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Research Article

Pilot Screening and Assessment of Microplastic Bioaccumulation in Wedge Clams *Donax trunculus* Linnaeus, 1758 (Bivalvia) from the Bulgarian Black Sea Coast

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Abstract: Pollution with plastic waste and its effects on marine organisms is a global problem, the Black Sea being no exception. This study aimed to assess for the first time the presence of microplastics (MPs) in wedge clams *Donax trunculus* Linnaeus, 1758 from different localities along the Bulgarian Black Sea coast. Three morphological forms of MPs were identified: pellets, fibers and fragments. Within each form, three size classes were recognised: 100–200 μm, 25–100 μm and ≤ 25 μm. MPs were present in all studied clams but with a different ratio (pellets – 94.7%; fibres – 64.9%; fragments – 21.1%). The mean number of MPs (per individual) in the wedge clams, collected from Ahtopol (4.46±1.09), Konstantin & Elena (3.66±1.09) and Sveti Vlas (3.56±1.07) was significantly higher, compared to the other localities. The lowest mean number of MPs was found in the southern localities Duni (0.31±0.07) and Arkutino (0.90±0.20). This is the first study to compare MPs in *D. trunculus* between two seasons and results showed significantly higher (t-test; $p<0.005$) number in summer. Multiple ANOVA proved interdependent effects of the categorical variables (form, locality, season) on the distribution and accumulation of MPs in the wedge clams. This confirmed that the local accumulation of MPs in the wedge clams was determined by multidirectional effects of many different factors, such as plastics sources, local environmental conditions, penetration pathways, physiological state of clams, etc.

Key words: Bulgarian Black Sea, *Donax trunculus*, microplastics bioaccumulation

Introduction

Microplastics (MPs) are unique pollutants that easily bioaccumulate in the marine ecosystems and can compromise the ability of the already stressed seas and oceans to deliver critical ecosystem services that support life on land. Unlike large plastic debris, MPs in the marine environment cannot be detected, collected for recycling and managed disposal cost-effectively (Andrady 2017). Pollution of the marine environment with plastic waste has become a global problem, the Black Sea being no exception (Moncheva et al. 2016, Simeonova & Chuturkova 2019, Pojar & Stock 2019, Berov & Klayn 2020,

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Eryaşar et al. 2021). Plastic deposition occurs from different sources, including also the river discharge of the Danube and Dniester containing a lot of garbage, of which 83% is plastics (Slobodnik et al. 2017, EMBLAS 2019, Simeonova & Chuturkova 2019). The highest pollution was established on the North-Western Black Sea shelf where the abundance of MPs was ten times higher than in sediments from the deep sea (Cincinellia et al. 2021).

Studies on the occurrence and accumulation of MPs in the Black Sea biota remain scarce. For the first time, presence of MPs was reported in Black Sea neuston (Aytan et al. 2016). Further, MPs were found in the Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 (Gedik & Eryaşar 2020) and in clams (*D. trunculus*, *Chamelea gallina* Linnaeus, 1758, *Anadara inaequivalvis* Bruguière, 1789, *Pitar rudis* Poli, 1795) from the Southern Black Sea (Şentürk et al. 2020, Gedik & Gozler 2022).

MPs present in sediments, water bodies, zooplankton and invertebrates can move up the Black Sea food webs and can be absorbed by higher marine organisms and humans (Aytan et al. 2016, Pojar & Stock 2019, Prinz & Corez 2020, Cincinelli et al. 2021, Eryaşar et al. 2021, Terzi et al. 2022). The effects of MPs on aquatic organisms are being intensively studied in recent years (de Sá et al. 2018), mainly in laboratory conditions. Most of these studies tend to use concentrations unlikely to be encountered in the field, usually on selected single species and with short observational times (Andrady 2017). The obtained data suggest that MPs may have significant negative impacts on the physiology of marine animals and, hence, affect also biotic communities and marine ecosystems (Gammaro et al. 2020). The MPs accumulated in the stomach and intestine of mussels were found to cause digestive tubule atrophy and a decrease in sex hormones and gonadal index (Choi et al. 2022). Furthermore, chronic exposure of bivalves to MPs can lead to changes in the pro-/antioxidant balance and neurotransmission in the digestive gland and gills; cellular immune apoptosis and DNA damage were also observed in mussels haemocytes (Hu & Palic 2020, Choi et al. 2022). These effects are manifested at the individual level by alterations in feeding behaviour, growth, development, reproduction and lifespan. In turn, such changes can have also negative effects at higher hierarchical levels, such as populations and ecosystems (Hu & Palic 2020). In addition, MPs can absorb toxic substances and pathogenic microorganisms on their surface and trigger even deeper negative consequences on marine biota and ecosystems (Mammo et al. 2020). Through the marine food chain, MPs can reach also humans, as the Black Sea is one of the major fishing areas in the world (FAO 2015).

At present, there are no studies regarding the spread and bioaccumulation of MPs in the marine biota of the Bulgarian part of the Black Sea. The present paper aims to provide the first preliminary data on the distribution and accumulation of MPs in wedge clams (*D. trunculus*) from selected localities along the Bulgarian Black Sea coast. In addition to its key role in coastal ecosystems, *D. trunculus* recently obtained significant economic importance in Bulgaria as an exported food item and this importance is quickly increasing (Chase 2018).

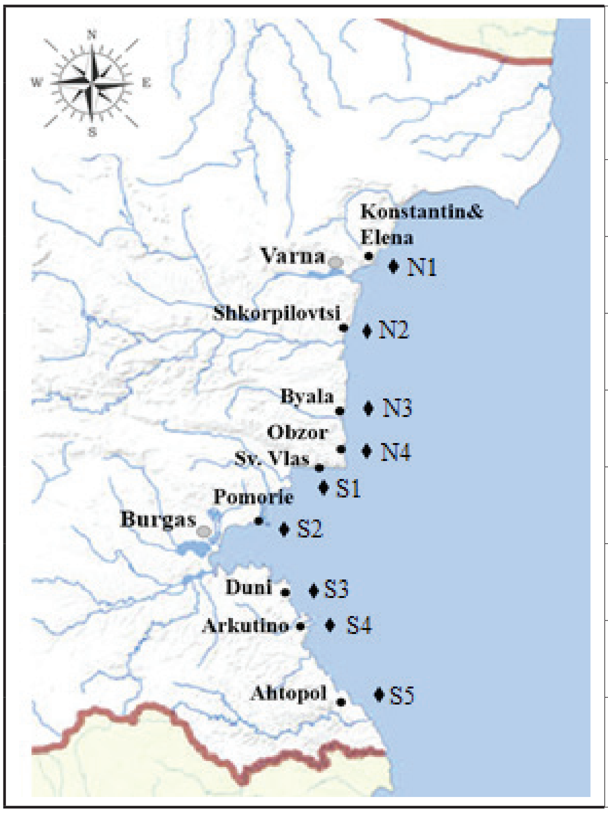
Materials and Methods

Sampling

For the purpose of this study, individual wedge clams were obtained mainly from commercial providers who gather them at depth 1.5–3.0 m and select them to have approximately the same size as required by trading standards. The samples for analyses originated from the natural habitats of the wedge clam: localities with sand beaches in the upper subtidal zone, known to contain a high abundance of this species. The samples were obtained in 2021 from nine localities situated both in the northern and southern coastal areas in two time periods: spring (March-April) and summer (June-July) (Fig. 1). From each sample (approximately 500 g), ten wedge clam individuals were digested and analysed for the presence of MPs. Specimens for analyses were selected with approximately the same size (29.32±2.59 mm shell length), which implies the same age and hence the same capacity of MPs accumulation. The wet weight of clams' soft tissue was measured but because of their similar size their weight was also quite similar (c. 0.9–1.1 g) and this was the reason to prefer to use the common format "mean number per individual" as more objective and illustrative. The selected wedge clams were frozen in dry ice before transportation to the laboratory and after that were stored at −20°C until analyses.

Tissue digestion and microscopic inspection

In order to avoid MPs cross-contamination during analysis, only glass vessels and metal tools were used and before the procedure, all laboratory glassware and tools were rinsed three times with filtered ultrapure water. All used reagents were preliminarily tested for the presence of possible MPs pollution using the black sample method. Tissue digestion was performed after Rosh & Brinker (2017).



Code	Locality	Longitude	Latitude
N1	Konstantin & Elena	43.232957°	28.015287°
N2	Shkorpilovtsi	42.961438°	27.898194°
N3	Byala	42.873656°	27.897987°
N4	Obzor	42.812863°	27.886932°
S1	Sveti Vlas	42.708304°	27.760431°
S2	Pomorie	42.562753°	27.606116°
S3	Duni	42.562753°	27.606116°
S4	Arkutino	42.330577°	27.736553°
S5	Ahtopol	42.106816°	27.920106°

Fig. 1. Localities, codes and geographical coordinates of wedge clam sampling sites along the Bulgarian Black Sea coast.

In brief, the shell of each clam was removed and the soft tissue was finely chopped and placed in an Erlenmeyer flask, which was covered with a watch glass to prevent contamination. Five ml of 1M NaOH was added and samples were heated to 50°C on a temperature-controlled shaker for 15 min. Then 17.5 ml HNO_3 (65 %) and 2.5 ml ultrapure water was added (final concentration of HNO_3: 10 M; ~49 %) and the samples were heated at 50°C for another 15 min. Thereafter the temperature was increased to 80°C for 15 min for removing the resistant suspended solids. Before filtration, the samples were diluted at a 1:2 ratio (v:v) with ultrapure water heated to 80°C. Filtration was performed on a vacuum pump through a cellulose nitrate filter (Ø 47 mm, 8 µm pore size, Sartorius Stedim Biotech, Goettingen, Germany). The flasks and filtration devices were rinsed with warm ultrapure water to ensure no loss of material. The filters were dried for 24 h at 37°C and subsequently analysed.

For MPs identification, filters were visually observed under a stereomicroscope SZM-D (OPTIKA Italy, 1000x magnification) coupled with Dino-Eye AM-4023X eyepiece camera (Dino-Lite ANMO Electronics, Taiwan). The digital images were examined using DinoCapture 2.0 software and the plastic particles were quantified by size (≤ 25 µm, 25–100 µm, and 100–200 µm) based on their largest cross section and shape (pellets, fibers and fragments).

For microplastics identification, filters were visually observed under a stereomicroscope SZM-D (OPTIKA Italy, 1000x magnification) coupled with Dino-Eye AM-4023X eyepiece camera (Dino-Lite ANMO Electronics, Taiwan). The digital images were examined by using DinoCapture 2.0 software and the plastic particles were quantified by size (≤ 25 µm, 25–100 µm, and 100–200 µm) based on their largest cross section and shape (pellets, fibres and fragments). For producing images, we used Dino-Eye AM-4023X digital camera (Dino-Lite ANMO Electronics, Taiwan). Each MP fragment was measured by the microscope operator and the measurements were performed by using DinoCapture 2.0 software. For producing images, we used Dino-Eye AM-4023X digital camera (Dino-Lite ANMO Electronics, Taiwan). Each MP fragment was measured by the microscope operator and the measurements were performed by using DinoCapture 2.0 software.

Statistical analyses

Multivariate ANOVA was applied to reveal the overall effects of the studied categorical variables. Meaningful post hoc and other two-group comparisons of means were made via the Student's t-test.

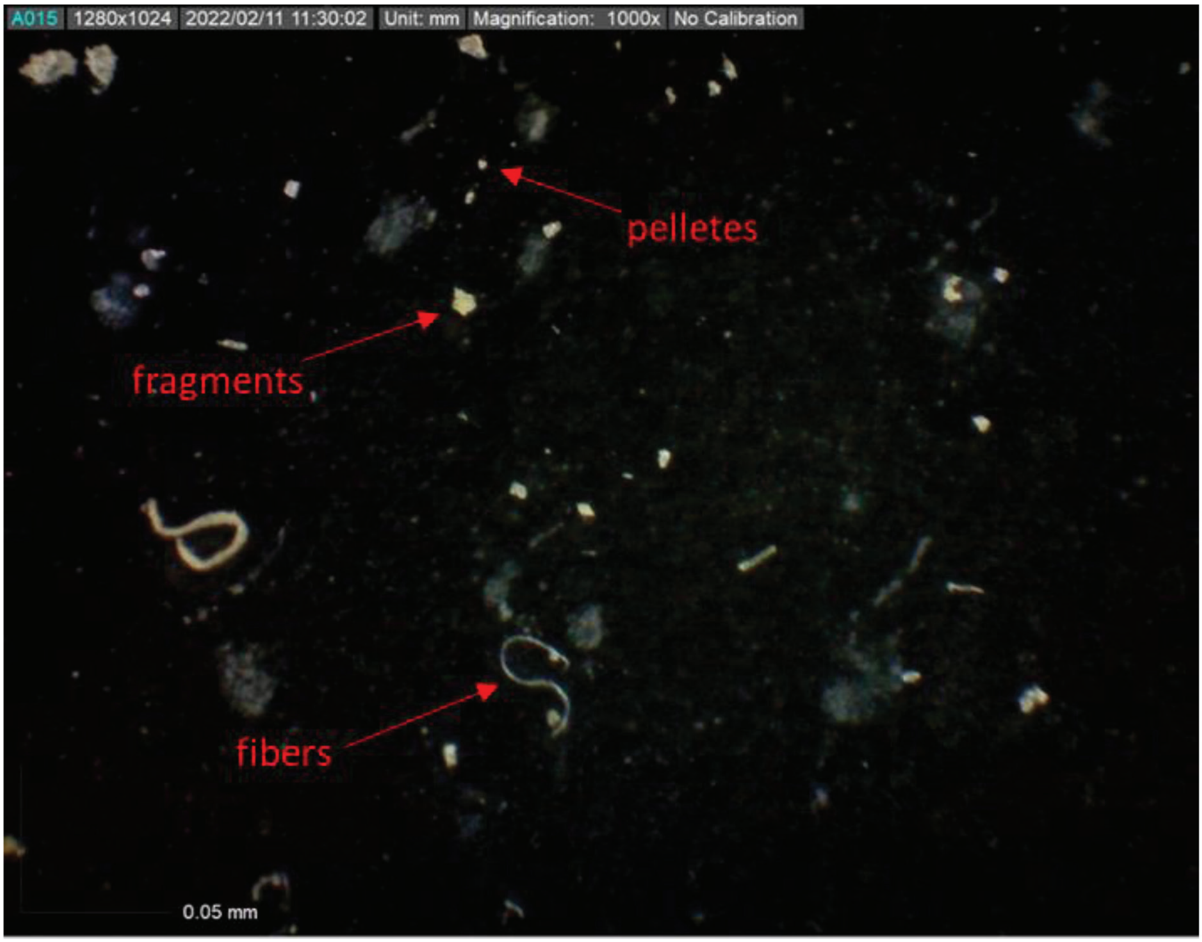


Fig. 2. Three morphological forms of microplastics recognised in the studied wedge clams.

Differences were considered significant at $p < 0.05$ in all statistical tests. The calculations were carried out using STATISTICA 10 package (Stat Soft Inc., USA).

Results

The first finding of our study was that different MPs were present in all of the tested individuals of wedge clams from all of the studied localities. However, the MPs found in the wedge clam specimens differed in number, form and size, and also among the localities and the two seasons. We identified three generally accepted forms (types) of MPs: pellets, fibres and fragments (Fig. 2).

Pellets have a spherical or slightly elongated shape with almost the same ratio of the long to the short axis (below 10:1); in fibres, the long axis is much longer than the short axis; fragments have an irregular shape (more ridged). The different types of MPs had a different occurrence in the studied wedge clams. The most common were the pellets (94.7%), followed by fibres (64.9%) and fragments (21.1%). The size of MPs was classified into three main size classes (Table 1). The abundance of MPs accumulated in the wedge clams of different forms and sizes varied greatly among the different localities and seasons. The data and statistical significance of differences are presented in Table 1.

Most numerous in the studied wedge clam individuals were the MPs pellets of the smallest size class (≤ 25 µm), which were present in the wedge clams from all localities (Table 1). Pellets belonging to the bigger size classes were not found in our samples.

MPs fibres were observed in the wedge clams from all studied localities (Table 1). As a whole, the fibres of the smallest size class (≤ 25 µm) were significantly (t-test, $p<0.005$) more abundant than those from the other size classes (Table 1). The fibres with the lowest mean number were of size 100–200 µm and were present only in samples from Ahtopol (Table 1).

Fragments of MPs were found in the soft tissue of wedge clams from all studied localities. The mean number of fragments belonging to the small-

Table 1. Mean number ± SD (per individual) of microplastics pellets, fibers and fragments of three size classes (100–200 µm, 25–100 µm, ≤ 25 µm) accumulated in wedge clam soft tissue from localities along the Bulgarian Black Sea coast with significance. of differences (* the locality significantly different at p<0.05 from localities with listed codes)

Code	**Sampling Sites**	**Pellets**			**Fibers**			**Fragments**			**Total MPs**
		≤ 25 µm	**25-100 µm**	**100-200 µm**	**≤ 25 µm**	**25-100 µm**	**100-200 µm**	**≤ 25 µm**	**25-100 µm**	**100-200 µm**	**Mean ±SD**
N1	Konstantin & Elena	23.33±4.64	0.00	0.00	1.66 ±0.47	0.33 ±0.27	0.00	7.67 ±4.61	0.00	0.00	3.66±1.09 *N3,N4,S3,S4
N2	Shkorpilovtsi	16.22±12.81	0.00	0.00	2.66±1.95	1.22±0.89	0.00	2.22±1.11	0.44±0.32	0.00	2.53±0.58 *S3,S4,S5
N3	Byala	12.83±7.57	0.00	0.00	0.17±0.11	0.18±0.11	0.00	4.33±3.13	0.00	0.00	1.94±0.48 *N1,S3,S4, S5
N4	Obzor	8.89±3.40	0.00	0.00	0.22±0.37	0.78±061	0.00	2.11±1.47	0.33±0.02	0.00	1.37±0.32 *N1,S1,S2,S3,S5
S1	Sveti Vlas	29.32±5.40	0.00	0.00	0.33±0.27	0.00	0.00	0.32±0.30	2.11±1.89	0.00	3.56±1.07 *S3,S4
S2	Pomorie	18.20±6.11	0.00	0.00	0.66±0.78	0.33±0.41	0.00	7.01±0.47	1.33±0.43	0.00	3.05±0.67 *S3,S4
S3	Duni	2.01±0.91	0.00	0.00	0.17±012	0.33±0.21	0.00	0.35±0.22	0.00	0.00	0.31±0.07 *N1,N2,N3,N4,S1,S2,S4,S5
S4	Arkutino	5.55±2.11	0.00	0.00	0.33±0.19	0.00	0.00	1.89±0.92	0.33±0.21	0.00	0.90±0.20 *N1,N2,N3,S1,S2,S3,S5
S5	Ahtopol	30.50±16.40	0.00	0.00	3.10±2.50	1.42±1.34	0.25±012	4.50±2.03	0.41±0.21	0.00	4.46±1.09 *N2,N3,N4,S3,S4
MPs form and size Mean±SD		16.31*±6.59	0.00	0.00	1.03±0.75	0.51±0.42	0.02±0.007	3.37±1.58	0.55±0.34	0.00	
% individuals with MPs		94.7%	0%	0%	45.6%	41.2%	0.03%	63.9%	31.4%	0%	

est size class (≤ 25 µm) was significantly greater than the mean number of other MPs. MPs fragments belonging to the largest size class (100–200 µm) were not found in the studied wedge clamp individuals (Table 1).

Significant differences were established in the mean number of MPs belonging to the studied forms and sizes accumulated in the wedge clams' soft tissue among the studied localities (Table 1). The mean number of pellets of the smallest size class was significantly higher in wedge clams from Ahtopol Bay compared to all remaining localities (Table 1). The mean number of pellets in the clams from localities Sveti Vlas and Konstantin & Elena were significantly higher compared to the remaining localities (excluding Ahtopol) (Table 1). The smallest mean number of pellets of the lowest size class (≤ 25 µm) was established in wedge clams from the coastal beach Duni and differed significantly from all the other localities (Table 1).

The mean number of MPs fibres of all sizes was significantly lower than the mean number of pellets in wedge clams from all localities (Table 1). The mean number of fibres (size ≤ 25 µm) present in the wedge clams from Ahtopol was significantly higher than the mean number of fibres of this size class compared to all other localities (Table 1).

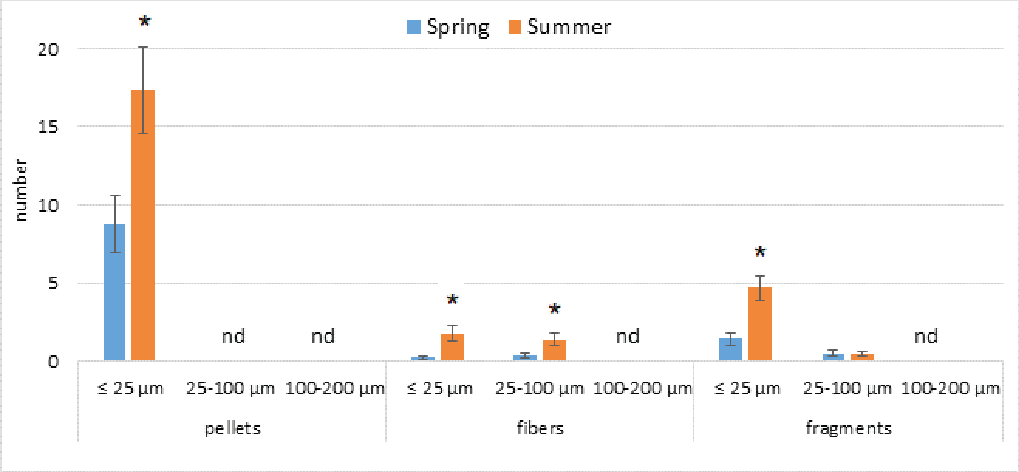
MPs fragments in wedge clams from the localities Konstantin & Elena and Pomorie had a mean number significantly higher than all the other localities (Table 1). No significant differences were found in the mean number of fragments (size 25–100 µm) among wedge clams from all the studied localities.

In an attempt to assess meaningful patterns of interaction between multiple dependent variables and to reveal the presence of associations, we used a multivariate (factorial) ANOVA design constructed from the available and suitable data. The results of the analysis are presented in Table 2.

The analysis clearly indicated the presence of some significant interactive effects among the three studied categorical variables "*Locality*", "*Form*" and "*Season*" on the dependent variable. The individual effects of the categories "*Form*" and "*Season*" were highly significant (Table 2). The individual effect of "*Locality*" was not statistically

Table 2. Results of multiple ANOVA of effects of categorical variables (form, locality, season) on the accumulated number of microplastics in wedge clams (sigma-restricted parameterisation, effective hypothesis decomposition) (significant effects underlined).

	Sum of Squares	Degrees of Freedom	Mean Squares	F -statistic	Significance (p)
Intercept	1683.547	1	1683.547	81.434	0.000000
Form	1605.262	2	802.631	38.823	0.000000
Locality	172.425	3	57.475	2.781	0.051048
Season	599.965	1	599.965	29.021	0.000002
Form*Locality	185.507	6	30.918	1.495	0.199910
Form*Season	514.483	2	257.241	12.442	0.000044
Locality*Season	255.076	3	85.025	4.112	0.011239
Form*Locality*Season	279.365	6	46.561	2.252	0.053899
Error	992.334	48	20.674		



* - difference significant at p<0.05

nd – non detected

Fig. 3. Average mean abundance of different forms and sizes of microplastics present in spring and summer in wedge clams from localities of the Bulgarian Black Sea coastal area.

significant although the p-value was bordering on significance (p = 0.051048) (Table 2. However, the interactive effects of ”*Locality*Season*” and also “*Form*Season*” were highly significant, thus indicating that “*Season*” was a significant factor for the microplastics accumulation in the wedge clams (Table 2). For this reason a post hoc comparison of the mean number of different MPs in the wedge clams from spring and summer samples was carried out (Fig. 3).

In both seasons the mean number of pellets of the smallest size (≤ 25 µm) was significantly higher than the other forms of MPs found. As a whole, the mean number of MPs present in the wedge clams from the spring samples was significantly lower than in the summer samples (Fig. 3). This was true for all tested MPs forms and size classes with only one exception concerning the group of MPs fragments of the middle-sized class (25–100 µm) where there were no significant difference between their mean number in spring and summer (Fig. 3).

Discussion

In this paper, we presented the first data on the distribution and bioaccumulation of MPs in wedge clams (*D. trunculus*) along the Bulgarian Black Sea coast.

Although there is no accepted size range of MPs, it is often believed this to be in the range of 10–5000 µm (0.01–5 mm) (Gamarro 2020). MPs are often categorised into two groups: primary and secondary. Primary MPs are with size less than

5 mm and are directly released into the environment either accidentally or mainly by wastewater. These may be pellets used in the production of larger plastic items or, for example, microbeads included in cosmetics and personal care products or used in industrial processes (Gamarro 2020). Secondary MPs on the other hand, originate from the gradual breakdown of larger products due to weathering of debris on shorelines and in the sea, UV-induced brittleness and subsequent fragmentation of plastics and others (Andrady 2017).

The first finding of MPs in Black Sea biota (neuston) was reported by Aytan et al. (2016) who found plastics in 92% of the samples they studied. Studies of Black Sea bivalves for the presence of MPs were carried out in the southern and south-western part of the coast of Turkey and published data showed that no MPs were found in *Abra alba,* while in *Mytilus galloprovincialis,* they were present in 48% of the sampled mussels (Gedik & Eryaşar 2020, Şentürk et al. 2020, Gedik & Gozler 2022).

Concerning the Bulgarian Black Sea part, several studies have previously reported the presence of high amounts of litter, containing also significant quantity of plastics, deposited in the coastal area and the marine environment (Moncheva et al. 2016, Simeonova & Chuturkova 2019, Berov & Klain 2020). However, at present there are no studies on the bioaccumulation of MPs in the biota of the Bulgarian Black Sea part.

Our results, although preliminary, clearly demonstrated that MPs of different form and size were present in all the studied individuals of *D. trunculus* gathered from several localities of their typical habitat along the Bulgarian Black Sea coast. The overall mean number of MPs items in the studied wedge clams was 2.52 (± 0.72) per individual. This number was higher than the reported 0.69 item/mussel in *Mytilus galloprovincialis* from 23 different locations along the Turkish coast of the Black Sea, the Sea of Marmara and the Aegean Sea (Gedik & Eryaşar 2020) and also that in clam species, i.e. *Chamelea gallina* from 15 sites along the south-western Black Sea coast, where MPs abundance ranged from 0.22 to 2.17 per individual (Gedik & Gozler 2022), as well as in *Donax trunculus*, *Chamelea gallina*, *Anadara inaequivalvis* and *Pitar rudis,* collected from the estuary of Yeşilırmak and Melet Rivers in the southern Black Sea, where the average number of MPs ranged from 1.69 to 4 per individual (Şentürk et al. 2020).

Our samples of wedge clams were dominated by pellets (94.7%), followed by fibers (64.9%) and fragments (21.1%). These data differ to some extent from the predominant morphological forms of MPs in bivalves reported in other published research. For example, fibres were found to dominate in clams from the Turkish coast of the Black Sea (Şentürk et al. 2020, Gedik & Gozler 2022), while in mussels (*M. galloprovincialis)* predominated fragments (67.6% fragments, 28.4% fibres, 4.05% films) (Gedik & Eryaşar 2020). The domination of pellets in the wedge clams in our study could be most probably the consequence of the predomination of primary MPs (mainly pellets less than 5 mm) in the marine environment of the Bulgarian part of the Black Sea. The quantity and distribution of different MPs are related to the predominant sources and types of the marine litter (ML) locally deposited, such as wastewaters, shipping, fishing, industrial processes and also the peculiarity of the habitat matrix and in the life cycle of the bivalves (Andrady 2021). It should be also kept in mind that the lack of harmonised analytical methods for microplastics, nanoplastics or compounds makes it difficult to directly compare analytical results between methods and/or laboratories and to obtain definite conclusions (Gamarro et al. 2020).

Concerning MPs size, in our study dominated the size class ≤ 25 µm (0.025 mm) with 93.4% in the wedge clam individuals. Other research reported that in samples of *Chamelea gallina* from the southern Black Sea dominated MPs with size <1000 µm (1 mm) (Gedik & Gozler 2022) and for other bivalve species the size was 1000–2000 µm (1–2 mm) (Şentürk et al. 2020). The most common size class of MPs found in *M. galloprovincialis* from the southern Black Sea was less than 500 µm (0.5 mm) (Gedik & Eryaşar 2020). Due to methodological challenges, the observations of the presence of smaller-sized MPs (for example <5 µm) in seafood were found to be very few (compiled by Barboza et al. 2018). However, at present it is difficult to make reliable comparisons between the results of published studies on the occurrence of MPs. Practically, there are no accepted standards for classification of type and size and of the different methodologies used to digest the tissues of organisms and identify microplastics and species (Andrady 2017).

We present the first preliminary data on the presence of some seasonal differences in the type and size of accumulated MPs in *D. trunculus* from the Bulgarian Black Sea coastal region. The mean number of MPs of all forms we identified was significantly higher in the studied samples from the summer season. At present, no special research on seasonal changes in MPs accumulation in Black Sea bivalves has been carried out. The higher number of MPs in the wedge clams in the summer samples observed by us could be a consequence of the significant increase in the marine environment of the released primary MPs (mainly small pellets less than 5 mm), such as microbeads included in cosmetics and personal care products in summer, mainly due to increased touristic activities, fishing and industrial processes (Gamarro 2020). It is well established that the amount of marine litter deposited in the Black Sea increases during the summer season mainly due to intensive tourism, wild camping, increased coastal population, wastewater discharge, active fishing, etc. (Simeonova & Chuturkova 2019), which inevitably increases also primary MPs deposition. The intensification of metabolic activity, feeding and reproduction of clams in the summer period most probably also play a role.

The results of the multivariate ANOVA demonstrated significant effects of the analysed categorical variables (factors), in particular MPs form, locality and season, and their associations on the dependent variables (mean number of MPs). In general, the analysis confirmed the presence of interdependent effects of the studied categorical variables (form, locality, season) on the observed pattern of distribution and accumulation of MPs in the wedge clams. This strongly suggested that the local accumulation of MPs in wedge clams from different localities and seasons was determined by multidirectional effects of different factors, e.g. MPs sources, local marine environmental conditions, penetration pathways, physiological state of clams.

Conclusion

In conclusion, the data reported here were the first to prove the presence of significant numbers of MPs in wedge clams from their natural habitats in the Bulgarian Black Sea coastal area. Obviously much more research is needed for a precise assessment of the patterns of bioaccumulation and effects of MPs in marine bivalves. This study, being a first step, is expected to provide a basis and encourage further research urgently needed to assess the ecological and socio-economic impacts of plastics on shellfish and human health in the Bulgarian Black Sea part.

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