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RESEARCH PAPER

The First Evidence of Microplastics Occurrence in Greater Pipefish (*Syngnathus acus* Linnaeus, 1758) in the Black Sea

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Abstract

Microplastics (MPs) occurrence in greater pipefish *Syngnathus acus* was assessed in the Black Sea for the first time. MPs were found in 21 % of total pipefish examined. MPs were mainly fibres in shape (89 %), black in colour (52 %) and 0.2- 1 mm (52 %) in size. The mean MP concentration was 0.30 mp. fish-1 (considering all the fish analysed) and was 1.38 mp. fish-1 (considering the fish that ingested them). The frequency of MP occurrence in male pipefish was higher (23.5 %) compared to females (20.7%) and immatures (16.7 %). 91.7 % of the polymers found were synthetic with the polyethylene terephthalate (PET) (29%) being the most common polymers. FTIR spectroscopy also confirmed the presence of the plastic-related compounds, butyl stearate (BS) and glyceraldehyde unnatural form (GA) in the analysed samples. Our results show that S. acus, which is an ecologically important demersal fish, is contaminated by MPs in the Black Sea and that there is an urgent assessment to better understand the environmental fate of MPs in the Black Sea and taking necessity measures to reduce the amount of plastics entering this basin.

Introduction

Plastic production has increased exponentially, reaching up to 390 million tons worldwide in 2021 (Plastics Europe, 2022). 10% of annual plastic production is estimated to end up in the ocean (OSPAR, 2007) due to poor waste management and low environmental awareness. The widespread use and permanent nature has made plastics ubiquitous pollutants in sea water, sediment and in marine organisms (e.g. Yang et al., 2015). Once plastics enter the marine environment, they break down into microplastics (MPs) (<5 mm) as a result of a series of physical, chemical and biological processes (Barnes et al., 2009; Hidalgo-Ruz et al., 2012). MPs can also enter the marine environment directly in the size of <5 mm such as fibres from laundering synthetic textiles, microbeads from personal care products and particles from car tires (Fendall & Sewell, 2009; Boucher & Friot, 2017).

Due to the ubiquity and small size of MPs (Cózar et al., 2014), a variety of marine organisms from zooplankton to fish to top predators have been reported to ingest MPs in marine environment (e.g. Burkhardt-Holm & N'Guyen, 2019; Duncan et al., 2019; Raju et al., 2019; Aytan et al., 2022 a,b). Global concern about the harmful effects of MPs on the environment and organisms has increased in recent years (Moore, 2008; Farrell & Nelson, 2013; Díaz Mendoza et al., 2020). Ingestion of MPs by marine organisms may cause weight loss, decline in growth rate and food consumption, and disruptions in physiological and behavioural activities such as respiration and reproduction (e.g. Rochman et al., 2015; Avio et al., 2015; Cole et al., 2015; Lusher et al., 2017). Microplastics can leach toxic additivities (Schrank et al, 2019; Luo et al, 2020; da Costa et al, 2023) and also adsorb heavy metals and persistent organic pollutants due to their hydrophobic properties (e.g. Koelmans, 2015; Brennecke et al., 2016; Patrício-Rodrigues et al., 2019; Liu at al., 2021; Kinigopoulou et al., 2022), thus, they pose a high toxicological risk to marine organisms (Shang et al., 2014; Jeong et al., 2016; Shi et al., 2020; Tang et al., 2020). These contaminants can eventually enter human diets through the food chain (Zarfl & Matthies, 2010; Tang et al., 2021).

The Black Sea is a semi-enclosed sea characterised by high river discharge and constitutes the drainage area of over ~170 million people from 21 countries in total (BSC, 2007). The Black Sea ecosystem has been drastically changed in the last half century due to increased pollution, introduction of invasive species, overfishing and climate change (BSC, 2007). Plastic pollution is a rapidly growing threat in the Black Sea environment (BSC, 2007; Aytan et al., 2016, 2020a; Bat et al., 2022). Vast amount of plastics enters to basin mainly from land-based sources (i.e. municipal sewage and industrial wastes, landfills, dumping areas, flood waters) (Aytan et al., 2020b). As a consequence of high plastic pollution in the basin, Black Sea is recognised as one of the hotspots of MPs pollution (Aytan et al., 2016). High levels of MPs have been reported from the sea water (Aytan et al., 2016; Öztekin & Bat, 2017), and sediment (Aytan et al., 2020b; Cincinelli et al., 2021; Pojar et al., 2021). MPs pollution places significant pressure on coastal ecosystems and biodiversity (Cole et al., 2011; Andrady, 2011). However, information on the occurrence of MPs on biota in coastal waters is still scarce (e.g. Şentürk et al., 2020; Atamanalp et al., 2021; Aytan et al., 2022a, Onay et al., 2023). The presence of MPs in zooplankton (Aytan et al., 2022b), invertebrates (Şentürk et al., 2020), commercial fish species (e.g. Atamanalp et al., 2021; Aytan et al., 2022a) and mammals (Mihova et al., 2023) in the Black Sea, provides evidence that MPs are highly available for each trophic level of both pelagic and benthic food web with a possible threat on biota and human health. Therefore, it is very important to understand the presence and effects of MPs on marine life in the Black Sea, to better manage practices and to take measures.

Syngnathus acus (Greater pipefish) is a demersal fish species that inhabits coastal and estuarine areas often associated with vegetation such as seagrass and seaweeds as breeding and feeding grounds and as shelter from prey (Dawson, 1986). It commonly occurs from the Black Sea through the Mediterranean Sea and the north-eastern Atlantic to Norway (Kullander et al., 2012). It is threatened by habitat loss and disturbance through anthropogenic activities such as destructive fishing gears (e.g. Trawls and dredges) and coastal development (e.g. Vincent et al., 2011; Caldwell & Vincent, 2012; Ceylan, 2014). These organisms are also threatened by pollution (Islam & Tanaka, 2004), since they are shallow coastal species, they are extremely susceptible to MPs pollution. *Syngnathus acus* are a typical component of fish communities in the Black Sea in shallow coastal waters, and have ecological function in pelagic and benthic food webs. *Syngnathus acus* can be affected by MP pollution by direct ingestion through their contaminated prey and the accidental ingestion of MPs present in the sea water. Although MP contamination by various commercial fish species has been reported in many parts of the world, the status of MP contamination of this ecologically important species is not known.

In this study, the presence of MPs in *S. acus* was investigated for the first time in the Black Sea. We hypothesised that MP ingestion by *S. acus* will be related to size and gender. This study adds up to current knowledge on the contamination of fish species by MPs and contributes to implementation of Marine Strategy Framework Directive (MSFD) Descriptor 10 by providing data to coastal managers and decision makers.

Material Method

Study Area and Sampling

The Southeastern Black Sea is characterised by a narrow continental shelf. Settlements in this region are concentrated along the coast. 60% of the Turkish commercial fisheries occur in the SE Black Sea. Local rivers constitute an important pathway of pollutants in particular plastics to the Black Sea (González Fernández et al., 2020). Due to the poor waste management, uncontrolled coastal dumping and landfills, intense fisheries and ship traffic, study area (Figure 1) is exposed to high plastic pollution (Aytan et al., 2016, 2020a).

To assess the occurrence of MPs in *S. acus* in the SE Black Sea, fish were obtained from small-scale fishing activities during July 2022. A total of 98 *S. acus* specimens as bycatch were caught using trammel nets at depths ranging from 15 m to 35 m. *S. acus* specimens were frozen immediately, and transported in iceboxes to the laboratory and stored at −20°C until further laboratory analysis.

Laboratory Analysis

For each individual, weight (W, nearest 0.1 g) and the total length (TL, nearest 0.1 g) (Lusher et al., 2013; Romeo et al., 2015) were recorded. Sex determination of specimens were made according to Vincent et al. (1995). Individuals with a pouch were defined as male and gonads were examined microscopically during dissection. To minimise the risk of contamination, the fish were opened with a scalpel and entire gastrointestinal tract (GIT) of each fish from the upper part of the oesophagus to the anal opening was dissected and weight (nearest 0.1 g) was recorded (Lusher et al., 2013).

GIT was rinsed with ultrapure deionised water and stored at −20°C in glass vials for further analysis. Then

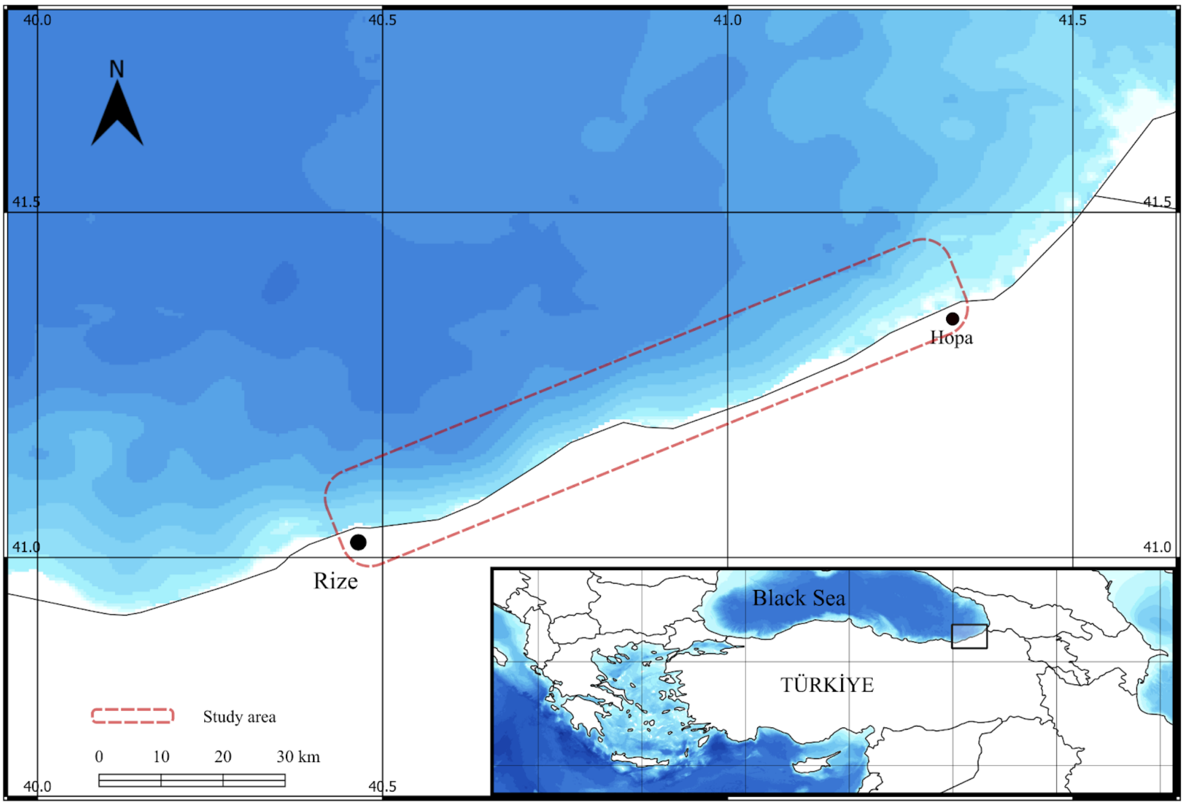

Figure 1. Sampling area in the SE Black Sea.

the whole GIT was digested to extract MPs. Approximately 10% of KOH solution with at least three times the volume of each sample was added to digest the organic matter (Bessa et al., 2018). Vials were covered with aluminium foil and kept at 40°C till full digestion occurred. After all the biological matter was removed, dissolved solutions were filtered on a glass microfiber filter (Whatman GF/C, 1.2 µm/pore, Ø=47 mm) and placed into petri dish and kept in oven (temperature <40°C) prior to microscopic examination. Presence of potential MPs were visualised under a Leica SAPO Stereomicroscope, and their images were taken with an image analysing system MIC 170 HD camera with Leica Application Suite (LAS) software. Plastics were classified by type (fibre, fragment, film, foam, pellet and microbead), and colour (black, blue, red, white, transparent, green, yellow, orange, grey, pink and purple). The largest cross sections of MPs were measured using their images and classified into classes as 0.2-1 mm, 1-2mm, and 2-5 mm. Suspected items were checked whether they were MPs or not using the hot needle test (Hermsen et al., 2018).

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier transform infrared spectroscopy (FT-IR) analysis was carried out on a Perkin Elmer Spectrum 100 FT-IR spectrophotometer equipped with attenuated total reflectance (ATR) apparatus to confirm the polymer origin of the particles found in pipefish GITs. The spectrum range was 4000-650 cm^{-1} and resolution of 1.0 cm^{-1} with 32 scans for each measurement. The identification of polymer type was done by comparing absorbance spectra to reference libraries using Perkin Elmer SEARCH Plus® software and only polymers showing >70% spectral similarity to reference spectra were considered.

Quality Assurance and Quality Control

To prevent contamination, 100% cotton lab coats and nitrile gloves were worn at all times. Working surfaces and all lab ware were cleaned with 75% ethanol before use and between specimens to prevent cross-contamination. The outer part of the fish was rinsed twice with ultrapure deionized water and once with ethanol to remove any potential particles attached to the fish body surface (Karami et al., 2017). In addition, procedural blanks using KOH were performed without tissues simultaneously. GIT sampling and content analysis were conducted under strict clean-air conditions. All filters were checked under microscope prior to use for the presence of MPs. To control air-born contamination, petri dishes with dampened filters were kept next to the sample during microscopic examinations and checked for presence of MPs.

Data Analyses

The number of MPs in each individual was counted and the mean MP ingestion considering all the fish analysed and considering the fish that ingested them was calculated (mp. $fish^{-1}$). The mean frequency of MP occurrence (FO%) was calculated for each gender following formula: FO% = (Ni / N) × 100, where FO% = frequency of occurrence of MPs; Ni = number of GITs that contained MPs; N = total number of GITs examined. To determine differences in the number of MPs ingested among female and male individuals independent samples t-test was performed. Normality of the data was checked prior to analysis. Immature individuals were not included in statistical analysis due to their low number. Spearman Rank correlation analysis was used to assess possible relations between the number of MPs and the total length, weight and GITs weight of fish. Significance level was considered for $p<0.05$ in all statistical analyses. Microsoft Excel 10 and Sigma Plot 12 were used in data analysis and visualization.

Results

A total of 98 *S. acus* specimens were examined for the presence of MPs. *Syngnathus acus* individuals comprised 59% of females, 35% males and 6% immatures individuals. Mean total length and weight of *S. acus* were approximately 25.19±2.47 cm and 24.5±2.33 cm, 17.72±2.27 cm and 4.85±1.69 g, 4.43±1.41 g and 1.55±0.61 g for females, males and immatures, respectively (Table 1). A total of 29 suspected MPs were extracted from the 21% of total fish examined. No MPs were found in the blanks. Fibres were the primary MPs found in GITs (90%) followed by fragments (10%), no films, foams, pellets and microbeads were found (Figure 2). In both female and male pipefish fibres (89%) were the most common MPs found in GITs, followed by fragments (11%), whereas only fibres were found in immature individuals.

A total of five different colours of MPs were found in GITs with black (52%) being the most common colour followed by blue (31%), transparent (10%), red (4%) and green (3%) (Figure 3). The variety of colours of MPs was higher in male *S. acus* than females (Figure 3). Only black MPs were found in immature individuals. The size of the MPs ranged between 0.35 and 4.17 mm (mean 1.25±0.88 mm) with the most common size being 0.2 -1 mm (52%), followed by 1-2 mm (31%), 2-5 mm (17%) (Figure 3). The size of MPs did not differ between the genders.

The mean MP concentration was 0.30 mp. fish^{-1} (considering all the fish analysed, n=98) and was 1.38 mp. fish^{-1} (considering the fish that ingested them, n=21) (Table 1). The maximum four MPs were found in a single individual. The frequency of MPs occurrence in male pipefish was higher (23.5%) compared to females

Table 1. Total length (mean±SD cm), weight (mean±SD, g), GITW (mean±SD, g), total number of fish analysed (N), total number of MPs (Total MP) found in GITs, frequency of MP occurrence (FO, %), number of MPs in all the fish analysed (A) and in fish that ingested them (B) (mp.fish^{-1})

	N	TL	W	GITW	Total MP	FO%	A	B
Female	58	25.19±2.47	4.85±1.69	0.23±0.12	19	20.7	0.33	1.58
Male	34	24.50±2.33	4.43±1.41	0.16±0.05	9	23.5	0.26	1.13
Immature	6	17.72±2.27	1.55±0.61	0.05±0.03	1	16.7	0.17	1.00
Total	98	24.49±2.97	4.50±1.73	0.20±0.11	29	21.4	0.30	1.38

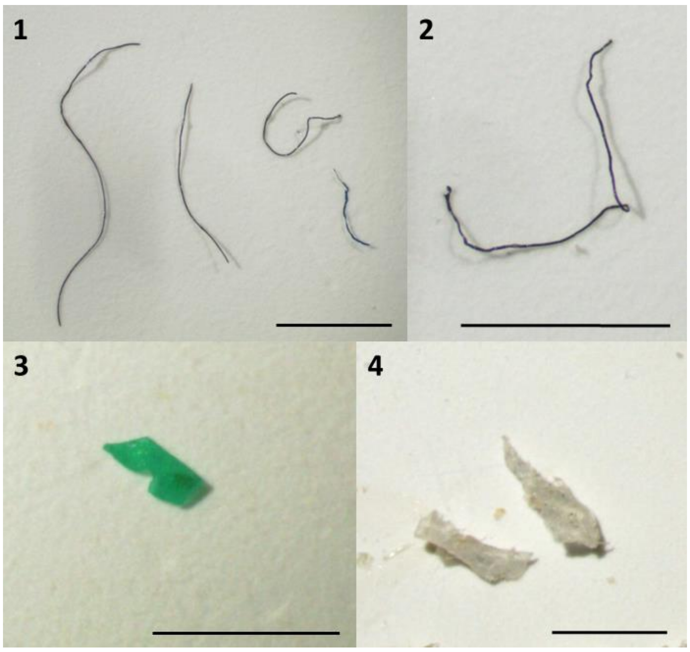

Figure 2. Representatives of MPs found in *S. acus* GITs (1-2: fibres, 3-4: fragments, scale bar=1 mm).

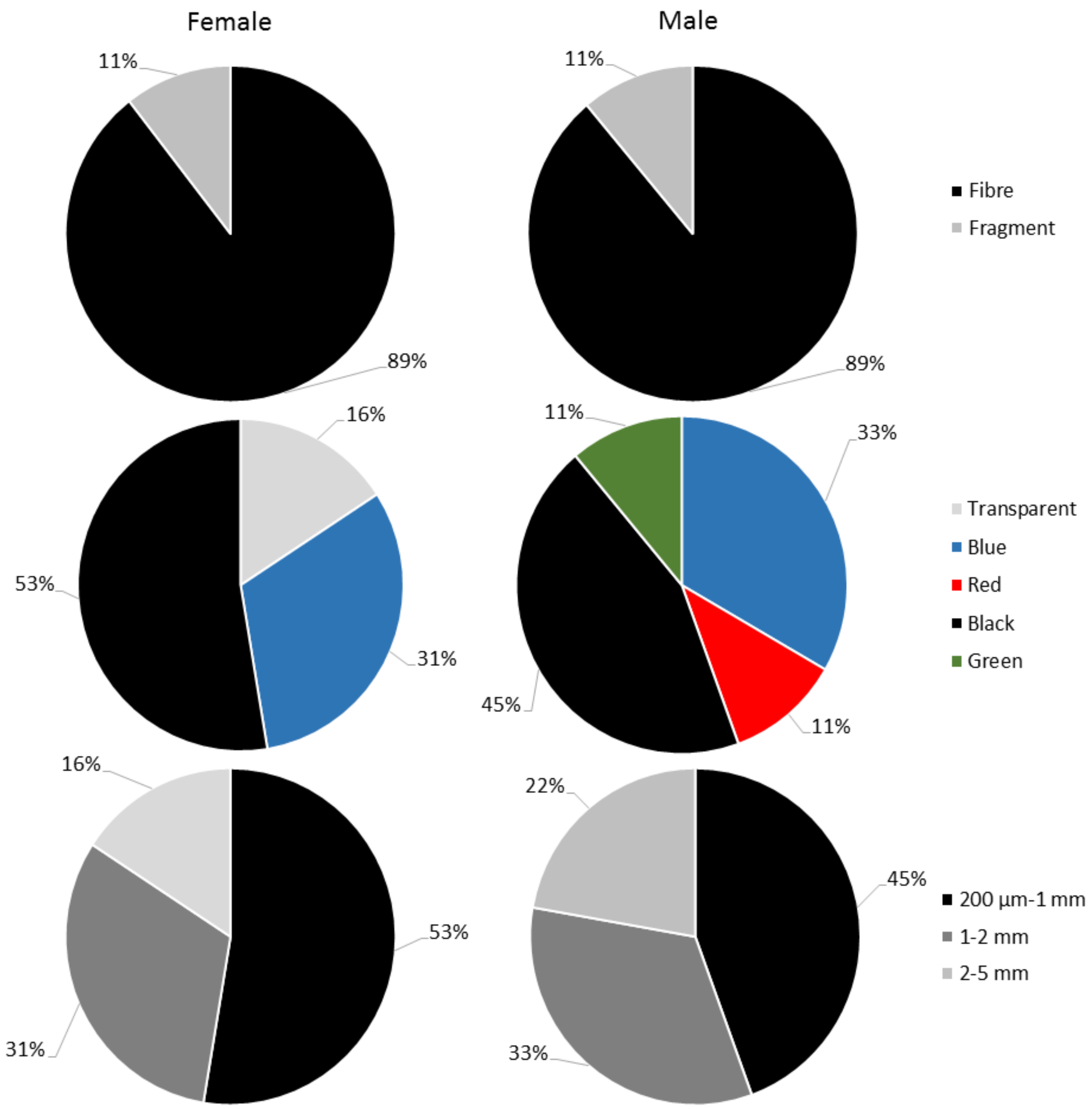

Figure 3. Percentage of types, colour and size of MPs ingested by female and male pipefish.

(20.7%) and immatures (16.7%) (Table 1). No significant correlation was found between TL, W, GITW of fish and number of MP ingested ($p>0.05$). Ingestion of MPs by females and males did not differ significantly ($F=0.31$, $p=0.73$).

83% of the MPs found in GITs analysed by FTIR spectroscopy. 91.7% of the polymer were synthetic and 8.3% was organic (cellulosic). 4.2% of the spectra (unidentified) did not match with the reference library, thus they were excluded from the analysis. The main synthetic polymers in the GITs were polyethylene terephthalate (PET) (29%), followed by polyethylene (PE) (14%), polypropylene (PP) (9%), ethylene-vinyl acetate (EVA) (9%), polystyrene-polyaluminum chloride (PS+PAC) (5%), and nitrile butadiene rubber (NBR) (5%) (Figure 4). FTIR spectroscopy analysis also confirmed the presence of the plastic-related compounds such as glyceraldehyde unnatural form (GA) (24%) and butyl stearate (BS) (5%) in the analysed samples.

Discussion

The present study provides detailed data on the presence of MPs in *S. acus* from the Black Sea for the first time. Pipefishes feed on small invertebrates and fish larvae including harpacticoid copepods, amphipods, cyprid larvae and decapod crustaceans (Taşkavak et al., 2010). Laboratory and field studies have shown the trophic transfer of MPs (Setala et al., 2014; Santana et al., 2017; Tosetto et al., 2017). A recent laboratory study showed ingestion of MP-contaminated copepods by seahorses (Domínguez-López et al., 2022). Microplastic ingestion by copepod species which were collected from their natural environment in the Black Sea (Aytan et al., 2022b) also provide evidence that these organisms from the lower food web can act as a vector of MPs and associated toxic chemicals to higher trophic levels including pipefishes.

Although there are field and laboratory studies on the ingestion of MPs by seahorses which is another genus from the Syngnathidae family (Jinhui et al., 2019: Liu et al., 2022a), no study was found in literature on the ingestion of MPs by pipefish. Therefore, our results are compared with the previous studies that reported MP ingestion by seahorses (Table 2). The mean MP ingestion in this study was similar to reported values of MPs ingestion by seahorses from the Black Sea (Table 2), however, lower than that reported extremely high number of MPs per individual of *Hippocampus* sp. from the China Sea (Liu et al., 2022b). The mean MP ingestion by pipefish were also lower than MP ingestion by pelagic and demersal fish in the Southern Black Sea (Atamanalp et al., 2021) and SE Black Sea (Aytan et al., 2022a). Although higher MP ingestion by female fish compared to males is reported (Horton et al., 2018), we did not find significant differences on the ingestion of MPs between genders.

Plastic ingestion by fish has been reported to correlate with their feeding behaviour and ambient MP concentration where fish were caught (e.g. Romeo et al., 2015; Battaglia et al., 2016; Sun et al., 2019). High MP pollution in surface water (Aytan et al., 2016; Öztekin & Bat, 2017; Pojar et al., 2021), water column (Aytan et al., 2020b) and sediment in the Black Sea (Aytan et al., 2020; Cincinelli et al., 2021; Pojar et al., 2021) can increase the bioavailability of MPs by *S. acus*. In this study, the most common size of MPs was 0.2-1 mm in agreement with the studies that reported the same size range of MPs from seahorses (Onay et al., 2023) and commercial fish (Aytan et al., 2022a) from the Black Sea. This provides evidence for the inclusion of MPs to the food chain in the Black Sea. Since they are in the same size range of plankton, MPs are bioavailable for many marine organisms (Jinhui et al., 2019).

In this study, fibres were the most common types of MPs found in pipefish GITs. Fibres are the dominant MP types reported from the variety of marine environment (e.g. Thompson et al., 2004; Noren, 2008; Browne et al., 2011; Desforges et al., 2014; Zhao et al., 2014; Güven et al., 2017; Taha et al., 2021) including the Black Sea (Aytan et al., 2016, 2020b; Pojar et al., 2021). The Black Sea constitutes the drainage area of many industrialised countries (BSC, 2007). The main source of fibres is recognised as synthetic textiles as a result of laundering which are transported to the marine environment by sewages, municipal waters, wastewater treatment plants, rivers (Browne et al., 2008) and atmospheric deposition (Liu et al., 2019; Yang et al., 2021). In the SE Black Sea, intense fishing operations take place along the narrow continental shelf near the coast, thus, ropes and nets used in fishing can also constitute an important fibre source in the region (Welden & Cowie, 2017; Aytan et al., 2022 a,b).

The dominant MPs colour was black in GITs of *S. acus* in the Black Sea in agreement with many previous reports from fish (Lusher et al., 2013; Bellas et al., 2016; Murphy et al., 2017; Sparks & Immelman, 2020; Bottari et al., 2022; Koraltan et al., 2022) including the Black Sea

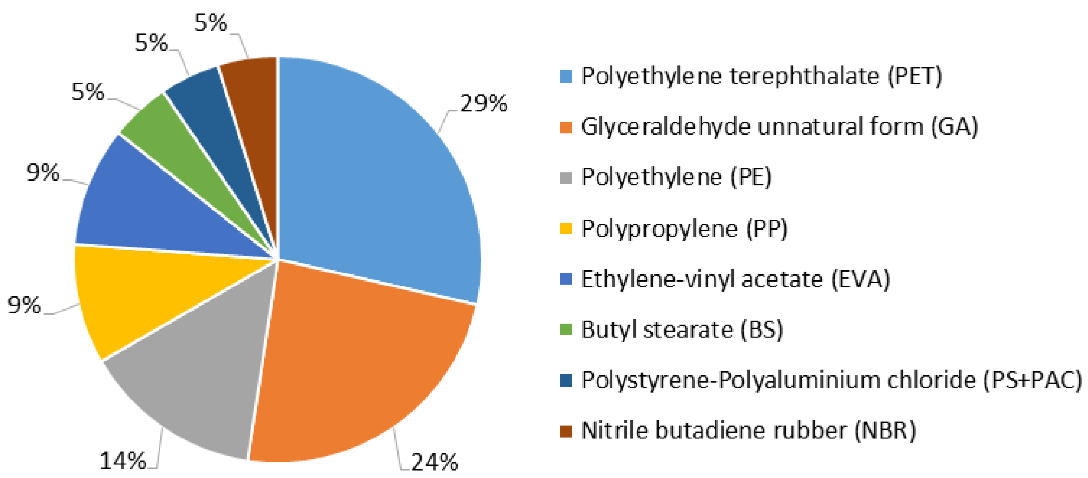

Figure 4. Chemical composition of MPs found in pipefish GITs.

Table 2. Comparison with previous studies

Location	Species	N	mp.fish^{-1}	Size (mm)	Type	Colour	Polymer	Reference
Southern Black Sea	Pelagic & Demersal Fish	82	2.01	0.05-0.2	Fibre	Black	CR, PA	Atamanalp et al., 2021
SE Black Sea	Pelagic & Demersal Fish	650	2.06±1.09	1.84±2.80	Fibre	Black	PP	Aytan et al, 2022a
China Coastal	*Hippocampus sp.*	30	92-322*	0.02-0.2	-	Green	PE	Liu et al 2022a
SE Black Sea	*H. guttulatus*	90	1.5**	1.39±1.76	Fibre	Black	PVC	Onay et al, 2023
SE Black Sea	*S. acus*	98	1.38	1.25±0.88	Fibre	Black	PET	This study

* considering the fish that ingested MPs

** showing data from 2022

(Aytan et al., 2022a). A recent study also reported black as one of the most prevalent MPs colour ingested by seahorses in the SE Black Sea (Onay et al., 2023). *Syngnathus acus* can ingest MPs by mistake as food or can be taken by contaminated prey. A recent study from the Black Sea reported that the dominant colour of the MPs ingested by copepod species in the Black Sea was also black (Aytan et al., 2022b). This provides evidence of one of the possible routes of trophic transfer of MPs and associated chemicals to the pipefish.

In this study, the most common polymers found in *S. acus* GITs were PET, PE, and PP. Polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET) are the most produced plastics globally and mainly used in packaging in particular single-use plastics (PlasticsEurope, 2021). As a result of wide applications and poor waste management, these polymers have been also reported as prevalent polymers in the marine environments (e.g. Erni-Cassola et al., 2019; Pojar et al., 2021; Aytan et al., 2020b) and in biota (e.g. Tanaka & Takada, 2016; Bessa et al., 2018; Aytan et al., 2022a, b, 2023; Onay et al., 2023). Since PE is also widely used in the production of fishing nets and ropes (Hung vd., 2022), intense fishing activities in the region can be another important source of plastic pollution. Low-density polymers PP and PE are expected to be more abundant in surface waters, whereas PET, which is a high-density polymer, is expected to be more abundant in subsurface waters (Erni-Cassola et al., 2019). However, MPs can sink faster in the Black Sea compared to other regions due to the less saline water in the upper layers. MPs can also accumulate in intermediate layers due to the permanent halocline separating the low-density upper layer from the more saline deeper layer in the Black Sea. Additionally, these polymers might be rapidly colonised by microorganisms (Esensoy et al., 2020) which may increase density and bioavailability of MPs for demersal fish and invertebrates.

Conclusion

This study presents a detailed evaluation of the MP contamination status of *S. acus* in the Black Sea, for the first time. Our results showed that *S. acus* is contaminated with plastics, mainly fibers. No difference in ingestion of MPs was found between female and male. MPs ingested by *S. acus* were mostly PET, PE and PP polymers which are mainly used in the production of single use plastics. In order to prevent this, urgent actions are needed for better waste management by municipalities in the Black Sea. Our results confirm that plastic is an increasing threat to coastal biodiversity which has already in the past been influenced by multiple anthropogenic pressures. Coastal habitats and biota are under high risk of MP pollution and its harmful effects and there is an urgent need to reduce the number of plastics entering the Black Sea and further multidisciplinary research to better understand the environmental fate of plastics in this ecosystem.

Ethical Statement

Not applicable Fish were obtained from local fishermen as a bycatch.

Author Contribution

YS: Investigation, Data Curation, Visualization, Writing – original draft

ME: Investigation, Data curation, Visualization, Writing – original draft

YC: Investigation, Visualization, Writing – original draft

ÜA: Conceptualization, Investigation, Visualization, Supervision, Funding acquisition, Writing – original draft

Conflict of Interest

The authors declare that they have no conflict of interest.

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