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Advances in quantification, degradation and ecotoxicology of microplastics in marine resources

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Advances in quantification, degradation and ecotoxicology of microplastics in marine resources

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Trapping of Microplastics in Halocline and Turbidity Layers of the Semi-enclosed Baltic Sea

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Microplastic pollution in semi-enclosed seas is gaining attention since microplastics are more likely to accumulate there. However, research on the vertical distribution of microplastics and impact factors is still limited. In this study, we focus on the Baltic Sea, which has distinguished salinity stratification, and we assume that the resulting strong density stratification (halocline) can influence the vertical distribution of microplastics in the water column. Therefore, we analyzed the vertical abundance distribution, the composition, and the sizes of microplastics (27.3–5,000.0 μm) in the Baltic Sea. The results showed that microplastics comprising fibers, fragments, and films occurred throughout the water column at an abundance of 1.1–27.7 items L^{-1}. The abundance of microplastics (3.2–27.7 items L^{-1}) at haloclines was significantly higher than those at other water depths except the near surfaces ($p < 0.05$), contributing 24.1–53.2% of the microplastics in the whole water column. Small microplastics (<100 μm) were more likely to accumulate in the water layers above halocline. Moreover, the current with high turbidity might be another carrier of microplastics in the near-bottom water layer due to its strong correlation with microplastics abundance. This study provides valuable evidence for the accumulation trend of microplastics in water columns and its influencing factors in the semi-enclosed marginal sea. Further research on the vertical distribution of microplastics under the control of multiple factors should be conducted in the future.

Keywords: Baltic Sea, halocline, microplastics, turbidity, vertical distribution

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INTRODUCTION

The widespread occurrence of microplastics as an anthropogenic fingerprint in the environment has received global attention (Rochman, 2018). Microplastics can persist for a long time in the marine environment owing to their durable properties and are readily transported over long distances from sources by wind and currents (Barnes et al., 2009; Isobe et al., 2014; Zhang, 2017; Zhang et al., 2021). During this period, there is an increased possibility of microplastics compounding environmental chemicals (i.e., metals or persistent organic pollutants) and microorganisms (Frias et al., 2010; Reisser et al., 2014; Brennecke et al., 2016). This may lead to changes in their density and influence their fate by sinking, further damaging marine organisms, living at different depths of the water column, or even ecosystems (Kowalski et al., 2016; Jeong et al., 2018; Paul-Pont et al., 2018; Zhang et al., 2020a; Uurasjärvi et al., 2021).

Until recently, most studies focus on the amounts of marine microplastic pollution in the surface or subsurface layers (Lusher et al., 2015; Song et al., 2015; Zhang et al., 2017; Kanhai et al., 2018). However, it is difficult to determine the number of microplastics in the ocean without investigating their abundance at different depths. Polypropylene (PP, ρ = 0.85–0.92 g cm^{-3}), polyethylene (PE, ρ = 0.89–0.98 g cm^{-3}), expandable polystyrene (EPS, ρ = 0.01–0.05 g cm^{-3}), polyethylene terephthalate (PET, ρ = 1.38 g cm^{-3}), and rayon (ρ = 1.46–1.54 g cm^{-3}) are considered common polymer types in the marine environment (Dai et al., 2018; Silvestrova and Stepanova, 2020; Zhang et al., 2020b). Although the densities of these plastics are lower or higher than the density of seawater, there is growing evidence that they can be transported up and down the water column by vertical mixing, and get to different layers of the water column (Kukulka et al., 2012; Gorokhova, 2015; Dai et al., 2018; Cincinelli et al., 2019; Rebeca et al., 2021; Uurasjärvi et al., 2021) and sediments (Van Cauwenberghe et al., 2013; Bergmann et al., 2017; Barrett et al., 2020; Reineccius et al., 2020) and do not just simply float on the surface. However, an understanding of the impact factors on the vertical distribution of microplastics is still limited.

The studies on microplastic vertical distribution are usually based on predictions and measurements made in laboratory experiments based on particle shape, size, and density, and also different environmental factors (Cole et al., 2016; Kowalski et al., 2016). For example, currents, aggregation with organic and inorganic particles, and biofouling can affect the transportation, sinking, and distribution of microplastics in the marine environment (Long et al., 2015; Suaria et al., 2016; Kaiser et al., 2017; Kooi et al., 2017; Wu et al., 2020). However, there is still not enough research based on the *in situ* data on microplastics to understand the vertical distribution in the water column and the factors determining it. More and more studies have indicated that the densities of virgin plastics cannot be considered as a decisive factor affecting the vertical distribution of microplastics in the seawater (Kaiser et al., 2017; Chen et al., 2021; Karkanorachaki et al., 2021). It seems that environmental factors play an important role in the vertical distribution of microplastics. Some studies show that microplastics accumulate mainly at the near-surface, the near-bottom, or specific layers due to environmental factors including storms, wind-driven mixing events, resuspension, and attachment (Lattin et al., 2004; Corcoran, 2015; Reisser et al., 2015; Katija et al., 2017; Martin et al., 2017; Dai et al., 2018; Song et al., 2018; Choy et al., 2019). For instance, Choy et al. (2019) found the highest abundance of microplastics at depths between 200 and 600 m (roughly 15 particles m^{-3}) in Monterey Bay because of the contribution of the pelagic particle feeders. Dai et al. (2018) reported that the turbulence caused microplastics to accumulate mainly at 5–15 m depth with an abundance of 0.6–23.0 items L^{-1} in the Bohai Sea. The halocline, which was caused by a strong, vertical salinity gradient within a body of seawater, usually is a distinctive feature of the semi-enclosed sea (Ferentinos et al., 2010; Virtasalo et al., 2011). It was believed to affect the vertical distribution of the particles including plastics in the water column, but there is little unequivocal evidence for the small microplastics (Gorokhova, 2015; Bagaev et al., 2017). Moreover, turbidity currents prevail at the near-bottom layers in the ocean including semi-enclosed seas, but the ability of bottomed currents to transport and accumulate plastics is essentially unknown (Pohl et al., 2020).

The Baltic Sea is a typical semi-enclosed sea. Microplastic pollution in the Baltic Sea has been reported in recent years, and most investigations have focused on the (near) surface water (Setälä et al., 2016; Gewert et al., 2017; Schönlau et al., 2020; Hänninen et al., 2021), seafloor, and beach sediments (Stolte et al., 2015; Talvitie et al., 2015; Graca et al., 2017; Zobkov and Esiukova, 2017; Kammann et al., 2018; Urban-Malinga et al., 2018). There are only a few studies focused on the vertical distribution of microplastics, but research based on the multi-regions, multi-depths, and relevant impact factors of microplastics distribution are limited (Gorokhova, 2015; Bagaev et al., 2017, 2018; Zobkov et al., 2019; Uurasjärvi et al., 2021). Moreover, as a semi-enclosed sea, the density of the seawater in the Baltic Sea varies considerably with temperature and salinity such that a microplastic that floats may sink as soon as the salinity or temperature changes (Bagaev et al., 2017; Uurasjärvi et al., 2021). Previous studies indicate that the halocline may affect the vertical distribution of the plastic particles in the Baltic Sea, but small microplastics (especially <100 μm) were ignored (Gorokhova, 2015; Bagaev et al., 2017; Uurasjärvi et al., 2021).

Here, we focused on the distribution characteristics of microplastics with a wide size range of 25.0–5,000.0 μm aiming to (1) clarify the differences of the regional and vertical distribution of microplastics. We tested the hypotheses that, (2) the strong salinity stratification can facilitate the accumulation of microplastics in the water column. Finally, (3) the turbidity can influence the abundance of microplastics at the near-bottom of the water column. This study provides valuable evidence for the accumulation trend of microplastics in water columns and impacts of halocline and turbidity on the vertical distribution of microplastics in the semi-enclosed Baltic Sea.

MATERIALS AND METHODS

The Study Area

The Baltic Sea (53°-66°N, 10°-30°E), with average depth of 55 m and a maximum depth of 459 m, is surrounded by nine countries and is the largest inland brackish adjacent sea of the Atlantic Ocean. The water temperature of the Baltic Sea varies significantly depending on the exact location, season, and depth. The salinity in the Baltic Sea is much lower than that of ocean water, mainly as a result of abundant freshwater runoff from the surrounding rivers and streams. There are more than 250 streams that drain a basin of about a volume of 660 km^3 year^{-1} to the Baltic Sea. Moreover, due to the inflow of the North Sea water with higher salinity from the west, the bottom of the Baltic Sea is saltier than the surface. It creates a vertical stratification named halocline of the water column, which acts as a barrier for the exchange of oxygen, nutrients, and particles (Maciejewska and Pempkowiak, 2014; Liblik and Lips, 2019).

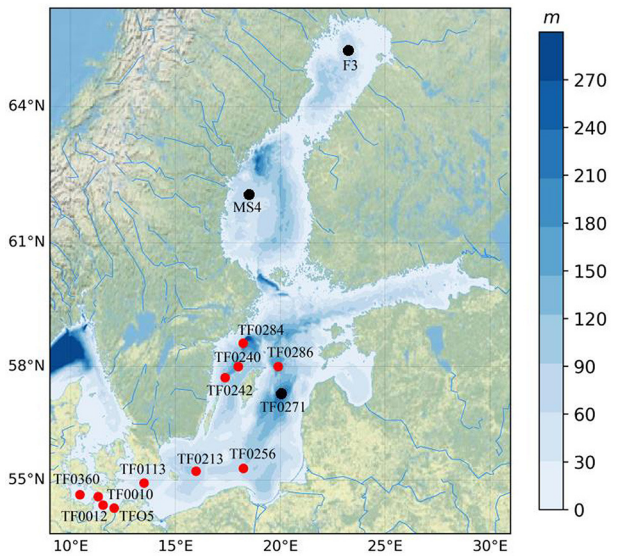

FIGURE 1 | Regional distribution of sampling stations for water and sediment in the Baltic Sea. (Red dot, both water, and sediment samples were sampled; black dot, only water samples were sampled. Sediment samples at stations TF0271, MS4, and F3 were not obtained due to bad weather conditions and resulting time limitations).

Vertical Sampling of Water and Sediment

The water samples were taken at 12 locations during a routine winter monitoring cruise of the Leibniz Institute for Baltic Sea Research (Warnemünde, Germany) in February 2019, and two locations in the northern Baltic Sea were added to the data set in May 2019 covering different basins of the Baltic Sea to obtain the spatial diversity of sampling. The sampling stations were located in the nearshore and offshore zones, covering Arkona Basin, Bornholm Basin, Gotland Basin, Gulf of Bothnia, Northern Baltic Proper, Lübeck Bay, Kiel Bight, and Fehmarn Strait as shown in **Figure 1** and **Supplementary Table 1**. About 10 L of seawater was collected at each selected water depth between the near surface (1–3 m from the seawater surface) and the bottom (1–5 m from the seabed surface with a maximum sampled depth of up to 437 m) using a 12-bottle rosette sampler system (SBE 9 Carousel Water Sampler) connected to a conductivity, temperature, and depth sensor (CTD) system (SBE911Plus, Sea-Bird, Bellevue, WA, USA) and equipped with 5-L free flow bottles. In addition to the water salinity, other water parameters including temperature, dissolved oxygen, chlorophyll a, and turbidity were also measured *in situ* by the CTD. Different water depths were chosen (the near surface and the depths of 10, 20, 50, 100, and 200 m until the near-bottom water layer) based on the station-specific depth and the depth of halocline for sampling (see **Supplementary Table 1**). About 5 L of water sample with two replicates collected at each depth and station was stored in a clean glass bottle for microplastic analysis.

The bottom sediments (top 3 cm) at each station were collected using a stainless multicore with tubes of 10 cm diameter. At each sampling site, three subsamples were collected and then bulked to form one composite sample. All the samples were put into the clean (pre-washed) aluminum containers respectively and stored at low temperature.

Microplastics Extraction From Water and Sediment

Extraction of the microplastics from the water samples was conducted using the method of Dai et al. (2018). Briefly, each 5-L water sample was filtered through a 47-mm diameter cellulose nitrate filter with 5 μm porosity (Whatman AE98, Cytiva, Marlborough, MA, USA) immediately by using the vacuum filtration device in the filtration laboratory on-board. The samples were not treated with hydrogen peroxide or enzyme-digested because they were needed for subsequent surface micromorphology analysis, and samples collected during the winter had low organic matter contents (Cole et al., 2014; Bagaev et al., 2018). Each filter was stored in cleaned glass Petri dishes with a diameter of 60 mm. The dishes with filters were sealed using glass lids, wrapped with aluminum foil, and stored at 4°C for further observation.

The microplastics were extracted from the sediment samples by density separation (Thompson et al., 2004; Vianello et al., 2013). All sediment samples collected were dried in a vacuum freeze dryer and filtered through a stainless-steel screen with a 2-mm aperture for removing large pieces of biological residues. Dried sediment (5.0 g) was placed in a pre-cleaned 250-ml tall glass beaker, mixed and dispersed thoroughly with 50-ml saturated sodium chloride solution ($\rho = 1.2\,\mathrm{g\ ml^{-1}}$, filtered through a 5 μm cellulose nitrate filter), and then more saturated sodium chloride solution was continually added to the beaker until the maximum mark line. Two replicates were conducted simultaneously. The glass rod was used to stir thoroughly for 2 min to make the sediment evenly dispersed in the solution, and then the substance adhered to the glass rod was rinsed into the beaker with sodium chloride solution. The beaker was covered with aluminum foil and settled for 24 h for microplastic separation. Floating materials were collected by filtration using a cellulose nitrate filter with 5 μm pore diameter (Whatman AE 98, Cytiva, Marlborough, MA, USA).

Observation, Identification, and Quantification of Microplastics

Preliminary observation and quantification of the microplastics in the filtered residues were conducted by visual examination under a Zeiss Discovery V8 stereomicroscope with ×80 magnification (Zeiss AG, Oberkochen, Germany). All filters were observed under the eyepiece with the aid of stainless-steel tweezers and dissection needles. Putative microplastics were counted based on the colors and morphology types of particles previously published in photographs and references (Nor and Obbard, 2014; Bagaev et al., 2017; Zhou et al., 2018). They were then photographed under the stereomicroscope equipped with a charge-coupled device (CCD) camera, and the shape type and color of every suspected microplastic were recorded. The quantification and the size measurement, including the length and the width of all potential microplastics, were carried

out using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). A total of 2,557 potential microplastics comprising 2,268 fibers, 280 fragments, and nine films were recorded. Due to time constraints, only a limited number of microplastics have been analyzed using micro-Fourier transform infrared spectroscopy (μ-FTIR). For doing it, we classified them into different groups and subgroups based on their color, shape, and morphologies (**Supplementary Table 3**). Microplastics and suspected microplastics comprising 227 fibers, 20 fragments, and four films were selected based on the different colors and shape morphologies from each group (**Supplementary Table 3**). They were identified using μ-FTIR spectrometry (Spotlight 400, Perkin Elmer, Waltham, MA, USA) with spectral region 750–4,000 cm^{-1} and a resolution of 4 cm^{-1} at a rate of 16 scans per analysis. The spectra obtained were compared with the standard database provided by the OMNIC software (Thermo Scientific Inc., Waltham, MA, USA). The analysis provided us with a proportion of the real number of plastic particles and the non-plastic particles in the original group and allowed us to correct our original counts in each subgroup in the same proportion according to the identification results and morphology information. Rayon is an artificial semi-synthetic material, which is different from natural materials, such as cotton and wood (Obbard et al., 2014; Gago et al., 2017). Rayon makes up a significant proportion of synthetic microparticles found in the marine environment (Lusher et al., 2013; Suaria et al., 2020) and has a large proportion in this study. Therefore, we classified rayon into a microplastics group for further examination and discussion. Moreover, in this study, we also considered the poly (N-methyl acrylamide) as a plastic polymer based on its chemical structure and acrylic properties. Finally, a total of 88 particles were not plastics in the groups of selected particles. Based on the proportion of identification results and the information on the recorded particles, all non-plastics were removed, and a total of 1,449 particles comprising 1,265 fibers, 176 fragments, and eight films were regarded as microplastics and analyzed further.

The microplastics from the near-surface water (two particles), haloclines (four particles), the near-bottom water (four particles), and sediments (three particles) were selected randomly to observe the surface substances using a MERLIN VP compact (Carl Zeiss, Oberkochen, Germany) in a vacuum. Samples were dried at room temperature first, and then placed on pin stubs with adhesive carbon pads and sputter coated with iridium by argon plasma at high voltage before analysis. The scanning electron microscope (SEM) was operated at 15.0-kV electron accelerating voltage.

Quality Assurance and Quality Control

All containers and instruments were cleaned with MilliQ water and covered with aluminum foil before use. Sample processing and separation were carried out in a laboratory specified for marine-particle analysis at the IOW (Germany), whereas the filtration of water samples was carried out onboard. Non-textile jumpsuits were worn during the experimental work to prevent contamination from fibers and other microplastic particles. Procedural blanks using MilliQ water were run in parallel with water samples. Blanks using filtered NaCl solution were run parallel with the processing of the sediment samples in the laboratory at IOW. Only blue fibers were observed in the blanks with one to four particles in 5-L MilliQ. Only two to three blue and transparent fibers were counted in the blanks during the sediment analysis. The quantities of the microplastics in all samples were calibrated based on the blanks.

Statistical Analysis

Microplastic abundances in water or sediments are presented as the number of microplastic particles per liter or per gram dry weight (items L^{-1} or items g^{-1} d.w.). Data analysis was conducted using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) and OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). The Pearson correlation was used for the correlation analysis between the abundance of microplastics and temperature, salinity, turbidity, dissolved oxygen, or chlorophyll a. One-way ANOVA was conducted to compare the difference of microplastics abundance among different regions and sampling depths using the IBM SPSS 20.0 software package (IBM Corp., Armonk, NY, USA), and the mapping of the spatial distribution of microplastics was conducted using ArcGIS10.2 (ESRI, Redlands, CA, USA).

RESULTS

Occurrence Characteristics of Microplastics in the Baltic Sea

The range of microplastic abundance was 1.1–27.7 items L^{-1} with the mean abundance of 5.8 $\pm$ 5.0 items L^{-1} in the water (for the original data, see **Supplementary Table 4**). The highest abundance of microplastics was found at station TF0286 in the Northern Baltic Proper and the lowest at station MS4 in the north of the Baltic Sea (Bothnia Sea). In the horizontal distribution, the microplastic abundance in the near-surface layers of the southern (8.8 $\pm$ 3.8 items L^{-1}) and central (6.0 $\pm$ 7.8 items L^{-1}) regions of the Baltic Sea was significantly higher than in the northern region (1.0 $\pm$ 0.8 items L^{-1}), ($p < 0.05$). The vertical distribution of microplastics showed different patterns that changed with depth among the stations (**Figure 2**). The coefficients of variation of microplastics abundance at different depths within the water column were 0.2–0.9 ($c_v < 1$, **Table 1**), showing small differences.

Three shape types of microplastics comprising fibers, fragments, and films were found in the Baltic Sea (**Supplementary Figure 1**). The fibers were the most popular type with a mean abundance of 5.2 $\pm$ 4.8 items L^{-1} ($\sim$90.4% of all microplastics) in the Baltic Sea water (**Figure 3A** and **Supplementary Figure 6**). Fragments were the second most abundant type with a mean abundance in the water of 0.5 $\pm$ 0.6 items L^{-1} ($\sim$9.4% of all microplastics), (**Figure 3A**). Films showed the lowest abundance of only 0.01 $\pm$ 0.07 items L^{-1} ($\sim$0.3% of all microplastics), (**Figure 3A**). Films were found only at two sampling stations in the southern area (TF0012 and TF0360) close to the coast. The microplastic shape types were less diverse in the sediments than in the water column. Here, only fibers (0.6 $\pm$ 0.4 items g^{-1} d.w.) and fragments (0.1 $\pm$ 0.2 items g^{-1} d.w.) were found (**Supplementary Figure 7**).

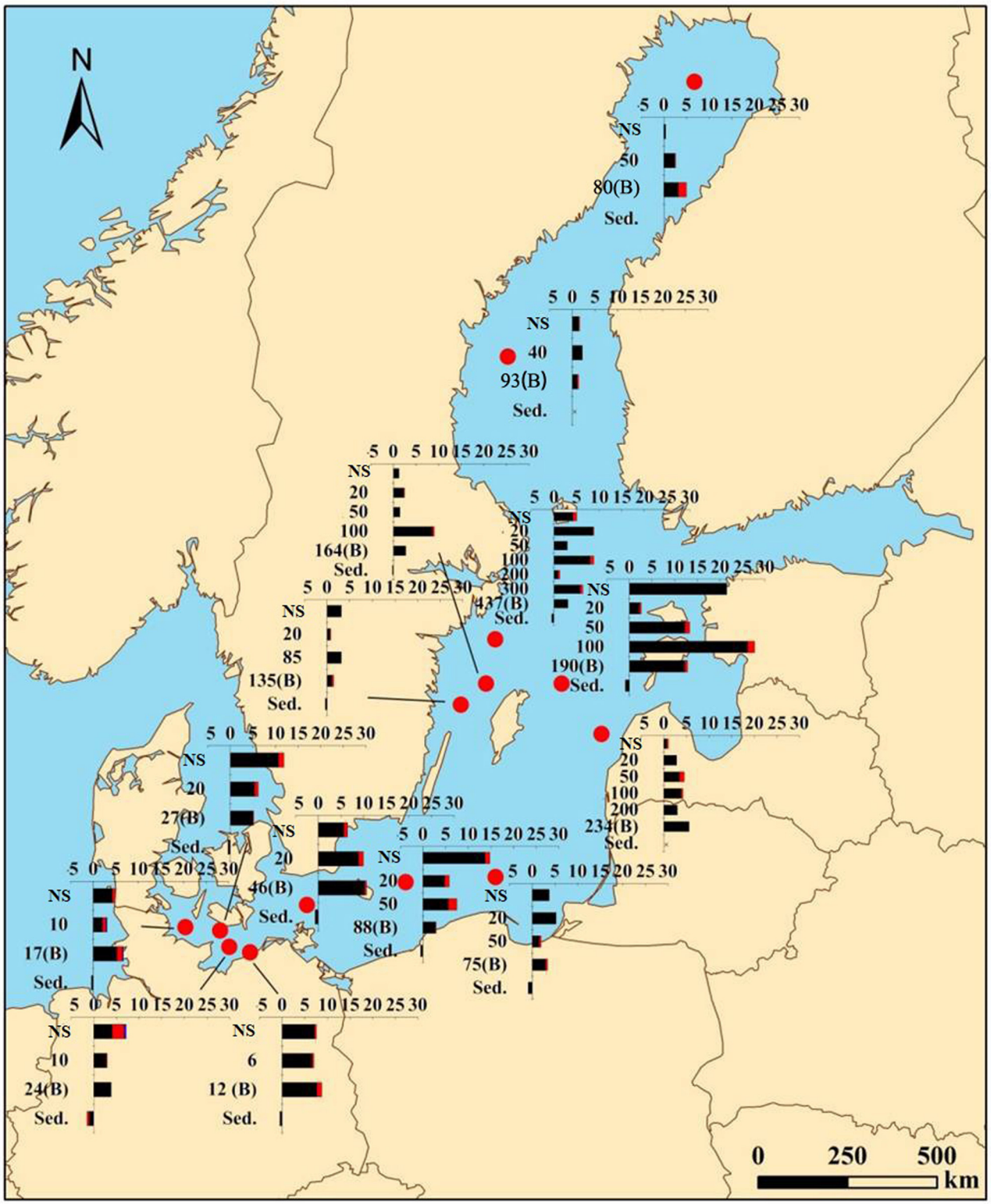

FIGURE 2 | Distribution of different microplastic types in the water and sediments in the Baltic Sea. The unit of the abundance of microplastics in the water column and sediments are items L^{-1} and items g^{-1} d.w.; "NS," near the surface; "B," near-bottom layer; "Sed.," sediment; the *Y*-axis represents sampling depth (m); the *X*-axis represents the abundance of microplastics.

TABLE 1 | Variation in the vertical distribution of microplastics (items L^{-1}) in the water column in different regions of the Baltic Sea.

Region	Station	Range	Average	SD*	CV*
Southern region	Rostock coast	TFO5	7.0–8.8	7.8	0.9	0.1
	Lübeck Bay	TF0012	3.0–7.1	4.7	2.2	0.5
	Kiel Bight	TF0360	3.0–6.7	4.9	1.8	0.4
	Fehmarn strait	TF0010	5.2–11.9	7.8	3.6	0.5
	Arkona basin	TF0113	6.5–10.7	9.0	2.3	0.2
	Bornholm basin	TF0213	2.9–14.8	7.8	5.1	0.7
Central region	Eastern Gotland basin	TF0256	2.0–5.3	3.6	1.4	0.4
		TF0271	1.1–5.6	3.6	1.6	0.4
	Northern Baltic proper	TF0286	2.6–27.7	15.6	9.5	0.6
	Western Gotland basin	TF0284	1.3–8.8	5.2	2.9	0.6
		TF0240	1.9–9.1	3.4	3.2	0.9
		TF0242	0.8–3.2	2.2	1.2	0.5
Northern region	Gulf of Bothnia	F3	0.4–5.0	2.7	2.3	0.9
		MS4	1.4–2.2	1.7	0.4	0.2
Total	–	**Total**	0.4–27.7	5.8	5.0	0.9

CV: coefficient of variation.

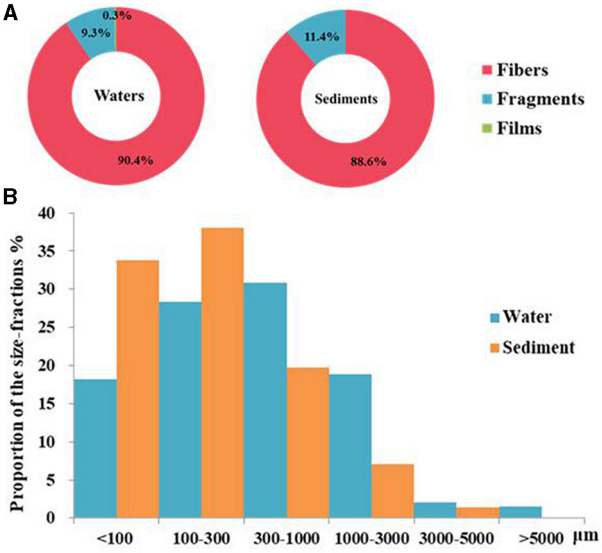

FIGURE 3 | The shape types (%) **(A)** and the size fractions (%) **(B)** of the microplastics in water and the sediments of the Baltic Sea.

Black and blue microplastic particles were dominant in both water and sediments (**Supplementary Figure 2**). The microplastic polymers consisted of rayon (81.0%), PET (4.9%), PP (4.3%), polyamide 6 (PA6), (3.7%), polybutene (PB), (2.5%), polytetrafluoroethylene (PTFE), (1.8%), and poly (N-methyl acrylamide), (1.8%) based on all identified polymer particles (**Supplementary Figures 3**, **4**). The histogram presents the size distribution of microplastics in waters and sediments (**Figure 3B** and **Supplementary Figure 8**). Microplastics with size range <1 mm dominated both in water (77.5%) and sediments (91.6%) sampled. However, the size distributions of microplastics showed one peak at 300–1,000 μm in water but 100–300 μm in sediments.

Microplastics at the Haloclines

The six sampling stations TF0360, TF0213, TF0286, TF0284, TF0240, and TF0242 (**Figure 1**) with strong haloclines (**Supplementary Table 1**) showed significant accumulation of microplastics in this distinct layer (**Figure 4**). By contrast, other sampling stations (e.g., TF0010 and TF0012) without evident haloclines showed microplastic accumulation at the near-surface layer. The microplastic abundance in the halocline at ∼16 m depth (a salinity difference of 3.2) of station TF0360 was 6.7 items L^{-1}, accounting for 45.9% of them in the entire water column. This was 1.4–2.2 times higher than at the other two water depths with microplastic abundances of 3.0 and 4.9 items L^{-1}, respectively (**Figure 4A**). At station TF0213, the abundance of microplastics at the halocline (7.5 items L^{-1}) was lower than at the near-surface layer (14.8 items L^{-1}) but significantly higher than at other layers (2.9–5.8 items L^{-1}), (**Figure 4B**). In the deeper basins at stations TF0286, TF0284, TF0240, and TF0242, the abundances of microplastics in the halocline at a depth of ∼100 m were all higher than at the other depths (**Figures 4C–F**). Among them, the highest microplastic abundance in the water columns of the Baltic Sea occurred in the halocline of station TF0286 with up to 27.7 items L^{-1}. In general, the microplastic abundance in the halocline at the above six stations ranged from 3.2 to 27.7 items L^{-1} (mean 10.5 ± 8.7 items L^{-1}), contributing 24.1–53.2% of the microplastics in the whole-water column and in total 33.8% at all sampling depths of the six stations.

In terms of types, fibers pre-dominated in the halocline, accounting for 90.3%, whereas fragments and films contributed only 9.4 and 0.3%, respectively, consistent with the overall distribution of microplastic types in the Baltic Sea. Heavy polymers (PET, ρ = 1.38 g cm^{-3}) with densities higher than seawater and light polymers (PP, ρ = 0.85–0.92 g cm^{-3}), together with the semisynthetic polymers (rayon), were detected in the halocline. The size of microplastics in the halocline showed a wide range of 30.0–4,972.0 μm, with a mean size of 694.0 ± 860.6 μm. The proportion of microplastics with size <300 μm accounted for nearly 50% in the halocline and almost 40% of microplastics at all water depths. Moreover, the percentage of microplastics with size <100 μm in the layers above the haloclines (including haloclines, 18.1%) was higher than those in the layers below the halocline (11.9%), but there were no discernible differences ($p > 0.05$), (**Supplementary Figure 5**).

Microplastics at the Near-Bottom Turbid Water Layer

The results here showed that microplastics tend to accumulate in the near-bottom layer in addition to the halocline in the Baltic Sea (**Figure 2**). The mean abundance of microplastics was 5.3 ± 3.5 items L^{-1} at the near-bottom layer, lower than at the near-surface layer (6.0 ± 6.5 items L^{-1}) and the halocline (10.5 ± 8.7 items L^{-1}), but higher than at the other water depths (4.4 ± 3.1 items L^{-1}), (**Supplementary Table 4**). Fibers accounted for

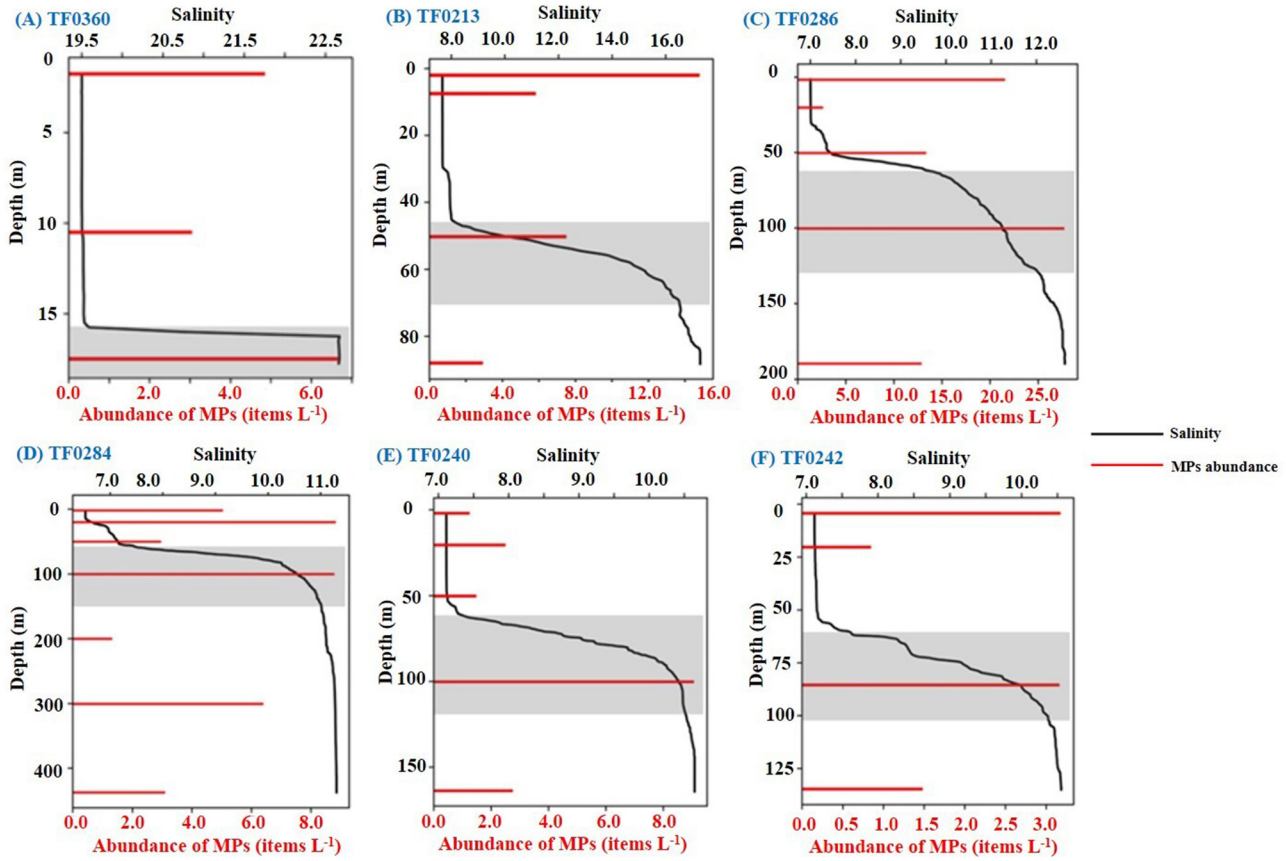

FIGURE 4 | Changes in microplastic abundance with salinity over depth in the water column at different stations **(A–F)**. Shadows in the figure indicate the locations of the halocline in the corresponding water column and their microplastics abundance. "MPs" refers to microplastics. The *Y*-axis represents sampling depth (m), the *X*-axis of the top (in black) represents the salinity, and the *X*-axis of the bottom (in red) represents microplastics abundance (items L^{-1}).

90.4% (4.8 ± 4.3 items L^{-1}), but fragments and films accounted for only 9.3% (0.5 ± 0.5 items L^{-1}) and 0.3% (0.01 ± 0.05 items L^{-1}), respectively, consistent with the distribution of the microplastic types at the haloclines. The most abundant polymer was semisynthetic rayon, accounting for 81.2%, whereas the polymers PET, PA6, and PB accounted for only 3.1–6.3%. The mean size of the microplastics at the near-bottom layer was 591.0 ± 840.0 μm with a high percentage (53.6%) of short particles (<300 μm).

DISCUSSIONS

High Levels of Microplastic Pollution in the Semi-enclosed Baltic Sea

Surprisingly, the abundance of microplastics in the Baltic Sea is one order of magnitude higher than that in the open sea such as the North Sea (23.0 items m^{-3}), (Roscher et al., 2021), (**Supplementary Table 2**). Similar microplastic abundances in the Baltic Sea were also reported by Bagaev et al. (2018) and Tamminga et al. (2018). Previous studies on the semi-enclosed seas, including the Bohai Sea and the Maowei Sea, showed high levels of microplastic pollution, with microplastic abundance up to 7,000 items m^{-3} (Dai et al., 2018; Zhu et al., 2019). They indicated that semi-enclosed seas are particularly susceptible to trapping microplastics, due to the emission of micropollutants from industrial and domestic uses, slow flushing, and restricted water exchange (Korpinen et al., 2012; Li et al., 2018; Schmidt et al., 2020). The microplastic abundance in the near-surface layers of the southern and central regions of the Baltic Sea was significantly higher than in the northern region in this study. This is because there are more intense anthropogenic activities including harbors, shipping, wastewater treatment plants, and the heavier load of pollutants in rivers caused by urban areas in the south than those in central and northern areas (Stankiewicz, 2010; Talvitie et al., 2015; Gewert et al., 2017; Rotander and Kärrman, 2019).

Fiber was the major contributor of types of microplastic pollution in the Baltic Sea. It shows clear domination of fibers at all depths of the water column and in the surface sediments, consistent with other surveys of microplastics in the Baltic Sea (e.g., Bagaev et al., 2017; 2018; Tamminga et al., 2018; Zobkov et al., 2019). Rayon fibers were the most prevalent type and distributed throughout the water columns and sediments in the Baltic Sea, which is consistent with the study on microplastics in

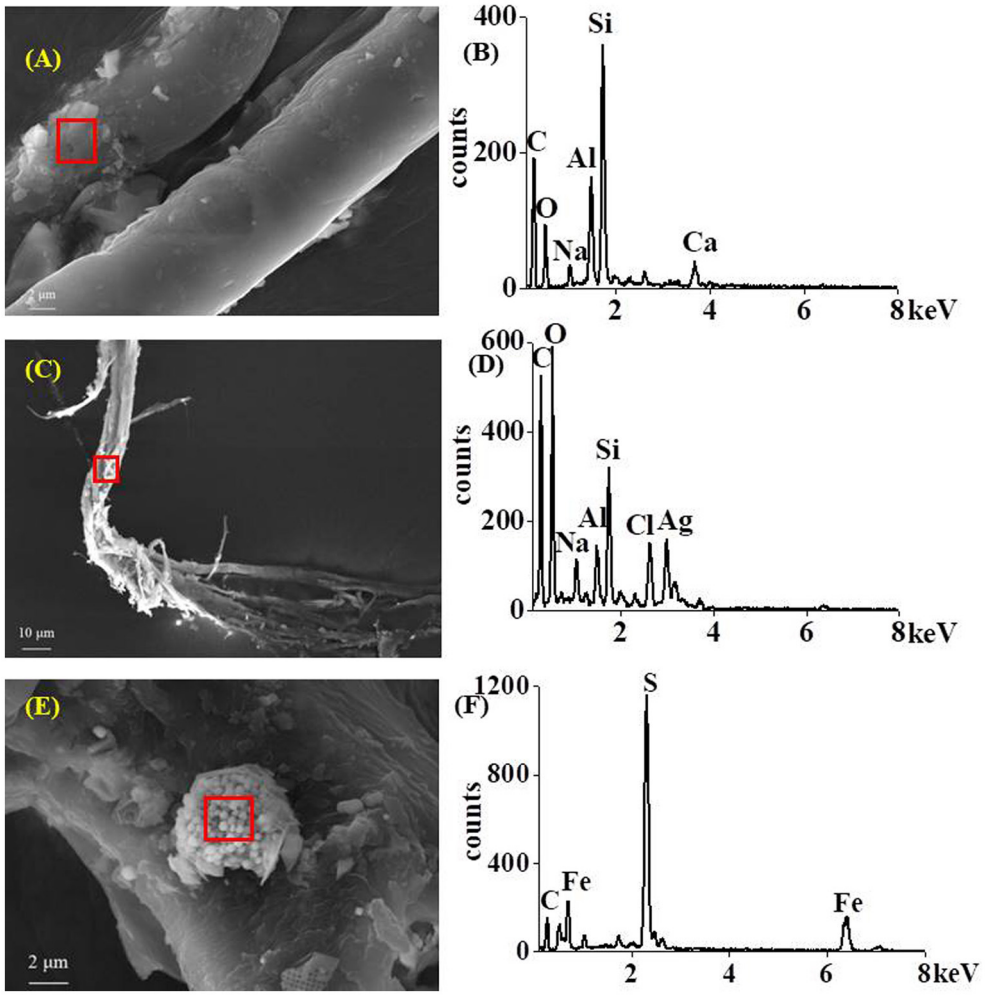

FIGURE 5 | Scanning electron microscope (SEM) images and energy spectra showing the substances on the surfaces of micro-fibers in the halocline [PP, **(A,B)**] and near-bottom layer [PB, **(C,D)**] and sediment [PP, **(E,F)**] in the Baltic Sea.

the Coral Reef Systems (Ding et al., 2019), Maowei Sea (Zhu et al., 2019), Arctic Sea ice (Obbard et al., 2014), and in Arctic polar waters (Lusher et al., 2015). Numerous studies have indicated the widespread occurrence of rayon fibers throughout marine environments (Lusher et al., 2013; Remy et al., 2015; Taylor et al., 2016; Sanchez-Vidal et al., 2018; Suaria et al., 2020). However, it is controversial to classify rayon as a microplastic worldwide (Peeken et al., 2018). Clothing made from synthetic and semisynthetic fibers, such as rayon, PET, and PA6 is common and therefore potential source of contamination in the Baltic Sea.

Haloclines Facilitate the Trapping of Microplastics

Interestingly, a distinct accumulation of microplastics at the specific sampling depth (the haloclines, most of them roughly at 50 or 100 m) at the sampling stations TF0360, TF0213, TF0286, TF0284, TF0240, and TF0242 was revealed. It indicated that the halocline is a key layer for the accumulation of microplastics in the Baltic Sea. The size distributions of microplastic particles at the halocline, the water layers above halocline, and the water layers below halocline were presented in **Supplementary Figure 5**. The proportion of microplastics with the size of $<100\,\mu m$ at halocline was 44.9%, which was higher than those at the water layers above (41.7%) or below (35.1%) halocline. This leads to the presumption that the halocline may act as a barrier to small microplastics ($<100\,\mu m$) and prevent them from sinking. Although previous studies have

TABLE 2 | Pearson correlation coefficients among different water parameters and microplastics in the near-bottom layer of the Baltic sea (significant correlation is given in bold).

	Temperature	Salinity	Dissolved oxygen	Chlorophyll a	Turbidity
Microplastics	−0.05	0.29	0.17	0.26	**0.70**

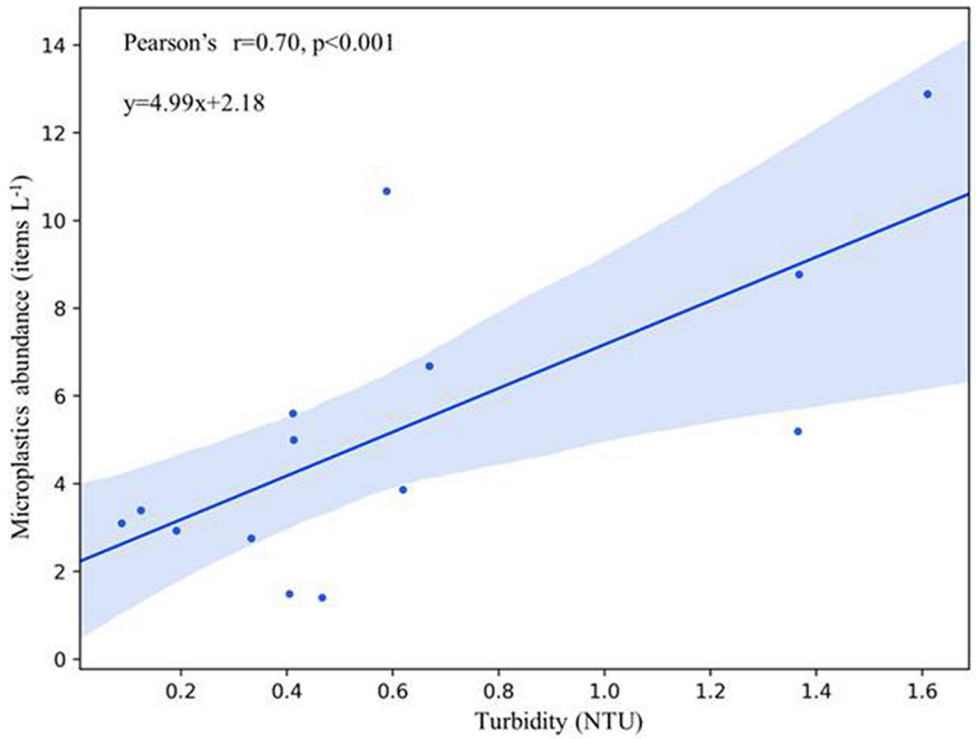

FIGURE 6 | Correlations between the microplastics abundance and turbidity at the near-bottom water layer. Linear least squares regression relationships were estimated.

given valuable evidence that microplastics can accumulate at the halocline, most of them have been based on the large microplastics (>175 μm) and overlooked the difference between the size of the microplastics in the haloclines and other water layers (Gorokhova, 2015; Bagaev et al., 2017; Uurasjärvi et al., 2021). In this study, the vertical distribution (including the halocline) of microplastics with a wider size range was analyzed and has confirmed that the halocline tends to accumulate small particles with size <100 μm. We suspect that this probably is related to the larger specific surface area of the small-sized microplastic particles by comparing with the larger ones. Because the small-sized particles with the larger specific surface area would lose buoyancy much more rapidly than larger ones, causing them easy to sink (Fazey and Ryan, 2016; Dai et al., 2018).

Furthermore, we selected the water column and sediment sampled at stations TF0286 and TF0284 as representative examples to observe the attachments on the microplastic surfaces at different depths, aiming for exploring the vertical migration of microplastics (**Figure 5**). The elements, including silicon and aluminum in addition to organisms, were detected on the surfaces of the micro-fibers (PP) found at the halocline (**Figures 5A,B**). However, fewer attachments were observed on the surface of particles at near-surface water layers by comparing with those at haloclines (**Supplementary Figure 9**). We assume that attachments may change the buoyancy of microplastics with low density and cause them to sink from upper layers and to access the halocline (Lattin et al., 2004; Katija et al., 2017; Uurasjärvi et al., 2021). However, they may not have enough negative buoyancy to escape from the halocline and continue sinking and ultimately accumulate in the halocline. It was assumed that microplastics first accumulate to halocline, when present, and sink slowly toward the bottom ultimately (Uurasjärvi et al., 2021). In this study, we detected metals, such as silver accumulating on the surface of the light fibers in the near-bottom layer (**Figures 5C,D**) and sulfur–iron mineral aggregates attached to the surface of fibers in sediments (**Figures 5E,F**), but they were not detected on the particle surfaces in the halocline. This indicates that microplastics with high-density elements (such as silver) attached might have enough negative buoyancy to overcome the halocline and sink continuously into the near-bottom water layer. However, this study did not consider the water hydrodynamic influence, which is also important to affect the microplastic transportation and accumulation (Zalesny et al., 2014; Zhang, 2017). Further studies are required to prove the hypothesis.

Turbidity Affects the Accumulation of Microplastics at the Near-Bottom Water Layer

The microplastic abundance and size distribution in sediments were analyzed for discussing microplastic accumulation at near-bottom layers. In sediments, the range of microplastic abundance was 0.2–1.5 items g^{-1} d.w. with the mean abundance of 0.6 ± 0.4

items g^{-1} d.w. Moreover, the higher proportion (53.6%) of short microplastics (<300 μm) at the near-bottom water is consistent with those in sediments (<300 μm, 71.8%), which indicated that the short microplastics at the near-bottom layers may come from bottom sediments in addition to upper water layers. We assumed that it is related to the water turbidity (e.g., particles resuspension from sediments). We further used the correlation analysis between the microplastic abundance and the salinity, dissolved oxygen, turbidity, and chlorophyll in the near-bottom water layers to explain the potential impact factor on the accumulation of microplastics (data refer to **Supplementary Table 1**). A strong correlation ($r = 0.70$) between the abundance of microplastics and the turbidity of the near-bottom water layer was found (**Table 2**, **Figure 6**). The near-bottom currents carrying turbid water usually occur when sediment particles are suspended above the seafloor by waves or incoming river plumes, and the turbid and relatively dense seawater cascades down due to the pull of gravity (Azpiroz-Zabala et al., 2017; Paull et al., 2018). The bottom current can transport sand, clay, organic carbon, and microplastics (Galy et al., 2007; Gwiazda et al., 2015). Therefore, bottom currents with high turbidity play a distinct role in the dispersal or accumulation of microplastics in the bottom water and sediments (Ballent et al., 2013; Pohl et al., 2020).

Moreover, we found heavy metals (silver and iron) attached on the surface of light microplastics in the near-bottom water layer and sediments (**Figures 5C–F**), which indicated that it is difficult to keep these microplastics retain in water even though there is high salinity in the near-bottom water. However, near-bottom currents can retain microplastics in resuspension, and stronger and sheared turbulent pulses on the surface of the seabed can prevent the microplastics from sinking (Lemckert et al., 2004; Bagaev et al., 2017). The process of resuspension can affect the turbidity of the near-bottom water by causing the particles, including microplastics, organic particles, and minerals, to reenter the water body. These suspended organic or inorganic particles can also hinder the microplastics from sinking. Therefore, the turbidity of the near-bottom water probably can indicate microplastic abundance at the near-bottom water in the Baltic Sea.

CONCLUSIONS

The vertical distribution of microplastics in the water column shows spatial heterogeneity in the Baltic Sea. Fibers and fragments were found throughout the water column, whereas films occurred only at the (near) surface water layer. The abundance of the microplastics at the near-surface, the halocline, and the near-bottom layer were considerably higher than those in other water layers. Strong halocline stratification and turbidity affected the vertical distribution of the microplastics and facilitated the accumulation of microplastics at those layers. The occurrence of microplastics in the surface sediments provides a potential source of particles to the near-bottom water. Studies on the sinking behavior of microplastics under multiple impact factors, especially material fouling in the marine environment, are needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

QZ collected samples, conducted the laboratory analysis of processing the samples, isolating and counting microplastics, the statistical analyses, and wrote the manuscript. JY and CF conducted spectral analysis on microplastics identification and generated the figures and maps. YL assisted with method development and contributed to editing. CT and JW conceptualized the study, contributed to editing, and acquired funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.761566/full#supplementary-material

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REVIEW
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Microplastics in Mollusks: Research Progress, Current Contamination Status, Analysis Approaches, and Future Perspectives

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Plastic fragments < 5 mm, known as microplastics (MPs), are ubiquitously present in the marine environment. Research on MPs pollution has gradually shifted from field investigations to laboratory studies. With the rapid growth of plastic consumption and the prevalence of aquaculture products, studies on marine MPs have focused on key marine species, such as mollusks. This review summarizes the recent knowledge including 77 important relevant literatures (from 2010 to 2021) on MPs contamination in mollusks with the objectives of (1) elucidating the current status of MPs pollution levels in mollusks, (2) highlighting the main methods and techniques for separation, extraction, and identification of MPs in soft tissues of bivalves and (3) presenting the current research progress and future directions. The review visually presents some of the important results in graphic form, which shows that the most common polymer plastics in bivalves are polypropylene, polystyrene, and polyethylene, and the shapes were mainly fiber and threadiness, mollusks are more likely to feed smaller MPs, most of the MPs in bivalves are less than 500 μm, and the abundance of MPs in seawater and the abundance of MPs in mollusks have a positive relationship, etc. This review will provide a comprehensive reference for studies of microplastics in marine organisms and the ecological pollution, and also has scientific guiding significance in the research method.

Keywords: microplastics, mollusks, bivalves, methods, contamination status

INTRODUCTION

Microplastics (MPs) refer to miniature plastic particles having a size of less than 5 mm, which form after large plastic waste enters the aquatic environment and breaks down due to the influence of photodegradation, physical degradation, and biodegradation (Browne et al., 2008; Moore, 2008; Cole et al., 2011). Recent studies have further defined plastic particles smaller than 0.1 μm as "nanoplastics" (Alimi et al., 2018). Plastic waste accounts for 80–85% of the total marine waste (Auta et al., 2017), and it is still increasing. Plastics are widely used in the commercial, industrial,

and pharmaceutical industries as they are lightweight, durable, inert, and resistant to corrosion. By 2016, global annual plastic production had surpassed 335 million tons, with disposable packagings such as plastic bags and soft drink bottles accounting for the majority of this total (Silva et al., 2018). It has been estimated that 4.8–12.7 million tons of MPs are released into the oceans every year, of which about 5 trillion MPs float on the surface (Eriksen et al., 2014). It has been reported that freshwater environmental input accounts for 70–80% of total MPs in the marine environment (Desforges et al., 2014). MPs inland and the atmosphere will eventually reach the ocean; therefore, oceans serve as the sink for all kinds of plastics including MPs (Xia et al., 2020). Increased MPs pollution has also resulted from the increased use of plastic products and insufficient management measures. In aquaculture systems, especially in industrially developed and densely populated areas, MPs pollution is more prominent. Household supplies, detergents, cosmetics, or drug carriers are the main sources of MPs (Fendall and Sewell, 2009; Patel et al., 2009). Synthetic fibers released from textiles are the main type of MPs in coastal sediments (Browne et al., 2011; Galvão et al., 2020). Therefore, MPs can enter aquaculture systems either directly or indirectly, negatively impacting the industry.

Mollusks are rich in nutrition, high economic value, and easy to breed. They are a highly regarded aquaculture resource, especially bivalves, which are typical filter feeders, constantly filtering out microbes and organic matter from the surrounding water (Xu et al., 2017). As a result, it is critical to monitor MPs in mollusks. MPs have been recorded in commercial mollusks, especially in *Mytilus edulis* (Graham et al., 2019). Based on the estimation of MPs found in two kinds of mussels sold on the European market, the annual dietary exposure of European mollusk consumers could reach 11,000 MPs (Van Cauwenberghe and Janssen, 2014). Bivalves are widely distributed, so some of them are considered as biological indicators for monitoring MP pollution in coastal areas, such as *M. edulis,* etc. (Beyer et al., 2017).

Great progress has been made in the study of MPs contamination in bivalves. However, there are still many challenges in quantifying, identifying, and characterizing MPs accurately. The increasing number of studies and the variability of methods used for particle separation, quantification, and identification have led to false comparability of data and conclusion. Since the study of MPs in bivalves is becoming more and more important worldwide, it is essential to analyze and compare the diversity and variability of analytical methods for the separation, quantification, and characterization of MPs in bivalves reported in the literature. However, little work has been done on this to date. Therefore, this article collates the recent knowledge including 77 relevant literatures (from 2010 to 2021) on MPs contamination in mollusks with the objectives of (1) elucidating the current status of MPs pollution levels in mollusks, (2) highlighting the main methods and techniques for separation, extraction, and identification of MPs in soft tissues of bivalves and (3) presenting the current research progress and future directions. This review will provide a comprehensive reference for studies of microplastics in marine organisms and the ecological pollution, and also has scientific guiding significance in the research method.

ABUNDANCE AND BIOACCUMULATION OF MICROPLASTICS IN MOLLUSKS

Compositional Profiles Microplastics in Mollusks

Microplastics ingested by mollusks differ in size, shape, color, and polymers. As the characteristics of MPs will affect their utilization rate by mollusks, we collected and analyzed information about the compositions of ingested MPs (**Supplementary Table 1** and **Figure 1**). According to their morphological characteristics, MPs are usually classified as fiber/line, fragments, films, pellets/balls, and foams. Fibers, fragments, films, and pellets, dominate in many freshwater and coastal areas, with fibers accounting for more than 50% (**Figure 1A**). It is not clear whether the fibers have a higher bioavailability or if they are the most abundant MPs in the sampling areas. MP's colors in mollusks are mainly blue, white, and black (**Figure 1B**). Plastics are synthetic polymers made from a variety of compounds with different properties. The types and content of MPs in bivalves are closely related to MPs in seawater. The main plastic components of MPs in seawater are polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polystyrene (PS), and polyethylene terephthalate (PET) (Andrady, 2011), which are also the most widely used plastics and account for about 90% of global plastic products (Andrady and Neal, 2009). The most common polymer plastics in bivalves are PP, PS, and PE (**Figure 1C**). The particle size of MPs directly affects their migration in water and whether they can be absorbed by organisms (Cole et al., 2020), which is closely related to biological safety. Mollusks are more likely to feed smaller MPs. Most of the MPs in bivalves are less than 500 μm (**Figure 1D**). The smaller the microplastics, the greater the damage to the mollusks (Wu et al., 2019; Bringer et al., 2020a). In addition, particle dosage (Song et al., 2020), charge (Junaid and Wang, 2021), exposure time (Song et al., 2020), type and additives also determine the effect (Banerjee and Shelver, 2021).

The abundance of MPs in seawater and the abundance of MPs in mollusks have a positive relationship. The main sources of MPs in marine aquaculture farms are the aging, water flow conditions, and disintegration of fishery equipment. Wind and upwelling also affect the distribution of plastic waste (David et al., 2009; Van Emmerik et al., 2019). MPs abundance in surface seawater is also positively correlated with river runoff, watershed area, population, and urbanization rate (Tang et al., 2018). MPs can largely disseminate in the environment, and the distribution of MPs varies among different spatiotemporal settings. When the density of MPs is greater than that of seawater, such as PS (1.04–1.1 g cm^{-3}) and PVC (1.16–1.58 g cm^{-3}), they will sink and eventually accumulate in marine sediments. When the density of MPs is less than that of seawater, such as low-density PE (0.89–0.93 g cm^{-3}), high-density PE (0.94–0.98 g cm^{-3}), PP (0.83–0.92 g cm^{-3}), plastic particles float on the sea surface,

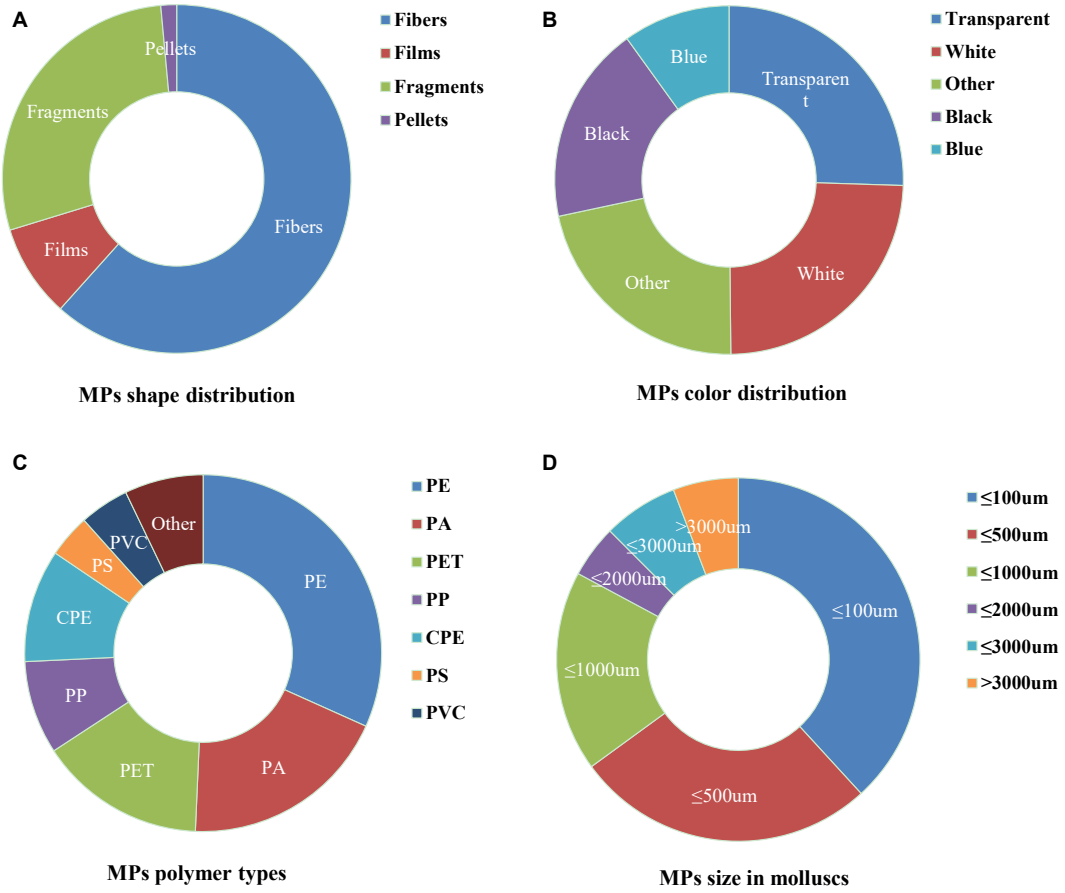

FIGURE 1 | Composition profiles (shape, color, polymer type, and size) of microplastics (MPs) in mollusks. **(A)** Description of shape is contained in the first panel; **(B)** Description of color is contained in the second panel; **(C)** Description of polymer type is contained in the Third panel; **(D)** Description of size is contained in the fourth panel.

but they are affected by migrating to the coastline under the influence of waves and tides, or sink into marine sediments under the influence of marine organisms (Suaria and Aliani, 2014; Junaid and Wang, 2021). Interestingly, MPs' intake amount and type differ between field and laboratory observations (Qu et al., 2018), while the units used in the report of MPs' abundance are inconsistent. Such discrepancies in results create difficulties to summarize the geographical distribution patterns and further comparison of MPs' pollution levels.

Bioaccumulation of Microplastics at the Tissue Level

Feeding is the main pathway by which MPs enter mollusks. When mollusks are exposed to MPs in the water, they remove most of the MPs through a process known as purification/filtration. The remaining MPs are mainly accumulated in the digestive glands and gills of mussels (Green et al., 2019). Then the MPs will enter mollusks through the surface microvilli or the ciliary movement of the gill and endocytosis (Pedersen et al., 2020). Some of the small plastic particles are swallowed by the cells in the entrails, move through the epithelial cells, and are transferred to the hemolymph (Scanes et al., 2019). Most MPs are excreted *in vivo* as pseudo feces. Some MPs entering the gills may also be identified by mollusks and directly discharged from the body. However, it has been reported that MPs can permeate the foot, mantle, and shells of mollusks through adherence (Kolandhasamy et al., 2018; Zhu et al., 2020). Different pollutants gather at different locations. In Pacific oysters (*Crassostrea gigas*), it was found that cytokinin tended to accumulate in gill, mantle, and muscle tissue, while polyester tended to accumulate in the entrails (Zhu et al., 2020). Therefore, the digestive tract of mussels cannot be regarded as the only sink for MPs.

RESEARCH PROGRESS IN MICROPLASTICS MEDIATED CONTAMINATION IN MOLLUSKS

Under certain environmental conditions, the biological uptake of MPs may be selective. It is generally believed that, compared with predatory species, mollusks absorb MPs more easily due to their filter-feeding behavior (Wesch et al., 2016) and efficient

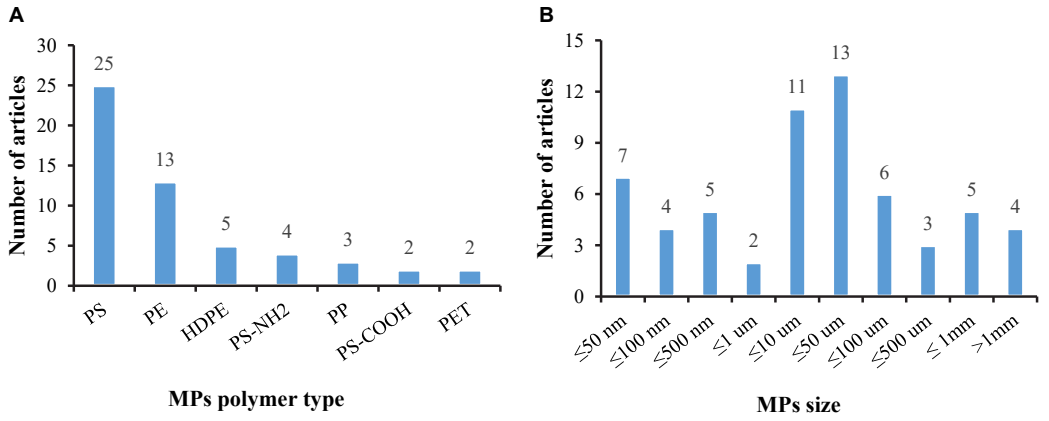

FIGURE 2 | The number of articles that have tested MPs in bivalves according to the polymer type and size: **(A)** Description of MPs polymer type is contained in the first panel; **(B)** Description of MPs size is contained in the second panel.

water purification capacity (Xu et al., 2017). Available studies have mainly focused on MPs' polymer type, size, exposure time, attached pollutants, and test-related indicators to reflect the impact of MPs on mollusks. Due to the small size of MPs, they are ingested by mollusks along with their food (e.g., microalgae) in the environment (Fernández and Albentosa, 2019a). Our data analysis showed that research has mainly focused on small-sized plastic particles with large yields (**Figures 2A,B**).

Microplastics can be transferred to the hemolymph, muscle, and other tissues or organs through phagocytosis (Scanes et al., 2019). Many of them are identified by bivalves as foreign elements then are removed (Birnstiel et al., 2019; Graham et al., 2019). It is suggested that MPs that reach the digestive tract are packaged into fecal particles and eliminated, but some of them will remain in the digestive tract (Fernández and Albentosa, 2019b), which are likely to affect the gut microbiota and damage internal organs (for example, hepatopancreas). Disorders of the gut microbiota usually lead to colitis and other abnormal behaviors (Glenny et al., 2017), and it has been confirmed that species exposed to microplastics for a long time suffer from gut biological disorders and inflammation (Jin et al., 2018; Fackelmann and Sommer, 2019), and the intestine may pass through selective removal and/or enrichment of certain bacterial taxa affects the related microbial community, thereby forming an obvious intestinal biofilm community on the particles (Kesy et al., 2016). Mollusks lack enzymes and have limited enzymatic pathways to decompose plastic (Wright et al., 2013), which will lead to long-term retention of MPs (Corami et al., 2020), affecting their reproductive, immune, and neurological systems (Prata et al., 2020). The absorption of MPs by clams varies with polymer type, concentration, color, and size (Li et al., 2019a), with particle size being a key factor (Cole et al., 2020). At the same time, it can be seen from **Figure 3** that the main focus is on short-term acute exposure experiments, while microplastics can exist for hundreds of years. Studies have found that the absorption (intake or adhesion) of MPs by mussels was positively correlated with the exposure time. It is implied that the amounts of MPs in mussels increase with the growth of mussels (Berglund et al., 2019).

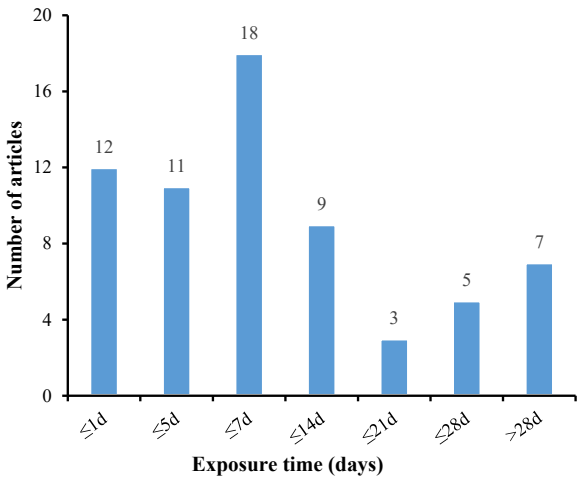

FIGURE 3 | The number of articles employing various exposure times to treat mollusks with microplastic.

Therefore, the focus of subsequent research will be the long-term harm of microplastics to organisms.

To understand the impact of MPs on mollusks under environmentally relevant conditions, some studies added anthropogenic pollutants and bacteria to their exposure solutions (**Figure 4**). Microplastics serve as carriers for multiple environmental pollutants in the aquatic environment. For instance, environmental pollutants such as metals and antibiotics adsorb and accumulate on the surface of MPs and may aggravate the hazards of those pollutants as well as those of MPs. Studies have reported the multifaceted mixture effects of MPs and other chemicals including biological oxidative stress, abnormal energy supply, and cell death in mollusks (Antunes et al., 2013; Canesi et al., 2015). Moreover, MPs have a large surface area that can carry microorganisms (Frère et al., 2018; Bowley et al., 2021), including bacterial pathogens (such as *Vibrio parahaemolyticus*, *V. vulnificus* etc.), which also pose a threat to human health. It has

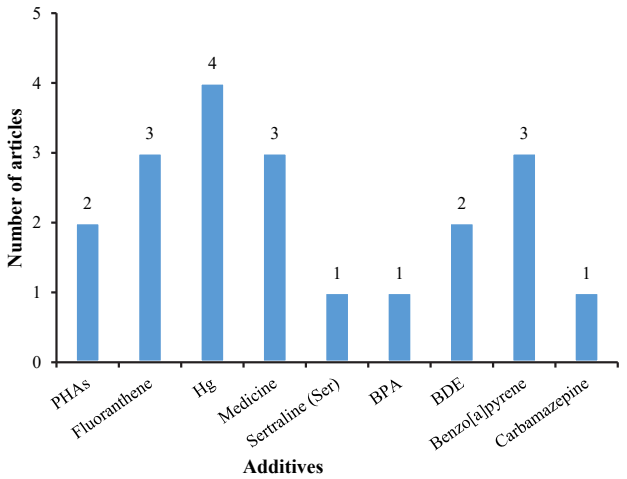

FIGURE 4 | The number of articles on the additives in mollusks exposed to microplastic.

been confirmed that when the mussels (*M. edulis*) contaminated with PS-MPs were fed to crabs (*Carcinus maenas*), then the PS-MPs were found in the stomach, hepatopancreas, ovaries, gills, and hemolymph of crabs, with the highest content in the hemolymph (Farrell and Nelson, 2013). This indicated that MPs could be accumulated in biological tissues and be transferred along with the food web (Fernández and Albentosa, 2019b).

Plastic additives are also environmental pollutants, which are chemicals added to improve the processing, physical, and chemical properties of plastic substrates. They are easily leached from plastic products and can be detected in both the aquatic environment and organisms (Huppertsberg and Knepper, 2018; Llorca et al., 2021). Plastic additives such as the flame retardant hexabromocyclododecane, polycyclic aromatic hydrocarbons and polybrominated diphenyl ethers were detected in plastic fragments floating in the ocean and expanded polystyrene buoys in the breeding area (Rani et al., 2017; Chen et al., 2018). The leachate in the disposable polyethylene plastic bag can significantly affect the growth of the clam (*Meretrix meretrix*) (Ke et al., 2019). Plastic additives also exhibit negative effects on mollusks, including oxidative stress, cytotoxicity, neurotoxicity, reproductive toxicity, growth and development toxicity, and disrupting their endocrine system (Zimmermann et al., 2019; Wang et al., 2020). Besides, MPs are small and light-weight particles, which make them easy to be transported to remote places through water flow or water currents.

Figures 5, **6** show that most of the previous studies, through analyzing changes in antioxidant defense system entities, such as malondialdehyde (MDA), superoxide dismutase (SOD), and reactive oxygen species (ROS), etc., found that MPs exposure can affect the immune system in mollusks. Several studies also used histopathological analyses to observe the tissue damage mediated by MPs in mollusks. Further, malformations of the nervous system, changes in growth and development (González-Fernández et al., 2018), physiological behavior alteration, and adverse effects on the reproduction and heredity of mollusks were also reported as toxic effects, amongst others (Bringer et al., 2020b).

METHODS FOR ISOLATION AND CHARACTERIZATION OF MICROPLASTICS IN MOLLUSKS

Dissolution

The initial stage in MPs separation is dissolution, which is the process of decreasing the organic content of mollusks and collecting MPs. The amount of time it will take the researcher to extract the MPs from the samples, as well as the potential dangers connected with the reagents, are critical concerns. The impact of the dissolution process on the color and shape of MPs is critical, as these characteristics provide information about the MPs' origins and the presence of additives. Chemical and enzymatic dissolution is the most widely utilized techniques nowadays.

Chemical dissolution mainly uses acid, alkali, and H_2O_2 to dissolve tissues. The acid decomposes organic matter such as proteins, carbohydrates, and oil, and also dissolves fragments such as bones and shells. Although strong acids have good dissolution effects, they will also destroy the structure of MPs. Polyamide and polyurethane are completely degraded during the HNO_3 dissolution method, and PET structure is destroyed and fused (Claessens et al., 2013). Alkalis can hydrolyze chemical bonds and denature proteins. One of the most commonly used strong alkali reagents is 10% KOH. It is typically incubated at 60°C and 30 rpm in 10% KOH at 1:5 (M: V) (Zhang et al., 2020a). This method has less reagent dosage and a short reaction time can quickly and effectively dissolve a large number of samples and has little effect on most polymer particles (Ding et al., 2018). It cannot dissolve cellulose, chitin, and silica, and can cause degradation of polymers such as polycarbonate, PA, and PET (Hurley et al., 2018). Therefore, 30% H_2O_2 is often used for the dissolution of organisms with chitin exoskeletons and organisms with high oil content in the organs (such as gonads, digestive tract, etc.). The dissolution temperature is generally 65°C, 200 mL 30% H_2O_2 is used to dissolve 5 g tissue (Li et al., 2019b). Although H_2O_2 can effectively dissolve biological tissues, a large volume of bubbles is produced during the dissolution process, which can easily cause the loss of MPs. It's worth noting that the oxidation of H_2O_2 will cause a variety of polymers to fade, and will slightly degrade PP and PE.

A single chemical agent solution could not completely dissolve the tissue, often leaving undissolved residues at the bottom of the beaker. The mixture of $H_2O_2$and acid is more effective, but 10% KOH is the most effective for the dissolution of biological tissues without affecting MPs, with a recovery rate of over 97% (Liu et al., 2020). The same conclusion was also reached by comparing H_2O_2, proteinase K, trypsin, and KOH to dissolve bivalve tissues, using the following indicators: filterability, dissolution efficiency, and recyclability of MPs. The MPs in the digestive system of bivalves can also be extracted with KOH (Ding et al., 2018). KOH can be used with spectroscopic analysis to detect MPs only several microns in size of bivalve tissues (Thiele et al., 2019).

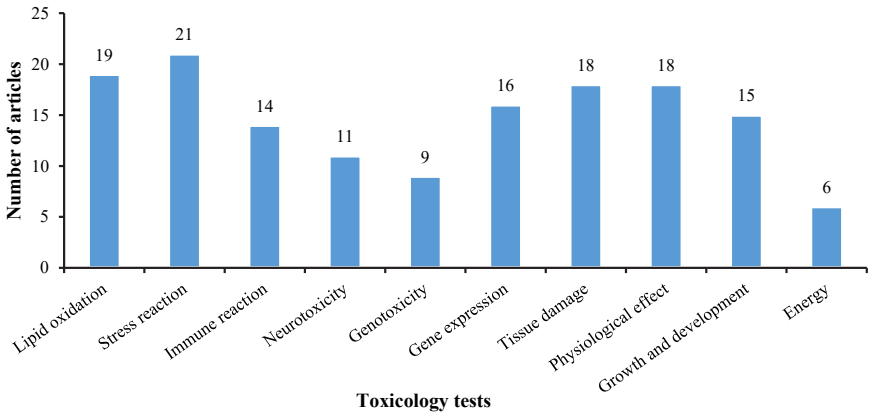

FIGURE 5 | The number of articles on the toxicological responses in mollusks exposed to microplastic.

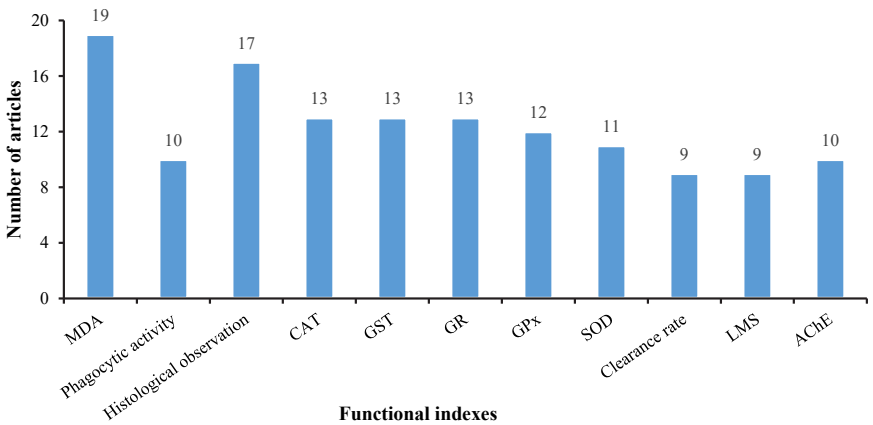

FIGURE 6 | The number of articles on functional responses in mollusks exposed to microplastics.

Enzymatic dissolution of biological tissues works by hydrolyzing proteins to obtain MPs in biological tissues. The enzymes used in this process are relatively mild, and the most commonly used enzymes are pancreatin (Von Friesen et al., 2019), lipase, amylase, and protease (Catarino et al., 2017). The cost of enzyme treatment is relatively high and the dissolution efficiency is relatively low, and it is thus only suitable for processing a small number of samples. In sum, for large quantities of samples, 10% KOH and 30% H_2O_2 are recommended as the most feasible dissolution methods for studying MPs in mollusks.

Separation and Extraction

When organic impurities are completely dissolved in mollusks samples, MPs are usually collected by density separation. The density separation approach uses density difference between MPs particles and environmental impurities for separation. For example, the density of MPs such as PE and PP produced by general industry is less than the density of water (1 g cm^{-3}), they will float on the surface of the water, and impurities will settle to the bottom of the water. Studies have used alcohol (0.8 g cm^{-3}), ultrapure water (1.0 g cm^{-3}), NaCl (1.12 g cm^{-3}), NaI (1.8 g cm^{-3}), and $ZnCl_2$ (1.8 g cm^{-3}) as flotation solutions to measure MPs with a density range of 0.8–1.8 g cm^{-3}. Alcohol and ultrapure water have a low density, it is impossible to collect plastic. NaI and $ZnCl_2$ are harmful to the environment. In addition, the $ZnCl_2$ solution has high surface

TABLE 1 | Density and advantages and disadvantages of flotation liquid.

Flotation fluid	Density g cm^{-3}	Advantage	Disadvantage
Alcohol	0.8	Non-toxic	Unable to float MPs
Ultra-pure water	1.0	Non-toxic	Unable to recover most MPs
NaI	1.8	Efficient	Toxic
$ZnCl_2$	1.8	Efficient	High surface tension, Toxic
NaCl	1.12	Non-toxic	Unable to recycle high-density plastic
NaH_2PO_4	1.4	Non-toxic, Efficient	Unable to recycle high-density MPs

The concentrations range of microplastics (MPs) is 0.8–1.8 g cm^{-3}.

TABLE 2 | Comparison analysis on the major methods for identifying MPs.

Identification methods	Principle	Advantage	Disadvantage	References
Microscope	Principles of optics	Simplicity of operation, Intuitive	low resolution, Low accuracy, Uncertain chemical composition of MPs	Gniadek and Dąbrowska, 2019
SEM	The electrons in the focused electron beam interact with atoms in the sample	High-resolution, high-resolution image, three-dimensional image, and non-destructive	Disturbed by water, complex pre-processing, required (0.5–1.5 kV), and low currents	Gniadek and Dąbrowska, 2019
Raman spectra	When monochromatic light irradiates the sample, the molecules (or atoms) cause Stokes to scatter the incident light, and the polarizability of the molecule changes	Rich information, simple sample preparation, high-resolution (1 μm), and non-destructive	Affected by fluorescence, The signal is susceptible to changes in a measurement parameter	Xu et al., 2019
FTIR	When infrared light irradiates a molecule, its chemical bond or functional group undergoes vibrational absorption, resulting in a change in the dipole moment	Rich information, high-resolution (10 μm), fast scanning speed, high sensitivity, and non-destructive	Disturbed by water, Fourier transform infrared spectra obtained in different modes are different, Effect of Chemical Degradation on Vibrational Spectral Band of Plastics	Xu et al., 2019
DSC	The changing heat capacities during the solid-liquid phase transition of a polymer	Simplicity of operation	required to identify polymer types, large particles can cause interferences, affected by production parameters, Damaged samples	Huppertsberg and Knepper, 2018
TGA	Measure the relationship between sample quality and temperature change under program temperature control	High accuracy, simplicity of operation, and measuring quickly	Affected by experimental conditions, damaged samples	Xu et al., 2019
GC-MS	GC separates mixed compounds; MS identifies molecular mass	Accurate identification of different polymer types, information on potentially toxic organic plastic additives	Cannot provide information on the quantity, type, and shape of plastics, Time consuming, rather for large debris (manually handled by tweezers), Damaged samples	Fries et al., 2013

SEM, scanning electron microscope; FTIR, Fourier transform infrared spectroscopy; DSC, differential scanning calorimetry; TGA, thermogravimetric analyzer; GC-MS, pyrolysis gas chromatography-mass spectrometry.

tension (Li et al., 2018). When it is hot without using flotation solution, as the flotation fluid cannot suspend all the MPs, but the undissolved grease in the digestion fluid can easily condense and block the filter holes, some researchers directly filter the digestion solution. Thus, NaCl has become the main flotation agent used in the laboratory because of its non-toxicity and effectiveness. However, NaCl is limited by density and cannot recycle higher-density plastics. The research found that NaH_2PO_4 solution can overcome this shortcoming. As the temperature increases, its solution density also increases. The density for saturated NaH_2PO_4 solution measured at 20, 30, and 40°C is 1.4, 1.46, and 1.51 g cm^{-3}, respectively (Zhang et al., 2020b). NaH_2PO_4 exhibits high extraction efficiency and non-toxic nature, indicating the potential application of this solution for extraction is broad.

Plastics are distributed uniquely. In surface water, the fraction of PP and PE is much larger than in other polymer compositions. The majority of polymers in soil are PA and PP, whereas PP, PE, and PS dominate in sediments (Xu et al., 2020). As a result, the experimental objectives and test purposes could be anticipated based on the density, benefits, and drawbacks of the flotation liquid, and a more suitable flotation solvent could be selected, as shown in **Table 1**.

Filter membranes are used to filter the supernatant containing MPs generated by density separation. For collection and post-identification, the filter membrane is crucial. The pore size and material requirements of the filter membrane change according to the equipment utilized for MPs' identification. The pore size and substance of the filter membrane should be chosen based on the identification instrument's resolution. A micro-infrared spectrometer, for example, has a spatial resolution of tens of microns, whereas a micro-Raman spectrometer has a spatial resolution of 1 μm. Glass fiber membranes, cellulose acetate membranes, nitrocellulose membranes, polycarbonate membranes, nylon membranes, metal membranes, and other materials are used to make filter membranes. Glass fiber membranes and polycarbonate membrane ester filter membranes are not suggested. The surface of the glass fiber filter membrane is rough, and fibers may fall out throughout the experiment. Polycarbonate membranes, on the other hand, are not hydrophilic, which makes MP retention difficult. Furthermore, polycarbonate has a strong infrared signal that will interfere with MPs' transmissions. The microplastic signal is present on the nylon membrane itself, interfering with the experiment. The best option is to use metal film. It has a flat surface, no infrared signal, and no micro-plastic signal.

Identification

After separation, MPs are identified and classified. This mainly involves physical form characterization and chemical component identification, such as size, shape, color, and polymer type. Commonly used techniques include microscopy, spectral analysis, and thermal analysis, etc. (**Table 2**).

Physical Form Characterization

For larger MPs, the characteristics of the physical form can be distinguished with the naked eye within the allowable range of experimental error. For MPs with no specific shape, small size, and light color, a microscope or scanning electron microscope (SEM) can be used to magnify the MPs. Although optical microscopes are simple to operate and can perform routine analysis on samples, they cannot accurately distinguish MPs from other particles (such as natural minerals). Therefore, researchers use the dyeing method to identify MPs. Nile-Red (NR) is a commonly used staining agent, which can specifically bind to neutral lipids, has strong fluorescence in a hydrophobic environment, and is an effective dye for highly hydrophobic MPs. A fluorescence microscope can be used to distinguish particles. This not only reduces the possibility of missing MPs but also reduces the time required to confirm each plastic particle with a spectrometer. Polymers having a particle size of less than 100 μm are identified using the NR staining technique. It works well with mixed samples comprising PE, PP, PS, and a lot of inorganic particles. However, because of its low specificity, it will cause other natural compounds to dye colors, such as natural fats in the sample. As a result, this technique is ineffective for identifying MPs in bivalves. The dye was a combination of calcofluor white and Evans's blue 3, and the samples were examined using a laser scanning confocal microscope. The biomass of arthropods is purple, while various polymeric polymers are red, green, and yellow using this approach (Maxwell et al., 2020). Fluorescent counterstaining has been used as a new technology for detecting terrestrial invertebrate biomass and microbes in feces. However, further research is required to determine whether it is suitable for detecting MPs in mollusks tissues. Therefore, it is necessary to select a higher resolution precision instrument for identification, such as SEM.

Chemical Composition Identification

The spectral analysis technologies commonly used for MPs in mollusks are Raman spectra (Frère et al., 2016) and Fourier transform infrared spectroscopy (FTIR) (Chen et al., 2020). Raman spectra employ laser scattering to identify the polarization of chemical bonds and are mostly used to evaluate materials or chemical compounds that contain aromatic bonds. Although color, fluorescence, additives, and contaminants adsorbed on or imbedded in MPs will impact Raman spectral analysis, the coupling of Raman spectra and microscope technology offers the benefits of employing extremely small sample sizes and spatial resolution (Fu et al., 2020). FTIR is mainly used to analyze polymers with polar functional groups (such as carbonyl). FTIR requires dried samples that must be made into thin films or ground into a powder before analysis. In addition, the premise of obtaining infrared absorption is the change of the dipole moment of the chemical bond. Therefore, to quickly and reliably analyze a large number of optical particles, a combination of Fourier transformed infrared FTIR and Raman microscopy, with a spectral database can determine the particle size, particle size distribution, and polymer types including particle color (Xu et al., 2019; Brandt et al., 2020). This method has been applied to the detection and analysis of mussel MPs (Vinay Kumar et al., 2021). Further, the chemical components of MPs are also identified by spectral analysis through SEM combined with energy spectrometer technology, and thermal analysis techniques.

Thermo-analytical methods are often considered complementary to spectroscopic methods, such as differential scanning calorimetry, thermogravimetric analysis (TGA) (Zainuddin and Syuhada., 2020), and Pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) (Picó and Damià, 2020), etc. They can identify specific comonomers, additives, and degradation products from plastic fragments (La Nasa et al., 2020). The advantage of Py-GC-MS over conventional spectroscopic methods is that it is possible to analyze polymer types and organic plastic additives in a single analysis. Researchers compared Py-GC-MS, TED-GC-MS, and TGA-FTIR, and found these methods can correctly identify all polymers and report reasonable quantitative results within the study concentration range (Becker et al., 2020). Mass spectrometry analysis requires that polymer samples are purified and concentrated, vaporized, and then entered into mass spectrometry analysis, as vaporization steps, such as laser ablation, may change or destroy the properties of plastic samples.

CHALLENGES AND PROSPECTS

Since MPs are a relatively new form of environmental contaminant, several scientific issues must be addressed immediately. To begin, consistent and standard detection methods for MPs in various environmental media must be established, as well as qualitative and quantitative detection methods for MPs and their combined contaminants. Various methods for analyzing and identifying MPs are employed, each with its own set of reporting units, resulting in poor comparability of findings. As a result, it's critical to enhance the accuracy, reliability, and comparability of detection data by optimizing qualitative and quantitative analysis methodologies for MPs coupled with contaminants, including standardizing technical procedures and parameters.

Second, image recognition technology has developed since the dawn of the artificial intelligence era. The development of machine vision-based automatic identification, categorization, counting, and measuring procedures for MPs improves data accuracy, lowers labor costs, and saves time. It is anticipated that it will aid in the transformation and advancement of MPs' analysis technology, the types of MPs examined are restricted, and the laboratory circumstances are not representative of actual situations. MPs are often made of fibers and spherical PE, PS, PVC, and other materials, although MPs made of various materials and forms may cause harm to organisms to varying degrees. As a result, future studies must take into account the current environmental circumstances in the area. The absorption and use of MPs by mollusks are influenced by the substance, size, shape, age, and quantity of microplastics. There are limited studies on the mechanisms through which MPs transport contaminants or microbes, as well as long-term dynamic changes and hazards in the maritime environment.

MPs are presently introduced into the environment at a far quicker rate than they are eliminated. MPs may be found in almost every aquatic habitat on the planet. As a result, several robust environmental protection measures, as well as increased research efforts, are required to curb the rise of microplastics. To begin, additional research into the toxicological impacts of microplastics on filter-feeding mollusks as well as on biological processes should be conducted.

Third, to decrease plastic waste at the source, individuals should reduce their use of plastic items and promote the manufacture and use of degradable plastic products and plastic alternatives. Enhance the usage rate of plastics by recycling and reusing items, and boosting information and scientific research to find microorganisms that can break down microplastics and minimize microplastic pollution.

CONCLUSION

The most recent research on MPs-mediated contamination in mollusks has been summarized and analyzed, focusing on three main topics: the main methods of MPs separation, extraction, and identification, the current state of shellfish microplastic pollution, and future research hotspots. It suggested that the most common polymer plastics in bivalves are PP, PS, and PE, and the shapes were mainly fiber and threadiness. Mollusks are more likely to feed smaller MPs. Most of the MPs in bivalves are less than 500 μm. The abundance of MPs in seawater and the abundance of MPs in mollusks have a positive relationship. Additionally, MPs exposure can affect the immune system in mollusks, and the tissue damage, malformations of the nervous system, changes in growth and development, physiological behavior alteration, and adverse effects on the reproduction and heredity in mollusks all mediated by MPs had also been confirmed in the current study. In terms of methodology, 10% KOH and 30% H_2O_2 are recommended as the most feasible dissolution methods for studying MPs in mollusks, and the best option for separation is to use metal film. Fluorescent counterstaining has been used as a new technology for detecting terrestrial invertebrate biomass and microbes in feces. It is necessary to select a higher resolution precision instrument for identification, such as SEM. Besides, the future research emphases are also summarized in the present review, including enhancing the accuracy, reliability, and comparability of detection data by optimizing qualitative and quantitative analysis methodologies for MPs, additional research into the toxicological impacts of microplastics on filter-feeding mollusks as well as on biological processes should be conducted for environmental protection, and decreasing plastic waste at the source.

AUTHOR CONTRIBUTIONS

RW and HM: conceptualization. BL: resources. XL and HZ: data curation. RW and HM: writing–original draft preparation. RW and MJ: writing–review and editing. JW: visualization. RW and JW: supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.759919/full#supplementary-material

Supplementary Table 1 | Data about plastic litter found in bivalves.

Supplementary Table 2 | Summary of data found on the literature for MPs and NP uptake, internalization, depuration.

Supplementary Table 3 | Summary of data on uptake, internalization, purification and effects of MPs and other pollutants on bivalves.

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Exploring the Occurrence Characteristics of Microplastics in Typical Maize Farmland Soils With Long-Term Plastic Film Mulching in Northern China

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Microplastics pollution has been threatening the global environmental security, in which agricultural activities are considered as a main source of microplastics occurrence in soils. However, little is known about the occurrence characteristics of microplastics in agricultural soils with long-term plastic film mulching. Therefore, the abundance, distribution, and composition of microplastics were investigated by analyzing 225 soil samples collected from typical maize (*Zea mays* L.) planting zones with and without long-term (>20 years) plastic film mulching in northern China. Microplastics abundance in mulched soils (754 ± 477 items kg^{-1}) was significantly higher than that in non-mulched soils (376 ± 149 items kg^{-1}), which indicated that plastic film mulching contributed half of microplastics in soils. Moreover, microplastics abundance was significantly positively related to the length of time with film mulching applied. The percentage of microplastics <0.5 mm in mulched soils (50.9%) was significantly lower than that in non-mulched soils (62.2%). Microplastics abundance and size in mulched and non-mulched soils decreased with increased soil depth. Most microplastics were fragments of polypropylene, films of polyethylene, and fibers of polyester. The proportion of films in mulched soils was significantly higher than in non-mulched soils, whereas that of fibers was significantly higher in non-mulched soils. This study confirmed that long-term plastic film mulching increases microplastics pollution in agricultural soils, warranting further evaluation of the associated ecological risks of microplastics in soil ecosystems.

Keywords: microplastics, maize, plastic film mulching, farmland soils, distribution characteristics

INTRODUCTION

Global plastic production has increased from 230 to 359 million tons between 2005 and 2018, and China accounted for 30% of that production (Statista, 2020). However, with recycling rates that are generally low, plastic waste is considered a global environmental pollution issue because of its low degradability (Barboza et al., 2018). Although plastics can remain in the environment permanently,

they decompose into increasingly smaller pieces of plastic debris under the actions of biological, physical, and chemical processes, such as decomposition by intestinal microorganisms and insects, agriculture cultivation, weathering, and oxidative degradation under ultraviolet irradiation (Sul and Costa, 2014; Rillig et al., 2017; Ahmed et al., 2018). Microplastics are plastic particles <5 mm, and because of their small size and large quantities, they are widely distributed in freshwater, marine, terrestrial, and other environments (Peng et al., 2018; Sighicelli et al., 2018; Gong and Xie, 2020). As new environmental pollutants, microplastics have received special attention and are listed as the second most important scientific issue in the ecology and environmental science (Farrell and Nelson, 2013; Horton et al., 2017). Much research has been conducted on the occurrence, fate, and effects of microplastics in aquatic environments (Vaughan et al., 2017; Tang et al., 2018; Wang and Wang, 2018). Although soils are the major and direct sources of microplastics, much remains unknown regarding microplastics in terrestrial environments (Rillig, 2012; Horton et al., 2017).

The major sources of microplastics in soils include residues of agricultural plastic film, sludge and sewage application, wastewater irrigation, organic fertilizer application, surface runoff, and atmospheric deposition (Zubris and Richards, 2005; Bläsing and Amelung, 2018; He et al., 2018; Zhang J. J. et al., 2021). The annual discharge of microplastics to terrestrial systems is 4 to 23 times that to the marine systems (Horton et al., 2017). When discharged from agricultural or industrial production activities, microplastics can remain in soils and be directly absorbed by various soil organisms, posing a threat to their reproduction and growth and accumulating in food chains and causing damage to soil biota in different trophic levels (Rillig, 2012; Rillig and Lehmann, 2020). In agricultural soils of Northwest China, the concentrations of microplastics range from 1,430 to 3,410 items kg^{-1} (Ding et al., 2020). According to Yu et al. (2020), the abundance of microplastics in vegetable farmland soils of northern China averaged 1,444 items kg^{-1}, ranging from 310 to 5,698 items kg^{-1}. Plant roots [i.e., wheat (*Triticum aestivum* L.) and lettuce (*Lactuca sativa* L.)] can absorb micron and submicron-sized microplastics through specific crack-entry modes and then transport the microplastics to shoots (Li et al., 2020). In addition, some microplastics migrate horizontally to rivers (in runoff), the atmosphere, or other parts of the land through the actions of wind and water, whereas others remain in the soil and migrate vertically to deeper soil (Huerta Lwanga et al., 2016; Scheurer and Bigalke, 2018). Microplastics can also absorb pollutants in soils, including antibiotics, pesticides, heavy metals, and persistent organic pollutants. These pollutants can cause serious toxic effects when transported into organisms, increasing the long-term harmful effects (Avio et al., 2015; Hüffer et al., 2018). Therefore, microplastics may threaten soil ecological functions and adversely affect food production (Turner and Holmes, 2015; Li et al., 2018). Because of the negative effects of microplastics on soil ecosystems, it is urgent to identify the distribution and characteristics of microplastics pollution in soils in order to maintain ecological security, promote green development, and ultimately implement effective mitigation and prevention measures.

However, most research on microplastics has focused on ocean and water pollution (Sul and Costa, 2014; Vaughan et al., 2017; Peng et al., 2018). Although microplastics have been investigated in soils of flood zones, industrial areas, vegetable fields, facility agricultural soils and in riverine soils, little research has been conducted in soils with grain crops (Fuller and Gautam, 2016; Scheurer and Bigalke, 2018; Amrutha and Warrier, 2020; Chen et al., 2020). Maize (*Zea mays* L.) is the largest grain crop in China, and Hebei Province is one of the main provinces for maize production (Zhang et al., 2017). Low accumulated temperature and little rain are the main factors limiting high maize yields in the northern of the province. Therefore, to overcome these constraints, the cultivation techniques of agricultural plastic film mulching have been adopted for more than 20 years. Long-term applications of plastic mulching can result in large amounts of plastic debris remaining in farmland soils that gradually decompose into smaller-sized fragments and form microplastics (Astner et al., 2019). However, the pollution status of microplastics in maize farmland soils that use plastic film mulching is unknown. The authors hypothesized that accumulated microplastics in soils with long-term plastic film mulching would be higher than that in non-mulched farmland soils because of the fragmentation of residual films, and microplastics size and shape would also be significantly different. Therefore, this systematical study was conducted with the purpose of investigating the distribution and characteristics of microplastics pollution in mulched and non-mulched maize farmland soils in northern China. The abundance, size, and others that indicate possible sources of microplastics such as polymer composition, color, and shape were determined.

MATERIALS AND METHODS

Study Area and Soil Sample Collection

Pingquan City (40°24′00″N to 40°40′17″N, 118°21′03″E to 119°15′34″E) is in the northeast part of Hebei Province and at the subsidence zone at the eastern end of the Yanshan Mountains. The elevation of the planting area is 450 to 800 m. The city has a continental monsoon climate. Springs are dry with little rain, summers are rainy and hot, autumns are cool with drought, and winters are cold with little snow. The average annual precipitation is 523 mm, and the average air temperature is 7.3℃. The frost-free period is 110 to 125 d per year, and the accumulated temperature is 2500 to 2850℃ (≥10℃). Maize is the main food crop in suburban farmlands and the most important source of agricultural products for circumjacent inhabitants. Maize is monocropped in Pingquan City. In the northern part of the city, due to low accumulated temperature and little rain, the cultivation techniques of agricultural plastic film mulching have been adopted for more than 20 years. However, plastic film mulching has never been used in the southern part. Maize is sown in late April to early May and harvested in late September to early October. In Pingquan City, the total area planted to grain crops in 2019 was

40.81×10^3 ha, and the annual production was 210.67×10^3 t. Maize accounted for 96.0% of the total area planted and 91.5% of the annual yield. As of 2019, the annual quantity of agricultural plastic film for maize planting in the city exceeded 50 tons and covered approximately 7×10^3 ha (Hebei Statistics Bureau, 2020). The thickness of plastics films used in this area generally ranged from 0.004 to 0.006 mm. Local farmers usually collect the plastic film after maize harvest, with a recycling rate of 70 to 80%.

Soil samples were collected in October 2020 from 15 villages, each with 10 km^2 of farmland planted to maize. Ten villages had mulched farmland soils and five had non-mulched farmland soils (**Supplementary Figure 1** and **Table 1**). Five representative sampling sites were selected from the maize farmlands of each village, and the soil samples from each site were composed of three subsamples. In these sites, farmland soils had broken plastic films on the surface and were surrounded by discarded plastics (e.g., polyfoams, nylon nets, plastic bags, and other domestic garbage) (**Supplementary Figure 2**). In each sampling site, soil samples were collected from the arable layer in a 0.5 × 0.5 m area using a stainless-steel shovel. Sampling was stratified, and soils were collected at 0–10, 10–20, and 20–30 cm depths, with depths measured accurately with calipers. Therefore, fifty and twenty-five sites were sampled in mulched and non-mulched farmland soils, respectively, and with the three depths, a total of 150 and 75 samples were obtained from the mulched and non-mulched farmland soils, respectively. After removing large visible garbage (>5 cm), approximately 2 kg of soil was collected from each soil layer in each sampling site. All soil samples were stored in labeled aluminum boxes and then transferred to the laboratory for analyses.

Sampling Processing

Microplastics were extracted from soil samples by a modified density separation method with saturated NaCl solution (1.2 g cm^{-3}) that has been demonstrated successfully applied for agricultural soils (Scheurer and Bigalke, 2018; Lv et al., 2019; Wang et al., 2020; Li et al., 2021). Specifically, the soil samples were air-dried and sieved through a 5-mm stainless steel sieve to remove plant residues and stones (or large debris). Air-dried soil subsamples of 50 g were weighted and mixed with 200 mL of saturated NaCl solution in 250 mL clean and dry glass bottles. The mixtures were stirred thoroughly for 30 min and then kept standing for 24 h. After that, the supernatants were collected in pre-cleaned beakers. To fully extract microplastics from soils, the density separation process was repeated three times. Finally, all collected supernatants were filtered through 0.45 μm GF/A membranes (Whatman, United States). All substances on filter papers were washed with 50 mL of 30% H_2O_2 solution into 100 mL glass beakers to digest organic matters, and the beakers were placed on a graphite electric heating plate for digestion at 60℃ for 24 h. After digestion, the solutions were filtered through 0.45 μm GF/A membranes and rinsed with deionized water. Finally, all filter papers with attached microplastics were put into glass petri dishes and dried at room temperature. Filter papers were observed under a microscope.

Observation and Identification of Microplastics

Microplastics on filter papers were inspected visually using a stereomicroscope (SZ760-DM500). In order to reduce the possibility of microplastics misidentification, the classification criteria developed by Hidalgo-Ruz et al. (2012) and Peng et al. (2017) were used in the visual identification. The particles with clear shapes and colors were selected and identified as potential microplastics according to the criteria. While other particles that were difficult to be identified but had similar shapes were considered to be suspected microplastics. All these particles were hand-sorted using stainless steel fine-tip tweezers under the stereomicroscope, and the suspected particles were stored separately. The number of these particles were counted (items kg^{-1}), and the shapes, sizes, and colors were recorded. To separate microplastics from other materials, the polymer composition was determined by a micro-Fourier Transform Infrared Spectrometer (μ-FTIR, Spotlight 400, PerkinElmer, United States). The spectrum ranging from 650 to 4,000 cm^{-1} was used to scan samples 32 times at a resolution of 4 cm^{-1} in transmission mode. The spectra of all selected samples were identified using the Spectrum software and compared with the standard spectral libraries of polymers (i.e., Sadtler Infrared Spectral library) to determine the polymer compositions. When the matching degree exceeded 70%, the sample corresponding to the spectrum was considered microplastics, and the corresponding polymer was confirmed accordingly (Liu et al., 2018).

Quality Assurance and Quality Control in Experiments

To avoid potential plastic pollution of artificial and airborne and ensure the reliability of the experimental results, strict quality control was performed during the experiment. All plastic materials were avoided during the entire pretreatment process, and all materials used for analysis were made of glass (e.g., stirrer rods, funnels, and petri dishes). All equipment and containers were carefully rinsed with deionized water for more than three times, and all samples were always sealed with aluminum foil between separate experimental steps to minimize possible pollution from indoor air. During the entire experiment, researchers wore cotton lab coats and gloves. In addition, blank experiments were performed to check the possible pollution of ultrapure water and air in the laboratory during the operation. No microplastics were detected in the blank controls.

A recovery experiment was performed to verify the separation method with saturated NaCl solution. Some commercial polyethylene (PE), polypropylene (PP), and polystyrene (PS) plastic products were selected and ground into microplastic particles (200 to 2000 μm) with a mill in the laboratory. A 50-g relative clean soil was mixed with 30 counts of the polymer

TABLE 1 | Abundance statistics of microplastics at different soil layers in mulched and non-mulched farmlands in Pingquan City, Hebei Province, China.

Soil type	Sample area No.*	Soil depth (cm)	Abundance statistics (items kg^{-1})			
			Min	Median	Average ± Standard Deviation	Max
Mulched farmland soils	1	0–10	320	440	600 ± 306	1080
		10–20	400	440	640 ± 325	1160
		20–30	280	400	400 ± 147	640
	2	0–10	1040	1400	1512 ± 541	2320
		10–20	960	1640	1744 ± 689	2590
		20–30	720	1080	1080 ± 348	1640
	3	0–10	400	760	864 ± 537	1680
		10–20	600	880	960 ± 419	1680
		20–30	320	560	632 ± 363	1240
	4	0–10	440	600	648 ± 249	1040
		10–20	200	640	704 ± 434	1400
		20–30	240	400	416 ± 166	680
	5	0–10	960	1400	1440 ± 391	2000
		10–20	520	640	688 ± 163	880
		20–30	320	520	616 ± 272	960
	6	0–10	1360	1600	1928 ± 602	2600
		10–20	960	1120	1360 ± 571	2360
		20–30	680	840	1024 ± 477	1840
	7	0–10	440	600	712 ± 370	1360
		10–20	560	640	816 ± 418	1560
		20–30	320	360	536 ± 395	1240
	8	0–10	200	360	368 ± 188	680
		10–20	160	360	376 ± 173	640
		20–30	200	400	376 ± 115	520
	9	0–10	160	280	280 ± 102	400
		10–20	280	320	368 ± 95	520
		20–30	400	440	512 ± 168	800
	10	0–10	160	440	360 ± 136	480
		10–20	120	360	360 ± 210	640
		20–30	120	280	296 ± 115	400
	Average		240	553	754 ± 477	2253
Non-mulched farmland soils	11	0–10	200	360	304 ± 78	360
		10–20	160	320	312 ± 91	400
		20–30	200	400	352 ± 87	400
	12	0–10	260	420	472 ± 186	720
		10–20	120	340	324 ± 136	480
		20–30	60	300	248 ± 143	400
	13	0–10	200	720	684 ± 340	1140
		10–20	160	320	404 ± 243	800
		20–30	140	220	244 ± 128	460
	14	0–10	300	560	540 ± 264	940
		10–20	140	500	432 ± 176	560
		20–30	100	260	356 ± 290	780
	15	0–10	200	280	424 ± 263	800
		10–20	160	320	336 ± 134	520
		20–30	120	200	216 ± 103	380
	Average		173	373	376 ± 149	800
Total	Average		173	493	628 ± 436	2253

**Each area included five sampling sites.*

particles. The mixture was pretreated and analyzed using the separation method as described above. Five replicates were settled for each type of microplastic. The recovered MP particles were counted under a stereomicroscope and the recovery rates for the MP particles were above 90%.

Statistical Analyses

Statistical analyses were performed using SPSS software (Version 23.0, IBM). The figures were plotted using Microsoft Excel (2016), SigmaPlot (14.0), and R ("ggplot2" package, version 3.3.3). Independent samples *t*-test was adopted to determine the differences in microplastics abundances, sizes, shapes, and colors between the mulched and non-mulched farmland soils at the 95% confidence level. The PROC GLM procedure was used to determine the differences in particle size of microplastics among different soil layers in the mulched and non-mulched farmland soils. One-way ANOVA was used to determine the differences in microplastics abundances among different soil layers and the differences in the percentages of microplastics among different soil layers in each size range in the mulched

and non-mulched farmland soils. Average values were compared using the least significant difference (LSD) test at $p < 0.05$. Linear regression analysis was used to quantify the relationship between the microplastics abundances and the length of time with film mulching applied.

RESULTS

Abundance and Vertical Distribution of Microplastics in Maize Farmland Soils

Microplastics were detected in different soil layers at all sampling sites. On average, the microplastics abundance of 754 ± 477 items kg^{-1} (range: 240 to 2,253 items kg^{-1}) in mulched farmland soils was significantly higher than the abundance of 376 ± 149 items kg^{-1} (range: 173 to 800 items kg^{-1}) in non-mulched farmland soils ($p < 0.05$, **Figure 1A** and **Table 1**).

Average abundances of microplastics at 0–10 cm, 10–20 cm, and 20–30 cm in mulched farmland soils were 871 ± 641 items kg^{-1} (range: 160 to 2,600 items kg^{-1}), 802 ± 561 items kg^{-1} (range: 120 to 2,600 items kg^{-1}), and 589 ± 363 items kg^{-1} (range: 120 to 1,840 items kg^{-1}), respectively (**Figure 1B**). In non-mulched farmland soils, at the same depths, the abundances were 485 ± 257 items kg^{-1} (range: 200 to 1,140 items kg^{-1}), 362 ± 158 items kg^{-1} (range: 120 to 800 items kg^{-1}), and 283 ± 164 items kg^{-1} (range: 60 to 780 items kg^{-1}), respectively. The vertical distributions of microplastics in mulched and non-mulched farmland soils were similar. Microplastics abundance in the two farmland soils decreased gradually with the increase in soil depth, and the abundance at 0–10 cm was significantly greater than that at 20–30 cm ($p < 0.05$, **Figure 1B**). Of all sampling areas, the highest concentration of microplastics was 2,600 items kg^{-1} at 0–10 cm in area 6 of the mulched farmland soils (**Table 1**). The lowest abundance of microplastics was only 60 items kg^{-1} at 20–30 cm in area 12 of the non-mulched farmland soils. In addition, microplastics abundance was significantly positively related to the length of time with film mulching applied ($R^2 = 0.43$, $p < 0.05$) (**Figure 2**).

Size Distribution of Microplastics

The size of microplastics particles ranged from 0.03 to 5 mm. The size distribution of microplastics in different soil layers in mulched and non-mulched farmland soils was generally similar, and the percentage of size distribution decreased with the increase of particle size (**Figures 3A,C** and **Supplementary Table 2**). In mulched soils, 50.9% of the particles were <0.5 mm, 18.5% were 0.5–1.0 mm, 17.9% were 1.0–2.0 mm, and 12.6% were 2.0–5.0 mm. In non-mulched soils, 62.2% of the particles were <0.5 mm, 13.3% were 0.5–1.0 mm, 12.8% were 1.0–2.0 mm, and 11.6% were 2.0–5.0 mm. The percentage of microplastics <0.5 mm in mulched soils was significantly lower than that in non-mulched soils (**Figure 3E**). In the mulched and non-mulched farmland soils, the highest percentage of microplastics <0.5 mm was at 20–30 cm, with averages of 57.9 ± 9.0% and 66.1 ± 9.8%, respectively. The percentage of microplastics <0.5 mm was significantly different between 0–10 and 20–30 cm in mulched farmland soils ($p < 0.05$, **Figure 3A**). For the particles 2.0–5.0 mm, their percentages tended to decrease with the increase of soil depth in mulched and non-mulched farmland soils, and the percentage at 20–30 cm was significantly lower than that at 0–10 cm in non-mulched farmland soils ($p < 0.05$, **Figure 3C**).

In mulched soils, the average size of microplastics was 1.03 ± 1.08 mm, 0.83 ± 0.85 mm, and 0.82 ± 0.89 mm at 0–10 cm, 10–20 cm, and 20–30 cm, respectively. In non-mulched soils, the average size was 1.07 ± 1.27 mm, 0.86 ± 0.87 mm, and 0.78 ± 0.72 mm at 0–10 cm, 10–20 cm, and 20–30 cm, respectively (**Figures 3B,D**). The average size of microplastics at 0–10 cm in the two farmland soils was significantly larger than that at 10–20 cm and 20–30 cm ($p < 0.05$, **Figures 3B,D**).

Shape Characteristics of Microplastics

Four shapes of microplastics were detected: fragment, fiber, film, and pellet (**Figure 4**). The distributions of microplastics shapes in mulched and non-mulched farmland soils were different (**Figures 5A,B**). Fragments were the highest percentage (37.7%) of microplastics in mulched and non-mulched soils, followed by films (33.9%), fibers (21.9%), and pellets (6.6%) in mulched soils, and fibers (29.6%), films (24.3%), and pellets (4.2%) in non-mulched soils. The percentage of films in mulched soils was significantly higher than that in non-mulched soils, whereas that of fibers was significantly higher in non-mulched soils ($p < 0.05$, **Figure 5C**). Fragments, fibers, and films were found in all soil samples, whereas pellets were detected in 70.0% of mulched soils and 60.0% of non-mulched soils (**Supplementary Table 2**).

Color Characteristics of Microplastics

Five colors of microplastics were detected: white, transparent, yellow, blue, and black. Overall, the distributions of different colors of microplastics at different soil depths in mulched and non-mulched soils were similar (**Figures 6A,B** and **Supplementary Table 2**). Most microplastics were white in mulched (40.2%) and non-mulched (43.3%) soils, followed by those that were transparent (36.4 and 39.5%, respectively), yellow (16.7 and 11.8%, respectively), and blue (4.4 and 3.2%, respectively). Black microplastics were detected at the lowest frequency, with average percentages of only 2.2 and 1.6% in mulched and non-mulched soils, respectively. No significant difference was observed in the proportion of different colors between mulched and non-mulched soils.

Polymer Composition of Microplastics

The polymer composition of microplastics included polyethylene (PE), polypropylene (PP), polyester, poly(ethylene terephthalate) (PET), rayon, poly(ethyl acrylate) (PEA), cellophane, and polyacrylonitrile/acrylic acid (PAN) (**Figure 7A**). Of the microplastics, 36.9% were composed of PP, 26.5% of PE, 17.1% of polyester, 10.9% of PET, 4.7% of rayon, 1.8% of PEA, 1.2% of cellophane, and 0.9% of PAN. Notably, the dominant polymer types were PP, PE, and polyester.

The polymer composition of microplastics varied with shape (**Figure 7B**). Polypropylene was the dominant polymer type in fragments, accounting for 66.4% of the total polymers, followed by PE (32.7%) and PEA (0.9%). The polymer types in pellets

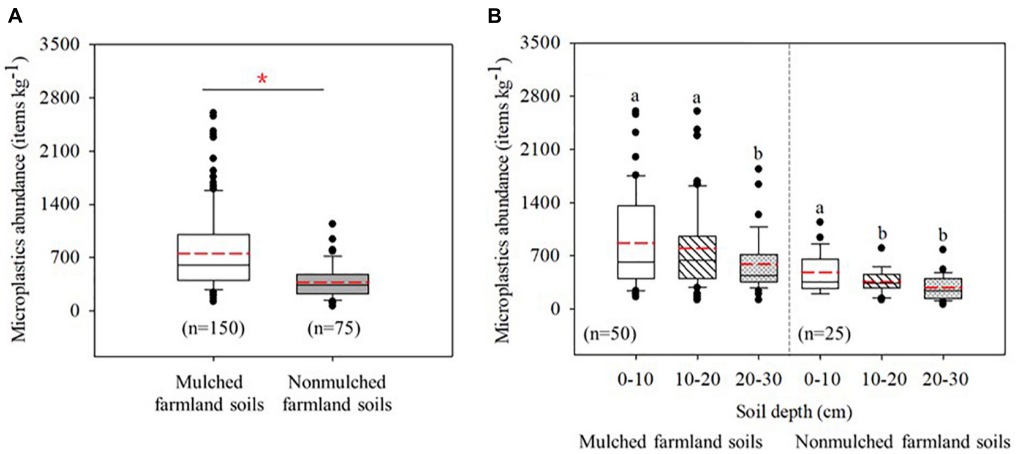

FIGURE 1 | Abundances of microplastics (items kg^{-1}) in mulched and non-mulched farmland soils. **(A)** Total abundance (*$p < 0.05$). **(B)** Abundances at different soil depths (cm), with different letters indicating significant differences at $p < 0.05$. Box-whisker diagrams show the average, 25th, 50th, 75th percentiles.

included PE (53.5%), PP (28.6%), and PEA (17.9%), whereas in films, only PE (67.4%) and PP (32.6%) were identified. In fibers, polyester (36.7%) was the dominant polymer, follow by PET (23.4%), PP (19.0%), rayon (10.1%), PE (6.3%), cellophane (2.5%), and PAN (1.9%).

DISCUSSION

Abundance and Vertical Distribution of Microplastics in Maize Farmland Soils

This study confirmed the occurrence of microplastics in maize farmland soils with long-term plastic film mulching in northern China. The abundance of microplastics in mulched soils was significantly higher than that in non-mulched soils (**Figure 1A**). This apparent distinction can probably be ascribed to the application of plastic mulching. Moreover, microplastics abundance was also different among the mulched farmlands, with the differences primarily due to the length of time with film mulching applied. A significant positive linear correlation was obtained between microplastics abundance and the length of time with film mulching applied (correlation coefficient $R^2 = 0.58$, $p < 0.05$, **Figure 2**). For example, area 6 had the highest concentration of microplastics (i.e., 2,600 items kg^{-1}) because of the longest period of plastic mulching (over 35 years) (**Table 1**). This was consistent with Huang et al. (2020) who found that microplastics abundances substantially increased with plastic mulching continuously employed. These results indicated that microplastics abundance increased greatly with long-term application of plastic films. In addition, a certain amount of microplastics (mainly fragments and fibers) was detected in non-mulched soils, which has no mulching history, indicating that plastic film mulch is not the only source of microplastics in agricultural soils. In the sampling areas, the residues of plastic package bags, pesticide bottles, broken fertilizer packaging bags, and residual agricultural film were found whether film mulching was used or not that could be also a potential source of microplastics. Apart from these, atmospheric deposition is also a route of fibrous microplastics entering soils (Bläsing and Amelung, 2018). All of these can lead to the appearance of microplastics in non-mulched soils.

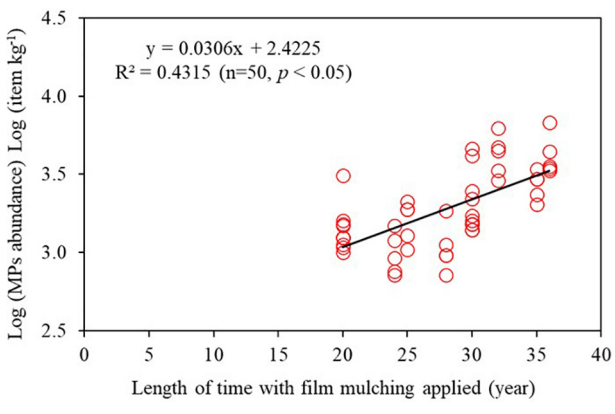

FIGURE 2 | Correlation between the length of time with film mulching applied and microplastics abundance.

To better understand the pollution status of microplastics in maize farmland soils, the results of this study were compared with the characteristics of microplastics in different terrestrial systems that were summarized in the current study (**Supplementary Table 3**). The average abundance of microplastics (i.e., 628 items kg^{-1}) in this study is comparable with that in Hebei coastal soils (i.e., 634 items kg^{-1}) and Swiss floodplain soils (i.e., 593 particles kg^{-1}) (Zhou et al., 2016; Scheurer and Bigalke, 2018). However, microplastics concentration in maize farmland soils is relatively high compared with that in other soil types, including greenhouse and orchard soils, rice–fish co-culture system soil, agricultural soils amended with pig manure, and riverine soils (Zhang et al., 2018; Lv et al., 2019; Amrutha and Warrier, 2020; Yang et al., 2020). However, the microplastics

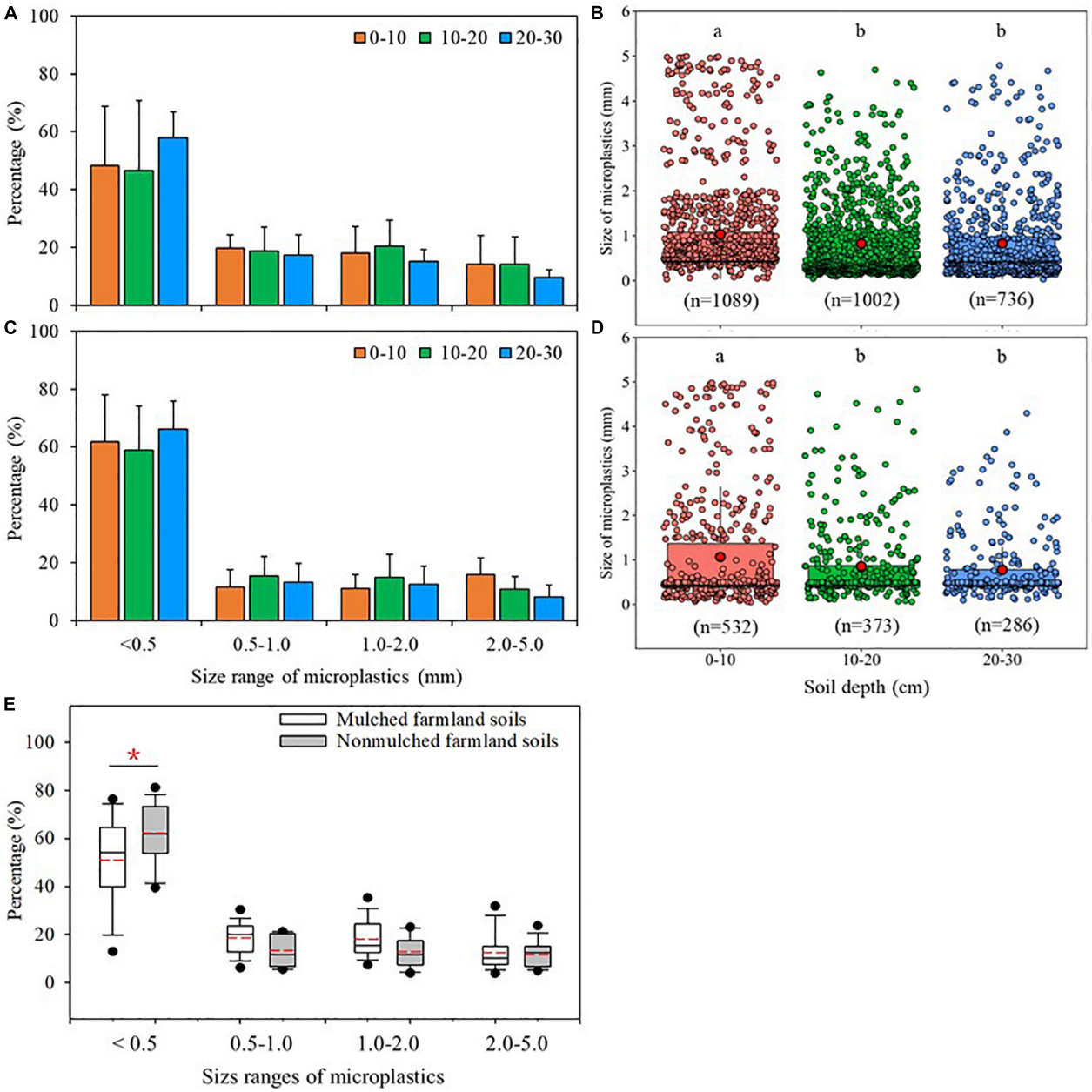

FIGURE 3 | Size (mm) distributions of microplastics at different soil depths (cm) in maize farmlands. Percentages of different size classes of microplastics at different depths in **(A)** mulched and **(C)** non-mulched soils [Columns represent the mean (n = 10 in mulched soils and n = 5 in non-mulched soils) and standard deviations]. Size of microplastics particles at different soil depths in **(B)** mulched and **(D)** non-mulched soils, with different letters indicating significant differences at $p < 0.05$. **(E)** Percentages of size of microplastics in mulched (n = 30) and non-mulched (n = 15) soils (*$p < 0.05$). Box-whisker diagrams show the average, 25th, 50th, and 75th percentiles.

abundance in this study is much lower than that in facility agricultural soils and vegetable farmland soils (Zhang and Liu, 2018; Chen et al., 2020). Therefore, as demonstrated by the above studies, microplastics are common contaminants in soil systems. Although most soils were collected from farmlands, the concentrations of microplastics varied widely in different regions, which may be due to many factors, such as crop rotation, fertilization, tillage, sampling sites, and extraction methods of microplastics in soils (Chen et al., 2020). In addition, the distribution of microplastics pollution is also associated with pollution sources and plastics content (Chen et al., 2020). Moreover, this study found that microplastics abundances in both mulched and non-mulched soils decreased gradually with increased soil depth (**Figure 1B**). During the maize planting in sampling areas, soils are usually ploughed once every two years in mulched farmlands and once per year in non-mulched farmlands, which might contribute to the vertical transfer of microplastics between shallow and deep soil

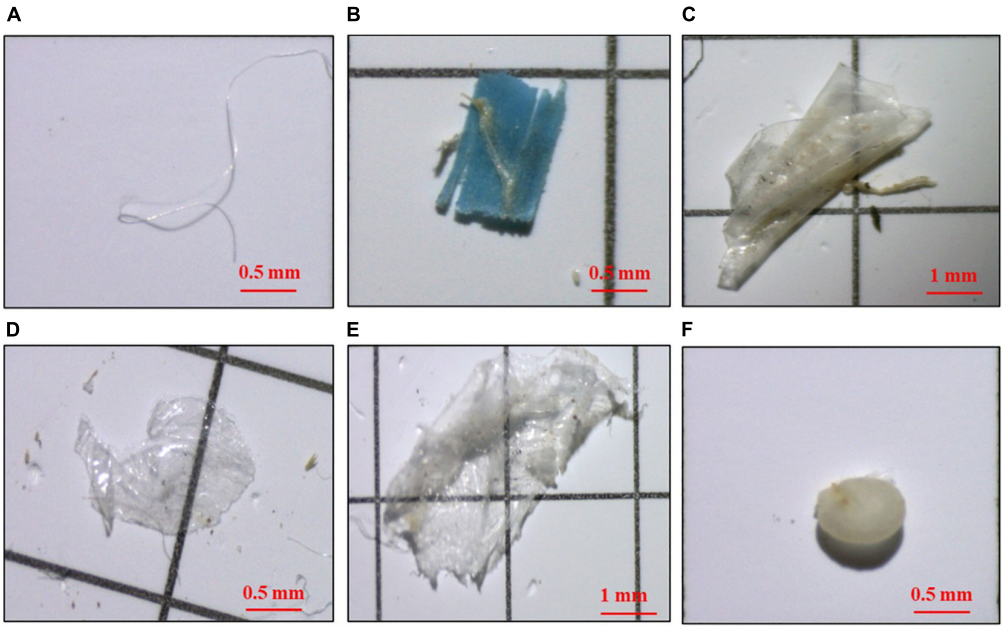

FIGURE 4 | Photographs of microplastics with different shapes under the stereomicroscope: fiber **(A)**, fragment **(B,C)**, film **(D,E)**, and pellet **(F)**.

layers. These results are consistent with those of previous studies on agricultural soils in Shanghai and Shandong, China (Liu et al., 2018; Yu et al., 2020). However, they are in contrast to those of Zhang et al. (2020) who found the abundance of microplastics at 20–30 cm (400 items kg^{-1}) was much higher than that in the shallow soil layer (0–20 cm, 100 items kg^{-1}) in suburban farm soils of Harbin, China. The differences in the vertical distributions of microplastics abundance in different studies may be related to many factors, including crop rotation, sampling depth, precipitation, and biological and mechanical disturbance.

Microplastics particles <0.5 mm were predominant in this study (**Figure 3**), and the percentage of size distribution decreased with increased particle size. This may be ascribed to the gradual fragmentation of large pieces into small pieces by mechanical abrasion, high temperatures, and ultraviolet radiation, among other factors (Song et al., 2017; Piehl et al., 2018). The percentage of microplastics <0.5 mm was significantly higher in non-mulched soils. This is because although the number of microplastics <0.5 mm in mulched soils is greater than that in non-mulched soils, the total abundance is significantly lower in non-mulched soils, leading to a high percentage of microplastics <0.5 mm. The average size of microplastics in topsoil of the mulched and non-mulched farmland was significantly larger than that at deep soil. In addition, the percentage of small-sized microplastics (i.e., <0.5 mm) increased with increased soil depth, whereas that of large-sized particles (i.e., 2.0–5.0 mm) decreased with increased soil depth (**Figures 3A,C**). This demonstrated that the mobility of smaller microplastics was higher than that of larger microplastics. These results are similar to those of Yu et al. (2020). This is partially because small-sized microplastics are liable to migrate to deeper soil layers under the influence of soil organisms; for example, earthworms can ingest small-sized microplastics and then excrete them with feces in deep soil channels (Huerta Lwanga et al., 2017). Small-sized microplastics are also more susceptible to soil erosion and runoff and can easily penetrate downward into deep soil layers through tiny cracks and pores (Bläsing and Amelung, 2018; Zhang et al., 2018). However, further research is needed on the transformation and migration of microplastics in soils.

Morphology and Composition Characteristics of Microplastics in Maize Farmland Soils

In this study, white (41.8%) and transparent (38.0%) were the dominant colors of microplastics in maize farmland soils, followed by yellow (17.5%), blue (3.8%), and black (1.9%). This is consistent with previous studies (Piehl et al., 2018; Amrutha and Warrier, 2020; Yu et al., 2020), although Liu et al. (2018) found translucent and black microplastics dominated in agricultural soils. The diversity of color in microplastics is also a reflection of the diversity of pollution sources (Zhang D. D. et al., 2021). White and transparent microplastics are from a wide range of sources, including plastic packaging materials, plastic bags, and plastic film (**Supplementary Figure 2**). In addition, polychromatic microplastics may be weathered and discolored under the actions of water, heat, and light to form white microplastics (Galafassi et al., 2019; Liu et al., 2020). However, multicolor microplastics may be sourced from colorful plastic consumer products, plastic packaging, clothing, and cosmetics used in daily life.

Across all sample sites, the dominant shapes of microplastics were fragments (39.8%), films (29.1%), and

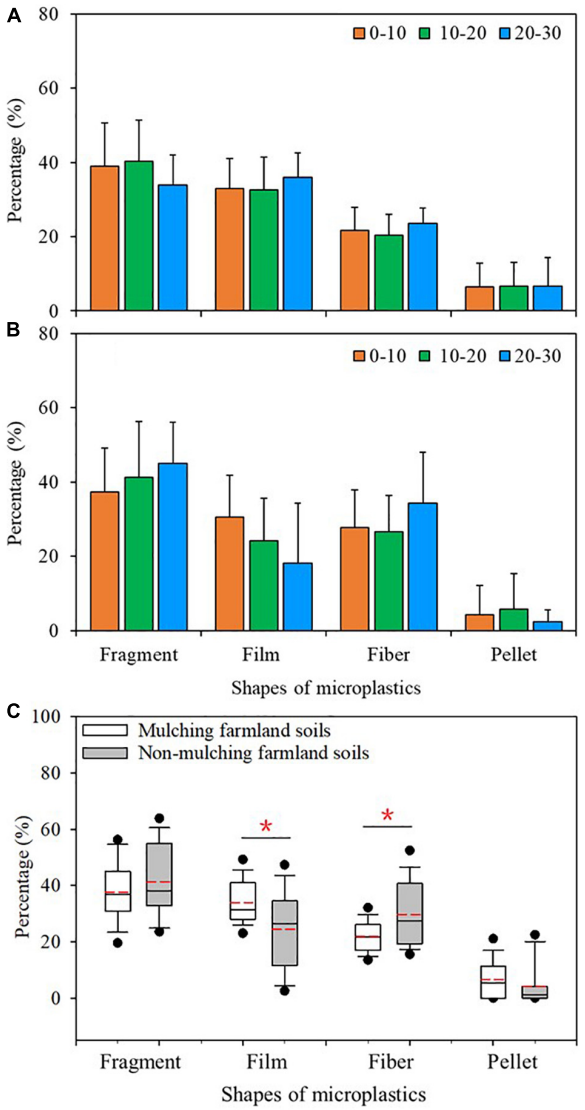

FIGURE 5 | Shape distribution of microplastics at different soil depths (cm) in maize farmlands. Percentages of different shapes of microplastics at different depths in **(A)** mulched and **(B)** non-mulched soils [Columns represent the mean (n = 10 in mulched soils and n = 5 in non-mulched soils) and standard deviations]. **(C)** Percentages of different microplastics shapes in mulched (n = 30) and non-mulched (n = 15) soils (*$p < 0.05$). Box-whisker diagrams show the average, 25th, 50th, and 75th percentiles.

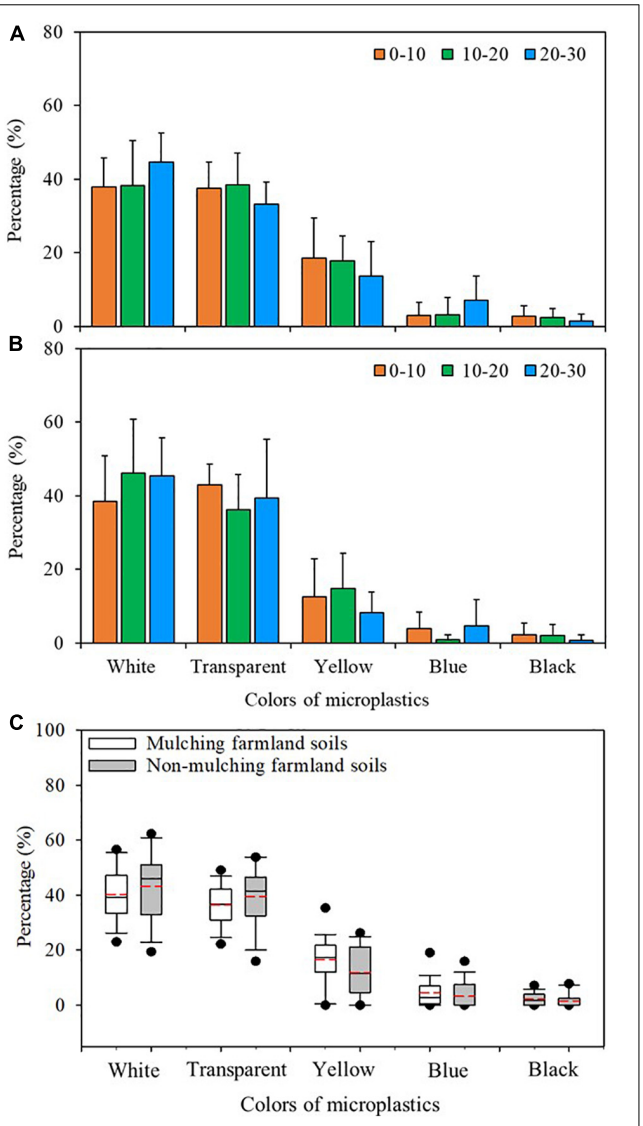

FIGURE 6 | Color distribution of microplastics at different soil depths (cm) in maize farmlands. Percentages of different colors of microplastics at different depths in **(A)** mulched and **(B)** non-mulched soils [Columns represent the mean (n = 10 in mulched soils and n = 5 in non-mulched soils]. **(C)** Percentages of different microplastics colors in mulched (n = 30) and non-mulched (n = 15) soils. Box-whisker diagrams show the average, 25th, 50th, and 75th percentiles.

fibers (25.7%) (**Figures 5A,B**). The result was similar to some findings previously reported (Amrutha and Warrier, 2020; Ding et al., 2020; Zhou et al., 2020), whereas it was differed from the results in which microbeads or flakes microplastics are dominant types (Zhou et al., 2018; Chen et al., 2020). Such differences in dominant shapes are likely because the sources of microplastics are different in different study areas. Polymer types in fragments were mainly PP and PE, accounting for 66.4 and 32.7%, respectively. Polypropylene is widely used in snack wrappers and food packaging, whereas PE is often used in plastic containers, food packaging films, reusable bags, agricultural plastic film, and trays. With UV exposure, fragmentation is more likely with PP, because the chemical bond dissociation energy is lower (Song et al., 2017). Therefore, the fragments microplastics in soils were likely mainly sourced from the degradation of residual plastic wastes under long-term weathering. Residues of plastic package bags, pesticide bottles, broken fertilizer packaging bags, and agricultural film were found whether film mulching was used or not around the sampling areas (**Supplementary Figure 2**). The presence of these residues suggests they are significant sources contributing to fragments microplastics in soils.

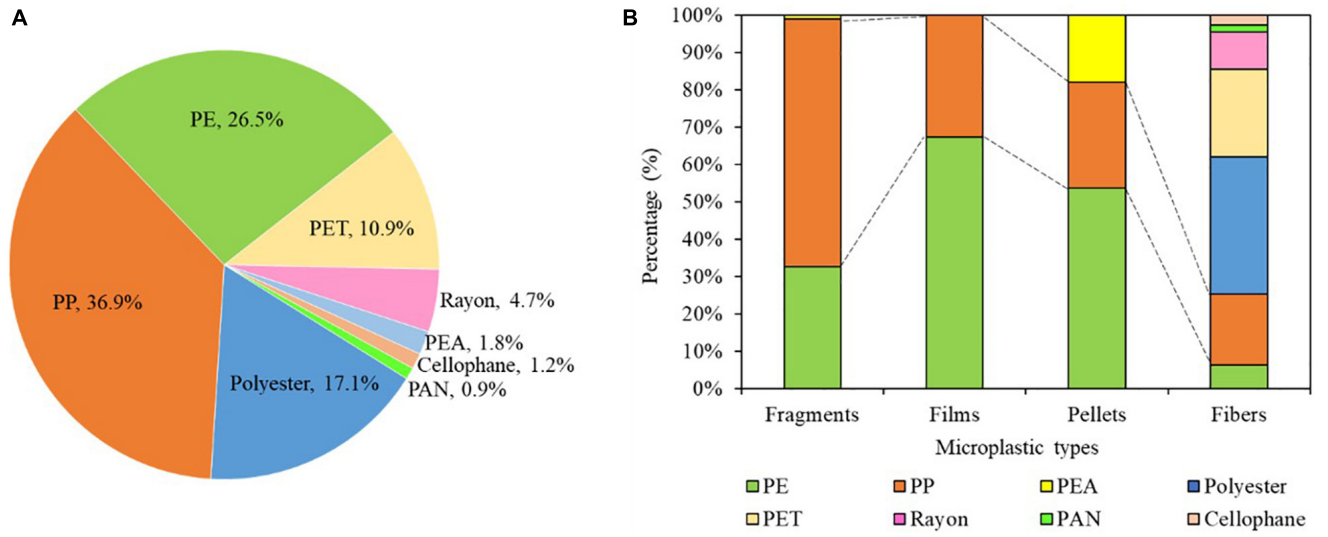

FIGURE 7 | Polymer composition of microplastics in maize farmland soils. **(A)** Overall percentages of different polymer compositions of microplastics. **(B)** Percentage contributions of different polymers of different shapes of microplastics. PP, polypropylene; PE, polyethylene; PET, poly(ethylene terephthalate); PEA, poly(ethyl acrylate); PAN, polyacrylonitrile/acrylic acid. Polymer composition was determined in 339 microplastics samples.

The polymer compositions of films included PE (67.4%) and PP (32.6%). The percentage of films was significantly higher in mulched soils (**Figure 5C**). In the present study, μ-FTIR determinations indicated that PE served as the main polymer composition of agricultural plastic mulching films (commercial products) used in maize farmland soils with plastic film mulching. The thickness of plastics films used in agricultural production in China is generally <0.006 mm, much thinner than that used in Japan or Europe (i.e., 0.02 mm), and therefore, films are easily torn during cultivation, and recycling is also difficult (Zhao et al., 2017). Although local farmers claimed they would recycle most plastic films after maize harvest, plastic films remaining in the farmlands may lose integrity and decompose into increasingly smaller plastic pieces of different sizes, of which some eventually form microplastics (Briassoulis et al., 2015; Steinmetz et al., 2016). Therefore, the application of plastic mulching film was a major contributor to films microplastics with a dominant component of PE in maize fields.

The polymer composition of fibers was widely variable in this study. They were composed primarily of polyester and PET; in addition, PP, rayon, PE, cellophane, and PAN were also identified in fibers. Notably, although polyester and PET materials are not widely adopted in agricultural production, they were found in maize farmland soils. These two polymers are mainly adopted in textile industries, and they have been detected at high contents in irrigation water that contains high levels of plastic fibers produced by laundry (Lusher et al., 2013; Zhou et al., 2020). Therefore, agricultural irrigation water may be an important pathway of fibers into the maize fields. In addition, atmospheric dry and wet deposition are also potential sources of fiber microplastics in soils (Dris et al., 2016; Leads and Weinstein, 2019; Liu et al., 2019). In this study, fibers showed substantially higher percentage in non-mulched soils. This may be due to the small difference in fibers from the above sources between mulched and non-mulched soils, and the much lower abundance of microplastics in non-mulched soils, resulting in a relatively high proportion of fibers. Although the density of polyester and PET is slightly higher than that of saturated NaCl, this study found that some PET and polyester fibers were extracted, similar findings were also reported by Li et al. (2021) and Yu et al. (2020). It may be attributed to that the density of microplastics was changed compared to theoretical plastics due to the changes of their physical and chemical properties and occurrence state in the actual environment (Scheurer and Bigalke, 2018). In addition, the combined action of the surface tension and buoyancy of small particles may make the saturated NaCl solution separate microplastics with slightly higher density (Li et al., 2021).

Pellets were a small percentage of the microplastics (5.4%) in the study areas, and the main polymer compositions included PE, and PP, which was consistent with the results reported by Han et al. (2020). Microplastics are added as abrasives to many products, including toothpastes and hand and facial cleaners, which can be discharged into the environment through domestic sewage (Duis and Coors, 2016). According to studies in Norway, Switzerland, and the European Union, approximately 6% of liquid skin-cleaning products contain microplastics, of which 93% are composed of PE (Gouin et al., 2015). Therefore, the microbeads in personal cleaning and care products might be the source of pellet microplastics (Lee et al., 2013; Ding et al., 2020). In brief, the results of this study demonstrated the different sources of microplastics in farmland soils. Apart from plastic film, other agricultural plastic materials should also be paid more attention to avoid excessive accumulation of microplastics in soils.

CONCLUSION

Microplastics pollution characteristics was revealed in typical maize farmland soils with and without long-term plastic film mulching. Mulched soils contained much higher abundances of microplastics than non-mulched soils. Microplastics were the most abundant and the largest in topsoil. Small microplastics (<0.5 mm) were dominant, and their percentage increased with increased soil depth, indicating that small-sized microplastics tended to migrate to deeper soil layers. Mulched soils had much lower proportion of microplastics <0.5 mm than non-mulched soils. Microplastics were mainly fragments, films, and fibers, and mulched soils had much higher percentage of films and lower percentage of fibers than non-mulched soils. Polypropylene, PE, and polyester were the dominated polymer compositions. In addition, the length of time with film mulching applied was greatly affect the accumulation of microplastics in soils. Overall, this study provides an important reference for future research on ecological risks of microplastics in agroecosystems. In future, more attention should be paid to related remediation and management strategies to reduce mulching-based microplastics pollution in agricultural soils, such as the development of biodegradable films and efficient recycle technologies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JZ conducted the laboratory analysis, formal analysis, and wrote the manuscript. GZ and YC co-supervised the project and assisted with various methodological of the associated manuscript. XW, BL, and YM conducted the investigation. LX assisted with methodological. WD, XZ, and LS contributed to editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Identification and Quantification of Microplastics in Aquaculture Environment

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The existence of microplastics (MPs) poses a potential threat to the entire ecosystem and has gained wide public attention. As an essential source of aquatic products, aquaculture industries are inevitably subjected to the pollution of MPs, particularly when the plastic products are widely used in aquaculture. Even so, the identification of MPs in aquaculture is rarely reported. Hence, high-efficient analytical methods for accurate detection of MPs in the aquaculture environment are of utmost significance. This review comprehensively summarizes the analytical methods for MPs in aquaculture, including sampling, extraction, and qualitative and quantitative analyses of MPs. MPs are identified and quantified mainly by visual inspection, spectroscopy, or thermal analysis. In addition, this review also points out the limitations of these methods and the accuracy of quality control. Finally, the need for establishing standard methods is emphasized, and suggestions for future research are also proposed.

Keywords: microplastics, analytical method, sampling, identification, aquaculture environment

INTRODUCTION

The ever-increasing human population has generated a remarkable amount of plastic waste. According to the statistical analysis, the global production of plastics has reached 370 million tons by 2019 (PlasticsEurope, 2020). Plastics are widely utilized in all aspects of our daily life due to their low cost, durability, good ductility, and lightweight, resulting in the accumulation of plastic wastes in the environment, which are inevitably introduced into the ocean through various pathways. Therefore, the ocean may become an immense reservoir for plastic wastes. The natural decomposition of plastics is extremely slow. The plastic waste will be broken into small plastic pieces after physical, chemical, and/or biological action (Wright et al., 2013). Among these, plastics with particle size <5 mm are defined as "microplastics" (MPs) (Erni-Cassola et al., 2017), which was first proposed by Thompson et al. (2004).

In general, MPs are classified into primary MPs and secondary MPs (**Figure 1**). The primary MPs are derived from microbeads in cosmetics, cleaning products, and air-blasting media, which can directly enter the environment (Du and Wang, 2021). The secondary MPs are derived from the

decomposition of larger plastic pieces. Pollution of MPs has gained significantly more research attention in recent years due to its persistence in the natural environment and potential adverse impacts on organisms. MPs can migrate in various environmental compartments, such as air (Dris et al., 2016), soil (Blaesing and Amelung, 2018), oceans (Andrady, 2011), and freshwater (Dris et al., 2015; **Figure 2**). They are easily ingested by organisms and translocated to higher trophic levels through food web. The exposure of MPs to humans aroused severe health concerns, encouraging to explore the removal techniques of MPs. At present, we have been plagued by the pollution of MPs. Therefore, techniques with high sensitivity and selectivity should be developed for the detection, identification, and localization of MPs. MPs can be detected either directly in environmental samples (e.g., aerosols, sediments, soils, and water) or within organisms originated from various habitats. So far, research on the detection of MPs in the aquatic environment has been conducted in some typical farms (**Supplementary Table 1**). These results exhibited that MPs have become a potential source of pollutants in the aquatic environment. Aquatic products are a significant source of human food. In recent years, world aquaculture production of cultured aquatic animals has grown steadily. To achieve the goal of sustainable aquaculture, some countries intend to produce more aquatic animals from aquaculture. Thus, aquaculture environments, such as ponds, lakes, rivers, and oceans, were inevitably contaminated by a plethora of pollutants including MPs that can be detrimental to the growth and development of aquatic organisms and, finally, end up in the human body through food chain, posing a serious threat to human health (**Figure 3**). In contrast, the review regarding the pollution of MPs in aquaculture systems is limited.

To better map the abundance of MPs in the aquaculture environment and potential damage to organisms, future research should focus on the identification and quantification of MPs across the world. The analysis of MPs, including the sampling, preparation, and identification, has been reported (**Figure 4**). Nonetheless, to date, the standardized protocols for sample collection and subsequent analysis have not been proposed. At present, the approaches utilized to detect MPs in the aquaculture environment are rarely reported.

In summary, the goal of this review is to (1) present the most common method for the identification and quantification of MPs in the aquaculture system, (2) discuss the limitations, quality control, and quality assurance of these methodologies, and (3) propose the existing knowledge gaps and recommendations for future direction on the detection of MPs in the aquaculture environment.

LITERATURE SEARCH STRATEGY

A systematic literature review was performed to retrieve literature regarding the detection of MPs in aquaculture environment resulting from Google Scholar, Science Direct, Web of Science, PubMed, and other commonly used databases using the combination of keywords, including MPs, detection, identification, quantification, and aquaculture system. The obtained items were further refined to peer-reviewed research articles. The special keywords "microplastic and aquaculture"

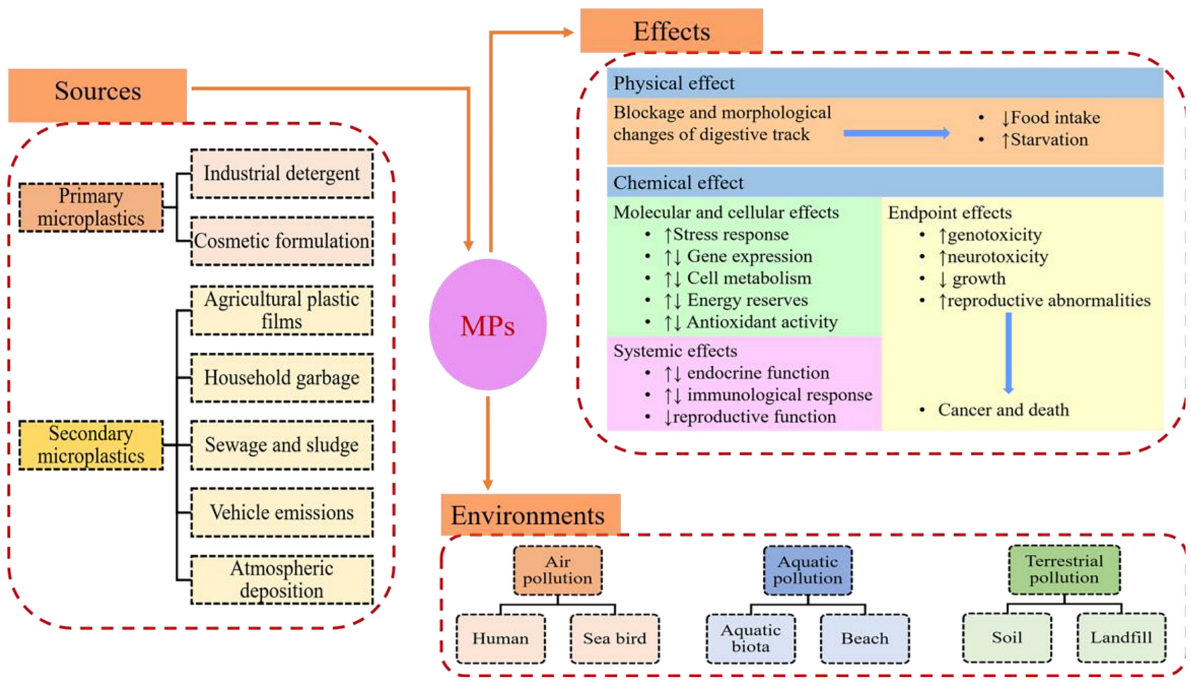

FIGURE 1 | The source and fate of microplastics (MPs).

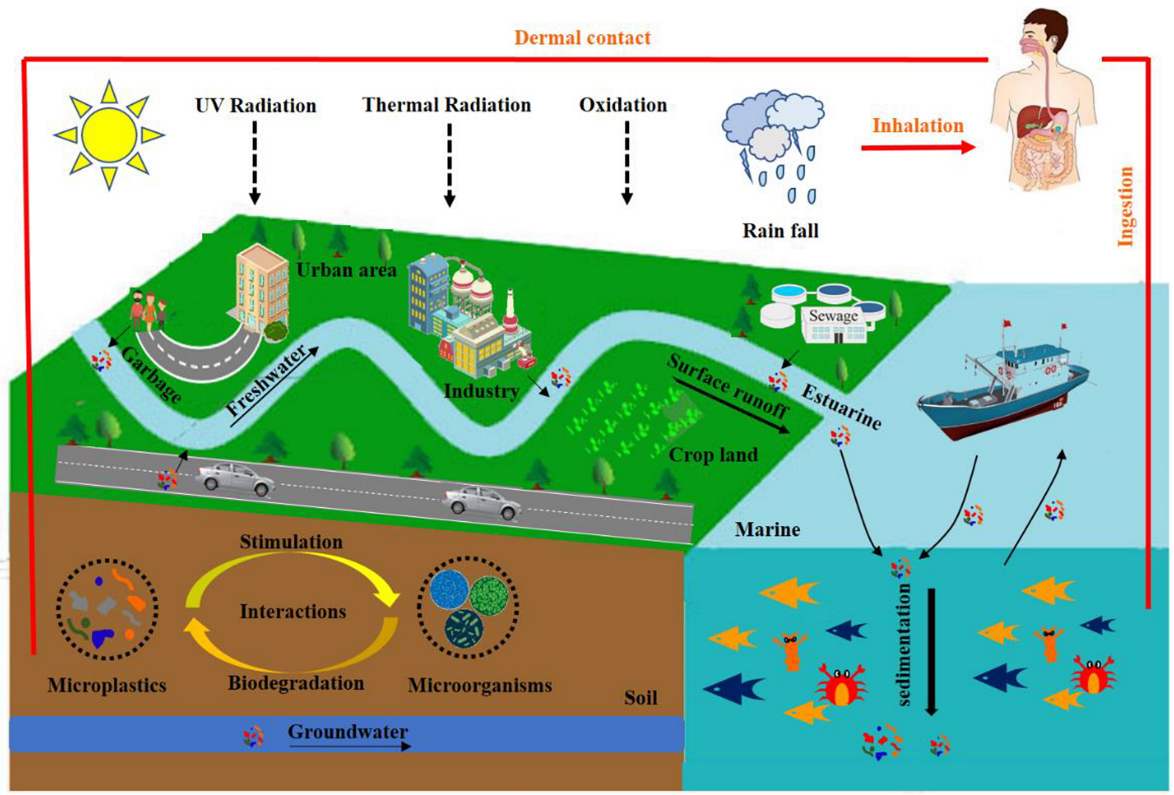

FIGURE 2 | The migration of microplastics (MPs) in the natural environment.

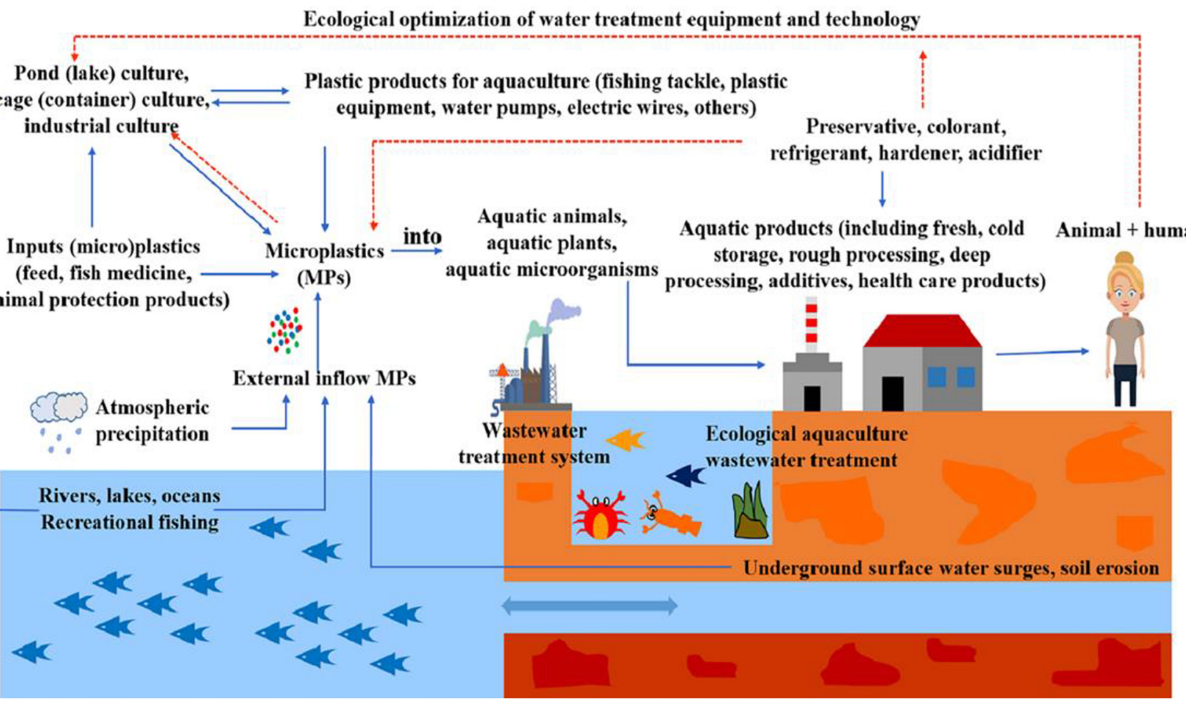

FIGURE 3 | The migration of microplastics (MPs) in aquaculture system (reproduced with permission from Zhou et al., 2021).

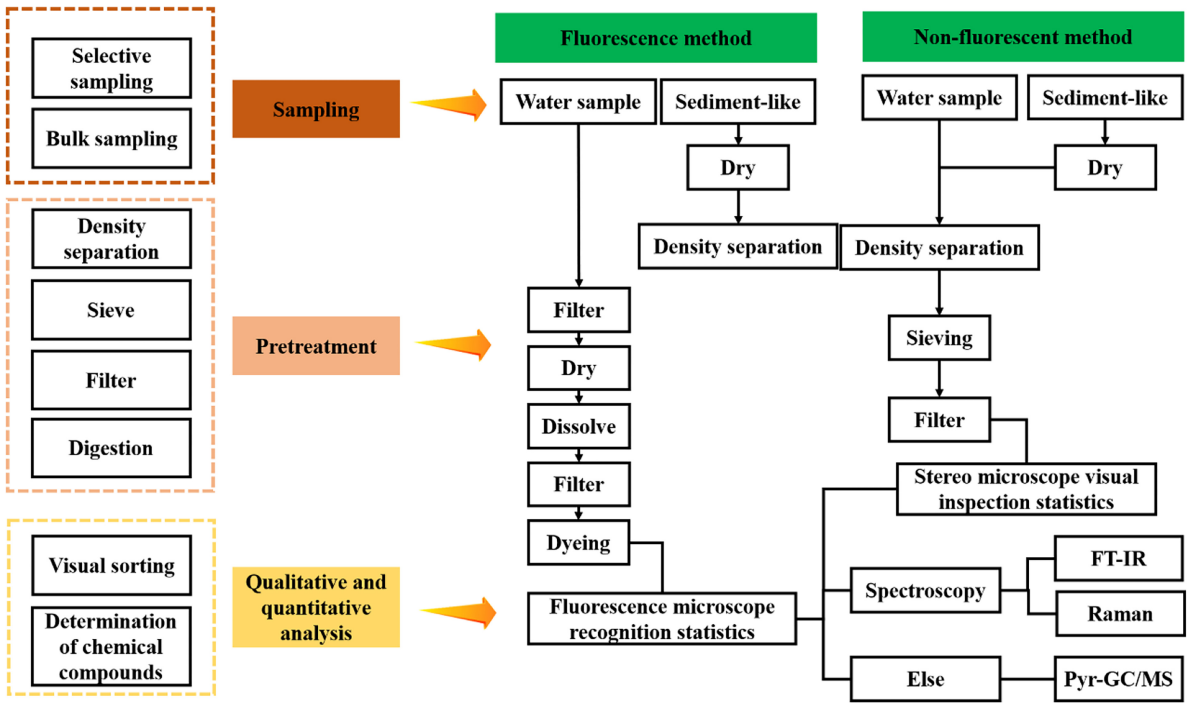

FIGURE 4 | Typical treatment and analysis process of microplastics (MPs).

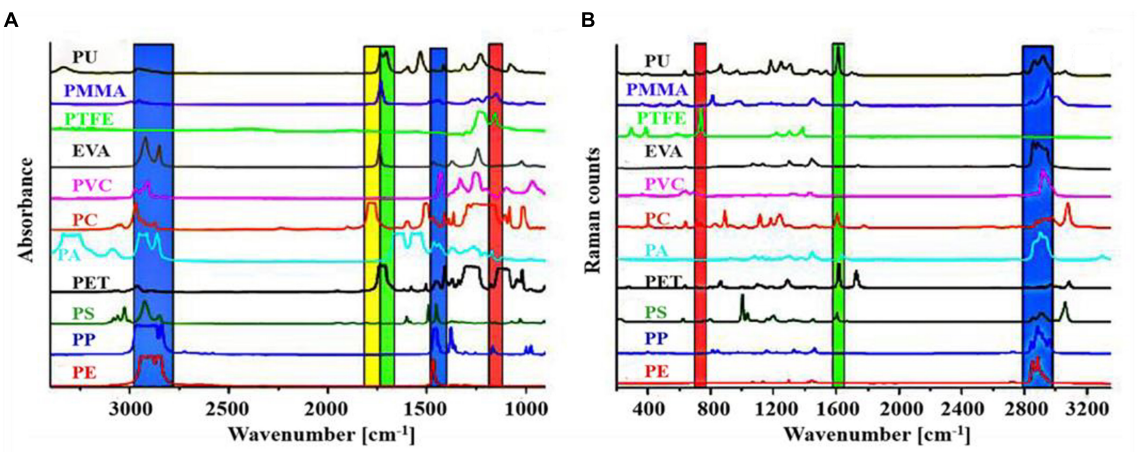

FIGURE 5 | (A) Fourier transform infrared (FTIR) (transmission mode) and **(B)** Raman spectra of various kinds of microplastics (MPs) (reproduced with permission from Käppler et al., 2016).

were applied as the key research criteria. We also examined and retrieved the reference list of each retrieved paper when it is necessary. Overall, research articles about the detection of MPs in the aquaculture environment were involved in this review.

SAMPLING COLLECTION

Collection of samples is the first step of sampling methods of MPs. The most common sampling methods of MPs include selective sampling, volume-reduced sampling, and bulk sampling (Hidalgo-Ruz et al., 2012). MPs are directly extracted from samples by visual identification, which is defined as selective sampling. Volume-reduced sampling indicates that samples are filtered and sieved, and thus the target components can be used for additional analysis at the sampling location. Bulk sampling does not separate components on-site and keeps all samples. For large water body areas, static sampling is carried out at each sampling point, and filtering sample collection is generally selected. For example, Wang et al. (2017) used a precleaned 12 V

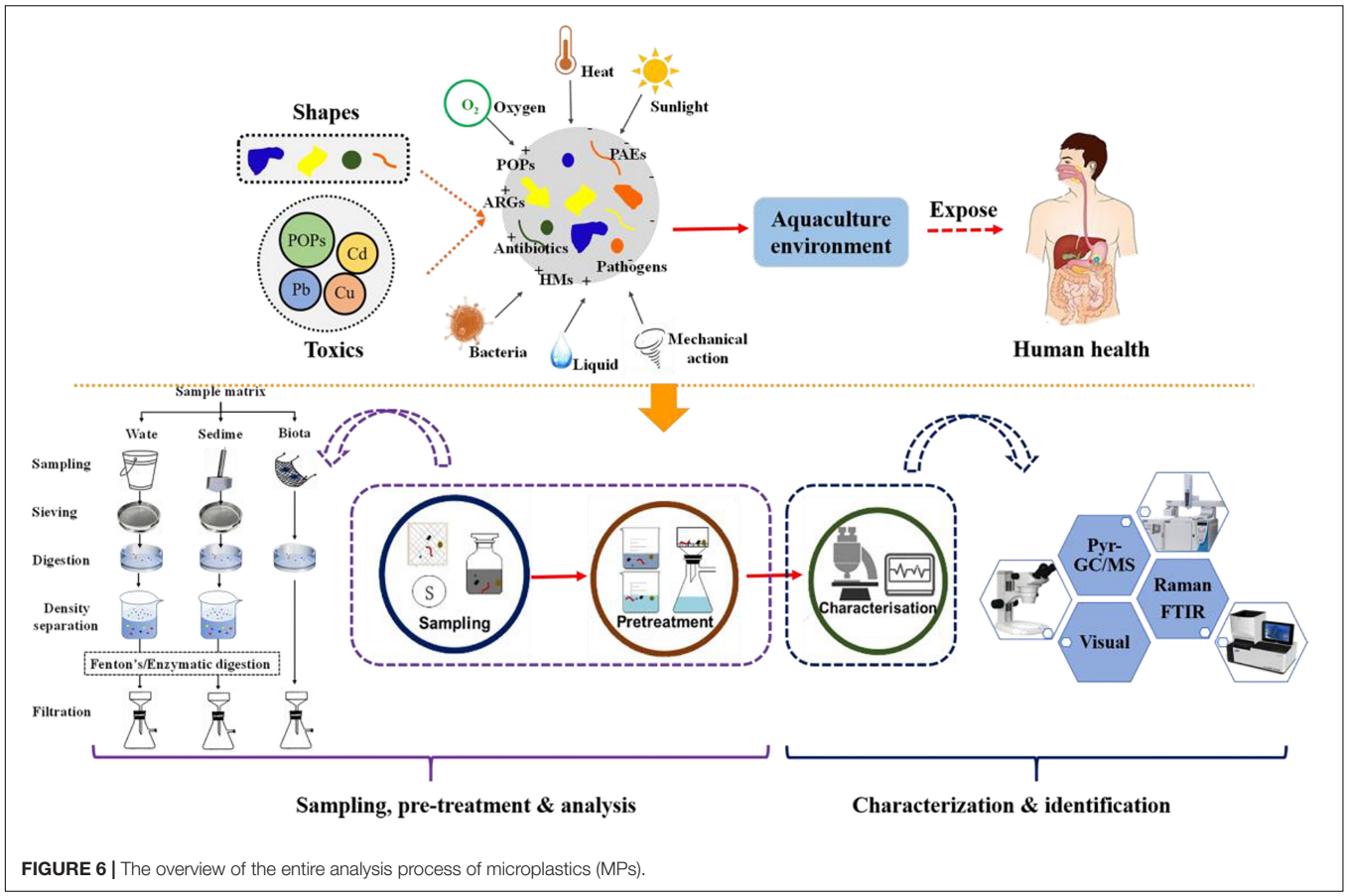

FIGURE 6 | The overview of the entire analysis process of microplastics (MPs).

DC Teflon pump to collect 20 L of surface water sample (0–20 cm in depth) and then the samples obtained were passed through a 50-μm stainless steel sieve.

Microplastics in aquaculture environment can be collected from the water surface or the water column at certain depths (Wang and Wang, 2018). For surface water sampling, neuston nets, and manta trawls are the most commonly utilized tools, but for water column sampling, near-bottom trawls, multiple opening–closing nets, continuous plankton recorders, bongo nets, and plankton nets are the main tools. Some alternative equipment are occasionally applied in surface water or water column sampling for MPs, such as water intake pumps, water collection bottles, or plankton traps. The smaller the size of the mesh, the smaller the particle size of the collected MPs (Uddin et al., 2020). The mesh can be blocked by algae or organisms if the aperture size becomes small. However, when the aperture size was increased, the toxic and small plastic particles may not be collected. In the aquatic environment, the depth of the water should be considered for sampling, and it is generally acknowledged that the abundance of MP surface is higher than that from water depth of 1–2 m. The used trawl nets varied between sampling depths. Samples from surface water used trawl-type sampling devices such as Manta trawl (Ta and Babel, 2020a,b). Bongo nets for mid-water samples and benthic trawls for deeper water from the bottom layer were applied. Typically, the mesh apertures most commonly used for the net are 333–335 μm (Du et al., 2021; Tirkey and Upadhyay, 2021).

SEPARATION AND PURIFICATION

Density Separation

Microplastics tend to float on the water surface due to their lower density than water. The target component and impurities can be separated by density flotation according to their density differences. To be specific, for density separation, the flotation solution was added to the sample, and then MPs were collected through a series of processes such as stirring, mixing, standing, and settling, and finally, the supernatant was separated. The density of most MPs is in the range of 0.80–1.40 g/cm^3 (Ivleva et al., 2017). Generally speaking, MPs with a density of 1.40 g/cm^3 can be obtained using a flotation solution.

The solution of NaCl is used extensively for the separation of MPs because it is cheap, readily available, green, and non-toxic (Eerkes-Medrano et al., 2015; Ma et al., 2020). Other flotation solutions are more efficient but limited to their expensive (SPT, NaI, etc.) or may pose a threat to the environment ($ZnCl_2$, etc.) (Ivleva et al., 2017). In recent years, researchers have developed several MP separators based on density separation to improve their recovery rate by optimizing flotation patterns (Imhof et al., 2012; Nuelle et al., 2014; Karlsson et al., 2017). Moreover,

Imhof et al. (2012) successfully developed a new device that can separate MPs <20 μm. Studies on the salt solutions for polymer separation are summarized in **Supplementary Table 2**.

Filtration and Sieving

Filtering or sieving is the most commonly used approach for separating the supernatant containing MPs from sediment samples and MPs from density separation of water samples. However, there exist some differences. For example, for filtration, the MPs onto filter membrane are obtained using a vacuum pump (Su et al., 2016), and sieving is performed directly onto screens with different pore sizes through gravity (Baldwin et al., 2016). The particle size of MPs collected depends on the size of the sieve and filter apertures. Generally speaking, the pore size (0.45–2 μm) of the filter membrane is smaller than that of the screen (Desforges et al., 2015; Fok and Cheung, 2015; Kim et al., 2015).

However, for the screen, some disadvantages, including severe blockage, its inapplicability to a wide range of sizes, and time-consuming (Mai et al., 2018), still exist. The separation efficiency of MPs can be improved by screens with various aperture sizes. However, the filtration may face the disadvantage that MPs may adhere tightly to the filter membrane, and it is difficult to remove the MPs. To address this issue, Hoffman and Turner (2015) found that C_3H_8O (50%, V/V) was a suitable detergent for the removal of components onto the filter membrane.

Digestion

Environmental samples contain biological materials that are often confused with MPs, resulting in the overestimation of environmental concentrations and increasing the number of MPs subjected to further analysis. The objective of digestion is to remove organic impurities that interfere with the identification of MPs. It is widely used in the preparation of biological, sewage, and sludge samples. Three common methods are used for the sample pretreatment, such as enzymatic digestion, alkaline digestion, and acid digestion. In particular, for enzymatic digestion, which is a time-consuming process, each enzyme works under its optimal temperature and pH condition, which must be monitored and retained through the experiment (Tirkey and Upadhyay, 2021). Wang et al. (2019) first extracted the MPs, then treated the dried sample with 30% H_2O_2 to digest the organic matter, and finally suspended and filtered the MPs by adding a saturated NaCl solution. Anderson et al. (2017) found that the solution containing H_2O_2 and Fe^{2+} was more beneficial to the oxidation of organic compounds. Researchers applied different kinds of digestion solutions including 100 g/L KOH, 10 mol/L NaOH, 30% H_2O_2, 69% HNO_3, HNO_3:HCl (1:1, V/V), and HNO_3:$HClO_4$ (4:1, V/V) to digest MPs, and they concluded that KOH (100 g/L, 60°C) posed severe damage to MPs (Zou et al., 2019).

MICROPLASTIC IDENTIFICATION

Visual Observation

Visual observation is suitable for plastic particles with large size (>1 mm) (Song et al., 2015). MPs are manually identified and sorted before being counted according to their physical characteristics (e.g., color, shape, and hardness). With the help of optical microscopes, electron microscopes, and scanning probe microscopes, it is possible to obtain more information on MPs. For example, scanning electron microscopy (SEM) can provide extremely high magnification and clear images with a resolution of up to 0.1 μm, which is capable of distinguishing MPs from organic particles. Ribeiro-Claro et al. (2017) believed that particles of MPs with various shapes (e.g., irregular polyhedral, hexagons, spheres, and fibers) and sizes can be accurately determined by SEM. Patterson et al. (2019) initially identified MPs from the Tuticorin coast, Gulf of Mannar, Southeastern India, by using stereomicroscope and then verified the composition of the polymers by Fourier transform infrared (FTIR)-attenuated total reflection (ATR). These results showed that polyethylene (PE) fiber (0.25–0.5 mm) is most common in oysters and seawater. Zhu et al. (2019) characterized the morphology of MPs with the assist of a stereomicroscope and then identified some plastics-like using μ-FTIR. They found that MPs were abundant in the Maowei Sea, a typical mariculture bay.

This method has the advantages of simple operation, low cost, and non-toxic. However, there are many substances similar to MPs in the environment, which readily cause misjudgment (Eriksen et al., 2013). Nile red (NR) can enhance the recognition efficiency. For example, Shim et al. (2016) demonstrated that MPs can be readily identified using an NR solution (5 mg/L) in *n*-hexane that can effectively dye plastics.

Fourier Transform Infrared

Three modes including ATR (Klein et al., 2015; Mani et al., 2015; Imhof et al., 2017), reflection (Harrison et al., 2012; Vianello et al., 2013; Ter Halle et al., 2017), and transmission (Frias et al., 2014; Löder et al., 2015) modes are applied for FTIR. FTIR mainly provides chemical bond information of compounds. The generation of peak types and specific spectrum rely on the bond structure. In comparison with the standard library, MPs can be distinguished from other organic and inorganic substances. The composition of MPs can be identified if the matching degree of MPs detected by infrared spectroscopy reaches more than 70% with the standard library.

The FTIR method was also widely used for the characterization of MPs because of simple operation and accurate identification. However, this method is time-consuming (Käppler et al., 2018), and it is easily affected by plastic inhomogeneity and material aging (Zhou et al., 2015). In addition, some plastic particles with size <20 μm cannot be detected. Micro-FTIR not only improved spatial resolution but also enabled the detection of smaller plastic particles. Garcia et al. (2020) used micro-FTIR to identify MPs isolated from fish tissues and found that the main polymers are PET, PES, and PE. Käppler et al. (2016) identified MPs in the environment using Raman spectroscopy and FTIR and compared and analyzed their advantages and disadvantages. They found that the samples were capable of being detected in different spectral ranges for synthetic polymers, as shown in **Figure 5**.

Raman Spectroscopy

The vibrational spectroscopy technique based on the inelastic scattering of light is defined as Raman spectroscopy. Based on the scattering spectra of different frequencies of the incident light, it is possible to obtain the molecular structure of substances (Chen et al., 2020). Characteristic spectral fingerprints can be achieved by detecting the molecular vibration of a sample through Raman spectroscopy, and the composition of the sample is identified by comparing it with a known reference spectrum.

Not only Raman spectroscopy can obtain information on the functional groups on the surface of MPs, but it also allows the observation of local microscopic features (Collard et al., 2015). Raman spectroscopy with a high spatial resolution (<l μm) (Oßmann et al., 2018) is not sensitive to interference signals from water and atmospheric carbon dioxide. Moreover, the fluorescence of the sample influences the Raman spectroscopy signal. Zhao et al. (2015) identified the MPs (polypropylene and PE) in the three estuaries of China using Raman spectroscopy. Collard et al. (2015) proposed a new extraction method based on hypochlorite digestion and ultrasonic treatment for the separation of MPs from membranes. This method is appropriate for the subsequent analysis of Raman spectra. It can avoid fluorescence and allows better identification of artificial particles in fish stomachs.

Raman spectroscopy has the advantages of higher spatial resolution and no interference from water, and some information can be obtained using Raman spectroscopy rather than infrared spectroscopy. However, the fluorescence effect from the pigment, additive, or contaminant in the environmental sample affects the measurements of sample with Raman spectroscopy, and the detection time of Raman imaging is remarkably higher than FTIR imaging.

Pyrolysis Gas Chromatography/Mass Spectrometry

Currently, pyrolysis gas chromatography/mass spectrometry (Pyr-GC/MS) is the most commonly applied method for the characterization of polymers. In the Pyr-GC/MS technique, the polymer was pyrolyzed under an inert atmosphere, and then the pyrolyzed products were fed to GC-MS, where GC separates them and pyrogram is formed. The pyrogram of the unknown samples is compared with developed or available reference pyrogram to know the composition of the polymer mass under investigation (Käppler et al., 2016). This technique has the advantages of small sample amount, qualitative and quantitative analyses, no additional reagents, and so on, but it requires stringent experimental conditions (Dekiff et al., 2014). Fischer and Scholz-Böttcher (2017) used the Pyr-GC-MS method to simultaneously identify and quantify many typical MPs including PE, PP, PS, PVC, PA6, PMMA, PET, and PC. Hermabessiere et al. (2018) characterized MPs with Pyr-GC/MS by optimizing the experimental conditions, and thus the detection signal was enhanced and detection time was shortened greatly.

TDS (thermodesorption)-GC/MS first heats the sample up to 800°C under inert conditions such as N_2 and then detects the sample composition by GC-MS (Li et al., 2017). It analyzes sample volumes of up to 100 mg, but it is limited to qualitative analysis. Dümichen et al. (2015) identified and quantified the characteristic decomposition products of spiked PE in complex environmental samples by combining thermogravimetric analysis coupled to solid-phase extraction (TGA-SPE) and TDS-GC/MS. This method with fast sample cleanup does not require any visual classification. However, it is only suitable for the samples that can be easily cleaned and for known polymers. In summary, the identification and quantification of MPs in the aquaculture environment by these analytical techniques are exhibited in **Supplementary Table 3**.

QUALITY ASSURANCE AND QUANTITY CONTROL

During the entire sampling process, quality assurance and quantity control are vital for data accuracy. Researchers should wear 100% cotton clothes and latex gloves. The collected samples are sealed in polythene bags and aluminum foil to avoid the interference of atmospheric MPs (Noik and Tuah, 2015). Since MPs are ubiquitous in the air, a series of blank tests must be conducted to minimize the impact of environmental pollution. The MPs collected on-site should be compared with those in the standards library (Ng and Obbard, 2006).

During the on-site sampling process, a procedure blank and standard addition blank should be prepared (Hanke et al., 2013). Non-plastic materials such as glass bottles should be used during the detection process. The glass bottle should be prewashed in a nitric acid bath and then rinsed with ultrapure water three times.

CONCLUSION AND FUTURE RECOMMENDATIONS

This review briefly summarized the sampling, separation, and purification of MPs in the aquaculture environment (**Figure 6**). In addition, we also compare the superiority and limitations of various characterization techniques. Nonetheless, some knowledge gaps still exist. Future recommendations for the detection of MPs are as follows.

a) During the sampling process, for surface water in lakes or oceans, manta trawls or nets are suitable. For the separation of MPs, density separation is recommended. For the purification of MPs, the Fenton reaction that can effectively oxidize the organic compounds was recommended.
b) It is very difficult to quantify and identify MPs with a single method. The combination of various techniques is recommended for the identification of MPs.
c) MPs can be detected using electrochemical method because surface of MPs with the similar properties to colloid is easy to be charged in the aqueous environment.
d) Establishing standards for quantitative and qualitative analyses of MPs is extremely essential.

AUTHOR CONTRIBUTIONS

SX and YX: conceptualization. XS: resources. SX and HD: data curation. SX and XS: writing—original draft preparation. JW and SX: writing—review and editing. JW: visualization, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of this manuscript.

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Development of a Binary Digestion System for Extraction Microplastics in Fish and Detection Method by Optical Photothermal Infrared

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Accumulating evidence indicates that aquatic organisms ingest microplastics (MPs), which may be a threat to essentially the entire global ecosystem. In current detection methods, even in cutting-edge nanoplastic technology, a major challenge for detecting microplastics (MPs) in aquatic organisms is removing complex biological matrices, such as fat. Herein we report combining HNO_3 and H_2O_2 to form a binary digestive reagent system to determine MPs in biological tissue. With insights obtained from a Gaussian model, the adding manners of two reagents were discussed. Thus, in the final protocol, we mixed MPs and tissue with 20 mL of 30% (v/v) aqueous H_2O_2, 10 mL 0.5 M NaOH,1 mL 5 mM Fe^{2+}, and 40 mL 11.5% (v/v) aqueous HNO_3, in sequence at different time intervals. What's more, sodium dodecyl sulfate (SDS) and ultrasound—alone or together—were explored to solve the problem of removing fat residues and thus membrane blockage during filtration. In this paper, we used the O-PTIR microscope to verify the feasibility of the protocol. Compared with traditional detection methods, the O-PTIR spectroscopy can significantly improve the lateral resolution, down to sub and super-micrometer, and the ability to quickly obtain high spatial resolution far-field non-contact infrared spectra, which provide a novel method for qualitative analysis of MPs. In field applications, in our attempt, the fixed wavenumber image by O-PTIR can realize sub and super-micrometer MPs *in situ*, far-field measurements. The present method is highly efficient, and facilitates the identification of plastic particles.

Keywords: microplastics, binary digestion system, O-PTIR, detection, extraction method

INTRODUCTION

Discarded bulk plastic products can eventually yield microplastics (MPs; < 5 mm in diameter), which are hazardous to aquatic environments (Collard et al., 2019; Gedik and Eryasar, 2020). Accumulating evidence indicates that there are plastic debris in hydrobiontes, such as zooplankton, fish, mussel, and crab (Wright et al., 2013; Watts et al., 2014; Hossain et al., 2019;

Süssmann et al., 2021). MPs may essentially threaten the entire global ecosystem, including human consumers, through the consumption of seafood products (Dehaut et al., 2016; Gedik and Eryasar, 2020; Süssmann et al., 2021).

To determine the ecological threat of MPs, the quantity of MPs in marine organisms should be known. Over the last 2 years, the detection and extraction of nanoplastics have been an especially active line of research (Nguyen et al., 2019; Zhou et al., 2019; Merzel et al., 2020; Ribeiro et al., 2020). Combining pyrolysis with gas chromatography–mass spectrometry (PY–GC/MS) has facilitated quantitation of nanoplastics in complex biological tissues for the first time (Zhou et al., 2021). However, the abundance of organic matter in biological matrices interferes with the extraction and PY–GC/MS determination of nanoplastics, necessitating adequate digestion of the biological matrices (Zhou et al., 2021).

Field investigations of MPs remain challenging. Although it is worth mentioning that nanoplastics are still in the research and development stage (Merzel et al., 2020), far from field applications, yet MPs detection is a part of routine environmental monitoring by relevant departments at home and abroad (Gorokhova, 2015). However, current detection methods are low-efficiency and high-loss, which substantially affect the efficiency of the work and the reliability of the experimental results (Eerkes-Medrano et al., 2015; Rodrigues et al., 2018; Süssmann et al., 2021). Both nanoplastics and MPs quantitation in environmental samples would benefit from an efficient means of digesting the biological matrix, especially the fat (Collard et al., 2019). However, to date, no highly efficient, standard method is available that completely removes tissues without affecting the integrity of the plastic polymers.

To improve the efficiency of quantitating MPs in fish, researchers have developed and applied various methods for digesting the soft tissues of marine organisms (Loder et al., 2017). The basic idea of such methods is to eliminate the biological materials and tissues that mask the MPs (Budimir et al., 2018; Collard et al., 2019; Nguyen et al., 2019). Four major classes of digestion agents to eliminate organic materials are acids (Dehaut et al., 2016; Karami et al., 2017), alkalis (Karami et al., 2017; Schirinzi et al., 2020), oxidative agents (Avio et al., 2015; Collard et al., 2015; Karami et al., 2017), and enzymes (Cole et al., 2014; Loder et al., 2017).

Some limitations of digestion agents are as follows. In acid treatment, the acid is generally in an especially high concentration, and the temperature is high, which may eliminate the organic tissue but may not be compatible with plastic preservation (Cole et al., 2014; Nguyen et al., 2019). For example, we demonstrate herein that polyamides in $HClO_4$ exhibit various degrees of corrosion or even complete degradation (Claessens et al., 2013) used HNO_3 for MPs extraction from mussels, wherein the treatment destroyed some of the fibers. The use of H_2O_2 has been limited because of the incomplete digestion of soft tissue fractions (Mathalon and Hill, 2014). Enzymatic digestion has been impeded by high prices and poor bone digestion (Simonsen et al., 2011). In summary, the research indicates that digestion based on a single type of digestive agent is insufficient.

In this paper, changes in the molecular integrity of plastic polymers across various treatments and field sample detection are analyzed by Optical Photothermal Infrared (O-PTIR) microscope. Traditional microplastics detection mostly uses Fourier transform micro-infrared (μFTIR) and micro-Raman spectroscopy (μRM). The μFTIR or μRM, in actual operation, requires complicated sample processing. The boundedness of μFTIR depends on the infrared wavelength, which is only 10–20 μm. μRM is impressionable to fluorescence interference, and the resolution is at least 1 μm (Kansiz et al., 2020; Olson et al., 2020). It can't characterize the chemical changes of the plastic surface at the sub-micron scale, nor can it identify individual nanoplastics (<1 μm) meanwhile making it very difficult to comprehensively detect and identify the type and composition of microplastics. A recent development on the O-PTIR, can significantly improve the lateral resolution, down to sub and super-micrometer. The unique pump-probe system architecture also facilitates submicron simultaneous μFTIR + μRM from the same spot with the same spatial resolution (Kansiz et al., 2020). O-PTIR is a still-emerging technique, the ability to rapidly obtain far-field non-contact μFTIR spectra at high spatial resolution facilitates the chemical identification of small organic contaminants that are not possible to measure with conventional μFTIR micro spectroscopy, which has been applied in such atmospheric particles, organic particles analysis and formation mechanism of micro-nano plastics (Olson et al., 2020; Fang et al., 2021; Su et al., 2021). In the field of microbiology, O-PTIR designed for (3D-) imaging living cells and organisms (Zhang et al., 2016), especially regarding fixed wavelengths images with the ultra-high image resolution (Ivleva, 2021). However, it has relatively few applications in MPs detection, limited studies indicator may be a better technique for detecting environmental plastics (nano) than other μFTIR techniques (Barrett et al., 2020; Merzel et al., 2020).

Thus, we optimized the fish tissue digestion protocol by designing a unitary digestion system composed of a series of single solutions (an acid, or a base, or an oxidizer) to find the most suitable digestion candidates and concentrations. On the basis of the corresponding results, we chose HNO_3 and H_2O_2 to form a binary digestion system. We performed chemical calculations with a Gaussian model to explain the reaction mechanism of digestion, guiding the design of a binary digestion system. To solve the problem of fat digestion, we tested the efficacy of ultrasonic treatment and sodium dodecyl sulfate (SDS). The O-PTIR was conducted to verify the feasibility of the protocol. What's more, we apply the fixed wavenumber image by O-PTIR in recovery experiments and in the field samples to explore its application in non-contact, far-field measurements that down to sub and super-micrometer plastic detection.

MATERIALS AND METHODS

Samples and Reagents

Fish Samples

Fish were brought from a local market in Shandong Province, collected from the Yellow Sea (detailed information of the fish

species is provided as **Supplementary Material**). The digestive tract characteristics of the fish we used are different, such as the fat content and the thickness of the digestive tract. Furthermore, because of different feeding habits the intestinal contents were quite different. For example, some contents were mainly small fish, shrimp, crab, and other arthropods; and some were mainly aquatic algae. Because of these differences, even if one uses the same experimental protocol, each kind of fish's digestion effect (the experimental result of the designed digestion experiment) is quite different. To eliminate such errors as much as possible, the tissues used in our study were derived from a mixture of all the fish digestive tracts and subsequently stored at −20°C.

Plastic Preparation

The 11 types of plastics tested in this study were supplied by Shanghai Yichen Industrial Co., Ltd. These include acrylonitrile–butadiene–styrene (ABS), acrylonitrile–styrene copolymer (AS), high-impact polystyrene (HIPS), high- and low-density polyethylene (HDPE and LDPE, respectively), polyamide (PA), polyethylene (PE), polyethylene phthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC). The following plastic samples differed in color: milky white (ABS, PE, LDPE, and HDPE), transparent (AS, PS, and PVC), semitransparent (PP, HIPS, and PA), and white (PET).

Our initial investigations used particle sizes from ∼3 to 4 mm in diameter. After confirming the digestion protocol, we used MPs (>60–500 μm in diameter) for the recovery experiments.

Subtle effects of the digestion system on the plastics were taken into consideration. A cutting knife was used to make an irregular cross-shaped incision on the surface of the particles. After marking, the particles were rinsed with Milli-Q water and dried to a constant mass at room temperature (RT).

For the recovery rate experiments, the aforementioned 11 plastic particles were polished with a pulverizer (HCP-100) for ∼1 min and a temperature below 40°C. The ground plastic particles were passed through a series of sieves with different mesh sizes (60, 100, 200, and 500 μm) and sorted into four grain size classes (<60, 60–100, 100–200, and 200–500 μm). The samples were measured with a microscope for further size determinations.

Chemicals

Hydrogen peroxide (H_2O_2), nitric acid (HNO_3), perchloric acid ($HClO_4$), sodium hydroxide (NaOH), and ferrous sulfate ($FeSO_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. SDS was supplied by Kalmar-reagent. Aqueous solutions of NaOH (0.5 M), HNO_3 (11.5% v/v), $FeSO_4$ (5 mM), and SDS (150 g/L) were prepared in Milli-Q water. All the digestion solutions, including the acid digests ($HClO_4$ and HNO_3) and oxidizing solutions (H_2O_2), were mixed with Milli-Q water to obtain the following proportions solutions: 1:0, 4:1, 2:1, and 1:1 (v/v), which is the high-concentration group [69, 55.2, 46, and 34.5% (v/v), respectively]; and 1:2, 1:3, 1:4, and 1:5 (v/v), which is the low-concentration group [23, 17.25, 13.8, and 11.5% (v/v), respectively]. The filter was obtained from Yancheng Prich Laboratory Instrument Co., Ltd. We used a filter of the following specifications: pore diameter, 5 μm; and filter diameter, 50 mm.

Digestion Protocol

To efficiently digest fish tissue without destroying the plastic polymers, we systematically investigated various protocols. In this paper, we investigated the effect of different kinds of digestion solution systems. We first studied a typical reagent at various concentrations; the results were not satisfactory. On the basis of the preliminary conclusions from typical single-reagent experiments, we continued to investigate the experimental effects of various combinations of digestion reagents. We accordingly differentiated the experimental batches as unitary and binary systems, respectively. We also optimized the experimental protocol by supplementing it with surfactants and sonication to overcome the fat-digestion problem to improve the experimental efficiency. We also used recovery experiments and field application to test whether the research protocol developed in this paper is feasible.

Validation of Method

Mixtures of fish digestive tracts were thawed at RT for 30 min. Samples of fish tissues (3 g ± 0.01 g) were weighed and spiked with three plastic particles each of ABS, AS, PA, PP, PE, PET, PS, PVC, HIPS, HDPE, and LDPE, the experiment was triplicate for each group. Our research investigated a series of digestion protocols to establish a high-efficiency digestion method, evaluated in four aspects: efficacy of digestion, impact on plastic particles, recovery rate, and field application.

Efficacy of Digestion

The effects of the digestion were determined by comparing photos taken before and after the reaction. Note that because the digestion solution bubbled as soon as the reagent was added, it was not possible to photograph the solution. Therefore, an equal volume of Milli-Q water was used to represent the digestive reagent. A corresponding photo was taken to represent the initial state. Then the Milli-Q water was poured out and replaced with a digestive reagent. At the end of the digestion, a photo was taken of the final state. Specifically, the mixtures were first added to 60 mL of Milli-Q water (start point) in a breaker to take photos to represent the state of the starting point of digestion. Next, the water was replaced by the same volume of various types of digestion solutions, and the digestion was maintained at RT for 12 h, if necessary, through ultrasonic (JP-040S, 180W) and/or SDS treatment (end point). At the ending point of digestion, the visual examination was performed and photographed, comparing with the starting point photo the initial digestion effect can be recorded. And the efficiency of the digestions was measured by the frequency of changing the filter membrane and the filtration duration.

Impact on Plastic Particles

The impact of digestion on the plastic particles was investigated in terms of their shape and chemical composition. The MPs were removed from the beaker, washed with Milli-Q water 3 × to

remove the impurities attached to the surface, and dried at RT for further examination.

In the initial investigation (sections "Unitary Digestion System," "Binary Digestion System," and "Protocol Optimization for Dispersion of Fat Problem"), the particles before and after the tests for the various digestion protocols were analyzed by a visual examination to check for signs of subtle changes in the cross marker (section "Plastic Preparation") which used by the digestion treatments. For unbiased comparisons, the observation point—set exactly at the intersection—was analyzed with a Raman spectrometer (Horiba, LabRAM, HR Evolution) with a 5 × Olympus objective.

The O-PTIR spectra were collected on the MP surface with an effective spectral resolution of 2 cm^{-1} and co-averaged for five spectral scans.

In field application investigations, the O-PTIR microscope was used to identify plastic particles. It is worth noting that the O-PTIR images were obtained at a 500-nm step size and 100-nm step resolution by tuning the QCL device to the frequencies that correspond to the specific vibrations of extraneous materials at various wavenumbers (1,023, 1,075, 1,641, and 1,725 cm^{-1}). The system was equipped with IR laser settings where the wavenumber was 1,247 cm^{-1}, the IP power was 4%, the pulse rate was 104.63 kHz, the duty cycle was 5%, and the pulse width was 480 ns. Regarding the probe settings, the probe power was 5% and the detector gain was 2 ×.

Determination of the Recovery Rate

In the recovery experiments, we counted the recovery rates for four particle sizes, and each particle size was spiked with 30 particles. The digests were then filtered with filter paper. The quantity of the MPs on the filter membrane was used to calculate recovery percentages using Equation 1:

$$Recovery(100\%) = \frac{R_a}{R_i} \times 100\% \quad (1)$$

where R_a = number of MPs recovered; and R_i = initial number of MPs.

Field Application

Fish species were collected from the Bohai Sea north of the Shandong Peninsula, and processed with the digestion method previously described and analyzed for MPs. The O-PTIR microscope was used to identify plastic particles.

Contamination Prevention

During all sample treatment and analysis steps, precautions were taken to avoid background plastic contamination. All equipment and glassware were washed with a commercial dishwashing liquid, and thoroughly rinsed with Milli-Q water before use. To avoid contamination of the samples by airborne fibers and other particles, vials were capped with aluminum foil during digestion. Within the laboratory, a cotton lab coat, nitrile gloves, and face masks were worn throughout the experiments.

RESULTS AND DISCUSSION

Unitary Digestion System

We investigated the digestion of 11 types of plastic particles—including ABS, PE, LDPE, HDPE, AS, PS, PVC, PP, HIPS, PA, and PET—and the effects of the plastic. Fish tissues were treated with a single solution at various concentrations ($HClO_4$, HNO_3, and H_2O_2; individually) at RT. Different protocols had different effects on the plastic particle integrity and fish tissue digestion. In terms of fish tissue digestion, high concentrations of acids were superior to those of low concentrations. Different types of digestion solutions had different effects on plastic particles (**Supplementary Figures 1–3**); we next elaborated on the effects of various treatments.

Acid Treatment

We explored each digestion reagent separately. Accordingly, we mixed $HClO_4$ (70% v/v) and HNO_3 (69% v/v) with water separately in various proportions, as detailed in section "Chemicals." One can visually observe the digestion of fish tissue and plastic particles in an aqueous acid solution. During digestion as per $HClO_4$, most of the plastic particles corroded yet the tissues were completely dissolved at various acid concentrations (**Supplementary Figure 1**). The use of $HClO_4$ in high concentrations is detrimental to nylon fibers (Claessens et al., 2013). We suggested that $HClO_4$ strongly corrodes plastics, rendering it unsuitable as a digestive agent. Regarding HNO_3, the corrosion of plastic particles by high concentrations of HNO_3 was obvious; the damage to PA was the most drastic. Low concentrations of HNO_3 had no obvious effects on the plastic particles except for PVC; only PVC had bubbles on its surface. However, fish tissue was poorly digested (**Supplementary Figure 2**). Among the plastics, we observed the most destruction for PA, which was completely destroyed by aqueous HNO_3 at proportions of 1:0, 2:1 and 4:1 v/v. As the concentration of HNO_3 decreased, PA initially dissolved, softened at lower concentrations, and at sufficiently low concentrations was undamaged. The results for other plastics were similar; that is, they retained their original shape when the concentration of HNO_3 was sufficiently low.

Our results are consistent with the literature of completely dissolved PA by acid treatment (Enders et al., 2017). Other polymers were affected to lesser extents, mainly color leaching and softening. Karami et al. (2017) digested biological materials in concentrated HNO_3 and produced optimum digestion efficiency only at RT. Concentrated HNO_3 had the most destructive effect on melted LDPE and PP fragments, and altered the color of most of the tested plastic polymers. Dehaut et al. (2016) recommended against using acid for studies of MPs because of its degradation of polyamide and its yellowing tendency to plastics; acid digestion may result in miscataloging of MPs during the optical examination. Thus, the use of acid to digest fish tissues for MP surveys is not straightforward. If acid treatment is to be implemented in practice, the experimental parameters (such as concentration and temperature) should be optimized or refer to Enders et al. (2017) to combine the treatment with other digestion solutions.

Oxidizing Treatment

Compared with the results of acid treatment, the results from H_2O_2 treatment were mild. The fish tissue gradually dissolved as per visual observations at the H_2O_2 proportions of 1:0 and 4:1 (v/v). The tissue was less well digested in accordance with decreasing H_2O_2 concentrations. Karami et al. (2017) reported that H_2O_2 did not completely dissolve biological material at either RT or 40°C. Li et al. (2015) dissolved bivalve tissues in 30% (v/v) aqueous H_2O_2, incubated the mixture at 65°C for 24 h, and then digested the mixture at RT for 24∼48 h. The tissues dissolved but the quantity of tissues is an important parameter. Cole et al. (2014) used H_2O_2 (35% v/v) at RT for 7 days, but did not observe much digestion. Avio et al. (2015) reported incubation with H_2O_2 (15% v/v) at 50°C overnight. Thus, temperature is an important parameter in digestion. Although increasing the temperature results in a greater extent of tissue digestion, there will be deterioration of the plastic particles.

In summary, the use of various concentrations of H_2O_2 was promising in that there was almost no effect on the plastics. Nevertheless, low concentrations of H_2O_2 poorly digested fish tissues, especially the digestive tract (**Supplementary Figure 3**). Therefore, the application of H_2O_2 as a digestive solution for extracting MPs from fish tissues is limited and requires improvement.

Binary Digestion System

In aforementioned unitary digestion system, $HClO_4$ dissolves fish tissue yet also deteriorates plastics. Low concentrations of HNO_3 should be used to maintain plastic integrity. H_2O_2 is comparatively mild and less corrosive to plastics than the aforementioned acids but poorly digests fish tissue. We also investigated experimental parameters such as time and temperature with a single digestion solution system, but the results were still unsatisfactory. Thus, a binary digestion system was necessary.

On the basis of the aforementioned results of the unitary digestion systems, considering the corrosivity of $HClO_4$, we chose HNO_3 and H_2O_2 for investigations of a binary digestive system (**Supplementary Figures 4**, **5**). In accordance with the experimented results under a single condition, we chose an 11.5% (v/v) concentration of HNO_3 (not expected to damage plastics). We thus added 11.5% (v/v) HNO_3 and 30% (v/v) H_2O_2 in accordance with the following proportions (all v/v) 1:0, 4:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5. We added the reagents either in combination or in sequence.

In accordance with the method of adding the two digestion reagents—i.e., whether to combine or separate, the results are discussed separately. The difference between these two methods of addition is whether the reagents are mixed before digestion or instead added in sequence.

Added in Combination

We first mixed 11.5% (v/v) HNO_3 with 30% (v/v) H_2O_2 in various proportions to form a mixture, and the tissue and plastic particles were digested by the added mixture. The digestion protocol did not substantially affect the plastic polymers; we observed no changes in shape. In accordance with increasing proportions of HNO_3, the digestion of the fish digestive tract epidermis gradually worsened. There were tissue residues in all the solutions except at the proportion of 1:0 v/v, especially in the low-concentration groups (**Supplementary Figure 5**).

It is odd that none of the binary digestive systems (HNO_3 and H_2O_2 added in combination) were as effective as any of the unitary digestion systems (**Supplementary Figures 1–3**). We hypothesized that these results for the binary digestion system may be because of a chemical reaction between HNO_3 and H_2O_2; after the reaction, the concentrations of H_2O_2 and HNO_3 decrease, resulting in an inferior digestion. To test this hypothesis, we calculated the reaction between H_2O_2 and HNO_3 (Equation 2) using Gaussian modeling. **Figure 1** shows the change in energy and material over the course of the reaction. The hypothetical reaction process and intermediate products in the reaction are shown in **Figures 1A,B**. As for **Figure 1A**, the A represents reactants HNO_3 and H_2O_2, which are optimized before calculation; as the reaction progresses, HNO_3 bond breaks and formed intermediate product B; the C represents the TS1 (NO_2, H·, OH·, and -O_2H) which was the transition state of the reaction, the D was the product of phase 1 which forming the final product NO_2, H_2O and the reactant of phase 2. For **Figure 1B**, the A represents the reactants OH· and -O_2H, which undergo chemical bond breaking and recombination (B, C) to form the final products O_2 and H_2O. The specific reaction path is shown in **Figure 1C**. The modeling results indicate that H_2O_2 and HNO_3 react under certain conditions, which will affect the concentration of the solution in the digestion system and ultimately affect the experimental results.

$$2HNO_3 + H_2O_2 = 2NO_2 \uparrow \ + O_2 \uparrow \ + 2H_2O \qquad (2)$$

Added in Sequence

Accordingly, to avoid a chemical reaction between HNO_3 and H_2O_2 that hinders digestion, we added 11.5% (v/v) HNO_3 and 30% (v/v) H_2O_2 in succession, in various proportions for tissue digestion. We first added H_2O_2 and then HNO_3. These treatments did not affect the polymers, whereas compared with the results in section "Added in Combination" the efficiency of the tissue digestion improved; large masses of tissue were not evident (**Supplementary Figure 6**). The proportion of 2:1 (v/v) afforded the best experimental results.

Although 11.5% HNO_3 and 30% H_2O_2 separately added in two steps can partly avoid both direct mixing reactions. The experimental results also indicated that, compared with directly adding, the digestion effect of tissues was significantly improved (**Supplementary Figure 6**), the fish tissues were not completely dissolved. The digestion efficiency was not as ideal as possible. This may be because of residual H_2O_2 in the digestion system, similarly to why the combination treatments were ineffective. Thus, we sought to decrease the quantity of residual H_2O_2. Accordingly, on the basis of the Fenton reaction principle, we added Fe^{2+} and NaOH after digestion by H_2O_2, yet before digestion by HNO_3. Other studies have also shown evidence that both enzymes and Fenton's reagent could achieve efficient, rapid, and inexpensive digestion of organic matter and other materials. Dissolving biological matrices to analyze the gut contents of

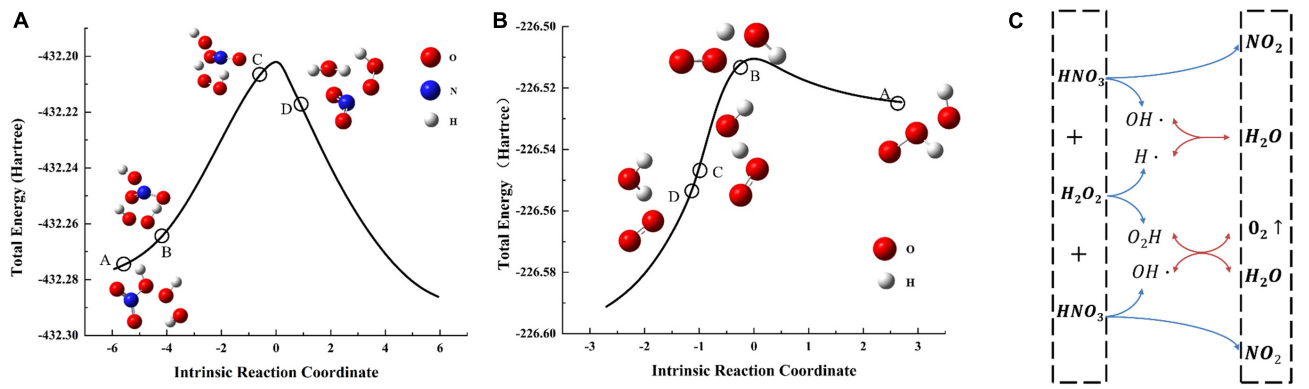

FIGURE 1 | Energy change over the course of the reaction: **(A)** reaction path 1, **(B)** reaction path 2. Bonds are broken and formed, affording substances in different reaction phases which shown in A, B, C, D of panels **(A,B)**. Panel **(C)** showed the specific reaction process of two phases and the intermediate products in the reaction process. (blue line) Reaction path 1, (red line) reaction path 2.

freshwater fish has literature precedent (Rodrigues et al., 2018; Collard et al., 2019).

After adding Fe^{2+} and NaOH, digestion was slightly improved compared with simply adding H_2O_2, perhaps because NaOH and Fe^{2+} catalyze digestion. Subsequent digestion as per H_2O_2–HNO_3 was considerably improved compared with the simple sequential H_2O_2/HNO_3 digestions, in which HNO_3 is used to react with the residual H_2O_2 rather than digestion. The plastics were not damaged.

Thus far, we had obtained optimum results for the following binary digestion: add 30% (v/v) H_2O_2 and allow to stand for 1 h; then add 10 mL of 0.5 M NaOH and 1 mL of 5 mM Fe^{2+}, and allow to stand for 4 h; then add 11.5% (v/v) HNO_3 and allow to stand for 6 h. Nevertheless, residual fat remained in the filter membrane, which decreased the filtration efficiency and recovery. We thus needed to optimize the protocol for dispersion of fat problem.

Protocol Optimization for Dispersion of Fat Problem

As aforementioned, sometimes the fat residue clearly affected the filtration speed. Previous study methods for overcoming the fat problem include high-temperature treatment, surface activation, and ultrasound. We studied only ultrasound and surface activation in this work because of corrosivity that resulted from our preliminary high-temperature studies. Sometimes, our initial experiments with ultrasound were unsuccessful. We observed granular fat residues after 1 h (**Supplementary Figure 7A**), despite the positive results reported by Lenz et al. (2015) within 10 min. This may be because of differences in the type of fish.

Sodium dodecyl sulfate is a common surfactant that has been used to disrupt organic matrices in marine MP research (Loder et al., 2017; Rodrigues et al., 2018). Lenz et al. (2015) revealed that applying SDS can facilitate the dissolution of coatings from hydrophobic surfaces. Budimir et al. (2018) studied Baltic herring purchased from their local supermarket as a fish sample. They added NaOH and SDS to a glass jar of fish tissue, and kept the mixture at 50°C for 24 h; their good dissolution results may be because of the low-fat content of herring. Our experiments indicate that the protocol of Budimir et al. (2018) is sufficient for the digestion of most fish samples, but not for fish that are high in fat (**Supplementary Figure 7B**).

Ultrasound by itself was insufficient and SDS in itself was insufficient, and therefore we combined the two methods for disrupting fat. We conducted experiments on the digestive tract, which contains a substantial quantity of fats. The fat gradually dispersed and the droplets' volume decreased. The effect was remarkable (**Supplementary Figure 8**). Thus, we combined our results into a binary resolution system.

Accordingly, we obtained an optimal protocol for extracting spiked MPs from tissues. First, thaw fish tissue to RT, then weigh ~3 g ± 0.01 g on an analytical balance. Dissolve the tissue and MPs in 20 mL of 30% (v/v) aqueous H_2O_2, and allow to stand at RT for 1 h. Then, add 10 mL 0.5 M of aqueous NaOH and 1 mL of 5 mM aqueous Fe^{2+}, and let stand for 4 h. Next, add 40 mL of 11.5% (v/v) aqueous HNO_3 and let stand for 6 h. The final step is to remove the remaining fat particles: first add 16 mL of 150 g/L SDS, then apply ultrasound for 1 h, and then let stand for a few minutes for subsequent filtration (**Figure 2**).

Verifying the Protocol

Evaluation of the Digestion Efficacy

We developed an efficient fish digestion protocol by visual inspection; it is difficult to conceive of a clear-cut quantitative metric. The literature indicates that the gravimetric method is pertinent to evaluating the efficiency of digestion, but compared with our method the weight change is not the most important factor. We found that the most important factor was the type of residue rather than the weight that affected the final test result, such as digestive tract dyspepsia. However, compared with the residual fat particles after digestion, the undigested digestive tract did not considerably interfere with the test results; the residual digestive tract can be cleaned on its surface and removed without participating in filtration. Therefore, by observing the digestion of fish tissues with unaided eyes, we can preliminarily test whether our digestion plan of fish tissues is feasible.

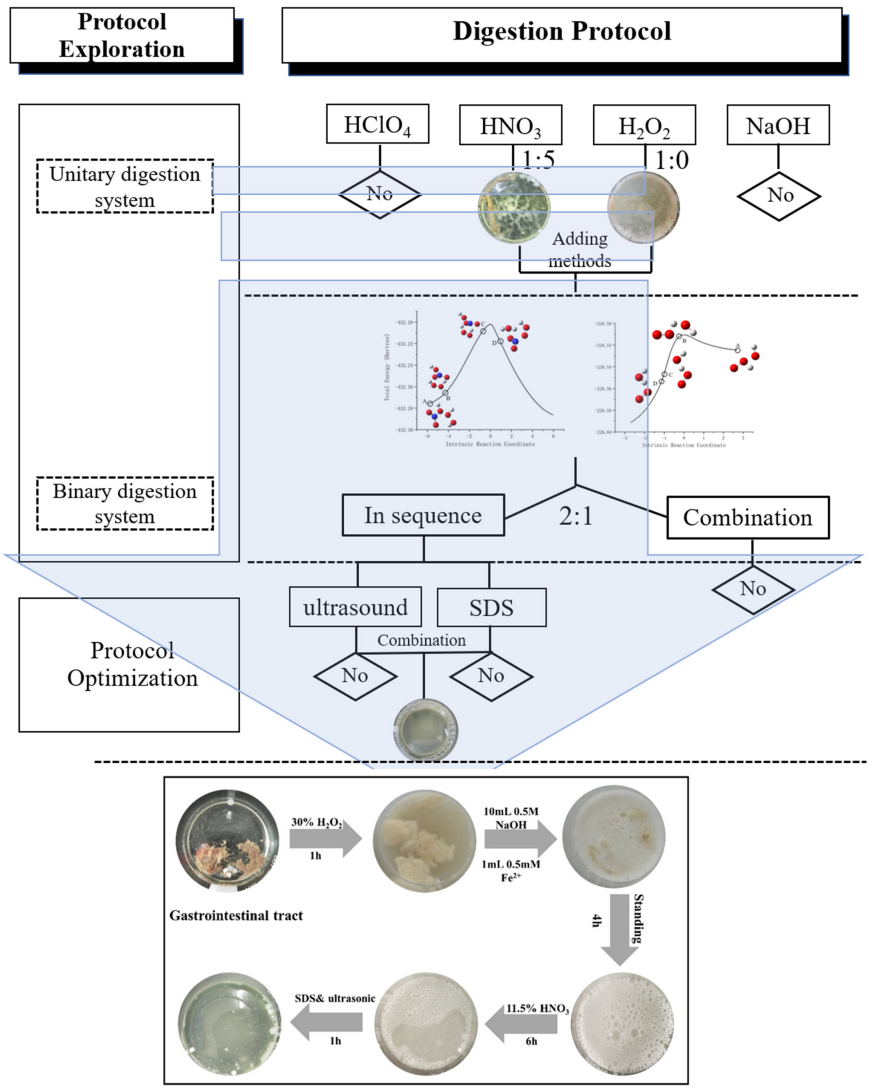

FIGURE 2 | Optimized digestion protocol.

We applied our protocol to the detection of 368 fish samples. We greatly improved the filtration speed. The last step of our experiment was to filter the digestion solution and observe the types of plastic particles on the filter membrane; thus, the filtration time is an important indicator to evaluate our method. After the application of our protocol, 40% roughly of the sample filtration time was controlled within 20 min, followed by 10 min (30%), 30–60 min (20%), and longer than 60 min (10%), thus proving that our binary digestion system improves efficiency and saves time.

The Impact of the Protocol on Plastic Particles

To further verify the feasibility of our protocol, its impact on MPs (micromorphology and chemical structure) should be evaluated. In order to improve the efficiency of the experiment, we used different methods to evaluate plastics in different experimental exploration stages. In initial investigations we used microscopes to observe changes in the details of the plastic and rule out methods that clearly affected the plastic. After obtaining a clear experimental protocol, we confirmed that the digestion process had no effect on the chemical structure of plastics by O-PTIR. The results for the recovery rate and field application finally proved that the method is feasible.

In initial investigations, we performed particle analyses by static image analyses with a Raman spectrometer. Artificial marks provide more details than smooth plastic particles; we made cross marks with a knife in each plastic particle. We used the position of the mark to compare readily identifiable changes before and after digestion. The cross marks on the surface of 11 types of plastic particles did not change appreciably after digestion compared with before digestion (**Figure 3**). The digestion solution did not corrode or dissolve the plastic particles.

We observed the changes in the chemical composition of the polymers by comparing the O-PTIR spectra before and after the

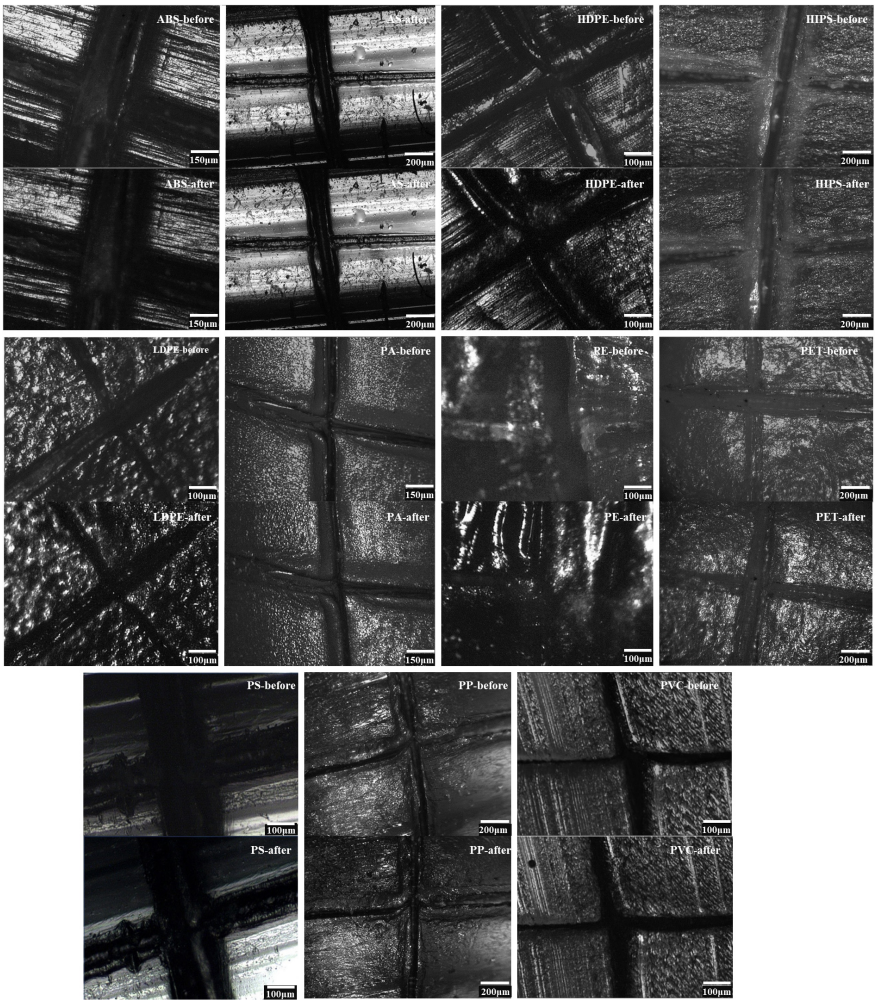

FIGURE 3 | Spectrometer photographs of 11 types of plastic particles before and after digestion, as per the final protocol. Microphotography of the following surface morphology: **(upper)** before particle digestion, and **(under)** post-digestion.

digestion (**Figure 4**). The peak positions of the 11 plastics before and after the reaction were not obviously changed, especially the HIPS, LDPE, PET, ABS, AS, and PVC particles. However, we observed a few differences in the spectra of individual plastic particles compared with the standard sample, such as for PA, PE, and PS. For PE, the peak intensity at the carbonyl stretching region around 1,720–1,770 cm^{-1} or 1,730–1,750 cm^{-1} was lower than that of the standard spectrum. Using O-PTIR Studio software, we determined that the corresponding stretching peak of these three positions represents two residues. One is attributable to the C = O stretching peak in ClCOOC and the other is attributable to the C = O stretching peak in COO. For PA, although an expected peak was not evident at 1,372 cm^{-1}, this peak represents the C = O stretching peak of RCOO; which indicates that the plastic has been reduced to produce carboxylic acids. Similarly, as for PS, the corresponding peak change was at 1,638 cm^{-1}, which indicates a C = O or C = N stretching peak. The functional group represented by the changed characteristic peak is either C = O or C = N. These two kinds of chemical bonds indicate that the plastic particles underwent a redox reaction over the course of digestion. This is an acceptable experimental error; the digestion reagents had no obvious effect on the molecular structure of the plastics and did not affect the particle quantitation results. Therefore, the structures of the plastics did not change appreciably. The digestion protocol had little impact on the plastic particles and did not affect the detection of MPs in fish tissue.

Recovery Rate

In our study (**Figure 5**), the recoveries of 11 types of plastic particles larger than 60 μm in diameter each reached more than 87%, as same as diameter < 60 μm. Larger-sized plastics (200–500 μm) each reached 90%; the recoveries of ABS, PE, PET, HIPS, and LDPE (200–500 μm) all reached 100%; PP reached 103% and PET reached 110%. The recoveries of PP, PVC, PE, and HDPE (100–200 μm) also each reached 100%; PE reached 103% and PET reached 107%. In the small size of 60–100-μm plastic particles, only ABS and HIPS reached

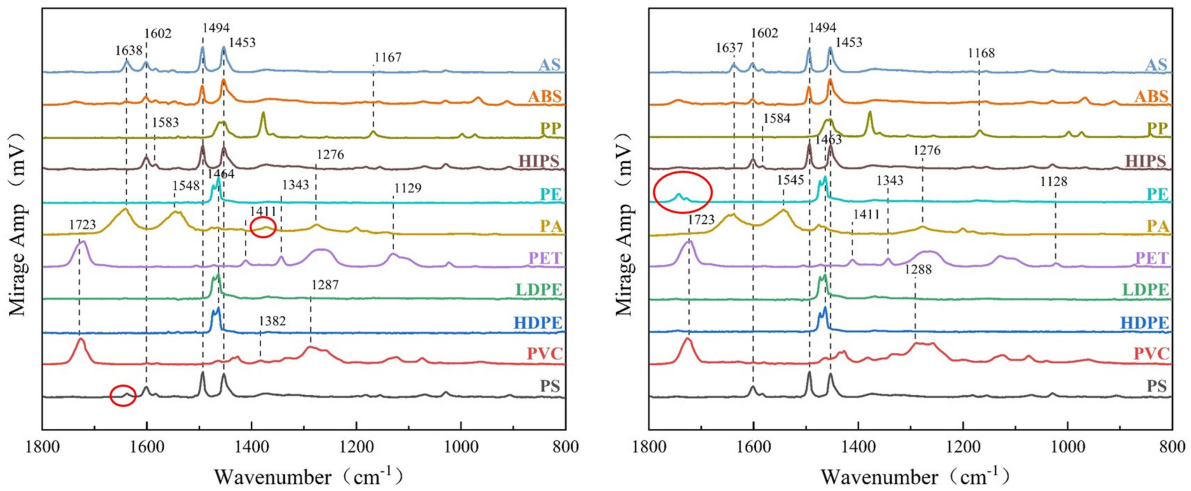

FIGURE 4 | Comparison of spectroscopy measured by O-PTIR spectra before and after digestion of 11 types of plastic particles, **(left)** before particle digestion, and **(right)** post-digestion.

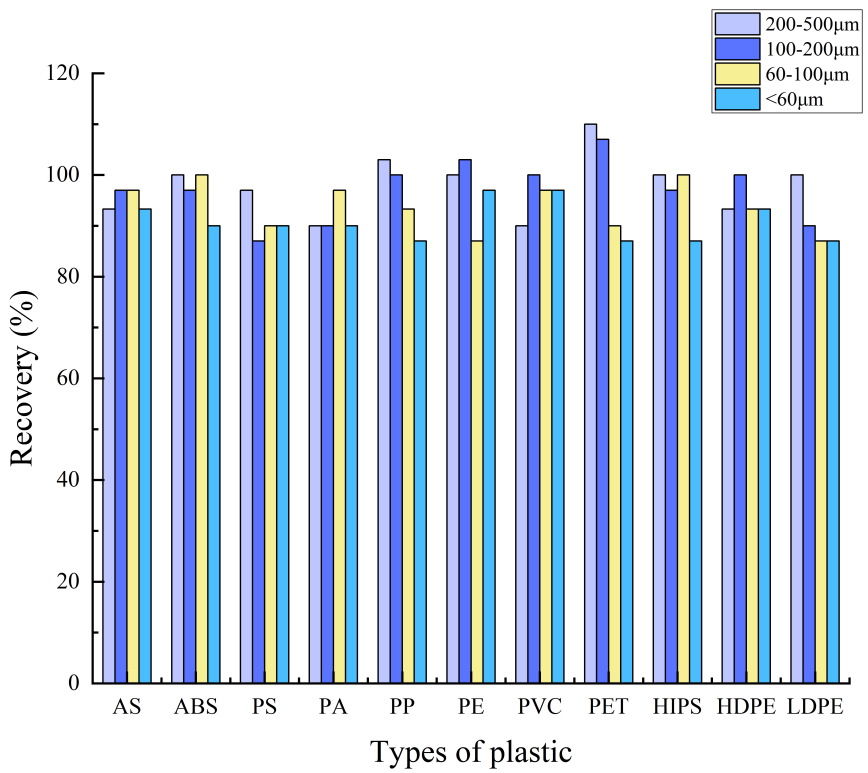

FIGURE 5 | Recovery of 11 types of plastics of various particle sizes.

100%. Regarding sizes less than 60 μm, the maximal recovery rate was 97%, which refers to PE and PVC. A recovery rate greater than 100% may be because of the influence of preexisting microplastics in the digestive glands of the fish. Similar results also appeared in Maes et al. (2017); Fraissinet et al. (2021) which is a relatively common situation. The recovery rate ranged from 87 to 100%, which indicated that the digestion protocol used in our experiments generally had relatively little influence on the recovery rate of MPs as a function of the type of plastic.

Field Application

Based on the verification of the protocol, we next analyzed field-collected fish from Shandong Tantai.

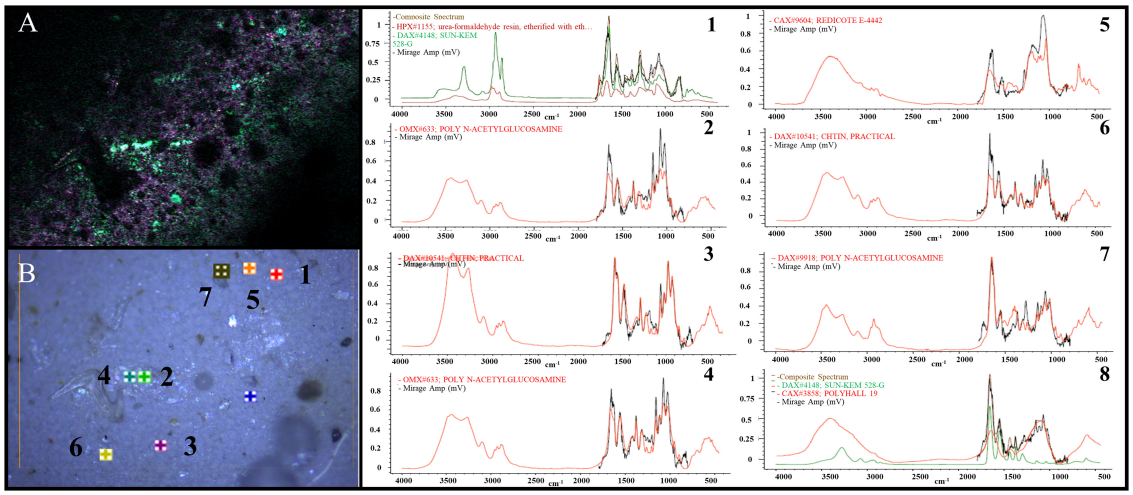

FIGURE 6 | Images of specific vibrations (1,023, 1,075, 1,641, and 1,725 cm^{-1}) of extraneous materials in various regions imaged by single-frequency O-PTIR was on the left **(A,B)**, the detailed analysis of eight materials in panel **(B)** was showed 1∼8.

As shown in **Figure 6**, we obtained single-frequency O-PTIR images at a 500-nm step size and 100-nm step resolution by tuning the QCL device to the frequencies that correspond to the specific vibrations of extraneous materials at various wavenumbers (1,023, 1,075, 1,641, and 1,725 cm^{-1}); detailed analysis of eight materials in **Figure 6**. In addition to PA, PET, and other plastic particles, we detected some crustacean bones and plastic additives.

We thus comprehensively confirmed the feasibility of our protocol. Our method is not only applicable to the digestion of fish tissues in the laboratory, but also applicable to practical analysis of real fish samples from the field, which has a practical value and promotion significance.

CONCLUSION

Because of the presence of MPs in many fish, albeit at low concentrations, it is critical to employ an extraction protocol that efficiently digests fish tissue without affecting the integrity of the plastic polymers. No single-component digestion solution was suitable for extracting MPs from fish tissues in our research because of inefficient digestion of fish tissues or corrosivity to plastics. Although $HClO_4$ had a high efficiency toward tissue digestion, it was highly corrosive to plastic particles. HNO_3 was effective toward tissue digestion but was strongly corrosive to plastic particles. The digestion protocol of H_2O_2 did not damage the tested particles but had poor digestion efficiency.

Using thorough experimentation, we obtained a plastic-safe and high-efficiency protocol (**Figure 2**): a synergistic binary digestion system that consists of 30% (v/v) H_2O_2 and 11.5% (v/v) HNO_3. In accordance with insights obtained from a Gaussian model, Fenton's reagent (Fe^{2+} and NaOH) should be added after digestion by H_2O_2, yet before digestion by HNO_3, to avoid the reaction between H_2O_2 and HNO_3. Based on the results of a single condition, the single-digestion reagent experiment confirmed that the reagents with a good digestion effect were 11.5% HNO_3 and 30% H_2O_2; thus, we combined the two reagents to construct a new binary digestion system. Under the guidance of a chemical calculation theory based on a Gaussian model, we successively added 11.5% HNO_3 and 30% H_2O_2 to solve the phenomenon of the synergistic reaction of HNO_3 and H_2O_2 that affects the digestion. We used SDS and ultrasound together to solve the problem of fat residue blockage of the membrane during filtration. We tentatively propose this protocol for the extraction and characterization of MPs from fish tissues.

We demonstrated the feasibility of our protocol by calculating the MPs recovery rate (**Figure 5**). We applied our protocol to field-collected fish samples and obtained considerable digestion results, which we verified by the mapping function of O-PTIR, and confirmed the practical applicability of this protocol. The results of our protocol may vary in accordance with the species of fish, but the protocol is efficient and reliable.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FfY: writing-original draft and confirmed the experimental results. XcW: experimental scheme design and completed the digestion experiment. HrS: field sampling. WhS: field investigation. ZxZ: O-PTIR analysis. XlS: Gaussian model analysis. JpZ: statistical analysis. LZ: pretreatment. XfW: instrumental analysis. MyL: editing. MgC: supervision. YZ: conceptualization, experimental scheme design, writing-review,

and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.845062/full#supplementary-material

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Baseline Study of Microplastics in the Gastrointestinal Tract of Commercial Species Inhabiting in the Coastal Waters of Karachi, Sindh, Pakistan

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A microplastics (MPs) emergence study in pelagic and mesopelagic species was carried out to delineate coastal degradation and ecosystem status around the Karachi metropolis. Species of high commercial and ecological worth were sampled using a gillnet of 1.5 cm knot-to-knot mesh size in November and December 2021. In total twenty-six individuals including *Liza subviridis* (15), *Thryssa dussumieri* (3), *Rastrelliger kanagurta* (2), and *Portunus sanguinolentus* (6) were used to perceive MPs. A strong linearity between body length and MPs (R^2 = 0.937, *SE* 0.071 and R^2 = 0.928, *SE* 0.104) were calculated for *L. subviridis* and *P. sangiuilatus,* respectively. However, the data of *T. dussummeiri* and *R. Kanagurta* showed minimization failure. The MPs in GIT were extracted using direct observation under a sophisticated binuclear microscope and chemical digestion (KOH) together with wet peroxide oxidation (H_2O_2+$FeSO_4$) methods. The MP materials were categorized as foam, film, fiber, fragment, and beads of three different sizes 170, 120, 100 μm in the stomach, intestine, and esophagus. Film-type MPs appeared frequently, whereas beads were rarely seen. It is hoped that this baseline research would help to minimize industrial release, recognize critical knowledge gaps, and demonstrate MP flux being released into the aquatic environment. The results will support mitigation of this emerging threat to the living resources around the Karachi coastal area.

Keywords: microplastic, foodchain, seafood, contaminants, Karachi coast

INTRODUCTION

Overharvest of fisheries resources and climate change has already threatened marine ecosystems globally. In recent years the emergence of microplastics in aquatic systems has augmented such threats, particularly in developing countries. In Pakistan, approximately 0.2 million tons of plastic garbage has been discharged into the Arabian Sea by the coastal inhabitants and through the Indus River (Dawn News, 2019). The Indus River is considered among the most plastic-polluted rivers in the world (Mairaj et al., 2021). About 6,000 manufacturers are involved in producing 0.6 million tons of plastic in Pakistan (Dawn News, 2019). Regretfully, plastic materials account for 65% of total garbage in Pakistan, with a 15% rise predicted each year (WWF Pakistan, 2021). In the case

of a developing country, it is hard to figure out future plastic load likely to be dumped into the marine environment. Dealing with urban trash, Pakistan has encountered grave problems owing to the Karachi metropolis. Nevertheless, more plastic is anticipated to be dumped in the sea by 2050 (WWF Pakistan, 2021).

The emergence of microplastic would not merely degrade aquatic habitats but would also deform food web structures. The continuous surge of pollutants can lead to livelihood risk by affecting fisheries and aquaculture activities (Plastic, 2014; Barboza et al., 2019). In this context, Barboza et al. (2019) affirmed that various coastal areas receive 61∼87% of their waste comprised of different forms of plastic. Nowadays the most widespread and persistent contaminants are plastics, which enter the coastal and marine environment by multiple routes comprising riverine and atmospheric transport, beach trash, and direct entry *via* aquaculture, shipping, and fishing (Lebreton et al., 2017; Villarrubia-Gómez et al., 2018). Gradually plastic waste is further worn into microplastics through processes of microbial degradation, extended UV exposure, and physical abrasion (Weis et al., 2015). The integration of marine litter steadily affects the coastal scenery, limiting recreational activities, causing a loss of touristic value, potentially harming the marine environment, and finally reaches the food chain. One of the primary concerns about the environmental consequences of microplastics is their interaction with the feeding of marine organisms (Botterell et al., 2019). As tiny as they are, plastics pose a problem for the environment since they are non-biodegradable and may find their way into marine food webs (Wright et al., 2013). Ingestion of microplastics, which may be mistaken for food and enter aquatic food webs, can occur by normal ventilation or swallowing them whole (Besseling et al., 2013; Setälä et al., 2014; Watts et al., 2014). Moreover, MPs may also cause indirect energy costs owing to their toxicity and tissue damage, and they may also be transmitted or enhanced *via* the food chain, presenting both ecological and human health hazards (Larue et al., 2021; Sokolova, 2021). Fish, bivalves, crabs, seabirds, phytoplankton, corals, and meiofauna have all been shown in many studies to bear adverse impacts of MPs accumulation (Wright et al., 2013; Mathalon and Hill, 2014; Watts et al., 2014; Van Cauwenberghe et al., 2015; Batel et al., 2016; Lusher et al., 2018).

In an aquatic ecosystem the food web plays a crucial role in energy shift among and within organisms through a series of organisms such as zooplankton to higher animals or grazers like fishes inhabiting the pelagic regimes. The entrance of MPs into the pelagic regime can shift up to the demersal organism as they prey on small organisms living around pelagic or demersal regimes.

Indigenous information on marine litter contamination is restricted to the abiotic environment (Balasubramaniam and Phillott, 2016; Qaimkhani, 2018; Tahira et al., 2020). Therefore, this study envisages the concentration of microplastic in the gastrointestinal tract of commercial and ecologically important species inhabiting around Karachi coastal waters. The findings will contribute to a better understanding of the propensity of microplastics to accumulate in fish and will help to fill a research gap for future evaluations of the health-related risks and food security concern in Pakistan.

MATERIALS AND METHODS

Sample Collection, Transportation, and Laboratory Handling

A total of 26 specimens were sampled from pelagic and mesopelagic regimes around Karachi coastal waters (N 240 52′ 0.38 E 067′ 00′ 0.99) using a gill-net with a mesh size of 1.5 cm knot-to-knot during November and December 2021. Specimens were immediately placed in iceboxes and transported to the laboratory. After fishes were thawed at room temperature each specimen was identified carefully using FAO field guides/identification keys. Total body lengths were taken in centimeters and wet weight in grams. Species inhabiting pelagic and mesopelagic regimes of high commercial and ecological value were selected for MPs analysis. The gastrointestinal tract was extracted and poured into beakers for further analysis. The intensity of MPs accumulation in the stomach, esophagus, and intestine were further separated.

Avoiding Contamination

The digestion and oxidation of each gastrointestinal tract were chemically processed through potassium hydroxyl (KOH), ferrous sulfate ($FeSO_4$), and the solution of hydrogen peroxide (H_2O_2). The solution of 4M KOH and $FeSO_4$ was prepared by dissolving in powder/pellet in ultra-pure distilled water. These were placed on cycling vibrator HY-4C (0∼350 rpm). For the separation of microplastics, each sample was passed through different sieves having mesh sizes of 170, 120, and 100 μm. Potential contamination during laboratory handling was minimized by the method adopted by Rasta et al. (2021). Glassware sterilization and cleaning was made using commercial dishwashing solutions to avoid contamination. These were further passed through ultra-pure distilled water, ethanol, and dried using an oven. The samples were further processed using a horizontal laminar flow cabinet to keep airborne MPs from mingling with it. To avoid contamination, the working surface was cleaned with 70% ethanol.

Microplastic Extraction and Analysis

Adopting a slight modification in the method used by Karami et al. (2017), MPs were extracted from the stomach, intestine, and esophagus separately. Here, we applied two types of treatments to extract the MPs from the GI tract. (i) Direct observation of microplastic in the stomach was made under a sophisticated binuclear microscope, but it was too vague to determine the type of microplastic. (ii) Digestion and oxidation of organic matter chemically treated was conducted to comprehend MPs and found to be a highly reliable approach. This approach allows us to examine intact plastic material without any damage.

Preparation of fixative was made with 4M KOH, H_2O_2, and $FeSO_4$. Solutions of KOH (4M w/v) and $FeSO_4$ were prepared by dissolving the content in ultra-pure distilled water. First, we

poured the stomach, intestine, and esophagus into 250 ml beakers separately. A blank control was adjusted to check for airborne contamination during experimental protocols. Precautionary measures were strictly applied during experimental work. A 30 ml (4M KOH) solution was added to each sample and placed on an electrical shaker for an hour at 350 rpm. After an hour intermission we added 5 ml of 30% hydrogen peroxide solution into each beaker and again placed it over the shaker for half an hour. Finally, the supernatant was filtered through a 125 μm sieve using a squirt bottle and particles collected back into the original beakers after a resting time of 2 h. Consecutively, wet peroxide oxidation for further extraction of MPs was applied. The reaction between the iron and oxygen turned the contents to a reddish-brown color. The contents were further passed through three sieves with mesh sizes 170, 120, and 100 μm. Consequently, contents were transferred into the corresponding Petri dishes for MPs identification and enumeration under a binuclear microscope (Model: SMZ 1,100∼Japan). As a first step, examining controls in empty Petri dishes was performed to rule out the possibility of airborne particle deposition in the laboratory. MPs were categorized according to their shapes, e.g., film, fiber, fragment, foam, and beads (**Figure 1**). The allocation and counting of plastic particles were maintained in separate containers and archived.

Statistical Analysis

Data of lengths and weights of each species were logarithmically transformed on a natural log. Mean, standard deviations, coefficient of determination, and standard error were calculated using excel spreadsheets.

RESULTS

This study envisages microplastic occurrence in the gastrointestinal tract (GIT) of the species of high commercial value dwelling in pelagic and mesopelagic regimes. A total of fifteen individuals of *Liza subviridis* (TL16.3 ± 1.1, TW 45.7 ± 6.8), *Thryssa dussumieri* (N = 3, TL 17.6 ± 2.3, TW 46.83 ± 16.1), *Rastrelliger kanagurta* (N = 2, TL 15.8 ± 0.3, TW 38.9 ± 1.4) and *Portunus sanguinolentus* (N = 6, TL 6.8 ± 0.66, TW 84.8 ± 38) were used to observe MPs. Strongly linearity between body length and MPs (R^2 = 0.937, SE 0.071 and R^2 = 0.928, SE 0.104 was calculated for *L. subviridis* and, *P. sangiuilatus,* respectively. However, data of *T. dussummeiri* and *R. Kanagurta* showed minimization failure (MF). Surprisingly, no individual was found to be completely free from microplastic in the gastrointestinal tract, however, variation and frequency of occurrence were differing. It is interpretable from the coefficient of determination that there is a strong relationship between fish body length and MPs consumed by the nektons (**Table 1**).

Some 808 microparticles were counted from the GIT of *Liza subviridis*, *Rastrelliger kanagurta* (590), *Thryssa dussumieri* (330), and *Portunus sanguinolentus* (96). The lengths, weights, feeding habits, and habitat are summarized in **Table 1**. The frequency of occurrence of MPs indicates that fishes inhabiting the pelagic region ingest more suspended microplastic than that of the mesopelagic regime. There is no recognizable pattern, though fish that ate more microplastic came from an area with higher ambient pollution. The occurrence of microplastic in three GIT parts (stomach, esophagus, and intestine) reveals that the plastic residues were categorically highest in the stomach and the lowest in the esophagus, this may be due to the low efficiency of digestion of plastic particles in the stomach where they accumulate and stay for a longer period (**Figure 2**). MPs were categorized as film, fragment, fiber, foam, and bead, based on their structure. Among them the frequency of film particles was the highest whereas bead-type particles were fewer (**Figure 3**).

A variety of shapes and colors of MPs in all gastrointestinal tracts was noticed, where transparent color particles were predominant. MPs particles were sieved in three sizes 170, 120, and 100 μm which were commonly found in all specimens. Nevertheless, particles measuring 170 μm were found significantly, whereas 120 μm were the lowest (**Figure 4**).

DISCUSSION

This is the first evaluation of the occurrence of microplastic (MP) particles ingested by highly economically valuable fishes and crabs caught by gill netter from the pelagic and mesopelagic regions of the Karachi coast. Overall outputs of this study have established baseline information that can be an input to underline an emerging dilemma in the coastal waters of Pakistan. The emergence of MPs in aquatic ecosystems seems a global problem. The consumption of MPs by microplankton, sessile organisms, and nektons can ultimately distress human health (Foley et al., 2018; Goswami et al., 2020). We found that both fishes and crab accumulated microplastics, although more accumulation of MPs was observed in the fishes rather than crabs; this might be due to dietary requirements and the fact that the stomach carrying capacity of fishes is larger than that of the crabs. Additionally, fishes constantly ingest seawater in search of food and ventilation, whereas crabs merely do so for ventilation. Wang et al. (2020) noticed that MPs accumulation is through more than the digestive tract, such as the hepatopancreas, guts, gills, and muscles. The gut and hepatopancreas components of the digestive system are the principal organs that chiefly function in digestion and absorption (Gibson and Barker, 1979). We observed that the fish esophagus, intestine, and stomach all contained MPs with varying frequency based on their holding capability, with the stomach having the most. This may be related to the inefficiency of plastic particle digestion in the stomach, where they aggregate and remain for a longer period. In this context, Ory et al. (2018) noted that microplastic particles are highly difficult for fish to digest, much moreso than food. They added that MPs reside in the guts of *Saurida violacea* for a week on average, and up to 7 weeks, which is far longer than the time needed by fish to digest and egest food pellets, which is 2 days at most. Similarly, Gassel et al. (2013) calculated microplastic clearance rates in juvenile yellowtail, *Seriola lalandi* to a maximum of 4 days, and Hoss and Settle (1990). declared that in striped mullet, *Mugil cephalus,* MP clearance rate was up to a maximum of 10 days. Several studies have reported the higher

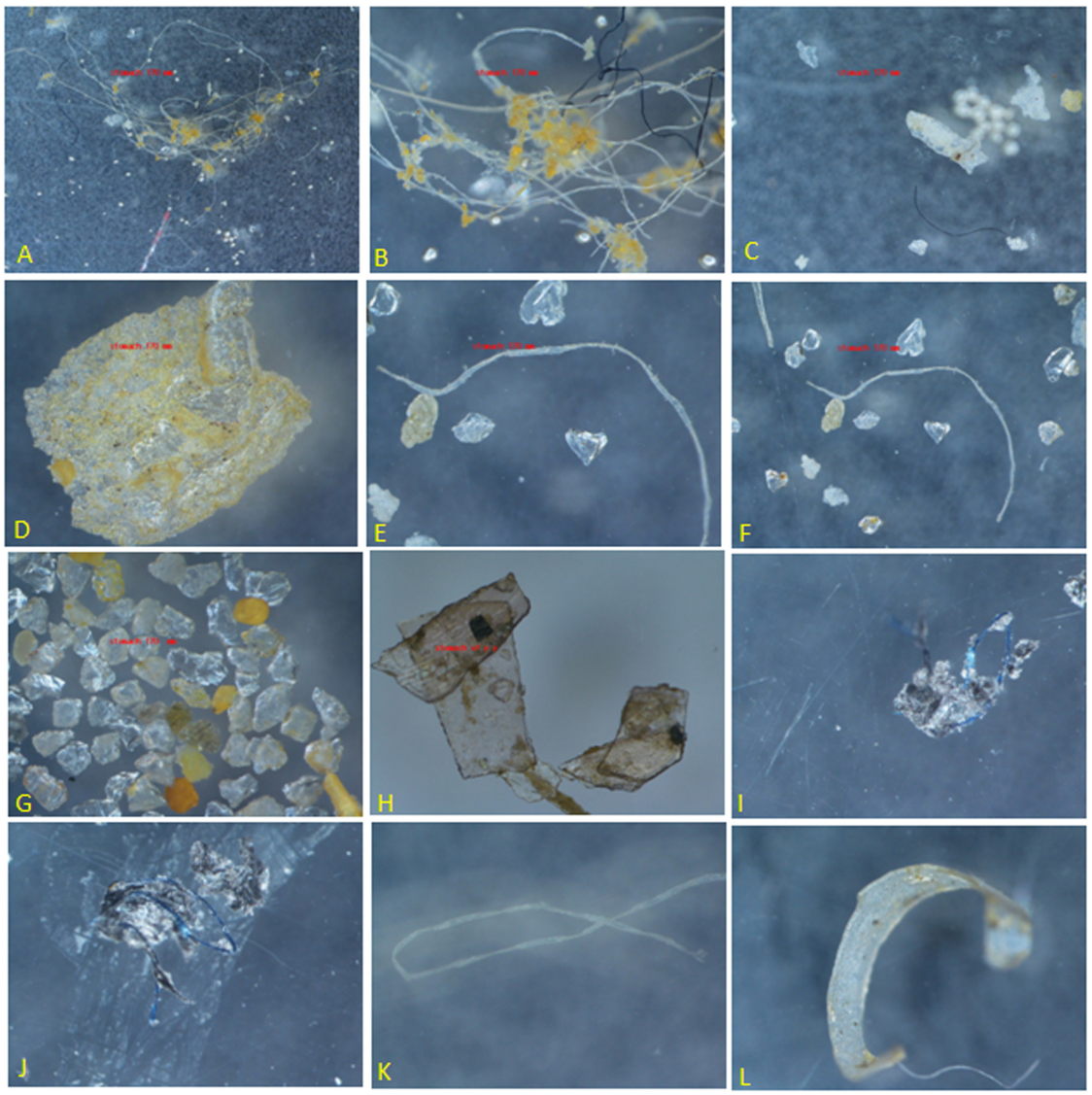

FIGURE 1 | Photomicrograph (170, 120, 100 μm) showing different shapes, sizes, and colors of microplastic **(A–L)** retrieved from the fishes and crabs inhabiting the waters around Karachi city (AG is beads; BEFKL fibers; CEFGIJ films; D foam and H is fragment).

TABLE 1 | Summary of the morphometric characters, lengths, weights, feeding habits, and habitat of four species used to evaluate MPs in gastrointestinal tracts (*N*, number of specimens; TL, total length; TW, total weight; R^2, coefficient of determination; SE, standard error; MF, minimization failure).

			TLcm	TWgm	Stomach		Intestine		Esophagus				
Species	***N***	***R*^{2} (SE)**	**Mean ± SD**		**W**	**L**	**W**	**L**	**W**	**L**	**MPs**	**Feeding Type**	**Habitat**
Liza subviridis	15	0.937 (0.071)	16.3 ± 1.1	45.7 ± 6.8	1.2	2.5	1.2	4.3	0.3	1.6	808	Generalist	Pelagic
Thryssa dussumieri	03	MF	17.6 ± 2.3	43.83 ± 16.1	1.8	3	1.2	4.2	0.18	2	590	Generalist	Pelagic
Rastrelliger kanagurta	02	MF	15.8 ± 0.3	38.9 ± 1.4	1.6	2.9	1	4.5	0.17	1.9	330	Opportunistic	Mesopelagic
Portunus sanguinolentus	06	0.928 (0.104)	6.8 ± 0.66	84.8 ± 38	0.8	2.2	0.5	4.4	0.38	1.2	96	Opportunistic	Mesopelagic

values of ingested MPs by the fish (Devriese et al., 2015; Peters and Bratton, 2016; Peters et al., 2017). In the northeastern Atlantic, there have been some reports of variations between distinct pelagic and deep-sea species (Pereira et al., 2020). Microplastic was found in substantially higher concentrations in pelagic species than in deep-water species.

Various types of microplastics were recovered from the specimens, including films, fragments, fiber, foam, and beads.

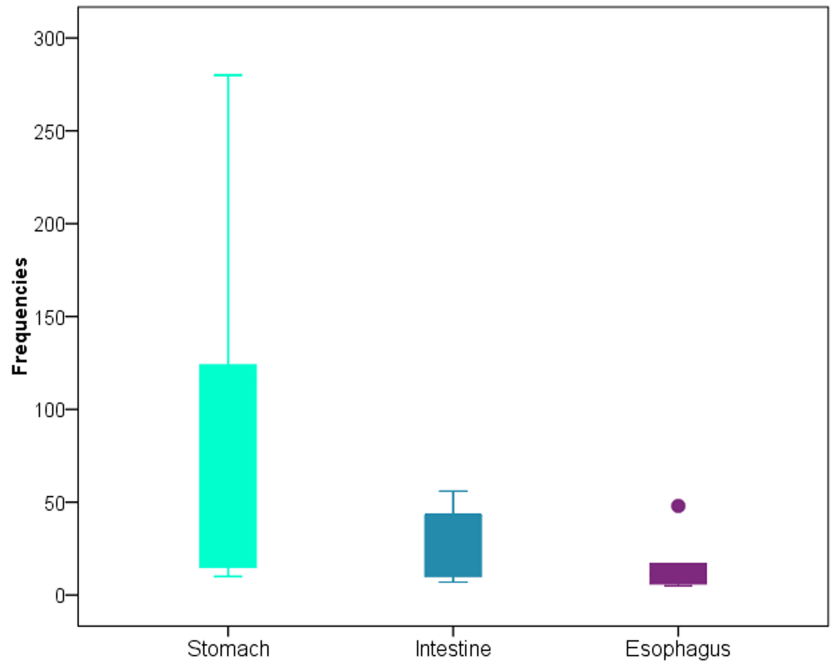

FIGURE 2 | Frequency of occurrence of microplastic detected from three gastrointestinal organs.

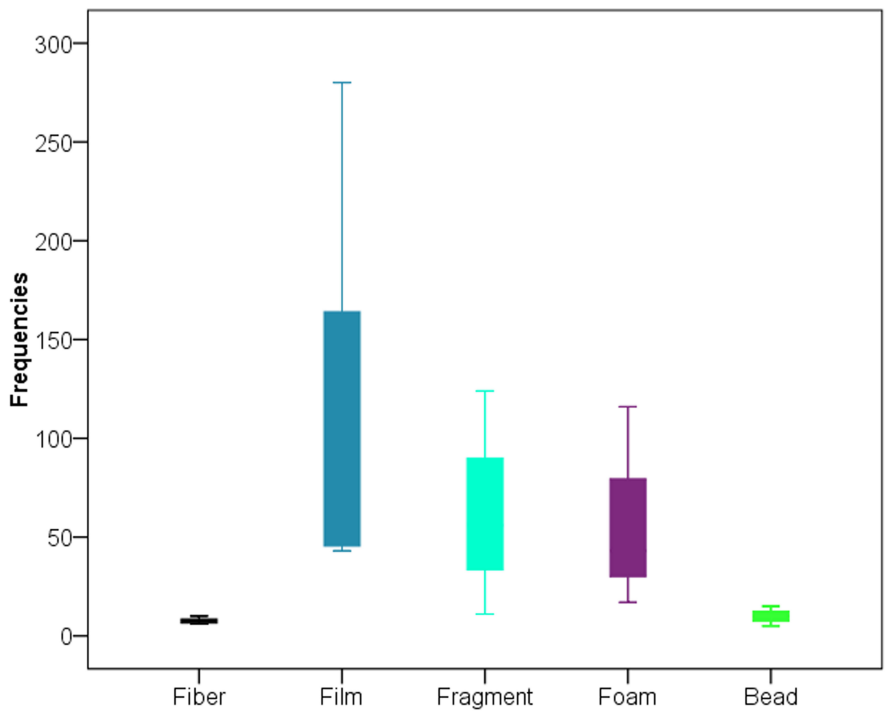

FIGURE 3 | Categorical distribution and frequencies of different forms of MPs identified in GIT of fishes and crab sampled from Karachi coastal waters.

Overall, the assessment reveals that film particles were the most common, whereas bead particles were rare in the gastrointestinal tract of organisms. Because films are secondary microplastics with weak polymers and low density, their microplastic concentration is larger in water than in sediments. Humans often employ films to assist their everyday life, such as food/beverage packing, soap packs or detergents, plastic bags, and so on (Browne et al., 2011). Plastic packaging trash and high plastic

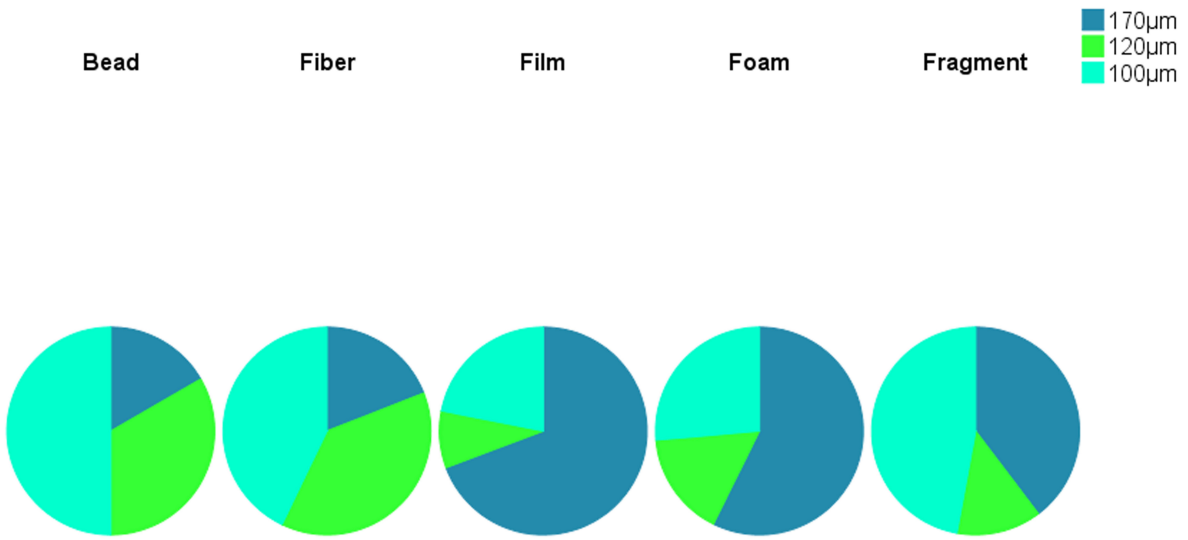

FIGURE 4 | Pie charts demonstrate microplastic particle of (170, 120, 100 µm) sizes counted and enumerated from the cabs and fishes in this study.

bag contamination in a region cause considerable biofilm growth over time, which is followed by changes in chemical-physical characteristics such as reduced buoyancy (Kershaw, 2015). Three sizes of MPs particles recorded in this study were common, this indicates that these types of plastic reach the aquatic ecosystem and chemically break into minute pieces that can easily be ingested by aquatic organisms. MP, which has a low density in comparison to seawater, tends to float on the water's surface. MPs, on the other hand, have a greater density than seawater and will sink and accumulate in sediments (Graca et al., 2017). However, we noticed a relatively lesser amount of MP fiber in the current study. Although, as Wright et al. (2013) pointed out, fibrous MPs are most prevalent in the aquatic realm.

Browne et al. (2011) mentioned that residential sewage including laundry waste may be a significant source of synthetic microfibers in the marine environment. The study area is in close vicinity of the metropolis of Karachi. Furthermore, the fisheries and shipping operations of the area are extensively established, and fishing may add to the occurrences of fiber in the marine ecology (Cole et al., 2011). It is worth mentioning here that the Karachi metropolis is closed to the sampling area can receive multiple inorganic pollutants. As a consequence, our findings may underestimate the overall concentration of MP fiber in the specimens. One possible explanation is that larger particles are more difficult for fish to eject and hence remain in their digestive tracts for extended periods of time. The longer the fiber, the more likely it is to get twisted in the gut wall and be difficult to eliminate from the body. Although, in the South China Sea, Zhu et al. (2019) studied microplastics in deep-sea fish and quoted that MPs are not limited to only the coastal ecosystem. Furthermore, Zhu observed no change in the contamination found from fish at different depths in terms of microplastics smaller than 1 mm in size, with film being the most abundant particle type followed by fiber and granules. Similarly, Wootton et al. (2021) reported that the MP detected in Fijian fish was dominated by plastic film sheets. Thus, our conclusion is consistent with a previous study from the South China Sea and Fiji about the quantity of film in fish.

The use of chemicals in the textile and leather industries has had a negative impact on the soil, air, and occupational health of Pakistan. Pollution issues have emerged primarily as a result of the indiscriminate discharge of effluent from industrial and agricultural sources, as well as the dumping of untreated liquid and solid wastes created in the home into the coastal environment. Karachi has around 8,000 small and large industrial units that may be classified into several industrial zones. The export businesses, which transport their products *via* Karachi Port, generate a considerable portion of the coastal pollution. Much of the wastewater from these enterprises is untreated and discharged straight into the port area. The occurrence of MPs in the organisms is associated with the intensity of MPs present in the aquatic environment. The effect of microplastics on animal health and human health through the consumption of seafood is yet to be defined (Ory et al., 2018; Walkinshaw et al., 2020). Keeping in mind the availability of microplastic in all species described in this study validates that organisms around the Karachi coast have no choice but to consume microplastics. Our findings offer preliminary information that may be linked with future data to produce a comprehensive profile of microplastic contamination emergence in the ecosystem and seafood in Pakistan.

CONCLUSION

This study provides an insight into the microplastic emergence in some species of high commercial worth living in two domains (the pelagic and mesopelagic ecosystems) around Karachi coastal waters. This research can be an important contribution to our

knowledge and understanding of microplastic emergence in the ecosystem that can create human health associated issues by seafood consumption. We assume that particle size of ≤ 100 μm can be assimilated in flesh and other body parts. Similarly, we strongly agreed with Ory et al. (2018) who noted that MPs are much more difficult for fish to digest than that of large food particles, therefore, we suppose that particles of ≥ 125 μm size could stay in the stomach for a longer time. Generally speaking, untreated waste disposal and effluent discharge from fishing and shipping operations have all been suggested as potential sources of MPs in the marine environment. It is hoped that this baseline research would help to minimize untreated industrial release, recognize a critical knowledge gap, and demonstrate MPs flux being released into the aquatic environment will support mitigation of this emerging threat to the living resources around the Karachi coastal area.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

Fish and crabs were sampled following the provincial code of conduct and HEC/CEMB/University of Karachi guidelines.

AUTHOR CONTRIBUTIONS

NA conducted the experiments and drafted the manuscript. SKP designed the research, supervised, and improved the quality of the manuscript.

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Microplastics: Global occurrence, impact, characteristics and sorting

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Stressors like microplastics (MPs) cause proliferating environmental pollution globally. Since plastics are continuously introduced into water bodies through numerous paths, novel solutions are required to segregate as well as decline their quantity in various environmental sectors. Numerous techniques have been used and proposed in the last 10 years to screen and enumerate MPs, define the particle's properties, for instance form, color, or size, and recognize the polymer material. This critical review aims to provide an overview of advanced procedures in MP investigation, provides illustrations of probable routes forward and lingering challenges, and categorizes present approaches as per their underlying research question. Methods presently employed for MP sampling, extraction, identification, characterization, and quantification were evaluated. Studies proposing use of precursors for removal of MPs from water *via* the sol–gel process were reviewed. Research on microfluidics systems finds application in environmental and industrial fields and has gained momentum in concentrating, sorting, classifying, focusing, and desegregating MPs. This review briefly discusses active and passive label-free microfluidic methods that are efficient in executing the desired particle separation and are gaining momentum in the ecological analysis of MPs. Although some sets of preliminary data of MPs at selected regions across the globe have been studied and obtained, the degree of MP contamination in most important rivers, nearshore inland areas, and air is yet to be understood completely. Along the Charleston Harbor Estuary, the MP concentration in intertidal sediment was found to be 0 to 652 MPs/m^2. In Asia, at the South Korean region, western Pacific Ocean, a high plastic concentration of 15–9,400 particles/m^3 was reported. In India, the MP concentration was identified as 288 pieces/m^3 in the Netravati River. In Turkey, ingestion of MPs was reported to be found in 458 out of 1,337 fish samples, indicating the polluted situation of the Mediterranean Sea. Despite the rapid development in MP analysis, no standardized technique for sampling along with separation has been approved. Therefore, for attaining a more inclusive picture of MPs' fate and abundance, this study highlights the importance of a standardized procedure for MP research that can be used globally and adequately enables comparisons around the world.

KEYWORDS

microplastics, global coastline, standard analytical methods, separation techniques, flow sensors

1 Introduction

Across the world, microplastics (MPs) are currently a major pollutant, especially in marine ecosystems. Plastics are synthetic polymers obtained from fossil fuels such as crude oil and natural gas which are the utmost prevalent type of marine debris found in oceans. Two major characteristics of plastics are durability and light weight, making them widely used. There has been a rise in global plastic production—up to 360 million tons of plastic production in 2018 from 1.5 million tons in 1950—and a portion of these plastics ranging from as high as 12.7 million tons to as low as 4.8 million tons through different pathways makes its way into the ocean. Hence, by 2050, production of plastics is expected to intensify up to 2,000 million tons (Barra et al., 2018). According to the U.S. National Oceanic and Atmospheric Administration (NOAA) and the European Chemicals Agency (EChA), any type of broken-down plastic fragment less than 5 mm in length is classified as "MP" (Dey et al., 2021). MPs are minuscule pieces of plastic that contaminate the environment and are far more pervasive than plastics which can be attributed to their persistent nature, imposing on marine, freshwater, and terrestrial ecosystems across the globe. The history of MPs is depicted in the timeline, as shown in Figure 1. Owing to the non-biodegradability of MPs and their minimal size, they are easily ingested by low trophic fauna. Apart from having adverse consequences on the organism ingesting it directly, it has far reached impacts on other organisms and ecosystems as low trophic fauna are almost always a part of a much longer food chain or food web (Veerasingam et al., 2020).

Plastics, including MPs depending on the polymer's physical and chemical characteristics and the environmental conditions, undergo biotic and abiotic processes of degradation (Sascha et al., 2018; Ali et al., 2020; Oliveira et al., 2020). Under favorable conditions, the general degradation processes of MPs include weathering triggered mechanical disintegration, photo- or thermal degradation linked to oxidation, and microbial and enzymatic degradation. Nevertheless, these become insignificant in the aquatic environment and additionally the complete breakdown or final state of reduced plastic particles is still not accurately understood; instead, they are assumed to be a constant (Ali et al., 2020).

MPs can further be classified into primary and secondary MPs (Duis and Coors, 2016; Smith et al., 2018). Primary MPs are MPs that are purposefully commercially manufactured. They are used in facial cleansers and other cosmetic products. Microfibers shed from clothes and other textiles such as fishing nets fall in the same category. Secondary MPs are obtained from larger plastic pieces or debris. Through fragmentation of such larger plastics by either physical or chemical or biological or photodegradation, these pieces lose their structural integrity and are reduced to sizes that are undetectable by the naked eye.

In modern times, plastics contribute an essential part of human society; their versatility, durability, and endurance are chief reasons for their widespread applications. The applications of plastics include making water bottles, tanks, containers, insulations, implants, and packaging material being, among others, composed of varied shapes (like pellets, fragments, films, foams), colors, sizes, polymer types, and specific densities (Hidalgo-Ruz et al., 2012). A significant constituent of electronic waste (e-waste) is plastic material. Recycling of e-waste frequently entails shredding and burning plastics, especially in developing nations, resulting in the release of MPs and additives locally (Hale et al., 2020). Chai et al. used FTIR spectroscopy to establish a new technique for assessing MPs in complex soil substrates and identified MPs in an e-waste dismantling location in Guangdong Province, China, suggesting that e-waste disposal sites have become MPs hotspots (Chai et al., 2020). E-waste generation is estimated to reach 52.2 million tons per year by the end of 2021 that will contribute as another source for MPs. Imminent technologies such as

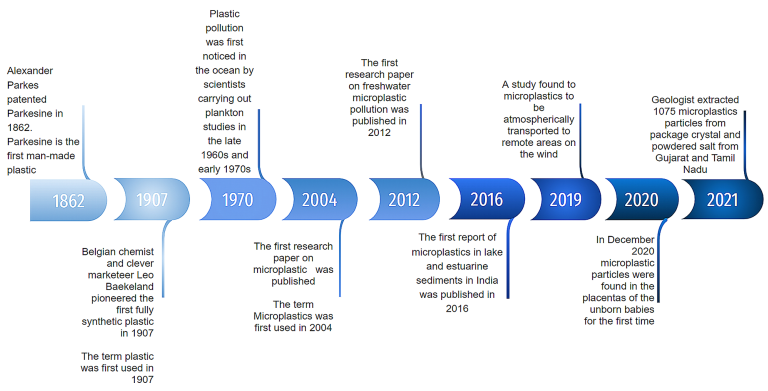

FIGURE 1
Flowsheet shows the milestones in evolution of plastic to microplastic over the years.

microfactories focus attention on a series of small devices and machines that use patented knowledge to accomplish restructuring of dilapidated commodity into novel and functioning products. This 3D printing technology is being used for recycling plastics into value-added products like industrial-grade ceramics, green steel, and plastic filaments (Sahajwalla and Gaikwad, 2018).

COVID-19 triggered a worldwide surge in single-use plastic and unrecyclable personal protection equipment, clogging sewage channels and ending up in seas and oceans *via* surface flooding and canals. According to WHO, almost 89 million procedural masks were required each month to prevent COVID-19, increasing the global manufacturing of face masks made from polymeric materials to an unprecedented level (Aragaw, 2020). The improper and unregulated disposal of face masks, composed of polypropylene (PP) and polyethylene (PE), as well as other polymeric materials including nylon and polystyrene, releases micro- and nanosized plastic fibers and silicon grains (Sullivan et al., 2021). Morgana et al. observed in their study that despite an overall modest level of fabric degradation (average 1.2 ± 1.3% of the initial weight), a consistently large number of micro/nanoplastics can be released from a single mask by replicating weathering circumstances under realistic intensity levels of mechanical deterioration (Morgana et al., 2021). Apart from face masks, personal protective equipment (PPEs), gloves, face shields, and many single-use plastics (SUPs) are not recycled or handled appropriately, resulting in mismanaged plastic waste (MMPW). Peng et al. also estimated that pandemic-associated MMPW would equal 11 million tons, resulting in a worldwide riverine discharge to the ocean of 34,000 tons (Peng et al., 2021).

Developing countries usually have a high percentage of mismanaged plastic waste and are the main contributors to plastic pollution in the marine atmosphere. Municipalities in these countries are often underfunded, lack institutional organization and interest, and have inadequate waste collection equipment; even when reuse and recycling activities exist, they sometimes lack a legal base and are thus carried out on an *ad hoc* basis. As a result, a large amount of solid trash winds up in landfills or is illegally dumped, and plastic garbage near freshwater systems has the potential to reach the aquatic environment, where further breakdown can create MPs (Wagner et al., 2018). For example, the current state of MP contamination in African countries' water systems results from poor management and weak implementation of applicable rules and regulations. To combat this, a top-down sustainability method has been proposed, which includes developing/adopting established policies and frameworks, and both corporate and government/public entities participate in microplastic research (Alimi et al., 2021; Aragaw, 2021; Deme et al., 2022). The geographical location and size of the water bodies had little effect on the dispersion of microplastic pollution (Chen et al., 2022).

Further, many chemicals and additives are added to plastics for altering their mechanical properties with approximately 4,000 chemicals in plastics used for food packaging alone. As consumption of plastic increases, accordingly concentrations of the debris and waste have increased as well, after the plastics have been used and discarded. MPs have been found and reported as early as the 1970s, and today it has become a major contributor to the plastic debris generated. MPs are being ingested by numerous smaller organisms like planktons and higher organisms such as fishes and thus entering the MP into the food chain (Andrady, 2011; Smith et al., 2018). Dumping of plastic litter off marine vessels remains the chief source of plastic debris in the marine environment. Despite regulations, dumping of plastic litter contributed as much as 6.5 million tons of plastic to the oceans in the early 1990s (Cole et al., 2011). Other sources include discarded fishing gears, microbeads from cosmetic and healthcare products running off *via* domestic or industrial drainage, and aquaculture. A large proportion of the plastics are present in the form of secondary MPs generated by abrasion and fragmentation of more significant plastic fragments. Cole et al. assessed that there were additionally 5 trillion plastic pieces of debris afloat on the sea, 90% of which were secondary MPs derived from fragmentation (Cole et al., 2011).

Although sewage treatment facilities are not designed to remove MPs, an average removal value of 88% for wastewater treatment plants (WWTPs) using preliminary/primary plus secondary treatment and 94% for WWTPs using preliminary/primary plus secondary and tertiary treatment has been reported, providing a clearer understanding of how WWTPs manage MP load and to what degree MPs reach river systems through WWTPs. The majority of the elimination occurs during the preliminary and main therapy stages. Although the overall removal is substantial, the residual quantity in treated effluent (10% of the MPs in influent wastewater) constitutes a significant release of MPs to the aquatic environment, considering the massive quantities of effluent involved (Iyare et al., 2020). The settling stage was shown to be responsible for a significant reduction in MPs reaching later stages of wastewater treatment. Tagg et al. observed that MPs larger than 600 μm were more likely to be removed at this stage, while a total of 1.5 MPs/l was identified in the final effluent (Tagg et al., 2020). In a similar study, Conley et al. assessed the removal efficiencies of three WWTPs discharging into Charleston Harbor, USA, over the course of a year. The authors calculated a total of 500–1,000 million MPs per day present in the effluent load of the three WWTPs combined. This resulted in an estimated 0.34–0.68 g MP per capita per year in treated wastewater, accounting for <0.1% of plastic debris input to the metropolitan area water bodies (Conley et al., 2019).

Gela et al. in their investigation to evaluate MPs from the urban ditches in Bahir Dar, Ethiopia, recorded a total of 239 MP particles,

demonstrating that a high quantity of MPs would wind up in the neighboring water bodies (Mhiret Gela and Aragaw, 2022). Sun et al. reviewed the removal of MPs in wastewater treatment plants. MPs have been found in both the influent and effluent of WWTPs, with reported influent quantities ranging from 1 to 10,044 particles/L and effluent concentrations ranging from 0 to 447 particles/L. Despite the comparatively low concentration of MPs in WWTP effluent, the overall discharge of MPs from WWTPs has been observed to have a median value of 2×10^6 particles/day, equating to an average annual efflux of 5×10^7 m^3/year (Sun et al., 2019). Figure 2 indicates the steps and techniques used in WWTP for detection of MPs.

Although there are several techniques and methods present to analyze MPs, a lack of standardized and uniform protocol makes it difficult to monitor MP samples of various sizes and types. Sascha et al. reported size ranges of MPs according to the sampling methods assumed in field studies (Sascha et al., 2018; Thomas et al., 2020).

2 Survey on the occurrence of MP

A literature survey was conducted on the presence of MPs in sediments, water, and biota samples across the globe. Retrieved articles were screened by study area, and only studies pertaining to beaches, rivers, estuaries, bays, and lakes were selected. A total of 74 reports were considered for this study, as represented on the world map in Figure 3. The geographical distribution of MP

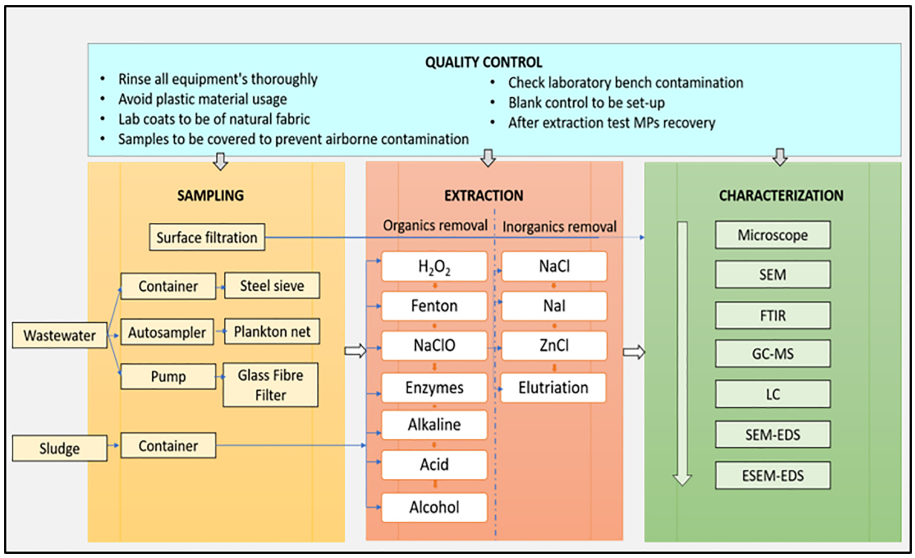

FIGURE 2
Diagram encapsuling the various steps and techniques used in WWTPs for microplastics detection (Modified from (Sun et al., 2018).

FIGURE 3
Map presenting the microplastic sampling in various areas outlined across the globe.

abundance observed in sediments, water, and biota across the globe is summarized in Tables 1–3.

Out of the 74 reports, 33 studies investigated the occurrence and distribution of MPs in the water column across the globe. A total of 50 papers have looked into the abundance of MPs in sediments from beaches, coasts, islands, and lakes. Lastly, there are 24 studies from field and laboratory investigations around the world that addressed the topic of MPs in various aquatic biota (zooplankton, fish, shrimps, mussel, oyster, bivalves, and invertebrates). The abundance of MPs in the subtidal sediment of the Charleston Harbor Estuary (3–4,375 MPs/kg wet weight) in South Carolina, USA, was higher than that recorded in the Kelvin River, UK (161–432 items/kg of dry sediment). In Europe, the River Elbe in Germany had the highest MP abundance in sediments ($3.35 \times 10^6 \pm 6.60 \times 10^6$ particles/m^3). China had the highest MP abundance in sediments in Asia (Vila Bay and Mila Bay: 33–33,300 particles/kg and 450–15,167 particles/kg). Based on the sample methods used, quantitative values of MPs in water are reported in various quantities (items/L, items/km^2, items/m^3, and percent). As a result, comparing the data is challenging. MPs in surface water along Rapa Nui, Chile, USA (64,907.5 ± 18,296.5 particles/km^2), are lower than those observed in the Mediterranean Sea, Turkey (16,339 to 5,20,213 items/km^2). The reporting units for MP abundance in biota are "items/individual" and "items/g"; fishes were frequently utilized to study MP intake in most of the publications analyzed. The South African coastline from the mouth of the Orange River to Mossel Bay had a 68% MP ingestion rate, while the Kerala coast in India had a 21% MP ingestion rate. Fish species in the Brazos River Basin, Texas, ingested 44.9 percent of the MP (Central Texas).

From the reports, it was observed that fragments, fibers, pellets, films, and foams have been found in diverse environmental matrices across the globe. The most common morphologies observed in research were fibers and fragments. According to the physical characterization of MPs, the majority of MPs identified were secondary MPs (fragments, fibers, films, and foams) rather than primary MPs (pellets), which might be generated by fragmentation of larger plastic products. In general, the shape of MPs is utilized to determine where they came from. MP fibers come from the use of degraded fishing gear (ropes, lines, and nets) at sea and land-based washing of synthetic fabrics, whereas films come from plastic bags and agricultural films. Both land-based (packing containers) and sea-based (thermocol buoys) sources are used to make the foams. Primary MPs (pellets) were discovered to be more abundant in beach sediments than in water and biota around the world. The most common MP forms detected in water and sediment samples are fibers and pieces. Fibers are the most common MPs in biota.

3 Various methods for analysis of MP samples

3.1 Sampling methods

3.1.1 Water column

There are different methods of sampling to get the sample for processing in the laboratory from water streams. For collection of MPs, water is first sampled using nets with a standard mesh size of either 335 (Bakir et al., 2020), 333 (Xiong et al., 2018), 330 (Ory et al., 2017), or 200 μm from depths of 10-cm to 200-m range. This choice is mainly due to the lower size limit for MPs of 333-μm size proposed by NOAA, USA. To quantify the volume of water sampled, a flow meter was mounted to the mouth of manta trawl nets. However, several studies did not specify whether a flow meter was put on the net, and measuring just the distance during sampling could result in significant errors if the net is not fully immersed or hindered by an abundance of suspended and floating objects. If the net is completely submerged in water, it may miss the surface layer, which is likely to contain a substantial number of floating MPs. Furthermore, sampling from the windward side or the back of a boat has an impact on quantification. The net was towed at a pace of one to four knots for 10–20 min in the majority of the investigations. Trawl nets have been frequently employed for collecting MPs in aquatic habitats since they enable the relatively rapid sampling of broad surfaces or quantities of water to get a representative sample of the investigated site (Sutton et al., 2016). The accuracy of the trawl, however, was discovered to be mostly dependent on the capacity to keep it level in the water and visually assess the water height during sampling. The actual trawled volume will also be influenced by the current and wind conditions at the time of sampling. This volume uncertainty is most likely the most significant uncertainty in trawl sampling (Karlsson et al., 2019). The Manta trawl is presently the most extensively used tool for surface sampling of MPs in saltwater and freshwater settings (Pasquier et al., 2022). The Neuston net has been the most routinely utilized trawl net in the marine environment for sampling and monitoring MP contamination after the Manta net and is used to collect samples located in the first few centimeters of the water column (Neuston net - Aquatic BioTechnology, 2022). The primary contrast between the nets is the height of the sampled water layer: Manta samples typically the first 15–25 cm, whereas the Neuston net samples a greater water layer (generally slightly less than 50 cm) (Pasquier et al., 2022). Other methods involve the surface microlayer process, hand-net array, and sampling of bulk water (Eriksen et al., 2018). Units used to express the quantity of MPs in water samples were, for instance, items/m^3 (Kazour et al., 2019), items/L (Su et al., 2016), and items/km^2 (Xiong et al., 2018). Hence, for sampling and data collection, vessels of diverse speeds, dimensions, and types are used.

TABLE 1 Studies on MP abundance in sediments summarized from selection of reports across the globe.

Study area	Mean observed abundance	References
Charleston Harbor Estuary, South Carolina, USA	Intertidal sediment: 0 to 652 MPs/m^2	(Leads and Weinstein , 2019)
Charleston Harbor Estuary, South Carolina, USA	Subtidal sediment: 3–4,375 MPs/kg wet weight	(Leads and Weinstein , 2019)
Virginia and North Carolina, USA	596–2,224 particles/kg	(Dodson et al., 2020)
Kelvin River, UK	161–432 items/kg of dry sediment	(Blair et al., 2019)
Tributaries of Thames River Basin, UK	88–1,190 items/100 g of dry sediment	(Lots et al., 2027)
Edgbaston Pool, England	26 particles/100 g of dried sediment	(Vaughan et al., 2022)
Deerness Sound, Orkney, Scotland	5.65 ± 0.55 number of MPs/kg of sediment	(Jones et al., 2020)
Irish Continental Shelf	7.67 ± 2.09 and 6.33 ± 4.91 MPs/m^3	(Martin et al., 2017)
Italy, France, Turkey, Greece, Israel, Bosnia, Portugal, Norway, Denmark, Sweden, Netherlands	72 ± 24 to 1512 ± 187 particles/kg of dry sediment	(Lots et al., 2017)
Lake Mjøsa and Lake Femunden, Norway	7.31 and 3.89 MPs/g	(Lusher et al., 2018)
Southern North Sea, Europe	421 particles/kg dry weight of sediment	(Maes et al., 2017)
River Elbe, Germany	$3.35 \times 10^6 \pm 6.60 \times 10^6$ particles/m^3	(Scherer et al., 2020)
Netherlands and Germany	100–2,071/kg dry weight of sediment	(Leslie et al., 2017)
Antuã River, Portugal	100–629 items/kg in March and from 18 to 514 items/kg in October	(Rodrigues et al., 2018)
Eastern Mediterranean, Lebanon	2,433 ± 2,000 MPs/kg d.w of sediment	(Kazour et al., 2019)
Lagoon-Channel of Bizerte, Northern Tunisia	7.96 ± 6.84 articles/g dry sediment	(Abidli et al., 2017)
Southeastern coastlines, South Africa	688.9 ± 348.2 particles/m^2 to 3,308 ± 1449 particles/m^2	(Nel et al., 2015)
Baltic Sea, Russia	34 ± 10 items/kg dw of sediment	(Zobkov and Esiukova, 2017)
Mumbai, India	68.83 items/m^2 (194.33 ± 46.32 items/m^2)	(Jayasiri et al., 2013)
Maharashtra, Goa, and Karnataka, India	43.6 ± 1.1 to 346 ± 2 items/m^2	(Maharana et al., 2020)
Chennai, Tuticorin, Tiruchenthur, Manapad, and Kanyakumari, India	439 ± 172 to 119 ± 72 items/kg in high tide; 179 ± 68 to 33 ± 30 items/kg in low tide	(Sathish et al., 2019)
Kanyakumari, India	43 particles/50 g dry sediment	(Sundar et al., 2020)
Mumbai, Dhanushkodi, and Tuticorin, India	45 ± 12 to 181 ± 60 items/kg	(Tiwari et al., 2019)
Nallathanni Island, Gulf of Munnar Biosphere Reserve, India	NA	(Krishnakumar et al., 2018)
Nethravathi River, India	Sediment: 96 pieces/kg Soil: 84.45 pieces/kg	(Amrutha and Warrier, 2020)
Sabarmati River, Gujrat, India	47.1 mg (MP in size range 75–212 μm) to 4 mg (MP in size range 212 μm–4 mm)	(Ram and Kumar, 2020)
Ganga River, India	107.57 to 409.86 items/kg	(Sarkar et al., 2019)
Silver Beach, India	204 particles/kg of sediment	(Vidyasakar et al., 2020)
Puducherry, India	72.03 ± 19.16 items/100 g	(Dowarah and Devipriya, 2019)
Kerala Coast, India	40.7 ± 33.2 items/m^2	(Robin et al., 2020)
Vembanad Lake, Kerala, India	252.80 ± 25.76 items/m^2	(Sruthy and Ramasamy, 2017)
Nattika Beach, Kerala, India	70.15 and 120.85 items/kg	(S.K and Varghese, 2019)
Tuticorin, Gulf of Mannar, India	8.22 ± 0.92 to 17.28 ± 2.53 items/kg	(Patterson et al., 2019)
Kochi, Kerala, India	10%–70%	(James et al., 2020)
Tuticorin, India	25 ± 18 to 83 ± 49 items/m^2	(Jeyasanta et al., 2020)
Qinghai Lake, China	50 to 1,292 items/m^2	(Xiong et al., 2018)
Xiangshan Bay, China	781.3 ± 258.3 items/kg	(Chen et al., 2018)
Hong Kong, China	3,242 ± 1,991 items/m^2	(Cheung et al., 2016)

(Continued)

TABLE 1 Continued

Study area	Mean observed abundance	References
Danjiangkou Reservoir, China	24 ± 9 particles/kg	(Di et al., 2019)
West Dongting Lake and South Dongting Lake, China	388.57 ± 66.19 to 501.43 ± 331.18 items/kg	(Jiang et al., 2018)
Yangtze River Basin, China	15–160 items/kg	(Su et al., 2018)
Vila Bay and Mela Bay, China	33–33,300 particles/kg and 450–15,167 particles/kg	(Bakir et al., 2020)
Yangtze Estuary, China	10–60 items/kg	(Li et al., 2020)
Qinzhou Bay, China	12–12,852 items/kg	(Li et al., 2018)
Taihu Lake, China	11–234.6 items/kg	(Su et al., 2016)
Red River Delta and Tien Yen Bay, Northern Vietnam	0–4,941 particles/kg	(Viet et al., 2021)
Eastern Gulf, Thailand	0–1,698 pieces/m^2 and 0–33 pieces/kg	(Jualaong et al., 2021)
Great Australian Bight	1.26 ± 0.68 fragments/g dry sediment	(Barrett et al., 2020)
Brisbane River, Australia	10–520 items/kg	(He et al., 2020)
New South Wales, Australia	83–350 particles/kg (inside port areas) and 59–281 particles/kg (background area)	(Jahan et al., 2019)
New Zealand	0–2,615 particles/m^2	(Bridson et al., 2020)

NA, Not available.

3.1.2 Sediment sampling

Currently, MPs are more frequently analyzed in sediments or beaches than in water columns.

MPs were collected in sediments from beaches and coastal areas by laying a metal or wooden frame on the sediment surface, pushing it down to a depth of 1–5 cm, scooping out the material, and removing the sample using a steel spoon or shovel. For sediment sampling, the frame sizes used are 25 × 25 cm, 30 × 30 cm, 50 × 50 cm, 100 × 100 cm, and 200 × 200 cm, with sampling depths ranging from 0 to 5 cm. MP pellets were handpicked or collected using stainless tweezers. Sampling location plays a vital role in determining the approaches for sampling, i.e., subtidal sediment sampling from a ship or sediment sampling directly on the beaches. Units used to express the occurrence of MPs in sediment samples were items/g (Lots et al., 2017; Dowarah and Devipriya, 2019), items/m^2 (Jayasiri et al., 2013; Cheung et al., 2016; Sruthy and Ramasamy, 2017; Robin et al., 2020), and items/kg (Patterson et al., 2019; Sathish et al., 2019; Sarkar et al., 2019; S. K. and Varghese, 2019; Tiwari et al., 2019; He et al., 2020).

3.1.3 Biota sampling

The ingestion of MPs by different aquatic invertebrates and vertebrates has been documented under laboratory and on-site conditions. Fishing nets, trawl nets, cages, and hand collection were used to acquire biota samples. Moreover, market-purchased biota samples were utilized. The biota samples were frozen at –20°C until further analysis. This review offers a brief overview of likely specimen species, emphasizing on-site sampling for the ingestion of MPs, as sampling approaches are complex and greatly rely on the targeted organism (Cole et al., 2013). Miniscule plastic beads of familiar polymer source are sometimes used in research work on the marine biota's consumption of MPs that can quickly be reconsidered and calculated in gut contents and excretions under the microscope or, in the organism itself, in the case of transparent planktonic species (Kumar et al., 2018; James et al., 2020). The recovery and enumeration of the MP particles are possible by the use of fluorescent particles (Dowarah et al., 2020). Study into the biota's intake of MPs on-site requires considerably greater exertion, and thus, studies are rare in this field of research. Digestive tract material or excretion of an organism is the target for sampling. Primarily fishes are sampled for MPs in larger species typically sampled by traps or networks.

In order to minimize contamination during a field campaign, intertidal sediment or sea surface microlayer samples are collected and stored using stainless steel, glass, or wooden containers to reduce contamination at each collection site. To reduce potential air deposition, sampling is carried out during calm conditions or from the site's downwind side. To prevent plastic contamination from clothing, nitrile gloves and white cotton laboratory jackets are worn at all times during sampling and in the lab. Blanks are used to measure potential plastic contamination in the lab.

3.2 Extraction techniques

After sampling, samples collected from water, sediment, and biota need to be segregated to extract the MPs present in the samples. MPs that are large in size can be extracted through visual sorting using tweezers, while small-sized MPs are extracted by size fractionation and density separation techniques. Popular consumer plastic polymers have densities ranging from 0.8 g cm^{-3} (silicone) to 1.4 g cm^{-3}, for example, polyvinyl chloride (PVC) and polyethylene terephthalate (PET), whereas long-drawn-out plastic foams, for instance, <0.05 g cm^{-3}

TABLE 2 Studies on MP abundance in water summarized from reports across the globe.

Study area	Mean observed abundance	References
Rapa Nui, Chile, USA	64,907.5 ± 18,296.5 particles/km^2	(Ory et al., 2017)
Chesapeake Bay, USA	1.0–563 g/km^2	(Yonkos et al., 2014)
San Francisco Bay, California, USA	15,000–2,000,000 particles/km^2	(Sutton et al., 2016)
San Francisco Bay, California, USA	0.086 MP particles/l (0.33 particles/gallon)	(Sutton et al., 2016)
Patagonia Lakes, South America	0.9 ± 0.6 MPs/m^3	(Alfonso et al., 2020)
The Solent Estuarine Complex, England	Total 2,759 MPs analyzed in the estuary	(Gallagher et al., 2022)
Coastal waters and supermarkets, UK	1.5–6.7 items/l	(Li et al., 2018)
Deerness Sound, Orkney, Scotland	7.50 ± 1.50 number of MPs/m^3	(Jones et al., 2020)
River Elbe, Germany	5.57 ± 4.33 particles/m^3	(Scherer et al., 2020)
Netherlands and Germany	1,400–4,900 particles/kg d.w of sediment	(Leslie et al., 2017)
Antuã River, in Portugal	58–193 items/m^3 in March and 71–1,265 items/m^3 in October	(Rodrigues et al., 2018)
Eastern Mediterranean, Lebanon	4.3 ± 2.2 items/m^3	(Kazour et al., 2019)
Mediterranean Sea, Turkey	16,339 to 5,20,213 items/km^2	(Guven et al., 2017)
Southeastern coastlines, South Africa	257.9 ± 53.36 particles/m^3 to 1,215 ± 276.7 particles/m^3	(Nel et al., 2015)
Ob and Tom Rivers in Siberia, Russia	44.2 to 51.2 items/m^3	(Frank et al., 2020)
Nethravathi River, India	288 pieces/m^3	(Amrutha and Warrier, 2020)
Kerala Coast, India	1.25 ± 0.88 items/m^3	(Robin et al., 2020)
Tuticorin, Gulf of Mannar, India	8.22 ± 0.92 to 31.05 ± 2.12 items/l	(Patterson et al., 2019)
Kochi, Kerala, India	10%–80%	(James et al., 2020)
Qinghai Lake, China	0.05–105 to 7.58–105 items/km^2	(Xiong et al., 2018)
Xiangshan Bay, China	8.9 ± 4.7 items/m^3	(Chen et al., 2018)
Danjiangkou Reservoir, China	2,594 ± 3,875 particles/m^3	(Di et al., 2019)
West Dongting Lake and South Dongting Lake, China	1,345.24 ± 560.81 and 1,464.29 ± 559.05 items/m^3	(Jiang et al., 2018)
Yangtze River Basin, China	0.5–3.1 items/l	(Su et al., 2018)
Vila Bay and Mela Bay, China	0.05–0.057 items/m^3	(Bakir et al., 2020)
Yangtze Estuary, China	0–259 items/m^3	(Li et al., 2020)
South China Sea, China	0.045 ± 0.093 and 2,569 ± 1,770 particles/m^3	(Cai et al., 2018)
Three Gorges Reservoir, China	4,703 ± 2,816 particles/m^3	(Di and Wang, 2018)
Bohai Sea, China	0.33 ± 0.34 particles/m^3	(Li et al., 2018)
Taihu Lake, China	3.4–25.8 items/l	(Su et al., 2016)
Yellow Sea, China	0.13 ± 0.20 pieces/m^3	(Sun et al., 2018)
South Korean region, Western Pacific Ocean	15–9,400 particles/m^3	(Eo et al., 2021)
Kyushu, Japan	0.49 ± 0.92 items/m^3	(Kobayashi et al., 2021)
Bacchus Marsh, Australia	38 ± 8 MPs/l	(Samandra et al., 2022)

expanded polystyrene (EPS), only have a fraction of the original polymer density. Isolation of MP particles can thus be carried out by flotation by saturated salt solutions such as NaCl, $CaCl_2$, NaI, and $ZnCl_2$ of high density from higher-density matrices, such as sediments with a 2.65-g cm^{-3} density (Patterson et al., 2019; Sathish et al., 2019; Sundar et al., 2020; Tiwari et al., 2019; Robin et al., 2020). For a certain period of time, dried sediment sample is combined with the concentrated salt solution and gets agitated (e.g., by stirring, shaking, aeration). Plastic particles remain dispersed in suspension or float to the surface.

3.2.1 Size fractionation

The fractionation of samples (sediment, biota, water) into at the minimum two divisions of a scale, for example, <500 and >500 µm, is appropriate regardless of the technique used for later detection of MPs. Recently, EU suggested a break into fractions of 20 µm–1 mm and 1–5 mm for monitoring purposes. The water samples can easily be fractionated through the sieving method. In case of large quantities in biotic form, for instance, large plankton, tissue, and gut contents obstructing the sieve, a purification process can be advantageous before the sieving step (Cole et al., 2013; Kumar et al., 2018; Patterson et al., 2019; Dowarah et al., 2020; James et al., 2020; Sathish et al., 2020). After extraction, MPs are readily size-fractionated from sediment samples. The fractionation step fails to distinguish between organic matter and MPs (Elkhatib and Oyanedel-Craver, 2020). Fractionation should be followed by the density separation method for effective extraction of MPs.

TABLE 3 Studies on MP abundance in biota summarized from selection of reports across the globe. .

Study area	Organism	Mean observed abundance	References
Rapa Nui, Chile, USA	Fish	2.5 ± 0.4 particles/fish	(Ory et al., 2017)
Brazos River Basin, Central Texas, USA	Fish	Of the 436 fishes examined, 196 fish stomachs contained MPs.	(Peters and Bratton, 2016)
Thames River, UK	Fish	0.69 particles ± 1.25 particles/fish	(Horton et al., 2018)
Coastal waters and supermarkets, UK	Mussels	1.1–6.4 items/individual	(Li et al., 2018)
Deerness Sound, Orkney, Scotland	Biota	4.50 ± 0.96 particles/individuals	(Jones et al., 2020)
Lake Mjøsa and Lake Femunden, Norway	Biota (duck mussels)	One out of 10 duck mussel had traces of MP	(Lusher et al., 2018)
Netherlands and Germany	Biota	0–105 particles/g of dry tissue	(Leslie et al., 2017)
Eastern Mediterranean, Lebanon	Biota	2.5 ± 0.3 items/individual	(Kazour et al., 2019)
Mediterranean Sea, Turkey	Biota	Ingestion found in 458 out of 1,337 fish sample	(Guven et al., 2017)
South African coastline from Orange River Mouth to Mossel Bay	Fish	Of the 593 fishes inspected, MPs were detected in 406 fishes	(Bakir et al., 2020)
Cape Town, South Africa	Mussels	3.83 ± 0.2 MPs/individual	(Sparks et al., 2021)
Kerala coast, India	Fish	Ingestion found in 15 out of 70 fish sample	(Robin et al., 2020)
Tuticorin, Gulf of Mannar, India	Oyster	5.21 ± 4.85 to 9.74 ± 8.92 items/individual	(Patterson et al., 2019)
Kochi, Kerala, India	Fish	Among 653 sample ingestion found in 4.6% of fishes	(James et al., 2020)
Tuticorin, Gulf of Mannar, India	Fish	Ingestion found in 12 fishes out of 40 fish samples	(Kumar et al., 2018)
Pondicherry, India	Bivalves (mussels and clams)	0.18 ± 0.04 to 1.84 ± 0.61 items/g; 0.50 ± 0.11 to 4.8 ± 1.39 items/individual	(Dowarah et al., 2020)
Tuticorin, India	Epipelagic and mesopelagic fishes	0.0002 ± 0.0001 to 0.2 ± 0.03 items/g; 0.11 ± 0.06 to 3.64 ± 1.7 items/individual	(Sathish et al., 2020)
Qinghai Lake, China	Fish	2 to 15 items/individual	(Xiong et al., 2018)
Yangtze River Basin, China	Clams	0.4–5 items/individual	(Su et al., 2018)
Vila Bay and Mela Bay, China	Fish	2.9 ± 4.6 items/individual	(Bakir et al., 2020)
Taihu Lake, China	Clams	1.3–12.5 items/g tissue wet weight in clams	(Su et al., 2016)
Pearl River Estuary, China	Biota	1.5–7.2 items/g tissue wet weight	(Li et al., 2018)
Melbourne Metropolitan Area, Australia	Fish	0.18 to 1.13 items/individual	(Su et al., 2019)
New South Wales, Australia	Oysters	0.15–0.83 particles/g of wet weight tissue (inside port areas) and 0.06–0.44 particles/g of wet weight tissue (background area)	(Jahan et al., 2019)

3.2.2 Density separation

Considering the nature of polymer in addition to the production method, MPs vary from 0.01 to 2.30 g cm^{-3} density. While organic matter and phyllosilicate minerals, for example, micas and clay minerals, can be witnessed dispersed apart from the particles, these values are typically lower than other minerals from the sediment. In particular, the process of density separation is necessary for sediments to differentiate between the residual of sample and MPs. Salinity-based density segregation employing an aqueous salt suspension promotes isolation of higher-density particles and inorganic material solids and flotation on the supernatant of the solution by lower-density MP particles (Veerasingam et al., 2020). Generally, for density separation processes, glass-separating funnels are used. The ability to extract and isolate MPs depends on their density, cost, and toxicity differences. The limitation of digestion and density separation technique is that they are time-consuming and

expensive compared to other extraction techniques (Elkhatib and Oyanedel-Craver, 2020).

3.2.3 Biological digestion

In brief, the soft flesh of fishes is rinsed with purified, distilled water to eliminate any MP that may have accumulated on the outside. After that, the tissue samples are kept in a glass bottle with 10% KOH added for digestion. The bottles are placed in an incubator and covered with aluminum foil till a clear solution is formed. After the digesting phase is completed, each bottle is filled with NaCl, $CaCl_2$, NaI, and $ZnCl_2$, which causes the MP particles to float due to density separation. The upper half of the solution is separated through filter paper after the samples have been allowed to remain at room temperature for 24 h, and the retrieved MPs are dried and stored for subsequent analysis. In 69 investigations, 30% H_2O_2, KOH, or NaOH was used to digest organic matter before or after density separation to dissolve it. After density separation and digestion, the supernatant was filtered through a variety of mesh sizes, including 0.22 µm [12 studies], 0.45 µm [18 studies], 0.7 µm [9 studies], 0.8 µm [7 studies], 1.2 mm [5 studies], and 38 µm [5 studies], and then dried naturally or in an oven for microscopic and spectroscopic investigation.

3.3 Characterization and quantification of MPs

Once MPs have been extracted, they need to be quantified and characterized. Occurrence and distribution of large MPs (1–5 mm) were recorded chiefly on beaches and, to a lesser degree, in surface waters before the word "MPs" became common. Due to this relatively vast size range accompanied by chemical characterization for confirmation of plastics, Figure 4 indicates various techniques available for MP detection and identification with respect to time, cost, and precision per sample analyzed. There are advantages and limitations of each technique and different combinations.

3.3.1 Visual sorting

In order to perform the analysis, MPs must be categorized from the residuary plastics after the completion of the purification and separation step. Noticeably, minute-sized MPs necessitate additional monitoring with the help of an optical microscope, while large plastics can be sorted out directly. The MPs were identified visually by their uniform color, brightness, and lack of cellular features. Even though visual sorting is performed, it is not always a reliable technique. Few studies have coupled visual sorting with hot needle tests to confirm the presence of MP (Sathish et al., 2019; Jeyasanta et al., 2020). Visual inspection is used to characterize MPs based on their size, color, and shape, as well as inferring their origin. Although visual sorting saves time when counting MPs, it can lead to dramatic over- or underestimation of plastic content based on plastic size ranges, as well as the possibility of counting non-plastic particles as plastic. Hence, Fourier transform-infrared spectroscopy (FT-IR) and Raman spectroscopy are done for further identification.

3.3.2 FT-IR spectroscopy

The FT-IR approach was employed in 70% of the 74 articles reviewed to detect the polymer types of MPs in various environmental matrices. FT-IR spectroscopy and its optimized technologies, such as µ-FT-IR, attenuated total reflectance (ATR) FT-IR (Ory et al., 2017), and focal plane array detector-based µ-FT-IR imaging, are most widely used to identify and quantify MPs (Krishnakumar et al., 2018; Sathish et al., 2019; Tiwari et al., 2019; S. K. and Varghese, 2019; Jeyasanta et al., 2020; Maharana et al., 2020; Robin et al., 2020). In MP research, the mid-infrared band (400–4,000 cm^{-1}) was the most commonly used FT-IR spectral region. MPs show absorption

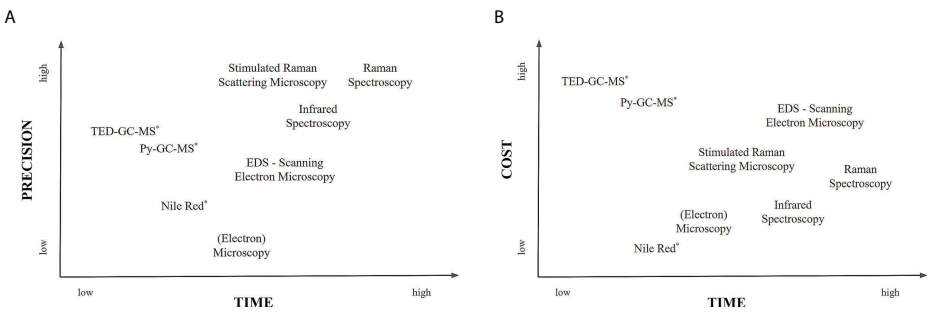

FIGURE 4
Identification and quantification techniques of microplastics based on precision/degree of detail, time and cost per sample to be analyzed. **(A)** A comparison between precision and time and **(B)** a comparison between cost and time. [The relative location of methods is based on literature data on required time for sample processing. Methods marked with an asterisk are destructive because they either pyrolyze the sample (Py-GC-MS, TDS-GC-MS) or stain the microplastic particles (Nile Red)]. [Modified from (Zarfl, 2019), (Primpke et al., 2020)].

peaks during analysis due to rocking deformation and CO stretching symmetric and asymmetric CH (-CH_2 or –CH_3) stretching, deformation, and bending whose characteristic wave numbers vary from material to material (Veerasingam et al., 2016). The most common FT-IR spectroscopy modes are attenuated total reflectance (ATR) and transmission. The big-size MPs (>500 mm) in sediments and water were characterized using the ATR-FT-IR method. The polymer types of tiny size MPs (500 mm) in biota were identified using FT-IR and a confocal microscope (known as μ-FT-IR imaging or chemical imaging). In addition to MP identification and characterization, the carbonyl index values were used to analyze the weathering pattern or ageing of MPs using the FT-IR technique. The μ-FTIR approach has grown in popularity to characterize materials that are too minute to be chemically examined using traditional FT-IR techniques. It gathers infrared signals with excellent spatial resolution (beam size as tiny as 5 μm) and has significant promise for the characterization of compositionally complex substances. More crucially, μ-FT-IR allows for *in situ*, non-destructive analysis without the need for sample purification and concentration, which is required in traditional FTIR analysis and almost always results in specimen loss and chemical property changes (Chen et al., 2015). Nonetheless, applying FT-IR for assessing fine plastic particles, for example, <1-μm-sized particles in addition to categorizing types of polymers from ecological samples, remains a challenge. Prior to FT-IR analysis, manual sorting is additionally required. Despite FT-IR being a promising advancement, additional optimization is essential for precise MP analysis (Xu et al., 2019).

3.3.3 Raman spectroscopy

Raman spectroscopy is a light scattering technique which provides in-depth information of the material under analysis. Details such as structure, crystallinity, molecular interactions and phase are obtained from this chemical analysis. The Raman technique (spectroscopy and microscope) is frequently used for identifying polymer kinds of MPs, and it was utilized in seven studies around the world to detect MPs in sediment and biota, with spectra ranging from 200 to 3,500 cm^{-1}. Raman spectroscopy is another imminent investigative method for MP recognition apart from the FT-IR technique (Sruthy and Ramasamy, 2017; Dowarah et al., 2020; Primpke et al., 2020; Sathish et al., 2020). Raman spectroscopy has a higher lateral resolution (1 *vs*. 20 mm) and a greater spectral coverage with a highly distinct fingerprint spectrum and less interference from water than FTIR spectroscopy. Raman spectroscopy can be used to analyze small particles up to 1 μm size. Another benefit is that in comparison to other analytical methods, Raman spectroscopy has improved responses to functional groups like non-polar plastic clusters (Xu et al., 2019). Micro-Raman is an optimized technology which couples Raman spectroscopy to an optical microscope, thereby enabling higher resolution of a sample (Di et al., 2019). The drawback, however, is that an FT-IR is more economically preferred than a Raman laser spectrometer.

3.3.4 Pyr-GC/MS and TDS-GC/MS

Organic additives as well as polymer type of MPs can be simultaneously analyzed by pyrolysis gas chromatography with mass spectrometry (Pyr-GC/MS) (Scherer et al., 2020). In order to determine the composition of each particle of the polymer by GC/MS, plastic particles are thermally decomposed, upon being removed from the environmental matrices. Further analysis of the MP composition in environmental samples is performed by the thermal desorption gas chromatography with mass spectrometry (TDS-GC/MS) method. The merits of TDS-GC/MS compared with Pyr-GC/MS are that it can measure more complex matrices and process larger sample mass. However, this technique has drawbacks such as it permits only a single unit at a period of time to drive through the pyrolysis since it is restricted by the tube's aperture size and is a time-consuming process. For a comprehensive analysis of MPs, TDS-GC/MS or Pyr-GC/MS may be used as opposed to FT-IR spectroscopy or Raman spectroscopy (Fries et al., 2013; Xu et al., 2019).

3.3.5 SEM/EDS

A scanning electron microscope coupled with an energy-dispersive X-ray spectrometer (SEM/EDS) provides topographical, elemental information, origin, and aging of MPs. SEM generates images of samples by scanning the surface with a focused beam of electrons. SEM additionally offers high-resolution data on surface condition as well as qualitative information on chemical composition. SEM/EDS was used to characterize MPs drawn out from water samples (Su et al., 2016; Patterson et al., 2019), sediment samples (Su et al., 2016; Patterson et al., 2019; Sathish et al., 2019; Tiwari et al., 2019), and biota samples (Su et al., 2016; Patterson et al., 2019; Sathish et al., 2020). SEM/EDX is a time-consuming and costly technique. Furthermore, because the separation of the MP is dependent on the researcher's expertise, chemical characterization may be prone to selection bias. Table 4 indicates the frequency of various plastic types identified using numerous characterization techniques across the globe. In most of the research examined, spectroscopic approaches were used to confirm the polymer types of MPs in various environmental matrices. Polyethylene (PE), polypropylene (PP), and polystyrene (PS) were the most frequent polymer types, as expected given that they accounted for 74% of worldwide plastic production (in 2015) and were commonly employed in short life-cycle products. Other polymers described in some research were polyethylene terephthalate (PET), polyvinyl chloride (PVC), nylon (NY), polyamide (PA), and polyurethane (PU). Since the major polymers (PE and PP) have a lower density than seawater (1.02 g cm^{-3}), they are widely disseminated in the water and its accompanying biota.

TABLE 4 Frequency of type of plastic as observed in the sediments, water, and biota samples across the globe and their common application (61 out of 74 studies reported the polymer type).

Type of plastic	Number of studies (*n*) that identified specific polymer	Specific gravity	Common applications
Polyethylene (PE)	57	0.91-0.94	Plastic bags, bottles, wires and cables, food packaging, sanitary napkins, netting, diapers, and drinking straws.
Polypropylene (PP)	57	0.83-0.85	Packaging for consumer products, plastic parts in automotive industries, bottle caps, diapers, rope, and netting.
Polystyrene (PS)	37	1.05	Food containers, diapers, plastic utensils, and general household appliances.
Polyamide (PA)	23	–	Fishing line, gears, guitar picks and strings, medical implants, electrical connectors, and nylon fabric.
Polyvinylchloride (PVC)	21	1.38	Films, containers, pipes, and packaging.
Polyethylene terephthalate (PET)	23	1.37	Containers for cosmetics and personal care products, packaging in pharmaceuticals, plastic bottles.
Polyester (PES)	21	–	Home furnishing material like bedsheets, carpets, pillowcases etc., polyester clothing fabric.

Thus, based on literature Figure 5 summarizes in the form of a schematic representation how various analytical methods are required for the analysis of MPs.

4 Discussion

4.1 Effect of various parameters on MP collection

4.1.1 Temperature and weathering

Degree of weathering of MPs is governed by hydrodynamic conditions such as wind, waves, currents, photo-oxidation by the UV rays from the sun, and biofouling. Figure 6 exhibits changes in primary MPs subjected to different weathering conditions (Andrady, 2017). The SEM characterization helps in estimating the extent of MP weathering. FT-IR is used to determine the extent of weathering/aging of MP based on carbonyl index value. For instance, Veerasingam et al. assessed the value of carbonyl index (CI values) to quantify photo-oxidation brought about by light in the marine environment (Veerasingam et al., 2016). Sathish et al. evaluated values of CI, with respect to the methylene group and carbonyl group area ratio in MPs (Sathish et al., 2019). Most studies use visual identification techniques despite the availability of sophisticated techniques such as FT-IR or Raman spectroscopy which is much more efficient and reliable, especially for small-sized MPs (Veerasingam et al., 2020).

4.1.2 Acoustic behavior of MP

In most of the cases, MPs are primarily segregated using sieving, digestion, and filtration step, but theoretical and

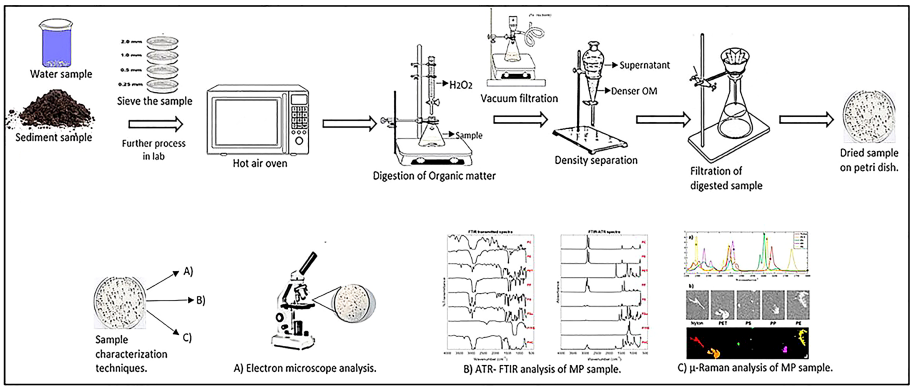

FIGURE 5
Schematic representation of various methods for analysis of MPs. Number of studies identifying the polymer type: 61 out of 74 studies reported polymer type.

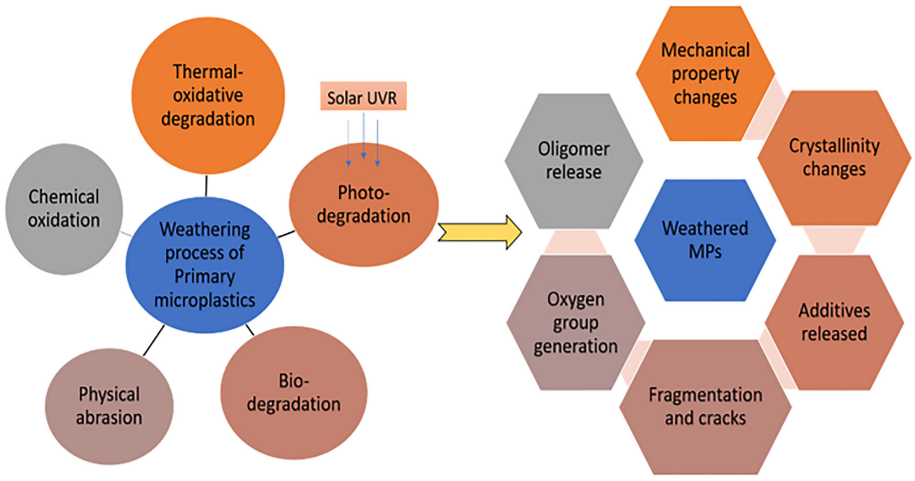

FIGURE 6
Diagram depicts weathering of primary MPs subjected to different weathering conditions.

practical views of acoustics for collection of MPs show great potential (Akiyama et al., 2020). Akiyama et al., in an experiment, used a microfluidic device with a bulk acoustic wave (BAW), and Oleh et al. used a device with a standing acoustic wave (SAW) for separation of MPs such as Nylon 6, polystyrene, and PET based on the principle of acoustic focusing using a piezo element attached to the microfluidic device (Akiyama et al., 2020; Oleh et al., 2020). Based on their physical properties and in accordance with theoretical considerations, MPs can be concentrated using acoustic focusing, and therefore acoustics can be used for separation of MPs of approximately 5-µm size. It also has potential to filter smaller-sized particles (<5µm) in aqueous suspensions such as laundry effluents.

4.1.3 Electrostatic behavior of MP

MP extraction becomes difficult as MPs come in different sizes, shapes, and properties. The acoustic properties of MPs can be used to filter the MPs below 5 µm, but for MPs of size >5 µm, the BAW device cannot be used. In such cases, the electrostatic properties of MPs can be used to filter MPs from the sample (Felsing et al., 2018). This process uses Hamo's electrostatic metals/plastic separator, which is known as the Korona–Walzen–Scheider (KWS) separator. The KWS separator uses a dry separation process where metal particles and other conductive materials introduced in the sample are separated from non-conductive materials like plastics of size range >5 µm in diameter. The absolute prerequisite is for samples to be dried well thoroughly before the separation. This process has high efficiency (has 99% yield), reduced time consumption, and manpower efficiency (Enders et al., 2020).

4.2 Additives and metal interaction with MPs

Plastic fragments can contain a wide range of chemicals which can be attributed to ingredients present in the plastic and chemicals that are absorbed. Chemicals or persistent organic pollutants (POPs) that are usually present in these plastic fragments are ultraviolet (UV) stabilizers, hydrocarbons, plasticizers, antioxidants, flame retardants, intermediates, lubricants, degradation products, and compounds of dyes and inks. These plastic-associated chemicals or additives can be analyzed by X-ray fluorescence analysis, chromatographic techniques, or mass spectroscopy–gas chromatography (MS/GC) (Hong et al., 2017). However, studies are limited to assessing adsorbed additives or POPs in MPs such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) like dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCHs), and polycyclic aromatic hydrocarbons (PAHs) (Ogata et al., 2009; Jayasiri et al., 2015; Sharma et al., 2020). It was reported that the frequency of detection and the total PCB concentration decreased with the increase in the IUPAC number of PCBs except for PCB-153, as shown in Figure 7. When plastics are suspended in a marine environment for an extended period of time, although inherently neutral, they tend to acquire a charge. The presence of metal elements such as Bi, Br, Cl, Cr, Cu, Fe, Ni, Pb, and Zn were noted in most of the MPs extracted from beaches (Robin et al., 2020). In another study, gastrointestinal tracts of fish revealed the presence of organic elements like Fe, Ni, As, Na, Sr, Ti, Mg, Si, S, Cl, and Ca present on the surface of fiber and fragment MPs (Sathish et al., 2020). The traces of these metal elements in MPs can be attributed to the fact that they are used as stabilizers,

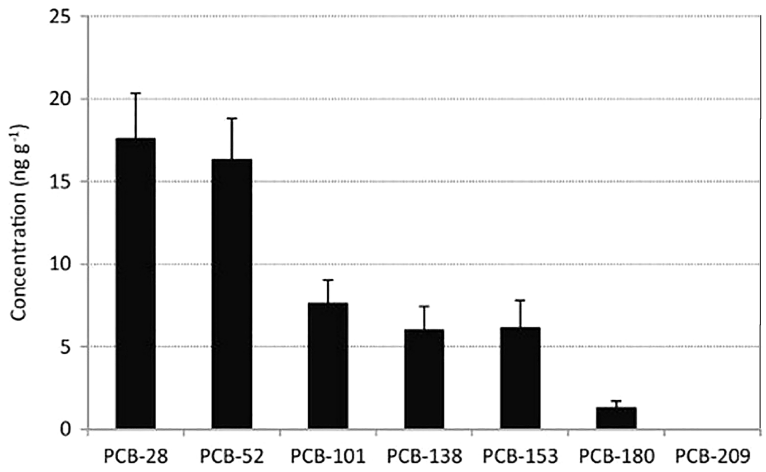

FIGURE 7
Figure represents the mean concentration of PCBs in plastic pellets (n=72) [Imprinted from (Jayasiri et al., 2015)].

colorants, antioxidants, flame retardants, pigments, and catalysts, which pose harm to the environment.

4.3 Separation of MPs

4.3.1 Removal of MPs by agglomeration

Herbort and Schuhen presented a novel idea where silicon-based precursors were used for agglomeration of MPs in water. This concept proposed to affix organic–inorganic hybrid silica gels *via* the sol–gel method as a precursor to remove hydrophobic stressors (MPs) from water in a cost-effective and straightforward approach. The proposed mechanism is shown in Figure 8 (Herbort and Schuhen, 2016). Another study presented the use of different branched and linear alkyl groups (chain length 1–18 C-atom) of alkyltrichlorosilanes to agglomerate MPs, a mixture of polyethylene (PE) and polypropylene (PP). This study evaluated the influence of the alkyl group in the precursor on the agglomeration behavior and reaction rate of the sol–gel process. Results of the study suggested that alkyl groups with intermediate chain length (three to five C-atoms) were best suited for removal of a synthetically prepared MP sample solution with removal efficiency >95%. The challenge is to transfer this concept to real-time samples (Sturm et al., 2020).

4.3.2 Sorting methods for MPs

The need to sort MPs in environmental, biomedical, and industrial applications is paving a way for the development of new microfluidic devices. Active sorting methods such as acoustophoresis or electrophoresis require the use of external differentiating forces to control sorting of particles in a microfluidic device. Passive sorting methods, on the other hand, sort particles under the influence of hydrodynamic forces due to an intense particle interaction suspended in the channel or actual structure of the microfluidic channel. The passive sorting approach is cost-effective, label-free, and straightforward in comparison to active sorting approaches. Notable techniques in this group include hydrodynamic filtration (HDF) (Yamada and Seki, 2005), pinched flown fractionation (PFF) (Yamada et al., 2004), cross flow filtration (CFF) (Altmann and Ripperger, 1997), deterministic lateral displacement (DLD) (Huang, 2004), inertial microfluidic (iMF) separation (Zhou et al., 2013), and shear-induced diffusion (SID) (Zhou et al., 2018). These techniques use physical properties such as density, shape, deformability, roughness, and size for sorting of micron-sized particles. The versatile mechanisms used in label-free passive techniques for particle sorting comprises intense particle interactions with a microfluidic structure or channel walls as in the case of DLD, with flow as observed in iMF, with fluid as witnessed in viscoelastic flow, and with other particles in SID or their combinations as noted in CFF, PFF, and HDF. Most common physical markers utilized in these passive sorting techniques are the differences in size. Out of these, high-purity separation >90% has been accomplished in DLD, viscoelastic iMF devices. The drawbacks of these passive methods are the on-demand separation activation and adaptability of parameters of separation, for instance, cutoff size. There is difficulty in separation with respect to the passive method since there are no external forces that can be adjusted. Hence, when flow conditions stabilize, the separation occurs. The challenges in using passive label-free microfluidic devices are that they have extensively been used only in biomedical practices. In areas like therapeutics, cell biology, and diagnostics (Gossett et al., 2010), passive sorting is yet to be utilized in the separation of MPs (Zhou et al., 2019).

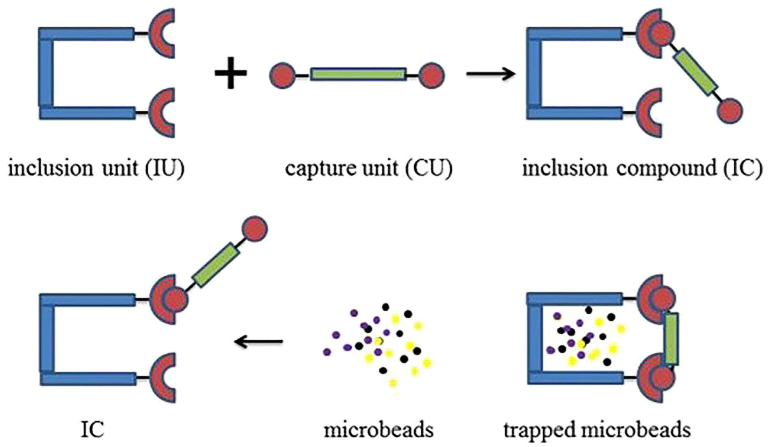

FIGURE 8
Schematic showing the capture mechanism of hydrophobic stressors (Imprinted from (Herbort and Schuhen, 2016).

4.4 Future challenges

Since MPs have been found in a variety of aquatic components, such as surface waters, oceans, and sediments, and even in the ice core of the polar regions due to their persistent nature, they have been a cause of great concern due to potential risks and toxicity posed to the organisms living in these ecosystems (Tang et al., 2021). Regardless of their density, MPs can act as colloid particles and remain suspended in the water bodies and their distribution depends on their hydrophobicity, surface currents, and wind forces (Wang et al., 2020). Hence, the presence of MPs in the marine environment makes it available to a wide range of aquatic organisms inhabiting in these ecosystems. Due to their small size, MPs are generally ingested by various aquatic organisms, including zooplankton, invertebrates, fishes, and seabirds. Lower trophic organisms, due to their indiscriminate feeding and limited ability to differentiate food from other particles, are more likely to ingest MPs (Cole et al., 2011). Figure 9 shows the interaction between organisms and MPs. Exposure to fishes occurs for the reason that they consume such MPs mistaking them for prey or through consumption of organisms that have themselves

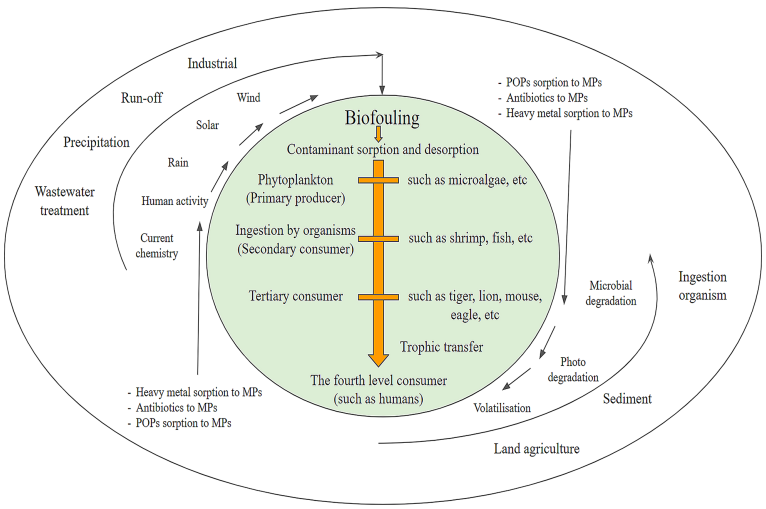

FIGURE 9
Diagram showing interaction between organisms and microplastics [Modified from (Tang et al., 2021)].

consumed MPs. An investigation carried out on the insides of gastrointestinal tracts of fish provided a conclusive proof regarding intake of MPs by fish species (Wang et al., 2020).

Many studies have been conducted under laboratory conditions to understand the toxicity effects of MPs in fishes and other organisms. Toxicity effects may include obstructions in the digestive system, inflammation, oxidative stress, and distress in the immune system. Studies have also shown that intake of MPs can have an impact on energy homeostasis and lead to reduced feeding activities in worms and crabs. Additives added to enhance plastic properties are introduced in the biological matrix of these organisms when ingested along with MPs and could lead to additional consequences. However, the extent of contribution to the transportation of lethal chemicals *via* MPs across diverse trophic levels are yet to reach a technical agreement (Wang et al., 2020). Furthermore, in the natural environment, an assortment of MPs is present, the overall toxicity of which has not been thoroughly studied under laboratory conditions. Therefore, this is an area for future work intending to obtain a more conclusive data on the toxicity of MPs in the environment. To address and overcome some of the problems highlighted thus far, the following section discusses the possible use of flow sensors and micro-liquid handling systems, which when implemented with the appropriate variables can detect and analyze MPs with high efficiency and precision.

4.4.1 Flow sensors

Flow sensors are devices used to regulate or measure flow of fluids through systems of pipes and tubes. They are connected to either digital devices or gauges to give the physical values of flow rate measured by electrical or mechanical means. Although there are many types of flow sensors, the working principle remains more or less the same. The flow sensors measure fluid flow rate in terms of either mass or volume (All About Flow Sensors, 2022). As fluid passes through the sensor, changes in velocity or area displayed help to determine and control the flow rate.

4.4.1.1 Types of flow sensors

Flow sensors can be broadly divided into two categories: contact and non-contact flow sensors. Processes where contact between the fluid being utilized and moving parts of the flow sensor can be tolerated are termed contact flow sensors. Non-contact flow sensors have no moving parts, hence used when risk of contamination of the liquid or gas would be high; otherwise, contact flow sensors are used. These sensors are used primarily in the food industry, where such risks cannot be permitted (What is a Flow Sensor?, 2022). Several parameters like the liquid or gas being measured, its temperature and pressure, corrosiveness, viscosity, and cost are taken into consideration for selecting a flow sensor in any particular process (Liquid Flowmeters, 2022).

4.4.1.2 Flow sensor and MPs

Recently, there has been thriving interest in the research and development of micro-liquid handling systems. The amount of fluid to be regulated in such systems is of utmost importance. Hence, flow sensors form an essential part of micro-liquid handling systems (SJ Lammerink et al., 1993). Extensive research has shown that the integration of microfluidics with sensor applications can be used to detect MPs effectively.

One such example that utilizes this very concept is microelectromechanical systems (MEMS) and nanoelectromechanical systems (NEMS). These devices can deal with fluids in small amounts, typically in micro- and nanoliters, and are used in separation, transport, purification, and sensing (Ashraf et al., 2011). MEMS devices have already been used successfully in various applications such as drug delivery systems, gas flow monitoring in industries, and marine hydrodynamic sensing technology. Small size and high sensitivity are what make them an attractive alternative to traditional sensing devices. In addition, various sensing principles can be used as thermal flow sensors, piezoresistive flow sensors, and piezoelectric flow sensors (Ejeian et al., 2019).

Since microfluidic devices deal with fluids at microscale, the behavior of fluid is quite different from its behavior in bulk systems. Several factors come into play that would otherwise have been negligible such as interaction of MPs with confining surfaces of the device, surface tension, and flow regime (Ashraf et al., 2011; Georgiev et al., 2020). Thus, detection and separation have become quite challenging in the field of MPs. Hence, a complete understanding of fluid behavior is necessary while designing a flow sensor for microfluidic applications. Moreover, precise flow control is essentially needed in automated meso–microfluidic applications. However, flow sensors are currently being used due to their large size and limited sensitivity that are inadequate for such applications. A trade-off is then made between the speed of a sensor and its specificity (Wu et al., 2001). Therefore, further research in this field is required to sense MP, and once sensed, additional research on whether the quality or quantity of MP can be determined in combination with sensing is an area that can be explored in the future.

Our understanding of MPs in the environment is fast evolving, as seen by information presented in this review. Nevertheless, there are significant knowledge gaps and numerous unanswered concerns. In summary, the following are the most pressing concerns:

1. How severe is MP pollution in diverse habitats right now, and how does it correlate with known contaminants in aquatic environments? Which polymers are the most abundant, and how does this differ depending on habitat and region?

2. To what degree do environmental variables and the properties of various plastic materials influence MP behavior and bioaccessibility in terrestrial and freshwater environments?

3. Do possible consequences result primarily from particle physical impacts, chemical toxicity, or a combined effect, and does this differ with polymer and species? Are there any parallels between what we know about the mechanisms of action of some nanoparticles and what we know about the mechanisms of action of others?

4. What are the potential ecological outcomes of plastics under practical exposure settings (i.e., MPs of the types and concentrations that organisms are likely to encounter)?

5 Conclusion

Over the last decade, a variety of techniques for sampling, extraction, identification, and quantification of MPs have been developed. Although a number of procedures and techniques are available for MP analysis, applied methods are subject to uncertainties, limitations being the structural resolution which dismisses miniscule MPs and restrictions in identifying natural particles from MP particles. This review offers a summary of the advanced procedures in MP analysis and the effect of various parameters on MP and gives examples of possible ways forward and challenges to be encountered ahead. Gaps in research in comprehending MPs' origin, its outcome, different conveyance pathways, standard operating procedures for analysis of MPs, and toxic effects of MPs in addition to the MP-related metal and POP's pollutants within the marine ecosystem still persist. The highly systematic separation of MPs is possible by meticulous manipulation of a particle in a microfluidic channel. MPs are heterogenous in composition and widely vary in size range. Therefore, MP particle separation necessitates tremendous effort in taking novel separation concepts into efficient, reliable sorting methods. In order to achieve separation of MPs, the integration of active and passive sorting methods is required to enable a more sophisticated and valuable platform. In an effort to obtain reliable data, it is recommended to use alternatives for plastic materials and to instead opt for glass- or metal-based materials for storage of samples, to use procedural blanks, and to use high-quality glass fiber filters. In the last decade, considerable progress has been made globally in understanding the toxicologic effects of MPs; the comprehension of MPs in water bodies across the globe is rather partial, and this offers room for research in the future. Therefore, intensive research for MP detection and separation is the need of the hour that paves a way for the development of a novel, rapid, and robust system for the same.

Author contributions

PP wrote the first draft of the manuscript. KP and PP wrote sections of the manuscript. JK contributed to early conception of the study. PP and KP organized the database. All authors contributed to manuscript revision. JK contributed for the funding source and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Polystyrene as a vector of heavy metals in hard clam *Meretrix lusoria* under various salinities

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Microplastics and heavy metals are the two main contaminants that are often found in aquatic environments and can lead to major issues for aquatic organisms. Polystyrene (PS) is a type of microplastic that is commonly found in aquatic environments. Hard clams are one of the organisms that are often used as a bioindicator of water pollution, and they can live in a certain salinity range. The objective of this study is to investigate the potential of PS particles as heavy metal vectors in *M. lusoria* influenced by differences in salinity. The result showed that the highest increase in concentrations of all heavy metals in hard clams was found in those placed at higher salinities. Hard clams that were placed at higher salinity required more water, allowing more PS particles to enter the clams' bodies. Hard clams placed at salinity 30‰ always gathered significantly more PS particles ($p<0.05$) than hard clams placed at the other two salinities (20 and 25‰). This is also corroborated by water depletion at a salinity of 30‰, which is significantly higher ($p<0.05$) than the other two salinities. Our findings indicate that PS particles have the potential as vectors for heavy metal pollutants in hard clams in environments of varying salinity.

KEYWORDS

microplastic, polystyrene, heavy metal, salinity, hard clam

1 Introduction

Microplastics of the polystyrene (PS) kind are frequently observed in aquatic environments (Andrady, 2017; Gambardella et al., 2017). Vinyl benzene, a styrene plastic monomer derived from petroleum and used to make PS, is renowned for its low weight and insulating properties. In the last two decades, the number of PS applications

has continued to increase. According to Lithner et al. (2011), 32.7 million tonnes of PS plastic were manufactured worldwide in 2011. This is one of the factors contributing to the highest level of plastic pollution in the ocean (above 60%) (Hahladakis, 2020; Galloway and Lewis, 2016). PS particles that accumulate can cause serious threats to aquatic organisms because they contain carcinogenic substances, namely styrene monomer (Wright et al., 2013).

One of the causes of the ocean's pollution, which makes up 8% of all pollutants, is heavy metals (Hahladakis, 2020). Heavy metals are difficult to eliminate, so it is easy to accumulate in the environment and their existence is naturally difficult to degrade. Heavy metals are absorbed by microplastics in both seawater (Brennecke et al., 2016; Gao et al., 2019) and freshwater (Holmes et al., 2014). Heavy metals and MPs can interact in two different ways: first, heavy metals dissolved in water can be absorbed by MPs, and second, heavy metals that have been absorbed on the surface of MPs can be released back into the water and pollute it. Our earlier research demonstrated that the two phases of interaction might be influenced by the salinity level of the water (Barus et al., 2021). These interactions can cause harmful effects to aquatic organisms. MPs particles that have absorbed heavy metals will be eaten by organisms and cause heavy metal contamination as well. The concentration of heavy metals in the bodies of organisms contained in MPs was higher than in organisms that were not found in microplastics (Vedolin et al., 2018; Mohsen et al., 2019). Besides, heavy metals that have been absorbed by MP particles can be released back into the water and cause the water to become polluted by heavy metals (Barus et al., 2021), which has an impact on the organisms that live in these waters.

Hard clams (*M. lusoria*) are the favored species of edible clam and one of the most economically important fisheries in Asia (Huber, 2010; Ju et al., 2020). *M. lusoria* is a sizeable component of the infaunal bivalve mollusk community that lives on the seafloor of fine sand, silt, or sandy silt and forms extensive and dense layers (Chen et al., 2007; Satheeswaran et al., 2019; Ju et al., 2020). These organisms are used as bioindicators of water quality because their lives settle in sediments (Satheeswaran et al., 2019). Hard clams are filter feeders that employ chiffon to draw in water, filter organic materials, and sift water and debris from one chiffon (Chien and Hsu, 2006). Hard clams are osmoconformers, meaning that the osmolality of their bodily fluids varies in the same direction as changes in the salinity of their surroundings. To deal with either hypo- or hyperosmotic stress, marine mollusks display a variety of osmoregulatory mechanisms (Lin et al., 2016). Heavy metals in hard clams can be transferred to humans through the consumption of these clams, causing harmful effects to human health and causing public health problems.

Studies have documented the buildup of heavy metals and microplastics in hard clams as well as the negative consequences. However, very few studies have examined the role of microplastics as carriers of heavy metals into the hard clam bodies, and as far as we are aware, no research has been done in various salinity levels. The purpose of this study was to investigate the effect of salinity on heavy metal contamination in hard clam using polystyrene particles as a carrier.

2 Materials and methods

2.1 Materials

The virgin PS particles with diameters of 50 and 250 μm used in this study were purchased from Tersulan Chemical Co., Ltd. (Guangdong, China). Lead (Pb), zinc (Zn), copper (Cu), and cadmium (Cd), four heavy metals used in this investigation, were bought from Merck (Darmstadt, Germany). The heavy metal stock solution was prepared at 1000 mg L^{-1} using 1 liter of distilled water. The stock solution was diluted to 1, 2, 5, and 10 mg L^{-1}. These heavy metal solutions with different concentrations were used for heavy metal adsorption on PS particles before exposure to biota.

2.2 Experimental animals

Adult hard clams (*M. lusoria*) with an average weight of 19.62 ± 0.04 g was bought from a local market (Keelung Fish Market, Taiwan). Hard clams were maintained at 1000 L of seawater with a salinity of 30‰ for 5 days. The seawater is pumped and filtered from the coast offshore by the National Taiwan Ocean University. After this time, some of the hard clams were maintained and adapted to salinities of 25 and 20‰. Salinities between 20 and 30‰ are thought to be hard clams' ideal physiological range (Malouf and Bricelj, 1989). The reduction in salinity was carried out by 1‰ per day to ensure that the clam could adapt. This group division aims to perform tests in environments with different salinities. Organisms were kept and acclimated at a constant temperature (22 ± 0.5°C) and with continuous aeration. During maintenance, *M. lusoria* was fed with green algae. After acclimatization and prior to PS particle exposure, the meat of the five hard clams was sampled to verify background contamination by heavy metals.

2.3 Experimental design

Before conducting the experiment, analysis of the heavy metal content of virnin particles was also carried out. The results show that all particles are metal free (undetectable). One gram of PS particles was introduced to a beaker glass along with 100 mL of a heavy metal solution, which was then agitated for 24 hours at 180 rpm using a magnetic stirrer. The concentrations of PS particles of size 50 and 150 μm with a weight of 1 g were 3 x 10^5

and 4 x 10^4 particles L^{-1}, respectively. Because the equilibrium point for the absorption of heavy metals into PS particles had been reached within that time frame, the duration of exposure to PS particles and heavy metals was chosen (Barus et al., 2021). Tests were carried out using different concentrations of heavy metals (1, 2, 5, and 10 mg L^{-1}). After 24 hours, filter the particles using filter paper of 0.45 µm. The clams *M. lusoria* that have been maintained and adapted to different salinities are put into a glass bath containing 1 L of seawater with a different salinity (20, 25, and 30‰) and then added PS particles that have been filtered from a heavy metal solution. Each glass tube contains 10 hard clams and is supported by continuous aeration to keep the clams alive and also to ensure that the particles in the tubes keep stirring. *M. lusoria* were collected after a contact duration of 6, 12, 18, and 24 hours, respectively. Each test was repeated 3 times.

The amount of heavy metal absorbed in PS particles of various sizes (50 and 250 µm) following interaction with heavy metals at various concentrations for 24 hours was also tested. Three times were run through the test. After 24 hours, the particles were filtered using 0.45 µm filter paper. Each sample's particles underwent sonication extraction (120 W, 10 min.) in nitric acid at a 2% concentration. A flame atomic adsorption spectrophotometer was used to measure the concentrations of heavy metals in the extraction solution (SpeactAA 240-FS, VARIAN, Palo Alto, CA, USA).

Five hard clams *M. lusoria* that have been in prolonged contact with the solution are taken, then the meat is taken and frozen at -20°C. The samples were freeze-dried (FD-20A2D, H.C.S., Taipei, Taiwan) at-60 °C. After 3 days, concentrated nitric acid (70%) was added to the samples, and they were digested in a microwave-accelerated reactor (MARS Xpress, CEM, Matthews, NC, USA). A flame atomic absorption spectrophotometer was used to measure the levels of heavy metals in clam bodies.

We examined the residual concentrations in PS particles and in seawater at each observation interval to determine the contribution of heavy metals adsorbed in PS particles on the rise in heavy metals in hard clams. After 24 hours, the particles were filtered using filter paper with a 0.45 µm particle size. Each sample's particles underwent sonication extraction (120 W, 10 min.) in nitric acid at a 2% concentration. A flame atomic adsorption spectrophotometer (SpeactAA 240-FS, VARIAN, Palo Alto, CA, USA) was used to measure the concentrations of heavy metals in the extraction solution. We also measured whether there was a change in water volume before and after exposure (water depletion) to determine the effect of salinity through the osmoregulation process.

2.4 The number of PS particles in *M. lusoria*

After the contact period, three hard clams were removed, and the flesh and clams were divided. 30% H_2O_2 was used to digest the flesh in accordance with the methods outlined by Galgani et al. (2013) and Li et al. (2015). Samples were treated with 250 mL of 30% H_2O_2 for 48 hours at 65 ^{0}C in an oscillation incubator running at 80 rpm. In order to keep the PS particles floating while the tissues settled to the bottom of each digestion bottle, 800 mL of filtered sodium chloride solution was added once the digestion process was complete. This concentrated saline solution (NaCl: 120 g L^{-1}) was created. The solution was then filtered with a Millipore vacuum pump onto Whatman® GF/C filters (0.45 µm pore size) to recover floating PS particles. In glass Petri plates with covers, each filter was put before being counted after it had dried at room temperature. The 40x stereo microscope was used to analyze the filtered microplastic particles. The primary characteristics used to count and distinguish PS particles from other particles were size, color, form, and surface roughness.

2.5 Data analysis

One- and two-way ANOVA were used to analyze all of the data in this study. Using Duncan's multiple range test, the significant differences between the treatments were determined. We tested for significant differences between the increasing heavy metal in the clam body (%) with respect to different water salinities. At the 0.05 level, statistical significance was recognized for all tests. The linear relationships among increasing heavy metal in the clam body with heavy metal concentration, salinity, and the number of accumulated PS particles were tested using a general linear model.

3 Results and discussion

3.1 PS particle adsorption capacity

The amount of heavy metal adsorbed on the PS particles was measured after a 24-hour period of contact. The results revealed that the adsorption amount varied depending on particle size. PS particles with a larger size absorb more heavy metals. Figure 1 shows that in all the initial heavy metal concentrations, PS particles of size 250 µm absorb more heavy metals than PS particles of size 50 µm. These results are in line with previous studies (Holmes et al., 2014; Fred-Ahmadu et al., 2019; Barus et al., 2021). Larger-diameter particles have a larger surface area, which serves as an adsorption site for heavy metals.

According to Figure 1, Pb has the highest rate of heavy metal absorption among PS particles, with Cu, Zn, and Cd having the lowest rates. These results were found for both PS particle sizes. Previous studies reported similar results (Turner and Holmes, 2015; Gao et al., 2019). Gao et al. (2019) reported that the heavy metal Pb was absorbed the highest in microplastic particles compared to other heavy metals, both in fresh water and sea

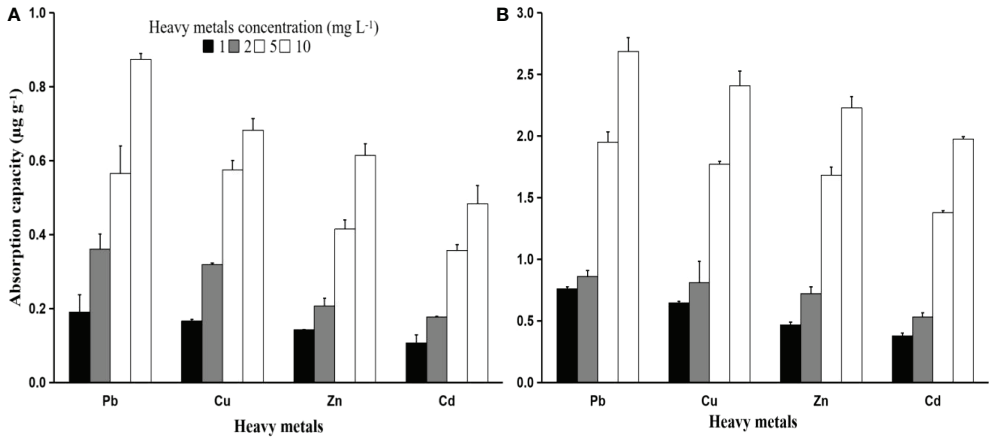

FIGURE 1
Heavy metals (Pb, Zn, Cu, and Cd) absorption capacity (μg g^{-1}) of PS of different sizes 50 μm **(A)** and 250 μm **(B)** after 24 hours of exposure to different heavy metal concentrations (1, 2, 5, and 10 mg L^{-1}).

water. This indicates that Pb has a higher potential to be a pollutant combined with MPs because Pb has a greater affinity for particle size and type compared to Zn, Cu, and Cd. Factors such as ionic radii, hydrolysis value, and softness cause differences in absorption rates for each type of heavy metal (Covelo et al., 2011; Hu et al., 2017). The absorption rate of heavy metals is inversely proportional to the ionic radius of the heavy metal. The smaller the ionic radius of a heavy metal, the greater the potential for adsorption (Covelo et al., 2011; Saha et al., 2002). The radii of the hydrated ions of Pb^{2+}, Cu^{2+}, and Cd^{2+} were 0.401, 0.419, and 0.426 nm, respectively. The relationship between the hydrated ionic radius and electrostatic interactions influences the degree to which metals adsorb on MPs. In comparison to other heavy metals, the Pb^{2+} ion has the lowest hydrated ionic radius and hence, the strongest electrostatic contact with MP.

The concentrations of heavy metals Pb, Cu, Zn, and Cd that were absorbed by PS particles of size 50 μm after 24 hours of contact duration with a heavy metal 1 mg L^{-1} were 0.257 ± 0.012, 0.236 ± 0.004, 0.165 ± 0.006, and 0.135 ± 0.006 μg g^{-1}, respectively. Meanwhile, the adsorption concentrations of 250 μm PS particles were 0.767 ± 0.021, 0.648 ± 0.008, 0.468 ± 0.007, and 0.375 ± 0.009 μg g^{-1}, respectively (Figure 1). The absorption of heavy metals in PS particles increases with the increase in the concentration of heavy metals in the solution. The highest adsorption capacity of PS particles was found in contact with heavy metals with a concentration of 10 mg L^{-1}, followed by 5, 2, and 1 mg L^{-1}. These results were found for both PS particle sizes (Figure 1). According to Hodson et al. (2017), the adsorption capacities reached 236-7171 mg g^{-1} at relatively high starting concentrations of Zn (0.1-100 mg L^{-1}), which was significantly more than what was seen at low beginning concentrations (Holmes et al., 2012; Turner and Holmes, 2015).

3.2 Heavy metal concentration in *M. lusoria* before exposure

Prior to contact with PS particles that had absorbed heavy metals, the value of heavy metals in hard clam meat was evaluated. The results show that the hard clam contains heavy metals with relatively low values. The heavy metal concentrations of Pb, Zn, Cu, and Cd in the hard clam body were 0.033 0.005, 8.572 0.892, 0.117 0.011, and 0.049 0.008 g g^{-1}, respectively. This indicates that the hard clam (*M. lusoria*) has been contaminated by heavy metals from the ocean. Previous studies have shown that Cd, Cr, Cu, Ni, Zn, and Pb metals can accumulate in hard clams (Satheeswaran et al., 2019). Hard clams are filter feeders that live on the bottom of the water, which use chiffon to attract water filtering organic matter (Chien and Hsu, 2006) and accumulate pollutants in their bodies (Satheeswaran et al., 2019).

3.3 Heavy metal concentration in *M. lusoria* after exposure

The concentration of heavy metals in the hard clam body has increased following contact with PS particles that have absorbed heavy metals. Larger particles (250 μm) increased the quantity of heavy metals in hard clam bodies in contact with PS more than small particles (50 μm) did (Figures 2 and 3). This was because the particles with a larger size have a higher capacity to absorb heavy metals based on Figure 1, so when these particles were eaten by clams, the heavy metals that were carried into the clams became higher. Additionally, heavy metals in the water become higher when they are released into the water (Figure 4). Extreme increases started to appear after the first six hours of observation.

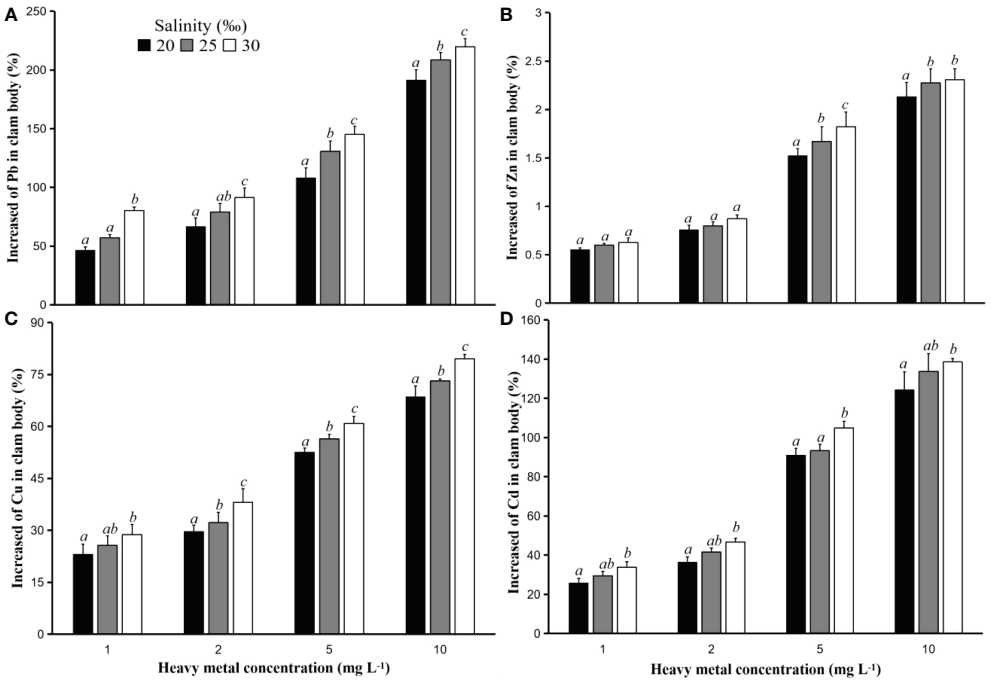

FIGURE 2
Increased heavy metal concentrations (%) Pb **(A)**, Zn **(B)**, Cu **(C)**, and Cd **(D)** in the hard clam (*M. lusoria*) body after 24 hours of exposure to PS particles with a size of 50 μm that had contact with heavy metals at various concentrations (1, 2, 5, and 10 mg L^{-1}) and salinities (20, 25, and 30‰). The data for increased heavy metal concentrations in clam body with different letters (a, b, and c) differed significantly (p<0.05) between salinities.

Additionally, the rise in heavy metals starts at a modest level and didn't change much until the observation's end (24 hours). This was most likely caused by the hard clams eating PS particles at the beginning up to 6 hours later, which still absorbed high levels of heavy metals before dispersing into the water. The concentration of heavy metals remaining in PS particles decreased dramatically at 6 hours of exposure and continued until the end of the observation, while the concentration in seawater increased even though in very low concentrations. This was found in tests using both PS particle sizes (Figures 4 and 5). This shows that the role of PS particles as heavy metal vectors in hard clams can occur through two pathways. First, PS particles can absorb heavy metals (Brennecke et al., 2016; Gao et al., 2019; Galloway et al., 2017) and enter the clams, causing the clams to be contaminated with heavy metals. Second, the heavy metals that have been adsorbed on the PS particles can be released back into the solution and cause the solution where the clam lives to be polluted by heavy metals (Costa, 2017; Barus et al., 2021).

Additionally, it was discovered that when hard clams came into touch with PS particles that had been exposed to higher concentration of heavy metals, their bodies' heavy metal concentrations increased. The value of the metal's adsorption into the PS particles increases with the concentration of heavy metal in contact with the particles. Oz et al. (2019) discovered that the value of heavy metal adsorption in microplastic particles was higher at an initial heavy metal concentration of 4 mg L^{-1} in comparison to concentrations of 1 and 2 mg L^{-1}. The highest increase in heavy metals in clam bodies in this study was found in tests using PS particles in contact with heavy metals with a concentration of 10 mg L^{-1}, followed by 5, 2, and 1 mg L^{-1}. These results were found both in tests with particles of size 50 and 250 μm, and in all types of heavy metals (Figures 2 and 3).

The concentration of heavy metals that build up in hard clam bodies is affected by the rise in water salinity. The higher the water salinity value, the greater the increased value of heavy metals in clam bodies. In addition, the number of particles accumulating in the hard clam bodies also has an important role in this case. The more PS particles that accumulate in the clams, the higher the increase in heavy metals.

The value of the increase in heavy metal accumulation in the hard clam (*M. lusoria)* in this study was determined by statistical analysis, which revealed that salinity, the concentration of heavy metals used in PS particle adsorption, and the quantity of accumulated PS particles all had a significant impact. According to this study, there is the following relationship between the increased rate (%) of each heavy metal and its concentration (C), salinity (S), and number of collected particles (P):

PS size 50 μm:

Increased Pb rate (%)= 69.693 + 13.094 C + 5.919 S + 0.357 P + 0.693 SP (R^2 = 0.764)

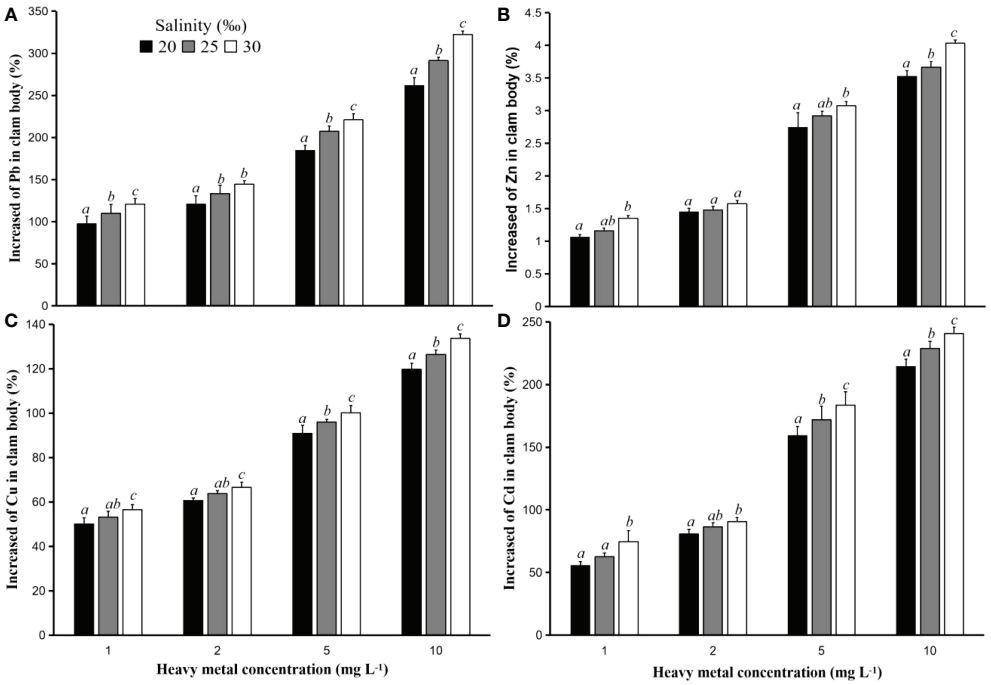

FIGURE 3
Increased heavy metal concentrations (%) Pb **(A)**, Zn **(B)**, Cu **(C)**, and Cd **(D)** in the hard clam (*M. lusoria*) body after 24 hours of exposure to PS particles with a size of 250 m that had contact with heavy metals at different concentrations (1, 2, 5, and 10 mg L-1) and salinities (20, 25, and 30‰). See Figure 2 for statistical information.

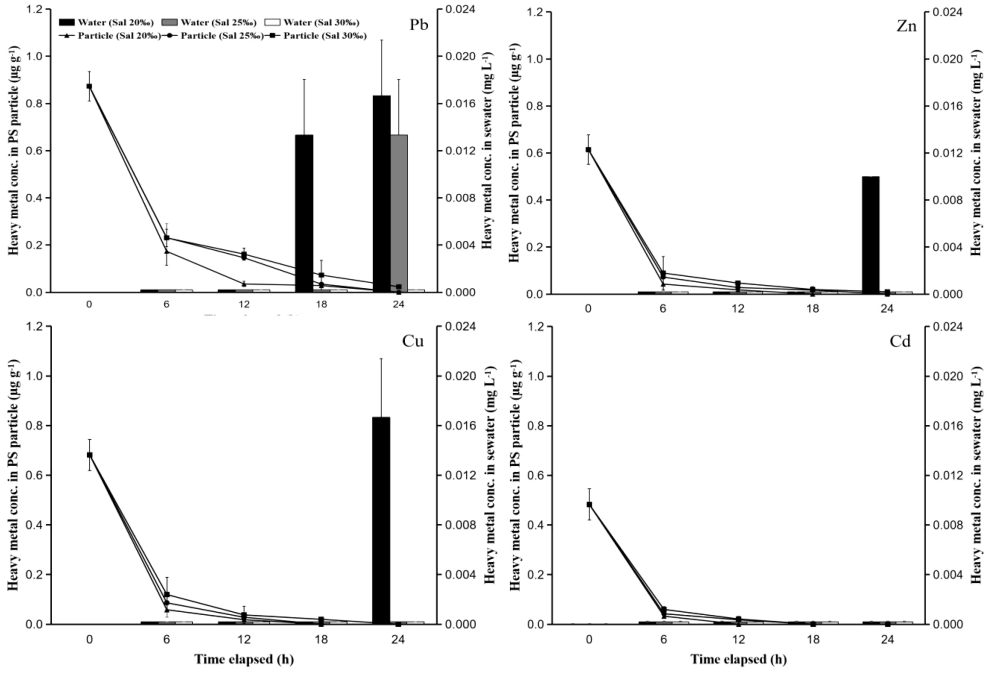

FIGURE 4
The time-course changes of heavy metal concentrations remaining in PS particles and water at the test used PS particles with a size of 50 μm that had contact with heavy metals at concentrations of 10 mg L^{-1} and different salinities (20, 25, and 30‰).

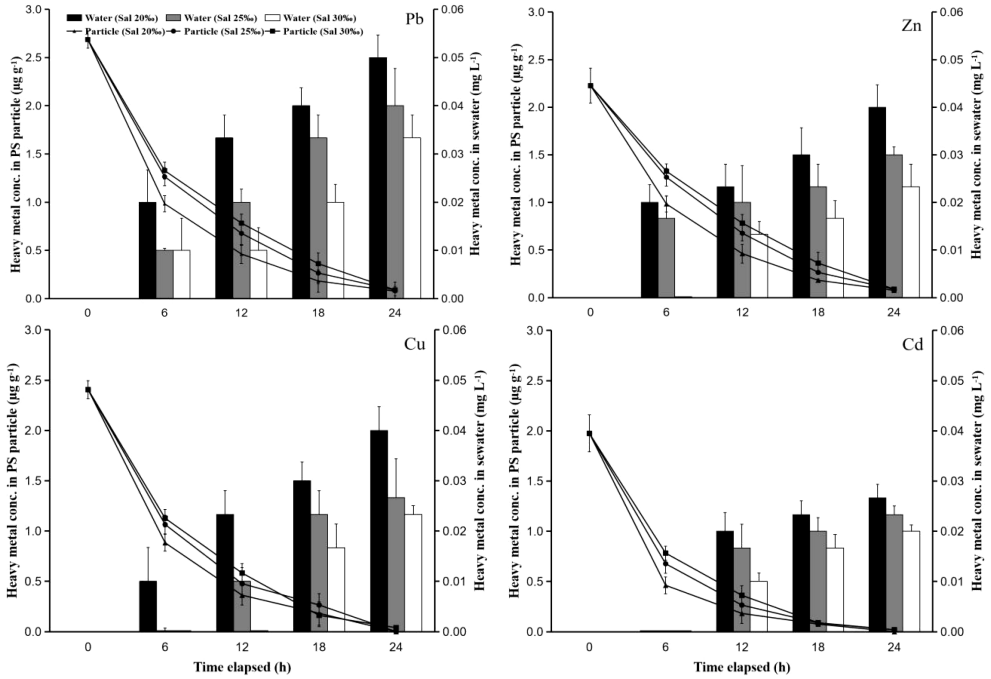

FIGURE 5
The time-course changes of heavy metal concentrations remaining in PS particles and water at the test used PS particles with a size of 250 μm that had contact with heavy metals at concentrations of 10 mg L^{-1} and different salinities (20, 25, and 30‰).

Increased Zn rate (%)= 7.765 + 2.408 C + 1.472 S + 0.135 P + 0.848 SP (R^2 = 0.854)

Increased Cu rate (%)= 20.774 + 6.266 C + 3.991 S + 0.433 P + 0.794 SP (R^2 = 0.960)

Increased Cd rate (%)= 56.324 + 13.724 C + 5.399 S + 1.710 P + 0.887 SP (R^2 = 0.851)

PS size 250 μm:

Increased Pb rate (%)= 37.159 + 22.681 C + 18.901 S + 0.702 P + 0.968 SP (R^2 = 0.889)

Increased Zn rate (%)= 41.503 + 4.495 C + 3.606 S + 1.252 P + 0.855 SP (R^2 = 0.746)

Increased Cu rate (%)= 52.995 + 9.203 C + 2.786 S + 0.698 P + 0.834 SP (R^2 = 0.798)

Increased Cd rate (%)= 48.482 + 20.528 C + 13.472 S + 0.480 P + 0.819 SP (R^2 = 0.964)

Figures 2, 3 demonstrate that, in tests employing particle sizes of 50 and 250 μm, the rise in heavy metal concentrations in the hard clam body varied according to the salt level of the water in which the clams were placed. All of the heavy metals utilized in this investigation produced results that were comparable. The concentration of heavy metals in the bodies of the hard clams increased as a result of being kept at a greater salinity.

In the test utilizing particle size of 250 μm, it was discovered that the difference in the increase in the concentration of heavy metals in hard clam bodies at changes in salinity was more evident. For example, the increase in heavy metal Pb in the test using particle size 250 μm in contact with heavy metal 1 mg L^{-1} and salinity 20‰ was less significant (p<0.05) compared to that at the other two salinities. The increase in heavy metals in clam bodies in the test with salinity 25‰ was also significantly lower (p<0.05) than that at salinity 30‰ (Figure 3). These results were found at all times of observation and at all initial Pb concentrations in this study. Meanwhile, the same test using particle size 50 μm showed that the increase in heavy metals in clam body placed at salinity 20‰ was not significantly different (p>0.05) from that at salinity 25‰, but significantly different (p<0.05) from that in hard clam placed at salinity 30‰ (Figure 2). These results were found at all times of observation. This is related to the concentration of heavy metals adsorbed on the PS particles. The larger the particle size, the higher the concentration of heavy metals that are absorbed, which causes the effect of the increase in heavy metals to be greater in the body of the hard clam.

In studies with a higher initial concentration of heavy metals in contact with PS particles, this impact was also observed. The increase in the concentration of heavy metal Pb in the body of hard clam placed at 20‰ salinity after 24 hours of exposure to PS particles in contact with heavy metal Pb with a concentration of 1 and 2 mg L^{-1} was not significantly different (p>0.05) with a salinity of 25‰, but significantly different (p<0.05) from that at

30‰ salinity. Furthermore, the test using concentrations of 5 and 10 mg L^{-1} in contact with PS particles showed that the increase in heavy metal Pb in hard clam bodies placed at different salinities was significantly different (p<0.05) from one another (Figure 2). This shows that the higher the concentration of heavy metals carried by the PS particles, the difference between the three salinities increases more clearly.

For other heavy metals, results with a nearly same trend were also discovered. The size of the particles used as carriers of heavy metals into the clam body had a strong influence on the increase in heavy metals Zn, Cu, and Cd in hard clam bodies placed at different salinities. The difference in the increase in heavy metals between different salinities is more pronounced the greater the particle size. The large value of heavy metal concentration used for contact with PS particles in the absorption process also has the same impact, namely the clearer difference in the increase in heavy metals in hard clam bodies with different salinities.

In this study, Zn showed the lowest rise in heavy metal content of any heavy metal in hard clam bodies. This was due to the fact that the clam also had the highest background heavy metal content. The effect of metal intake through particles and water is quite weak because to the substantial starting amount of heavy metals. According to Jaffar and Pervaiz (1989), the significant zinc accumulation in fish can be attributed to certain metabolic pathways and zinc-containing coenzyme catalytic reactions. In metal biomolecules with amino acid side chains having illustrious N, O, and/or sulfur donors, zinc additionally functions as a catalyst (Singh and Steinnes, 1994).

The content of heavy metals in the bodies of hard clams increased according to the salt level of the water to which they adapted. This was closely tied to the hard clams' osmoregulation mechanism. Clams that live in more salinized settings need and consume more water to keep their bodies in balance (Baker et al., 2005; Lin et al., 2016). If the water has been contaminated with heavy metals, this is consistent with the rise in the concentration of heavy metals in their bodies. In contrast, organisms that thrive in less salinity-saturated habitats need less water and release a lot of water to maintain their internal equilibrium. The effect of salinity on the osmoregulation process is increasingly visible from the results of measurements of water volume before and after exposure. The volume of water at higher salinity decreased (water depletion) more than that at lower salinity. In general, water depletion in each salinity is significantly different for almost the entire exposure time. The water depletion in the experiment with salinity 30‰ was significantly higher (p<0.05) than that in the other two salinities (Figure 6). The hard clams allow their blood salinity to vary. They need to keep the ion concentration in their cells relatively constant to maintain the function of important metabolic enzymes. In this study, clams living at a higher salinity drank more water than those living at a lower salinity. This caused PS particles mixed in the solution to have a higher probability of entering the hard clam body. This is indicated by the interaction between salinity and the number of particles accumulated in the clams in all the equations of the relationship above. The more PS particles caused an increase in the concentration of heavy metals in the hard clam bodies. However, maintaining a consistent salinity in cells presents a number of challenges. The salinity of the blood will be lower than the salinity of the cells when the salinity of seawater declines. Osmosis is the process by which water (but not ions) passes from the blood's high concentration of water into the cell's low concentration of ions (Berger and Kharazova, 1997;

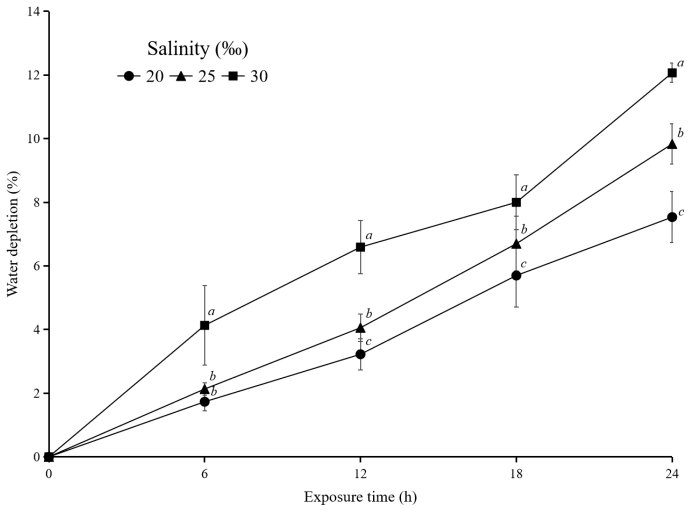

FIGURE 6
Water depletion (%) after exposure with hard clam (*M. lusoria*) (n=10) at different times dan salinities (20, 25, and 30‰). The data for water depletion (%) with different letters (a, b, and c) differ significantly (p<0.05) between salinities.

Baker et al., 2005). According to Jones (1975), *Idotea baltica*, *Idotea neglecta*, *Idotea emarginata*, and *Jaera albifrons* plants exposed to greater salinity saltwater showed higher amounts of Cd and Zn metals than those exposed to lower salinity seawater. The same results were found for the mercury content in the body of *Jaera albifrons*.

The rise in the number of accumulated particles in the first observation (6 h) did not differ significantly from the end of the observation (24 h). This was due to the limited body size of the clam to accumulate these particles. PS particles with a size of 50 µm accumulated more than those of 250 µm. This result was found in all tests. For example, the number of PS particles of size 50 µm that accumulated in the test with heavy metal Pb with a concentration of 1 mg L^{-1} and a salinity of 20‰ was 63 ± 5 particles (Figure 7A), while the size of 250 µm was 32 ± 4 particles (Figure 7B). The number of accumulated PS particles was also different in each test with a different salinity. The higher the salinity value, the more accumulated particles there are. These results were found in tests with both PS particle sizes and all tests with different heavy metals. In general, the number of particles accumulated in clam bodies placed at a salinity of 20‰ was significantly lower ($p<0.05$) than that at a salinity of 25‰. The number of particles accumulated in the clam body placed at a salinity of 25‰ was significantly lower ($p<0.05$) than that at a salinity of 30‰ (Figure 7). For example, after 24 hours of exposure to metal Zn at 1 mg L^{-1} concentrations and salinities of 20, 25, and 30‰, the number of PS particles of size 50 µm accumulated in the clam body was 62 ± 6, 70 ± 6, and 98 ± 6 particles, respectively (Figure 7A) whereas there were 37 ± 4, 55 ± 4, 71 ± 5 PS particles with a diameter of 250 µm (Figure 7B).These results indicate that salinity affects the number of particles that enter the hard clam bodies, causing the concentrations of heavy metals in their bodies to also increase. According to Viera et al. (2021), there may be a connection between the concentration of heavy metals and the particle MPs in oyster bodies. Oysters polluted with microplastics were shown to have increased heavy metal contents.

The manner in which heavy metals are released into the water varies depending on the salinity of the water. The release of heavy metals at higher salinity would be slower than that at lower salinity (Barus et al., 2021). Figures 4 and 5 shows that the concentration of heavy metals remaining in the PS particles at the lower salinity test was lower than that at the higher concentration. This indicates that more heavy metals in the particles are released into the water. This is supported by the results of observations of heavy metals remaining in seawater at each observation time. Heavy metal concentrations in seawater with higher salinity have lower values. This was due to ion competition in the water. Competition and higher ion density are found in solutions with higher salt content (Holmes et al., 2014; Liu et al., 2018), which causes heavy metal ions that are absorbed on the surface of the PS particles to become harder to release. The increase in heavy metal concentrations in seawater was found to be higher in the test using PS particles with a size of 250 µm compared to the test with PS particles of 50 µm. This was due to the particles with a larger size absorbing more heavy metals, so that more heavy metals were released into the seawater (Holmes et al., 2014; Fred-Ahmadu et al., 2019). The higher concentration of heavy metals in the test with lower seawater salinity caused the increase in heavy metals in the clam body after 6 hours of observation to be also higher than that in the test with higher salinity.

Heavy metal levels in hard clam will be elevated due to the ongoing accumulation of heavy metals in hard clam. This will

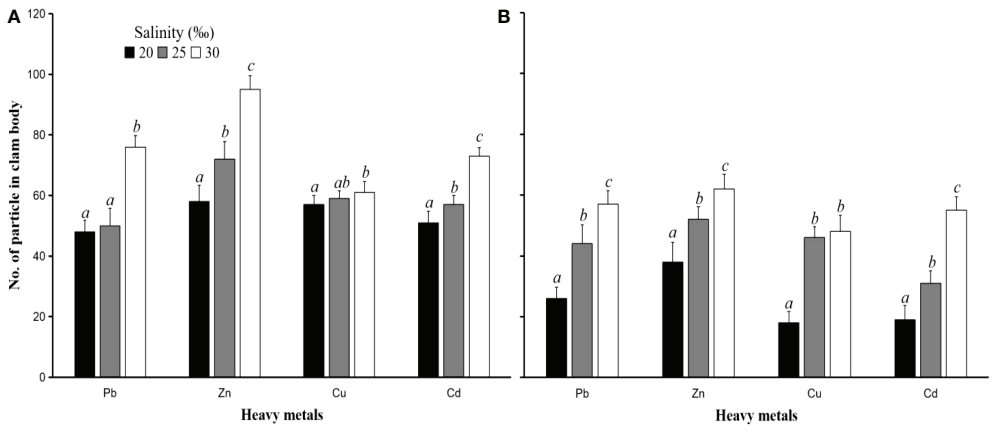

FIGURE 7
The number of PS particle sizes 50 µm **(A)** and 250 µm **(B)** accumulated in the hard clam body (*M. lusoria*) after contact with heavy metals (Pb, Zn, Cu, and Cd) with an initial concentration 10 mg L^{-1} at various salinities of 20, 25, and 30‰ after 24 hours of exposure time. The number of particles in the clam body with different letters (a, b, and c) differs significantly ($p<0.05$) between salinities.

cause the concentration value in the body of the clam to exceed the tolerable standard limit. This situation will be even more dangerous when the clam is consumed by humans. Hard clam is a food ingredient that contains nutrients needed by humans. Hard clams are a protein-rich food that has nutritional significance since they are rich in minerals and amino acids (Karnjanapratum et al., 2013). Heavy metals that accumulate in the clam would be transferred to the human body and cause the human body to become contaminated with heavy metals.

4 Conclusion

Our finding shows that salinity has an effect on the rate of accumulation of heavy metals in hard clams (*M. lusoria*) carried by PS particles. The results of this study also demonstrate that heavy metals that collect in clam bodies can be transported by PS particles. These findings point to a process that has never been explored in other studies—a continuation of the interaction between heavy metals and microplastics that contaminates organisms. All the variables employed in this study, such as PS particle size, the initial concentration of heavy metals used in the absorption process by PS particles, and water salinity, have a significant role in determining the increasing value of heavy metals in hard bodies. Hard clam bodies from higher salinity environments were observed to accumulate more heavy metals. In order to demonstrate the role of microplastics as heavy metal vectors in the real environment, further research is advised. However, this study is restricted to a laboratory size, and other environmental elements are advised to be added.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

KC, MC, and S-YC contributed to conception and design of the study. BB and ZZ wrote the first draft of the manuscript. C-YC, KC, and JW wrote the sections of the manuscript. BB and HW organized the database. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Natural and synthetic microfibers alter growth and behavior in early life stages of estuarine organisms

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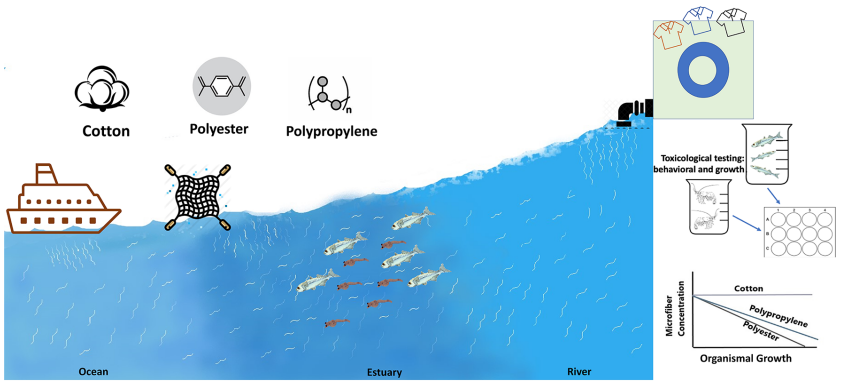
Increasing shares of microfibers are being detected in environmental samples and a closer look to identify the risk associated with them using ecologically relevant endpoints, especially at sensitive early life stages, is needed. To assess exposure hazards, we used rope samples representative of fiber types ubiquitous in coastal systems, where microfibers are often the most common debris type found in the water column. To compare responses to natural vs. synthetic microfibers, we used rinsed "natural" cotton, polyester, and polypropylene microfibers (80-150 µm length, 8-20 µm width) created from the rope. Larval and juvenile estuarine indicator species Inland Silverside *(Menidia beryllina)* and mysid shrimp *(Americamysis bahia)*, respectively, were exposed to these three microfiber types at three concentrations (3, 10, 30 particles/ml) along a 5-25 PSU salinity gradient to mimic estuarine conditions. Behavioral responses, growth, and ingestion were measured. The cotton microfibers were not detected in the digestive tracts of Silversides, however, both the polyester and polypropylene microfibers were detected in the Silversides' stomach and gut lining. None of the fiber types were detected in mysid shrimps. Mysids exposed to cotton microfibers had fewer behavioral effects compared to Silversides, who responded more to cotton. Cotton exerted no effect on growth in Silversides but did cause reduced growth in the mysids at the two lower salinities. In contrast, polyester and polypropylene were identified to have a significant dose dependent effect on mysid and Silverside behavior as well as growth was affected in at least one of the three salinities at concentrations as low as 3 particles/ml. Cotton impacted both the organism's behavior more at higher salinities, whereas polyester and polypropylene had more impacts at lower salinities. This raises concerns for microfiber impacts on estuarine ecosystems and the need for policies to limit microfiber production and outfall into the aquatic environment.

KEYWORDS

rope, cotton, polyester, polypropylene, inland silversides, mysid shrimp, sublethal, marine



GRAPHICAL ABSTRACT

1 Introduction

There are a wide range of contaminants entering the environment from anthropogenic sources, with the largest component being from marine litter (Bergmann et al., 2015; Auta et al., 2017). Recently, micro-sized particles (<5 mm), particularly microfibers, have gained great attention due to increased identification in samples and identification of adverse impacts on organisms and ecosystems (Mishra et al., 2020; Granek et al., 2022; Kwak et al., 2022). In coastal systems, most microfiber are produced by the ropes and fishing gear that contribute 1277 ± 431 microplastic pieces m^{-1}, with an estimated 44% from fishing rope and 49% from nets (Wright et al., 2021). Additional sources of microfibers are natural and synthetic textiles (Granek et al., 2022). Synthetic microfiber consist of persistent polymers including nylon, polyester, polyethylene terephthalate (PET), polypropylene, acrylic or spandex (Athey and Erdle, 2021; Granek et al., 2022), while natural fibers can be cotton, wool, linen, etc. The ubiquity of microfibers in the environment ranges from the atmosphere to the deepest parts of the ocean (Krüger et al., 2020; Mishra et al., 2020; Reineccius et al., 2020; Suaria et al., 2020; Acharya et al., 2021; Brahney et al., 2021, Caldwell et al., 2022).

From 1975 to 2020, total global fiber production increased from 32 to120 million metric tons, and is expected to increase to 146 million metric tons by 2030 (Truscott, 2020). Microfibers are released into the environment due to their propensity for shedding and are sourced from household washing machines, cloth dryers and also from vacuum cleaning (Hartline et al., 2016; Sillanpää and Sainio, 2017; Yang et al., 2019), and most recently from protective personal equipment during the pandemic (e.g., masks) (Akhbarizadeh et al., 2021; Morgana et al., 2021). Of the total fabric produced in 2015, approximately 75% either went to a landfill or was incinerated (Ellen MacArthur Foundation, 2017). Accumulation of microfibers is of great concern due to their potential environmental impacts as well as their carbon footprint (Zhu, 2021; O'Brien et al., 2022).

Natural microfibers (e.g., cellulose based), may be less persistent and can break apart during metabolic processes as well as through aerobic biodegradation processes (Zambrano et al., 2019; Zambrano et al., 2020). However, their fate and role in the aquatic ecosystems due to different chemical dyes, functional finishes (e.g., fluorinated compounds) coupled with a higher propensity for shedding compared to synthetics is unknown, and they may provide more surface area to adsorb contaminants (Salvador Cesa et al., 2017; Henry et al., 2019; Mishra et al., 2019; Athey et al., 2020). For synthetic microfibers, their nonbiodegradable nature and high tensile strength likely leads to increased persistence in the environment (Li et al., 2010), entrapment in the gills or digestive tract, or once ingested a false sense of satiation and subsequent loss of nutrition (Watts et al., 2015; Watts et al., 2016; Stienbarger et al., 2021). Given organisms are exposed to both natural and synthetic fibers (e.g. Caldwell et al., 2022, Granek et al., 2022), it is essential to study both.

Estuaries have high variability in physio-chemical conditions and provide a wide range of favorable habitat for a diverse suite of taxa (Costanza et al., 1997). Being first to receive direct inflow from rivers transporting contaminants into surface waters, they can concentrate microplastics (Browne et al., 2010; Wright et al., 2013). Regardless of this, data on estuarine organisms for microplastic ingestion and effects are fairly limited (Possatto et al., 2011; Vendel et al., 2017; Bessa et al., 2018; Athey et al., 2020) and this data gap has been noted (Granek et al., 2020; Kutralam-Muniasamy et al., 2020; Kwak et al., 2022).

Additionally, behavioral endpoints are very poorly studied in relation to emerging contaminants, including microplastics and especially for microfibers, even though behavioral alterations are demonstrated to impact fitness and survival in the wild

(McCormick et al., 2020; Brodin et al., 2014; Scott and Sloman, 2004; Weis et al., 2001). To address these knowledge gaps, this study describes behavioral and growth impacts of natural cotton and synthetic (polyester, polypropylene) microfiber (80-120 µm) in two estuarine indicator species (mysid shrimp and Silversides) across a salinity gradient. We hypothesized that sublethal effects would be caused following exposure to both synthetic and non-synthetic fibers. To the best of our knowledge, this is the first paper to measure and compare natural verses synthetic microfiber responses in the early life stages of both fish and invertebrates.

2 Methods

2.1 Chemicals

Suwanee River Natural organic matter (NOM) - 2R101N used to create suspensions of microparticles in exposure wells was purchased from the International Humic Substance Society, St. Paul, MN. Tissue-Clearing Reagent CUBIC-R+ [for Animals] (T3741) and Tissue-Clearing Reagent CUBIC-L [for Animals] (T3740) for visualization of particles within organisms following exposures were purchased from Tokyo Chemical Industry Co., Ltd. Ropes were purchased from SGT knots and the "natural" cotton rope was purchased from the local craft store.

2.2 Microfibers preparation

Detailed microfiber preparation protocol has been provided in Figure S1. Various studies reported microfiber production in the lab (Saborowski et al., 2019; Ward et al., 2019; Gambino et al., 2020; Knauss et al., 2021; Ma et al., 2021); however, very few demonstrated efficient microfiber generation with limited equipment. In this study, we adapted microfiber production methods described by Cole (2016). Briefly, microfibers were untwisted and cut into short sections. After adding a freezing solution (Surgipath® FSC 22® Frozen Section Embedding Medium, Leica), each cut section was frozen in a -80°C freezer for 5 minutes. Next, each frozen block was shredded through a coffee grinder (>1 mm) with some filtered DI water. The shortest microfibers from the wall of the coffee grinder cup were collected and mixed with warm DI water (40 °C) before filtering through 120 micron filter paper (polycarbonate). Filtered microfiber of <120 µm in length was collected and washed with ethanol. The resultant stock solution was then used to prepare the required amount for the exposures. The micron (80-120 µm) sample particle count is determined by triplicate sampling of the suspension and the particle count analysis confirmed using a light microscope (Leica EZ4) and Sedgwick rafter slide without a coverslip to avoid miscounting (Stienbarger et al., 2021). The microfiber size range found in the natural environment is from 50 to 100 µm (about 53% of total sampled microfiber) (Pirc et al., 2016; Conley et al., 2019). The concentration of microfibers used in this study was 3-30 particles/ml. Since these microfibers are generated from ropes, which are used in the coastal environment during commercial and recreational fishing activities, their bioavailability increases with more use. They are potentially a higher cause of concern, as they do not go through wastewater treatment process, which tends to reduce microfibers significantly in treated effluent (Granek et al., 2022). In part the aim of these concentrations was also to make microfibers available for the organism to ingest at levels comparable to what is found in larval fish in the wild (e.g. Lasdin et al. in revision), reported later in this paper. Using this method, cotton microfiber generation was higher compared to polyester and polypropylene fiber. This is a general trend reported by others, that generation of microfiber per gram of textile laundered is significantly higher in cellulose-based fabrics compared to polyester (Sillanpää and Sainio, 2017; Cesa et al., 2020; Zambrano et al., 2021).

2.3 Model organisms, their sources, and experimental setup

Americamysis bahia larvae were purchased from Aquatic Biosystems in Fort Collins, Colorado, and reared in three tanks to adulthood at 15, 20, and 25 PSU salinities with filtered artificial seawater prepared (AFSW). Following EPA protocol 833-C-09-001 (USEPA, 2009; Siddiqui et al., 2022). when adult *A. bahia* reproduced, larvae were moved to additional tanks of the same salinity and reared for seven days. Microfiber exposures with mysids were initiated at seven days post fertilization (dpf) (n=9) under static renewal conditions for seven days. *Menidia beryllina* embryos were harvested from broodstock held at the OSU Hatfield Marine Science Center and placed into three acclimation aquaria of 5, 15, and 25 PSU salinities with filtered AFSW following modified methods from Middaugh et al. (1987), as done in previous studies in the Brander lab (DeCourten et al., 2020; Hutton et al., 2021; Siddiqui et al., 2022). Larvae were placed into exposure vessels at 6 ± 1 days post fertilization (dpf) (n=6) and maintained under static renewal conditions for 96 h.

Each model species was exposed to a total of 26 treatments: each in covered beakers containing water control, NOM control with each of the three-microfiber types with concentration treatments (3,10 and 30 particles/ml) across three salinities per species as described above (Figure S2). Nominal water concentrations with detailed QA/QC are provided in SI table 1. Water quality parameters were measured daily just prior to and following 80% water renewal. Cumulative hatching and mortality were recorded daily. *A. bahia* were fed concentrated brine shrimp (*Artemia franciscana*) ad libitum, and *M. beryllina* were fed Gemma Microdiet 0.2 mg/beaker/day (Skretting,

Westbrook, Maine). Both organisms were fed daily and allowed to feed for at least two hours before water was changed. Tables SI 2, 3 provides water quality parameters maintained throughout the experiment. A control filter was set up to measure background contamination in the air.

2.4 Behavioral assays

Following microfiber exposures of 7d (*A. bahia*) and 96 h (*M. beryllina*), behavioral assays were performed post-exposure for each treatment using a DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands) for the Dark: Light cycle as described previously (Mundy et al., 2021; Segarra et al., 2021; Siddiqui et al., 2022). Briefly, *A. bahia* and *M. beryllina* larvae were randomized and placed in individual 10 ml glass beaker in a 12 well plate frame in the Ethovision Observation Chamber (EOB) to observe natural photo motor response. Larvae were allowed to be acclimatized for at least 1 hour before placing into the EOB. After acclimatization outside, another 5-minute acclimatization time was provided inside the dark chamber, followed by three 2-minute intervals of dark stimuli and three 2-minute intervals of light stimuli. Behavior and activity were recorded and tracked by a Basler Gen 1 Camera using Ethovision XT15 software. Velocity thresholds were determined for swimming parameters between 0.5 cm/s (freezing) – 2.0 cm/s (moving) (Segarra et al., 2021; Siddiqui et al., 2022). A virtual center zone (1.6 cm diameter) was established to measure the time that larvae spent in the center of the 2.2 cm diameter in the beaker. All behavioral tests were conducted between 09:00 and 18:00 h. The resolution was set at 1280 x 960, light cycles were programmed at 10,000 lux, and the frame rate was set at 25/s. A total of eight variables were analyzed in this study, the p values and other statistics for which are included in Table 1. Following behavioral analysis, organisms were euthanized humanely, Silversides per IACUC protocol #0035, and fixed in paraformaldehyde (PFA) to preserve tissues for examination of microfibers internalization.

2.5 Growth and Microfibers internalization

Per Siddiqui et al. (2022), at least three individuals from each species per replicates were collected for growth. Length and width measurements were collected *via* dissecting scope equipped with Moticam visual software, and particle uptake was visualized on a Zeiss Axio Observer inverted microscope (Carl Zeiss, White Plains, NY). Growth data were assessed by creating a growth index with the following formula:

$$\frac{W}{L} X \ d$$

Where W is the Width of the organism, L is the length, and d is the number of days the organism is exposed to the microfiber. This relationship provides the index used to plot the final growth curve. Organisms were then cleared using a protocol adapted for larval organisms with CUBIC^{TM} clearing reagents (Susaki et al., 2015; Ohnuma et al., 2017). Briefly, to remove pigmentation and allow visualization of internalized microplastics (1-20 um), individual organisms fixed in 3% PFA were washed in 5 ml phosphate-buffered saline (PBS) for 30 minutes and incubated in 5 ml CUBIC-L at 37° C for seven days to encourage lipid removal. Following this step, organisms were washed again in 5 ml PBS for an additional two hours and then transferred to CUBIC-R + for an additional seven days to clear the remaining tissue.

2.6 Statistical analysis

Statistical analysis was performed using RStudio Version 1.0.153. Dose-response curves were generated to evaluate larval swimming behavior and growth effects across concentration treatments. The growth data and concentration dependent dose response curves (C-DRC) for behavioral data were prepared by drm function in r using the DRC package by Ritz

TABLE 1 Behavioral variables from Noldus ethovision software used in this study to analyze Mysid shrimp (*A. bahia*) larvae and Silverside (*M. beryllina*) larvae behavioral response.

Variable	Unit	Description
Distance (Total)	cm	Total distance moved inside the well throughout the video recording time.
Angular velocity	Deg/sec	Dividing the Turn angle by the sample interval
Velocity	Cm/sec	Velocity for the center point
Freezing	Sec	The mean of total time fish moving less than 2 seconds
Movement	Sec	Duration for which the selected body point was changing location with respect to center
In Zone duration	S	The total time spent in the beaker center part (zone)
Meander	Deg/cm	Turning in animals moving at different speed.
Turn Angle	Deg	Difference in heading between two samples.

et al. (2010) (Ritz, 2010) and lines were fit *via* ggplot (Wickham, 2005). The Shapiro–Wilk test was used to test normality, and Levene's test was used for homogeneity testing. After confirming normality and homogeneity of data, a 3-4 parameter model using a nonlinear regression approach was used to prepare the model at each salinity and combined using ggplot2 function to create graphs in R. Analysis of Variance (ANOVA) was used to evaluate differences among treatment groups. A Tukey HSD *post-hoc* test was used to compare particle concentrations between treatments, ($\alpha =< 0.05$). Responses between the NOM control and water only control where combined into only one control treatment per salinity for statistical analysis. Radar (or spider web) plots were used to visualize behavioral effects of microfibers across multiple salinities. To effectively convey the large amount of data produced by behavioral assays, radar plots are commonly employed (Mundy et al., 2020; Segarra et al., 2021). Radar plots are particularly useful when trying to compare multiple variables to each other with highly dynamic data, such as those presented from behavioral assays. For radar plots, all data were normalized to a 0-1 scale per behavioral analyses published in Segarra et al. (2021).

3 Results and discussion

Average *A. bahia* larvae survival for control and exposure treatments was 99 ± 1% and 95 ± 2%, respectively, with no significant difference across the treatments (ANOVA (Normal distribution, Tukey HSD *post-hoc*, $p > 0.05$). Average *M. beryllina* larvae survival for control and exposure treatments was 98 ± 2% and 93 ± 1%, respectively, with no significant difference across the treatments (ANOVA (Normal distribution, Tukey HSD *post-hoc*, $p< 0.05$). As such, these were truly sublethal exposures.

3.1 Internalization of microfibers

When Silversides were observed under a Zeiss model microscope (Carl Zeiss, White Plains, NY) following tissue clearing for microfiber presence, cotton microfibers were not detected in the Silverside stomach or gut (Figure S3A). However, short pieces (15-60 µm length, shorter than length of added fibers ranging 80-100 µm) of synthetic microfiber (polyester and polypropylene) were observed in both the stomach and gut in the samples examined (Figures S3B, C). Similar partitioning results were reported by other studies in different aquatic organisms (Kolandhasamy et al., 2018; Woods et al., 2018). Other model species (invertebrates and fish) when exposed to synthetic microfibers, showed particle ingestion (Bour et al., 2020; Knauss et al., 2021; Ma et al., 2021). When looking at body tissue partitioning, blue mussel (*Mytilus edulis*), accumulated 81.3% of ingested microfibers in the digestive gland, followed by 14.4% in gills and 4.3% in soft tissues (Woods et al., 2018). No fibers of either type, synthetic or natural, could be detected in the cleared mysids. This finding is in line with other studies showing that crustaceans are more effective than fishes in breaking down microplastics (Cau et al., 2020; Matteos-Cárdenos et al., 2020).

Aquatic organisms seem to be more interested in capturing and consuming plastic microfibers, which may relate to visual or tactile misidentification or be a result of flavoring by organic compounds on their surfaces (Brillant and Macdonald, 2002; Moore, 2008). Other studies suggest plastics may act as phagostimulants that cause organisms to ingest them (Allen et al., 2017). Cotton is primarily made up of enriched cellulose which can be easily digested and broken down by living organisms (Stickney and Shumway, 1974), whereas polyester is a hydrophobic and non-sugar based compound that is not as easily degraded (Li et al., 2010). Additionally, the breaking load of cotton yarn at the dry and wet stage is much higher when compared to polyester (Zambrano et al., 2019), which can increase digestibility in organisms when internalized.

Another aspect of internalization can lead to bioaccumulation, uptake, and particle movement to higher trophic levels. Low feeding rates were reported in freshwater diving beetle *Cybister japonicus* (a top predator in freshwater ecosystems) after consumption of zebrafish (*Danio rerio*) exposed to polyethylene microspherest (diameter- 247.5 ± 14.4 µm; concentration 92± 7 particles/ml) with a trophic transfer rate of 13-18% (Kim et al., 2018). The observation of internalization of synthetic fibers in the larval fish studied herein, but not in juvenile shrimp, suggests a longer residence time in comparison to the mysid shrimp. This should be further investigated over longer exposure times to determine if sublethal effects caused by internalization are more severe over longer timescales. The lack of detection for cotton in either taxa is perhaps not surprising given the comparably easier digestibility of natural materials.

3.2 Growth impact

Although it was not detected in their digestive tract, mysids exposed to cotton had significantly lower growth relative to the control in the lowest and middle salinities (Figure 1A). However, silversides displayed no decrease in growth following cotton exposure at any salinity (Figure 2A). While the mysids did have decreased growth compared to the controls it was not decreased in a concentration dependent manner, nor was there a concentration dependent response in Silversides (Figures 1B, 2B). Polyester decreased growth in all three salinities in mysids but only the lowest and highest salinities in Silversides (Figures 1A, B). Polyester also caused a significant concentration dependent decrease in mysid growth from the medium and high salinity exposures and in Silversides in the highest salinity (Figure 1). When both organisms were exposed to polypropylene both mysids and silversides had decreased growth relative to the controls from all three salinities.

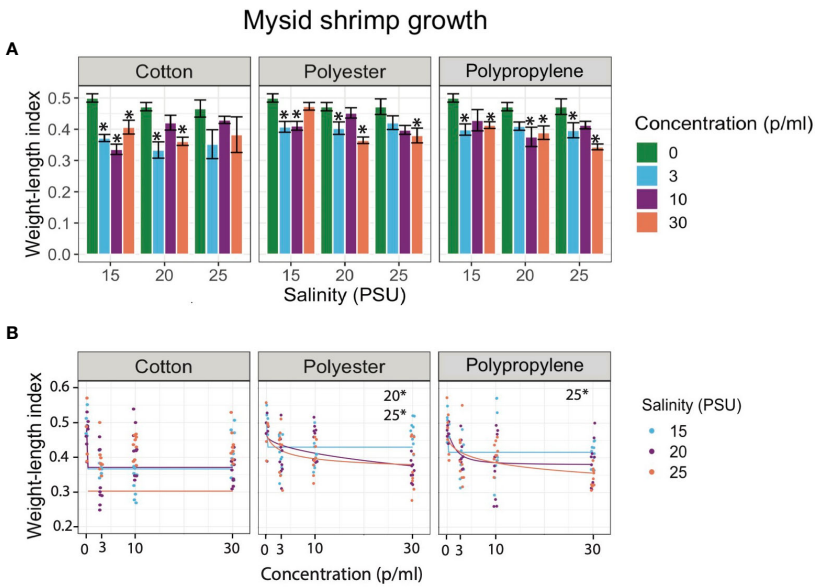


FIGURE 1
(A) Bar graphs of juvenile mysid shrimp (*A. bahia*) larvae growth exposed to cotton, polyester, and polypropylene microfibers across 0 - 30 p/mL concentration and 15-25 PSU salinity gradient respectively. Asterisks represent statistically significant differences relative to the controls. Only effects relative to the control are shown ($\alpha < 0.5$; TukeyHSD). **(B)** Dose response curves (DRCs) of juvenile mysid shrimp (*A. bahia*) larvae growth exposed to cotton, polyester, and polypropylene microfibers across 0 - 30 p/mL concentration and 15-25 PSU salinity gradient respectively. Each circle in the DRCs represents the rescaled growth index mean of one larva (n=9) and regression lines are plotted from the DRC model from the R drc package. Data are presented on a log scale.

Polypropylene exposed mysids had a concentration dependent decrease in growth in the highest salinity as did Silversides in the lowest salinity (Figures 1B, 2B). The decrease in mysids but not Silversides may be a result of species differences in feeding, where in mysid shrimp feed prolifically from hatching while Silversides can feed following hatching, it is not necessary until 6-7 dph (personal observation). Different feeding rates may explain the differences in growth if microfiber ingestion results in satiation without caloric value. Salinity may also play a role in altering the agglomeration, density, and therefore settling of microfibers, contributing to varied growth responses. Microplastic agglomeration differs across salinities (Shupe et al., 2021), and this may also hold true for microfibers, however few data currently exist on the sinking rates of fibers across salinities, although a recent paper suggests there may be a strong influence of salinity on the transport and fate of microfibers in the global ocean (Lima et al., 2021).

Contaminants, microplastics in particular, can impair feeding behavior by demotivating feeding preferences due to false satiation, resulting in reduced search effectiveness and prey capture ability. Various studies have linked foraging behavior alterations to reduced feeding resulting in lower growth (Little et al., 1990; Chapron et al., 2018; Lo and Chan, 2018; Cole et al., 2019; Coppock et al., 2019; Naidoo and Glassom, 2019). In general, when organisms are exposed to microplastics, reduced growth and energy reserves are reported (Yin et al., 2018; Jacob et al., 2020). Studies specifically examining microfibers reported similar results to ours in other organisms (crab, *Carcinus maenas*) where they observed reduced energy available for growth (scope for growth) when exposed to polypropylene rope (1–5 mm in length) (Watts et al., 2015). Other studies also show the selection preference depends upon the type of microplastic fibers in addition to the presence and absence of food. For example, sea anemone (*Exaiptasia pallida*) ingested a higher percentage of nylon microfiber compared to the other polymers in the absence of brine shrimp, whereas; anemones ingest all types of synthetic microfibers when offered in the presence of brine shrimp (Romanó de Orte et al., 2019). In another study, Norway lobster (*Nephrops norvegicus*) demonstrated reduced body mass in addition to blood and stored lipid when exposed to polypropylene rope microfibers (Welden and Cowie, 2016). Similarly, growth impacts on fishes, including Silversides, were observed following exposure to other microplastic types (Critchell and Hoogenboom, 2018; Athey et al., 2020; Siddiqui et al., 2022). When planktivorous reef fish (*Acanthochromis polyacanthus*) were exposed to polyethylene terephthalate (300-125 µm diam), they showed decreased growth under limited food availability conditions (Critchell and Hoogenboom, 2018). Collectively these results show that there may be more the negative health impacts of synthetic microfibers compared to cotton, however additional studies comparing synthetic fibers to cellulose-based fibers across

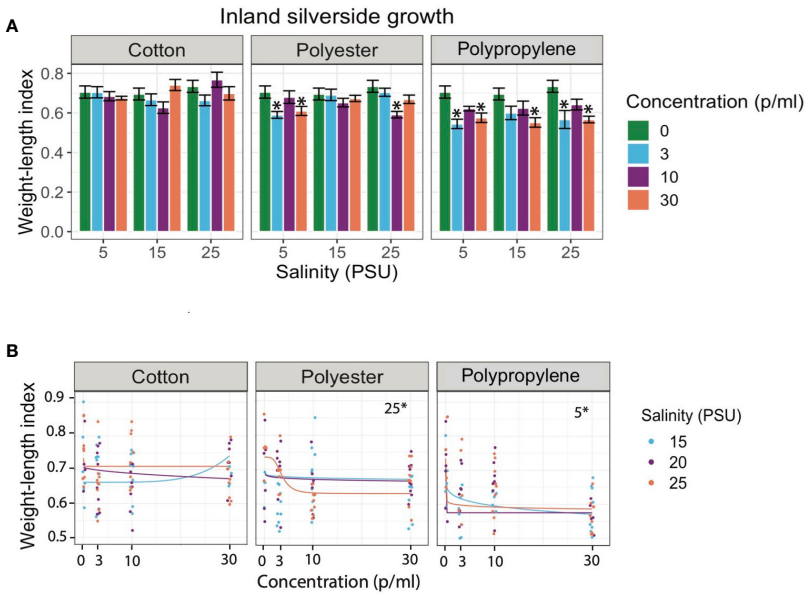


FIGURE 2
(A) Bar graphs of juvenile Silverside (*M. beryllina*) larvae growth exposed to cotton, polyester, and polypropylene microfibers across 0 - 30 p/mL concentration and 15-25 PSU salinity gradient respectively. Asterisks represent statistically significant differences relative to the controls. Only effects relative to the control are shown ($\alpha < 0.5$; TukeyHSD). **(B)** Dose response curves (DRCs) of juvenile Silverside (*M. beryllina*) larvae growth exposed to cotton, polyester, and polypropylene microfibers across 0 - 30 p/mL concentration and 15-25 PSU salinity gradient respectively. Each circle in the DRCs represents the rescaled growth index mean of one larva (n=9) and regression lines are plotted from the DRC model from the R drc package. Data are presented on a log scale.

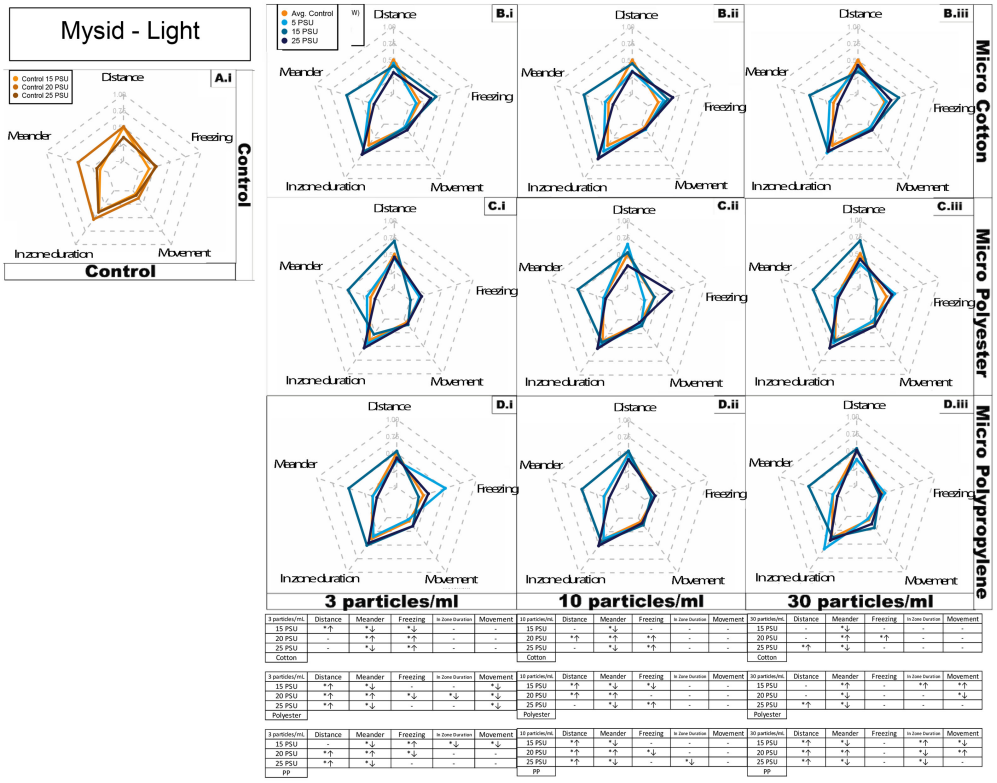
many types and origins (e.g. clothing, rope, carpet, etc.) are needed, especially given the variability in composition, additives used, tensile strength, and other industrial treatments fabrics undergo prior to shedding into the environment (Athey and Erdle, 2021; Granek et al., 2022)

3.3 Behavioral impacts

In the case of the cotton treatment, out of eight mysid behavioral activities (Table 1), only 20% of behavioral variables were affected in a concentration dependent manner in at least one salinity (Figures 3, S4A). In contrast, 90% of behavioral variables measured in mysids exposed to polyester were affected in a concentration dependent manner at least at one salinity (Figure S4B). For polypropylene, 50% of behavioral variable measured in mysids were impacted in a concentration dependent manner in at least at one salinity (Figures 3, S4C).

To summarize the significantly- impacted behavioral endpoints collectively described above, cotton microfiber exposed mysids exhibited a significant concentration dependent increase in freezing activity from medium to highest concentration within the highest salinity under dark cycle (Figures 3, S4A). There was a significant concentration dependent increase in time spent in the central portion of the beaker (change in zone) at medium salinity (dark cycle) and highest salinity (light cycle). The lowest salinity demonstrated a significantly higher turn angle activity under dark conditions, but with no concentration dependence. Overall, cotton exposed mysids exhibited more significant behavioral variations at highest salinity (50% of total activity; Figures 3, S4), compared to lowest salinity (40% of total activity; Figures 3, S4).

A concentration dependent decline in angular velocity during light cycle conditions was observed in polyester microfiber-exposed mysids, with a significant increase at the medium salinity from lowest to highest concentration (Figures 3, S4B). Mysids exposed to polyester also demonstrated a significant increase in total distance moved at the medium salinity from the lowest to the highest concentration. There was a significant concentration dependent increase in freezing within the lowest and highest salinity in both dark and light cycles, with a significant decline in medium salinity in the light cycle. Mysids in the medium salinity exhibited a significantly higher incidence of meandering activity under the light conditions with no concentration dependence. In the case of turn angle, mysids held at the lowest salinity (15 ppt) demonstrated significantly higher activity with no concentration dependent activity, whereas the highest salinity (25 ppt) demonstrated a concentration dependent increase from the medium to highest concentration under dark conditions. In contrast, mysids exposed at a medium salinity demonstrated significantly decreased



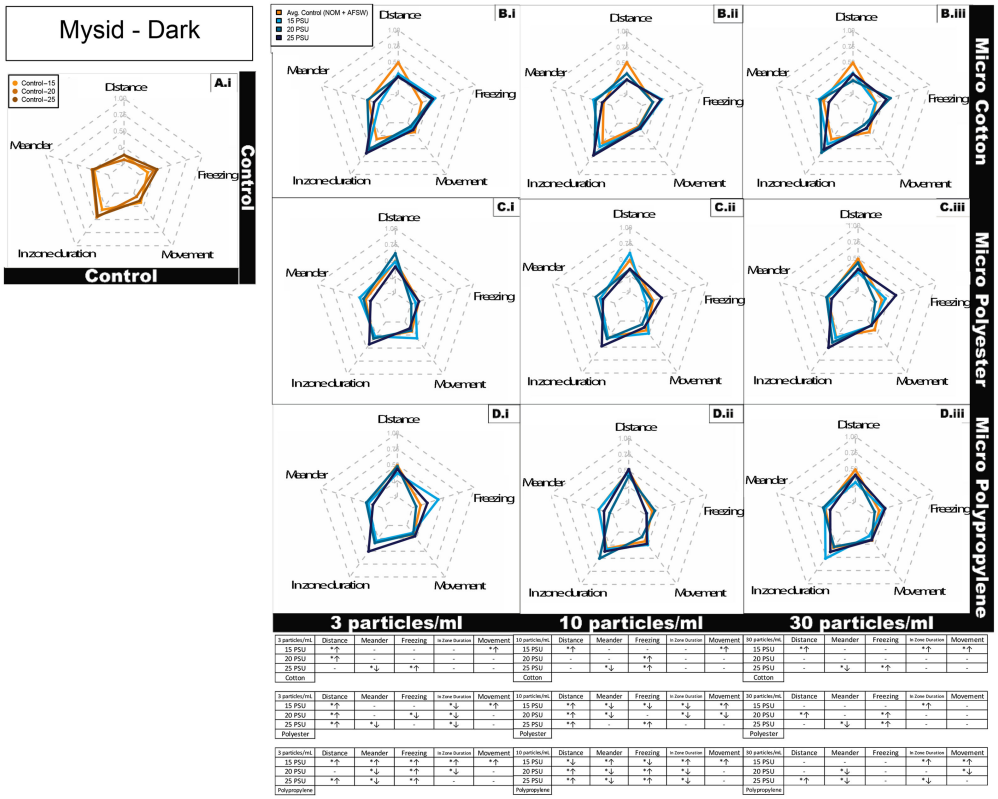


FIGURE 3 (Continued)

FIGURE 3 (Continued)
Juvenile Mysid shrimp (*A. bahia*) behavioral response represented as radar plot after 7 days exposure to cotton, polyester, and polypropylene microfibers in dark and light cycles across a salinity gradient 15PSU – 25PSU. **(A)** Control in water and NOM, combined for each salinity; **(B)** Cotton microfiber with average control; **(C)** Polyester microfiber with average control; **(D)** Polypropylene microfiber with average control. Data are representative of the calculated Z-scores normalized to controls (per Segarra et al., 2021), which are presented on the 0 axis in the middle of each figure in the panel. In the tables, asterisks represent statistically significant differences relative to the controls. The arrows indicate an increase or decrease in response for that particular behavioral endpoint. Only effects relative to the control within each salinity are shown ($\alpha < 0.5$; TukeyHSD).

concentration dependent activity under light conditions. Mysids in the lowest salinity had significantly higher activity under the dark condition with no concentration dependent activity (Table SI4). Overall, polyester-exposed mysids had more significant behavioral variations at highest salinity (94% of total activity; Figures 3, S4), compared to lowest salinity (56% of total activity; Figures 3, S4).

Polypropylene microfiber exposed mysids demonstrated a significant concentration dependent decrease in freezing within the lowest salinity in both dark and light cycles (Figures 3, S4C). There was a concentration dependent increase at the lowest salinity observed in time duration in the center of the beaker (zone) at both dark and light cycle conditions. Mysids in medium salinity demonstrated significantly higher meandering activity under light conditions with no concentration dependent activity. In contrast, mysids in the lowest salinity demonstrated significantly decreasing concentration-dependent velocity under the dark condition with no concentration dependent activity in the light cycle. Overall, polypropylene exposed mysids displayed similar behavioral variations at both highest and lowest salinity (56% of total activity; Figure S4).

Micro cotton, polyester and polypropylene all demonstrated a significant decrease in meander at all particle concentrations, except in polyester at 15 ppt in highest concentration, where it increased over time (Figure 3A). There was no impact observed on freezing behavior in all microfiber types at two highest concentrations. In medium particle concentration, there was no effect on movement observed across all microfiber types. In both synthetic microfiber treatments, there was increased distance moved by mysids in at least at one salinity. Meander was affected at all salinities in both the synthetic microfiber treatments.

Silversides exposed to the cotton microfiber treatment had 30% of measured behaviors affected in a concentration dependent manner in at least one salinity (Figure 4, S5A). In the case of polyester microfibers, 90% of behavioral endpoints exhibited a concentration dependent effect in at least one salinity (Figure S4B). For polypropylene microfiber exposed Silversides, 100% of behavioral activities demonstrated a concentration dependent effect in at least one salinity (Figure S5C). The behavioral variables are discussed below with each treatment. To summarize specific impacts on fish behavior, Silversides exposed to cotton microfiber had overall significantly higher activity at the medium salinity in light conditions (Figure 4, and Figure S5A). In the medium salinity total distance moved had significantly increasing concentration dependent activity under the dark condition in contrast to significantly lower activity in the light cycle. Silversides in the lowest salinity demonstrated significantly increased concentration dependent freezing under both dark and light conditions. Silversides also spent increasing time in the zone (center of beaker, as described above for mysids) in a significant concentration dependent manner in the lowest salinity under both light and dark conditions. Overall, at the lowest salinity Silversides demonstrated significantly lower meandering activity under both, light and dark conditions compared to other salinities. Silversides at the lowest salinity also demonstrated a significant concentration dependent decreasing movement under both light and dark conditions, whereas the medium salinity only demonstrated decreasing concentration dependent activity in the light cycle. At the medium salinity Silversides exhibited significant concentration dependent increasing velocity under dark conditions. Overall, cotton exposed silversides shown significant behavioral variations at highest salinity (94% of total activity; Figures 4, S5), compared to lowest salinity (56% of total activity; Figures 4, S5).

Polyester microfiber exposed Silversides in medium and high salinity demonstrated significantly increasing concentration dependent angular velocity under both dark and light conditions with increasing concentration dependent activity only within highest salinity in dark cycle (Figures 4, S5B). Total distance moved was significantly increased in a concentration dependent manner in both the dark and light cycles. Both the medium and high salinities Silversides demonstrated significantly increasing concentration dependent freezing under both, light and dark condition. Silversides exposed at all three salinities showed decreasing activity in the zone in a significant concentration dependent manner under both light and dark conditions. In the highest salinity, Silversides showed significant concentration dependent increasing meandering activity in both light and dark conditions. The high salinity also showed significantly decreasing concentration dependent movement in the dark cycles. In contrast, Silversides in low and medium salinity exhibited significantly increasing concentration dependent movement under light conditions. Silversides in highest salinity also showed significant concentration dependent increasing turn angle under both, light and dark conditions. Velocity was only altered in the medium salinity under light conditions, where Silversides demonstrated significant concentration dependent

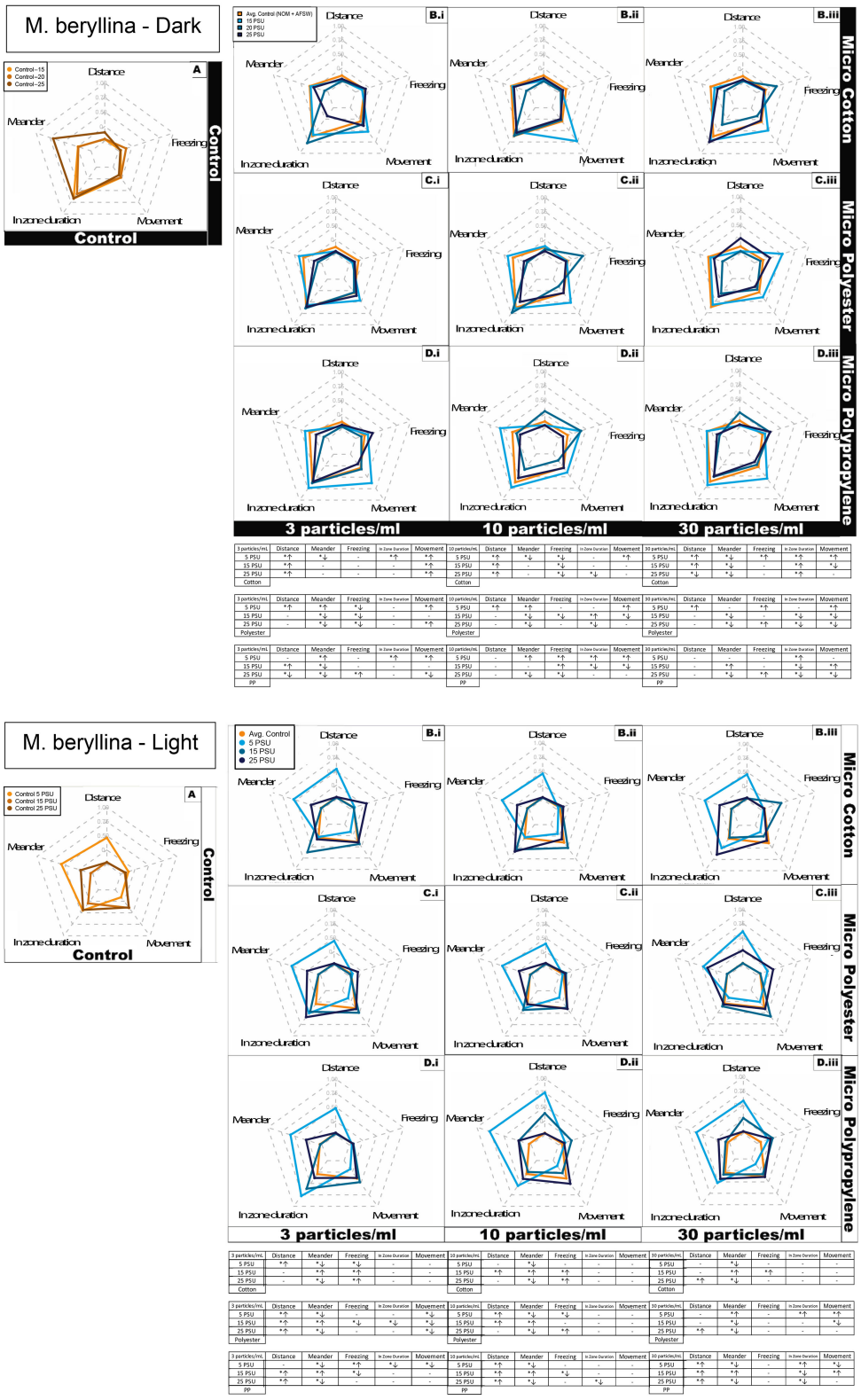


FIGURE 4 (Continued)

FIGURE 4 (Continued)
Silverside (M. beryllina) larvae behavioral response represented as radar plot after 4 days exposure to cotton, polyester and polypropylene microfibers in dark and light cycles at both the cycles across a salinity gradient 5 PSU – 25 PSU. S = salinity, arrows indicate significant increases or decreases for that particular behavioral endpoint. **(A)** Control in water and NOM, combined for each salinity; **(B)** Cotton microfiber with average water and NOM control; **(C)** Polyester microfiber with average water and NOM control; **(D)** Polypropylene microfiber with average water and NOM control. Data are representative of the calculated Z-scores normalized to controls (per Segarra et al., 2021), which are presented on the 0 axis in the middle of each figure in the panel. In the tables, asterisks represent statistically significant differences relative to the controls. The arrows indicate an increase or decrease in response for that particular behavioral endpoint. Only effects relative to the control within each salinity are shown ($\alpha < 0.5$; TukeyHSD).

increasing velocity. Overall, polyester exposed silversides shown similar behavioral variations at both, highest and lowest salinities (62.5% of total activity; Figures 4, S5).

Polypropylene microfiber exposed Silversides showed significantly increasing concentration dependent angular velocity in the lowest salinity under dark conditions whereas in the light cycle, highest and medium salinities demonstrated concentration dependent increasing angular velocity (Figures 4, S5C). Silversides in the lowest salinity showed significantly higher total distance moved in the light conditions followed by the highest and medium salinity. In the case of freezing activity, fish exposed at the lowest salinity demonstrated significantly increasing concentration dependent activity under both, light and dark conditions whereas, the highest salinity demonstrated significantly increasing concentration dependent activity in the light condition only. In zone duration was decreased in all three salinities in a significant concentration dependent manner under dark conditions. Meandering activity was significantly increased in the medium salinity dark cycle, in contrast to the lowest activity in the light cycle. Silversides at lowest salinity showed a significant concentration dependent increase in turn angle under both, light and dark conditions. At the lowest salinity Silversides' velocity was significantly decreased in a concentration dependent manner under light conditions. Overall, polypropylene exposed silversides exhibited significant behavioral variations at lowest salinity (87% of total activity; Figures 4, S5), compared to lowest salinity (50% of total activity; Figures 4, S5).

In case of overall behavioral variation, there was significant increased distance observed in all three microfiber types in at least one salinity (Figure 3b). Meandering activity decreased in all concentrations at the highest salinity in all three microfiber types. Increased freezing behavior was observed in polypropylene at all the salinity whereas cotton caused increased freezing at highest salinity in both the lowest and medium concentration. There was no increase in zone (center of beaker) duration observed in cotton at lowest concentration compared to both the synthetic microfiber types. At lowest salinity all microfiber types caused increased movement over time at all concentration ranges. During medium concentration range cotton microfiber caused least behavioral variations compared to both the synthetic microfibers. At highest concentration overall behavioral variation was less in all microfiber types.

It is important to discuss the implications of these mixed behavioral responses in the context of the current body of literature, which is still somewhat limited in terms of describing and understanding behavioral responses to microplastics. Rather than being caused by a specific molecular interaction, such as in the case of pesticides which are designed to act as neurotoxicants, microplastic and microfiber effects from particles that are too large to translocate may be caused by a combination of particle ingestion causing an altered physiological state, coupled with encountering foreign objects in the water column (Jacob et al., 2020). Plastics of other types besides microfibers, such as polystyrene microbeads (from 0.001 to 10 mg L^{-1}) reduced mobility and altered swimming speed in sea urchin, *Paracentrotus lividus*, and also reduced mobility (with increased mortality) in the rotifer *Brachionus plicatilis* (Gambardella et al., 2018). Similarly, for blue mussel (*Mytilus edulis*) reduced filtering activity was reported following exposure to 100-nm polystyrene beads (Wegner et al., 2012). Another study reported that swimming area and a total distance of Chinese rice fish (*Oryzias sinensis*) and Korean dark chub (*Zacco temminckii*) were affected by nano-sized polystyrene exposure (Chae et al., 2018). Juvenile jacopever (*Sebastes schlegelii*) had reduced swimming speed and range of movement following exposure to polyesterene microplastics (Yin et al., 2018).

When organisms are exposed to synthetic microfibers, in particular, several behavioral effects have been observed. For example, blue mussels (Mytilus edulis) fed concentrations up to 30 MPF mL^{-1} had reduced filtration rates with a concentration dependent increase in microfiber tissue accumulation (Woods et al., 2018). Similarly, Kolandhasamy et al. (2018) reported microfiber retention by mussel foot and mantle that affected their adherence properties. In another study, copepods (*Calanus finmarchicus*) had altered prey selectivity when exposed to 50 particles/ml nylon microfibers for 6 days (Cole et al., 2019). Long term exposure to polypropylene microfibers are reported to be toxic at high concentrations (3 particles/L, ~1000 μm length) to crab (*Emerita analoga*), in addition to reducing egg clutches retention and affecting embryonic development (Horn et al., 2020).

Typically, behavior in fish is triggered by an external stimulus acting *via* neural networks (Weber and Speiler, 1994). If these neural networks disrupt by some external stimuli in the

form of contaminants, that can result in altered behavior. Silverside behavior, for example, is also impacted by tire particle exposure with salinity-dependent differences (Siddiqui et al., 2022). Another study on planktivorous reef fish (*Acanthochromis polyacanthus*) exposed to PET (300-125 µm diam), under limited food availability conditions showed altered behavior (Critchell and Hoogenboom, 2018). Similarly, juvenile European seabass, *Dicentrarchus labrax* exhibited reduced swimming velocity and resistance time when exposed to fluorescent red polymer microspheres (1–5 µm diameter) (Barboza et al., 2018). Another study on Crucian Carp (*Carassius carassius*) reported groups exposed to live Daphnia enriched with nanoparticles moved much more slowly, exhibiting stronger shoaling behavior, occupied less space in the aquarium and did not hunt as actively as control fish (Mattsson et al., 2015). Due to limited studies reporting behavioral variability in fish following microfiber digestion, these results can be considered as the first to report behavioral alterations in fish subsequent to microfiber exposure, particularly considering the inclusion of cellulose-based fibers which is novel. Traditionally, growth, development and reproduction are considered important ecotoxicological endpoints that can help identify the severity of risk associated with contaminants. However, microplastics and microfibers are often present below the quantities that can cause significant toxicity, such as mortality or deformities. Under such low doses, endpoints such as behavior serve as a link between physiological and ecological processes to study environmental contaminants, and changes in behavior can affect an organism's chances of surviving, as well as its fitness (Weis et al., 2001). The need to include behavioral indicators for ecologically relevant monitoring has been proposed from the late 80s (Atchison et al., 1987) and has continued to date (Ford et al., 2021).

In summary, natural microfibers were identified as least toxic when compared to the other two synthetic microfiber types in this study, but they still caused adverse responses in terms of growth in mysids and altered behavior in both taxa. Similar results were reported in brine shrimp *Artemia franciscana* when exposed to two commonly synthetic microfibers (polypropylene and polyethylene terephthalate) and one natural fiber (lyocell) (Kim et al., 2021). Based on behavioral variability mysids shrimps are more affected by polyester microfibers whereas Silverside larvae were more impacted by polypropylene microfibers. Both organisms demonstrated the lowest impact from the cotton microfibers. When considering salinity, cotton impacted both fish and invertebrate behavior more at higher salinities, whereas polyester and polypropylene had higher impacts at lower salinities. Also, overall, in terms of internalization and effects on growth, cotton has a lesser impact compared to synthetic fibers. This study contributes to our understanding of impacts to growth and the behavioral ecotoxicology of microfiber exposure and identifies potential risks associated with each. Our results support proposed efforts to reduce the loading of microfibers into the environment, such as potentially requiring filtration devices on washing machines and clothes dryers (Erdle et al., 2021). The impact of microfibers on growth and behavior in both organisms tested here is concerning, and further investigations are needed to better understand the behavior of microfibers across salinities, as well as the potential relationship between changes in apical endpoints, such as growth, and altered behavior.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Data and code used in this analysis can be found at https://github.com/Brander-Harper/microfiber_acute_ss_mysid. For further inquiries please contact the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Oregon State University IACUC.

Author contributions

Conceptualization, SB, SH. Methodology, investigation, formal analysis, data curation, writing SS, JD, SB. Review, editing and investigation SJH, EP, SB. Visualization SJH, SS. Supervision, project administration, funding acquisition, SB. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.991650/full#supplementary-material

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Occurrenceand characteristics of microplastics in benthic species from mangrove wetlands of Hainan, South China

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Microplastics (MPs) are emerging contaminants that pose a global threat to the environment. Mangrove ecosystems, which contribute to biogeochemical cycles, are vulnerable to various anthropogenic disturbances and chemical pollutants. In this study, the abundance and the characteristics of MPs were investigated in 10 species of benthic organisms, including crabs, bivalves, and snails, from seven typical mangrove distribution areas, with a total of 15 sampling sites in Hainan, South China. The abundance of MPs in each sampling site ranged between 0.83 ± 1.32 and 12.00 ± 0.00 items/individual, with an average of 3.90 ± 3.31 items/individual, while the abundance of MPs varied between 0.17 and 2.00 items/individual for the different species. Fibers (80.13%) were the most abundant MPs, most of the MPs were brown (37.18%) or blue (26.64%), and more than 80% were small-sized plastic (<2 mm). Raman analysis showed that polypropylene (94.44%) was the most dominant type of polymer. In addition, crabs (with an average abundance of 1.10 ± 0.59 items/individual) showed a higher potential for accumulating MPs than the other species investigated in the present study. This study shows that MPs are widely distributed throughout benthic species in the mangrove wetlands of Hainan.

KEYWORDS

microplastics, mangrove wetland, invertebrates, contaminates, Hainan Island

1 Introduction

Microplastic (MP; particles <5 mm) pollution is one of the emerging threats to both aquatic and terrestrial ecosystems, even in polar regions, due to their long-distance migration in the environment (Wright et al., 2013; de Souza Machado et al., 2018). Their widespread presence in different environmental media, including atmospheric (Cai et al., 2017; Liu et al., 2019), terrestrial (He et al., 2018; Zhou et al., 2018; Corradini et al., 2019; Chia et al., 2021), freshwater (Wang et al.,

2017; Fu and Wang, 2019; Mintenig et al., 2019; Meng et al., 2020; Yang et al., 2021), and marine (Cincinelli et al., 2017; Wang et al., 2019; Zhang et al., 2020; Gao et al., 2022; Jiang et al., 2022) ecosystems, has been increasingly reported. MPs are ingested directly and indirectly by fish, bivalves, crustaceans, and other animals (Chan et al., 2019; Teng et al., 2019; Carlin et al., 2020; Savoca et al., 2020; Sequeira et al., 2020; Pequeno et al., 2021; Yin et al., 2022). MPs may transfer from lower to higher trophic levels along the food chain and may cause potential threats to human health (Santillo et al., 2017).

Mangrove forests are saline and tidal habitats and are considered one of the most carbon-dense ecosystems on Earth (Bai et al., 2021). Mangrove wetlands provide numerous ecological services and functions, including water purification, coastal protection, and marine animal habitats (Lovelock and Duarte, 2019). Several studies have reported the distribution of MPs in global mangrove forests. Li et al. (2018) found that the abundance of MPs ranged from 15 to 12,852 items/kg in sandy beaches and mangrove wetlands. The average abundance of MPs in mangrove sediments in Singapore was 9.2 ± 5.9 particles/250 g (Mohamed Nor and Obbard, 2014) and varied from 0.6 to 8.0 items/individual in fish species collected from the mangrove wetland of Zhanjiang (Huang et al., 2020). In addition, Zhou et al. (2020) investigated the distribution of MPs along the coast of China and found that the abundance of MPs in mangrove sediments was 8.5 times higher than that in mangrove-free sediments. Nevertheless, in the Muara Angke Wildlife Reserve of Indonesia, the concentrations of MPs in sediments were higher outside than inside mangrove areas (Cordova et al., 2021). However, few studies have focused on MP pollution in benthic species, especially invertebrates, in mangrove wetlands. Hainan Island represents nearly 20% of the mangrove forest areas in China, and these areas are distributed in Dongfang, Danzhou, Lingao, Chengmai, Dongzhaigang, Wenchang, and Sanya. Among them, the Dongzhaigang National Mangrove Reserve, which was established in 1986, was designated as one of the most important wetlands in the world in 1992 (Qiu et al., 2011; Wu et al., 2013).

In this study, wild benthic species were collected from 15 sites along seven typical mangrove wetlands in Hainan Island, and their abundance, morphotype, size, color, and polymer composition were investigated. The main objectives were to quantify and characterize the MPs in benthic organisms from mangrove areas. This study provides basic data on the contamination level of MPs in benthic organisms in the mangrove wetlands of Hainan.

2 Materials and methods

2.1 Study area

Seven typical mangrove distribution areas around Hainan Island, including Danzhou, Lingao, Chengmai, Dongzhaigang, Wenchang, Sanya, and Dongfang, were chosen as the sampling sites for mangrove benthos. A total of 15 sites were included: Huachong, Caiqiao, Fuli mangrove bay, Beijie, Kunshan, Wuli, He Harbor, Changwan, Nanjie, Dongye, Pai Harbor, Xiachang, Sanya River, Qingmei Harbor, and Dongfang. The layout of the stations in each area is shown in Figure 1.

2.2 Sample collection

A total of 10 species of mangrove benthos were collected from the Hainan mangrove area in April 2019. All the benthos were sieved from sediments collected with a Van Veen grab at 30 cm depth from a

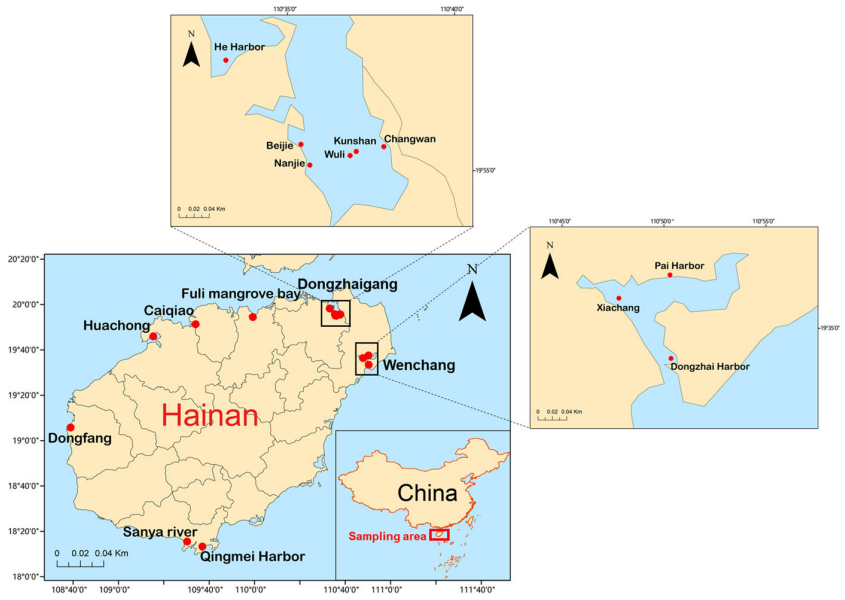

FIGURE 1
Sampling locations in typical mangrove areas of Hainan, China.

~10 m × 10 m area in each sampling site, using a five-point sampling method according to previous studies, with slight modifications (Ryan, 2004). A total of 135 individuals were identified down to the species level (Table 1) by referring to the *Atlas of molluscs in Dongzhai bay, Hainan*, and the *Atlas of marine animals in mangrove wetland in Beibu Gulf, Guang Xi*. There were six species of crabs (n = 80), three species of bivalves (n = 49), and one species of snails (n = 6). To avoid contamination of MPs during transportation, the collected samples were packed in aluminum foil, transported to the laboratory under −4°CC, and stored at −20°CC.

2.3 Microplastic extraction

The MPs were extracted as described by Munno et al. (2018), with some modifications. Briefly, the soft tissues of animals (crabs, bivalves, and snails) were collected and weighed and then placed in 500-ml glass beakers individually. Subsequently, 180 ml of 10% (m/v) KOH and 20 ml of 30% H_2O_2 were added for the digestion process. Each beaker was covered with aluminum foil and placed in an oven at 60°CC for at least 48 h. To ensure complete digestion, the beaker was shaken every 6 h. The digestate was then cooled and vacuum filtered through a GF/F glass microfiber filter (0.7 mm pore size, 47 mm diameter; Whatman plc, Maidstone, UK). Afterward, the filters were placed in clean Petri dishes and dried at room temperature (25°CC) until analysis.

2.4 Microplastic identification

Suspected plastic particles were observed using a stereoscopic microscope (GL6545T; Guilin, China) equipped with a high-resolution digital camera. The MPs were classified and counted according to shape (classified into fibers, granules, fragments, pellets, and films), size (classified into <1, 1–2, 2–3, 3–4, and 4–5 mm) (Cui et al., 2022), and color (Nie et al., 2019). In addition, a laser confocal microscope and a Raman spectrometer (DXR2; Thermo Fisher Scientific, Waltham, MA, USA) were used to analyze the suspected plastic particles according to the methods of Di and Wang (2018). To identify the chemical composition, a spectral database based on OMNIC software (Thermo Fisher Scientific) was used to compare the spectra of the samples, and the level of certainty was set to 60% (Woodall et al., 2014; European Commission, 2013).

2.5 Quality assurance and quality control

All of the experimental equipment used in this study were made of non-plastic materials and were rinsed carefully with filtered distilled water several times to avoid potential contamination from other sources. All solutions, including the distilled water, KOH, and H_2O_2, were filtered through a 0.45 μm filter paper under vacuum before use. During all experimental processes, all containers were covered with aluminum foil, and polymer-free gloves and cotton lab coats were worn. In addition, the blank samples were corrected for potential procedural contamination.

2.6 Statistical analysis

A location map of the sampling areas was drawn using ArcGIS 10.2. All data were analyzed using Microsoft Excel and are shown as the mean ± standard deviation (SD). The abundance of MPs in benthic species at each site was expressed as items per individual (items/individual). In addition, the characteristics of the MPs were plotted using GraphPad Prism software, and SPSS 16.0 was used for statistical analysis. Differences in the abundance of MPs were determined using one-way ANOVA with Dunnett's test, and significance was set at $p < 0.05$.

3 Results and discussion

MP pollution in the benthos of Hainan's mangrove wetlands was studied for the first time. A total of 135 benthic organisms, including six species of crab, three species of bivalves, and one species of snail from 15 sampling sites in seven typical mangrove wetlands of Hainan, were analyzed to determine the abundance and characteristics of MP contamination. The different sizes, shapes, colors, and chemical compositions of the MPs were examined in the benthos samples from different mangrove wetland areas.

3.1 MP abundance in benthos from different mangrove wetland areas

The abundance of MPs in the benthos from different mangrove areas is shown in Table 2. The abundance of MPs ranged between 0.83 ± 1.32 and 12 ± 0.00 items/individual (average, 3.90 ± 3.31 items/individual), with the highest abundance in Changwan, Dongzhaigang (12.00 ± 0.00 items/individual), while the lowest abundance (0.83 ± 1.32 items/individual) was found in Fuli mangrove bay, Chengmai. This result was similar to the number of MPs observed in organisms collected from the mangrove region of Zhanjiang (0.6–8.0 items/individual) (Huang et al., 2021). Our results also demonstrated that there were differences in the MP abundance between each sampling site (Figure 2A). The abundance of MPs in the benthos collected from Changwan was significantly higher than that from other sites in the Hainan wetlands ($p < 0.05$), which was largely due to the input of plastic debris from the tourism industry and the semi-closed bay with weak hydrodynamic conditions. It has been reported that Dongzhaigang is a mangrove wetland nature reserve in China and is the biggest bay in Hainan Island (Li et al., 2020). Furthermore, the abundance of MPs in Pai Harbor, He Harbor, and Sanya River was relatively high, with values of 9.00 ± 8.08, 7.50 ± 0.58, and 6.33 ± 6.66, respectively, which may due to port transportation and the urban communities around these areas.

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TABLE 1 Abundance of microplastics in different benthic species.

Family	Species	Benthos samples (*n*)	Microplastics (items)	Abundance (items/individual)
Crab	*Uca vocans*	9	2	0.22
	Uca arcuata	19	22	1.16
	Perisesarma bidens	15	16	1.07
	Uca dussumieri	6	12	2.00
	Helicana wuana	9	7	0.78
	Sesarma plicata	22	30	1.36
Bivalve	*Vignadula atrata*	23	5	0.22
	Geloina erosa	13	3	0.23
	Saccostrea echinata	13	24	1.85
Snail	*Littoraria melanostoma*	6	1	0.17

TABLE 2 Abundance of microplastics in benthos samples from different sample sites.

Mangrove areas	Sampling sites	East longitude North latitude	Abundance (items/individual)
Danzhou	Huachong (HC)	109°C15′21" 19°C46′3"	4.40 ± 4.45
Lingao	Caiqiao (CQ)	109°C34'0" 19°C51′23"	2.40 ± 1.82
Chengmai	Fuli mangrove bay (FL)	109°C59 '13" 19°C54′32"	0.83 ± 1.32
Dongzhaigang	Beijie (BJ)	110°C35'24" 19°C55′47"	1.00 ± 0.00
	Kunshan (KS)	110°C37 '3" 19°C55′35"	1.50 ± 0.58
	Wuli (WL)	110°C36'53" 19°C55′27"	3.00 ± 0.00
	He Harbor (HH)	110°C33'9" 19°C55′27"	7.50 ± 0.58
	Changwan (CW)	110°C37'53" 19°C55′43"	12.00 ± 0.00[a]
	Nanjie (NJ)	110°C35'40" 19°C55′10"	3.00 ± 1.15
Wenchang	Dongye (DY)	110°C50'21" 19°C33′32"	1.00 ± 1.46
	Pai Harbor (PH)	110°C50'18" 19°C37′37"	9.00 ± 8.08
	Xiachang (XC)	110°C47'47" 19°C36′29"	3.50 ± 3.20
Sanya	Sanya River (SR)	109°C30'17" 18°C15′33"	6.33 ± 6.66
	Qingmei Harbor (QH)	109°C36'60" 18°C13′21"	2.00 ± 2.68
Dongfang	Dongfang (DF)	109°C38'54" 19°C5′50"	1.00 ± 1.22

[a]Significant differences in microplastic abundance in the benthos between sampling sites.

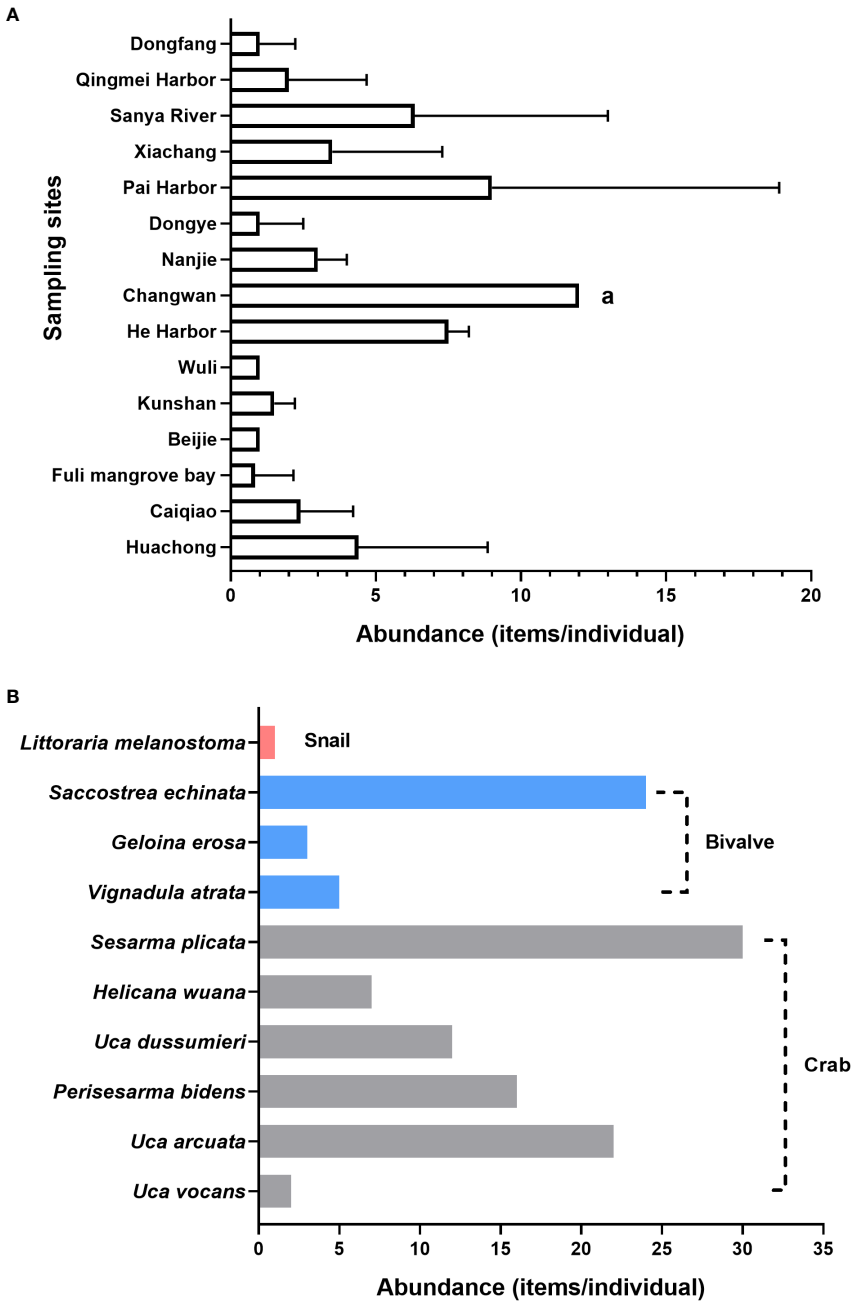

FIGURE 2
Microplastic abundance in the different sample sites **(A)** and in each benthic species **(B)** from the mangrove areas of Hainan. Lowercase letter "a" indicate significant differences between sampling sites.

In addition, we calculated the abundance of MPs in the different benthic species (Table 1 and Figure 2B) and found that it varied from 0.17 to 2.00 items/individual in each species, with the highest MP abundance found in crabs (including *Uca vocans*, *Uca arcuata*, *Perisesarma bidens*, *Uca dussumieri*, *Helicana wuana*, and *Sesarma plicata*), which showed an average abundance of 1.10 ± 0.59 items/individual; among them, *S. plicata* had the highest abundance (1.36 items/individual), followed by *U. arcuata* and *P. bidens* (1.16 items/individual for both). The average abundance of MPs in bivalves (including *Vignadula atrata*, *Geloina erosa*, and *Saccostrea echinata*) was 0.77 ± 0.94 items/individual, with the highest found in *S. echinata* (1.85 items/individual), followed by *G. erosa* (0.23 items/individual) and *V. atrata* (0.22 items/individual); the snails *Littoraria melanostoma* had the lowest MP abundance (average, 0.17 items/individual). We compared our results with those of previous studies that focused on the abundance of MPs in benthos from different areas (see Table 3). The MP abundance values found in our study were consistent with those detected in crabs from the English Channel and the Atlantic Ocean (Welden et al., 2018), but the concentrations of MPs in crabs and bivalves from the Arctic and sub-Arctic regions (Fang et al., 2018) were slightly lower than those in the present study. Moreover, the bivalve species from Qingdao (Ding et al., 2021),

TABLE 3 Comparison of microplastic abundance in benthic species with previous studies.

Species and sources	MP concentrations (Items/individual)	Dominant types and polymers of microplastics	Reference
Crabs, bivalves, snails Hainan mangrove wetlands, South China	0.17–2.00	Fibers (80.13%) PP (94.44%), PS (5.56%)	This study
Spider crab (*Maja squinado*) English Channel and Atlantic Ocean	(1.39)[a]	–[b] –	Welden et al., 2018
Crab (*Chionoecetes opilio*), bivalves (*Astarte crenata* and *Macoma tokyoensis*) Arctic and sub-Arctic regions	0.17 ± 0.12–0.83 ± 0.43	Fibers (87%) PA, PE	Fang et al., 2018
Four locally cultured bivalve species Qingdao, China	0.5–3.3	Microfiber PVC and rayon	Ding et al., 2021
Nine bivalve species Shanghai's biggest fishery market, China	4.3–57.2	Fibers –	Li et al., 2015
Four oyster species Coastline of China	(2.93)[a]	Fibers CP, PE, and PET	Teng et al., 2019
Predatory snail (*Thais mutabilis*) Persian Gulf	3.70–17.70	Fibers PE, PET, and PA	Naji et al., 2018

PP, polypropylene; PA, polyamide; PE, polyethylene; PVC, polyvinyl chloride; CP, cellophane; PET, polyethylene terephthalate.
[a]Average abundance.
[b]Data not shown in the article.

Shanghai's biggest fishery market (Li et al., 2015), and the coastal areas of China (Teng et al., 2019) have been reported to have considerably higher MP abundance compared to this study. The average abundance of MPs in snails from the Hainan mangrove areas was lower than that in predatory snails from the Persian Gulf (Naji et al., 2018). Our results suggest that the levels of MPs in biota species from Hainan's mangrove wetlands were low to moderate compared to those reported in previous studies.

3.2 Morphological properties of the MPs in benthos from different mangrove areas

Five different morphotypes of MPs—fibers, granules, fragments, pellets, and films—were observed in the sampled benthic organisms from these mangrove areas. The most common type of MP in all collected benthic species was fiber (80.13%) (Figure 3A), which was consistent with those detected in benthic organisms from other areas (Table 3) and in fish species from the mangrove wetland of Zhanjiang (70%) (Huang et al., 2020), as well as in mussels from 25 sites along the coastal waters of China (Qu et al., 2018). In addition, the site proportion of fibrous MPs in Sanya River, Dongye, Wuli, Beijie, Fuli mangrove bay, and Caiqiao accounted for 100% (Figure 3D), all of which are close to urban communities and fishing areas. It was speculated that the high levels of fibrous MPs may be associated with human activities such as the disposal of municipal wastewater and the fishery.

The sizes of the MPs in the present study were classified into five ranges, i.e., <1, 1–2, 2–3, 3–4, and 4–5 mm, which accounted for 48.08%, 32.69%, 10.90%, 5.13%, and 3.21% of the MPs, respectively (Figure 3B). The proportion of MP size in benthos from the Beijie site accounted for 100%, while Changwan contained more smaller-sized MPs (<1 mm, 86.61%), which also had the highest abundance of MP in benthic species (Figure 3E). The main size ranges were <1 mm (48.08%) and <2 mm (>80%), which were similar to the proportions of small MPs found in benthic species from other mangrove and coastal areas of China (Courtene-Jones et al., 2017; Wang et al., 2019; Filgueiras et al., 2020). The size range of the MPs can be explained by the feeding habits of benthic species (Bour et al., 2018). It has been reported that mussels are more likely to ingest smaller MPs (Qu et al., 2018).

In addition, the color can affect the ingestion of MPs by aquatic species (Filgueiras et al., 2020). MPs of five different colors—brown (37.18%), blue (25.64%), white (13.46%), red (1.28%), transparent (1.28%), and other artificial colors (21.15%)—were observed (Figure 3C). Among the examined species, brown and blue MPs were the predominant ingested items, similar to other studies on mussels (Digka et al., 2018) and sea snails (Courtene-Jones et al., 2017). The differences in the colors of the MPs in each site are shown in Figure 3F, with multicolor or brown and blue MPs being the most prevalent. Moreover, in the present study, crabs were the dominant species with the highest proportion of MPs (72.95%), followed by bivalves (22.95%) and snails (4.10%) (Figure 4A). The higher intake of small and colorful particles may be explained by their feeding habits, as crabs are visual predators and may confound plastic particles with their natural food (Nanninga et al., 2020).

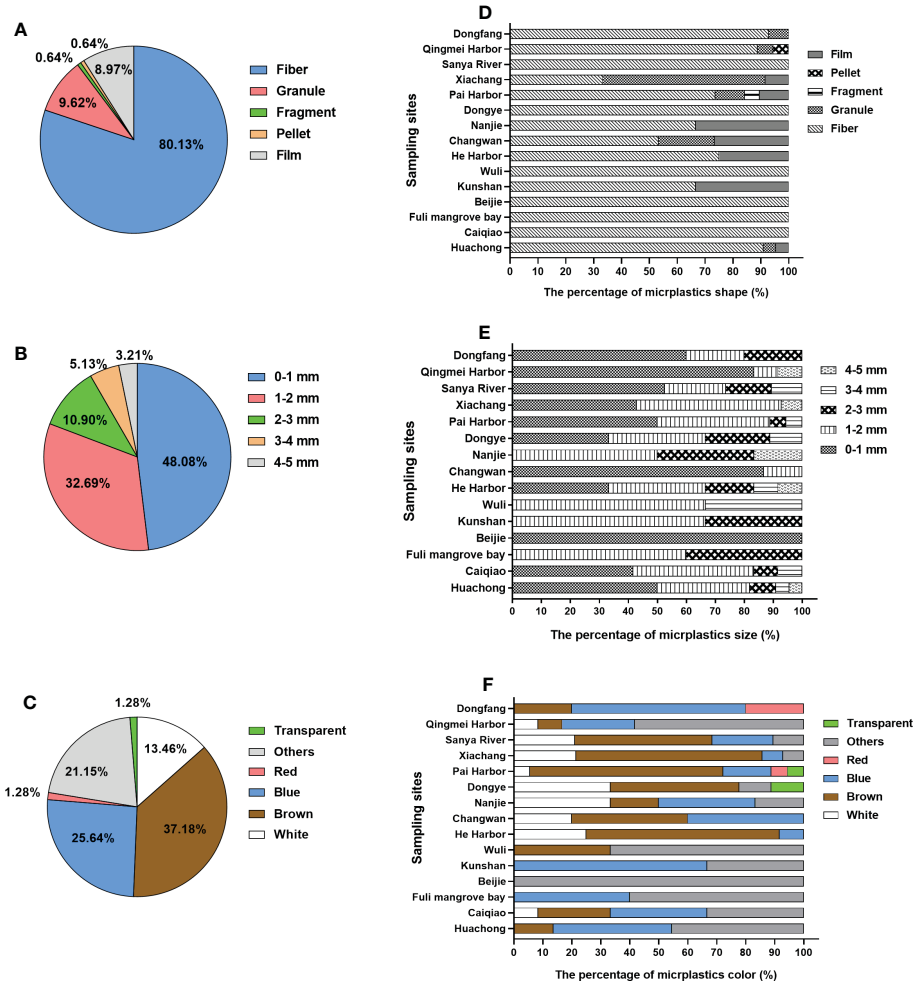

FIGURE 3
(A–C) Shape **(A)**, size **(B)**, and color **(C)** of the microplastics (MPs) in the benthos. **(D–F)** Distributions of the shape **(D)**, size **(E)**, and color **(F)** of the MPs in benthic species from the different sample sites in Hainan's mangrove areas.

3.3 Chemical properties of the MPs in the benthos from different mangrove areas

A laser confocal microscope and a Raman spectrometer were used to identify the polymer types of the MPs ingested by the benthic species in Hainan's mangrove wetland areas. As shown in Figures 4B, C, two types of MPs, polypropylene (PP) and polystyrene (PS), were identified. PP accounted for 94.44% of the MPs in benthic species, which was inconsistent with previous studies reporting that polyethylene (PE), polyamide (PA), and/or polyethylene terephthalate (PET) were the major polymer types of MPs in benthic organisms collected from other areas (Fang et al., 2018; Naji et al., 2018). On the contrary, other studies reported that PP fibers were the most abundant in sediments from the Beibu Gulf Sea (Xue et al., 2020). PP is commonly used in packaging, containers, pipes, textiles, and fishing equipment (Park et al., 2004; Cai et al., 2018). Around the mangrove region, there are a number of fish ports and mariculture sites in the harbor; concurrently, the mangrove wetlands are tourist areas. The present study suggests that artificial disturbance, including urban wastewater treatment, mariculture, and port transportation, might be the sources of MP contamination in the Hainan mangrove wetlands.

4 Conclusion

Mangrove ecosystems are important coastal resources that create unique ecological environments hosting various species. This study is the first to quantify MP pollution in the benthic species from Hainan's mangrove wetlands. In this study, the MPs were extensively characterized in 10 benthic species collected from 15 sampling sites within seven typical mangrove wetlands in Hainan Island. The average abundance of MPs ranged between 0.83 ± 1.32 and 12.00 ± 0.00 items/individual in each sampling site. According to Raman analysis, most detected MPs were PP, with an abundance rate of 94.44%, mainly in the form of fibers (80.13%). Ingesting MPs and associated contaminants in other organisms through the food chain is a great risk to human health. Our results are indicative of the

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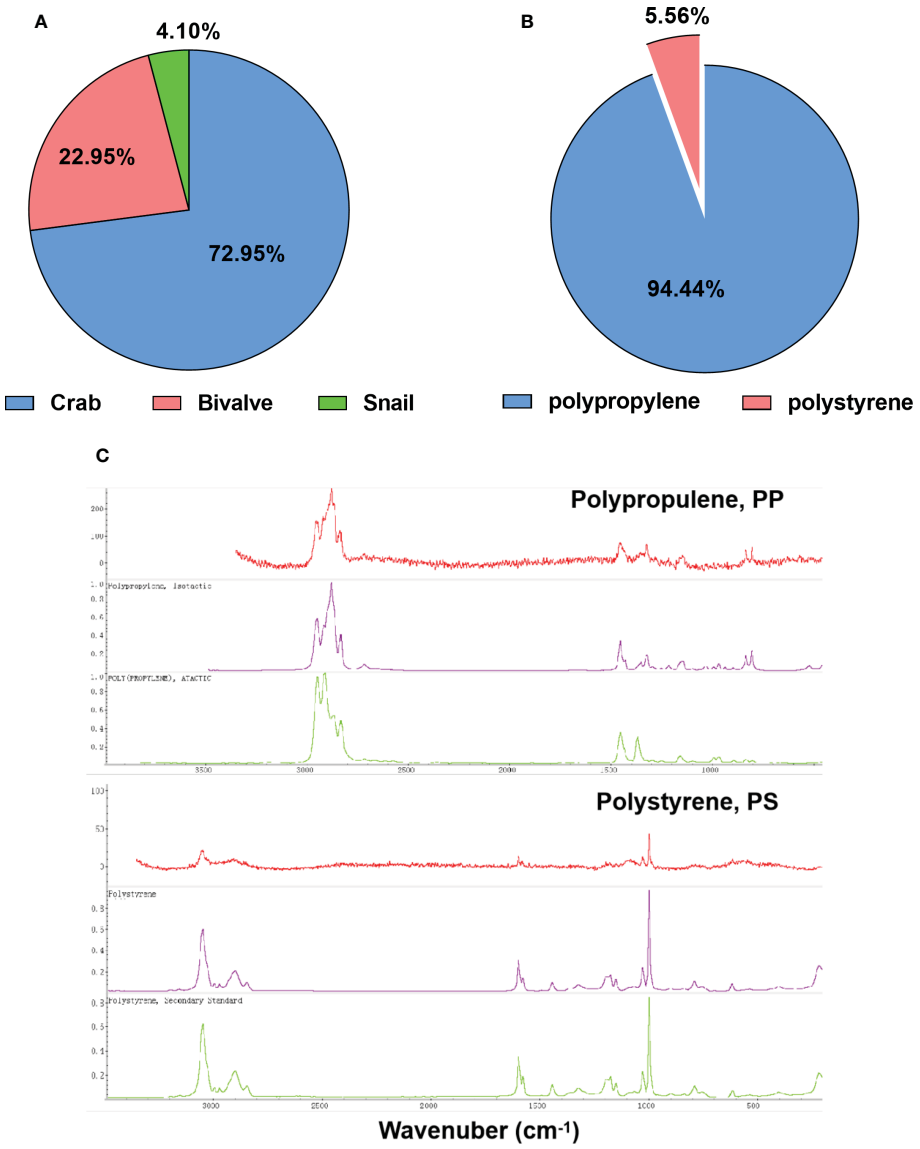

FIGURE 4
(A, B) Number and percentage of abundance of microplastics (MPs) **(A)** and polymer types **(B)** in the different benthic species. **(C)** Raman spectra of the selected MPs in benthic species.

bioavailability of MPs to benthic marine organisms. Future studies on the abundance and distribution of MPs in various organisms from different geographical locations are needed to assess the risk of MPs to public health and ecosystems.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

QZ: Conceptualization, data curation, formal analysis, and writing—original draft. JX: Conceptualization, data curation, formal analysis, and writing—reviewing and editing. SM: Investigation, methodology, and data curation. YC and FL: Investigation. XD: Conceptualization, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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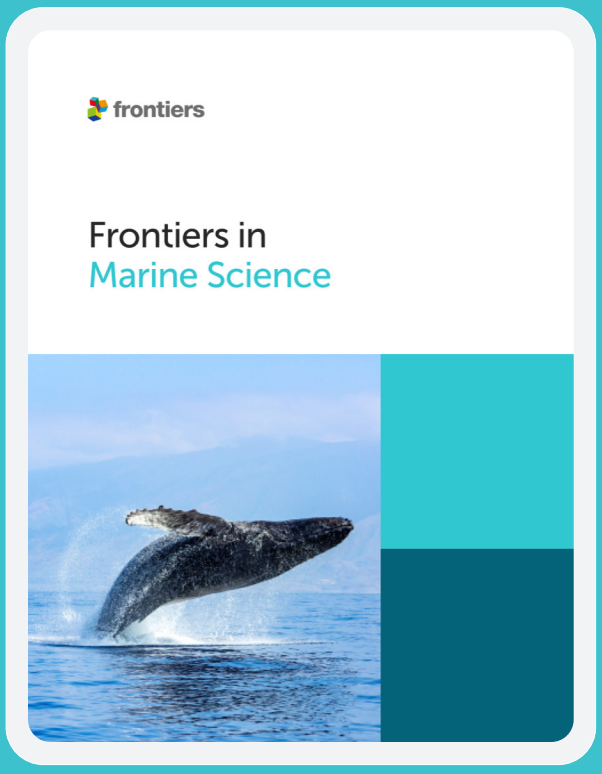

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