessandria, 3.III.2007 (Cabella C., pers. comm.); Gravere, Torino, 18.IV.2018 (Cabella C., pers. comm.); torrente Scrivia, Villa Ivernia, Alessandria, 3.IV.2022 (Cabella C., pers. comm.); Magnano, Biella, 45.4656°N, 8.0298°E, 23.III.2022 (1 male), recorded by Simone Bocca (iNaturalist); Greggio, Vercelli, 45.4586°N, 8.3957°E, 27.II.2022 (1 male), Front, Torino, 45.2628°N, 7.6800°E, 29.II.2021 (1 ex.), recorded by Alessandra Serini (iNaturalist); Rubiana, Torino, 45.1837°N, 7.3479°E, 10.III.2019 (1 male), recorded by Vuillermoz (iNaturalist); Borgone Susa, Torino, 45.1303°N, 7.2406°E, 10.III.2021 (1 male), recorded by Francesca de Leo (iNaturalist); Mompantero, Torino, 45.1477°N, 7.0948°E, 09.III.2020 (1 male), Gravere, Torino, 45.1175°N, 7.0118°E, 30.III.2019 (1 female), Usseaux, Torino, 45.0661°N, 7.0610°E, 17.II.2020 (1 male), Roure, Torino, 45.0567°N, 7.1149°E, 08.III.2019 (1 male), Oulx, Torino, 45.0276°N, 6.8003°E, 01.III.2019 (1 male), Giaveno, Torino, 45.0100°N, 7.3047°E, 10.III.2021 (1 male), recorded by bferrero (iNaturalist); Perrero, Torino, 44.9249°N, 7.1791°E, 16.III.2021 (1 male), recorded by Andrea Pane (iNaturalist).

Lombardia: Colli di San Fermo, Bergamo, mid-March-May (Föhst 1991); Veddasca, Varese, 46.0824°N, 8.7979°E, 22.II.2020 (1 ex.), recorded by giuss91 (iNaturalist); Cantello, Varese, 45.8230°N, 8.8742°E, 13.III.2020 (1 ex.), recorded by Mirko Tomasi (iNaturalist); Malnate, Varese, 2.IV.2021 (1 female), recorded by Mirko Tomasi (naturamediterraneo); Cesano Maderno, Monza e Brianza, 45.6274°N, 9.1296°E, 29.III.2019 (1 male), Cesano Maderno, Monza e Brianza, 45.6274°N, 9.1250°E, 16.III.2019 (1 female), Cesano Maderno, Monza e Brianza, 45.6253°N, 9.1228°E, 25.III.2019 (1 male), recorded by alilibere (iNaturalist); Acquanegra Sul Chiese, Mantova, 45.1411°N, 10.4421°E, 08.III.2020 (1 ex.), Acquanegra Sul Chiese, Mantova, 45.1382°N, 10.4361°E, 21.II.2021 (1 ex.), 02.II.2019 (1 male) recorded by Francesco Cerere (iNaturalist); Motta Baluffi, Cremona, 45.0342°N, 10.2409°E, 24.II.2019 (1 male), recorded by Fausto Leandri (iNaturalist); Motta Baluffi, Cremona, 45.0311°N, 10.2299°E, 28.II.2019 (1 male), recorded by Matteo (iNaturalist); Casalmaggiore, Cremona, 44.9557°N, 10.4894°E, 13.IV.2017 (1 larva), 19.II.2020 (1 ex.), recorded by Tiziana Dinolfo (iNaturalist); Voghera, Pavia, 45.0344°N, 8.9852°E, 01.III.2019 (1 female), recorded by Associazione Naturalistica Codibugnolo (iNaturalist); Bannio Anzino, Verbanio-Cusio-Ossola, 650 m elev., 28.III.2018 (1 male), recorded by Bantorp (naturamediterraneo); Viadana, Mantova, 26.II.2020 (some specimens), recorded by Tiziana Dinolfo (naturamediterraneo).

Trentino: Südtirol (Kitschelt 1925); surroundings of the Lago di Garda (Wolfsberger 1965).

Alto Adige: Ulten (Hinterwaldner 1867); Sonnenberghang, Naturns (Daniel and Wolfsberger 1957); Taufers/Waalweg, 1250 m elev., 46.657°N, 10.477°E, 14.IV.2013 (2 exx.), Huemer P. leg (BOLD); Bolzano, Bolzano, 46.4792°N, 11.3483°E, 10.III.2021 (1 ex.), recorded by Zenzi Martin (iNaturalist); Bolzano, Bolzano, 46.4788°N, 11.3491°E, 07.III.2021 (1 ex.), recorded by Verena Trockner (iNaturalist); Segonzano, Trento, 46.1896°N, 11.2509°E, 15.V.2020 (1 larva), recorded by Karol Tabarelli de Fatis (iNaturalist).

Veneto: Valdstico, Vicenza, 24.III.2010 (2 males), recorded by Archimede24 (naturamediterraneo).

Friuli: San Leonardo, Udine, 150 m, 12.III.1994 (1 maschio), C. Morandini leg. (Cicerale and Sciarretta 2005).

Venezia Giulia: Strazig, Gorizia, 8.III (1 es.) (Hafner 1910); Salcano, Gorizia, 15–30.III (some specimens) (Hafner 1910).



Figure 5. Collected specimen of *Boudinotiana notha*: Contrada Rocchi, Rende, Cosenza, 10.III.2022, male, wingspan: 32 mm.



Figure 6. Calabrian collection site of *Boudinotiana notha*. Blue and white square indicates the collecting point, where the specimen was found resting on the soil. Photo: Giuseppe Rijillo.

Emilia-Romagna: Modena, 24.III.2010 (1 ex.), recorded by Enrico Ferrari (naturamediterraneo).

Toscana: Torniella, Torrente Farma, Grosseto, 300 m elev., 2.IV.1991 (1 femmina) (Fabiano and Zilli 1998); Iesa, Siena, 350 m elev., II–IV (Dapporto et al. 2005); Bagni di Petriolo, Siena, 160–300 m elev., II–IV (Dapporto et al. 2005); Torniella, Grosseto, 300–400 m elev., II–IV (Dapporto et al. 2005); Carpineto, Siena, 200 m elev., II–IV (Dapporto et al. 2005); Civitella Paganico, Grosseto, 43.0847°N, 11.3131°E, 17.III.1999 (1 ex.), recorded by L. Dapporto (iNaturalist).

Lazio: Parco Nazionale del Circeo, foresta planiziale, 24.III.1995 (1 male) (Fabiano and Zilli 1998).

Puglia: Torre Fantine, Chieuti, Foggia, 27-II-2002 (1 female), T. Cicerale leg. (Cicerale and Sciarretta 2005).



Figure 7. Italian distribution of *Boudinotiana notha*. Red circle: new finding in south Italy.

Conclusions

Our findings confirm the great importance for diversity conservation of semi-natural habitats imbedded within cultivated and urbanised areas. They can represent either primary habitats where the species can complete their biological cycle or corridors enabling the dispersal of species across unsuitable habitats. During our study, we recorded only males, which are usually better fliers than females. This observation led us to hypothesise a primary role of corridors for such remnants, but we cannot exclude the role of primary habitat at least for *Boudinotiana notha* as its larvae feed on trees characterising its sampling site. The finding of one specimen only makes necessary an *ad-hoc* monitoring programme to clarify the role of semi-natural remnants for this species, as well as its range and its population size in the Crati Valley.

Even though our conclusions are based on three specimens only, they refer to very rare species for which also the finding of one specimen is of great importance and deserves to be valorised. In fact, our findings represent the second specimen of *B. notha* and the second and the third specimens of *Amphipyra cinnamomea* ever found in south Italy. As suggested by the high number of records concerning the day-active *B. notha* in north Italy deriving from citizen's observations, *B. notha* in south Italy can be considered very rare and its populations threatened by increasing anthropogenic and climate pressures. On the other hand, the lack of data deriving from citizen's observations concerning

the night-active *A. cinnamomea* could be due to both its rarity and its low detectability also when light traps were used. In fact, it might be better surveyed using sugar-based baits as observed for congeneric species, but there is no evidence of this.

In addition, our records also highlight the need for implementation of monitoring programmes of wild diversity in non-natural areas as well as the need of protection for the remnant patches of semi-natural habitats for both the diversity of species having restricted habitat preferences, as *B. notha* and *A. cinnamomea* documented here and for the maintenance of ecosystem services. The implementation of monitoring programmes with a variety of sampling methods can certainly help to better define the areas of species occurrence and abundance. Moreover, scientific programmes can be profitably integrated by citizen science, which contribute to refine the species distribution patterns, as demonstrated by data concerning *B. notha* for which literature data are very scarce.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

LB, GZ, SS write the manuscript; KG, IM supported field surveys; IM, SS conceived the study; LB, GZ, KG, SS data analysis; LB, GZ studied references.

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Data availability

All of the data that support the findings of this study are available in the main text.

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