MICROPLASTICS IN THE MARINE ENVIRONMENT: SOURCES, DISTRIBUTION, BIOLOGICAL EFFECTS AND SOCIO-ECONOMIC IMPACTS

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Editorial: Microplastics in the Marine Environment: Sources, Distribution, Biological Effects and Socio-Economic Impacts

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Keywords: plastic pollution, environmental monitoring, ecotoxicology, waste management, policy recommendations

Editorial on the Research Topic

Microplastics in the Marine Environment: Sources, Distribution, Biological Effects, and Socio-Economic Impacts

INTRODUCTION

From all the synthetic materials ever produced, plastic is the most versatile, overthrowing both glass and metal in many applications, due to its low weight and cost. Global plastic production started shortly after WWII, around the 1950's (PlasticsEurope, 2010), and became a popular household item around the same time (Time, 1955). Since then, global production has been exponentially increasing at a rate of 8% *per annum* (PlasticsEurope, 2020). Notably, it took only 10 (1965) to 17 (1972) years until researchers started noticing the first evidence of plastics in the marine environment (Carpenter and Smith, 1972; Ryan, 2015). Between the 1960's and the 1990's, several studies reported direct consequences of plastic interaction with vessels, particularly entanglement of propellers, and with wildlife, *via* entanglement or ingestion (Ryan, 2015). Consistent findings throughout the world led to calls for action, due to the likelihood that over time the problem would be amplified by fragmentation of larger plastic items into smaller pieces (Carpenter and Smith, 1972). Microplastic research is now a well-established research field, with at least 2,500 papers published so far on this topic (Zhang et al., 2020).

Despite being a relatively recent research field, microplastic pollution has gone beyond the realm of academia into the general public. Several stakeholders with different vested interests are involved in this topic, from standardization bodies to grassroot movements, from national agencies to research institutions. Plastic has become a social issue, due to its economic and environmental consequences, which affect human activities and the natural cycles of the planet. In order to contribute to the debate, this Research Topic (RT) highlights recent research developments in the microplastic field, in a diverse set of topics that cover relevant aspects from methodologies to modeling, and from impacts on fauna to legislation. A total of 23 research papers from 43 primary and partner institutions, in four continents and spread across 15 countries (**Figure 1A**), reveal the prevalence of this global problem, and report on some of the solutions ahead.

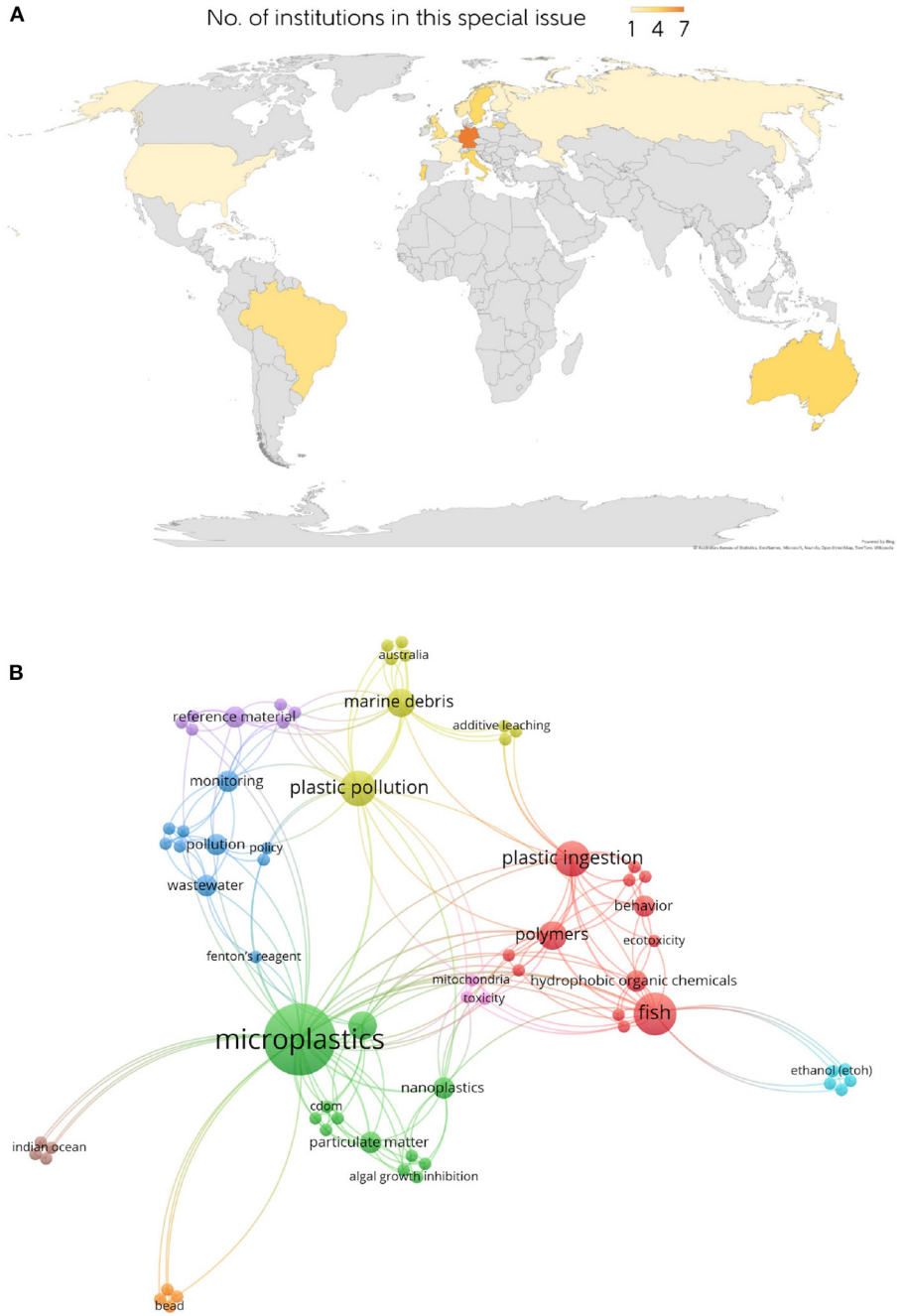

FIGURE 1 | (A) Number of institutions primary and partner institutions per manuscript ($N = 43$) contributing to this RT. **(B)** Distance-base map (based on association strength) of a set of 67 keywords retrieved from 21 papers. Keywords are grouped into 9 clusters of different colors.

A SHORT BIBLIOMETRIC ANALYSIS OF PAPERS PUBLISHED IN THIS RT

The author's keywords from each paper were compiled and analyzed in the software VOS viewer to illustrate the diversity of topics explored here (Van Eck and Waltman, 2010). The set of 21 papers (with their final versions published by 01.03.2021) in this RT had a total of 152 keywords. To standardize keywords describing the same concept, a thesaurus was created (**Table 1**). For example, polyethylene, polypropylene and other polymer types were all grouped under the keyword "polymers." As such, a total of 67 keywords are presented in the final set. The

TABLE 1 | Thesaurus of alphabetical ordered author's keywords ("Label") and standardized concepts.

Label	Replace by
Anthropogenic litter	Marine debris
Atlantic chub mackerel	Fish
Atlantic salmon (*Salmo salar* L.)	Fish
Benzo(a)pyrene	Hydrophobic organic chemicals
Chlorpyrifos	Hydrophobic organic chemicals
Combined sewer overflow	Wastewater
Cost-effective marine litter monitoring method	Monitoring
EE2	Hydrophobic organic chemicals
Extraction techniques	Methods
FT-IR spectroscopy	Spectroscopy
GIT analysis	Plastic ingestion
Horse mackerel	Fish
Hydrodynamic dispersion model	Model
Hydrodynamic model	Model
Infrared imaging	Spectroscopy
Ingestion	Plastic ingestion
Lates calcarifer	Fish
Long-term monitoring	Monitoring
Lutjanus argentimaculatus	Fish
Marine litter	Marine debris
Microplastic	Microplastics
Microplastic (MP)	Microplastics
Microplastic pollution	Microplastics
pe	Polymers
Peppery furrow shell	Bivalve
Pet	Polymers
Plastic and plastics	Plastic pollution
Plastic polymers	Polymers
Plectropomus leopardus	Fish
Polyethylene	Polymers
Polyethylene terephthalate	Polymers
Polyethylene(PE)	Polymers
Polypropylene	Polymers
Polystyrene	Polymers
Polystyrene(PS)	Polymers
pp	Polymers
Reflectance micro-FTIR	Spectroscopy
Sand	Reference material
Selachians	Fish
Silica	Reference material
Sodium iodide	Density separation solution
SPM	Particulate matter
Stickleback	Fish
Storage	Methods
Suspended matter	Particulate matter
Three-spined stickleback (*Gasterosteus aculeatus*)	Fish
Top marine beach litter items	Marine debris
Uptake	Plastic ingestion
Wastewater treatment plant (WWTP)	Wastewater
Wastewater treatment plants	Wastewater
Zebrafish	Fish

Authors keywords are listed in alphabetical order.

most popular keyword is "microplastics" ($N = 10$ occurrences), followed by "fish" ($N = 5$), "plastic pollution" and "plastic ingestion" ($N = 4$). The keywords "polymers," "spectroscopy," and "marine debris" appeared in three papers each ($N = 3$). All other keywords appeared in one or two publications only, indicating a generally very low frequency of used keywords and therefore a variety of studied topics (**Figure 1B**).

This RT included a relatively high number of papers using fish as a model organism (see "fish" in **Figure 1B**), either by exploring combined effects of (nano-micro) plastics and organic pollutants in teleost (Trevisan et al.; Bour et al.; Ašmonaite et al.; Abihssira-García et al.) or by improving extraction and analysis methods for predicting plastic ingestion in fish (Dawson et al.; Pedà et al.; Pequeno et al.). In the same cluster, the keyword "plastic ingestion" included papers that explored the transfer of microplastics particles among successive levels in marine trophic webs or potential transfer of plastic additives and chemicals from plastics to biota when ingested (Costa et al.; Kühn et al.). Also grouped together are papers using the keyword "polymers" showing works that explore polymer-specific effects of particles in model-animals (Santana et al.).

Papers with more general approaches are clustered around keywords such as "plastic pollution" and "marine debris" (**Figure 1B**, in yellow). These are papers related to legislations

to mitigate plastic (marine) pollution (Da Costa et al.; Galaiduk et al.), potential bioindicators of (micro)plastic pollution (Reichelt and Gorokhova; Fossi et al.), but also to method development with potential to be used over large geographical areas (Enders et al.; Haseler et al.; Rodrigues et al.; Tagg et al.) and modeling of microplastic sources into the environment (Balthazar-Silva et al.; Gorman et al.; Schernewski et al.; Piehl, Atwood et al.; Piehl, Hauk et al.).

CONCLUSION

We considered this special issue to be very successful both in terms of number of papers published and variety of studies targeting several microplastic pollution issues. Notorious research advancements and science breakthroughs, as well as technological developments, are highlighted here based on the efforts of the microplastic scientific community over recent years. Manuscripts in this RT aim at fulfilling knowledge gaps while creating new research questions to fully understand the ubiquitousness of plastics in the environment. Although there is still a long way to go within this research extensive knowledge gathered so far [see for example Galgani et al. (2021)] will allow decision makers to make better decisions surrounding this global problem, while consolidating microplastic pollution as a permanent research field.

Plastic pollution is intrinsically linked to consumption habits and waste management practices globally. Therefore, recommendations need to be aligned with regulations and with the adequate use of market-based instruments, so that solving this problem is addressed holistically. One thing that the global pandemic brought to sight is that behavior change is possible, and when we work together reduction and prevention can be achieved. For example, understanding how to tackle losses and emission throughout the entire supply-chain will effectively reduce the abundances of plastic marine litter in the environment. That is an excellent way to start to flatten the current plastic pollution scenario worldwide.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Can Water Constituents Be Used as Proxy to Map Microplastic Dispersal Within Transitional and Coastal Waters?

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Due to high spatiotemporal variability of aquatic systems, relationships between microplastic sources and sinks are highly complex and transportation pathways yet to be understood. Field data acquisitions are a necessary component for monitoring of microplastic contamination but alone cannot capture such complex relationships. Remote sensing is a key technology for environmental monitoring through which extrapolation of spatially limited field data to larger areas can be obtained. In this field study we tested whether microplastic distribution follows the same transport pattern as water constituents depictable from satellite images, namely chlorophyll-a, suspended particulate matter, and colored dissolved organic matter, and discuss their applicability as proxies. As rivers are a major source for marine microplastic contamination, we sampled three example river systems: the lower courses and river mouths of the Trave and Elbe estuary in Germany and the Po delta in Italy. For a full quantitative analysis of microplastics (>300 μm), ATR- and FPA-based μFT-IR spectroscopy and NIR imaging spectroscopy were utilized. Comparing water constituents with in-situ data using regression analysis, neither a relationship for the Elbe estuary nor for the Po delta was found. Only for the Trave river, a positive relationship between microplastics and water constituents was present. Differences in hydrodynamic conditions and spatiotemporal dynamics of water constituents and microplastic emissions among the river systems are possible explanations for the contrary results. Based on our results no conclusions on other river systems and likewise different seasons can be drawn. For remote sensing algorithms of water constituents to be used as microplastic proxy an adaption for each system as well as for different seasons would thus be necessary. The lower detection limit of 300 μm for microplastics could also have influenced relationships as microplastic abundance exponentially increases with decreasing size class. Further studies with improved sampling methods are necessary to assess our proposed method.

Keywords: microplastic, spectroscopy, remote sensing, HySpex, SPM, chlorophyll, CDOM

INTRODUCTION

Pollution of aquatic ecosystems with microplastic (MP) has recently gained particular attention due to its ability to accumulate in food webs and its potential threat to human health (Sharma and Chatterjee, 2017; Wang et al., 2019). To combat MP pollution in marine systems, MP (synthetic polymer particles <5 mm) litter has been included in several local and global directives for the protection of the marine environment (Directive of the European parliament, 2008; UN Resolution, 2020). Further, member states are required to adapt operational monitoring programs to assess sources, transport, and accumulation of MP within marine systems. As most plastic products are produced and consumed on land, rivers are a major transport pathway for MP litter to the oceans (van Wijnen et al., 2019). But to date, only limited data on MP abundances in estuaries and adjacent coastal areas exist (Rezania et al., 2018). Reported concentrations are highly variable, from less than one to over a thousand particles per cubic meter (Rezania et al., 2018) and are often accompanied by high standard deviations. Especially estuaries, as transition zone between fresh- and saltwater, are highly dynamic systems influenced by both local and global meteorological and hydrodynamical factors (e.g., local surface wind and waves, tidal influences, basin specific currents, regional precipitation). Consequently, in-situ sampling of MP within estuaries and adjacent coastal areas requires sufficient replication to cover the high spatiotemporal variability. Nevertheless, current field data collection of MP samples from estuaries and adjacent coastal areas are associated with high-cost ship expeditions. Further, the use of state-of-the-art sample processing and analytical methodologies for MP identification in environmental samples remains very time consuming (Enders et al., 2020). Thus, existing data of MP abundance at the water surface exhibits high uncertainties and are both spatially and temporally limited.

Remote sensing offers a promising monitoring tool to obtain data on large spatial and temporal scales, and thus could provide valuable data for improved microplastic monitoring approaches. One drawback is that the direct detection of MP is not possible due to its size and concentrations within the water surface layer which are too low to sufficiently influence the water surface reflectance signal. An indirect approach to investigate plastic litter transport was first conducted by Pichel et al. (2007) studying the sub-tropical convergence zone (STCZ) within the North Pacific. The STCZ accumulates floating materials due to its convergent current patterns, including plastic litter (Lebreton et al., 2012). Pichel et al. (2007) used chlorophyll-a (Chl-A) concentration as well as sea surface temperature (SST) derived from satellite images to identify the location of the STCZ. With this approach they were able to extrapolate single in-situ measurements of Chl-A and SST to a larger area. Moreover, a spatial correlation could be detected for Chl-A and SST with macroplastic litter (Pichel et al., 2007) but MP litter was not investigated. Additionally, a further spatial relationship of Chl-A and MP seems to be possible as it was shown that several species of algae attach to floating polymer particles (Masó et al., 2003) possibly due to the formation of extracellular polymeric substances enhancing aggregate formation (Xiao and Zheng, 2016). Besides Chl-A, further water constituents that can be derived from satellite images are suspended particulate matter (SPM) and colored dissolved organic matter (CDOM). As SPM comprises the same size range as MP and exhibits the same diversity of characteristics, i.e., consisting of a heterogenous mixture of particles of various sizes, densities and shapes, a spatial relationship due to similar transport pathways seems reasonable. A spatial correlation between CDOM (consisting of both decaying material of terrestrial origin and/or in-situ biological activity in the respective area) and MPs is further assumed likely as terrestrial inputs are thought to dominate the CDOM source in coastal areas (Stedmon and Nelson, 2014; Harvey et al., 2015) similar to MP litter inputs. Assuming that algae (Chl-A), SPM, CDOM and MPs are passive drifters which are mixed and transported by the same physical mechanisms (i.e., wind, waves, and currents) we tested the hypotheses that with (i) increasing Chl-A, (ii) SPM, and (iii) CDOM concentration, the concentration of MP increases. Moreover, an alternative indirect monitoring approach for floating MP through mapping proxies (the water constituents) measurable with remote sensing methods is proposed.

MATERIALS AND METHODS

Study Area

Sampling was conducted at three different terminal river systems (Trave, Elbe, and Po) entering different seas (North Sea, Baltic Sea, Northern Adriatic Sea), which were chosen as to cover a broad range of expected MP abundances (**Figure 1**).

The Trave river in northeastern Germany has a length of 113 km, a watershed of 1,804 km^2 (LM-MV and LM-SH, 2011) and exhibits an average annual discharge of 7.37 m^3/s into the western Baltic Sea. Samples were taken from 22.05.2014 to 25.05.2014 at the brackish river mouth, a riverine shallow water firth with an average depth of 5.5 meter. Sampling was conducted along a distance of approximately 5 km with river width varying from approximately 22 to 535 m (see **Supplementary Information 4**). Water exchange with the Baltic Sea is limited through a narrow connection at Lübeck Bay with salinity ranges from 0.2 to 17.3 PSU. Oscillations of the sea level (<20 cm) is a result of wind-induced water movements as well as the effect of buildup of water reaching the coast. The Trave drainage area is highly influenced by agriculture, and tourism is an additional important economic factor. The harbor of Lübeck, located ∼18 km inland of the river mouth, serves as a shipyard location and a nationally important transfer point for passenger ferries and commercial goods.

The Elbe river in northwestern Germany is one of the largest rivers of Central Europe with a length of 1,100 km, an average yearly discharge of 844 m^3/s into the North Sea and a watershed of 148,000 km^2 which is home to 25 million people. Samples were taken from 19.08.2015 to 21.08.2015 within transitional waters of the tidally influenced river mouth (widths between ∼0.2 and 21 km) and adjacent coastal waters spanning a total

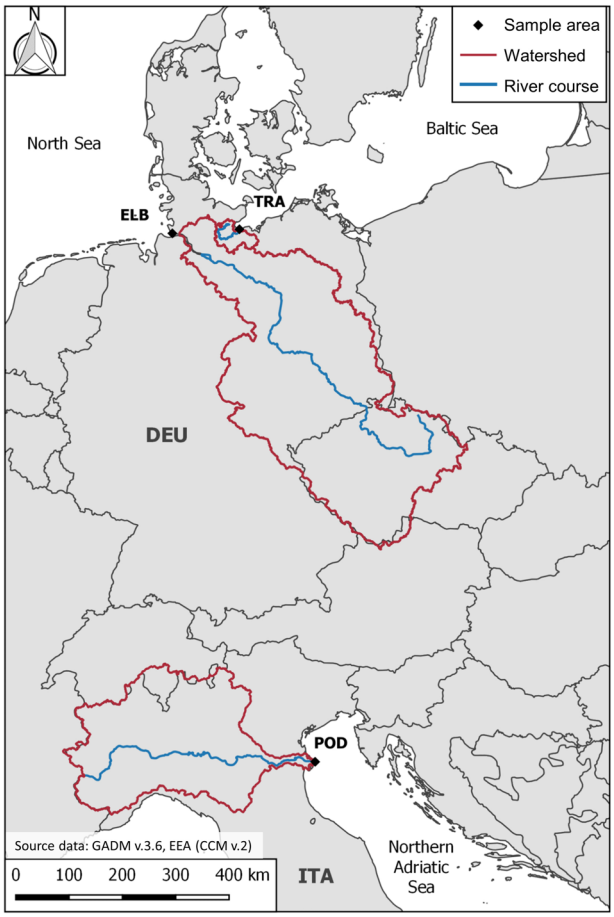

FIGURE 1 | Study areas of the Trave (TRA) and the Elbe estuary (ELB) in Germany (DEU) and the Po delta (POD) in Italy (ITA), course of the rivers and their respective watersheds.

length of ∼32 km (see **Supplementary Information 5**). The Elbe estuary is a partly- to well-mixed mesotidal estuary with a tidal amplitude ranging between 2 to 4 m. Between a single tide, an average distance of 20 km is traveled between capsize points. As a hyper-synchronous estuary, the tidal amplitude increases to a maximum between the coast toward the city of Hamburg and thereafter decreases. The Elbe estuary morphology and hydrology leads to a so called "maximum turbidity zone" (MTZ) where concentrations of suspended matter reach their highest value. Inputs from upstream are supplemented with additional inputs from adjacent areas such as dike-free river branches and wastewater discharges. The investigated area is significantly influenced by ship traffic, as the harbor of Hamburg is the third largest container port in Europe.

The Po river in Italy has a watershed of 74,000 km^2 (Artioli et al., 2005) and a length of around 652 km (Lanzoni et al., 2015). Although exhibiting a smaller watershed as compared to the Elbe river, it contains a much higher population density representing almost half of the Italian population (∼30 million people). It is the longest Italian river and has an average annual discharge of 1,500 m^3/s (Falcieri et al., 2014) flowing into the Northern Adriatic Sea (**Figure 1**). Given these factors, the Po river is thought to be the second most important marine litter source to the Adriatic basin (Liubartseva et al., 2016). Potential sources for MP include run-off and wastewater from intensively practiced agriculture, several industrial parks as well as large cities. The terminal river splits into seven active branches before entering the sea (**Supplementary Information 6**). As deltaic system, it exhibits a net sediment transport from the river to the delta. The Northern Adriatic Basin is influenced by tidal fluctuation of around 30 cm but under specific conditions can reach up to 140 cm (Maicu et al., 2018). Sampling was conducted from 10.06.2016 to 23.06.2016 at the terminal river branches with varying width from ∼63 to 795 m and extending ∼7.8 km offshore (see **Supplementary Information 6**).

Sampling

At each sampling location, water surface samples for MP and water constituents (Chl-A, SPM, CDOM), were simultaneously taken as described in detail by Atwood et al. (2019). Microplastic samples were concurrently collected from the top 15 cm of the water surface alongside motorboats and sailing ships using a mini-manta trawl equipped with a flowmeter (30 × 15 cm opening, 2 m long net with a mesh size of 300 μm). Water samples were always taken with flow direction of the river and in zigzag pattern perpendicular to the flow direction except for the Elbe estuary, where this maneuver was not possible due to intense ship traffic. Samples were stored in glass jars until processing in the laboratory. In total, 13 locations were sampled at the Trave river and 20 locations each at the Elbe estuary and the Po delta (see **Supplementary Information 4, 5, and 6**) as to cover a wide range of water constituents and MP concentrations. To assure similar conditions over the length of one trawl, water clarity was measured with a secchi disk at the beginning and the end of each trawl. For water constituents, 2 L water samples were concurrently collected with a glass flask from the water surface (top 40 cm) and kept both dark and cold until further processing. Later that same day, samples were separated and filtered on GF/F glass microfiber filters (0.7 μm pore size) for Chl-A following the JGOFS protocol (Knap et al., 1996) or pre-weighted cellulose acetate filters (0.45 μm pore size) for SPM following the protocol of Lindell et al. (1999).

Water Constituent Analysis

Chlorophyll pigments were extracted from the filters using 90% acetone [following JGOFS protocol (Knap et al., 1996)]. Extracted chlorophylls from the Trave river were analyzed with a fluorometer (Turner Designs, TD-700) with an excitation wavelength of 340–500 nm and an emission wavelength >665 nm. For the Elbe estuary and the Po delta, chlorophylls were extracted using 96% ethanol and extracts analyzed with a fluorometer (JASCO, FP-8600) at an excitation wavelength of 435 nm and an emission wavelength of 670 nm. The fluorometer was calibrated using a photometer (JASCO, V-670) and a Chl-A standard (Sigma-Aldrich, C6144-1MG). After each first measurement, samples were acidified with HCl and again measured to allow the subtraction of phaeopigments in order to obtain Chl-A concentration following the JGOFS protocol (Knap et al., 1996). Although extraction protocols slightly

differed, obtained results do not influence the general results, as regression analysis with MPs were performed separately for each river system.

Samples for SPM analysis were dried for 2 h at 60–80°C and cooled in a desiccator for 10–15 min before weighted on an analytical balance (Sartorius, R 200 D).

Absorbance analysis of CDOM was conducted using the filtrate of the SPM samples (material <0.45 μm). Duplicate samples were stored in glass flasks covered with aluminum foil and stored at a temperature of 7°C until spectrophotometric analysis. Measurements were taken with a UV-VIS-NIR spectroradiometer (PerkinElmer, LAMBDA 950) using 5 cm glass cuvettes. Absorbance measurements were performed at the wavelength positions 400, 412, 420, and 440 nm.

Microplastic Sample Processing

Each sample was first fractionated into two size classes: 5000–500 and 500–300 μm. All potential MP particles >500 μm were visually pre-sorted under a stereomicroscope (Leica, M50) using a counting chamber for zooplankton. For a more efficient visual pre-sorting, samples with a high organic content were additionally treated with a wet peroxide oxidation (Masura et al., 2015). Potential MPs were photographed (Olympus, DP 26), and stored in Eppendorf tubes for analysis with Attenuated Total Reflectance (ATR)-Fourier transform-infrared (FT-IR) spectroscopy (Löder et al., 2015).

The smaller size fraction (500–300 μm) of all samples was treated with an enzymatic purification protocol (Löder et al., 2017) and afterward with wet peroxide oxidation (Masura et al., 2015) to remove organic matter, which would interfere with subsequent spectroscopic analysis. As remaining particle numbers were still very high, subsamples were filtered onto 25 mm aluminum oxide filters (Whatman, Anodisc 25). Detailed information on the subsamples can be found in the **Supplementary Information** (see **Supplementary Information 1**). Subsamples were analyzed with a Focal Plane Array (FPA) based micro FT-IR (μFT-IR) spectroscope (Bruker, Tensor 27 FT-IR spectrometer equipped with a Hyperion 3000 FT-IR microscope with a 15× Cassegrain objective and a 64 × 64 FPA detector) according to Löder et al. (2015). In order to conduct a full quantitative analysis, the remainder of each sample was filtered onto glass fiber filters (Macherey-Nagel, MN 85/90 BF) and analyzed with a newly developed hyperspectral imaging methodology (Schmidt et al., 2018) using a near-infrared (NIR) imaging spectrometer (Norsk Elektro Optics AS, HySpex SWIR-320m-e).

To account for potential airborne contamination of the samples with small microplastics (500–300 μm), negative controls with filtered water were processed together with the microplastic samples for each set of samples from a river. In total, six negative controls were measured with the HySpex imaging spectrometer on which no MP particles were found. Mostly fibers were found (0.7 to 11.6 fibers on average for the three investigated data sets) within a total of 15 negative controls analyzed with FPA based μFT-IR spectroscopy (**Supplementary Information 3**). Only for the Po delta was one polypropylene particle found in one out of six negative controls corresponding to 0.17 particles on average. This amount was subtracted from measured polypropylene abundances in each sample within the Po delta data set. Synthetic fibers were generally not considered within this study as synthetic fibers normally exhibit diameters between 6 and 175 μm (Cole et al., 2015; Dris et al., 2016, 2018) which could not be sampled quantitatively with the utilized manta net. As no fibers or particles >500 μm were observed within the negative controls, it is assumed that airborne contamination of the larger size class can be neglected. Finally, obtained MP particle numbers from all three methods were added for each sample and standardized according to the filtered water volume to MP particles per cubic meter.

Statistical Analysis

All statistical analyses were performed using the software R v.3.5.2. Linear regression analyses were conducted to test the unidirectional null hypothesis that MP abundance will not increase with increasing water constituent abundance. To meet model assumptions for parametric linear regression analysis, the Trave data were square root transformed and for the Po delta data a log transformation was applied. Correlation analyses among all investigated water constituents were conducted to check for collinearity. Relationships were accepted as significant at $p < 0.05$, and all results are shown as average ± one standard deviation (SD).

To further investigate potential patterns within the datasets, principal component analyses (PCA) were applied. The packages "FactoMineR" (v.1.34) and "factoextra" (v.1.0.7) were used for PCA and biplots for visualization of the data. First the predictor variables were scaled to have a SD of one and a mean of zero. Two missing values within the Po delta dataset were excluded from the analysis, although imputing the two data points by predictive mean matching produced similar results. The function "prcomp" was used to calculate principal components (PCs) via singular value decomposition on the original data matrix.

To investigate potential spatial relationships between MP and water constituent abundance, subgroups according to sampling location were defined. For the Trave, the categories included river, wastewater treatment plant (WWTP), and coastal samples. For the Elbe data, a distinction between coastal and river samples was made, whereas the categories river, mouth (representing the seven distal branches of the delta), and coastal samples were defined for the Po delta data.

RESULTS

The three chosen river systems exhibited the assumed wide range of MP concentrations, ranging from 1 to 78 particles/m^3 at the Po delta, followed by the Elbe estuary with 0–11 particles/m^3 to only a few particles at the Trave river (0–1 particles/m^3; **Figure 2**, specific values in table **Supplementary Information 1**). For the Trave river generally higher MP concentrations were observed within the river than the coastal area (**Supplementary Information 4**) whereas for the Elbe estuary MP concentrations showed an increasing trend toward the coast (**Supplementary Information 5**). At the Po delta elevated MP concentrations

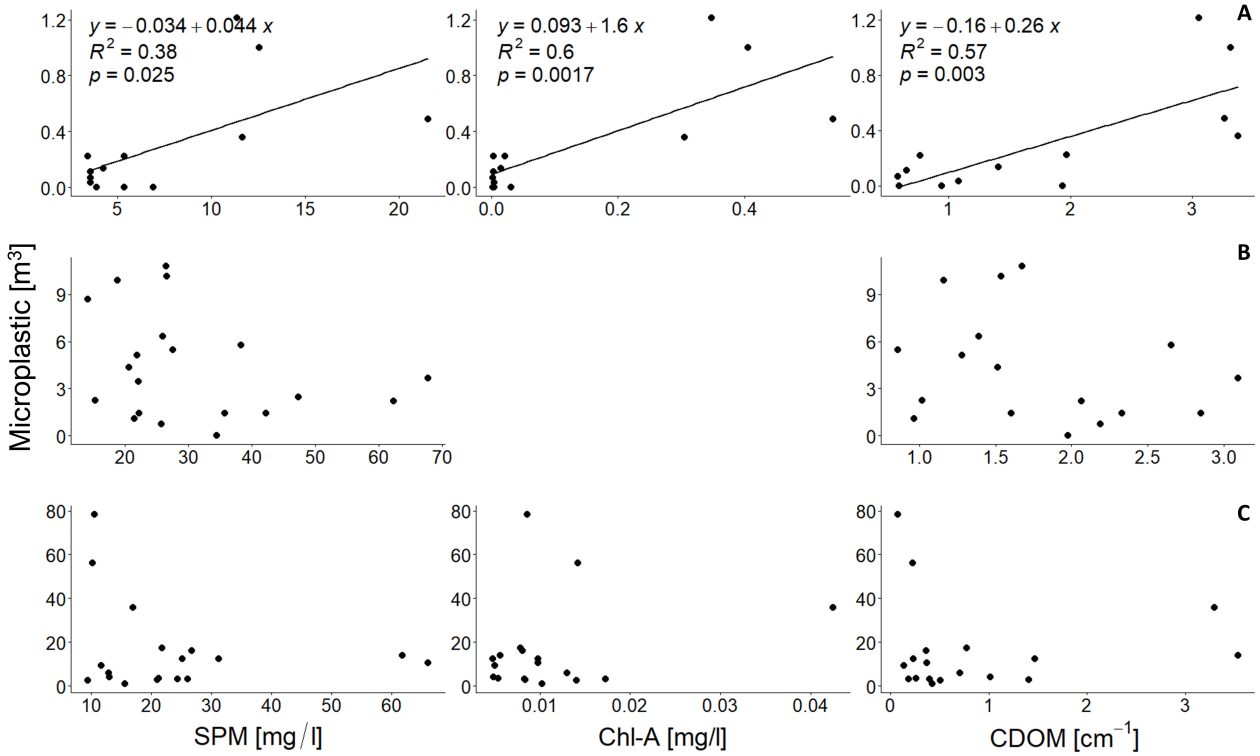

FIGURE 2 | Relationship between measured water constituents and microplastic (0.3–5 mm) at the Trave river **(A)**, the Elbe estuary **(B)**, and the Po delta **(C)**. Linear regression analysis revealed a significant positive relationship among the abundance of all three water constituents and microplastic abundance for the Trave river **(A)**. Chl-A, chlorophyll-a, SPM, suspended particulate matter, CDOM, colored dissolved organic matter.

were observed within the river branches, as well as the outer river plume (**Supplementary Information 6**). Linear regression analysis revealed a significant relationship between the abundance of MP and all three investigated water constituents for the Trave river, whereas Chl-A explaining the largest portion of the variance (**Figure 2**). Nevertheless, this result has to be interpreted with caution as MP abundances were generally low within this river system (**Figure 2**, **Supplementary Information 1**). PCA was conducted and results displayed within biplots to reveal further patterns within the data sets (**Figure 3**).

PCA of the Trave data revealed that the first PC, explaining 93.8% of the variance within the data, is almost equally represented by all three water constituents: Chl-A (35%), SPM (34%), and CDOM (32%) (**Figure 3A**). The second PC (explaining 5% of variance) instead is mostly represented by CDOM (65%) and SPM (28%). Microplastic abundances in coastal areas are better represented by CDOM with only a small angle between the main axis of the ellipse and the CDOM vector (**Figure 3A**).

Considering PCA results of the Elbe estuary a similar pattern can be seen with coastal samples better represented by CDOM (**Figure 3B**). The two predictor variables SPM and CDOM are equally represented (each 50%) by both PCs (**Figure 3B**).

Contrarily, none of the investigated water constituents could predict MP abundance for either the Elbe estuary (SPM: $F = 1.72$, $p = 0.21$ and CDOM: $F = 1.25$, $p = 0.28$) or the Po delta (Chl-A: $F = 1.14$, $p = 0.30$, SPM: $F = 1.71$, $p = 0.21$, and CDOM: $F < 0.001$, $p = 0.99$). For the Elbe estuary, the lacking relationship was further indicated by a second field data acquisition in June. Here, one of the highest MP values (4.44 MP particles/m^3) correlated with one of the highest SPM concentration (323.87 g/l) but the very highest MP value (4.60 MP particles/m^3) was sampled on a spot with a low SPM concentration (20.78 g/l) (**Supplementary Information 1**).

Furthermore, all water constituents showed a positive correlation among each other for both German river systems, whereas for the Po delta none of the investigated predictor variables correlated (**Supplementary Information 2**). Conducting PCA for the Po delta, the first PC is best represented by CDOM (57%) and Chl-A (31%), but only explaining around 49% of the variance (**Figure 3C**). The second PC is mostly represented by SPM (62%) and Chl-A (37%), explaining around 37% of the variance (**Figure 3C**). No clear differences in MP abundances between river mouth, river, and coastal samples can be seen (**Figure 3C**).

DISCUSSION

A clear relationship among water constituents and MP for all three investigated river systems could not be identified. The positive relationships between the three proxy water constituents

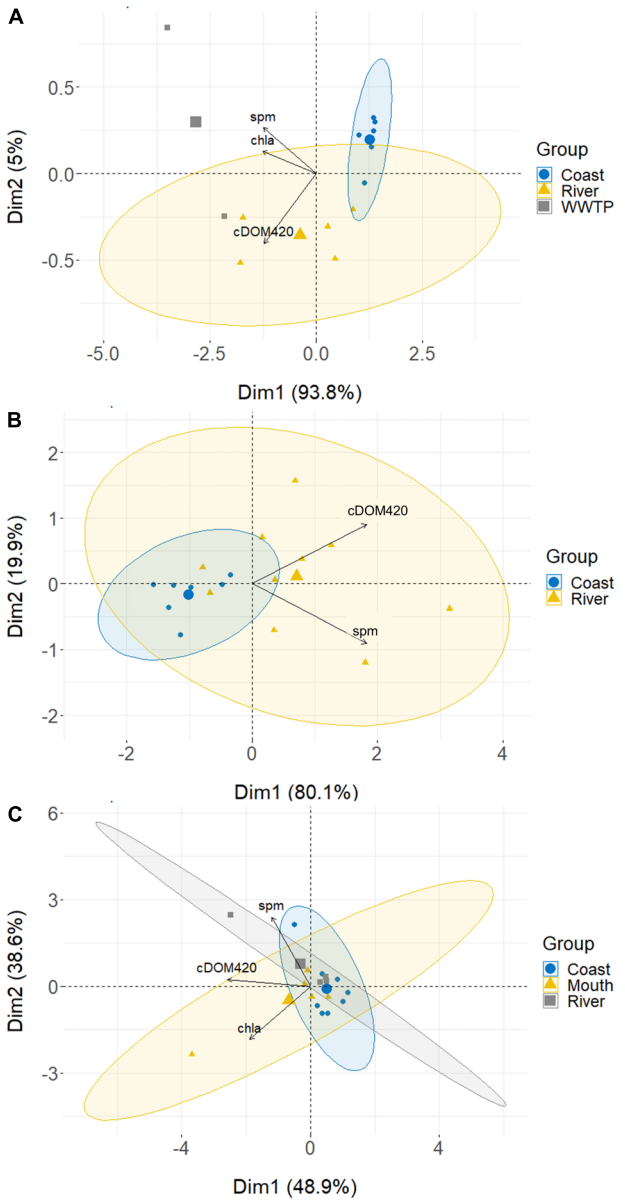

FIGURE 3 | Biplots of the Trave river **(A)**, the Elbe estuary **(B)**, and the Po delta **(C)** showing the scores of individual samples grouped by different symbols and colors. Loadings of the predictor variables chlorophyll-a (chla), suspended particulate matter (spm), and colored dissolved organic matter (cDOM420) on the first two principal components (Dim1 and Dim2) are represented as vectors. Larger symbols depict the center of the confidence ellipse (95% confidence level) drawn around the groups.

and MP abundances found for the Trave river needs to be verified in future studies, as the results are based on very low MP abundances within the obtained water samples (**Supplementary Information 1**). As indicated by the biplots, depending on the estuary type, spatial relationships between water constituents and MPs are further spatially limited. Thus, potential relationships need to be evaluated for various river systems separately and additionally for the river course and the coastal zone.

One factor influencing possible relationships between water constituents and MPs are the presence and location of their emission sources. In the Trave river, higher concentrations of MPs were found within the river, specifically close to the WWTP (**Supplementary Information 4**) and gradually decreasing toward the Baltic Sea. For the Baltic Sea, it is assumed that land-based litter inputs are dominating (Haseler et al., 2018) and thus the lower MP water surface concentrations at the coastal area may be a dilution effect. For the Elbe estuary, results show a tendency of higher abundance of MPs within the adjacent coastal area compared to the inner parts of the river (**Supplementary Information 5**). The increasing MP concentrations at the Elbe estuary toward the coastal area may be a consequence of additional inputs of sea-based sources. Both the study system of the Elbe estuary and the Po delta are highly influenced by fishing and aquaculture related litter inputs (Simeoni and Corbau, 2009; Kammann et al., 2018). Nevertheless, for the Po delta with its diverse terminal branches no pattern in MP abundance could be detected (**Figure 3C** and **Supplementary Information 6**). The protruding form of the Po delta plays an important role for dispersion of suspended material, as MP and water constituents emitted by the branches exhibit different hydrodynamical and meteorological conditions. The hydrodynamical conditions spanning from a mixed mesotidal estuary (Elbe) to nearly stratified systems (Trave, Po) generally influence spatial patterns of suspended materials and thus, further may explain observed differences.

For example, SPM showed no spatial relationship with MPs at the Elbe estuary. Nevertheless, a study on larger litter collected with bottom trawls and at the shore within an estuary in South America, reported that larger litter was accumulating in the MTZ (Acha et al., 2003). In our study, we focused on water surface MP concentrations at the Elbe estuary which also exhibits an MTZ. Even though our sampling did not cover the MTZ in August, during another sampling in June samples were taken closer to Hamburg city (**Supplementary Information 5**) and the MTZ was covered according to salinity measurements (**Supplementary Information 1**; located between 0.1 and 4 PSU; Herman and Heip, 1999). Here, a correlation analysis revealed a relationship between SPM and MPs (Spearman's rank correlation, $S = 154$, $p = 0.01$, rho = 0.66). The positive relationship of SPM and MPs at the Trave, explaining 38% of the variance, could be due to dominating riverine inputs for both. The missing relationship at the Po delta on the opposite could be the result of differing sources of SPM and MPs among the several distal branches at the investigated site. Suspended particulate matter dynamics are coupled to meteorological conditions and will vary on small temporal scales. For example, higher SPM inputs occur during high precipitation events due to increased terrestrial runoff as well as mobilization of riverbed sediments. Likewise, high MP export was already associated with high flow events in rivers (Hurley et al., 2018). Considering CDOM as proxy water constituent for MPs, only at the Trave river was a positive relationship found, whereas PCA showed that CDOM explained most of the variance in coastal samples (**Figure 3A**). A similar pattern was found for the Elbe estuary (**Figure 3B**) even though a relationship could

not be verified by regression analysis (**Figure 2B**). The absence of a relationship for the other two investigated sites can be explained by differing DOM dynamics among systems. For example, Berto et al. (2010) has shown an influence on DOM dynamics on a monthly and sub-monthly time scale at the Po delta. Processes leading to higher CDOM concentrations in spring and summer vary with higher inputs due to melt-water run-off from rivers in spring (Ferrari and Dowell, 1998; Stedmon and Nelson, 2014). Moreover, higher photo-bleaching in summer can alter the optical properties of surface waters and lead to decreased CDOM absorption and fluorescence (Vodacek et al., 1997). Both processes influence potential spatial as well as temporal relationships, in particular on a seasonal scale, of CDOM and MPs.

Even though a positive relationship between water constituents and MP abundance was found for the Trave river, the results need further validation since results are based on generally low MP abundances (**Supplementary Information 1**) as well as only one sampling campaign. Following linear regression, most variance was explained by Chl-A (62%). Contrarily, a correlation between MPs and Chl-A could not be detected for the Po delta. Although the slightly different extraction protocols used should not have influenced the observed results as samples were treated equally within a dataset, this source of potential error cannot be definitely excluded. Even though standard protocols for the analysis of chlorophyll exist, they vary in their results. A relationship would be further influenced by seasonal patterns, as well as the ability of some algae to influence their vertical position in the water column to counteract sinking out from the euphotic zone as well as resource acquisition (Raven and Doblin, 2014).

Remote sensing of water constituents in coastal systems is difficult due to the overlapping spectral signal from different optical components. The Trave provided the clearest CDOM signal of all three river systems, in that the SPM concentration was relatively low and the CDOM concentration relatively high. The Po delta was found to have the lowest CDOM concentrations and some of the highest SPM concentrations of all three study systems. Even though the least amount of variance was explained by SPM within the Trave data, from an in-situ monitoring point of view, SPM fulfills the requirements for an operational monitoring in being cost- and labor-effective, as well as easy to assess. A relationship between MP concentrations and sediment grain size as well as organic matter deposition has been proposed in previous studies (Vianello et al., 2013; Maes et al., 2017; Haave et al., 2019). Haave et al. (2019) further reported that particles <100 µm made up $>95\%$ of the MP concentration, whereas particles >500 µm showed different spatial distribution patterns. This points toward the importance of MP particles <300 µm not covered by our MP sampling method. Thus, it would be interesting in future studies to test the relationship of SPM and MPs with recently developed pumping systems which are able to extract MPs down to 1 µm (Lenz and Labrenz, 2018). In conjunction with the development of faster identification methods of MPs, higher sample replication can be achieved, resulting in more precise model results.

Further research in the field is desirable as satellite imagery has the potential to complement spatial and temporal limited point measurements and thus improve our current understanding of

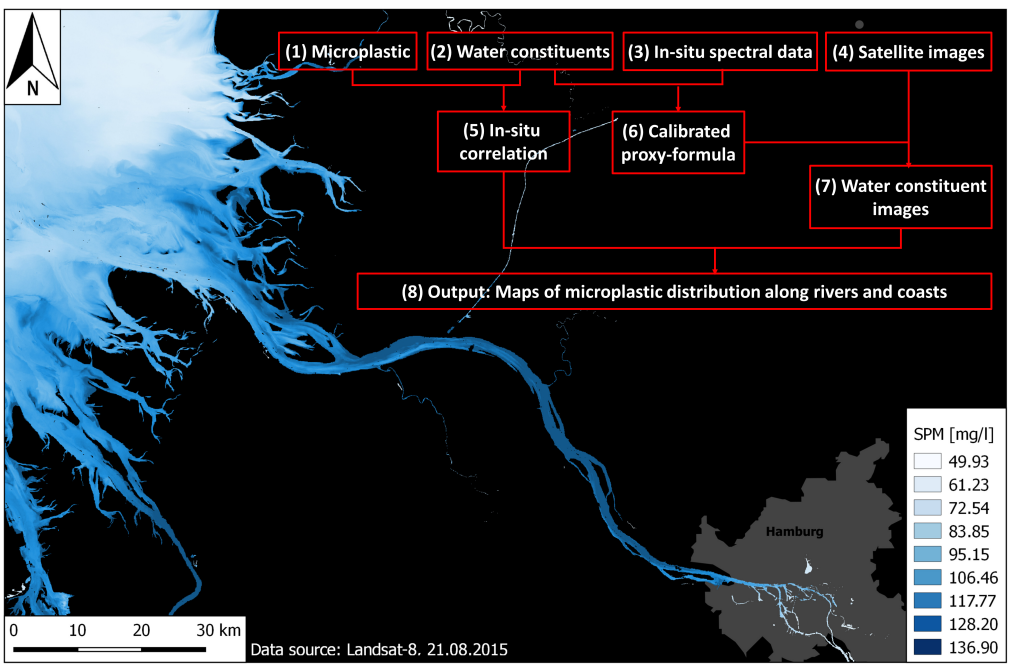

FIGURE 4 | Satellite image of suspended particulate matter (SPM) at the Elbe esturay. The diagram illustrates the proposed framework to track microplastic distribution via proxy water constituent maps (software: QGIS 2.14.8).

sources, transport, and accumulation of MPs within river courses and adjacent coastal areas.

In this study, we analyzed a spatial relationship (**Figure 4**) between in-situ MPs (**Figure 4**, component 1) and in-situ water constituents (**Figure 4**, component 2). Further steps are necessary to transfer remotely sensed water constituents into MP abundance to produce MP distribution maps along rivers and coasts which is described in detail by Atwood et al. (2019) and shown in **Figure 4** (components 3, 4, 6, and 7). Near-range spectral measurements (**Figure 4**, component 3) taken concurrent to sample collection would be needed to build regionally calibrated remote sensing spectral reflectance water parameter algorithms (**Figure 4**, component 6) for different satellite platforms (**Figure 4**, component 4) as specified in Atwood et al. (2019). Through the adaption of the remote sensing imagery of water constituents (**Figure 4**, component 7) with the calibrated algorithms, maps of the water constituents could then be used as proxy for MP. Existing single point measurements could thereby be extrapolated to large spatial scales to produce maps of MP distribution along rivers and coastal areas (**Figure 4**, component 8).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MB, JF, FS, and CL initiated the study. All authors designed the study. SP, EA, MB, and HI conducted the field sampling. SP prepared the microplastic samples with the help of four technichal assistants, conducted the microplastic particle identification with FT-IR spectroscopy, and wrote the first draft of the manuscript. MB conducted the NIR microplastic particle identification as well as did the analysis of CDOM. SP and HI did Chl-A and SPM analysis. EA processed the remote sensing data. SP analyzed the data. SP, EA, MB, HI, JF, and CL discussed and interpreted the data. SP and EA created the figures and tables. All authors were involved in revision and rewriting 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/fenvs.2020.00092/full#supplementary-material

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Microplastic Vector Effects: Are Fish at Risk When Exposed via the Trophic Chain?

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In aquatic organisms, trophic transfer is a relevant exposure route for microplastics (MPs). Despite their relevance, effect studies on fish exposed via trophic chains are currently very scarce. MPs are known to contain many chemicals that could be transferred to organisms and induce deleterious effects. However, there is currently no consensus on whether MPs represent a significant exposure pathway to chemicals in contaminated habitats. Here, we exposed three-spined sticklebacks (*Gasterosteus aculeatus*) to polyethylene MPs via prey ingestion, in a one-month experiment. MPs were either pristine or spiked with chlorpyrifos (CPF), and a CPF control was included to§compare vector effects of MPs and natural prey. Following exposure, we assessed AChE activity and fish behavior (feeding, locomotion, environment exploration and reaction to the introduction of a novel object). No effect was observed in fish exposed to pristine MPs. CPF accumulation was observed in fish exposed to CPF-spiked MPs (MP-CPF), confirming the vector potential of MPs. However, CPF accumulation was more important in fish exposed to CPF via prey. In fish exposed to MP-CPF, we observed significant AChE inhibition and hyperactivity, which could result in increased vulnerability to predation. CPF organ distribution differed between groups, suggesting that chemical exposure via MPs could alter organ distribution of chemicals. This can result in a change in the organs most at risk, likely increasing intestine exposure.

Keywords: ecotoxicity, chlorpyrifos, behavior, uptake, stickleback

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INTRODUCTION

Increasing numbers of field and laboratory studies have shown that most lower trophic level organisms are able to ingest microplastics (MPs) (Lusher, 2015; Scherer et al., 2018). Ingestion of MP-contaminated prey by predator species is therefore very likely and trophic transfer has been identified as a relevant contamination pathway for MPs (Farrell and Nelson, 2013; Nelms et al., 2018). Despite their relevance, MP trophic transfer and its impacts on upper trophic level organisms are still poorly investigated, especially in studies involving fish. MPs are known to contain many additives, such as plasticizers, flame retardants, stabilizers, surfactants and pigments (Lambert and Wagner, 2018), as well as environmental contaminants, such as PCB, PAHs and PBDEs (Koelmans, 2015). A major concern is therefore their potential to act as vectors and to transfer chemicals to organisms (Rochman, 2019). However, modeling studies have questioned this hypothesis, arguing that the role of MPs in chemical transfer to organisms could be minor in a context of contaminated environments (Teuten et al., 2007; Gouin et al., 2011; Koelmans et al., 2013). Especially,

the ingestion of contaminated prey and/or natural particles could result in greater chemical uptake, compared to MPs. Despite the importance of comparing MPs to other natural vectors of contamination, such as natural prey, these alternative exposure pathways are still poorly investigated in MP ecotoxicity studies (Koelmans, 2015), especially those focusing on aquatic organisms.

To explore these major knowledge gaps, the present study aims to investigate (i) the effects of MPs on fish exposed via prey ingestion, (ii) the potential of MPs to transfer chemicals (i.e., vector effect) to fish exposed via prey ingestion, (iii) the relative importance of MPs' vector effect, in comparison with contaminated prey, and (iv) the consequences of MPs' vector effect on organisms' performance. For this purpose, we studied an experimental trophic chain comprising the three-spined stickleback (*Gasterosteus aculeatus*) as predator species, and brine shrimps (*Artemia sp.*) as natural prey. The three-spined stickleback naturally occupies a wide range of aquatic habitats (Bell and Foster, 1994) and has been widely used in ecotoxicity and behavior studies (Girvan and Braithwaite, 1998; Sturm et al., 2000; Dingemanse et al., 2007; Jutfelt et al., 2013; Fürtbauer et al., 2015; Thompson et al., 2016; Marchand et al., 2017). For this study, stickleback individuals were fed during 4 weeks with brine shrimps previously exposed to pristine MPs, chemical-spiked MPs or chemical-contaminated water. To study the vector effect of MPs, we selected polyethylene (PE) as a model plastic polymer. PE is the most produced polymer type (PlasticsEurope, 2017) and is commonly found in environmental matrices (Bour et al., 2018). Chlorpyrifos (CPF) was selected as a model chemical compound. Its intermediate partition coefficient (log K_{ow} = 4.66) suggests the possibility to bind to MPs in aqueous environments, while potentially allowing for desorption in biological matrices. CPF is a commonly used organophosphate pesticide that inhibits acetylcholine esterase (AChE) (Ware, 1999), an enzyme involved in neurotransmission, which can result in behavioral disorder in fish, as previously shown in *Gambusia* (Rao et al., 2005). In addition to AChE inhibition, we assessed behavioral changes to study the effects of MPs and CPF on stickleback. Behavior is a sensitive endpoint likely to be affected at low contaminant concentrations, and behavior alterations can have consequences at the ecosystem level (Galloway et al., 2017). The study of behavioral endpoints is therefore very relevant in ecotoxicity studies. Here, we specially focused on feeding, locomotion and fish reaction to the introduction of a novel object.

MATERIALS AND METHODS

Materials

Particles and Chemicals

Microplastics were purchased from Cospheric (Santa Barbara, United States; lot #120328-2–1). Opaque blue polyethylene microspheres were selected to ease quantification of exposure. According to the manufacturer, MPs are spherical (>90% of particles), with a 27–32 μm diameter (>90% of particles in the indicated size range) and a density of 1.00 g/cc. CPF was purchased from Sigma-Aldrich as powder (purity >98%). High-purity methanol (99.89%) was purchased from VWR. All the chemicals used for the determination of AChE activity were of the highest purity available.

Model Organism

Three-spined sticklebacks (*G. aculeatus*) were collected in a reference site (Skaftö, Sweden [58°13′55.9″N 11°28′18.2″E]; water salinity: 16–18‰) with a hand-operated net, and immediately brought to the laboratory in aerated, thermally isolated boxes containing water from the sampling site. They were then acclimatized to and kept in artificial sea water for a month (13 ± 1°C, 30‰, pH = 7.9) prior to start of the experiment. Fish were fed daily with red mosquito larvae. Continuous water flow and aeration ensured good water quality, and environmental enrichment was provided (gravel substrate pictures glued on the outer side of the tanks bottom). Fish were sexually mature, with a size range of 3.3–5.6 cm (median: 4.2 cm; average: 4.2 ± 0.5 cm; N = 96 individuals).

Spiking of Microplastics With Chlorpyrifos

CPF solubility in water is very low, therefore CPF dissolution was performed in methanol. To spike MPs, a CPF solution was prepared in methanol at the concentration of 30 mg/ml. Glass material was used for the spiking to limit chemical sorption on the walls of the vials. CPF solution was added to MP batches of approx. 100 mg, to obtain a final ratio between MPs and solvent (methanol) of 1:1 (w:w) (Smedes and Booij, 2012). The use of methanol to spike PE MPs was previously validated in a pilot experiment: no melting, color loss or other alteration of the MPs was observed (unpublished). MPs were left in contact with CPF solution for 10 days, and gentle agitation was provided to ensure homogeneous spiking of MPs. To force the partitioning of CPF to the particles, milli-Q water was gradually added during the whole spiking process to eventually reach 90% of the final volume. After the last day of spiking, MPs were filtered using 10 μm nylon filters, rinsed with 50 ml of milli-Q water, and filtered again for recovery. Five filtration-recovery cycles were performed before storage (4°C).

Five MP batches were prepared in total, each batch being sufficient for 1 week of organism contamination. A new batch of spiked MPs was prepared before every week of organismal exposure and used immediately for the exposure. Therefore, spiked MPs were stored for a maximum duration of 1 week. Analysis of spiked MPs subsamples shows an average CPF concentration of 17.7 ± 8.4 μg/mg (46.6% of CPF sorption, on average; see section 2.4 for quantification method).

Experimental Setup

The exposure was carried out in September, once stickleback's breeding season is over. Fish were exposed to pristine MPs (further referred to as "MPs"), CPF-spiked MPs ("MP–CPF") or CPF only ("CPF") via prey ingestion during 4 weeks. Exposure was performed by feeding individualized fish with pre-exposed prey every second day, three times a week, to limit handling of the fish. The number of Artemia fed to the fish increased over

time, and a total of 51 *Artemia*/fish was reached at the end of the exposure (2 *Artemia*/fish/contamination day on weeks 1,2, 3 on week 3, and 5 on weeks 4,5). Every contamination day, live adult brine shrimps (*Artemia spp.*; approx. 1 cm) were placed for 15 min in Eppendorf tubes (2 ml; 15 *Artemia* individuals per tube) belonging to one of the experimental conditions (i.e., control, MPs, MP-CPF or CPF). Eppendorf tubes were prepared as follows, before the addition of *Artemia*: artificial seawater (ASW) only (control condition), approx. 2 mg of MPs or MP-CPF in ASW followed by strong manual shaking (MP and MP-CPF conditions, respectively), or CPF solution prepared in ASW (100 mg/L, solvent <10% total volume; CPF condition). After exposure, *Artemia* individuals were rinsed twice in ASW and fed to the fish from the respective experimental conditions less than 5 min later. Extra individuals were also kept and stored at −20°C after every contamination day, for further CPF concentration analysis. Exposure method is presented in **Figure 1**.

Every experimental condition, including control, was performed in triplicates, each replicate (randomly allocated aquarium; 20L) comprising eight fish. Fish size range was 3.3–5.6 cm, with random allocation to exposure groups and no significant size differences between groups (ANOVA, $p = 0.2$). Exposure was performed under controlled temperature (14°C) and light (12:12, light:dark cycle) conditions. Half of the water was renewed every second day and aeration was provided in every aquarium, to ensure good water quality. Fish were individualized, during the feeding to ensure equal prey ingestion between fish, then placed back together in their respective aquaria. Outside exposure days, red mosquito larvae were provided *ad libitum* without individualization of the fish.

After two weeks of exposure, significant mortality was observed in fish from the CPF group. The exposure was therefore stopped for this group, behavior tests performed on both control and CPF groups, then fish from the CPF group were euthanized (48 h after the last contamination). For MPs, MP-CPF and control groups, behavior assessment started after four weeks of exposure and lasted for a week. Exposure via prey continued during that week to prevent depuration of the fish and differing contamination levels between the first and last trial days. Behavioral trials were performed during the mornings and feeding in the afternoons, to prevent stress related to fish handling. After the last behavior trial (96 h after the last contamination), fish were euthanized, measured, weighed and organs (gut, liver, gonads, muscle, gills, and brain) were sampled for further analysis. Chemical analysis (CPF quantification) was performed on pooled fish that were found dead during the experiment (last contamination performed 48 h before) and immediately stored at −20°C. Biochemical analyses (enzymatic activity, protein content) were performed on single, euthanized fish; samples were snap-frozen in liquid nitrogen and stored at −80°C.

Determination of Microplastic Ingestion and Chlorpyrifos Quantification

MPs selected for this study are blue beads that could easily be seen through *Artemia* cuticle under microscopic condition. Therefore, *Artemia* individuals ($n = 47$) were observed and photographed (Leica EZ4HD stereomicroscope with integrated HD camera) without prior sample preparation, immediately after their exposure and before being fed to the fish. Images were further analyzed with ImageJ software to count particles. Fish intestines ($n = 17$) were digested overnight in 10% KOH at 50°C (Bour et al., 2018). Extracts were then filtered on 10µm nylon mesh, filters observed under the stereomicroscope and particles counted.

CPF was quantified in MPs and organism samples by UHPLC-MS, using CPF-(diethyl-d10) as internal standard. Extraction and instrumental analysis were performed at the Swedish Environmental Research Institute (IVL). Additional information concerning chemical analysis can be found in supporting information.

Toxicity Assessment

Acetylcholinesterase (AChE) Activity

AChE activity was determined in fish liver and brain, following an adapted procedure of Ellman's method (Ellman et al., 1961; Sturve et al., 2016). Protein content was determined according to Lowry's method (Lowry et al., 1951).

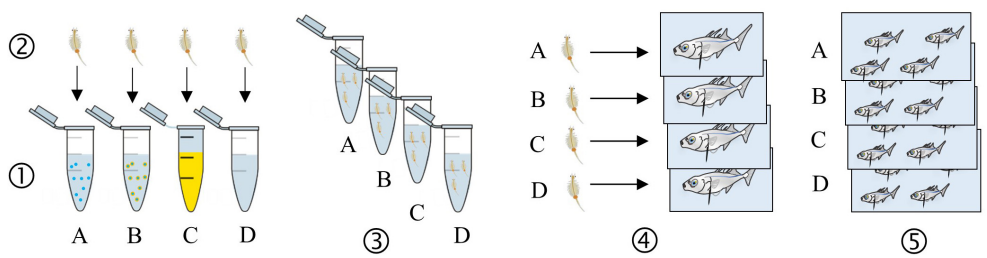

FIGURE 1 | Schematic representation of the exposure method. Colors, organisms and MPs numbers and size are not accurate. **(1)** Eppendorf ($n = 3$) preparation for each condition: MPs in Artificial Sea Water (ASW) **(A)**, MP-CPF in ASW **(B)**, CPF in ASW **(C)** and control (ASW) **(D)**. **(2)** Artemia ($n = 15$ per tube) are added and exposed for 15 min. **(3)** Exposure medium is removed and replaced by ASW to rinse Artemia (twice). **(4)** Artemia are fed to individualized fish (2–5 Artemia per fish. The number of Artemia increased over time, but was the same for every fish). **(5)** Fish belonging to the same exposure condition are grouped once all the Artemia have been ingested (eight fish per replicate, three replicates per condition).

Behavior Assessment

Feeding, locomotion, environment exploration and reaction to the introduction of a novel object were assessed. For the feeding trial, fish were individualized, allowed to acclimatize for 10 min then fed two frozen mosquito larvae. The whole trial was video recorded and recordings were visually analyzed to determine the time required for each fish to ingest both larvae.

Locomotion, environment exploration and reaction to a novel object were assessed during a second trial, following the procedure described by Thompson et al. (2016). Fish were individualized in containers comprising one shelter (piece of tile) each, and allowed to acclimatize for 10 min. Video recording started after the acclimation period. After 10 min of recording, a novel object (bolt attached to a transparent fishing line) was gently introduced in the center of the container and the fish reaction was recorded for 10 more minutes. Locomotion (immobility, total distance traveled, average speed, average acceleration, maximum speed, maximum acceleration) were determined over the first 10 min of recording, using idTracker software. Time spent in shelter and fish behavior following the introduction of a novel object were determined by visual analysis. Fish containers were virtually divided between the area close to the shelter (half container) and the distal part of the container, to determine the total time spent inside the shelter, close to the shelter and far from the shelter, during the first 10 min. After introduction of the novel object, the assessed endpoints were (i) fish immediate reaction (i.e., freezing: immediate immobility and slight curving of the tail; escape: fast swimming on the opposite direction of the novel object; no specific reaction), (ii) delay in returning to normal behavior after the immediate reaction, (iii) delay in active observation of the novel object (i.e., fish facing the novel object), (iv) delay in approaching the novel object after active observation, and (v) whether the fish actively touches the novel object or not.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8.1.1 software. Group comparisons of qualitative data (i.e., immediate reaction to a novel object and touching it or not) were performed with Chi-square tests. For quantitative data, normal distribution and homoscedasticity of residuals were verified with Shapiro-Wilk's and Bartlett's tests, respectively. Mann-Whitney tests were performed to compare CPF group and control group assessed after two weeks of exposure. One-way ANOVA tests were performed to compare MPs, MP-CPF and control group assessed after four weeks of exposure, when both normal distribution and homoscedasticity were verified. In cases of non-normal distribution, Kruskal-Wallis tests on rank were performed instead. Dunn's test was performed to compare groups when significant differences were detected. Detailed data on the performed statistical analyses is presented in **Supplementary Material** (**Supplementary Table 1**). Levels of significance were set at $p < 0.05$. For behavior endpoints, trends were considered from $p < 0.1$.

RESULTS

Trophic Transfer of MPs and CPF

MP Ingestion and CPF Accumulation in Artemia

Artemia ingested 204 ± 13 MPs/individual (average ± SE) before being fed to the fish. Based on MP ingestion and CPF concentrations in different MP batches, the expected (theoretical) CPF concentration in *Artemia* is 62.1 ng/individual. CPF quantification in *Artemia* samples from the MP-CPF group shows measured concentration of 97.3 ng/mg, equivalent to 57.8 ng/individual. Measured concentration in *Artemia* from the CPF group is 405.5 ng/mg, equivalent to 293.2 ng/individual.

Trophic Transfer of MPs and CPF in Fish

Each fish was fed 51 *Artemia* in total, therefore ingesting theoretically 10 404 MPs in total (equivalent to 140 µg of MP). However, after four weeks of exposure MPs were found in only two fish (samples contained two and three particles, respectively).

CPF concentrations were measured in gonads, viscera (i.e., intestine, liver and gall bladder), body muscle, gills and brain (**Table 1**). Daily checks ensured that dead fish were removed from the water and stored in less than 18 h.

CPF transfer to fish via *Artemia* ingestion, both with and without the inclusion of MPs, was quite low (**Figure 2**), with total values (addition of organ values) of 3 and 5.3%, for the MP-CPF and CPF groups, respectively. Single organ values ranged from 0.2 to 2% of total CPF ingested by the fish, depending on exposure conditions and organs. The relative distribution of CPF in the internal organs differed between MP-CPF and CPF groups (**Figure 3**). While the organs showing the highest concentrations were the viscera and gonads for the CPF group (33 and 34%, respectively), most CPF was detected in the viscera (68%) in fish from the MP-CPF group. Compared to the CPF group, gonad samples from the MP-CPF group showed low percentage of CPF uptake (8%). Percentages indicated here correspond to the ratio (x100) between the total amount of CPF measured in organs (ng) and the total amount of CPF ingested via *Artemia* (ng; theoretical values).

TABLE 1 | CPF concentrations measured in fish organs from different exposure groups.

	CPF (ng/mg)			
	Ctrl	**MPs**	**MP-CPF[1]**	**CPF[2]**
Gonads	< LOD	< LOD	0.21	3.51 ± 0.21
Viscera	< LOD	< LOD	0.51	1.05 ± 0.20
Muscle	< LOD	< LOD	0.14	0.67 ± 0.06
Gills	< LOD	< LOD	0.07	0.49 ± 0.10
Brain	< LOD	< LOD	0.27	1.66 ± 0.09

[1]Values from one pool of fish (n = 3). [2]Average values (±SE) of two pools of fish (n = 4).

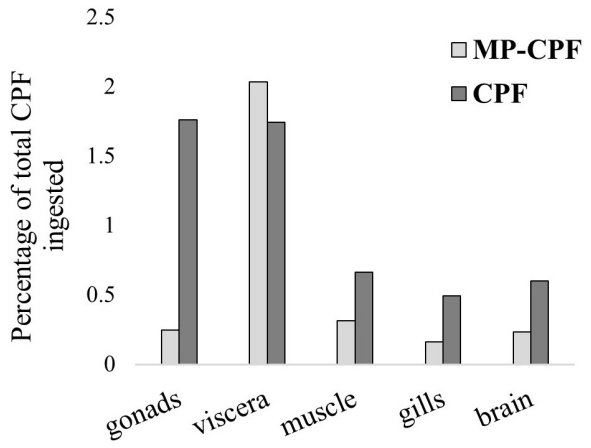

FIGURE 2 | CPF transfer to fish, expressed as the ratio (×100) between the total amount of CPF measured in organs (ng) and the total amount of CPF ingested via *Artemia* (ng; theoretical values). CPF was quantified in one fish pool (n = 3) and two fish pools (n = 4), for the MP-CPF and CPF groups, respectively.

Effects on Fish

Mortality

Significant mortality (58%) was observed in fish from the CPF group after two weeks of exposure. The exposure was therefore stopped for this condition after 16 days. The mortality recorded over the four weeks of exposure for control, MPs and MP-CPF groups was attributed to natural sensitivity of the species and handling stress, and considered non-significant (average mortality ± SE: 1.7 ± 0.3, 2.3 ± 0.9, and 2.7 ± 0.3 individuals, respectively).

AChE Activity

Decreased brain AChE activity was observed in fish exposed to MPs, MP-CPF and CPF (30.6, 46.9, and 85.5% lower than control, respectively). However, the difference compared to the control group was statistically significant only for fish exposed to CPF alone ($p < 0.01$). In liver, 10 and 55% decreases were observed for the MP-CPF and CPF groups, respectively. An increase in liver AChE activity was observed in fish exposed to MPs (28.2% increase, compared to control). However, the differences observed between groups were not statistically significant ($p > 0.05$). AChE activities are presented in **Figure 4**.

Behavior

Behavior endpoints were grouped under four categories: feeding, locomotion, time spent in shelter and reaction to a novel object. Fish exposed to MPs did not present any behavioral changes compared to control fish. Fish exposed to MP-CPF exhibited changes in environment exploration and in their reaction to the introduction of a novel object. They spent less time in the shelter, compared to control fish (Δt = −54%; p = 0.1), and returned faster to a normal behavior after their first reaction following the introduction of the novel object (Δt = −47 %; p = 0.1). Stronger behavioral changes were observed with fish exposed to CPF via prey, with changes in all four endpoint categories. CPF fish exhibited significantly longer feeding time and immobility (Δt = +309 and +97%, respectively; p = 0.05), they spent less time in the shelter (Δt = −73%; p = 0.05) and active looking at and proximity with the novel object were delayed (Δt = +138 and 205%, respectively; p = 0.05). Detailed results are presented in **Supplementary Material** (**Supplementary Table S2** and **Supplementary Figures 1**, **2**).

DISCUSSION

Experimental Trophic Chain

Trophic transfer of MPs has been identified as a most relevant contamination pathway for predators (Lusher, 2015). In the present study, the selected trophic chain allows to control MP ingestion by preys and therefore fish exposure to MPs. We observed a consistent number of MPs in *Artemia* individuals throughout the experiment (204 ± 13 MPs per individual on average) and CPF concentrations measured in *Artemia* from the MP-CPF group (57.8 ng/individual) were very close to the expected concentrations (62.1 ng/individual). These results indicate that fish exposure to MPs and CPF was consistent throughout the experiment and validate the use of the present trophic chain as an appropriate method for controlled fish

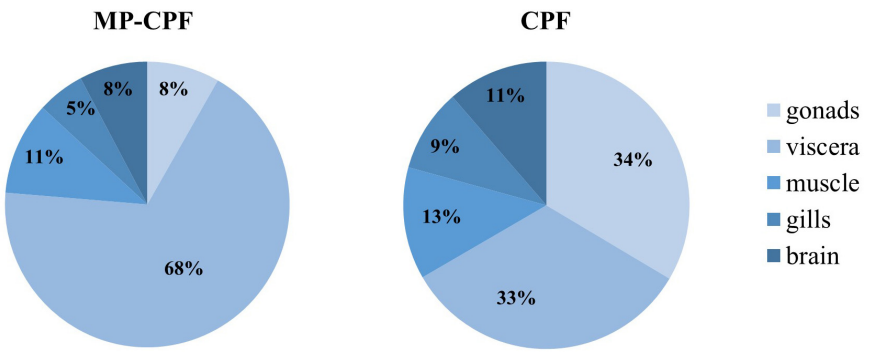

FIGURE 3 | CPF relative distribution in different fish organs, expressed as the percentage of total CPF in fish. CPF was quantified in one fish pool (n = 3) and two fish pools (n = 4), for the MP-CPF and CPF groups, respectively.

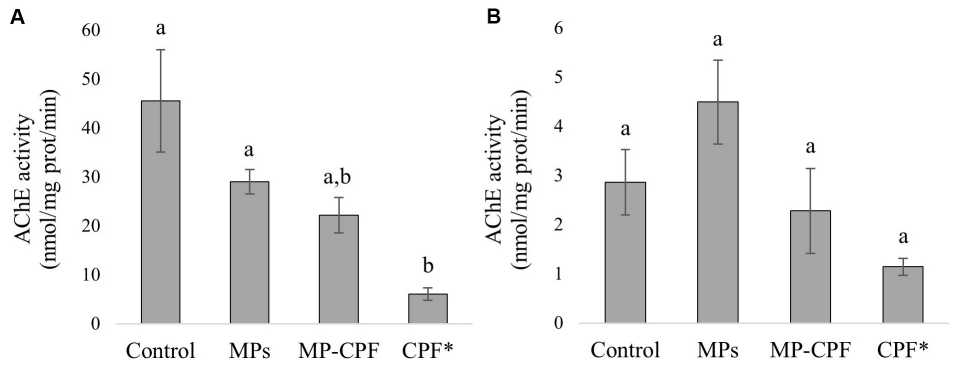

FIGURE 4 | AChE activity (nmol/mg protein/min, mean values ± SE) in fish **(A)** brain and **(B)** liver. Different letters (a,b) indicate statistically different groups ($p < 0.01$). *Fish from the CPF group were euthanized and sampled after two weeks of exposure.

exposures. The absence of MPs in most fish samples is explained by the short particle retention time in three-spined stickleback (<48 h; Bour et al., 2020): the last contamination before fish were sampled was performed more than 48 h and total egestion of MPs could be expected.

In the present study, the MP concentration used to expose *Artemia* (∼1 mg/ml) is very high and not environmentally relevant. This concentration is not intended to represent realistic contamination conditions, but was chosen to maximize interactions between MPs and *Artemia.* Similarly, the average numbers of MPs ingested by *Artemia* and fish are too high to be environmentally relevant. A lower vector effect can therefore be expected in the environment. The MPs used for this study, pristine microspheres, were selected as model particles and do not represent the majority of MPs present in aquatic environments. The selection of these model particles can influence the observed effects: particle shape, specific surface area and the presence of other contaminants (i.e., "non-pristine" particles) highly influence sorption and desorption of chemicals (Heinrich et al., 2020), and therefore MP vector effect.

Chlorpyrifos is widely used in agriculture and in urban areas, and this compound and/or its metabolites are present in waters and sediments of streams, rivers, ponds, lakes and estuaries (Ware, 1999). The high concentration used to spike MPs is not representative of environmental concentrations (Müller et al., 2000; Marino and Ronco, 2005; Arain et al., 2018) but was chosen to compensate for potential loss of chemical during the spiking process (Smedes and Booij, 2012). For the same reasons, a high CPF concentration was used to contaminate *Artemia* (CPF group), as they were exposed in CPF solution for only 15 min. Pre-test were performed to ensure that these exposure conditions did not alter *Artemia* survival and swimming behavior (data not shown). Altered swimming ability of *Artemia* could have indeed influenced fish predation and resulted in fewer prey ingested.

Chlorpyrifos Uptake in Fish and Vector Effect of Microplastics

CPF uptake varies markedly between organs (**Table 1**), with bioaccumulation factors ranging from 0.001 (gills) to 0.009 (gonads) for fish from the CPF group. A previous study showed much higher bioaccumulation of CPF in *Aphanius iberus* exposed via contaminated *Artemia*, with a bioaccumulation factor of 0.3 (Varó et al., 2002). These results are not contradictory since CPF accumulation is highly dependent on fish species: other studies have investigated CPF accumulation in fish exposed via water and observed values ranging from 0.004 to 380 ng/mg (Thomas and Mansingh, 2002; Tilak et al., 2004; Rao et al., 2005). The detection of CPF in fish from the MP-CPF group shows that MPs can act as a vector for organic contaminants when ingested via the trophic chain. As ingested CPF quantities were initially different between MP-CPF and CPF groups, CPF transfer was expressed as the percentage of ingested CPF (**Figure 2**). These values show that except in viscera, CPF accumulation in fish was much lower in the MP-CPF group. This result shows that although MPs can act as vector of contamination, contaminant transfer is limited compared to other exposure routes. The same phenomenon has been observed in most studies comparing contaminant uptake (PCBs, BFRs, PFCs, PBDEs, PAHs, and organic contaminants) via MPs and other matrices (Browne et al., 2013; Grigorakis and Drouillard, 2018; Rainieri et al., 2018), although one study showed higher contaminant transfer in fish when exposed via MPs, compared to spiked food (Granby et al., 2018). Our results therefore confirm previous exposure and model studies, which concluded that MP vector effect could be negligible compared to natural pathways. This phenomenon has been explained by lower fugacity gradients between plastics and biota, compared to gradients between biota lipids (Koelmans et al., 2016). In our study, another factor could also contribute to this phenomenon: the digestion of natural prey likely resulted in the total release of CPF accumulated in prey tissue, while MPs were not digested, therefore limiting CPF release.

CPF accumulation varies between organs in both exposure conditions (**Figure 2**). Previous studies showed CPF distribution patterns in fish similar to what was observed here in fish from the CPF group, gonads, brain and viscera being the most exposed organs (Thomas and Mansingh, 2002; Tilak et al., 2004). In fish exposed via MPs, CPF desorbed from the polymer and was mostly detected in the viscera. Batel et al. came to similar conclusions after exposing zebrafish via the trophic route to benzo[a]pyrene

(BaP) sorbed on MPs: partial desorption of BaP from MPs was observed, with most of the BaP being detected in the intestinal tract and some detected in the liver, to a lower extent (Batel et al., 2016). Interestingly, the strongest BaP signal was detected in fish fed with Artemia contaminated via water, in line with a limited vector effect of MPs.

In other organs, initial sorption of CPF on MPs not only decreases CPF uptake, but also changes CPF distribution among organs (**Figure 3**). While gonads seem to be the most exposed organ in the CPF group, the relative CPF concentration in gonads decreases in the MP-CPF group and reaches values below viscera and body muscle. This phenomenon can be explained by two different CPF release scenarios, either fast and total (CPF group) or low and constant (MP-CPF group), based on the combination of three factors: the low fugacity of CPF sorbed on MPs, the fast natural prey digestion versus the absence of digestion of MPs, and the degradation of CPF over time. Fish from CPF group rapidly digested their prey, which resulted in a fast and total release of CPF. This high CPF gradient between intestine and secondary organs resulted in fast and important transfer to secondary organs, especially gonads that are fat tissues and therefore accumulate hydrophobic contaminants more than muscle or gills. On the contrary, the low fugacity gradient of CPF sorbed on MPs resulted in low release. It can therefore be hypothesized that most CPF was degraded before reaching secondary organs. However, since MPs were not digested by fish, CPF release was constant and resulted in constant exposure of intestine, explaining the high concentrations found in this organ, in the MP-CPF group. CPF can be quickly degraded by organisms: in a previous study, total elimination of CPF was observed after only one day in fish exposed via the trophic route (Varó et al., 2002). Overall, our results suggest that when chemical exposure occurs via MPs, the organs most at risk can be different compared to exposure via water or via natural prey, decreasing gonads exposure in the case of CPF and dramatically increasing intestine exposure. However, fugacity gradients and release of sorbed chemicals depend on the properties of the considered chemical(s) (partition coefficient) and polymer(s) (binding capacity), and our findings might not hold true for every chemical-polymer combination. Studies involving different combinations of chemical and MP properties (partition coefficient and polymer type) are therefore needed to better understand MP vector effects.

Effect of Contaminants

Similarly to our results, studies assessing the ecotoxicity of pristine PE MPs on fish exposed via trophic chains also reported no effects (Rochman et al., 2014; Mazurais et al., 2015; Jovanović et al., 2018). Moreover, most studies exposing fish via water showed either no effects (Ferreira et al., 2016; Karami et al., 2017; Batel et al., 2018; Malinich et al., 2018; Rainieri et al., 2018) or mild effects, which include inhibition of AChE activity, impaired energy reserves, decreased swimming and predatory performances, changes in plasma biochemistry and histological changes in gills (Oliveira et al., 2013; Luís et al., 2015; Karami et al., 2016; Choi et al., 2018; Wen et al., 2018). The adverse effects observed with pristine PE MPs in these studies could be due to the presence of monomers or additives (Lambert and Wagner, 2018) sorbed on MPs but that were not reported, either because no chemical analysis was performed or because concentrations were below limits of detection. Different hypotheses could explain the absence of effect following exposure via trophic chain, reported both in the scientific literature and in the present study. First, it could be explained by a faster elimination of MPs, directly related to gut retention time, while MPs could have a longer retention time when directly ingested or could get stuck in the gills when present in water. Another explanation could be that in trophic chain experiments, the additives potentially present on pristine MPs affect the preys but not the fish; the prey would therefore "protect" the predator species against the effects of MPs. One hypothesis is that the prey partially metabolizes the contaminant, thereby limiting the toxicity for predators.

In contrast with fish from the pristine MPs group, strong effects were observed on fish from the CPF group. As most organophosphate pesticides, CFP is a neurotoxic that inhibits AChE activity. Fish metabolize CPF to multiple metabolites, including CPF-oxon, which is the most efficient AChE inhibitor among the activated forms of CPF (Ware, 1999). It has been shown to be acutely toxic to fish, with 96 h LC_{50} ranging from 300 to 650 μg/L (Tilak et al., 2004). The significant mortality observed here shows that CPF trophic transfer was important enough (up to 3.5 ng/mg; **Table 1**) to induce acute toxicity in stickleback. In a previous study, authors reported no acute toxicity in Tilapia despite CPF accumulation values exceeding 100 ng/g (Thomas and Mansingh, 2002), which highlights important differences in species sensitivity to CPF. Unsurprisingly, significant brain AChE inhibition (84% decrease in activity) was observed in fish from the CPF group (**Figure 4**). This strong inhibition is likely to be a cause of the important behavior impairment observed (Peakall et al., 2002). Taken together, the behavior changes observed show a dramatic hypoactivity, in comparison to control fish. Slow feeding is a sign of decreased predatory performance that could result in population changes for fish and their major prey (Weis et al., 2001). Increased immobility and time spent in the open field (the shelter being considered as a safe location) increase fish susceptibility to predation (Lafferty and Morris, 1996; Seppälä et al., 2004). Finally, fish behavior following the introduction of a novel object is indicative of their interaction with their environment. Previous studies observed changes in inspection of a novel object by fish, including stickleback, following exposure to chemicals (Maximino et al., 2010; Jutfelt et al., 2013). Here, the decreased curiosity, highlighted by the delay in observing and approaching the novel object, is likely to be a result of the severe hypoactivity in fish from the CPF group. These results show that CPF can severely impact stickleback behavior, with potential consequences at the population level. The reported behavior results should be interpreted with caution, given the small number of individuals left in the CPF condition.

While strong effects were observed in the CPF group with a general decrease in movement patterns, suggesting hypoactivity, fish exposed to CPF via MPs (MP-CPF) exhibited altered behavior to a lower extent, and in the opposite direction. Indeed, their faster reaction following the introduction of a novel object, combined with decreased time spent in the shelter,

suggests hyperactivity. CPF has previously been shown to induce hyper excitability in exposed fish, increasing their vulnerability to predation (Little, 2002; Tierney et al., 2007; Halappa and David, 2009). It has also been shown that different behavior alterations occur at different thresholds of AChE inhibition (Tierney et al., 2007), resulting from different chemical exposure concentrations. This explains the differences in behavioral responses between CPF and MP-CPF exposed fish, since different levels of AChE inhibition were observed between the two groups (**Figure 4**). These results show that the quantities of CPF transferred from MPs to fish are high enough to induce behavior impairment, potentially resulting in increased exposure to predation and increased energy expenditure, which should be considered when evaluating consequences at the ecosystem level (Galloway et al., 2017).

CONCLUSION

Taken together, our results show that while PE MPs do not seem to cause adverse effects when ingested via prey, they can act as a vector for chemicals. Although lower transfer occurred when CPF was sorbed on MPs, compared to CPF accumulated in prey, it was important enough to induce adverse effects in stickleback. Moreover, CPF organ distribution in fish differed between exposure conditions, due to different CPF release scenario: either fast and total released from prey, or low and constant when released from MPs. Our results suggest that chemical exposure via MPs could alter the organ distribution of chemicals and result in a change in the organs most at risk, with a likely increase of intestine exposure.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Animal husbandry and experiments were conducted in compliance with ethical practices from the Swedish Board of Agriculture (Ethical permit number # 15986-2018).

AUTHOR CONTRIBUTIONS

AB, JS, and BC conceived the study. AB conducted the experiments and wrote the manuscript. JH contributed to the data analysis of fish behavior. All authors approved the manuscript before submission.

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SUPPLEMENTARY MATERIAL

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Microplastics as a Vector for Exposure to Hydrophobic Organic Chemicals in Fish: A Comparison of Two Polymers and Silica Particles Spiked With Three Model Compounds

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The role of microplastics as chemical vectors delivering environmental contaminants into biota has been proposed, but their environmental relevance remains an issue of a debate. In this paper we compared the propensity and relative importance of synthetic polymer microparticles [glassy polystyrene (PS) and rubbery polyethylene (PE)] and silica glass particles (SG) to act as vectors for hydrophobic organic chemicals (HOCs) into fish after ingestion. Particles were spiked with three HOCs [17α-ethinylestradiol, chlorpyrifos and benzo(α)pyrene], which differ in hydrophobicity and induce well-known biomarker responses. Three-spined stickleback were exposed to 8 different diets: control diets (1), diets with non-spiked particles (2–4), diets containing a mixture of particles spiked with 3 model contaminants (5–7) and, finally, diets loaded with only the chemical mixture (8), for 14 days. Chemical sorption onto the particles was quantified and chemical transfer into the fish was investigated via biomarkers (CYP1a, ERα, VTG, and AChE) in fish intestine, liver and brain and quantification of HOCs in fish muscle. Results demonstrated particle-mediated chemical transfer of moderately hydrophobic contaminants into fish. While PS and PE particles mediated higher chemical transfer and tissue accumulation of 17α-ethinylestradiol and chlorpyrifos than SG, the overall chemical transfer was found to be very low. The present work suggested that chemical sorption, desorption and subsequent transfer of chemicals *in vivo* depends on multiple interconnected factors, including physicochemical properties of particles and contaminants, as well as toxicokinetic and toxicodynamic interactions. The biomarker approach was, however, suboptimal for assessing chemical transfer when addressing particle-associated chemical mixtures.

Keywords: microplastics, vector effects, hydrophobic organic chemicals, fish, benzo(α)pyrene, EE2, chlorpyrifos, three-spined stickleback (*Gasterosteus aculeatus*)

INTRODUCTION

Microplastics (MPs) are widely known to sorb and concentrate hydrophobic organic chemicals (HOCs) from the ambient environment (Mato et al., 2001; Teuten et al., 2007; Rochman et al., 2013a; Prokic et al., 2019). Dozens of potentially toxic and persistent chemicals have been found associated with plastic debris, stimulating hypotheses indicating MPs as potential carriers, or vectors for hazardous environmental chemicals into aquatic organisms (Mato et al., 2001; Teuten et al., 2009; Hirai et al., 2011). While some field and laboratory studies demonstrate the capacity of MPs to facilitate chemical transfer into biota, the ecological importance of this phenomenon remains poorly understood and widely debated. Some experimental data suggest that certain polymers have the ability to sorb and accumulate HOCs to a greater extent than sediments and suspended organic particles (Mato et al., 2001; Teuten et al., 2007; Wang and Wang, 2018), however the comparative importance of chemical sorption to synthetic versus natural particulates, and subsequent transfer into organisms, remains relatively unknown.

Modeling studies strongly suggest that MPs play a negligible role for chemical influx into biota and that the magnitude of MP-mediated chemical exposure is low compared to other exposure pathways, such as uptake via food, other particulates or aqueous exposure (Gouin et al., 2011; Koelmans et al., 2016, 2013). The abundance of various mineral or organic particulates in the aquatic environment drastically exceeds the current reported levels of MPs (Ogonowski et al., 2017; Guzzetti et al., 2018; Alimba and Faggio, 2019). However, MPs concentrations in the aquatic environment are expected to increase in the future due to projected increase in plastics emission (Jambeck et al., 2015; Burns and Boxall, 2018; Everaert et al., 2018). The propensity of vector phenomenon remains an issue of discussion, especially while addressing the environmental relevance compared to other naturally occurring particulates (Koelmans et al., 2016; Ziccardi et al., 2016). Thus, it is important to improve our understanding about the role and relative importance of MP-mediated contaminant transfer, compared to naturally occurring particulates.

Also, knowledge regarding the potential for different polymers to first sorb and then transfer contaminants into aquatic animals is limited. Partitioning of hydrophobic environmental chemicals into plastic polymers is governed by both sorbent and sorbate properties (Lee et al., 2014). In polymers, the sorptive capacity of HOCs is highly dependent on the properties of the polymer matrix, such as segmental mobility and degree of cross-linking and crystallinity, porosity, as well as surface area and structure (Ngoc et al., 2010). Plastic polymers can be differentiated into glassy (adsorptive) or rubbery (absorptive) polymers, depending on their glass transition temperature (Hüffer and Hofmann, 2016; Hartmann et al., 2019). Rubbery polymers, such as polyethylene (PE), have amorphous regions where polymer segments are more mobile and flexible, allowing chemical sorption into the plastic (absorption), whereas in glassy polymers, such as polystyrene (PS), polymer chains are more condensed and cross-linked, thus favoring adsorption of HOCs onto their surfaces, or partitioning of chemicals into the nano-sized pores (Teuten et al., 2009). Also, the hydrophobicity of chemicals has been identified as one of the main properties governing chemical sorption onto polymer particles (Lee et al., 2014; Hüffer and Hofmann, 2016; Hartmann et al., 2017; Müller et al., 2018) and is important for not only for chemical sorption, but also for desorption, determining the influx of the chemicals into biota.

Ingestion of MPs has been suggested as an alternative pathway for chemical exposure and uptake of HOCs into aquatic organisms (Rochman et al., 2013b). As ingested particles can reside and, to some extent, accumulate in the gastrointestinal tract (Lu et al., 2016; Skjolding et al., 2017; Ding et al., 2018), the intestinal lumen represents an important interface between ingested particles and associated chemicals and the organism (Hartmann et al., 2017). Artificial intestinal fluids have been shown to augment chemical desorption from MPs (Bakir et al., 2014), suggesting that physiological conditions in the gastrointestinal tract can enhance chemical desorption, leading to subsequent intestinal absorption and distribution into an organism. However, the biological fate and processes governing chemical desorption of contaminants associated with MPs and other solid indigestible microparticles in an organism following ingestion remain largely unknown (Batel et al., 2016), and require further investigations.

The present work explored the propensity and relative importance of two types of synthetic MPs (glassy PS, rubbery PE) and one type of naturally occurring particles (silica glass) to act as vectors for pollutants into aquatic organisms. We quantified the chemical sorption and chemical transfer of three model HOCs into the fish. The chemicals used differed in hydrophobicity: a synthetic estrogen - 17α-ethinylestradiol (EE2; log K_{ow} = 3.87), a chlorinated organophosphorous pesticide - chlorpyrifos (CPS; log K_{ow} = 4.66) and a commonly used model PAH - benzo(α)pyrene (BαP; log K_{ow} = 5.99). The spiked particles were used in a feeding experiment with three-spined stickleback and subsequent chemical transfer of model chemicals was investigated using well-established genetic and enzymatic biomarkers. We analyzed biological responses in different tissues of the fish to address local effects at the site of particle intake (intestine) and systemic chemical distribution (liver, brain). The accumulation of the chemical compounds was investigated in fish muscle.

MATERIALS AND METHODS

Fish

Three-spined stickleback (*Gasterosteus aculeatus*) were chosen as a model species in this study for several reasons. The species are widely spread; they are present in both salt and fresh water systems throughout the Northern Hemisphere. They can be easily caught and kept under laboratory conditions, and due to high tolerance for handling, fish can successfully be used in experimental studies involving sublethal markers (Katsiadaki et al., 2006). Fish were caught in a shallow creek at the west coast of Sweden (Skaftö, Sweden) in April, 2017. The fish were housed in the Department of Biological and Environmental Sciences, University of Gothenburg, in artificial sea water (13 ± 1°C,

30%, pH = 7.9) under a constant photoperiod - light:dark (12:12 h). Fish were fed daily *ad libitum* with red mosquito larvae. Fish husbandry and feeding experiments were conducted in compliance with ethical practices from Swedish Board of Agriculture (Ethical permit number: 220-2013).

Microparticles and Model Chemicals

To assess the relative importance of MPs and naturally occurring particles to sorb and transfer HOCs, we used two types of synthetic polymer particles: glassy polystyrene (PS) and rubbery polyethylene (PE), and silica glass microparticles (SG) as a reference model of natural particles (**Figure 1**, and **Supplementary Table S1**). Commercial PE particles (reported size: 212–250 μm) were purchased from Cospheric (Santa Barbara, CA, United States) and PS particles (reported size: 250 μm) were purchased from Goodfellow Cambridge Ltd. (Huntington, United Kingdom). Silica lime glass particles (reported size: 250 μm) were also obtained from the commercial supplier (Cospheric, Santa Barbara, CA, United States).

The synthetic estrogen 17α-ethinylestradiol (EE2; log K_{ow} = 3.87) acts as a ligand for estrogen receptor (ER), upregulating egg yolk protein vitellogenin (VTG). Chlorpyrifos is a chlorinated organophosphorous pesticide (CPS; log K_{ow} = 4.66) and is known to act as an acetylcholine esterase (AChE) inhibitor (**Supplementary Table S2**). Benzo(α) pyrene (BαP; log K_{ow} = 5.99) is a well-studied polycyclic aromatic hydrocarbon (PAH), and commonly used compound in MPs vector studies (Batel et al., 2016; Donovan et al., 2018; Pittura et al., 2018). BαP is a potent aryl hydrocarbon (AhR) receptor agonist, inducing cytochrome P450 (CYP1a) metabolism. Octanol-water partitioning coefficients for the model compounds were determined with EPI (Estimation Programs Interface) Suite KOWWIN Program (United States Environmental Protection Agency, United States, v. 1.67, 2000–2012) at pH = 7.

Particle Characterization

Particle morphology and surface topography were determined using scanning electron microscopy (SEM) (DSM 982 Gemini, Zeiss, DE) in a backscattered mode. Prior to imaging, particles (PS, PE, and SG) were spatter-coated with a gold layer (5 nm) (Turbomolecular-pumped coating system, Q150T ES, Quorum Technologies Ltd., United Kingdom). To determine particle size distribution and particle numbers (**Figure 1**), a single subsample of each type of commercial particle was analyzed with CAMSIZER (Retch Technology, DE) using *Xarea* model estimations, as described previously (Ašmonaite et al., 2018). Additionally, N_2-BET surface areas and porosity of particles were estimated (3Flex Surface Characterization Analyzer, Micromeritics, United States) (**Figure 1**). Cumulative particle volume estimation was obtained using spherical approximation, applied on Camsizer data in each particle size category (**Supplementary Table S1**).

Chemical Spiking and Chemical Analysis

To ensure detection of chemicals in the tissues after the feeding experiments, the amount of chemicals to be used in the chemical spiking process was back-calculated from the chemical-analytical detection limits, amount of food ingested, body weight, with expected loss of 30% of each chemical (Smedes and Booij, 2012). Microparticles (PS, PE, and SG) were spiked with each model HOC to a nominal concentration of 1.2 mg chemical/g of particles in separate spiking experiments. Prior to spiking, each chemical was dissolved in a solvent: for BαP a mixture of acetonitrile (ACN):methanol (MeOH) (1:1) was used, and for CPS and EE2 solely MeOH was used. The final ratio between particles and solvent was adjusted to 1:1 (w:w) (Smedes and Booij, 2012). The spiking experiments were performed in glass tubes (100 mL) initially containing 6 mL solvent-particle suspension to which Milli-Q water was added to force the partitioning of chemicals into the particles. Water was added to the particle/chemical mixture-suspension daily with an increasing volume: 2; 4; 8; 16, and 20 mL to reach final water content of 90% after a period of 5 days. Tubes were kept on a shaking table throughout the entire procedure at room temperature. After the final addition of water, the particles were dried by filtering the solutions using a cartridge with a metal frit.

To investigate the final amounts of substances attached to the particles, liquid chromatography–tandem mass spectrometry (LC/MS-MS) was used for the EE2 and CPS, and High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) for BαP. A single aliquot of the particles was extracted using MeOH (for EE2 and CPS) or MeOH:ACN (1:1) (for BαP) followed by ultra-sonication for 40 min. The extractions were pooled and internal standards (IS) added: EE2-d_4 (14 μg) for EE2, carbamazepine-carboxamide-^{13}C,^{15}N (100 ng) for CPS before analysis. No IS was used for BαP. For EE2 and CPS, a LC-20 Prominence high performance LC system (Shimadzu, Kyoto, Japan) coupled to an API 4000 Q TRAP triple quadropole MS (Applied Biosystems/MDS Sciex, Toronto, CA) was used. CPS was analyzed using positive electron spray ionization (ESI^+) and EE2 was analyzed using negative electron spray ionization (ESI^-). The LC column was a C_{18}_XBridge (2.5 μm i.d., 3.0 × 50 mm, BEH Technology, USA) with a guard column. The mobile phases used for the CPS and EE2-analysis were: A: 1 mM ammonium fluoride (NH_4F) in H_2O and B: ACN:MeOH (1:1 v/v). The acquisition and processing of data were performed using Analyst software (v. 1.6.3, Applied Biosystems/MDS Sciex instruments, CA).

To compare sorption between different types of particles, the sorption efficiency was estimated for each model HOC (Eq. 1):

$$Sorption\ (\%) = \frac{Mass\ of\ chemical\ on\ particles\ (\mu g)}{Total\ mass\ of\ chemical\ used\ for\ spiking\ (\mu g)} \times 100 \quad (1)$$

HOCs sorption was estimated primarily using mass units (**Figure 2A**). Complementary, mass-based sorption estimations were used to calculate sorption per particle surface area (**Figure 2B**) and particle volume (**Figure 2C**).

Feeding Experiment

In the study, a non-equilibrium experimental design was used, where uncontaminated fish were fed with contaminated diet (Koelmans et al., 2016). Eight different types of experimental

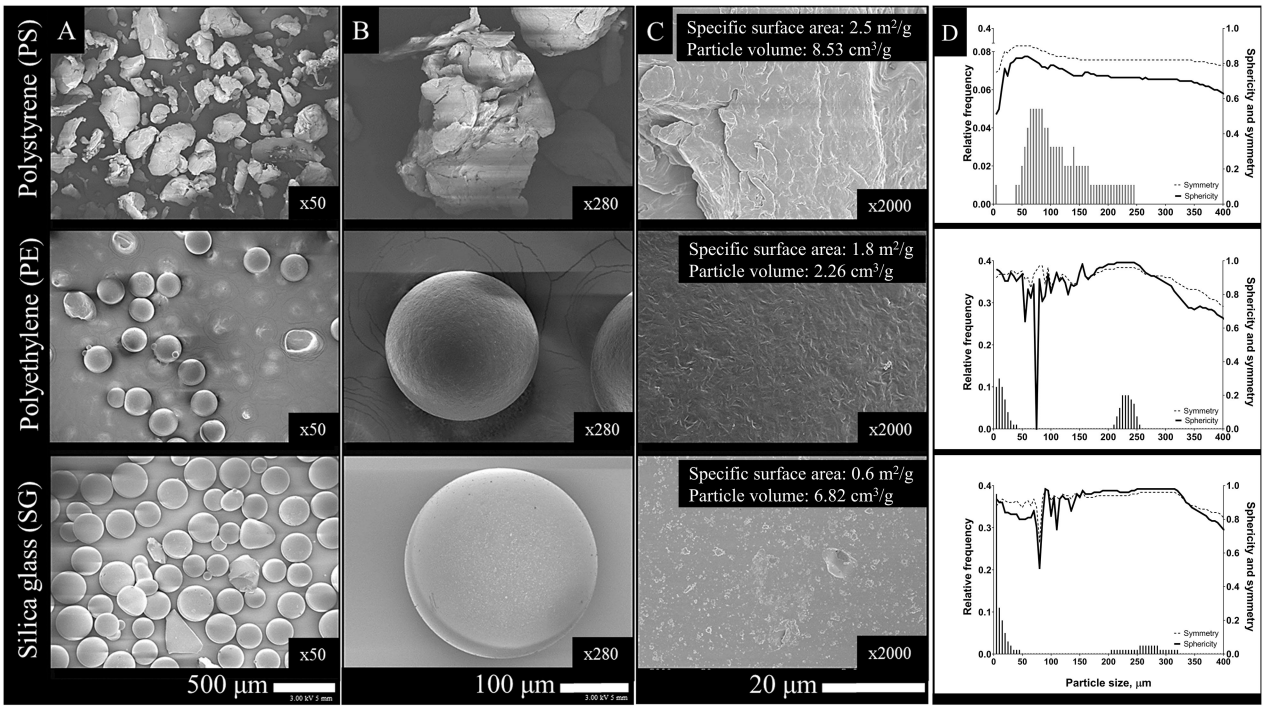


FIGURE 1 | Morphometric characterization of experimental microparticles (PS, PE, and SG): particle morphology and surface topography **(A–C)** and particle size distribution, sphericity and symmetry **(D)**. Specific N_2-BET surface area and particle volume is complementarily provided **(C)**.

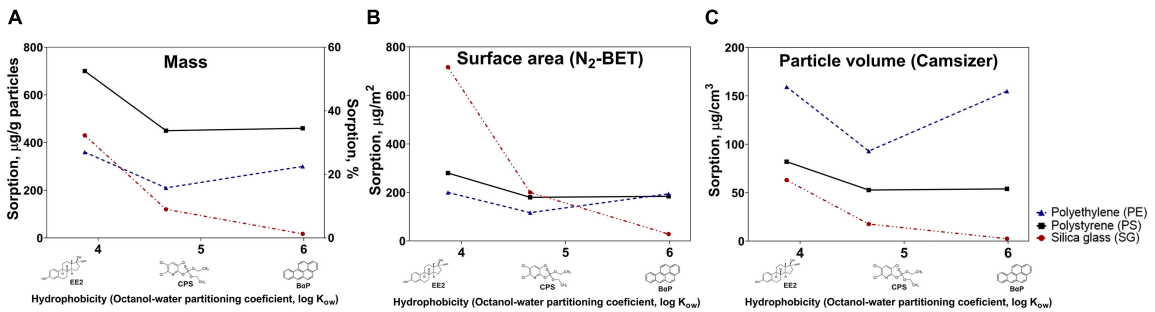


FIGURE 2 | Sorption of model compounds (EE2, CPS, and BαP) on experimental microparticles (PE, PE, and SG) measured per mass of particles (**A**; µg/g and %) and sorption estimates normalized for specific N_2-BET surface area (**B**; µg/m^2) and particle volume (**C**; µg/cm^3).

feeds were prepared for feeding trials: (1) negative control, i.e., fish food without added particles or chemicals; (2–4) feeds each containing one type of virgin particles (5% of daily food intake; PS, PE, SG) without added chemicals; (5–7) feeds each containing one type particle spiked with EE2, CPS, and BαP (subsequently labeled PS_{Mix}, PE_{Mix}, SG_{Mix}); (8) feed loaded with a mixture of the three chemicals but without added particles (Mix). For each exposure treatment, fish ($N = 8 \times 2$; 1.45 ± 0.32 g) were randomly allocated into in two (replicate) glass aquaria (19 L, $35 \times 21 \times 26$). Experiments were conducted in well-aerated artificial seawater (35 PSU) under semi-static conditions with manual water exchange every second day. During the experiment, fish were fed daily (6% of their body weight) for a period of 14 days. The fish consumed all food rapidly (in less than 1 minute), and no particle dissolution into the water was observed prior to ingestion. Maximum daily exposure doses to model HOCs (associated with spiked particles) were estimated (Eq. 2):

$$Maximum\ daily\ exposure\ dose = ng\ chemical/g \times daily\ plastic\ intake\ (g) \quad (2)$$

Preparation of Fish Feed

Red mosquito larvae were used as basis for experimental feeds (crude protein 3%, crude fat 3%, ash 0.8%, fiber 0.9%) (Imazo AB, Vara, SE). For feed preparation, thawed red mosquito larvae (40 mL) were homogenized in batches using a high performance

dispersing instrument Ultra-Turrax® (Labasco, Janke and Kunkel KG, IKA Werk, DE) (50 Hz, u/min 20 000, 30 s), then centrifuged at 700 rpm for 1 min and re-homogenized for 30 s. To eliminate non-grinded material, the homogenate was sieved with a fine-threaded net (Ø 1 mm). Gelatin sheets were soaked in cold Milli-Q water for 10 min, the excess water was drained, and the gelatinous solute was microwaved (20 s) and allowed to cool. To prepare the experimental diets, particles spiked with each of the three model compounds were mixed in equal parts by weight, and added into food preparation. Particles were added to the liquid gelatin and were thoroughly mixed to obtain a homogenous particle-gelatin mixture, prior adding it to red mosquito larvae homogenate. For preparation of the chemical mixture feed, model chemicals (EE2, CPS, and BαP) were dissolved in solvents: MeOH (EE2 and CPS) and DMSO (BαP). Solvents were allowed to evaporate prior mixing with melted gelatinous solute, which then was added into red mosquito larvae mixture. Estimated daily intake of each model compound was 0.5 μg/fish/day. Thereafter, the prepared experimental feed mixtures were loaded into sterile syringes (10 mL; Plastipak, Becton Dickinson, ESP), which were refrigerated at 4°C until the mass reached jelly consistency. Feeds were individually shaped and portioned into worm-shaped pellets (Ø 2 mm), frozen on dry ice in foil packages in individual portions, and stored at −20°C until use. With this food preparation methodology we mimic dietary exposure via contaminated prey. Each portion constituted the daily food intake in a single exposure aquarium (N = 8 fish; 6% fish body weight). Selected exposure levels of PS and PE MPs, as well as concentrations of model HOCs, were not intended to represent exposure scenarios that fish are likely to encounter in the natural environment. Chemical analysis was additionally performed to confirm the presence of model HOCs in experimental fish feed.

Sampling

After the exposure, fish from each treatment were sacrificed, weighed and measured. No physical changes were observed on fish during the feeding experiment. The intestinal tracts, livers and brains were immediately dissected and preserved frozen in liquid nitrogen until use in biomarker analysis (N = 6). Ingested particles were observed in the dissected gut at the final sampling time (also confirming the ingestion of particles during the experiment). The remaining carcasses was stored in – 80°C until chemical analysis.

Biomarker Approach

Gene Expression Using Quantitative Real-Time PCR (qPCR)

Gene expression of CYP1a, ERα, VTG, and AChE were analyzed in liver and intestines using quantitative real-time PCR (qPCR) (**Supplementary Table S3**). RNA extraction was performed using the RNeasy® Plus Mini kit (Qiagen, Hilden, DE) according to the manufacturer's instructions. Liver and gut samples (10 mg) were taken directly from liquid nitrogen and added to RLT buffer plus β-mercaptoethanol. Samples were homogenized at 25 HZ for 2 × 3 min using the TissueLyser II (Qiagen, Hilden, DE). Quality and quantity of extracted RNA was measured with a Nanodrop 2000 Spectrophotometer (Thermo scientific, United States) and automatic electrophoresis RNA ScreenTape System 2200 (Agilent Technologies, Inc., Waldbronn, DE). Synthesis of cDNA was performed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.), using 1000 ng of mRNA. The following thermal protocol for the synthesis was used: (1) 5 min at 25°C, (2) 30 min at 42°C, and (3) 5 min at 85°C. Each qPCR reaction was performed with a final reaction volume of 5 ng/μL containing 20 ng cDNA. The final primer concentrations for CYP1a and ERα were 500 nM, 700 nM for VTG and 300 nM for AChE. The genes used as references for normalization was RPL13 and HPRT (intestinal tissue), and HPRT and β-tubulin (hepatic tissue).

Enzymatic Assays

Ethoxyresorufin-O-deethylase (EROD) fluorometric assay was used to measure the enzymatic activity of cytochrome P450 1A (CYP1a) in the S9 of the liver tissue, according to the established procedure (Förlin et al., 1984). Acetylcholine esterase (AChE) activity was measured in fish brain using an established methodology (Ellman et al., 1961). Enzymatic measurements were normalized with protein content, assessed with the Lowry method, using bovine serum albumin (BSA) as the protein standard (Lowry et al., 1951).

Quantification of Model Compounds in Fish Muscle

To quantify the chemicals in the fish muscle, samples from individual fish (N = 6; 1.06 ± 0.28 g) were homogenized in polypropylene tubes followed by the addition of an internal standard mixture (ethinylestradiol-d_4, carbamazepine-(carboxamide-^{13}C,^{15}N) and benzo[a]pyrene-d_{12}, 100 ng of respective compound). Chemicals were extracted twice with 6 mL of ACN, followed by spinning for 1 min, ultra-sonication for 20 min and centrifugation at 2500 × g for 5 min. Thereafter, the supernatants were transferred to a new tube. A third extraction was performed by the addition of 6 mL methyl tert-butyl ether (MTBE), spinning for 1 min, ultra-sonication for 20 min and centrifugation at 2500 × g for 5 min. The supernatants were combined and concentrated with N_2 and gentle heating to a volume of 6 mL. To remove the lipids, a dispersive solid phase extraction kit from Agilent (QuEChERS Enhanced Matrix Removal – Lipid) was used and each tube was spun for 1 min and then centrifuged at 2500 × g for 5 min. The supernatant was collected and concentrated with N_2 and gentle heat to a final volume of 1 mL ACN. The extracts were left overnight at -20°C and filtrated (with polypropylene micro-filters) before analysis. To quantify transfer of the model compounds CPS and EE2 into fish muscle, LC/MS-MS in multiple ion monitoring (MRM) mode was used following the methodology described in section "Chemical Spiking and Chemical Analysis." For BαP quantitative analysis was carried out using a GC/MS-MS Agilent 7000 (Agilent Technologies, Santa Clara, California, United States) in Electron Impact mode. The GC/MS column used was a DB5-MS 30 m + 10 m guard column. The GC/MS was operated with electron energy of 70 eV and the ion source was set to 230°C. Both quadrupoles were set to 150°C and a pulsed splitless

injection was used. The acquisition and processing of data were performed using MassHunter (version B.08.00, 2016). BαP levels were below detection limit, thus were not considered further in the analysis. To estimate transfer of model HOCs (EE_2 and CPS) from particles (PS_{mix}, PE_{mix}, SG_{mix}) into the fish, the following equation was used (Eq. 3):

$$Chemical\ transfer\ (\%) = \frac{chemical\ in\ tissue\ (ng/g\ \ fish)}{maximum\ daily\ exposure\ dose\ (ng/g\ fish) \times\ duration\ (days)} \times 100$$

Due to the small size of this fish species (and limited organ weight), we performed chemical analyses in fish muscle, and used other target organs in biomarker analyses.

Statistical Data Analysis

Prior to statistical analysis, data were tested for the normality (Shapiro-Wilk test) and for the homogeneity of variance (Levene's test). Data that did not comply with these assumptions was log-transformed. Data from replicate tanks were pooled for subsequent analyses, as no tank-specific effects were observed (*T*-tests). One way analysis of variance (ANOVA) was used to compare biomarker responses across exposure groups and Dunnett's *post hoc* test was used to assess differences between the control and exposure treatments, and Bonferroni *post hoc* test was used to obtain multi-comparisons between all the groups. A factorial analysis using a two-way ANOVA was used to assess particle-specific effects, and test whether particles (PS, PE, and SG) induced different effects than their chemically spiked counterparts (PS_{mix}, PE_{mix}, and SG_{mix}) (**Supplementary Table S4**). Also, a principal component analysis (PCA) was performed to visualize particle-chemical interactions (**Supplementary Figure S1**). For analyses, SPSS Statistics (v.25, IBM Corporation, United States, 2017) and open-access software R (v. 3.3.2) and R studio (v. 1.1463©, 2009–2018 RStudio, Inc.).

RESULTS

Sorption of HOCs Onto Microparticles

The sorption efficacy of each model HOC on three types of microparticles (PS, PE, and SG) was primarily estimated using mass-based units (**Figure 2A**), which are commonly used in MP spiking studies (Hüffer and Hofmann, 2016; Batel et al., 2018). Additionally, surface area and particle volume estimations were performed (**Figures 2B,C**). Mass-based results showed that synthetic plastic polymer particles (PS and PE) absorbed more of CPS and BαP compared to inorganic SG (**Figure 2A**). In regards to chemical sorption of HOCs on different types of synthetic polymers, we found that the glassy PS particles sorbed more chemicals than rubbery PE particles per mass unit (**Figure 2A**). The similar pattern was observed when sorption was normalized to particle surface area (**Figure 2B**), but not to particle volume (**Figure 2C**). No clear relationship was observed between the chemical sorption and compound's hydrophobicity (**Figures 2A–C**). On contrary to what was expected, the sorption of EE2, which had the lowest degree of hydrophobicity, in many instances, exceeded that of CPS and BαP.

Chemical Transfer of HOCs Into Fish

Biomarker Responses

Changes in the expression of target genes in fish intestine (mRNA of CYP1a, ER, VTG and AChE) were observed (**Figure 3A** and **Supplementary Figure S1**), suggesting the chemical release of model HOCs from test particles and subsequent activation of intestinal metabolism. The upregulation of CYP1a expression in the intestinal tissue ($p < 0.05$) suggested release of aryl hydrocarbon receptor (AhR) agonist, i.e., BαP, from all spiked particles. The observed increase of ER and VTG mRNA levels ($p < 0.05$ and $p > 0.05$, respectively) suggested desorption and uptake of EE2 into intestinal lumen and tissue, whereas marginally elevated AChE mRNA levels ($p > 0.05$) indicated potential dissociation of CPS.

Similarly, changes in hepatic gene biomarker responses indicated uptake of EE2 and CPS in fish liver (**Figures 3B,D**). An increase in gene expression of ER and VTG ($p > 0.05$ and $p < 0.05$, respectively) were observed in fish from PS_{mix}, PE_{mix}, and SG_{mix} treatments. A trend towards upregulation of AChE was observed, but these changes were not statistically significant ($p > 0.05$). Cytochrome P450 metabolism was not induced in the liver, as indicated in the down-regulation of CYP1a mRNA ($p < 0.05$ for PS_{mix}, SG_{mix}, and $p > 0.05$ for PE_{mix}) and suppression of EROD activity was observed for all treatments containing BαP ($p < 0.05$) (**Figures 3B,D**).

Regarding chemical transfer in brain, AChE inhibition has been indicated, suggesting transfer of the CPS - AChE inhibitor, into this region (**Figure 3C**, $p < 0.05$ for PS_{mix}, PE_{mix}, $p > 0.05$ for SG_{mix}).

Chemical Quantification in Fish Muscle

Quantification of chemical burden in fish muscle revealed that two of model compounds (EE2 and CPS; log K_{ow} = 3.87 and 4.66, respectively) were taken up and further systematically distributed in an organism (**Table 1**), confirming the accumulation of model HOCs beyond the gastrointestinal tract. It was not possible to detect BαP in the muscle in all treatments containing this chemical. Overall, results indicated that microparticle-mediated transfer of chemicals with low to moderate hydrophobicity (EE2 and CPS) is quite low (**Table 1**). When the accumulation potential for HOCs was compared across different particle types, it can be seen that synthetic MPs (PS and PE) mediated greater chemical transfer into the fish than SG.

DISCUSSION

To address the ongoing discussions concerning the propensity and relevance of MPs to act as vectors for environmental contaminants, we examined the sorption of three common hydrophobic environmental pollutants (EE2, CPS, BαP) to two types of synthetic MPs and one type of naturally occurring

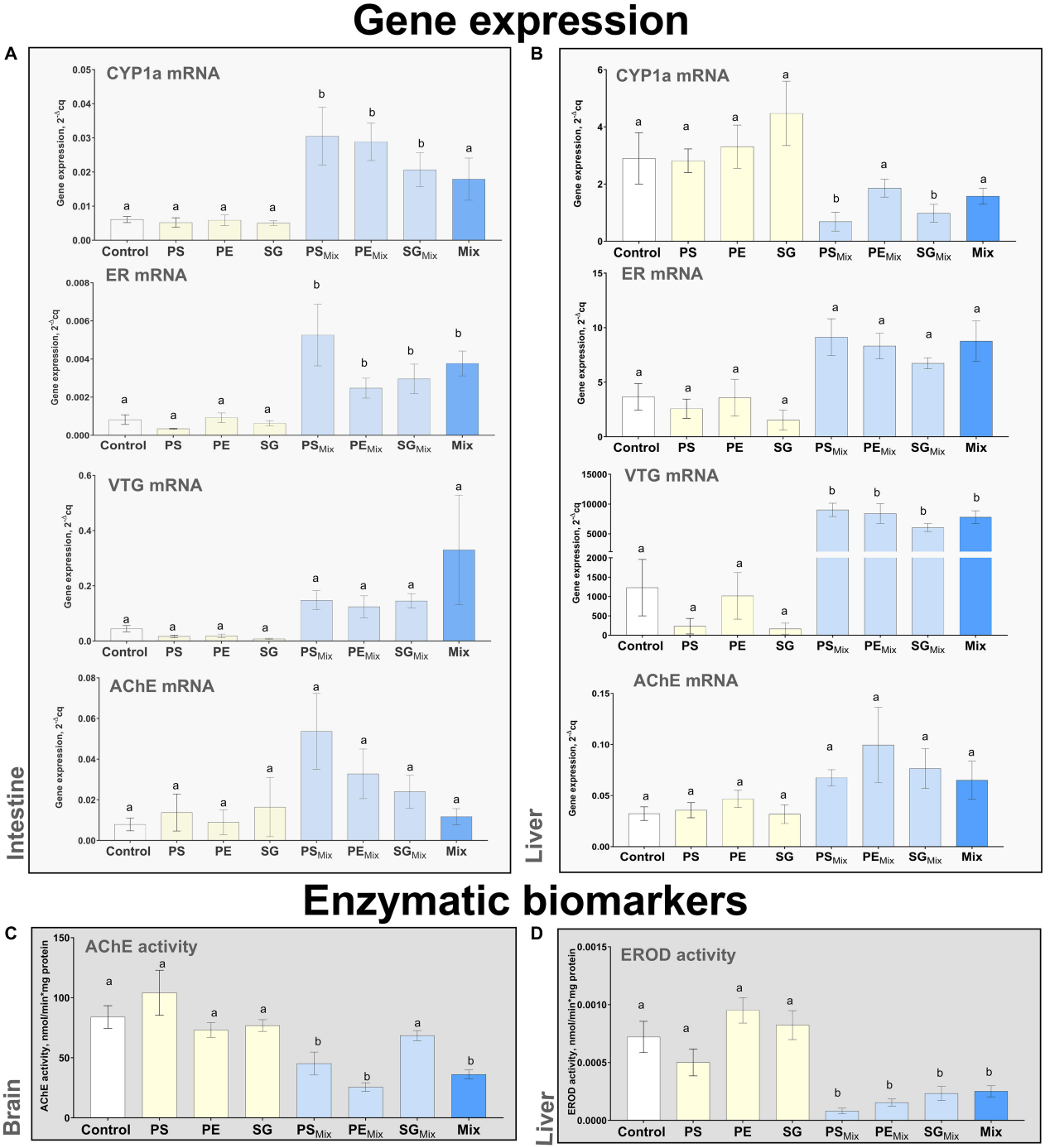


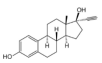
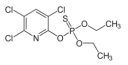
FIGURE 3 | Biomarker responses: gene expression **(A,B)** and enzymatic activities **(C,D)** in different organs: intestine **(A)**, liver **(B,D)** and brain **(C)**. Data expressed as means ± SE, $N = 6$. Different letters indicate statistical differences between the control and exposure treatments (one-way ANOVA, *post hoc* Dunnett test, $p < 0.05$).

particles, and followed their subsequent transfer into the fish upon ingestion.

Sorption of HOCs Onto Microparticles

In the first part of the study, we performed artificial chemical spiking with selected HOCs and investigated the capacity of test chemicals to sorb onto test microparticles (PS, PE, and SG). The findings suggested that synthetic particles (PS, PE) sorb more of two of the model compounds: CPS and BαP than SG (**Figure 2A**). Herein, we used silica glass particles as a proxy for naturally occurring particles, a comparison which is crucial but often overlooked in experimental MP studies

TABLE 1 | Accumulation of model HOCs compounds in fish muscle, expressed by average amount chemical (ng) per g fish in the end of the experiment ± SE.

Exposure treatment	 EE2 (log K_{ow} = 3.87)	 CPS (log K_{ow} = 4.66)	 BαP (log K_{ow} = 5.99)
Polystyrene (PS_{mix})	20.23 ± 5.36 **(0.206 ± 0.055%)**	11.89 ± 2.20 **(0.189 ± 0.035%)**	<LOD
Polyethylene (PE_{mix})	7.13 ± 1.45 **(0.142 ± 0.029%)**	10.86 ± 1.55 **(0.370 ± 0.053%)**	<LOD
Silica glass (SG_{mix})	3.48 ± 0.24 **(0.058 ± 0.004%)**	1.11 ± 0.19 **(0.067 ± 0.011%)**	<LOD
Chemical mixture (Mix)	3.61 ± 0.51 **(0.094 ± 0.014%)**	4.58 ± 1.18 **(0.557 ± 0.144%)**	<LOD

Chemical transfer (%) estimates are presented in the brackets. Data provided as means ± SE, N = 6; LOD, limit of detection.

(Ogonowski et al., 2017). When comparing chemical sorption of HOCs on natural occurring inorganic particulates, it has been observed that sorption onto MPs exceeds sorption to natural sediments (Teuten et al., 2007). Our findings agree with such observation (with an exception with EE2, **Figures 2A,B**). While naturally occurring organic particulates have been demonstrated as having higher affinity to HOCs than synthetic particles (Ghosh et al., 2000; Beckingham and Ghosh, 2017), the sorption to natural inorganic (non-porous) solids is generally smaller, and likely limited to chemical adsorption onto the surface. While our results suggested that PS and PE MPs were prone to higher sorption of two test HOCs (CPS and BαP) than SG particles, we can argue that sorption of HOC on microparticles depends on complex interplay between the properties of sorbate and sorbent, as well as on general laboratory spiking conditions (e.g., duration, availability of sorbate, solvent choice, etc.). In this study it was difficult to elucidate a clear relationship between chemical sorption and hydrophobicity of HOCs. It is generally accepted that sorption affinity to sorbent increases with increasing hydrophobicity of the compound (Müller et al., 2001; Smedes et al., 2009). The highest mass-based sorption onto all types of particles was observed for EE2, which had the lowest log K_{ow} among the selected model compounds. Chemicals with lower hydrophobicity are known to reach chemical equilibrium faster than more hydrophobic compounds (Endo et al., 2013), thus the observed trend may indirectly indicate faster sorption kinetics onto different particles during the artificial spiking procedure. Also, the lower sorption of more hydrophobic substance onto particles could be explained by the loss of compound during the spiking procedure (e.g., due to attachment to the vessel). On the other hand, EE2 have hydroxyl functional group that can interact with the surface of the substrate, thereby increasing or decreasing the sorption (Magner et al., 2009).

In regards to chemical sorption of HOCs on synthetic polymer particles, glassy PS revealed higher chemical association than rubbery PE (**Figure 2A**), which somewhat contradicted with aforementioned glassy-rubbery state theory. Glassy polymers possess dual sorption mechanisms: partitioning or dissolution of HOCs in the amorphous polymer regions and adsorption (hole-filling) into internal polymer nanovoids (Teuten et al., 2009). Although PS is considered as a glassy polymer, higher sorption capacity than predicted by its glass transition temperature has been previously reported (Alimi et al., 2018). The presence of the benzene ring in the polymeric backbone of PS monomer was suggested to distend the distance between adjacent polymer chains, facilitating diffusion into the polymer (Pascall et al., 2005; Velzeboer et al., 2014). While adsorption is the dominant sorption mechanism for PS MPs, the larger surface area of the PS MPs (**Figure 1C**) could have facilitated a faster interaction and greater sorption of HOCs compared to the PE MPs. Particle characterization revealed high numbers of the smallest size particles (<200 μm) in PS MPs (**Figure 1D**). Such fine particulate fractions are known as considerably more effective sorbents due to higher abundance of binding sites on larger surface area (Ngoc et al., 2010). On the other hand, when the sorption was normalized for particle volume, a property facilitating absorption, it could be seen that PE sorbed more HOCs compared to PS (**Figure 2C**). Structurally, glassy amorphous polymers (PS) are dense with little void spacing, whereas rubbery polymers (PE) can encapsulate larger volumes between the molecules allowing greater diffusion of contaminants into the polymer (Bakir et al., 2012; Hartmann et al., 2017; Alimi et al., 2018). Having larger (free) volume available for chemical absorption, PE may be viewed as a more prominent sorbent for HOCs than PS. However, it is important to note that for smaller polymer particles having large surface-to-volume ratio, adsorption of HOCs onto surface may be more important mechanism than absorption into the polymer. It is also important to consider that the methodology used here, with a relatively short sorption time, might favor surface adsorption (as opposed diffusion into the polymer phase), in a potentially non-equilibrium situation (Koelmans et al., 2016; Mohamed Nor and Koelmans, 2019). Importantly, in this experiment particles were neither weathered nor exposed to biofilms, which are, of course, an important factors that can affect the binding affinity to chemicals under environmentally realistic conditions (Velez et al., 2018). Also, herein, artificial spiking was used to load particles for feeding experiment, and did not intend to describe sorption kinetics, which notably could be very valuable for better understanding HOCs and microparticle interactions. While there are no established guidelines for MPs spiking experiments in vector studies, the characterization and presentation of test materials remain of high importance for results' interpretation. It is important to consider not only the

mass, but also particle morphology (size, shape, surface area), and/or other properties that relate to sorptive capacity of the materials (crystallinity, surface-to-volume ratio, surface polarity).

Chemical Transfer Into Fish

The second part of the study addressed the chemical transfer from spiked microparticles into the fish following ingestion. By measuring the changes in metabolic pathways, specific to the model compounds, we could infer the release of particle-associated HOCs into fish intestine and subsequent chemical distribution to peripheral organs. It is known that intestinal surfactants can enhance desorption of hydrophobic compounds from MPs (Bakir et al., 2014), liberating and making them bioavailable for metabolism, and/or for intestinal absorption. While we could document uptake and transfer of EE2 and CPS, the transfer of BαP into fish was not detected. This could be explained that BαP released from particles may not have been transferred across intestine and was largely contained (and/or metabolized) in the intestine or/and rapid metabolism of the compound took place in the hepatic tissue (**Table 1** and **Figure 3**). While number of studies suggest rapid transformation of BαP in liver (thus limited transfer of the mother substance in to the muscle) (Lemaire et al., 1990; Van Veld et al., 1997; Oris et al., 2004), some studies indicate metabolism and storage of BαP metabolites in intestinal epithelia. For instance, it has been shown that BαP administrated via dietary exposures associate with lipids, and its hydrolysis products can be found absorbed by enterocytes, and can be stored in the lipid droplets (Vetter et al., 1985). Due to extensive biotransformation, or storage, occurring in the gut, dietary exposure to BαP can increase CYP1a in intestinal mucosal epithelia and vasculature (Van Veld et al., 1997). The levels of active compounds, such as BαP can subsequently be reduced, thus limiting its translocation to other tissues. Some studies suggest that the efficiency of gastrointestinal absorption of hydrophobic chemicals (log K_{ow} > 5.5) is thought to decrease (Gouin et al., 2011). The uptake of lipophilic compounds (log K_{ow} > 6.3) following dietary intake is considerably less efficient and is limited due to slow diffusion in unstirred water layer adjacent to brush border membrane of enterocytes (Kelly et al., 2004). It has been predicted that the bioaccumulation of HOCs, associated with MPs, having log K_{ow} higher than 5 could lead to the reduction of body burden concentrations, also known as the "cleaning" effect (Gouin et al., 2011). The desorption of HOCs, chemical uptake and potential organismal distribution can be influenced by a number of factors, such as chemical loading, hydrophobicity of ab/ad-sorbed chemicals, intrinsic particle characteristics (surface area, porosity, etc.), desorption kinetics and physiological conditions and metabolism in the intestine (Teuten et al., 2009; Bakir et al., 2014; Wardrop et al., 2016; Sleight et al., 2017). In addition to this, the importance of gut retention time is becoming increasingly recognized in studies investigating MP-mediated chemical transfer (Mohamed Nor and Koelmans, 2019). In stickleback, due to short retention time (<48 h) of MPs in the gastrointestinal tract (Bour et al., 2020), the desorption of HOCs from ingested plastics could be rate-limited (Mohamed Nor and Koelmans, 2019), limiting the uptake of particle-bound chemicals into the fish. The metabolization of a compound could also play an important role in mediating not only the influx of HOCs from ingested plastic into organism, but also its bioaccumulation potential (Diepens and Koelmans, 2018). For example, metabolization of PAHs is thought to diminish fugacity in the lipids, leading to attenuated transfer into an organism (Diepens and Koelmans, 2018). The metabolic capacity of various HOCs in fish intestine is not well known, and generally is excluded from consideration in MP-vector studies, but can be crucial for chemical release kinetics and organismal distribution. While the results, regarding BαP transfer, remain inconclusive, EE2 and CPS-based results suggest that gastrointestinal tract is an important physiological compartment, where the chemical desorption and metabolism of particle-bound chemicals take place, and that microparticles act as carriers for certain HOCs into intestine and further into an organism.

Application of Biomarker Approach in Vector Studies

It is known that estrogenic compounds can interfere with AhR signaling and can suppress PAH-mediated CYP1a mRNA transcription and EROD activity (Förlin et al., 1984; Arukwe et al., 1997; Navas and Segner, 2001). As our results suggested, EE2 was taken up and reached hepatic tissue (**Figure 3B** and **Table 1**), however, an inhibitory effect on AhR signaling was suspected, as transcript levels of CYP1a enzyme and CYP1a catalytic activity in liver were significantly suppressed (**Figures 3B,D**). The above-mentioned example pointed towards the potential interference between model HOCs in biomarker responses. Effect-based biomarker approaches provide estimations of biologically active, or bioavailable fractions of the compound, but may be hindered by the specificity (or lack thereof) of the selected biological response. With that, we argue that solely effect-based approaches for vector studies may be insufficient to understand the causality and interactions between compounds, as chemicals can interact with different receptors, and metabolism can occur via the same pathways. While the biomarker approach was a valuable tool to determine bioavailability of desorbed model HOCs on enzyme and gene expression level, however, it also revealed its potential limitations in the context of mixture toxicology. BαP is commonly used in MPs vector studies (Batel et al., 2016; Donovan et al., 2018; Pittura et al., 2018; González-soto et al., 2019; Wang et al., 2019); however, herein it revealed some experimental implications for making conclusions regarding particle-mediated chemical transfer of this chemical into the fish. Therefore, it is important to address that toxicokinetic interactions between contaminants can occur, and potentially could impede the assessment not only of chemical transfer, but also of biological effects. As chemical sorption, desorption and subsequent biological uptake and distribution of chemicals *in vivo* depends on multiple interconnected factors including physiocochemical properties of particles and contaminants, and toxicokinetic and toxicodynamics, and these variables need to be carefully considered while designing vector studies with animals.

Comparative Importance of Chemical Transfer Mediated via Natural and Synthetic Particles

As results suggested, chemical transfer of moderately hydrophobic metabolizable chemicals of both glassy PS and rubbery PE MPs, as well as natural SG microparticles is likely, however, was low. SG are non-polymer mineral particles that generally very abundant, yet have low levels of sorption, thus it is not surprising that low-level chemical transfer was observed for these particles. However, given their wide occurrence, inorganic particles may also serve as an exposure pathway of these chemicals into biota, and this could be relevant for species foraging in benthic habitats. The relative importance of microparticle-mediated chemical exposure remains the subject of continued discussions and calls for more experimental studies to better understand particle-chemical interactions, especially in comparison to other solid particulates present in the environment. Whether MPs are of considerable importance for transfer of HOCs compared to other sources, e.g., prey, organic matter other colloidal particulates, or uptake directly from the water through the gills is still a matter of a debate (Bakir et al., 2012; Koelmans et al., 2016, 2015). We suggest that the low level chemical transfer demonstrated in this study, and low MPs encounter rates in the natural environment to date, should render MPs an insignificant exposure pathway for HOCs into fish.

CONCLUSION

This study explored the capacity of MPs and silica microparticles to sorb and transfer chemicals into fish and provided experimental insights and considerations for vector studies using fish. Results suggested that microparticle-mediated chemical transfer of moderately hydrophobic contaminants into fish is likely. The synthetic particles demonstrated higher chemical sorption (of CPS and BαP) than silica particles. PS and PE MPs mediated higher chemical transfer and accumulation of model HOCs (EE2 and CPS) into fish muscle compared to silica particles, but the overall dietary transfer was low. Considering the sheer abundance of these inorganic particles in the natural environment, compared to the relatively low levels of MPs, could still indicate that the amount of chemicals sorbed to inorganic particles would greatly exceed the amount of chemicals bound to MPs (Koelmans et al., 2016), reducing the relative importance of MPs as vectors under natural environmental conditions. We also concluded that while the biomarker approach was valuable tool to determine biological availability of contaminants in fish, however, it was determined to be suboptimal for assessing biological effects and chemical transfer when addressing chemical mixtures associated with microparticles.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This animal study was reviewed and approved by the Swedish Board of Agriculture (Ethical permit number: 220–2013).

AUTHOR CONTRIBUTIONS

BC, TB, JM, and EW designed the study. EW performed the artificial particle spiking, as well as chemical analysis (particles and fish tissue samples). MT and GA prepared the experimental diets. MT and BC carried out the feeding experiment. MT performed the biomarker measurements. GA performed the particle characterization, assisted in biomarker analysis, analyzed the data, and took the lead in writing the manuscript. All authors provided critical feedback and helped to shape the research, analysis, and 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/fenvs.2020.00087/full#supplementary-material

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Evaluation of Electrostatic Separation of Microplastics From Mineral-Rich Environmental Samples

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Reliable, easy, cost-effective and reproducible ways of extracting microplastics (MP) from environmental samples remain important requirements for MP research. In this context, electrostatic separation is a new proposition, especially for extracting MP from mineral-rich samples and large sample volumes. However, there is little research evaluating the reliability of the technique. This study has evaluated the effectiveness of the Korona-Walzen-Scheider (KWS) system; a small-scale version of larger machines designed to sort recycling materials. Recovery rates of a variety of sizes of MP, spiked in beach sediments, were found to be highly dependent on the MP size. MP $\geq$ 2 mm achieved 99 – 100% recovery (with the exception of fibers: ∼80%), MP of 63–450 μm achieved ∼60–95% recovery and MP of 20 μm achieved ∼45% recovery. For particle-based analysis, additional density separation is still inevitable for the analysis of small MP after KWS separation and further reduces the overall recovery rates. Mass reduction rates of beach and commercial reference sand greatly differed, 93 and 17%, respectively. Mineral analysis using SEM-EDX suggested that lower reduction rates found in commercial sand was due to high presence of small (<50 μm) calcite particles. Tests based on environmental soil samples revealed comparatively low mass reduction rates (∼1%), suggesting that KWS treatment was inefficient for soils due to high levels of fine particulates. Sieving to remove fine particles improved mass reduction, though only to ∼15%. To specifically test for influence of fine particulates, recovery rates were determined for sand samples spiked with a defined amount of silicate dust, resulting in a reduction of certain MP recovery rates, especially medium-sized (450 μm) MP. Conclusively, several key influential parameters were identified, such as mineral composition and grain size, that can negatively effect sediment mass reduction as well as MP recovery rates. Given the variability in recovery rates, the use of internal standards is recommended when using the KWS, particularly for smaller MP (<500 μm). For large-volume (beach) sand samples, where interest is mainly in MP > 450 μm, electrostatic separation is a reliable and fast approach for MP extraction from the environment.

Keywords: electroseparation, method test, protocol, anthropogenic litter, plastic, monitoring, reference material

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INTRODUCTION

Current limitations in the field of microplastics (MP) extraction from complex matrices foster the development of, and search for, new approaches. Electrostatic separation is one of the techniques that has recently been proposed by Felsing et al. (2018) for MP purification of different sample matrices, ranging from beach sand to more complex matrices like freshwater suspended particulate

matter and freshwater sediments. In their study, a set of six different commodity polymers were tested at four different size ranges (2–5 mm, 0.63–2 mm, 200–630 μm and 63–200 μm) using a Korona-Walzen-Scheider (KWS-XS, Hamos GmbH) and yielded recovery rates of 90–100%.

The intended effect of electrostatic separation is to selectively separate non-MP particulates from MP particles, thereby reducing the non-MP particulate portion. This reduces the amount of material submitted to the subsequent steps and hence allows for preparation of larger initial sample volumes. The subsequent treatment of the electrostatically reduced sample is completed with already-established methods (e.g., density separation and digestion).

Density separation alone is the most widely used reduction method of the inorganic fraction. By using a variety of different density salt solutions (e.g., $ZnCl_2$, NaI, sodium polytungstate) it can reduce bulk particulate material in many samples (Hidalgo-Ruz et al., 2012). However, there is a general lack of efficient separation procedures targeting large sample volumes of several kilos which is often needed when dealing with relatively low MP numbers in order to increase statistical robustness. The Munich Plastic Sediment Separator has been designed to density separate larger volumes, though, it appears to be a rather labor-intensive method (Imhof et al., 2012). Electrostatic separation is not as exhaustive as density separation in eliminating the entire mineral fraction (99.98 ± 0.03%, Enders et al., 2020), although approximately 98% mass could be reduced in previous tests in just 5 h (Felsing et al., 2018). However, due to deviating objectives a direct comparison to other established methods is difficult (though respective reviews exists, see Stock et al., 2019). Most other purification techniques where chemicals (acidic or basic) or mechanical forces (e.g., stirring during density separation) are involved require elaborate testing of MP resistivity (Lenz et al., 2020). Electrostatic separation can be considered a very gently treatment and such tests can be omitted as no effect on the MP integrity is to be expected.

The working principle of electrostatic separation using the KWS is based on the different electrical conductivity of the sample particulates, with mineral particulates being generally more conductive than plastics. As water content fundamentally changes the electrostatic behavior of particles, an initial (freeze) drying step is needed. The dried sample is then entered at the sample-entry funnel of the instrument and transported by a vibrating conveyor plate toward the Corona electrode system. For better visualization of this process, see the KWS-XS schematic in the supporting information (**Supplementary Information, Figure S1**). The particles become charged in a high-voltage electrical field (max. 35 kV, DC) between the grounded drum and an above-mounted rake-shaped electrode. More conductive mineral particles discharge quicker and jump off the drum with the dividing flap guiding them into the "sediment container." Less conductive plastics discharge slower and remain adhered to the rotating drum and only detach later into a separate collection container (the herein named "plastic container"). All particles that remained adhering to the drum are mechanically removed by a scraping plate, which lets them fall into the plastic container as well. By recycling the content of the plastic container into the top (i.e., repeated runs), the mineral fraction is further reduced and MP are further refined. According to the prior study, three repetitions were found to be most efficient. As the working principle is already thoroughly explained by Felsing et al. (2018), in the following, the focus is concentrated only on the additional findings of this method e.g., concerning influential factors on the recovery rates as well as on the working steps necessary to allow a replication of the method.

Based on the results by Felsing et al. (2018), the aim of the present study was the validation of the instrument-functionality for current sample matrices and the determination of their standard error to be reported in future studies. The main scope was to use electrostatic separation for beach samples, as they (by experience) often require relatively large initial volumes (up to several kilos) to reach statistically robust MP numbers. Beach sediments are usually on the less complex end (low in organics, relatively homogeneous grain size distribution) of possible environmental matrix compositions and reached highest enrichment rates in the prior study mentioned above. However, more complex matrices (freshwater suspended particulate matter) also appeared to reach reasonable recovery rates and mass reductions. This is why we also report observations on the extraction efficiency of soil samples, a matrix which typically challenges other methods due to their complex compositions (rich in organics, heterogeneous grain size distribution, high levels of aggregation; Möller et al., 2020).

The extraction efficiency was evaluated based on three requirements:

- a reasonable mass reduction of the initial sample mass that allows for a subsequent quick and easy density separation in a separation funnel [as presented in Enders et al. (2020)]
- reasonable and reproducible (i.e., consistent) recovery rates of MP
- low influence of matrix-related variables (i.e., grain size, mineral composition) on recovery rates and mass reduction

As the prior study showed no differences in recovery rates between polymer types, the present study focuses only on different particle sizes with a broader overall size range from 20 μm to 4 mm, as well as with finer steps between each size range compared to prior tests (Felsing et al., 2018).

MATERIALS AND METHODS

Test Material

Recovery rates were determined using the same model of electrostatic separator (2nd prototype, KWS-XS, Hamos GmbH) as presented in Felsing et al. (2018). Sets of different recovery-test MP of various sizes, polymers and shapes (**Table 1** and **Supplementary Information, Figure S2**) were prepared and the test sediment samples spiked. The smaller the particles, the higher the number of spiked test MP due to the expected lower recovery rates. Spiked MP

TABLE 1 | Set of spiked MP of various size (longest dimension), color, polymer type and shape.

Number added	Size	Color	Polymer	Shape
20, 40	4 mm	black	PA66 (Polyamide)	pellet
20, 40	2 mm	white	HDPE (High-density Polyethylene)	pellet
20	2 mm	green	PE	fiber
20, 40	450 μm	red	PA6	pellet
60 - 80	125–150 μm	green (florescent)	PE	sphere
60 - 220	63–74 μm	blue (florescent)	PE	sphere
20 - 201	20–27 μm	green (florescent)	PE	Sphere

*An image of the listed particles can be found in the **Supplementary Information, Figure S2**. Added numbers of spiked MP varied (see 1st column).*

were counted visually (≥450 μm) aided with a binocular microscope (Zeiss Stemi 2000) and an ultraviolet light source (Tattu U2, 395 nm) where necessary (≤150 μm, fluorescent microspheres, Cospheric LLC).

Test samples were composed of either:

(a) 100 g (d/w) beach sand from the Baltic Sea (Warnemünde beach, 0–2 cm surface, d50 = 319 μm, 197–530 μm [10, 90 th percentiles], **Supplementary Information, Figure S3A**)

(b) 100 g (d/w) commercial sand (Aquarienkies, Rosnerski Quarz Verpackungswerk, d50 = 310 μm, 193–512 μm [10, 90th percentiles], **Supplementary Information, Figure S3B**)

(c) 100 g (d/w) sand as described in (a) added with 30 g micro glass beads with a size range of 40–70 μm (Strahlgut, Samore GmbH)

(d) 500 g w/w (∼440 g d/w) agricultural soils (0–30 cm surface mixed-core soil, from two test fields, one with 10 tons ha^{-1} of sludge from a waste water treatment plant previously applied in 2014, the other without sludge).

Preparation

Test samples (a)–(c) were baked for 5 h at 500°C to eliminate any MP contamination present in the sample which would potentially hinder a quick determination of the recovery rates. The soil samples (d) were not baked but freeze dried (according to Enders et al., 2020) as both the organic fraction was intended to be preserved and the determination and thus conservation of environmental MP was targeted in addition to the recovery rates of the spiked MP standards.

The test sediments were added together with the spiked MP into the entry-funnel at the top of the KWS and a test run started according to the attached protocol (**Supplementary Information, Text S1**). The procedures described here were written based on the steps reported by Felsing et al. (2018) and experiences made by the authors of this study. For example, run repetitions were set to five (instead of three) in order to increase the mass reduction. Subsequently, a small-scale density separation was performed according to Enders et al. (2020) to allow unhindered identification of the spiked test-MP. A schematic overview of the separation steps can also be found in Felsing et al. (2018, **Figure 3**).

Optimal Instrument Settings

At first, optimal instrument settings had to be identified to perform recovery tests. Those initial tests were performed using the test MP between 4 mm and 450 μm in size (**Table 1**), as they could be quickly visually identified without performing a subsequent density separation. For beach sands (a, c) two different high voltages (20 kV, 22 kV) were tested and the full set of MP recovery rates and mass reduction determined. The voltage optimum was found to be at 22 kV. While higher voltages generally improved the separation efficiency, when further increased (up to 25 kV), smaller particles were strongly dissipated and scattered in an uncontrollable manner. The drum speed was set relatively slow, 5 rpm (4%), as otherwise centrifugal forces and particle-particle interactions prevented independent separation of particles. To ensure the maintenance of a mono-layer of sample material on the drum, the terminal vibration strength of the conveyor plate was set between 60 and 120 rpm (1–2%). Initially, higher vibration strength might be needed as it depends on the sample weight on the conveyor plate which has to be transported. Depending on the sample type, drum speed and vibration strength can be changed but must be configured to ensure mono-layering of particulate matter on the drum. The sample-entry funnel on top of the KWS comes with a relatively large output opening (12 cm), which caused large variations of sample weight present on the conveyor plate and thus a lot of manual adjustment of the required vibration intensity to achieve a mono-layer (as a function of the sample being processed). Since, in this original design, failure in properly adjusting the vibration strength during sample-processing could influence the results (by impacting drum mono-layering), the funnel was modified with additional tapering using an adjustable stainless steel plate. The new opening can be adjusted to approximately 5–10 mm (**Supplementary Information, Figure S1**). It is further noted that the original particle shield (Polyvinyl chloride) above the vibration plate and drum, built to reduce the loss of particles to the KWS interior, was unmounted due to several reasons. These are: (1) Both sand and MP particles were found adhering to the shield surface and were thus lost from the sample; (2) The PVC shield constitutes a potential source of plastic contamination, and (3) It hindered control of mono-layering. Given these benefits, it is not expected that the shield removal negatively effects results. As an additional modification, a hardboard sheet was added above the sediment container to reduced the loss of sediment to the KWS interior (**Supplementary Information, Figure S1**). The metal dividing flap that determines the boundary between sediment and plastic container was set to an angle of 19.5°, the optimally determined position where maximum sediment would be separated without any large MP falsely entering the sediment container (standard procedures and parameters in **Supplementary Information, Text S1, Figure S4**).

Mineral and Grain Size Classification

SEM-EDX analyses were completed at the IOW by the authors using a Zeiss Merlin VP Compact Scanning Electron Microscope (SEM) combined with an Oxford X-Max 80 energy-dispersive

x-ray (EDX) spectrometer to analyse mineral content of two exemplary sand samples (software: Oxford AZtec 3.3). Samples were vacuum sputter-coated with elemental carbon to provide good electrical conductivity (Cressington Carbon Coater 108carbon/A, TESCAN GmbH, 10 nm layer thickness). Measurements were taken with an aperture of 30 μm, a working distance of 8.5 mm and a kV of 15.00 using Inca feature 5:04 software. An upper limit of 1000 features (i.e., particulates) were measured per sample. Mineral classification was applied according to the in-house-developed Baltic Sea Standard. Homogenized and freeze dried subsamples were grain size analyzed in two replicates using the laser-sizer Mastersizer 3000 by Malvern (10 s ultrasound applied).

RESULTS

Recovery Rates

Across the different types of sand sediment matrices (a–c), MP recovery rates decreased with MP size (**Figure 1**, data in **Supplementary Information Table S1**). The standard deviation increased for smaller MP as well as fibers.

For beach sand (a), the MP recovery rates decreased from 100 to 10% median recovery, including losses generated during density separation (**Figure 1**, red box plots; **Supplementary Information Tables S1A–D**). The recovery rates presented here were determined after the density separation, a necessary procedural step, as otherwise remaining sediment particles hindered a direct analysis, especially of the particles ≤150 μm. To determine KWS specific recovery rates, the recovery rates of the density separation only were determined separately and subtracted from the total (**Figure 1**, opaque bars; **Supplementary Information Tabled S1L–M**), resulting in a corrected recovery rate declining from 100 to 45% with size. There was no difference in recovery rates between the two different voltages (20 and 22 kV), thus recovery rates were averaged (**Supplementary Information Tables S1A–D**).

Median recovery rates for the commercial sand (b) were slightly lower compared to the beach sands (**Figure 1**, turquoise box plots; **Supplementary Information Tables S1E–G**), with the exception that the recovery rates of the smallest spiked MP (20–27 μm) were significantly higher (*t*-test, $p = 0.03$).

The presence of the fine (40–70 μm) glass beads (c) resembling fine sediment grains or, more generally, dust, partly distorted the previously described recovery-rate pattern for beach sand sediments (**Figure 1**, dark blue box plots; **Supplementary Information Tables S1H–K**). While the large particles (>2 mm) were unaffected, the recovery rate of the intermediate size class (450 μm) was significantly reduced (*t*-test, $p = 0.04$) by nearly 40%. Spiked MP (125–150 and 63–75 μm) closer to the size spectrum of the added dust particles showed a slightly increased recovery rate compared to the previous test without additional dust. It seems reasonable to assume that the added dust particles cannot sufficiently adhere to MP particles of similar size and thus do not negatively affect the recovery rate. However, larger MP which can be sufficiently surface-coated by dust can obtain a higher net-conductivity and enter the sediment container more frequently. This negative effect on the intermediate size class seems to amplify when using glass beads as sole sample matrix (one observation only; **Supplementary Information Table S1O**).

Mass Reduction

The main reason of applying the KWS is the selective MP refinement by reduction of the mineral mass fraction. In case no considerable mass-reduction is achieved for a specific sample, electrostatic separation would simply constitute an increase in methodological efforts and resources (as well as an extra step for possible contamination and loss of particles), without any prominent benefit. Therefore, besides MP recovery rates, the mass reduction is the ultimate measure of treatment effectiveness.

The mass reduction of beach sand was influenced by the voltage applied and resulted in 74% at 20 kV and 93% at 22 kV. Comparable numbers were acquired with dust added, 70% at 20 kV and 94% at 22 kV. Thus, a higher voltage generally increases the mass reduction. However, as already mentioned in the above section "Optimal instrument settings," a further increase strongly affected the smaller particles that scattered in an uncontrollable manner.

The effectiveness of the method in terms of mass reduction for the commercial sand was substantially lower compared to the beach sand, resulting in a 17% average (at 22 kV), MP recovery rates were within the same ranges. The lower mass reduction, and thus a larger remaining volume, of the commercial sand probably explains the higher recovery rates of the smallest spiked MP, which appear not to be actively separated. An increase in voltage (to 25 kV) did not have a measurable effect on the mass reduction of commercial sand, as the majority of the material remained adhered to the drum before being collected into the plastic container by the scraping plate. As the grain size distribution of both sand types (a, b) were very similar, likely mineral compositions (e.g., mineral specific electrical conductivity) plays a larger role.

The mass reduction can also be influenced by the angle of the dividing flap between the sediment and plastic containers. The larger the angle, the more of the mineral fraction will be separated (and ultimately eliminated). However, over-increasing the flap angle comes at the cost of MP recovery rates, as certain (particularly large) MP will start to be eliminated from the sample if the angle is too extreme. As stated above, an optimum angle was determined to be 19.5°; the highest angle where no large MP were lost to the sediment container (runs: $n = 3$). It has been observed that an initially higher angle (starting at 28°) lead to an increased loss of MP, particularly the 4 mm black pellets and the 2 mm fibers (up to 10%), to the sediment container. The rounded morphology of these MP occasionally initiated rolling movement on the drum which, combined with drum-centrifugal force, likely explains the higher trajectory and loss to the sediment container of these MP. For the sands used in this study, a higher flap angle of 28° did not further increase mass reduction rates. It was observed that the eventual non-separated sediment fraction was adhering to the drum, indicating that further increase of the angle would have a negligible effect. While initial instrument settings, such as the angle of the dividing flap or high voltage, can influence the separation efficiency, the

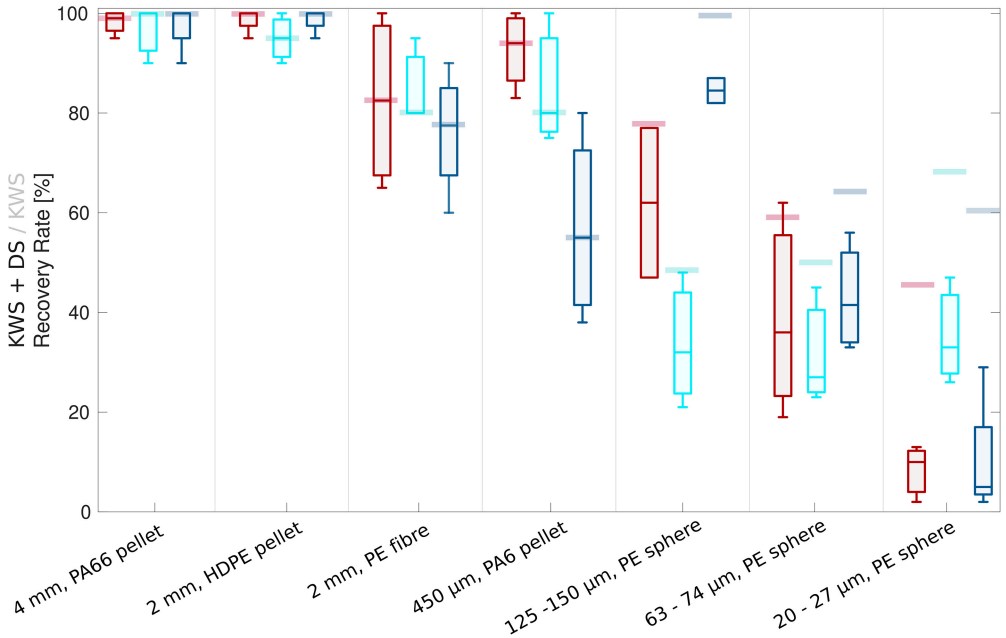

FIGURE 1 | Box plot series showing recovery rates (incl. density separation, DS) of MP of different size classes from beach sand (red), commercial sand (turquoise) and beach sand with additional presence of dust (dark blue). Whiskers represent the 25 and 75 percentiles. KWS-specific median recovery rates of MP of different size classes, mathematically corrected for density separation, are shown as opaque bars behind the box plots.

determining factor for the final mass reduction seems to depend on sediment composition.

Sediment Composition

It was not initially clear why beach and commercial sand had different mass reduction rates, since they are expected to be compositionally similar (i.e., mainly quartz(SiO2)-based). However, given there was a clear difference in mass reduction, and that the two sands had a different visual appearance (beach sand brown in color while commercial sand was white, **Supplementary Information, Figure S3**), it was necessary to further analyse the mineral composition of the sediment to better understand if, and how, mineral composition influences reduction rates. SEM-EDX spectroscopy was applied to the two sand samples. It was apparent that the commercial sand contained higher levels (50.5%) of small (<50 µm) calcite particles in comparison to beach sand (23.5%). A visual demonstration of this difference can be seen in **Figure 2**. Aside from this, the elemental analysis showed slightly lower levels of certain metal elements (e.g., aluminum, potassium and magnesium) in the commercial sand.

From the obtained data we can only presume that either the actual mineral composition differences lead to changes in the material conductivity and thus different electrostatic separabilities. Or, that the compositional differences lead to distinct hygroscopic properties that entail varying material humidities at the time of separation. The latter may be tested in a humidity controlled environment, which is, however, beyond the scope of this study.

Soils

Initially, soil samples (d) achieved ∼1% visually estimated reduction after two runs. This ineffectiveness of the KWS was postulated to be due to the high levels of silt and clay particulates present in soils (especially agricultural soils). Therefore, dry-sieving to remove the <100 µm quotient was applied (according to Enders et al., 2020). After a further six runs of the sieved sample, the mass was reduced overall by ∼15% (total eight runs taking ∼7 h per 500g w/w sample, not including reductions of the sieved-out fraction). This indicates that removal of the dust-sized quotient of the soil samples improved the performance considerably (from ∼1 to ∼15% reduction); however, this reduction rate was far below that observed for beach sand. Due to such a comparatively poor reduction, it was considered redundant to test recovery rates for KWS treatment of soil. This is because to enable MP analysis, other methods, such as sieving, and multiple cycles of density separation and H_2O_2 digestion were required. This makes KWS-specific recovery rates difficult to accurately determine, and ultimately without value, given the lack of usefulness of the KWS approach for these samples going forward.

DISCUSSION

Influential Factors

Microplastics recovery rates for different mineral-rich samples using the KWS scaled with MP size, with generally the highest rates achieved for the largest particles. In consequence, a general standard error for overall MP recovery using the KWS is not

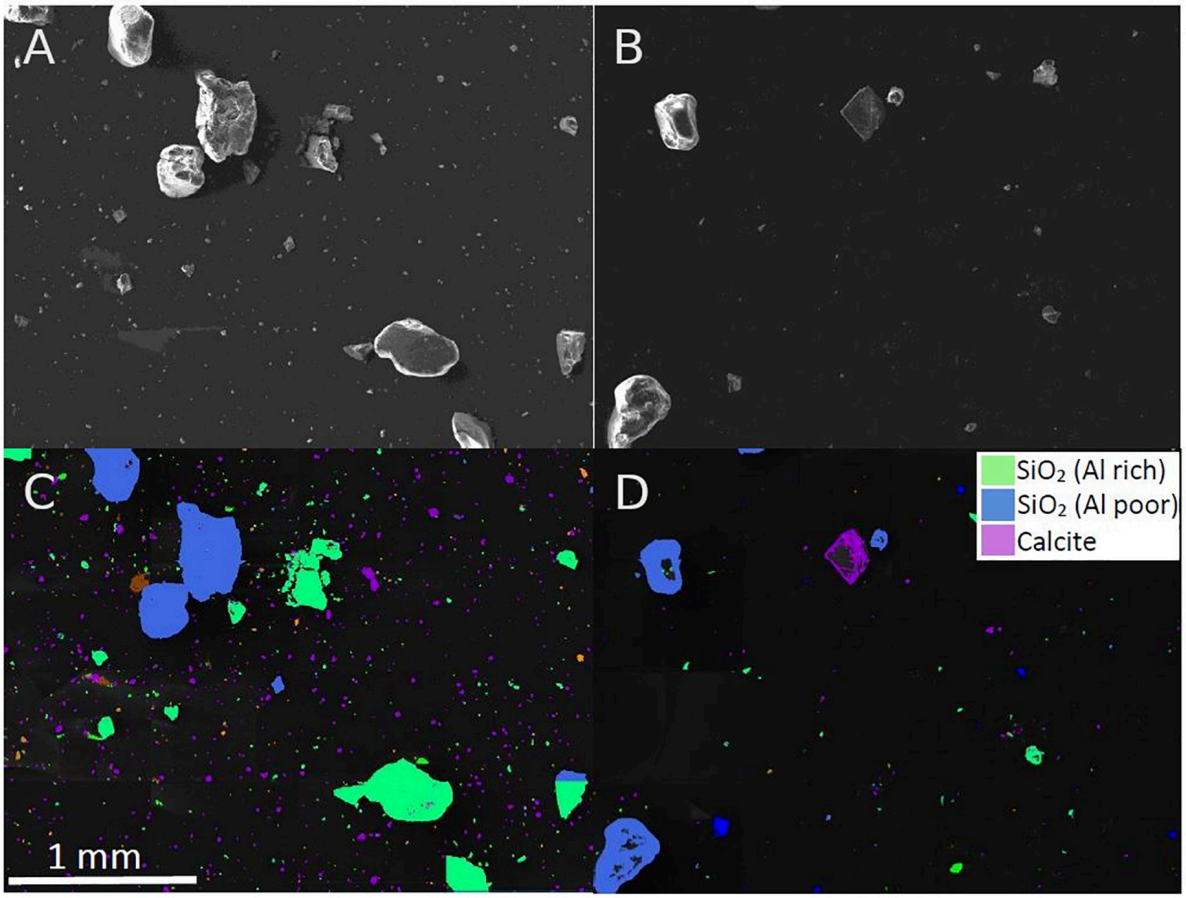

FIGURE 2 | SEM images of **(A)** commercial sand and **(B)** beach sand. Mineral classification of sediment particles for **(C)** commercial sand and **(D)** beach sand. High amounts of small calcite particles can be seen in the commercial sand samples (purple in **C**), which are largely absent in the beach sand sample **(D)**.

useful and thus not determined, suggesting to report a standard error per size range (see **Figure 1**).

The results demonstrated that, aside from customisable instrument settings, many factors concerning the composition of the sediment matrix can potentially influence MP recovery rates obtained by electrostatic separation. While some of these factors can be easily adjusted, such as sample condition e.g., humidity by prior freeze-drying, factors determining sample composition are more complex to control for. Among those are grain size distribution (especially with respect to the smaller "dust" fraction), mineral composition as well as organic matter content (potentially causing aggregation). This study showed that increased presence of dust of one specific grain size strongly influenced the recovery rate of certain MP sizes. An explanation for this effect could be an increased surface-coating of MP by smaller silicate particles causing a higher net-conductivity and increased loss to the sediment container. SEM-EDX analysis suggested that presumably the high presence of calcite dust (as seen in the commercial sand, **Figure 2**) or lower presence of metal-rich minerals reduced sediment reduction rates, lowering the usefulness of the approach. It is, however, still unclear which dust sizes or mineral compositions have the greatest impact on size-dependent recovery rates and mass reduction rates. The amount of dust likely negatively scales with the specific recovery rates, as observed when using the dust particles as the sole sample matrix. In contrast to the freshly produced and clean test MP, environmental MP are usually coated with organics and fine dust. Therefore, the actual recovery rates for environmental MP are likely below those presented here.

Soil observation results also suggest that high presence of fine particulates and the presence of agglomerates (as typical for soils) in samples limits KWS applicability. As the primary purpose of implementing electrostatic separation is for the bulk reduction of sample volume, it turned out that KWS treatment, even with a large amount of fine particles being removed by dry-sieving, enabled only a reduction of the original volume of ∼15%. Taken into account that the time taken to perform this reduction was 7 h, the value of the applied method was low for our purpose. Indeed, even after sieving and KWS treatment, an advanced density separation was still required to process the samples. Methods for the refinement of MP in environmental samples benefit from having the least steps necessary, given the problems of particle loss and contamination (Dehaut et al., 2019; Enders et al., 2020). As such, the method applied to soils within this study

offers limited promise. However, it should be considered that dry-sieving does not completely remove dust, as conglomerates of fine material and those adhering to larger particles may not pass through the sieve. Wet-sieving (performed before freeze-drying), which uses water to carry material through the sieve, is likely to be more efficient at dust-removal. However, it is unknown how much of a pronounced difference this might have on KWS reduction rates on agricultural soils. Given issues surrounding agglomeration of particulates in soils, reduction rates are still anticipated to be well below those of sand, and progressing directly to a larger-scale density separation is likely to produce better results than wet-sieving combined with KWS.

The material requirements reported by the manufacturer of the KWS (hamos GmbH) are in line with our findings in view of a conservative perspective. The company recommends sizes of 2–12 mm for plastic separation and >100 μm for mineral separation. Ideally, the material is dust-free and completely disintegrated (no composites/agglomerates).

Other Limitations

As already mentioned the KWS has originally been developed for large-scale recycling material separation, focusing on plastic particles of several millimeters to centimeters (Tilmatine et al., 2009; hamos GmbH, 2020). Although the KWS-XS has been downscaled in size which allows an application of smaller samples in a usual scientific lab, certain limitations derive from the design of the instrument and related physics of particles. The KWS has a relatively closed outer hull which prevents contamination from outside. However, when working with particles down to the smaller micrometers in size, the arrangement of the functional inner parts would obviously require a more closed system of higher precision, with less surfaces and corners causing potential particle losses. It has frequently been observed that larger particles, especially fibers and smaller MP, repeatedly remained adhered to the drum as a result of not being dislodged by the scraping plate which is installed to provoke particles, still adhered once passing the sediment (waste) container, to fall into the plastic container. The scraping plate also unintentionally collects particles on the rear side over time, and requires manual opening and scraping to ensure all relevant particles are collected in the MP container. This process is, however, less effective the smaller the MP. In the case of soil samples, clay presence was so high that, given the inefficiency of the scraping plate at consistently removing small particles, after a number of runs the drum was permanently coated in fine particulates. This made mono-layering highly challenging and probably contributed to the lower mass reduction rates. In general, the large surface areas inside the KWS offer small particles plenty of space to stick to due to electrostatic forces of attraction. This effect can be observed after sample runs that contained relatively small grain sizes, after which all surfaces gathered dust. Accordingly, cleaning between sample runs is exhaustive. A size measurement of the dust collected on the inner surfaces of the KWS after a beach sand separation showed an average size of 54 μm, ranging from 33 to 79 μm as the 10 and 90th percentiles, respectively. Occasionally, larger MP (>450 μm) fell to the bottom of the instrument, outside the remit of the collection containers.

The MP container collecting the refined material has to be thoroughly rinsed and the rinse water collected following sample transferral to the density separation (or any other desired next experimental step). In some cases, more than 50% of the spiked MP < 150 μm were found in this rinsate (and included in the results).

Scope of Application

The results of the present study showed that a reduction of MP recovery rates correlated with size, with the smallest fraction (20–27 μm) achieving consistently less than 20% recovery (incl. KWS and density separation) for sands that were sufficiently reduced in mass. In contrast, Felsing et al. (2018) found as high as 100% for all MP size fractions tested, from their largest (2–5 mm) to their lowest size fraction (63–200 μm), in both quartz and beach sand. Although this is a much larger and broader range size category than in the present study, the most comparable size class to their smallest size fraction is 63–74 μm, which achieved less than 60% for beach and commercial sands, when corrected for the density separation (for best comparability as Felsing et al. (2018) counted recovery rates without density separation).

Instrument settings were overall comparable between the two studies. Although drum speed and vibration of the conveyor plate were slightly faster in the prior study, as long as the mono-layering is ensured, no influence on the recovery rate is expected. The angle of the metal flap divider between the collection containers was also in a similar range (20°) in the Felsing et al. (2018) study (Kochleus, 2020). However, in the present study, it has been shown that, rather than the instrument settings, sample matrix variance influences the mass reduction to the greatest degree, as demonstrated by SEM analysis of the two sand samples as well as the ineffectiveness of KWS treatment on the soil samples.

A difference between the designs of the two studies concerns the shape of the spiked MP used. Whereas the prior paper used MP with a flake-like (flat) morphology, the present study used mainly pellet or round MP. Flat particles have a larger surface area-volume ratio which presumably better-adhere to the drum, whereas round MP, due to the relatively small contact area compared to mass, are more likely to fall from the drum prematurely if the electrostatic force is insufficient compared to gravity acting upon the particle. As mentioned above, this fall-off effect was observed especially for the larger MP (>2 mm). However, it is unlikely that shape significantly affects the recovery of the smaller MP. The small MP fraction reported by Felsing et al. (2018) describes particles within a comparatively larger size range (e.g., 63–200 μm) and the actual size distribution of particles within that range is unknown. This makes it difficult to compare to the finer small-MP size ranges used in the present study (125–150, 63–74, and 20–27 μm). The MP sample number was lower in the previous study ($n = 10$), compared to the present study ($n > 20$), for the individual spiked MP which increases the risk of statistical outliers.

However, an important factor to consider when comparing studies testing electrostatic separation is that we now know that mineral composition plays an important role. SEM analysis of the two sand samples within the current study showed that

TABLE 2 | Summary of advantages and limitations of electrostatic separation (using the KWS) for MP purification from sediments.

Advantages	Limitations
• relatively fast and easy method • reduction of a potentially large sediment fraction • no polymer resistivity tests needed due to non-destructive separation mechanism • generally good recovery rates for large MP	• additional (established) treatment steps needed if a particle-based analysis is targeted • mass reduction depends on multiple matrix-related factors which can negatively impact the efficiency: (1) mineral composition, presence of less conductive or less hygroscopic minerals; (2) grain size, presence of fine sediment (<100 μm) • decreasing MP recovery rates with smaller MP sizes • fine sediments (<100 μm) can reduce MP recovery rates • improvements on the instrument design needed

Findings are elaborated in more detail above.

both the size and composition of particulate matter can have a marked difference on mass reduction. Therefore, comparing reduction or recovery rates between samples where mineral composition is unknown is a challenge. Yet, the difference in results between the two studies are so pronounced that, even with a difference in mineral composition, it is likely that more unknown factors are in play.

Microplastics samples treated with electrostatic separation require further subsequent treatment steps such as density separation and digestion. Ultimately, the recovery rates of the respective procedural steps add up. The sum of all this can be quite considerable for some size classes, as shown with the example of combined electrostatic and density separation (**Figure 1**, box plots). The more methodological handling steps, the more errors occur and it is generally recommended to keep the individual treatment pipeline as short and simple (but robust) as possible. Therefore, it has to be evaluated case by case, whether the requirements shown in the present paper fit both the sample type and the research question. Less complex sediment matrices with expectantly low MP content that statistically require larger initial sample volumes (>1 kg) may justify such a first volume reduction step. In any case, the additional financial investment, as well as per-sample time and effort related to electrostatic separation has to be taken into account. A simple density separation (hand-shaking) in a separation funnel is efficient to up to ∼50 g of sediment (lower for organic-rich samples). For samples up to 500 g (or even up to 1 kg if the sample is split), a density separation, combined with advanced stirring, such as the spiral sediment conveyor (Enders et al., 2020) is most-likely more efficient and robust for a greater range of research questions. However, samples significantly larger than this can make density separation too challenging as a first step. In these cases, the KWS approach could be used to reduce the starting volume, especially for sand samples. However, given the variance in recovery rates due to MP size and sample composition, the use of internal standards is highly beneficial, especially for the smallest sizes targeted (something typically determined by analysis limitations). MP that are highly unusual (so as not to be confused with real environmental MP) and easy to distinguish (i.e., brightly colored) are most recommended. UV-fluorescent MP are highly beneficial for this purpose.

Despite the above, open questions still remain from the current state of knowledge, and of particular concern are the recovery rates of environmental (degraded) MP and the influence of MP shape. Larger systematic tests would be needed to better understand these questions, as well as to quantify the impact of the identified key influential parameters of the sediment matrix. As a concise summary of the above findings, advantages and limitations of electrostatic separation (using the KWS) for MP purification from sediments are listed in **Table 2**.

CONCLUSION

Based on the presented results, the application of the KWS for MP purification can be recommended for sediments of relatively large grain sizes (above 100 μm), in absence of dust and for large MP sizes between 0.5 and 5 mm, especially where large sample volumes (>1 kg) are desired. It should be tested in advance whether the mass reduction of the targeted matrix achieves the required volume for further processing, given that although mineral composition has shown to be of influence, the extent of this influence is unknown. The use of internal standards is highly recommended, especially for small MP, as recovery rates vary with MP size as well as sediment composition.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KE and ML: study design. KE: data acquisition and analysis (KWS). AT: data acquisition and analysis (SEM). KE and AT: writing—original draft. ML: writing—review and editing, supervision, and project administration. KE: visualization of KWS. AT: visualization (SEM). 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/fenvs.2020.00112/full#supplementary-material

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POLICY BRIEF
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Transnational Plastics: An Australian Case for Global Action

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Mitigating plastic pollution requires strong international cooperation because significant volumes of plastic waste are transported across jurisdictions both as waste exports and drifting ocean plastics (OP). Here we estimate which nations are (1) sources for overseas OP reaching Australian waters and (2) destinations receiving OP from Australian sources. We then provide actionable recommendations for mitigating plastic pollution in Australian waters and beyond. We estimated that the vast majority of overseas OP reaching Australia is from Indonesia, and that most of the Australian-sourced OP reaching overseas territories is entering New Zealand. Key actions for mitigating the OP issue in Australia include better governance, upgraded enforcement, and increased investments to reduce fossil fuel-based plastic production and to drastically improve both domestic and international waste management infrastructure and operations.

Keywords: marine pollution, marine debris, plastic pollution, Australia, connectivity, ocean currents

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INTRODUCTION

Plastic pollution is a global environmental concern due to its widespread damage to ecosystems, economies, and human well-being. Under a "business as usual" scenario, plastic impacts will keep increasing as both the production and mismanagement rates of these materials are high and rising. Globally, we are currently producing ∼380 million tons of plastic per year, with just 9% of it being recycled; the rest is burned, landfilled, or dumped in the environment (Geyer et al., 2017). Notoriously, around 40% of the plastics we produce are used for packaging that becomes waste immediately after its single use, contributing to more than 60% of global beach litter (Schweitzer et al., 2018). Currently, more than 15 million tons of plastic enter oceans each year (Forrest et al., 2019). Plastics of all kinds—from microscopic packaging fragments to giant fishing nets—have accumulated in many marine ecosystems, including beaches (Lavers and Bond, 2017), coastal and oceanic waters (Ryan, 2013; Lebreton et al., 2018), marine canyons (Schlining et al., 2013), coral reefs (Lamb et al., 2018), mangroves (Martin et al., 2019), and even sea ice (Peeken et al., 2018). Pronounced marine plastic pollution impacts include the often lethal consequences of entanglement and ingestion interactions between ocean plastics (OP) and marine wildlife (Vegter et al., 2014). Other more subtle OP implications include transport of invasive species across ocean basins (Barnes, 2002) and release of persistent bioaccumulative toxic (PBTs) chemicals into environments and organisms, with the potential bioaccumulation of plastic-associated PBTs up food webs that include humans as seafood consumers (Setälä et al., 2014; Rochman et al., 2015; Chen et al., 2018). Aside from impacting oceans, plastics are also causing significant damage during their upstream lifecycle. The vast majority of today's plastics are made of fossil fuels (∼99%) and the release of greenhouse gases during their production and incineration is costing around

US$ 700 billion per year to the global economy (Forrest et al., 2019). Additional damage is created by the release of toxic chemicals from plastics to air, soil and water resources (UNEP, 2014) as well as human exposure to plastic particles and additives linked to cancer, obesity, diabetes, and endocrine system disorders (Brophy et al., 2012; DeMatteo et al., 2013; Manikkam et al., 2013; Lehner et al., 2019).

To solve plastic pollution, countries around the world need better governance at the municipal (e.g., Cohen et al., 2015), national (e.g., Commonwealth of Australia, 2018a), regional (e.g., European Commission, 2019), and international levels (Borrelle et al., 2017; Weikard, 2019). The last of these is particularly complex but essential given the transboundary nature of plastic contamination, particularly as wide OP dispersal (Lebreton et al., 2012) and waste trade (Brooks et al., 2018) (**Table 1**). Most of the global plastic waste is generated by high-income countries (HICs), where per capita plastic consumption is particularly high (Hoornweg and Bhada-Tata, 2012). As recycling is a low margin business, the majority of HICs plastic waste intended for recycling is exported to lower income "East Asia and Pacific" nations with low labor costs (Locock, 2017). Australia is a classic example of a HIC "plastic waste exporter" as it ships large volumes of plastic waste to low- to middle-income countries (LMICs). In 2018 alone, Australia exported ∼127,400 tons of plastic waste (UN Trade Statistics, 2018; code 3915 "waste, parings and scrap of plastics"), the majority of which was exported to Malaysia (35%), Indonesia (22%), and Thailand (18%; **Figure 1**). Historically, plastic waste trade has been associated with serious environmental and social issues, including export of low-quality plastic bales by HICs and illegal practices for processing imported materials by LMICs, including labor exploitation, burning, and discard of low-value plastic waste into dumps, rivers, and oceans (GAIA, 2019; Sarpong, 2020). A significant disruption to global plastic waste trade occurred in 2017, when China banned the import of large volumes of plastic waste into its territory (Liu et al., 2018; Walker, 2018) and neighboring LMICs started receiving this rejected waste (Wang et al., 2019). These LMICs lack the infrastructure to manage their own plastic waste, let alone a rapid increase in plastic waste supplied by HICs. The international plastic waste crisis is now quite evident, with waste-importing countries such as Malaysia and Indonesia starting to ship contaminated plastic waste back to its HICs sources (CNN, 2019; Walden and Renaldi, 2019). Without improved plastic waste governance, there is a risk that increasing quantities will ultimately become plastic pollution.

Plastic waste is not only exchanged between nations via trade. Once leaked into rivers and oceans, it can be transported by currents, winds, and waves, often polluting other nations' exclusive economic zones (EEZs) (Lavers and Bond, 2017; Lebreton et al., 2017). It is quite difficult to track OP back to sources, with the degree of difficulty increasing over time as OP items undergo environmental weathering and fragmentation into progressively smaller pieces known as microplastics when <5 mm and nanoplastics when <0.1 μm (Gigault et al., 2018). Previous studies attempting to quantify OP connectivity dynamics have utilized 2D numerical models forced by sea surface current velocity fields to simulate OP transport at different scales (Lebreton et al., 2012; Eriksen et al., 2014; Critchell and Lambrechts, 2016). To the best of our knowledge, there are no studies to date attempting to quantify fluxes of OP between nations. This information is key to better inform stakeholders involved in designing cost-effective strategies for decreasing OP pollution. In this policy brief, we used ocean modeling to estimate which nations are (1) sources for overseas OP reaching Australian waters and (2) destinations receiving OP of Australian origin. Using our findings and incorporating an extensive literature review, we then describe governance and policy recommendations for mitigating plastic pollution in Australia and beyond.

TABLE 1 | Top five nations with the highest mass of plastic waste exported ("*Top Exporters*") and leaked to oceans ("*Top Emitters*").

Rank	*Top exporters*	*Tons*	*Top emitters*	*Tons*
1	Germany	1,266,787	China	2,425,422
2	United States	1,217,145	Indonesia	884,635
3	Japan	1,035,421	Philippines	518,006
4	United Kingdom	756,106	Vietnam	504,300
5	France	441,705	Thailand	282,628

Numbers are mass estimates of nations' plastic waste exports in 2018 (UN Trade Statistics, 2018) and emitted to oceans in 2010 (Jambeck et al., 2015; after correction for Sri Lanka data, as described in Lebreton et al., 2017).

ORIGIN AND DESTINATIONS OF OCEAN PLASTICS CROSSING THE AUSTRALIAN EEZ

We used a Lagrangian model that simulated inputs and surface transport of OP in the world's oceans (Lebreton et al., 2012) to estimate connectivity between the Australian EEZ and other world countries' EEZs. Our analysis indicated that the vast majority of overseas OP reaching the Australian EEZ is coming from Indonesia (∼70.5%; **Figure 2**), followed by Fiji (∼8%), Vanuatu (∼4%), Papua New Guinea (∼3%), Philippines (∼2.5%), Vietnam (∼2.5%), East Timor (∼2%), South Africa (∼2%), China (∼1.5%), Malaysia (∼1%), Solomon Islands (1%), and other minor contributions (∼3%) from 19 nations: Sri Lanka, Thailand, Samoa, Maldives, Tanzania, Comoro Islands, New Zealand, Myanmar, Mozambique, Peru, Mauritius, Singapore, Pakistan, Kenya, Madagascar, and Ecuador. In relation to Australian-sourced OP reaching overseas EEZs, we predicted that ∼78% is entering New Zealand waters, with the remaining reaching the EEZs of New Caledonia (∼17.5%), Fiji (∼4%), and Vanuatu (∼1%).

Our model released "virtual OP" particles in the world's EEZs at rates proportional to estimated volumes of mismanaged plastic waste per country (Jambeck et al., 2015) and derived their transport from sea surface current velocity fields sourced from the HYCOM + NCODA global reanalysis (Fox et al., 2002; Cummings, 2005; Cummings and Smedstad, 2013). For every day between 1993 and 2012, we documented the number of particles inside the Australian EEZ arriving from each foreign country as well as the number of particles of Australian origin inside the EEZ

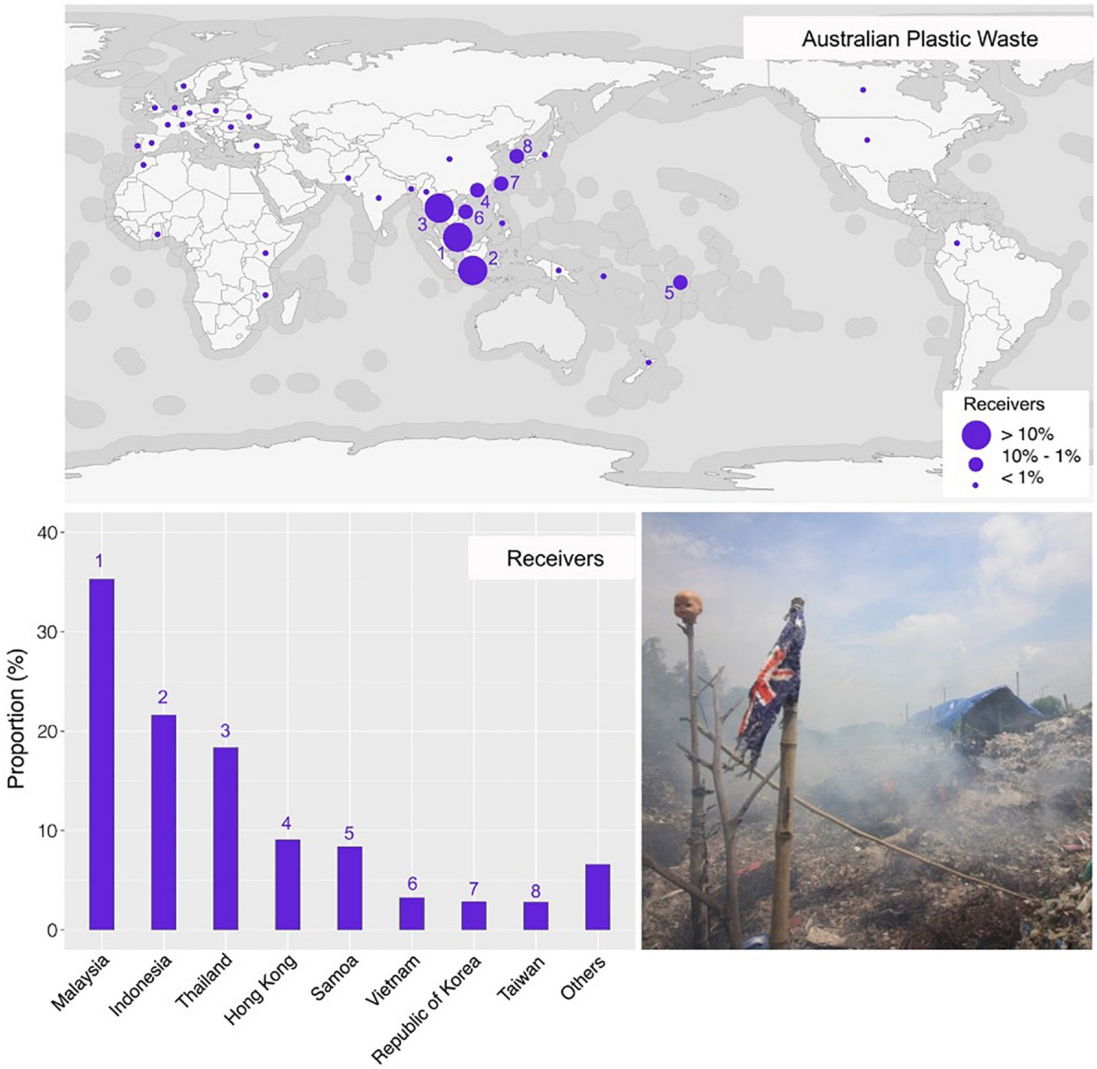

FIGURE 1 | Countries identified as destinations for plastic waste exported by Australia in 2018 (UN Trade Statistics, 2018). The map indicates countries receiving Australian plastic waste, with circle sizes proportional to amounts exported in 2018. Top Australian waste importers are (1) Malaysia, (2) Indonesia, (3) Thailand, (4) Hong Kong, (5) Samoa, (6) Viet Nam, (7) Taiwan, and (8) Republic of Korea. Histogram shows the proportion of exported Australian plastic waste reaching its major destinations in 2018. Image shows mismanagement of Australian waste (i.e., burning of low-value plastics) in Surabaya, Indonesia. Image source: Ecoton—http://ecoton.or.id/en/home/.

of every other country. We averaged the daily number of visits per emitting country over the total number of particles present in the model and over the full 20-year period. It is important to emphasize that our model considered an infinite lifetime for virtual OP particles floating at the sea surface. As a consequence, significant OP removal processes such as beaching (Olivelli et al., 2020) and sinking (Porter et al., 2018) were not accounted for. Nonetheless, we know that a proportion of floating OP is persistent enough to travel long distances (Lebreton et al., 2018). Therefore, assuming removal processes have similar magnitudes across countries of origin, our predictions of international OP connectivity are reasonable.

The occurrence of Indonesian-based plastics reaching the Australian EEZ and shorelines is in line with field observations (Reisser et al., 2013). Significant OP accumulation zones dominated by Indonesian-based debris from both on-land (e.g., mismanaged single-use packaging) and at-sea (e.g., dumped fishing nets) sources have been recorded in a range of Australian regions such as Gulf of Carpentaria (Wilcox et al., 2013; Dhimurru Aboriginal Corporation, 2018), Christmas Island (Lavers et al., 2020), Cocos Island (Lavers et al., 2019), and Ashmore Reef (Lavers et al., 2013; **Figure 3**). This is not surprising since the waters and shorelines of North West Australia, North Queensland, and Northern Territory are exposed to OP originated in Indonesia's EEZ due to both geographic proximity and ocean hydrodynamics (Sprintall et al., 2019). The accumulation of OP in remote regions of Australia is quite concerning given ingestion and entanglement interactions

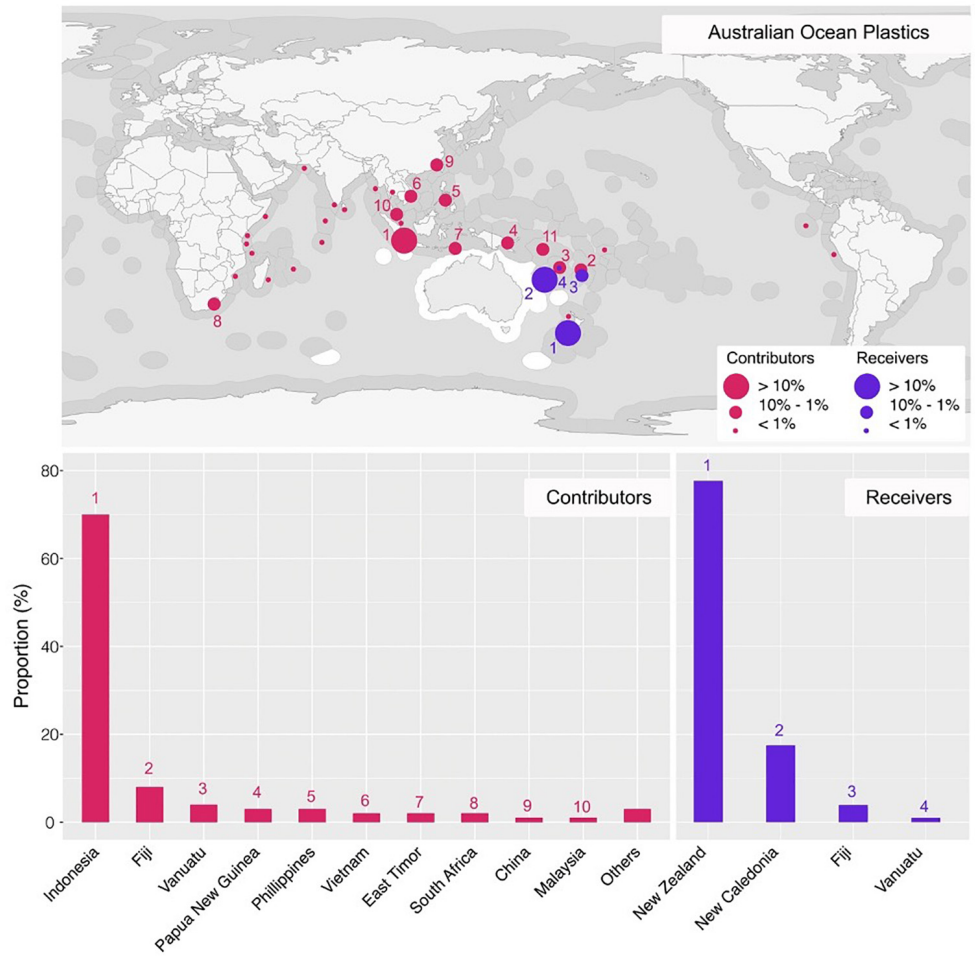

FIGURE 2 | Countries identified as sources (pink) and destinations (blue) for ocean plastics (OP) crossing the Australian Economic Exclusive Zone (EEZ) boundary. The map indicates country locations, with circle sizes proportional to amounts of OP reaching the Australian EEZ (pink) or receiving Australian-based OP (blue). The Australian EEZ is highlighted in white. Top "source countries" are (1) Indonesia, (2) Fiji, (3) Vanuatu, (4) Papua New Guinea, (5) Philippines, (6) Vietnam, (7) East Timor, (8) South Africa, (9) China, (10) Malaysia, and (11) Solomon Islands. Ranking of "destination countries" is (1) New Zealand, (2) New Caledonia, (3) Fiji, and (4) Vanuatu. Histogram in the left shows the contributions of "source nations" for overseas OP reaching Australian waters. Histogram in the right shows the proportion of OP from Australian sources reaching "destination countries."

with endangered marine wildlife (e.g., sea turtles; Wilcox et al., 2013), cross-boundary transport of pollutants and organisms (Reisser et al., 2014; Yeo et al., 2015; Lamb et al., 2018), damage to tourism, and remote clean-up costs (Commonwealth of Australia, 2018b).

GOVERNANCE AND POLICY RECOMMENDATIONS

This study highlights the transboundary nature of a major issue associated with our broken "plastics economy": persistent waste that creates high and ever-increasing levels of wide-spreading pollution. As of today, there are only a few global interventions dealing with specific plastic pollution issues (**Table 2**) and although the United Nations Convention on the Law of the Sea (UNCLOS, 2020) requires the adoption of laws and standards to prevent, reduce, and control land-based pollution, these have not yet eventuated (Article 207). The existing initiatives are quite fragmented and uncoordinated, with no overarching binding commitment to jointly tackle all facets of our transnational plastics crisis. Like others (Borrelle et al., 2017; Simon and Schulte, 2017; Haward, 2018; Raubenheimer and McIlgorm, 2018; Vince and Stoett, 2018; Tessnow-von Wysocki and Le Billon, 2019; Weikard, 2019; Raubenheimer and Urho, 2020), we believe it is important to create a new global convention that establishes a legally binding framework for global plastics governance. As suggested by Simon and Schulte (2017), the form of such convention should reflect both great ambition and political feasibility, as to ensure agreeability between and finally compliance by state parties. It could be built on five pillars: (1) a clear and binding goal to eliminate plastic contamination of water, food, air, land, and oceans; (2) strategic options

FIGURE 3 | Examples of plastic pollution hotspots in Australia, dominated by Indonesian-based debris. Top right: Christmas Island waters (Image source: Tangaroa Blue); Top left: Cocos Island (Image source: University of Tasmania): Bottom left: Gulf of Carpentaria (Image source: Parley for the Oceans); Bottom right: Ashmore Reef (Image source: Minderoo Foundation).

for national implementation; (3) supporting mechanisms, with a strong multilateral financing component; (4) stringent monitoring, review, compliance, and enforcement processes; and (5) strong involvement of non-governmental stakeholders. To truly solve plastic pollution, we need better worldwide governance, compliance, enforcement, and investments both downstream, by improving waste management systems, and upstream, by decreasing production of fossil fuel-based plastics. The latter can be achieved by incentivizing the replacement of fossil fuel-based plastics with recycled content and/or renewable bio-benign polymers. Setting a "plastic price" that incorporates the external costs of plastic pollution into the price of fossil fuel-based plastics would certainly incentivize their replacement with renewable and/or recycled materials (Raubenheimer and McIlgorm, 2018). Non-binding business-led proposals for implementing a transnational "plastic price" based on a production levy (Forrest et al., 2019) and credit scheme (3R Initiative, 2020) have been made in the past, but so far with very little implementation traction. We encourage policymakers to consider the implementation of a similar, but legally binding, intervention; perhaps under a "Global Extended Producer Responsibility" scheme (Raubenheimer and Urho, 2020) and/or via the introduction of phased targets reducing international trade of fossil-fuel based plastics (Raubenheimer and McIlgorm, 2017).

Mitigating plastic pollution in Australia and further afield also requires a systemic improvement to existing law, policy, and practice at the national level (**Table 3**). Australian government agencies and regulators need to move beyond the identification of barriers, capacity gaps, needs, and measures and start to scale implementation of state-of-the-art standards in waste segregation, collection, sorting, exports, composting, and recycling practices across its states and territories. While the country's waste collection infrastructure and operations are of relatively high quality, its waste sorting, recycling, and composting operations require considerable improvement. Furthermore, efforts are needed to reduce production and consumption of virgin fossil fuel-based plastics, including incentivizing development and use of substitutes. We believe that key regulatory measures requiring prioritized implementation include:

- Creation of a national bin system that enables citizens to separate waste into standardized higher value material streams (Mühle et al., 2010);

TABLE 2 | Examples of international initiatives to mitigate plastic pollution.

Name	Year	Description
Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter; also known as London Convention (LC)[1]	1972/1996	Protects the marine environment from pollution. The LC was amended by the London Protocol to prohibit the deliberate disposal of waste at sea unless it is listed on Annex I, and permits are needed to dump waste on Annex II. This "reverse list" approach essentially banned the dumping of plastic at sea.
International Convention for the Prevention of Pollution from Ships (MARPOL)[2]	1973/1978	Prevents pollution of the marine environment by ships. Annex V prohibits at-sea disposal of plastics by vessels in both exclusive economic zones and waters beyond national jurisdictions.
United Nations Convention on the Law of the Sea (UNCLOS, 2020)[3]	1982	Establishes rules governing all uses of the oceans and their resources. It does not specifically mention plastics, but broadly calls on states to "protect and preserve the marine environment" (Art 192), including marine pollution (Art 194). In addition, states are required to "adopt laws and regulations to prevent, reduce, and control pollution of the marine environment from land-based sources. . ." (Art 207).
Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal (BC)[4]	1989/2019	Aims to prevent environmental and health damage by hazardous waste. It was recently amended to classify certain types of traded plastic waste as hazardous and therefore subject to prior informed consent (PIC) procedure, which is a mechanism for obtaining decisions from importing nations as to whether they wish to receive shipments and for ensuring compliance with these decisions by exporting nations. This amendment will become effective in early 2021.
Code of Conduct for Responsible Fisheries (the Code)[5]	1991	Non-binding instrument to establish principles for responsible fishing and fisheries activities initiated by the Food and Agriculture Organization of the United Nations (FAO). Although plastics are not specifically mentioned, it makes ports and harbors responsible for providing adequate disposal systems for a very problematic type of plastic: fishing nets.
Agenda 21[6]	1992	Non-binding plan of action to be taken "in every area in which human impacts on the environment." It acknowledges the degradation of the marine environment by litter (17.18) and encourages the transfer of technology to maximize environmentally sound waste use and recycling (para 21.23).
Global Program of Action for the Protection of the Marine Environment from Land-based Activities (GPA)[7]	1995	Non-binding mechanism addressing the connectivity between terrestrial, freshwater, coastal, and marine ecosystems. It advises authorities to reduce the amount of litter produced, improve the management of waste, and up-scale recycling (Art 144).
Stockholm Convention on Persistent Organic Pollutants (POPs)[8]	2001	Protects human health and the environment from POPs. The production, use, and disposal of harmful plastic additives can be regulated by this instrument, but the control is limited to plastic substances listed in the convention.
Honolulu Strategy[9]	2011	Non-binding instrument that sets out a number of "soft" goals to reduce marine debris; e.g., capacity building and waste monitoring. It does not define binding plastic pollution reduction targets.
Manila Declaration[10]	2012	Non-binding commitment to develop policies for reduction and control of wastewater, marine litter, and fertilizer pollution. It contains actions to be taken between 2012 and 2016 at international, regional, and local levels.
2030 Agenda for Sustainable Development[11]	2015	Non-binding Plan of Action for people, planet and prosperity. Commits to prevent and significantly reduce marine debris by 2025 (Sustainable Development Goal SDG 14.1). It also has goals related to the treatment of wastewater (SDG 6), waste management (SDG 11), reduction in use of hazardous chemicals (SDG 3), sustainable production and consumption (SDG 12).
New Plastics Economy Global Commitment[12]	2018	Non-binding instrument with 450 + signatories (e.g., companies representing 20% of all plastic packaging produced globally) committed to (1) eliminate all problematic and unnecessary plastic items; (2) innovate to ensure plastics are reusable, recyclable, or compostable; and (3) circulate plastic items to keep them in the economy and out of the environment.

[1]www.imo.org/en/OurWork/Environment/LCLP/Pages/default.aspx; [2]www.imo.org/en/KnowledgeCentre/ReferencesAndArchives/IMO_Conferences_and_Meetings/MARPOL/Pages/default.aspx; [3]http://www.imo.org/en/OurWork/Legal/Pages/UnitedNationsConventionOnTheLawOfTheSea.aspx; [4]www.basel.int/Implementation/Plasticwaste/Overview/tabid/8347/Default.aspx; [5]http://www.fao.org/3/v9878e/v9878e00.htm; [6]https://sustainabledevelopment.un.org/content/documents/Agenda21.pdf; [7]https://www.unenvironment.org/resources/toolkits-manuals-and-guides/global-programme-action-protection-marine-environment-land; [8]http://www.pops.int/TheConvention/Overview/TextoftheConvention/tabid/2232/Default.aspx; [9]http://wedocs.unep.org/handle/20.500.11822/10670; [10]https://www.unenvironment.org/resources/report/manila-declaration-draft; [11]https://sustainabledevelopment.un.org/post2015/transformingourworld; [12]www.newplasticseconomy.org/projects/global-commitment.

- Use of advanced plastic sorting laser technologies in Material Recovery Facilities (MRFs) for production of higher quality polymer bales (Hopewell et al., 2009);
- Banning of unnecessary plastic items, hazardous additives, and polymers (World Economic Forum et al., 2016);
- Support to novel "reuse" business models (Ellen MacArthur Foundation, 2019);
- Investments in innovations to:
  - Switch from fossil fuel-based plastics to renewable and bio-benign alternatives (Zheng and Suh, 2019).
  - Redesign problematic items (e.g., multi-layered packaging, plastic films) to make their recycling or composting economically and technically possible;
  - Improve technologies able to turn plastic waste into food-grade recycled pellets (Pohjakallio and Vuorinen, 2020).

TABLE 3 | Examples of Australian national initiatives to mitigate plastic pollution.

Name	Year	Description
Environment Protection (Sea Dumping) Act 1981[1]	1981	Aims to minimize marine pollution threats by (1) prohibiting ocean disposal of waste considered too harmful to be released in the marine environment and (2) regulating permitted waste disposal to ensure environmental impacts are minimized. Plastic waste is not directly addressed in this Act.
Protection of the Sea (Prevention of Pollution from Ships) Act 1983[2]	1983	Prohibits discharge of certain types of garbage into the sea, including plastics such as ropes, fishing nets, garbage bags, and ashes from burned plastics.
Hazardous Waste (Regulation of Exports and Imports) Act 1989[3]	1989	Regulates the export, import, and transit of hazardous waste to ensure that they are dealt with appropriately so that human beings and the environment, both within and outside Australia, are protected from its harmful effects. Plastic waste is not directly mentioned in the Act.
National Strategy for Ecologically Sustainable Development[4]	1992	Facilitates a coordinated and co-operative approach to ecologically sustainable development in Australia. It does not mention plastics directly. This commitment forms the basis for the 2009 National Waste Policy for Australia (see below).
Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act)[5]	1999	Provides a legal framework to protect and manage nationally and internationally important flora, fauna, ecological communities, and heritage places. It lists "Injury and fatality to vertebrate marine life caused by ingestion of, or entanglement in, harmful marine debris" as a key threatening process, and considers that at least 20 endangered and vulnerable species listed under the EPBC Act may be adversely affected by marine debris.
Australian Packaging Covenant (the Covenant)[6]	1999	The Covenant is an agreement between the packaging industry and Australian governments to reduce the environmental impacts of packaging. Its 2019 strategic plan has four targets to be achieved by 2025: (1) 100% of packaging to be reusable, recyclable or compostable, (2) 70% of packaging recycled or composted, (3) 30% average recycled content across all packaging, and (4) phase out problematic and unnecessary single-use plastic packaging through redesign, innovation, or alternative delivery methods.
Threat Abatement Plan for the Impacts for Marine Debris on Vertebrate Marine Life[7]	2009/2018	Postulated under EPBC Act (above), this plan incorporates actions to abate sources of marine debris under a coordinated national approach. It binds the Commonwealth and its agencies to respond to the impact of marine debris on vertebrate marine life, and identifies the research, management, and other actions needed to reduce the impacts of marine debris on the affected species.
National Waste Policy[8]	2009/2018	Provides a framework for collaborative action by businesses, governments, communities, and individuals for waste and resource recovery in Australia. It identifies five overarching principles: (1) avoid waste, (2) improve resource recovery, (3) increase use of recycled material, (4) better manage material flows, and (5) improve information to support innovation, guide investment, and consumer decisions.
National Environment Protection (Used Packaging Materials) Measure 2011[9]	2011	Aims to reduce environmental degradation arising from the disposal of used packaging and conserve virgin materials through the encouragement of waste avoidance and the re-use and recycling of used packaging materials by supporting and complementing the voluntary strategies in the Covenant (see above) and by assisting the assessment of the performance of the Covenant.
Reef 2050 Plan[10]	2015	Overarching framework for protecting and managing the Great Barrier Reef, which was recognized as a World Heritage property in 1981. It includes actions to protect the marine environment and wildlife from harmful materials and debris and allocation of funds toward increasing community engagement in Reef protection through activities such as coastal clean-ups.

[1]https://www.legislation.gov.au/Details/C2016C00778; [2]https://www.legislation.gov.au/Details/C2004C00098; [3]https://www.legislation.gov.au/Details/C2017C00194; [4]http://www.environment.gov.au/about-us/esd/publications/national-esd-strategy; [5]https://www.environment.gov.au/epbc; [6]https://www.environment.gov.au/protection/waste-resource-recovery/publications/australian-packaging-covenant-2017; [7]https://www.environment.gov.au/system/files/resources/e3318495-2389-4ffc-b734-164cdd67fe19/files/tap-marine-debris-2018.pdf; [8]https://www.environment.gov.au/protection/waste-resource-recovery/national-waste-policy; [9]https://www.legislation.gov.au/Details/F2011L02093; [10]https://www.environment.gov.au/marine/gbr/long-term-sustainability-plan.

As demonstrated in this study, Australia is both an emitter and receiver of plastic pollution to/from neighboring countries. Therefore, it needs a robust international engagement strategy to complement current National Policies (e.g., Australian Government, 2019). If building responsible recycled plastic trade is deemed to be more beneficial than solely relying on isolated Australian-only operations, agreements involving transboundary deployments of technical and financial resources in waste management and recycling ecosystems should become major priorities. This would ensure regional-scale economic, social, and environmental targets are reached and associated risks minimized (Government of Indonesia, 2017; ASEAN, 2020; DFAT, 2020). Of paramount importance here is for Australia to be accountable for the quality and appropriate management of shipped waste by the receiving entities. This could be effectively implemented via the application of traceability technologies (e.g., Blockchain) enabling tracking of plastic waste movements and transactions under a harmonized material code classification and requiring trade players to adhere to a comprehensive set of requirements in order to participate (e.g., sufficient waste management infrastructure). This type of tracking technology could also assist LMICs with implementing a plastic waste import tax to fund the development of their domestic solid waste management infrastructure. If successfully implemented, responsible international recycled plastic trade could be economically and environmentally beneficial to both Australia and importing nations due to a holistic improvement in regional waste management ecosystems. By improving waste management both domestically and in LMICs importing recyclables, Australia could simultaneously (1) decrease the

proportions of Australian plastic waste mismanaged and landfilled, (2) contribute to LMICs sustainable development via boosting their waste management and creating jobs, and (3) substantially decrease loads of plastic leakages to oceans. Of upmost importance to the success of international plastic recycling systems is for HICs such as Australia to support LMICs by drastically improving the collection and sortation of their own domestic plastic waste; otherwise, the local recycling operations will continue to rely on overseas feedstock and the leakages to the environment will remain substantial.

Finally, even if Australia stops exporting plastic waste overseas as recently announced by the federal government (Australian Government, 2019; CoAG, 2020), there would still be a need for strong foreign engagement given the substantial OP sources surrounding Australia and the occurrence of overseas OP in Australian waters (MOFA, 2011; ASEAN, 2017, 2019a,b). It is time for Australia to show stronger environmental leadership on the international stage by facilitating and supporting existing and future transnational cooperation (IORA, 2017) and perhaps co-funding waste management infrastructure where it is most needed. In the same way Australia and other HICs use foreign policy aid to help build schools and hospitals in LMICs, we can contribute to implementing better waste management in places where the gap between social development and said infrastructure is the highest (Schnurr and Walker, 2019). Of particular relevance here is our study's demonstration of the strong connectivity between the Australian and Indonesian plastic crises. Indonesia is not only a major player in the Australian plastic waste trade (i.e., ∼22% of Australian plastic waste exports reach Indonesia) but also a major source of OP (e.g., ∼70% of the overseas OP reaching Australia is from Indonesia). Therefore, strong bilateral approaches with Indonesia are particularly important for reducing plastic pollution in Australian waters and surroundings seas (Government of Indonesia, 2017; Marsdon, 2019; DFAT, 2020). Supporting Indonesia will not only benefit Australia's own national interests but may also induce a knock-on effect by encouraging other HICs to show greater global responsibility (Schnurr and Walker, 2019).

FINAL REMARKS

Plastic emissions into the environment have accelerated at a pace commensurate with plastic production. This, combined with the serious flow-on effects of plastic pollution on wildlife, economies, and human well-being, necessitates the urgent development and implementation of legislation and legally binding agreements on the production, use, and management of plastics, capable of keeping pace with the scale of this growing problem. Of upmost importance is for governments to move beyond recommendations and long-term non-binding commitments, and to start supporting transformative options, their implementation, and enforcement efforts, with clear and measurable progress targets and indicators. Since many issues found throughout the lifecycle of plastics are transboundary in nature, eradicating them will require a clear and binding global commitment to eliminate plastic contamination of our water, food, air, land, and oceans. An advantage related to plastic pollution when compared to other globally connected problems is that this issue is highly visible, has no deniers, and is receiving increasing public attention globally. Perhaps the wide acceptance of the urgency to act upon the transnational plastic crisis will act as a lever to pioneer an effective global regime that rapidly and cost-effectively (1) mitigates a serious transboundary problem and (2) serves as a flagship to catalyze similar undertakings on an even more serious issue that also results from our reliance on fossil fuels: climate change. The pace and scale of our solutions to pollution must start to match the pace and scale of emissions.

AUTHOR CONTRIBUTIONS

JR, RG, and LL designed the study and analyzed the data. JR and RG prepared figures. JR, RG, and ET wrote the manuscript. All authors reviewed the manuscript.

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The Role of Legislation, Regulatory Initiatives and Guidelines on the Control of Plastic Pollution

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There has been an exponential interest in the occurrence and potential ecotoxicological consequences stemming from the growing prevalence of (micro)plastics in the environment. This has been especially evident by the increasing concern regarding the visible effects on marine ecosystems, with multiple local, regional, and *trans*-national initiatives developed toward the mitigation of what has been construed as an environmental disaster. However, it is not clear what the benefits – if any – of the multitude of norms, regulations, laws and recommendations that have been proposed and/or implemented in recent years are. Furthermore, many of the proposed laws may be of limited applicability, particularly considering the extent to which plastic occurs in everyday life. Herein, the current regulatory instruments are overviewed, focusing on the existing proposals and the extent to which these are based on the currently available scientific data, as well as the foreseen challenges that may restrain the relevancy and suitability of such legislative proposals.

Keywords: legislation, pollution, plastics, policy, microplastics

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Abbreviations: ABNJ, Areas Beyond National Jurisdiction; ASEAN, Association of Southeast Asian Nations; CBD, Convention on Biological Diversity; CCAMLR, Commission for the Conservation of Antarctic Marine Resources; COBSEA, Coordinating Body on the Seas of East Asia; ECHA, European Chemicals Agency; FAO, Food and Agriculture Organization; GEF, Global Environment Facility; GES, Good Environmental Status; GPA, Global Program of Action; GPML, Global Partnership of Marine Litter; HELCOM, Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area; IOC, International Oceanographic Commission; MARPOL, Convention for the Prevention of Pollution from Ships; MDP, Marine Debris Program; MPPRCA, Marine Plastic Pollution Research and Control Act; MSFD, Marine Strategy Framework Directive, or Directive 2008/56/EC; NGOs, non-governmental organizations; NMDMP, National Marine Debris Monitoring Program; NOAA, National Oceanic and Atmospheric Administration; NOWPAP, Action Plan for the Protection, Management and Development of the Marine and Coastal Environment of the Northwest Pacific Region; OSPAR, Convention for the Protection of the Marine Environment of the North-East Atlantic; PACOL, Pacific Ocean Pollution Prevention Program; PAME, Protection of the Arctic Marine Environment; PEMSEA, Partnership in Environmental Management for the Seas of East Asia; PRF, Port Reception Facility; RAPMaLi, Regional Action Plan on Marine Litter Management; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; ROPME, Regional Organization for the Protection of the Marine Environment (Persian Gulf and the Gulf of Oman); RSC, Regional seas conventions; SAICM, Strategic Approach to International Chemicals Management; SAPEA, Science Advice for Policy by European Academies; SEC, U.S. Securities and Exchange Commission; SPREP, Secretariat of the Pacific Regional Environment Program; UNCLOS, United Nations Convention on the Law of the Sea; UNEP, United Nations Environment Program; UNEP-CAR/RCU, UNEP's Program-Caribbean Regional Coordinating Unit; WHO, World Health Organization.

INTRODUCTION

Perhaps one of the most characteristic features of the proposed Anthropocene epoch is the marked increase of the influence of human activity on Earth since the 1950's (Lewis and Maslin, 2015), reflected, among others, by the beginning of the large-scale manufacture of plastics. The production of these materials has undergone a steady exponential increase in past decades, as highlighted in **Figure 1**, growing from 15 million tons in the early 1960s to approximately 359 million tons in 2018 (PlasticsEurope, 2019), while its production is expected to triple by 2050 (WEF, 2016).

The prevalence of these materials, across an ever-expanding number of industrial sectors, has led to its accumulation in the environment, and, particularly, in Oceans, where it accounts for about 80% of all marine litter (Ryan et al., 2009). The current global pervasiveness and environmental consequences – both known and unknown – of plastics material appear to be drawing an increasing level of interest by scientists, the general public and policy makers. Propelled by the growing number of news reports detailing the visible consequences to marine life, fueled by the immediacy of social networks and popular documentary TV shows, the public concern over the effects of plastics in the oceans has led to the implementation of strict guidelines and policies, although the efficacy of said regulations remains undetermined and no convention focused solely on solving the problem of marine plastic pollution exists, as well as no unified and integrated mechanisms to regulate and control the spread of these materials. Considering that some plastics are classified as carcinogenic and/or mutagenic, including polyacrylonitriles, polyurethanes, polyvinyl chloride (PVC) and epoxy resins (Lithner et al., 2011), and that plastic-associated chemicals have also been shown to be hazardous (Groh et al., 2019), there is the need to create and implement legislation aimed at curtailing, and, ideally, eliminating, the continual growing threats of plastic waste. Hence, these existing regulatory instruments and initiatives, voluntary or legally binding, are overviewed, and the inherent limitations of these, as well as the approaches to overcome such potential restraints, are discussed.

INTERNATIONAL REGULATORY INSTRUMENTS AND INITIATIVES

The United Nations Convention on the Law of the Sea (UNCLOS) of 1982, entered into force in 1994, and commonly referred to as a "Constitution for the Oceans" (Gagain, 2012), constituted an unprecedented attempt at regulating "all aspects of the resources of the sea and uses of the ocean, and thus bring a stable order to mankind's very source of life" (United Nations, 1982). Focusing on a wide range of topics, including navigational rights, territorial sea limits, economic jurisdiction, legal status of resources on the seabed beyond the limits of national jurisdiction, conservation and management of living marine resources, protection of the marine environment, a marine research regime and a binding procedure for settlement of disputes between States, it did not contain any provisions regarding plastic pollution specifically, but rather considered plastic as all other wastes potentially hazardous for the marine environment. UNCLOS is composed of 320 articles, of which 46 (Articles 192-237, Part XII) cover the protection and preservation of the marine environment. For example, Article 210 obliges states to develop frameworks to "prevent, reduce and control pollution of the marine environment by dumping," while simultaneously stating that any signatory State "has the right to permit, regulate and control such dumping after due consideration of the matter with other States which by reason of their geographical situation may be adversely affected thereby" (United Nations, 1982). Hence, because ocean pollution is a transnational issue unrestrained by boundaries and the sources of marine waste, in general, and

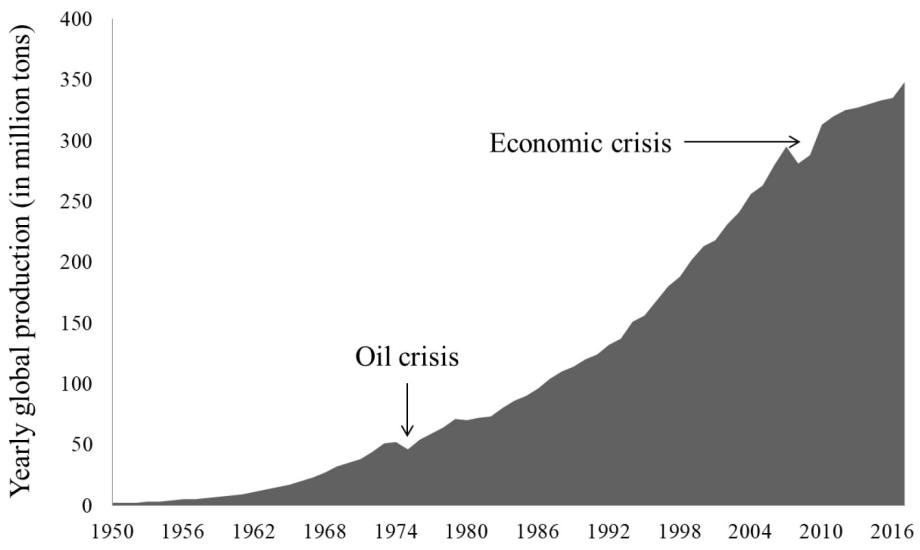

FIGURE 1 | The yearly global production of plastics, from 1950 to 2017. Adapted from Schlanger (2018).

plastic debris, in particular, are often difficult to identify, the measures envisioned by UNCLOS to address the problem of plastic pollution in the ocean seem to be ineffective. Additionally, this rather large and complex document has inherent limitations that mostly stem from basic regional, historic and economic conflicts. Moreover, the United States, a key player in the regional maritime security and environmental protection, is not a signatory state (Bateman, 2007). Non-compliance with the norms and principles of this Convention is somewhat recurrent, and flag states frequently do not fulfill their responsibilities. This sometimes arises from grievances caused by, among others, the added responsibilities by coastal states used for international navigation in, for example, pollution prevention, as well as search and rescue or navigational information and infrastructures, for which UNCLOS makes no provision for compensation. Yet, UNCLOS remains an important landmark and constitutes a *de facto* basis for communication between member states and to initiate processes that, in the long run, may actively contribute to reducing plastic litter entering the environment, depending on the will of member states. Specifically devised toward the prevention, reduction and management of marine debris, the United Nations Environment Program (UNEP) and the Marine Debris Program (MDP) of the U.S. National Oceanic and Atmospheric Administration (NOAA) have jointly developed a global agenda within the last decade. The document includes possible actions aiming at combating the increasing prevalence of marine litter, focusing on "preventing, reducing and abating the ecological, human health and economic impacts of marine debris worldwide" (UNEP/NOAA, 2011). Its application, however, is restricted to the will of participating nations, owing to its non-binding nature to meet the recognized challenge of plastic pollution. Similarly developed for dealing with ocean-based litter pollution, the Convention for the Prevention of Pollution from Ships (MARPOL), (73/78) Annex V, revised in 2012, under the International Maritime Organization, is another key international regulatory instrument (IMO, 1988). This convention requires that all ships to dispose of the generated waste at land based wasted facilities (Vince and Hardesty, 2018), and, as of mid-2016, over 154 states have ratified MARPOL, effectively accounting for approximately 99% of the global annual shipping tonnage. Complimentarily to MARPOL, guidelines have also been devised on the surveying and monitoring of marine litter and on lost, abandoned or discarded fishing gear by the International Oceanographic Commission (IOC) and the Food and Agriculture Organization (FAO) (Crawford and Quinn, 2017). Although flag states – i.e., the jurisdiction under which laws the vessel is registered or licensed, considered as the nationality of the vessel – possess the authority to enforce marine pollution restrictions and prohibitions in international waters, they often lack the resources and/or the will to fulfill their duty (Dewey, 2018). However, it is possible that multinational agreements, such as Free Trade Agreements, could enhance MARPOL compliance (Huang and Hu, 2018), by playing a key role on trade, investment and dispute resolution. More recently, the United Nations Environment Assembly of UNEP gathered in Nairobi (Kenya), and passed a draft resolution on marine litter and microplastics (UNEP, 2017), i.e., plastic particles < 5 mm (NOAA, 2015) that can be either directly discharged into the environment, in the form of, for example, pellets or ingredients of numerous cleaning or hygiene products, or formed once in the environment, by fragmentation of larger particles (da Costa et al., 2017). Broadly, this draft document reflects the recognition that there are multiple "challenges (. . .) addressing marine plastic pollution in the face of increasing production and consumption of plastic in products and packaging." It also urges "all countries and other stakeholders to make responsible use of plastic while endeavoring to reduce unnecessary plastic use, and to promote research and application of environmentally sound alternatives." It is also underlined that both public and private initiatives aimed at curbing pollution are of vital importance, and, in fact, some cross industry agreements have been reached and some companies have also independently have developed efforts in this direction. These include, for example, the agreement for "the prevention of microplastic release into the aquatic environment during the washing of synthetic textiles," proposed by a group of European industry associations representing a collective membership of approximately 180.000 companies (AISE et al., 2017). Individually, some companies are also taking strides toward more environmentally friendly practices, by phasing out single-use plastics, or by cutting down the use of plastic in their products and actively replacing these with refillable recipients (Beament, 2018; Butler, 2018; Eschener, 2019). In the same year (2017), the United Nations proclaimed a Decade of Ocean Science for Sustainable Development, to be held from 2021 to 2030 (United Nations, 2020). The goal of this initiative is broader than the subjects of pollution and plastic litter, focusing on creating and fostering active science-policy interfaces, aimed at facilitating and encouraging the sustainable management of oceans and coastal areas (United Nations, 1982). Whilst still in its preparatory phase (2018–2020), this initiative may greatly benefit from the current increasing goodwill toward the protection of oceans, and the development of science-based policies stemming from multiple, integrated areas of research – physical, geological and chemical sciences, as well as marine biology – may constitute an unique opportunity in ocean conservation.

The Group of 7 (G7) and Group of 20 (G20) have also addressed the issue of marine plastic pollution. From these two groups, action plans have been devised (G20, 2017; G7, 2018). Although the declarations, by the members of these groups, stress the need to develop strategies that promote waste prevention, resource efficiency, sustainable waste management and raising awareness, most of the actions carried out have been, so far, in the form of workshops, organized by the country under which the presidency is and that have served the purpose of highlighting the need to identify better solutions to the issue of marine litter.

REGIONAL REGULATORY INSTRUMENTS AND INITIATIVES

As of June 2019, the European Union (EU) has in force the Directive on the reduction of the impact of certain plastic products on the environment (EuropeanParliament, 2019a), which requires all member states "to ensure environmentally

sound waste management to prevent and reduce marine litter from both sea and land sources." Different strategies are envisioned for the numerous plastics used, including market restrictions and consumption reduction, ultimately leading to the promotion toward the gradual transition to a circular economy of plastics (EuropeanParliament, 2018), through the development and implementation of innovative and sustainable business models, materials and products. However, perhaps the most relevant EU directive relating to marine pollution and debris is the Marine Strategy Framework Directive (MSFD), or Directive 2008/56/EC (EuropeanParliament, 2008), which is an integrated policy aiming at achieving Good Environmental Status (GES) of the European marine environment by 2020. Briefly, the goal of the MSFD is to protect the "resource base upon which marine-related economic and social activities depend" with the specific and explicit regulatory objective of maintaining biodiversity as the cornerstone of such goals. Therefore, the MSFD considers 11 descriptors to define GES, of which marine litter, namely, microplastics (Gago et al., 2016), is included. Furthermore, the GES of all aquatic ecosystems within the European Union are covered by the Directive 2000/60/EC, commonly referred to as Water Framework Directive (EuropeanParliament, 2000b), which has a direct impact over marine litter pollution owing to the fact that it encompasses both coastal waters and estuaries up to one nautical mile from mainland. However, despite the formal definition of GES – "The environmental status of marine waters where these provide ecologically diverse and dynamic oceans and seas which are clean, healthy and productive" – member-states may interpret what GES means in practice, although the EU has made efforts to set out eleven qualitative descriptors describing the environment when GES has been achieved. Moreover, in a subsequent Communication (EuropeanParliament, 2017), the EU added, revised and defined criteria and methodological standards for some of the qualitative descriptors detailed in the MFSD. Nonetheless, the language continues to allow different interpretations, and, therefore, this allows for some discrepancies between the used definitions by member-states. Attendant to MARPOL's Annex V, the EU has put forth specific laws aimed at curtailing and enforcing the adequate disposal of ship-generated litter, known as the Port Reception Facility (PRF) (2000/59/EC) (EuropeanParliament, 2000a), based on the "polluter pays" principle. There has also been a marked effort by the EU in the last 25 years in the development and enactment of regulations devoted to the harmonization of measures regarding the management of packaging and packaging waste, in order to "prevent any impact thereof on the environment and (. . .) [to] ensure the functioning of the internal market" (Directive 94/62/EC) (EuropeanParliament, 2004). However, both the MFSD and the PRF are not without limitations. In the case of the former, for example, it has been noted that EU countries have been responsible for the development of the required tools to implement the devised marine strategies, which may lead to difficulties when comparing the assessments performed by different member-states (Bellas, 2014). Recent proposed changes to the PRF have also led to some concerns as well. For example, a new policy leading to the introduction of a 100% fixed fee (indirect fee) for garbage, as well as for passively collected waste in fishing nets, may lead to the delivery of vast quantities of garbage, including dangerous waste, for a fixed fee. As some have noted (ESPO, 2018), this would constitute a severe divergence from the "polluter pays" principle. Nonetheless, presently, this provision is in force: "no direct fee shall be charged for such waste, in order to ensure a right of delivery without any additional charges based on the volume of waste (. . .); passively fished waste shall be covered by this regime" (EuropeanParliament, 2019b). Similarly, the Convention for the Protection of the Marine Environment of the North-East Atlantic – OSPAR – is a statutory mechanism signed and ratified by the EU and 15 governments intended to promote the cooperation toward the protection of the marine environment. Stemming from this collaborative initiative, specific guidelines for monitoring marine litter on beaches have been devised, which include not only practical advices, but photographic guides and standardized methodologies for the quantification and identification of the sampled litter (OSPAR, 2010). Within the OSPAR Convention, a series of Annexes for specific areas are contained, included for the Prevention and elimination of pollution from land-based sources (Annex I), Prevention and elimination of pollution by dumping or incineration (Annex II), Prevention and elimination of pollution from offshore sources (Annex III), Assessment of the quality of the marine environment (Annex IV), and On the protection and conservation of the ecosystems and biological diversity of the maritime area (Annex V). In the Southern hemisphere, and, more precisely, in Antarctica, the Commission for the Conservation of Antarctic Marine Resources (CCAMLR) was established in 1982 with the patronage of the Fisheries and Aquaculture Department of the Food and Agriculture Organization of the United Nations, 2013 (FAO, 2013). This Convention develops sets of conservation procedures that determine the use of aquatic living resources in the Antarctic, and, given the reported presence and apparent accumulation of microplastics in the Antarctic region (Waller et al., 2017), measures designed to curtail the amount of debris entering this system and to mitigate their impact in the Convention Area have been formulated (CAMLR, 2018). UNEP's regional seas conventions (RSC), launched in 1974, are perhaps the most comprehensive efforts toward the protection of coastal and marine environments, encompassing 18 regions of the World – Antarctic, Arctic, Baltic, Black Sea, Caspian, Eastern Africa, East Asian Seas, Mediterranean, North-East Atlantic, North-East Pacific, Northwest Pacific, Pacific, Red Sea and Gulf of Aden, Persian Gulf, South Asian Seas, South-East Pacific, Western Africa and Wider Caribbean. These programs – which include UNEP and non-UNEP-administered initiatives – engage neighboring countries in comprehensive and specific actions aimed at protecting their common marine environment, through a "shared seas" approach (Neretin, 2018). It comprises a multi-sectorial approach to coastal and marine areas, as well as associated environmental problems, and aims at including governments since the design and inception of the programs, as these states are, ultimately, those that mostly benefit from the implementation of such strategies (Campbell et al., 2017).

Also as a part of UNEP's Regional Seas Program, an Action Plan for the Protection, Management and Development of the Marine and Coastal Environment of the Northwest Pacific Region

(NOWPAP) was adopted by Japan, China, the Republic of Korea, and the Russian Federation in September 1994. The medium-term strategy (2018–2023) envisioned in NOWPAP includes the protection of biodiversity and the active monitoring of marine pollutants, namely, plastic debris (Kim, 2015).

The Wider Caribbean Region is also covered by the Regional Action Plan on Marine Litter Management (RAPMaLi), an initiative conducted by UNEP's Program-Caribbean Regional Coordinating Unit (UNEP-CAR/RCU) with financial support from the Regional Seas Program and the Global Program of Action from UNEP. Reports produced under the RAPMaLi have mostly focused on the need to continuously develop efforts in the education and awareness of the "public, government, NGOs and community groups," encouraging "persons to dispose of waste properly and address the issues of illegal dumping on abandoned beaches and gullies" (Corbin et al., 2014). This action plan also focuses on Small Islands Developing States (SIDS), increasingly more intently engaged in marine pollution issues, owing to the fact that waste disposal infrastructures and resources are limited and inadequate in many of these countries (Vince and Hardesty, 2018). Under the Secretariat of the Pacific Regional Environment Program (SPREP), the Pacific Ocean Pollution Prevention Program (PACOL) Strategy and Work Plans have been developed aimed at a "Cleaner Pacific 2025." This comprehensive blueprint details the waste and pollution management priorities of the region, focusing on the "strengthening [of the] institutional capacity, promotion of public-private partnerships, implementation of sustainable best practices, development of human capacity, dissemination of outcomes and experiences, and promotion of regional and national cooperation" (SREP, 2016). Ideally, the outcomes of the devised strategies and work plans will contribute to reducing and preventing the generation of wastes and pollution in the region, while simultaneously allowing for the recovery of resources.

Mostly centered in the Persian Gulf and the Gulf of Oman, the Regional Organization for the Protection of the Marine Environment (ROPME), formerly known as Kuwait Action Plan, is comprised of legal instrument binding the signatories – Bahrain, Iran, Iraq, Kuwait, Oman, Qatar, Saudi Arabia, and the United Arab Emirates – "to coordinate their activities toward the protection of their common marine environment" (ROPME, 1979). Presently, under the framework of this convention, a protocol concerning the conservation of the biological diversity and the establishment of protected areas is being prepared.

For the East Asian region there are also numerous agreements, projects, and actors, and different action plans are in development. These are under the purview of multiple partnerships and strategies, such as the East Asian Seas Action Plan, the Sustainable Development Strategy for the Seas of East Asia, UN's Global Environment Facility (GEF) International Waters projects, the Coordinating Body on the Seas of East Asia (COBSEA), the Association of Southeast Asian Nations (ASEAN) and the Partnership in Environmental Management for the Seas of East Asia (PEMSEA). Although the initial purposes of these collaborations were mostly focused on the acceleration of both economic growth and social progress, as well as the regional cultural development in through joint endeavors, these have since included international efforts toward arresting the environmental degradation in the region of the Seas of East Asia, with tangible benefits to the environment and to the local inhabitants. At the moment, the East Asian Seas Action Plan is "geared toward cooperation with non-government and government organizations," as well as with the private sector to achieve the goals of a pragmatic "management, conservation, restoration and sustainable use" of the regional marine environment (UNEP-GPA, 2018).

The Baltic Sea is governed by the Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area (HELCOM), signed in 1992. Entered into force in 2000, HELCOM's main goals are the prevention and elimination of pollution, for the ecological restoration of the Baltic Sea, the promotion of the use of Best Environmental Practice and Best Available Technology and to apply the polluter-pays principle. Additionally, the Convention clearly states that its implementation should not cause transboundary pollution outside the Baltic Sea Area (Ehlers, 1993). Under the umbrella of HELCOM, numerous guidelines have been made publicly available on wide range of topics, including for the periodical compilation and reporting of waterborne pollution inputs and monitoring of radioactive substances, to the determination of "heavy metals" in sediments and reprotoxic substances (i.e., substances that may induce reproductive toxicity) (HELCOM, 2019).

The global coverage of these regional action plans on marine litter is summarized in **Figure 2**. RSC and Action Plans are essential for supporting the overall implementation of the Global Program of Action, or GPA, at the national/regional levels. Although the action plans usually consist of identical approaches, each has been devised by the participating governments and local organizations and, therefore, should theoretically reflect their regional, specific environmental challenges, although, consequently, their strategies may vary in scope, legal structure and effectiveness. As noted, some countries are active participants in different action plans, which may be legally binding or merely suggestive of the best practices aimed at curtailing the prevalence of marine litter, and, specifically, plastic debris. However, the described regional plans do not constitute an exhaustive list of the currently implemented programs, although they are certainly illustrative of the existing initiatives and the extent to which multi-national efforts have been put forth toward achieving GES throughout the multiple regions of the world. In **Table 1**, additional regional regulatory instruments and conventions are listed, as well as a brief description of their goals and mechanisms of action.

From the previous paragraphs, it becomes clear that there are numerous regulations, recommendations and laws pertaining to pollution in the Ocean and its regulation. However, for most of these international and regional laws, conventions, agreements and regulations, the main issue remains compliance, particularly in Areas Beyond National Jurisdiction (ABNJ) (Vasilevskaia, 2018; Vince and Hardesty, 2018). ABNJ comprise over 40 percent of Earth's surface, making up 64 percent of oceanic surface and constitute approximately 95 percent of its volume (Kimball, 2005). In these areas, commonly

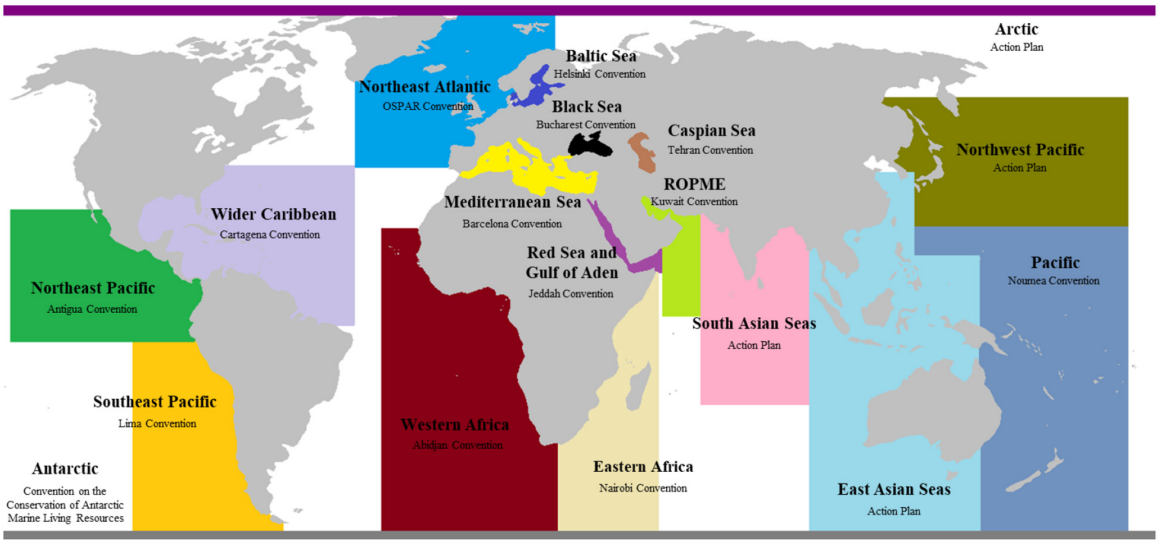

FIGURE 2 | Regional action plans on marine litter. These may vary in the aspects and extent of actions suggested to the states. For example, regional action plans in the Mediterranean include legally binding measures, while, for the Baltic and North Atlantic, these plans are based on sets of essential principles.

dubbed "high seas," no sole nation has the responsibility for its policing, monitoring and management. Consequently, fragmented legal frameworks result in increased vulnerability in these regions. Although UNCLOS encompasses an international legal regime that governs the ocean, it does not effectively aiming at the conservation of the marine environment, it does not include nor describe the specific mechanisms or instruments through which this can be achieved in ABNJ. On the other hand, regional approaches are also insufficient to address this issue, owing to the immense interconnectivity of marine ecosystems, including ocean currents and long migratory pathways. Presently, negotiations are underway to create an "Implementing Agreement" to UNCLOS. If implemented, this agreement could help closing the existing ABNJ governance gaps, providing a tool toward the conservation and sustainable biodiversity in these areas, including environmental impact assessment and pollution prevention (Gjerde et al., 2013). Strengthening cooperation and coordination, as well as regional capacities and the inter-regional cooperation are essential and promising stepping-stones for a successful ecosystem based management approach at a regional scale, including in ABNJ (Rochette et al., 2014). Nonetheless, international and regional accords and regulations should be supported by effective measures implemented at the national level, as these constitute key catalysts for the development of more aware and participating societies in combating pollution.

NATIONAL REGULATORY INSTRUMENTS AND INITIATIVES

At the national level, numerous governments have created legislation focusing on litter, and, particularly, on marine pollution. For example, in the United Kingdom, the Scottish legislature put forth the Marine Litter and the National Litter Strategies, in response to the EU's MSFD (Chen, 2015). To be implemented until 2020, the main goals of these legal instruments are to reduce or – ideally – prevent the incidence of litter through an outreach approach to their citizens, educating them to the dangers posed by these debris, especially within aquatic systems. Furthermore, these goals are set to be achieved by developing both tools and infrastructures, as well as deterrence and enforcement mechanisms (ScottishGovernment, 2013). Concomitantly, the United Kingdom has also developed legislation specifically targeting microplastics (Tagg and Labrenz, 2018), although not yet been subject to a vote (O'Halloran, 2017). In Canada, following the report of up to the significant presence of microbeads in Lakes Eerie and Ontario (Vermaire et al., 2017), the federal government announced its intent to add these materials to its list of toxic substances and declared a ban on the sale, import and production of personal care products containing microplastics (Pettipas et al., 2016). The US has also developed several legal instruments focused on marine litter, namely, the MDP, the Marine Plastic Pollution Research and Control Act (MPPRCA), the National Marine Debris Monitoring Program (NMDMP), the Beaches Environmental Assessment and Coastal Health and Shore Protection Acts (Crawford and Quinn, 2017). Taken together, these consist of multi-disciplinary approaches that aimed at the conservation of the marine and coastal environments, but that also take into consideration public health and human safety, as well as the economy. Federally, the Microbead-Free Waters Act of 2014 has been implemented since mid-2017, with respect to manufacturing, and, as of July 2018, it has been enforced on the introduction into interstate commerce (USCongress, 2015). The Australian government has drafted a proposal aiming at the severe mitigation of marine debris at associated impacts. The document, issued by the Australian Department of the

TABLE 1 | Additional regional treaties, agreements and conventions for the management of marine litter and pollution.

Accord	Brief description
London Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter	In force since 1975, the main objective is to promote control over all sources of marine pollution, whilst simultaneously taking practicable steps to prevent sea pollution. At present, 87 States are Parties to this Convention, which predicts regional cooperation based on mechanisms of action that allow for the full and open exchange of information (IMO, 1996).
Protection of the Arctic Marine Environment (PAME) Working Group	First established under the 1991 Arctic Environmental Protection Strategy, it was continued by the 1996 Ottawa Charter that established the Arctic Council. It is currently developing a Regional Action Plan on Marine litter, with a special focus on plastic pollution.
Basel Convention on the Control of *Trans*-boundary Movements of Hazardous Wastes and Their Disposal	Entered into force in 1992, the Convention is presently signed by 187 of the 195 countries in the World. Its main goal is to reduce the production and toxicity of hazardous wastes, while promoting environmental management and enforcing restrict and highly regulated transboundary movements of hazardous wastes. Recently amended (BC-14/12) in May 2019 enhancing the control of the transboundary movements of plastic waste (UNEP, 2019a).
Convention on Biological Diversity (CBD).	CBD entered into force in 1993 and signed by 168 countries, it is devoted to biological conservation. In 2016, the Conference of Parties urged members to implement within national jurisdiction measures to prevent and mitigate the impacts of marine debris on marine and coastal biodiversity.
Food and Agriculture Organization (FAO) of the UN's Code of Conduct for Responsible Fisheries	Adopted in 1995, this voluntary "code of conduct" seeks to promote long-term fisheries, setting the principles and standards of behavior for the responsible practice of fisheries to ensure the conservation, management and development of living aquatic resources. The Code includes the principle that fisheries should be conducted in manners that reduce the generated waste and minimize the negative impacts on the environment. The voluntary nature of the accord has, however, resulted in limited compliance by the signatory parties (Pitcher et al., 2006).
Strategic Approach to International Chemicals Management (SAICM)	Voluntary, SAICM was adopted in 2006 as a policy framework to promote chemical safety. Presently, SAICM is assessing the possibility of considering plastics as materials of concern, as well as certain plastic additives, that constitute endocrine disruptors (Ripley, 2019).
The Honolulu Strategy	One of the main outcomes of the Fifth International Marine Debris Conference, the Strategy consists of a "framework for a comprehensive and global effort to reduce the ecological, human health, and economic impacts of marine debris globally" (NOAA, 2011). The three main goals include the reduction of the amount of land- and ocean-generated waste, minimization of their environmental impact and reduction of their accumulation on shorelines. As a framework document, the Strategy does not supplant or supersede activities of national authorities, municipalities, industry, international organizations, or other stakeholders; rather, it calls for collaboration and cooperation between all agents based on the common goal and developed and implemented tools.
Global Partnership of Marine Litter (GPML)	Under the auspices of UN Environment, GPML is a multi-stakeholder partnership launched at the UN Conference on Sustainable Development Rio + 20. Its main goal is to "by 2025, prevent and significantly reduce marine pollution of all kinds" (GPML, 2018)
Regional Seas Conventions (RSC)	Currently, 18 RSC exist. These serve as platforms for information exchange and international cooperation on pollution issues. Seven are administered by UN Environment (Wider Caribbean, Northwest Pacific, Mediterranean, East Asian and Caspian Seas, Eastern Africa and Western Africa) and an additional 7 are managed by other organizations [Red Sea and Gulf of Aden, Black Sea, Northeast Pacific, ROPME Sea Area (Persian and Oman Gulfs), South Asian Seas, Southeast Pacific and Pacific] and 4 are independent regional seas (Antarctic, Arctic, Baltic and Northeast Atlantic) (Campbell et al., 2017).

A brief description of each accord is included. The list does not purport to be exhaustive, but merely indicative of the numerous and varying regulatory initiatives in place.

Environment and Energy, specified that over 80% of the surveyed marine debris was comprised of plastic and that the reduction of the release of these materials into the environment, as well as the removal of the existing litter, is not only intended, but necessary (Tilley, 2017). However, perhaps the most emblematic and well recognized measure implemented by governments is the phasing out of lightweight plastic bags or the widely publicized bans on plastic straws (Mosquera, 2019), achieved through either the complete ban or charges on these products. Bangladesh and Denmark were among the first countries in the World to either introduce a charge for these products or a complete ban. In Portugal, for example, a plastic bag tax was implemented in February 2015. Results showed that, 2 years later, a 74% reduction of plastic bag consumption was observed, but accompanied by a simultaneous 61% increase of reusable plastic bags. Furthermore, the consumption of garbage bags also increased, by 12%, due to the fact that, in the past, consumers used the previously untaxed carrier bags as garbage bags (Martinho et al., 2017).

When reviewing the existing public policies on plastic bags, Nielsen and colleagues concluded that 66% reduction in usage was observed in Denmark, and that this value could be as high as 90%. Reduction on the use of plastic bags ranged between 75–90% in South Africa, Hong Kong, Belgium, and the United Kingdom, while in Botswana and China this reduction was of approximately 50% (Nielsen et al., 2019). In India, a ban on plastic bags has been enforced since 2016, with the goal of eliminating all disposable plastic products by 2022 and, in Costa Rica, all single-use plastics, including water bottles, bags and cups, are to be banned by 2021 (Vasilevskaia, 2018). However, perhaps the most evocative and dramatic example on this increasing action against single use carrier bags comes from Kenya, where the local government has not only implemented a total ban on plastic bags, but has also introduced of $40,000 or imprisonment of up to 4 years for the production, sale or use of plastic bags (Harchekar and Kandalgaonkar, 2018), a measure that has also been implemented by Rwanda regarding polyethylene bags (Froidbise, 2015). However, particularly in the case of Kenya, this

ban has created a black market for these products, and smuggling from the neighboring countries, especially across the Ugandan border along Lake Victoria, has threatened to undermine Kenya's ban on plastic bags (UNEnvironment, 2018). Literature detailing specific policies developed for other nations can be found elsewhere (Dauvergne, 2018; Lam et al., 2018; Schuyler et al., 2018) and a recent report by UNEP details the existing bans classified according to continent and country (UNEP, 2018).

Nearly 150 countries have implemented some form of legislation aimed at phasing-out the use of single plastics, and these are summarized in **Figure 3**, which does not illustrate measures not yet in effect.

Determining the actual, measurable effects in public awareness, and changes in behavior is challenging, and, although the strict enforcement of ban may ensure compliance, incremental approaches, such as the introduction of levies and charges, could, in the long term, yield more effective modifications in the motivation and attitudes of consumers, which is necessary for enduring changes in the consumption of single use plastics, such as carrier bags. Particularly in the case of emerging economies, while bans could temporarily alleviate some environmental issues, they fail to address the underlying problems, such as inadequate waste management infrastructures and management. Nevertheless, emerging economies, with little or no plastic production, and very limited recycling capabilities, have led the way an, in fact, proposed ambitions plans, at the international stage, to reduce the production and use of these plastics. For example, at the United Nations Environment Assembly (UNEA) in 2019, India piloted a resolution aimed at phasing out single-use plastics by 2025. The final March 15th declaration removed the "decisive" intentions of the proposed measure and extended to timeline to 2030, committing solely to a "reduction" by that time (UNEP, 2019b).

LOCAL INITIATIVES

At the local level, most actions developed have focused on larger plastics, and, in particular, plastic bags. These include efforts such as those developed, for example, in the city of Buenos Aires, Argentina, which has put in place a measure that allowed for supermarkets to charge for plastic bags, which, according to some estimates, lead to the reduction in their consumption of about 50% (Télam, 2016). Following the apparent success of this enterprise, the city expanded this to a full ban on plastic bags. Other cities and provinces have also implemented bans on the distribution of plastic bags in supermarkets, a measure also enforced by approximately 80 municipalities in Chile. In the US, for example, San Francisco was one of the first cities to introduce plastic bag bans at groceries and supermarkets checkouts. Soon thereafter, bans on single-use plastics were enforced by over 135 state municipalities (Romer, 2007). However, for microplastics, there have been comparatively fewer initiatives developed at the local level. In the US, at the State level, Illinois developed proactive efforts that resulted in this state as the first to ban cosmetics containing microplastics. Soon, other states and counties approved legislation specifically aimed at phasing-out and/or completely eliminating synthetic microplastics, including the counties of Albany, Erie, Chautauqua, Cattaraugus and Suffolk, in the state of New York, and the states of Maryland, Indiana, Colorado, New Jersey, and Wisconsin (Xanthos and Walker, 2017). In Canada, the province of Ontario passed legislation banning the production of microplastics in 2015

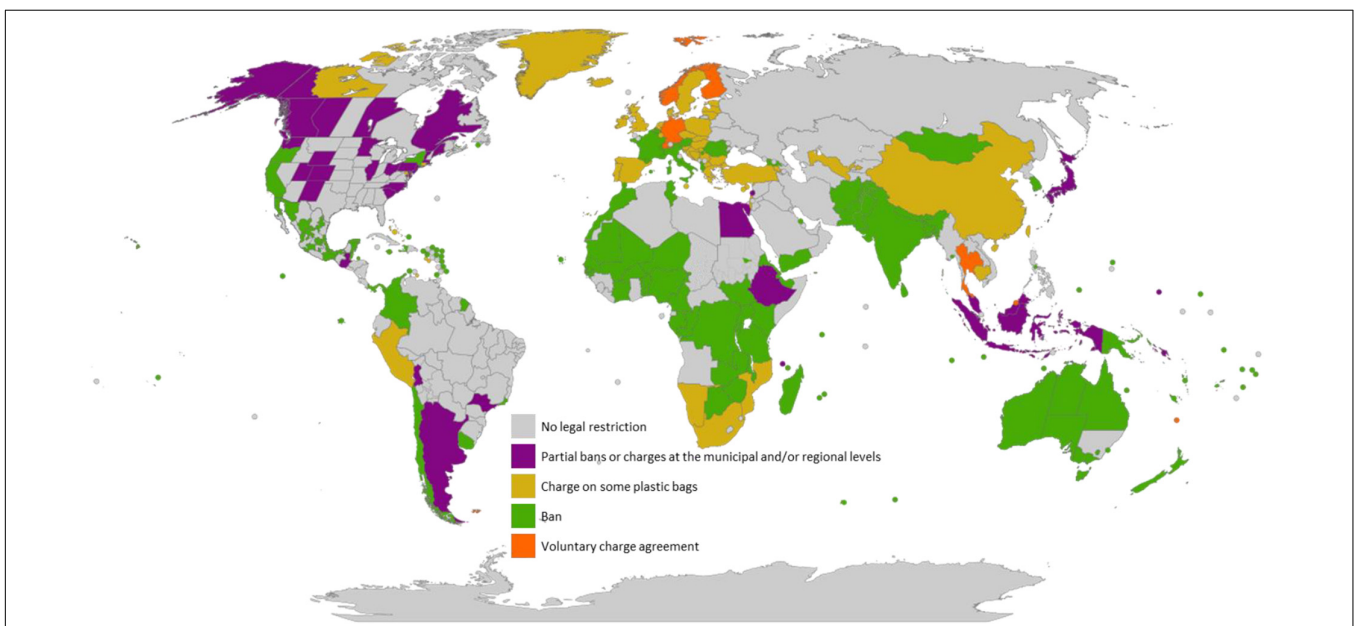

FIGURE 3 | Global current legislative efforts (March 2020) regarding lightweight plastic bag laws. Available from Wikipedia (2020) and available under Attribution-ShareAlike 3.0 Creative Commons (CC BY-SA 3.0).

(Lalonde, 2015). Contrary to all these legislative efforts, in some states in the US, there have been some attempts at classifying the current bans as illegal, effectively imposing a "ban on bans." In fact, some legislators in at least 17 states make such a claim and four states created preemptions during 2019, with two narrowly failing in South Carolina and Alabama. Presently, eight other states (Texas, Colorado, Arizona, Idaho, Minnesota, Michigan, Wisconsin, and Florida) are evaluating the implementation of preemption measures making it illegal to ban single-use plastics (Gibbens, 2019). Simultaneously, legislators defending these bans have begun to draft anti-preemption bills, putting to a vote what can best be described as a "ban on ban of bans." Presently, however, no such bill has significantly progressed, which may pave the way to significant roll-backs and regression of the policies so far imposed.

Though sparse, these local efforts, when combined with those developed at the national, regional and national levels are encouraging policies that may have beneficial environmental impacts. However, the current phasing-out period or recent implementation of these initiatives render their efficiency uncertain, and some doubts remain on how such bans will be implemented and enforced.

REGULATORY INSTRUMENTS CORRELATION AND EFFICACY

All regulatory instruments devised, whether at the local, regional or international level, address pollution through one or more venues of intervention, which, broadly, may be classified as (Chen, 2015):

(1) Preventive – focuses on the 3R rule: reuse, reduction (at sources) and recycling, as well as on multiple land-based management actions;
(2) Removal – debris monitoring and clean-up initiatives;
(3) Mitigation – litter disposal and development of discharge regulations;
(4) Educational – covers awareness campaigns and economic/incentive approaches.

However, such strategies are often developed within specific frameworks and/or by certain organizations or groups with limited coordination between all stake holders. For example, when social movements pushed for the ban of microbeads in the US, companies such as Procter & Gamble and Johnson & Johnson lobbied for legislation that allowed for the inclusion of "biodegradable" microbeads (Dauvergne, 2018). Nonetheless, such "biodegradable loophole" would fail to consider that not only there is an ongoing scientific debate regarding the supposed biodegradability of such materials (Van den Oever et al., 2017), thus undermining the applicability of the ban; furthermore, these biodegradable microbeads would still pose a severe risk to the environment owing to their low degree of biodegradation in deep, cold, and dark waters and the retained ability to leach associated toxins and chemicals. A profound discussion and collaboration between all interested parties could have perhaps lead to a more productive and timely solution, as the proposed use of soluble polyhydroxyalkanoate (PHA) microbeads (Bhattacharya, 2016), or, more recently, the use of salt, jojoba (*Simmondsia chinensis*) beads, ground coffee and diatomaceous earth (Mastrup et al., 2018) in some exfoliating products. This stems from the apparent lack of integration and interaction between the policies developed at the different levels of intervention, often applied in isolation, as depicted in **Figure 4A**. A more desirable approach is depicted in **Figure 4B**, where the focus of the applied regulatory tools and instruments percolate from international to regional, national and local levels. This could help in filling some of the existing regulatory and legislative gaps that exist. If addressed, it may actively contribute to the desired reduction of the prevalence of plastic, in particular, and litter, in general, in the environment. Such gaps include regulatory insufficiency on the scope regarding the existing main sources of plastic pollution (Gold et al., 2014), which vary greatly at the levels of intervention, the present lack of implementation and enforcement of existing regulations and management actions (Chen, 2015), poor international cooperation and insufficient participation of states in regional initiatives (Interwies et al., 2013) and, perhaps more limiting in the development of these legal tools, the current lack of sufficient scientifically gathered data on the prevalence of plastic debris in the environment (da Costa et al., 2017). But there are also inherent national and regional challenges, owing to societal changes, namely, rapid economic growth, urbanization, and changing production and consumption behaviors, that may affect the efficiency of efforts to curtail the prevalence of these materials (Akenji et al., 2020). Moreover, in spite of the implementation of bans, for example, these have sometimes been done with little to no consultation and, in some cases, without national campaigns and/or limited notification (Adam et al., 2020).

THE ROLE OF THE MEDIA AND SOCIAL NETWORKS

In spite of all the aforementioned initiatives, plastic pollution levels continue to increase, and, concomitantly, so has the awareness of not only the scientific community, but of the general public as well. This mindfulness has been propelled by both the "traditional" and "social media," which plays a growing role in everyday life. In the past decade, information technology has fundamentally changed communication, and, presently, social media platforms have been firmly established as immediate and effective forms of sharing information. In fact, this effectiveness has been recognized by regulators, such as capital market regulators, as viable disclosure channels for key information, as evidenced by the decision by SEC (U.S. Securities and Exchange Commission) to allow companies to announce important information in compliance with Regulation Fair Disclosure (Jung et al., 2017). Similarly, the World Health Organization (WHO) has developed Social media toolkits for the release of the WHO reports, such as the "Air Pollution and Child Death" (World Health Organization [WHO], 2018). As a consequence, the public has become more cognizant of the current levels of plastic contamination and of their ultimate consequences, a consciousness that has been continuously

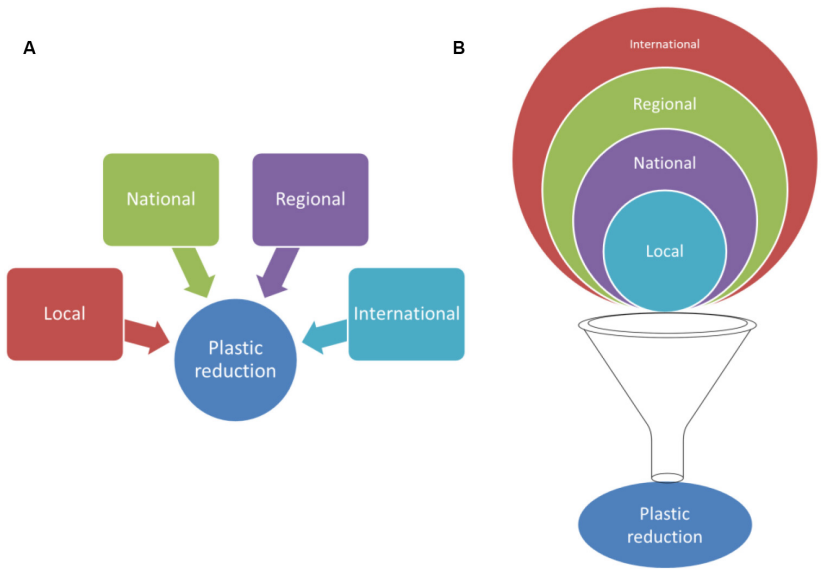

FIGURE 4 | In **(A)**, how, currently, the different levels of regulatory instruments are applied. In **(B)**, how, ideally, such instruments should interact and correlate, effectively constituting the framework of a global approach toward the reduction of plastic waste in the environment.

sustained by continual information dissemination of the hazards these materials pose to the environment (e.g., Carrington, 2016, 2018; Rodrigues and Firmino, 2017; Gosling, 2018). Hashtags are metadata tags that are used on social networks services that allow users to apply user-generated dynamic tagging that facilitates finding messages with specific content or within a specific theme. In the recent past, these tags have been used to popularize different challenges that rapidly reached the status of viral, i.e., that circulated vary fast and widely on the Internet (Baghel et al., 2018). Environmentally, this impact is reflected, for example, in initiatives such as the so-called "cleanup challenge," circulated online through the hashtags "#cleanup" and/or "#trashtag." This challenge, classified as viral, encourages people to clean up litter and participation requires simply the search for a litter-filled area or overflowing trashcan, cleaning, and subsequently sharing "before" and "after" photos online using the aforementioned hashtags. This challenge has been taking place for some years and, according to HashtagsForLikes, a website dedicated to measuring exposure for content and social media profiles, these hashtags are "long-lived," with a total of over fifty thousand unique online posts, on one social media platform alone (Hashtagsforlikes, 2019). As of January 2019, brand24.com estimates a 2.2 million regarding social media reach, based on number of authors, respective followers and average visibility percentage (Brand24, 2019). On a regulatory level, social media has reportedly have shown some traction for tackling air pollution in China. Following the initiative of the Embassy of the United States in Beijing to publish the results of its air quality sensor in 2008, Chinese microbloggers focused their attention on the subject of air pollution and, in 2012, the Chinese government introduced new air quality standards. This was hailed as a victory for these online activists, and broadly considered a significant step toward the emergence of social media as a democratizing force (Kay et al., 2015). Complementarily, web-based and smartphone technologies allow not only to disseminate, but also to gather information. Hence, such technologies can be used, for example, to reach wider numbers of participants in studies focused on assessing pollution, but also the consequences, on a physical and/or psychological level (Zhang et al., 2014a), although such approaches remain largely underexplored and have been mostly used under detailed conditions, such as specific crises (Zhang et al., 2014b). Ultimately, although such online movements may have helped inspiring people of all ages, but particularly young people to save the natural world, the question remains: can social media-based initiatives solve plastic pollution? No, but they can propel much needed changes that are the core of plastic pollution, and these are not at the end of the life cycle of these products, but rather at the beginning. In other words, these movements that are gaining momentum could drive the transition from a plastic-based economy toward an alternative one.

THE ROAD AHEAD

Notwithstanding the growing number of regulatory and legislative initiatives, as well as the willingness to address the already mentioned existing gaps, mostly propelled by an increasing awareness of the general public regarding the risks microplastics pose to the environment, and, ultimately, health, the development of regulatory instruments developed specifically aimed at curtailing the prevalence of these materials is significantly hindered by the lag time between reporting of research results and the subsequent implementation of evidence-based strategies, commonly defined as the "enlightenment function" (da Costa, 2018). Additionally, policies and research are developed within different operating settings, which are

frequently delimited by diverse professional resources, culture and timeframes. Consequently, it becomes necessary to more proactively intertwine the different stakeholders toward the development of evidence-based approaches that are not the end-point of a linear method, but rather a stage of a more circular, dynamic and integrative process, often described as "knowledge brokering" (van Kammen et al., 2006) that may lead to continuous improvements – tweaks – aimed at better achieving the proposed goals. The brokering of knowledge may be accomplished by actively fostering communication between the involved parties, by setting common goals and agendas, by organizing joint forums for researchers and policy makers alike and by establishing the current and future informational needs. In fact, in this regard, there is much that remains unknown. Recently, SAPEA (Science Advice for Policy by European Academies) published a Report reviewing the current scientifically available evidence on the issue of microplastics with the aim of informing the European Union Commission's Group of Chief Scientific Advisors (SAPEA, 2019). The conclusions detail the mix of consensus on the pollution and impacts of microplastics and how, in spite of the informed extrapolation, significant speculation and many unknowns remain, reflecting "both the immaturity of the field and its intrinsic complexity" (SAPEA, 2019). The overall scientific conclusion does point toward the (temporary) conclusion that microplastic pollution does not constitute a widespread risk. However, if uncontrolled, microplastic pollution, combined with its long-term persistence and irreversibility, will lead to effect concentration thresholds that will effectively constitute a widespread risk within a century. It is therefore not surprising that the report exhorts the development of "reasonable and proportional measures" aimed at preventing the direct release of microplastics into the environment and their formation from the fragmentation of larger plastics. Policy tools, including bans and taxation, are just some of the solutions required to address this growing global concern. In the long-term, education, outreach and awareness initiatives regarding the issue of plastic pollution and microplastics, may represent the best strategies and approaches. This can be achieved by actively developing and implementing education and outreach programs to modify behavior (Kershaw et al., 2011). Ocean, pollution, and waste management education in schools could prove to be of great value, particularly, in the long term, as education and behavioral changes of children represent an important source of social influence among their parents, peers and local communities (Hartley et al., 2015).

Another important step is that under consideration by the European Chemicals Agency (ECHA). Currently under "opinion development," a proposal has been submitted considering restriction options under REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) to address the potential risks of microplastics. The proposed restriction scenario aims at restricting the use of intentionally added microplastics to consumer or professional use products of any kind (ECHA, 2019). Furthermore, the considered working definition was deemed to be applicable to all polymers, and not solely to thermosets and thermoplastics – i.e., plastics that are and are not irreversibly molded after the initial forming, respectively (Rennie, 1999). The document focuses on numerous fields of applications, including agriculture, cosmetics, paints and coatings, as well as medical/pharmaceutical applications, detergents and the oil and gas sectors. Although only aimed at intentionally added microplastics, if entered into effect, this restriction will greatly contribute to the reduction of primary microplastics into the environment.

For secondary microplastics, reduction strategies are essential tools to reduce the emission of the larger plastic materials from which these smaller particles derive. Consistent and complimentary measures must be continuously implemented to help mitigate plastic pollution.

CONCLUSION

There has been a steady increase in awareness of the environmental, economic, social, public safety and individual health risks posed by (micro)plastic pollution. This has led to the development of numerous and diverse sets of regulatory tools at local, national, regional, and international levels. Whether voluntary or compulsory, bottom-up governance, whilst highly fragmented, has paved the way and made clear advances in reducing some forms of plastic pollution at the global scale. Internationally, the developed efforts vary in scope and range, focusing on the manufacture, commercialization and use of microplastics, while, at the national and regional levels, most initiatives endeavor to curtail plastic pollution by imposing either levies or bans, whether full or partial. Yet, such instruments have insofar been deemed insufficient. Multiple jurisdictions, producers and retailers lag behind and the industry continues to actively fight some of the legislative propositions. At the fundamental research level, there is also the need to gather more data regarding the real prevalence and effects of these materials in both biota and the environment as only such a detailed knowledge will allow the suitable development of adequate and efficient regulations. Bans, corporate commitments and bioplastics will not curtail the current global plastic pollution problem. Ultimately, the best approach for dealing with this issue will include a multitude of multi-tiered approaches. These will inevitably include bottom–up governance, local, national, regional and international hard and soft laws. Better waste management, as well as better infrastructures, are needed. Corporations will have to reconsider the design of their products based on the implementation of a closed, circular economy, considering all stages of their products, from "cradle to the grave." Consumers will also have to adjust their behaviors, and, together with manufacturers, shift toward a culture of reduction, reuse, and recycle. Significant strides will then be possible for the reduction of plastic entering the environment, though the question remains: will that have been enough and on time?

AUTHOR CONTRIBUTIONS

JC researched the subject and prepared the document, based on the idea put forth by AD and TR-S. CM and MC supported

the preparation of the manuscript. All the authors discussed the results and contributed to the final manuscript.

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PAH Sorption to Nanoplastics and the Trojan Horse Effect as Drivers of Mitochondrial Toxicity and PAH Localization in Zebrafish

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Plastics are world-wide pollutants that pose a potential threat to wildlife and human health. Small plastic particles, such as microplastics and nanoplastics, are easily ingested, and can act as a Trojan Horse by carrying microorganisms and pollutants. This study investigated the potential role of the Trojan Horse effect in the toxicity of nanoplastics to the vertebrate model organism, zebrafish (*Danio rerio*). First, we investigated if this effect could affect the toxicity of nanoplastics. Second, we analyzed if it could contribute to the biodistribution of the associated contaminants. And third, we focused on its effect on the mitochondrial toxicity of nanoplastics. We incubated 44 nm polystyrene nanoparticles with a real-world mixture of polycyclic aromatic hydrocarbons (PAHs) for 7 days and removed the free PAHs by ultrafiltration. We dosed embryos with 1 ppm of nanoplastics (NanoPS) or PAH-sorbed nanoplastics (PAH-NanoPS). Neither type of plastic particle caused changes in embryonic and larval development. Fluorescence microscopy and increased EROD activity suggested the uptake of PAHs in larvae exposed to PAH-NanoPS. This coincided with higher concentrations in the yolk sac and the brain. However, PAH-only exposure leads to their accumulation in the yolk sac but not in the brain, suggesting that that the spatial distribution of bioaccumulated PAHs can differ depending on their source of exposure. Both nanoplastic particles affected mitochondrial energy metabolism but caused different adverse effects. While NanoPS decreased NADH production, PAH-NanoPS decreased mitochondrial coupling efficiency and spare respiratory capacity. In summary, the addition of PAHs to the surface of nanoplastics did not translate into increased developmental toxicity. Low levels of PAHs were accumulated in the organisms, and the transfer of PAHs seems to happen in tissues and possibly organelles where nanoplastics accumulate. Disruption of the energy metabolism in the mitochondria may be a key factor in the toxicity of nanoplastics, and the Trojan Horse effect may amplify this effect.

Keywords: plastic pollution, nanoplastics, microplastics, carriers of organic pollutants, polystyrene, mitochondria, toxicity, zebrafish

INTRODUCTION

Plastics are of significant concern for water contamination and constitute the largest amount of man-made debris in aquatic environments (Koelmans et al., 2015). Degradation of plastics via biotic and abiotic weathering leads to the formation of plastic particles at the micro (≤5 mm) and nanoscale (≤1 μm) (Gigault et al., 2018). Concentrations of microplastics in freshwater and marine ecosystems are very variable, ranging from a few particles to thousands of particles per cubic meter (Hamid et al., 2018). Modeling studies suggest that microplastics ranging from 0.333 to 4.75 mm represent the vast majority of the plastic particles in the ocean, accounting for more than 90% of the total number of plastics in ocean surface waters (Eriksen et al., 2014). But modeling studies may underestimate the potential environmental threat of microplastics and nanoplastics. They are limited by the technical challenge on detecting small microplastics (<0.333 mm) and nanoplastics, the inadequate understanding of aggregation and deposition patterns for microplastics and nanoplastics, and the lack of robust models. For instance, a recent study (Brandon et al., 2019) indicated that microplastic levels can be 5–7 orders of magnitude higher than previously thought (Goldstein et al., 2013) when microplastics as small as 10 μm are included. These values can reach a few million to tens of millions of particles per cubic meter in open ocean waters and nearshore areas (Brandon et al., 2019). These findings suggest that small microplastic particles, and presumably nanoplastics, are very abundant plastic particles and may account for a significant fraction of the problem of plastic pollution. Due to the small size of nanoplastics, detection and removal prove to be difficult, and it facilitates ingestion, tissue penetration, trophic transfer, and interactions with organic matter and other contaminants. Research addressing organismal accumulation and associated adverse effects is of crucial importance. Nanoplastics are probably the least understood compound of marine litter but potentially also the most hazardous one (Koelmans et al., 2015).

The toxicity of nanoplastics has been studied using different animal models, including the zebrafish (*Danio rerio*). Research indicates that polystyrene nanoparticles (NanoPS) smaller than 200 nm are able to cross the zebrafish embryonic chorion through pore canals and they appear to accumulate at first in the yolk sac, brain, retina, and blood vessels (Pitt et al., 2018a; Lee et al., 2019). NanoPS are then further transported to different organs, such as the heart, pancreas, gall bladder, liver, and the intestine. They can ultimately cause bradycardia and larval hypolocomotion (Pitt et al., 2018a). Uptake of nanoplastics can lead to disturbances in energy metabolism, including impaired glucose homeostasis and mitochondrial ATP production (Brun et al., 2019; Trevisan et al., 2019). These findings show that nanoplastics accumulate in early developmental stages, reach different organs throughout development, and have the potential to cause physiological disorders. Most of these results come from animals exposed to nanoplastics through the water. But nanoplastics can also be transferred through the diet (Chae et al., 2018) and via maternal transfer (Pitt et al., 2018b). Thus, their small size can also result in multiple routes of exposure.

Nanoplastics are generally composed of inert polymers but the adsorption of various molecules to their surfaces creates a coat called the surface corona. This has the potential to greatly increase their biological reactivity. The corona can consist of metal ions, polysaccharides, proteins, lipids, nucleic acids, microorganisms, and organic pollutants (Paul-Pont et al., 2018). Their high surface area and significant affinity to hydrophobic compounds suggest that nanoplastics will likely have organic compounds sorbed to their surface in the environment (Koelmans et al., 2015). Thus, nanoplastics can carry toxic environmental pollutants through a Trojan Horse effect mechanism. For example, plastic particles have a high affinity for hydrophobic organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Lee et al., 2014). This Trojan Horse effect can be potentiated by specialized feeding strategies such as filter feeding, what can significantly increase the ingestion of plastic particles and associated contaminants (Fossi et al., 2014). In a previous study from our group, we found that NanoPS interact with a complex mixture of PAHs. This results in decreased levels of free PAHs available for uptake and reduces the acute toxicity of the NanoPS + PAHs mixture to zebrafish embryos (Trevisan et al., 2019). The present study aimed to investigate the sole contribution of the Trojan Horse effect to the transfer of PAHs. This was achieved by using an approach in which NanoPS are the only source of PAHs. We exposed zebrafish embryos to virgin or PAH-sorbed nanoplastics and analyzed their development, accumulation of PAHs, and mitochondrial energy metabolism. We hypothesized that plastics could expose the sorbed contaminants to aging and weathering, resulting in altered toxicity. We also hypothesized that exposure to PAH-sorbed plastics would cause signs similar to PAH exposure, such as cardiotoxicity, increased EROD activity, and PAH bioaccumulation. And as both nanoplastics and PAHs can modulate energy metabolism, we hypothesized nanoplastics loaded with PAHs could cause greater disruption of mitochondrial energy production when compared to virgin nanoplastics.

MATERIALS AND METHODS

Plastic Nanoparticles and an Environmental Mixture of PAHs

Non-functionalized polystyrene nanoparticles (NanoPS) were obtained from Bangs Laboratories, Inc. (Fishers, IN, United States), as a 10% (w:v) stock solution containing 0.1% sodium dodecyl sulfate (SDS) and 0.05% sodium azide. According to the manufacturer, the stock solution (100 ppt) contains 2.158E+15 particles/ml. These same NanoPS were recently characterized in exposure medium (30% Danieau) by our research group, with a hydrodynamic diameter of 44.73 nm, a surface charge of −38.0 mV, a relatively low polydispersity index (0.328), and polymer composition of polystyrene (Trevisan et al., 2019).

The environmental mixture of PAHs consisted of a sediment extract from the Atlantic Wood Industries Superfund site, located in the Elizabeth River (VA, United States), called Elizabeth River

Sediment Extract (ERSE). It was previously collected, processed, and chemically characterized by our laboratory with a total PAH content of 5,073 ng/mL PAHs as a result of 36 different analyzed PAHs (Fang et al., 2014). This solution has been extensively studied in our laboratory as a model environmental PAH mixture with different fish species, including zebrafish (*D. rerio*), Atlantic killifish (*Fundulus heteroclitus*) and medaka (*Oryzias latipes*) (Brown et al., 2016; Riley et al., 2016; Lindberg et al., 2017; Mu et al., 2017; Trevisan et al., 2019). This extract was obtained from sediments collected at the Atlantic Wood Industries Superfund Site (VA, United States), which contained ˜122.6 μg/g dry sediment of total PAHs (Clark et al., 2013). These values are extremely high and close to the values obtained in highly industrialized or urbanized areas across the globe as reviewed by Clark and Di Giulio (2015): Tokyo Bay (∼300 μg/g dry weight), Sidney Harbor (∼400 μg/g dry sediment), and Boston Harbor (∼400 μg/g dry sediment).

Animal Husbandry and Embryo Collection

Laboratory zebrafish (*D. rerio*) were maintained in a recirculating Aquatic Habitats system (Pentair Aquatic Eco-systems, Apopka, FL, United States) on a 14:10 h light: dark cycle. Water quality was maintained at 27.5–28.5°C and pH 7.0–7.5, through the use of carbon-filtered water supplemented with commercial sea salts (60 mg/L; Instant Ocean, Foster & Smith, Rhinelander, WI, United States). The fish were fed twice daily with brine shrimp (INVE Aquaculture, Inc., Salt Lake City, UT, United States) in the morning and Zeigler's Adult Zebrafish Complete Diet (Zeigler Bros., Inc., Gardners, PA, United States) in the afternoon. Breeding crosses of two males and three females were set at 5 PM and embryos were collected the following morning within 1 h of spawning between 9 and 10 AM and kept in a medium of 30% Danieau water (17 mM NaCl, 2 mM KCl, 0.12 mM $MgSO_4$, 1.8 mM $Ca(NO_3)_2$, 1.5 mM HEPES, pH 7.6).

Sorption of PAHs to the Surface of NanoPS

For each exposure independent experiment, two glass flasks containing 40 ml of 30% Danieau water were prepared 1 week before the experiment and NanoPS were added at the final concentration of 10 ppm. One of the flasks also received the ERSE solution at the final concentration of 5% (PAH-NanoPS group), while the other flask did not receive ERSE (NanoPS group). These values were chosen based on data from a previous experiment that suggested the sorption of PAHs to NanoPS at these concentrations (Trevisan et al., 2019). The flasks were incubated at 28°C and 60 rpm for 7 days with a 14:10 h light: dark cycle (**Figure 1A**), after which the solutions were transferred to Vivaspin 300 KDa MWC filters (**Figure 1B**) and centrifuged as recommended by the manufacturer. The samples were washed three times with 30% Danieau to remove free PAHs and the retained fraction containing the nanoplastics was resuspended in 30% Danieau to the nominal concentration of 1 ppm NanoPS and 1 ppm PAH-NanoPS. At this concentration, the expected number of particles is 2.158E+9 particles/ml, close to the highest predicted environmental concentrations for 50 nm nanoplastics particles (between 1E+3 to 1E+9 particles/L) (Lenz et al., 2016).

Exposure to NanoPS and PAH-NanoPS

Zebrafish embryos at 6 hpf (hours post-fertilization) were dosed with freshly prepared NanoPS or PAH-NanoPS solutions as described in section "Sorption of PAHs to the Surface of NanoPS," and an additional group was kept in the exposure solution (30% Danieau) as a control (Ctl) (**Figures 1C,D**). The exposures were carried out in glass Petri dishes at a density of 1 zebrafish embryo/ml. The total number of embryos per group varied according to each assay, as described in the following sections. Animals were exposed to the solutions in an incubator at 28°C and 60 rpm until further analysis.

Exposure to Different Ultrafiltration Fractions of the Fresh and Aged PAH Mixture

Preliminary experiments were carried out to account for possible interferences in the toxicity assays. To investigate if the 7-day pre-incubation period (hereafter called aging) could affect the toxicity of the PAH-NanoPS solution due to alterations in the chemical structure of the PAHs, preliminary acute toxicity assays were carried out with unfiltered (total fraction) 5% fresh ERSE and 5% aged ERSE (**Figures 1E,F**). The aged ERSE solution was prepared in 30% Danieau under the same conditions as described in section "Sorption of PAHs to the Surface of NanoPS": one flask containing 40 ml of 5% ERSE (diluted in 30% Danieau water) was prepared 1 week before the experiment. The flask was incubated at 28°C and 60 rpm for 7 days with a 14:10 h light: dark cycle. Animals were then exposed to this solution (unfiltered 5% aged ERSE) or freshly prepared unfiltered 5% ERSE (freshly prepared in 30% Danieau).

Another set of experiments was performed to investigate the possible accumulation of free PAHs in the filter, which could contaminate and increase the toxicity of the PAH-NanoPS solution. Acute toxicity assays were tested with the retained and eluted fractions of 5% fresh and aged ERSE (**Figures 1E,F**): 30 ml of freshly prepared 5% ERSE (diluted in 30% Danieau) or 5% aged ERSE (prepared as described above) were transferred to Vivaspin 300 KDa MWC filters, centrifuged as recommended by the manufacturer, and the eluted fraction was collected. The retained fraction was washed three times with 30% Danieau to remove free PAHs and resuspended in 30 ml of 30% Danieau. Animals were exposed to these solutions as described above.

Preliminary Assessment of the Developmental Toxicity of Fresh and Aged ERSE Solutions

Zebrafish embryos exposed to the total, retained, and eluted fractions of freshly prepared or aged ERSE solutions were analyzed for survival and deformity rates at 96 hpf. Each experiment consisted of 2 Petri dishes of 10 embryos per group, and each petri dish was considered a biological replicate in a total of three independent experiments (n = 6). Results

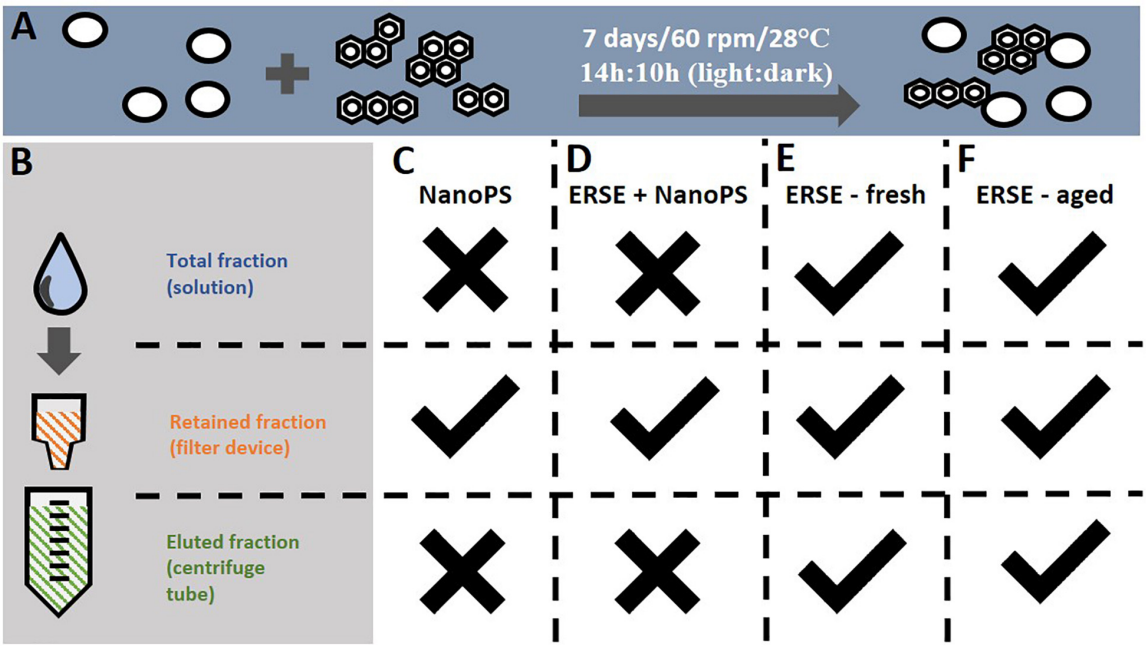

FIGURE 1 | Procedures for the sorption of PAHs to nanoplastics and the exposure groups analyzed in this study. **(A)** Polystyrene nanoparticles (44 nm) were incubated at 10 ppm with a mixture of PAHs (Elizabeth River Sediment Extract, ERSE – 5% v:v) in a glass flask for 7 days at the described conditions. Incubations containing only NanoPS or ERSE were carried out in the same conditions. **(B)** Free PAHs unbound to nanoplastics were removed from the solution using an ultrafiltration device, followed by resuspension of the retained nanoplastics to the final concentration of 1 ppm (NanoPS and PAH-NanoPS groups). **(C,D)** Zebrafish embryos were exposed only to the retained fractions of the NanoPS and PAH-NanoPS solutions. For comparison, embryos were also exposed to the total (unfiltered), retained and eluted fractions of **(E)** fresh 5% ERSE (non-incubated) or **(F)** aged 5% ERSE (incubated for 7 days in the absence of nanoplastics). Checkmark indicates that the respective fraction was analyzed for toxicity while an X indicates the opposite.

of these preliminary experiments were used for design of subsequent studies.

Assessment of the Developmental Toxicity of NanoPS and PAH-NanoPS

Zebrafish embryos exposed to 1 ppm NanoPS or PAH-NanoPS underwent a deformity assessment in the form of yolk sac edema, pericardial edema, and curved tail every 24 h until 96 hpf. The survival rate and hatching rate was determined at the same time points, except that the hatching rate was additionally analyzed at 56 hpf. Each experiment was carried out with three Petri dishes per group, and each petri dish was considered a biological replicate, in a total of three independent experiments (n = 9). The heartbeat rate was measured in these same experiments at 48 hpf in 12 embryos per group (four embryos from each petri dish) (n = 36).

Analysis of PAH Exposure by the EROD Assay

In vivo Ethoxyresorufin-O-Deethylase (EROD) activity was analyzed in 96 hpf zebrafish larvae to estimate PAH exposure in Ctl, NanoPS, and PAH-NanoPS exposure groups. At 24 hpf, embryos were dosed with 0.02 mg/L PTU (phenylthiourea) to inhibit pigmentation. At 96 hpf, embryos were individually transferred to solid white 384 well plates and incubated with 1.5 μM 7-ethoxyresorufin. The plate was incubated in a plate reader at 28°C, and the fluorescence was quantified after 8 h. The assay was adapted from a previously published method (Noury et al., 2006), and the relative EROD activity was calculated by normalizing the fluorescence values to the control group. The experiment was performed with 10 larvae per group per experiment, in three independent experiments (n = 30).

Analysis of PAH Uptake by Fluorescence Microscopy

PAH uptake was estimated in 96 hpf zebrafish larvae in Ctl, NanoPS, and PAH-NanoPS exposure groups via fluoresce microscopy, utilizing the natural fluorescence of PAHs. At 24 hpf, embryos were dosed with 0.02 mg/L PTU (phenylthiourea) to inhibit pigmentation. Larvae were individually transferred to a black clear bottom 96 well plate, anesthetized in 200 μg/mL tricaine (prepared in 30% Danieau), and analyzed for green fluorescence using a BZ-X700 automated fluorescence microscope (Keyence Corporation of America, Itasca, IL, United States) with 470/40 (em) and 525/50 nm (ex) filters. *In vivo* quantitative determination of PAH uptake was performed relative to the control group. Fluorescence was quantified using ImageJ software and expressed as fold change normalized to the control group. The experiment was performed with 6–7 larvae per group per experiment, in three independent experiments (n = 18–21).

To estimate the concentration of PAHs bioaccumulated by the end of the PAH-NanoPS exposure, the fluorescence fold change obtained as described above was compared to the values obtained from animals exposed to unfiltered (total fraction) aged ERSE solution at the concentrations of 0.1, 0.5, 1, 2, and 5%. This experiment was performed with four larvae per group per experiment, in three independent experiments ($n = 12$).

Bioenergetics Analyses

Assessment of oxygen consumption rate and extracellular acidification rate was performed via the XFe24 Extracellular Flux Analyzer (Agilent Instruments, Santa Clara, CA, United States) in 24 hpf embryos or 96 hpf larvae according to previously published protocols (Stackley et al., 2011; Raftery et al., 2017). Animals were staged in an islet capture microplate (24 wells) containing filtered water supplemented with commercial sea salts (65 ppm), at a density of two embryos or one larva per well. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin A, and sodium azide were used to estimate the bioenergetics partitioning, as described in **Supplementary Material**. Each experiment consisted of 7–8 animals per group, in three independent experiments ($n = 21–24$).

Animals were also assayed *in vivo* for NADH production according to a previously published protocol using Alamar Blue (Thermo Fisher Scientific, Waltham, MA, United States) (Williams and Renquist, 2016). Embryos at 24 hpf or larvae at 96 hpf were transferred to a 96 well white plate at a density of two embryos per well or one larva per well, and the fluorescence was analyzed over 24 h at 28°C. The change in fluorescence values between the end and the start of the assay was calculated, normalized to the control group for each experiment, and expressed as relative fluorescence change. Each experiment consisted of 10 animals per group, in three independent experiments ($n = 21–24$).

Statistical Analysis

Data were analyzed for normal distribution and homogeneity of variability by Shapiro-Wilk and Bartletts, respectively. Survival rate and deformity rates after exposure to different ultrafiltration fractions of fresh or aged ERSE or different concentrations of unfiltered aged ERSE were analyzed by Kruskal-Wallis followed by the two-stage set-up method of Benjamin, Krieger, and Yekutieli for controlling the false discovery rate (5%). Additional experiments with NanoPS and PAH-NanoPS exposure groups were analyzed by one-way ANOVA followed by Tukey's *post-hoc* or Kruskal-Wallis followed by Dunn's *post-hoc*. Values were considered statistically significant at $p < 0.05$.

RESULTS

Preliminary Assessment of Aging and Ultrafiltration in the Developmental Toxicity of the PAH Mixture

Initial experiments investigated if the incubation period of 7 days at 28°C could cause aging and weathering effects on the PAHs, affecting their developmental toxicity. Zebrafish embryos were exposed to the total fraction (unfiltered) of freshly prepared or aged 5% ERSE solution. The results shown in **Figure 2** indicate that the total fraction of the fresh PAH solution is highly toxic to early development zebrafish. Exposure to it caused high frequencies of animals containing pericardial edemas (98%) or curved tails (34%). On the other hand, the toxicity of the aged PAHs solution appears to be lower. After exposure, fewer animals presented pericardial edema (36%), but a similar number of them had curved tails (28%).

The effects of the removal of free PAHs by ultrafiltration on the toxicity of the fresh and aged ERSE solution were analyzed. This experiment aimed to investigate if PAHs could unintentionally accumulate in the retained fraction during the ultrafiltration of the PAH-NanoPS solution. Such accumulation could contaminate and overestimate the biological effects of the PAH-NanoPS. No signs of toxicity were detected when animals were exposed to the retained fractions of fresh or aged PAHs solutions (survival rate $> 86\%$ and deformity rates $< 3\%$). These findings suggest that no significant amount of PAHs accumulates in this fraction after the washing steps. Yet, the toxicity of the eluate fraction was slightly lower than the total fraction for both PAHs solutions, probably due to some loss of free PAHs during the ultrafiltration.

Developmental Toxicity Assays With NanoPS and PAH-NanoPS

The preliminary tests ruled out the significant accumulation of free PAHs in the retained fraction. After such confirmation, an initial daily screening for alterations in developmental and morphological parameters was performed in zebrafish embryos and larvae exposed to 1 ppm NanoPS or PAH-NanoPS (**Figure 3**). Results indicated no significant differences for survival, hatching, and deformity rates from 24 to 96 hpf. No signs of cardiotoxicity were detected when the heartbeat was assayed in 48 hpf animals (**Figure 3**).

We measured the larval EROD activity as a qualitative indication of the transfer and bioaccumulation of PAHs. A 1.8-fold increase in EROD activity was detected in larvae exposed to PAH-NanoPS (**Figure 3**). Despite the absence of classic signs of PAH developmental toxicity, this result suggests that bioaccumulation of PAHs occurs after exposure to PAH-NanoPS.

PAH Uptake in Organisms Exposed to Nano-PS or PAH-NanoPS

We used the natural fluorescence of PAHs to further investigate the bioaccumulation of PAHs. Green fluorescence levels were analyzed in 96 hpf larvae through fluorescence microscopy. In line with the EROD data, we detected high fluorescence levels in animals exposed to PAH-NanoPS (**Figures 4A,B**). A 3D surface plot analysis of the green fluorescence (**Figure 4C**) indicates the yolk sac as the main organ for PAH bioaccumulation. This same analysis also indicates that the area corresponding to the brain appears as a possible target. Quantitative analysis indicates a 2.6-fold increase in the fluorescence levels in the whole animal, a

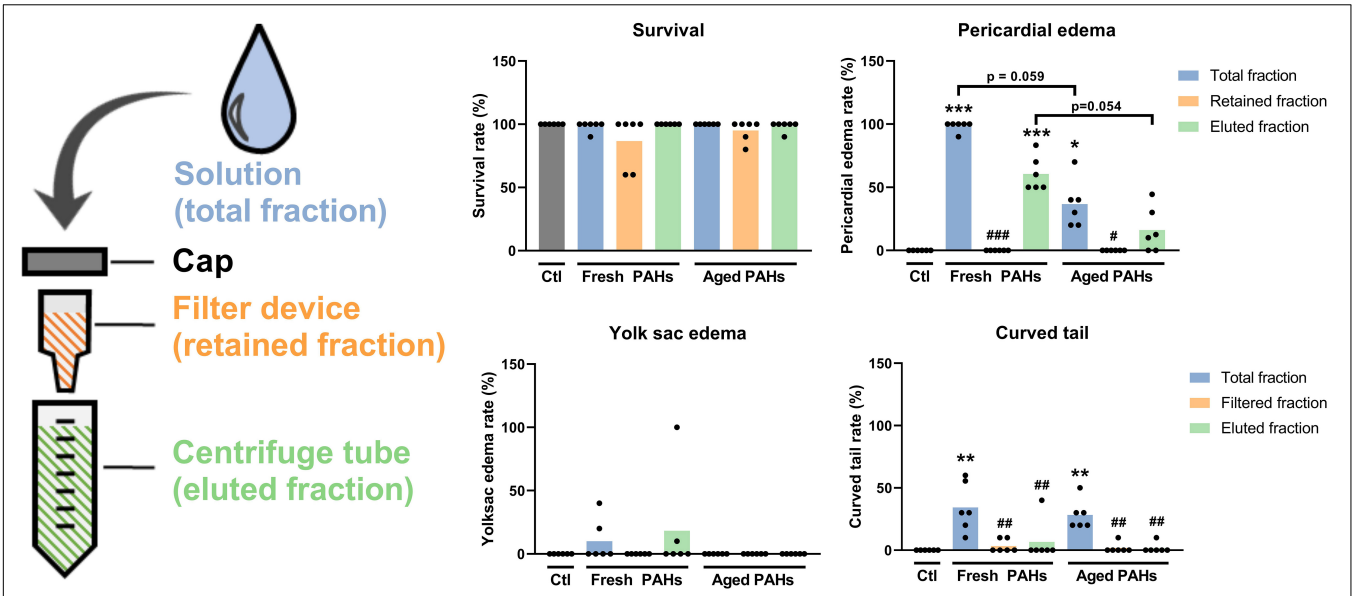

FIGURE 2 | Analysis of the incubation and ultrafiltration on the acute toxicity of the PAH solution to embryonic and larval zebrafish. Animals at 6 h post-fertilization (hpf) were exposed to different ultrafiltration fractions of freshly prepared (fresh) 7 days old (aged) 5% Elizabeth River Sediment Extract as a model environmental PAH solution. The total fraction represents an unfiltered solution, while the retained and eluted fractions are collected by ultrafiltration. Animals were analyzed at the end of the exposure (96 hpf). Data were analyzed by Kruskal-Wallis followed by the two-stage set-up method of Benjamin, Krieger, and Yekutieli for controlling the false discovery rate. Differences relative to the Ctl are shown as *, **, or *** ($p < 0.05$, 0.01, or 0.001) on top of the bars, while differences relative to the respective "total fraction" are shown as $^{\#}$, $^{\#\#}$, or $^{\#\#\#}$ ($p < 0.05$, 0.01, or 0.001) (all p-values were adjusted to the false discovery rate of 5%).

4.0-fold increase in the yolk sac, and a 1.25-fold increase in the brain region of larvae exposed to PAH-NanoPS.

We also estimated the amount of PAHs accumulated in larvae exposed to PAH-NanoPS (**Figure 4D**). The fluorescence levels of organisms exposed to PAH-NanoPS were compared to the ones obtained from direct exposures to aged ERSE. The fluorescence increase in the whole animal and the yolk sac in PAH-NanoPS animals is similar to the increase after exposure to 0.5% aged ERSE. Yet, we did not detect significant accumulation of PAHs in the brain region with aged ERSE concentration as high as 5%. Moreover, the levels of total PAHs accumulated after exposure to 5% aged ERSE were still slightly lower than the ones from PAH-NanoPS animals.

Mitochondrial Bioenergetics of Animals Exposed to NanoPS and PAH-NanoPS

Both nanoplastics and PAHs can affect the mitochondria (Meyer et al., 2013; Lee et al., 2019; Trevisan et al., 2019). Thus, we analyzed the mitochondrial energy metabolism in zebrafish embryos and larvae *in vivo*. A summary of the mitochondrial bioenergetics partitioning can be seen in **Figure 5**. The top panel (**Figure 5A**) shows the different metabolic fractions of the oxygen consumption rates (OCR) analyzed *in vivo* in this study. The total maximum respiration of the organism is comprised of a non-mitochondrial fraction, a mitochondrial basal respiration fraction, and a mitochondrial spare capacity fraction. We can also divide the mitochondrial basal respiration into ATP-linked and proton leak fractions. The bioenergetics fractions in 24 hpf embryos and 96 hpf larvae are shown as the corresponding percentage of the total maximum respiration (sum equals 100%). The statistical analyses of the absolute values of these fractions are shown in **Supplementary Figures S1, S2**.

Figure 5B indicates the effects of NanoPS and PAH-NanoPS on the bioenergetics fractions in 24 hpf embryos. Both exposures seem to increase the fraction associated with proton-leak at the expense of ATP-linked OCR. This can be further analyzed by looking at the mitochondrial coupling efficiency. This parameter represents the percentage of mitochondrial respiration linked to ATP synthesis. Only exposure to PAH-NanoPS caused a significant decrease, from 85 to 79% (**Figure 5C**).

It is not possible to estimate the proton-leak and ATP-linked OCR in 96 hpf larvae. Under our experimental conditions, oligomycin A is not effective at this developmental stage. Thus, only the basal mitochondrial OCR fraction is shown. At this developmental stage, PAH-NanoPS decreased the mitochondrial spare capacity from 29 to 21% of the total maximum respiration. It also decreased the non-mitochondrial OCR fraction from 12.7 to 9.2% (**Figure 5D**).

In addition to the OCRs, we measured the NADH production and extracellular acidification rate (ECAR) *in vivo* in embryos and larvae. In these whole organisms, the ECAR values do not reflect the glycolysis rate due to lactic acid excretion. They are indicative of the excretion of CO_2 from the tricarboxylic acid (TCA) cycle (Stackley et al., 2011). CO_2 will then generate bicarbonate and carbonate in the medium, changing the pH value of the medium. We did not see differences in either of these assays in 24 hpf embryos (**Figure 6**). Yet, 96 hpf larvae had lower NADH production rates when exposed to NanoPS. It was not possible

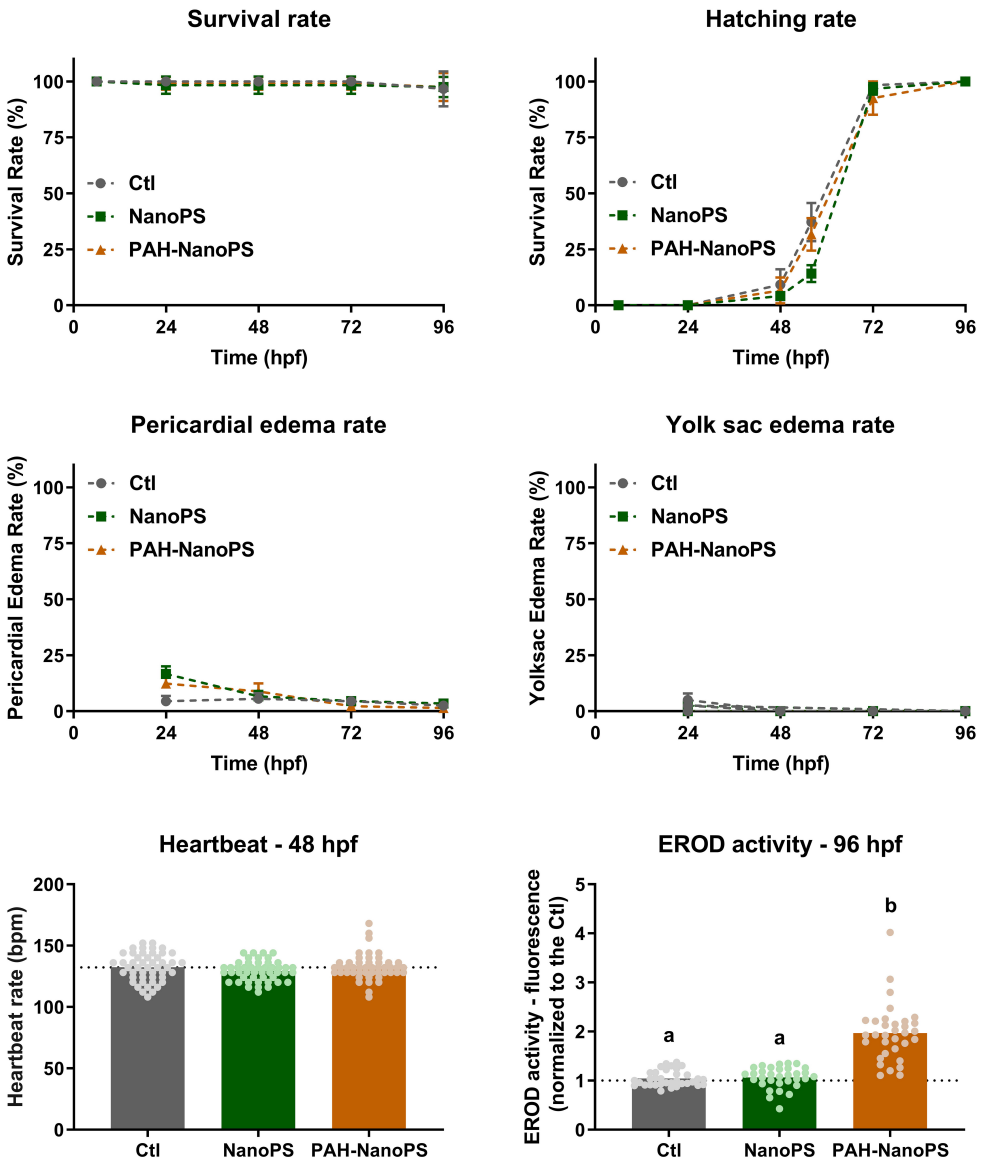

FIGURE 3 | Morphological screening and EROD activity of zebrafish embryos and larvae. Animals were exposed at 6 h post-fertilization (hpf) to 1 ppm polystyrene nanoparticles (NanoPS) or 1 ppm polystyrene nanoparticles with PAHs sorbed to their surface (PAH-NanoPS) (please see **Figure 1** and section "Sorption of PAHs to the surface of NanoPS" for further details). Survival, hatching, yolk sac edema, and pericardial edema were assessed throughout the exposure, while the heartbeat was assessed at 48 hpf and EROD activity was analyzed *in vivo* at 96 hpf. Data are presented as average ± standard deviation (n = 9–12), except for heartbeat (n = 48) and EROD activity (n = 32–35) which are shown as average and scatter plot of individual replicates. The dotted line represents the average of the control group. Survival rate was analyzed by Mantel-Cox log-rank test; hatching, yolk sac edema, and pericardial edema rates were individually analyzed at each time point by one-way ANOVA followed by Tukey's *post-hoc* or Kruskal-Wallis followed by Dunn's *post-hoc*; heartbeat was analyzed by one-way ANOVA followed by Tukey's *post-hoc*. Groups not sharing letters are statistically different ($p < 0.01$).

to measure the ECAR/CO_2 release rates in larvae, as the values were negligible.

DISCUSSION

As previously discussed, plastics have been detected globally in a wide range of environments, including freshwater and marine environments. Land-based activities are major players for marine plastic pollution. Thus, it is not surprising that rivers are classified as main transporters of plastics into the estuaries and the ocean, and that estuarine benthic sediments are important sinks for plastic accumulation (Simon-Sánchez et al., 2019). During this transport, the sorption of organic pollutants to plastic particles can result in the accumulation of chemicals in the estuaries (Fraser et al., 2020) and ultimately, the ocean. In freshwater systems, nanoplastics quickly interact with dissolved organic matter to form microgels, which will be transformed

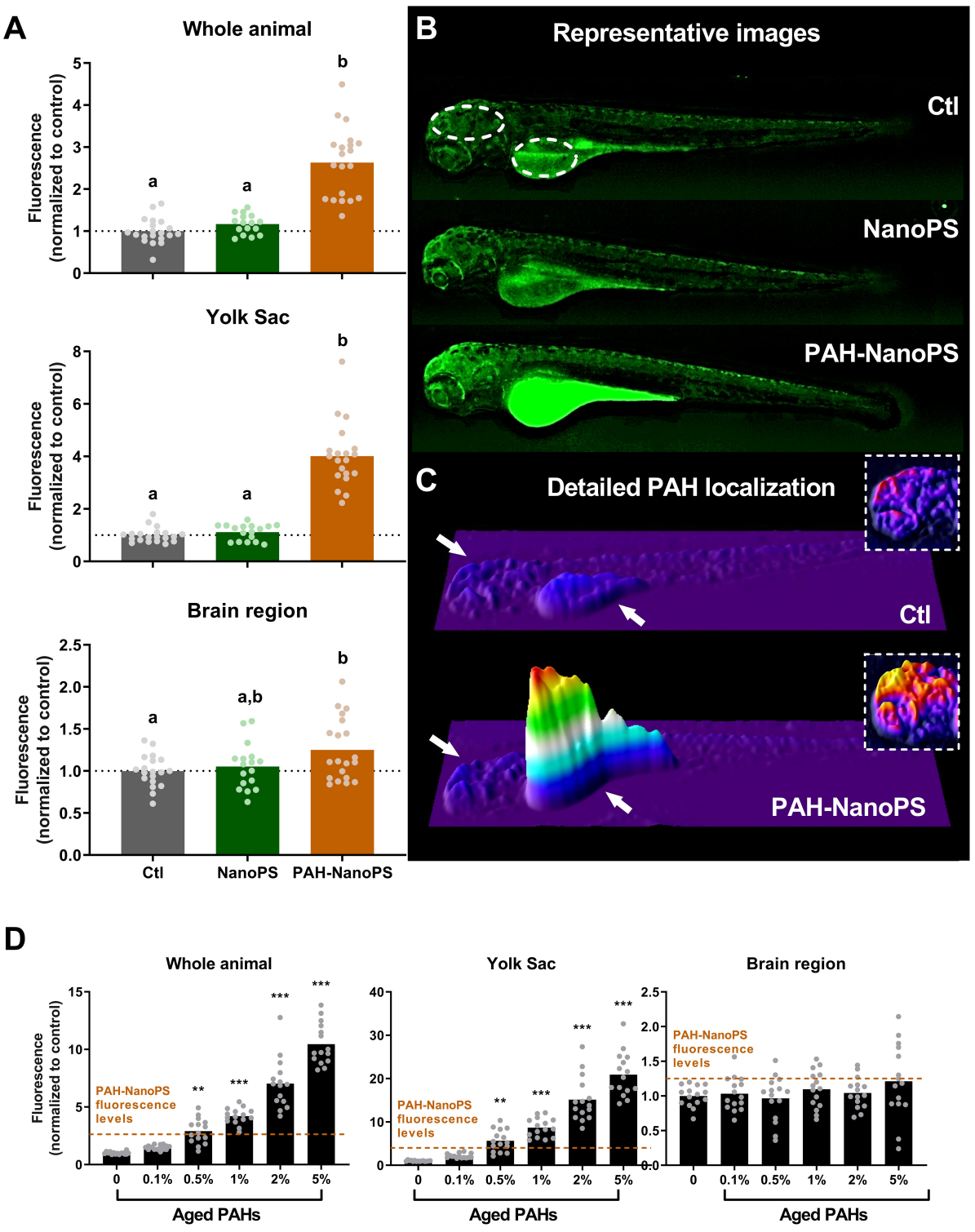

FIGURE 4 | PAH uptake estimated by green fluorescence microscopy in 96 hpf larvae. Animals were exposed at 6 h post-fertilization (hpf) to 1 ppm polystyrene nanoparticles (NanoPS) or 1 ppm polystyrene nanoparticles with PAHs sorbed to their surface (PAH-NanoPS) (see **Figure 1** and section "Sorption of PAHs to the surface of NanoPS" for further details). **(A)** Green fluorescence was quantified in the whole animal, in the yolk sac, and in the brain region, and is shown as average and scatter plot of individual replicates (n = 17–20). **(B)** Representative images are shown in the top, and the dashed circles indicate the individual areas analyzed. **(C)** 3D surface plot representations of the green fluorescence levels indicate that the fluorescence increase related to PAHs uptake occurs mostly in the yolk sac (arrow) and to some extent in the brain region (arrow). The insets (dashed line) represent an estimate of PAH uptake in the brain region with the color scale adjusted

(Continued)

FIGURE 4 | Continued
to that region. **(D)** Green fluorescence levels in larvae exposed to aged ERSE (n = 15). The black dotted line represents the average of the control group, while the dashed orange lines indicate the fluorescence values obtained from animals exposed to PAH-NanoPS. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* or Kruskal-Wallis followed by Dunn's *post-hoc* for **(A)**, and one-way ANOVA followed by Dunnet's *post-hoc* or Kruskal-Wallis followed by Dunn's *post-hoc* for **(D)**. Groups not sharing letters are statistically different ($p < 0.01$) when compared to the Ctl group **(A),** and ** ($p < 0.01$) or *** ($p < 0.001$) represents statistical differences against the Ctl group **(D)**.

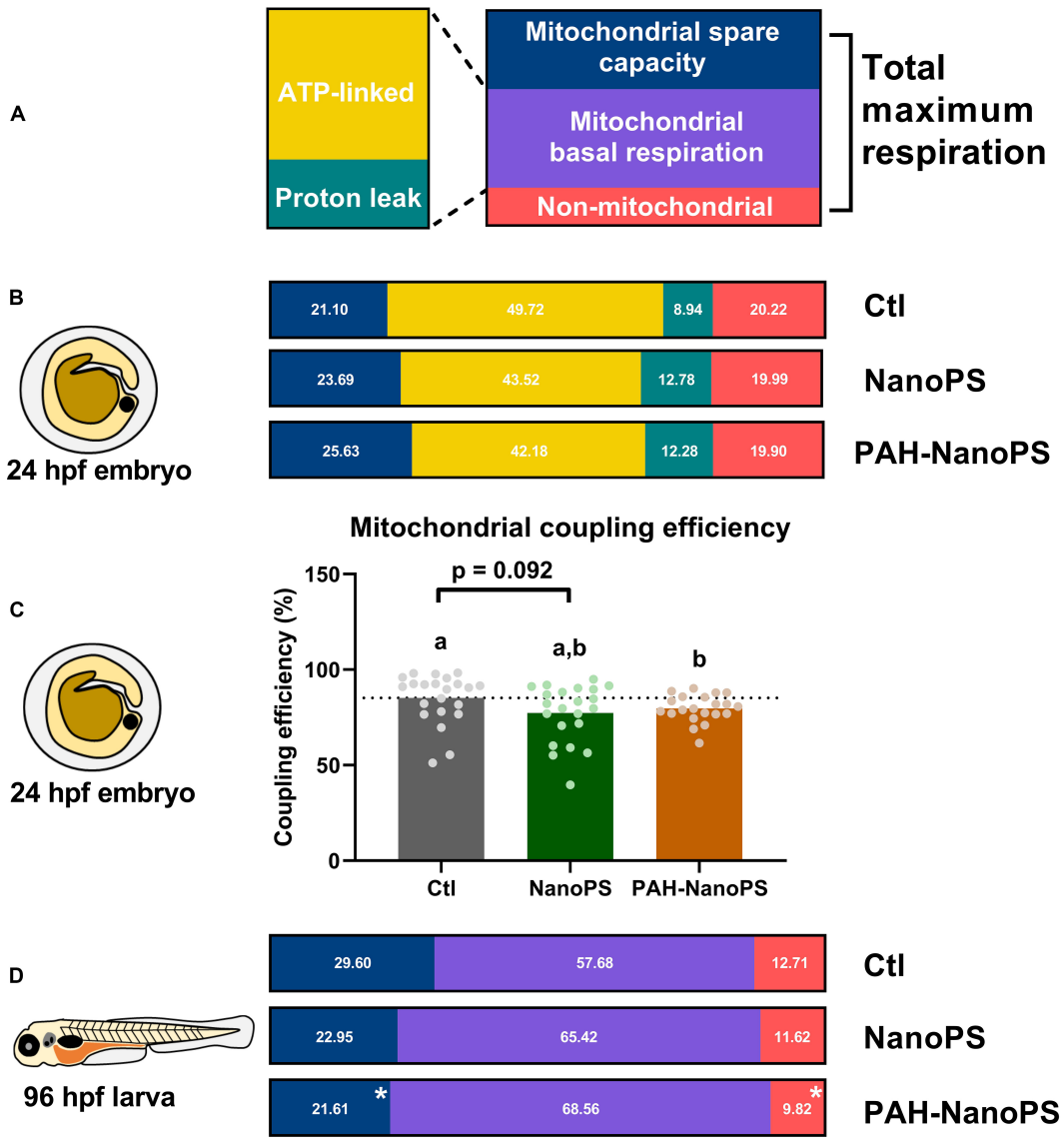

FIGURE 5 | Mitochondrial bioenergetics profiles of zebrafish embryos and larvae. Animals were exposed at 6 h post-fertilization (hpf) to 1 ppm polystyrene nanoparticles (NanoPS) or 1 ppm polystyrene nanoparticles with PAHs sorbed to their surface (PAH-NanoPS) (please see **Figure 1** and section "Sorption of PAHs to the surface of NanoPS" for further details). **(A)** *In vivo* oxygen consumption rates (OCR) in 24 hpf embryos, shown as the proportion of OCR due to the mitochondrial spare capacity, ATP synthase (ATP-linked), proton leak, and non-mitochondrial respiration. The percentages were calculated based on the *in vivo* total maximal OCR values (100%, sum of non-mitochondrial, mitochondrial basal and spare capacity). **(B)** Mitochondrial coupling efficiency of oxidative phosphorylation in 24 hpf zebrafish embryos, shown as average and the scatter plot of individual replicates (n = 21–22). The dotted line represents the average of the control group. **(C)** *In vivo* oxygen consumption rates (OCR) in 96 hpf larvae, shown as the proportion of OCR due to mitochondrial spare capacity, basal mitochondrial respiration (sum of ATP-linked and proton leak), and non-mitochondrial respiration. ATP-linked respiration, proton leak respiration, and mitochondrial coupling efficiency could not be determined in larvae due to their lack of response to the chemical drugs used in the assay. **(D)** Metabolic partitioning of whole animal OCR used in the present study. For the bioenergetics partitioning, * represents statistical difference when compared to the control group ($p < 0.05$), while for the mitochondrial coupling efficiency groups not sharing letters indicate statistical differences ($p < 0.05$). Please see **Supplementary Material** for additional information on the assay, equations for the bioenergetics partitioning, and additional statistical analyses.

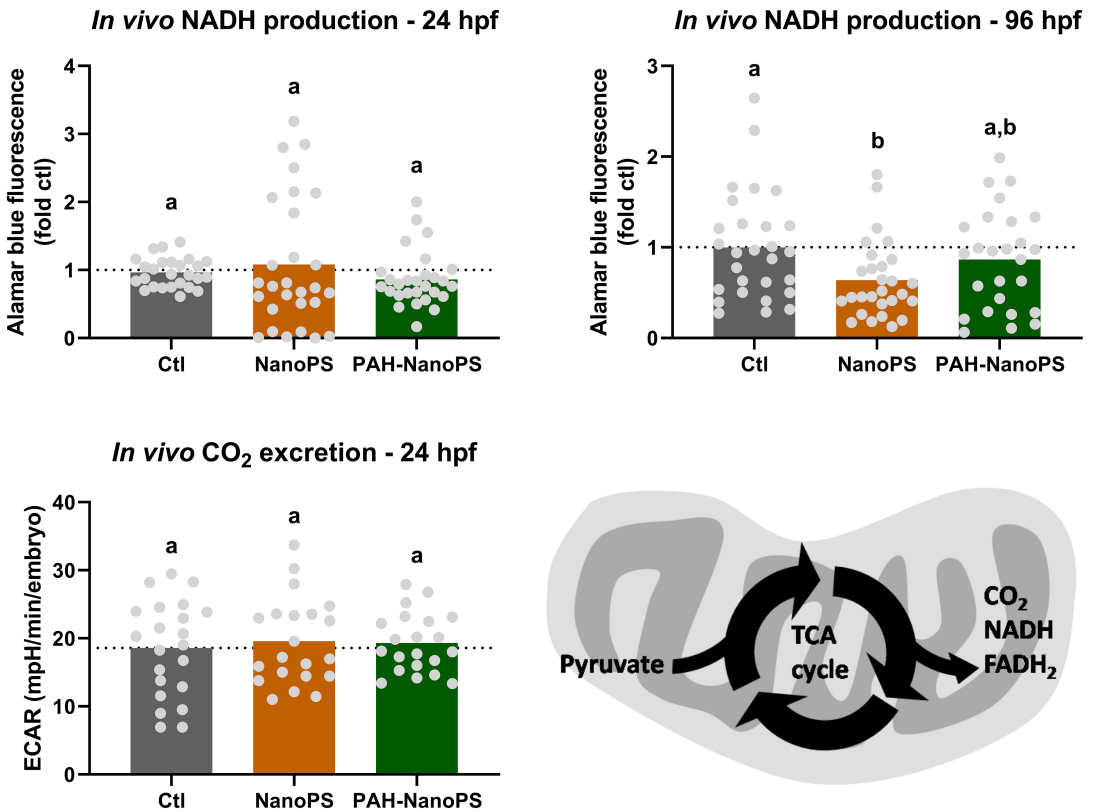

FIGURE 6 | Assessment of the TCA cycle through NADH production and CO_2 release in zebrafish embryos and larvae. Animals were exposed at 6 h post-fertilization (hpf) to 1 ppm polystyrene nanoparticles (NanoPS) or 1 ppm polystyrene nanoparticles with PAHs sorbed to their surface (PAH-NanoPS) (please see **Figure 1** and section "Sorption of PAHs to the surface of NanoPS" for further details). *In vivo* NADPH production was estimated in 24 hpf embryos and 96 hpf larvae (n = 26–28), while CO_2 release from the TCA cycle was analyzed only in 24 hpf embryos (n = 21–22). The dotted line represents the average of the control group. The bottom right image indicates how NADH and CO2 productions can be indicative of the activity of the TCA cycle. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* or Kruskal-Wallis followed by Dunn's *post-hoc*. Groups not sharing letters are significantly different ($p < 0.05$).

into particulate organic matter and facilitate sedimentation and pollutant transport (Shiu et al., 2020). Despite this role of rivers in the fate and transport of microplastics and co-contaminants, there is a large gap between research on freshwater and marine plastic pollution as little is known about plastic particles in freshwater systems. This study aims to provide information on how nanoplastics can be a source of other pollutants in freshwater systems and potentially in estuaries and marine environments. It aims to describe how the transport of such contaminants by nanoplastics in these environments can affect the energy metabolism of embryonic and larval fish, as we used zebrafish as a model organism commonly used to investigate the potential adverse effects of freshwater and marine contaminants.

Many studies have shown that virgin nanoplastics can cause adverse effects in a range of organisms. This includes bacteria, algae, zooplankton, bivalves, copepods, and fish, as reviewed elsewhere (Shen et al., 2019). Many authors suggest that the use of virgin and homogeneous micro or nanoplastics is a limitation for toxicological studies (Horton et al., 2017). But such studies can provide valuable information for future research using other plastic particles and more realistic exposure scenarios. For example, many studies using virgin plastics have investigated the potential role of the Trojan Horse effect. And they have shown that it can increase the overall toxicity of plastics particles (Ma et al., 2016; Yu et al., 2019). Previous data from our group indicated that NanoPS decreases the uptake and the overall toxicity of free PAHs in a co-exposure scenario. This is likely due to the sorption of the PAHs to the NanoPS (Trevisan et al., 2019). The present study further explored this topic by investigating if exposures to PAH-sorbed NanoPS could cause the transfer of PAHS and induce signs of PAH-dependent toxicity in developing zebrafish. In specific, we were interested if such transfer would happen in areas suggested to accumulate NanoPS during early development. And we aimed to investigate if such bioaccumulation could lead to disruption of the energy metabolism.

Our experimental design used nanoplastics as the only source of PAHs during the exposure. The exposure of nanoplastics to PAHs happened at 5% ERSE, ∼253.65 μg/L. These values are considered high when compared to the PAHs levels detected in rivers in urban or industrial areas across the world, such as the Seine River (France, 0.004–0.036 μg/L), Mississippi River (United States, 0.063–0.145 μg/L), Tonghui River (China, 0.192–2.651 μg/L), and Pearl River (China, 6.960–26.920 μg/L)

(reviewed by Cao et al., 2010), but can be found in highly contaminated estuarine and marine environments such as the Niger Delta (Nigeria, 24.390–283.600 μg/L) (Duke, 2008). It is important to note that the total levels of PAHs detected in each study are a result of the PAHs selected for analysis, and therefore the values cannot be easily compared between studies. In our experimental approach, after the sorption of PAHs to the nanoplastics, we removed any remaining free PAHs from solution by ultrafiltration. Preliminary toxicity assays estimated the efficiency of such removal. The results indicated no signs of acute toxicity in the retained fraction of both the fresh or aged ERSE solution in the absence of nanoplastics. This finding suggests that the levels of free PAHs were low or absent after the washing steps. Thus, the use of ultrafiltration should not interfere with the toxicity of the PAH-NanoPS particles. In fact, we detected a possible loss of free PAHs after the ultrafiltration. This is expected as PAHs can sorb to the filter resin (polyethersulfone) (Endo and Matsuura, 2018). But it is important to highlight that this should not affect the toxicity of the PAH-NanoPS solution, as it should not contain free PAHs after the ultrafiltration.

The effects of the 7 days incubation period on the toxicity of the PAHs solution were also analyzed. The aged ERSE solution was less toxic to developing zebrafish than the respective fresh solution. Aging and weathering effects such as volatilization, dissolution, and photodegradation can have direct effects on PAHs. They can decrease their concentrations, change their physicochemical properties, and alter the final composition of PAH mixtures (Stout et al., 2016). The exact mechanisms underlying the lower toxicity of the aged ERSE solution were not investigated in the present study. But our results suggest that weathering and aging of the associated pollutants must be taken into account for laboratory studies. They also point to a significant role of these events on the toxicity of plastics in the environment.

The initial toxicity screening of NanoPS and PAH-NanoPS indicated no signs of adverse effects of these particles. We did not detect changes in the survival, hatching, and deformity rates in zebrafish using the same NanoPS in previous studies (Pitt et al., 2018a; Trevisan et al., 2019). A decrease in heartbeat rate after exposure to 1 ppm NanoPS has been reported previously by our group (Pitt et al., 2018a). These results cannot be easily compared to the present study, in which the NanoPS were pre-incubated for 7 days. But these findings suggest that 35–45 nm NanoPS at the low ppm range do not cause major effects on zebrafish early development. Other studies of NanoPS of similar sizes had comparable results (Lee et al., 2019; Liu et al., 2019).

Sorption of NanoPS with PAHs for 7 days before the exposure period did not lead to acute toxicity or teratogenicity. The environmental mixture of PAHs used in this study (ERSE) is a potent inducer of cardiac toxicity in developing fish (Brown et al., 2016; Riley et al., 2016; Lindberg et al., 2017; Mu et al., 2017; Trevisan et al., 2019). The lack of such findings in animals exposed to PAH-NanoPS suggests, at least, two possible scenarios. One is the low transfer of PAHs through exposure to PAH-NanoPS. And the other is the decrease of the PAH-related toxicity of the PAH-NanoPS due to aging and weathering during the incubation period.

Analysis of EROD activity indicated a possible increase in the body burden of PAHs in animals exposed to PAH-NanoPS. ERSE exposure is known to cause an increase in EROD activity through the activation of the AhR pathway (Fleming and Di Giulio, 2011; Lindberg et al., 2017; Trevisan et al., 2019). Although it does not necessarily correspond to the developmental toxicity of PAHs, EROD activity is a very sensitive biomarker response to exposure to many PAHs (Shankar et al., 2019). Fluorescence microscopy confirmed the bioaccumulation of PAHs in animals exposed to PAH-NanoPS. It occurred mostly in the yolk sac and at minor levels in the brain. This increase in PAH levels in the whole animal and the yolk sac is equivalent to exposures between 0.1 and 0.5% of aged ERSE. At these concentrations, aged ERSE is not expected to affect the development of zebrafish embryos (**Supplementary Figure S3**). This supports the acute toxicity data obtained from the PAH-NanoPS group. Fluorescence microscopy cannot discriminate between PAHs accumulated as free molecules or still sorbed to the nanoplastics. It remains unclear what is the extent of PAH desorption after the uptake of PAH-NanoPS. Regardless, EROD activity serves as a biomarker for increased bioaccumulation of reactive PAHs. Our findings show that, at least, a significant fraction of the bioaccumulated PAHs should be available as free PAHs within the zebrafish. It also remains unclear which PAHs present on the ERSE mixture sorb to the nanoplastics and bioaccumulated in the organisms. And how aging and weathering may have affected the PAH composition of the plastic particles and the profile of PAHs bioaccumulated. Further studies on this topic could offer insights into the organic contaminant buildup in aquatic environments and biota by nano and microplastics.

The bioaccumulation of PAHs in the yolk sac and the brain is not surprising. Both organs can be a target for hydrophobic contaminants due to their high lipid content. An interesting finding was that no significant amount of PAHs bioaccumulated in the brain of larvae exposed to aged ERSE at concentrations as high as 5%. This is the opposite of the data obtained from animals exposed to PAH-NanoPS. PAHs seem to bioaccumulate at higher levels in the brain when the exposure happens through nanoplastics rather than through the water. Nanoplastics accumulate at high levels in the yolk sac and can be further transported to other organs, including the brain and digestive organs (Pitt et al., 2018a; Sokmen et al., 2019). This should ease the transfer of associated contaminants to these organs. Unfortunately, the bioaccumulation of PAHs in the digestive organs of larval zebrafish was not investigated due to the high fluorescence levels in the yolk sac in animals exposed to PAH-NanoPS. Co-exposures of nano and microplastics with other chemicals can alter the biodistribution of the co-contaminants (Chen et al., 2017; Trevisan et al., 2019). Ma et al. (2016) showed nanoplastics accumulate in the gut of daphnids and that co-exposures with phenanthrene increase the accumulation of this PAH in the whole animal (Ma et al., 2016). However, these studies relied on co-exposures of plastics and other contaminants. They could not address if the Trojan Horse effect of plastic particles could cause the transfer of contaminants to specific areas of the organisms. This possibility of targeted transfer of associated contaminants needs to be further investigated. Such an effect can

help to understand the potential risks of plastic particles in a more realistic environmental scenario.

Mitochondria are also targets of both nanoparticles and PAHs (Meyer et al., 2013; Jayasundara, 2017), and our results show that both NanoPS and PAH-NanoPS cause mitochondrial stress. The effects of NanoPS were limited to decreased NADH production in larvae. On the other hand, PAH-NanoPS decreased embryonic mitochondrial coupling efficiency and larval mitochondrial spare capacity. Virgin NanoPS at higher concentration (10 ppm) can decrease embryonic mitochondrial coupling efficiency and increase larval NADH production in zebrafish (Trevisan et al., 2019). Nanoplastics smaller than 100 nm can be easily taken up by cells. After uptake, they interact with the tubulin cytoskeleton and localize within the mitochondria (Johnston et al., 2010). Live-cell imaging indicates that exposure to virgin NanoPS causes rapid uptake and accumulation of nanoplastics in the cytosol. This is followed by interaction and accumulation in reticular and vesicular structures such as mitochondria. Diffusion to the nucleus can also happen, but at a much slower rate (Hemmerich and Von Mikecz, 2013). The mitochondrial toxicity of polystyrene nanoparticles is not limited to effects on energy metabolism. It also includes mitochondrial swelling, loss of mitochondrial membrane integrity, and release of cytochrome c. This toxicity depends on nanoparticle's surface chemistry and corona (Ruenraroengsak and Tetley, 2015).

The effects on mitochondrial bioenergetic fractions happened only in animals exposed to PAH-NanoPS. PAHs can decrease ATP production and content by negatively affecting mitochondrial coupling (Du et al., 2015; Lindberg and Di Giulio, 2019). PAH exposure can also decrease mitochondrial spare capacity *in vitro* and *in vivo* (Bansal et al., 2014; Raftery et al., 2017). As the effects on these parameters were limited to the exposure to PAH-sorbed polystyrene nanoparticles, we suggest that the Trojan Horse effect can also determine the fate of associated contaminants at the subcellular level. It is possible that nanoplastics can transfer environmental pollutants to target organelles, including the mitochondria. The release of these chemicals could then cause more severe adverse effects. Mitochondria are associated with numerous metabolic pathways, not only with ATP production and the TCA cycle, but also fatty acid oxidation, amino acid metabolism, nitrogen balance and elimination, calcium signaling, and the biosynthesis of heme, steroid hormones, pyrimidines and purines (Hu et al., 2019). The potential toxicity of nanoplastics and associated contaminants to the mitochondria suggest that exposure to these plastic particles can have direct effects on many cellular systems. For example, exposure to virgin NanoPS decreased the levels of metabolites related to the TCA cycle and amino acid metabolism in the nematode *Caenorhabditis elegans* (Kim et al., 2019). It also disrupted the expression of genes associated with energy metabolism in the copepod *Daphnia pulex* (Liu et al., 2018). But further studies are necessary to shed light on the effects of nanoplastics on mitochondrial metabolism and their physiological consequences, and how the Trojan Horse effect can aggravate this scenario.

As recently suggested, there is an urgent need for a more comprehensive view of ecotoxicology with a focus on the biological fitness of an organism instead of lethal and sublethal effects (Straub et al., 2020). While additional studies with multigenerational and reproductive approaches would be necessary to achieve such a goal, this study indicates that energy production can be a key component of the ecological impacts of pollutant-sorbed nanoplastics. It is known that mitochondrial integrity and function are essential for organismal performance and fitness and that mitochondria can be more sensitive to pollutants than natural stressors (Sokolova, 2018). Impaired mitochondrial bioenergetics can lead to decreased larval growth (Bolser et al., 2018), altered larval behavior (Zhang et al., 2017), and possibly lower reproduction rates (as discussed by Dreier et al., 2019). These are key ecological outcomes linked to animal fitness. Based on our data and the use of mitochondrial Adverse Outcome Pathways (as reviewed by Dreier et al., 2019), we hypothesize that the exposure of freshwater organisms to high concentrations of pollutant-sorbed nanoplastics (at the ppm range) can potentially impact the ecosystem via the disruption of energy homeostasis at the organismal level. And that long-term exposures to this scenario could cause populational effects by decreased reproduction rates and population decline. It is important to note that in estuarine and marine environments, sedimentation of nanoplastics due to salinity and particulate organic matter can partially protect free-swimming organisms from waterborne exposures to nanoplastics. But it remains unclear if the trophic transfer of nanoplastics and associated contaminants or their accumulation in sediments in these environments could trigger similar effects in energy homeostasis of free-swimming or sessile organisms, respectively.

In summary, our results suggest that aging and weathering events can play an important role in plastic toxicity. They can not only affect the plastic particles but also the chemical structure and composition of contaminants bound to them. This can result in different toxicity profiles and should be taken into consideration for laboratory and field studies. The sorption of PAHs to nanoplastics has significant effects on their toxicity. In this study, it did not affect fish early development, but as expected, nanoplastics acted as carriers of environmental pollutants. Like nanoplastics, PAHs accumulated in lipid-rich environments. It occurred primarily and in the yolk sac, but significant levels were also found in the brain. We hypothesize that the Trojan Horse effect can also happen at the tissue and organelle level, with plastic particles mediating the transfer of PAHs to specific parts of the organism. The sorption of PAHs also increased the toxicity of the nanoplastics to the mitochondria through a decrease in their efficiency to produce energy. Our findings also indicate that developing fish exposed to nanoplastics maintain their ability to survive. However, the presence of PAHs in their surface could potentially affect the nervous system and the ability of organisms to operate in high-energy demand scenarios.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Duke University Animal Care and Use Program.

AUTHOR CONTRIBUTIONS

RT designed the experiment and wrote the main manuscript. RT and DU performed the experiments. RD contributed with the reagents, materials, and analysis tools. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTAL MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00078/full#supplementary-material

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Coupling Gastro-Intestinal Tract Analysis With an Airborne Contamination Control Method to Estimate Litter Ingestion in Demersal Elasmobranchs

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This study aims to assess the litter ingestion in some demersal elasmobranchs, combining a classical gastro-intestinal tract (GIT) analysis with a procedure methodology to reduce airborne fibers contamination. In order to prevent the overestimation of litter ingestion, we applied severe mitigation measures to avoid airborne contamination during the analyses, integrating a new approach for the correction of estimates of fibers abundance using control procedure. In this study, we assessed the anthropogenic litter ingestion in four elasmobranch species from the southern Tyrrhenian Sea: *Scyliorhinus canicula* (n = 27), *Etmopterus spinax* (n = 16), *Galeus melastomus* (n = 12), and *Raja clavata* (n = 6). The GIT of each specimen was analyzed by visual sorting and the polymers identified by Fourier transform infrared spectroscopy technique. Overall, 19 litter particles were found in the GIT of 13 demersal elasmobranchs (%O = 21) and for the first time, evidence of litter ingestion by *R. clavata* in Mediterranean waters was also reported. In *G. melastomus* and *R. clavata* all anthropogenic particles were plastics, whereas in *S. canicula* other litter categories were also found. No litter ingestion was instead observed in *E. spinax.* More than 50% of litter particles belonged to microlitter category (<5 mm). Polyamide was the only polymer typology found in all examined species. We described the procedures to control the airborne contamination applied at each step of laboratory analysis and, thanks to the application of our control method, it was possible to exclude the 95% of fibers found in samples from the assessment. Moreover, we compared fibers abundances observed in samples and controls. This study, combining an approach for minimizing the bias associated to airborne fiber contamination, provided a reliable assessment of marine litter ingestion in demersal elasmobranchs.

Keywords: selachians, GIT analysis, microplastics, FT-IR spectroscopy, polymers, airborne contamination control, Mediterranean Sea

INTRODUCTION

In the last decades, the marine litter (ML) pollution has generated many concerns about the potential global implications on marine environment and organisms (Laist, 1997; Kühn et al., 2015; Rochman et al., 2016). The increasing development of both coastal and maritime human activities is one of the main reasons of the ML ubiquity in all marine habitats, from the beaches to the open ocean and seafloor (Galgani et al., 2015).

Ingestion of anthropogenic debris represents one of the main threats for marine fauna (Galgani et al., 2013a; Fossi et al., 2018) and, in particular, plastics are the most common litter found in the stomach contents of marine organisms (Anastasopoulou and Fortibuoni, 2019). Organisms can intentionally ingest ML, because debris particles are mistaken or confused as prey, or accidentally eat debris during foraging activity (e.g., filter feeders) (Kühn et al., 2015; Romeo et al., 2015, 2016). It is also known that secondary ingestion occurs in the marine environment during the predator–prey interaction, when predators consume ML contaminated prey (Chagnon et al., 2018; Nelms et al., 2018; Welden et al., 2018). Ingested ML may cause physical/mechanical damages in tissues and induce toxicological harm in marine species, affecting several levels of the trophic web (Rochman et al., 2013, 2014; Pedà et al., 2016; Fossi et al., 2018). The semi-enclosed basin of the Mediterranean Sea is largely affected and threatened by ML pollution and several monitoring programs and mitigation actions have been launched in order to reduce its impacts. In this context, the Marine Strategy Framework Directive (MSFD; EC 2017/848) aims to achieve the Good Environmental Status (GES) in European waters, i.e., to ensure that "*the amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned*" (Descriptor 10, criterion D10C3). Moreover, Member States are required to follow standardized methods for monitoring and assessment the amount and composition of litter and micro-litter ingestion in the following groups: birds, mammals, reptiles, fish, or invertebrates.

In the Mediterranean basin, ML ingestion in marine organisms has been documented in both invertebrates (Fossi et al., 2014; Alomar et al., 2016; Digka et al., 2018a) and vertebrates (Anastasopoulou et al., 2013a; Romeo et al., 2015, 2016; Battaglia et al., 2016; Giani et al., 2019; Schirinzi et al., 2020), including endangered species (Campani et al., 2013; Fossi et al., 2014), and a large number of these studies regarded fish, including cartilaginous species (Deudero and Alomar, 2015; Fossi et al., 2018; Anastasopoulou and Fortibuoni, 2019).

So far, several methods have been used for the extraction of ML from fish [e.g., visual sorting and chemical digestion protocols of gastro-intestinal tract (GIT)] as well as for ML quantification, categorization and polymer identification (e.g., visual identification, infrared or Raman spectrometry) (Romeo et al., 2015, 2016; Alomar and Deudero, 2017; Digka et al., 2018a; Giani et al., 2019; Rios-Fuster et al., 2019; Capillo et al., 2020; Schirinzi et al., 2020). However, despite the growing number of scientific publications, data on the occurrence, amount and categorization of litter ingested by fish are often not comparable due to the lack of harmonized protocols (Hermsen et al., 2017; Bray et al., 2019; Giani et al., 2019). In addition, another crucial issue concerns the need for the application of standardized methodologies to ensure quality assurance and quality control for the ML analysis in biota (Torre et al., 2016; Hermsen et al., 2018; Kühn et al., 2020). Some authors have shown that contamination by airborne fibers may represent a serious problem in studies on ML ingestion in marine fauna (Torre et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020), leading to the overestimation of ML pollution and also to potential erroneous conclusions (Torre et al., 2016). Contamination prevention measures have only recently been introduced in ML studies (Lusher et al., 2013; Romeo et al., 2016; Hermsen et al., 2017; Digka et al., 2018a; Giani et al., 2019; Capillo et al., 2020; Schirinzi et al., 2020). However, these procedures are not often applied during all steps of laboratory analysis or they are poorly described or reported (Hermsen et al., 2017; Kühn et al., 2020).

For these reasons, this study aims to couple a standard GIT analysis with an airborne contamination control method, in order to estimate ML ingestion in some demersal elasmobranchs from the southern Tyrrhenian Sea (GFCM Geographical Sub-Area – GSA 10): *Scyliorhinus canicula* (Linnaeus, 1758), *Etmopterus spinax* (Linnaeus, 1758), *Galeus melastomus* Rafinesque 1810, and *Raja clavata* Linnaeus, 1758. This methodology is applied in order to reduce ML overestimation originated from airborne fiber contamination.

This paper also provides useful information on the typology and features of ML ingested by these Mediterranean demersal elasmobranchs, providing data which could be used for the assessment of the ML impact on these predators.

MATERIALS AND METHODS

Study Area and Fish Collection

A total of 61 individuals of demersal elasmobranchs (27 *S. canicula*, 16 *E. spinax*, 12 *G. melastomus*, and 6 *R. clavata*) were collected from bycatch in trawl and longline fisheries in the western Mediterranean Sea (southern Tyrrhenian Sea, GSA 10; **Figure 1**) during 2015. *R. clavata, G. melastomus*, and *E. spinax* were caught by bottom trawl on a seafloor ranging from 570 to 680 m, whereas *S. canicula* was caught by bottom longline at 340 m depth.

Elasmobranch species were identified according to taxonomic features reported by Compagno (2001); Serena et al. (2010) and individuals were measured to the nearest 0.1 cm (total length, TL) and weighed to the nearest 0.1 g (total weight, TW; Tab.1). Then, they were stored at −20°C, before the laboratory analyses.

Visual Sorting and Litter Quantification

In the laboratory, the GIT of each specimen was removed, transferred to a glass petri dish and analyzed by visual sorting, performed under a stereomicroscope Zeiss Discovery V.8. Ingested ML items were separated and categorized following the Litter Categories for marine Biota, reported in the Marine Strategy Framework Directive (MSFD) protocol (MSFD Technical Subgroup on Marine Litter, 2013). Then, ML items were counted, weighed (in grams to the nearest 0.0001 g),

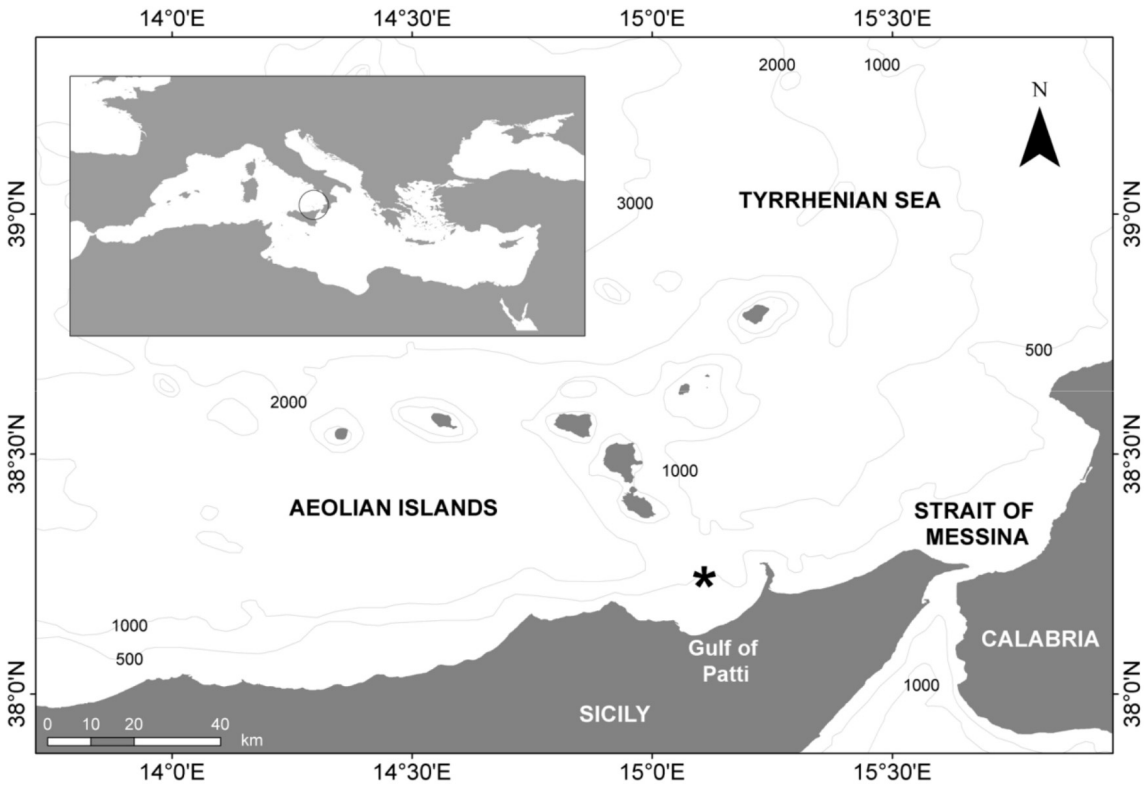

FIGURE 1 | Map of the study area in the western Mediterranean Sea (southern Tyrrhenian Sea), showing the sampling location (*).

measured (length and width in mm) and photographed using a stereomicroscope Zeiss Discovery V.8. coupled with AxioVs40 version 4.8.2.0 digital image processing software.

Marine Litter Identification and Classification

Marine litter items were analyzed by Fourier transform infrared (FT-IR) spectroscopy technique to identify their polymer nature. Fourier transform infrared polymer analysis was carried out using the Agilent Cary 630 spectrophotometer supplied with specific polymer libraries (Agilent Polymer Handheld ATR Library, Agilent Elastomer Oring and Seal Handheld ATR Library, Agilent Polymer, POLY_D, ATR Demo Library). According to Fossi et al. (2017) and Bernardini et al. (2018), three repeated measures were performed for each ingested ML item, setting up to 80% the level of similarity, in order to compare the sample spectra with ones contained in the software database. Only polymers matching reference spectra for more than 80% were accepted. Identified plastics were classified based on their color, shape (sheetlike, threadlike, foam, fragment, and other typologies), and size range (micro: <5 mm; meso: 5–25 mm; macro: >25 mm), according to literature (Galgani et al., 2013b; MSFD Technical Subgroup on Marine Litter, 2013; Romeo et al., 2015; Schirinzi et al., 2020). Marine litter abundance indices were calculated for each species as follows:

1. Litter and plastic percentage of occurrence (O%) was estimated as the proportion, on the total sample, of the individuals which ingested litter and plastics: (%O = N. individuals which ingested litter and plastics/N. total samples × 100);
2. Average number of plastic items found in the GITs, calculated on the total number of individuals (N. plastic items/N. all examined individuals);
3. Average number of plastic items found in the GITs, calculated on the total number of individuals which ingested plastics (N. plastic items/N. individuals which ingested plastics).

Airborne Fiber Contamination Control Method

Rigorous contamination mitigation measures were adopted during the laboratory analysis in order to reduce the risk to overestimate fibers (**Figure 2**). Air currents were reduced through closing of windows, doors, and air conditioners and all researchers wore 100% cotton lab coats. During the study, only glassware and metal equipment were used and all laboratory instruments, including dissection tools such as tweezers, scalpels and scissors, were cleaned with filtered water whenever samples were examined. Specimens were washed using filtered water (0.45 μm) before GIT removal and each step of analysis (from the GIT dissection to the GIT content separation) was performed

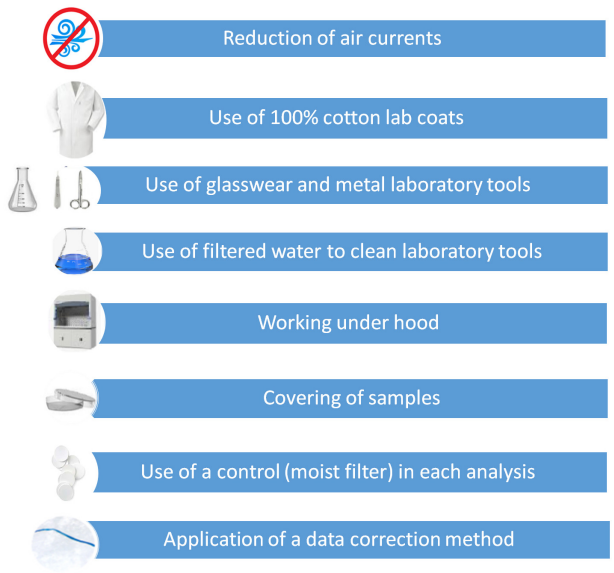

FIGURE 2 | An overview of adopted mitigation measures to reduce airborne contamination in samples.

under the fume hood. In order to limit airborne contamination, Petri dishes containing samples were kept covered using a clean glass cover, while moving from the fume hood to the stereomicroscope.

In parallel to the analysis of each sample, a control procedure was used for the assessment of airborne fiber contamination: a cleaned filter, moistened with filtered water (to simulate the same conditions of the wet GIT content), was put in a control glass Petri dish and maintained near the Petri containing the sample, during all operations under the fume hood and during visual sorting at the stereomicroscope. The number of fibers observed in both samples and control Petri dishes was recorded (and here named as observed fibers and control fibers).

The airborne fiber contamination control followed the procedures reported in **Figure 3**. In order to exclude the airborne fiber contamination, for each species, we statistically tested the difference in fiber abundance between samples and controls, using the Wilcoxon rank sum test. When p-value is <0.05, the comparison indicates that there are significant differences between observed and control fibers; then, they may have different origin and observed fibers should be considered in the ML assessment, without control fiber detraction. Otherwise, when p-value is > 0.05 the output indicates that control and observed fibers are probably coming from the same source, suggesting a potential contamination. Therefore, the correlation test (we used Kendall's rank based on the nature of our data) was conducted to establish the best method to detract the number of control fibers from the observed values. If the results of test Kendall's rank test do not show significant correlation, the mean number of fibers in the control sample should be detracted from observed fibers, according to Kühn et al. (2018). In contrast, if significant correlation is observed, the total number of control fibers should be detracted from observed fibers in samples. In addition, as last step, to further reduce the potential bias associate to the entire detraction of fibers, according to Hermsen et al. (2017) and Schirinzi et al. (2020), only the control fibers having similar features (i.e., structure, color) to observed fibers in GITs are detracted (**Figure 3**).

This statistical approach allows to exclude the potential airborne fiber contamination from the results of ML assessment, achieving a more reliable assessment of ML ingestion. Statistical analyses were performed using R and R-Studio software (R Core Team, 2019; RStudio Team, 2019).

RESULTS

Marine Litter Ingestion

A total of 61 specimens of demersal elasmobranchs were examined. **Table 1** shows the size and weight ranges of individuals and the corresponding mean values for each species. Overall, 19 ML particles were found in the GITs of 13 individuals (%O = 21.3): 9 items in *S. canicula* (%O = 22.2), 6 items in *G. melastomus* (%O = 33.3), and 4 items in *R. clavata* (%O = 50), whereas no litter ingestion was observed in *E. spinax*. In *G. melastomus* and *R. clavata*, the 100% of anthropogenic particles found in the GIT were identified as plastics, while in *S. canicula* only the 56% of them belonged to this category and the remaining 44% were chicken remains and polyacrylamide particles, classified as other rubbish (%O = 11.1) and pollutants waste (%O = 33.3), respectively (**Table 2**).

A total of 15 plastics items (0.25 items/specimen; range: 0–2 items per specimen) have been ingested by 11 elasmobranch specimens (%O = 18) (**Table 2**). In **Table 2**, the length, width and weight ranges of ingested plastics for each species are also reported. The average length and width of all plastics recovered from fish were 19.42 ± 50.44 and 1.42 ± 1.37 with a range from 1.28 to 200 mm and from 0.01 to 3.91 mm, respectively. The average weight was 0.01 ± 0.02 g, varying between <0.0001 and 0.0738 g (**Table 2**).

Characteristics and Polymers Typology of Plastics

Based on their size, plastic items found in the GITs of demersal elasmobranchs mainly belonged to microplastics category (53.4%), but also mesoplastics (33.3%) and macroplastics (13.3%) were found (**Figure 4A**).

The shape of plastic items (**Figure 4B**) was quite variable between species; threadlike was the most common plastics in stomachs *G. melastomus* (50%) and foam was the most abundant shape category in *S. canicula* (40%). The proportion of each of the following shape categories in GITs of in *R. clavata* was 25%: sheetlike, threadlike, fragment, and other plastic typologies (dense rubber).

White and transparent were the most frequent colors of the ingested plastics, but also blue, red, and brown items were found (**Figure 4C**).

The FT-IR spectroscopic analysis allowed to identify the following plastic polymers items: 2 polyethylene (PE), 2

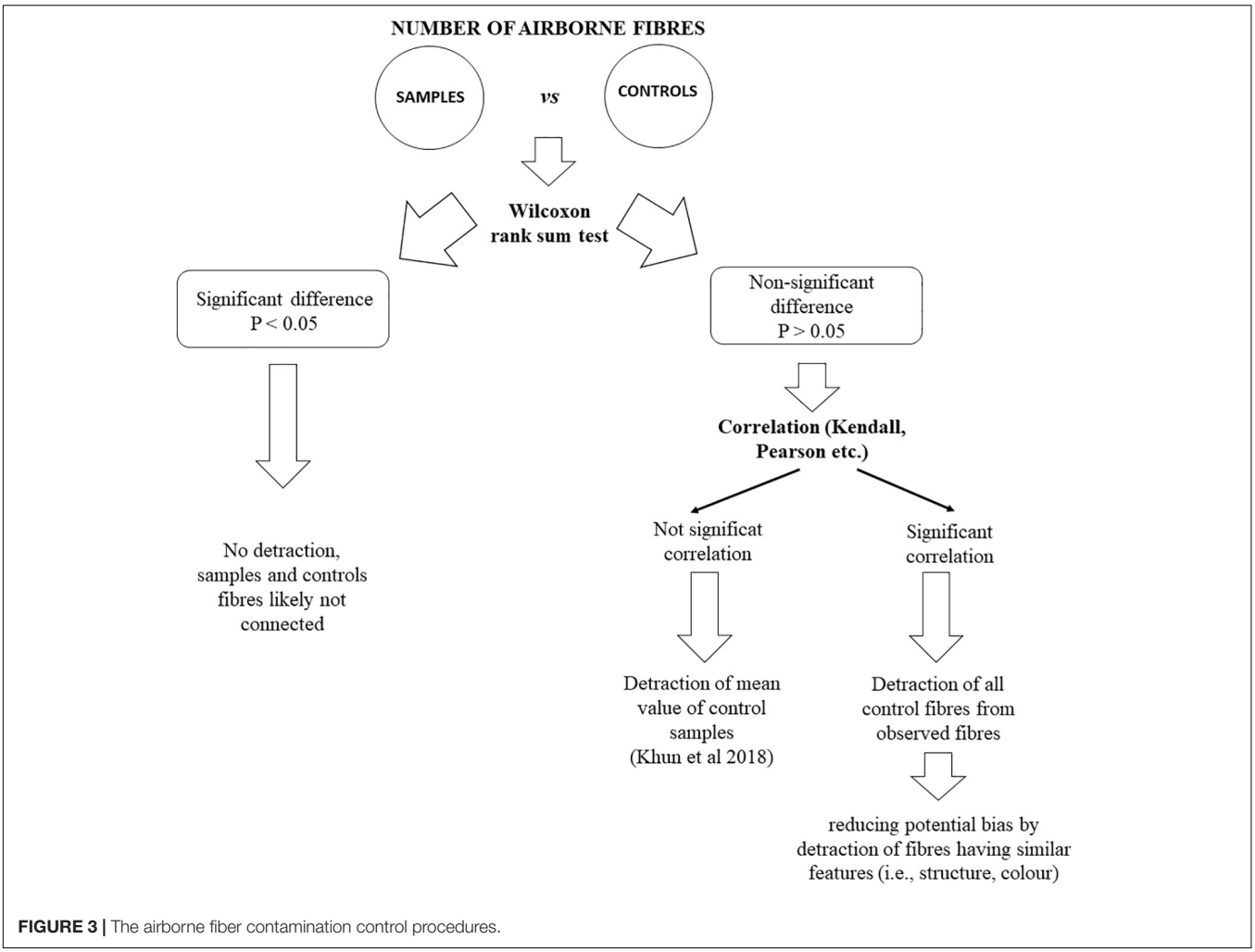

FIGURE 3 | The airborne fiber contamination control procedures.

TABLE 1 | Number of examined gastro-intestinal tracts (GITs), size (TL, cm), and weight (TW, g) ranges for each species.

Species	Number of GITs examined	Mean TL ± SD (cm)	TL range (cm)	Mean TW ± SD (g)	TW range (g)
SYC	27	40.5 ± 6.3	26.2–51.2	187.4 ± 96.3	32.5–378.5
ETX	16	21.2 ± 7.7	14.3–39.3	55.7 ± 58.5	11.6–197.2
SHO	12	20.7 ± 3.0	17.3–27.9	24.9 ± 10.8	12.7–51.1
RJC	6	65.8 ± 2.8	62.9–69.8	31.7 ± 1.3	30.0–34.0

SYC, Scyliorhinus canicula; ETX, Etmopterus spinax; SHO, Galeus melastomus; RJC, Raja clavata; SD, standard deviation.

polypropylene (PP), 2 polystyrene (PS), 5 polyamide (PA; including nylon and aromatic polyamides), 1 polyester (PL), 1 polyurethane (PUR), and 1 rubber (**Figure 4D**). Polyamide was the only polymer found in all species. In *S. canicula* 40% of plastics analyzed were identified as PS followed by PE, PP, and PA (all having 20%). Polyamide (50%) was the most frequent polymer in GITs of *G. melastomus,* while PE and PL were ingested to a lesser extent (16.7%).

In addition, one fiber (16.7%) was also recorded in the gut of this species, but it was impossible to analyze this sample by FT-IR because it was too thin (0.03 mm) and, then, it was considered as not determined (N.D.). Finally, PP, PA, PUR, and rubber had a frequency value of 25% in GITs of *R. clavata*. Images of some plastic samples found in the GITs of demersal elasmobranchs are reported in **Figure 5**, together with the corresponding FT-IR spectra.

Fiber Contamination in Samples

A total of 21 fibers were found in 25% of the demersal elasmobranchs GITs. **Table 3** shows the information on fibers abundance in the examined samples, with the comparison between the observed values in GITs (0.34 ± 0.66 items/individual) and controls (0.61 ± 0.99 items/individual). The number of fibers per fish GIT ranged between 0 and 2, and in controls between 0 and 4. According to the results of Wilcoxon rank sum test, no significant differences were found between

TABLE 2 | Results on the occurrence, abundance and size of marine litter ingested by demersal elasmobranchs (*Scyliorhinus canicula* = SYC, *Galeus melastomus* = SHO, *Raja clavata* = RJC).

Marine Litter	SYC	SHO	RJC	Total
Number of GITs with ML	6	4	3	13
Number of ML items	9	6	4	19
Percentage of occurrence (%O) of ML items	22.2	33.3	50	21.3*
Number of GITs with plastics	4	4	3	11
Number of plastic items	5	6	4	15
Percentage of occurrence (%O) of plastic items	15	33.3	50	18*
Plastics' abundance:				
(i) N. plastic items/N. all examined individuals (average ± SD; range)	0.19 ± 0.48 (0–2)	0.50 ± 0.80 (0–2)	0.67 ± 0.82 (0–2)	0.25 ± 0.57* (0–2)
(ii) N. plastic items/N. individuals which ingested plastics (average ± SD; range)	1.25 ± 0.50 (1–2)	1.5 ± 0.58 (1–2)	1.33 ± 0.58 (1–2)	1.36 ± 0.50 (1–2)
Plastics' length (average ± SD; range; mm)	8.64 ± 10.62 (1.28–27.29)	38.25 ± 79.45 (1.46–200)	4.64 ± 2.59 (2.31–8.29)	19.42 ± 50.44 (1.28–200)
Plastics' width (average ± SD; range; mm)	2.11 ± 1.45 (0.34–3.91)	0.42 ± 0.43 (0.01–0.94)	2.07 ± 1.51 (0.14–3.83)	1.42 ± 1.37 (0.01–3.91)
Plastics' weight (average ± SD; range; g)	0.02 ± 0.01 (0.0002–0.0738)	0.001 ± 0.001 (<0.0001–0.0016)	0.01 ± 0.01 (0.0004–0.014)	0.01 ± 0.02 (<0.0001–0.0738)

*No litter ingestion was observed in E. spinax = ETX. *Data calculated on 61 examined stomachs (i.e., also including E. spinax samples).*

samples and controls ($p > 0.05$) in each species, indicating that likely fibers in samples may be due to airborne contamination (**Table 4**). According to the results of Kendall correlation test, the correlation was significant for all species (**Table 5**). Based on these results, the number of observed fibers was corrected by detracting the total number of control fibers. The last step of our control method consisted in the comparison of structure and color of observed and control fiber, in order to confirm the exclusion of those fibers having the same features (**Figure 6**). According to our approach, only in *G. melastomus* the presence of one ingested fiber can be confirmed.

DISCUSSION

Marine Litter Ingestion in Demersal Elasmobranch Species

The present study provided information on the ML ingestion in some elasmobranch species (*S. canicula, G. melastomus*, and *R. clavata*) collected as bycatch in trawl and longline fisheries in the southern Tyrrhenian Sea (GSA 10). ML ingestion has been observed in 21.3% of individuals, although one of the investigated species (*E. spinax*) did not show the presence of ML.

The present study reports, for the first time, data on the litter ingestion by *R. clavata* in Mediterranean waters. Although only six specimens were analyzed, half of them resulted affected by ML ingestion. Previous investigations on *R. clavata*, collected off Cephalonia Island (Greece, Eastern Ionian Sea) and central Tyrrhenian Sea, did not show any sign of ML pollution (Anastasopoulou et al., 2013a; Valente et al., 2020). However, recently, in a close-related species (*Raja miraletus*), Capillo et al. (2020) observed ML debris in GIT of just one individual, caught in the southern Tyrrhenian Sea. So far, the impact of ML on species belonging to the family Rajidae has been poorly studied and this aspect is worth of further investigations.

The occurrence of ML (22.2%) in GITs of *S. canicula* in the study area was lower than data previously observed on the same species from the Tyrrhenian Sea; Valente et al. (2019) and Capillo et al. (2020) reported %O values of 66.7 and 33%, respectively. The highest level of plastic ingestion in *S. canicula* was observed by Mancia et al. (2020) in the Strait of Sicily (about 80% of occurrence).

Among the analyzed species, *G. melastomus* is the most investigated demersal elasmobranch for ML ingestion in the Mediterranean Sea. In the present study, the ingestion of ML (%O = 33.3) was higher than values previously observed by Capillo et al. (2020) in the same study area (%O = 8), but lower than the one (%O = 78.1) reported by Valente et al. (2019) in the central Tyrrhenian Sea. Data on ML ingestion in *G. melastomus,* from studies carried out in other Mediterranean areas, revealed a lower interaction with this species, reporting %O values ranging from 3.2 to 12.5% in the eastern Mediterranean Sea (Anastasopoulou et al., 2013a,b; Madurell, 2003) and from 6.3 to 16.8% in the western Mediterranean Sea (Carrassón et al., 1992; Cartes et al., 2016; Alomar and Deudero, 2017).

In this study, no litter particles were found in the GITs of *E. spinax,* although other authors had already observed ML ingestion in this elasmobranch. Indeed, in the western

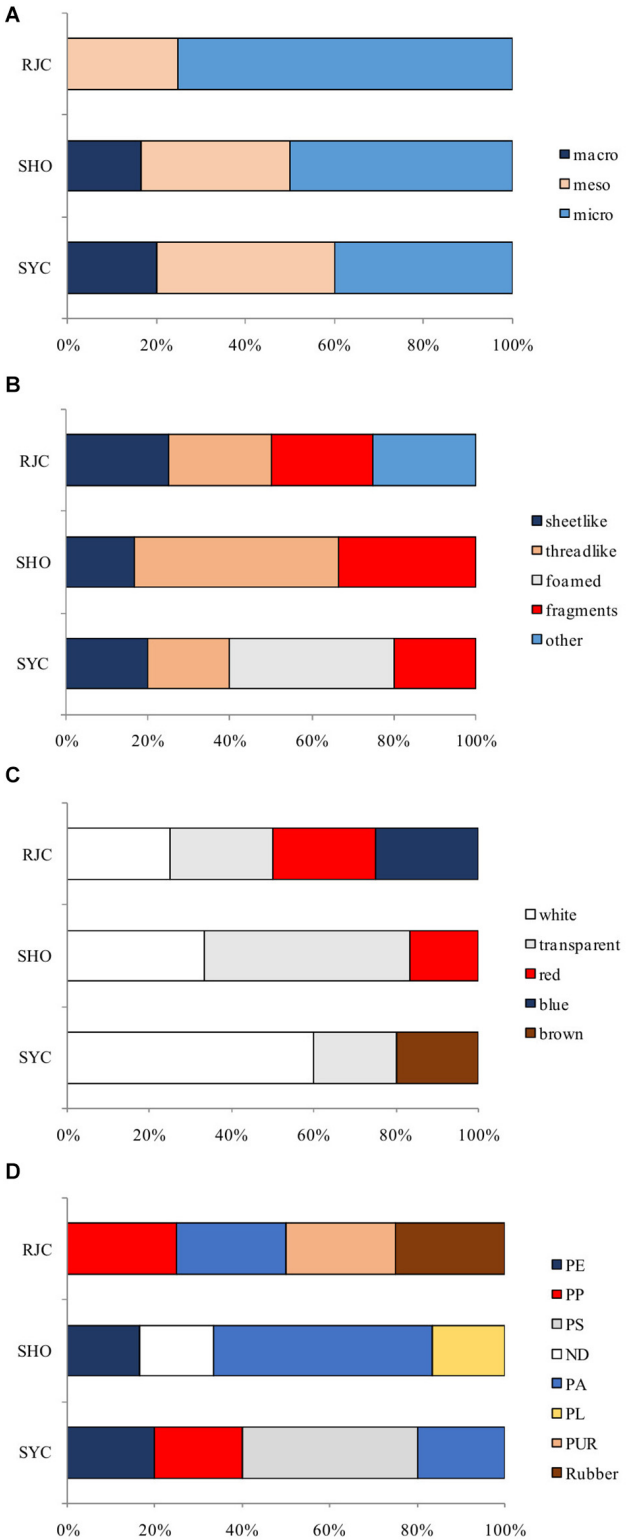

FIGURE 4 | Classification by size, shape, color and polymer type of plastics detected (n = 15) in demersal elasmobranchs (*Scyliorhinus canicula* = SYC, n = 5; *Galeus melastomus* = SHO, n = 6; *Raja clavata* = RJC; n = 4). **(A)** Percentage of size classes; **(B)** Percentage of shapes; **(C)** Percentage of colors; and **(D)** Percentage of polymer types.

Mediterranean Sea, Cartes et al. (2016); Alomar and Deudero (2017) and Valente et al. (2019) reported %O values of 7.8, 50, and 61.8%, respectively, while ML ingestion in the eastern Mediterranean Sea resulted around 7% (Anastasopoulou et al., 2013a; Madurell, 2003).

From the analysis of these results and bibliographic information, it is possible to observe that ML ingestion in Mediterranean demersal elasmobranchs shows high variability in terms of occurrence. These differences may be related to the mutability of environmental factors and features (i.e., river inputs, convergence currents, etc.) and anthropic pressure in the study areas, but also, more probably, to the different methods used to assess ML ingestion. The main source of variability is due to the use of different plastic extraction methods and contamination control procedures. For instance, Anastasopoulou et al. (2013a; 2013b), Alomar and Deudero (2017), and Capillo et al. (2020) analyzed the stomach contents by visual sorting, whereas Valente et al. (2019) and Mancia et al. (2020) digested stomach contents by chemical digestion protocols. Sometimes, data on ML ingestion are not the main focus of a research program and information are additionally collected during studies on trophic ecology of different marine species (Madurell, 2003; Anastasopoulou et al., 2013b) and, for this reason, they may lack of airborne contamination control procedures or quality assurance measures. Moreover, the polymer identification by IR or Raman spectrometry is a procedure only recently used in ML studies.

In addition, the diverse sample sizes considered in Mediterranean studies is another important limit to data comparability.

The finding of ML particles in the GITs of demersal elasmobranchs is certainly related to their feeding behavior and their strong relationship with the seafloor, as also suggested by other authors (Alomar and Deudero, 2017; Fossi et al., 2018; Valente et al., 2019; Capillo et al., 2020). Indeed, these species live in direct contact with the seafloor (Fanelli et al., 2009; Valls et al., 2011), are characterized by a generalist feeding behavior and mainly feed on the bottom invertebrates and benthic fish (Fanelli et al., 2009; Valls et al., 2011; Šantić et al., 2012; Anastasopoulou et al., 2013b). Furthermore, *R. clavata* is able to find its food by excavating in soft sediments were prey is usually buried (Gray et al., 1997; Saglam et al., 2010; Šantić et al., 2012); this feeding behavior may determine an increase of the risk to ingest ML, which could be accumulated inside the sediments. Indeed, the seafloor represents an important sink for the accumulation of ML and their density is often greater in deep waters along the continental shelf edge than in shallow waters (Galgani et al., 1995, 2000; Barnes et al., 2009). The density of ML in the seafloor of the study area may be also affected by the absence of bottom trawl fishing up to a bathymetry of about 500 m, due to access restrictions established since 1990 (Battaglia et al., 2017).

Debris particles may be accidentally ingested during feeding activity or confused with their prey, but secondary ingestion cannot be excluded. For instance, it is known that *G. melastomus* is able to feed also on lanternfish (Fanelli et al., 2009; Valls et al., 2011), which have been reported as species affected by ML ingestion (Romeo et al., 2016). Elasmobranchs are also known

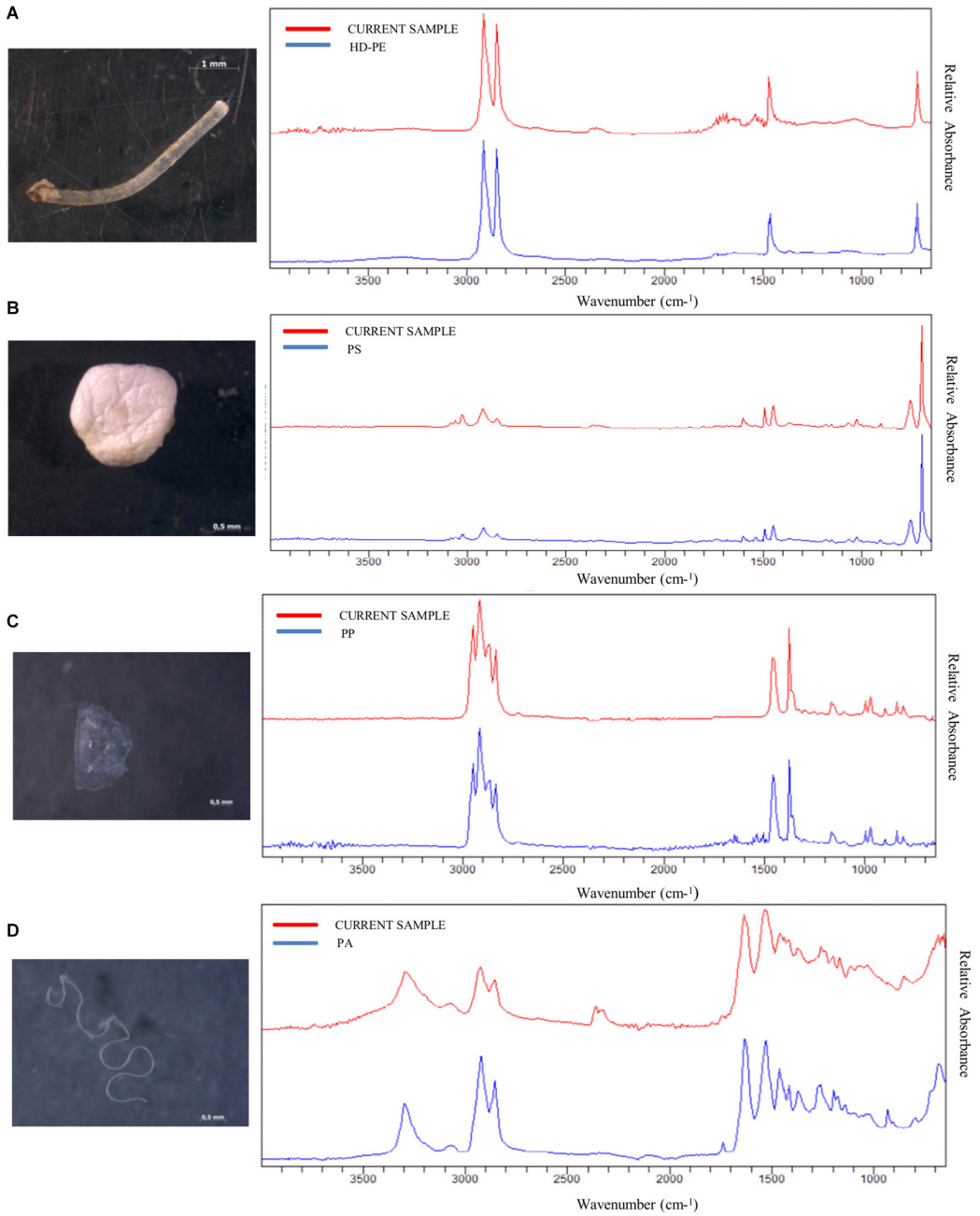

FIGURE 5 | Images of some plastics samples found in the GITs of demersal elasmobranchs (*Scyliorhinus canicula* = SYC, *Galeus melastomus* = SHO, *Raja clavata* = RJC) with the respective FT-IR spectra. **(A)** HD-PE threadlike in SYC; **(B)** PS foam in SYC; **(C)** PP sheet in RJC; and **(D)** PA threadlike in SHO.

as scavengers, feeding opportunistically on carrions and preying on dying, dead or decomposing individuals (Olaso et al., 1998), which may have potentially ingested ML in upper waters and that sink toward seafloor (Olaso et al., 1998; Valente et al., 2019).

Plastic Characterization

Plastic represented the main litter category found in the GIT of examined demersal elasmobranchs, as also observed in previous studies on Mediterranean demersal sharks (Anastasopoulou et al., 2013a; Kühn et al., 2020; Cartes et al., 2016; Alomar and Deudero, 2017; Valente et al., 2019; Capillo et al., 2020; Mancia et al., 2020) and on a wide range of marine organisms (Campani et al., 2013; Romeo et al., 2015, 2016; Battaglia et al., 2016; Bernardini et al., 2018; Digka et al., 2018a; Bottari et al., 2019; Savoca et al., 2019, 2020; Schirinzi et al., 2020). *G. melastomus* and *R. clavata* ingested only plastics while *S. canicula* ate also chicken remains (probably used as bait by local artisanal fishermen targeting common octopus) and polyacrylamide

TABLE 3 | Information on the presence of fibers in all examined species (*Scyliorhinus canicula* = SYC, *Etmopterus spinax* = ETX, *Galeus melastomus* = SHO, *Raja clavata* = RJC).

Species	n fish	Fiber in controls			Fiber in samples (observed)			Fiber in samples (corrected)		
		n fiber (range/specimen)	Average number ± SD	%O	n fiber (range/specimen)	average number ± SD	%O	n fiber (range/specimen)	Average number ± SD	%O
SYC	27	16 (0–3)	0.593 ± 0.931	33.3	11 (0–2)	0.407 ± 0.747	25.9	0	0.000 ± 0.000	0.0
ETX	16	8 (0–4)	0.500 ± 1.095	25	3 (0–2)	0.188 ± 0.544	12.5	0	0.000 ± 0.000	0.0
SHO	12	8 (0–3)	0.667 ± 0.985	41.7	5 (0–2)	0.417 ± 0.669	33.3	1 (0–1)	0.083 ± 0.289	8.3
RJC	6	5 (0–3)	0.833 ± 1.329	33.3	2 (0–1)	0.333 ± 0.516	33.3	0	0.000 ± 0.000	0.0
Total	61	37 (0–4)	0.607 ± 0.988	34.4	21 (0–2)	0.344 ± 0.655	24.6	1 (0–1)	0.016 ± 0.128	1.6

The abundance (average number ± standard deviation, SD) and percentage of occurrence (%O) of fibers observed in samples and controls are provided together with the corrected values after application of control procedures.

TABLE 4 | Wilcoxon rank sum test between samples and controls.

Species	Parameters (W)	*p*-value
SYC	332.5	0.4977
SHO	64	0.6149
ETX	111.5	0.3755
RJC	16	0.7745

TABLE 5 | Kendall's rank correlation Tau of each species between samples and controls.

Species	*z*	Tau	*p*-value
SYC	4.72	0.86	2.4e-06
SHO	2.16	0.60	0.03061
ETX	2.81	0.67	0.004912
RJC	2.19	0.94	0.02846

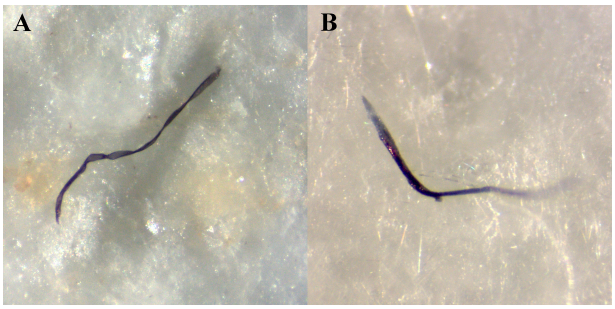

FIGURE 6 | Example of fibers having similar characteristics, found in samples **(A)** and in the respective control filter **(B)**.

particles. Polyacrylamide (a high molecular weight polymer) are widely used in industrial processes to aid flocculation and complexation and in oil production processes (Hansen et al., 2019). The potential effects of polyacrylamide on marine organisms are still not well investigated (Hansen et al., 2019).

The analysis of ML size revealed that the largest amount of plastics found in the stomachs belongs to microlitter category (53%). This result could be related to the morphological traits of the investigated elasmobranchs, which have a small mouth more suitable for the ingestion of small prey. Information on microplastics' levels in sediments of the study area is not available, but data from an adjacent zone (Aeolian Islands) indicates that this ML category is quite abundant on the seafloor (Fastelli et al., 2016; Martellini et al., 2018). On the other hand, macroplastics were only found in few stomachs of *S. canicula* and *G. melastomus*, in agreement with findings of Capillo et al. (2020) and Valente et al. (2020), which had previously observed low levels of macrolitter ingestion in such species. According to Valente et al. (2020), these demersal elasmobranchs may regurgitate macrolitter, being the intestinal spiral valve an obstacle to their transit toward their intestinal tract.

Threadlike and fragment were the most abundant plastic shape categories, as observed by other authors in different Mediterranean areas (Digka et al., 2018a,b; Capillo et al., 2020; Valente et al., 2020). However, these studies reported a

considerably higher abundance of fibers, whereas in our research, the threadlike category included almost exclusively filaments (most of them were remains of fishing lines) and only one fiber. This is probably due to the application of airborne contamination control method, which allowed to exclude fibers derived from secondary contamination.

The color of ingested plastics was mainly transparent or white, even though also red, blue and brown particles were found. The prevalence of clear color could mirror the real ML patterns in the marine environment, otherwise it could be related to their resemblance of elasmobranchs' potential prey. Indeed, according to Kühn et al. (2015), specific colors might attract predators which may confuse ML for their prey.

The characterization of plastic polymers through FT-IR analysis showed that PA (aliphatic and aromatic polyamides) was the most abundant plastic compound found in the stomachs. The PA polymers have a large number of applications. For instance, nylon is an aliphatic polyamide of high commercial importance (Aoki et al., 1979), highly used for fishery purposes. Aromatic polyamides (Nomex and Kevlar fiber) are utilized for firefighting and in fire-resistant clothing, automotive, nautical, planes, and space sectors but also employed in several sports equipment (Aoki et al., 1979; Baker, 2018). Although PA is not among the most used and produced plastic polymers at global level (Geyer et al., 2017), their high use in industrial production is probably the main cause of their presence in the marine environment and then in the GITs of examined species, and the occurrence in the study area could be related to the specific vocation toward recreational and professional fishing activities, as well as to the presence of touristic harbors and nautical activities, that are highly developed also for their closeness to the Aeolian Archipelago (Savoca et al., 2019).

The other most common polymers (polyethylene, polypropylene, and polystyrene), identified in the GITs, are quite abundant in the marine environment (Andrady, 2011; Cózar et al., 2014; Suaria et al., 2016; Geyer et al., 2017; Digka et al., 2018b). Although these polymers have positive buoyancy, after density modifications due to abiotic (e.g., currents and water circulation) and biotic (biofouling) factors they could sink in the sediments becoming potentially available for demersal marine organisms (Andrady, 2017).

Finally, the study area is characterized by an intense fishing activity (Battaglia et al., 2017), and then, PE, PP, and nylon particles may derive by the degradation of lost or abandoned fishing gears (Consoli et al., 2018).

Contamination Control Method

The airborne fiber contamination is a crucial and complex issue. Several studies suggest that the risk of contamination by airborne fibers, both natural or synthetic, is very high and unavoidable during the sampling and laboratory analysis (Torre et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020; Schirinzi et al., 2020). For instance, air currents, operators' clothing and a not accurate cleaning of laboratory tools can lead to samples contamination and, then, to overestimation of fibers ingestion in biota (Hidalgo-Ruz et al., 2012; Torre et al., 2016).

To date, some studies on ML ingestion in marine organisms have demonstrated that, if mitigation measures are correctly applied, the assessment of this phenomenon is lower thanks to the reduction of airborne fibers in samples and controls (Rummel et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020). Furthermore, although the quality assurance criteria are applied in almost all studies, details on fiber contamination and correction of estimates through the application of a control contamination method are rarely provided (Kühn et al., 2018, 2020).

For these reasons, our study applied severe mitigation measures to avoid airborne contamination during the analysis, integrating a methodology for the correction of estimates of fibers abundance using control procedures. In this regard, we also suggest in future studies to carry out a separated analysis for the estimation of fibers presence and to provide clear information on both fibers abundance observed in samples and controls. This will be useful for the procedure and data standardization and will help the comparison between different studies on ML ingestion.

The integrated method applied in the present study achieved a reliable assessment of ML ingestion in demersal elasmobranchs, having excluded potential airborne fiber contamination from the results. Indeed, the large part of observed fibers was excluded by our assessment, after the comparison with fibers found in the control filters, and only the presence of one fiber was confirmed as anthropogenic litter in the GIT of a *G. melastomus* individual. Other studies, using different methodologies, reported a consistent amount of fibers in GITs of elasmobranch species from some Mediterranean areas: *S. canicula* and *G. melastomus* from the southern Tyrrhenian Sea (Capillo et al., 2020); *S. canicula, G. melastomus* and *E. spinax* from the central Tyrrhenian Sea (Valente et al., 2019) and *G. melastomus* from the Balearic Island (Alomar and Deudero, 2017).

Although it is impossible to totally avoid airborne contamination, our control procedures aims to avoid the overestimation of ML, being this issue a crucial point in studies on ML ingestion.

Based on these results and considerations, it is possible to recommend in future studies to provide a detailed description of mitigation measures, applied at each step of laboratory analysis and aimed to tackle airborne contamination: reduction of air currents, use of 100% cotton lab coats, use of glassware and metal laboratory tools, use of filtered water to clean laboratory tools, working under the fume hood, covering of samples, use of a control (moist filter) in each analysis and application of a data correction method (**Figure 2**).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we used dead fish collected from bycatch of local fisheries.

AUTHOR CONTRIBUTIONS

CP organized the database, performed the data analysis, and wrote the manuscript. PB contributed to the conception and design of the study, wrote, commented on, and edited the manuscript. MD'A performed the GITs analysis and revised the manuscript. FLa performed the GITs analysis. DM performed the statistical analysis and revised the manuscript. PC, TV, FA, TB, and SG contributed to the manuscript revision. FLo contributed to the sample collection. MG, MB, and MF performed the FTIR analysis and revised the manuscript. TR provided funds, contributed to conception and design of the study, commented on, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Rainfall and Tidal Cycle Regulate Seasonal Inputs of Microplastic Pellets to Sandy Beaches

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Microplastic contamination of coastal environments is a global problem and pellets used in industrial processes are a persistent and worldwide form of microplastic pollution. Regions that host port and petrochemical facilities are well known sources of plastic pellets to local and adjacent coastal areas. This study assessed pellet densities over regional and local scales to provide a greater understanding of the spatio-temporal variation in inputs to sandy beaches. Pellets were used as a proxy to undertake a multi-scale assessment of the spatial (local vs. regional) and temporal (tides and weather) stranding of microplastics. Microplastic variability differed between local and regional scales. Regional variation was driven by weather (i.e., rainfall) and distance from source. Local-scale variability was driven by distance from source (along shore), tidal cycle, and beach hydrodynamics. Our results address the drivers of large spatio-temporal variability in microplastic pollution and provide useful information for monitoring programs by pointing to the need to consider variability in inputs over both regional and local scales.

Keywords: microplastic, bead, input, multi-scale variability, long-term monitoring

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HIGHLIGHTS

- This study assessed plastic pellet inputs over regional and local scales.
- Microplastics variability differed between local and regional scales.
- Regional scale variation was driven by rainfall and distance from source.
- Local-scale variability depended on distance from source, tides and beach hydrodynamics.
- Understanding spatio-temporal variability of microplastic pollution can aid management.

INTRODUCTION

Contamination of coastal environments such as sandy beaches by plastic material is a major environmental problem worldwide (Browne et al., 2015; Jambeck et al., 2015). Microplastics, i.e., particles smaller than five millimeters (Andrady, 2011; Cole et al., 2011), including pellets used in industrial processes, are amongst the most abundant forms of this material even in relatively pristine areas (Turner and Holmes, 2011) and can represent up to 53% of the overall plastic debris present in beaches (Antunes et al., 2013).

Plastic pellets most commonly enter marine and coastal ecosystems through transportation losses in port terminals (Turner and Holmes, 2011). The persistence and biological impacts of microplastics can have severe economic and environmental effects through direct ingestion by biota, lost fisheries production and declines in tourism and recreation (Rochman et al., 2013; UNEP, 2014). Moreover, the environmental risks to humans (e.g., vector of pollutants such as PCBs and PAHs; Fisner et al., 2013a,b), demands monitoring of long-term variations in the abundance and inputs of these particles to beaches and other coastal and marine habitats.

The spatial variability in the distribution of beach litter is often related to season and site-specific factors. River supplies (Asensio-Montesinos et al., 2019) as well as hydrometeorological features (Cordova and Nurhati, 2019) generate seasonal variability in beach litter. This varibility in beach litter is also related to factors such as the proximity to densely populated areas (Browne et al., 2011; Ryan et al., 2018), river-mouth (Williams et al., 2016) and hydrodynamics (Williams et al., 2017). Similar factors are related to the spatial variability of plastic pellets within coastal ecosystems.

Recent works have demonstrated the often profound spatial variability in the distribution and accumulation of plastic pellets within coastal and marine habitats; a reality that constitutes a major conundrum to the development of assessment and monitoring programs (Turra et al., 2014; Moreira et al., 2016a,b; Fisner et al., 2017). At a broader or regional scale, catchment characteristics (i.e., proximity to sources and local weather) as well as oceanographic currents are likely to influence the stranding of particles in certain areas (Browne et al., 2011; Browne, 2015; Ryan et al., 2018). The occurrence of seasonal climatic features (e.g., wind and rainfall) can also interact with the relative loads stored in catchments to result in pulsed inputs to estuarine and coastal environments (Rech et al., 2014; Krelling et al., 2017; Gorman et al., 2020 in review).

At a local-scale (i.e., individual beaches) pellets can accumulate in zones spanning the shoreline to the backshore, the coastal dunes (often associated with strandlines; Moreira et al., 2016b) and throughout the sediment column to depths of up to 2 m (Turra et al., 2014). In addition, tidal cycles interfere with the dispersal of microplastics from estuaries to open waters, given that greater amounts of particles leave these environments during spring tides (Sadri and Thompson, 2014). Further, plastic pellet distribution might also be spatially variable in the along shore direction of sandy beaches (Bowman et al., 1998) as a result of hydrodynamics (Andrady, 2015; Thompson, 2015) and relative proximity to sources (Karapanagioti and Klontza, 2008; Turner and Holmes, 2011). These environmental processes are responsible for variations in the stranding rates (input) and accumulation (standing stock) of plastic pellets in sandy beaches (Turra et al., 2014; Moreira et al., 2016a,b).

A key requirement to understanding processes of stranding and accumulation of plastic pellets, is knowledge of the variability in inputs over both spatial and temporal scales (Duis and Coors, 2016). In this context, we conducted a multi-scale assessment of the spatio-temporal inputs of plastic pellets to sandy beaches along the central coast of São Paulo state, Brazil. The initial aim was to assess variation in the inputs of plastic pellets driven by regional-scale characteristics such as distance from potential sources and weather. We tested the hypothesis that inputs would show variability among regional (i.e., "beaches") and temporal replicates (i.e., "months"). Having identified some of the principal drivers of variability at the regional scale (i.e., climate and rainfall) we then focused on local-scale phenomenon by testing the effects of season (i.e., the onset vs. end of the rainy season), along shore beach position (along shore) and consecutive tidal cycles. Here we tested the hypothesis that variation in plastic pellet inputs differ along the shore and over consecutive tidal cycles and season. Overall, our goal was to present useful information describing the spatio-temporal dynamics of plastic pellet contamination of sandy beaches for monitoring programs tasked with evaluating the environmental risks to the biota (Nelms et al., 2015) and to the physical environment (Antunes et al., 2013). This approach is also useful as a baseline information for monitoring programs that could evaluate the efficiency of the adoption of the "zero pellets loss" programs (in before and after impact design) (e.g. Operation Clean Sweep; Plastics & ACC, 2017).

MATERIALS AND METHODS

We used a hierarchical sampling design to test regional and local scale variability in the input of plastic pellets to sandy beaches of the Santos region of Brazil (**Figure 1**). The rationale was to understand better the drivers of the often high variability observed in estimates of plastic pellet input and standing stocks (see Moreira et al., 2016a,b). The sampling focused on recently stranded pellets by the last tidal cycle, present only in the intertidal up to the last high tide mark. We began by investigating variability in the density (i.e., numbers of particles per linear meter along transects) of stranded plastic pellets at regional scale, among beaches located at different distance from known sources of plastic pellet inputs (Pereira, 2014) (the port of Santos, **Figure 1C**). In this regional scale, we evaluated the interference of the rainy season on the dispersion and occurrence of plastic pellets, so we developed a novel sampling design to accomplish surveying in the dry season (April, May, June, July, August, and September) and in the rainy season (October, November, December, January, February, and March). We then focused on the influence of local-scale phenomena including, along-beach distribution, tidal cycles (semidiurnal), and hydrographic processes within a heavily impacted and relatively well-studied beach arc (**Figure 1C**; Santos Bay; Turra et al., 2014).

Regional-Scale

At regional scale, the spatial and temporal variability of plastic pellet inputs was evaluated at four beaches along the central coastline of São Paulo (**Figure 1B**) which are located at different distances from a known major source (i.e., the port of Santos) (Fisner et al., 2013a,b; Taniguchi et al., 2016). The selected beaches (ranked by distance from Santos) included Canal cinco, Ocian, Iporanga and Itaguaré, all of which can be characterized as dissipative/intermediate morphodynamic with a NE-SW

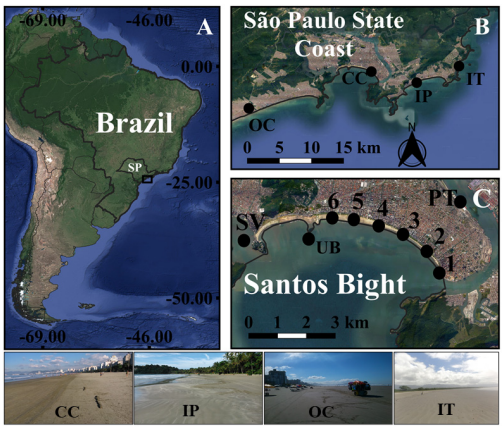

FIGURE 1 | Study sites included in the multi-scale assessments of spatio-temporal variation in plastic pellet pollution; **(A)** Brazil, showing the location of São Paulo state (SP). **(B)** Regional-scale study including Ocian (OC), Canal cinco (CC), and Iporanga (IP) and Itaguaré (IP). **(C)** Small scale replicates within Santos Bight, dots with number represent the sampling locations (1, 2, 3, 4, 5, 6), PT indicates the location of Santos Port Terminal, UB is Urubuqueçaba Island and SV is São Vicente Channel.

orientation. Although differing in their catchment characteristics and coastal geomorphology, these beaches experience similar hydrodynamic and morphodynamics processes. Sampling was done between February 2014 and February 2015 to capture rainy and dry seasons.

Local-Scale

Having investigated variability in pellet inputs at a regional scale, we then focused our attention on processes operating at local-scale by assessing the influence of along shore position, tidal cycle and hydrodynamic process within Santos Bay itself (**Figure 1C**). The study area for this component incorporated a 7 km stretch of exposed sandy beach with an east-west orientation sheltered and a degree of protection at the western end (i.e., due to the blocking effect of Urubuqueçaba Island; Elliff et al., 2013). The area receives estuarine waters discharged both from the Santos Channel in east and the São Vicente Channel in the west (Gregorio, 2009). The coastline also receives urban and industrial runoff via seven drainage channels that are interspersed along the beach approximately every 1 km (Italiani, 2014). Sampling was done in November 2014 and again in March of 2015 to capture seasonal variation; representing the beginning and end of the wet-season in Brazil, respectively. Within these seasonal replicates, sampling was done during the spring tides, at six sampling points along shore (at increasing distances from the Santos channel, a potential source of pellets).

Sampling Approach

Sampling at both scales was done during low tide (between 0 and 0.5 m), using five 2-m wide transects oriented in a cross-shore direction (up/down shore). The across-shore extension of transects varied according to beach morphology and the amplitude of the tides (Moreira et al., 2016a). Each transect was pre-prepared by cleaning all plastic pellets along marked transects prior to sampling. This had the effect of creating a clean surface, so that subsequent sampling would only collect newly stranded pellets within the last tidal cycle. The sampling procedure involved gathering all stranded pellets in the low tides prior and after to the tidal cycle by scraping the surface of the sediment with a squeegee from the water line to the high strandline. Processing of collected particles involved washing the mix of sediment and the other material through a 0.1 mm sieve. Isolated pellets were packaged on-site and transported to the laboratory for counting. At the Regional-scale, sampling was repeated during different sampling periods (dry and rainy seasons) and spanned a full year (i.e., February 2014 and February 2015). For the local scale, sampling was done in November 2014 and March 2015, over three subsequent low tides at the same time at all sites by different teams. Transects sampled in each site were randomly established at the beginning of each season and were resampled during the three consecutive low tides.

Data Analysis

Variability in plastic pellet densities at both scales was assessed using analysis of variance (ANOVA). At the regional scale, data were analyzed using two-way ANOVA, considering the factors "weather," with two levels, representing the sampling conducted in dry and rainy seasons, and replicate beaches, with four levels, representing catchments of differing characteristics and distance from source (Santos port terminal). Data were checked for heterogeneity of variance using Cochran's test and *ln* $(x + 1)$ transformed where necessary. The significant differences were *a posteriori* evaluated with the Student-Newman-Keuls test (SNK) (Underwood, 1997). At the local scale, data were analyzed using three-way repeated measures ANOVA, considering the factors "zones," with six levels, representing along shore beach position (along shore), "cycles", with three levels, representing three consecutive low tide cycles and "season," with two levels representing the onset and the end of the rainy season (November and March respectively). The significant differences were evaluated with Bonferroni test (Quinn and Keough, 2002).

RESULTS

Regional-Scale

At the regional scale, there was substantial variability in the density of pellets across the year and among beaches (**Figure 2A**), with strong peaks observed for Canal cinco between November 2014 and February 2015 (i.e., coinciding with the rainy season) and Iporanga during April and August 2014. In general, the lowest densities of plastic pellets were observed during dry season, while the higher estimates were observed during wet season. There was also an overall trend of decreasing inputs with increasing distance from the Port of Santos (**Figure 2B**). When comparing the density of pellets between dry and wet seasons, sites demonstrated different patterns (**Figure 3** and **Table 1**, "Weather × Beach" interaction term) with those closer to the Port of Santos showing the greatest difference between dry and rainy season.

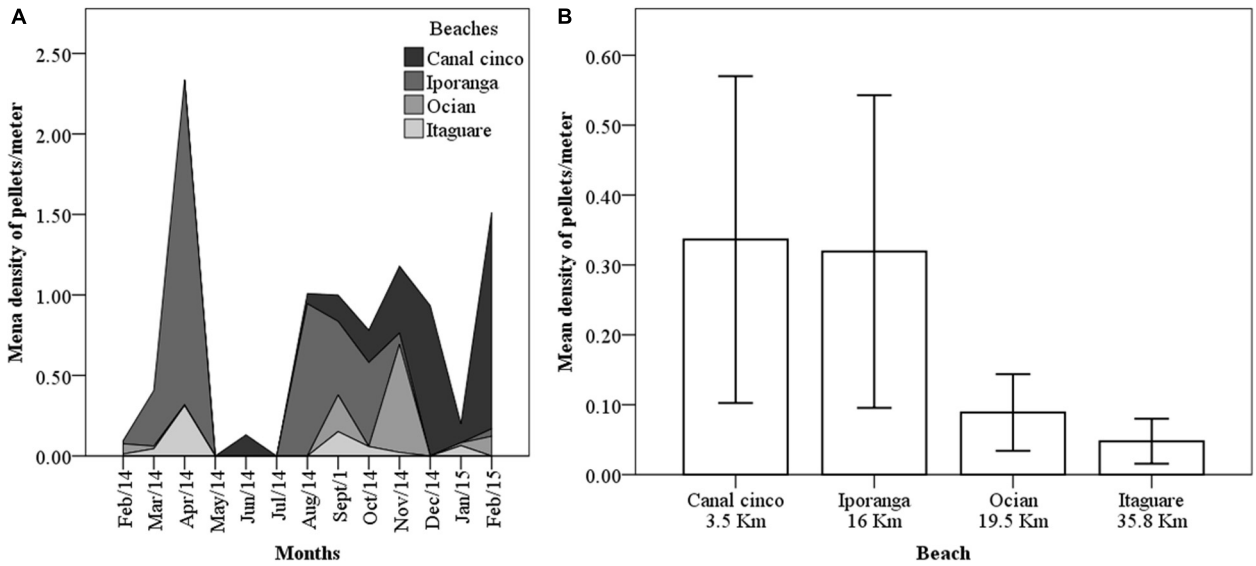

FIGURE 2 | Estimates of plastic pellet inputs to replicate beaches along the coastline of São Paulo state; **(A)** over 1 year, and **(B)** with *x*-axis reflecting the distance of each beach from the Port of Santos. The bars represent mean (±SE) for sampling sites for each replicate beach.

Local-Scale

Analysis of plastic pellet density revealed a smaller quantity of pellets prior to the rainy season (**Figures 4A,B**). Further, the analysis of the local scale revealed a significant variation according to the sampling season and an interaction between tidal cycles and the along-shore location. An interesting observation was that there was no interaction between season and zones or cycles, suggesting that these patterns were relatively consistent (**Figure 4** and **Table 2**). The significant interaction between "zones and cycles" suggested that the variability in pellet density over consecutive cycles is different in the along-shore beach position. Indeed, the pairwise test revealed significant differences among cycles.

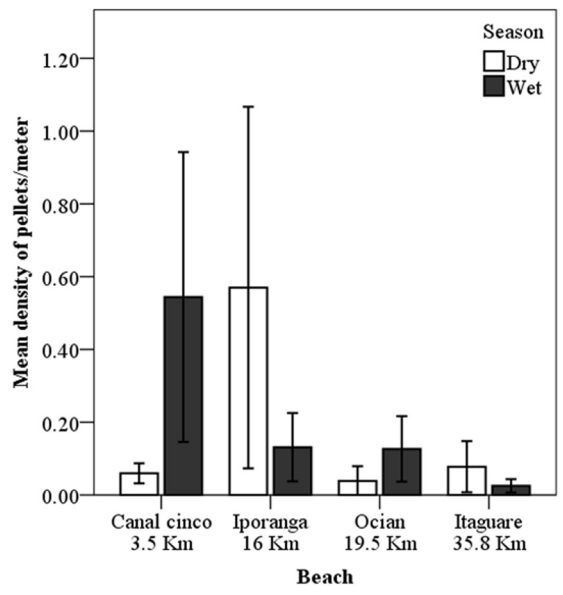

FIGURE 3 | Estimates of plastic pellet inputs to replicate sandy beaches in dry and wet seasons. Bars represent mean (±SE) for each sampling zones in Santos bay. The *x*-axis reflects the distance of each beach from the Port of Santos.

DISCUSSION

The aim of this study was to investigate the drivers of spatio-temporal variability in microplastic pellet inputs to sandy

TABLE 1 | Two-way ANOVA comparing plastic pellet inputs to four regional beaches along the coast of São Paulo state during different weather conditions (dry vs. wet).

Source	*df*	MS	*F*	*P*
Weather	1	42.13	52.43	<0.001
Beach	3	23.6	29.36	<0.001
Weather × beach	3	5.37	6.68	<0.001
Error	182	0.804	–	–
Total	190	–	–	–

SNK-test results

Dry:	**Wet:**
Canal cinco > Iporanga	Canal cinco > Iporanga
Canal cinco > Itaguaré	Canal cinco > Itaguaré
Canal cinco = Ocian	Canal cinco > Ocian
Iporanga = Itaguaré = Ocian	Iporanga > Itaguaré
	Iporanga = Ocian
	Itaguaré = Ocian

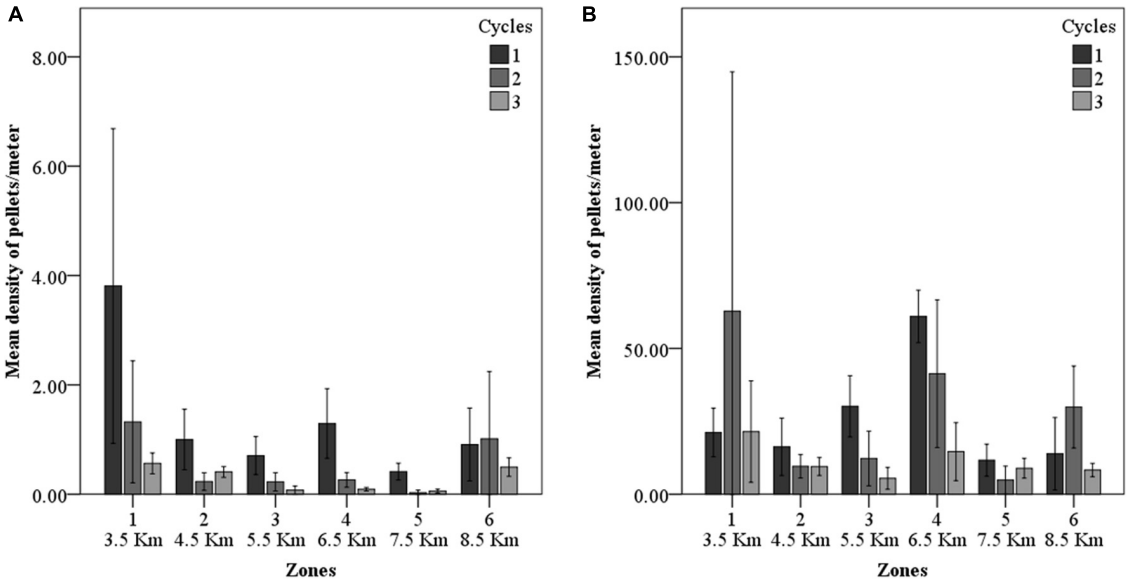

FIGURE 4 | Estimates of plastic pellet inputs to sandy beaches at the "local-scale" during; **(A)** the onset of the rainy season (November), and **(B)** at the end of the rainy season (March). Data are grouped by zones (with increasing distance from the Santos channel in *x*-axis) and by consecutive tidal cycle (following mean high tide of spring). The bars represent mean (±SE) for each sampling zones.

beaches. We assessed the influence of regional characteristics (i.e., rainfall and distance from known source) and local-scale phenomena (i.e., along shore zonation and tides) on variability in the density of plastic pellets stranded on sandy beaches. Our results suggest that factors operating at both scales can have strong and predictable consequences for patterns of pellet deposition and might need to be considered when designing and conducting monitoring programs that are often hampered by the large variability in standing stocks of microplastic contamination (Moreira et al., 2016a).

At a regional scale, inputs of plastic pellets varied considerably across an entire year and showed significant correlation with the geographical (distance) and local weather conditions (rainfall) of various beaches. Overall, Canal cinco showed the greatest inputs, which is not surprising given its proximity to the Port of Santos (i.e., a known source) and its position within the Santos Bay. Our results thus concur with other recent studies on the abundance of plastic litter indicating that the temporal dynamics can be influenced by the distance from industrial and urban centers (Laglbauer et al., 2014; Davis and Murphy, 2015; Gorman et al., 2019; Izar et al., 2019). Inputs declined as a function of distance, although not in a direct linear fashion, and were ∼15% (cf. Canal cinco) at the beach Itaguare located ∼38 km to the northeast. This pattern emphasizes the importance of coastal currents, wind and circulation patterns as factors that influence the dispersal of plastic particles (Law et al., 2014; Gorman et al., 2020 in review). The variability across relatively large spatial scales observed in the present study (tens of kilometers) indicates that long-term surveys of the plastic pellets input should consider the distance from potential sources as part of the sampling procedure.

We also demonstrate the strong influence of rainfall on the input of pellets and probably other microplastic particles to sandy beaches. In the rainy season, the rate at which pellets were stranded increased, with the highest values again observed for Canal cinco. The hydrological and sedimentary processes occurring in this beach are under the influence of strong tidal currents that originate from the Santos estuary. The currents in this location are influenced by the progressive tidal waves inside of the channel and are North-South oriented (Harari and de Camargo, 2003). It appears that the "flushing" of plastic materials accumulated within the catchment (during comparatively dry periods) out of the estuary and into coastal waters (Gorman et al., 2020 in review) results in a "pulse" of stranded pellets on adjacent beaches. Overall, our results suggest that long-term monitoring programs should consider the differences between chronic and episodic inputs as failure to account for pulsed inputs may lead to substantial errors in estimates, even among samples taken in consecutive days (also see Moreira et al., 2016a).

TABLE 2 | Three-way repeated measures ANOVA comparing plastic pellet inputs across different local-scale phenomena; including sampling season (onset vs. end of the rainy season), beach zone (along shore beach position) and tidal cycle (three consecutive low tides).

Source	SS	*df*	MS	*F*	*P*
Season	42.57	1.00	42.57	48.87	0.00
Zones	2.91	2.16	1.35	3.94	0.06
Cycles	2.12	2.00	1.06	51.70	0.00
Season × zones	1.06	2.85	0.37	2.72	0.10
Season × cycles	0.24	1.34	0.18	3.42	0.12
Zones × cycles	1.83	2.39	0.76	5.22	0.03
Season × zones × cycles	1.21	2.97	0.41	3.13	0.07

Pairwise comparisons on cycles: 1 > 2; 1 > 3; 2 > 3

Local-Scale

Having established the importance of regional-scale characteristics (specifically distance from source and rainfall) as determinants of pellet inputs to sandy beaches, we then focused on the effect of local-scale drivers that may help to explain accumulation processes at the scale of individual beaches. Recently, Moreira et al. (2016a) recommended that surveys investigating input dynamics should include small-scale spatial and temporal stratification in order to identify patterns and provide more reliable estimates. Our data show that different degrees of wave action along the beach, tidal cycles, and season (i.e., the onset vs. end of rainy season) can all contribute to the variability in plastic particle estimates.

The first thing to note were the seasonal differences in inputs, which were higher at the end of the rainy season (March) than they were at the onset (November). This is likely to reflect variation in important physical distribution mechanisms that can influence the sources and sinks of plastic particles at a local-scale (Vermeiren et al., 2016). Estimates of the particle flux for microplastics in estuaries have previously shown that they are correlated to weather conditions, such as wind and rainfall (Yonkos et al., 2014), and to seasonal hydrodynamic features (Lima et al., 2015). Indeed, a recent modeling study within the Santos region has shown seasonal variation in the movement of plastic particles, where greater amounts of debris coincide with rainfall events (Gorman et al., in review). It is likely that the material released from the estuary at the onset of the rainy season, takes some time to disperse to the adjacent beaches of Santos. This time lag is another factor that should be considered when attempting to understand patterns of microplastic pollution of coastal habitats.

Local-scale variability in pellet density was also influenced by an interaction between beach zone (reflecting beach morphodynamics) and tidal cycle. At the onset of the rainy season, zones 1–5 had greater inputs in the initial tidal cycle. Contrastingly, the density of stranded pellets increased at zones 1 and 6 over the subsequent two tidal cycles. These observations are likely to be governed by seasonal differences in the depositional environment along the beach (onset vs. end of the rainy season). The volume of water exiting the channel along with the high-energy tidal events likely generate conditions that are favorable to particle deposition close to the channel (Magini et al., 2007). On the other hand, the influence of Urubuqueçaba Island on hydrodynamic features of Santos Bay generates favorable conditions of deposition in zones 4 to 6 (see Turra et al., 2014). The beach dynamics also explain the different variation of pellet inputs over consecutive tidal cycles from zone 1–6. Indeed, after three low tide cycles, inputs had declined on average from the initial pulse. This result helps to clarify the interference of physical processes on microplastics settling in the sediment column and in the promotion of differences in local spatial scales (Turra et al., 2014). Future work should consider this relationship in order to evaluate the impacts of local distribution of plastic particles on physical environmental processes (Carson et al., 2011) and to the biota (Koelmans et al., 2014).

In conclusion, we show that the large variability often observed in the density of plastic pellets (see Turra et al., 2014) can be partially explained by large variability in the input-dynamics, operating across both regional- and local-scales. Geographic location (distance from potential sources), weather, tidal cycles and oceanographic processes are all likely to influence the density of plastic particles that become stranded on sandy beaches and potentially other coastal habitats. We suggest that these factors need to be incorporated into the design of the risk assessment and monitoring protocols to improve the accuracy and efficiency of estimates of microplastic contamination. Failing to incorporate (or at least report) the conditions under which sampling was conducted (e.g., before or after rain?) could lead to spurious and unreliable results that limit our ability to compare trends over time and space. Overall, while we address the considerable variation in microplastic density estimation, it is clear that more work needs to be done to fully understand the distribution patters, processes of accumulation and implications of microplastics contamination within marine and coastal habitats (e.g., Hidalgo-Ruz et al., 2012; Ivar do Sul and Costa, 2014).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DB-S is the first author, he contributed in field work, data analysis and writing. AT was DB-S advisor. FM also was DB-S advisor. RC, AO, and LB contributed in field work. DG contributed in data analysis and writing. All authors contributed to the article and approved the submitted version.

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Transfer of Additive Chemicals From Marine Plastic Debris to the Stomach Oil of Northern Fulmars

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For this study, the transfer of plastic additives to stomach oil of northern fulmars (*Fulmarus glacialis*) has been investigated. Procellariiform seabirds retain oily components of their prey in theirs stomach as a means to store energy. A marine litter-derived microplastic reference mixture and separately a marine litter-derived polystyrene sample were added to stomach oils in an experiment. A total of 15 additives, including plasticizers, antioxidants, UV stabilizers, flame retardants, and preservatives, were identified in the original plastic mixtures and monitored in the leachates. These substances include those known for endocrine disruptive, carcinogenic, and/or other negative effects on organisms. Stomach oil was exposed to these plastic materials and was sampled during a long-term experiment (0, 14, and 90 days' exposure of plastic particles in stomach oil) and a subsequent short-term detailed study (8 h and 1, 2, 4, 8, and 21 days). Five of the monitored substances were shown to strongly leach from the microplastic reference mixture into the stomach oil during the experiment. Four substances were identified in a marine litter-derived polystyrene foam, of which two leached into stomach oil. Leaching of harmful plastic additives to the stomach oil of fulmars may be of concern, as fulmars regularly ingest plastics that are retained and gradually ground in the gizzard before passage to the intestines and excretion.

Keywords: plastic ingestion, marine litter, additive leaching, gastric fluid, *Fulmarus glacialis*

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INTRODUCTION

Plastic pollution in the marine environment is ubiquitous in all ecosystems (Galgani et al., 2015; van Sebille et al., 2015) and represents a size continuum of items from macroplastic (> 5 mm), through microplastic (5 mm to 1 μm) to nanoplastic (< 1 μm; Arthur et al., 2009; Gigault et al., 2018). In addition, plastic pollution also represents a diverse range of polymer types that can contain a broad range of additive chemicals that provide specific properties and functionalities to plastic products (Rochman, 2015; Rochman et al., 2019). Northern fulmars (*Fulmarus glacialis*) are seabirds of the order of tubenoses (Procellariiformes). They are non-specialized foragers, opportunistically hunting for fish, squid, crustaceans, and jellyfish at or close to the seawater surface (Camphuysen and Van Franeker, 1997; Ojowski et al., 2001; Byrkjedal and Langhelle, 2019), but they also scavenge on ship offal and carrion (Camphuysen and Garthe, 1997). Fulmars regularly ingest plastics in high quantities, with 95% of birds studied in the North Sea containing an average of 31 plastic particles

at an average mass of 0.28 g (OSPAR, 2019). These plastic loads decrease with latitude and reflect local abundances of plastics in the environment (Mallory, 2008; Van Franeker et al., 2011; Kühn and Van Franeker, 2012; Trevail et al., 2015). In contrast to other seabirds, most Procellariiformes store energy reserves both in adipose fat and in oil accumulated in the proventriculus of the bird (Wang et al., 2007). This light yellow– to dark orange–colored oil is produced from dietary remains and is not a product of stomach excretions (Lewis, 1969; Clarke and Prince, 1976; Imber, 1976). The composition of stomach oils can vary greatly, depending on prey species and the most recent diet, but typically consists of different types of wax esters, diacyl glycerol ethers, and triglycerides (Lewis, 1969; Imber, 1976). Stomach oil is found in all life stages of Procellariiform seabirds and is an efficient way to store highly concentrated caloric food in low volumes (Place et al., 1989). According to Place et al. (1989), this is more efficient and corresponds more flexibly to the energy demands of the birds as metabolism of fatty acids to the adipose fat reserves is unnecessary. When threatened, fulmars also spit out stomach oil as an effective deterrent. Hydrophobic organic pollutants such as petroleum hydrocarbons (Clarke and Prince, 1976), polychlorinated biphenyls (PCBs), and pesticides (Foster et al., 2010) are lipophilic, meaning that they preferentially partition and dissolve in stomach oil. As hydrophobic organic pollutants can be obtained via contaminated natural food, stomach oil has been suggested as a suitable monitoring medium for marine pollution (Clarke and Prince, 1976).

Plastic debris can contain a broad range of additive and adsorbed chemicals with associated degradation products (Andrady and Neal, 2009). There are two main pathways in which chemicals are associated with plastic debris. First is the addition of chemicals during their production process to enhance specific characteristics of the plastics (e.g., flexibility, flame resistance, or color) and residual chemicals from this production process (Rani et al., 2015). The other pathway occurs when plastics are exposed to dissolved chemicals already present in the (marine) environment (Teuten et al., 2009). Substances can be adsorbed to the plastic surface, especially smaller plastic items, which exhibit a comparably larger surface-to-volume ratio (Barnes et al., 2009). Weathering of plastics in the marine environment may enhance this adsorption process (Jahnke et al., 2017). The bioavailability of these plastic-associated chemicals following ingestion of plastic debris by marine organisms remains unclear, but has been suggested to be a function of gut conditions, gut residence time, environmental concentrations, and previous exposure/existing chemical accumulation in individual organisms (Koelmans et al., 2016; Sørensen et al., 2020).

The combination of both chemical exposure pathways, together with the physical characteristics of plastic, may harm marine wildlife when plastic debris is ingested. The capacity for wildlife to take up a range of plastic associated chemicals has been demonstrated in laboratory experimental setups (e.g., Teuten et al., 2009; Browne et al., 2015; Hermabessiere et al., 2017; Tanaka et al., 2018; Roman et al., 2019a). However, unrealistic exposure scenarios are often applied during experiments with regard to the type and shape of plastic, degree of degradation, and associated toxic substances, as most studies use homogeneous-shaped, pristine plastics (Phuong et al., 2016; Sørensen et al., 2020). Furthermore, most studies lack environmental relevance because of artificially loading the plastic materials with high concentrations of chemicals prior to study, and very few studies consider factors such as background levels of chemicals or existing chemical levels in organisms (Heinrich et al., 2020).

In 2020, the ingestion of plastics by marine organisms had been reported in at least 701 species (Kühn and van Franeker, 2020). Procellariiform seabirds, in particular, were found to regularly ingest plastics, possibly confusing them with natural diet items (Kühn et al., 2015; Ryan, 2016). Of 144 Procellariiform seabird species, 63.2% have been recorded with ingested plastics (Kühn and van Franeker, 2020), sometimes in frequencies of occurrence higher than 90% (e.g., Van Franeker et al., 2011; Roman et al., 2016; Rapp et al., 2017; Rodríguez et al., 2018). Procellariiformes ingest a wide variety of plastics, including different shapes (Van Franeker et al., 2011) and colors (Kühn et al., 2015), with the size of the ingested plastics related to body size (Roman et al., 2019b).

The first prolonged contact of ingested plastic (and natural food) with Procellariiform seabirds occurs in the proventricular stomach where gastric juices are produced to initiate the digestion process. Once in the smaller and muscular gizzard, plastic items are gradually worn, and pieces small enough to pass to the intestines are excreted (Fisher, 1952; Warham, 1996). However, the intensity and pace of the wearing process are not fully understood. In northern fulmars, hard plastic particles have to be reduced to just a few millimeters in size before they can pass from the gizzard to the intestines (Bravo Rebolledo, 2011; Terepocki et al., 2017). Terepocki et al. (2017) indicated different sizes of plastics along the digestive system from the proventriculus, via the gizzard, to the gut. The average particle mass reduced from 66 to 25 mg and finally 7 mg, respectively. The retention time of plastic in seabirds is unknown and may vary between species and by type of plastic item (size, shape, and flexibility). For the northern fulmar and its close relatives, Van Franeker and Law (2015) have suggested 75% of ingested plastic disappears within a month. However, for other species, retention times of several months to even years have been suggested (Ryan, 2015).

The leaching of chemical components from particulates to the surrounding medium is known to increase as a function of decreasing particle size and the corresponding increase in surface area (Rochman, 2015). The grinding process in seabird digestive systems increases the surface area of ingested plastics in the stomach, which consequently increases the available surface area for additive chemicals to partition into the stomach oil. As the medium in this case is an oil, it is expected that hydrophobic chemicals in particular will preferentially partition from the plastic (Tanaka et al., 2015). This process has been described previously by Tanaka et al. (2015), where the uptake of polybrominated flame retardants to stomach oil of Procellariiformes has been recorded. Once plastic particles have been reduced to a size that they can be easily excreted, it is likely that any further partitioning of additive chemicals is limited owing the short residence times of particles < 1 mm.

There has been limited focus in previous studies on the partitioning of additive chemicals. The current study aims to document the potential uptake of harmful plastic additive chemicals into fulmar stomach oil using plastic debris sampled from the marine environment as test materials. The mechanism of uptake of substances from plastic into seabirds is a crucial step to understand potential harm of plastic on seabirds, both at the individual and population levels.

MATERIALS AND METHODS

Plastic debris was collected and carefully characterized in terms of polymer type, shape, and size and milled into a microplastic mixture (Kühn et al., 2018). A comparable sample was prepared using beached polystyrene foam only. Stomach oil collected from fulmars was exposed to these plastics under realistic gut conditions. The additive chemical profiles were determined in the source materials and the stomach oil samples after exposure.

Plastic Source Materials

A marine litter-derived microplastic reference material (PTX001) was used. The collection and production of the material have been described in detail in Kühn et al. (2018). Briefly, 351 macroplastic litter items were collected from a Dutch beach (Texel, April to August 2016), equaling the mass composition of plastics during an earlier large beach clean-up. The sample comprised a mixture of rigid and flexible items (ca. 37 and 63%, respectively). The collected material was cryomilled (Retsch ZM 200, Carat GmbH) to a mix of variable sizes less than 3.0 mm in diameter. The size distribution of these particles was determined by sieving the mixture through a stacked sieve system. One gram of the PTX001 material contained around 400,000 plastic particles. The produced microplastics are irregular in shape and exhibit a broad size distribution, being more environmentally representative than the uniform spherules often used in exposure studies (Phuong et al., 2016). The polymer composition comprised mainly polyethylene (PE; 60.9%) and polypropylene (PP; 27.7%), but with many other polymers present in small amounts that provide a distribution similar to that of global polymer production (Geyer et al., 2017) and that found in seabirds (e.g., Tanaka et al., 2019). A detailed chemical analyses confirmed the presence of various heavy metals (Cd, Cr, Cu, Fe, Ga, In, Mn, Mo, Ni, Pb, Pd, Sn, Zn) and light metals (Al, Ba, Ca, K, Mg, Na, Sr, Ti), as well as additive chemicals and chemicals associated with plastic production processes (Kühn et al., 2018). A summary of the plasticizer, UV stabilizer, and flame retardant additives found in the mixture is listed in **Supplementary Table 5**. To determine the contribution of a different plastic type to the total process of additive leaching, three pieces of weathered expanded polystyrene foam (PS) were collected at the same time from the same beach as the macroplastic litter items used in production of the PTX001 mixture. The PS foam was cut manually to small particles of ∼0.5 mm in size, because cryomilling of the foamed material proved unsuccessful.

Stomach Oil Preparation

Stomach oil was collected from northern fulmars on the Faroe Islands in the north Atlantic Ocean, where fulmar fledglings are harvested for human consumption (Jensen, 2012). Fledglings are caught from the sea surface with a long-handled net ("fleygg"). Shortly after fledging, most of the young birds are too heavy to take off and are easily caught. Once caught, birds are immediately killed by breaking the neck. The fledglings not only have large fat deposits but often also contain considerable quantities of stomach oil (some tens to well over 100 mL). For this project, hunters prevented the loss of the oil by tying a small rope around the neck and provided us with the undamaged stomachs. The oil was drained directly from the stomach into glass bottles and frozen at −20°C. The stomach oil used in the current study was a homogenate combined from more than 50 different chicks collected between 2014 and 2016. As natural foods may contain contaminants, and as chicks already contain plastics transferred by their parents, a basic load of chemicals in the stomach oil was already expected at the start of the experiments.

Experimental Setup

Two exposure experiments were conducted: a long-term experiment (LTE) with three sampling points (0, 14, and 90 days) and a detailed short-term study with sampling at 8 h and 1, 2, 4, 8 and 21 days. The procedure and setting for both studies were the same, and the same batches of homogenized stomach oil and plastic test materials (PTX001 and PS) were used in both studies. Plastics were added to the stomach oil and stirred continuously at 120 revolutions/min in a shaking bath (Julabo SW23) to mimic stomach contractions and to keep the plastics in suspension. The oil was kept in amber glass vials at 40°C, the common body temperature in Procellariiform seabirds (Warham, 1996). The exposure design comprised two bottles of the PTX001 microplastic mixture, two bottles of PS, and two control bottles without added plastic, with each exposure and control sample containing 40 mL of stomach oil. The added quantity of plastics was 1.0 g for the PTX001 samples (25 g/L) and 0.3 g for the PS (7.5 g/L). A lower exposure concentration was used for the PS foam (7.5 g/L) because the particles had a very low density by high volume relative to the available volume of stomach oil. A 5 mL aliquot of oil was removed from each bottle at each sampling time point. Care was taken to ensure oil and plastics were removed in proportional quantities by using a large glass pipette where plastics were retained together with the oil. The oil was then vacuum-filtered through a glass microfiber filter (GFF, pore size 0.7 μm; GE Whatman) in a glass filtration system to remove the plastic particles (**Figure 1**). The filtered oil from each 5-mL sample was then divided between three glass vials of ˜1 mL each and directly frozen at −20°C until further analysis. This corresponds to 3 × 1-mL test vials being retrieved at each time point from each duplicate bottle, resulting in a total of six subsamples per plastic type and time point. All laboratory materials and Teflon-capped sample containers were carefully rinsed with hexane prior to use to reduce contamination.

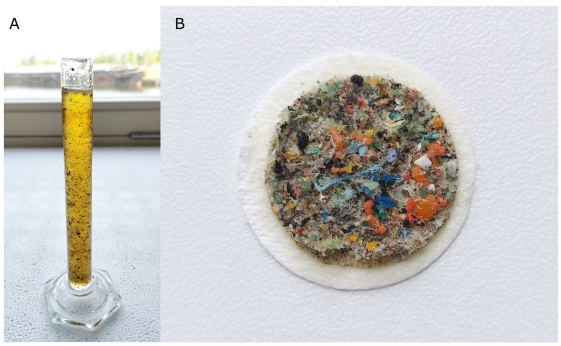

FIGURE 1 | (A) Marine derived plastic litter (PTX001) in stomach oil before filtration. **(B)** PTX001 on a GFF filter (diameter 25 mm) after filtration. Note the different plastic shapes, sizes, and colors.

Sample Analysis

In total, three sets of chemical analyses were conducted:

1. The LTE: Oil samples collected and analyzed on day 0 (control only) and next after 14 and 90 days, with controls repeated at both dates. The sampling point of 90 days was chosen as the retention time of plastics in bird species has been reported to be between 2 months (Terepocki et al., 2017) and many months (Ryan and Jackson, 1987; Ryan, 2015).
2. The short-term detailed experiment (STD): Replicated the LTE setup, but over a shorter timescale. STD oil samples were taken on day 0 (control) and next after 8 h and 1, 2, 4, 8, and 21 days. Controls were measured in the first three and last samples.
3. The long-term replicate (LTR): These analyses represented a check on the replicability of the initial (LTE) methods. The 14-day, 90-day, and control samples from the LTE were reanalyzed.

Plastic Extraction

Samples of the plastic materials (∼500 mg PTX001 mixture, ∼18–30 mg PS foam) were solvent extracted in triplicate with two different solvents; dichloromethane (DCM, Rathburn) and ethyl acetate (EtOAc, Fluka). In each case, 4 mL of solvent and an internal standard mixture (0.2508 μg naphthalene-*d*8, 0.0500 μg phenanthrene-*d*10, 0.0486 μg chrysene-*d*12) were added to each sample prior to bath sonication for 30 min (Bandelin Sonorex Super RK 510 H, 640 W, 35 kHz) at either room temperature (DCM) or 65°C (EtOAc). The solvent extract was then filtered through a pipette packed with Bilsom cotton to remove plastic particles and a small amount of anhydrous Na_2SO_4 to remove any moisture. The extracts were then concentrated by solvent evaporation (40°C under a gentle flow of N_2) to ˜500 μL, and a recovery internal standard (0.0984 μg fluorene-*d*10 and 0.1064 μg acenapthene-*d*10) was added prior to gas chromatography–mass spectrometry (GC-MS) analysis. Prior to clean-up by gel permeation chromatography (GPC), samples extracted by DCM were readjusted to 1 mL volume with additional DCM.

Stomach Oil Extraction

Samples of fulmar oil (50 mg in the LTE and 100 mg in the LTR and STD) were transferred from the 1-mL vials to a glass tube and dissolved in 1 mL DCM:*n*-hexane (1:1). An internal standard mixture (0.2508 μg naphthalene-*d*8, 0.0500 μg phenanthrene-*d*10, 0.0486 μg chrysene-*d*12) was added, and the sample vortexed (30 s). The sample volume was then adjusted to 1 mL by solvent evaporation (40°C under a gentle flow of N_2).

Gel Permeation Chromatography

Both fulmar oil extracts and DCM polymer extracts were subject to instrumental clean-up by GPC (Agilent). Samples (500 μL) were injected with DCM as the mobile phase (0.5 mL/min in the LTE, 5 mL/min in the LTR and STD), and components separated using either an Agilent PLGel column (7.5 × 300 mm, 5 μm; LTE) or a Waters Envirogel column (19 × 300 mm, 15 μm; LTR and STD). Chromatograms were monitored at 210, 254, and 280 nm UV. After initial optimization, analyte fractions were collected from 16 to 35 min (LTE) or 10.5 to 15 min (STD) with preadded *n*-hexane in the collection vials as a keeper. The sample volume was adjusted to 0.5 mL by solvent evaporation (40°C under a gentle flow of N_2), and recovery internal standards (0.0984 μg fluorene-*d*10 and 0.1064 μg acenapthene-*d*10) were added prior to GC-MS analysis.

GC-MS Full-Scan Analysis

The GC-MS system comprised an Agilent 7890A GC equipped with an Agilent 5975C Mass Selective Detector. The inlet was set to 250°C, the transfer line to 300°C, the ion source to 230°C, and the quadrupole to 150°C. The carrier gas was helium at a constant flow of 1.1 mL/min. Samples of 1 μL were injected by pulsed splitless injection. The GC column was an Agilent DB5-MS ultra-inert column (30 m, 0.25-μm film thickness, 0.25-mm internal diameter). The GC oven was held at 40°C (2 min), ramped at 6°C/min to 320°C, and held at that temperature for 20 min. Mass spectra were recorded in full scan mode over the mass range 50 to 500 m/z, after a 12-min hold time.

GC-MS Selected Ion Monitoring Analysis

Using compounds identified from the full-scan analysis of the PTX001 and PS material extracts, a selected ion monitoring (SIM) method was developed to enable a more detailed, targeted analysis of chemicals present in the stomach oil extracts. This approach increases the sensitivity of the analysis and helps to reduce background noise and interference from biogenic compounds derived from the stomach oil. The same GC-MS system and instrumental conditions as above were applied. Selected ions representative of the tentatively identified organic additive compounds were monitored according to **Supplementary Table 1**.

Data Treatment

For non-target screening, chromatograms and mass spectra were recorded using Chemstation software, investigated in

Masshunter Qualitative Navigator B.08.00, further processed using Masshunter Unknowns Analysis ("Unknowns") followed by export to csv format using Python and data processed in R. After initial inspection of chromatograms, peaks were deconvoluted using Unknowns algorithms, and best hits from NIST 2017 library were extracted. Compounds were filtered based on their observed presence in at least three of six replicates for each polymer and a > 90% match to NIST 2017 library mass spectra. Biogenic compounds, or compounds of possible biogenic origin, were removed from the data set. All compounds found in the control samples were also removed from the data set, leaving only those that could be confidently attributed to coming from the PTX001 and PS materials.

For targeted analysis, the selected tentatively identified compounds were recorded by their retention time and major ions (**Supplementary Table 1**) in GC-MS SIM mode. Masshunter Quantitative Analysis was further used to integrate peak areas of the selected compounds and the added internal standards. The area of each tentatively identified compound was normalized by dividing by the area of one internal standard in each sample and the normalized relative intensities used to compare samples.

Control-Derived Limits of Detection

Given the exploratory and non-quantitative nature of the analysis, there was a lack of reference standard chemicals for the identified additives, and therefore, it was not possible to establish individual calibration curves for each chemical. Control-derived limits of detection (LOD) were established based on control measurements for each of the three treatments separately. The LOD was calculated as the average of the controls of each treatment plus three standard deviations. As expected, results showed that even the unexposed control stomach oil samples contained some level of additive chemicals already present when the oil was harvested.

RESULTS

Characterization of Plastic Material

In the current study, non-target screening with a 90% confidence match to library spectra (>90% match to NIST 2017 library) permitted identification of 15 different organic chemical additives in the solvent extracts produced from the two test materials, with 14 identified in PTX001 and 4 identified in the PS (**Table 1**). Three of the identified compounds were found in both materials (acetophenone, propanediylbisbenzene, and triphenylbenzene). Full names for each compound are given in **Table 1**. These substances include common additives such as plasticizers, antioxidants, UV stabilizers, flame retardants, and preservatives (**Table 1**). For some chemicals identified in the samples, however, the use or origin is unclear. Chemical properties and estimated biodegradability, bioaccumulation, and biotransformation rates of these compounds are given in **Supplementary Table 6**. Estimates have been calculated using the BIOWIN^{TM} and BCFBAF^{TM} packages of EpiSuite (US EPA, 2012). For both the long-term experiments (LTE, LTR) and the short-term experiment (STD), the temporal concentration trends of each target compound in the stomach

TABLE 1 | Compounds monitored by GC-MS SIM analysis.

No.	Substance short name	CAS number	Full substance name	Detected in	Known uses
1	Acetophenone	98-86-2	Acetophenone	PS, PTX	Precursor to resins/copolymers, used in coatings, inks, and adhesives
2	*p*-Benzoquinone	719-22-2	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)	PTX	Used in synthesis
3	Dibutylphenol	96-76-4	2,4-Di-tert-butylphenol	PTX	Antioxidant
4	Propanediylbisbenzene	1081-75-0	Benzene, 1,1'-(1,3-propanediyl)bis	PS, PTX	
5	Phenyl benzoate	93-99-2	Benzoic acid, phenyl ester	PS	Preservatives used in cosmetics, film, foods
6	TCEP	115-96-8	Tri(2-chloroethyl) phosphate	PTX	Plasticizer, flame retardant, viscosity regulator
7	TCPP (3:1)	13674-84-5	2-Propanol, 1- chloro-, phosphate (3:1)	PTX	Flame retardant
8	BCPP	137888-35-8	Bis(3-chloro-1-propyl) (1-chloro-2-propyl)phosphate	PTX	
9	7,9-Di-*tert*-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	82304-66-3	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	PTX	Antioxidant (degradation product)
10	DBP	84-74-2	Dibutyl phthalate	PTX	Plasticizer
11	TPhP	115-86-6	Triphenyl phosphate	PTX	Plasticizer, flame retardant
12	Triphenylbenzene	28336-57-4	Cyclohexane, 1,3,5-triphenyl	PS, PTX	Packaging migration residue, polystyrene impurity
13	DEHP	117-81-7	Bis(2-ethylhexyl) phthalate	PTX	Plasticizer
14	Bumetrizole	3896-11-5	Bumetrizole	PTX	UV stabilizer
15	Di(2-ethylhexyl) terephthalate	6422-86-2	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	PTX	Plasticizer

The abbreviations or alternative names of the substances, CAS numbers, detection in PS or PTX001 material, and known applications are given for each identified chemical.

oil are shown in **Supplementary Material** (Chapters 3.1–3.15).

Exposure Conditions During Measurements

Stomach oil extract samples from long-term exposures to PTX001 and PS (0, 14, and 90 days) were analyzed twice (LTE and LTR) to evaluate analytical reproducibility, because the LTE and STD results were generated using two different GC-MS analysis methods. The results of the LTE and LTR analyses showed mostly consistent responses relative to the internal standard (phenanthrene-*d*10) and how it relates to the LOD. For five substances (acetophenone, propanediylbisbenzene, triphenylbenzene, DEHP, and di-(2-ethylhexyl) terephthalate), the response scale increased for the LTR. The relative LOD was comparable for all substances during the LTE and the LTR.

For most samples, the two bottles representing identical treatments (bottles A and B; indicated in **Supplementary Material** graphs by two samples at the same time point) show similar averaged results, indicating the replicability of the chosen approach. The highest variation between A and B samples was observed for the compound TCPP (3:1) for PTX001 and PS (but not for the controls) during the LTE and the LTR experiments (**Supplementary Material** Chapter 3.7, page 13). Within these bottles, the replicability was generally good (indicated in the graphs with error bars per sample). Different results between pairs of A and B sample bottles were mainly observed in the STD experiment. For PTX001, a high deviation was observed in TCEP and bumetrizole, whereas for PS, a high deviation was observed in 7,9-di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione and DBP. Among control bottles, the difference between the A and B samples was pronounced for only one compound, di-(2-ethylhexyl) terephthalate.

Another indication of the reproducibility of the measurements is demonstrated by the control values, which remained relatively stable over time during all measurements with only a few exceptions. Variable control values over time were observed mainly during the first days of the STD experiment, with the strongest variation found in DEHP (**Supplementary Material** Chapter 3.13 page 19).

Additive Leaching From Plastic to Stomach Oil

All experimental results are summarized in **Table 2** and presented in detail in the graphs in **Supplementary Material** Chapters 3.1–3.15. A selection of results is shown and discussed here, subdivided into (i) additive compounds that exhibited clear signs of leaching to the stomach oil and (ii) compounds for which there was no evidence of leaching. Other substances showed weak or inconclusive results (**Table 2**). For improved

TABLE 2 | Summary of the leaching results for different substances detected in marine litter-derived microplastic reference mixture (PTX001) and marine litter-derived polystyrene foam (PS) to stomach oil of northern fulmars (0 = no, + moderate, + + strong leaching).

No.	Substance short name	Compound identified in	Leaching behavior from PTX001 marine plastic debris mix		Leaching behavior from marine PS foam debris	
1	Acetophenone	PS and PTX	Moderate leach in initial weeks, then stable or minor decrease	+	Strong leach in initial weeks, then stable or minor decrease	+ +
2	*p*-Benzoquinone	PTX	Strong increase in initial weeks followed by disappearance at 90 days	+ +	No initial effect, and slightly decreasing on long term	0
3	Dibutylphenol	PTX	Persistent leaching to day 90	+ +	Initially leaching but not persisting on long term	+
4	Propanediylbisbenzene	PS and PTX	Possibly light initial leach, but unclear pattern	0	No indications for leaching	0
5	Phenyl benzoate	PS	Possibly minor initial leach, but disappears afterward	0	Strong leaching in first few weeks, but compound then largely disappears	+ +
6	TCEP	PTX	Rapid initial leaching then more or less constant on longer term	+ +	No indications for leaching	0
7	TCPP (3:1)	PTX	Initially leaches and remains constant on longer term	+	Variable data, possibly slight initial leach but disappears	0
	BCPP	PTX	Leaches in first weeks, but then stabilizes	+ +	Leaches in first weeks, then slightly decreases	+ +
9	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	PTX	No evidence for leaching	0	No evidence for leaching	0
10	DBP	PTX	Moderate leaching	+	No signals of leaching	0
11	TPhP	PTX	Exponential leaching slowing down on long term	+ +	No evidence for leaching	0
12	Triphenylbenzene	PS and PTX	No good evidence for leaching	0	No evidence for leaching	0
13	DEHP	PTX	Long term continued leaching	+ +	Initial slight leaching, but reduces on longer term	+
14	Bumetrizole	PTX	Rapid initial leach, persists on longer term	+ +	No evidence for leaching	0
15	Di-(2-ethylhexyl)terephthalate	PTX	Moderate leaching quickly stabilizes	+	Moderate leaching unclear on longer term	+

Conclusions are based on three independent experiments. The behavior details of each compound are shown in the Online Supplement.

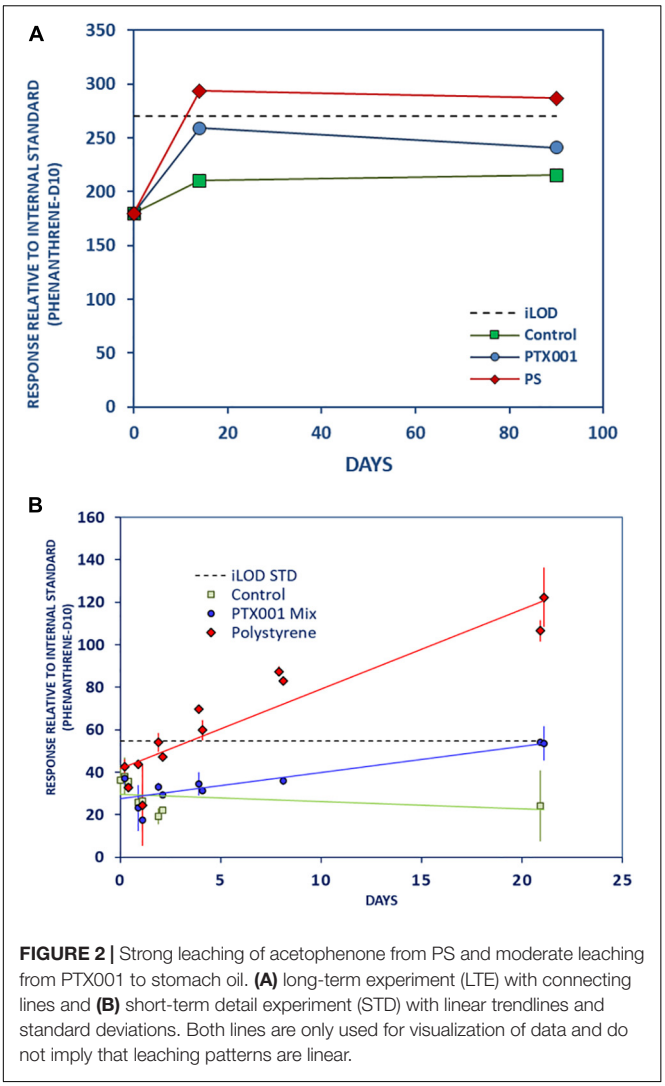

FIGURE 2 | Strong leaching of acetophenone from PS and moderate leaching from PTX001 to stomach oil. **(A)** long-term experiment (LTE) with connecting lines and **(B)** short-term detail experiment (STD) with linear trendlines and standard deviations. Both lines are only used for visualization of data and do not imply that leaching patterns are linear.

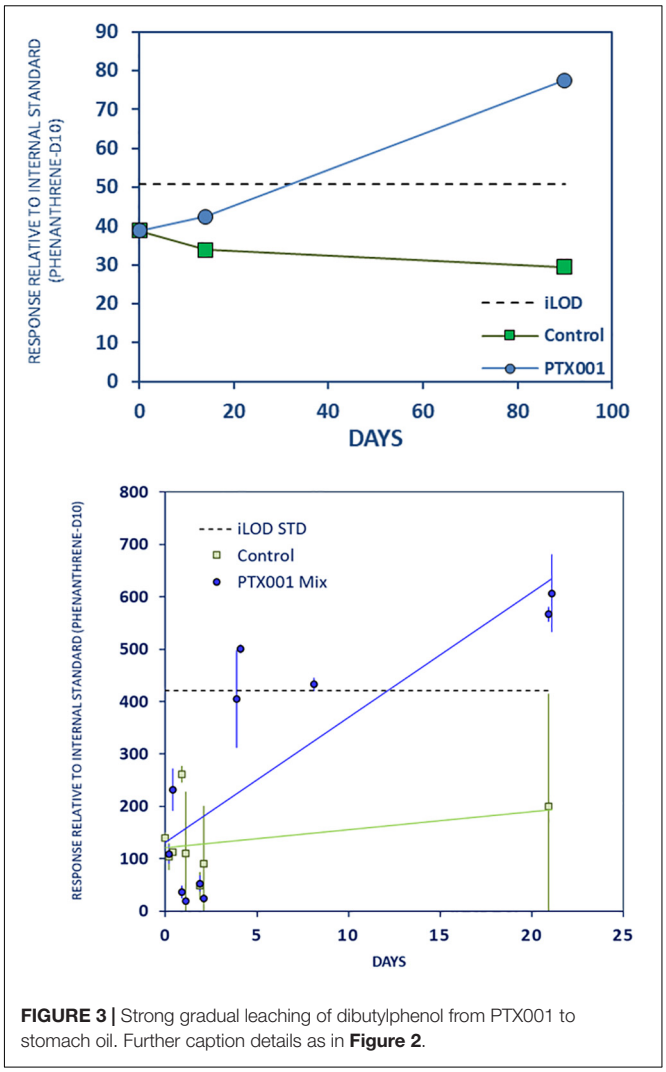

FIGURE 3 | Strong gradual leaching of dibutylphenol from PTX001 to stomach oil. Further caption details as in **Figure 2**.

visual interpretation, graphs for the LTE show connection lines from the 0-day measurement (control) to the 14- and 90-day measurements. STD graphs include linear trendlines and standard deviations of the duplicate measurements. In **Supplementary Material**, all graphs include linear trendlines and standard deviation.

Leaching Observed

Fast leaching of acetophenone (a precursor to resins and copolymers) from both PTX001 and PS foam to the stomach oil was evident during the first 2 weeks (**Figure 2** and **Supplementary Material** Chapter 3.1 page 7), with PS foam especially exhibiting a high level of leaching. STD results show that acetophenone started leaching from PS almost immediately, followed by strong increase in relative concentration up to day 8 and reached the highest level by day 21. The LTE experiment supports that pattern, showing a high increase during the first 14 days followed by a stabilization until day 90. For PTX001 in the LTE experiment, the acetophenone level increased above the level in the control samples after 14 days and subsequently decreased slightly at day 90. During the STD experiment, acetophenone showed gradual leaching from PTX001 until day 21, however, leaching was less pronounced than in PS.

Strong leaching of dibutylphenol, an antioxidant additive, was observed to occur from the PTX001 plastics (**Figure 3**). LTE and LTR results suggest gradual leaching for up to 3 months. Results from the STD experiment are rather variable but do support leaching also in the initial weeks. Some leaching of dibutylphenol may also occur from PS, but results are rather variable (**Supplementary Material** Chapter 3.3, page 9).

Phenyl benzoate, a preservative, showed an initial strong leaching from PS foam to the stomach oil in all three experiments (**Figure 4**). Results from the LTE and the LTR experiments indicated a decrease of phenyl benzoate leachate concentrations between 14 and 90 days, although the substance was still above the control levels at 90 days (**Supplementary Material** Chapter 3.5, page 11).

TCEP, a plasticizer, flame retardant, and viscosity regulator, showed an initial rapid leaching from PTX001 during the LTE and the LTR experiments, which decreased only slightly after

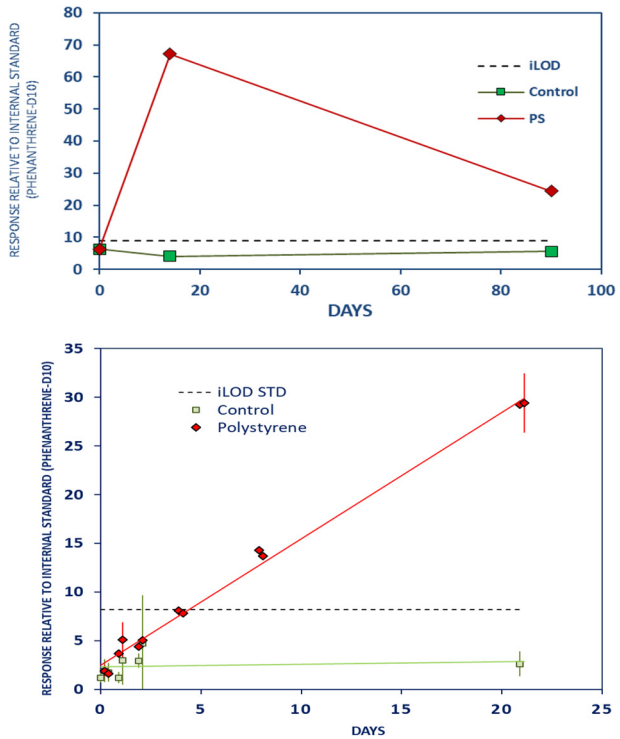

FIGURE 4 | Strong initial leaching of phenyl benzoate from PS to stomach oil with decrease in leachate concentrations between 14 and 90 days. Further caption details as in **Figure 2**.

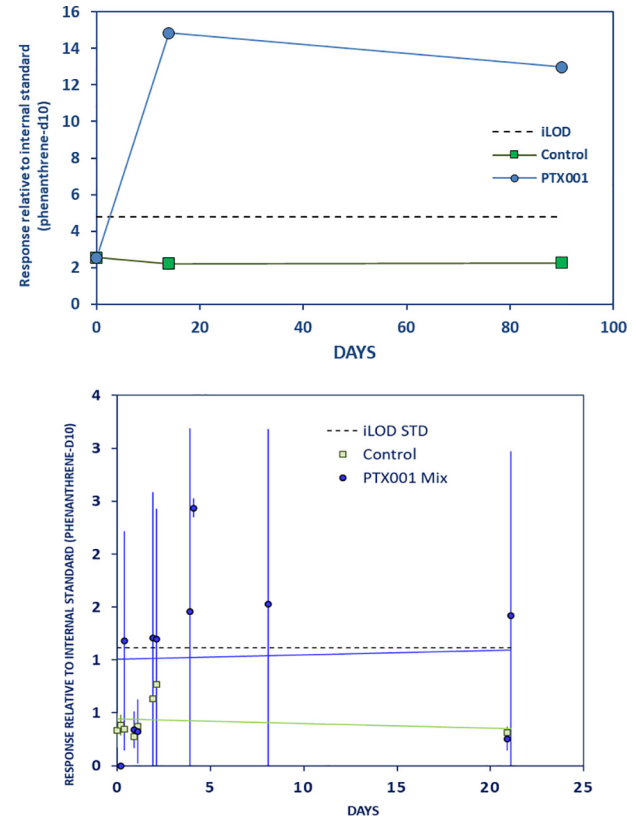

FIGURE 5 | Strong initial leaching of TCEP from PTX001 to stomach oil. Left: long-term experiment. Further caption details as in **Figure 2**.

90 days (**Figure 5** and **Supplementary Material** Chapter 3.6, page 12). During the STD experiment, high variation within both replicate samples (bottles A and B) was observed, and although all data points are above the control level, the results are probably less reliable.

DEHP, a plasticizer (**Figure 6** and **Supplementary Material** Chapter 3.13, page 19), showed strong leaching from PTX001 during both the LTE and LTR experiments, with concentrations continuing to increase strongly until day 90. These findings are not supported in the STD experiment, where the control samples showed a highly varied pattern over the initial days and thus indicating lower reliability of the results.

Bumetrizole, a UV stabilizer also known as Tinuvin 326 (**Figure 7** and **Supplementary Material** Chapter 3.14, page 20), rapidly leached from PTX001 to a great extent during the first days of exposure in the LTE and LTR experiments. The levels appear to stabilize after 14 days of exposure, demonstrated by results from all three experiments. Data from the STD experiment indicate that leaching to the maximum observed concentration occurs almost instantly after the plastic is exposed to the stomach oil.

No Leaching Observed

Although identified in the solvent extracts of the PTX001 and PS test materials, some of the tentatively identified additive chemicals were not found in the corresponding stomach oil leachates in any of the studies. In the case of PTX001, no detectable leaching was measured for phenyl benzoate (preservative; **Supplementary Material** Chapter 3.5, page 11), propanediylbisbenzene (unknown use; **Supplementary Material** Chapter 3.4, page 10), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (antioxidant; **Supplementary Material** Chapter 3.9, page 15), DBP (plasticizer; **Supplementary Material** Chapter 3.10, page 15), and triphenylbenzene (packaging migration residue; **Figure 8** and **Supplementary Material** Chapter 3.12, page 18). For the PS foam, propanediylbisbenzene and triphenylbenzene (**Figure 8**) were only found in the parent material and not in the corresponding leachates.

DISCUSSION

Results from this study confirmed that different types of additives leached from the marine litter-derived microplastic test materials to the fulmar stomach oil. The relative amounts varied across the different chemicals and reflected their individual properties. As a result, the additive chemical profiles of the test materials and leachates exhibited some substantial differences to each other. For the microplastic mixture (PTX001), leached chemicals included precursors to resins and copolymers, antioxidants, plasticizers, flame retardants, and UV stabilizers. For PS, leaching of precursors to resins and copolymers, antioxidants, and

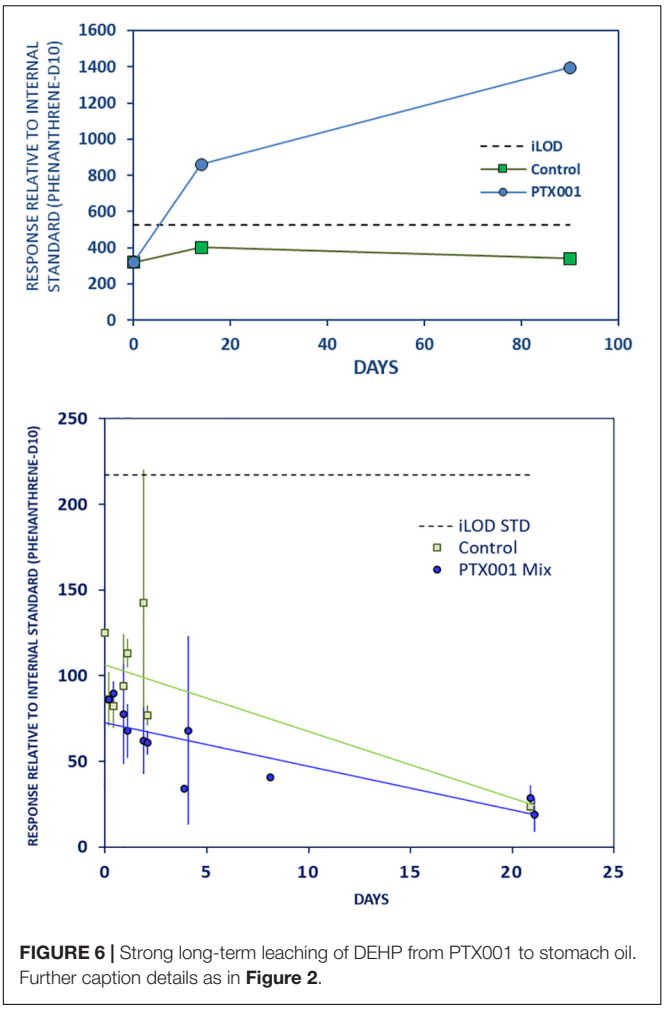

FIGURE 6 | Strong long-term leaching of DEHP from PTX001 to stomach oil. Further caption details as in **Figure 2**.

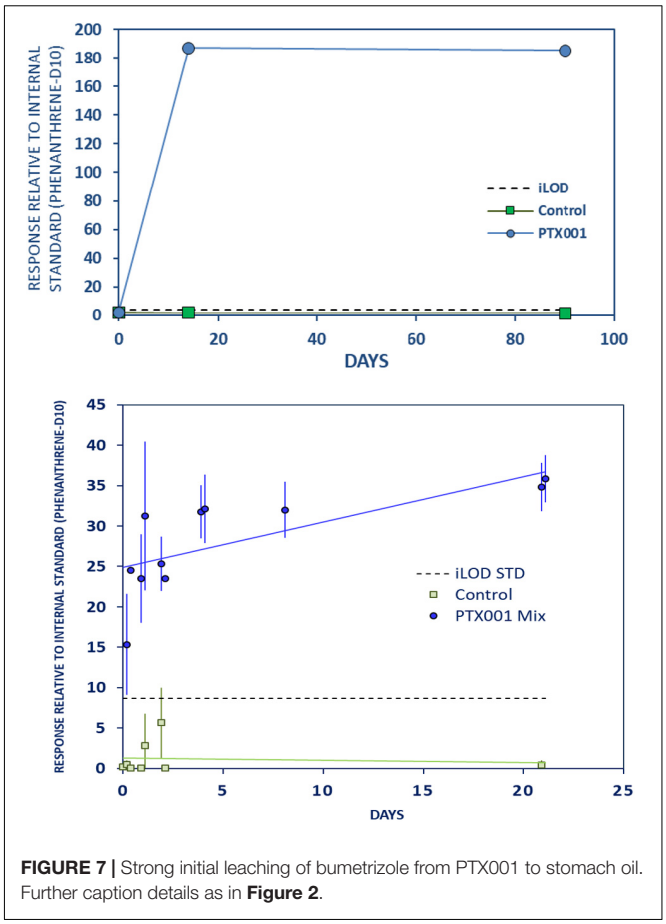

FIGURE 7 | Strong initial leaching of bumetrizole from PTX001 to stomach oil. Further caption details as in **Figure 2**.

preservatives was observed. According to Smedes et al. (2017), increasing lipophilicity (log K_{OW}) of organic hydrophobic contaminants would lead to increased lipid–polymer partition coefficients. In the current study, we observe no clear pattern between log K_{OW} and which compounds leach to a greater extent. The higher number of chemicals present in the PTX001 material reflects its composition of multiple plastic types, each with its own additive chemical profiles. Three of the chemicals were present in both materials, suggesting these may derive from the PS component of PTX001 or that different polymer types sometimes contain the same additives.

Many studies looking at the leaching of additives from plastic have utilized chemical analyses to investigate the concentrations of specific target chemicals and chemical groups. While this approach can provide useful information, it risks filtering out other compounds that may be present and contributing to any observed leachate toxicity. The current study used non-target screening as start point for identifying as many chemicals as possible in marine litter-derived microplastic test materials (PTX001 and PS foam). This enabled the development of analytical methods for quantifying these same chemicals in the corresponding leachates generated in a series of exposure studies (LTE and STD) conducted with northern fulmar stomach oil.

Exposure Conditions During Measurements

In the STD experiment, some of the substances showed high variation and unclear patterns of leaching during the first days (0–4 days) of exposure. For example, *p*-benzoquinone (**Supplementary Material** Chapter 3.2, page 8) and TCPP (3:1) (**Supplementary Material** Chapter 3.7, page 13) show varying concentration patterns for all measurements during 8 h and days 1 and 2 stomach oil leachate samples (high variation between bottles A and B and high variation within each bottle). This could be caused by compound instability in the sample matrix during processing, storage, or transportation. In addition, minor differences in sampling time and sample treatment may influence the data more significantly in the early stages of the exposure. Despite the sometimes erratic concentration patterns during the first days of the STD experiment, the longer-term results and trends overlap between the three experiments (LTE, LTR, and STD), providing the necessary confidence in the results presented.

Some of the additive chemicals (e.g., *p*-benzoquinone and triphenylbenzene for PTX001 and phenyl benzoate for PS)

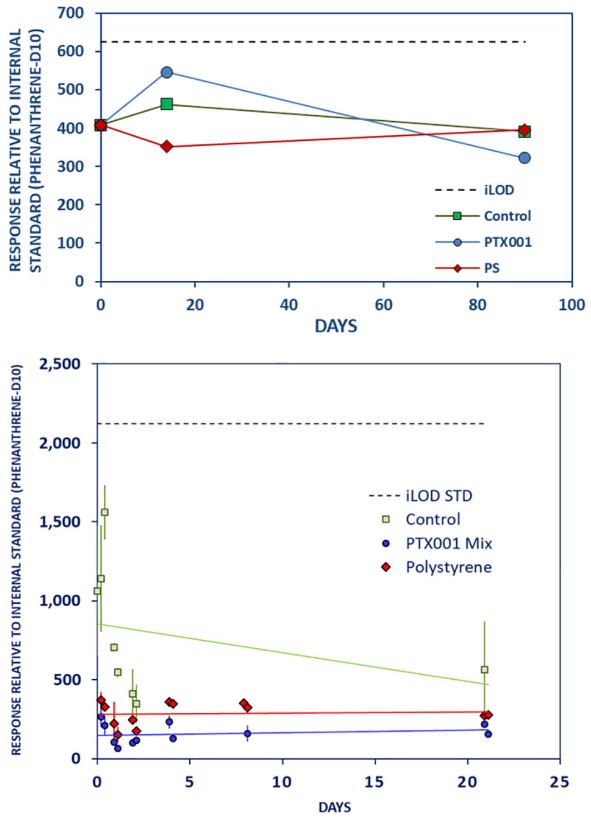

FIGURE 8 | No leaching of triphenylbenzene from PTX001 or PS to stomach oil. Further caption details as in **Figure 2**.

exhibited an initial increase in stomach oil followed by a subsequent decrease at 90 days. The data for such chemicals suggest that equilibrium has been reached prior to the sampling point at 90 days, but that a secondary process is occurring that results in the concentration decrease observed between 14 and 90 days. It is not possible to identify exactly what process or processes have contributed to this trend, but a number of viable mechanisms are possible given the long timescales used in the LTE experiment. Several of the identified compounds are readily biodegradable according to estimates made using BIOWIN^{TM} (**Supplementary Table 6**; US EPA, 2012). However, as some of the compounds that are readily biodegradable do not see a decrease in relative concentrations of the duration of the experiments, it is unlikely that biotic degradation has influenced the results of the current study. It is important to consider that the exposure systems were not sterile and that microbial biotransformation and biodegradation of additive chemicals might occur once they have partitioned to the stomach oil. These processes only require a small modification to the chemical structure (e.g., partial degradation) and the resulting biotransformation products would not be measured using the targeted analytical chemical methods developed for leachate characterization and quantification. It is possible that specific chemicals will be more or less susceptible to such different processes, but also that more than one of these mechanisms may act on an individual additive chemical at the same time.

In our attempts to document the leaching of different organic chemical additives from marine litter-derived microplastic test materials into the stomach oil of northern fulmars, we encountered issues with the comparability of data from the three experiments. The results in the LTE experiment and the STD experiment were sometimes inconsistent, and we are unable to propose a satisfactory explanation for such discrepancies. Long-term samples were analyzed twice, initially as part of the LTE experiment and then reanalyzed (LTR) together with the samples generated in the STD experiment. All GC-MS analyses were performed in randomized order, and blank samples were frequently analyzed to ensure there was no carryover of chemicals between samples and analyses. Subsamples of fulmar stomach oil, PTX001, and PS foam samples from the same batches (common source) were used in both the long- and short-term studies, and it is suggested that any inhomogeneity in oil or plastic materials is relatively small and not responsible for the observed differences in the leachates as the comparability of A and B samples demonstrates. The stomach oil was stored frozen prior to the LTE and quickly refrozen for storage between the LTE and STD exposure experiments. Furthermore, there were no changes in the sampling protocol between the two studies. Therefore, we have chosen to regard the LTE experiment and STD experiment as separate studies representing short- and long-term exposures. Despite the inconsistencies described, the strong similarities between many of the leaching profiles from the LTE experiment and the STD experiment samples, together with the stable levels of contamination (in most cases) observed in the control samples and good overlap of A and B samples, suggest sufficient reliability in the reported outcomes of this study.

Additive Leaching From Plastic to Stomach Oil

The results in the current study clearly show that several additive substances can leach from plastics ingested by fulmars into stomach oil under environmentally relevant conditions and over timescales estimated to be within the gut residence time (Ryan and Jackson, 1987; Van Franeker and Law, 2015; Ryan, 2015; Terepocki et al., 2017). Once leached into the stomach oil, there are well-established mechanisms that can facilitate the uptake of some of these chemicals by the birds (Galloway, 2015; Garvey, 2019; Tanaka et al., 2020). However, whether an individual additive chemical is subsequently transferred into specific organs or tissues and whether it will accumulate are influenced by a range of factors. The properties of a specific chemical will determine the partitioning between uptake or excretion in the feces (Tourinho et al., 2019; Ribeiro et al., 2019). For chemicals that are absorbed, some may undergo metabolization prior to or during storage and accumulation in specific tissues or removal from internal organs via kidney function or to feathers (Letcher et al., 2010; Provencher et al., 2018). It must be emphasized that leaching of chemicals to stomach oil in the current study was observed to occur in stomach

oil that was already contaminated with chemicals from food and plastics ingested by the fulmar chicks during the 7-week nestling period. The basic load of selected additives can be seen in the 0-measurements in this study, as these represent the oil before any treatment.

It is difficult to find relevant toxicity data for the specific 15 chemicals identified in the current study. However, several of the compounds that were observed to leach into the stomach oil belong to groups of chemicals that may have serious impacts on the health of the animals (Zimmermann et al., 2019). For example, certain UV stabilizers (e.g., UV320, UV326-328) have been shown to bioaccumulate, act as endocrine disruptors, and cause mutagenic toxicity responses (Rani et al., 2015). Phthalates, widely used as plasticizers, have been found to be endocrine disruptors, as well as affecting reproduction (Oehlmann et al., 2009; Geueke and Muncke, 2017). DEHP is very common in the environment (Hermabessiere et al., 2017), but our results indicate that it can also leak directly from marine plastic litter to the stomach oil of fulmars, offering an additional pathway of uptake. The uptake of DEHP from plastics is enhanced by the natural conditions in seabirds' digestive systems, such as high temperatures and low pH values (Bakir et al., 2014). Negative effects of plastic-associated substances on other organisms have been observed in other experimental studies (e.g., Lithner et al., 2009; Capolupo et al., 2020). Coffin et al. (2019) provided strong experimental evidence of increase of the biological estrogenicity of cells from ingestion of some plastic items by both birds and fishes.

In our study, leaching of chemicals from the plastic test materials is presented relative to the initial occurrence. We cannot assess if the leached quantities of chemicals would lead to direct health impacts in fulmars, and this should be a focus of future studies. Importantly, any effects from plastic-associated chemicals on marine organisms are likely to be influenced by the complex interplay of multiple intrinsic and extrinsic factors, including environmental conditions, specific chemical profiles associated with individual plastic items, polymer type, amount of plastic ingested, form and origin of the plastic ingested, and preexisting contaminant levels in organisms and the surrounding environment. Although the sublethal effects of plastic-related compounds on the health of populations or species remain difficult to substantiate (Browne et al., 2015; Werner et al., 2016), our results give grounds for concern.

Tanaka et al. (2019) showed that plastics ingested by fulmars and albatrosses contain UV stabilizers, flame retardants, and styrene oligomers, similar to those found in the PTX001 and PS foam test materials. PE and PP were the most common plastic types encountered in the studies by Tanaka et al. (2019) and in the PTX001 samples (Kühn et al., 2018). Tanaka et al. (2013, 2015) documented that specific congeners of polybrominated flame retardants leached from ingested plastic to the stomach fluids and were subsequently transferred to tissues in short-tailed shearwaters (*Ardenna tenuirostris*). This organism-level detection of polybrominated flame retardants shows a pathway that may occur in fulmars and other seabirds, comparable to the stomach oil leaching mechanism in fulmars as described in our experiment. Recently, Tanaka et al. (2020) fed artificially spiked plastic pellets to streaked shearwater (*Calonectris leucomelas*) chicks and concluded that in seabird species that consume plastics as frequently as fulmars, leaching of additives represented a considerably more important pathway of specific pollutants to the bird tissues than through accumulation of pollutants in food. Importantly, in the experiment of Tanaka et al. (2020), additives were built in the polymer matrix, and not just added to the surface. Together with organism-level detection of such chemicals by Tanaka et al. (2020), our results provide the evidence that such leaching of additives or their degradation products from degrading plastic litter actually occurs in the marine environment, from plastics ingested by a range of marine wildlife.

The studies by Tanaka et al. (2013, 2019, 2018, 2020) and our work on embedded additive leaching cannot be compared to some published model approaches and seabird investigations focusing on plastic surface adsorption and desorption of persistent organic pollutants. Model approaches by, e.g., Gouin et al. (2011), Koelmans et al. (2014, 2016), and Bakir et al. (2016) have indicated that plastics ingested by seabirds are not acting as a relevant source of pollutants in comparison to food. It has even been implied that during gut passage plastics could act as passive samplers for pollutants already present in the organisms, thereby reducing contaminant concentrations in the body. Seabird studies by Herzke et al. (2016), Provencher et al. (2018), and Provencher et al. (2020) tend to be seen as support for such models because no correlation could be demonstrated of selected pollutants on plastics in the stomachs of individual birds and the concentration of such substances in their tissues. As such, the models and seabird studies represent a quite different process to that of the leaching of a wide range of plastic additives embedded in the polymer matrix, and results should not be compared.

CONCLUSION

In the current approach, we attempted to avoid inclusion of foodweb-related chemical pollutants, either additives or adsorbed, by excluding substances found in the untreated stomach oils. The relative importance of both pathways seems difficult to quantify. As additive chemicals are distributed throughout the entire polymer matrix and not just at the surface, grinding of plastics in seabird stomachs makes such substances increasingly available for leaching due to the increased surface area. Although modeling studies can provide a useful indication of the bioavailability of plastic additive and adsorbed chemicals to organisms following ingestion (Koelmans et al., 2014; Koelmans, 2015), they are not necessarily able to consider the ingestion of a broad spectrum of highly variable consumer debris items in combination with the unique gastric environment in Procellariiformes, with high temperatures, low pH values, the occurrence of stomach oil, and the grinding activity in the gizzard. Our results clearly add evidence to earlier studies by Tanaka et al. (2015, 2020). Results of both studies suggest major value in further work to evaluate impacts at the cellular, tissue, and organism levels. Plastic ingested by Procellariiformes can be a vector of several harmful additive chemical compounds (e.g.,

plasticizers, flame retardants, etc.) over environmentally relevant gut residence times.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This animal study was reviewed and approved by the Environment Agency of the Faroe Islands (Reference number: 18/00440-1).

AUTHOR CONTRIBUTIONS

JF conceptualized the study and collected the fulmar stomach oil on the Faroe Islands. AO and colleagues at Carat GmbH developed the grinding technology and provided the microplastic mixtures used for the experiments. SK executed the exposure experiment and wrote the first draft of the manuscript. LS and AB analyzed the samples and wrote sections of the manuscript. All authors contributed to manuscript revisions and read and approved the submitted version. A previous version of this manuscript is included in the Ph.D. thesis of SK (https://doi.org/10.18174/509638).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00138/full#supplementary-material

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Conflict of Interest: AB and LS were employed by the research institute SINTEF Ocean. AO was employed at the company CARAT GmbH.

The remaining 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.

frontiers in Environmental Science

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Micro- and Nanoplastic Exposure Effects in Microalgae: A Meta-Analysis of Standard Growth Inhibition Tests

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Background: Ecological impacts of micro- and nanoplastics particles (MNP) are among the most discussed environmental concerns. In algae, MNP are commonly hypothesized to reduce growth, which is a standard ecotoxicological endpoint. However, the reported test outcomes vary, with both growth inhibition and stimulation being observed. Due to this conflict of information, a data synthesis for MNP potential to cause growth inhibition in toxicity testing is needed.

Methods: We performed a meta-analysis study to assess the effect of MNP exposure on algal growth. Twenty studies published between 2010 and 2020 and representing 16 algal species and five polymer materials administered as particles in size range 0.04–3,000 μm were included in this meta-analysis. A random-effect model was used to estimate the effect size in three datasets: (1) *Low concentration* range (<100 mg/L), (2) *High concentration* range (≥100 mg/L), and (3) *Full range model* (0.004–1,100 mg/L), which encompassed all studies using the combination of experimental settings (test species, MNP concentration, polymer material, and particle size) yielding the highest effect size within a study.

Results: The exposure to MNP was not significantly associated with growth inhibition in any of the models tested. However, a high heterogeneity between the studies was found in all three models. Neither MNP concentration nor polymer material contributed significantly to the heterogeneity, whereas polymer density had a significant moderating effect, with a higher risk of growth inhibition at lower densities. We also identified a publication bias, with small studies that reported significant inhibition being overrepresented in our dataset.

Conclusions: The meta-analysis found limited evidence for MNP effect on microalgal growth in the standard algal growth inhibition test. The heterogeneity and varying methodological quality of studies limited the interpretation and the confidence in the findings. For hazard assessment, standardization and controlled exposure are needed as well as more sensitive endpoints that can inform us about the effect mechanisms. Finally, using particle-free controls in such tests cannot account for the presence of inert

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particulates in the test system, and, hence, does not allow to attribute observed effects to the test polymers.

Keywords: metaanalysis, algal growth inhibition, hazard assessment, nanoplastics, microplastics, particulate matter, suspended solids

INTRODUCTION

The pollution by plastic litter is ubiquitous in aquatic environments, both freshwater (Rodrigues et al., 2018) and marine (Anderson et al., 2016). Once released in the environment, plastics undergo weathering and fragmentation due to chemical, mechanical, and biological degradation. These processes generate smaller particles collectively known as secondary micro- (MP; <5 mm) and nanoplastics (NP; <0.1 μm), the main contributors to an environmental load of plastics (Andrady, 2011). Primary MP (e.g., microbeads) manufactured for use in industry, personal care products, and medicine are also contributing to the plastic pollution worldwide, although to a lesser extent than secondary MP and NP.

Given the global nature of the plastic littering, accurate characterization of the impacts of these environmental contaminants is essential for risk assessment. The current concern is that both MP and NP (MNP) are harmful to aquatic biota. However, while some studies report adverse effects (Casado et al., 2013; Della Torre et al., 2014; Au et al., 2015), no-effect observations in laboratory tests with plants and animals are also common (Watts et al., 2016; Long et al., 2017; Chae et al., 2018). Moreover, the effect mechanisms specific for MNP in microorganisms, plants, and animals are largely unknown. Due to this conflict of information, more studies on environmental fate and biological effects of these emerging environmental contaminants in various organisms are needed.

The exposure to anthropogenic polymer particles is particularly relevant for lower trophic levels within food webs (Yokota et al., 2017), because (1) this is the entry point into the food web affecting the baseline load of MNP in consumers, (2) negative impacts on primary producers and primary consumers may change food resources and translate into adverse effects on higher consumers and food web functions, and (3) interactions of MNP with bacterio- and microplankton may have implications for biogeochemical cycling in ecosystems (Rogers et al., 2020). Moreover, similar to other particulates in the water, MNP form aggregates with detritus and microorganisms, which facilitate sedimentation and transfer of these contaminants in the food web but can also affect microbial growth and physiology (Ward and Kach, 2009; Long et al., 2017; Mao et al., 2018) and downward fluxes in the system (Rogers et al., 2020). Therefore, exposure of algae and other microorganisms to MNP should be a part of the risk assessment for plastic litter.

It has been shown that exposure to MNP can inhibit algal growth (Casado et al., 2013; Besseling et al., 2014; Bergami et al., 2017), chlorophyll content (Besseling et al., 2014; Zhang et al., 2017), and photosynthetic activity (Bhattacharya et al., 2010). For example, Besseling et al. (2014) reported a typical dose-response for the freshwater alga *Scenedesmus obliquus* exposed to polystyrene (PS). However, the particle concentrations inducing significant growth inhibition are much higher than MP concentrations reported from the aquatic environments (Eerkes-Medrano et al., 2015; Li et al., 2018). When the test concentrations resemble the levels of suspended solids that are typical for surface waters, the observed effects on algal growth and physiology appear to be limited (e.g., Long et al., 2017; Prata et al., 2018) or even positive, i.e., growth stimulation can be observed (e.g., Yokota et al., 2017; Canniff and Hoang, 2018). Also, the observed responses may vary during the exposure; for instance, for *Chlorella pyrenoidosa,* growth inhibition was observed in the exponential phase of the growth curve, whereas during the stationary phase, this trend was reversed, and growth exceeded that in control (Mao et al., 2018). Such biphasic responses indicate that algal populations are able to adapt to the presence of particulates, including MNP, and sustain high productivity (Sjollema et al., 2016; Long et al., 2017; Yokota et al., 2017; Zhang et al., 2017; Mao et al., 2018; Prata et al., 2018). What is more important for the risk assessment is that it is not always clear whether the observed effects can be attributed, at least partially, to changes in the experimental conditions related to the presence of particulate material in the test system, such as high turbidity, nutrient limitation, and poor light penetration (Besseling et al., 2014; He et al., 2017), which are not necessarily specific to the polymers as test materials.

When testing MNP effects on biota, it is surprising that the general effects of suspended solids in the exposure systems are largely ignored, although there is a consensus that suspended particles of a particular geochemistry and size range induce various responses in aquatic organisms (Gordon and Palmer, 2015). In algae, sediment particles, and other organic and inorganic solids can cause growth inhibition (Cahoon et al., 1999). Consequently, the concentration of suspended solids in effluents is recognized as an important parameter that should be under control in toxicity testing (Hatch and Burton, 1999; He et al., 2017). Levels of suspended solids that cause adverse effects vary depending on the algal species and other components in the test system (Bilotta and Brazier, 2008). With respect to setting thresholds beyond which impacts on biota would be negligible, the estimates vary from 20 to 100 mg/L (Bilotta and Brazier, 2008; Gordon and Palmer, 2015; Chapman et al., 2017). A threshold value of 100 mg/L suggested by U.S. EPA (2015), has been used in the comparative analysis of the fossil-based polymers and natural particles (Ogonowski et al., 2018). In the latter study, it was found that median toxic concentrations were very similar between natural particles (such as clay and cellulose) and MP; moreover, these concentrations were within

environmentally acceptable levels for suspended solids (Gordon and Palmer, 2015).

A recent review and meta-analysis by Bucci et al. (2019) based on 66 studies evaluating microplastic effects in aquatic organisms suggested that the crucial parameters influencing the test outcome are particle concentration, their shape, and size. In particular, fibers were found to be more hazardous than beads and fragments; moreover, for smaller particles, the adverse effects were more likely to be detected (Bucci et al., 2019). This meta-analysis provided a useful synthesis of the experimental data; unfortunately, only six studies examining effects in algae were included in the data set. Other critical reviews for various effects of MP in biota (Foley et al., 2018) and the effect concentrations (Ogonowski et al., 2018) also indicate a high variability across the reports and a lack of comprehensive data sets for algae compared to invertebrates and fish (Bucci et al., 2019). As effect mechanisms are likely to differ between the producers and consumers, it is necessary to conduct a comparative analysis focusing on specific groups of biota.

Given the variability of the responses, it has been recommended to use meta-analysis to support the risk assessment of plastic litter and the development of the appropriate test systems (Bucci et al., 2019). Indeed, single trials with often contradicting reports, small sample sizes, variable exposure conditions, and a diversity of species used in the experiments are difficult to synthesize. Meta-analysis permits an evaluation of the effect sizes across different studies by increasing the statistical power and analyzing the variability between studies. In toxicology, the meta-analysis techniques allow generalizing tendencies, resolving uncertainties, and identifying promotors of the effects. Based on the outcome of the meta-analysis, new hypotheses, and experimental designs can be generated (Fagard et al., 1996) to improve mechanistic understanding of the hazard potential of the plastic litter.

To critically evaluate the current experimental evidence for adverse effects of MNP in algae, we reviewed available studies and conducted a meta-analysis. The aim was to determine the associations between the exposure to MNP and the risk of growth inhibition in unicellular freshwater and marine algae, with a particular focus on the responses observed below and above the threshold concentration of 100 mg/L adopted by US EPA. Based on the current literature suggesting inhibitory effects of MNP on algal growth, we put forward the following hypotheses: (H1) exposure to plastic particles causes growth inhibition, which is concentration-dependent, (H2) particle size, shape, and polymer material moderate to the variations in the effect size, (H3) the inhibitory effect decreases with increasing exposure duration; and (H4) freshwater and marine species respond similarly to the exposure. When testing these hypotheses, we focused on both nano- and microplastics, owing to their global relevance, public concerns, and ecologically plausible interactions with algae in both freshwater and marine ecosystems. Also, from the ecological perspective, there is NP-MP size continuum that organisms are exposed to *in situ* and, therefore, considering the entire spectrum in the meta-analysis is environmentally relevant.

METHODS AND MATERIALS

Data Sources and Search Strategy

Studies examining the response of algae to nano- and microplastic exposure were retrieved from *Web of Knowledge* and *Google Scholar* databases. We used broad-range search combining keywords: *microplastic*, *nanoplastic*, *exposure*, *algae*, and *experiment*, and targeting article title, keywords, abstract, and subject headings. When screening the retrieved studies, we paid attention to the experimental design, including information on the physicochemical properties of the test particles reported in the paper. In some cases, when the polymer material was specified but its density was not, we used a polymer-specific value obtained from Omnexus Plastics Database[1].

The publication date was not restricted; however, the last search was conducted on January 14, 2020. Further studies were sought by manually searching reference lists of the selected articles. In addition, given the paucity of the published reports, we included one study deposited in the *bioRxiv*[2] manuscript repository (Gorokhova et al., 2020) and one M.Sc. thesis (Rogstad, 2019); both studies met the selection criteria.

The completed search yielded 28 studies, which were further screened according to the following criteria: (i) effects of exposure were quantified in a controlled experiment, (ii) experimental results were reported with basic statistical measures, including sample size, mean, and variance for the endpoint measured, (iii) primary information on the polymer material used in the exposure experiments, test concentrations of the particle suspension, and particle size were presented within the paper or in its **Supplementary Materials**, and (iv) the endpoints used in the experiment were relevant for standard growth inhibition test with the growth measured either by cell count or fluorescence (OECD, 2011). We used growth inhibition because it is a standard endpoint in current toxicity testing with microalgae. This screening resulted in 20 studies that met the selection criteria and, hence, were included in the meta-analysis; see **Figure 1**, **Table 1**, and **Supplementary Table S1** for an overview of the studies included in the final data set and those that failed to meet the selection criteria. In most cases, the grounds for not including a study in the final selection were related to a lack of appropriate growth measurements, insufficient reporting of primary results (e.g., missing variance metrics), and insufficient description of the experimental setup (**Supplementary Table S1**).

Data Extraction

From each study, we extracted the following data: (1) authors and year of publication, (2) test species, (3) observed response in the treatment (exposure to the test material in the culture medium) and control (no exposure to any suspended solids, culture medium only), (4) method used to measure a change in algal population size by cell counting or fluorescence-based techniques, (5) a set of exposure system characteristics (salinity and exposure duration), and (6) microplastic characteristics

[1] https://omnexus.specialchem.com/polymer-properties/properties/density
[2] www.biorxiv.com

FIGURE 1 | Workflow for data selection and grouping. To investigate effects at the low and high levels of MNP in the tests, the studies were assigned to three models: *Low concentration model* (<100 mg/L), *High concentration model* (≥100 mg/L), and *Full range model* (0.004–1,100 mg/L). The threshold of 100 mg/L was used following the recommendations for suspended solid concentrations in stormwater (U.S. EPA, 2015).

(polymer material, particle size, concentration, shape, and density). For the continuous responses representing a change in the algal population abundance, values for mean, variance (standard deviation, SD), and sample size (number of experimental units in treatment and control) were used. When possible, the mean and variances were extracted from tables and text of the publication; however, when these results were presented only as graphs, they were retrieved using ImageJ version 1.52a (Abramoff et al., 2004). If SD in control was set to zero, the SD of the treatment was used together with both control and treatment mean (Wiebe et al., 2006).

Selection Criteria and Data Structure

Studies included in this analysis were conducted to measure algal growth inhibition following—at least to a large extent—the OECD guidelines (OECD, 2011) (**Table 1**). Multiple experiments per study were used when they were conducted independently, e.g., with different polymer materials (e.g., Bergami et al., 2017; Rogstad, 2019; Gorokhova et al., 2020) or different algal species (e.g., Long et al., 2017). Given the focus of our analysis, this was considered appropriate as long as the exposure effect was measured in independent trials within a study.

The study entries were grouped in relation to the threshold for exposure concentration that was set to 100 mg/L, the level below which no adverse effects on aquatic biota would be expected; this value was based on the regulations for the total suspended solid concentration in stormwater and various effluents (U.S. EPA, 2015). Based on the particle concentration in the experimental system, we generated three datasets: (1) *Low concentration model* (<100 mg/L); here, no growth inhibition due to non-specific effects of suspended solids, such as shading or nutrient sorption, was expected to occur, (2) *High concentration model* (≥100 mg/L); under these conditions, the non-specific effects were likely and, thus, growth inhibition was expected. Therefore, we expected to observe a more pronounced inhibition in the *High concentration model* compared to the *Low concentration model*. In both datasets, when study design included multiple test concentrations and exposure durations, we aimed at a conservative estimate from the environmental perspective and used experimental settings that yielded the most considerable

TABLE 1 | Overview of studies testing micro- and nanoplastic effects on algal growth that were included in the meta-analysis.

References	Polymer	Exposure time (d)	Parameter measured	Analysis method
Bergami et al. (2017)	PS-COOH PS-NH2	3 3	Cell count	Hemocytometer, light microscopy
Besseling et al. (2014)	PS	3	Cell count	Automated cell counter
Canniff and Hoang (2018)	PE	5	Cell count	Hemocytometer, light microscopy
Chae et al. (2019)	PE	6	Fluorescence	Fluorescence microplate reader and flow cytometry
Chae et al. (2018)	PS	3	Fluorescence	Fluorescence microplate reader
Davarpanah and Guilhermino (2015)	PE	4	Cell count	Hemocytometer, light microscopy
Fu et al. (2019)	PVC Aged PVC	10 10	Cell count Cell count	Hemocytometer, light microscopy
Gambardella et al. (2018)	PS	3	Cell count	Hemocytometer, light microscopy
Garrido et al. (2019)	PE	3	Cell count	Coulter counter
Gorokhova et al. (2020)	PET Aged PET	3 3	Fluorescence	*In-vivo* fluorometry
Guo et al. (2020)	PE PVC	4/9 4/9	Cell count	Hemocytometer, light microscopy
Long et al. (2017)	PS	15/22/23	Cell count	Fluorescence and flow cytometry
Nolte et al. (2017)	PS	3	Cell count	Coulter counter
Prata et al. (2018)	PE	4	Fluorescence	Fluorescence Microscopy
Rogstad (2019)	PES PA	13 13	Cell count	Electronic particle counter
Sjollema et al. (2016)	PS	3	Fluorescence	Fluorometry and flow cytometry
Su et al. (2019)	PS	7	Cell count	Sedgwick-Rafter chamber, light microscopy
Yokota et al. (2017)	PS	21	Cell count	Light microscopy and electronic particle counter
Zhang et al. (2017)	PVC	4	Cell count	Hemacytometer, light microscopy
Zhao et al. (2019)	PVC	4	Cell count	Hemacytometer, light microscopy

Test polymers, exposure time (days), measured parameter used to derive growth and analysis methods used in the trials are specified. PE, polyethylene; PES, polyester; PA, polyamide; PS, polystyrene; PET, polyethylene terephthalate; PVC, Polyvinyl chloride.

effect size for a given polymer material or a species tested. In addition, a dataset that included all studies was used as (3) *Full range model* (0.004–1,100 mg/L), which included entries with the combination of the experimental settings (MNP concentration, polymer material, particle size, etc.) yielding the highest effect size for a specific study. In this model, we expected to see a stronger evidence for the effect size being affected by MNP concentration and particle size because of the broader span of values for these parameters in the dataset.

Calculation of Effect Sizes

The effect size (Cohen's *d*; Equation 1) was calculated using observations for treatment (i.e., exposed algae) and control (i.e., algae that were grown in pure culture medium):

$$d = (Mean_T - Mean_C) / \sqrt{\frac{(n_T - 1) * SD_T^2 + (n_C - 1) * SD_C^2}{n_T - n_C - 2}} \quad (1)$$

where n_T and n_C are sample sizes in treatment and control, respectively; SD_T and SD_C are standard deviations in treatment and control, respectively; and *df* is degrees of freedom.

The calculated Cohen's *d* values were adjusted using a correction factor *J* (Equation 2):

$$J = 1 - \frac{3}{4 * (df - 1)} \quad (2)$$

This correction accounts for a small sample size (Nakagawa and Cuthill, 2007; Borenstein et al., 2009; Hedges and Olkin, 2014); the resulting estimate is referred to as Hedge's *g* (Equation 3).

$$g = J * d \quad (3)$$

If only standard error (SE) and sample size (*n*) were reported, we calculated SD for each group as:

$$SD = SE * \sqrt{n} \quad (4)$$

The sampling variances corresponding to the observed outcome (Hedges' *g*) were calculated as:

$$V = \frac{(n_C + n_T)}{n_C * n_T} + \frac{g^2}{2 * (n_C + n_T)} \quad (5)$$

The standardized unbiased effect size estimated as Hedges' *g* and the related sampling variance were used in the meta-analysis.

Meta-Analysis

The meta-analysis was conducted with the R package *metafor* version 2.1.0 (Viechtbauer, 2010) using the R studio version 3.6.0 (R Core Team, 2019) and the computed Hedges' *g* and *V* values. Because study design and test species varied across the studies, a random-effects analysis was used to allow for heterogeneity of the true effect sizes among the studies (Borenstein et al., 2010). The impact of each entry was weighted based on sample size and illustrated by the size of the symbol in the figures. Pooled

estimates of the outcomes were reported as a forest plot depicting the mean effect size and the associated 95% confidence intervals (95% CI). Prediction interval (95% PI) was calculated to estimate the interval that a new observation would fall into (IntHout et al., 2016) as opposed to the uncertainty estimated by the 95% CI of the effect size. The mean effect size, 95% CI and 95% PI were estimated for each model.

Exploratory random-effects meta-regression analysis was used to investigate the moderating role of exposure time, MNP concentration, particle size, polymer density, and temperature. In addition to the particle size, the ratio between the longest linear dimension of the algal cell (**Supplementary Table S2**) and the nominal size of the test particle (cell-to-particle size ratio) as a continuous predictor of the effect sizes was evaluated. The effect was considered significant when the regression slope was significantly non-zero. To estimate the percentage of variance accounted for by the moderator, an R^2 index was calculated (López-López et al., 2014). While the R^2 index has a range of 0–100%, it is possible for sampling error to yield a value of R^2 that falls outside of this range. In that case, the value is truncated to either 0% (if the estimate falls below 0%) or to 100% (if it falls above 100%). Categorical parameters, such as polymer material (PS vs. PE vs. PVC), particle shape (spherical vs. non-spherical), particle size category (MP vs. NP), and origin of the test algae (freshwater vs. saltwater species), were also explored as a source of heterogeneity. Finally, we tested whether different approaches for growth measurement (cell count by light microscopy or electronic means vs. fluorescence-based methods) contributed to the heterogeneity because these approaches have different precision and sensitivity; they also address different aspects of algal growth (Butterwick et al., 1982). The effect of a categorical predictor was considered significant when the 95% CI range did not include zero.

Assessing Publication Bias and Heterogeneity

Funnel plots were used to assess publication bias by plotting the effect size vs. the standard error (Sterne and Egger, 2001; Peters et al., 2008). Using these plots, we tested whether there is a relation between the effect size of a study and its precision. First, the Egger's test was applied to evaluate the asymmetry of the plot (Egger et al., 1997), which may result from excluding non-significant studies or presence of studies with small sample sizes. Further, we applied the trim-and-fill method, which is an iterative rank-based method, to estimate the number of missing studies in the dataset and their effect on the meta-analysis outcome (Duval and Tweedie, 2000). Lastly, a sensitivity analysis was applied to assess the importance of bias.

Between-study heterogeneity in study-specific effects sizes was evaluated with the Q statistic (Higgins et al., 2002). The Q-test is based on the chi-square distribution and provides a measure of the sum of the squared differences between the observed and the expected results in each study under the assumption that each study estimates the same treatment effect (i.e., growth inhibition). When all studies share the same effect size, the expected Q value is equal to the degrees of freedom (the number of studies minus 1), whereas a higher Q value indicates heterogeneity. The I^2 statistics was calculated as a measure to assess the percentage of variation due to heterogeneity between the studies rather than chance alone (Higgins and Thompson, 2002).

RESULTS

Overview of the Datasets

The set of studies used for meta-analysis included 20 publications, with 67 data entries (**Figure 1**) allocated to three datasets: (1) *Low concentration model* (<100 mg/L; 0.004–99 mg/L; 27 entries, 19 studies), (2) *High concentration model* (≥100 mg/L; 100–1,100 mg/L; 12 entries; 10 studies), and (3) *Full range model* (0.004–1,100 mg/L; 28 entries; 20 studies). The majority of entries (65%) presented tests with saltwater algae, among which Dinophyceae and Prymnesiophyceae were the most common (13 and 9%, respectively), whereas Chlorophyceae were the most frequent species (36%) used in the studies with freshwater algae; only four out of 16 species were used in more than a single study (**Supplementary Figure S8**).

Across the selected studies, the particle size and MNP concentration ranged from 0.04 to 3,000 μm and 0.004 to 1,100 mg/L, respectively (**Figure 2**). The mean and median values for the particle size were 213 and 2 μm, respectively. Only six studies used particles in the nano-range, whereas 70% of studies used particles larger than 0.1 μm. The mean and median values for MNP concentration used in the exposure experiments were 182 and 50 mg/L, respectively.

Many studies used polystyrene (PS; 45%) followed by polyethylene (PE; 25%) and polyvinyl chloride (PVC; 20%); others used polyethylene terephthalate (PET), polyester (PES) and polyamide (PA). The polymer densities ranged between 0.99 and 1.40 g/cm^3. In most cases, the plastics were virgin, and only two studies used weathered PET and PVC; moreover, one study used functionalized PS. With regard to the particle shape, 85% of studies used spherical particles. Due to the limited number of studies that used fibers and irregular fragments and the similarity of the effects observed in these studies, these entries were grouped as *non-spherical particles* for moderation analysis.

To assess changes in algal growth, the majority of the studies used cell count (75%; either microscopy-based or using electric counters), whereas fluorometry was less common (25%). Moreover, the fluorometry-based methods varied among the studies and included flow cytometry, *in vivo* fluorometry of Chlorophyll *a*, and fluorescence microscopy with image analysis.

Effects of Exposure to MNP on Algal Growth

Effects Observed at MNP Concentrations Below 100 mg/L

In the *Low concentration model,* the effect size was overlapping with zero in 66% cases, whereas significant growth inhibition and stimulation were observed in 26 and 7% cases, respectively (**Figure 3**, upper panel). The mean effect size of the MNP exposure was −0.22, indicating decreased algal growth compared to the particle-free controls; however, the 95% CI for the mean effect size overlapped with zero [−0.90–0.45]. Therefore, mean growth in the exposed algae was not significantly different from that in controls. The 95% PI ranged from −3.20 to 2.75,

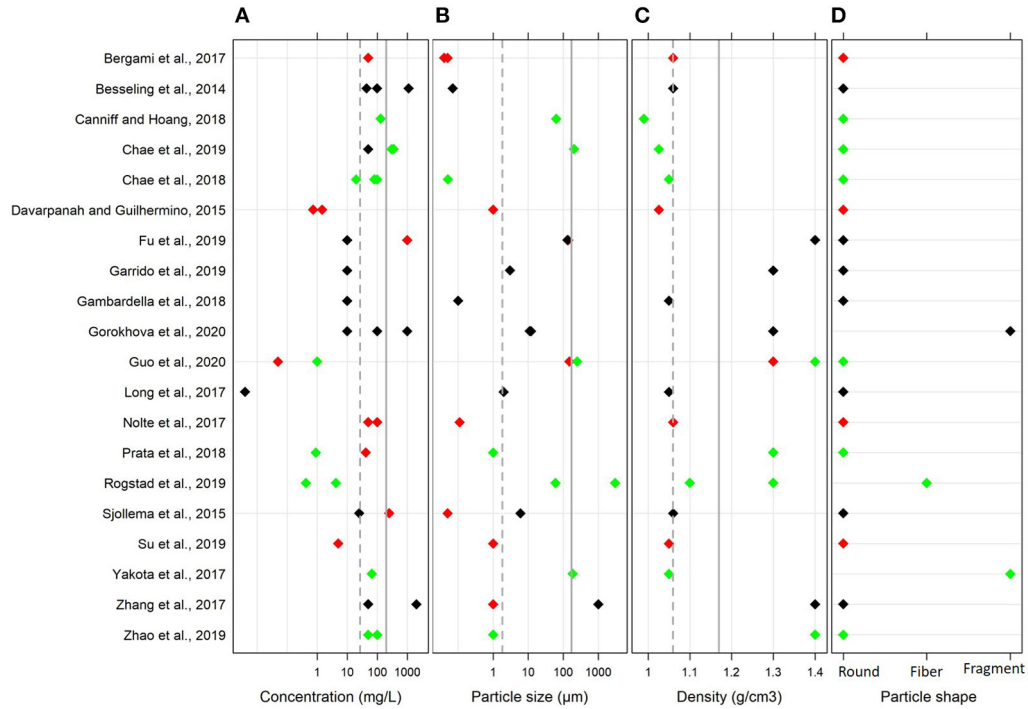

FIGURE 2 | Variability of the main parameters in the global dataset used in the meta-analysis: **(A)** MNP concentration (mg/L), **(B)** particle size (μm), **(C)** polymer density (g/cm^3), and **(D)** particle shape. The mean and median values are shown by solid and dotted lines, respectively. The effects on algal growth reported by the authors in each study are indicated by the color coding; red, negative effect; black, no effect; and green, positive effect. Note logarithmic scale on the x axes for **(A,B)**.

indicating that both positive and negative effect sizes are nearly equally plausible for a future test employing similar design.

The heterogeneity was high and significant ($Q = 100.20$; $I^2 = 75.8\%$; $p < 0.001$), indicating divergent responses among the studies that cannot be attributed to chance alone. Results of the exploratory meta-regression showed that polymer density was a significant positive predictor ($p < 0.03$; **Figure 4A**), i.e., with increasing density, the growth inhibition was less likely. The moderator explained 37% of the heterogeneity in the model. Particle size showed a similar trend, albeit explaining only 14% ($p > 0.07$; **Supplementary Figure S2A**), implying that growth inhibition was more likely when algae were exposed to smaller particles. By contrast, neither MNP concentration nor exposure time nor cell-to-particle size ratio were significant as predictors (**Supplementary Figures S1A**, **S3A**, **S4A**). Among the categorical predictors, a significant influence on the effect size was only found for the particle shape (**Figure 5A**): non-spherical particles (fibers and fragments) were more likely to induce growth stimulation compared to the spherical particles (**Figure 5A**). Of the three polymer materials (PS, PE, and PVC) that were sufficiently represented to allow for the comparison, none were found to cause significant growth inhibition (**Supplementary Figure S5A**). NP had slight adverse effects on growth compared to MP, albeit, not significant (**Supplementary Figure S6A**). The test outcome was not affected by the freshwater or saltwater origin of the test species (**Supplementary Figure S7A**).

Effects Observed at MNP Concentrations Above 100 mg/L

In the *High concentration model,* the effect size was overlapping with zero in 41% cases, whereas significant growth inhibition and stimulation were observed in 41 and 17%, respectively (**Figure 3**, lower panel). The mean effect size was −1.11, with the 95% CI overlapping with zero [−3.17, 0.95]. The 95% PI was also wide, ranging from −8.01 to 5.79; thus, both positive and negative effects will be observed in future tests employing similar design.

The heterogeneity was high and significant ($Q = 68.73$; $I^2 = 89.9\%$; $p < 0.001$). However, none of the continuous predictors (i.e., concentration, exposure duration, polymer density, particle size, and cell-to-particle size ratio; **Supplementary Figures S1B, S2B, S3B, S4B**; **Figure 4B**, respectively) were significant ($p > 0.07$ in all cases). MNP concentration explained 29% of the heterogeneity in the effect size, with a higher probability of growth inhibition at higher MNP levels; the effect was not, however, significant ($p > 0.07$; **Supplementary Figure S1B**). Also, neither polymer material nor particle size category nor species origin were significant as predictors (**Supplementary Figures S5B–S7B**). The effect of particle shape could not have been evaluated due to the lack of observations for the non-spherical particles.

Full Range Model

The *Full range model* was based on 28 entries from all 20 studies selected for the meta-analysis, 42% of which had the effect size

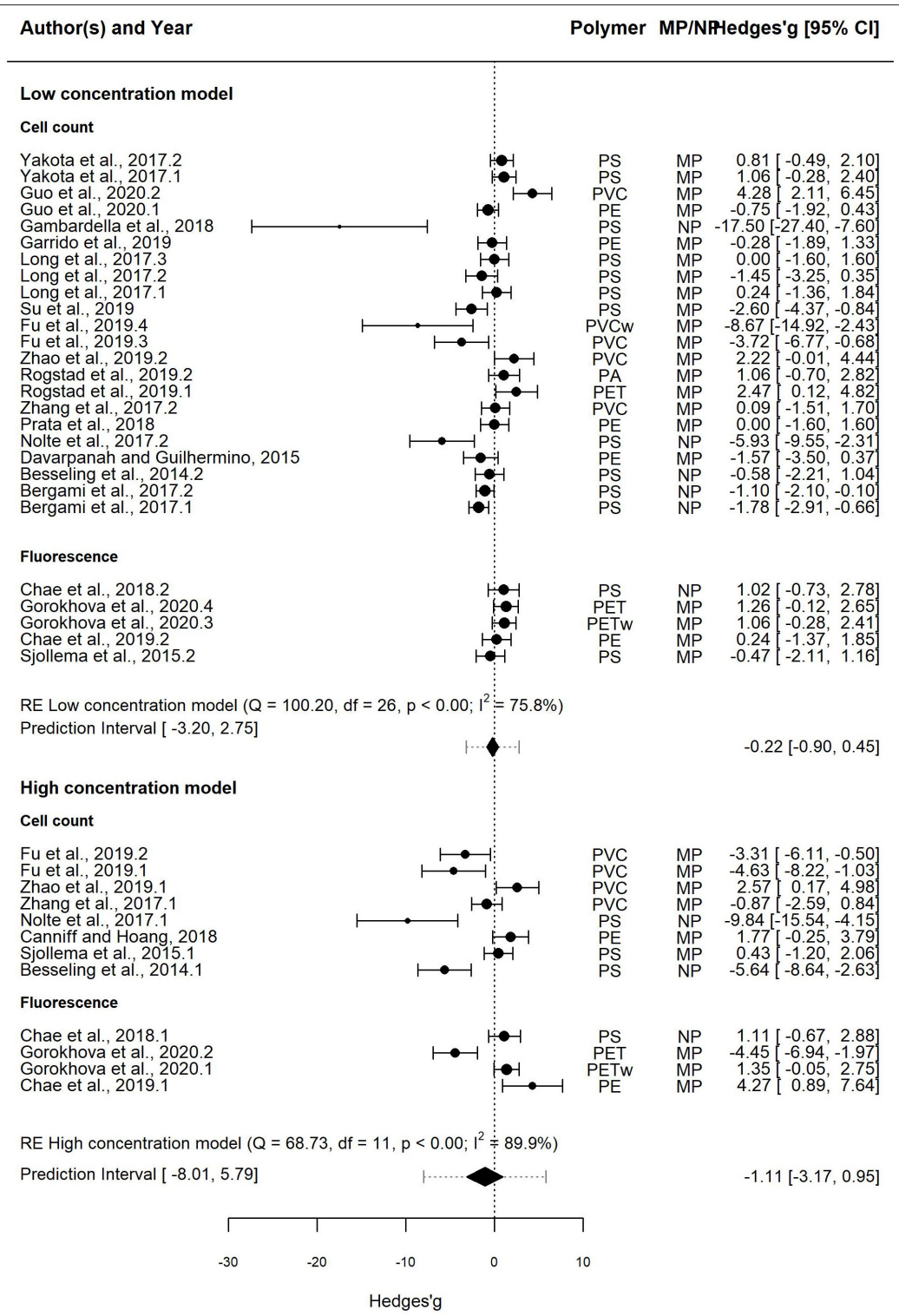

FIGURE 3 | Forest plot of the random-effect models for effect sizes estimated as Hedges' *g* and the associated 95% confidence interval (95% CI) in the *Low concentration model* (upper panel; 20 entries) and the *High concentration model* (lower panel; 11 entries). The negative and positive values for Hedges' *g* correspond to growth inhibition and stimulation, respectively. In each model, the overall effect (i.e., pooled estimate) is depicted as a diamond. The prediction interval shown by lines extending from the overall effect diamond captures the expected true effect for a future study. Symbol size is proportional to the study weight. Multiple entries for some studies correspond to independent trials for different polymers and/or different species. In each model, studies that employed cell count and fluorescence methods for measuring growth are shown separately.

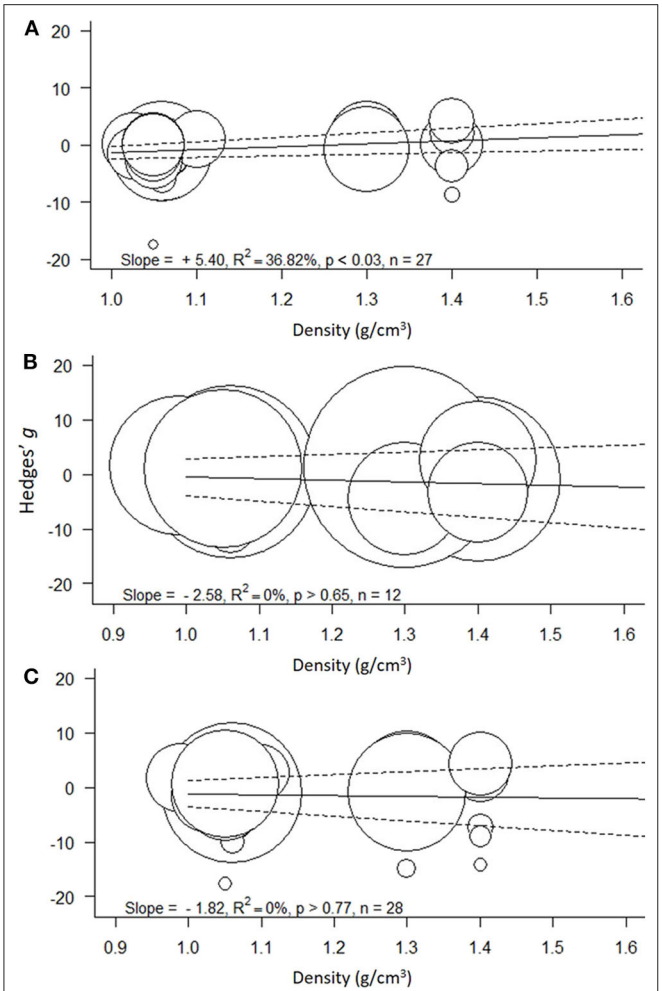

FIGURE 4 | Meta-regressions for the effect size as a function of polymer density (g/cm^3) across the models: **(A)** *Low concentration model* (<100 mg/L), **(B)** *High concentration model* (≥100 mg/L), and **(C)** *Full range model* (0.004–1,100 mg/L). Circle size indicates the study weight in the effect size calculation; note that the scale for the weights varies among the models. Solid line represents model prediction and dashed line is the corresponding 95% CI.

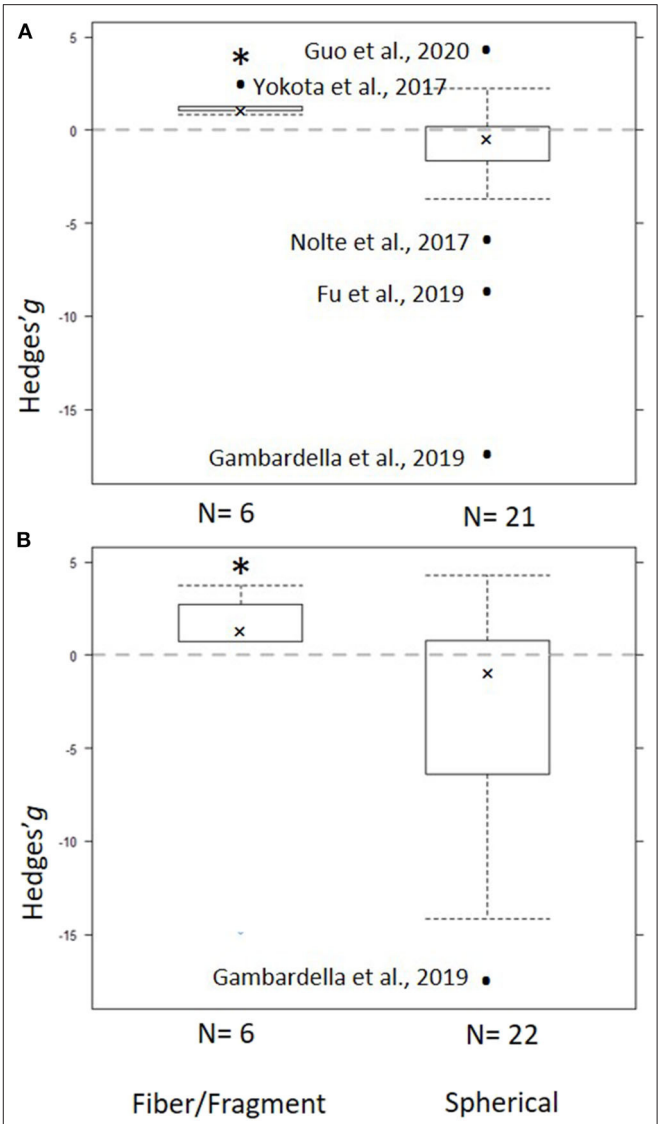

FIGURE 5 | Subgroup analysis for the particle shape as a predictor for the effect size across the models: **(A)** *Low concentration model* (<100 mg/L) and **(B)** *Full range model* (0.004–1,100 mg/L). Crosses represent the median, the boxes are the 95% CI of the median, and whiskers show the overall 95% CI. The black dots indicate outliers. The horizontal dashed line indicates no effect. N values on the *x* axis indicate number of entries in each subgroup. The asterisk indicates statistical significance at $\alpha = 0.05$.

overlapping with zero, whereas significant growth inhibition and stimulation were observed in 32 and 17% cases, respectively. The mean effect size was negative (−1.34), with the 95% CI [−2.88, 0.20]. The prediction interval was very broad, ranging from −8.94 to 6.26, indicating that both negative and positive effects will be observed in the future tests even if only maximal effect sizes across the treatments in each study are considered.

The heterogeneity was high and significant (Q =156.88 I^2 = 94.1%, $p < 0.001$), reflecting the variability in the data. None of the continuous predictors (MNP concentration, exposure duration, polymer density, polymer size, and cell-to-particle size ratio) were significant in explaining the heterogeneity ($p > 0.09$ in all cases; **Supplementary Figures S1–S3C**, **S4C**; **Figure 4C**). Among these predictors, the cell-to-particle size ratio contributed most to the observed heterogeneity (17%, $p > 0.09$; **Supplementary Figure S4C**), with a higher probability of growth inhibition at higher cell-to-particle size ratio. Particle shape was a significant predictor; we found that exposure to fibers and fragments was associated with growth stimulation (**Figure 5B**). Neither polymer type nor particle size category nor species origin contributed significantly to the observed heterogeneity (**Supplementary Figures S5C–S7C**).

This dataset was also used to assess the effect of the growth measurement method, i.e., cell count (22 entries) vs. fluorescence (6 entries) on the effect size (**Figure 6**). The mean effect size was not significantly different from zero regardless of the method

used, with the mean effect size for the cell count model and the fluorescence model being −1.26 [−2.82, 0.29] and −1.68 [−6.62, 3.27], respectively. The prediction interval for the true treatment effect was broader in the fluorescence model (−14.22–10.86) compared to that based on cell counts (−8.05–5.53), which at least partly was related to the fewer entries in the former.

Publication Bias

Funnel plots revealed some low-precision studies (**Figure 7**). Moreover, low precision studies that reported significant inhibitory effects were present, whereas those reporting stimulatory effects were not. All studies reporting stimulatory effects of the exposure had high precision and most of them were significant, whereas the negative effect studies had greater effect size but lower precision. In all three models (**Figure 7**), a significant asymmetry was found using Egger's test, with z-scores of −4.79 ($p < 0.0001$), −3.09 ($p < 0.002$), and −5.17 ($p < 0.0001$) for the *Low Concentration, High concentration* and the *Full range* models, respectively (**Supplementary Table S3**). Thus, the negative effect sizes appeared to be reported by studies with smaller sample sizes (**Figure 7**). This asymmetry indicates that a bias toward the publication of large negative effects may stem from the sample size of the study.

With high between-sample heterogeneity (as in all three models), the use of funnel plot for publication bias detection may yield false-positive results, i.e., detect publication bias when none is present. To address this concern, we first inspected the funnel plot distribution. Large moderating effects, i.e., effects of the moderators that were not tested, should result in a bimodal distribution, which did not occur (**Figure 7**). Therefore, the existence of the publication bias and small study effect was accepted, albeit with caution.

Second, the trim-and-fill method was applied to estimate the number and impact of missing studies in the dataset (**Figure 7**). Five studies were identified to be missing in the *Low concentration model* and seven in the *Full range model* on the right side of the funnel, and none in the *High concentration model*. The imputation changed the model results to the mean effect size and 95% CI of 0.10 [−2.09, 2.29] and PI [−10.09, 10.9] for the *Low concentration model* and −0.41[−1.74, 2.55] with PI [−11.71,12.52] for the *Full range model*. In both cases, the corrected values were similar to the original output; thus, no significant effect of the exposure across the studies was found even when the results were corrected for the publication bias.

DISCUSSION

Using meta-analysis, we found that exposure to micro- or nanoplastic was not associated with significant growth inhibition in unicellular algae. In the 20 studies based on standard growth inhibition assay, all possible outcomes were reported, ranging from inhibition (up to 57%) to stimulation (up to 56%) of growth rate. Neither *Low concentration model* nor *High concentration model* nor *Full range model* produced a significant mean effect size; moreover, prediction intervals indicate that tests following this type of experimental design are likely to yield both inhibitory and stimulatory responses. Thus, our meta-analysis lends limited support to the hypothesized growth inhibition of algae by exposure to micro- and nanoplastic (hypothesis H1). However, caution should be taken when interpreting these results owing to substantial heterogeneity within the models and a relatively low number of studies.

Notably, we found no clear indication that the exposure effect is related to MNP concentration (H1). The exposure to MNP concentrations of <100 mg/L resulted in mean inhibition by 0.22 standard deviations (*Low concentration model*), whereas at the concentration ≥100 mg/L (*High concentration model*), the average inhibition was more pronounced (1.11 standard deviations). Although the *High concentration model* suggested more adverse effects compared to the *Low concentration model*, which was in line with our expectations, neither mean effect sizes were significantly different from zero (**Figure 3**; **Supplementary Figures S1A,C**). The only weak indication of a possible concentration-dependent response was found for the *High concentration model*, where the negative trend indicated that at higher MNP concentrations, growth inhibition might be more likely ($p > 0.07$; **Supplementary Figure S1B**). However, the *High concentration model* had the lowest number of entries; therefore, to investigate this trend and establish effect concentrations, more studies would be needed.

The effect concentrations reported in the experimental studies are very variable and often biphasic. Dose-response relationships using growth as the endpoint (Besseling et al., 2014; Sjollema et al., 2016; Gambardella et al., 2018; Wu et al., 2019; Zhao et al., 2019) indicate that the lowest concentrations which induced inhibition (LOEC) varied 0.001–50 mg/L, i.e., within the range of our *Low concentration model*. Also, similar concentrations of 1-μm PVC (≤50 mg/L) caused significant growth inhibition (Zhang et al., 2017). However, in some cases, PVC induced less inhibition at higher concentrations (≥100 mg/L) compared to 10 mg/L (Fu et al., 2019). Moreover, for unplasticized PVC, Guo and co-workers showed growth stimulation at 50 g/L compared to significant inhibition (37%) at 0.05 mg/L (Guo et al., 2020). The main reason for these counter-intuitive observations could be a reduced exposure during the test because of the uncontrolled particle aggregation and settling, which are enhanced at higher concentrations. These methodological shortcomings in the experimental design and uncertainties with regard to the stability of the exposure would preclude meaningful evaluation of the concentration effects not only across the studies but also within a study unless sufficient measures were taken to prevent particle sedimentation.

An indication of the particle size influence on algal growth was found in the *Low concentration model*, with growth inhibition being more likely in trials with smaller particles ($p > 0.07$; **Supplementary Figure S2A**). In the *High concentration* and *Full range* models, the regression slopes for the particle size effects were also positive, albeit non-significant and with low explanatory power (**Supplementary Figures S2B,C**), which might also be related to the less pronounced effect due to particle aggregation at higher MNP concentrations. The subgroup analysis contrasting MP and NP effects corroborated the meta-regression outcome as growth inhibition in algae exposed to NP appeared to be more pronounced compared to

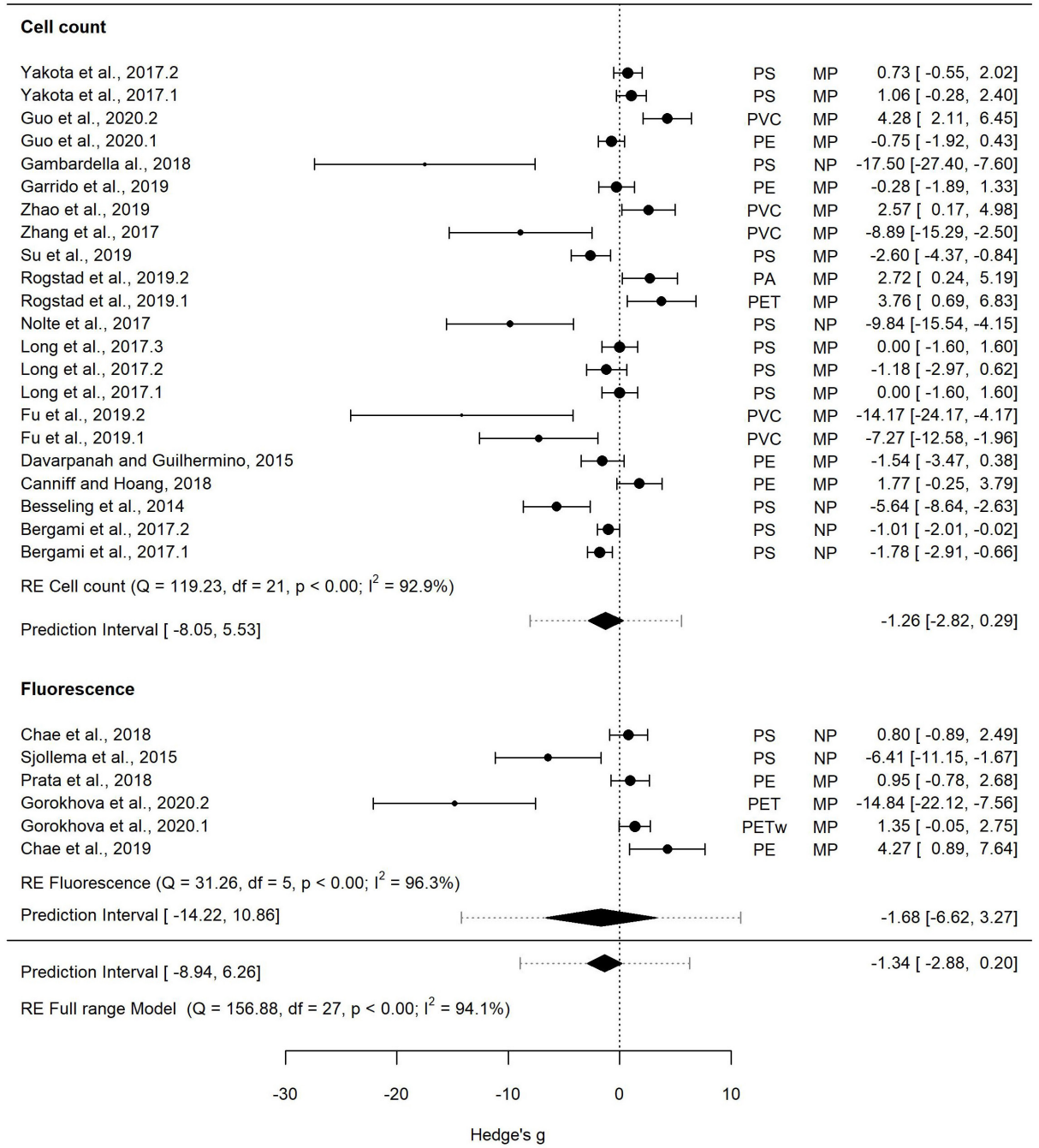

FIGURE 6 | Forest plot of the random-effect model for effect sizes estimated as Hedges' *g* and the associated 95% confidence interval (95% CI) in Forest plot for the *Full range model* (28 entries). Symbol size for individual entries is proportional to the study weight. The overall effect (pooled estimate) on growth according to the random-effect model is depicted as a diamond. The prediction interval (PI) is shown with lines extending from the diamond. Multiple entries for some studies correspond to different polymers tested within a study or several species used in the tests. Studies that employed cell count and fluorescence methods for measuring growth are shown separately.

that in MP exposures, albeit neither effect size was significant (**Supplementary Figure S6**). In algae and filter-feeders, particle size has been considered a crucial driver of the adverse effects (Cole et al., 2013; Lee et al., 2013; Besseling et al., 2014; Davarpanah and Guilhermino, 2015; Jeong et al., 2016). However, even in a controlled experimental system with appropriate

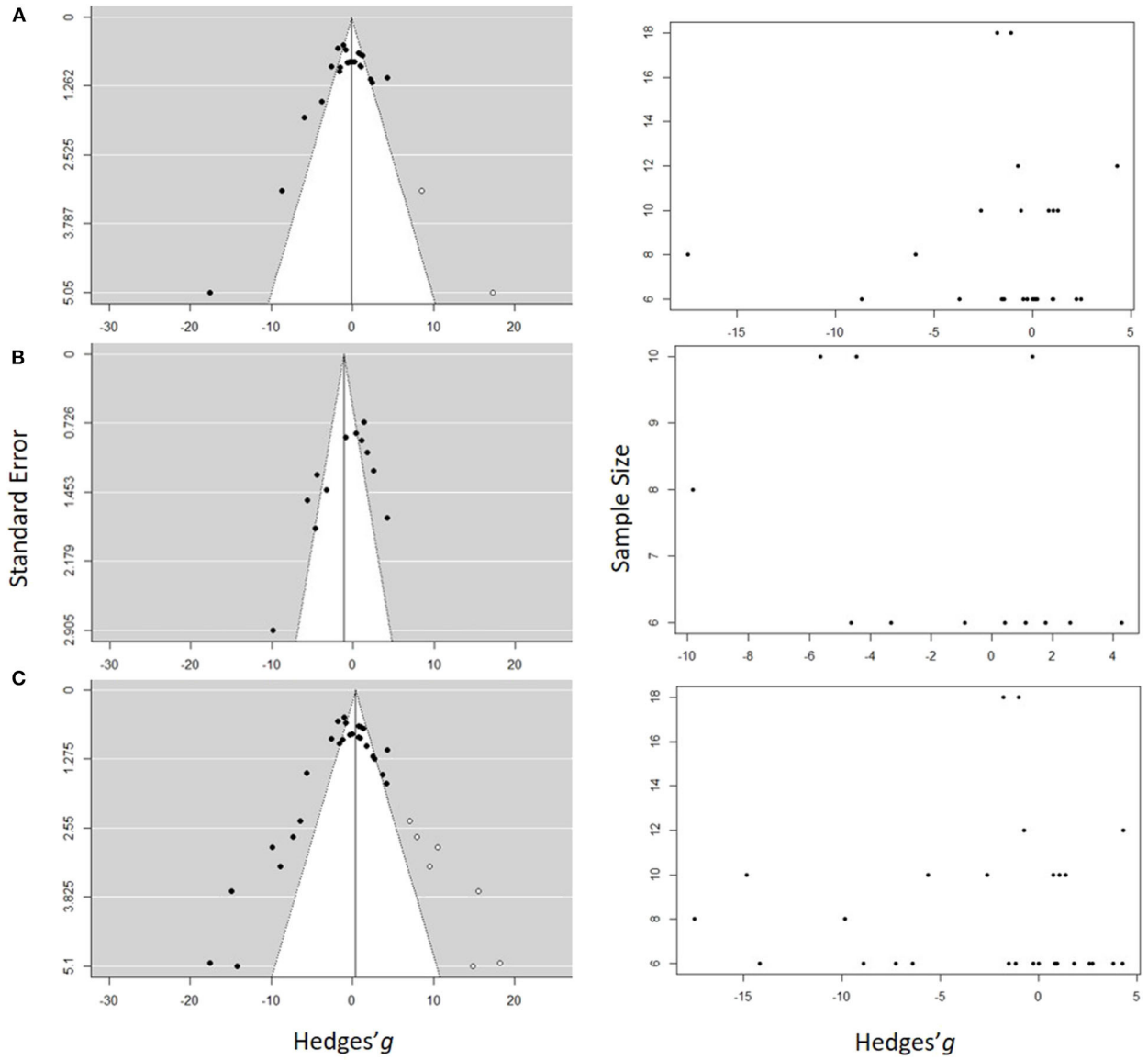

FIGURE 7 | Funnel plots estimating probability of a publication bias for the studies used in the meta-analysis for **(A)** *Low concentration model*, **(B)** *High concentration model,* and **(C)** *Full range model*. In the panels on the left side, the standard error is plotted against the effect size (Hedges' *g*), and in the panels on the right side, the sample size is plotted against the effect size. Each dot represents an entry in the model. The hollow symbols in the funnel plots are the studies filled by the trim-and-fill procedure, which would balance out the asymmetry of the funnel. The white area in the funnel plots depicts the significance with $0.05 < p \leq 1.00$ and the gray area is for $0.00 < p \leq 0.05$.

mixing, particle size is affected by aggregation during the exposure (Gorokhova et al., 2020), which makes it a challenging parameter to consider in the risk assessment. In algae, particles of 0.05–1 μm were reported to cause growth inhibition (Sjollema et al., 2016; Zhang et al., 2017; Gambardella et al., 2018), whereas those of ∼200 μm had a stimulatory effect (Chae et al., 2019). Moreover, the cell size of the test algae has also been suggested to contribute, with adverse effects on MNP observed at higher cell-to-particle size ratio (Chae et al., 2019). The mechanisms of these effects have been linked to particle adsorption to algal cell walls leading to compromised permeability, inhibition of growth, and photosynthetic activity (Bhattacharya et al., 2010; Mao et al., 2018). Although we found no significant influence of the cell-to-particle ratio on the effect size within the cell size range used in the dataset (1–78 μm; **Supplementary Table S2**), the observed negative trend was in agreement with the suggested effect direction and the mechanisms (Chae et al., 2019), and in the *Full range model*, this moderator explained 17% of the model heterogeneity.

Of the three polymers used in the subgroup analysis, none was found to induce a significant growth inhibition; thus, no support to the hypothesized effect of polymer material as a

significant predictor of the effect size was obtained (H2). PS was the most commonly used polymer (45% of studies) followed by PE (25%) and PVC (20%). In experimental studies, the high toxicity of styrene for algae and invertebrates has been related to the adverse effects of PS (Sussarellu et al., 2016; Mueller et al., 2020). Moreover, PS with anionic and cationic groups can undergo strong aggregation, which was suggested to mediate embryotoxicity in sea urchin embryos (Della Torre et al., 2014). Similar mechanisms of the adverse effects in alga *Dunaliella tertiolecta* were suggested by the same team (Bergami et al., 2017). However, the same polymer (PS-COOH) at comparable concentrations (40–50 mg/L) had differing effects on algal growth, ranging from no-effect (Bergami et al., 2017) to strong inhibition (Nolte et al., 2017), which suggests that other exposure characteristics, such as media and species, may alter the outcome. In particular, higher aggregation potential for positively charged polymer particles was suggested to affect biological availability and, thus, exerted effects, which is in line with the higher toxicity observed for cationic amino-modified particles (Bhattacharya et al., 2010).

Across the studies, PE and PVC were found to have both inhibitory and stimulatory effects resulting in the non-significant mean effects. These polymers have relatively low ($\leq$1 g/cm^3) and high ($\sim$1.4 g/cm^3) density, respectively, which makes them more likely to either float on the surface (low-density PE) or sink to the bottom (PVC) during the experiment. Therefore, static tests are not likely to provide a controlled exposure and, hence, comparable results for polymers with negative or positive buoyancy (Karami, 2017), emphasizing the need for methodological developments and search for adequate exposure systems.

Support for the hypothesized effect of the polymer material (H2) was provided by the meta-regression showing that polymer density was a significant predictor of the effect size in the *Low-concentration* model but not in the other two models encompassing higher exposure concentrations. At MNP concentrations <100 mg/L, low-density polymers are more likely to inhibit algal growth compared to high-density polymers, which is most likely due to the lower sedimentation of the former. At higher MNP concentrations, the enhanced contact between the polymer particles could facilitate aggregation and sedimentation, thus masking the effect of the polymer density.

Non-spherical particles, such as fibers and irregular fragments, were found to have stimulating effects on algal growth, unlike spherical particles that tended to exert inhibition. However, these findings must be interpreted with caution because studies using non-spherical particles are very scarce, and in our meta-analysis, all entries for such particles originated from only three studies (Yokota et al., 2017; Rogstad, 2019; Gorokhova et al., 2020). Although fragments and fibers are the most commonly observed MP in the field (Hidalgo-Ruz et al., 2012; Eriksen et al., 2014; Avio et al., 2015), spherical beads are used in the vast majority of the experiments, which has been broadly criticized (Wagner et al., 2014; Ogonowski et al., 2018). Given the observed differences in the effects caused by spherical and non-spherical particles, studies on MNP shaped as fibers and fragments are particularly needed, if we are to obtain environmentally relevant hazard assessment. More testing is also needed to evaluate weathering effects on the hazard potential of MNP because both stronger inhibition of growth (Fu et al., 2019) and no-effect outcomes (Gorokhova et al., 2020) have been reported for weathered particles compared to virgin ones. Plastic weathering changes the particle shape and size, surface area, and near-surface functional groups leading to the increasing frequency of hydroxyl groups (Liu et al., 2020). Unfortunately, the low number of studies that employed weathered plastics precluded evaluation of this factor as a predictor of the effect size for growth inhibition.

The hypothesized positive effect of the prolonged exposure on the effect size (i.e., lower inhibition at longer exposure duration; hypothesis H3) was not statistically significant. In the *Full range model*, however, 92% less inhibition was observed when exposure time increased from 72 to 168 h ($p > 0.13$). For such time-related effects, there are several possible explanations, related to algae adaption to the experimental conditions and changes in the exposure levels as well as culture conditions with time. Under prolonged incubation, different trade-offs and adaptations can occur in the exposure systems with algae (Andriukonis and Gorokhova, 2017) in response to deficiencies in light and nutrient regimes due to increased turbidity and concomitant nutrient depletion. If mixing is not adequate, particle aggregation and sedimentation will decrease the exposure and, hence, the response. Indeed, several studies reported the inhibition effect to be more severe in shorter incubations (e.g., $\leq$4 vs. >5 d; Fu et al., 2019; Zhao et al., 2019). Linking this pattern to the growth curve, Mao et al. (2018) explained growth inhibition during the exponential phase with compensatory growth as a sign of adaptation in the populations approaching the stationary phase. Moreover, changes in cell morphology and lipid accumulation can be indicative of such adaptations. For example, Su et al. (2019) observed an increase of cell density over a 10-d period, with a concomitant reduction in the cell size suggestive of nitrogen limitation (Kilham et al., 1997). Also, no growth inhibition has been reported after a 4-d exposure but a significant inhibition and lipid accumulation after 9-d, which could be explained by nutrient depletion due to algal growth but also sorption to the polymers (Guo et al., 2020). Therefore, time-related changes in the growth conditions may mask true response to the exposure, and the OECD-recommended duration of 72–96 h for the algal growth inhibition test design must be followed.

The effect sizes were not significantly different between the tests with fresh- and saltwater algae, thus supporting the hypothesized similarity (H4); however, freshwater algae had more negative effects in all three models (**Supplementary Figure S7**). The saltwater algae were used in 65% of the studies, which means that we have less information for conducting MNP hazard assessment in freshwaters compared to marine environments, where the plastic pollution has been studied more actively (Barboza and Gimenez, 2015; Eerkes-Medrano et al., 2015). Moreover, the taxonomic diversity of the used test species is relatively low (16 species), with the majority of species belonging to Dinophyceae, Prymnesiophyceae and

Chlorophyceae. Yet, the similarity of the observed effects suggests that the test results are transferable between the freshwater and marine algae; thus, all available information can be used for risk assessment of plastic debris in lakes and rivers until more data for freshwater microalgae become available.

Given that plastic litter has become a hot topic in the current environmental research fuelled by keen public interest, discovering indications of the publication bias was not particularly surprising. The magnitude of the bias was, however, not sufficiently large to invalidate the meta-analysis outcome. Nevertheless, the publication bias in testing MNP effects indicates that a study reporting inhibitory effects is more likely to be published, even though the results would be less reliable, and the sample size is small. There are several potential causes of this bias, including the fact that we used entries representing treatments with the largest effect size when compiling the datasets. However, all studies were treated equally, and small effect studies were well-represented in all three models. Thus, the bias appears to be related to the selection of an effect size associated with statistical significance for reporting. It is also a possibility that variables that have not been accounted for as moderators, particularly methodological parameters, such as elements of test design, physicochemical properties of the particles, or different levels of measurement error across the studies, might explain some of the observed funnel plot asymmetries. To alleviate some of these concerns, improve the comparability of the test results and, thus, their suitability for meta-analysis, it is critical to standardize the test protocols and report all the details of the experimental design as well as any outcomes of the testing, both positive and negative, regardless of their significance and sample size.

Since adverse effects can occur in turbid environments with a variety of particulates, both natural, such as sediment, and anthropogenic, such as microplastic, it is relevant to compare responses between the microplastic and other suspended solids present at ecologically relevant concentrations. Our findings, including model heterogeneity and lack of significant effects rated to the variation of the nominal particle concentrations, strongly suggest that analysis of the particle size distribution in the test systems should be included in the experimental design to control for the aggregation and sedimentation as potential drivers of algal growth. Needless to say, that these processes occur in mixtures of any suspended solids, both natural and anthropogenic.

We hope that our study would stimulate a debate on the methodology of microplastic hazard assessment and possibly influence further research efforts toward this goal. Following the critical analysis of the recent publications on the subject (**Table 2**), we propose practical recommendations for researchers for conducting transparent and methodologically sound test protocols and reporting routines. This should help to consolidate the search for convergent assays informing the risk assessment of plastic litter in aquatic ecosystems. Current standard toxicity testing is mostly developed for chemicals, not particulates with complex behavior resulting in particle aggregation and physical interactions with test organisms (Ogonowski et al., 2018; Gerdes et al., 2019). As a standard ecotoxicological endpoint, algal growth alone might be insufficient to provide insights into the ecological impacts of MP (Gambardella et al., 2019). Therefore, more sensitive endpoints, such photosynthetic efficiency (e.g., Wu et al., 2019), electron transport (e.g., Mao et al., 2018), and biochemical changes (e.g., Bhattacharya et al., 2010), together with reliable test protocols, are needed to evaluate how microalgae might be affected by polymer particles. At present, it was not possible to include these subcellular responses in the meta-analysis, because of the low number of such studies and considerable variation in the methods employed. One should also keep in mind that subcellular-level responses in these tests must be linked to those at the higher levels of biological organization (Martyniuk, 2018) if we are to protect wild populations exposed to environmental contaminants, including plastic litter.

TABLE 2 | Practical recommendations for standardization of algal bioassays with micro- and nanoplastic and reporting routines based on the outcome of the meta-analysis.

Test aspects	Recommendations
Test materials, species, and endpoints	• Well-characterized test materials must be used in terms of the polymer and particle size distribution
	• More studies on polymer materials other than PS and PE as well as non-sphaerical and weathered plastic particles are needed
	• Phylogenetically diverse taxa, both marine and freshwater, need to be used; more data on the freshwater taxa are needed
	• Apical endpoints considered in an accepted guideline test should always be included when using subcellular endpoints to assess effects
Equipment considerations and exposure conditions	• Controls should include both particle-free controls (growth media only) and particle controls, in which either natural particles or benchmark plastic particles are used
	• Care must be taken to keep the exposure vessels in suspension; use of plankton wheels or other rotating devices must be obligatory to provide constant exposure over time
	• Levels of nutrients and light intensity must be sufficient to ensure no limitation during the exposure in the highest concentration of the test material
	• Exposure duration should be adjusted to the growth curve of the algae and the experiment should be terminated before approaching the plateau; the recommended 72–96 h are in most cases optimal for detecting growth limitation in the exponential phase
Reported parameters	• All test results, both positive and negative, regardless of the statistical significance of the outcome must be reported
	• The primary data must be made available for use in meta-analysis and data synthesis
	• All available information on the physicochemical properties of the test and reference particles must be provided; an effort should be made to obtain this information
	• Particle size distribution during the exposure should be assessed and reported as a parameter affecting actual exposure levels in the system
	• The test parameters should be carefully presented to allow for intercomparisons and meta-analysis

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

EG conceived the study idea. EG and SR designed the study and wrote the manuscript. SR collected data and performed the analysis. Both authors contributed to the data interpretation and writing.

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SUPPLEMENTARY MATERIAL

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Microplastic Monitoring at Different Stages in a Wastewater Treatment Plant Using Reflectance Micro-FTIR Imaging

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While the presence of microplastics (MP) has been reported in aquatic habitats across the globe, the pathways through which they enter the environment are still poorly understood. Studies investigating the fate of MP in wastewater are gaining attention but are still scarce, despite the urgent need to understand the role of wastewater treatment plants (WWTP) as point sources of aquatic MP pollution. A likely reason for the limited number of WWTP-associated studies is that working with a biogenic organic matter (BOM)-rich sample matrix like wastewater is challenging. Here, we investigated the presence of MP throughout several stages of a WWTP at multiple depths, employing Fenton's reagent and focal plane array-based reflectance micro-Fourier-transform infrared spectroscopic (FPA-based reflectance micro-FTIR) imaging, a protocol that allows the automated detection and identification of MP in complex samples with high organic matter content, without the need for previous visual sorting, or reducing considerably the thickness of the sample, or the use of IR-transparent transmission windows. It was found that the number of MP fragments detected at downstream stages of the WWTP notably decreased following the primary settlement stage, with primary settlement stage samples responsible for 76.9% of total MP detected. Despite the marked reduction in the number of MP particles following the primary settlement stage, an average total of 1.5 MP L^{-1} were identified in the final effluent of the WWTP.

Keywords: microplastics, reflectance micro-FTIR, wastewater, Fenton's reagent, infrared imaging

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INTRODUCTION

While microplastic (MP) pollution is an important environmental concern and its presence has been extensively studied globally (Browne et al., 2011; Cole et al., 2011), the pathways by which microplastics enter aquatic environments remain understudied. Research on the fate of microplastics in wastewater have started to gain more attention only during recent years. However, a likely reason for the still scarcity of such studies is that working with a biogenic

organic matter (BOM)-rich sample matrix like wastewater is very challenging. Most aquatic-based investigations have assessed microplastics in seas, rivers and lakes, where the challenges associated with the separation of microplastics from other organic content are often less pronounced (Hidalgo-Ruz et al., 2012).

Despite the difficulty of detecting microplastics in BOM-rich matrices, there is a growing trend to investigate the fate of these pollutants in wastewater to help build a better picture of how wastewater treatment plants (WWTPs) cope with microplastic load and to what extent microplastics enter river systems through WWTPs (Lares et al., 2018; Sun et al., 2019). However, the majority of the studies have focused on the comparison between raw and treated wastewater samples (inlet and outlet only), and only a few have started addressing the different stages within the WWTP. Murphy et al. (2016) investigated microplastic abundances in various wastewater treatment stages and found that the majority of microplastics were removed during the grease removal (settlement stage), yet 0.25 ($\pm$0.04) MP L^{-1} were found in later stages including final effluent, where extrapolation of this data suggested 65 million microplastics could be released into natural waters every day from the WWTP studied. Carr et al. (2016) found differing results, suggesting that tertiary wastewater effluent is not a significant source of microplastics in the environment, finding one microplastic particle per 1,140 L (or 0.0009 MP L^{-1}). Mintenig et al. (2017) sampled 12 WWTPs in Germany and estimated yearly discharges ranging between 9×10^7 to 4×10^9 MP particles and fibers from the WWTPs studied. Simon et al. (2018) estimated that ten of the largest Danish WWTPs discharge around 3 tonnes per year of MP in the size range 10–500 μm. Murphy et al. (2016) collected bulk samples before a sieving step (65 μm mesh size), Carr et al. (2016) fixed stacked sieves (400–445 μm) in a wastewater stream directly without taking bulk samples, Mintenig et al. (2017) applied enzymatic-oxidative purification in combination with focal plane array (FPA)-based transmission micro-FTIR, and Simon et al. (2018) used sieve meshes to eliminate particles and fibers larger than 500 μm in the raw and treated wastewater only. Different studies used diverse techniques to sample, extract, treat and detect microplastic presence in wastewater, and were conducted at different wastewater sites that may use alternate methods for wastewater treatment and support different population sizes and structures. Additionally, as the studies were conducted in areas with differing climate, rainfall and other geographical factors, it is perhaps unsurprising that the results show disparity.

Visual selection has been a commonly used technique for separating microplastics from a sample and relies on the user to visually determine what may be plastic debris before further analysis is undertaken. Visual selection is likely to bias study results, particularly when microplastics occupy the lowest section of the micron range or have a color that is similar to a background or the surrounding medium. The concern that visual selection may be inaccurate is well established (Reddy et al., 2006; Corcoran et al., 2009; Harrison et al., 2012; Hidalgo-Ruz et al., 2012; Rocha-Santos and Duarte, 2015; Tagg et al., 2015, 2017). While it may be possible to avoid analytical bias by treating every particulate solid as a potential microplastic, this may not be possible or practical in complex sample media such as active biologically-treated wastewater.

Over the past years, several studies have investigated the use of spectroscopic imaging as a method to detect microplastics in environmental samples without the need for a visual selection step (Löder et al., 2015; Tagg et al., 2015; Käppler et al., 2016; Mintenig et al., 2017). For example, FPA-based micro-Fourier-transform infrared spectroscopic (FPA-based micro-FTIR) imaging has been used to detect these pollutants in both wastewater and seawater, following their filtration onto membrane filters. Due to its semi-automated nature, this approach is much less user-intensive than approaches involving a visual selection step (Löder et al., 2015; Tagg et al., 2015; Mintenig et al., 2017). An additional advantage is that FPA-based micro-FTIR imaging can be used for the approximate sizing of microplastics using the chemical images produced. There are, however, issues surrounding microplastic sizing, and these are discussed in more detail below.

Infrared imaging can be performed either in transmission or reflectance mode, and both approaches have been successfully used to identify microplastics down to a size of ∼20–25 μm (Löder et al., 2015; Tagg et al., 2015; Mintenig et al., 2017). Although analyses in transmission mode provide comparatively well-resolved spectroscopic and imaging results (Löder et al., 2015), this mode may be unsuitable for samples containing thick and/or opaque plastic fragments. Indeed, in a recent study that employed FTIR imaging in transmission mode to detect microplastics in WWTP effluents, fragments of >500 μm had to be analyzed following a visual sorting step (Mintenig et al., 2017).

Although reflectance micro-FTIR imaging could be used as a stand-alone method to monitor microplastic concentrations in wastewater, this technique has mainly been employed in "proof-of-principle" studies involving either spiked particles or limited volumes of wastewater (Tagg et al., 2015, 2017) or in a subset of samples to detect larger particles only (Simon et al., 2018). In this study, we use reflectance micro-FTIR spectroscopy as the sole spectroscopic tool to investigate the presence of microplastics within three different treatment stages of a WWTP. A pre-treatment step using Fenton's reagent enabled the effective filtration of wastewater for the rapid isolation of microplastics from these BOM-rich samples, while not impacting the size of the microplastics or affecting the presence and positions of the key FTIR absorbance bands for plastic identification (Tagg et al., 2017). By imaging the entire membrane filters directly, with no need for a visual pre-selection step or requirement of IR-transparent transmission windows, we demonstrate that reflectance micro-FTIR can be used as a rapid and reliable tool to detect microplastics in all of the treatment stages examined (including highly challenging sample types such as biologically activated wastewater).

MATERIALS AND METHODS

Sampling

Wastewater samples were collected from a wastewater treatment facility in the East Midlands (United Kingdom) in summer 2015

between 12 and 4 pm (BST) and in spring 2016 between 12 and 4 pm (GMT). This is a major WWTP serving a population of 200,000. Samples were collected from both the surface (top 5 cm) and subsurface from three different treatment stages (primary settlement, activated biological anoxic treatment, and activated biological aerobic treatment) and the final effluent at point of release. Surface samples were collected using an aluminum telescopic sampling pole, extendable up to 6 m (Telescoop, Waterra Ltd., Solihull, United Kingdom, with a bottle holder scoop container model TSB-0750). Subsurface samples were collected using a hand-operated suction pump and weighted nozzle (Burkle Uni-Sampler, Bad Bellingen, Germany) set at a depth of 2.5 m, with the exception of the final effluent where the sampling depth was ∼60 cm. A total of 10 L was collected at each treatment stage in each visit, giving a total volume sampled of 80 L per visit (4 sampling sites × 2 different depths), with a total of 160 L in the two sampling visits (summer 2015 and spring 2016). Samples were regularly mixed via inversion during storage (no more than a month until analysis). For the activated biological aerobic treatment stage (where aeration occurs on site) a constant air flow was maintained using 0.2 μm membrane filters (VWR, Leicestershire, United Kingdom) on in and out air lines to prevent any contamination.

Sample Preparation

Samples were homogenized (via inversion mixing) and 1 L was extracted for analysis. Each 1 L sample underwent centrifugation at 2,038 *g* for 2 min in a Thermo Scientific Heraeus 400R Labofuge Refrigerated Centrifuge. The supernatant was retained for filtration and the solid fractions were treated using a 7-day 30% (v/v) H_2O_2 pretreatment (Tagg et al., 2015) to enable the solids to be effectively filtered afterward. Where this proved ineffective (in samples with very high levels of BOM, i.e., samples from the activated biological treatment: aerated and anoxic tanks), Fenton's reagent ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + HO^-$) pretreatment was used for 10 min (see Tagg et al., 2017 for a complete description of the methodology). Filtration was performed using a Millipore vacuum filtering assembly through 47-mm Isopore polycarbonate membrane filters (Millipore Corporation, Billerica, MA, United States), with a pore size of 5 μm at −40 kPa.

Sample Analysis

Membrane filters were imaged using FPA-based reflectance micro-FTIR using a PerkinElmer Spotlight micro-FTIR spectroscope (Beaconsfield, United Kingdom) equipped with a mercury–cadmium–telluride FPA detector (consisting of 16 gold-wired infrared detector elements). A per-pixel aperture size of 25 × 25 μm was used with two co-added scans per pixel and a spectral resolution of 16 cm^{-1}. To identify microplastic types, chemical images of the entire 47 mm (diameter) membrane filter (see **Figure 1**) were generated for polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and nylon, using the approach described in Tagg et al. (2015). Approximate microplastic size was determined by averaging *x* and *y* values (horizontal and vertical cross-section values taken at widest points) of each imaged microplastic using ImageJ v.1.50g (Abràmoff et al., 2004). Further details on the micro-FTIR methodology can be found in the **Supplementary Material**. Lab coats made of natural fabrics were used at all times during sampling and sample analyses to avoid contamination. Lab materials were carefully cleaned and covered directly with tin foil when not in use. Negative controls using MilliQ water as opposed to wastewater did not show presence of plastic particles or fibers.

RESULTS AND DISCUSSION

Microplastic Count

Examples of the chemical images produced by FPA-based micro-FTIR imaging are shown in **Figure 1**. Two of the microplastics shown in **Figure 1** resemble "microbeads" [PE (A) and nylon (D)], while one microplastic [PP (B)] resembles a fiber. When examining total microplastic counts across the various sampling points, the number of microplastics was found to decline from the first sampling point (primary settlement) to the final sampling point (final effluent; see **Figure 2** and **Table 1**). This reduction was most marked directly following primary settlement and was more pronounced in the surface sample sets. The most likely reason for the reduction in microplastic counts is that the primary settlement (also referred to as the grease-removal stage by Carr et al., 2016) is designed for the removal of floating debris (James, 1971). Many common polymer types (such as PE and PP) have a lower density than water (Hidalgo-Ruz et al., 2012). Therefore, it is expected to see relatively high numbers of microplastics in settlement stage surface samples, where both floating and settling debris collects. Furthermore, microplastic abundance can be anticipated to decline in downstream samples because of the removal of floating debris at this treatment stage. A similar decline (from settlement stage to further downstream aquatic stages) was also observed by other authors (Carr et al., 2016; Murphy et al., 2016).

An important trend to consider is the difference in count data between surface and subsurface samples. It can be seen in **Figure 2** that the surface counts were consistently higher than those for subsurface samples at the settlement stage (Sg 1). This suggests that surface samples are unlikely to give an accurate overall estimate of microplastic abundance within the water column, as this would greatly overestimate the overall microplastic content. However, counts following this point (**Figure 2**; Sg 2–4) were similar, indicating that the difference between surface and subsurface count data did not continue following the primary settlement stage. When the summer 2015 and spring 2016 sample sets were compared (**Table 1**), a similar reduction in plastic abundance was observed between both surface samples. However, this reduction, while evident to a lesser extent in the spring 2016 subsurface sample set, was not detected in the summer 2015 sample set. The lower microplastic count in settlement surface samples from summer 2015 compared to spring 2016 (1 compared to 10 microplastics found) may be due to variation within the water column. A possible reason for this variation may be that settlement stages tend not to be vigorously mixed to allow

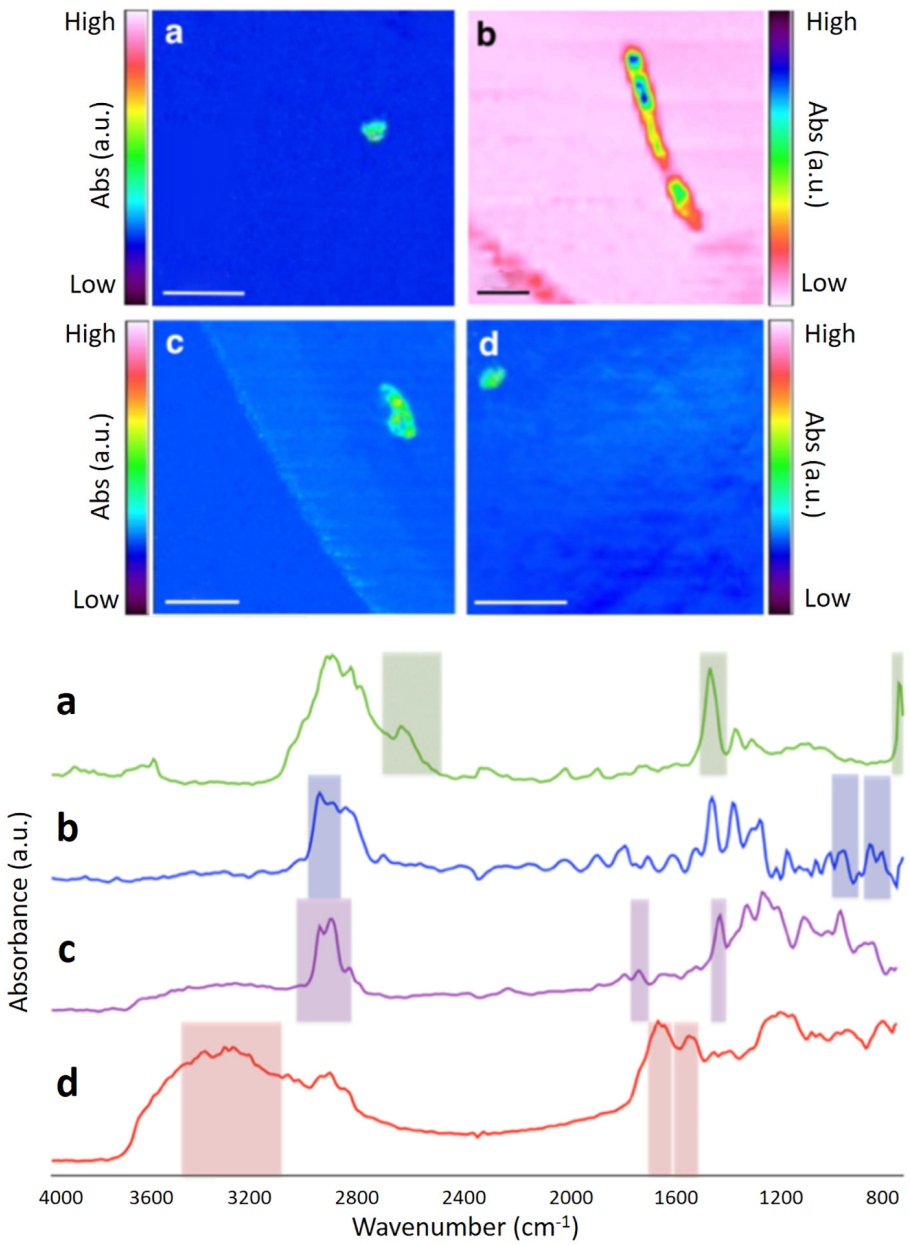

FIGURE 1 | FPA-based micro-FTIR false-color images of microplastics found in the WWTP, generated using key spectral-peak selections (see **Supplementary Material** for details). Corresponding spectra of different polymer types are shown below the false-color images. **(a)** PE; **(b)** PP; **(c)** PVC; **(d)** Nylon. Scale bar = 1 mm.

low density particulates and oils to congregate at the surface (James, 1971). However, a more plausible explanation could be lower/higher usage of water between Summer and Spring, or variations in rainfall.

Another observation was that, in multiple samples (surface summer 2015 and subsurface summer 2015 and spring 2016), microplastic count increased slightly between stages 2 and 3 (activated biological anoxic and activated biological aerobic). This difference may be due to the downstream stage being aerated, as the introduction of air streams within the aerobic stage may cause additional mixing and upwelling of particulate matter. This could therefore cause microplastics to be more dispersed.

Microplastic Type

The majority of microplastics identified were PE (50%; see **Table 1**). This finding is in general agreement with most studies; a meta-analysis of 68 environmental microplastic studies (Hidalgo-Ruz et al., 2012) found PE (along with PP) to be the most commonly identified polymer type. For studies conducted within WWTP, the data are less conclusive. A study of microplastic presence within a relatively small WWTP in Lysekil, Sweden

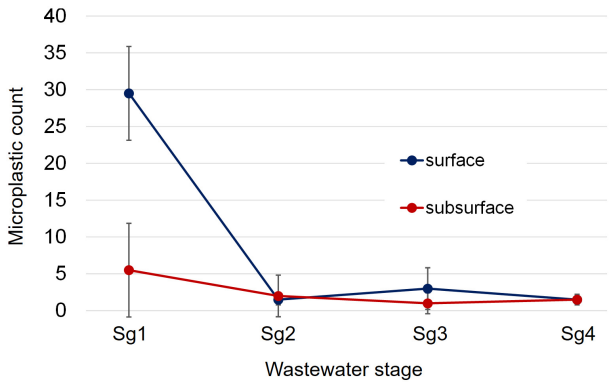

FIGURE 2 | Total microplastic count data across the four wastewater stages sampled in parity with the progress of wastewater treatment from influent to effluent in both surface and subsurface samples. Stage 1 (Sg1) refers to the primary settlement stage, Stage 2 (Sg2) to the activated biological anoxic stage, Stage 3 (Sg3) to the activated biological aerobic stage, and Stage 4 (Sg4) to the final effluent. Reported values are mean microplastic counts based on the two sampling visits, with the error bars being one time the standard deviation.

(serving a population of approximate 14,000), conducted by the Swedish Environment Institute (Magnusson and Norén, 2014) identified two out of the total five microplastic particles tested as PE using FTIR. Mintenig et al. (2017) also found PE to be the most prevalent microplastic in the WWTPs sampled in Germany; while Simon et al. (2018) found that the most abundant type in raw wastewater from the largest Danish WWTPs was acrylates, and PE and Polyester for treated wastewater. Murphy et al. (2016) studied a larger WWTP in Glasgow, United Kingdom (serving a population of approximately 650,000), and found alkyds (a type of polyester resins that is often used in paints; Hofland, 2012) to be the most common microplastic in many sample sets. Another study analyzing WWTP effluent from two sites in New South Wales, Australia (2 × 750 mL; five particles) found polyester fibers to be most abundant (Browne et al., 2011).

There are many reasons why different studies investigating microplastic occurrence in wastewater can show different results. Variables pertaining to both time of year and time of day, population size associated with the WWTP, speed and volume of effluent produced per unit time, primary, secondary or tertiary treatment, stages selected for sampling and closely located plastic-producing or -utilizing industries may be attributed to variation in study results. However, the disparity between some of these findings (for microplastic type abundance) may also be partially explained by the fact that, in all studies, differing techniques were used and different amounts of wastewater were sampled. Murphy et al. (2016) used an alternate sampling approach, similar to this study, by bulk sampling rather than sieving the flow over an extended period, and tested 140 L total wastewater per replicate (30 L × 3 stages; 50 L × 1 stage) where samples were collected from surface wastewater only. Similar to Carr et al. (2016), some of these samples could not be fully analyzed, with three out of the four stage samples being partially examined, which makes the total coverage examined difficult to compare. Our study sampled 160 L of wastewater across the different stages and depths (80 L in summer and 80 L in spring), and examined 8 L per sampling trip (1 L surface and subsurface samples for each of the four treatment stages; 16 L total), where each 1 L sub-sample was fully analyzed in all cases. Therefore, disparity between results of these studies (without considering the possible discrepancy associated with experimental procedures such as visual selection, limited use of FTIR and partial sample analysis) may be attributed to differing sampling techniques and sample volumes analyzed. Even if techniques and sample volumes were more comparable, differences in results may well be expected due to the innate differences in treatment approaches of WWTPs and differing populations and industries they serve. Given these potential differences between different WWTPs, variation in microplastic abundance data may be expected to reflect differences in the overall composition of wastewater samples, depending on where the samples were obtained. However, it is still difficult to determine whether differences in results observed between studies analyzing microplastic fate in wastewater are due to differences in methodologies or not,

TABLE 1 | Microplastic count data and polymer type across the four wastewater stages, in both surface and subsurface samples. Samples were collected from both the surface (top 5 cm) and subsurface (depth of 2.5 m, except for the final effluent where the sampling depth was ~60 cm).

		Polyethylene		Polypropylene		Polyvinyl chloride		Nylon		Polystyrene		Total	
		Summer 2015	Spring 2016	Summer 2015	Spring 2016	Summer 2015	Spring 2016	Summer 2015	Spring 2016	Summer 2015	Spring 2016	Summer 2015	Spring 2016
Surface	Primary settlement	8	17	0	3	15	12	2	2	0	0	25	34
	Activated biological anaerobic	1	2	0	0	0	0	0	0	0	0	1	2
	Activated biological aerobic	4	1	0	0	1	0	0	0	0	0	5	1
	Final effluent	0	1	0	0	1	1	0	0	0	0	1	2
Subsurface	Primary settlement	1	4	0	0	0	6	0	0	0	0	1	10
	Activated biological anaerobic	0	2	0	1	0	1	0	0	0	0	0	4
	Activated biological aerobic	2	0	0	0	0	0	0	0	0	0	2	0
	Final effluent	1	1	0	0	1	0	0	0	0	0	2	1
	Total MP counts	17	28	0	4	18	20	2	2	0	0	37	54

until a standardized approach is adopted and applied across multiple sites.

It is possible that innate differences in wastewater composition (discussed above) may explain the relatively high percentage (42%) of PVC microplastics present in this study, particularly in comparison with nylon (4%) and PP (4%) and a complete absence of PS. Although PVC is a commonly produced and used plastic, it is much less common in the environment than other types of microplastics generally found in environmental microplastic studies (Hidalgo-Ruz et al., 2012). It is unclear why a relatively higher amount of PVC microplastics was found in this study, and additional research would be required to determine why PVC microplastics were common in this specific WWTP during the sampling period. While one origin of these fragments could be due to a high number of PVC-made pipes in the houses' draining systems adjacent to the WWTP, it is also recommended to sample this WWTP for a longer period of time (years), with more replicates, and at different times of the year. This could provide more robust data and a much longer and continuous description of the type of plastics received by the WWTP, as external factors such as temporary industrial activity (where PVC plastics may be present in high amounts) could also play a role. A recent study by Wagner et al. (2019) found, for example, an unusually high abundance of PS microfragments while monitoring plastic concentrations in the rural subcatchment and downstream of the urban subcatchment of the River Parthe in Leipzig, Germany. It was found that the high PS abundance was due to building construction projects during the sampling period, particularly low-energy modernization, and construction with extensive use of PS building insulation material. Another potential factor to consider is that the approach in this study involved no visual pre-selection step. It is possible that microplastics in complex, BOM-rich substrates which share characteristics similar to non-plastic sample debris, such as color of morphology, would be systematically overlooked when using visual sorting of samples for analysis (Murphy et al., 2016). As a result, the higher PVC presence found in the present study might also be because the use of reflectance micro-FTIR imaging decreases the risk of underestimating microplastics that could otherwise be left unnoticed by visual selection, particularly given the small size of many of the polymer particles that were found.

Microplastics in Effluent

Another important factor to consider is the amount of microplastics released in effluent. In this study, the mean microplastic concentration in the effluent was 1.5 L^{-1}. While these estimates can be useful for comparative purposes with other WWTP-focused microplastic studies (since similar abundances have been reported), the extrapolative approach used to produce such estimates may not be sufficiently accurate. In all current studies which have examined wastewater effluent, no two surveys have produced the same estimate. Our study produced an estimate of 1.5 MP L^{-1} of effluent, similar to the value of 1 MP L^{-1} reported by Browne et al. (2011). Within the site studied by Murphy et al. (2016), microplastic abundances were found to be 4× lower than this (0.25 MP L^{-1}). Other studies have reported even lower rates of 0.009 MP L^{-1} (Magnusson and Norén, 2014) and 0.0009 MP L^{-1} (Carr et al., 2016). Microplastic counts for several WWTPs in Germany (Mintenig et al., 2017) found between 0 and 0.04 large (<500 μm) MP L^{-1}, 0.08 and 9 small (<500 μm) MP L^{-1} and 0.1 and 5 MP fibers L^{-1}; whereas at three WWTPs in Charleston Harbor, SC, United States (with different treatment sizes, operations and service compositions) counts ranged between 1 and 30 MP L^{-1} across all three WWTPs (Conley et al., 2019). As can be seen, this high variability in particle counts between sampling sites and differences in microplastic abundances are likely to be strongly influenced by location-specific factors that affect the overall microplastic load as previously discussed. In addition, each WWTP may have different stages and characteristics, also affecting their MP removal performance. For example, the use of a final filtration step undertaken at tertiary treatment plants, such as granular sand, pile fabric or microfiltration has been reported to reduce microplastic presence in effluent (Michielssen et al., 2016; Mintenig et al., 2017).

Microplastic Size

Determining the size distribution of microplastics in environmental samples is of importance since differently sized microplastics may respond differently to different waste removal treatments (Talvitie et al., 2017). The microplastics detected in our study had a mean size of 392 μm (±27 μm SE), with the smallest particle being 54 μm and largest being 1,277 μm. An interesting trend in the size data was the absence of microplastics >600 μm in samples after the primary settlement stage (**Figure 3**). This was consistent between both sampling visits and indicates that the primary settlement stage may be responsible for the removal of

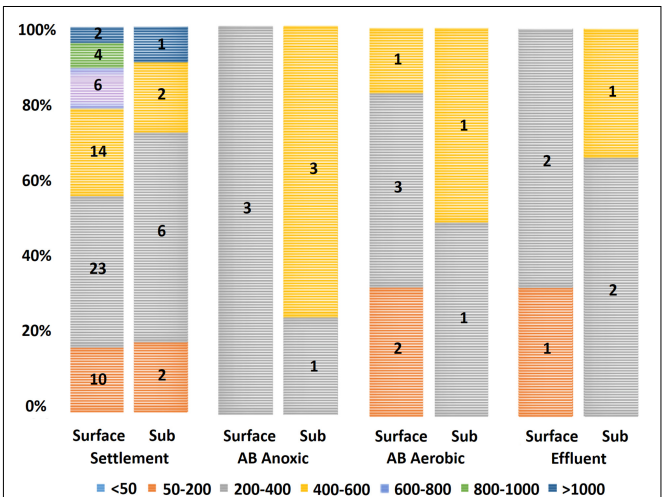

FIGURE 3 | Microplastic size data by wastewater stage (total count of the two sampling visits). Legend entries refer to grouped sizes in micrometers (μm). Microplastics with sizes 200–400 μm had the highest count, with 45% (41/91) falling within this group. No microplastics larger than 600 μm appeared in samples downstream of the settlement stage. Sub: subsurface; AB: activated biological.

larger microplastics. It should also be considered that the settlement stage removes most microplastics present at this stage (as suggested by 76.9% of all microplastics identified in this study corresponding to the settlement stage; see Carr et al. (2016) and Murphy et al. (2016) for similar results).

Direct comparisons of microplastic size between studies remain challenging. Some earlier wastewater-associated studies do not provide sufficient details on the sizes of the microplastics found in each stage (Browne et al., 2011; Magnusson and Norén, 2014; Carr et al., 2016), and in other publications, microplastics are grouped by size class, with microplastics <1 mm being the minimum reported size group (Doyle et al., 2011; Hidalgo-Ruz et al., 2012). Murphy et al. (2016) found plastic particles within liquid fraction samples to have a mean size of 598 μm (±89 μm). Mintenig et al. (2017) reported the occurrence of large (>500 μm) microplastics (1–5 particles in the range 500–7,200 μm per sample) and a larger amount of small (<500 μm) microplastics (3–12 microplastics per sample, 59% of which were between 50 and 100 μm and 96% of which were below 250 μm) in the WWTPs studied, which is more in agreement with what has been found in the present study.

Sampling protocols may explain why smaller microplastics were more common in both the present study and that of Mintenig et al. (2017) when compared with other studies. For example, Magnusson and Norén (2014) analyzed particles in wastewater collected on a filter with a mesh size of 300 μm. It is possible that microplastics smaller than the sieve apertures could not be retained. Since this mesh size is relatively close to the average particle size found in this study, this might explain why the present survey found a higher frequency of particles per liter of effluent (1.5 MP L^{-1} compared with 0.009 MP L^{-1}). Studies by Doyle et al. (2011) and Lattin et al. (2004), for example, used plankton net trawls with mesh sizes of 505 and 333 μm, respectively. These sampling methods could also leave out microplastics smaller than the mesh sizes. Although these studies were not related to WWTPs but pelagic ecosystems, and hence have a different scope, it again points out the discrepancy in sampling protocols, and the need of a standardized approach for the analysis of microplastics in the environment.

Similar to our study, Mintenig et al. (2017) also used FPA-based micro-FTIR imaging allowing the authors to accurately identify microplastics down to a size of 20 μm. Although the authors used a visual selection step to identify larger particles and only imaged a portion of 11 mm-diameter filter membranes, this still represents a much more robust approach for identifying small microplastics than using visual selection alone. The use of chemical imaging likely explains why both our study and that by Mintenig et al. (2017) report such comparatively high numbers of small microplastics. However, it must also be noted that in our study, data suggests that larger microplastics (>600 μm) are removed during primary settlement. Therefore, the small fractions found by Mintenig et al. (2017) in treated WWTP effluents may be indicative of the sizes of microplastics typically found in latter treatment and effluent stages.

Limitations and Future Recommendations

While this study has improved our understanding of microplastic presence and composition in wastewater and confirmed the suitability of FPA-based reflectance micro-FTIR imaging for detecting microplastics within multiple wastewater treatment stages, there are several further ways in which research into this topic could be improved. An automated microplastic spectral data processing pipeline has been published for FTIR imaging analyses performed in transmission mode (Primpke et al., 2017; Brandt et al., 2020), and future work could involve extending it to data produced in reflectance mode. In addition, while bulk sampling was used without visual selection or need for physical transfer of microplastics from the membrane filter, there is still a lower detection limit of 25 μm, where microplastics smaller than this may be overlooked due to the minimum spatial resolution currently available for FPA FTIR detectors. However, methods utilizing rapid Raman imaging are now also emerging (Ando et al., 2016; Lares et al., 2018; Wolff et al., 2019) and recent work suggests that the combination of a FPA-based FTIR imaging approach with Raman imaging may allow for analysis of particles down to 1 μm (Käppler et al., 2016). While rapid Raman imaging currently requires specialist equipment, further developments in this research area could eventually make this technique suitable for routine microplastic monitoring purposes.

Finally, this study presents a snapshot of microplastic presence in a specific WWTP, but much more work is still required to obtain an accurate estimate of microplastics likely to be released/prevented from release into aquatic systems. Longer and continuous studies, with multiple replicates and visits throughout the year, comparing multiple stages of different WWTPs with different approaches to treatment, different population sizes and the effect of storm water overflow on microplastic release (with a consistent sampling and analytical protocol) are urgently required. Since no studies focusing on the release of microplastics from WWTPs have yet applied the same methods, more studies are required using a standardized approach to sampling and analyzing microplastic presence and size without the use of visual selection or partial-membrane filter analysis to improve the understanding of the fate of microplastics in wastewater. To improve our understanding of the temporal dynamics of microplastics in wastewater, more complex monitoring schemes would be required. Ideally, such schemes would involve studies conducted over several temporal ranges (investigating changes over the course of hours, days and seasons) in order to more fully understand microplastic load in wastewater effluent.

CONCLUSION

This study demonstrates the effectiveness of FPA-based reflectance micro-FTIR imaging for detecting microplastic present throughout key stages of wastewater treatment, by imaging the entire membrane filters directly, with no need for a visual pre-selection step or requirement of very thin samples and IR-transparent transmission windows. It was observed that the settlement stage (grease removal stage) was responsible for

a considerable reduction in microplastics reaching latter stages in wastewater treatment. It was also found that microplastics >600 μm were particularly likely to be removed at this stage (since no microplastics >600 μm were found downstream of this stage). Microplastic counts at this stage were consistently higher in surface samples than subsurface. Nevertheless, analyzing only surface-samples may underestimate microplastic numbers if subsurface sampling is ignored. In this study, samples were collected at surface and subsurface from three different wastewater treatment stages (primary settlement, activated biological anoxic treatment, and activated biological aerobic treatment) and the final effluent, during Summer 2015 and Spring 2016. Ten liters were collected at each treatment stage in each visit, giving a total volume sampled of 80 L per visit (four sampling sites × two different depths), with a total of 160 L in the two sampling visits. A total of 1.5 MP L^{-1} was found in the final effluent (average combination of surface and subsurface samples, and the two visits). While these results show a "snapshot" of microplastic presence in a specific WWTP, more accurate or reliable values could be obtained by the inclusion of additional replicates, longer and continuous studies, and additional visits at different times of the year. This study helps to further the insight into the fate of microplastics in WWTPs using FPA-based reflectance micro-FTIR imaging, but further work is needed to obtain an improved understanding of this topic by using consistent and accurate sampling methodologies and extensive temporal-based monitoring schemes at a variety of sites.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AT, JO, and MS designed and performed the sample collection at the WWTP. AT and JO developed the reflectance micro-FT-IR protocol. AT performed the Fenton's treatment and the reflectance micro-FT-IR analyses. MS, JH, and YJ-N contributed to discussions and improvement in the data collection/analysis and methodology. YJ-N provided extra expertise in Fenton's treatment. CS and EB contributed to the structure and editing of the manuscript. The manuscript was authored by AT with all authors contributing to manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.00145/full#supplementary-material

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The remaining 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.

frontiers in Environmental Science

BRIEF RESEARCH REPORT
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Trophic Transfer of Microplastics From Copepods to Jellyfish in the Marine Environment

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Microplastics (MPs) can be ingested by marine organisms directly or indirectly through trophic transfer from contaminated prey. In the marine ecosystem, zooplankton are an important link between phytoplankton and higher trophic levels in the marine food web. Among them, copepods and gelatinous species have been recently reported to ingest MPs, but no potential MP transfer has been verified yet. In this study, a simplified two-level trophic chain – formed by nauplii of the *Tigriopus fulvus* copepod as prey, and the ephyrae stage of *Aurelia sp.* as predator – was selected to investigate MP trophic transfer. The experimental setup consisted in feeding ephyrae with nauplii previously exposed to fluorescent 1–5 μm polyethylene MPs and evaluating two ecotoxicological end-points: jellyfish immobility and pulsation frequency. After 24 h, the jellyfish ingested nauplii contaminated with MPs; however, neither immobility nor behavior was affected by MP transfer. These findings show that MPs can be transported at different trophic levels, but more research is needed to identify their potential effects on the marine food web.

Keywords: behavior, ephyrae, immobility, ingestion, polyethylene, zooplankton

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INTRODUCTION

Microplastics (MPs, <5 mm, Arthur et al., 2009) are bioavailable to marine organisms, through direct or indirect ingestion by trophic transfer from contaminated prey (Lusher, 2015; Nelms et al., 2018). Marine organisms can ingest unknown quantities of MPs using different feeding strategies since MPs occupy the same size range as plankton (Cole et al., 2013; Farrell and Nelson, 2013).

MP uptake is primarily due to their particle size in relation to natural prey and particle density, which determines their position in the water column and the possibility of encountering the organisms (Desforges et al., 2015). MP ingestion and accumulation have been successfully demonstrated in several marine species belonging to different trophic levels, from zooplankton to fish and marine mammals (Boerger et al., 2010; Avery-Gomm et al., 2012; Goldstein and Goodwin, 2013; Frias et al., 2014; Desforges et al., 2015; Bellas et al., 2016). Many zooplankton species feed on phytoplankton and pass this energy upward through the food web. Moreover, they play a key role in ecosystem functioning, including nutrient, and carbon cycling and the production of sinking fecal pellets (Botterell et al., 2019).

Marine zooplankton are most susceptible to MPs, since their ingestion has been recorded in more than 30 species from 28 taxonomic orders (Zheng et al., 2020). Among them, crustaceans

have been widely investigated and MP ingestion has been reported in several copepod species (i.e., *Tigriopus fulvus*, *Acartia clausi*, *Centropages typicus*, *Calanus helgolandicus*, *Temora longicornis*, *Neocalanus cristatus*; Cole et al., 2013, 2015; Beiras et al., 2018; Botterell et al., 2019; Coppock et al., 2019), accounting for a high proportion of the total zooplankton carbon biomass. In these organisms, MP ingestion affects their fecundity and reduces their feeding capacity, being able to discriminate natural algal prey from MPs (Cole et al., 2015; Botterell et al., 2019; Coppock et al., 2019). Recently, also gelatinous zooplankton (i.e., jellyfish, tunicates, salps), feeding on crustaceans and fish larvae and a key food source for pelagic top predators (Purcell et al., 2007), have been reported to internalize MPs with further impairment of swimming, lower feeding rate, growth, and oxygen consumption (Macali et al., 2018; Paffenhöfer and Köster, 2018; Wieczorek et al., 2019; Brandon et al., 2020; Costa et al., 2020).

Among gelatinous zooplankton, jellyfish (i.e., *Aurelia* sp., *Pelagia noctiluca*) are very vulnerable to plastic pollution: marine litter may be transferred through jellyfish to pelagic predators that may confuse floating plastics for prey (Macali et al., 2018). While many studies are available for both crustaceans and gelatinous zooplankton (Beiras et al., 2018; Macali et al., 2018) on MP ingestion and its effects on individual zooplankton species either under laboratory conditions or in the field, very little is known about MP transfer from lower to higher trophic levels. The latter may cause potential cascading effects in marine food webs (Frias et al., 2014; Setälä et al., 2014; Botterell et al., 2019; Wieczorek et al., 2019), since zooplankton play a key role in the link between primary producers and higher trophic organization (Sun et al., 2017).

To date, MP transfer among zooplankton has only been reported from polychaete larvae and copepods to mysid shrimps (Setälä et al., 2014). In general, MP potential to enter the marine food chain has been reported from adult mussels to crabs (Farrell and Nelson, 2013), from plankton to fur seals (Eriksson and Burton, 2003) and benthic filter feeders (Van Colen et al., 2020), from crustaceans to fish (Batel et al., 2016), from beach hoppers to ray-finned fish (Tosetto et al., 2017), and in marine top predators (i.e., from Atlantic mackerel to gray seals, Nelms et al., 2018).

Our current knowledge is insufficient to understand MP potential for bioaccumulation and biomagnification within the marine food chain, due to a lack of robust data describing MP transfer from prey to predator. Many questions remain when considering ecosystem-level dynamics, such as MP trophic transfer in the marine environment (Au et al., 2017). Since marine zooplankton are an important link between primary producing phytoplankton and higher trophic levels in food webs (Turner, 2004), more research is needed to clarify MP transfer among them. To fill this gap, a simplified two-level trophic chain was set up, consisting of a zooplankton species such as the nauplii of *T. fulvus* copepod as prey and the ephyrae stage of *Aurelia* sp. jellyfish as predator, to investigate MP trophic transfer. These species were selected since they are quite abundant and easy to culture in the laboratory and are promising models to evaluate MP pollution. To achieve this goal, nauplii were previously exposed to polyethylene MPs. Once contaminated, they were fed to ephyrae jellyfish. Finally, the ecotoxicological effect of MP trophic transfer in ephyrae jellyfish was investigated by evaluating two end-points (Immobility and Pulsation Frequency), recently proposed for this model organism (Faimali et al., 2014; Costa et al., 2015, 2020; Giussani et al., 2015).

METHODS

Predators and Prey

Tigriopus fulvus nauplii and *Aurelia* sp. ephyrae were used as preys and predators, respectively. *T. fulvus* nauplii, a harpacticoid copepod widely distributed in the Mediterranean, originated from synchronized cultures (24 h) obtained from ovigerous adult females at CNR-IAS laboratory, following UNICHIM protocol.

Ephyrae of *Aurelia* sp. were released by strobilation from polyp cultures in the laboratories of the "Acquario di Genova, Costa Edutainment S.p.A.," and transported to CNR-IAS, according to Costa et al. (2020).

Microplastics

Fluorescent green 1–5 μm polyethylene MPs (1.3 g/cm^3 density, 414 nm excitation/515 nm emission) μm polyethylene MPs (CPMS-0.96, 0.99 g/cm^3 density) were purchased from Sigma-Aldrich (Germany). Stock solutions of MPs (10 mg/L) were prepared in 0.22 μm of Filtered Natural Sea Water (FNSW).

Artificial Food Chain

A simplified two-level trophic chain was set up by feeding ephyrae jellyfish with nauplii of *T. fulvus* previously exposed for 6 h to different concentrations (0–1–10 mg/L) of fluorescent 1–4 μm polyethylene MP, according to Beiras et al. (2018). Briefly, 2 mL of nauplii (25 organisms per mL) was transferred by using a Pasteur pipette into small glass vials containing 2 mL of control FNSW, or serial dilutions (1–10 mg/L) of MPs. Vials were then placed in 50 mL centrifuge tubes (5 vials/tube), fixed on a rotatory wheel (2 rpm speed) and kept at 20°C. After 6 h, the nauplii were transferred from glass vials into a multiwell plate, to verify MP ingestion under an epi-fluorescence microscope (Olympus). Each treatment, including controls, was prepared in triplicates.

Then, nauplii were offered to ephyrae in a semi-dynamic condition according to Costa et al. (2020) for 24 h. More in detail, 10 ephyrae collected immediately after strobilation were incubated in a glass beaker filled with 100 mL of FNSW, with constant aeration for oxygen supply, in order to ensure current balance to the organisms (Widmer, 2008). For each tested MP concentration, each batch of 10 ephyrae was fed with previously contaminated 50 *T. fulvus* nauplii. The control group of ephyrae was fed with untreated (MP-free) nauplii. The prey (nauplii)/predator (ephyrae) ratio was previously defined in order to avoid an excess of food in the beaker considering that *Aurelia* sp. ephyrae can capture only live preys (Suchman and Sullivan, 2000; Bamstedt et al., 2001). Indeed, the non-consumed nauplii setting on the beaker bottom could have caused overfishing conditions. For each MP dilution, 3 replicates were prepared. Constant temperature was maintained by keeping the glass beaker in a thermostatic room at 20°C ± 0.5 for 24 h.

MP Transfer

In order to analyze only the MP transfer from copepods to ephyrae, after 24 h, the ephyrae were recovered and washed three times with fresh FNSW to remove any microspheres which, if released in the water by nauplii, could bind to the gelatinous body. The organisms were anesthetized with the addition of a few menthol crystals, fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4), and mounted in glycerol–PBS (1:1) to be visualized by immunofluorescence under an epi-fluorescence microscope (Olympus) according to Williams and Van Syoc (2007) and Costa et al. (2020).

Ecotoxicological Responses

Before anaesthetization, each ephyra was transferred from the glass beaker into a single Petri dish (containing clean FNSW) in order to evaluate immobility (acute end-point) and pulsation frequency (subacute response). This was done using an automatic recording system coupled with a specially designed video graphics analyzer: Swimming Behavioral Recorder (SBR; Faimali et al., 2014; Costa et al., 2020). Completely motionless ephyrae were counted as immobile organisms, and the immobility percentage (I%) was calculated for each dilution and compared to controls. Pulsation frequency was calculated by recording the number of pulsations made by each ephyra in 1 min.

Statistical Analysis

Significant differences between treated samples and controls were evaluated using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, the non-parametric Kruskal–Wallis test and Mann–Whitney test were used. Data were considered significantly different when $p < 0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

RESULTS

MP Transfer

Copepod nauplii ingested 1 and 10 mg/L MPs after 6 h of exposure. Particles were shown to have accumulated in the gut (data not shown). Jellyfish fed with contaminated copepods for 24 h showed to have ingested them, thus indicating that MP trophic transfer had occurred (**Figure 1**): both control (**Figure 1A**) and treated (**Figures 1B,C**) organisms ingested a

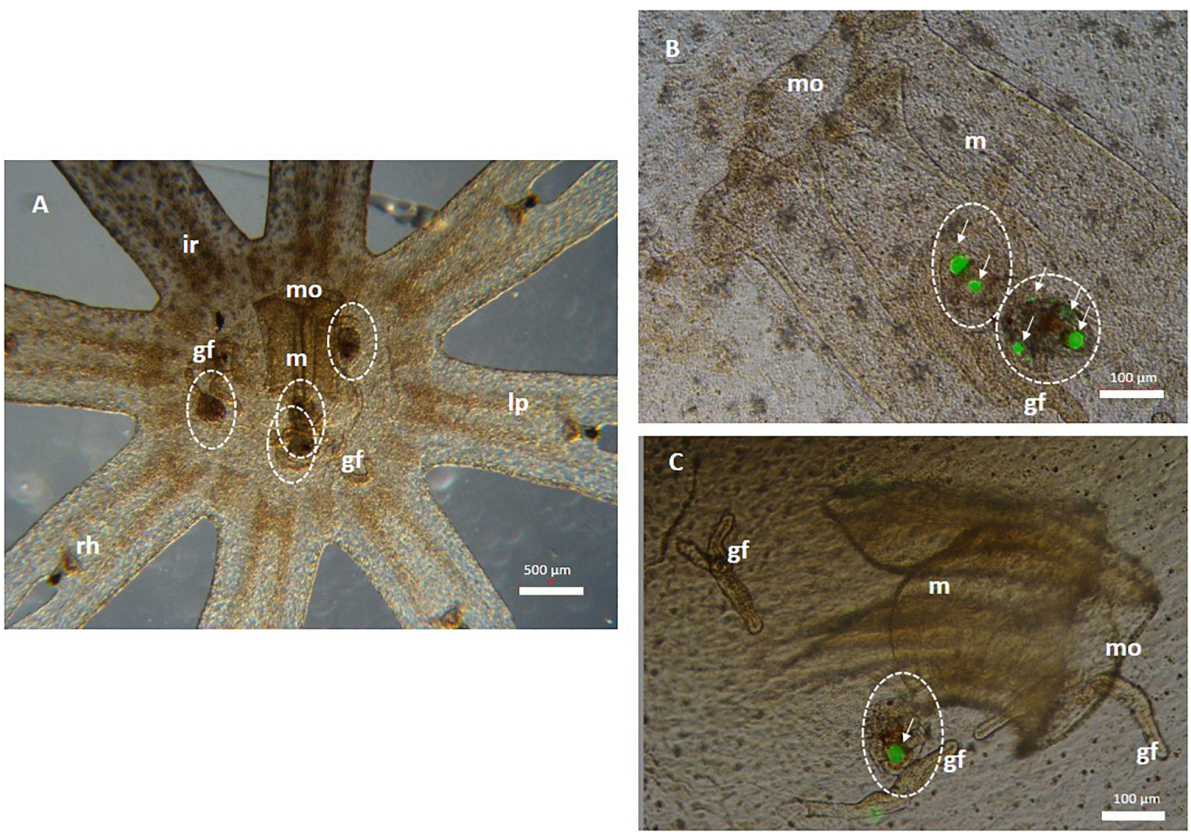

FIGURE 1 | Microplastic (MP) trophic transfer from *T. fulvus* nauplii (prey) to *Aurelia* sp. Ephyrae (predator) after a 24 h exposure. **(A)** Control *Aurelia* sp. ephyrae that ingested uncontaminated copepods (white dotted circles). **(B,C)** Copepods (white dotted circle) containing fluorescent polyethylene MPs (white arrows) are clustered in the manubrium of the mouth and near gastric filaments. mo, mouth; m, manubrium; ir, inter-radial; lp, lappets; rh, rhopalia; gf, gastric filaments; white dotted circle, copepod; white arrows, MPs (or aggregate).

variable number of nauplii (from 1 to 4). In detail, control ephyrae that ingested untreated copepods are indicated in **Figure 1A**. Details of the ephyra mouth, manubrium, and gastric filaments are seen in **Figure 1B**, where two copepods were found that had internalized MPs in an aggregate form. **Figure 1C** highlights one copepod, containing aggregates of fluorescent MPs, close to the ephyra's gastric filament.

Ecotoxicological Responses

Ingestion of MP-contaminated preys did not cause any, either acute or subacute, ecotoxicological effects. Immobility percentage in ephyrae fed with *T. fulvus* nauplii contaminated with 1 mg/L and 10 mg/L polyethylene MPs was not significantly affected (0%). Further, the subacute end-point (pulsation frequency) was not significantly affected ($p > 0.05$) in ephyrae fed with *T. fulvus* nauplii contaminated with both polyethylene MPs dilutions. Actually, after the ingestion of 10 mg/L MP-contaminated copepods (**Figure 2**), only a very slight decrease in the number of pulsations (vs. controls) was observed.

DISCUSSION

In this study, MP trophic transfer has been successfully demonstrated between copepod nauplii and ephyra jellyfish. Polyethylene MPs were ingested indirectly as a result of trophic transfer, whereby contaminated prey was consumed by predators. The potential of MPs to be transferred between trophic levels, from copepods to macrozooplankton (mysid shrimps), following ingestion, has been already observed by Setälä et al. (2014). An indirect MP transfer has also been demonstrated from mussels to crabs (Farrell and Nelson, 2013), from Atlantic mackerels to gray seals (Nelms et al., 2018), from plankton to fur seals (Eriksson and Burton, 2003), and from crustacean larvae to fish (Batel et al., 2016) by either laboratory experiments or field observations. Taken together, these studies suggest that trophic transfer is an indirect major pathway of MP ingestion for any species whose feeding ecology involves the consumption of whole prey (Nelms et al., 2018). Zooplankton may incorporate several elements or toxic compounds, including persistent organic pollutants (POPs), either directly, by absorption from water, or indirectly, by ingestion of contaminated algae and other zooplankton (Nizzetto et al., 2012; Bettinetti and Manca, 2014). POPs and other hydrophobic organics can adsorb to plastics and MPs in the environment, being bioavailable to aquatic organisms (Au et al., 2017). Among zooplankton, copepods are a potential carrier of organic hydrocarbon compounds (i.e., dioxins), by dietary uptake and bioaccumulation of contaminated phytoplankton (Wallberg et al., 2001; Zhang et al., 2011).

Our results demonstrate that copepods can also transfer MPs to the higher trophic chain levels, since MP-contaminated nauplii were ingested in ephyrae gelatinous tissues. Jellyfish have a strong top-down control and influence on plankton communities (West et al., 2009). In addition, as a group of gelatinous plankton, they are considered part of marine ecosystems' microbial – crustacean – fish – carnivorous macrozooplankton pathway (Richardson et al., 2009; Epstein et al., 2016; Boero et al., 2019). Jellyfish feed on fish eggs and larvae as well as on crustaceans. Therefore, they may transfer contaminants from other zooplankton prey to predators. Jellyfish can accumulate several kinds of contaminants in their gelatinous tissues, such as heavy metals (i.e., cadmium, chromium, copper;

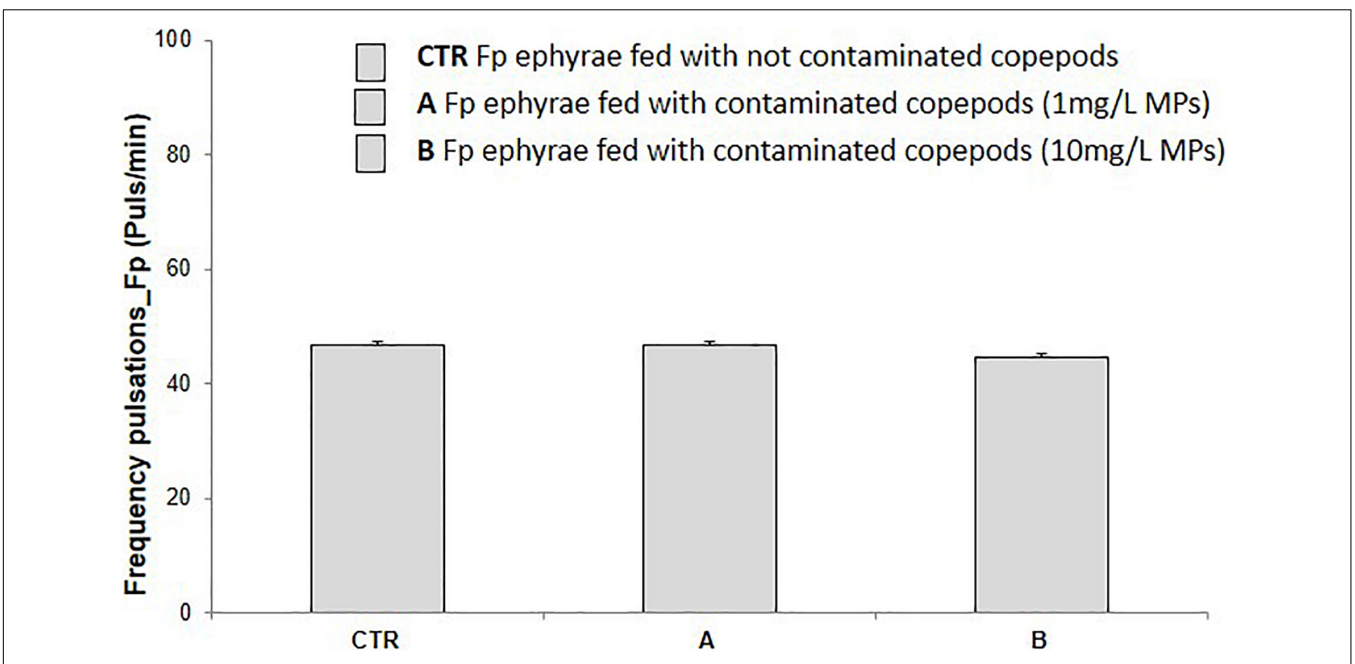

FIGURE 2 | Pulsation frequency (pulse/min) recorded for *Aurelia* sp. ephyrae after feeding uncontaminated *T. fulvus* nauplii (CTR) and nauplii contaminated with 1 mg/L (A) and 10 mg/L (B) of polyethylene MPs. (M ± SE, $n = 3$). No significant difference was observed between treatments and controls in any of the tests (A,B).

Richardson et al., 2009; Templeman and Kingsford, 2010, 2015; Klein et al., 2015), polycyclic aromatic hydrocarbons (PAHs; Almeda et al., 2013), and MPs (Macali et al., 2018; Costa et al., 2020). However, transfer of these contaminants to their natural predators has been suggested only for some contaminants. In this regard, Caurant et al. (1999) linked jellyfish cadmium accumulation and transfer to leatherback turtles, while Almeda et al. (2013) proposed that crude oil PAHs accumulated in jellyfish may be potentially transferred up the food web, thus affecting apex predators. Since in our study MP transfer occurred in a simplified two-level trophic chain, formed by copepods and jellyfish, further investigations are needed to verify any subsequent bottom-up implications for higher trophic levels, considering that jellyfish may play a role as a carrier of plastics along the food web. Although MP transfer occurred from zooplankton to jellyfish larvae (as reported in "Predators and Prey"), it did not affect ephyrae immobility and behavior. A longer feeding exposure timeframe (>24 h) may be required in order to observe any ecotoxicological effect from MP trophic transfer from prey to predator. It has been reported that direct MP exposure in jellyfish ephyrae temporarily affects both their survival and behavior due to mechanical disturbance caused by MPs triggering a loss in radial symmetry (Costa et al., 2020). Conversely, no measurable response could be detected after indirect MP exposure through ingestion of MP-contaminated copepods. The short feeding time (24 h) used in this study might have not been enough to determine any measurable effects in jellyfish ephyrae. However, studies on freshwater food web (crustaceans and zebrafish) have demonstrated that even indirect MP transfer through chronic exposure (up to 2 weeks) poses no serious harm along the trophic chain, since no signs of stress or disease were observed (Batel et al., 2016).

Jellyfish are involved in MP transfer through zooplankton; however, MP transfer toward high trophic levels still needs further investigations. Kakani et al. (2017) and Brandon et al. (2020) demonstrated that filter feeders and gelatinous zooplankton – namely, larvaceans and salps – can ingest large MP amounts from near surface water and move MPs to the deep sea, by sinking fecal pellet aggregates. Therefore, they are biological carriers for MP transport in the marine ecosystem through the water column, from sea surface to the seabed. Likewise, gelatinous zooplankton such as jellyfish may contribute to MP transfer from zooplankton to pelagic top predators. This study for the first time reports an indirect MP trophic transfer in a simplified food chain formed by copepod nauplii and jellyfish ephyrae. While no ecotoxicological responses were observed in jellyfish fed with MP-contaminated preys, this study shows that MPs are indirectly ingested in predators: further studies are needed to detect any potential MP transfer effects on the marine food web.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

EC and CG conceived the study and wrote the manuscript. EC and SL collected the jellyfish. VP, EC, and CG performed the experiments and analyzed the results. VP, SL, MF, and FG edited and reviewed the text. All authors approved the final version of the manuscript.

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Solving a Sticky Situation: Microplastic Analysis of Lipid-Rich Tissue

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Given current concerns regarding the extent of microplastic contamination in the environment, routine monitoring for microplastics in biological tissues is becoming increasingly common place. However, complex sample matrices, such as lipid-rich tissues, require multiple pre-treatment steps which may lead to increased sample processing time and costs, and a reduction in microplastic recovery rates thereby hindering monitoring efforts. Lipid-rich (fat) tissues often pose difficulties for traditional potassium hydroxide (KOH) digestion methods due to saponification. This reaction produces a suspension of glycerol and fatty acids (soaps), which may entrap microplastics inhibiting their recovery and clog filters thus reducing the efficiency of the filtration or inhibiting it altogether. In this study, the incorporation of 100% ethanol (EtOH) to existing KOH digestion methods was found to completely redissolve the viscous saponified gel formed in these reactions, with a digestion efficiency greater than 97% for all treated lipid-rich tissue samples. Recovery of spiked polyethylene and polystyrene fragments, and rayon and polyester fibers, ranged from 93% to 100%. The addition of EtOH did not induce physical or chemical degradation on these polymers. The inclusion of an *ad hoc* decision-making tool within the digestion workflow reduced pre-processing time for samples and allowed for solid saponified samples to be completely redissolved. This validated workflow facilitates high through-put sampling of biota, by enabling lipid-rich tissues to be filtered with a high degree of efficiency thereby successfully separating microplastics from their gelatinous matrix.

Keywords: potassium hydroxide (KOH), ethanol (EtOH), fat deposit, saponification, gel, *Plectropomus leopardus*, *Lates calcarifer*, *Lutjanus argentimaculatus*

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INTRODUCTION

Once considered an emerging contaminant, microplastics (MPs; a heterogenous mixture of plastic particles <5 mm), have proliferated in the global marine environment (Akdogan and Guven, 2019). As such reliable and regular monitoring for marine MPs is now advocated by many national and international agencies (Lusher et al., 2017a; GESAMP, 2019) to better understand the extent, sources, fate and impacts of contamination throughout a diverse array of marine ecosystems. Typical matrices of interest for these monitoring programs include seawater, sediments and marine biota.

A variety of methods have been developed to separate and identify MPs present in these environmental matrices (Miller et al., 2017). In general, for marine biota, the most common

method to verify MP intake is to dissect the gastrointestinal tract (GIT) and chemically digest the tissue, leaving only indigestible remains, including putative MPs (Lusher et al., 2017b; Hermsen et al., 2018; GESAMP, 2019). Alkali tissue digestions using potassium hydroxide (KOH) are recommended as the ideal method to dissolve tissue and retain the integrity of ingested microplastics (GESAMP, 2019). Notably, KOH has minimal impact on most common polymer types under ambient to moderate temperatures (<40°C) for short periods (<2 weeks) (Dehaut et al., 2016; Karami et al., 2017), is inexpensive and is low risk to laboratory users. As such, KOH has been utilized in many MP studies for multiple marine organisms (Lusher et al., 2017b), but most commonly for fish. Further, many studies have sought to optimize KOH digestions to increase digestion efficiency whilst decreasing reaction times (e.g., Dehaut et al., 2016; Karami et al., 2017; Kühn et al., 2017). Digestion periods typically range from 24 hr (Dehaut et al., 2016) up to 2–3 weeks (Foekema et al., 2013; Munno et al., 2018), with tested reaction temperatures ranging from 18°C (Kühn et al., 2017) through to 60°C (Dehaut et al., 2016; Karami et al., 2017; Munno et al., 2018). If significant indigestible material remains following the KOH digestion step, the solution can be further clarified with a density separation (Coppock et al., 2017; Karami et al., 2017; Herrera et al., 2018). The solution is then passed through a suitable pore-sized filter to isolate the intact putative MPs for subsequent physical and chemical analysis (Hermsen et al., 2018; Lusher and Hernandez-Milian, 2018).

Marine biota with a high lipid (fat) content pose a challenge for separation of MPs from their tissues. Fatty tissues can be excised from the GIT prior to MP separation (e.g., Caron et al., 2018a), however, this lipid-rich tissue may itself be of interest for examination, such as liver tissue (e.g., Collard et al., 2017). Lipids in the presence of an aqueous alkali salt undergo a saponification reaction to form soap (Kent, 2003; Mabrouk, 2005). Sodium-based salts form a hard soap while potassium-based salts form soft (liquid suspension) soaps (Konkol and Rasmussen, 2015). In a typical 10% aqueous KOH digestion, saponification will occur if the lipid content is sufficiently high within the solution, even at room temperature. This has led to some authors suggesting that 10% aqueous KOH digestions should not be used on samples with high lipid content or with large fat deposits (Bessa et al., 2019).

This study optimized the alkali KOH digestion method to facilitate recovery of MPs from lipid-rich tissues prone to saponification. There are many variations of the KOH digestion method reported throughout the literature, with digestion time and temperatures being the primary parameters considered (Dehaut et al., 2016; Kühn et al., 2017). In this study, two digestion methods, one using room temperature (Foekema et al., 2013) and the other using 40°C heating (Karami et al., 2017), were optimized for lipid-rich fish tissue. Ethanol (EtOH) is a versatile polar solvent capable of dissolving polar (due to its hydroxyl group) and many non-polar (due to its ethyl group) substances, but has been shown to have minimal impact on MPs (Courtene-Jones et al., 2017; Herrera et al., 2018). Therefore, a simple treatment with EtOH post-KOH digestion was investigated as a means of dispersing and redissolving the resultant gelatinous soap suspension. Specifically, the objectives were to design a suitable workflow to expedite the recovery of MPs from lipid-rich saponified biota samples. The workflow was optimized using farmed fish due to their fatty composition and regulated homogenous diet, and then applied to wild caught fish with unknown lipid content and multifarious diet. Both wild and farmed fish are regularly consumed by humans globally and are of particular interest for studies evaluating MPs in human seafood.

MATERIALS AND METHODS

Materials

Potassium Hydroxide pellets (Thermo Fisher Scientific UNILAB CAS No. 1310-58-3) were dissolved with Milli-Q (Millipore) water to form a 10% *w/v* aqueous solution. Absolute ethanol AR grade (Thermo Fisher Scientific CAS No. 64-17-5) was used undiluted. Stainless steel (316 grade) filters (547 μm pore size - 55 mm Ø, Plain Weave; 263 μm pore size - 19 mm Ø, Plain Weave; and 26 μm pore size - 19 mm Ø; Twill Weave) were punched from woven mesh. Polypropylene filters (30 μm - 47 mm Ø Millipore) were sourced from Merck.

Irregular polyethylene (PE) particles were cut into nominal 1 mm lengths from a green laboratory rope using a scalpel. Irregular polystyrene (PS) particles (1–2 mm) were purchased from Sigma Aldrich. Polyester (PES) fibers (1–5 mm) were abraded from a fluorescent safety vest using commercial sandpaper. Commercial rayon thread (Gütermann CA 02776) was cut into nominal 1 mm lengths and individual fibers were then teased apart. All polymers had their chemical composition confirmed using Fourier Transform Infrared Spectroscopy (FTIR) with a PerkinElmer Spectrum 100 universal ATR (16 scans at 4 cm^{-1} resolution, wavenumber range = 4000–600 cm^{-1}). Spectra were searched against a commercial library (NICODOM) following Kroon et al. (2018).

Lipid-Rich Fish Tissue

Local fish shops provided adult farmed barramundi (*Lates calcarifer*) samples for analysis. These were either viscera removed from the fish frame (n = 3), or whole fish frames with viscera intact (n = 6). Frames and viscera were transported to the laboratory then frozen at −20°C for storage until processing. Samples were defrosted at ambient room temperature (RT; ∼22°C) and fish frames were dissected to extract the viscera. The swim bladder was removed from all samples. Viscera were then either analyzed whole (n = 6) (Experiment A and C) or dissected further to separate the stomach and intestine (n = 3) (Experiment B). Visual assessment of Experiment C samples revealed they contained extremely large solid lipid deposits when compared with samples prepared for Experiment A and B. Therefore, some deposits that were not well attached to the viscera were removed to ensure samples were comparable between the three experiments (see **Supplementary Figure S1**). All tissues were weighed individually and placed in glass vessels for digestion.

KOH Digestion of Lipid-Rich Fish Tissues

To examine the applicability of EtOH resuspension to both RT and elevated temperature (40°C) digestion methods, two

TABLE 1 | Methodological approach used to optimize potassium hydroxide (KOH) digestion of lipid-rich fish tissues and recovery of spiked microplastics.

Experiment	*Species*	*N*	*Digestion method*	*Period (days)*	*Temp.*	*Tissue*	*KOH (tissue: volume)*	*EtOH Conc. (EtOH: KOH)*	*Spiked MP*
A	Barramundi	3	I	14	RT	Viscera	1:4	1:1, 1:2, 1:4, 1:10	No
B	Barramundi	3	I	14	RT	Stomach	1:4	1:10	Yes
						Intestine	1:4	1:10	No
C	Barramundi	3	II	3	40°C	Viscera	1:10	1:4	Yes
D	Mangrove jack	1	II	3	40°C	Viscera	1:10	1:10	No
	Coral Trout	1	II	3	40°C	Viscera	1:10	None	No
	Barramundi	1	II	3	40°C	Viscera	1:10	1:4	No

RT = room temperature, N = sample size, Digestion Method I - Foekema et al. (2013) and II - Karami et al. (2017).

different aqueous KOH digestions were conducted (**Table 1**). For the RT method (Foekema et al., 2013), the whole viscera (Experiment A), or the individual stomach and intestine (Experiment B), were digested with 10% (*w*/*v*) KOH solution with a final concentration of 1:4 (*tissue weight*/KOH *volume*) (**Supplementary Table S1**) at ambient RT for 14 days. In Experiment B, the stomach and intestine from each sample were analyzed separately. Sample vessels (1 L glass Schott bottles or a 1 L beaker) were gently swirled by hand once a week throughout the digestion period.

For the elevated temperature method (Karami et al., 2017) (Experiment C), viscera were digested in 10% (*w*/*v*) KOH solution with a final concentration of 1:10 (*tissue weight*/KOH *volume*) and incubated at 40°C for 3 days in an Innova 40 benchtop incubator. Sample vessels (3 L glass volumetric flasks) were gently swirled by hand after 48 h.

Treatment of Saponified Solutions

Lipids in the presence of KOH undergo saponification to form a soft weak gel (Kent, 2003) that exhibits no flow when in the steady-state (Ferry, 1980). All samples from Experiment A, B and C were visually inspected at the beginning and midway through the digestion period for the formation of a viscous soft gel at the solution's surface, signifying saponification. Experiments A, B and C were performed sequentially, with the results and general observations influencing the design of the subsequent experiments. For each of the three experiments, the methods used to redissolve the gel were optimized (**Figure 1**), then incorporated into a generic workflow applicable to any lipid-rich tissue sample.

The objective of Experiment A was to establish a suitable method to redissolve saponified gel present in lipid-rich samples whilst still achieving high digestion efficiencies (> 95%), typical of 10% KOH digestions where saponification does not occur. For samples presenting with a viscous gel, two methods of gel resuspension were tested sequentially. First, samples were vigorously agitated by stirring with a glass rod in an effort to mechanically breakdown the gel. However, this process proved counterproductive to the desired result and was discontinued. EtOH treatments post-KOH digestion were then examined as a means of redissolving the resultant soap (**Table 1**). To determine the lowest possible concentration of EtOH needed to achieve complete resuspension of the viscous gel, 5 subsamples (approximately 5 ml) of gel were taken from each sample. Subsamples were diluted with either a 0 (control), 1:1, 1:2, 1:4, or 1:10 volume of EtOH (100% EtOH/gel subsample, *v*/*v*), respectively. After adding the EtOH, subsamples were stirred with a glass rod until dispersed or up to 1 min at RT, then visually assessed for the presence of a homogenous liquid, indicating resuspension. Subsamples were then vacuum filtered through a 26 μm stainless steel filter. The control subsamples were not treated with EtOH before filtration. The remainder of each of the three samples was then treated with the lowest EtOH concentration required to achieve redissolution, stirred briefly and filtered, sequentially through a number of pre-weighted stainless steel filters to determine digestion efficiency, as described below.

Experiment B was designed to assess whether a reduction in sample weight, by removal of non-target tissue and solid lipid deposits prior to digestion, prevented or reduced the likelihood of saponification. Therefore, all non-target tissue surrounding the GIT was excised and only the individual stomach and individual intestines were digested separately with KOH as described above. The EtOH concentration, determined from Experiment A, was added to each of the samples 24 h before termination of the reaction. For these samples EtOH was not stirred with a glass rod, but the vessel was swirled gently by hand for <1 min to evenly distribute the EtOH. After 24 h, the suspension was then filtered to determine digestion efficiency, as described below.

Experiment C was designed to examine whether EtOH treatment could successfully redissolve saponified samples generated during a 10% KOH digestion conducted at elevated temperatures. Viscera were digested at 40°C for 3 days. The EtOH concentration determined from Experiment A was added to the digestion solution 1 h before termination of the reaction. The vessels were gently swirled by hand for <1 min to circulate the EtOH, then left stationary for the remainder of digestion period. The resuspended solution was then filtered to determine digestion efficiency, as described below.

Digestion Efficiency

All samples treated with 100% EtOH were filtered sequentially onto 547 μm (55 mm Ø), 263 and 26 μm (19 mm Ø) pre-weighed stainless steel filters. Using a Teflon wash bottle, these were then flushed liberally and sequentially with Milli-Q water and 80% EtOH, to reduce the possibility of particulates adhering

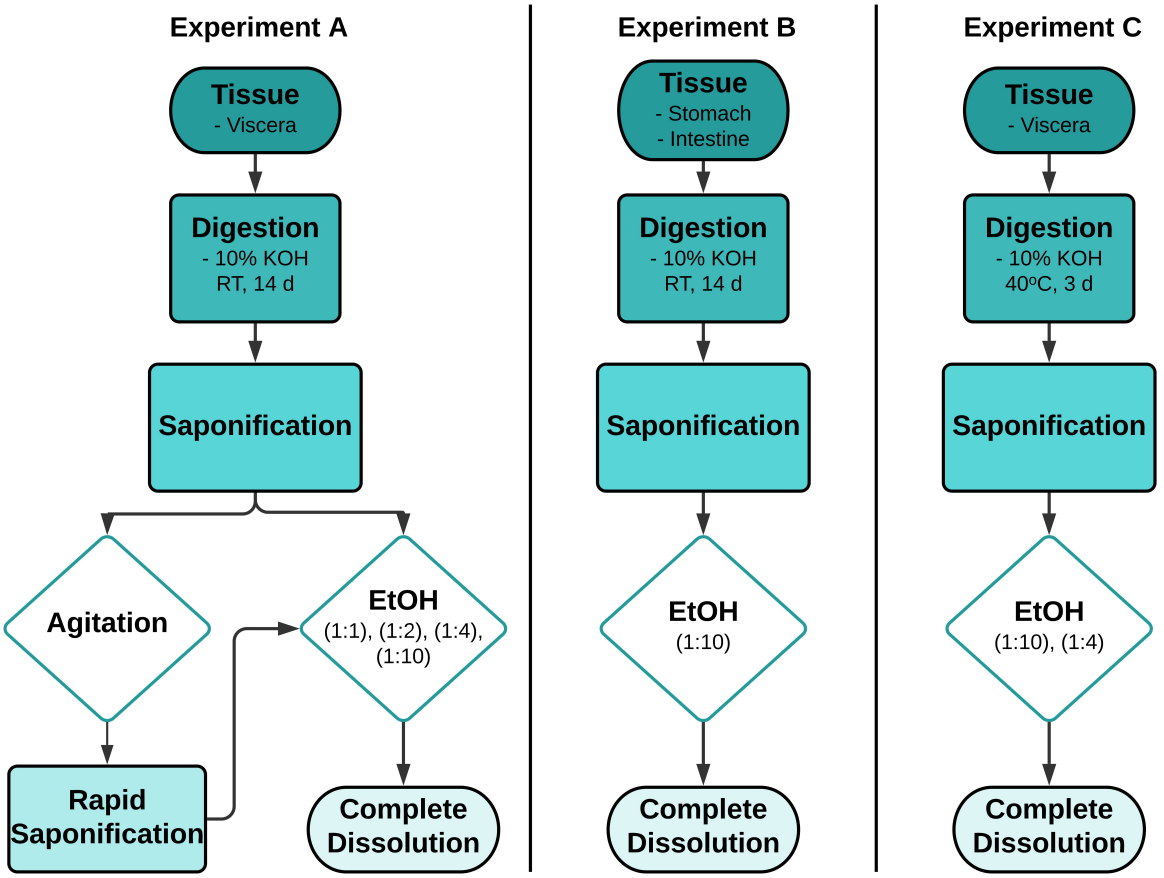

FIGURE 1 | Workflows outlining the process of optimizing the treatment of 10% potassium hydroxide (KOH) digested lipid-rich fish tissues. RT -room temperature, EtOH - ethanol, d- day, solvent ratios - 100% EtOH: Biota/KOH solution, *v/v*.

to the sides of the funnel and to remove any chemical residue. Filters and the filtration system were flushed at least 3x or until there were no particulates adhering to the system. Filters were dried overnight in an oven at 40°C then stored in a desiccator, thereafter weights were recorded to calculate the digestion efficiency (Equation 1).

$$\textit{Digestion efficiency}\ \% = \frac{W_i - (W_a - W_b)}{W_i} \times 100$$

where W_i = Initial weight of lipid-rich materials (w.w), W_a = Weight of dry filter membrane after filtration (d.w), and W_b = Weight of dry filter membrane before filtration (d.w).

The initial sample weight was measured with a Sartorius TE3102S balance (2 d.p). Filter weights before and after filtration were measured with a Sartorius CP224S balance (4 d.p). The samples were allowed to stabilize on the balance before recording the final weight.

Effect of KOH and EtOH on Microplastics

Model MPs were exposed to KOH alone or combined KOH and EtOH to examine whether one or both reagents would significantly degrade polymers, potentially inhibiting recovery and identification. To account for any KOH-induced degradation of the spiked polymers, MPs were exposed to 10% KOH for 14 days at RT or 10% KOH for 3 days at 40°C, in the absence of biological tissue and EtOH. Model MPs comprised of 10 black rayon fibers, 10 green PE fragments, 10 transparent PS fragments and 10 yellow PES fibers.

Lipid-rich tissues from Experiment B and C were spiked with model MPs (**Table 1**) to investigate whether the additional EtOH treatments would result in MP degradation when combined with KOH. Individual barramundi stomachs (Experiment B) were spiked with 5 black rayon fibers, 5 transparent PS irregular fragments and 5 green PE irregular fragments. Additionally, Experiment C viscera were also spiked with 5 yellow PES fibers per sample as PES fibers are known to degrade following KOH digestion at elevated temperatures, particularly above 60°C (Karami et al., 2017).

Following exposure, all samples were vacuum filtered onto stainless steel filters and retained MPs were assessed for physical and chemical changes by comparing color, shape and infrared spectral profiles with non-treated virgin MPs. MPs were visually identified using a Leica Stereo microscope MZ16A. Polymer spectra of virgin ($n = 3$) and exposed particles were acquired using ATR-FTIR. Spectral comparisons were conducted using the COMPARE analysis in the PerkinElmer Spectrum IR

software (Kroon et al., 2018). Further, carbonyl indices for both non-treated virgin MPs and MPs exposed to KOH and EtOH (Experiment B and C) were compared (A detailed description of the spectral analysis is provided in **Supplementary Material**).

Establishing a Treatment Workflow

Results obtained throughout Experiments A, B and C were used to develop a tailored workflow to ameliorate the digestion of lipid-rich tissues. The workflow utilized the following three criteria to establish the complete dissolution of the gel post EtOH treatment: (i) solution appeared homogenous (see **Table 2** for guidance in visual assessment), (ii) ability of the treated sample to pass through a 26 μm pore size stainless steel filter in <5 min, and (iii) a digestion efficiency of equal efficacy to KOH digestion without saponification (i.e., >95%). The criteria used to verify the workflow were: (iv) recovery rates of spiked MPs above 90%, and (v) no EtOH-induced changes in physical or chemical characteristics in recovered MPs.

Application of the Workflow: Case Study Using Wild Caught Fish (Experiment D)

The tailored workflow, based on the results of Experiment A, B, and C, was validated using wild fish (**Figure 2**). Wild fish were hypothesized to be less fatty than farmed fish and were used to determine if the workflow was suitable for analyzing fish with variable and unknown lipid content. The viscera of coral trout (*Plectropomus leopardus*), barramundi (*Lates calcarifer*), and mangrove jack (*Lutjanus argentimaculatus*), were obtained from fish shops or provided by local fishers from Townsville, Australia.

Viscera was digested in 10% KOH as per Experiment C (3 days at 40°C) in glass Schott bottles. One hour before termination of the digestion, the reaction solution was visually assessed and if a solid gel had developed (**Table 2**), was processed using the tailored workflow. Using this workflow, EtOH was added to samples with a saponified gel in 1:10 EtOH:KOH ratio. After gently swirling by hand, the samples were returned to the incubator for 1 h. If the gel was visually assessed as having completely dissolved, it was filtered as described above, if not, then additional EtOH was added to achieve a concentration of 1:4 EtOH:KOH. The samples were gently swirled, then visually assessed again. If necessary, the process was repeated using a 1:2 or a 1:1 concentration of EtOH, until the solution presented as a homogenous liquid. Only after complete resuspension was achieved, based on visual assessment, was the sample filtered to assess digestion efficiency.

Copious amounts of prey items and sediment were present in the wild fish GIT, which was visible as a thick layer of sediment in the bottom of the Schott bottle. Thus, these were filtered onto larger 47 mm Ø filters (30 μm pore size). The increased surface area accommodated a larger retentate load before clogging. Digestion efficiency was a calculated for these samples as outlined above.

QA/QC

The objective of this study was to assess recovery of spiked polymers, and not to quantify environmental MP burdens. Therefore, although dissection and analyses followed recommended clean laboratory procedures to prevent laboratory contamination, procedural blanks were not collected. These procedures included wearing a 100% cotton lab coat, cleaning glassware and utensils with detergent (if needed) and Milli-Q water before use and between samples, the use of plastic utensils

TABLE 2 | Saponification visual assessment criteria.

Physical appearance of sample (Viscosity)		Description of sample phase	EtOH treatment recommended
Petroleum jelly	INCREASING VISCOSITY ⇒	Semi-solid; when inverted there is no flow or falls in a solid single movement; very difficult to stir, may break into smaller chunks but doesn't return to liquid phase (e.g., **Figure 3B**)	Yes
Honey to Molasses		Extremely viscous but soft; when inverted pours slowly; significant resistance when stirred (e.g., **Supplementary Video**)	Yes
Floating solid (transparent or opaque) resembling petroleum jelly		Floating mass is semi-solid; when inverted underlying liquid may be trapped and unable to be poured (either briefly or permanently); easily stirred but mass does not redissolve, may break into smaller chunks but doesn't return to liquid phase; chunks will reform into solid layer if left untreated (e.g., **Figure 4A**)	Yes
Transparent skin adhering to the liquid surface		Thin solid skin on the surface of a mostly homogenous liquid; when inverted sample is temporarily restricted from flowing, skin bulges and easily breaks from the weight of the liquid; easily stirred, skin may redissolve	EtOH treatment may not be necessary, but is still recommended to prevent filter clogging and release microplastics potentially trapped in the skin
Sticky residue adhering to the liquid/glass meniscus		Small solid deposits on glass along the meniscus of a mostly homogeneous liquid; when inverted sample flows freely but sticky residue remains at the site of the liquid/glass meniscus; easily stirred, residue may redissolve	EtOH treatment may not be necessary, copious rinsing of the vessel and filter with Milli-q and EtOH may remove the sticky residue
Liquid with or without small particulates settled on the bottom		Homogeneous liquid; when inverted sample flows freely; easily stirred, sample may foam slightly (e.g., **Figure 4B**)	No EtOH treatment necessary Or EtOH treatment has been successful

The physical appearance of sample is inspected, paying particular attention to the top third of the solution, especially along the meniscus. The results of the assessment will determine if ethanol treatment of the sample is necessary.

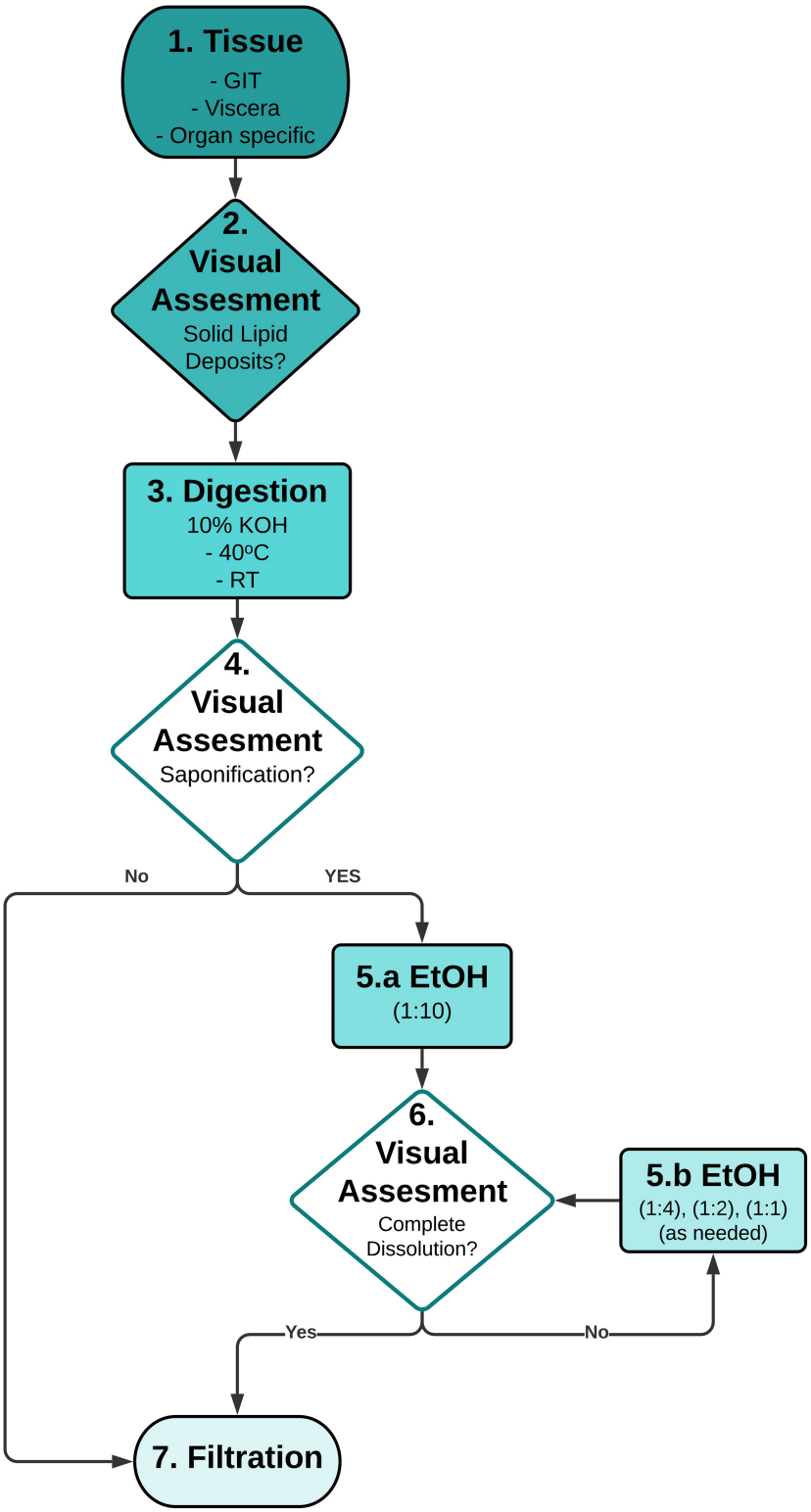

FIGURE 2 | Recommended workflow for dissolution of the gel formed in saponified potassium hydroxide (KOH) digestions. 1. Method is applicable to any desired fish tissue; 2. Optional: Removal of any excess solid lipid (fat) deposits attached to the tissue is recommended to reduce KOH/EtOH reaction volume; 3. Method is valid for both ambient and elevated temperature (≤40°C) KOH digestions; 4. Stage-gate using visual assessment (**Table 2**) to process sample via filtration, or if saponification has occurred to treat with EtOH; 5a. Lowest concentration of EtOH is added; 6. Stage-gate using visual assessment to process homogenous solution via filtration, or if gel is still evident treat with EtOH; 5b. Sequentially add excess EtOH to dissolve the gel. 7. Filtration of homogeneous solution over suitable pore size filters to retain microplastics. GIT – gastrointestinal tract, EtOH – ethanol, RT - room temperature, solvent ratios - 100% EtOH: Biota/KOH solution, *v/v*.

was avoided, laboratory benches were cleaned with 70% EtOH and samples were kept covered with aluminum foil (Lusher and Hernandez-Milian, 2018). Samples were not processed inside a clean air device (Wesch et al., 2017). To apply this workflow to environmental samples, it is recommended to follow established QA/QC method, such as those described in Karami et al. (2017), Bråte et al. (2018), or Hermsen et al. (2018).

RESULTS

Ethanol Workflow for Resuspension of Saponified Fish Fat

All farmed barramundi tissue subjected to 10% KOH digestion resulted in the formation of a viscous gel, which visually resembled gelatin (jelly) (**Table 2** and **Figure 3**). These saponified samples (Experiment A: viscera; Experiment B: stomach/intestines; Experiment C: viscera) could not be easily poured from the glass vessels in this state.

In Experiment A, agitation in the form of shaking and inverting, resulted in the gel breaking into smaller clumps, however, it could not be redissolved. Further, the gel layer reformed shortly after shaking ceased. Continued vigorous stirring with a clean glass rod induced an extremely rapid saponification reaction, with the physical properties of the entire sample changing from a biphasic mixture (lower liquid layer, and upper floating gel), to a homogenous thick gel (**Figure 3** and **Supplementary Figure S2**), which could not be poured, nor broken apart with vigorous shaking. Attempted vacuum filtration of the control (untreated) subsample through a 26 μm filter was unsuccessful, thus no further analysis of untreated saponified samples was carried out. Treatment of the gel subsamples with EtOH (1:1, 1:2, 1:4, and 1:10) resulted in complete dissolution, with all resulting solutions able to pass through the 26 μm filter, with only minimal gelatinous residue being retained. Thus, the lowest dilution, 1:10, was selected to redissolve the remainder of the samples from Experiment A, and for Experiments B and C.

In Experiment B, the complete removal of the stomach and intestines from lipid-rich organs (e.g., liver) and solid lipid deposits did not prevent saponification. The individual stomachs and intestines all presented with floating soft weak gels when digested with KOH. The samples were treated with 1:10 EtOH

FIGURE 3 | Saponified viscera samples of barramundi (*Lates calcarifer*) (Experiment A) after treatment with 10% potassium hydroxide (KOH) at ambient room temperature (~22°C) for 14 days, **(A)** sample with a single layer of gel floating atop a liquid layer, gel is approximately 0.5 cm thick, and **(B)** entire sample (~ 500 mL) has saponified due to agitation and formed a solid soft gel.

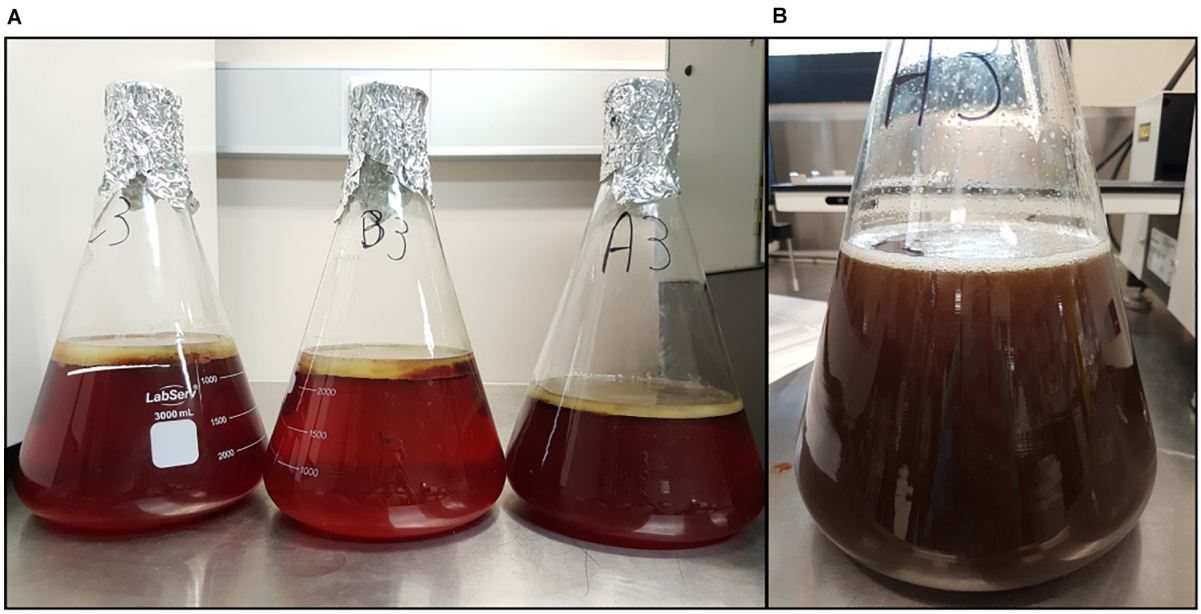

FIGURE 4 | Saponified and resuspended samples of barramundi (*Lates calcarifer*) viscera (Experiment C) after digestion with 10% potassium hydroxide (KOH) at 40°C for 3 days, **(A)** samples showing the formation of a white gel floating on top of the KOH digested tissue (brown underlaying liquid), gel is approximately 2 cm thick, and **(B)** the same sample after the addition of ethanol (1:4), the floating gel is completely resuspended.

and visual assessment determined that gel was redissolved. As dissection was not an effective method of preventing or reducing the level of saponification, complete viscera were used in all following tests to reduce sample preparation time.

The formation of a solid gel following KOH digestion was also observed in Experiment C (viscera 1:10 tissue:KOH (*w/v*) 40°C) (**Figure 4A**). Visual assessment revealed this solid gel did not completely dissolve after initially adding EtOH (1:10). Increasing the volume of EtOH (1:4) was successful in redissolving the remaining gel and allowed for filtration without any gel residue (**Figure 4B**).

The results of Experiment A, B and C demonstrated that an *ad hoc* visual assessment of the physical appearance of the sample (**Table 2**) was a simple and effective method to establish the presence of saponified gels. Visual inspection was also effective at verifying dissolution, with all samples that were judged redissolved able to pass through a 26 μm filter after EtOH treatment. Hence, visual assessment combined with EtOH treatment was applied in the wild fish case study (Experiment D).

Inspection of wild-caught coral trout viscera found it to be relatively free of fat deposits. Digestion of this tissue under Experiment D conditions yielded a clear solution with no obvious saponified gel observed. As a result, addition of EtOH was deemed unnecessary and the sample was filtered as is. Wild-caught mangrove jack and barramundi viscera were observed to have solid fat deposits (although notably less than farmed barramundi, **Supplementary Figure S3**). Digestion with 10% KOH formed a viscous gel, therefore both samples were further treated with EtOH (1:10, as per the tailored workflow). Gel-like solids in the mangrove jack sample dissolved completely resulting in a clarified solution that was easily filtered. Treatment of the wild-caught barramundi sample with EtOH (1:10) resulted in only partial dissolution of the gel, as was also noted in Experiment C. Increasing the volume of EtOH to 1:4 redissolved the remaining gel, and the solution was filtered.

Digestion Efficiency

The digestion efficiency, notwithstanding the limited sample replication, for all farmed barramundi (Experiment A, B, and C) was greater than 99% (**Table 3**). Very little prey and sediment residue was present on the filters for these samples (**Supplementary Figure S4**). Conversely, the wild-caught fish (Experiment D) contained substantial prey items in their GIT, including whole animals as well as bones, crustacean exoskeletons, shells and algae, all of which were retained on the filter. Sediments were also observed in the digested viscera samples obtained from mangrove jack and barramundi. Thus, viscera samples from wild fish required multiple large (47 mm Ø; 30 μm pore size) filters to prevent clogging (**Supplementary Figure S5**). Although the digestion efficiency was still considered acceptable for fish tissues (>97%), and the gel was able to be resuspended by the EtOH, the abundance of material remaining on the filter would impede visual examination of the digestate for MPs.

Effect of KOH and EtOH on Microplastics

Examination of the MPs exposed to 10% KOH for 14 days at RT found there was no change in color or shape of PS or PE. However, black rayon fibers were bleached and changed from opaque to transparent. Chemical analysis using FTIR found no substantial changes in the spectral profiles compared to virgin plastics (PE – 99.0%, PS- 98.7%, Rayon – 94.9.0% match). Despite

TABLE 3 | The digestion efficiency and recovery of spiked MPs (polyethylene PE, polystyrene PS, polyester PES and rayon) in the four Experiments (A–D).

Experiment		Sample weight (g)	Digestion efficiency			Substantial residue on filter?*	Spiked MP recovery (%)	Spiked MP changes
			Range (%)	Mean (%)	Std. Dev.			
A	- Viscera	116.04	99.20–100.00	99.73	0.37	Negligible	na	na
B	- Stomach	23.45	99.98–100.00	99.99	0.01	Negligible	PS - 100%	PS -No
							PE -93.3%	PE- No
							Rayon – 93.3%	Rayon- Yes∧
	- Intestine	16.38	99.99–100.00	100.00	0.01	Negligible	na	na
C	- Viscera	212.62	99.98–100.00	99.98	0.01	Moderate	PES - 100%	PES - No
D	- Viscera	78.18	97.94–99.72	98.97	0.75	High	na	na

*Residue on filters was typically inorganic (e.g., sediment) or resistant to potassium hydroxide (KOH) (e.g., chitin). Spiked microplastics were assessed for changes in shape, color and chemical spectra after exposure to 10% KOH and EtOH.*See **Supplementary Figures S4**, **S5** for residue. ∧color change.*

a slightly lower % match for rayon, it was still above the accepted similarity threshold of > 70% (Lusher et al., 2013; Kroon et al., 2018). Exposure to 10% KOH at 40°C for 3 days also did not result in significant changes in the chemical spectra for the four polymers (PE – 98.5%, PS- 99.6%, Rayon – 97.9%, PES – 97.6% match). There were no changes in color and shape, apart from rayon, which underwent similar color changes as per the RT KOH exposure.

Recovery of spiked MPs from KOH digested and EtOH treated barramundi tissue was 100% for PS particles, 93.3% for PE particles, 93.3% for rayon fibers, and 100% for PES fibers (**Table 3**). All spiked samples that produced saponified gels were treated with EtOH. Thus, there were no attempts to recover MPs directly from the gel itself. For all MPs, shape was unchanged after the combined KOH and EtOH exposure. Color was also unaffected by the combined exposure for PS, PE and PES. However, rayon fibers became transparent, likely due to KOH exposure prior to the addition of EtOH (**Table 3**). Spectral analysis revealed no major change in profile compared to MPs exposed to KOH only. All polymer spectra returned correlations > 90% with the virgin MP after the combined KOH and EtOH exposure (PE – 98.6%, PS- 99.3%, Rayon – 97.1%, PES - 98.0% (**Supplementary Table S2**). Although not all recovered MPs were able to be FTIR analyzed. Carbonyl indices for all polymers treated with KOH and EtOH were not significantly different to untreated virgin MPs (PE: Mann–Whitney $U = 15$, $n_1 = 14$, $n_2 = 3$, $p > 0.05$, two tailed) (PS: Mann–Whitney $U = 16$, $n_1 = 15$, $n_2 = 3$, $p > 0.05$, two tailed) (PES: Mann–Whitney $U = 10$, $n_1 = 8$, $n_2 = 3$, $p > 0.05$, two tailed) (Rayon: Mann–Whitney $U = 11$, $n_1 = 8$, $n_2 = 3$, $p > 0.05$, two tailed) (**Supplementary Table S3**).

DISCUSSION

Saponification (or alkaline hydrolysis) of triglycerides (e.g., lipids/animal fat) in the presence of a strong base (e.g., KOH) is a reaction where both reactants are consumed to form the salt of a carboxylic acid (otherwise known as soap) and an alcohol (Mercantili et al., 2014). This process has not been widely addressed in the literature concerning tissue digestion in terms of impacting MP extraction. It is possible that this is because few studies have attempted to extract MPs from large lipid-rich biota, where saponification is more likely to occur. Studies that have investigated larger biota (e.g., tuna, turtle, dolphin, whale) have either focused on stomach contents and not the stomach tissue (Besseling et al., 2015; Lusher et al., 2015; Chagnon et al., 2018; Hernandez-Gonzalez et al., 2018) or strategically avoided digestion with KOH (Caron et al., 2018b). One of the few studies to note the occurrence of saponification reported that KOH digestion of cod stomachs produced a "Layer of black/brown slime afloat" and recommended using a combined KOH: NaClO digestion (Enders et al., 2016). However, implementation of this method relies on prior knowledge that saponification will occur in the samples. One advantage of the workflow presented here is that it uses an existing method for tissue digestion (10% KOH) (Lusher and Hernandez-Milian, 2018; GESAMP, 2019), does not rely on prior knowledge of sample lipid content or the propensity for saponification, and can be applied to samples with the option of modification only if saponification occurs.

Larger biota such as barramundi and mangrove jack, with a high lipid content complicate the application of recommended KOH digestion methods (Enders et al., 2016; Karami et al., 2017; Bessa et al., 2019; GESAMP, 2019). Agitation and stirring the saponified fish sample did not clarify the solution, rather it accelerated saponification producing a homogenous soft gel (**Supplementary Video**). This can be explained in terms of reaction kinetics, where stirring and agitation have a positive effect on the conversion and rate constant (Mercantili et al., 2014; Danish et al., 2015). The addition of EtOH was effective at resuspending the gel within all samples that were initially deemed intractable, including those in which the entire volume (∼500 mL) had completely saponified due to agitation. Thus, EtOH treatment was found to be a suitable process to redissolve saponified samples and as such was included in the tailored workflow for treating gels formed in KOH digestions. A workflow based on visual assessments allowed for the flexibility required to process variable samples. Simple visual assessment was demonstrated to be a satisfactory decision tool for determining whether a sample, either treated or untreated, would pass through a fine pore filter with no

gel residue. Thus, reducing the need for complex analytical testing of precious samples or troubleshooting after filtration has commenced. Further modification of the workflow, from a single predefined EtOH concentration (1:10), to a decision node triggered by visual assessment with clearly defined criteria, allowed for greater flexibility and the ability to handle samples of unknown complexity within a structured process. Each species of the wild fish exhibited varying levels of saponification (coral trout < mangrove jack < barramundi), and the workflow facilitated a tailored process for each species. Further, digestion efficiencies for all saponified, lipid-rich tissue samples were comparable to published values of biological materials using 10% KOH at 40°C without saponification. For example, reported digestion efficiencies include 96.8% for fish muscle and skin (Karami et al., 2017), 91.2% and 98.0% for bivalve tissue (Thiele et al., 2019). The addition of EtOH to saponified samples did not assist digestion or increase the efficiency, rather it decreased the sample viscosity, dissolving the solid gels and facilitated the final processing steps of filtration, which was not possible without treatment. Thereafter, putative MPs present on the filters can be further identified and characterized using established procedures [e.g., Kroon et al. (2018)].

The addition of EtOH in this study had no discernible effects on the spiked polymers. Exposure of some polymers to alcohols, including EtOH, may cause stress cracking or stiffening due to the dissolution of plasticizers, but do not impact on the polymer itself (Harvey, 2012). The recovered PS, PES and PE particles were visually unchanged, and comparison of their infrared spectral profiles with that of non-treated polymers revealed a >97% match. Bleached rayon fibers were present in all treated samples including those exposed to only KOH; confirming that EtOH did not contribute to the bleaching process under the conditions used. Although the infrared spectral profile of the treated rayon was changed, likely due to the leaching of the dye, an acceptable spectral match of 86–99% to the untreated rayon was still achieved. A previous study also found no visual effect of 70% EtOH on rayon when used to preserve spiked wild mussel samples for 7 days (Courtene-Jones et al., 2017). They also reported no effect on the abundance or diversity of polymers able to be recovered. Similarly, Herrera et al. (2018) utilized EtOH for the separation of MPs from vegetal material with no effect on the recovered polymers. In the current study, recovery of rayon and PE from spiked farmed barramundi tissue was 93.3%. This slight reduction in recovery rate from the expected 100% is unlikely a result of either KOH or EtOH exposure, as established above, but more likely a result of mishandling the samples or insufficient rinsing of the filtration system with Milli-Q to ensure particles were not adhered to the walls. As the aim of this study was to establish a workflow to clarify KOH digested samples, there was no attempt to quantify the recovery of MPs from untreated saponified tissue samples. Low density MPs are likely to float in 10% KOH (specific gravity 1.09 g mL^{-1}) and thus be incorporated into the gel. Given most saponified samples were too viscous to pour and the gel would not pass through a fine pore filter, MPs would need to be hand-picked, making this process impractical. Importantly, in this study KOH tissue digestions were limited to 40°C, as significant damage to some polymers has been observed at higher temperatures (Karami et al., 2017; Munno et al., 2018). As such, the effects of exposing polymers to heated EtOH (>40°C) were not investigated. Although it is unlikely that EtOH would exacerbate the effects already induced by heating KOH (Van Krevelen and Te Nijenhuis, 2009; Harvey, 2012), it would be prudent to conduct further validation before applying this workflow to KOH digestions > 40°C. Moreover saponification reactions are endothermic thus elevated temperatures increase the saponification reaction (Danish et al., 2015; Eze et al., 2015). Therefore, KOH digestions above 40°C should be avoided for lipid-rich tissue.

Following digestion of wild fish viscera, a surprising amount of biological material remained on the filters, reflecting their multifarious diet. This included crustacean exoskeletons that had remained almost completely intact, but their soft tissue was completely digested. In contrast, retentate from farmed barramundi were much cleaner suggesting their GITs contained little ingested material. Although not an objective of this study, the results also demonstrate that chitinous material is particularly resistant to 10% KOH, at both RT and 40°C. Although ingested MPs were not quantified in the wild fish, based on the abundance of KOH resistant material (e.g., chitin, bone) remaining post digestion, it is likely that visual separation of putative MPs from the GIT content would be difficult to achieve. To facilitate this, further treatment with a hypersaline solution (e.g., Coppock et al., 2017) to separate MPs from the undigested residue is recommended for future work with similarly complex samples.

Finally, digestion efficiency is calculated as a function of sample weight pre- and post-treatment and reported as a percentage. However, achieving a high efficiency can be an artifact of sample size. With large samples it becomes possible to achieve a high digestion efficiency and still have significant undigested materials retained on the filter. For example, a single prawn contains a significant amount of chitin, which is highly resistant to KOH digestion. A single prawn digested in KOH will have a lower digestion efficiency than a fish stomach containing the same single prawn, even though the amount of undigested residue retained on the filter would contain the same amount of chitin. This was observed in the wild barramundi and farmed barramundi samples, where there was <0.3% difference in the digestion efficiencies but marked visual differences in the retentate burden. For future studies, visual inspection of retentate coupled with calculated digestion efficiency may be an appropriate method to determine if further sample clarification is needed to facilitate MP extraction from digestate.

CONCLUSION

Following established workflows for estimating microplastic contamination in environmental samples is key to ensuring global consistency. Overall, potassium hydroxide is one of the most recommended, and utilized, digestion techniques for isolating microplastics from biological tissue, being highly effective on soft tissue. The workflow developed in this

study proposes a variation of the traditional potassium hydroxide digestion, which is applied only when samples exhibit saponification. An *ad hoc* decision, based on visual assessment, to determine the extent of ethanol treatment was demonstrated to be a suitable workflow for resuspending saponified samples, with demonstrated applicability for a variety of experimental conditions. Using this method, all samples in this study achieved a digestion efficiency >97%. Treatment with ethanol was established to be an inexpensive, simple and relatively safe method to optimize existing potassium hydroxide digestion procedures, thus enabling filtration of lipid-rich saponified samples. This technique also allows for the whole viscera to be digested and filtered, negating the need to remove non-target tissue during the initial dissection, thus reducing sample preparation time and associated contamination risk. However, it is worth noting that for particularly fatty samples, such as large farmed fish, removal of some excess lipid deposits will decrease the working volume of both potassium hydroxide and ethanol and may decrease the potential for complete saponification. We further suggest that biota possessing substantial stomach content resistant to digestion be further refined using a density separation to allow for microplastic extraction and visual identification.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because deceased animal parts were commercially purchased or donated.

AUTHOR CONTRIBUTIONS

ALD conceptualized the study, carried out the laboratory analysis, analyzed the data, and wrote the original draft. CAM contributed to the methodology and reviewed and edited the manuscript. FJK acquired funding and contributed to reviewing and editing the manuscript. 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/fenvs.2020.563565/full#supplementary-material

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Predicting the Dispersal and Accumulation of Microplastic Pellets Within the Estuarine and Coastal Waters of South-Eastern Brazil Using Integrated Rainfall Data and Lagrangian Particle Tracking Models

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Understanding how microplastic particles move and accumulate within estuarine and coastal waters requires consideration of primary inputs (e.g., raw materials from industrial zones) as well as secondary inputs resulting from fluvial processes (i.e., materials carried into coastal waters by rivers and streams). This study presents a novel approach to achieve this aim, by comparing the individual and combined ability of Particle Tracking Models (PTMs) and seasonal rainfall data, to explain observed inputs of microplastic pellets to the ocean beaches of Santos City (south-eastern Brazil). A Lagrangian PTM based on high-resolution hydrodynamic models was used to simulate seasonal patterns of pellet dispersal from five release points within the Santos Estuarine System (SES) and nearshore waters which are known contributors to the regions microplastic debris problem. Model outputs suggested that the debris field is likely to be small within the estuary (ranging from 3.6 to 8.1 km^2), intermediate at the river mouth (mean 34 km^2) and greatest for near- and offshore sites (ranging from 34 to 40 km^2). The spatial footprints were strongly modulated by season (and rainfall), with simulations alone unable to reconcile daily inputs of pellets observed on the beaches of Santos Bay (ranging from 2 to 51 particles $m^2 \cdot d^{-1}$). Given this discrepancy, a Generalized Additive Modeling approach was employed to integrate the PTM outputs with rainfall data to improve predictions of beached particles. Results confirmed that considering fluvial processes, could significantly improve the ability to predict rates of pellet accumulation (raising the explained deviance in observed inputs from 41 to 93%). Thus, the study highlights the potential to couple widely used dispersion models with metrics that describe fluvial forcing (rainfall and estuarine flushing) in order to better understand the spatio-temporal dynamics of microplastic debris transport and accumulation within dynamic coastal environments.

Keywords: microplastic, pellet, hydrodynamic dispersion model, fluvial processes, marine debris

INTRODUCTION

The accumulation of plastics in the biosphere is emerging as one of the most pervasive and intractable pollution problems facing the planet (Barnes et al., 2009). It has recently been estimated that 359 million tons of plastic are produced annually (PlasticsEurope, 2019) and a proportion of this waste ultimately finds its way into coastal and marine ecosystems (GESAMP, 2020). Microplastics, which are a subset of plastic particles that have an upper size limit of 5 mm (GESAMP, 2019) can include primary plastics (e.g., resin pellets for manufacturing, microbeads used in personal care products), as well as secondary materials that have broken down from larger items (Barnes et al., 2009). These particles, are now found in all of the world's oceans, beaches and deep sea environments (Lebreton et al., 2012; Van Sebille et al., 2015; Zhang C. N. et al., 2020; Zhang D. D. et al., 2020) and can affect the health and survival of biota through accidental ingestion as well as by transferring hazardous chemicals (GESAMP, 2020). Indeed, there is increasing evidence that microplastics are entering marine foodwebs through zooplankton communities (Frias et al., 2014), mussels (Browne et al., 2008), corals (Hall et al., 2015), pelagic and demersal fish (Lusher et al., 2013), seabirds (Thiel et al., 2018), whales (Fossi et al., 2012) and even human beings (with a recent report suggesting that the average person ingests the equivalent of a credit card worth of plastic per year; De Wit and Bigaud, 2019).

A major impediment to managing and mitigating the problem of microplastic pollution in the marine environment is the incomplete understanding of processes that govern dispersal and accumulation (GESAMP, 2015). Although microplastics enter coastal ecosystems from both land and sea as a result of accidental spills (e.g., at stages of production, transportation and processing; Ogata et al., 2009) and the release of residential and industrial wastes (EPA, 1990; Cabral, 2014; Do Sul et al., 2014), they can also be swept into rivers, estuaries and the sea by fluvial (river born) processes and stormwater runoff (GESAMP, 2015) that are strongly influenced by seasonal rainfall. Indeed, a recent study suggested that up to 80% of the plastics entering the oceans do so through river networks (Lebreton et al., 2017). Not only this, but because a considerable proportion of microplastics are buoyant (i.e., often showing exponential decreases with depth; Kooi et al., 2016), they disperse widely in the open ocean as a result of phenomena such as surface currents, stokes drift and mesoscale eddies (Iwasaki et al., 2017; Onink et al., 2019). Closer to shore, patterns of accumulation on beaches and other habitats can be modulated by a range of factors including; terrestrial runoff, release point, hydrodynamics (i.e., tides and currents), weather, coastal geomorphology and a subsequent suite of physical, chemical and biological processes (Frere et al., 2017). Additionally, they can become trapped in sediments, riparian vegetation (e.g., mangroves and tidal marshes; Viehman et al., 2011; Debrot et al., 2013) and even human infrastructure (e.g., coastal dwellings that are common in many poorer countries), only to be remobilized as a result of fluvial processes driven by rainfall. This may be more pronounced in tropical and subtropical regions that experience strongly seasonal rainfall (Krelling et al., 2017) that can release microplastic particles that have been trapped in often dense estuarine vegetation and deep sediments (which characterize these regions) during more stable and dry periods (Ockelford et al., 2020). The failure of dispersal models to include such processes, may present an impediment to gauging risks to the environment, animal and human health, and may be exacerbated in the future given an increase in global flood risk and increased likelihood of extreme weather events.

Although understanding the spatial and temporal dynamics of microplastic accumulation has proven notoriously difficult (Fisner et al., 2017), the increasing resolution and performance of hydrological models is helping to address such challenges and inform policy (Braunschweig et al., 2003; Mateus et al., 2012; Krelling et al., 2017). While early studies focused on identifying point sources (Martinez et al., 2009; Lebreton et al., 2012; Maximenko et al., 2012; Reisser et al., 2013), there has been a shift toward understanding the effect of diffuse entry points and the consequences for debris accumulation within estuarine, coastal and marine habitats (Hardesty et al., 2017). Nevertheless, relying on simulation models alone can lead to a decoupling with reality if predictions are not validated against field observations and/or do not take into account stochastic processes like fluvial forcing (e.g., stormwater and surface runoff) which govern the input and/or reintroduction of particles from terrestrial sources that contribute to patterns of accumulation. Matching high resolution hydrodynamic models, with particle tracking approaches and local catchment characteristics such as rainfall may hold the potential to better reflect the spatio-temporal complexity of microplastic debris within coastal ecosystems to better inform management and intervention strategies (Ballent et al., 2013).

The goal of this study was to use hydrodynamic models and a simple Particle Tracking Model (PTM) to simulate the dispersal of microplastic pellets through the estuarine and coastal waters of the Santos region (south-eastern Brazil). The site was selected due to its complex geomorphological characteristics, strongly seasonal climatic and oceanographic conditions, and because it is home to the largest port in South America (Lamparelli, 1998) which contains several important manufacturing zones that involve the production and/or use of microplastic pellets that are often accidentally released into the environment. The aims of the study were threefold: (1) to simulate the effect of season and different release points on the dispersal footprint (km^2) of pellets within the region, (2) to examine whether predicted dispersal area correlates with rainfall, and (3) to combine the outputs of the PTM with rainfall data and beach morphology (zonation) in order to improve predictions of daily rates of pellet accumulation (no. particles $m^2 \cdot d^{-1}$) on the beaches of Santos Bay.

MATERIALS AND METHODS

Study Area

The study involved the estuarine channels and ocean beaches of the Santos region, Brazil (**Figure 1**) that included five simulated release points in the upper and middle estuary (sites A and B), river mouth (site C), inshore and offshore zones (sites D and E). The Santos Estuarine System (SES) is formed by the confluence of the Bertioga Channel Estuary, Port of Santos

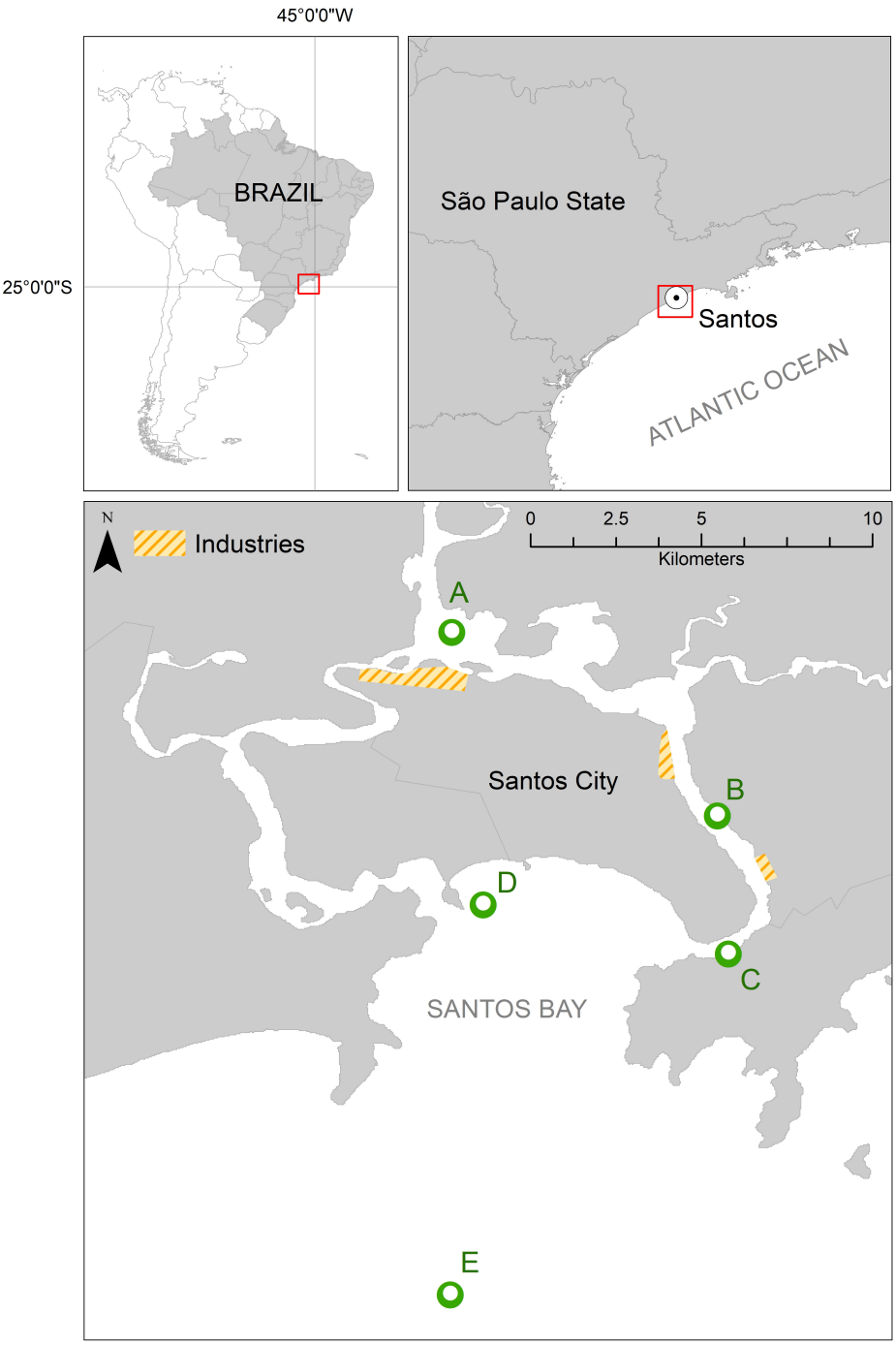

FIGURE 1 | Details of the Santos Estuarine System (SES), within the Central Region of São Paulo State, in south eastern Brazil. Letters give modeled release points: A (upper estuary), B (mid estuary), C (estuary mouth), D (inshore beach) and E (offshore) for microplastic pellets. Hashing indicates areas with a high concentration of industries that produce and/or consume microplastic pellets.

Channel Estuary and São Vicente Estuary and covers an area of approximately 44 km^2 (Moser et al., 2005). The dominant vegetation type is mangrove forest (CETEC, 2000), of which there has been substantial loss since the 1980s as a result of urban and industrial development (Gorman, 2018). The SES is home to the largest port in Latin America and is heavily impacted by industrial activity from the Cubatão industrial complex (refineries and petrochemicals) located less than 20 km inland, in addition to three main industrial areas within the SES itself.

Tide is the most important hydrological factor, with tidal ebb currents having a mean speed of ca. 1 $m{\cdot}s^{-1}$ (Moser et al., 2005), but may reach up to 1.5 $m{\cdot}s^{-1}$ as a result of strong winds from the NE and SW directions. Winds and resulting currents show marked seasonal difference with winds from the east, water moving to south during August to November, while winds predominate from the south, resulting in water moving to the west/coast during April to July. The ocean-shelf dynamics off the Santos region is influenced by coastal winds and tidal currents that are governed by coastline geomorphology and bathymetry (Harari et al., 2000, 2006; Harari and Gordon, 2001). The ocean beaches of Santos Bay have a mean tidal range of 1.5 m and a study by Harari et al. (2008) suggested that in absence of significant meteorological effects, tidal circulation is a good approximation of total circulation. The outflow of materials from the SES to Santos Bay is also influenced by the dynamics of the estuary mouth which is approximately 500 m wide and 12 m deep. The region experiences strong seasonal weather patterns, with a typically dry and stable period from August to November, a pronounced summer "wet season" from December to March (thunderstorms) and a winter period of strong frontal systems (sea storms) that peak in April to July. During the summer wet season, stormwater is discharged directly onto the beaches of Santos Bay through a series of six canals which has the effect of creating distinctly different beach morphological types (i.e., becoming wider with a more gradual slope toward the west) which has been shown to have a profound effect on the accumulation patterns along the beach (Balthazar-Silva, 2016).

Port and industrial activities are known sources of pellets to the SES and broader Santos region, with four of Brazil's largest plastics producing factories being located within the study area. The greatest concentration of industries that produce and/or use polyethylene, PVC and polypropylene pellets (estimated production of 350,000 tons $year^{-1}$) is centered in the industrial district within the inner SES called Alemoa (Manzano, 2009). In order to assess the influence of other potential release points, a preliminary survey was conducted [following the protocols of the U.S. Environmental Protection Agency (1992)] of the number of industries that produce or use pellets within the industrial districts of Alemoa and the city of Cubatão; the middle reaches of Santos Harbor and Guarujá; those that enter via terrestrial runoff and stormwater outflow through beach channels and potential offshore inputs resulting from shipping activities (Manzano, 2009). Overall, 5 potential release points were chosen (**Table 1**) that coincide with the "upper estuary" (i.e., adjacent site A), "middle estuary (site B), "river mouth" (site C), "inshore beach" (site D) and "offshore" (site E) and attribute the main sources of inputs to each. While in reality, the contribution of primary inputs from these points is likely to differ (due to the spatial concentration of industries), no weighting was applied in order to provide an unbiased assessment of the cumulative input throughout the SES and adjacent coastal waters of Santos Bay.

Modeling Particle Dispersal

The Lagrangian particle tracking model developed by Harari and Gordon (2001) was used to simulate the dispersal of plastic particles from the five release points. This model incorporated the hydrological features of the SES and neighboring coastline (i.e., tides, currents, particle density and wind) to simulate seasonal patterns of dispersal over a single year (see: Harari et al., 2000). The adaptations of the current model to Harari et al. (2000), involved the use of flexible code to combine hydrological models of different spatial resolutions (i.e., a coastal shelf model with a resolution of 1 km, and a nested estuarine model with a resolution of 100 m) into a single model sufficient to resolve the particle tracking within the predicted debris field (described below). The typical formulation of a Lagrangian model treats any pollutant as a set of particles moving through a bi-dimensional space (here representing the water surface), with zonal and meridional directions (x, y). Particles are randomly released from a sub-region within the domain (here, potential release points within the SES) at the initial time of the simulation. Subsequently, for each time-step Δt, each individual particle P has its original position $x(P)$ and $y(P)$ updated to a new position $x(P_{\text{new}})$ and $y(P_{\text{new}})$, depending on the interpolated current vectors (u, v), at that time and at that point and including a random variation of the particles position $x(P_{\text{random}})$ and $y(P_{\text{random}})$ which represent the diffusion effect. The model can be summarized using the equations:

$$x\,(P_{\text{new}}) = x\,(P) + u \times \Delta t + x\,(P_{\text{random}}) \text{ and } y\,(P_{\text{new}}) = y\,(P) + v \times \Delta t + y\,(P_{\text{random}})$$

where u and v are computed by the underlying hydrodynamic model and $x(P_{\text{random}})$ and $y(P_{\text{random}})$ are derived from a random number generator with an interval $[-1,+1]$, multiplied by the maximum displacement allowed for any particle in one time step Δt. In this way, the model employed polynomial interpolations, both horizontally and vertically, to estimate the value of currents between grid meshes and store the origin and

TABLE 1 | Summary of simulated release points within the SES and nearshore zones, potential sources, and modeled dispersal area.

			Modeled dispersal area (km^2)		
Site	**Release zone**	***Site*, potential sources**	**Mean**	**Min (month)**	**Max (month)**
A	Upper estuary	*Alemoa industrial complex*, discharge from factories, accidental loss during loading	8.1	6.7 (November)	11 (April)
B	Middle estuary	*Santos Harbor*, discharge from factories, accidental loss during loading and transport	3.6	3 (November)	4.1 (June)
C	River mouth	*Santos Harbor Cargo terminal*, accidental loss and industrial waste	33.6	23.5 (December)	40.6 (July)
D	Inshore beach	*Santos beach*, storm water runoff, spillage during transit, accidental loss, ocean sources	39.3	26.9 (November)	61.2 (June)
E	Offshore	*Santos Bay*, offshore transport, accidental spills, ship maintenance (blasting of ballast tanks)	32.9	25.3 (November)	41 (October)

trajectory information (u and v). While the effect of waves and Stokes drift can be important when modeling microplastics for open ocean areas and at higher latitudes such as the Arctic and Sea of Japan (Iwasaki et al., 2017), their effect in subtropical regions is thought to be less (Onink et al., 2019) and was therefore not considered in the present study.

The particle tracking model employed a different formulation for the horizontal eddy diffusivity than the underlying hydrodynamic model. The approach considered the relationship between maximum displacement in a time step and the diffusivity coefficient, while the hydrodynamic model considered the Smagorinsky formulation for horizontal diffusion. Particle diffusion was simulated using the well-known random walk method (Rubinstein and Kroese, 2016), considering a function of uniform probability density, with zero mean and unitary standard deviation which meant that the maximum displacement of each particle, for each time interval, is about 50 m, corresponding to a typical diffusion coefficient in this area between 10 and 100 $m^2\ s^{-1}$, as computed by Ghisolfi and Garcia (1996). This computation is equivalent to a coefficient of the diffusion equation corresponding to ($N\ L^2/_2$) where N is the number of displacements per time unit and L is the maximum displacement allowed at every walk (Bowden, 1983). The model included "beaching" whenever an individual particle reached dry cells (i.e., crossing a land boundary and leaving the fluid field), whereby it was fixed in space and not subject to any further advection, diffusion or decay (i.e., and thus the model did not include wetting and drying). The model did not incorporate sinking of particles, as most types of polyethylene resin pellets encountered in the study region float on or just below the seawater surface. Overall, the simulations were designed to evaluate the intra-annual (seasonal) variability in pellet dispersal and forecast the progress of the debris field across a typical year.

The current field was provided by the three-dimensional hydrodynamic numerical model adapted by Harari et al. (2000). This numerical model is based on the Princeton Ocean Model (POM) developed by Blumberg and Mellor (1987) and presented in detail by Mellor (1998). POM is a three-dimensional model of primitive equations, with time-varying free surface and sigma vertical coordinates. The prognostic variables are the three components of velocity, in addition to temperature and salinity. The model grid for the São Paulo State shelf contains 150 × 450 points and 11 sigma levels, with a uniform resolution of 1 km in the horizontal plane, and time steps of 180 and 6 s, for the internal and external modes. The main advantage of the implemented model configuration is the possibility of using nested grids in estuarine regions, such as the Santos study region.

The model was run for the entire year of 2008 considering mean monthly conditions of temperature and salinity, extracted from Levitus and Boyer (1994); typical river discharges (DAEE, 1999); the tidal elevations at the boundaries based on results of the global tidal model (Leprovost et al., 1994), offshore tidal measurements (De Mesquita and Harari, 2003); mean sea level oscillations from the Oceanographic Institute of São Paulo University and surface wind conditions from the global atmospheric model of NCEP / NCAR, available at http://www.cdc.noaa.gov/cdc/data.ncep.reanalysis.html. The bathymetry of the grid layer was obtained from the global database GEBCO bathymetry (General bathymetric chart of the Oceans)[1], which was merged with local bathymetric charts obtained from the Brazilian Geography and Statistics Institute database[2]. The total prediction area encompassed 232 km^2 of coastal and estuarine waters within the SES. Model outputs were integrated into ArcGIS version 10.1 (Environmental Systems Research Institute, Inc., Redlands, CA, United States) and used to calculate the total dispersal area (km^2) for each month of the year. Because the model represented a simulation of particle transport (i.e., no actual particles were released) outputs were calibrated and validated only qualitatively, based on the assessments of pellets inputs along the beaches of Santos Bay (described in the next section). The discrete particle field was mapped into a continuous field using the "Point Density" function in the ArcGIS Spatial Analyst toolbox, which calculates a magnitude-per-unit area (m^2) from points using a kernel function to fit a smoothly tapered surface to each point.

Predicting Rates of Pellet Accumulation on the Beaches of Santos Bay

To test if the dispersal models could predict rates of pellet accumulation on the beaches of Santos Bay, model outputs were compared with daily inputs of pellets (no. $m^2 \cdot d^{-1}$) sampled at six points along the beaches of Santos Bay during "stable weather" (November), at the "end of the wet season" (March), and during the "storm season" (July). Pellet accumulation was quantified according to the standard protocols for micro-litter (GESAMP, 2019) which involved the marking out and preparation (24 h prior to sampling), of 2-m wide fixed transects ($n = 5$ replicates) established at 6 sites along the beaches of Santos Bay (separated by ∼1 km). Preparation involved pre-cleaning all surface debris (plastics, wood, vegetation) from the water's edge to the high strandline using a 50 cm wide squeegee to establish a virgin surface from which to evaluate the daily accumulation rate. Sampling was done using the same squeegee, to concentrate and collect all beached debris for separation and identification. This approach has been used previously to measure pellet inputs to the beaches of Santos Bay (Balthazar-Silva et al., 2020) and conforms to the requirements of "rapid repeated surveys of accumulation" outlined by GESAMP (2019). Separation involved washing the mix of sediment and plastic particles through a 0.1 mm sieve, packaging all retained material on-site and transportation to the laboratory for classification and counting. Sampling was repeated over three consecutive days following the high tide of spring (full moon) for each month to provide a seasonal comparison of daily pellet inputs (no. $m^2 \cdot d^{-1}$). The relationship between observed inputs of pellets, PTM outputs, rainfall and beach position were investigated using Generalized Additive Models (GAMs), fit with the "mgcv" package (Wood, 2006) in the R statistical software environment (R Development Core Team, 2011). Models employed a Poisson distribution with significance assessed using the test criterion ($\alpha = 0.05$) and significant

[1] http://www.ngdc.noaaGov/mgg/GEBCO/

[2] http://mapas.ibge.gov.br/bases-e-referenciais/basescartograficas/

combinations of predictor variables reported in terms of their level of explained deviation.

RESULTS

Pellet Dispersal Footprint

The dispersal footprint of microplastic pellets was strongly contingent on release point and month (**Table 1**). The smallest dispersal area coincided with the middle estuarine release point (site B), which showed a mean footprint of only 3.6 km^2 and exhibited little change across the year. The upper estuary release point (site A) showed a larger mean dispersal of 8.1 km^2 and comparatively greater seasonal variation. Nevertheless, the majority of microplastic particles released from these two estuarine points concentrated close to their point of origin, rapidly becoming static (dry cell). In contrast, river mouth, inshore and offshore release points showed significantly greater dispersal (**Table 1**). Pellets released from the estuary mouth (point C) exhibited broad dispersal across the year (mean 34 km^2) but showed marked seasonal variation (ranging from 24 km^2 in December to 41 km^2 in July). The inshore and offshore release points produced the greatest and most consistent dispersal areas (39 and 33 km^2, respectively), with inshore releases from the beach exhibiting comparatively greater seasonal range (ranging from 39 km^2 in November to 61 km^2 in June) when compared to the offshore release point (ranging from 33 km^2 in November to 41 km^2 in October).

The effect of season on pellet dispersal is clearly illustrated by comparing the footprints of points A, C and D, for the three most characteristic periods of the year (i.e., November, March and July; **Figure 2**). Again, releases from the upper estuary (site A) during November did not appear to lead to major downstream transport when compared to March, or the more dynamic period of winter storms. Releases from the river mouth (site C) showed large seasonal variation and a pattern likely to lead to significant inputs of stranded pellets to the ocean beaches of Santos Bay. Likewise, simulated releases from inside the bay itself (site D), show that this could also be a major contributor of accumulated debris, especially at the western end, where beach morphology (typically wider and more gently sloping) and inputs from the stormwater canals can lead to more pronounced accumulation (see also Turra et al., 2014).

The dispersal footprint of pellets also highlighted interesting correlations with seasonal rainfall across a typical year (**Supplementary Figure 1**). Overall, the greatest period of rain occurred between December and March, the period May through to July was comparatively dry, and the period from August through to November was intermediate. The simulated dispersal of pellets from each release point differed considerably with monthly average rainfall. Site A showed a strong positive relationship with rainfall (with the greatest dispersal coinciding with the highest rainfall records over the first 4 months of the year); while inshore site D showed a negative relationship (high dispersal during lower rainfall during the winter months due to frontal systems from the south). Interestingly, the scale of dispersal from the river mouth (site C) did not present either a significant positive or negative relationship with rainfall.

Microplastic Litter Accumulation on the Beaches of Santos Bay

Pellet inputs rates to Santos beach varied with season and distance along the beach (zonation). Inputs during November (mean: 2 pellets m^2) were substantially lower than July (14 m^2), which was lower than March (51 m^2); and for all periods there was a pronounced decrease with increasing distance westwards from the Santos river mouth (showing an average decline of 13 pellets m^2 for every kilometer). The output of the Generalized Additive Model highlights the value of Lagrangian predictions, but also the effect of rainfall and beach zone (**Table 2**). Overall, the hydrodynamic dispersal model performed well in predicting the observed pellet inputs to Santos Beach (explaining 46% of the deviation), however, the inclusion of total rainfall and beach zonation (i.e., distance from river mouth) improved the predictive power substantially (93%) leading to a relationship between observed and predicted inputs that did not differ greatly from a slope of 1 (**Figure 3**).

DISCUSSION

The goal of this study was to improve predictions of the dispersal and accumulation of microplastic particles within the estuarine and coastal waters of the Santos region (south-east Brazil). This information is crucial for regional policy development, given that this stretch of the Brazilian coast continues to be profoundly impacted by microplastic pollution (Turra et al., 2014) and there is a broad desire to identify and control further inputs (Izar et al., 2019). Simulations of the dispersal footprint (in km^2) from different release points within the SES, suggest that microplastics disperse more broadly from river mouth and nearshore points than they do within the estuary. By combining PTM predictions with data that describe fluvial processes (governed by rainfall) and beach zonation (reflecting morphology and the influence of stormwater canals), it was possible to achieve a more accurate account of observed daily rates of pellet accumulation. This improvement in predictive power, suggests that integrated models might help to reconcile the interplay between raw material inputs from known point sources and the reintroduction of particles from diffuse sources through fluvial forcing and thereby guide management and mitigation policy relating to microplastic debris within linked estuarine and coastal systems (Ballent et al., 2013; Critchell et al., 2015).

Pellet Dispersal Footprint

The dispersal footprints generated for each release point (upper and middle estuary, mouth, inshore and offshore) reflect the likelihood that pellets released from these zones will be deposited along the beaches and coastal environments of Santos Bay. Pellets released from the upper and middle reaches of the SES (points A and B) appear more likely to interact with the edge of the predicted debris field (i.e., the river bank or shoreline) and are therefore likely to become beached or retained by vegetation,

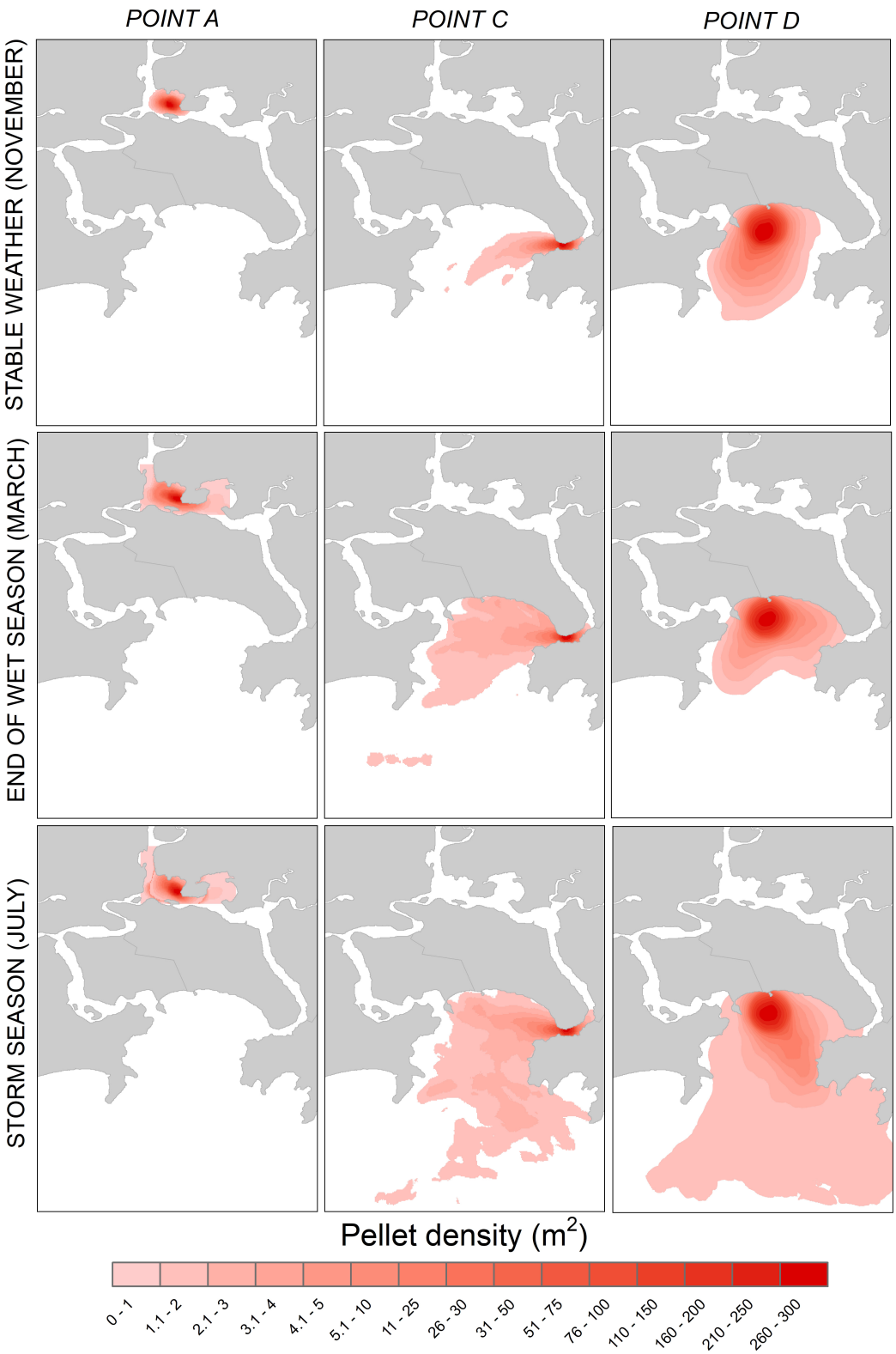

FIGURE 2 | Dispersal simulations for plastic pellets during the stable weather conditions of November (top), the end of the rainfall period (March, middle) and the period of peak sea storm activity during June (bottom) considering 5 different release points within the SES and coastal waters of Santos Bay. Polygons show the area of dispersal, with the color ramp indicating the number of accumulated particles predicted using a "density plot" function that calculates a magnitude-per-unit area (m^2) from points using a kernel function to fit a smoothly tapered surface to each point.

TABLE 2 | Model selection based on explained deviance related to the inclusion of different predictors: Particle Tracking Model (PTM) outputs, rainfall and beach longshore zonation to explain rates of observed input of microplastic pellets to the open coastal beaches of Santos Bay.

(a) Model selection

Variables	Deviance explained (%)
PTM + rainfall + zone	93.1
PTM + rainfall	89.7
PTM + zone	79.5
PTM	45.5
Rainfall	40.9
Zone	13.0

(b) Model output

Fixed effect	Estimate	SE	*z*-value	*P*
Intercept	0.51	0.41	1.25	0.211
PTM	0.01	0.00	18.27	<0.001
Rainfall	0.01	0.00	4.71	<0.001
Zone	−0.32	0.03	−9.54	<0.001

sediments or coastal infrastructure. From a modeling perspective, this means that they are subsequently excluded from further consideration, even though they might be re-introduced into the aquatic environment through rainfall events, flooding or extreme events that release them from the terrestrial environment, back into the debris field. Indeed, the retention and exportation of plastic debris from mangroves has been observed throughout the world, including the northeast and southern coasts of Brazil (Do Sul et al., 2014; Gorman and Turra, 2016).

Release points located at the river mouth, inshore (beach) and offshore areas of Santos Bay, showed significantly greater dispersal footprints across an entire year (ranging from 34 to 41 km^2). Pellets released near the river mouth (point C) flowed out into the bay during all seasons, but there was a noticeable peak in March (coinciding with highest seasonal rainfall) that led to greater diffusion into coastal waters when compared to November and July. Although arguably less important than terrestrial inputs of microplastics, the release of particles from ships has been recognized as a pervasive source of marine litter in some regions (Derraik, 2002). This is true of Santos Bay, where continued illegal dumping and ship maintenance (e.g., blasting of tanks; Manzano, 2009) of anchored vessels is believed to contribute pellets to the regions beaches, especially during winter, when these buoyant particles are washed up in high concentrations (Magini et al., 2007).

Our models suggest that pellets released from inshore (beach) and offshore zones (points D and E) disperse broadly during winter months and make a significant contribution to the microplastic debris accumulating on the beaches of Santos Bay (see; Turra et al., 2014; Balthazar-Silva et al., 2020). These patterns of accumulation however, are unlikely to be uniform and will be governed by sometimes strong tidal currents (up to 100 cm s^{-1}) that include the westward drift vortex and the Itararé vortex (Maggi et al., 2012) which coincide with the passage of winter storm fronts. This type of alongshore variability in plastic debris accumulation in other parts of the world has been attributed to a combination of complex coastline geomorphology, oceanic forcing and resuspension of floating debris by waves (Critchell et al., 2015). It is equally likely that a significant proportion of the pellets released from offshore sources are exported to neighboring beaches (Turra et al., 2014; Moreira et al., 2016) and even as far afield as other states hundreds of kilometers away (e.g., Paraná; Gorman et al., 2019).

Overall, the data presented suggests that the factors governing dispersal and accumulation of microplastics in the Santos region may differ spatio-temporally between estuarine and nearshore zones. While the origin of plastic debris in estuaries may be easier to delineate spatially (i.e., they tend to "beach" close to their origin), the temporal contribution of these materials may be harder to resolve because they may become alternatively "beached" and then "reintroduced" as a result of fluvial processes. In contrast, nearshore and offshore sources may be easier to delineate if there is detailed knowledge of the timing and intensity of oceanographic circulation and winter storms but may show weaker links to rainfall and fluvial possesses. Contrasting the relative importance of these processes in estuaries vs. open coasts is an intriguing topic and one that may help reconcile the role of local rivers vs. ocean processes as transport pathways for plastic debris in coastal and marine environments (Zhao et al., 2019).

While dispersal models accurately predict the behavior of a particle in the water column under a given set of internal conditions (i.e., wind, currents, density, etc.), they may be improved substantially by including external drivers such as rainfall and beach characteristics (morphology and terrestrial inputs). The fact that a high proportion of simulated particles released from the upper and middle zones of the estuary become rapidly beached and were considered no longer active, highlights a potential limitation to the modeled behavior (similar to model limitations at the scale of oceans, see; Iwasaki et al., 2017). Accounting for the re-introduction of these materials during rainfall events using additive modeling approaches (e.g., GAMs), might help to reconcile and improve our predictive capacity. The GAM outputs highlight marked but contrasting relationships between simulated dispersal and total monthly rainfall for different release points. This is likely to reflect flood events that reintroduce trapped particles by dislodging riparian vegetation and eroding sediments. In this way, by including rainfall data into models, it was possible to increase current understanding of the temporal cycles that capture pulse-driven inputs of debris to coastal and adjacent offshore areas. While, there have been attempts to quantify the magnitude of plastic items exported from coastal wetlands during increased periods of runoff (Ryan et al., 1990; Coe and Rogers, 1997; Do Sul et al., 2014) this field of research remains a challenge because the response is likely to be decoupled in space and time and moreover confounded by shoreline complexity (i.e., the density of shoreline vegetation and shoreline topography).

Coupling PTM's With Fluvial Processes

Although several studies have validated the output of particle tracking models using survey data over large spatial scales (e.g.,

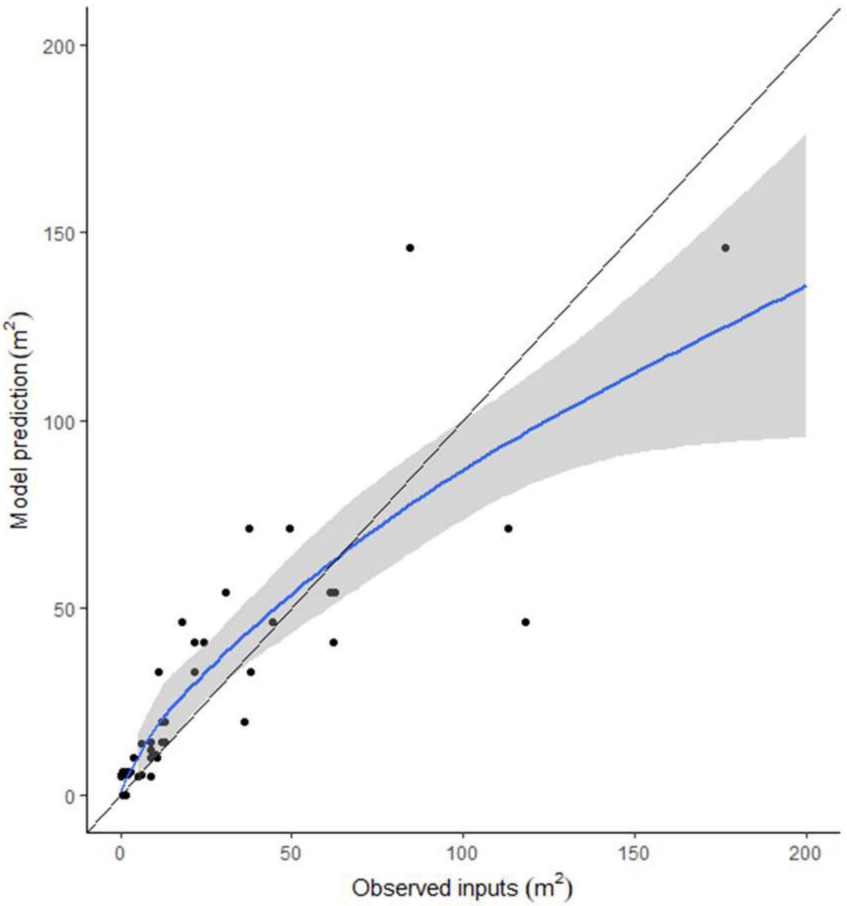

FIGURE 3 | Relationship between observed pellet inputs to the beaches of Santos Bay (particles $m^2 \cdot d^{-1}$) and predicted pellet inputs from the best performing model that incorporated the factors; PTM, rainfall and beach zone. Shading is the smooth term of the fitted model and the dashed line represents a 1:1 ratio between observed and predicted values.

the entire Sea of Japan; Iwasaki et al., 2017), the present study is one of few to compare model predictions with observed particle inputs at the scale of an individual beach. Results show that pellet inputs vary both seasonally (March having particle counts 19 × greater than November) and that there is consistent variation with distance from the river mouth (density declining by 7% per km) that reflects beach morphology and the level of terrestrial inputs. While this finding is not new for the region (see previous studies by‘: Turra et al., 2014; Fisner et al., 2017) the present data points to the value of combining PTM outputs with fluvial processes using a stepwise correlative approach. Indeed, the Generalized Additive Model emphasizes the need to consider such factors to accurately and consistently predict microplastic accumulation on ocean beaches. The marked effect of rainfall for the study region is to be expected, as heavy falls can occur over relatively short periods of time (Gorman et al., 2017) washing material from roads, parks and drainage networks (canals) into the marine environment. Indeed, the effect of fluvial forcing on the dispersal of microplastics from rivers is highly dependent on season, proximity to sources and sedimentary dynamics (e.g., China; Wan et al., 2018; Italy; Guerranti et al., 2017; Korea; Seo and Park, 2020 and the Atlantic coast of France; Frere et al., 2017). A previous study by Manzano (2009), suggested greater inputs of plastic pellets to Santos beaches during winter (average of 102 pellets/m^2 and maximum of 377 pellets/m^2). This matches similar reports across the globe, whereby the highest accumulation of plastic waste on beaches typically occurs during winter months and after storm events (Shiber, 1987; Moore et al., 2002). Indeed, recent data from Brazil, demonstrates that short-term episodic events can be just as important as seasonal variation in terrestrial runoff as a driver of debris accumulation in subtropical systems (i.e., a 20-fold increase in pellet inputs per m^2, before and after rainfall events; Balthazar-Silva et al., 2020).

In conclusion, this study highlights the value of hydrodynamic models for mapping the spatial footprint of microplastic debris in complex coastal systems with strong seasonal climate. Further, by coupling the predictions from dispersal models with seasonal trends in rainfall and beach zonation, it is possible to explain variability in the input of pellets to open coastal beaches at an even finer spatial resolution. This will not only help to delineate the sources of

microplastic pollution in complex marine and coastal systems (permitting effective local intervention and mitigation; Vegter et al., 2014), but can improve the ability of models to explain and replicate real-time spatial patterns. This information will not only join with efforts around the world to better understand the growing problem of plastic pollution across a range of spatial scales (Maximenko et al., 2019) but will help to reconcile processes occurring within dynamic estuarine systems where the interaction of processes occurring on land and water make predicting the dispersal and accumulation of microplastics a challenge.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DG: conceptualization, methodology, formal analysis, writing – original draft, project administration, and funding acquisition. AG: methodology, software, formal analysis, and writing – original draft. AT: conceptualization, writing – review and editing, project administration, and funding acquisition. AM: conceptualization and investigation. DB-S: investigation, validation, and writing – review and editing. NO: investigation and writing – review and editing. JH: conceptualization, resources, methodology, software, formal analysis, 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/fenvs.2020.559405/full#supplementary-material

Supplementary Figure 1 | Relationship between monthly rainfall and modeled dispersal area (km^2) of microplastic pellets from three different release points within the estuary and open coastal waters of the Santos region. Correlations are Pearson's correlation (*r*) and two-tailed probability (*P*).

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Transport and Behavior of Microplastics Emissions From Urban Sources in the Baltic Sea

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Urban sources, wastewater treatment plants (WWTPs), untreated wastewater (not connected to WWTPs), and especially combined sewer overflow systems (CSS) including stormwater are major pathways for microplastics in the aquatic environment. We compile microplastics emission data for the Baltic Sea region, calculate emissions for each pathway and develop emission scenarios for selected polymer types, namely polyethylene (PE)/polypropylene (PP) and the polyester polyethylene terephthalate (PET). PE/PP and PET differ with respect to their density and can be regarded as representative for large groups of polymers. We consider particles between 20–500 μm with varying shapes. The emission scenarios serve as input for 3D-model simulations, which allow us to estimate transport, behavior, and deposition in the Baltic Sea environment. According to our model results, the average residence time of PET and PE/PP in the Baltic Sea water body is about 14 days. Microplastics from urban sources cause average concentrations of 1.4 PE/PP (0.7 PET) particles/m^2 sea surface (20–500 μm size range) in the Baltic Sea during summer. Average concentrations of PET, resulting from urban sources, at the sea floor are 4 particles/m^2 sediment surface during summer. Our model approach suggests that accumulation at the shoreline is the major sink for microplastic with annual coastal PE/PP and PET accumulation rates of up to 10^8 particles/m each near emission hot-spots and in enclosed and semi-closed systems. All concentrations show strong spatial and temporal variability and are linked to high uncertainties. The seasonality of CSS (including stormwater) emissions is assessed in detail. In the south-eastern Baltic, emissions during July and August can be up to 50% of the annual CSS and above 1/3 of the total annual microplastic emissions. The practical consequences especially for monitoring, which should focus on beaches, are discussed. Further, it seems that PET, PE/PP can serve as indicators to assess the state of pollution.

Keywords: wastewater treatment plants, stormwater runoff, combined sewer overflow, pollution, hydrodynamic model, monitoring, sedimentation, accumulation

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INTRODUCTION

In the United Nations Environment Programme, marine litter is defined as "persistent, manufactured or processed solid material that is discarded, disposed of, or abandoned in the marine and coastal environment[1]." Marine litter is regarded as a global concern, affecting seas and

[1]https://www.unenvironment.org/

oceans worldwide and causing environmental, economic, and aesthetic problems. Marine litter is commonly separated into macro- (> 25 mm), meso- (5–25 mm), and micro-litter (< 5 mm). The most important fraction of marine litter is plastics and especially microplastics (MP) is of major concern (GESAMP, 2016). The European Union Marine Strategy Framework Directive (MSFD 2008/56/EC) addresses this problem and has the aim to "ensure that properties and quantities of marine litter do not cause harm to the coastal and marine environment." As a consequence, the state-of-pollution of the marine environment has to be assessed, environmental targets and associated indicators have to be developed, major emission sources have to be identified and quantified and effective measures leading to reductions in marine litter pollution have to be tested (JRC, 2011).

Sources for microplastics in the environment are primary microplastics in cosmetic and cleansing products entering wastewater systems, emissions through accidental spills and during the life cycle of a product (clothing, tires) as well as secondary microplastics arising from the fragmentation and degradation of meso- and macroplastics. Plastics enter the marine environment via riverine systems, coastlines, from vessels and platforms or the atmosphere (GESAMP, 2016). Since human activities are the source for microplastics, wastewater is considered as a major emission pathway (e.g., Mintenig et al., 2016; Ziajahromi et al., 2016; Kay et al., 2018; Prata, 2018). For untreated wastewater, high microplastic concentrations between 10^1–10^4 particles/L are reported (Gatidou et al., 2019; Sun et al., 2019), but municipal wastewater treatment plants (WWTPs) are efficient in removing microplastics (Carr et al., 2016; Talvitie et al., 2017; Gies et al., 2018). For WWTPs in the Baltic Sea region, Baresel and Olshammar (2019) assume, depending on their treatment stages, a microplastics retention between 85 and 98%. Despite this relatively good overall removal efficiency, WWTPs are still considered as a major microplastic emission pathway in the Baltic Sea region because of the high wastewater quantities treated (Baresel and Olshammar, 2019).

Combined sewer overflow water, that consists of storm water and untreated wastewater can be another major source of microplastics, even though overflow events happen rarely. For the Baltic Sea region, Baresel and Olshammar (2019) conclude that the annual discharge of microplastics from sewer overflows can be in the same magnitude as from treated wastewater. In addition, poorly known emissions result from untreated wastewater as a consequence of technical failures or capacity limitations at the WWTPs and the sewer system (Magnusson et al., 2016; Dris et al., 2018). In the Baltic Sea region wastewater and stormwater drainage systems are often separated. However, wash off during intensive precipitation can cause high plastic concentrations in stormwater and represent another important emission pathway for the aquatic environment. For example, in the water of seven Danish urban and highway storm water ponds, microplastics concentrations between 0.5 and 23 particles/L were found (Liu et al., 2019).

Objectives of our study are to (a) compile emission scenarios from urban sources for the selected representative polymer types polyethylene/polypropylene (PE/PP) and polyethylene terephthalate (PET) into the entire Baltic Sea (b) perform 3D-model simulations on transport, behavior, and deposition in the Baltic Sea environment and (c) assess the practical consequences and implications for policies and the development of a microplastic monitoring. We consider microplastics with a size between 20 and 500 μm and varying shapes. We take into account the emissions from three pathways, namely WWTP, combined sewer overflow systems (CSS) including urban stormwater runoff as well as untreated wastewater (not connected to WWTPs).

MATERIALS AND METHODS

Plastic Polymers and Properties

According to Geyer et al. (2017) the amount of non-fiber plastics manufactured worldwide since 1950 is about 7300 Mt compared to fibers with 1000 Mt. Fibers usually consist of polyester, polyamide, and acryl and are mainly used for clothing. The largest groups in total non-fiber plastics production are PE (36%), PP (21%), polyvinyl chloride (PVC, 12%), and PET(< 10%). 42% of all non-fiber plastics, mainly PE, PP, and PET, is used for packaging, e.g., plastic bags, plastic films, and bottles. 19% of all non-fiber plastics, mainly PVC, is used in the building and construction sector, e.g., for pipes, doors and windows.

The density of plastics (artificial polymers) is an important parameter that determines its transport, behavior and deposition in the aquatic environment. According to the density, we separate floating and sinking polymer types. The group of floating polymers includes low and high density PE (0.915–0.97 g/cm^3 density) and PP (0.89–0.92 g/cm^3 density). The group of sinking polymers covers rigid PVC (1.3–1.45 g/cm^3 density) and PET (1.38 g/cm^3 density). The most common polyester fibers are made of PET. Acrylic and polyamide fibers have a density of only 1.18 g/cm^3 and 1.14 g/cm^3, respectively. According to Sun et al. (2019) PP, PE, and PET are the most abundant polymers in WWTPs.

In literature, the PE/PP and polyester (PET) fraction of the total microplastics particles in stormwater and raw wastewater varies in a wide range (e.g., Kang et al., 2018), but both are usually in the same order of magnitude (e.g., Liu et al., 2019; Olesen et al., 2019; Sun et al., 2019). Kooi and Koelmans (2019) provide the shares most common microplastics polymer types in the aquatic environment: PE 25%, PET 16.5%, and PP (14%). Based on literature (Talvitie et al., 2015; Murphy et al., 2016; Lares et al., 2018; Simon et al., 2018; Wolff et al., 2019; Long et al., 2019), review papers (Kang et al., 2018; Gatidou et al., 2019; Sun et al., 2019) and taking into account the data quality assessment by Koelmans et al. (2019), we calculated an average minimum of 511 and an average maximum of 18,196 PE/PP particles/m^3 raw wastewater and an average minimum of 119 and an average maximum of 21,967 PE/PP particles/m^3 in stormwater. Based on Murphy et al. (2016); Long et al. (2019) and Wolff et al. (2019) we calculated an average minimum of 401 and an average maximum of 14,492 PET particles/m^3 raw wastewater and stormwater. The average minimum and average maximum are based on the lower and upper concentrations reported in the

literature. In our simulation scenarios, we applied the average maximum concentrations.

Beside its density, the floating and sinking behavior of microplastics is influenced by particle size and shape. For one simulation, we separated the size classes 20–200 and 200–500 μm and, based on data in Ziajahromi et al. (2017), assumed that 90% of microplastics in WWTPs belongs to the size class 20–200 μm. According to a comprehensive review by Sun et al. (2019), we separated microplastics into fibers and particles and assumed that fibers have an average proportion of 50%. All other shapes are considered as (spherical) particles.

Microplastics Emission Calculations

Model simulations about the transport, behavior and deposition of microplastics in the aquatic environment require the concentrations of microplastics in and the amount of discharge water as model input. CSS, which are common in urban areas of the Baltic Sea region, collect surface water runoff, domestic sewage, and industrial wastewater. Baresel and Olshammar (2019) compiled data about and quantified the amount of sewage water for 3,525 WWTPs in the Baltic Sea region. As average microplastics removal efficiency in WWTPs depending on the treatment technology we used 85% for primary treatment, 90% for secondary treatment, 95% for tertiary treatment (N and P removal), 97% for sand-filtration and 98% for micro-filtration. For details see Baresel and Olshammar (2019). The concentrations of each polymer in influent sewage water, the waste water discharge and the removal efficiency of each WWTP allowed the quantification of the annual microplastics-discharge from WWTPs into the river systems of the Baltic Sea region. We complemented missing data for single WWTPs based on country specific average amounts of wastewater per person and day as well as taking into account the country specific percentage of the population connected to WWTPs.

In CSS, sanitary sewer overflows take place, where untreated wastewater is discharged from a sanitary sewer into the aquatic environment. Usually this happens due to a temporary insufficient hydraulic capacity after heavy precipitation. Baresel and Olshammar (2019) assumed that in the Baltic Sea region, weather related sanitary sewer overflow accounts for 1.5% of the total WWTP inflow. This value was applied and for seasonal overflow calculations, we assumed that this is equivalent to 1.5% of the time of the year. We assumed that sanitary sewer overflows resulting from technical problems are included in the 1.5%. Separated sewer systems (SSS) collect storm water and wastewater in separated systems. In these systems storm water is always released into the aquatic environment usually without treatment. For the Baltic Sea region reliable numbers about the water discharge from SSS are lacking. We assume that SSS have a share of 50% in the Baltic Sea region. SSS emissions are not treated as separate pathway but are integrated into CSS emissions.

Sanitary sewer overflows are a result of heavy rains, which do not have the same likelihood throughout the year and differ regionally in the Baltic Sea region. To reflect realistic conditions, the emissions were calculated on a monthly basis for each WWTP. For each WWTP, daily precipitation amounts were extracted from the German Weather Service (DWD) 3-h forecasts. For the years 2009–2016, output of the LME model was used, and for the years 2017–2018 the predictions originate from the ICON model. The resulting daily time series over 10 years was created by adding large-scale and convective rainfall predicted by the model. Solid precipitation (snow) was neglected because we assumed, that snow and snow melt plays only a minor role for sanitary sewer overflows. For each WWTP, we identified the 98.5% percentile, meaning the amount of rain which is exceeded on 1.5% of the days only. We then attributed the rainfall on those days to the calendar month to create a monthly climatology of heavy rainfall at each WWTP. The resulting seasonal pattern describes the monthly likelihood of sewer overflow at each WWTP. The total annual microplastics emissions were allocated to the single months according to their overflow likelihood.

Our model approach allows a scaling of the plastic polymer concentrations in the environment, by post-processing the simulation results. This means the absolute concentrations emitted via each pathway, polymer type and size class potentially can be adjusted if new insights or better field data are available. This is possible as long as the relative spatio-temporal emission pattern remains the same.

Model Approach

The modeling approach follows Osinski and Radtke (2020). We use the uncertainties in ensembles of regional reanalyses (UERRA) high-resolution atmospheric reconstruction, provided by sveriges meteorologiska och hydrologiska institut (SMHI), to drive both a third-generation wave model (WAVEWATCH 3) and a hydrodynamic model for the Baltic Sea general estuarine transport model (GETM). Both models have a horizontal resolution of one nautical mile. A microplastics transport module is integrated online into the hydrodynamic model following Osinski et al. (2020). The wave model provides wave properties required for the calculation of bottom shear stress, and the hydrodynamic model provides the current field used for the passive transport of the particles, which are represented in an Eulerian framework as a concentration per grid cell. The size, density, and shape of the particles determine the vertical velocity relative to the ambient water and the critical shear stress for the resuspension. The actual shear stress at each time step is calculated from the bottom current velocity and the significant wave height which is provided from the wave model. Settled particles are re-suspended when the actual shear stress exceeds the critical value. Tidal currents are generally taken into account, but do not play a role because the Baltic Sea is micro-tidal with a tidal range of a few centimeters only.

Sinking velocities are determined from the Stokes parameterization assuming a spherical shape. For each size class, we use the lower size limit as the particle diameter assumed for the Stokes formula, since (a) smaller particles typically have a higher abundance and (b) deviations of larger particles from the spherical shape would cause a reduction in the vertical velocity, such that they would behave like smaller spherical ones in this sense. The critical shear stress is calculated from the Shields curve (Shields, 1936). Exception are fibers, for which we chose the empirically determined parameterizations by Waldschläger and Schüttrumpf (2019a) for the sinking velocity

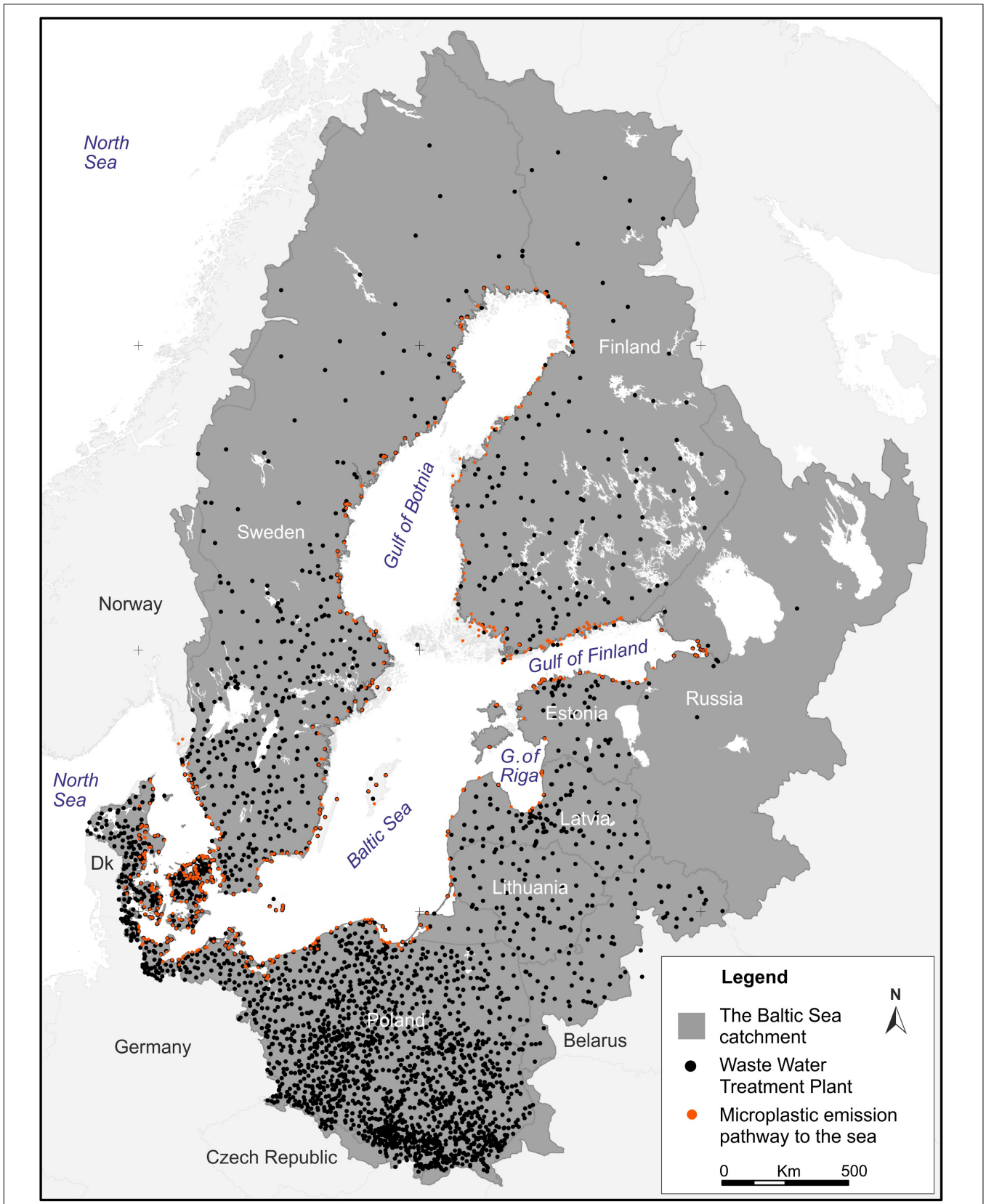


FIGURE 1 | Location of the about 3500 wastewater treatment plants (WWTPs) in the Baltic Sea catchment (black dots) and microplastics emission pathways/points (rivers and direct discharge) to the Baltic Sea.

and Waldschläger and Schüttrumpf (2019b) for the critical shear stress. The restriction of our approach to the 20–500 μm size class results from model limitations. Large PET particles show a higher sinking velocity and the faster transfer from one vertical model layer to the next one cannot be resolved with the applied model calculation time steps and would cause model instability. The actual values used for sinking velocity and critical shear stress are given in the online supplement.

Beach accumulation is parameterized as follows: Particles entering a grid cell adjacent to a land cell are immediately removed from the model and counted as beached. An exception are those grid cells acting as a source, such as rivers, here we do not assume beach accumulation. A possible resuspension and further transport of the particles is neglected. The model simulations covered 2 years, the period from March 2016 until February 2017. Additional 2 months before were used for model spin-up.

Scenarios for PP/PE and PET

Spatially resolved microplastics emission scenarios serve as model input. The emission takes place at the land/sea interface. Every (sub-)scenario serves as input for one model run. Scenarios 1 and 2 assume constant daily microplastics emissions during the year. Scenario 1 deals with PE/PP, takes into account the 20–500 μm microplastics size fraction and considers emissions from treated wastewater (WWTP effluents; sub-scenario 1.1), untreated wastewater from populations not connected to WWTPs (sub-scenario 1.2) and CSS (including both stormwater and untreated wastewater; sub-scenario 1.3).

Scenario 2 focusses on PET but assumes different size classes and particle shapes. Similar to scenario 1, the sub-scenarios 2.1–2.3 take into account the 20–500 μm microplastics size fraction and emission separated into WWTPs, untreated wastewater and CSS including stormwater. Sub-scenarios 2.4–2.6 assume the emissions from all three urban emission sources but distinguish the size fractions 20–200 and 200–500 μm and separate fiber and spherical shaped particles.

Scenario 3 deals with PE/PP and the 20–500 μm microplastics size fraction but is restricted to emissions from CSS including stormwater. CSS emissions are resolved on a monthly basis according to spatio-temporal distribution of heavy rain events. Every scenario is simulated separately.

RESULTS

Spatio-Temporal Emission Pattern to the Baltic Sea

The calculated PE/PP emissions from the three urban sources, namely treated wastewater, untreated wastewater (not connected to WWTPs) and CSS (always including stormwater) into the entire Baltic Sea are presented in **Figures 2A–D**. The compiled dataset shows for PE/PP, that CSS are the most important emission source followed by treated and untreated wastewater (**Figure 2F**). Large rivers are the major pollution pathway followed by coastal cities. In Germany, Denmark, Sweden, and Finland practically 100% of the population is connected to WWTPs and the average cleaning efficiency is around 95%. In the south-eastern countries Poland, Lithuania, Latvia, Estonia, and Russia, between 78 and 100% are connected to WWTPs as well, but the cleaning efficiency on average is lower (85%). As a consequence, the WWTP emissions from these countries are relatively high. Since in most of these countries not all wastewater is treated, microplastics emissions with untreated wastewater are relatively high in the south-eastern Baltic, as well. Especially Kaliningrad and St. Petersburg regions (Russia) are hot-spot in this respect. CSS systems are common in many countries and high emissions take place all around the Baltic, despite the fact that sewer overflow and stormwater events are usually taking place only during a few days per year.

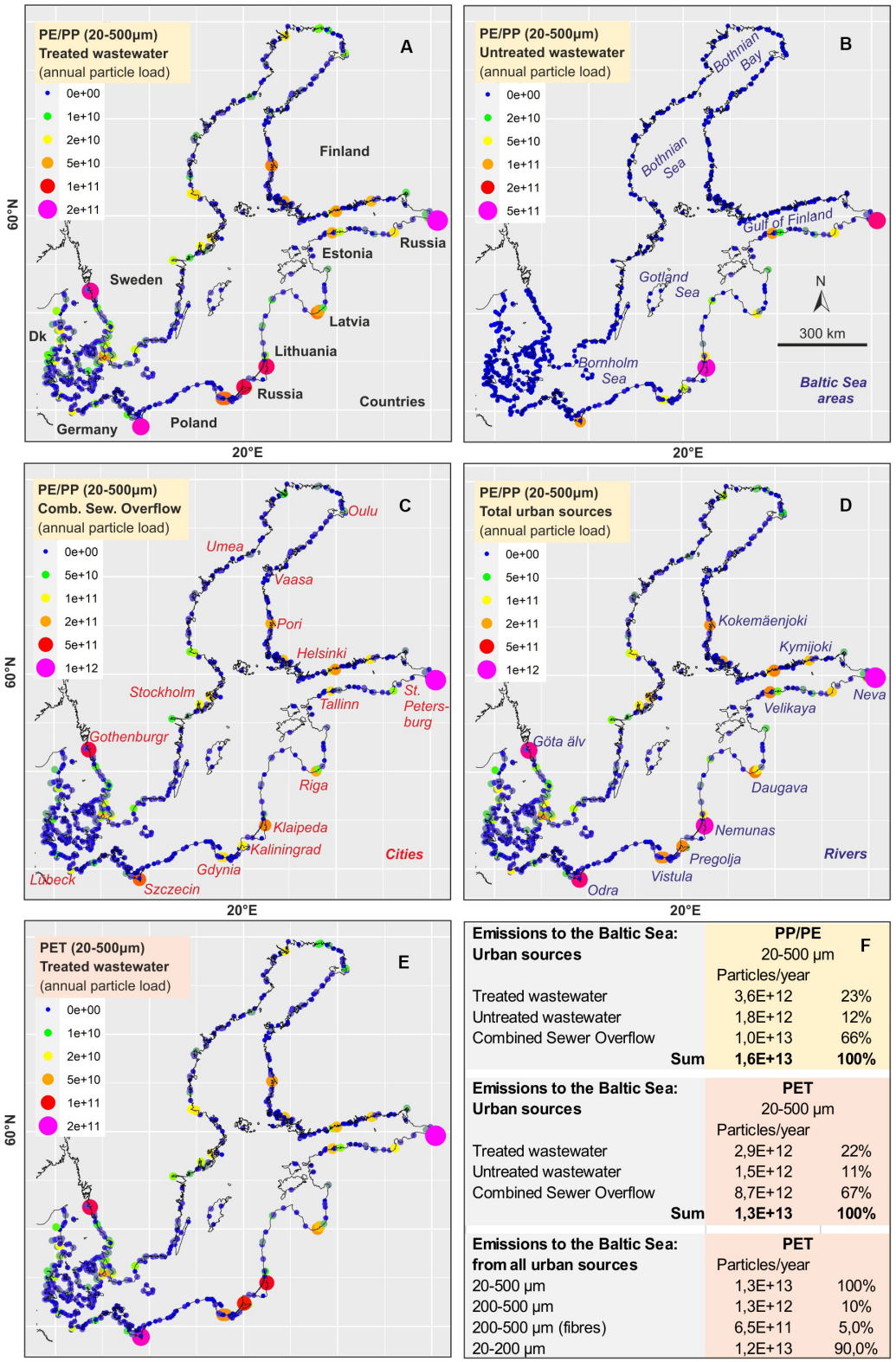
With respect to PET, the emission amounts and pattern are very similar and only exemplary shown for treated wastewater (**Figure 2E**). **Figure 2F** provides an overview about the calculated total emitted quantities for each of the urban pathways.

Residence Time and Relevance of Size and Shape

Our model approach suggests, that once a PE/PP microplastic particle enters the Baltic Sea, it stays in average about 14 days in the water column before it is washed ashore (**Figure 3A**). The residence time is about 1 day longer in summer and autumn as well as about 1 day shorter in spring and winter. This corresponds to the average meteorological conditions during the seasons (stronger wind speeds and more wave action in spring and winter) as well as to the hydrographic conditions (thermal stratification separates surface and bottom waters during summer and autumn).

While PE/PP is floating, PET has a density of 1.38 g/cm^3 and is heavier than water. Despite that it shows a similar average residence time of 13–14 days in the water column (**Figure 3B**). The coastal Baltic Sea is shallow and turbulence does not allow a settling close to the coast. Only after a transport to deeper waters, a temporary settling at the sediment surface is possible. The sandy sediments of the coastal Baltic Sea do not allow a burial of deposited PET. The seasonality of the residence time is less pronounced, because PET can either be washed ashore or be accumulated at the sea floor. The latter is favored by summerly calm weather conditions. Especially in winter, the residence time can, in rare cases, exceed 50 days. At that time, previously accumulated microplastics at the sea floor is re-suspended and can cause microplastic concentrations in the water column that exceed the 50-fold of the daily emissions. Apart from winter season, the residence time of larger PET particles (200–500 μm) in the water body is, with 3–7 days, much shorter because of a faster deposition at the sea floor. The results do not show a significant difference in residence times between spherical and fiber shaped particles (**Figures 3C–E**).

Figure 4 gives an insight into the behavior of PET entering the Baltic Sea. The short residence times indicate that only a small share of PET can be found in the water body, smaller (20–200 μm) and larger PET (200–500 μm) differs with respect to their behavior in the sea. While in spring,



Emissions to the Baltic Sea: Urban sources	PP/PE 20-500 µm Particles/year	F
Treated wastewater	3,6E+12	23%
Untreated wastewater	1,8E+12	12%
Combined Sewer Overflow	1,0E+13	66%
Sum	**1,6E+13**	**100%**

Emissions to the Baltic Sea: Urban sources	PET 20-500 µm Particles/year	
Treated wastewater	2,9E+12	22%
Untreated wastewater	1,5E+12	11%
Combined Sewer Overflow	8,7E+12	67%
Sum	**1,3E+13**	**100%**

Emissions to the Baltic Sea: from all urban sources	PET Particles/year	
20-500 µm	1,3E+13	100%
200-500 µm	1,3E+12	10%
200-500 µm (fibres)	6,5E+11	5,0%
20-200 µm	1,2E+13	90,0%

FIGURE 2 | Emissions of Polyethylene (PE)/Polypropylene (PP) and Polyethylenterephthalat (PET) microplastic particles (20–500 µm size fraction) from different urban sources to the Baltic Sea assuming no retention. Treated wastewater covers emissions from effluents of wastewater treatment plants (WWTPs; **A,E**). Untreated wastewater means emissions from populations not connected to WWTPs **(B)**. Emissions from combined sewer overflow systems (CSS), largely untreated, are kept separately **(C)**. Total urban emissions **(D)** are the sum of WWTPs, untreated wastewater and CSS. Total annual emissions from urban sources to the Baltic Sea **(F)**.

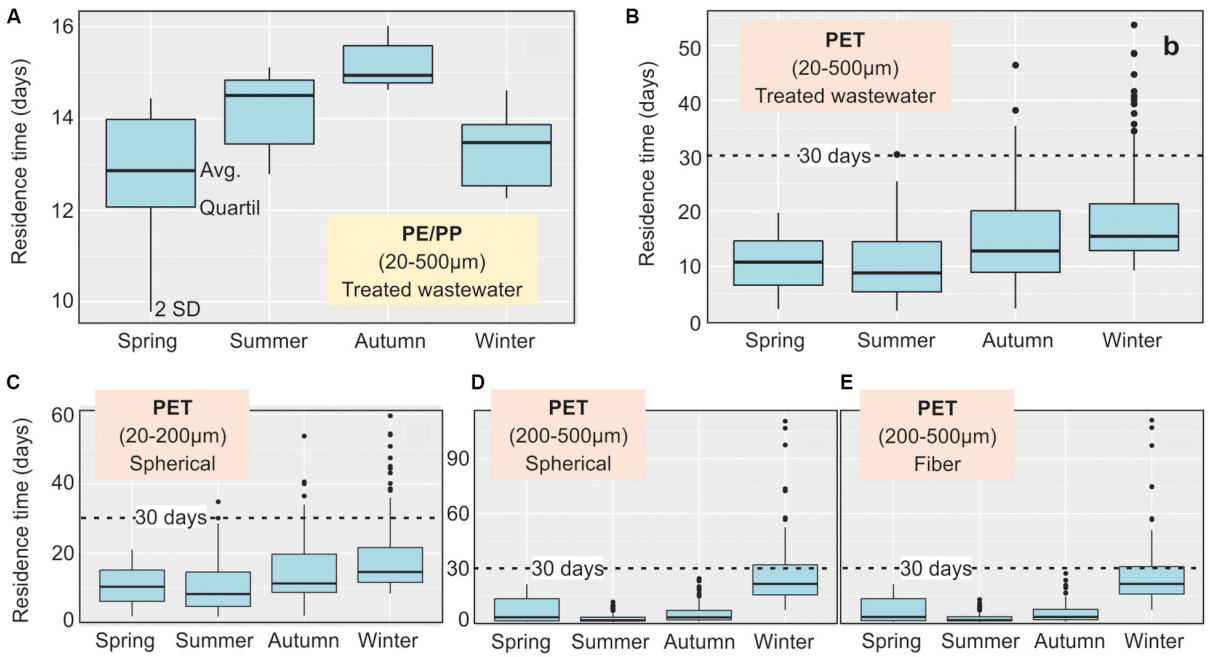


FIGURE 3 | Residence time of Polyethylene (PE)/Polypropylene (PP) and Polyethylenterephthalat (PET) microplastic particles in the Baltic Sea in different meteorological seasons. The results are based on simulations with a 3D hydrodynamic model using meteorological data of the years 2016/2017. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources. **(A,B)** cover the 20–500 µm size fraction; **(C)** focusses on the small 20–200 µm fraction and **(D,E)** address the larger fraction (200–500 µm), but separate between spherical and fiber shaped particles. The Box-Plots indicate the median, the upper and lower quartile and 2*standard deviation as well as extreme values.

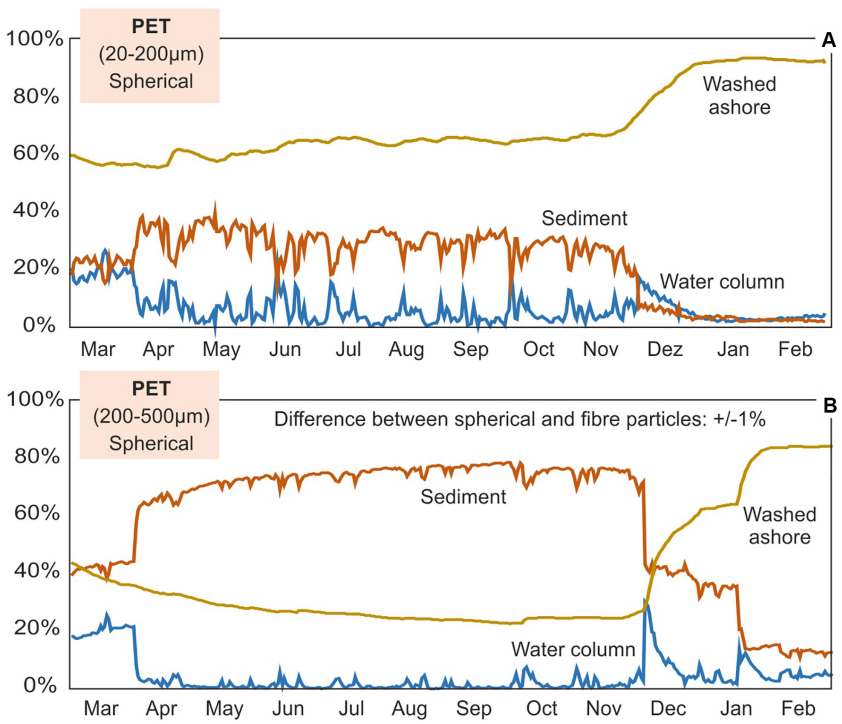


FIGURE 4 | Relative shares of emitted Polyethylenterephthalat (PET) microplastic particles in the water column, on the sediment surface and washed ashore during the course of a year. The results are based on simulations with a 3D hydrodynamic model using meteorological data of the years 2016/2017. The emissions cover all three urban sources and spherical particles of the size fractions **(A)** 20–500 µm and **(B)** 200–500 µm.

summer, and autumn about 30% of the smaller fraction are accumulated at the sea bottom, this is true for over 70% of the larger fraction. Over 60% of the small fraction and less than 30% of the large fraction are washed ashore. This pattern changes in winter. Storms cause a resuspension of particles accumulated on the sediment surface and wash them ashore during winter. Smaller particles nearly entirely end-up at the shore, while a share of about 10% of the larger PET particles stays on the sea bottom. It is likely, under our assumptions of a high shoreline-accumulation efficiency, that very heavy storms re-suspend practically all deposited plastic, cause a sediment surface cleaning, temporally high concentrations in the water column and a sub-sequent shoreline pollution.

Our model approach suggests that plastic entering the Baltic Sea does not stay long in the sea. The relatively short residence times of both very different plastic polymers suggests that this is true for other plastic polymers with a comparable density, as well.

Spatial Pattern in Sediments and the Water Column

We focus the spatial model results in terms of the horizontal patterns for the selected particle types. Our 3-D model also provides vertical distributions in the water column, but these are shown in the online supplement for selected stations.

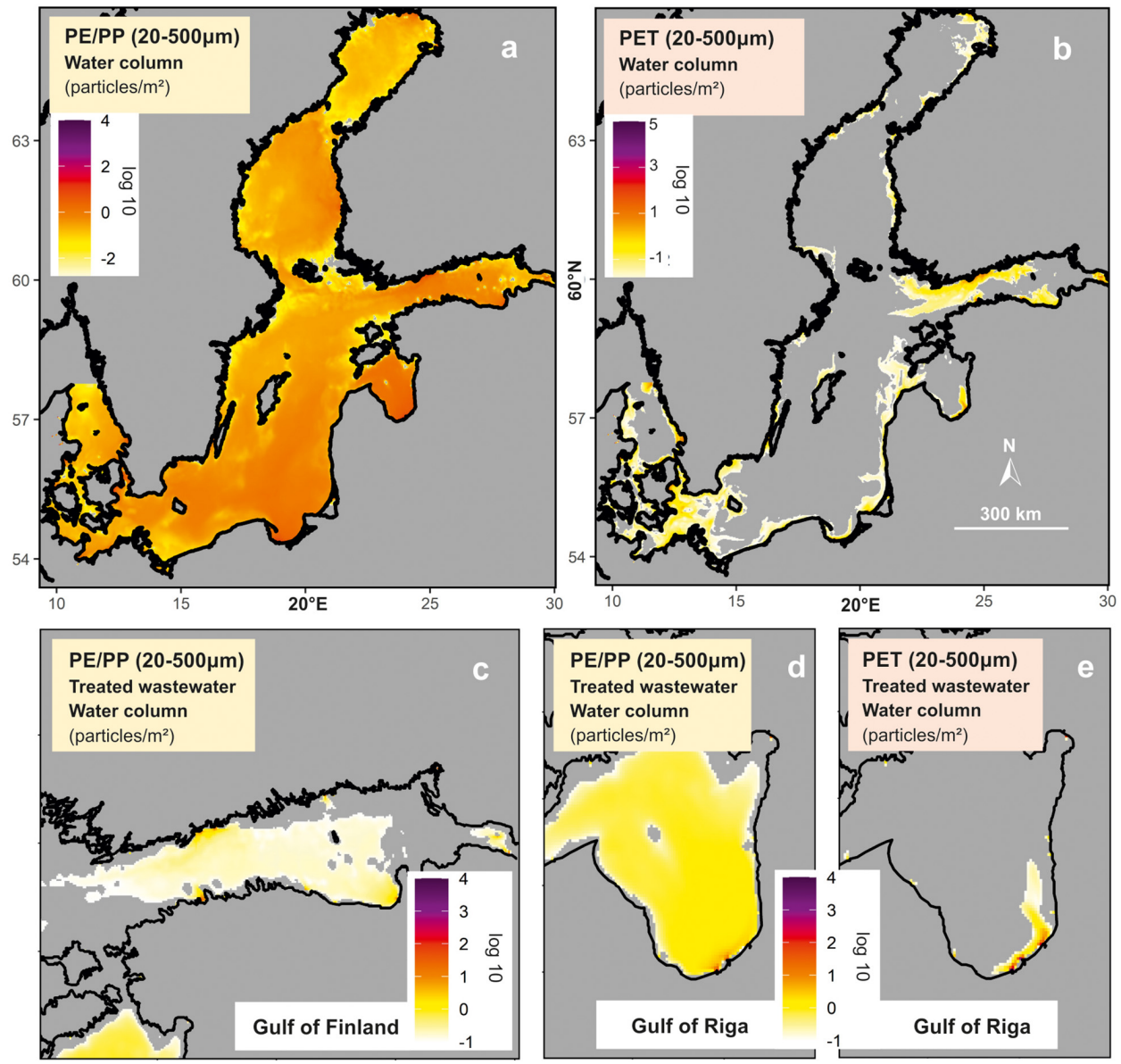


FIGURE 5 | Spatial concentration of Polyethylene (PE)/Polypropylene (PP) **(a)** and Polyethylenterephthalat (PET) **(b)** microplastic particles (20–500 µm size fraction) in the water column of the Baltic Sea and in selected sub-basins **(c–e)** based on simulations with a 3D hydrodynamic model. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources. Gray areas in the sea indicate concentration below the color scale.

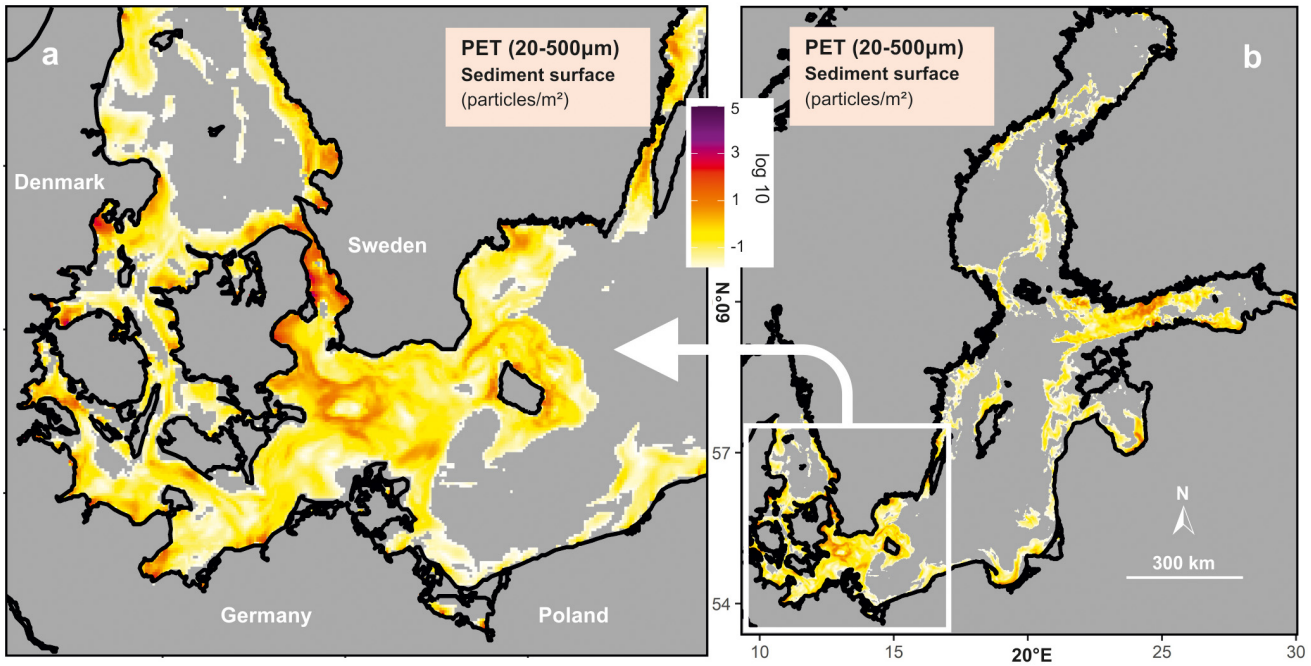


FIGURE 6 | Spatial concentration of Polyethylenterephthalat (PET) microplastic particles (20–500 µm size fraction) at the sediment surface of the Baltic Sea **(a)** and in the western Baltic Sea **(b)** based on simulations with a 3D hydrodynamic model. PET represents sinking plastic. The emissions cover all three urban sources. Gray areas in the sea indicate concentrations below 0.01 particles/m^2.

Polyethylene (PE)/PP with its low density is floating and as consequence a certain amount of the emitted particles are distributed all around the Baltic Sea (**Figure 5**). Our model simulations suggest that there is hardly any area in the Baltic Sea without microplastics pollution. Wind-driven currents spread particles within a few weeks even to central and remote sea regions. However, the highest concentrations are observed near the emission points and in semi-enclosed bays with high emissions and restricted water exchange. The Gulf of Riga is an example for that (**Figure 3E**). Because of the short residence time of PE/PP particles, already after a few months, the model shows stable conditions, and rough estimates for PE/PP and PET particle concentrations for different sea areas can be provided. The calculated average over the entire Baltic Sea water body is 1.4 PE/PP particles/m^2 sea surface during summer. In the central parts of the Gulf of Gdańsk and Gulf of Riga the annual average concentrations are around two PE/PP particles/m^2 and in the central Gotland and Arkona Sea as well the Gulf of Finland the concentrations of particles that entered via the investigated pathways are close to 0.5 PE/PP particles/m^2 in the water column. The concentrations refer to PE/PP from urban sources only.

Polyethylene terephthalate with its higher density behaves differently and emitted particles are sinking soon after entering the sea. As a consequence, PET in the water column is only observed near emission spots and in a near-shore belt around the Baltic Sea. In sheltered areas, such as the Gulf of Riga (**Figure 2E**), where wind and wave energy and subsequent resuspension is lower, PET is not transported far into the sea but spread with the currents along the coast. The model suggests an average concentration in the Baltic Sea water body of 0.7 PET particles/m^2 sea surface in summer. However, in all central sea areas and even in central parts of the Bay of Riga and the Bay of Gdańsk concentrations stay below 0.001 PET particles/m^2 sea surface in the annual average.

Polyethylene (PE)/PP is floating, kept in the water column and not accumulated on the sediment surface. PET particles are deposited at the sediment surface soon after entering the sea. As a consequence, PET is accumulated in a coastal water belt around the Baltic Sea. Strong local accumulations especially take place, in sheltered areas, such as the Gulf of Riga (**Figure 2E**), where wind and wave energy and subsequent resuspension is limited. The model suggests an average concentration in the Baltic Sea water body of nearly four PET particles/m^2 sediment surface during summer. In Øresund, the strait between Denmark and Sweden, concentrations above 10–20 PET particles/m^2 sediment surface can be expected on annual average (**Figures 6a,b**).

Spatial Accumulation Pattern at Shores

The vast majority of emitted PE/PP is washed ashore in the first few kilometers around the emission source (**Figure 7**). At the coasts around St. Petersburg above 10^8 particles/m are washed ashore every year according to our model approach. The example of Riga Bay with the river Daugava and the cities Riga and Jūrmala as major emission sources, show annual accumulations up to or slightly above 10^8 particles/m, as well, but high accumulation rates are limited to a 10 km stretch of coastline (**Figure 7B**). The mouth of the Odra/Oder river in the Szczecin/Oder Lagoon,

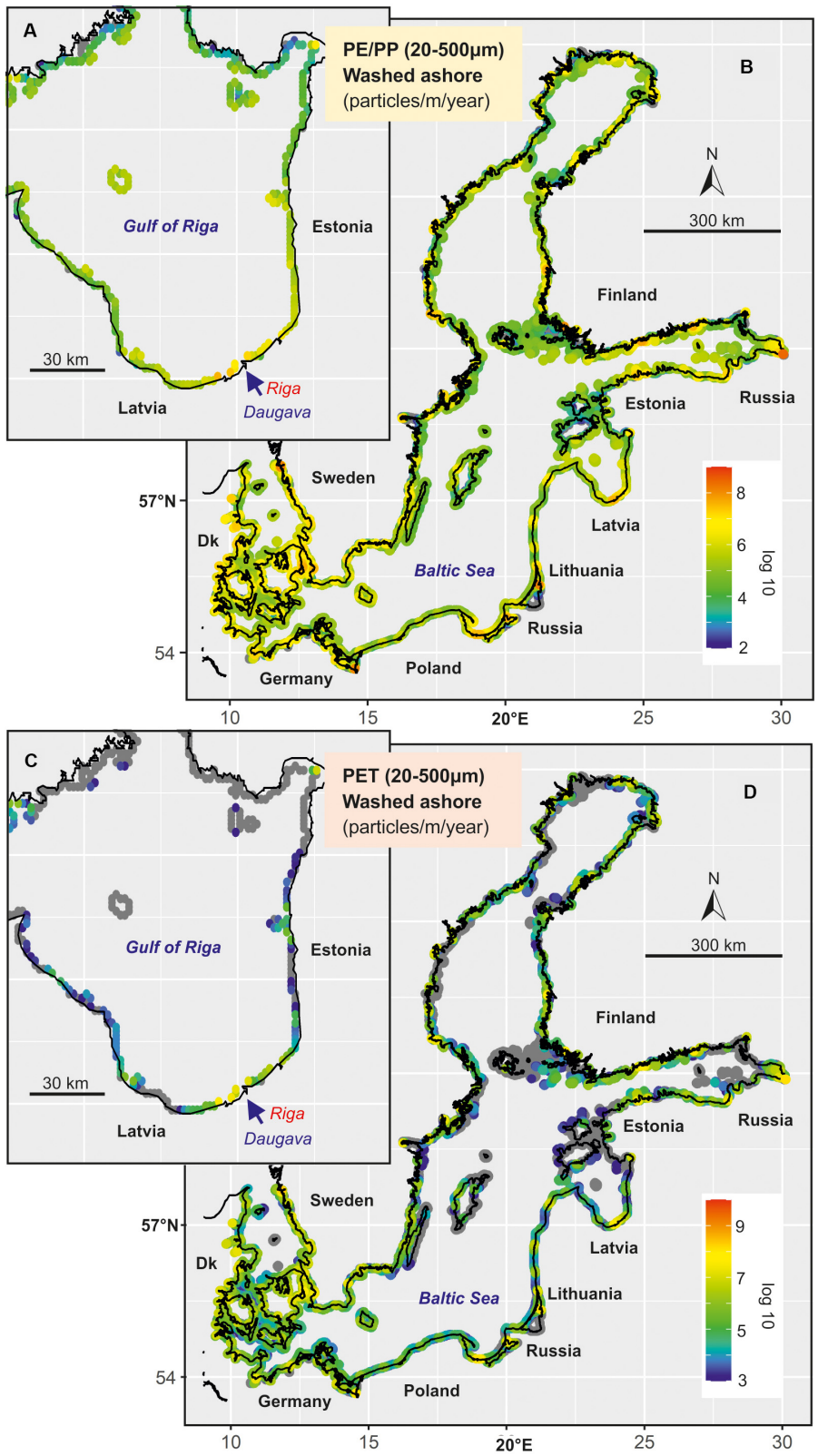


FIGURE 7 | Accumulation of Polyethylene (PE)/Polypropylene (PP) and Polyethylenterephthalat (PET) microplastic particles (20–500 µm size fraction) at shores of the Baltic Sea **(B,D)** and in the Gulf of Riga **(A,C)** based on simulations with a 3D hydrodynamic model. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources.

the Curonian Lagoon near Klaipeda, the Vistula Lagoon near Kaliningrad, the Gulf of Gdańsk coast as well as fjords near Gothenburg, Stockholm, Helsinki are predicted as other hot-spots with annual accumulations of up to 10^8 particles/m^2 coastline. Our model suggests that few remote sheltered areas in the Bothnian Sea and Bay do not face significant accumulation. For PET, this pattern is even more pronounced and an even higher share is accumulated in the vicinity of the emission sources. The accumulation hot-spots of PET and PE/PP are the same and the annual accumulation numbers are comparable to PE/PP (**Figure 7D**).

In detail, bottom morphometry, coastline structure, shelter, and exposition play a very important role for the accumulation of micro-litter at the coast. As a consequence, a strong spatial patchiness is predicted. The limitations of the model approach and the spatial resolution of one nautical mile do not allow detailed analyses of pollution gradients and small scale pattern in the vicinity of single emission sources. The model suggests very high beach accumulations in nearly closed and semi-closed systems, such as lagoons and fjords. It is very likely that these systems serve as important sinks for microplastics and reduce the pollution of the Baltic Sea. Their 'cleaning' efficiency should depend on several factors, and the water exchange rate should be of major importance. However, our present approach does not allow a reliable quantification of this retention.

Realistic Sewer Overflow Emission Pattern

In the previous scenario simulations, emissions from CSS were calculated on an average annual basis. However, it is known that overflow emissions are associated to heavy rain event and that these events are not evenly distributed throughout the seasons of a year. **Figure 8A** gives an overview about the average monthly spatial distribution of heavy rain events in the Baltic Sea catchment for selected months. Between December and April, the likelihood of heavy rain events is close to zero in the entire Baltic Sea catchment. This likelihood increases in spring until July and August and decreases in autumn again. In the north-western catchment (Germany, Denmark, Sweden), the maritime climate causes a likelihood of heavy rains above 10% in all months between May and November. In contrast, the continental climate in south-eastern countries (Poland, the Baltic States, Belarus, and Russia) is responsible that about 50% all heavy rains of a year take place in July and August. This has consequences on the CSS emissions. **Figures 8B,C** show the resulting hypothetical monthly emissions of microplastics particles to the Baltic Sea exemplary for May and July. Especially in the south-eastern Baltic, the emissions of PE/PP in July and August from CSS account for up to 50% of the annual CSS emissions. CSS is by far the most important pollution pathway for PE/PP and the same is true for PET, if the assumption of 50% combined treatment of rain- and wastewater is correct. As a consequence, our results suggest that in the south-eastern Baltic, more than 1/3 of the total annual microplastics emissions take place during July and August. Our results are based on several assumptions and simplifications and the results have to be treated with care. However, it is very likely that microplastics emissions show a strong seasonality and that this seasonality differs between the Baltic Sea regions.

DISCUSSION

Uncertainties and Limitations

We used the present knowledge about location and emissions from WWTPs, CSS systems including stormwater and untreated wastewater (not connected to WWTPs) in the entire Baltic Sea region, estimated the emissions of PET and PE/PP based on a comprehensive literature search and most recent publications and applied an up-to-date 3D model approach. Our approach refines existing microplastics mass emission estimates for the Baltic Sea (Siegfried et al., 2017; Bollmann et al., 2019). However, the uncertainties of our approach are very high. Uncertainties related to the emissions of microplastic from WWTPs, CSS systems, and untreated wastewater are discussed in Baresel and Olshammar (2019). Taking into account the wide range of PP/PE and PET concentrations in raw wastewater found in the literature, the calculated specific emissions can over or underestimate the reality easily by ± one order of magnitude. Talvitie et al. (2015), for example, report extreme values above 600,000 and Simon et al. (2018) even above 7,000,000 microplastics particles/m^3 in raw water.

Apart from two simulations, we do not distinguish between fibers, fragments, beads, spheres, flakes, and films. The share between fibers and other fragments is based on literature and uncertain. The same is true with respect to different microplastics size fractions. It is well known, that beside density, shape and size play an important role for the sinking velocity (e.g.,; Kowalski et al., 2016; Kooi and Koelmans, 2019). Waldschläger and Schüttrumpf (2019a; 2019b) provide an overview of data and formulas, which were adapted by us. However, differences in shape and size did not play an important role for the temporal and spatial resolution we used in our model approach. As soon as the behavior of particles within days and transport on a spatial scale of meters are in the research focus, size and shape need to be taken into account. Further we do not assume that during the relatively short transport time of weeks in the environment, plastic particles are significantly modified in their properties (e.g., density changes because of bio-films or aggregation).

Several publications deal with retention in rivers (e.g., Nizzetto et al., 2016; Besseling et al., 2017; Kooi et al., 2018) which depends on particle size, shape and density. Besseling et al. (2017) carried out scenario studies with a hydrological model and conclude that in 40 km river practically all particles [> 100 μm spherical polystyrene (PS)] are kept back. But this study does not cover a long time period and it remains unclear what happens to sedimented plastic particle in river beds during flood events. However, some studies take retention into account in emission calculations (e.g., Siegfried et al., 2017) and in others do not (e.g., van Wijnen et al., 2019). Windsor et al. (2019) state that still little is known about the residence time of plastics in rivers and

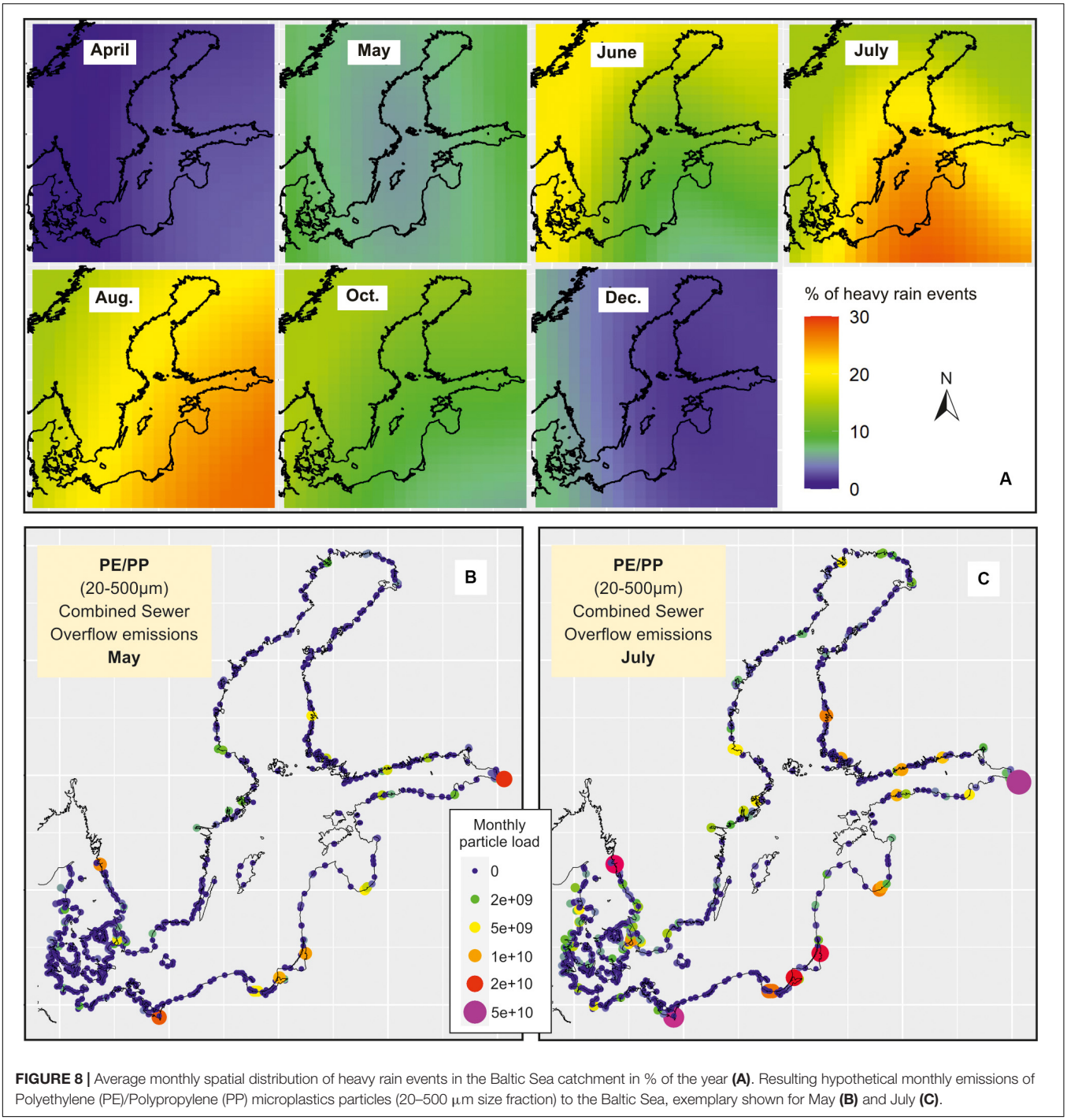


FIGURE 8 | Average monthly spatial distribution of heavy rain events in the Baltic Sea catchment in % of the year **(A)**. Resulting hypothetical monthly emissions of Polyethylene (PE)/Polypropylene (PP) microplastics particles (20–500 µm size fraction) to the Baltic Sea, exemplary shown for May **(B)** and July **(C)**.

their role as temporary sinks. Further, our own unpublished data of the Warnow River does not indicate a significant retention. To our mind, field data is still too incomplete and contradicting (e.g., Dikareva and Simon, 2019; Wagner et al., 2019; Schmidt et al., 2020) to enable the calculation of retention rates that can be used in a reliable way in emission calculations on large spatial scales. We assume that degradation during the transport is unlikely and permanent sinks (e.g., lakes and reservoirs) are of minor and/or only temporal relevance. However, the application of a retention factor would affect the amount of emissions and the spatial emission pattern. The calculation of monthly sewer overflow emissions based on the monthly likelihood of heavy rains is a simplified and theoretical approach. It can solely provide a rough impression of the average seasonality of emissions.

Model approaches and simulations always provide a simplified picture of the reality. In general, the spatial transport pattern in the open sea are much more reliable compared to the microplastics accumulation pattern at the coast. The spatial

and temporal resolution of the weather input data has, especially during extreme events, significant effects on transport, resuspension and accumulation. We used the best available data, but are aware of the limitations. The model grid with a size of about 1.8 km edge length reflects only a simplified coastline and does not represent the morphometry of smaller structures, such as lagoons and fjords.

Our most simplifying assumption in the model is that particles that enter a grid cell adjacent to land are assumed to be washed ashore immediately. This means that near-shore processes are neglected. It is obvious that the surf-zone physics cannot be dynamically represented in a model with our grid resolution, so the effects of, e.g., wind speed and wave height on the accumulation efficiency are not taken into account in the model. The result might be an overestimation of beach accumulation efficiency and a corresponding underestimation of open-water microplastics concentrations, residence times, and sediment concentrations. To quantify the impact of this simplification on the overall results, future studies should investigate the sensitivity of the model to the chosen beach accumulation rate in dedicated sensitivity experiments. A resuspension from the beach after a previous accumulation is not assumed, but it is known that especially particles with a density above 1 g/cm^3 can travel along the coast, before strong wave action causes a permanent burial at the beach. The model assumes that particles can be washed ashore at all kinds of coasts, even the rocky coasts of Scandinavia. Some additional uncertainty is added by the need to translate the number of accumulated particles in the coastal model cell into accumulated particles per meter of coastline, whose length in the model (where it is straight in every single grid cell) may differ from reality.

Representativity

In a global review, Boucher and Friot (2017) assessed the primary microplastics sources in the Oceans and conclude that 98% of the emissions result from land-based activities. Beside tire ware, urban wastewater can be considered as one of the major sources of microplastics (e.g., Dris et al., 2018) world-wide (Boucher and Friot, 2017) and in the Baltic Sea region (Siegfried et al., 2017). It is very likely that our emission scenarios reflect the most important inputs to the Baltic Sea.

A recent study in China (Ly et al., 2019) analyzed raw wastewater and found the following polymer shares: PET (47%), PS (20%), PE (18%), and PP (15%). However, the shares of polymers in wastewater vary in a wide range. An indication which plastic polymers can be expected in the aquatic environment provide the production volumes. PE, PP, PVC, PS, and PET belong to the six most commonly produced polymers worldwide (Vermeiren et al., 2016; Geyer et al., 2017; Kooi et al., 2018). We used PE, PP, and PET as exemplary polymers, because of abundance in the environment and because they cover a density spectrum from around 0.9 to 1.3 g/cm^3. We can assume that PET, PE, and PP, and the size-class up to 500 μm, which are considered in our study constitute to over 50% of the total microplastics emissions into the Baltic Sea, when neglecting road runoff (tires).

With respect to the behavior in the marine environment, a differentiation between low and high density PE (0,915–0,97 g/cm^3) and PP (0.89–0.92 g/cm^3) is not necessary, because it does not affect the spatial pattern and in the water column and coastal accumulation significantly. This is at least true for our spatial model resolution. The results of the polyester PET can be transferred to PVC (1.38 g/cm^3), polyvinyl acetate (1.19 g/cm^3), polyamide (1.14 g/cm^3) as well, because of a comparable density. Our simulations show only minor differences between fibers and spherically shaped particles. However, original polymers and the final processed plastic products often show very different densities and behaviors in the sea. In general, the results give an impression, how other common polymers, such as PS (0.96–1.05 g/cm^3) and polyurethane (PU; 1.2 g/cm^3) may behave in the marine environment, as well, but these polymers are often used in form of foams with densities below 0.1 g/cm^3. For plastic products below a density of about 0.8 g/cm^3 our simulations are not representative, because with decreasing density, transport and accumulation is increasingly determined by wind instead of water currents.

Our model approach shows a strong accumulation of microplastics particles around the emissions pathways and in enclosed and semi-encloses coastal waters. Generally, this is well supported by literature. Gewert et al. (2017) found nearly ten times higher abundance of plastics near central Stockholm than in offshore areas. Yonkos et al. (2014) reported the highest microplastics concentrations near densely populated areas of Chesapeake Bay and comparable results exist for other estuaries and lagoons (Vianello et al., 2013; Song et al., 2015; Vermeiren et al., 2016; Blašković et al., 2018; Gray et al., 2018; McEachern et al., 2019). A concrete comparison of our results with field data is problematic, because we restrict ourselves to only three polymers but cover the 20–500 μm particle size class. Field studies often address the size fraction above 333 μm, using a phytoplankton net for sampling, and therefore did not collect the majority of microplastics (Setälä et al., 2016; Tamminga et al., 2018).

Similar to our results, Pedrotti et al. (2016) observed a differentiation between microplastic particles in the Mediterranean Sea, were the floating polymers PE, PP, and polyamides dominated at all distances off-shore and accounted for 86–97% of the total items. The concentration in water bodies vary in wide ranges and reflect the local emissions and the physical situation, such as the water exchange (e.g., Song et al., 2015; McEachern et al., 2019) and can hardly be compared to the Baltic Sea. McEachern et al. (2019) observed higher microplastic concentrations after intense rainfall events in the summer. This indicates the importance of stormwater and supports our results.

Vermeiren et al. (2016) and Wessel et al. (2016) consider beaches as a major sink for microplastic, as well, and burial at beaches as a potential mechanism for long term storage. Claessens et al. (2011) suggests that the depth profile of sediment cores from beaches may even reflect the global plastic production increase. At sandy shorelines in a northern Gulf of Mexico estuary microplastics was abundant and the concentrations were

66–253 times larger than reported for the open ocean (Wessel et al., 2016). A model study by Liubartseva et al. (2016) in the Adriatic reports a relatively short half-life time of microplastics in the sea of 43.7 days with a significant seasonality and considers the shoreline as major sink, too.

A large amount of literature about microplastics in marine sediments exists (e.g., Claessens et al., 2011; Willis et al., 2017; Blašković et al., 2018; Gray et al., 2018; McEachern et al., 2019). Microplastic is found in types of sediments and highest concentrations are observed near populated areas and near emission sources, especially in semi- and enclosed systems (Claessens et al., 2011; Vianello et al., 2013; Gray et al., 2018). Some authors consider the sediments even as major sink for microplastics (Boucher et al., 2019) and suggest that microplastics and fine sediments show similar sinking and accumulation behavior (Vianello et al., 2013). This questioned by other authors. Especially high loads of microplastics fibers on deep seafloor may be largely due to sample contamination (Willis et al., 2017). However, how long microplastics are accumulated on the seafloor depends on hydrodynamic conditions and turbulence. Our results indicate that PET is accumulating over months in shallow coastal waters. In the Baltic, these coastal waters usually show sandy sediments reflecting deep reaching wave induced turbulence and in the coastal Baltic Sea microplastic will hardly be able to accumulate over years.

All our results certainly reflect the specific conditions in the Baltic Sea. The enclosed character does not allow a washout into other seas, its shallowness favors wave-induced resuspension and its long and ragged coastline facilitates fast accumulation at beaches. However, the results are affected by the model approach and several simplifications as well.

CONCLUSION – PRACTICAL IMPLICATIONS

Despite all uncertainties and simplifications, the results allow some practical conclusions and recommendations. The short residence time of PP/PE as well as PET in the Baltic environment of estimated 14 days and the high annual accumulation rates calculated for shores indicate that most microplastics is not transported over long distances but washed ashore soon after the emission. A consequence is that beaches and shores are the major sink for plastic and that the highest pollution takes place close to the emission source. This means that a microplastic monitoring should focus on surface accumulations in the flood zone/tidal seam of beaches and that sampling should take place in the surrounding of emission sources. To catch the spatial gradients, several sampling spots along a beach with increasing distance from the emission source are recommendable.

The model calculates the highest particle accumulations at the shores of semi- or enclosed water bodies, such as fjords, bays, and lagoons. It seems that these systems serve as effective sink and retention unit for microplastics and protect the open Baltic Sea from pollution. To get a better insight into this retention function and capacity a monitoring of these systems is especially recommended.

Our simulations suggest that microplastic particles on the sediment surface stay only for weeks and few months. They are presumably washed ashore after each storm event because of wave-induced resuspension and subsequent accumulation at the coast. A sampling of the water column or the surface or the sediment surface of the coastal sea is less recommendable, because it only reflects concentrations resulting from the most recent emission events and the concentrations largely depend on previous weather conditions as well as resuspension events. Because of hydrodynamic conditions, bottom bathymetry, water depths, and the course of coastline, concentration patterns show a strong spatial variability. From our point of view, single observed concentrations in the water column or on the sediment surface can hardly be regarded as spatially or temporally representative and cannot serve as an indicator for the general state of pollution, at least in the Baltic Sea. This is especially true when additionally taking into account the methodological problems and uncertainties associated to existing field data.

It is well known that nutrients entering the Baltic Sea have a residence time of around 30 years and emission reductions only cause a slow recovery from eutrophication. This is different with respect to microplastics particles. Their short residence time in the Baltic Sea means that concentrations observed in the water body and on sediment surfaces reach a steady state already after a few months. Therefore, microplastics emission reductions would have an immediate positive effect on the pollution of the Baltic Sea. Our simulations suggest that preventing all emissions would result in a practically microplastics unpolluted sea already within 1 year. The coastal areas would contain and absorb the overwhelming majority of the microplastics loads.

Combined sewer overflow systems seems to be not only the most important quantitative urban emission pathway, but seems to have a strong seasonality. The summer months, especially in the south-eastern Baltic Sea seems to be most important with respect to emitted amounts. To efficiently reduce the microplastics load into the Baltic Sea requires measures tackling CSS as a major pathway. High emissions after heavy rain events and subsequent accumulations at nearby beaches call for a complementary event based monitoring. An event-based monitoring is recommended after heavy storms, as well, because a large share, even of heavier particles will be washed ashore. A flood zone beach monitoring after storms will provide a good insight into type and abundance of polymers polluting the Baltic Sea.

The majority of plastic polymers have a density between 0.8 and 1.5 g/cm^3. Of course, density, size, and shape do affect their behavior in the Baltic Sea, but on a time-scale of months not too much. Proximity to the emission source appears to be the most important driver for differences in beaching rates for every particle class investigated. This has two implications: A few polymer-types, e.g., PET, PE/PP, can serve as indicators for the state of pollution. If the emissions of other plastic polymers

are known, the overall pollution of different parts of aquatic environment can be estimated based on these few indicator polymers because spatial distribution pattern can be calculated based on the model results. If the size of particles, on time-scales of months, does not play a major role, then larger particle can be used as indicators as long as it is known that they originate from the same sources (such as a WWTPs) and the ratio between their emission rates remains stable. The large micro- (1–5 mm) and mesoplastic size fraction (5–25 mm) can be easily monitored in a flood zone and the observed larger polymer types can be determined at relatively low costs. The concentration of these larger particles can potentially be used as indicator for the pollution with microplastic particles of a size below 1 mm. We encourage future studies to look further into the feasibility of using larger plastic particles as pollution indicators.

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

GS developed the manuscript concept and the emission scenarios, took care of data analyses, and did the manuscript writing. HR carried out the model simulation and the data processing. RH prepared the input data, carried out a literature analysis, and provided complementary calculations for the model emission scenarios. CB and MO compiled and provided all data about the sewage treatment plants and emission calculations. RO provided the model setup. SO coordinated the overall work within project MicroPoll and contributed comments and proofreading. 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/fenvs.2020.579361/full#supplementary-material

FIGURE S1 | Western Baltic: emissions of Polyethylene (PE)/Polypropylene (PP; **b)** and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) from all urban sources to the Baltic Sea.

FIGURE S2 | Gulf of Finland: emissions of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) from all urban sources to the Baltic Sea.

FIGURE S3 | Gulf of Riga: emissions of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) from all urban sources to the Baltic Sea.

FIGURE S4 | Western Baltic: spatial concentration of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) in the water column of the Baltic Sea. The emissions cover all three urban sources. Gray areas in the sea indicate concentration below the color scale.

FIGURE S5 | Gulf of Finland: spatial concentration of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) in the water column of the Baltic Sea. The emissions cover all three urban sources. Gray areas in the sea indicate concentration below the color scale.

FIGURE S6 | Gulf of Riga: spatial concentration of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) in the water column of the Baltic Sea. The emissions cover all three urban sources. Gray areas in the sea indicate concentration below the color scale.

FIGURE S7 | Spatial concentration of Polyethylenterephthalat (PET) microplastic particles (20–500 mm size fraction) at the sediment surface of the western Baltic Sea **(a)**, the Gulf of Finland **(b)** and the Gulf of Riga **(c)** based on simulations with a 3D hydrodynamic model. PET represents sinking plastic. The emissions cover all three urban sources. Gray areas in the sea indicate concentrations below 0.01 particles/m^2.

FIGURE S8 | Western Baltic: accumulation of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplasticparticles (20–500 μm size fraction) at shores based on simulations with a 3D hydrodynamic model. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources.

FIGURE S9 | Gulf of Finland: accumulation of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) at shores based on simulations with a 3D hydrodynamic model. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources.

FIGURE S10 | Western Baltic: accumulation of Polyethylene (PE)/Polypropylene (PP; **b**) and Polyethylenterephthalat (PET; **a**) microplastic particles (20–500 μm size fraction) at shores based on simulations with a 3D hydrodynamic model. PE/PP represents floating and PET sinking plastic. The emissions cover all three urban sources.

FIGURE S11 | (a) Sinking velocities and critical shear stress: Both sinking velocities and critical shear stresses for microplastics particles depend on viscosity, therefore they vary with temperature. The example shows values for 10°C water temperature. **(b)** Vertical profiles of MP distribution: The following image shows mean vertical profiles (March 2016–February 2017) for two different types of microplastics for station HELCOM121 (Gulf of Riga, 23.617°E, 57.6167°N) as an example.

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Fluorescent Microplastic Uptake by Immune Cells of Atlantic Salmon (*Salmo salar* L.)

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The ubiquitous presence of microplastics and their marine ecotoxicity are major public concerns. Microplastics are ingested accidentally by the marine fauna or are taken up indirectly through the food chain. These particles can accumulate in cells and tissues and affect the normal biological functions of organisms, including their defense mechanisms. There is limited information available about the response of immune cells to microplastics; the degree of uptake by the cells, the response of different organs or the impact of environmental concentrations of microplastic are matters that remain unclear. Moreover, very little is known about the toxicity of different polymer types. This study aimed to shed light on the physical impact of small microplastics (1–5 μm) on cells from Atlantic salmon. Immune cells from intestine, blood, and head kidney were exposed to green fluorescent polyethylene microplastic (PE-MP), yellow fluorescent polystyrene microplastic (PS-MP) and both. High (50 mg/L), medium (5 mg/L), and low (0.05 mg/L) concentrations were tested for 1, 24, 48, and 72 h to study cell mortality and microplastic uptake. Quantitative data of microplastic uptake by fish immune cells were obtained for the first time by imaging flow cytometry. Salmon immune cells showed a relatively low ability to phagocytose microplastics. Less than 6% of the cells ingested the particles after 48 h of exposure to high concentrations. Cells also phagocytosed microplastics at low concentrations although at low rates (<0.1%). PE-MPs was phagocytosed by higher percentage of cells compared to PS-MPs and the former bioaccumulated in time while the latter decreased over time. However, each cell generally phagocytosed more PS-MPs particles than PE-MPs. Cells from different tissues showed different responses to the microplastic polymers. In conclusion, this study shows that immune cells of Atlantic salmon can phagocytose microplastics, and the impact is dependent on the microplastic type. PE-MPs, the most abundant polymer in the oceans and a widely used plastic in salmon aquaculture, was more easily taken up than PS-MPs. Furthermore, the study demonstrates how imaging flow cytometry can be applied in microplastics research.

Keywords: microplastic (MP), ecotoxicology, phagocytosis, imaging flow cytometry (IFC), Atlantic salmon (*Salmo salar* L.), polystyrene (PS), polyethylene (PE), immune cells

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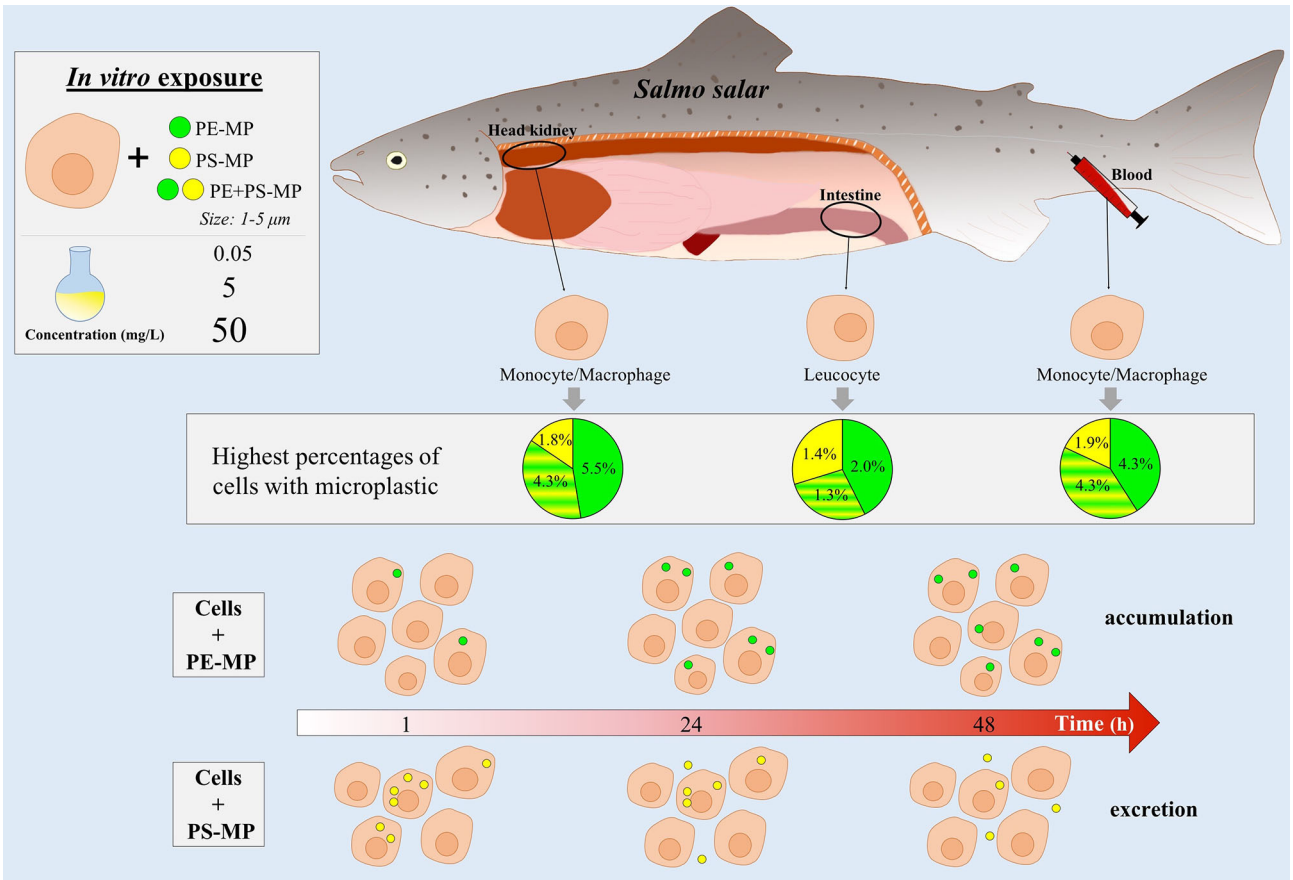

GRAPHICAL ABSTRACT |

INTRODUCTION

The increasing presence of microplastics (MPs) in the marine environment is of global concern. MPs are defined as plastic particles smaller than 5 mm (Arthur et al., 2009), although it has been recently proposed to redefine the limits of MPs to 1 to <1,000 μm to fit the SI nomenclature and for a better consensus among scientists (Hartmann et al., 2019). MPs in the marine environment mainly come from the degradation and fragmentation of different plastic items (GESAMP, 2015), resulting in rough and irregular shaped particles (Choi et al., 2018). Plastic debris mainly consist of fossil fuel-derived synthetic polymers, which can be classified into thermoplastic and thermoset (UNEP, 2016). Thermoplastic polymers are the most abundant plastic types found in the marine environment; a study conducted in 2014 found that almost half of the MPs collected in the North Atlantic ocean was polyethylene (PE) and polypropylene (PP) (Enders et al., 2015). Similarly, a study carried out in 2015 found that PE accounted for 73% of the small MPs (20–999 μm) collected in the North Atlantic Subtropical Gyre, followed by PP, PVC, and PS (Ter Halle et al., 2017). Other synthetic polymers such as polyethylene terephthalate (PET) and polyamides (PA) are also widely detected in the marine environment (Suaria et al., 2016; Erni-Cassola et al., 2019).

MPs are ingested by a wide range of marine organisms (Lusher et al., 2017b; Alimba and Faggio, 2019). Particles can accumulate in the digestive tract of animals and affect, among other parameters, their behavior, growth and survival (Von Moos et al., 2012; Choi et al., 2018; Foley et al., 2018; Assas et al., 2020). For example, a 7-days exposure of zebrafish (*Danio rerio*) to 5 μm PS-MPs resulted in their accumulation in the gills, liver, and gut of the fish, leading to increased oxidative stress; underlining the potential toxic effect of the MP (Lu et al., 2016). A few studies have documented that fine MPs can adhere to tissues [for example fish skin] and translocate across living cells into the circulatory system, dispersing the particles to other organs (Wang et al., 2020) and impacting the health of the cells and the immune system of the organisms (Wright and Kelly, 2017). Small MPs (<3 μm) have been found in both the digestive tract and muscles of painted comber (*Serranus scriba*) fish collected in the coast of Tunisia (Zitouni et al., 2020). MPs can affect several immune parameters such as the phagocytic activity, respiratory burst and the expression of immune-related genes in fishes (Espinosa et al., 2017; Choi et al., 2018). Therefore, it is important to study

the impact of MPs on fish immune cells. An important aspect to consider when studying the toxicity of microplastics is the polymer type. The type of polymers ingested by an organism depends on its ecological niche. Pelagic fish tend to ingest low-density polymers since they normally float in seawater, while demersal fish preferably ingest polymers with higher densities than seawater as such particles sink and are mainly present in deep waters (Bråte et al., 2017). A few studies have reported that different MP polymers induce different toxicities in fish. A study on fathead minnow (*Pimephales promelas*) showed that both PS and PC nanoparticle aggregates (41 nm−1,710 μm) induced degranulation of primary granules of neutrophils, but only PC nanoparticles affected the oxidative burst of the cells (Greven et al., 2016). PVC-MP and PE-MP (40–150 μm) induced different responses in gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*); while PVC-MPs decreased the phagocytic capacity of leucocytes, high concentrations (100 mg/ml) of PE-MPs significantly increased oxidative stress (Espinosa et al., 2018). In zebrafish, 10 days of exposure to 10 mg/L of PP-MP decreased the survival rate of the fish by 27%, while exposure to the same concentration of PA-MP, PE-MP, PVC-MP, and PS-MP (70 μm) did not affect survival (Lei et al., 2018). Recently, a study on Japanese medaka (*Oryzias latipes*) showed that environmental concentrations of a mixture of virgin PE-MP and PP-MP (∼400 μm in size) affected development and behavior of the larvae. After 30 days of exposure to the plastic mixture, fish had a higher head/body length ratio than the control group, and their swimming speed was also affected (Pannetier et al., 2020). These findings highlight the need to study polymer-specific impacts on fish immunity.

Even though publications related to MP accumulation in fish and invertebrates have increased considerably since 2010 (Lusher et al., 2017b), research into how MPs affect living organisms is still in its infancy, and many questions remain unanswered. For instance, the effects of various types of polymers, the degree of uptake of MP by immune cells, the response of different tissues and organs to MP, and the response of the organisms to environmentally relevant concentrations of MPs, have to be delineated through further research.

In this work, we studied the impact of two MPs, PE and PS, on Atlantic salmon (*Salmo salar* L.), the commercially most important farmed marine fish species (FAO, 2018). A recent study revealed that PE accounted for ∼40% of the MPs ingested by five fish species from the North and Baltic Sea (Rummel et al., 2016). Moreover, marine litter in Arctic, North Atlantic, North Sea, Skagerrak, and Baltic Sea is dominated by PS plastic (Strand et al., 2015). In addition, the weathering of plastic equipment used in aquaculture, such as cage floats or feed pipes that are mainly made of PE, is littering the marine environment (Lusher et al., 2017a). Hence, it is important to understand how Atlantic salmon, a farmed species that is produced mainly in open sea cages, is affected by PE and PS. We examined the physical impact of MPs in terms of uptake by cells. For this purpose, immune cells from three tissues, distal intestine (DI), blood, and head kidney (HK), were exposed to two types of polymers, PE-MP and PS-MP; either to each polymer singly or to a combination of the two, at three concentrations (50, 5, and 0.05 mg/L) for 1, 24, 48, and 72 h. The DI is the main entry point of MPs in the body, and blood cells can act as vehicles to carry MPs to other organs (Ding et al., 2018). The HK is one of the main organs where the hematopoiesis occurs and it is considered as a lymphoid organ, where immune cells are produced (Zapata et al., 2006; Whyte, 2007). After the exposure of leucocytes to the MPs, the mortality of the salmon immune cells as well as their phagocytic ability and capacity to ingest MP were determined by employing imaging flow cytometry. The present study sheds light on (1) the quantitative uptake of MPs by fish immune cells, (2) the response of immune cells exposed to two different polymers, (3) the effect of relatively low MP concentrations on immune cells, and (4) the organ-specific response of immune cells to MP exposure.

MATERIALS AND METHODS

Microplastics and Reagents for Cell Studies

PS (2.1 ± 0.12 μm) and PE (1–5 μm) microspheres, were acquired from Magsphere Inc. (California, USA) and Cospheric (California, USA), respectively. PS-MPs had a more uniform sphericity than PE-MPs. The agglomeration rate of PS particles was 2.04 ± 2.12%, and of PE particles, 3.22 ± 1.32% (**Supplementary File 1**). In order to distinguish the two polymers, we obtained PS-MPs and PE-MPs that emit yellow and green fluorescence, respectively. For the exposure studies with each polymer, stock solutions (2.5 g/L, 250 mg/L, and 2.5 mg/L) were prepared prior to the experiments.

Leibovitz's L-15 Medium (L-15) (Sigma, Oslo, Norway), employed for cell isolation, was adjusted to a pH of 7.3 and osmolarity of 380 mOsm by adding a solution consisting of 5% (v/v) 0.41 M NaCl, 0.33 M $NaHCO_3$, and 0.66 (w/v) D-glucose, and supplemented with 100 μg/mL gentamicin sulfate (Sigma), 2 mM L-glutamine (Sigma), 15 mM HEPES (Sigma), and 50 U/mL penicillin, 50 μg/mL streptomycin (Sigma). An enriched L-15 medium supplemented with 2% fetal bovine serum and 10 U/mL heparin (L-15+) was used to isolate and culture the cells from DI, blood and HK tissues. Percoll solutions (Sigma) adjusted to 380 mOsm by adding NaCl were also prepared for the cell isolation process. DTT (0.145 mg/mL dithiothreitol + 0.37 mg/mL EDTA in Ca^{2+} & Mg^{2+} free HBSS, Sigma), DNAse I (0.05 mg/mL; Sigma) and digestive (0.37 mg collagenase IV/mL washing medium, Thermo Fisher Scientific, Oslo, Norway) solutions were employed to extract cells from the DI.

Leucocyte Harvesting From Atlantic Salmon

Atlantic salmon smolts (60 g) were procured from Sundsfjord Smolt, Nygårdsjøen, Norway and transferred to the Research Station of Nord University, Bodø, Norway. The fish were maintained in a seawater flow-through system (7–8°C) and fed on commercial feeds (Skretting AS, Stavanger, Norway) for several months. Fish in the size range 500–800 g were then employed for the study. The selected fish were starved for 24 h and euthanized with an overdose of MS-222 (Tricaine methane

sulphonate; Argent Chemical Laboratories, Redmond, USA; 160 mg/L) prior to cell extraction.

Immune cells from DI, blood and HK tissues were extracted as described by Attaya et al. (2018), Haugland et al. (2012), and Park et al. (2020), respectively, with some minor modifications. Briefly, 2 mL of blood from each individual was withdrawn with a heparinized syringe and mixed in 6 mL of L-15+. HK was dissected and placed in 4 mL of L-15+, and the cells were harvested by passing the tissue through a 40 μm cell strainer and washing twice with L-15+ by centrifugation (500 × g, 5 min, 4°C). The monocytes/macrophages from both the blood and the HK cell suspensions were then separated using Percoll 34/51% (by centrifugation; 500 × g, 30 min, 4°C) and then washed twice with L-15 by centrifugation (500 × g, 5 min, 4°C). The resulting cell layer was carefully collected and transferred to a petri dish for a 2-day incubation at 12°C to allow the macrophage-like cells to adhere. Thereafter, the supernatant was removed and the adherent cells on the petri dish were detached with ice-cold PBS supplemented with 5 mM EDTA. The collected cells were centrifuged (500 × g, 5 min, 4°C) and employed for the exposure experiment. As for the DI tissue, it was dissected out, washed with 1x PBS, and then incubated in 6 mL of DTT solution for 20 min at RT in order to remove as much mucus as possible. The tissue was washed with L-15+ supplemented with DNAse I to avoid cell clumping and remove excess DTT. The tissue fragment was then transferred into 6 mL of the digestive solution and placed on a shaking incubator (200 rpm) for 60 min at RT. The tissue and supernatant were then harvested by passing through a 100 μm cell strainer and the resulting cell suspension was washed twice with L-15+ by centrifugation (500 × g, 5 min, 4°C) and layered on a Percoll solution of 25/75%. After centrifugation (500 × g, 30 min, 4°C), the leucocyte-rich Percoll intermediate layer was collected, washed twice with L-15 by centrifugation (500 × g, 5 min, 4°C) and later used in the exposure experiment. For the exposure study, cells from 36 individuals were employed to prepare the 6 replicates of the experiment, each of which consisted of a random pool of cells from six fish.

Exposure Experiment

To understand the polymer-specific response of salmon immune cells, they were exposed to either PE-MP or PS-MP or a mixture of both (PE+PS-MP). The treatment employing a combination of PE-MP and PS-MP was selected to ascertain the synergetic or antagonistic effect of the two polymers. Three different concentrations of the polymer types were tested: high (50 mg/L), medium (5 mg/L), and low (0.05 mg/L) concentration. The low concentration was selected based on the highest concentration of MP reported for the North Pacific Gyre (California Current Ecosystem LTER and Goldstein, 2017). However, this concentration does not reflect the environmental concentration of the microplastic size used in this experiment since mesh size used for the sampling was 333 μm. The medium and high concentrations were higher than the concentrations found in the environment, but still lower than what is being used in several MP exposure experiments. A negative control (C), where cells were not exposed to MP, was also set up to ascertain the viability of the cells. DI (0.2×10^6 cells/100 μL), blood (0.5×10^6 cells/100 μL), and HK (0.5×10^6 cells/100 μL) cells were incubated in L-15+ along with the MPs at the different concentrations mentioned above, and the tubes with the cells were maintained at 12°C on a rotator (Rotator PTR-60, Grant-bio, Cambridge, IK) for 1, 24, 48, or 72 h. Six replicates per treatment were employed for the study.

After the exposure, the cells were analyzed by ImageStream$^{®X}$ Mk II Imaging Flow Cytometer (Luminex Corporation, Austin, TX, USA) as described by Park et al. (2020). Briefly, cells were washed by centrifugation (500 × g, 5 min, 4°C), resuspended in 50 μL of 1x PBS, and 1 μL of propidium iodide (PI, 1 mg/mL, Sigma) was added prior to loading the sample in the imaging flow cytometer to assess the mortality. A total of 30,000 images per sample were acquired with the 488 nm argon-ion laser set up at 0.15 mW to detect MP particles and dead cells. Cell mortality, phagocytic ability and phagocytic capacity were analyzed using IDEAS 6.1.822.0 software (Luminex) (Park et al., 2020). Cell mortality was determined as the percentage of cells with red fluorescence (stained by propidium iodide) among the total cells. The phagocytosis assay, consisting of phagocytic ability and phagocytic capacity, was used to study the uptake and bioaccumulation of MPs in the immune cells. The phagocytic ability was defined as the percentage of cells with MP particles inside (i.e., cells with green fluorescence, yellow fluorescence, or both). The phagocytic capacity was defined as the number of MP particles ingested by each phagocytic cell, which were detected by the function "spot count" of the IDEAS software. The phagocytic capacity was calculated and normalized using the following equation:

$$Phagocytic\ capacity = \frac{\sum_{i=1}^{7} \mathrm{i} \cdot \mathrm{Ci}}{\mathrm{n}} \cdot 100$$

Where *Ci* is the number of phagocytic cells with *i* phagocytosed particles and *n* the total number of phagocytic cells. Cells with surface binding particles were counted as phagocytic cells and included in the phagocytic ability and capacity data analysis. Surface binding is considered an early stage of the phagocytosis since it triggers the physical exploration of the particle by the extension of pseudopods by the cells, the intracellular signaling and eventually the internalization of the particle (Underhill and Goodridge, 2012).

Statistical Analysis

Statistical analyses were done with the software RStudio 1.1.463. Poisson regression models were fitted to the data on cell mortality, phagocytic ability and phagocytic capacity. This approach helped us to understand the relationship between the response variable and the predictor variables. Data from each tissue were analyzed separately and statistical differences were considered significant at $p < 0.05$.

For the cell mortality ($n = 6$), two factor regressions were carried out, considering *Time* (1, 24, 48, and 72 h) and *Treatment* (C, PE-0.05, PE-5, PE-50, PS-0.05, PS-5, PS-50, PE+PS-0.05, PE+PS-5, and PE+PS-50) as the predictor variables. The factors *Polymer type* and *Concentration* were grouped into *Treatment* to avoid incorrect comparisons. For both the phagocytic ability (*n*

= 6) and phagocytic capacity ($n = 6$), three factor regressions were carried out, considering *Time* (1, 24, and 48 h; 72 h was excluded), *Polymer type* (PE-MP, PS-MP, and PE+PS-MP) and *Concentration* (50, 5, and 0.05 mg/L) as the predictor variables. For the DI data, the *Concentration* 0.05 mg/L was excluded from both phagocytic ability and phagocytic capacity analyses due to the fewer number of samples with phagocytic cells.

For all three response variables and for each tissue, several Poisson regression models were fitted to detect the differences of interest. For this purpose, one specific level of each factor was fixed as first line in each model, with which the comparisons were made.

RESULTS

Cell Mortality

Cell mortality was assessed to examine the response of leucocytes to MPs, in terms of time, polymer type and MP concentration. MP type or concentration was not found to have a significant effect on cell mortality. On the other hand, time affected the cell mortality significantly, based on the statistical analyses described below (**Figure 1**; **Supplementary File 2**, **Supplementary Tables 1–3**).

The mortality of DI cells increased significantly at 24 and 48 h compared to 1 h ($p < 0.05$) in 90% of the cases, while only 40% increased at 72 h compared to 1 h ($p < 0.01$). On the other hand, mortality was higher at 72 h for blood and HK cells. In the case of blood, mortality at 72 h was significantly higher in almost 50% of the cases ($p < 0.05$), and 24 h was the timepoint with the lowest mortality. For the HK cells, the mortality increased significantly at 72 h compared to 1 and 24 h ($p < 0.05$) in 95% of the cases, and at 48 h the mortality was significantly higher than 1 h ($p < 0.05$) in 40% of the cases. Yet the mortality at this timepoint was still lower than at 72 h.

Considering the general trend in mortality, the 72 h timepoint was not considered for the subsequent studies.

Microplastic Uptake

Immune cells from all the three tissues (DI, blood, and HK) of Atlantic salmon phagocytosed PE-MPs and PS-MPs of 1–5 μm in size (**Figures 2–5**). Phagocytosis was documented at different concentrations including the lower MP concentration (0.05 mg/L) (**Figure 3**). Single cells from blood and HK, but not from DI, had the capacity to phagocytose both PE-MPs and PS-MPs simultaneously (**Figure 5**).

Phagocytic Ability to PE-MP and PS-MP

The ability of immune cells from all three tissues to phagocytose different MP polymer types is shown in **Figures 3**, **4**. The cells had higher phagocytic ability toward PE-MP than PS-MP, and in some cases the values for the former MP were six times higher than those observed for the latter polymer type. The phagocytic ability was found to increase with the MP concentration. In **Figure 3**, the phagocytic ability of the three concentrations at 48 h is shown, the timepoint with most distinct differential responses. Cells from all the three tissues presented higher MP uptake when the concentration increased independently of the polymer type; there were statistical differences for comparisons based on concentrations at all timepoints and polymer types ($p < 0.001$) (**Supplementary File 2**, **Supplementary Tables 4–6**).

The ability of DI immune cells to phagocytose MP varied with the polymer type (**Figures 4A,B**). The phagocytic ability of cells exposed to high and medium concentrations of PS-MP decreased significantly with time ($p < 0.05$; * and • in **Figures 4A,B**). In contrast, at high concentrations of PE-MPs and PE+PS-MPs the phagocytic ability increased at 24 and 48 h compared to 1 h ($p < 0.001$; * in **Figure 4B**). PE-MPs were more phagocytosed than PS-MPs by DI cells exposed to high and medium concentrations at both 24 and 48 h ($p < 0.001$; ***a*** and ***b*** in **Figures 4A,B**). Similarly, cells exposed to high and medium concentrations of PE+PS-MPs had higher phagocytic ability than those exposed to PS-MPs at 48 h ($p < 0.001$; ***a*** and ***b*** in **Figures 4A,B**).

The phagocytic ability of blood cells exposed to high and medium concentrations of PE-MPs and PE+PS-MPs was significantly higher than to PS-MPs at all three timepoints ($p < 0.001$; ***a***, ***b***, and ***c*** in **Figures 4C,D**). However, differences in the ability to phagocytose PE-MPs and PE+PS-MPs varied depending on the concentration. Cells exposed to the medium concentration of PE-MPs presented a higher phagocytic ability than the mixture at all times ($p < 0.05$; ***a*** and ***b*** in **Figure 4C**). Nevertheless, at 48 h, the phagocytic ability of cells exposed to high concentrations of PE-MPs and PE+PS-MPs were similar (***a*** at 48 h in **Figure 4D**). Overall, the ability of blood immune cells to phagocytose all the three polymer types increased at 24 and 48 h compared to 1 h ($p < 0.05$; * in **Figures 4C,D**). However, cells exposed to high and medium concentrations of PS-MPs had less phagocytic cells at 48 h compared to 24 h ($p < 0.001$).

The differences in the phagocytosis of different polymer types by the HK immune cells were distinct as shown in **Figures 4E,F**. The phagocytic ability of the cells exposed to high and medium concentrations of PE-MPs was significantly higher than those of PE+PS-MPs and PS-MPs at all times ($p < 0.01$; ***a***, ***b***, and ***c*** in **Figures 4E,F**). Moreover, low concentrations of PE-MPs induced a significantly higher phagocytic ability than PS-MPs ($p < 0.05$). Cells exposed to high and medium concentrations of PE+PS-MPs showed a higher phagocytic ability than those of cells exposed to PS-MPs ($p < 0.001$; ***b*** and ***c*** in **Figures 4E,F**). Different trends in time depending on the polymers were evident in HK cells (**Figures 4E,F**). The phagocytic ability to PE-MPs increased significantly with time after exposures to high and medium concentrations ($p < 0.01$; * and • in **Figures 4E,F**). By contrast, the phagocytic ability to medium and high concentrations of PS-MPs decreased with time ($p < 0.05$; * and • in **Figures 4E,F**). However, the phagocytic ability of HK cells exposed to high concentrations of PE+PS-MPs increased at 24 and 48 h ($p < 0.001$; * in **Figure 4F**) while that of the cells exposed to medium concentrations had lower phagocytic ability after 48 h of exposure ($p < 0.001$; *• in **Figure 4E**).

Overall, blood and HK cells had higher abilities to phagocytose MP (**Figure 3**). HK cells exposed to 50 mg/L of PE-MPs were found to have the highest phagocytic ability, observed at 48 h, with an average value of 5.5% (SD 2.0). The highest ability to

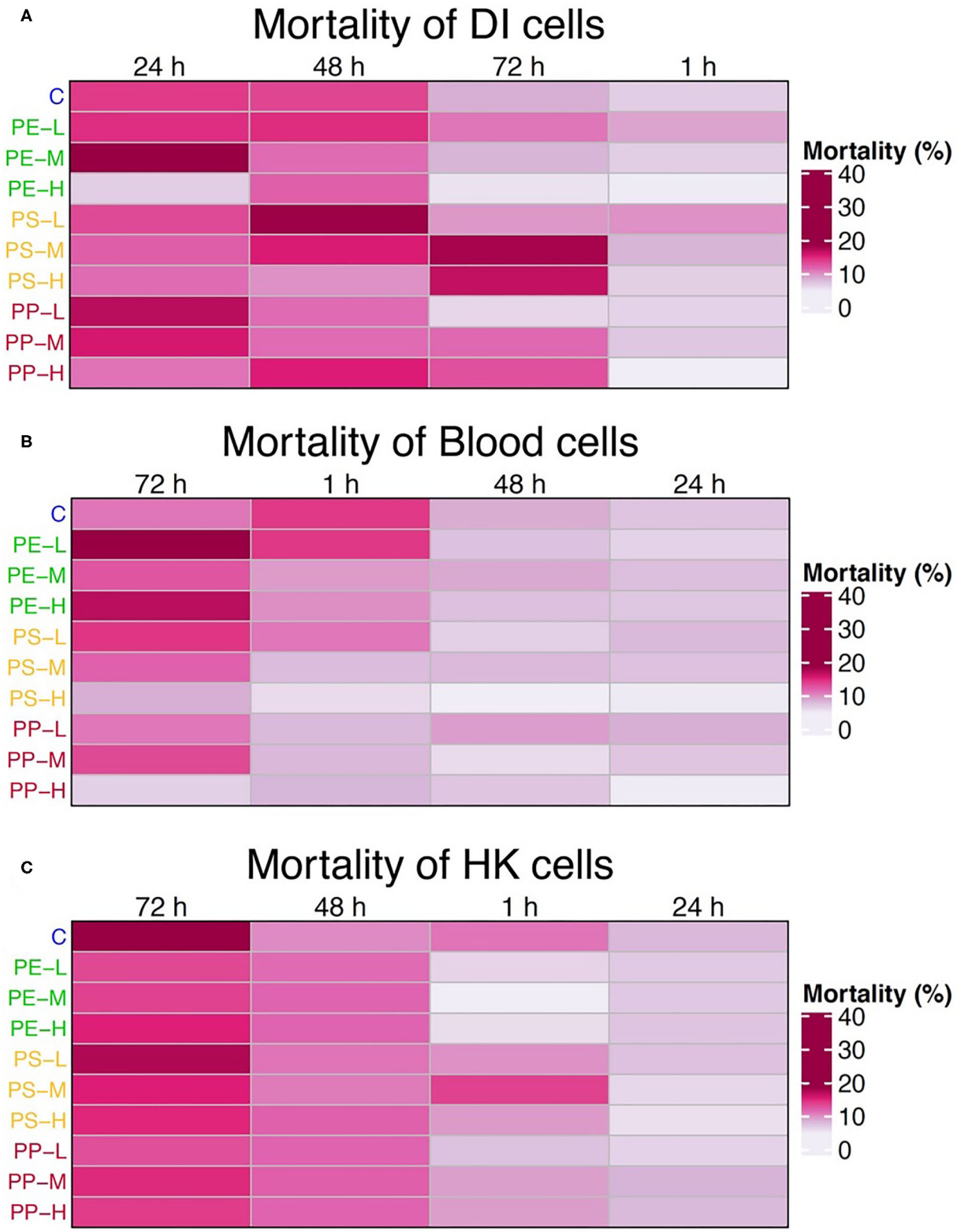

FIGURE 1 | Heatmaps showing the average mortality of **(A)** intestinal, **(B)** blood, and **(C)** HK cells at 1, 24, 48, and 72 h for each treatment group. Time is arranged based on the decreasing level of mortality, from left to right. Cell treatments on the left of the map are color coded based on the polymer types: blue = control, green = PE, yellow = PS, and red (PP) = PE + PS; and ordered by concentration (L = 0.05 mg/L, M = 5 mg/L, and H = 50 mg/L). The mortality is expressed as the percentage of dead cells (cells with red fluorescence) from the total number of cells.

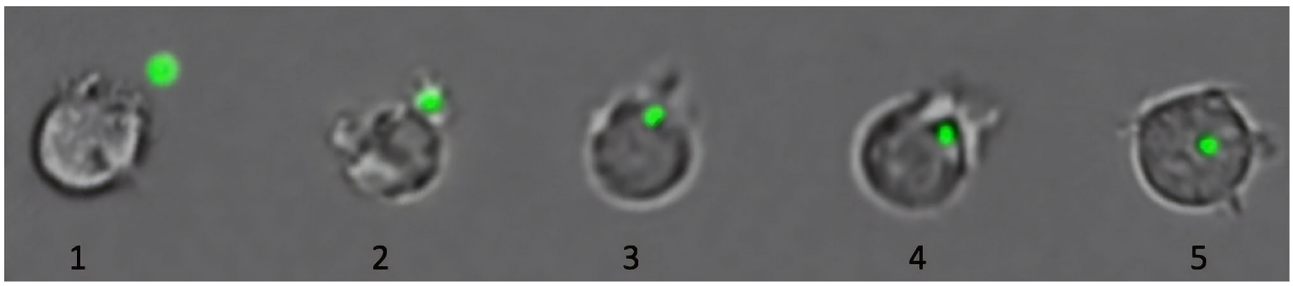

FIGURE 2 | Phagocytosis of microplastics (MPs) by immune cells of Atlantic salmon. As shown in this figure, cells ingest MP (green fluorescent particle) by performing the first steps of phagocytosis: detection and recognition of the MP (1), attachment of the cell to the MP by formation of pseudopods (2), and internalization of the MP (3–5) (Esteban et al., 2015). The last steps of the phagocytosis, involved in the digestion of the particle, are not present due to the lack of specific pathways to degrade plastic (Gewert et al., 2015). The collated images are from representative pictures of five different cells captured at different phagocytic stages. Cells are not to scale.

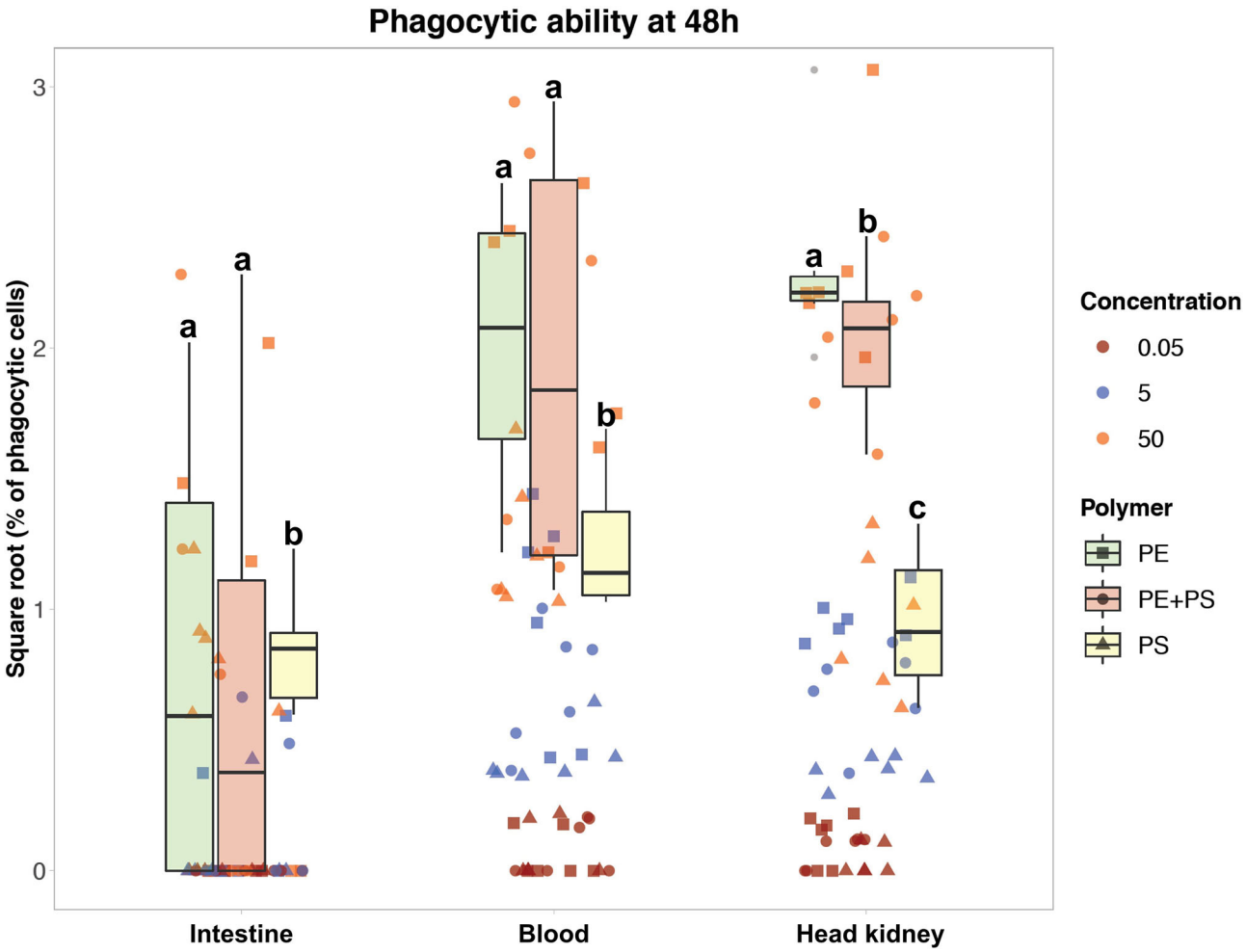

FIGURE 3 | Phagocytic ability of Atlantic salmon immune cells after 48 h of exposure to MP. The color codes for the three MP concentrations are: maroon (0.05 mg/L), blue (5 mg/L), and orange (50 mg/L). The boxplots represent the phagocytic ability of the cells exposed to the high MP concentration (50 mg/L) of PE (green), PE + PS (red), and PS (yellow) at 48 h. Different letters above the box plots represent statistical differences in the ability of the cells from the same tissue to phagocytose different polymers.

phagocytose PS-MPs was observed at 24 h in blood cells exposed to 50 mg/L (1.9%, SD 1.0), and the highest ability to phagocytose PE+PS-MPs was found at 48 h in blood cells exposed to the high concentration (4.3%, SD 3.3). DI cells had the lowest phagocytic ability among the three tissues; those exposed to 50 mg/L of PE-MP had the highest phagocytic ability (2.0%, SD 1.6), at 24 h.

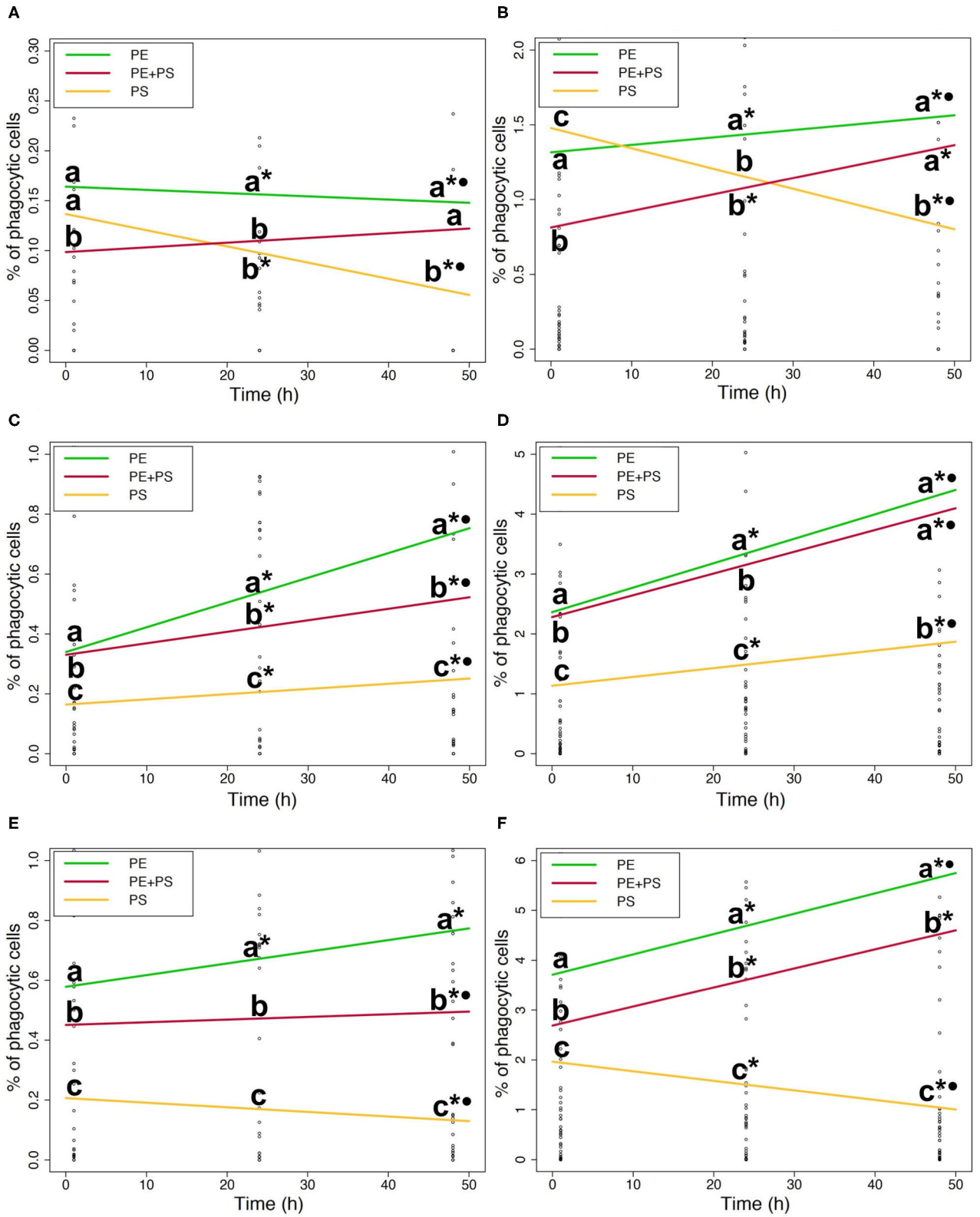

FIGURE 4 | (A). Phagocytic ability of DI cells to microplastic (5 mg/L). **(B)**. Phagocytic ability of DI cells to microplastic (50 mg/L). **(C)**. Phagocytic ability of blood cells to microplastic (5 mg/L). **(D)**. Phagocytic ability of blood cells to microplastic (50 mg/L). **(E)** Phagocytic ability of HK cells to microplastic (5 mg/L). **(F)** Phagocytic ability

(Continued)

FIGURE 4 | of HK cells to microplastic (50 mg/L). Phagocytic ability of DI **(A,B)**, blood **(C,D)**, and HK **(E,F)** cells exposed to the medium (left) and the high (right) MP concentrations. The lines show the general MP accumulation trends in time. The color codes for the MPs are: PE (green), PS (yellow), and PE + PS (red). Different letters above the lines represent statistical differences between polymer types at each time point. Asterisk and dot represent statistical differences between time points of a polymer type; *shows that there are differences compared to 1 h and •shows that there are differences compared to 24 h.

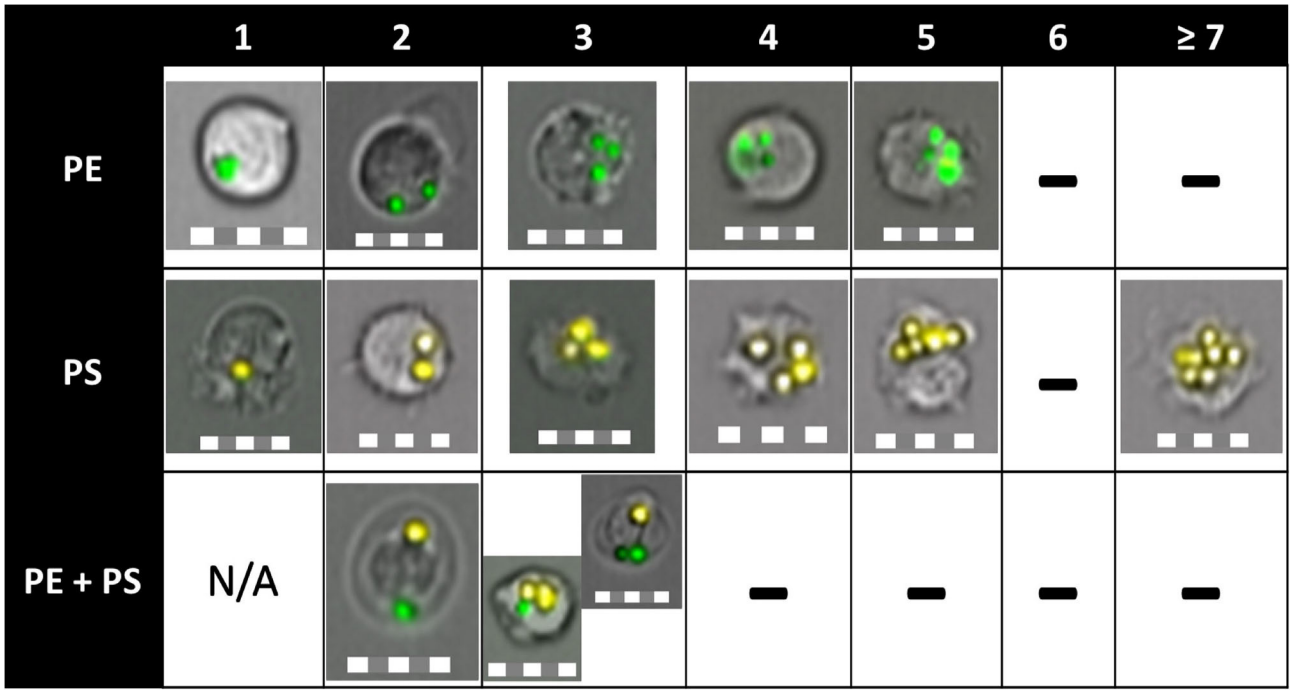

FIGURE 5 | Phagocytic capacity of Atlantic salmon immune cells to ingest PE-MP, PS-MP, or both. The column names indicate the number of particles inside a cell. "-" indicate the absence of cells that engulfed that number of particles. Cells were able to ingest up to five PE particles and up to eight PS particles, which translated to a higher phagocytic capacity of the cells toward PS-MP. HK and blood cells were able to phagocytose two different polymer types at the same time (shown on 3rd row, engulfed PE-MP and PS-MP), a fact proven for the first time in this study. Pictures were obtained by imaging flow cytometry from all tissues, timepoints, and MP concentrations. Scale = 10 μm.

Cells from all three tissues had a low degree of phagocytosis at 0.05 mg/L, the lower concentration of MP; few cells were able to phagocytose MP (0.08% was the highest phagocytic ability).

Phagocytic Capacity to PE-MP and PS-MP

The phagocytic capacity (i.e., the number of particles ingested by an immune cell) of Atlantic salmon to MP differed from tissue to tissue (**Supplementary File 2**, **Supplementary Tables 7–9**). DI immune cells had high phagocytic capacity to PE-MPs and PE+PS-MPs as compared to PS-MPs after 48 h of exposure to the highest MP concentration ($p < 0.05$). However, unlike the phagocytic ability, the phagocytic capacity of DI cells was not affected by the MP concentration.

Blood immune cells had higher phagocytic capacity to engulf PE-MPs at 24 h, while it was at 48 h for the other MPs ($p < 0.05$). However, statistical differences in the phagocytic capacity of blood cells to take up the different polymers were not evident, although PS-MPs were, in average, more ingested than the other MP types at both medium and high concentrations.

HK immune cells had the highest capacity to phagocytose MPs. While more than 7 PS-MP particles were observed inside these cells at 1 and 24 h of exposure, a maximum of five particles were seen in cells exposed to PE-MP for 24 h (**Figure 5**). Yet, the capacity of the cells to ingest the different MP polymer types did not vary significantly. The phagocytic capacity of HK cells increased with the MP concentration. Cells exposed to high concentrations of PE-MPs and PE+PS-MPs had higher phagocytic capacity than cells exposed to low concentrations at all timepoints ($p < 0.05$), as well as cells exposed to the medium concentration at 48 h ($p < 0.05$). However, HK cells exposed to 50 mg/L of PS-MPs only showed high phagocytic activity as compared to 0.05 and 5 mg/L at 1 and 24 h ($p < 0.01$).

DISCUSSION

The adverse effects of microplastics (MPs) on the immune system of animals have been intensively studied in recent years. Nevertheless, the physical response of cells to these small particles is poorly understood. This study aimed to provide new insights into the impact of MPs on the fish immune system at the cellular level. For this purpose, Atlantic salmon immune

cells from DI, blood and HK were exposed to high, medium and low concentrations of PE-MPs, PS-MPs, and PE+PS-MPs for 1, 24, 48, and 72 h. Here we report for the first-time quantitative data on the accumulation of different MP polymer types in fish leucocytes, analyzed by imaging flow cytometry. The present study shows that Atlantic salmon immune cells can phagocytose MPs, although to a relatively low degree. Less than 6% of the cells exposed to high MP concentrations phagocytosed them, and a maximum of 0.08% of cells was found to phagocytose the MPs at low concentrations. The concentration had a clear effect on the response of immune cells exposed to MP, as observed in other organisms, including humans (Kögel et al., 2019). Increased release of MPs into the environment can therefore have more serious consequences than previously thought.

From the results of the present study, it is clear that the phagocytic ability and capacity of the cells from the different tissues of salmon are influenced by the type of polymer to which it is exposed. Overall, salmon immune cells had a higher ability to phagocytose PE-MPs compared to PS-MPs. PE-MPs bioaccumulated in the cells, while the number of cells with PS-MPs decreased over time. This indicates that the cells are able to discriminate the MP types, as discussed below. The decrease in the ability of the cells to phagocytose PS-MPs may be attributed to the process of exocytosis. Macrophages have been shown to exocytose nanoparticles of different nature (Oh and Park, 2014). In human cells, a time dependent exocytosis was observed, and ∼66% of the endocytosed nanoparticles were exocytosed after 48 h of exposure (Asharani et al., 2009). However, exocytosis of particles, compared to endocytosis, is poorly understood and, to our knowledge, no studies are found that report exocytosis of MPs on fish. Nevertheless, a few recent studies have reported the excretion of PS-MPs by living organisms. Accumulated PS nanoparticles decreased after a few days of exposure of zebrafish embryos and larvae to the particles (Pitt et al., 2018). The authors suggested that 72 hpf larvae were able to excrete the PS through the gastrointestinal tract. Japanese medaka exposed to 10 μm PS-MPs was reported to egest 0.5–2% of the ingested microplastic per day (Zhu et al., 2020). Nevertheless, Japanese medaka exposed for 3 weeks to 2 μm PS-MPs still had significant amounts of particles in their intestine (Assas et al., 2020). A study carried out with Pacific oyster (*Crassostrea gigas*) found that PS-MPs did not accumulate in the gut and that the organism could egest these particles through the feces (Sussarellu et al., 2016). The shape of the microbeads was suggested to be the main factor determining the egestion of those particles by the oyster, and could, in turn, explain the higher phagocytic ability of the cells to PE-MPs than PS-MPs observed in this study. A study carried out on rat macrophages found that shape is the main factor regulating the phagocytosis, rather than the particle size. Spherical particles were phagocytosed less successfully than other shapes because the angle formed between the particle and the cell in the initial contact (Ω) was bigger (Champion and Mitragotri, 2006). The PS-MPs used in the present study had a uniform shape, with more than 80% of the particles showing a perfect sphericity, while the PE-MP particles were more irregularly shaped, with only 25% of the particles being spherical (**Supplementary File 1**). Hence, the higher ability of the cells to phagocytose PE-MPs could potentially be due to their lower Ω compared to PS-MPs. This would also explain the similar phagocytic response observed between cells exposed to PE+PS-MPs and PE-MPs. Cells exposed to PE+PS-MPs phagocytosed PE-MPs faster and more successfully than PS-MPs, relatively masking the effect of the latter particles on the cells. This hypothesis became evident after looking into the cells exposed to PE+PS-MPs at 1 h and comparing the number of phagocytic cells with internalized PE-MP with the ones with internalized PS-MPs (**Supplementary Figure**). The roughness and shape are hence important factors to consider when assessing the toxicity of MPs, as it has also been reported in *in vivo* studies (Choi et al., 2018). This is especially relevant since <1% of the MPs found in marine waters are spherical (Isobe, 2016). The higher phagocytic ability of the cells to PE-MPs could also be due to the less uniform size of these particles (1–5 μm) compared to the PS-MPs (2.1 μm). However, analyses of the images of the cells with internalized PE-MPs did not reveal any evidence of cells ingesting smaller particles (1 μm) compared to larger particles (5 μm). Similarly, mice macrophages exposed to 1.2 and 5.2 μm PS-MPs did not show differences in the phagocytic ability to these particles (Tomazic-Jezic et al., 2001). Nevertheless, the authors found differences when they assessed the ability of bigger particles (12.5 μm). Another important factor to consider when analyzing the results of the phagocytic ability is the number of particles/L. The concentration of microplastic added to each sample was calculated in mg/L, but since the density of PE and PS is different, cells incubated with PE-MP were not exposed to the same number of particles as cells exposed to PS-MP. An estimation of the number of particles/L was done to assess whether the higher phagocytic ability of the cells toward PE-MP could be due to the higher number of particles/L. Our calculation revealed that cells exposed to 50, 5, and 0.05 mg/L of PE-MP were exposed to 4.8×10^9, 4.8×10^8, and 4.8×10^6 particles/L respectively, while cells exposed to PS-MP were exposed to more particles/L in comparison (1.75×10^{10}, 1.75×10^9, and 1.75×10^7, respectively). These results underline that cells had a higher response to PE-MPs than to PS-MPs.

As for the phagocytic capacity, blood and HK cells generally ingested more PS-MP particles than PE-MPs. The higher phagocytic capacity of cells toward PS-MPs could be related to the polarity of the polymer, as previously suggested for PS and PC micro- and nanoparticles (Greven et al., 2016). PS tends to become positively charged when it is in contact with water, while PE is consider a non-polar polymer and its charges remain stable (Albrecht et al., 2009). The positively charged PS have higher ability to interact with the cells, stimulating its uptake by cells. Positively charged particles show higher interaction with the cells because of electrostatic attraction, given the negative charge of the cell plasma membrane. Moreover, the binding of PS could be associated to hydrogen bond formation between the charged particles and the hydrophilic region of the phospholipids from the plasma membrane, as suggested for cellulose (Bhattacharya et al., 2010; Ma et al., 2017). Thus, the higher phagocytic capacity of the cells toward the PS-MPs could partly be explained by the polarity of the polymer.

Immune cells from the three tissues responded differently to MPs. Blood and HK cells showed a higher ability to phagocytose MPs compared to DI cells (**Figure 3**). DI immune cells consisted of different leucocytes, and not all of them were phagocytic. On the other hand, the harvested adherent cells from blood and HK were mainly monocytes/macrophages, the main phagocytic cells composing the leucocytes along with the neutrophils (Delves and Roitt, 2000). This abundance in macrophages would explain the main difference in phagocytic ability observed between tissues. However, in Atlantic salmon, the percentage of monocytes/macrophages found in the leucocytes is only 10% lower than the percentage of monocytes/macrophages found in the adherent cells isolated with the protocol used in the present study (Park et al., 2020). Hence, differences in the phagocytic ability of cells from different tissues could also be explained by the principle of organ-specific innate immunity. This principle states that the activation of innate immunity differs between different organs, which have specialized mechanisms to achieve a more efficient response (Raz, 2007). The lower ability of fish DI cells to phagocytose MPs could be a beneficial adaptation to prevent or reduce the uptake of particles from the food. Several fish species ingest and accumulate MPs in the digestive tract (Bråte et al., 2017) as well as in several other tissues, such as the gills, liver and even in the brain (Ding et al., 2018). It has been suggested that MPs translocate from the gut into the circulatory system in fish, which in turn will transport them to other tissues and organs (Ding et al., 2018; Wang et al., 2020). The present study provides new understanding of the MP accumulation potential in Atlantic salmon immune cells and sheds light on potential entry routes for MP particles.

Cell mortality increased at 72 h in all the groups, including the control group. Hence inferences about the cytotoxicity of the MPs based on the mortality of the control group cannot be drawn. Nevertheless, **Figure 2** suggests that MP did not induce any clear polymer- or concentration-dependent signs of cytotoxicity in the studied salmon immune cells. A study on gilthead seabream and European sea bass leucocytes reported no decrease in viability of cells exposed to high concentrations (1, 10, and 100 g/L) of PE-MP and PVC-MP for 1 and 24 h. However, immune parameters such as respiratory burst and phagocytic capacity of the cells were affected (Espinosa et al., 2018). A sublethal effect of MP on fish immune cells was also observed in fathead minnow, where exposure of neutrophils to PS or PC micro- and nanoparticles induced cell degranulation as well as oxidative burst activity (Greven et al., 2016). In the present study, we used fluorescent-dyed MPs and therefore did not measure any additional parameters other than cell mortality. Earlier studies have shown that some fluorochromes can leach and accumulate in tissues (Catarino et al., 2019), and influence the superoxide production as well as other immune parameters directly (De Clerck et al., 1994). Our focus was to understand the physical impact of salmon immune cells to different types of MP polymers, by assessing the phagocytic ability and capacity of the cells.

In the present study, we found that MPs can impact the immune cells of Atlantic salmon even at relatively low concentrations and that different polymers can induce distinct responses on the cells. Exposure of farmed salmon to MP can have economic implications for this prime aquaculture industry (FAO, 2018), especially if the MP particles act as vectors for contaminants (Browne et al., 2013). Moreover, plastic is widely used in aquaculture and MP released from sources such as feeding pipes into the surrounding waters may potentially be ingested by the farmed fish (Lusher et al., 2017a; Gomiero et al., 2020). We found that PE-MP accumulates more easily than PS-MP in the immune cells of the Atlantic salmon. PE is the most abundant MP polymer in the marine environment (Enders et al., 2015; Ter Halle et al., 2017; Erni-Cassola et al., 2019; Pannetier et al., 2020) and, therefore, the most often ingested polymer by fishes (Rummel et al., 2016; Bråte et al., 2017). Hence, the impact of PE-MPs on wild populations of salmonid species cannot be ignored.

CONCLUSION

The present study used an imaging flow cytometry approach to understand how different MP polymers are taken up by fish cells. This technique enabled us to prove for the first time that a single cell can phagocytose two different microplastic polymer types simultaneously. In conclusion, Atlantic salmon immune cells isolated from different tissues phagocytosed microplastic polymers, even at relatively low concentrations. Polyethylene microparticles were more easily ingested than polystyrene ones. With increasing amounts of plastic debris in the marine environment, the study highlights how their uptake into the tissues can lead to damaging effects on aquatic life. The methodology adopted in the present study opens new possibilities for observing the impact of micro and nanoplastic on several species, including humans.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because, in accordance the Norwegian regulation of the Research Animal Act (FOR-2015-06-18-761). The Approval of trials regulation §6 states that approval requirement does not apply to experiments involving only the killing of animals to use organs or tissues from them. Live fish was handled by personnel with FELASA-C course, based on the policies by the Federation of European Laboratory Animal Science Association.

AUTHOR CONTRIBUTIONS

IA-G, PO, and VK: conceptualization and design of the study. IA-G and YP: methodology, protocols, and experiment set up. IA-G: analysis of data and writing. IA-G, YP, VK, and

PO: review and editing. 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/fenvs.2020.560206/full#supplementary-material

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Marine Litter Pollution in Baltic Sea Beaches – Application of the Sand Rake Method

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Most marine litter monitoring methods used on beaches focus on macro-litter (>25 mm) only and show shortcomings regarding smaller litter classes (<25 mm), especially at Baltic Sea beaches. Therefore, we used a sand rake method developed for large micro- (2–5 mm), and meso- (5–25 mm) litter to quantify the overall pollution status of Baltic Sea beaches and to test if the method is useful in terms of the requirements of the Marine Strategy Framework Directive (MSFD). Between July 2017 and October 2019, 197 sand rake method surveys were carried out at 35 regions around the Baltic Sea. In total, 9345 litter pieces were found on an area of 10,271 m^2 of which 69.9% were 2–25 mm in size. Artificial polymers (4921 litter pieces) were predominant (mean 52.7% ± 13.3). Abundance of litter was 0.91 pieces/m^2 ± 1.50 (median 0.40 pieces/m^2). The most common litter were industrial pellets (19.8%), non-identifiable plastic pieces 2–25 mm (17.3%), cigarette butts (15.3%), and paraffin (11.9%). At 15 surveys at the German North Sea island of Sylt the litter abundance ranged from 0.45 pieces/m^2 (median) to 0.59 pieces/m^2 ± 0.37 (mean). Here, 69.2% of the litter was 2–25 mm in size and paraffin was predominant (69.2%). Beaches show a high pollution level with large micro- and meso-litter (2–25 mm) and our data can serve as a Baltic-wide pollution baseline. In contrast to the naked eye OSPAR method for macro-litter, the sand rake method is generally applicable on all sandy beaches, both urban and remote. This method also allows for the provision of a full spatial pollution pattern and can serve for assessing the effectiveness of marine litter mitigation measures.

Keywords: Marine Strategy Framework Directive, top marine beach litter Items, plastic pellets, cost-effective marine litter monitoring method, Baltic Sea

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INTRODUCTION

Marine litter is any discarded or lost material entering the marine environment from human activities (Cheshire et al., 2009). It is found in all marine habitats (UNEP, 2005; Ivar do Sul and Costa, 2014) and it is a growing threat for the marine environment (Pham et al., 2014) all over the world. It occurs in all size ranges from commercial shipping containers to plastic bottles, cigarettes butts, industrial pellets, all the way down to particles in nanometer size. Plastic as the vast majority of anthropogenic litter (Reisser et al., 2013) is found on the surface of the open sea

(Law et al., 2010; Cozar et al., 2014; Eriksen et al., 2014), in the deep sea (Peng et al., 2020), in the Arctic (Tekman et al., 2017), and on beaches worldwide (UNEP, 2015; Matsuguma et al., 2017). To protect the marine environment across Europe, the Marine Strategy Framework Directive (MSFD) was adopted, to achieve a 'Good Environmental Status' (GES) of European waters through the use of 11 descriptors by 2020 (MSFD, 2008/56/EC). Descriptor 10 specifies, that the GES is achieved only when "Properties and quantities of marine litter do not cause harm to the coastal and marine environment" (MSFD, 2008/56/EC). This requires the assessment of "trends in the amount of litter washed ashore and/or deposited on coastlines, including analysis of its composition, spatial distribution and, where possible, source" (MSFD TSG ML, 2013). Marine litter on coastlines and beaches is obvious and omnipresent (JRC, 2011). Therefore, the monitoring of sandy beaches is a common method to assess the pollution of marine systems (Browne et al., 2015), as beaches are considered to be a major sink for marine litter (Cauwenberghe et al., 2015).

To assess the pollution of beaches in the European Union, a standardized macro-litter (>25 mm) monitoring method (OSPAR, 2010) is largely used. This 'naked eye' OSPAR method is carried out on rural and unmanaged beaches of the North-East Atlantic (Schulz et al., 2017) and at Baltic Sea beaches (Balčiūnas and Blažauskas, 2014; LUNG M-V, 2015). However, this method is less suitable for many Baltic Sea beaches (Schernewski et al., 2017) because many of them are managed and cleaned and therefore do not meet the OSPAR criteria for the selection of reference beaches. Additionally, an increase in public awareness leads to more beach litter collections (by locals, non-government organizations, beach users, etc.) that could mask actual variations in litter abundance and composition (Addamo et al., 2017; Schöneich-Argent et al., 2019). Furthermore, naked eye methods neglect most of meso- (5–25 mm) and large micro-litter (1–5 mm) at the beach, as it is simply overlooked, due to its small size (JRC, 2011). Therefore, most litter (<25 mm) remains in the sediment, even if beaches are cleaned (Laglbauer et al., 2014; Lee et al., 2017). Accordingly, higher amounts of litter (<25 mm) are found at urban beaches (Okuku et al., 2020).

Meso- and large micro-litter are important size fractions that are numerically abundant and require an appropriate and harmonized monitoring approach in order to understand litter degradation at beaches (Hanke et al., 2019). Subsequent analysis of material and polymer types of meso- and large micro-litter can help to determine the origin or the initial product (Addamo et al., 2017). In general, new monitoring methods that deliver reliable data for smaller litter (<25 mm) at affordable costs are needed (MSFD TSG ML, 2013; Addamo et al., 2017). To address this issue, Haseler et al. (2017) tested a sand rake method at German and Lithuanian Baltic Sea beaches. They concluded that meso- and large micro-litter are a major problem at Baltic beaches as it was responsible for 65% of the litter found in terms of size.

The objective of this study is to test the general applicability of the sand rake method on a wide range of sandy beaches. Therefore, we (a) applied the sand rake method on a large scale, in all bordering states of the Baltic Sea and at tidal beaches at the German North Sea, (b) documented beach pollution with litter items in the size classes of large micro-, meso-, and macro-litter, (c) identified the polymer types of plastic litter using a mobile infrared spectrometer (Microphazir), and based on this information, we (d) provide cost estimations and discuss the suitability of the sand rake method for a MSFD monitoring, complementing the existing macro-litter OSPAR method.

STUDY SITES AND METHODS

With 377,000 km^2, the enclosed, micro-tidal (<15 cm) (Sztobryn et al., 2005) Baltic Sea is one of the largest brackish water bodies worldwide and is surrounded by nine countries, with 85 million inhabitants living in the drainage basin (Leppäranta and Myrberg, 2009). Limited water exchange takes place with the North Sea (HELCOM, 2010). Coastal types in the Baltic Sea region are highly diverse, including (but not limited to) soft moraine cliffs, sandy beaches/dunes, rocky cliffs, meadows, and organic wetlands (Łabuz, 2015), with moraine material dominating in the south and southeast, and hard bottom and rocky shores at the northern coasts (Schiewer, 2008).

In order to investigate marine litter (>2 mm) around the beaches of the Baltic Sea, the sand rake method was applied 205 times, in 35 regions, in all of the nine bordering states of the Baltic Sea. Additionally, the island of Sylt region (North Sea, Germany) was surveyed 15 times (**Figure 1**). Survey areas were limited to sandy beaches. The regions were chosen to present a spatial overview of the abundance of beach litter at Baltic Sea beaches. Therefore, a first screening of possible regions was carried out online via Google Earth. Here, photos of the beaches were checked to investigate if the beach sediment in the regions is sandy and if beaches were accessible by car. This clustered monitoring approach (for the different regions) was chosen, as it was not possible to investigate the same beaches several times. At different beaches, the small-scale distribution of litter was investigated. Thus, two surveys were taken at the same beach with a distance (length of the beach) of 50–100 m in between. The abundance of litter in the 36 different regions (**Table 1**) was surveyed with a minimum of two and a maximum of 18 surveys (in average = six) per region. Six surveys per region were considered to be feasible within 2 days. Only two or three beach surveys could be taken in four regions (**Table 1**) because of circumstances outside of researcher control (thunder storm and heavy rains). In other regions close to the author's institutes, additional surveys were conducted. All surveys were taken during daylight hours (no designated time). Tides were not considered during the surveys of the Baltic Sea. North Sea surveys were taken during outgoing tide to avoid a flooding of the survey area. Rainy weather and wet beach sediment was avoided, as the sand rakes clogs faster. Two people, each with a sand rake, conducted the surveys. All surveys were carried out by the authors in the months of June–October between 2017 and 2019.

The length of the examined beaches differed, from around 50 m to a few kilometers. The beach width, from the waterline to the back of the beach (vegetation, cliff, etc.) varied between <5 and 220 m. The exact survey area location on each beach was randomly chosen. When two surveys were conducted at one beach, a gap of around 50–100 m was maintained between the

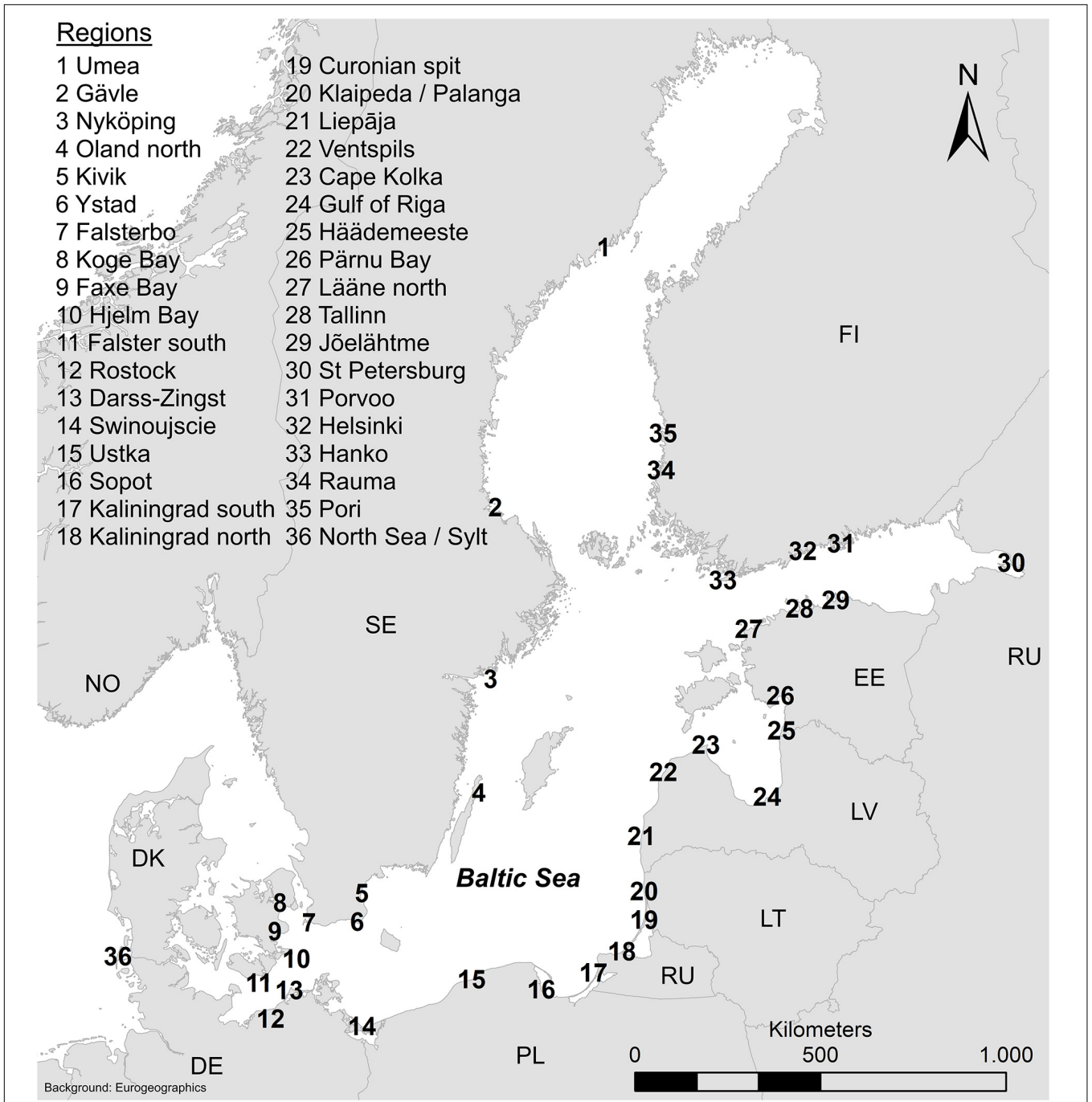

FIGURE 1 | Overview map of the Baltic Sea and part of the North Sea with the 36 survey regions (Eurogeographics, 2019).

surveys. The manual used sand rake has an operational width of 0.5 m and a mesh size (MS) of 2 mm. During sediment raking, maintenance of a 3–5 cm penetration depth was attempted, although depth wasn't actively measured and depended on beach sediment properties.

In general, the backshore was sampled. Sand raking started at the first occurrence of dry sand by the waterline. The beach was raked in columns until the landward end of the beach (dune, vegetation, wall, etc.). Each column is further divided into subsections of 5 m length, with an area of 2.5 m^2 (**Figure 2**). Since the entire beach width should be surveyed, this resulted in different amounts of subsections. The minimum surface area per survey was 50 m^2. Therefore, if the area of one column was less than 50 m^2, more columns were necessary. All columns were sampled in full, even if the minimum area of (50 m^2) was reached, meaning most surveys sampled an area >50 m^2. Due

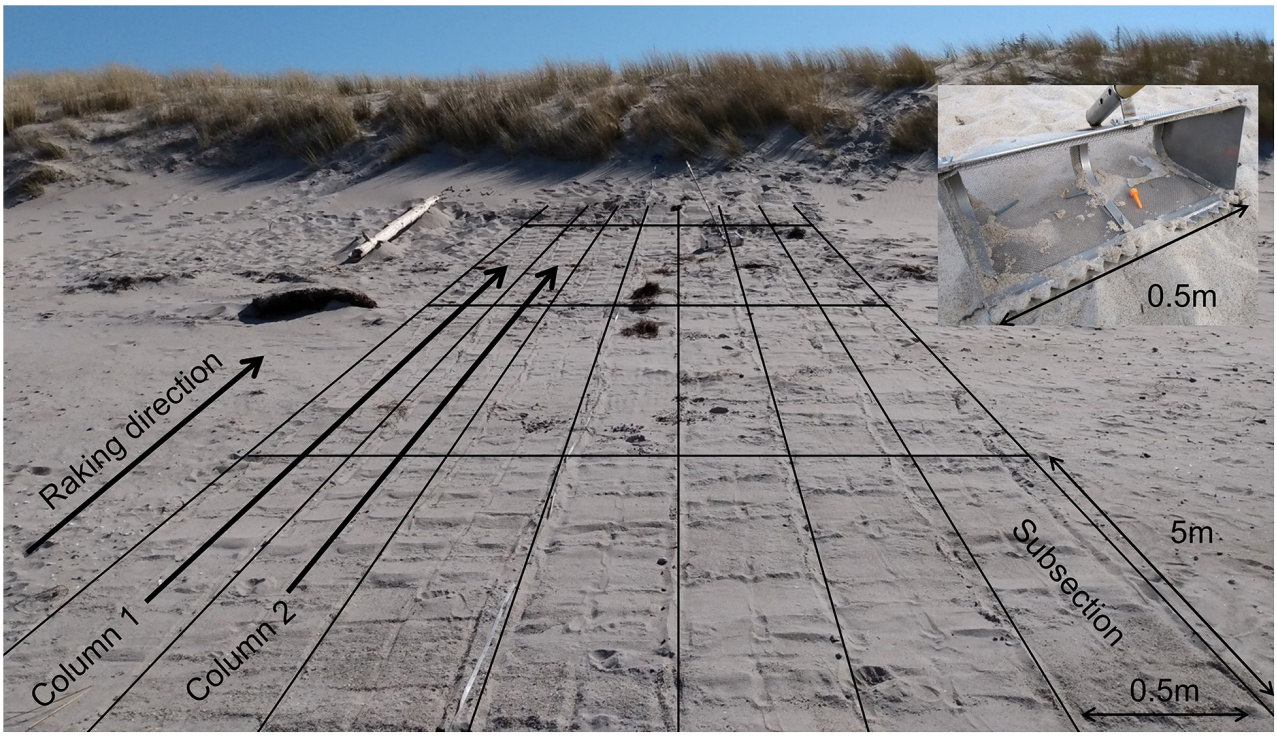

FIGURE 2 | Sand rake method survey on a sandy beach. Raking direction is from the waterline to the back of the beach (backshore). Operation width of the sand rake is 0.5m; the mesh size used is 2 mm. Each survey is divided in subsections of 0.5 m × 5 m resulting in an area of 2.5 m^2. Subsections at the back of the beach may be smaller. One survey consists of one or more columns, with different number of subsections. The minimum area per survey is 50 m^2.

to thunderstorms, heavy rain, occurrence of beach visitors or other beach characteristics, this was not always possible and led to eight surveys with just one entire row surveyed, with sampled areas between 25 and 28 m^2. The average surveyed area was 52 m^2 (maximum 110 m^2 and minimum 25 m^2; **Supplementary Table S1**). For more information on how the sand rake method works see Haseler et al. (2017).

All litter size classes were collected with the sand rake method at the beach. Litter was later photographed, counted, measured (mm-paper), separated into the micro- (2–5 mm), meso- (>5–25 mm), and macro- (>25 mm) litter fractions and identified based on the list of litter (MSFD TSG ML, 2013). In this study, the list of litter was adapted and included 183 litter items in seven categories (Artificial polymers including rubber (referred as: artificial polymers); cigarette butts; paraffin; paper/cardboard; glass/ceramics; metal; and other). The adaption of the list was necessary to classify different litter items according to their size class. Cigarette butts were counted as an independent category due to their high occurrence (**Supplementary Table S2**). Non-identifiable plastic pieces were further divided by size, referred to as: micro-plastic pieces (2–5 mm), meso-plastic pieces (>5–25 mm), and macro-plastic pieces (>25 mm) (HELCOM, 2015).Their color was visually determined and the polymer type was analyzed with the Microphazir PC by Analyticon[1]. The Microphazir uses near-infrared spectroscopy (NIR). The analysis per particle provides two results within five seconds: firstly, the polymer type, and secondly, a percentage of accuracy. In this study, only particles with a percentage accuracy ≥95% are classified into different polymer types. All particles with an accuracy less than 95% were counted as unidentified. Exclusion of black particles from the polymer analysis was necessary, as they do not scatter visible light and absorb the wavelength of the laser in the near infrared.

The Microphazir is a battery-operated, handheld device, using near infrared spectroscopy (NIR) with a resolution of 12 nm (pixels) and 8 nm (optical). The spectral range is 1600–2400 nm. For calibration, an internal reference standard is placed on a glass plate above the light bulb and an analysis is performed. The internal library of the Microphazir covers more than 30 different synthetic polymers and it is possible to import further created libraries by the user. To increase the detection efficiency of dirty plastic particles, the particle was cleaned beforehand (fresh water or ethanol) or a fresh internal surface was exposed by breaking the particle and this surface was used for the analysis. During analysis, spectral results of the particle of interest are compared against library spectra. In this study, only particles that matched the library spectra (≥95%) were classified into corresponding polymer types. Even if the analyzed particle is not an artificial polymer, there is always a percentage result with regard to the most likely polymer type as the Microphazir compares the

[1]https://www.analyticon.eu/en/microphazir-pc.html

TABLE 1 | The average beach litter density for the 36 different regions in mean numbers of litter pieces/m^2 ± SD.

Nr.	Country	Region	Mean number of litter pieces/m^2 ± SD	Median number of litter pieces/m^2	Minimum number of litter pieces/m^2	Maximum number of litter pieces/m^2	Surveys per region (n)	Litter categories in %						
								Artificial polymer	Cigarette butts	Paraffin	Glass/ Ceramic	Paper/ Cardboard	Metal	Other
1	Sweden	Umea	1.14 ± 0.78	1.07	0.10	2.32	5	61.4	12.2	2.0	0.3	2.4	3.4	18.3
2	Sweden	Gävle	0.55 ± 0.27	0.49	0.25	0.97	4	28.6	8.4	0.0	7.6	0.0	2.5	52.9
3	Sweden	Nyköping	0.53 ± 0.55	0.28	0.10	1.48	4	22.2	50.0	11.1	2.8	2.8	1.9	9.3
4	Sweden	Oland north	0.21 ± 0.06	0.25	0.10	0.27	5	43.1	22.4	0.0	0.0	20.7	1.7	12.1
5	Sweden	Kivik	1.24 ± 0.69	1.14	0.40	2.26	4	80.6	1.6	3.6	10.1	1.6	1.6	0.8
6	Sweden	Ystad	0.18 ± 0.16	0.12	0.02	0.44	5	47.9	8.3	2.1	14.6	2.1	0.0	25.0
7	Sweden	Falsterbo	1.46 ± 1.55	0.72	0.28	4.12	4	64.0	1.3	30.3	1.3	0.3	0.7	2.0
8	Denmark	Koge Bay	0.28 ± 0.20	0.22	0.00	0.60	6	46.6	35.2	1.1	0.0	10.2	1.1	5.7
9	Denmark	Faxe Bay	0.25 ± 0.13	0.26	0.07	0.42	6	27.1	50.0	0.0	10.4	2.1	0.0	10.4
10	Denmark	Hjelm Bay	0.05 ± 0.05	0.04	0.00	0.15	6	40.0	0.0	0.0	30.0	0.0	0.0	30.0
11	Denmark	Falster (south)	0.04 ± 0.02	0.05	0.00	0.06	6	41.7	16.7	0.0	8.3	0.0	16.7	16.7
12	Germany	Rostock	0.75 ± 0.59	0.52	0.18	1.89	12	28.2	45.2	0.0	11.0	4.2	3.0	8.3
13	Germany	Darss-Zingst	0.27 ± 0.16	0.22	0.10	0.51	6	50.6	24.1	0.0	2.4	1.2	1.2	20.5
14	Poland	Swinoujscie	0.68 ± 0.43	0.52	0.36	1.59	6	22.9	24.1	0.0	0.8	5.9	9.9	36.4
15	Poland	Ustka	0.64 ± 0.58	0.33	0.13	1.70	6	32.7	20.9	0.5	1.5	15.3	8.7	20.4
16	Poland	Sopot	2.93 ± 2.09	2.66	0.39	6.65	6	48.6	21.0	1.0	0.6	11.6	5.4	11.9
17	Russia	Kaliningrad south	1.19 ± 0.74	1.02	0.58	2.78	6	64.6	19.9	5.9	2.2	3.4	1.4	2.5
18	Russia	Kaliningrad north	2.69 ± 0.28	2.59	2.38	3.26	6	50.0	4.8	38.1	3.3	1.5	0.7	1.5
19	Lithuania	Curonian spit	0.67 ± 0.38	0.74	0.06	1.26	18	54.9	1.2	38.4	1.5	0.3	0.3	3.2
20	Lithuania	Klaipeda/ Palanga	1.42 ± 0.69	1.30	0.66	2.69	10	31.6	11.0	44.0	5.5	2.0	1.2	4.6
21	Latvia	Liepāja	2.03 ± 3.33	0.26	0.14	9.32	6	79.8	11.3	0.5	5.7	0.3	1.0	1.3
22	Latvia	Ventspils	0.14 ± 0.08	0.17	0.00	0.21	4	75.6	16.3	0.0	2.0	2.0	2.0	2.0
23	Latvia	Cape Kolka	0.39 ± 0.11	0.44	0.22	0.50	5	80.8	7.1	5.1	4.0	1.0	0.0	2.0
24	Latvia	Gulf of Riga	0.26 ± 0.14	0.20	0.10	0.54	12	55.6	23.8	1.3	0.6	6.3	5.0	7.5
25	Estonia	Häädemeeste	0.1 ± 0.08	0.10	0.02	0.18	2	30.0	20.0	20.0	0.0	0.0	30.0	0.0
26	Estonia	Pärnu Bay	0.28 ± 0.15	0.32	0.04	0.44	4	52.6	24.6	0.0	10.5	1.8	5.3	5.3
27	Estonia	Lääne north	0.19 ± 0.08	0.17	0.06	0.30	5	37.5	50.0	0.0	0.0	4.2	0.0	8.3
28	Estonia	Tallinn	4.46 ± 4.47	2.89	0.82	13.96	6	68.7	2.2	0.0	25.7	0.7	1.0	1.7
29	Estonia	Jõelähtme	0.14 ± 0.04	0.13	0.08	0.20	4	60.7	7.1	0.0	3.6	10.7	10.7	7.1
30	Russia	St. Petersburg	2.5 ± 2.30	1.81	0.68	8.14	8	87.6	2.3	0.0	7.6	0.2	0.4	1.9
31	Finland	Porvoo	1.48 ± 0.10	1.48	1.37	1.58	2	24.5	28.5	45.0	0.0	0.0	1.3	0.7
32	Finland	Helsinki	1.2 ± 0.52	0.96	0.56	1.93	5	40.1	34.6	0.0	15.4	4.7	2.0	3.2
33	Finland	Hanko	2.11 ± 1.54	1.90	0.34	4.10	3	64.4	26.5	0.0	3.5	0.6	3.5	1.6
34	Finland	Rauma	0.78 ± 0.17	0.68	0.65	1.02	3	54.8	23.3	0.0	6.8	2.7	6.8	5.5
35	Finland	Pori	0.22 ± 0.19	0.14	0.08	0.60	5	31.9	35.3	0.0	4.2	5.0	5.0	18.5
36	Germany	North Sea/ Sylt	0.5 ± 0.37	0.45	0.02	1.35	15	24.5	2.1	69.2	1.4	0.5	0.7	1.6

The median beach litter density, the maximum, and the minimum per region. In the second part the litter categories in percentage (%) for artificial polymers, cigarettes butts, paraffin, glass/ceramics, paper/cardboard, metal, and other.

spectrum of the measured particle with the 30 internal spectra of the internal library.

The costs of future, long-term, sand rake monitoring on German Baltic beaches were calculated for two scenarios (**Figure 5**). First, the cost of sand rake monitoring on 15 beaches that are not suitable for an OSPAR method were calculated. These 15 beaches were chosen because they met one or more of the following conditions: (a) urban beaches; (b) managed beaches, partially cleaned; (c) close to river mouth; (d) touristic beaches. Furthermore, these 15 beaches should be distributed along the German Baltic coast to provide a spatial overview of the litter abundance. At the German Baltic Sea nearly all accessible sandy beaches are used for touristic purposes. To calculate the amount of tourists visiting a certain beach is difficult, as official numbers are only available for overnight stays at the community level. Here, the "touristic beaches" are located in communities where the governmental statistical services indicate between 0.14 and 2.40 million overnight stays occur per year (Statistisches Amt Mecklenburg-Vorpommern, 2018; Statistisches Amt für Hamburg und Schleswig-Holstein, 2018). For the second scenario, the costs of sand rake monitoring at 14 beaches suggested for the OSPAR method (see Schernewski et al., 2017) were calculated (**Figure 5**). For both scenarios, the costs of one year of monitoring with a seasonal approach with four surveys (summer, autumn, winter, spring) were calculated. Once for an expert-based approach, here all work steps (WS 1-5) are carried out by experts (German pay scale group E9 Level 1 - 37.50€/h) (**Table 4**) and second for a volunteer-based approach. In the second approach, trained volunteers shall carry out the beach related work (WS 1-2) only, and the following steps (WS 3-5) are carried out by experts (**Table 4**). The hours (h) calculated to travel to the beach and back (WS 1; 4 h) are based on the average distance between the Leibniz-Institute for Baltic Sea Research (IOW) and the suggested survey beaches in Mecklenburg-Vorpommern. The averaged hours calculated for the works steps (WS 2-5): the sand rake surveys at the beach (WS 2; 5 h), litter analysis (WS 3; 2.5 h), data processing (WS 4; 1.5 h); and reporting (WS 5; 1 h) are based on the experience of more than 200 sand rake surveys performed by the authors in this study.

RESULTS

Each member state of the EU is responsible for the development of a strategy to reach the GES of its own waters (MSFD, 2008/56/EC). Here, voluntary national actions can be helpful (HELCOM, 2015). Therefore, we decided to present our results on a national level (**Tables 2**, 3). The results on the beaches of Russia (non EU member state) are divided in the two geographically separated regions named after their central cities - Kaliningrad and St. Petersburg. The results on German beaches are divided in the Baltic Sea and the North Sea surveys. Since geomorphological, social, and other differences may affect the litter abundance on the beaches, we further present our results in different regions per nation (**Figure 1** and **Table 1**).

Country Specific Marine Litter Densities

Sweden

In 31 surveys (1624.5 m^2) a total of 1176 litter pieces were found (mean 0.72 pieces/m^2 ± 0.87; median 0.40 pieces/m^2), with a minimum of 0.02 pieces/m^2 and a maximum of 4.12 pieces/m. The litter was distributed as follows: 297 micro-litter pieces (0.18 pieces/m^2-25.2%), 603 meso-litter pieces (0.37 pieces/m^2-51.3%) and 276 macro-litter pieces (0.17 pieces/m^2-23.5%) (**Table 3**). On 14 beaches where 2 surveys were conducted, the small-scale spatial variation of litter per beach was between 0.02 and 3.84 pieces/m^2 (mean 0.75/m^2 ± 0.99; median 0.42 pieces/m^2). The highest pollution (4.12 pieces/m^2) was found in the region of Falsterbo and the lowest in the region of Ystad (0.02 pieces/m^2) (**Table 1**).

Denmark

The abundance of litter found in Denmark was the lowest in this study. In 24 surveys (1035 m^2) 158 litter pieces were found (mean 0.15 pieces/m^2 ± 0.17; median 0.07 pieces/m^2). During three surveys no litter was found. Overall, 12 micro-litter pieces (0.01 pieces/m^2-7.6%), 50 meso-litter pieces (0.05 pieces/m^2-31.6%), and 96 macro-litter pieces (0.09 pieces/m^2-60.8%) were collected (**Table 3**). The highest abundance of litter found were 0.6 litter pieces/m^2 in Koge Bay (**Table 1**). The small-scale spatial variation of litter (12 beaches with 2 surveys) was between 0.001 and 0.35 pieces/m^2 (mean 0.13/m^2 ± 0.12; median 0.07 pieces/m^2).

Germany

Altogether 554 litter pieces were collected in 18 surveys on an area of 944 m^2 (mean 0.59 pieces/m^2 ± 0.54; median 0.43 pieces/m^2). The lowest amount of litter (0.10 pieces/m^2) was conducted during a survey in Darss-Zingst. The highest concentration of litter found was 1.89 pieces/m^2 during a survey in Rostock (**Table 1**). Macro-litter pieces (0.31 pieces/m^2-52.5%) were predominant, followed by meso-litter (0.26 pieces/m^2-43.7%) and micro-litter (0.02 pieces/m^2-3.8%) (**Table 3**). The small-scale spatial variation of litter (9 beaches with 2 surveys) was between 0.003 and 1.71 pieces/m^2 (mean 0.33 pieces/m^2 ± 0.53; median 0.18 pieces/m^2).

Poland

The number of litter pieces collected in 18 surveys (997.5 m^2) was 1450 (mean 1.45 pieces/m^2 ± 1.67; median 0.67 pieces/m^2), with a minimum of 0.13 pieces/m^2 (Ustka region) and a maximum of 6.65 pieces/m^2 (Sopot region) (**Table 1**). 220 micro-litter pieces (0.22 pieces/m^2-15.2%), 686 meso-litter pieces (0.69 pieces/m^2-47.3%) and 544 macro-litter pieces (0.55 pieces/m^2-37.5%) were collected (**Table 3**). On 9 beach areas where 2 surveys each were conducted, the small-scale spatial variation of litter per beach was between 0.04 and 2.38 pieces/m^2 (mean 0.60 pieces/m^2 ± 0.68; median 0.36 pieces/m^2).

Russia – Kaliningrad

A total of 1164 litter pieces were found (12 surveys) on an area of 600 m^2, with a minimum of 0.58 pieces/m^2 and a maximum of 3.26 pieces/m^2 (mean 1.94 pieces/m^2 ± 0.94; median 2.48 pieces/m^2). The litter size distribution was the

TABLE 2 | Top ten litter items per country, for all Baltic Sea surveys (cumulative) without St. Petersburg, and for the island of Sylt, Germany in the North Sea.

	Baltic surveys (without Saint Petersburg)	**Sweden**	**Denmark**	**Germany**	**Poland**	**Kaliningrad**
1	Industrial pellets	Plastic pieces (meso)	Cigarette butts	Cigarette butts	Cigarette butts	Plastic pieces (meso)
	1852/19.8%	266/22.6%	57/36.1%	233/42.1%	312/21.5%	180/15.5%
2	Cigarette butts	Industrial pellets	Plastic pieces (meso)	Plastic pieces (meso)	Plastic pieces (meso)	Paraffin (micro)
	1426/15.3%/35.1%	180/15.3%/37.9%	16/10.1%/46.2%	43/7.8%/49.8%	230/15.9%/37.4%	166/14.3%/29.7%
3	Plastic pieces (meso)	Cigarette butts	Crisp packets/sweet wrappers	Bottles incl. pieces	Industrial pellets	Paraffin (meso)
	1405/15.0%/50.1%	125/10.6%/48.6%	7/4.4%/50.6%	30/5.4%/55.2%	166/11.4%/48.8%	161/13.8%/43.6%
4	Paraffin (meso)	Slack/Coal	Plastic construction waste	Other glass items	Paper fragments	Industrial pellets
	815/8.7%/58.8%	100/8.5%/57.1%	7/4.4%/55.1%	19/3.4%/58.7%	90/6.2%/55.0%	151/13.0%/56.5%
5	Other glass items	Paraffin (meso)	Paper fragments	Slack/coal	Slack/coal	Cigarette butts
	424/4.5%/63.4%	74/6.3%/63.4%	7/4.4%/59.5%	19/3.4%/62.1%	85/5.9%/60.9%	110/9.5%/66.0%
6	String and cord (<1 cm)	Plastic pieces (micro)	String and cord (<1 cm)	Firework plastic pieces	Oil/Tar/Paint particles	String and cord (< 1 cm)
	293/3.1%/66.5%	63/5.4%/68.7%	6/3.8%/63.3%	17/3.1%/65.2%	65/4.5%/65.4%	55/4.7%/70.7%
7	Paraffin (micro)	Paraffin (micro)	Industrial pellets	String and cord (<1 cm)	Other textiles (incl. rags)	Plastic pieces (micro)
	259/2.8%/69.3%	39/3.3%/72.0%	6/3.8%/67.1%	15/2.7%/67.9%	37/2.6%/67.9%	39/3.4%/74.1%
8	Slack/coal	Plastic pieces (macro)	Slack/coal	Plastic pieces (macro)	Foil wrappers, aluminum foil	Plastic pieces (macro)
	224/2.4%/71.7%	33/2.8%/74.8%	6/3.8%/70.9%	14/2.5%/70.4%	36/2.5%/70.4%	30/2.6%/76.6%
9	Plastic pieces (macro)	String and cord (< 1 cm)	Bottles incl. pieces	Food waste (galley waste)	Bottle caps, lids and pull tabs	Foam sponge/pu foam
	216/2.3%/74.0%	31/2.6%/77.5%	4/2.5%/73.4%	14/2.5%/72.9%	34/2.3%/72.8%	27/2.3%/79.0%
10	Plastic pieces (micro)	Other glass items	Other glass items	Paper fragments	Plastic pieces (macro)	Sheets, Industrial packaging
	210/2.3%/76.2%	26/2.2/79.7%	4/2.5%/75.9%	11/2.0%/74.9%	33/2.3/75.0%	25/2.1%/81.1%
top ten	7124; 76.2%	937/79.7%	120/75.9%	415/74.9%	1088/75.0%	944/81.1%
	Lithuania	**Latvia**	**Estonia**	**Finland**	**St. Petersburg**	**Sylt island**
1	Paraffin (meso)	Industrial pellets	Industrial pellets	Cigarette butts	Industrial pellets	Paraffin (meso)
	507/36.4%	314/34.2%	742/48.5%	305/30.4%	381/38.1%	198/46.6%
2	Plastic pieces (meso)	Plastic pieces (meso)	Other glass items	Industrial pellets	Plastic pieces (meso)	Paraffin (macro)
	245/17.6%/54.1%	143/15.6%/49.8%	274/17.9%/66.4%	188/18.7%/49.1%	349/34.9%/72.9%	85/20.0%/66.6%
3	Industrial pellets	Cigarette butts	Plastic pieces (meso)	Plastic pieces (meso)	Plastic pieces (micro)	Industrial pellets
	95/6.8%/60.9%	122/13.3%/63.1%	156/10.2%/76.6%	126/12.5%/61.7%	96/9.6%/82.5%	45/10.6%/77.2%
4	Cigarette butts	String and cord (< 1 cm)	Cigarette butts	Paraffin (meso)	Bottles incl. pieces	Plastic pieces (meso)
	90/6.5%/67.4%	76/7.3%/70.4%	72/4.7%/81.3%	54/5.4%/67.0%	44/4.4%/86.9%	19/4.5%/81.6%
5	String and cord (< 1 cm)	Plastic pieces (macro)	Bottles incl. pieces	Other glass items	Other glass items	String and cord (< 1 cm)
	63/4.5%/71.9%	37/4.0%/74.5%	71/4.6%/85.9%	38/3.8/70.8%	31/3.1%/90.0%	17/4.0%/85.6%
6	Paraffin (micro)	Other glass items	Plastic pieces (micro)	Bottles incl. pieces	Cigarette butts	Paraffin (micro)
	42/3.0%/74.9%	24/2.6%/77.1%	26/1.7%/87.6%	33/3.3%/74.1%	23/2.3%/92.3%	11/2.6%/88.2%
7	Plastic pieces (macro)	Sheets, Industrial packaging	Oil/Tar/Paint particles	String and cord (< 1 cm)	Oil/Tar/Paint particles	Plastic pieces (macro)
	32/2.3%/77.2%	18/2.0%/79.1%	21/1.4%/89.0%	27/2.7%/76.8%	19/1.9%/94.2%	10/2.4%/90.6%
8	Plastic pieces (micro)	Bottles incl. pieces	Construction material	Plastic pieces (macro)	Cotton bud sticks	Cigarette butts
	30/2.2%/79.4%	15/1.6%/80.7%	18/1.2%/90.1%	22/2.2%/79.0%	15/1.5%/95.7%	9/2.1%/92.7%
9	Other glass items	Crisp packets/sweet wrappers	String and cord (< 1 cm)	Processed timber with paint	Foam sponge/pu foam	Other wood < 50 cm
	30/2.2%/81.5%	13/1.4%/82.1%	14/0.9%/91.1%	18/1.8%/80.8%	7/0.7%/96.4%	4/0.9%/93.6%
10	Paraffin/Wax macro	Cotton bud sticks	Foam sponge/pu foam	Foil wrappers, aluminium foil	Plastic caps/lids unidentified	Bottles incl. pieces
	27/1.9%/83.5%	12/1.3%/83.4%	12/0.8%/91.8%	14/1.4%/82.2%	7/0.7%/97.1%	4/0.9%/94.6%
top ten	1161/83.5%	765/83.4%	1406/91.8%	825/82.2%	972/97.1%	402/94.6%

St. Petersburg results do not include macro-litter pieces. For every litter item the total amount and the percentage is shown and the cumulative percentage of the top ten litter items.

following: 370 micro-litter pieces (0.62 pieces/m^2-31.8%), 497 meso-litter pieces (0.83 pieces/m^2-42.7%) and 297 macro-litter pieces (0.50 pieces/m^2-25.5%) (**Table 3**). On six beach areas with two surveys each, the small-scale spatial variation of litter per beach area was between 0.18 and 1.72 pieces/m^2 (mean 0.61 pieces/m^2 ± 0.52; median 0.44 pieces/m^2). The vast majority of litter along the northern region was paraffin (mean 1.03/m^2 ± 0.81; median 0.68 pieces/m^2). Lower amounts of paraffin (mean 0.07/m^2 ± 0.03; median 0.08 pieces/m^2) were found along the southern region (**Table 1**).

Lithuania

During 28 surveys (1502.5 m^2) altogether 1391 litter pieces were collected (mean 0.93 pieces/m^2 ± 0.63; median 0.80 pieces/m^2). These were composed of 199 micro-litter pieces (0.13 pieces/m^2-14.3%), 889 meso-litter pieces (0.59 pieces/m^2-63.9%) and 303 macro-litter pieces (0.20 pieces/m^2-21.8%) (**Table 3**). In Klaipeda/Palanga region, the highest pollution detected was 2.69 and 2.3 pieces/m^2. The lowest pollution of litter found was at the Curonian spit with 0.06 and 0.16 pieces/m^2 (**Table 1**). On 14 beach areas with two surveys each, the small-scale spatial variation of litter per beach area ranged between 0.04 and 0.76 pieces/m^2 (mean 0.28 pieces/m^2 ± 0.23; median 0.19 pieces/m^2).

Latvia

The overall pollution detected during 27 surveys (1525 m^2) was 917 litter pieces (mean 0.60 pieces/m^2 ± 1.74; median 0.23 pieces/m^2), with a minimum of 0.00 pieces/m^2 and a maximum of 9.32 pieces/m^2. The litter was nearly equally distributed within the size classes: 334 micro-litter pieces (0.22 pieces/m^2-36.4%), 264 meso-litter pieces (0.17 pieces/m^2-28.8%) and 319 macro-litter pieces (0.21 pieces/m^2-34.8%) (**Table 3**). On 13 beach areas (with 2 surveys each) the small-scale spatial variation of litter per beach area was between 0.05 and 7.28 pieces/m^2 (mean 0.71 pieces/m^2 ± 1.90; median 0.18 pieces/m^2). By far, region Liepaja was the most polluted one, due to the presence of 9.32 litter pieces/m^2. Lowest amount of litter recorded was in Ventspilis; between 0.00 pieces/m^2 and 0.21 pieces/m^2 (**Table 1**).

Estonia

In 21 surveys (1075 m^2) a total of 1531 litter pieces were found (mean 1.42 pieces/m^2 ± 3.1; median 0.25 pieces/m^2), with a minimum of 0.02 pieces/m^2 and a maximum of 13.96 pieces/m^2. The size distribution was 808 micro-litter pieces (0.75 pieces/m^2-52.8%), 426 meso-litter pieces (0.40 pieces/m^2-27.8%) and 297 macro-litter pieces (0.28 pieces/m^2-19.4%) (**Table 3**). On 8 beach areas with 2 surveys each, the small-scale spatial variation of litter per beach area was between 0.00 and 8.86 pieces/m^2 (mean 1.27 pieces/m^2 ± 2.87; median 0.22 pieces/m^2). Around Tallinn the highest quantity of litter found was13.96 pieces/m^2 and 5.10 pieces/m^2. Lower numbers of litter were found in the region of Häädemste (0.02 pieces/m^2 and 0.18 pieces/m^2) (**Table 1**).

Finland

During 18 surveys (967.5 m^2) the amount of litter found was 1004 pieces (mean 1.04 pieces/m^2 ± 0.95; median 0.78 pieces/m^2). The

TABLE 3 | Litter sizes in pieces/m^2 and the litter categories in total numbers and in percentage for each country.

Country	Litter sizes in pieces/m^2			All litter sizes in pieces/m^2	Litter categories in total numbers and in percentage							
	Micro	Meso	Macro		Artifical polymer	Cigarette butts	Paper/Cardboard	Metal	Glass/ceramics	Paraffin	Other	Total
Sweden	0,18	0,37	0,17	0,72	679 (58%)	125 (11%)	28 (2%)	22 (2%)	49 (4%)	119 (10%)	154 (13%)	1176
Denmark	0,01	0,05	0.09	0.15	63 (40%)	57 (36%)	10 (6%)	3 (2%)	9 (6%)	1 (1%)	15 (9%)	158
Germany	0,02	0,26	0,31	0,59	175 (32%)	233 (42%)	21 (4%)	15 (3%)	54 (10%)	0 (0%)	56 (10%)	554
Poland	0,22	0,69	0,55	1,45	608 (42%)	312 (22%)	161 (11%)	96 (7%)	11 (1%)	11 (1%)	251 (17%)	1450
Russia Kaliningrad	0,62	0,83	0,50	1,94	634 (54%)	110 (9%)	24 (2%)	11 (1%)	35 (3%)	329 (28)%	21 (2%)	1164
Lithuania	0,13	0,59	0,20	0,93	591 (42%)	90 (6%)	17 (1%)	11 (1%)	51 (4%)	576 (41%)	55 (4%)	1391
Latvia	0,22	0,17	0,21	0,60	692 (75%)	122 (13%)	14 (2%)	15 (2%)	41 (4%)	10 (1%)	23 (3%)	917
Estonia	0,75	0,40	0,28	1,42	1022 (67%)	72 (5%)	16 (1%)	23 (2%)	364 (24%)	2 (0.1%)	32 (2%)	1531
Russia St. Petersburg	1,33	1,05	0,13	2,51	877 (88%)	23 (2%)	2 (0.2%)	4 (0.4%)	76 (8%)	0 (0%)	19 (2%)	1004
Finland	0,23	0,40	0,41	1,04	457 (46%)	305 (30%)	26 (3%)	31 (3%)	74 (7%)	68 (7%)	43 (4%)	1001
Germany North Sea	0,08	0,33	0,18	0,59	104 (24%)	9 (2%)	2 (0.5%)	3 (1%)	6 (1%)	294 (69%)	7 (2%)	425

Macro-litter and all litter sizes results of St Petersburg are underlined because during the surveys it came to a loss of most of the macro-litter pieces and therefore only the large micro-litter and the meso-litter fraction are presented correctly.

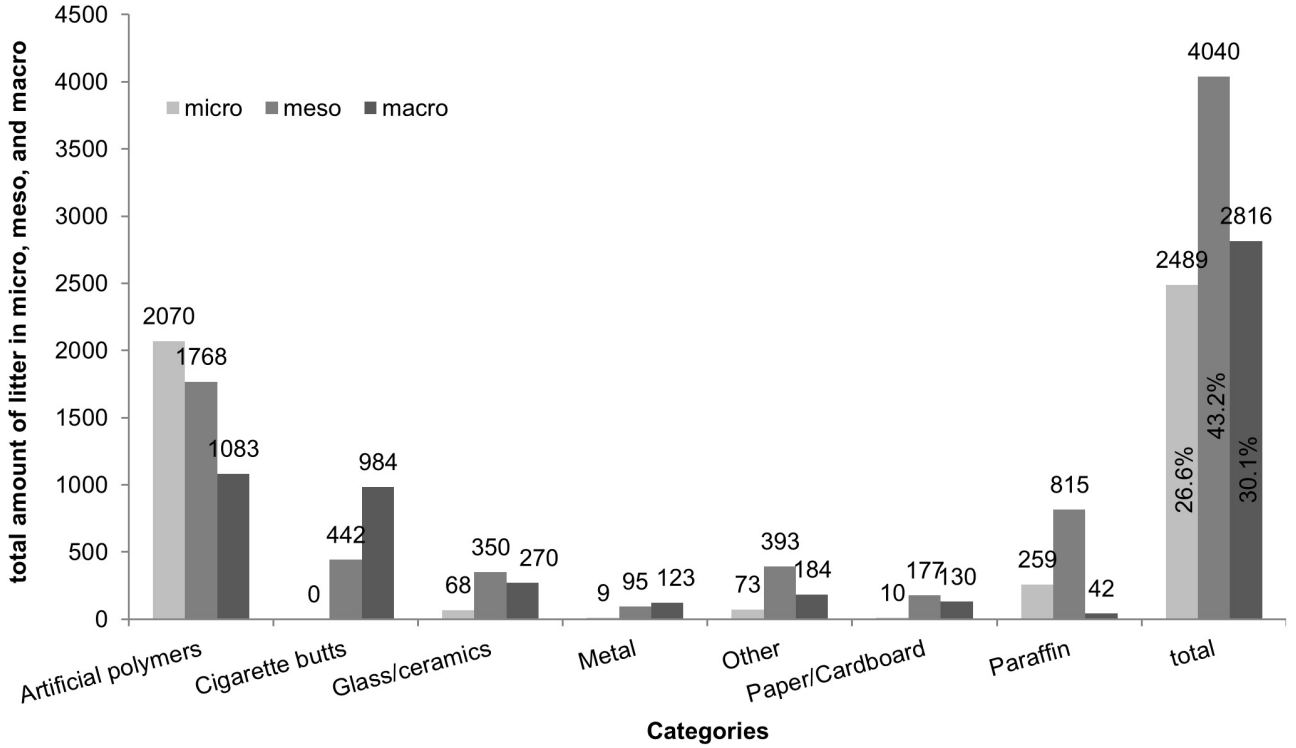

FIGURE 3 | The amount of micro-, meso-, and macro-litter per category and the total amount of litter at the beaches of the Baltic Sea (n = 197).

highest pollution (4.1 pieces/m^2) found was around Hanko. The lowest quantity of litter was found in the region of Pori (0.08 pieces/m^2) (**Table 1**).The size class distribution was 227 micro-litter pieces (0.23 pieces/m^2-22.6%), 383 meso-litter pieces (0.40 pieces/m^2-38.1%) and 394 macro-litter pieces (0.41 pieces/m^2-39.2%) (**Table 3**). On 4 beach areas with 2 surveys each, the small-scale spatial variation of litter per beach area was between 0.06 and 0.40 pieces/m^2 (mean 0.29/m^2 ± 0.14; median 0.35 pieces/m^2 ± 0.14; median 0.35 pieces/m^2).

Russia – St. Petersburg Region

During the surveys it came to a loss of most of the macro-litter pieces and therefore only the large micro-litter and the meso-litter fraction are presented correctly. Accordingly, the amount of 52 macro-litter pieces (0.13 macro-litter pieces/m^2) is too low as many more macro-litter pieces were found but could not be quantified due to their loss. That also influences the total number of litter found (**Table 3**); here the numbers would be higher too.

In 8 surveys (400 m^2) a total of 1001 litter pieces were found (mean 2.50 pieces/m^2 ± 2.30; median 1.81 pieces/m^2). The size class distribution was 530 micro-litter pieces (1.33 pieces/m^2-52.9%), 419 meso-litter pieces (1.05 pieces/m^2-41.9%) and 52 macro-litter pieces (0.13 pieces/m^2-5.2%) (**Table 3**). The highest quantity of litter found was 8.14 pieces/m^2. The lowest amount of litter recorded was 0.68 pieces/m^2 and 0.82 pieces/m^2 (**Table 1**). In two surveys (RU 13 and RU 14) hundreds of non-identifiable rusted metal pieces (2-10mm) were found. These were found nowhere else, and as there was no reference to the original source, the results of this outlier event were not included in the following results and figures.

Germany North Sea/Sylt Island

A total of 425 litter pieces (mean 0.51 ± 0.37 pieces/m^2; median 0.45 pieces/m^2) were found in 15 surveys (829 m^2) on the island of Sylt composed of 56 micro-litter pieces (0.07 pieces/m^2-13.2%), 238 meso-litter pieces (0.29 pieces/m^2-56.0%) and 131 macro-litter pieces (0.16 pieces/m^2-30.8%) (**Table 3**). Paraffin occurred in 13 of 15 (87%) surveys and 198 pieces (67.3%) of the paraffin belonged to the meso-litter size class (**Table 2**).

General Results Baltic Sea

Due to the loss of macro-litter data (disappearance/loss of the plastic bags with litter), the eight St. Petersburg surveys were not included in the following results. The litter collected in 197 surveys (10.271 m^2) on the Baltic beaches (without St. Petersburg) of nine countries totaled up to 9345 pieces (mean 0.91/m^2 ± 1.50; median 0.40 pieces/m^2) of which there were 2489 micro-litter pieces (0.24 pieces/m^2-26.6%), 4040 meso-litter pieces (0.39 pieces/m^2-43.2%), and 2816 macro-litter pieces (0.27 pieces/m^2-30.1%) (**Figure 3** and **Table 3**). The artificial polymers category was the main contributor to the overall pollution (mean 52.7% ± 13.3) in nearly all countries, followed by cigarette butts (mean 15.3% ± 13.0), and paraffin (mean 11.9% ± 14.1). Glass/ceramics (mean 7.4% ± 6.4), other (mean 7.0% ± 5.2), paper/cardboard (mean 3.4% ± 3.1), and metal (mean 2.4% ± 1.7) were only found in lower amounts (**Table 3**).

Artificial polymers in the micro-and meso-litter size class had an especially high share (41.1%) of the overall pollution. In Lithuania (41%) and Kaliningrad, Russia (28%), paraffin was found in large numbers.

Industrial Pellets, Cigarette Butts, Non-identifiable Plastic Pieces and Paraffin

In total, 1852 industrial pellets found in 102 of 197 (52%) surveys were the most common litter item in this study (19.8%), and ranged from 0 to 8.86 industrial pellets/m^2 (mean 0.18/m^2 ± 0.79; median 0.02 pieces/m^2). With one exception (Germany), industrial pellets were found in all countries within the top ten items (**Table 2**). Most industrial pellets 45.6% (in three surveys) and 73.3% (in ten surveys) were found close to the urban/industrial regions.

Cigarette butts were the second most abundant litter item (15.3%), with a total of 1426 cigarette butts found in 150 out of 197 (76%) surveys and their quantity ranged from 0 to 1.51 cigarette butts/m^2 (mean 0.14/m^2 ± 0.22; median 0.06 pieces/m^2). A total of 984 (69.0%) cigarette butts found belonged to the macro-litter size class, the other 442 (31.0%) were meso-litter.

With a quantity of 1405 pieces, meso-plastic pieces were the third most common litter, found in 145 of 197 surveys (73.6%), with a range of 0 to 2.0 meso-plastic pieces/m^2 (mean 0.14/m^2 ± 0.25; median 0.05 pieces/m^2). 210 micro-plastic pieces were found in 76 of 197 surveys (38.6%), with a range of 0 to 0.29 pieces/m^2 (mean 0.02/m^2 ± 0.04; median 0.00 pieces/m^2).

In total, 1116 paraffin pieces (all size classes) were found in 58 of 197 (29.4%) surveys. Most of the paraffin pieces were collected in Lithuania (62.2%), in Russia, Kaliningrad (19.8%), in Sweden (9.1%) and in Finland (6.6%). Lowest numbers of paraffin pieces were found in Poland (1.1%); Latvia (0.9%); Estonia (0.2%); and Denmark (0.1%), while no paraffin pieces were found in Germany and in Russia, St. Petersburg. Paraffin was found in all size classes. Combined results of the three size classes lead to a quantity range from 0 to 2.28 paraffin pieces/m^2 (mean 0.11/m^2 ± 0.33; median 0.00 pieces/m^2).

Near-Infrared Polymer Analysis With the Microphazir

Including the results of St. Petersburg region, a total of 2283 non-identifiable plastic pieces were found (223 micro-pieces - 9.8%, 1754 meso-pieces - 76.8%, and 306 macro-pieces - 13.4%). For 1925 (84.3%) of these non-identifiable plastic pieces the polymer type could be identified with an accuracy of ≥95%. The majority of these plastic pieces were composed of polyethylene (PE: 890 pieces, 39.0%), polypropylene (PP: 595 pieces, 26.1%), and polystyrene (PS: 417 pieces, 18.3%). Altogether for 358 plastic pieces (15.7%) the polymer type could not be identified, for 141 particles (6.2%) it was because of their black color. The other 0.9% consisted of polyvinyl chloride (PVC), acrylonitrile butadiene styrene (ABS), polyamide (PA), polycarbonate (PC), polyethylene terephthalate (PET) and poly-methyl methacrylate (PMMA) (**Figure 4**). Most of the non-identifiable plastic pieces had a white color followed by transparent pieces, blue pieces and other colors (**Figure 4**). Most frequent were white PS (388 pieces/17.0%), white PE (180 pieces/7.9%), transparent PE (159 pieces/7.0%), unidentified black pieces (141 pieces/6.2%), and white PP (124 pieces/5.4%).

Costs of a Long-Term Monitoring With the Sand Rake Method at German Baltic Beaches

Based on the experience gathered during the surveys, we calculated the costs of future long-term monitoring campaign at German Baltic beaches using the sand rake method (**Table 4**). The cost of one survey (WS 1-5) conducted by experts (37.50€/h) is 7,104.30€ including all material costs. The costs for one survey (WS 1-5) of the volunteer-based approach including all material costs and the one-time training workshop are 7,050.75€. After the training workshop the beach related work steps (WS 1-2) can be performed independently by the volunteers. The same material (sand rake, Microphazir, etc.) can be used during the long-term monitoring campaign. Therefore, with every further replicate at the beach the labor costs only increase by 749.70€ (experts) and 267.75€ (volunteer). Altogether, the costs of one year of monitoring campaign at 15 non-OSPAR beaches (**Figure 5**.) surveyed four times a year are 51,336.60€ (expert) and 27,846.00€ (volunteer). Note that the volunteer approach includes costs for 15 sand rakes, measuring tapes, etc. At the 14 German Baltic beaches, suggested for OSPAR method monitoring, the sand rake method monitoring costs would amount to 48,337.80€ (expert) or 26,418.00€ (volunteer).

DISCUSSION

With an average of 0.91 pieces/m^2 for all the Baltic surveys (n = 197) the pollution of the Baltic Sea beaches is lower compared to other areas of the world. Comparable sieving methods often focused on plastics only and reported higher pollution levels, such as 185 plastic pieces/m^2 in Portugal (MS of 2.5 × 3.5 mm) (Martins and Sobral, 2011), >430–1600 micro-litter pieces/m^2 at the Canary islands (Herrera et al., 2018), >19.000 plastic pieces/m^2 at beaches of South Korea (MS between 1 and 5 mm) (Lee et al., 2013). The results of former sand rake method studies at the Baltic Sea are comparable to our results. In Haseler et al. (2017), pollution of German beaches was between 0.5 and 3.0 pieces/m^2, and in Lithuania at 0.56 pieces/m^2. Sand rake method results along the Curonian spit are in the same order of magnitude (0.63–2.34 pieces/m^2) (Esiukova et al., 2020). Artificial polymers (52.7%) have the largest share of pollution on the Baltic Sea beaches which is identical to the finding of other studies worldwide (Aniansson et al., 2007; Marlin, 2013; Oosterhuis et al., 2014; LUNG M-V, 2015; Ríos et al., 2018). The most common polymer types (PE, PP, and PS) and their percentages (39, 26.1, and 18.3% respectively) are comparable to previous studies (Hidalgo-Ruz et al., 2012; Urban-Malinga et al., 2020). The high amounts of cigarette butts (15.3%) are a known problem at the Baltic beaches (Schernewski et al., 2017; Veiga et al., 2016) and paraffin occurs

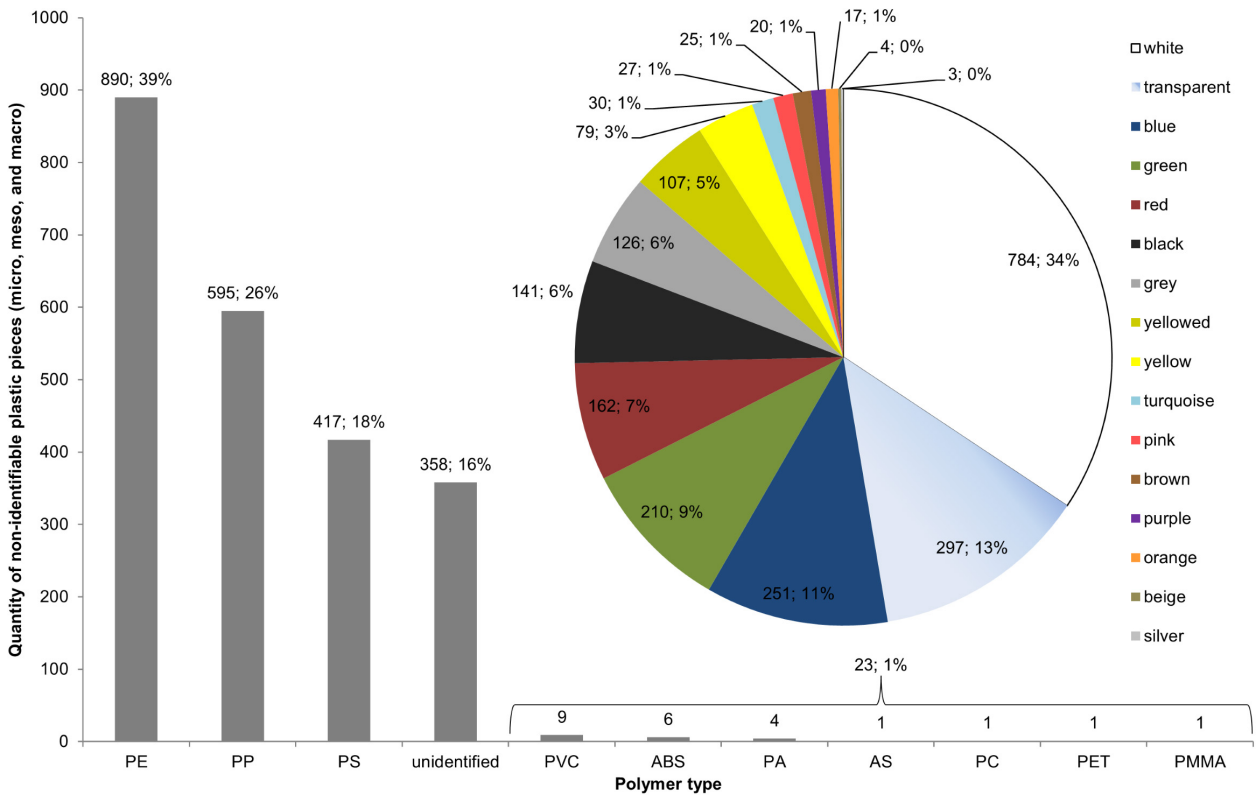

FIGURE 4 | The quantity per polymer type (gray bars) and the color (pie chart) of 2283 non-identifiable plastic pieces (micro, meso, and macro) from the 205 surveys of the beaches around the Baltic Sea (including St. Petersburg).

regularly on beaches of the Curonian spit (Esiukova, 2016). Lower amounts of paper/cardboard, glass/ceramics, metal and other categories are in line with former results (Marlin, 2013). This shows that our method is able to detect the common litter categories. Our method can also be used on urban/managed beaches. Furthermore, recovery rates were already calculated for different litter items and categories (Haseler et al., 2017). This enables a future assessment of the number of litter pieces that were probably missed and left at the beach. To the best of our knowledge no such recovery rate experiments exist for any naked eye methods, which makes an evaluation of these visual methods difficult, as it is unknown how much and what kind of litter is missed at the beach.

One intent of this study was to assess the meso-, and large micro-litter pollution; which has a combined share of 69% at our surveyed beaches of the Baltic Sea (n = 197) and the North Sea (n = 15). This is mostly due to the high amounts of industrial pellets, cigarette butts, non-identifiable plastic pieces and paraffin. As to the best of our knowledge, no other broad scale studies using a similar sieving method (2 mm MS) at the Baltic Sea beaches exist, and therefore the best possible comparison of our results are to a Joint Research Center (JRC) Report (Addamo et al., 2017). In this report, the aggregated results of ∼30.000 litter pieces collected with visual survey methods (Marlin, 2013; OSPAR, 2010) on beaches in different countries around the Baltic Sea are presented. As described in the report, the investigated area was typically a transect of 100 m length, which was surveyed three or four times a year; all visible litter pieces on the beach surface were picked up by volunteers, later on counted and entered in the MSFD Master list of litter items (Addamo et al., 2017).

In our study, industrial pellets account for 19.8% of all litter, yet in contrast no industrial pellets were found in the JRC Report. Industrial pellets are quickly mixed with or buried in the sediment (Abu-Hilal and Al-Najjar, 2009), and, if further large amounts of litter are present at the beach, it is easy to miss them (Velander and Mocogni, 1999) with naked eye methods. This is especially so, when pellets are colorless, as in our study (∼90%), and pebbles and shells are frequent on the beach. As a consequence, the number of industrial pellets found in our study is higher compared to the JRC report. However, industrial pellets were found only in 52% of our surveys. This might already be the effect of the regulatory measures and legislative framework of the European countries. Industrial pellets leach into the environment mainly due to spills during transport or production (Karlsson et al., 2018). The first scientific reports, documenting the occurrence of plastic pellets in the marine environment, were published in the 1970's (Carpenter et al., 1972), and therefore, half a century of public awareness might have been enough to reduce their leakage in the European region. The abundance of industrial pellets is decreasing with further

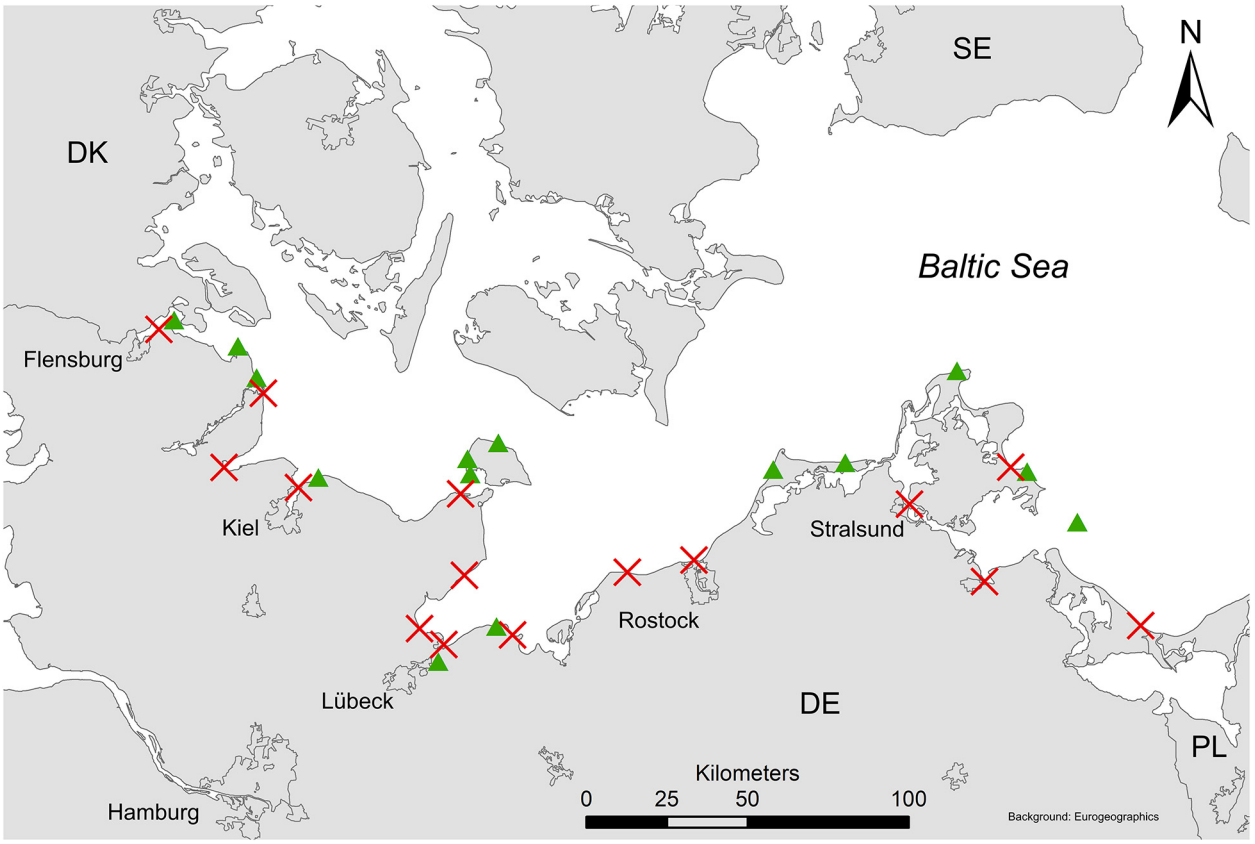

FIGURE 5 | Map of the 15 beaches suggested for a seasonal sand rake method monitoring only (red crosses) and the 14 beaches suggested for the sand rake monitoring implemented into the naked eye OSPAR method monitoring (green triangles).

distance from ports, etc. (Abu-Hilal and Al-Najjar, 2009). This aligns with our results, where the maximum contamination by pellets was found near large ports and in industrial regions close to the cities (Tallinn, Liepaja, Sopot, St.Petersburg and Hanko). Industrial pellets are washed ashore from the sea (Ivar do Sul and Costa, 2014) and in a German study they were mostly found after storm surges (Haseler et al., 2019). Therefore, their abundance is most probably low during summer times with fewer storms. Furthermore, in the micro-tidal Baltic Sea their abundance is most probably decreasing toward higher parts of the beach, leading to lower amounts of pellets found with our method.

The amount of cigarette butts in the JRC report is half (7.74%) of what we found (15.3%). Cigarette butts occur in high numbers even at manually and mechanically cleaned beaches (Laglbauer et al., 2014; Zielinski et al., 2019) and therefore they accumulate and get buried over time (Loizidou et al., 2018). Our surveys took place mostly during summer. Therefore, it might be that more beach users led to the higher amount of cigarette butts found. Similar results with more cigarette butts found during summer were already reported for the German Baltic coast (LUNG M-V, 2015). Nevertheless, 35.1% of cigarettes butts we found are meso-litter which makes it difficult to see them using naked eye methods, especially if they are partially or entirely below the surface.

The number of plastic pieces (<25 mm) found in the JRC report (1.45%) is smaller by one order of magnitude compared to our results (15.0% meso + 2.3% large micro). However, these differences are not surprising; 64% of the non-identifiable plastic pieces in our study have a colorless (white, transparent, gray, black or yellowed) appearance. Besides the litter color, the surveyor's ability to detect plastic litter (≤20 mm) depends on the sand color and presence of shell fragments, which in an experimental study resulted in an undercounting of plastic in 72.8% of 2472 counts, with 103 observers (Angelini et al., 2019).

Paraffin (10.8% including all size classes) was lower compared to the JRC report (31.85%), which is probably due, both to single surveys with extremely high abundances of paraffin and to the categorization of 'paraffin/wax' in the JRC report which includes a number of additional chemical compounds (Addamo et al., 2017). Nevertheless, in our study, paraffin was observed in large quantities in Lithuania (41%) and in Russia, Kaliningrad (28%). Paraffin is discharged at sea through tank washing. This is legal under current legislation, with specific restrictions (Rijkswaterstaat, 2018) leading to an increase of paraffin mass beaching events along European coasts (Suaria et al., 2018). Lots of vessels are permanently in the ports of Klaipeda (Lithuania) and Baltiysk (Kaliningrad, Russia), wash their tanks there, and the prevailing winds transport paraffin toward the shores of

TABLE 4 | Hours (h) and costs (€) for a single sand rake method survey for an expert- and a volunteer-based approach (left) and the costs of further replicates at the same beach.

Work step						Urban beaches (non-OSPAR)			Rural beaches (OSPAR)		
	hours [h] for one survey	Costs [€] for one survey (Expert)	Costs [€] for one survey (Volunteer)	Costs [€] for one replicate (Expert)	Costs [€] for one replicate (Volunteer)	Annual hours [h year-1] 15 beaches 4x per year	Annual cost [€ year-1] 15 beaches 4x per year (Expert)	Annual cost [€ year-1] 15 beaches 4x per year (Volunteer)	Annual hours [h year-1] 14 beaches 4x per year	Annual cost [€ year-1] 14 beaches 4x per year (Expert)	Annual cost [€ year-1] 14 beaches 4x per year (Volunteer)
1 x Training workshop for volunteers (conducted by two experts)	4	/	300.00	/	/	/	/	300.00	/	/	300.00
Travel by car to the beach and back	4	150.00	/	150.00	/	240	9,000.00	/	224	8,400.00	/
Sand rake survey (2x~50m^2) at one beach	5	187.50	/	187.50	/	300	11,250.00	/	280	10,500.00	/
Litter analyses steps: counting, measuring, categorizing, polymer analysis	2.5	93.75	93.75	93.75	93.75	150	5,625.00	5,625.00	140	5,250.00	5,250.00
Data processing	1.5	56.25	56.25	56.25	56.25	90	3,375.00	3,375.00	84	3,150.00	3,150.00
Reporting	1	37.50	37.50	37.50	37.50	60	2,250.00	2,250.00	56	2,100.00	2,100.00
Material expenses: Sand Rake, measure tape, consumables		250.00	250.00	/	/	/	250.00	3,750.00	/	250.00	3,500.00
Material: Microphazir (21,000 €, life-time 5 years)		4,200.00	4,200.00	/	/	/	4,200.00	4,200.00	/	4,200.00	4,200.00
Net sum		4,975.00	4,937.50	525.00	187.50	/	35,950.00	19,500.00	/	33,850.00	18,500.00
20% Overhead		995.00	987.50	105.00	37.50	/	7,190.00	3,900.00	/	6,770.00	3,700.00
19% VAT		1,134.30	1,125.75	119.70	42.75	/	8,196.60	4,446.00	/	7,717.80	4,218.00
Gross sum		7,104.30	7,050.75	749.70	267.75		51,336.60	27,846.00		48,337.80	26,418.00

Shown are also the hours and costs of a sand rake method monitoring (expert and volunteer) on 15 urban beaches (middle) and on 14 rural beaches suggested for a regular macro-litter (OSPAR) monitoring at the German Baltic Sea (right).

the region. For the hundreds of metal pieces found around St. Petersburg no origin could be identified.

In general, it is doubtful that meso- and large micro-litter (JRC, 2011), industrial pellets (MSFD TSG ML, 2013), and cigarette butts (Kataržytė et al., 2020) can be monitored effectively if only the beach surface is visually investigated (OSPAR method) without having a closer look (sieving methods) in the upper layers of the beach sediment. This is especially so, given the greatest limitation for a precise quantification at the beach is the human eye (Vegter et al., 2014), which overlooks most of this litter without any sediment reduction step. Furthermore, plastic that is exposed long-term to UV radiation at the beach fragments faster than in seawater (Andrady, 2011). This (relatively) rapid size reduction leads to particles which can be even harder to see. Compounding this is the fact, most of the secondary micro- and meso-plastic pieces are white-to-colorless (Hidalgo-Ruz et al., 2012; Zobkov and Esiukova, 2018; Lacerda et al., 2019; Jeyasanta et al., 2020), as it was the case in our study. Many of these colorless pieces (<25 mm) are undercounted using naked eye methods (Angelini et al., 2019), even by experienced observers (Lavers et al., 2016) and professional beach cleaners. Therefore, they remain in the sediment (Laglbauer et al., 2014; Zielinski et al., 2019), which leads to an accumulation and further fragmentation over time (Loizidou et al., 2018) and to a direct threat to marine organisms through to ingestion (JRC, 2011). Ultimately, these factors compound to make naked eye (OSPAR method) surveys less likely to reliably sample challenging micro- and meso-litter demonstrate the value of methods, such as the sand rake approach, which help to aid litter determination.

Industrial pellets, cigarette butts, non-identifiable plastic pieces 2–25 mm and paraffin (all sizes) were responsible for ∼64% of all our findings. Exactly these items/size classes were neglected by the JRC to calculate the "EU marine beach litter baselines" (Hanke et al., 2019). They were not included because the naked eye methods used to gather the baseline data delivers either incomplete coverage data, not comparable results and they are not made to collect meso-litter (Hanke et al., 2019). Yet as our study shows, these are important contributors to beach litter in the Baltic and North Sea environment and need a harmonized monitoring approach to quantify their amount. Avoidance and mitigation strategies to minimize the amount of paraffin (Marine Environment Protection Committee (MEPC), 2019a,b), prevent spillage of industrial pellets (Plastics Europe, 2018); and introduce reduction measures for cigarette butts (European Commission, 2018; Kataržytė et al., 2020; Schneider et al., 2011) are important steps for a cleaner marine environment. However, such steps need to be evaluated to test if they lead to a quantifiable success. Therefore, suitable and cost effective long-term monitoring methods, usable on a broad scale of beaches, are needed that cover these litter items and size classes (2–25 mm). Here, the sand rake method can be an effective tool.

Beach Litter Baseline

Our results can be used as a starting point to calculate the average litter abundance per m^2 (2–25 mm) on sandy Baltic beaches. However, the high variation of litter quantity makes it necessary to consider further environmental influences such as wind, tides and site exposure (Schöneich-Argent et al., 2019) when choosing survey areas for long-term monitoring. The small-scale distribution of litter makes the choice of location an important decision (Bergmann et al., 2015). Furthermore, an expanded survey approach over a longer period of time at different beaches (replicates) delivers in all likelihood more reliable results. Ultimately, a statistical calculation of the minimum number of surveys (based on the amount of monitoring beaches and replicates) is needed for a baseline calculation of the average litter pollution. It is important to determine if only urban/cleaned beaches that do not meet the criteria for selecting reference beaches should be surveyed or if a combined approach, including rural OSPAR method beaches, is used. We assume that a combined approach is more reliable, because urban regions are more polluted in general, as shown by our study. A baseline calculated solely on the litter abundance at urban beaches would be too high, and many rural beaches would reach this target without the implementation of mitigation measures.

Evaluation of the Sand Rake Method Regarding Monitoring Needs of the MSFD

Our results showed that the sand rake method is able to detect large micro-, and meso-litter on the top layer (3-5 cm) of sandy beaches that are usually missed during the OSPAR method (naked eye) surveys. The sand rake method works best when the sediment is dry and fine (no pebbles, gravel, etc.) having a recovery rate of ∼65% regarding meso-litter (cigarette butts, etc.) and ∼50% for larger micro-litter (Haseler et al., 2017). Therefore, the weather should be considered while planning a survey because wet sediment makes sieving complicated and requires more time. On Sylt Island the tides left half the beach wet, which made sieving very hard as the sediment got stuck in the sand rake. Therefore, the hands had to be used to rub/press it through the rake. In Russia, St. Petersburg the sediment grain size was very coarse and a lot of sediment remained in the sand rake. That made it harder for the observer who was looking for litter pieces between the stuck sediment. In such cases a larger mesh size (5 mm) might be helpful or the sediment could be rinsed out with seawater but this ideas were not further considered in this study. For further strength and weaknesses of the method see Haseler et al. (2017). Considering most beaches we surveyed, the sand rake method could be used without major problems. To gather quality data that is comparable across regions and countries, training of volunteers is essential. Further recovery rate experiments with different litter items and size classes tested by volunteers could lead to more precise recovery rates and estimations of the "true beach pollution." With minor adjustments the list of litter and the "OSPAR Marine Litter Monitoring Survey Form" can be used for sand rake monitoring as well, guaranteeing comparable results in terms of litter items. Polymer analysis of litter with the Microphazir is fast and easy, and information about polymer types might provide knowledge about litter degradation on beaches (Hanke et al., 2019). It should be noted that the polymer type distribution shown in **Figure 4** characterizes just non-identifiable plastic pieces,

i.e., those plastics which were much degraded/fragmented in the marine environment. Litter items made of harder polymers, like, e.g., PET bottles or PVC packaging, are typically well identifiable on the beach, and were thus counted as the corresponding litter item. Indeed, the distribution of polymer types of plastic particles shows the predominance of PE, PP, and PS – the polymers widely used for cheap disposable products. This is one more argument in favor of legislative restrictions against single-use plastic products; their elimination should significantly reduce the amount of meso- and micro-plastic items on the beaches. Therefore, a reliable monitoring method for meso- and large micro-litter is needed to evaluate the success of such restrictions.

We think that the sand rake method is suitable as a monitoring method that fits the requirements of the MSFD. Therefore, we discuss how sand rake monitoring can be used at beaches where the OSPAR method is not suitable. Furthermore, we consider a combined approach where the sand rake method and the OSPAR method are used at the 14 OSPAR beaches. The 15 beaches selected for sand rake monitoring cover beaches where an OSPAR method monitoring is not suitable. These beaches can be used as a representative case study of the litter pollution (2–25 mm) of urban, managed, touristic and river mouth beaches at the German Baltic Sea. In order to obtain comparable results to former studies, we suggest that a seasonal monitoring approach is most suitable. The monitoring at the 15 beaches should be standardized (temporally and spatially) in communication with local authorities, as they should have the best knowledge concerning the beach management. In any case, many external factors (like voluntary cleaning activities, beach access, littering behavior, etc.) cannot be controlled for, not even by local authorities and as a result, the abundance of litter is influenced by these external circumstances.

A combined sand rake method and OSPAR method monitoring at the 14 German beaches of the Baltic Sea would sufficiently cover all size classes of litter (>2 mm). This allows for the gathering of more data about the overall litter abundance and for results comparisons between both methods. Such monitoring could be organized as follows: at both ends of a pre-defined 100 m OSPAR monitoring section (surveyed with the naked eye), a sand rake survey (each $\sim 50\ m^2$) is made. This approach is considered for reference beaches that fit the pre-determined beach selection requirements of OSPAR monitoring.

With regard to costs, only average values can be proposed, as the survey time at the beach depends on the external circumstances and the litter analysis steps mostly on the litter quantity found, which differs from survey to survey. Nevertheless, the volunteer-based approach makes it possible to survey on a broad scale over a long time period at reasonable costs. This is necessary to detect possible changes in beach pollution over time. To guarantee a high standard of data acquisition (of the beach litter abundance), all involved actors/volunteers should be trained in how to use the sand rake method properly. Contact person need to be identified, both to help to solve possible problems on the fly and to also take responsibility for the quality assurance and quality control.

CONCLUSION AND RECOMMENDATIONS

Industrial pellets, large micro and meso-plastic, paraffin, cigarettes butts, and other litter items (2–25 mm) represented more than 69% of the litter found in our study. In terms of plastics, colorless pieces (barely visible for the naked eye) predominated. Such litter are particularly likely to have been underestimated by the application of the OSPAR method along European beaches historically. Furthermore, urban pollution hot-spots are currently not taken into account with the OSPAR approach. This leads to an incomplete pollution pattern and an underestimation of the present pollution. Due to the focus of the sand rake method on the 2–25 mm size fraction, monitoring on urban and rural beaches, without impact from clean ups and/or other disturbing activities is theoretically possible. The sand rake method is a cost-effective approach that meets policy demands. It allows for the involvement of volunteers and supports awareness-rising with respect to the litter problem. Our data can serve as basis for calculating pollution baselines and for defining the Good Environmental Status for Baltic beaches, as required by the MSFD. Furthermore, integration of the sand rake method into the existing OSPAR method monitoring is possible at low costs. Mobile spectrometers for rapid polymer analysis can provide additional information on pollution sources and on items that can serve as indicators, but here more research is needed. We recommend the application of the sand rake method over several years at different beaches, including seasonal sampling. This data would help to optimize the monitoring approach and would provide insights in the temporal dynamics of large micro-, and meso-litter pollution on Baltic beaches.

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

MH: conceptualization, methodology, field work, litter analysis, data analysis, and writing – original draft. AB: conceptualization, field work, and litter analysis. RH and AE: field work, litter analysis, and review. VS: field work and litter analysis. IC: review, editing, and funding acquisition. GS: supervision, conceptualization, writing, review, editing, and funding acquisition. 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/fenvs.2020.599978/full#supplementary-material

Supplementary Table 1 | List of the 220 beach surveys divided in the 36 regions with survey date, total amount of litter and litter/m^2.

Supplementary Table 2 | List of litter (n = 183). Amount of litter is divided in Baltic Sea (including St. Petersburg) and North Sea surveys.

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Distribution Patterns of Microplastics in Seawater Surface at a Portuguese Estuary and Marine Park

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Measuring local levels of marine pollution by microplastics (MP) and identifying potential sources in coastal areas is essential to evaluate the associated impacts to environment and biota. The accumulation of floating MP at the sea surface is of great concern as the neustonic habitat consists of a feeding ground for primary consumers (including filter-feeders) and active predators, which makes these organisms a relevant via of MP input into the marine trophic chain. Here, a baseline evaluation of MP accumulation at the sea surface was conducted with a neuston net (335 μm mesh) at the Arrábida coastal area, in Portugal. The study site encompasses a marine protected area and an estuary, both under strong anthropogenic pressures due to multiple activities taking place. A short-term investigation on local spatiotemporal distribution, concentration and composition of MP was performed for the first time, through the monthly collection (summer 2018 to winter 2019) of samples at 6 stations. All the neuston samples contained MP and their mean concentration was 0.45 ± 0.52 items m^{-3} (mean ± SD). Both the averaged MP:neuston and MP:ichthyoplankton ratios were higher in December, when concentrations of organisms decreased. Temporal distribution patterns followed expected trends, as MP concentration was clearly higher in winter months due to precipitation and runoff. Although mean MP concentrations did not vary significantly between sampling stations, there was a spatial distribution of MP in relation to particle shape and size. Fragments were the most abundant shape and MP belonging to 1–2 mm size class were dominant. Amongst a diversity of 10 polymers identified by FTIR analysis, polyethylene (PE), polypropylene (PP) and copolymer PP/PE were the most abundant. Potential links between local sources/activities and the different polymers were suggested. Altogether, the information provided in this study aims to raise awareness among the identified sectors and consequently to act toward the prevention of MP inputs in the region.

Keywords: microplastics, distribution, Sado estuary, marine park, MP:neuston ratio, MP:ichthyoplankton ratio, plastic polymers, Portugal

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INTRODUCTION

Tackling marine plastic pollution became a major planetary challenge of the 21st century. Besides the worldwide scientific contribution to the topic for more than one decade and the increasing public awareness, governments have proven their commitment by implementing more sustainable measures and encouraging both initiatives and changes (European Commission, 2018; UNEP, 2018). Yet, although plastic production has recently decreased in Europe from 64.4 MT in 2017 to 61.8 MT in 2018, it has continued to grow at a global level, from 348 MT in 2017 to 359 MT in 2018 (PlasticsEurope, 2019). This tendency largely relies on the persistent high demand for such a low-cost, lightweight, versatile, and durable material (Barnes et al., 2009). Consequently, and adding to the excessive consumption of disposable items (Napper et al., 2015) and poor waste management (Frias et al., 2014), plastic pollution represents a significant threat to the marine environment (Laskar and Kumar, 2019). The latest estimations pointed out between 1.1 and 8.8 MT of mismanaged plastic waste being generated annually by land-based human activities at each country (Jambeck et al., 2015). From this waste amount, a considerable part ends up in the marine environment, mainly through wastewater treatment plants discharges, land runoff or transported by the wind, rivers and tides (Andrady, 2011; Jambeck et al., 2015). Despite the greater relevance of terrestrial sources, there are several sea-based activities, such as fishing, aquaculture, maritime traffic, offshore platforms and recreational uses, which may also be considered as additional sources of plastic pollution (Browne et al., 2011; Jambeck et al., 2015; UNEP, 2016; Gewert et al., 2017).

Pioneer studies focused on plastic debris abundance and distribution in the marine environment inevitably verified that plastic pollution could act at a wide size range (from macro to nanoplastics), at a broad spectrum of impacts, as skin injuries or smothering from entanglement, gastrointestinal tract lesions or blockage from ingestion, and event act as vectors of pathogens and chemicals (Laist, 1987; Teuten et al., 2009; Kühn et al., 2015; Bowley et al., 2020). Indeed, the potential of smaller plastics to be ingested by marine biota (Barnes et al., 2009) and to be transferred throughout the trophic chain (Eriksson and Burton, 2003; Farrell and Nelson, 2013; Setälä et al., 2014), was rapidly recognized. This perception shifted the focus of investigation onto microplastics [hereafter MP; defined as particles between 1 μm and 5 mm (Arthur et al., 2009)] which developed into a new research topic addressed worldwide. In addition, the critical concern about the potential impacts of MP in human health through oral, dermal and inhalation exposure has triggered an increase of investigation on this subject although it remains poorly understood (Thompson et al., 2009; Galloway, 2015; Revel et al., 2018).

Regarding the origin of MP, it was considered to be either primary, if manufactured in microscopic size ranges (as industrial pellets and abrasives or microbeads from personal care products); or secondary, if resulting from fragmentation of larger objects (fishing gear, packaging, fibers from synthetic textile washing, paint flakes from nautical coating and dust from vehicle tire) (Cole et al., 2011; Hidalgo-Ruz et al., 2012; GESAMP, 2016; Rochman et al., 2019). The fragmentation of plastic may occur by photo-degradation, mechanical, chemical, and biological action (Barnes et al., 2009; Andrady, 2011; van Sebille et al., 2015). Regardless of its origin, an evident spatial distribution of MP in the water column occurs vertically, from the water surface to the seabed (Thompson, 2004). This mainly relies on polymers density and biofouling level, as both affect particles buoyancy (Gregory, 2009; Kaiser et al., 2017). Horizontal distribution of MP is also known to occur as a result of hydrodynamic forces, mainly by wind (Kukulka et al., 2012), tides, waves and thermohaline gradients (Zhang, 2017). In addition, both vertical (Choy et al., 2019) and horizontal distribution may be influenced by biota, through ingestion and egestion of MP in different compartments of the water column and different locations.

Regarding the impacts on marine biota upon MP ingestion, besides physical harm [e.g., damage in the gastrointestinal tract with inflammatory responses (von Moos et al., 2012) or false sense of satiation (Kühn et al., 2015)], toxicological effects have also been reported (Rochman et al., 2013; Wright et al., 2013). These rely on potential load of harmful chemicals adsorbed from seawater onto plastic and on the toxic additives incorporated during manufacture (Teuten et al., 2009). As a result, MP are suggested to act as trophic vectors of contaminants (Teuten et al., 2007; Garcia-Garin et al., 2020), although their contribution for bioaccumulation (and bioamplification) in organisms tissues may not be as relevant as other contamination pathways, such as prey ingestion or dermal uptake (Koelmans et al., 2016).

Understanding the exposure of primary consumers to MP became essential to evaluate the consequent implications in the marine trophic chain (including eventual detrimental impacts on human health due to seafood contamination). This triggered an increase in research aiming at calculating encounter rates between MP and primary consumers, based on their concentrations and ratio (Collignon et al., 2012, 2014; Hitchcock and Mitrovic, 2019). Yet, such research has been scarcely conducted in Portuguese waters (Frias et al., 2014; Rodrigues et al., 2019), being insufficient for a country where fisheries have a large cultural and social importance (FAO, 2017) and where seafood constitutes a very important diet component (FAO, 2010; Almeida et al., 2015; EUMOFA, 2020).

Both the Sado estuary and Professor Luiz Saldanha Marine Park, located at the Portuguese west coast, are important nursery areas for fish larvae (Borges et al., 2007, 2009) and constitute valuable artisanal fishing grounds (Horta e Costa et al., 2013a,b; Batista et al., 2015). However, there are multiple anthropogenic activities taking place at this coastal zone, potentially contributing to local and regional MP pollution and thus posing a threat to this hotspot of biodiversity (Cunha et al., 2014). In this context, this study aims to contribute with baseline data on MP pollution at a Portuguese estuary and marine park by assessing temporal and spatial variations in concentration, distribution, and composition of MP particles. Two hypotheses are tested: (i) MP concentration decreases at sampling stations far away from the metropolitan area of Setúbal; and (ii) MP concentration increase in winter months when

compared to summer and autumn months. Additionally, since these are important nursery areas for fish larvae, the ratio of MP to ichthyoplankton was calculated separately from the MP to neuston ratio, aiming to be useful either for comparing with other regions or as a simple and clear take-home message at science outreach activities.

MATERIALS AND METHODS

Study Area

The study area, located on the west coast of Portugal, encompasses the south-facing coastal area between the city of Setúbal and the village of Sesimbra (**Figure 1**). It comprises both the mouth of Sado estuary [designated as the transitional water body Sado-WB1 (ARH Alentejo, 2012)] and the Professor Luiz Saldanha Marine Park (from its eastern side - Figueirinha beach – until the buffer area contiguous to Sesimbra). The meso-tidal homogeneous Sado estuary has a mainly tidally driven flow (Martins et al., 2002) with an annual average flow of 40 m^3s^{-1} (Vale et al., 1993). It is under considerable anthropogenic pressure due to numerous activities (mostly occurring in its northern margin), from urban and industrial (including maritime traffic), to agriculture and animal production, fisheries and tourism sectors (APA, 2016). Nevertheless, this estuary (Ramsar site no. 826) encompasses a Nature Reserve [RNES (ICNF Reserva Natural do Estuário do Sado)], where birds and habitats are, respectively, protected by a Special Protection Area (PTZPE0011; Birds Directive) and a site classified under the Habitats Directive (PTCON0011), both belonging to Natura 2000 network. Located outside these conservation areas, the Sado-WB1 is adjacent to the city of Setúbal (ca 119.000 inhabitants: Statistics Portugal 2013), being close to a multipurpose terminal port, ship repair yard and to the submarine outfall of Setúbal wastewater treatment plant. Along the Sado-WB1 margins, where two streams discharge (Comenda and Livramento), there are diverse nautical and tourist facilities, an important cement industry, an Orthopedic Hospital and beaches of high demand.

Established westward from the estuary, is Professor Luiz Saldanha Marine Park (hereafter marine park), a sheltered

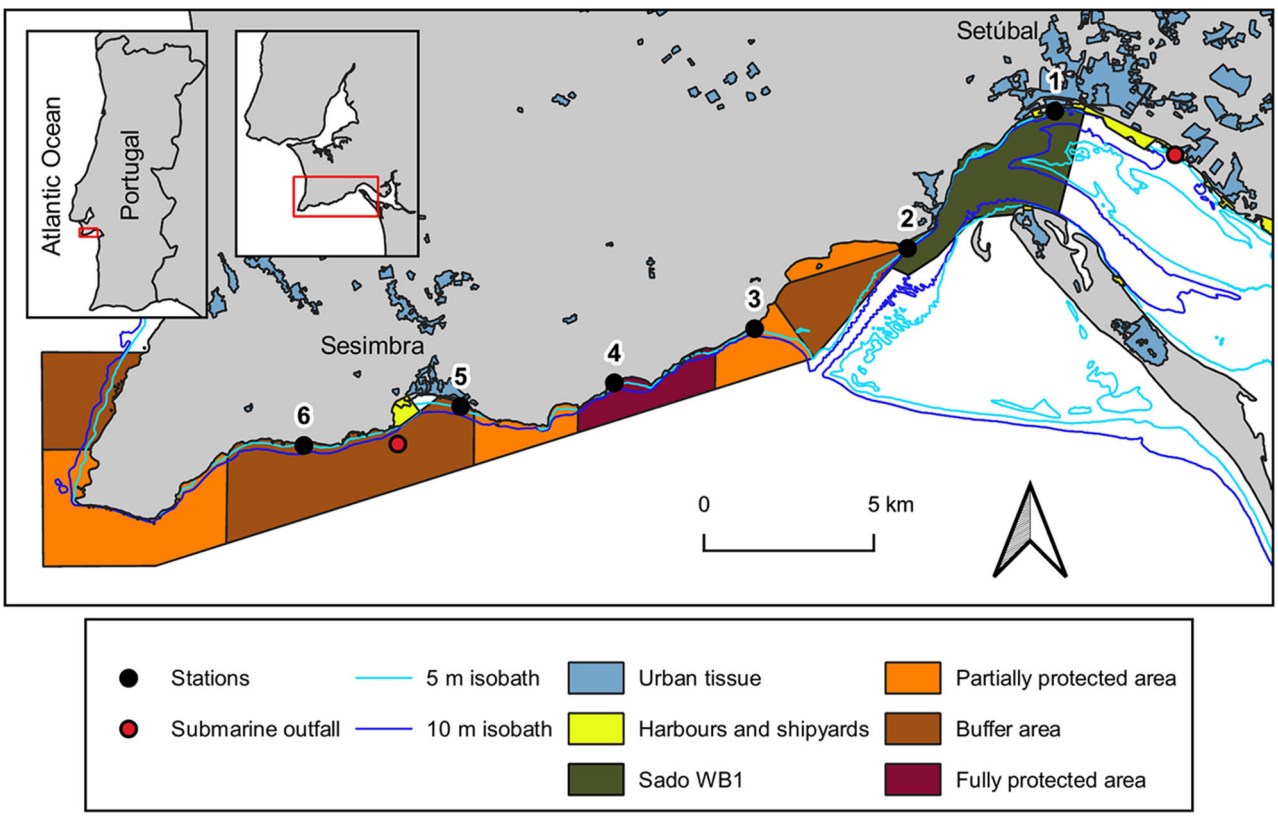

FIGURE 1 | Map of the study area with the location of sampling stations at the coastal area between Setúbal city and Sesimbra village, at the Portuguese west coast. Urban tissue includes industry and tourist facilities. The isobath lines were provided by Instituto Hidrográfico and the submarine outfalls location was given by Águas do Sado (Setúbal WWTP) and SIMARSUL (Sesimbra WWTP). Map creation was based on 2 information layers: (1) land use and occupation of 2018[1] and (2) transitional surface water bodies of Portugal mainland[2], respectively developed by Direção-Geral do Território and by Agência Portuguesa do Ambiente. Marine park borders and protection areas were depicted according to the POPNA spatial plan regulation (no. 141/2005).

[1]https://snig.dgterritorio.gov.pt/rndg/srv/por/catalog.search#/metadata/b498e89c-1093-4793-ad22-63516062891b
[2]https://snig.dgterritorio.gov.pt/rndg/srv/por/catalog.search#/metadata/0F67303C-5822-4D91-80F3-D217FD33667F

coastline from the prevailing north and north-west winds by the Arrábida mountain chain (Henriques et al., 1999). The different protective measures established in this marine park aim to minimize the impacts of nautical, recreational and fishing activities on its biological and ecological patrimony (Henriques et al., 1999). The erosion of the adjacent cliffs of Arrábida (Gonçalves et al., 2002; Costa et al., 2013) contribute to the complex substratum found in this subtidal rocky reef, which is expected to export MP to the adjacent marine environment due to the breakdown of larger items through physical abrasion on rocks (Eriksson and Burton, 2003; Cheshire et al., 2009). One of 2 submarine outfalls regularly used for effluent discharges of wastewater treatment plants of Sesimbra Municipality (ca. 50.000 inhabitants; Statistics Portugal 2013) is located within the study area.

Sampling Methods

Six sampling campaigns were conducted from August 2018 to February 2019 (summer to winter), at 6 stations (**Table 1**). These were located at the 5 m isobaths and distributed 5 km apart from each other, from the mouth of Sado estuary through the marine park (**Figure 1**). In each station, a 30 min neuston trawl (following Directive, 2013) was performed in the E-W direction, at a constant speed of 1-3 knots. Initial and final GPS positions were registered and enabled trawl length and area calculations to allow posterior standardization of MP data (following Law et al., 2014). Sampling campaigns were specifically scheduled to days with calm weather conditions (Beaufort wind scale $\leq$3) and tows were performed out of the vessel wake zone (ca 25 m behind the vessel). Precautions intended to reduce vertical mixing of buoyant plastic particles and consequently increase the efficiency of the selected equipment (neuston net). The 3 m long neuston net (Aquatic Biotechnology) had a stainless steel 0.8 $\times$ 0.3 m (width $\times$ height) rectangular opening and a 335 µm polyamide mesh. Its floatation system assured that only half of the opening frame was submerged (therefore collecting MP floating in the top 15 cm of the water column). The flowmeter (Hydro-bios) attached to the lower third of the net opening enabled the calculation of the volume of filtered water. As only half of the net opening is submerged, the volume was calculated with the following formulae:

$$\text{Volume} = \frac{\text{net opening area}}{2} \times \text{Tow length, where}$$
$$\text{Tow length} = \text{flowmeter revolutions} \times \text{hydraulic pitch}$$

Following each tow, the content in the cod end container was thoroughly poured into a 250 µm stainless steel mesh sieve (where larger pieces of biological material as sticks, seagrass leaves and algae, were rinsed with filtered seawater before being discarded) and then stored in glass jars. A small aliquot (ca 50 ml) per sample was collected and preserved separately, in 100 ml of 70% ethanol, to allow the identification of neustonic organisms and the calculation of the MP:neuston and MP:ichthyoplankton ratios. The neuston samples (n = 36) were transported in ice coolers to the laboratory and then frozen at −20°C.

TABLE 1 | Name, distance from the estuary (km) and GPS (datum WGS-84) coordinates of each sampling station.

Station	Name	Distance from the estuary (km)	LAT (°)	LON (°)
St1	Setúbal	0	38.51970	−8.89348
St2	Figueirinha beach	5	38.48294	−8.94286
St3	Portinho da Arrábida	10	38.46124	−8.99428
St4	Fully Protected area	15	38.44652	−9.04146
St5	Sesimbra	20	38.43987	−9.09325
St6	Mijona beach	25	38.42905	−9.14605

TABLE 2 | Particle shape definition.

Shape	Definition
Fragment	Hard or soft irregular particle
Film	Thin and malleable, flimsy particle
Foam	Lightweight, sponge-like particle
Fiber	Thin line, equally thick throughout its entire length, frequently curled
Filament	Thicker and straighter than fiber
Bead	Spherical particle

Laboratory Procedures

Sample Processing and Microplastics Characterization

Due to the considerable volume of biological material present, samples were processed according to Gago et al. (2018). After thawing, the sample was transferred to a 2 L glass beaker where the biovolume was measured after 1 h of sedimentation. Then, the organic content digestion was performed by adding a 10% KOH solution, with volume equivalent to at least 3 times the sample biovolume. Following the 48 h of digestion at room temperature, density separation was conducted by adding 1 L of a hypersaturated NaCl solution (1.2 g cm^{-3}). After manual stirring, it was left to settle for 1 h before filtration of the supernatant with a vacuum filtration system. After filtration of every 500 ml (approx.), the sample was stirred and allowed to settle again before the next filtration. Each filter (MFV2 glass fiber filter with 47 mm Ø and 1,0 µm pore; FILTER-LAB) was stored in a covered Petri dish until observation under a stereoscopic microscope (Leica MZ12.5) equipped with a camera (MOTICAM 10+). Particles were measured with the Motic Images Plus 3.0 software, considering the 0.335-5 mm size range (the lower limit corresponds to mesh size of the neuston net) and then attributed to one of the following size classes: 0.335-1, 1-2, 2-3, 3-4, and 4-5 mm. Characterization consisted of registering both color and shape. Particles were assigned to one of six shapes: fragment, film, foam, fiber, filament, and bead [**Table 2**; adapted from Lusher et al. (2017)]. The particles selected to follow polymer identification were isolated in covered concave slides. MP concentration was reported as items m^{-3} and items km^{-2} to enable comparisons with similar studies.

TABLE 3 | Total of particles and number of MP selected for FTIR per shape.

Shape	Total	FTIR
Fragment	1,480	220
Film	557	26
Foam	638	6
Fiber	109	12
Filament	61	27
Bead	75	18
	2,920	309

Polymer Identification

Selection of particles for polymer identification, from all shapes (**Table 3**), was based on the best expert judgment according to similarity, texture, thickness, shine and reaction to touch (following Lusher et al., 2017).

Polymer identification was achieved by Fourier Transformed Infrared Spectroscopy (FTIR). The majority of the particles (mainly between 1 and 5 mm) were analyzed in attenuated total reflectance (ATR) mode. Spectra were acquired using an Agilent Handheld 4300 FTIR Spectrometer with a DTGS detector, with controlled temperature and a diamond ATR sample interface; the analyses were performed at the sample surface. Spectra were acquired with a resolution of 4 cm^{-1} and 32 scans. For fibers and smaller particles (mainly at the 0.335-1 mm size range), analyses were carried out in a Nicolet Nexus spectrophotometer coupled to a Continuμm microscope (15x objective) with an MCT detector. Spectra were collected in transmission mode, with a resolution of 8 cm^{-1} and 128 scans. The spectra are shown here as acquired, without corrections or any further manipulations, except for the occasional removal of the CO_2 absorption at ca. 2,300–2,400 cm^{-1}. The identification of polymers was first made by searching in the extensive polymer spectral database of the Department of Conservation and Restoration (FCT NOVA) and the assignments were confirmed by analysis of the polymers characteristic bands (Hummel, 2002).

Quality Assurance (QA) and Quality Control (QC)

The airborne contamination was analyzed by exposing wet filters to the air (procedural controls; blanks), both during field (inside a hanging open glass jar, at the boat deck, one per sampling campaign) and lab work (inside Petri dishes, one at the left and one at the right of the working area, per group of 3 samples). All the fibers extracted from a sample which were similar to those found in the respective blanks (from field and lab work) were excluded from results. Sources of contamination were also minimized both during field and lab work by using glass, stainless steel and aluminum materials. Samples were kept covered at all times, both cotton lab coat and nitrile gloves were always worn, and benches and equipment were rinsed before use with Milli-Q filtered water and ethanol.

MP:neuston and MP:ichthyoplankton Ratios

The biovolume of neuston aliquots was registered after 1 h of sedimentation in the graduated cylinders and then homogenized (manual stirring). Three subsamples of 2 ml each were analyzed under a stereomicroscope using a Bogorov counting chamber. Apart from insects, neuston organisms mainly consisted of zooplankton. Dominant groups (fish larvae and eggs, Mysidacea, Polychaeta, Chaetognata, Apendiculata, Bivalvia larvae, zoea and megalopa of Brachyura, Cladocera, naupli of Cirripedia, Copepoda, Echinodermata larvae, Amphipoda, Isopoda and Insecta), rather than individual species or genera (Di Mauro et al., 2017), were counted with the support of a hand tally counter, enabling the calculation of each group abundance. Mean counts (all dominant groups were considered for MP:neuston ratio, whereas only fish larvae and eggs were considered for MP:ichthyoplankton ratio calculation) were extrapolated according to the aliquot and sample biovolume and then converted to individuals m^{-3}.

Statistical Analysis

To evaluate how the MP:neuston ratio varied temporally (along 6 months) and spatially (between the 6 stations), a Kruskal-Wallis test was performed. This non-parametric test, conducted after the invalidation of parametric assumptions, was followed by *posthoc* multiple comparisons with the Dunn's test. The same tests were applied for MP:ichthyoplankton ratio.

A two-way ANOVA without replication was performed to assess whether temporal (6 campaigns) and spatial (6 stations) variation occurred in MP concentration (dependent variable). This parametric test was used after Box-Cox transformation of original data to meet normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) assumptions. *Posthoc* Bonferroni's test ($p < 0.05$) were used to identify the sources of significant differences. Analysis were conducted in Statistica 13 (Statsoft) software.

The effect of campaigns and stations (fixed factors; with 6 levels each) in MP concentration of each particle shape (multivariate data) was tested by a permutational multivariate analysis of variance (PERMANOVA), with 999 permutations. Data were square-root transformed and the resemblance matrix between samples was calculated based on Bray-Curtis similarities. When differences were statistically significant, pair-wise comparisons among levels were analyzed. Then, to determine which particle shape most contributed to explain the dissimilarity amongst each pair of samples, the similarity percentages routine (SIMPER; with a cut-off percentage of 90% for low contributions) was conducted. These statistical procedures, which were conducted in the Primer 6 software with the Permanova+ add-on (Clarke and Gorley, 2006; Anderson et al., 2008), were similarly applied to understand the response of MP concentration of each size class to both factors (campaigns and stations).

RESULTS

Presence and Concentration of Microplastics

From the total of particles (3,317) extracted from the 36 neuston samples, 353 (11%) were discarded for being considered airborne contamination fibers and 44 (1%) were excluded after

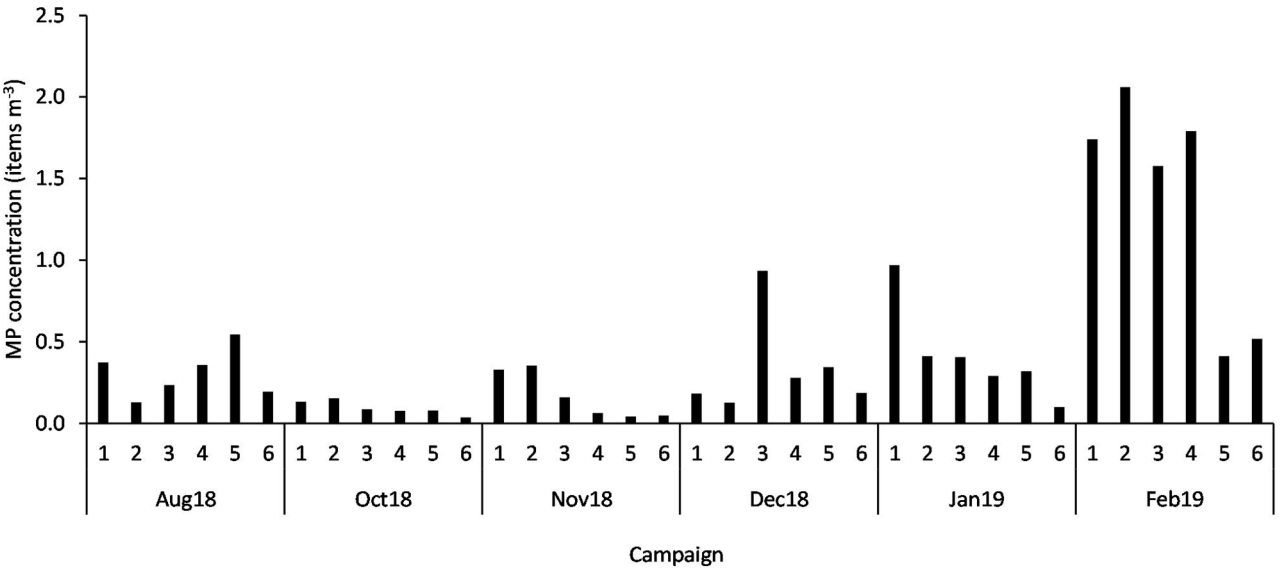

FIGURE 2 | MP concentration (items m^{-3}) in each sample ($n = 36$; 1 to 6 stations; Aug 18 to Feb 19 campaigns).

TABLE 4 | MP number and relative abundance (%) assigned to each polymer.

MP	%	Polymer
176	66.42%	Polyethylene (PE)
48	18.11%	Polypropylene (PP)
25	9.43%	Copolymer PP/PE
5	1.89%	Polystyrene (PS)
3	1.13%	Polyvinyl alcohol (PVA)
3	1.13%	Rayon
2	0.75%	Polyester
1	0.38%	Polyurethane (PUR)
1	0.38%	Poly(acrylic acid) (PAA)
1	0.38%	Polyamide (PA)

being identified as non-plastic particles by Fourier Transformed Infrared Spectroscopy. Therefore, the assessment of the temporal and spatial distribution of MP (size range 0.335 to 5 mm) was based on a total of 2,920 particles. All samples contained MP, with a mean concentration of 0.45 ± 0.52 items m^{-3} (mean ± SD) and 40,822.58 ± 43,578.63 items km^{-2}. While the highest concentration per cubic meter was found in February at Figueirinha beach (St2; 2.06 items m^{-3}), the highest concentration per square kilometer was verified at Setúbal (St1; 203558.50 items km^{-2}). Conversely, the lowest concentration (0.04 items m^{-3} or 2,068.85 items km^{-2}) was observed at Mijona beach (St6) in October (**Figure 2**). The number of MP ranged from 405 at St1 in February to 5 MP at St6 in October.

Fourier Transformed Infrared Spectroscopy (FTIR) Analysis

Among 265 particles confirmed as microplastics by FTIR analysis, a total of 10 polymers were identified (**Table 4**; **Figure 3**), including a Copolymer PP/PE. Despite the diversity of polymers identified, three of them (PE, PP and Copolymer PP/PE) represented more than 90% of the particles. Kaolin was also identified associated with PS and Copolymer PP/PE.

MP:neuston and MP:ichthyoplankton Ratios

Considering all samples, the MP:neuston ratio was 0.0009 ± 0.0013, with the highest ratio 0.0059 (or 1:168.398) occurring in December (**Figure 4**), when neuston concentrations reached minimum levels (76.61 individuals m^{-3}). The average MP:ichthyoplankton ratio was 0.091 ± 0.146, with the highest ratio 0.773 (or 1:1.294) being observed in November. A statistically significant variation at the MP:neuston ($H = 20.80$, $p < 0.001$) and MP:ichthyoplankton ($H = 17.32$, $p < 0.05$) ratios was found between campaigns (**Figure 5**) but not between stations.

Temporal and Spatial Distribution

MP concentration in February was significantly higher than those found in all other campaigns (**Figure 6A**), except for January (Bonferroni test, $p < 0.05$). In October, concentration was the lowest and significantly different from January. MP concentration did not vary significantly between stations ($p = 0.06$; **Figure 6B**).

Distribution Variations According to Particle Shape

The relative abundance of six MP shapes (**Figure 7**) had the following decreasing order: fragment (51%) > foam (22%) > film (19%) > fiber (4%) > bead (3%) > filament (2%). The PERMANOVA results showed significant differences in the

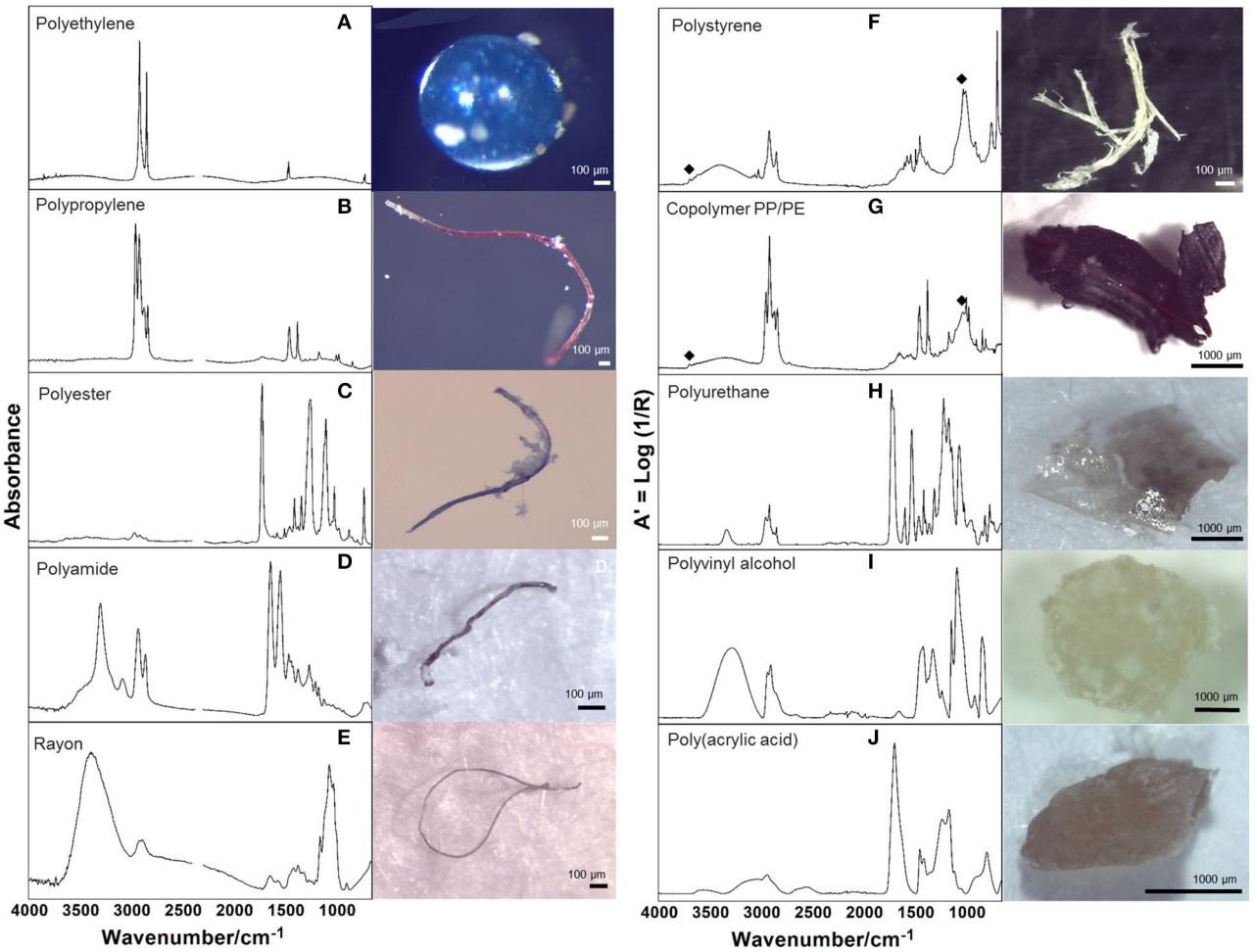

FIGURE 3 | Representative infrared spectra of the identified polymers, analyzed in transmission (left column) and ATR mode (right column); ♦ identifies the presence of kaolin. The image assigned to each spectrum corresponds to the particle analyzed by FTIR. **(A,I)** Bead; **(B–E)** Fiber; **(F,G,J)** Fragment; **(H)** Film shapes.

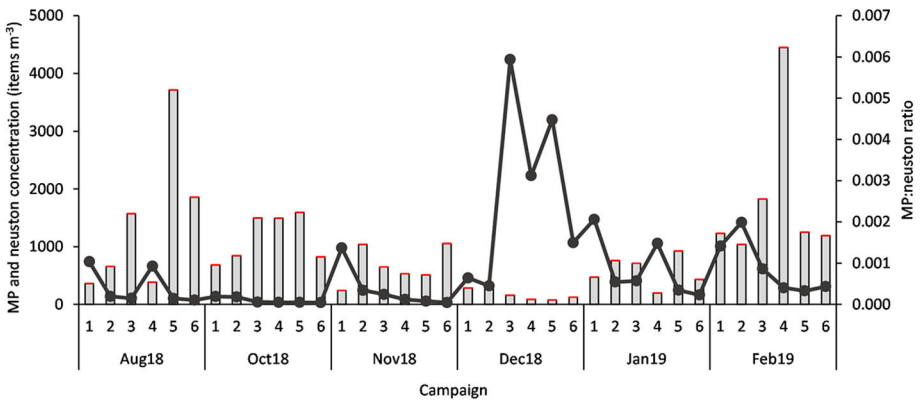

FIGURE 4 | MP concentration (items m^{-3}) (red bar), neuston concentration (items m^{-3}) (gray bar) and MP:neuston ratio (dark gray dots), in each sample ($n = 36$; 1 to 6 stations; Aug 18 to Feb 19 campaigns).

MP concentration of each particle shape, between sampling campaigns (Pseudo-F = 6.57, P(perm) = 0.001) and stations (Pseudo-F = 2.11, P(perm) = 0.008). MP concentrations per particle shape differed mainly between October and February, but also between each of these 2 months and all the other campaigns. The combination of the 3 predominant shapes:

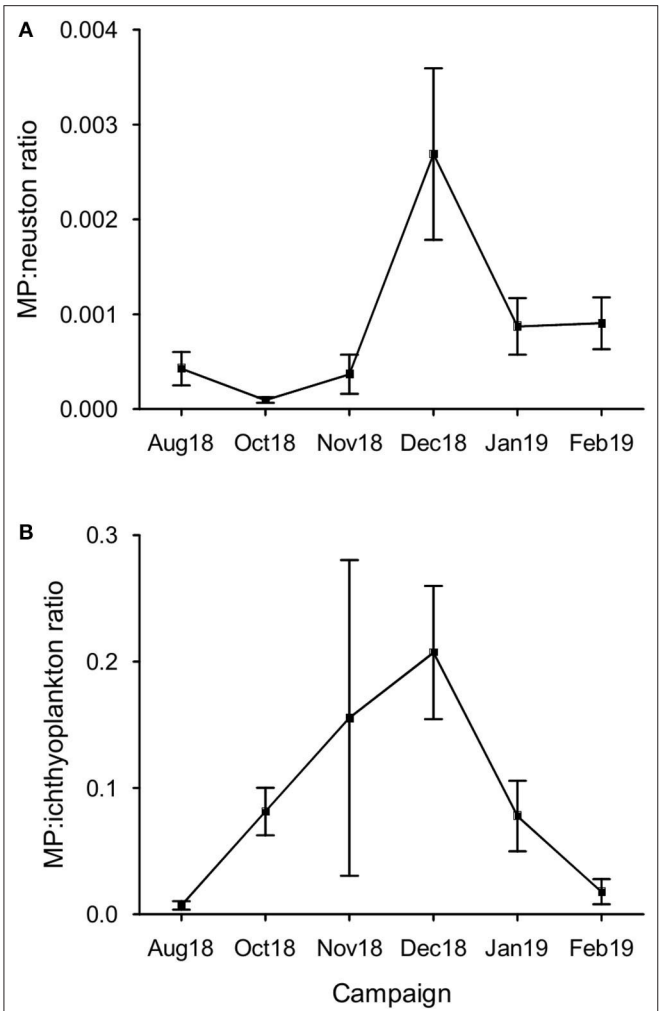

FIGURE 5 | Variation of the MP to primary consumers ratio in the sampling period. **(A)** MP:neuston ratio per campaign (mean ± SE, $n = 6$); **(B)** MP:ichthyoplankton per campaign (mean ± SE, $n = 6$).

fragments, films and foams contributed with more than 71% (cumulative percentage) for the dissimilarities between all pairs, with concentrations being always higher in February (**Figure 8A**). An additional result from pair-wise comparisons concerned the dissimilarities between November and January campaigns, which were based on the higher concentration of fragments, foams and beads (with contributions of 27.87, 24.54, and 14.28%, respectively), found in January. Regarding spatial variation, the relatively higher foam and bead concentrations at St1 (estuary) explained dissimilarities found between this station and both St5 (Sesimbra; foam-24.82% and bead-14.00% contributions) and St6 (Mijona beach; foam-27.44% and bead-13.26% contributions) (**Figure 8B**). Moreover, at St6 concentrations of fragments were significantly lower than St3 (Portinho da Arrábida). No plastic pellets were collected in this study and all beads belonged to the smaller size class (0.335-1 mm).

Distribution Variations According to Size Class

By decreasing order, the relative abundance of each size class (mm) was: 1-2 (36%) > 2-3 (24%) > 3-4 (16%) > 0.335-1 (15%) > 4-5 (9%). According to PERMANOVA results, MP concentration varied according to size class between campaigns (Pseudo-F = 7.69, P(perm) = 0.001) and stations (Pseudo-F = 2.55, P(perm) = 0.005). MP belonging to the 1-2 and 2-3 mm size classes explained (with more than 46% of cumulative contribution) the dissimilarities found between February and all the other campaigns and also between January and both November and October months (**Figure 9A**). In addition, while the 0.335-1 mm size class largely contributed (ca. 29%) to distinguish August from October (being more represented in August), the higher concentration of MP at the 1-2 and 3-4 mm class sizes in December, compared to October, contributed more than 48% for their differences. The particle size range at St6 (the furthest station from the estuary) was distinct from all the others, mainly due to its low concentration of MP belonging to the 3-4 mm size (contributions between 21 and 25%) and particularly different from St1 and St4 due to the smaller concentration of MP at the 4-5 and 1-2 mm size ranges, respectively (**Figure 9B**).

DISCUSSION

Presence and Mean Concentration of Microplastics – Comparison With Other Studies

The presence of MP in all coastal samples collected in this study is in accordance to reported MP pollution levels close to shore and to estuaries, either at Portuguese (Frias et al., 2014), European (Pedrotti et al., 2016; Frère et al., 2017), Gulf of Mexico (Di Mauro et al., 2017) or Indonesian (Germanov et al., 2019) waters. The mean MP concentration found in this study (0.45 ± 0.52 items m^{-3}) was higher than levels found in other Portuguese locations such as the Douro estuary (0.17 ± 0.16 items m^{-3}; Rodrigues et al., 2019) and others (Aveiro: 0.002 ± 0.001 items m^{-3}; Lisboa: 0.033 ± 0.021 items m^{-3}; Costa Vicentina: 0.036 ± 0.027 items m^{-3}; Algarve: 0.014 ± 0.012 items m^{-3}; Frias et al., 2014), but was lower than values reported by Bessa et al. (2018) for the Mondego estuary (1.53 ± 1.04 items m^{-3}).

In addition, if compared with surface waters of estuaries and contiguous coastal areas from other countries, our study area presents higher MP concentrations than those quantified by Lima et al. (2014) at the Goiana estuary in Brazil (0.26 items m^{-3}). Conversely, mean MP concentration at Arrábida was more than one order of magnitude lower than the 3 estuaries in Australia east-coast investigated by Hitchcock and Mitrovic (2019) (with a range of 23–198 items m^{-3} at the Clyde estuary, the one with the lowest MP concentration). However, comparisons must be performed cautiously as local environmental conditions, levels of anthropogenic pressure, and methodologies applied may differ among studies (Lima et al., 2014). In fact, the lack of methodologies standardization has been often

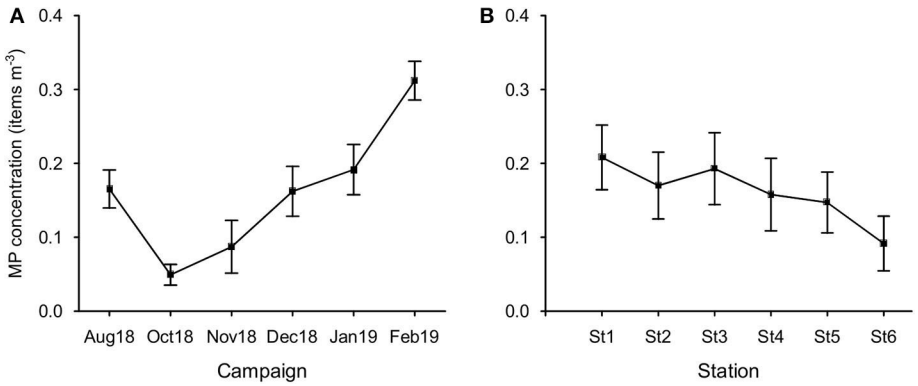

FIGURE 6 | Variation of MP concentration (items m^{-3}; mean ± SE) per campaign **(A)** and per station **(B)**.

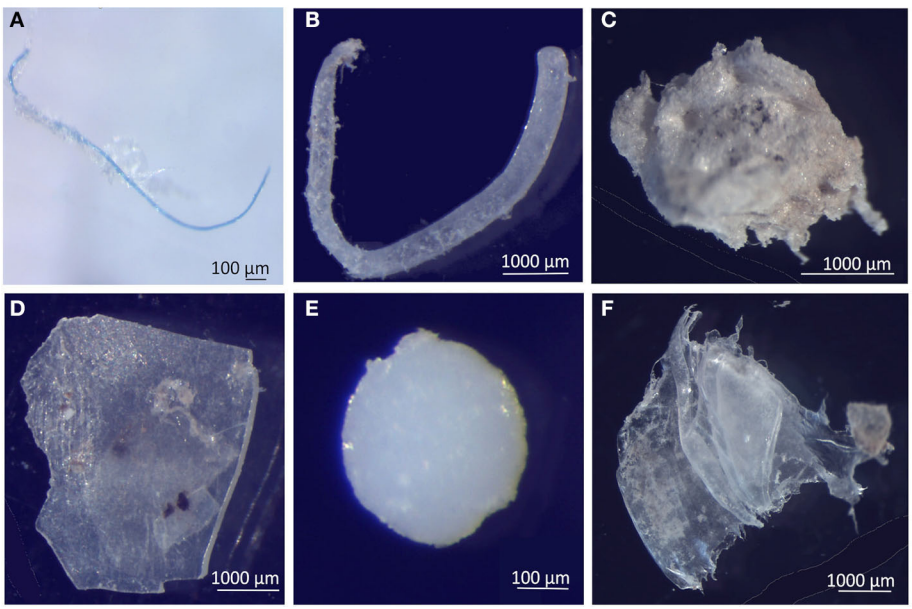

FIGURE 7 | Selected microplastics from each particle shape, in neuston samples from the Sado estuary and Professor Luiz Saldanha Marine Park. **(A)** Fiber; **(B)** Filament; **(C)** Foam; **(D)** Fragment; **(E)** Bead; **(F)** Film.

highlighted (Gago et al., 2018; GESAMP, 2019) and remains a current challenge.

MP:neuston and MP:ichthyoplankton Ratios

The accumulation of floating MP at the seawater surface layer leads to concerns about the exposure of neustonic organisms, such as zooplankton (including ichthyoplankton), to these synthetic particles and, consequently, of their active predators and filter-feeding biota (Collignon et al., 2012).

As expected, the increasing tendency of MP concentration observed in winter months and the simultaneous decline of zooplankton and larval fish abundance (Cunha, 1993; Primo et al., 2011) increased both MP:neuston and MP:ichthyoplankton ratios in this time of the year. Regarding the MP:ichthyoplankton ratio, although MP have never exceeded ichthyoplankton in number at any sample, their similar proportions suggest a higher potential for MP to be ingested either by fish larvae or by ichthyoplankton's predators [crustaceans: crabs, shrimps, euphausiids, amphipods, and copepods; ctenophores; fishes; medusae (Bailey and Houde, 1989; Paradis et al., 1996)]. As a consequence, it would be expected to find critical variations in the following spring at these important nursery areas. Further studies focused on MP:ichtyoplankton ratio and on MP ingestion by wild fish larvae would be essential to confirm possible impacts, as their survival largely influence fish recruitment success and population fluctuations (Houde, 1987).

The average MP:neuston ratio verified in this work (0.0009) was low when compared to other studies: 0.002 at the Bay

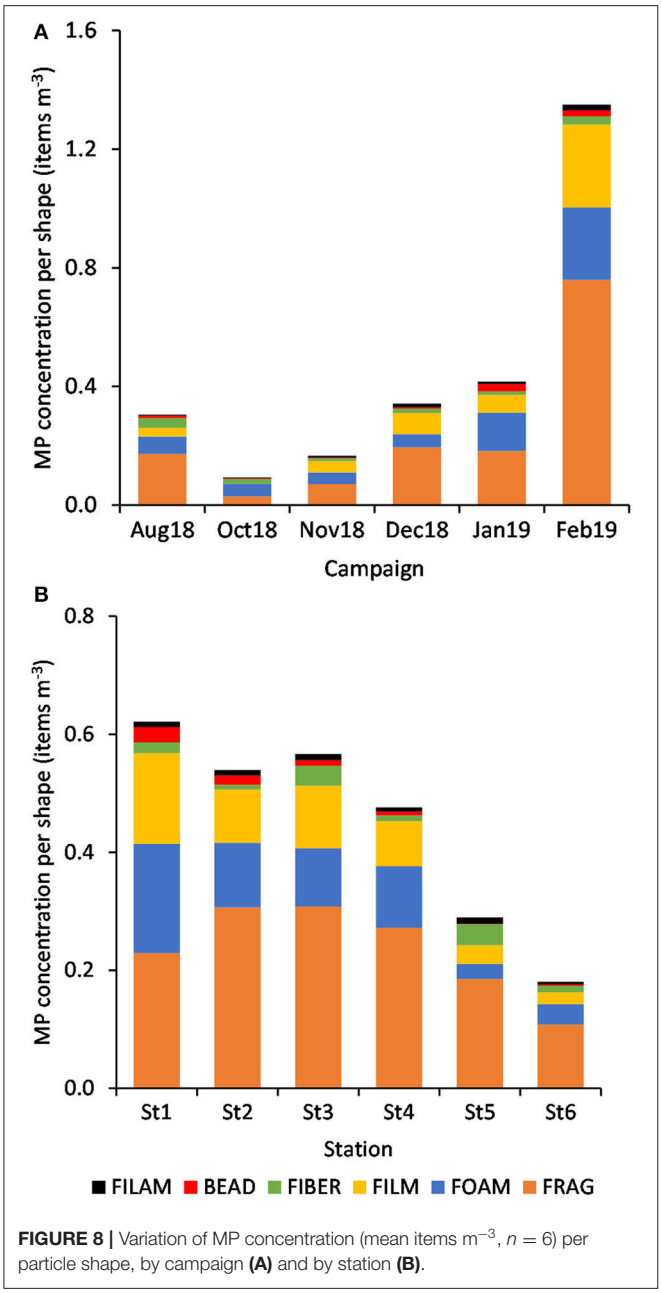

FIGURE 8 | Variation of MP concentration (mean items m^{-3}, $n = 6$) per particle shape, by campaign **(A)** and by station **(B)**.

of Calvi (Collignon et al., 2014) and 0.2 at the Ligurian Sea (Pedrotti et al., 2014). To our knowledge, besides the MP to fish larvae ratio (1.5:1.0; fish eggs excluded) found in Douro river, Portugal (Rodrigues et al., 2019), no studies have assessed the proportion between MP and ichthyoplankton alone (0.091 in average; 1:1.294 maximum), as it has been pooled together with all other zooplankton organisms.

Temporal and Spatial Distribution

Both temporal and spatial distribution variations were verified for MP concentration in our study site. As expected, MP concentrations increased significantly in winter months, achieving a maximum in February. This is in agreement with the reported increase of MP concentrations in marine coastal waters after storms and heavy rainfall, typically frequent in winter season for Mediterranean-type climatic conditions (Santos et al., 2005), which induces frequent floods and increase river discharges (Veerasingam et al., 2016; Gündogdu et al., 2018; Hitchcock, 2020). Regarding the spatial distribution, it was anticipated a clear seaward decrease in MP concentration at stations further away from the metropolitan area of Setúbal (Sado estuary), with an eventual increase at the station close to Sesimbra. Instead, MP pollution level found at stations located between Setúbal and Sesimbra municipalities kept similar orders of magnitude, although with a slight decrease tendency. Such retention of MP, which might be related with the shelter provided by Arrábida mountain chain against the prevailing north and north-west winds, may impact the high biodiversity of this Marine Park. Therefore, the continuous input of MP in the estuary (at St1, the closest station to the urban area of Setúbal) is suggested to partially accumulate in the sheltered Arrábida nearshore area.

Further explanations could rely on the hydrodynamics at the Arrábida rocky reef which may potentially enhance fragmentation of both MP or even larger items, by mechanical action against rocks (Eriksson and Burton, 2003; Cheshire et al., 2009) contributing for the increase of secondary MP. Subsequently, the continuous exportation of these MP by local currents could explain the considerable concentration of particles at st5, despite being distant from the estuary. Concentrations calculated at this station may also result from Sesimbra village input of MP yet, due to the fragmentation potential at the sandy surf zone of this sheltered bay, particles may easily achieve sizes which are not retained by the neuston net.

Lastly, fragmentation enhanced during retention at the Arrábida nearshore may also contribute to export MP in the coastal drift, explaining the unexpected high concentration of MP reported further south by Frias et al. (2014) at Costa Vicentina.

Distribution Variations According to Particle Shape

Bead and foam shapes presented distinct patterns in their distribution at the study area, unlike the other MP shapes. Both were predominantly collected in station 1 (Setúbal), contrasting with station 5 and 6 (Sesimbra and Mijona beach), with concentrations being higher in the January and February campaigns. The preponderance of foam shape (expanded polystyrene) in the estuary is potentially related to fisheries activities, consisting of secondary MP from the breakdown of buoys and cooler boxes for bait and catches, which despite the decrease of fishery activities during winter (DGRM, 2018) are frequently kept close to the seashore and left exposed to adverse weather conditions till the next fishing season. Conversely, beads (primary MP) are suggested to enter in the marine ecosystem by wastewater treatment plant (WWTP) effluents after domestic use (Fendall and Sewell, 2009), as these particles may not be retained in the treatment processes. The predominance of fragments in

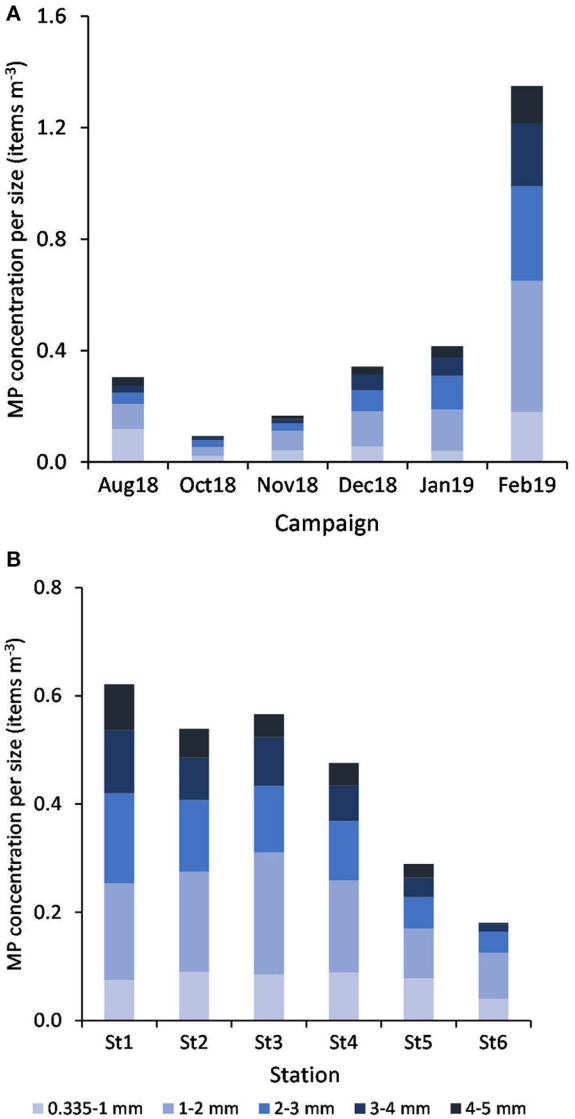

FIGURE 9 | Variation of MP concentration (mean items m^{-3}, $n = 6$) per size class, by campaign **(A)** and by station **(B)**.

this study is in line with results from a similar study performed in Portugal, in the Douro estuary (Rodrigues et al., 2019) and in Australia, at Clyde, Bega and Hunter estuaries (Hitchcock and Mitrovic, 2019). This suggests that secondary sources of MP prevail, rather than primary sources, and are related with the diverse activities taking place in the nearby urban area, including littering. Our findings differ from studies reporting fibers as the predominant shape detected (Beer et al., 2018; Bessa et al., 2018), usually attributed to fishing ropes degradation (Ramos et al., 2012) and to the inefficient retention of fibers from textile laundry by the WWTP (Browne et al., 2011). In fact, fibers represented only 4% of the total of MP found in our study, after the exclusion of airborne contamination (11%) from the original MP amount. The small abundance of fibers reported here may rely on the retention efficiency of treatment processes of WWTW (Gies et al., 2018) at both Setúbal (advanced secondary treatment) and Sesimbra (tertiary treatment) Municipalities, or be related to the sampling method applied, as neuston nets are suggested to underestimate the concentrations of fibers when compared with other methods (Barrows et al., 2017; Green et al., 2018).

Distribution Variations According to Size Class

Distribution patterns of MP according to their size were noticed both in time and space. In fact, the predominance of bigger sized MP (3-4 and 4-5 mm size) inside the estuary, the abundance increase of MP in December (beginning of winter), particularly MP belonging to the 3-4 mm size class and the high concentration of MP from intermediate size classes (1-2 and 2-3 mm) at january and mostly in February, suggest that MP inputs in this Portuguese region occur mostly close to Setúbal and mainly consists of larger particles which undergo fragmentation over time.

The preponderance of 1-2 mm sized particles among the 5 size classes, instead of the expected smallest size class (0.335-1 mm), according to Norén (2007) and Kang et al. (2015) findings, may be essentially related with the sampling method used here. As mentioned before, the use of neuston nets may underestimate fibers concentrations, which are more malleable and easier to escape through the net mesh, explaining the low concentrations of fibers collected here and in particular those belonging to our smallest size class (0.335-1 mm). Secondly, the reduced concentration of MP belonging to the 0.335-1 mm size class could be related with the retention time spent at sheltered stations, which could enhance biofouling levels and consequently cause smaller particles to sink (Kaiser et al., 2017) or to be ingested, as biofilms are suggested to increase MP palatability (Vroom et al., 2017).

As several studies have already highlighted (Song et al., 2014; Lenz et al., 2016), MP concentrations at surface waters are potentially underestimated due to the lower size limit of the range considered for monitoring, usually *ca.* 330 μm (net mesh used). Consequently, as there is a tendency over time for continuous fragmentation of plastic and permanent input into the marine ecosystem, studies are missing the size fraction which is potentially more abundant and easily ingested by primary consumers (Cole et al., 2013). Therefore, the selection of a sampling method that efficiently collects smaller MP in further studies would be required to clarify the abundance patterns found at this coastal area.

Polymer Diversity

Polymer identification of particles in plastic pollution studies is essential to confirm visual identification processes (Löder and Gerdts, 2019), to characterize the diversity of polymers available and to assist in identifying potential local sources, as it will empower authorities and stakeholders to tackle this global concern by implementing efficient prevention measures.

The high polymer diversity (10 polymers) detected mirrors the diverse activities performed in the area, both on land (domestic, commercial, industrial and tourism) and at sea (fishing and recreational activities, intense maritime traffic to shipyards). As expected, polyethylene (PE) and polypropylene (PP) showed higher percentages, since they are widely used in many applications (mainly packaging of consumer goods and single-use items). Nevertheless, there was also a considerable amount of particles identified as copolymer PP/PE, which occurs as an industrial way to recycle both PE and PP by giving origin to other high demanding applications and expanding market options (Graziano et al., 2019), as containers, outdoor decking or sack bags (Aumnate et al., 2019). Polystyrene (PS) particles, can be related to fragmentation of disposable cutlery, cups and Styrofoam® items (expanded and extruded PS, EPS and XPS), which are currently used in fishing activities, in food trays and other disposable items (Farrelly and Shaw, 2017). A note should be mentioned regarding the presence of kaolin in PS and copolymer PP/PE particles, which is used as a filler to improve the strength of the plastic material. Particles of polyvinyl alcohol polymer (PVA), considered of low environmental impact, may have been originated from medical and sanitary devices, as well as from food packaging. In fact, this polymer is considered appropriate for orthopedic applications (Baker et al., 2012) potentially linked to the Orthopedic Hospital located close to St2. Fibers were identified as Rayon, a cellulose-based semi-synthetic fiber frequently found in similar studies (Comnea-Stancu et al., 2017); polyester, widely used in packaging, textile, automotive, medical, electronic, and construction sectors (Camlibel, 2018); and polyamide (PA), predominantly used in fishing nets, but also used in the automotive sector and as a bone tissue scaffold in the medical sector (Winnacker, 2017; Atayeter and Atar, 2018). Polyurethane (PUR) is widely used in coating epoxy resins to protect boat hulls from deterioration and used as rigid foams to insulate boats from extreme temperatures and noise, besides biomedical, construction, and automotive applications (Akindoyo et al., 2016). Finally, polyacrylic acid (PAA) is used in the manufacture of household cleaning products, but also for enhancing the mechanical properties of hydrogels used as biological glues in the medical and tissue engineering sector (GVR - Grand View Reasearch, 2017).

CONCLUSION

As expected, MP pollution in this study was higher during the winter months, co-occurring with the usual decrease of primary consumers abundance in this season. The consequent increase of both MP:neuston and MP:ichthyoplankton ratios suggests therefore a critical time period for marine biota feeding in the neustonic habitat. Regarding MP spatial distribution, instead of a clear decreasing gradient from the estuary (area with higher human impact) to further coastal stations, a slight decline in concentrations was observed, suggesting a retention effect close to the Arrábida shore. Although fragments were the dominant shape, only foam and beads presented distinct variation in space, according to the location of their potential sources (fishing harbor and WWTP submarine outfall). The predominance of particles at the 1-2 mm size range instead of the smaller size range (0.335-1 mm), is suggested to be related with the sampling method used, although further studies would be required to clarify this hypothesis. The diversity of polymers reflects the multiple activities occurring in the estuary and in the marine park, highlighting the urgent need to disseminate findings locally, namely on fishing communities and in tourism, industrial and marine traffic sectors. Sharing scientific findings with society aims to increase public awareness about MP pollution and to inspire actions toward the prevention and reduction of plastic entering the marine environment.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DR conducted fieldwork sampling, laboratory procedures (MP extraction and characterization, neuston identification), statistical analysis, and wrote the manuscript. JA performed FTIR analysis, collaborated in the discussion and selection of the best method for MP extraction from neuston samples, provided assistance with laboratory procedures and at reporting FTIR analysis, and results. VO performed micro - FTIR analysis, assisted in the interpretation of spectra, and at reporting FTIR analysis and results. PS co-ordinated the study, discussed results, gave important contributions to the writing and to the English review of the text. MC reviewed and made important contributions to the text. All authors contributed to the article and approved the submitted version.

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Cetaceans as Ocean Health Indicators of Marine Litter Impact at Global Scale

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Marine litter is a growing concern for marine animals, including cetaceans for which there is a developing body of evidence showing impacts of both entanglement and ingestion. Better understanding is needed of the current and predicted scales of impacts on cetacean species of both macro- and micro-litter. Some emerging methodological approaches, such as the "threefold approach," will help address data gaps. The relationship between this form of pollution and some cetaceans is strong and the particular feeding habits, and widespread distribution of two whale species means that they can be proposed as ocean health indicators for macro- and micro-litter impacts at global scales, helping steer research. The species concerned are sperm whales (*Physeter macrocephalus*), for macro-litter at depth, and fin whales (*Balaenoptera physalus*), for micro-debris. Once appropriate techniques have been fully developed for non-lethal assessment, other whale species might also be used as indicators of litter pollution in their specific feeding zones.

Keywords: cetaceans, marine litter, microplastics, ocean health, indicators, research perspectives

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INTRODUCTION

The effects of marine debris on marine wildlife have been documented since the 1960's (CBD, 2016; Germanov et al., 2018). However, the production of plastics and associated pollution has subsequently increased greatly, and marine debris is now been recognized as a global problem (CBD, 2016), with more than 800 species known to have been adversely affected (Fossi et al., 2018c; Kühn and van Franeker, 2020). For cetaceans, impacts from entanglement or ingestion can be acute or chronic (Laist, 1997, IWC., 2020). Almost two-thirds of cetacean species have been found to have ingested plastic macro-litter (2.5 cm+) and this affects species across many different habitats and which exhibit differing feeding techniques (Walker and Coe, 1990; Laist, 1997; Katsanevakis, 2008; Cornish et al., 2011; Simmonds, 2011, 2012; Baulch and Perry, 2014, Fossi et al., 2018b, IWC., 2020, Kühn et al., 2015). This paper focuses on ingested materials and plastic items are the most recorded type of ingested debris, including large pieces of netting and sheets of plastic.

In December 2019, the International Whaling Commission (IWC) held its third international workshop on cetaceans and marine litter (IWC., 2020) and took an in-depth look at the relationship between cetaceans and marine litter. Alongside other aims the workshop sought to review the latest evidence on interactions with cetaceans and identify best protocols for gross pathology, including

for micro-debris. Based on its review of both published and unpublished sources, the workshop agreed that "the scale of the actual and projected increase in plastics" was "alarming," noting that cetaceans can be killed by ingestion because of gastric impaction/occlusion and perforation or as a result of the associated lesions. It was also noted that chronic health concerns could result if plastics persisted in the gastrointestinal tract (GIT) where they might reduce the space for food, adversely affecting nutrition and ultimately the animal's condition. Ingested plastic debris can also cause inflammatory changes and act as a vector of pathogens or pollutants.

The 2019 IWC workshop also considered entanglement, noting that ∼640,000 tons of Abandoned, Lost and otherwise Discarded Fishing Gear (ALDFG) arrives in the oceans annually. Among its recommendations, the workshop highlighted how important long-term studies are and the need for uniformity in post-mortem studies. At the present time, the most universally used method to examine effects and occurrence in cetaceans is the examination during necropsy of the GI tract of stranded individuals. This can demonstrate the type of exposure of the species but has limitations in terms of identifying all the adverse effects on both the individual and at the population level. Problems with this approach include that:

i few bodies are retrieved;
ii of these, even fewer are in good enough condition to be examined; and
iii an apparently low associated rate of reporting.

In our experience, obtaining accurate samples from necropsies is also often problematic and so are the practical issues that arise from analyzing the large quantities of material from the GI tract of the larger whales. Studies on microplastics are notably rare, although they have been systematically determined in seven small cetaceans to date: *Tursiops truncatus*, *Delphinus delphis*, *Stenella coeruleoalba*, *Phocoena phocoena*, *Orcinus orca,* and *Ziphius cavirostris* (Lusher et al., 2018; van Franeker et al., 2018) and one stranded humpback whale (*Megaptera novaeangliae*) (Besseling et al., 2015).

Microplastic uptake by cetaceans can occur by various mechanisms, including:

i ingestion when feeding;
ii inhalation when taking a breath at the surface; or
iii transfer via prey items (IWC., 2013).

Planktivorous (Collard et al., 2015), pelagic and demersal fish have all been shown to uptake microplastics (Lusher et al., 2013; Murphy et al., 2017), as have copepods and euphausiids, some of which are common prey species for baleen whales (Desforges et al., 2015). Uptake has also been demonstrated in some shellfish and other benthic organisms (e.g.,Pellini et al., 2018), potentially providing a further contaminated link in the food chain to other species that feed on the seabed.

Here we report on the scientific evidence and discuss the emerging gaps in understanding related to both filter feeder baleen whales (such as fin whales, *Balaenoptera physalus*) and deep diving odontocetes (such as sperm whales, *Physeter macrocephalus*) in relation to, respectively, micro- and macro-litter impacts, including their related toxicological effects. We also consider emerging methodological approaches and, in particular, the threefold approach, which can contribute to new diagnostic tools. Finally, we propose these species as potential ocean health indicators of macro- and micro-litter impact at a global scale.

EMERGING METHODOLOGICAL APPROACHES TO DETECT THE IMPACT OF PLASTIC POLLUTION AND PLASTIC ADDITIVES IN CETACEANS

Investigating the impacts of plastic pollution on cetaceans presents several significant challenges, including access to materials (which need to be in an appropriate condition to be examined), having adequate knowledge about the biology and distributions of the species concerned and also the multiple potential physical and ecotoxicological effects of marine debris interactions. The challenge of finding carcasses in a good state of preservation and the difficulties inherent in conducting strict ecotoxicological investigations in the field are leading to the development of novel integrated approaches such as the new threefold approach. This approach can add to the data on both the rate of ingestion in cetaceans and the multiple sublethal stresses that marine debris ingestion can cause in the short and long terms. Each of the three levels of investigation tools that make up the threefold approach can be applied independently or simultaneously and whether the animals concerned are stranded or free ranging (Fossi et al., 2018a).

The threefold approach consists of:

a) **GI content analysis:** the rate of occurrence of ingested litter (with a focus on plastics and micro-debris) and associated lesions determined from stranded or bycaught cetaceans;
b) **Plastic additives analysis:** in theory, tissue concentrations of plastic additives and associated Persistent Bioaccumulative and Toxic (PBT) compounds could be used as a proxy for ingestion via the examination of samples, from stranded or bycaught animals, or biopsies, from live ones. This approach requires considerable ground-truthing, including because such compounds can be ingested from other sources, although promising progress has been made.
c) **Ecological end-point analysis: which** would apply biomarkers (for example, gene expression biomarkers, CYP1A and CYP2B expression, or endocrine disrupters end-points) to look at the toxicological effects of additives or PBT in stranded or bycaught animals (within a few hours of death) or wild ones (again via biopsies) (Fossi et al., 2016).

The further development of the threefold approach will allow a fuller consideration of the sublethal effects of ingestion. This approach and its development is further discussed in Fossi et al. (2018a) and it can be applied to the indicator species that we describe below.

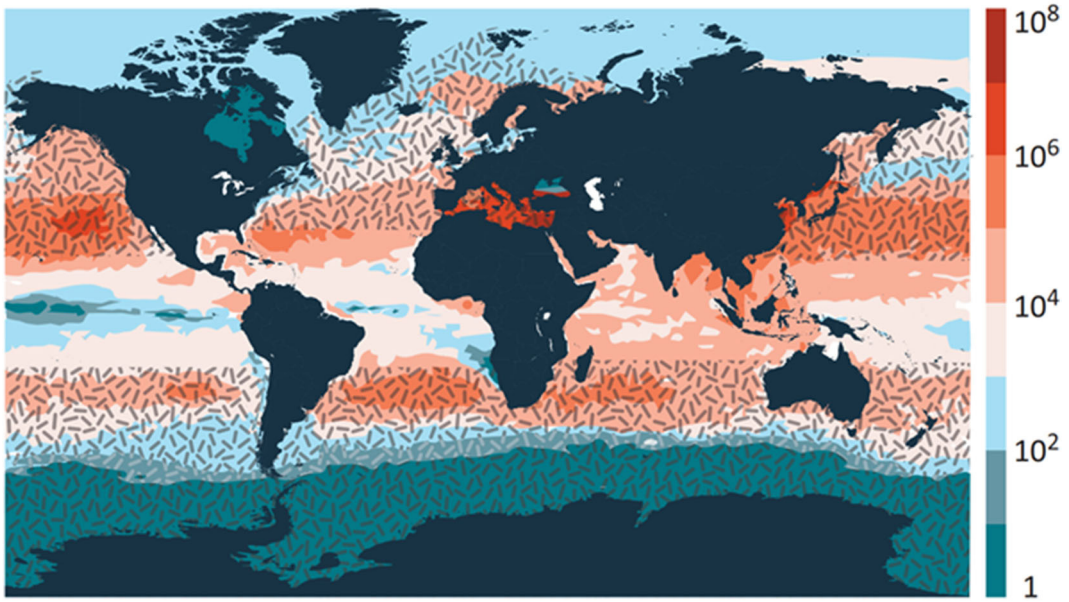

FIGURE 1 | Key buoyant microplastic (microplastic items per km 2 log10 scale) overlap with habitat range of fin whales. The habitat range is indicated by thatched lines and regions containing high levels of buoyant microplastic pollution shown in red/pink. This is adapted from models of buoyant microplastic concentrations from Germanov et al. (2018) (modified; used with permission from the publisher, Elsevier) and the habitat range of fin whales from the IUCN (2018).

FIN WHALES: A OCEAN HEALTH INDICATOR OF MICRO-LITTER IMPACT AT GLOBAL SCALE

In this, and the following section, we explore how two large and wide-ranging species might be used as indicators of marine debris at a global scale and the available information that underpins this. We think that the development of their use in this way will assist global assessment of the impacts of marine debris, although we also accept that further research is needed to help underpin this idea.

The fin whale *(Balaenoptera physalus)* occurs across all oceans and is the second biggest whale species, weighing from 40 to 80 tons and reaching up to 85 feet in length (NOAA Fin whale, 2020). Fin whales, like all large whales, were hunted commercially and especially during the mid-1900's, when hundreds of thousands were killed. The species remains "endangered" according to the United States Endangered Species Act (ESA) and the US Marine Mammal Protection Act (MMPA) has it as "depleted." The IUCN re-classified it as "vulnerable" in 2018, having listed it previously as "endangered" in 2008 (Cooke, 2018), and there are thought to be in the region of 100,000 mature individuals alive. Fin whales typically feed in the warmer months of the year on small schooling fish (such as sand lance, herring, and capelin), squid and krill. They engulf prey, filtering their prey out from the water using the 260–480 baleen plates which hang down from their upper jaws. They may mainly fast in the winter when some make migrations to warmer waters. Currently, only a few studies on marine litter impact on fin whales have been published. Two papers report ingestion in individuals from North Atlantic water (Lusher et al., 2018) and from the sea off East Asia (Im et al., 2020).

More comprehensive studies on the accumulation of micro-litter by this species have so far been focused on two populations in the Mediterranean and the Sea of Cortez and, hence, our evidence of micro-debris accumulation comes from these regions. The emergent threat of micro-litter for large filter-feeding marine animals was recognized by Fossi et al. (2012, 2014, 2017) for baleen whales and later for whale sharks (*Rhincodon typus*) and basking sharks (*Cetorhinus maximus*) (Fossi et al., 2012, 2014, 2017). Fossi et al. (2014) found organochlorines and a phthalate metabolite were higher in a stranded Mediterranean fin whale than in a basking shark, suggesting that fin whales are more heavily impacted by micro-litter and therefore a stronger candidate as an indicator species than other filter feeders. These marine animals are susceptible to high levels of microplastics ingestion and potential exposure to associated toxic compounds due to their feeding strategies and the overlap between their habitats and microplastic hot spots. For example, the SPAMI Pelagos Sanctuary in the Mediterranean Sea is a site where high concentrations of microplastics and cetaceans co-occur and specific end-point responses have been found in skin biopsies taken there [this is further discussed in Fossi et al. (2016), Baini et al. (2017), Fossi et al. (2017, 2018b)].

Filter-feeding cetaceans in areas, such as the Pelagos Santuary, need to sieve thousands of liters of water each day to obtain

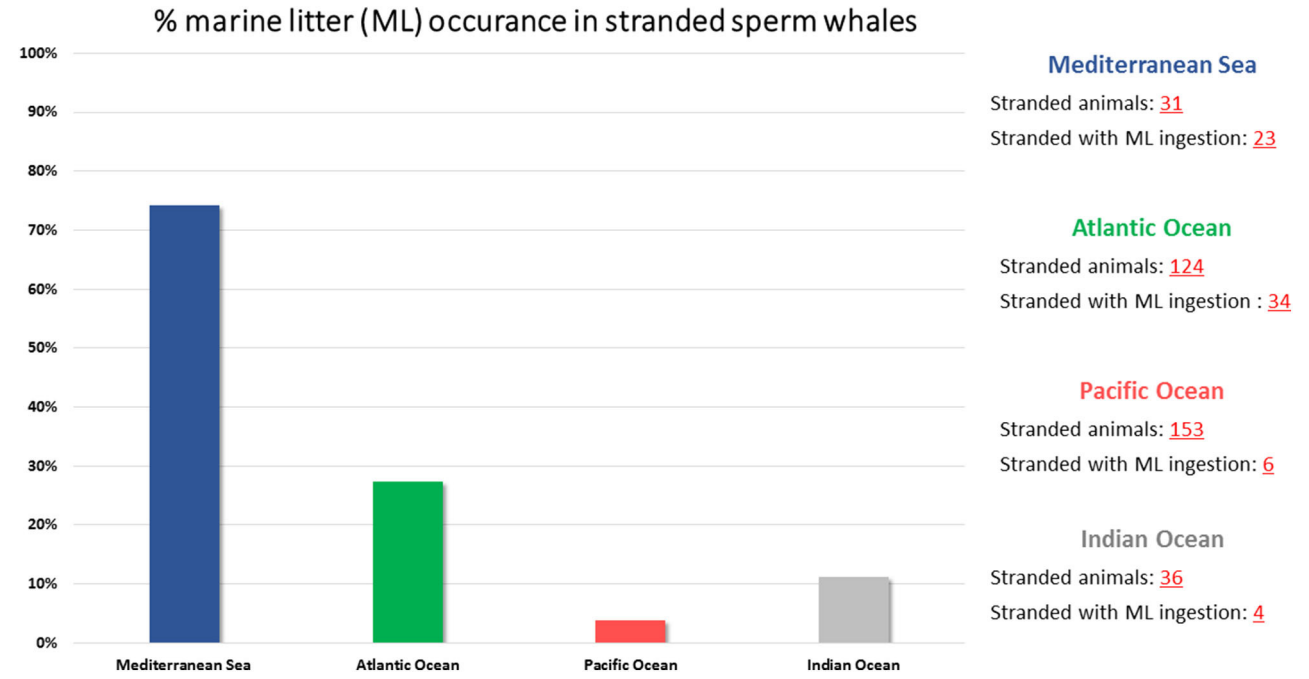

FIGURE 2 | Percentage occurrence of marine litter ingested by stranded sperm whales around the world. Data derived from 20 papers published worldwide, from 1967 to 2019, reporting on a total of 344 stranded specimens.

their food and will, unfortunately, simultaneously ingest plastics and other debris. The high plastic/plankton ratio in the Mediterranean Sea means that a fin whale there will ingest some 3,000 pieces of microplastic each day (Fossi et al., 2014). The region of the Mediterranean known as the Ligurian Sea, where fin whales feed, has very high microplastic contamination which is comparable to that recorded in the North Pacific Gyre (Fossi et al., 2017).

A comparison of micro-debris contamination between the Sea of Cortez and the Mediterranean, which are both semi-enclosed basins, showed higher plastics pollution in zooplankton in the latter, with associated higher biomarker responses and plastic additives and PBT contamination (Fossi et al., 2016). The potential threat to fin whale health and its potential as an indicator species for this form of contamination around the world (**Figure 1**) have been highlighted in previous work (Fossi and Panti, 2017; Fossi et al., 2018b) and were supported by the recent IWC workshop (IWC., 2020). Further research into microplastic contamination in this species around the world should take into account feeding grounds and possible differences in feeding between ages and sexes.

SPERM WHALES: A OCEAN HEALTH INDICATOR OF MACRO-LITTER IMPACT IN THE DEEP SEA AT A GLOBAL SCALE

The sperm whale (*Physeter macrocephalus*) is another large whale and is amongst the most cosmopolitan of cetaceans with populations in all deep oceans (NOAA Sperm Whale, 2020). Mature females weigh some 15 tons, and are around 40 feet in length, and males weigh around 45 tons, reaching 52 feet in length. Sperm whales can live for up to sixty years. Heavily hunted for their oil in preceding centuries, the species is categorized as "endangered" by the ESA and "depleted" by the MMPA. The IUCN categorizes it as vulnerable, noting that its population trend is unknown (IUCN., 2019). Sperm whale dives can take them to depths of more than 1,200 m (Amano and Yoshioka, 2003) and they prey on deep sea fish, including sharks and skates, and also squid. The feeding mechanism of sperm whales is not fully understood. Their relatively small lower jaw and large peg-like teeth that fit into sockets on the upper jaw reflect the fact that they can grasp items, including prey, but suction is also very probably involved (Fais et al., 2016), which may explain their seemingly high levels of ingestion of marine debris. In other words, they cannot avoid such ingestion where plastics are in the water column alongside prey. Part of their hunting range will include marine canyons, which have been widely recognized globally as among the areas of maximum marine litter accumulation (Angiolillo et al., 2015; Fischer et al., 2015; Peng et al., 2020), and there is also evidence that plastics are accumulating in deep sea trenches (e.g., the Mariana trench, Marceau Trench, and New Britain Trench). This suggests that hadal trenches may be the ultimate sink for a significant proportion of the plastics entering the ocean (Peng et al., 2020).

Mediterranean sperm whales appear to be especially badly affected (e.g., Roberts, 2003; Mazzariol et al., 2011; IUCN., 2012; de Stephanis et al., 2013; Alexiadou et al., 2019) in comparison to other oceanic areas (e.g., Martin and Clarke, 1986; Evans and Hindell, 2004; Jacobsen et al., 2010; Unger et al., 2016), as shown in **Figure 2**, and this is likely to be because of the relatively

high level of marine litter contamination in this sea area. A quantitative assessment of debris on the Mediterranean seabed found fishing gear was the dominant type of debris present (89%) (Angiolillo et al., 2015) and this may therefore be a particular problem in this region for this species, although more research is needed. While other deep diving cetacean species, including for example Cuvier's beaked whales, *Ziphius cavirostris*, also seem to be highly susceptible to the ingestion of marine litter (e.g., Baulch and Perry, 2014; Fossi et al., 2018a; IWC., 2020), we propose that the long-lived sperm whales are the better indicator because they are more cosmopolitan, their bodies are probably more likely to be retrieved and their biology is better known. Hence, the Sperm Whale is proposed here as ocean health indicator of marine litter impact in deep seas at a global scale.

DISCUSSION AND CONCLUSION

Recently, the idea of cetaceans as indicators of oceans health has attracted the attention of the scientific community (as evidenced by the recent IWC workshop), other stakeholders and the media. Here we have emphasized the potential of some cetaceans to provide important information about marine litter impact at a global scale. Whale sharks and baleen whales are prone to microplastics ingestion and potentially exposed to associated toxic compounds due to their feeding strategies and habitat overlap with microplastic hot spots, as seen in the Mediterranean Sea. As noted by the IWC workshop, skim feeders, like right and bowhead whales (Balaenidae), might also be monitored for their potential susceptibility (IWC., 2020), but species with a wider distribution appear better candidates as global indicators. Humpback whales have also been promoted as possible candidates for this type of monitoring but are generally faithful to discrete feeding grounds, whereas fin whales are more wide-ranging in their foraging, except for some unique, segregated populations (for example in the Mediterranean and the Gulf of California). The gray whale (*Eschrichtius robustus*), which feeds almost exclusively on the seabed, could be a good candidate for monitoring microplastic impact from the benthos at appropriate depths (IWC., 2020).

As outlined, development of the threefold approach, which is based on the detection of new plastic tracers in tissues and the identification (through omics techniques) of the potential ecotoxicological effects caused by plastic debris ingestion in indicator species, is a promising new diagnostic methodology. More research is needed, including investigations into the potential ecotoxicological effects caused by the ingestion of plastics and consideration will also need to be given to the effects of potential differences in the feeding behavior of different cetacean age classes and sexes. We would also like to emphasize the importance of more coordinated effort on debris ingestion and entanglement in cetaceans, to aid a better understanding of the issues that this presents both in terms of macro- and micro-debris. In this context, we reemphasise here the recommendations from the IWC workshop concerning standarised approaches to necropsies and recording and measuring plastics and other debris (IWC., 2020). This will allow better comparisons to be made between investigations around the world and this relates equally to whether the debris is ingested or is associated through entanglement.

In conclusion, we are increasingly concerned about the health, welfare and conservation implications of the growing amounts of marine debris entering the oceans for cetaceans and other species, and we recommend the development of appropriate programmes of research to further consider sperm whales as a global indicator of macro-litter at depth and fin whales as a global indicator of micro-debris.

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

MF participated in the sampling, conceived the study, and coordinated the writing of the manuscript. MB contributed to the analysis of the data. MS supported the writing of the manuscript and language revision of the ms. All authors contributed to the writing process, reviewed critically the drafts of the manuscript, and gave final approval for publication.

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Microplastics in Marine and Estuarine Species From the Coast of Portugal

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Microplastics (MP) have been confirmed as emerging pollutants in the marine environment due to their ubiquity, bioavailability, persistence and potential toxicity. This study contributes with valuable data regarding the abundance and characteristics of the MP found in five species collected from Portugal. The mussel *Mytilus galloprovincialis* (*n* = 140) was collected from the Tagus estuary and Porto Covo coastal area, the peppery furrow shell *Scrobicularia plana* (*n* = 140) and the polychaete *Marphysa sanguinea* (*n* = 30) both from the Sado estuary, and *Trachurus trachurus* (*n* = 82) and *Scomber colias* (*n* = 82) fished off Figueira da Foz and Sesimbra. Soft tissues of all individuals were digested using a KOH (10%) solution, which allowed the extraction of MP. All studied species presented MP. In a total of 502 MP observed from all samples, 80% were fibers and 20% were fragments, with a size range of 73 μm–4,680 μm and blue was the most common color recorded (46%). The frequency of occurrence of MP was higher in *T. trachurus* (70%) and lowest in *M. sanguinea* (17%). MP abundance ranged from 0.30 ± 0.63 MP. ind^{-1} in *S. plana*, to 2.46 ± 4.12 MP. ind^{-1} in *S. colias*. No significant correlation was found between the individual biometric parameters and total MP, fibers and fragments ingested by each species. The FTIR analysis revealed that polyester and polyethylene were the most common polymers present. These results can be used as a reference for future studies regarding the use of indicator species for monitoring MP pollution in the coast of Portugal.

Keywords: microplastics, plastic pollution, mussels, peppery furrow shell, polychaetes, horse mackerel, atlantic chub mackerel, coastal waters

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HIGHLIGHTS

- Microplastics were recorded in *Mytilus galloprovincialis, Scrobicularia plana*, *Marphysa sanguinea, Trachurus trachurus* and *Scomber colias* from Portugal;
- *S. plana* presented the lowest quantities of ingested MP;
- *T. trachurus* presented the highest percentage of individuals contaminated with MP;
- Fibers were the most common MP in mussels, peppery furrow shell and fish, accounting for approximately 80%;
- In the polychaete *M. sanguinea*, plastic fragments were dominant (83%);
- Blue microplastics were dominant over other detected colors.

INTRODUCTION

Plastic production and consumption have been increasing since the 1950s (GESAMP, 2016), which completely changed the profile of the waste produced (Sheavly, 2005) and leads to plastic accumulation in the environment. In 2015, the global plastic production was 322 million tons (GESAMP, 2016), including high levels of production of specific polymers such as polyethylene (PE) and polypropylene (PP) (PlasticsEurope, 2018), which coincides with the two most common polymers found in marine debris (Erni-Cassola et al., 2017). It is estimated that 80% of marine litter is composed by plastics and that about 5–13 million metric tons of plastic end up in the oceans each year (Jambeck et al., 2015). It is also estimated that there are more than five trillion plastic pieces floating in the oceans, weighing over 250,000 tons (Eriksen et al., 2014). Due to their properties, plastics can last up to hundreds of years in the environment (Thompson and Moore, 2009). Microplastics (MP) are defined as any plastic particle with less than 5 mm in size (Arthur et al., 2009) and can be classified as primary or secondary, according to their source. Primary MP can be found in cosmetic and personal healthcare products, such as exfoliants (Godoy et al., 2019) and tooth pastes (UNEP, 2016), house cleaning products (Napper et al., 2015) and in the form of virgin or recycled plastic pellets used as raw material for production (Browne et al., 2011). Secondary MP are a result of the fragmentation and degradation of larger plastic debris on land or sea (GESAMP and Kershaw, 2016), which can be induced by factors such as light (and ultraviolet light), higher temperatures, availability of oxygen and mechanical actions and also by biological interactions (Veiga et al., 2016). These MP include fibers from synthetic fabrics that can be released during laundering, in which a single piece of clothing can release up to 1900 fibers per wash (De Falco et al., 2019). More recently, Frias and Nash (2019) proposed a new definition for MP: "*Microplastics are any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water.*"

MP have been found in several aquatic environments such as oceans (Pan et al., 2019), rivers (Jiang et al., 2019), estuaries (Hitchcock and Mitrovic, 2019; Rodrigues et al., 2019; Yan et al., 2019) and regions from the Arctic (Kanhai et al., 2019) and the Antarctic (Suaria et al., 2020). Being sampled from the water surface (Cincinelli et al., 2019; Tan et al., 2019), beaches (Retama et al., 2016; Piperagkas et al., 2019), marine sediment (Reed et al., 2018) and biota (Stock et al., 2019), they pose a challenge in terms of determining sources, pathways and potential effects (Veiga et al., 2016). In fact, MP enter the ocean through wide variety of land- and sea-based sources, rivers, wastewater and coastline run-offs, losses or discharges at sea and atmospheric transport, and at every level of plastic's life cycle (Fahrenfeld et al., 2019), and there is a general trend toward finding more MP near densely populated coastal environments (Ivar Do Sul and Costa, 2014). Once in the oceans, MP have complex dynamics determined by factors such as currents, waves, wind and their polymer composition types (Triebskorn et al., 2019). Due to their physicochemical properties, MP have the ability to sorb persistent organic pollutants (POPs) (Fred-Ahmadu et al., 2020). There are also other low-molecular weight chemical species that raise concern in MP, such as the additives used in the manufacture of plastics (stabilizers or flame-retardants) (Sun et al., 2019). These chemicals may be released when in contact with organisms and be a potential chemical hazard (Wang et al., 2018; Prokić et al., 2019). MP have been found in a wide variety of different organisms with different feeding strategies and trophic levels (Gall and Thompson, 2015). These include zooplankton (Desforges et al., 2015), sponges, cnidaria and echinoderms (de Sá et al., 2018), annelids (Hurley et al., 2017), molluscs (Su et al., 2018), fish (Bessa et al., 2018) and also seabirds (Tanaka et al., 2013) and turtles (Hoarau et al., 2014). More than 220 different species have been reported to ingest MP debris in wildlife and in some species, ingestion levels are as high as 80% of the sampled individuals (Ory et al., 2017). MP can be taken up by organisms via direct ingestion (Lusher, 2015), indirect ingestion through ingested prey (Farrell and Nelson, 2013), ventilation (Watts et al., 2014), absorption (Long et al., 2015) or adherence to soft tissues (Kolandhasamy et al., 2018). Contamination with these particles can lead to negative health effects in the individuals exposed to them (Galgani et al., 2010; Besseling et al., 2013; Avio et al., 2015).

Monitoring MP and identifying the potential sources is essential for the assessment of the levels, composition and type of plastic polymers entering the marine environment, to provide knowledge about the behavior and impacts of MP and to create mitigation methods to reduce their inputs (GESAMP, 2019). There are several international and national actions under development, focused in protecting the marine environment and minimizing MP pollution impacts, namely the European Plastics Strategy (European Commission, 2018a) and Single-use Plastics Directive to reduce marine litter (European Commission, 2018b). In Europe, the Marine Strategy Framework Directive is establishing environmental targets for marine litter (and in particular MP) and associated indicators to achieve Good Environmental Status (GES) until the present year.

This study investigates the presence of MP in *Mytilus galloprovincialis*, *Scrobicularia plana*, *Marphysa sanguinea*, *Trachurus trachurus* and *Scomber colias*, from the Portuguese coast. The species were selected due to their different feeding strategies and habitats, as well as economic importance. The study also aims to contribute to the Marine Strategy Framework Directive (MSFD) 2008/56/EC with baseline data and knowledge.

M. galloprovincialis are benthic bivalves, with a filter feeding strategy and wide geographical distribution (Gosling, 1992) and can survive under polluted conditions and accumulate pollutants (Arienzo et al., 2019). All these characteristics make mussels a successful indicator of marine pollution (Li et al., 2019). Mussels also have a high economic interest due to their use in gastronomy in Portugal.

S. plana is an endobenthic bivalve with a deposit feeding strategy and can accumulate contaminants from both sediments and water (González-Domínguez et al., 2016). *S. plana* has commercial value as a human food resource in Portugal and its ecological importance, extensive distribution and sedentary lifestyle makes it a valuable

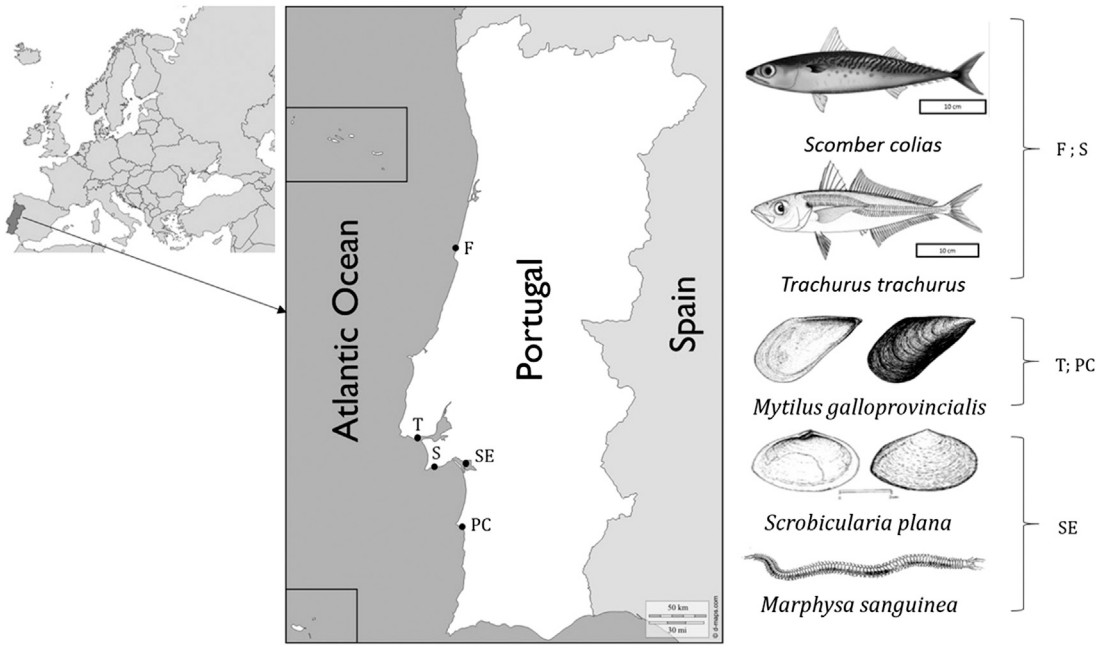

FIGURE 1 | Sampling sites and the species collected along the Portuguese coast—F: Figueira da Foz; T: Tagus Estuary; S: Sesimbra; SE: Sado Estuary; PC: Porto Covo.

biomonitor organism for contaminants (Langston et al., 2007) including MP (Ribeiro et al., 2017).

M. sanguinea is a large-sized omnivore annelid (Fauchald and Jumars, 1979) that lives in the sediment (Prevedelli et al., 2007). This polychaete has an ecological value due to its sediment turnover and an economical value due to its use as live bait for line fishing (Seo et al., 2016).

T. trachurus and *S. colias* are characterized for being pelagic oceanodromous fish, however, *T. trachurus* also displays benthopelagic behavior (FAO, 2005). Their geographic distribution and depth ranges are similar. The feeding behaviors of *S. colias* are based on zooplankton (fish larvae, small crustaceans and pteropods) and *T. trachurus* feeds on crustaceans (copepods), shrimps, small fish and squids. *T. trachurus* tend to be in demersal waters during the day and at night they rise to the surface for feeding, while *S. colias* are in the pelagic zone and occurs in schools close to surface waters, feeding on living organisms and other organic particles present in these areas (FAO, 2005).

MATERIAL AND METHODS

Study Area and Sampling

M. galloprovincialis were collected by hand directly on site at the Tagus estuary (Portinho da Costa beach, on the South bank) and Porto Covo (**Figure 1**). Clams and polychaetes were collected from the Sado estuary (near Carrasqueira, on the South bank). Fish were made available by Docapesca, S.A. at Figueira da Foz and Sesimbra fishing ports. To prevent the possible loss of MP via physiological activities, all individuals were frozen within 1 h after being collected. All species were analyzed for MP presence. The total number of individuals sampled was 474: 70 *M. galloprovincialis* from the Tagus estuary (T); 70 *M. galloprovincialis* from the Porto Covo coastal area (PC); 140 *S. plana* and 30 *M. sanguinea* from the Sado estuary (SE), 82 fish (41 *T. trachurus* and 41 *S. colias*) from Sesimbra (S); and 82 fish (41 *T. trachurus* and 41 *S. colias*) from Figueira da Foz (F). Sampling campaigns were held in May 2017 and all sites were chosen primarily due to the ease of access.

Laboratory Procedures

Samples were processed in the laboratory for MP detection and identification. The shells of mussels and clams were measured to determine their length and width and, after dissection, the wet weight of each individual was recorded, as well as for polychaetes. Fish were measured (standard length and total length) and weighed (total wet weight) and the wet weight of individual gastrointestinal (GI) tracts was obtained after dissection.

All individuals (clams, mussels and polychaetes) and GI tracts from fish were stored in glass flasks for alkaline digestion. All the equipment used in the dissection was pre-washed using distilled MilliQ water. The samples were chemically digested by a solution of potassium hydroxide at 10% (KOH). This method was chosen after reviewing the works of other authors that confirmed the efficiency of KOH in removing biogenic material while preserving the polymers (Foekema et al., 2013; Kühn et al., 2017), and that it has no significant impact in polymer mass or form, except for cellulose acetate, which makes it suitable for the digestion of molluscs and fish tissues and considered one of the best methods for extraction and identification of MP from biota (Dehaut et al., 2016; Karami et al., 2017; Bessa et al., 2019). The jars were covered

with aluminum foil and stored at room temperature for 2 days. The jars were not stirred or shaken to prevent damaging of MP by other hard particles such as sand or other inorganic compounds. On average, after 48 h, a complete digestion of the biological material was observed.

Once digestion of the biological material was completed, the solution was filtered with a vacuum filtration system onto Fiorini and Whatman glass fiber filters (~1 μm pore size). Filters were stored in covered Petri dishes, dried at room temperature and observed under a Leica® stereoscopic microscope equipped with a Leica Microsystems DFC480 digital camera. MP were classified into two different types: fibers and fragments and counted for each species. All MP were measured using ImageJ® software and their color was noted, except for fish, where a subsample of 183 MP was pooled for both species. To account for airborne contamination, the number of fibers in the controls was subtracted from the total of fibers in the samples. Visual identification of MP is open to bias and chemical confirmation of the polymers present must be performed. In this work, and as suggested by Hanke et al. (2013), a subsample of 10% of the total MP observed was randomly selected and analyzed by Fourier transformed infrared spectroscopy in attenuated total reflectance mode (FTIR). Spectra were acquired using an Agilent Handheld 4300 FTIR Spectrometer with a DTGS detector, with controlled temperature, and a diamond ATR sample interface; the analysis was performed at the sample surface. All spectra were obtained with a resolution of 4 cm^{-1} and 32 scans. Spectra are shown as acquired, without any further manipulation. The identification of the samples relied on the match over 80% between the sample and the library data (Agilent FTIR Spectral Libraries and Nicolet™ Condensed phase Sampler FTIR Spectral Library), and on best expert judgment from the presence of specific absorption bands for degraded polymers or copolymers.

Quality Control

Special caution was taken regarding contamination by airborne MP, with the use of cotton lab coats and controls. During the dissection and digestion procedures, one control was created for each 5 samples processed, by following the same steps described for biological samples to account for possible airborne MP contamination. The control filters were then examined for MP. New blanks consisting of wet filters were placed close to the stereoscopic microscope (2 controls for each group of 10 samples examined) to assess airborne fibers contamination during microscope observation.

Statistical Analysis

All data was tested for normality using Kolmogorov-Smirnov and tested for homoscedasticity using Levene's test. Statistical analysis was made using $\alpha = 0.05$. As data was not normally distributed (Kolmogorov-Smirnov: $p < 0.05$) and not homoscedastic (Levene's test: $p < 0.05$), non-parametric tests were performed. The Mann-Whitney *U* test was used for pairwise comparisons between the total number of MP found in *M. galloprovincialis* collected in the Tagus estuary and Porto Covo coastal area. The Spearman correlation coefficient was used to assess correlations between the individual biometric parameters and total MP, fibers and fragments ingested by species. In fish, Kruskal-Wallis H test was used for comparisons between the number of fibers and fragments for each species and sampling site, followed by the post-hoc Dunn's test for pairwise comparisons. Significance level established was 95% ($\alpha = 0.05$) for all the analysis. All calculations were performed with Statistica® software.

TABLE 1 | Biometric parameters of the studied species (average ± standard deviation (SD), n—number of individuals).

Species	n	Length (cm)	Width (cm)	Wet weight ($g.ind^{-1}$)
M. galloprovincialis	140	5.57 ± 1.18	2.84 ± 0.53	3.36 ± 2.08
S. plana	140	4.50 ± 0.33	3.41 ± 0.28	4.30 ± 0.76
M. sanguinea	30	–	–	2.45 ± 0.62
T. trachurus	82	23.37 ± 2.68	–	124.74 ± 21.10
S. colias	82	26.78 ± 1.71	–	174.22 ± 33.84

RESULTS

Biometric parameters for all species are shown in **Table 1**. Microplastics (fibers and fragments i.e., irregular shaped particles) were registered in all the species analyzed and showed variations in length, size and color. **Figure 2** shows some examples of MP selected for polymer identification by FTIR.

A total of 502 MP were registered, 80% being fibers and 20% fragments. **Table 2** presents the number of MP per individual wet weight ($MP.g^{-1}$) and per individual ($MP.ind^{-1}$) for each species (average ± SD) as well as the percentage of individuals with MP.

From all the studied species, polychaetes showed the lowest percentage of individuals with MP (17%) while *T. trachurus* had the highest percentage (70%). *S. colias* had the highest average of MP per individual (2.46 ± 4.12 $MP.ind^{-1}$) and the highest average of fragments per individual (0.72 ± 1.24 Fragm. ind^{-1}). The number of MP found in a single individual ranged from one to three in mussels, one to two in polychaetes and clams and 1 to 20 in fish. **Figure 3A** presents the type of MP collected for each species, in percentage. As shown in **Table 2**, polychaetes showed the lowest average size of MP (223 ± 233 μm) and the highest average size was observed in fish (1,090 ± 1,011 μm). Average MP size for mussels was 890 ± 489 μm and for the clams was 927 ± 479 μm. MP sizes ranged from 90–2,574 μm in mussels, 90–1827 μm in *S. plana*, 73–822 μm in *M. sanguinea* and 87–4,680 μm in fish.

Fragment ingestion in mussels from Porto Covo was significantly higher when compared to mussels from Tagus (Mann-Whitney U, $p < 0.05$). No correlation was found between the individual biometric parameters and total MP, fibers and fragments ingested by each species (Spearman test, $p > 0.05$).

The presence of fibers in *S. colias* was significantly higher in Figueira da Foz, when compared with the same species from Sesimbra (Kruskal-Wallis H test, $p < 0.05$). At Sesimbra, fibers ingested by *T. trachurus* were significantly higher when compared to *S. colias* (Kruskal-Wallis H test, $p < 0.05$). At Figueira da Foz,

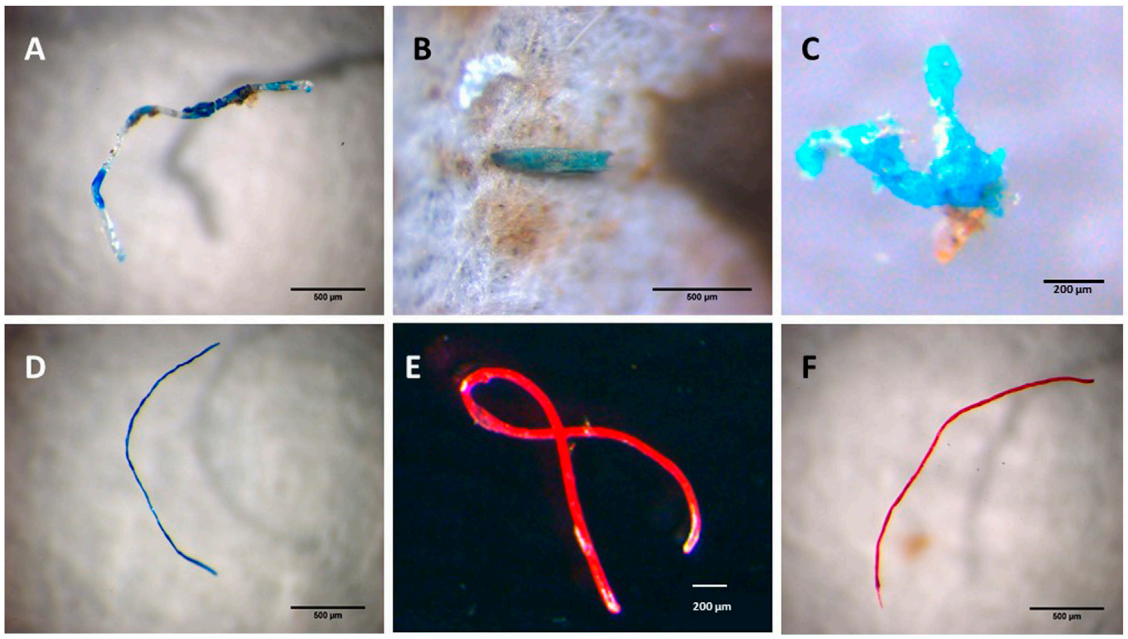

FIGURE 2 | Different types of MP. **(A)** - PET fiber found in *S. plana*; **(B)** - PVC fragment found in *S. plana*; **(C)** - PE fragment found in *S. colias*; **(D)** - PET fiber found in *M. galloprovincialis*; **(E)** - PP fiber found in *S. colias*; **(F)** - PET fiber found in *S. plana*.

TABLE 2 | Microplastics in the five species analyzed: MP per individual wet weight (MP.g^{-1}) and per individual (MP.ind^{-1}), fibers and fragments per individual (average ± SD and total number of each), percentage of individuals with ingested MP and size range of MP (µm), n—number of individuals.

Species (n)	MP.g^{-1} average ± SD	MP.ind^{-1} average ± SD	Fibers.Ind^{-1} average ± SD (total)	Fragm.Ind^{-1} average ± SD (total)	Indiv.With MP (%)	MP size average ± SD (µm)
M. galloprovincialis (140)	0.18 ± 0.31	0.45 ± 0.67	0.41 ± 0.61 (57)	0.036 ± 0.22 (5)	44	889.55 ± 488.87
S. plana (140)	0.07 ± 0.15	0.30 ± 0.63	0.26 ± 0.59 (37)	0.04 ± 0.22 (5)	23	926.73 ± 478.69
M. sanguínea (30)	0.19 ± 0.43	0.40 ± 0.88	0.06 ± 0.25 (2)	0.33 ± 0.0.84 (10)	17	223.08 ± 232.77
T. trachurus (82)	0.018 ± 0.016	2.24 ± 2.05	1.96 ± 1.95 (170)	0.28 ± 0.55 (23)	70	1,090 ± 1,011*
S. colias (82)	0.015 ± 0.026	2.46 ± 4.12	1.74 ± 3.47 (143)	0.72 ± 1.24 (60)	55	

**pooled sample.*

the ingestion of fragments was significantly higher in *S. colias* when compared with *T. trachurus* (Kruskal-Wallis H test, $p < 0.05$).

Except for *M. sanguinea*, ingested fibers were dominant over fragments (**Figure 3A**). MP colors found were blue, black, red, green, brown and transparent (**Figure 3B**). Overall, blue was the most common color, representing 46% of all MP, followed by black with 26%. MP in polychaetes were only blue and black.

The FTIR spectral matches identified polyester (PET), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and nylon (PA). PET was the most common polymer found in MP extracted from *M. galloprovincialis* (60%), *S. plana* (65%) and *T. trachurus* (64%). In contrast, in polychaetes, PVC was the most common polymer (83%) and in *S. colias* the most common was PE (47%). Additionally, in mussels, 60% of the MP were identified as PET, 20% as PP and 20% as PS; in clams 75% as PET and 25% as PVC; in polychaetes 17% as PET and 83% as PVC; in the Horse mackerel 64% as PET, 27% as PE and 9% as PP; and in the Atlantic chub mackerel 34% as PET, 47 as PE, 16% as PP and 3% as PA. Selected spectra from the analyzed MP are shown in **Figure 4**.

DISCUSSION

This study reports MP presence in five different species (*Mytilus galloprovincialis, Scrobicularia plana, Marphysa sanguinea, Trachurus trachurus* and *Scomber colias*), with different feeding strategies and habitats, collected in different locations from the coast of Portugal. The results showed that there was a constant and widespread presence of MP in these species, during the studied period. The plastic particles have been detected in several aquatic environments

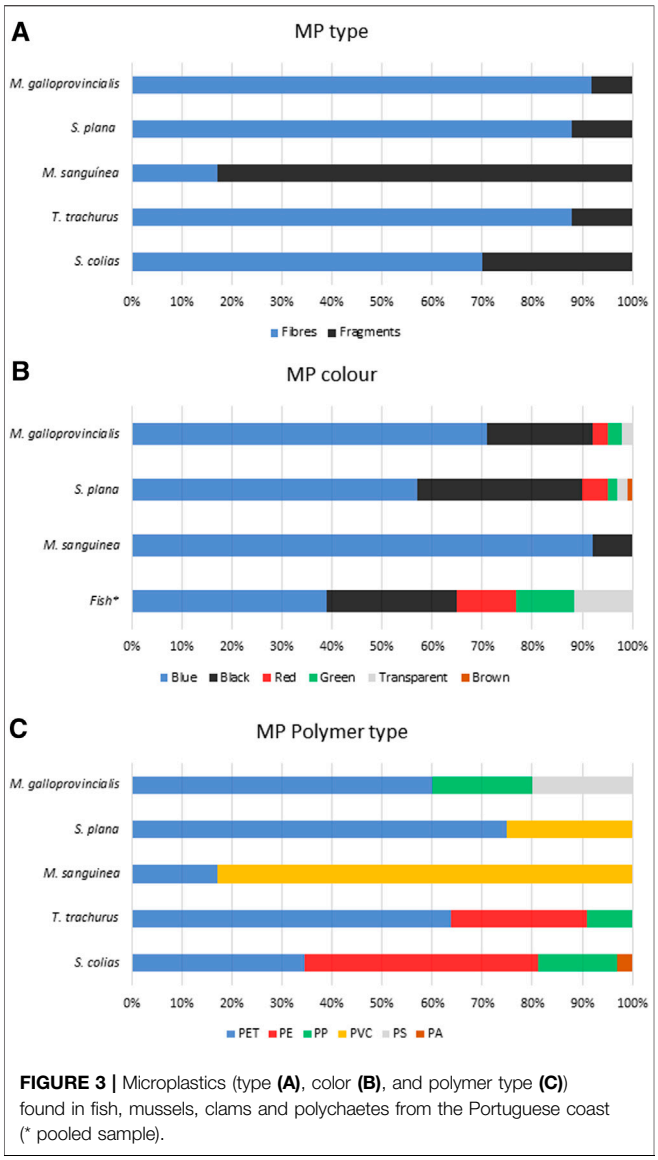

FIGURE 3 | Microplastics (type **(A)**, color **(B)**, and polymer type **(C)**) found in fish, mussels, clams and polychaetes from the Portuguese coast (* pooled sample).

and in wild conditions in fish, mussels and other species of polychaetes, as already documented by several authors (Li et al., 2015; Van Cauwenberghe et al., 2015; Vandermeersch et al., 2015; Digka et al., 2018; Qu et al., 2018; Fernández and Albentosa, 2019; Li et al., 2019). MP in *S. plana* have also been recently reported (Piarulli et al., 2020).

A total of 502 MP was recorded in all the 474 individuals analyzed, and their size varied from 73 to 4,680 µm. Fibers were the most common MP recorded in mussels, clams and fish, accounting for approximately 80% of the total counted, which is consistent with other studies for aquatic species (Murphy et al., 2017; Li et al., 2018). The overall abundance of blue MP in all species (64%) might have occurred due to the attractiveness of this color shown by some marine organisms (Ory et al., 2017; Weis, 2020).

MP higher presence in bivalves comparatively to polychaetes probably occurred due to their feeding strategies. Mussels and clams are filter feeding organisms and filter high volumes of water, which can increase their exposure to MP ingestion (Filgueira et al., 2013).

Microplastics in *M. galloprovincialis*

Microplastic concentrations in mussels did not differ significantly between sites (Tagus and Porto Covo), although the contrary was expected as estuaries are generally more polluted (Vandermeersch et al., 2015), except for fragments which were higher in *M. galloprovincialis* from Porto Covo.

Mussels are sentinel organisms used for biomonitoring and are commercially important as seafood for human consumption. MP presence in wild mussels has been documented in field studies worldwide (De Witte et al., 2014; Mathalon and Hill, 2014; Li et al., 2015; Van Cauwenberghe et al., 2015; Vandermeersch et al., 2015; Qu et al., 2018). Our results showed a concentration of 0.18 ± 0.31 MP g^{-1} and 0.45 ± 0.67 MP.ind^{-1}, with a frequency of occurrence of 44%. Fibers were the most common MP type and blue was the most observed color.

Our concentrations are lower than the ones reported in mussels from Belgium (0.51 fibers g^{-1} and 0.26 fibers g^{-1}), the North Sea (0.36 ± 0.07 MP g^{-1}), Tagus estuary (0.34 ± 0.33 MP g^{-1}), China (1.52–5.36 MP g^{-1} and 0.77 to 8.22 MP ind^{-1}) and the United Kingdom (between 0.7 and 2.9 MP g^{-1} and 1.1 to 6.4 MP ind^{-1}) (De Witte et al., 2014; Vandermeersch et al., 2015; Li et al., 2018; Qu et al., 2018). Vandermeersch et al. (2015) reported 0.12 ± 0.04 MP g^{-1} in mussels collected in Europe and Van Cauwenerghe et al. (2015) 0.2 ± 0.3 fragments g^{-1} in mussels from the North Sea Coast. These concentrations are similar to the ones reported in this study. The prevalence of fibers as the most common MP is in accordance with other studies (Li et al., 2015; Li et al., 2018; Qu et al., 2018; Scott et al., 2019). The presence of MP in the mussels collected provides further evidence that mussels can be used as MP pollution bioindicator in coastal waters and estuaries (Li et al., 2019).

Microplastics in *S. plana*

The average concentrations of MP recorded in *S. plana* were the lowest recorded overall, probably due to the location they were collected, in a lower contaminated zone of Sado estuary (Carrasqueira) (Caeiro et al., 2005). Fibers were the most common MP recorded in clams, similarly to mussels and fish. Blue was the most common color registered in MP ingested by clams, probably due to the presence of intensive fish farms (Caeiro et al., 2005) using blue fishing nets.

Piarulli et al. (2020) studied the presence of MP in different salt marsh species, in which 10 *S. plana* were sampled from the Schelde estuary in the Netherlands. One MP was found in the *S. plana* sample: a polyacrylonitrile fiber. Due to the difference in the sample size, this result is not comparable to ours.

There were no significant correlations between the biometric parameters and total MP, fiber and fragment presence in *S. plana* (Spearman test, $\alpha = 0.05$). This suggests that MP presence occurs regardless of the size, weight of the clams. In accordance with other authors (Ribeiro et al., 2017), it is suggested to use *S. plana* as a future biomonitor for MP environmental risks.

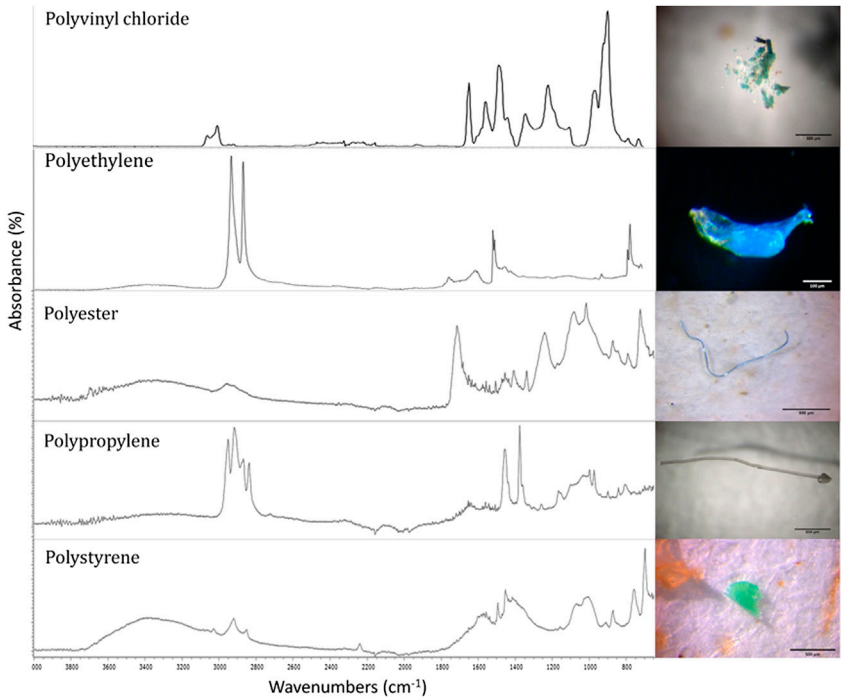

FIGURE 4 | Selected infrared spectra of microplastic in the studied species: PVC fragment found in *S. plana*; PE fragment found in *S. colias*; PET fiber found in *S. plana*; PP fiber found in *M. galloprovincialis*; PS fragment found in *M. galloprovincialis* (scale bar: 500 μm).

Microplastics in *M. sanguinea*

In polychaetes, fragments were the most common MP, representing 83% of the total MP observed. Accumulation of MP in lugworms has already been studied by some authors that also have detected impacts of exposure to chemicals (Wright et al., 2013; Besseling et al., 2017). Jang et al. (2018) reported 131 ± 131 particles.ind^{-1} and 24 ± 15 particles.g^{-1} in *M. sanguinea*. These results are much higher than the ones reported here and can be explained by the use of EPS buoys for *M. sanguinea* to live in.

No published work was found about MP presence in *M. sanguinea* in the field. In this study a total of 12MP was observed in 30 polychaetes, with average concentrations being 0.19 ± 0.40MP.g^{-1} and 0.40 ± 0.88MP.ind^{-1}. Unlike the other species in this study, fragments were the most common MP in *M. sanguinea* (83%). The polychaetes also registered the lowest MP average size (223 ± 233 μm), which could be explained by fragments being, in general, smaller than the fibers and might suggest that polychaetes will ingest fragments more easily than fibers. The results obtained from *S. plana* (sampled from the same site and with a deposit feeding strategy) also seem to support this idea, since only 12% of MP in *S. plana* were fragments, when compared with 83% fragments found in *M. sanguinea*.

This study contributes with valuable data regarding the abundance and characteristics of MP found in wild *M. sanguinea* for the first time, suggesting it as a potential biomonitoring species for MP contamination in sediments.

Microplastics in *T. trachurus* and *S. colias*

T. trachurus and *S. colias* registered a frequency of occurrence of MP of 70% and 55% respectively. This result is higher than the ones observed in *T. trachurus* (30%) and *Scomber* spp. (27%) captured between Cape Cantin and Cape Boujdour, Central zone of the Atlantic (Maaghloud et al., 2020) and *T. trachurus* (42%) from the North East Atlantic Ocean (Barboza et al., 2020). Sparks and Immelman (2020) studied seven fish species including *T. trachurus*, from the Agulhas Bank, South Africa and reported a frequency of occurrence of 87%, and Herrera et al. (2019) conducted a study on *S. colias* from the Canary Islands with a 78,4% MP occurrence. Barboza et al. (2020) also studied *S. colias* and reported a frequency of 62% in the North East Atlantic Ocean. These results are more similar with the result reported by this study. Lopes el al (2020) studied *T. trachurus* and *S. colias* from the Western and Southern Iberia and reported a frequency of 100% and 64%, respectively. While the frequency in *T. trachurus* was higher, in *S. colias* the result obtained is similar to the one reported here.

Most of the MP found were fibers (79%). This finding is supported by previous studies where fibers were also the most common MP type for several fish species (Neves et al., 2015; Güven et al., 2017; Bessa et al., 2018; Compa et al., 2018; Herrera et al., 2019; Valente et al., 2019; Koongolla et al., 2020; Lopes et al., 2020; Sparks and Immelman, 2020). In more detail, Herrera et al. (2019) found that 74.23% of MP collected in *S. colias* were fibers, while Barboza et al. (2020) reported that *T. trachurus* and *S. colias* specimens from the North coast of Portugal had more fragments

(76%) than fibers (22%) and pellets (2%) in the gastrointestinal tract, which is a different result than the one reported in this study. However, other studies conducted in fish from Portuguese waters show a prevalence of fibers as the most common MP even in different habitats and areas (Neves et al., 2015; Bessa et al., 2018; Lopes et al., 2020). Differences in results from different locations could be related to different sources of pollution and waste management strategies (Rochman et al., 2015), and should be monitored.

Blue and black were the most common colors in ingested MP. The predominance of these colors in microplastics has also been previously reported in *T. trachurus* and *S. colias* in different parts of the world (Herrera et al., 2019; Barboza et al., 2020) but also in Portuguese waters for the same species (Lopes et al., 2020) and other species of fish (Neves et al., 2015; Bessa et al., 2018; Lopes et al., 2020), which is a widely reported pattern.

Polymer Types

Polymer analyses revealed the presence of polyester (PET), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and nylon (PA), which is in accordance with the polymers commonly found in the environment, namely PET, PE and PP (Browne et al., 2011), and reflect the recently reported polymer diversity globally described for MP in water and sediments (Gago et al., 2018), since these polymers are the three most abundant worldwide (White et al., 2018). Polyester fibers were found in the majority of the individuals and blue was the predominant color and have probably originated from their massive use in clothing worldwide. These fibers are leached into the environment (Browne et al., 2011) and contribute to ocean plastic pollution (Napper and Thompson, 2016), with sediments being known as sinks for microplastic fibers (Law et al., 2010; Morét-Ferguson et al., 2010; Cózar et al., 2014). Polyester has already been found in mussels (Li et al., 2018) and fish (Rochman et al., 2015; Lefebvre et al., 2019; Koongolla et al., 2020). Our results are also comparable to previous studies in fish collected in Portugal (Neves et al., 2015; Bessa et al., 2018; Barboza et al., 2020). It is worth noticing that in this study we report ingestion of PVC by two endobenthic species, *S. plana* and *M. sanguinea*, which can be related to PVC's deposition on the sediment due to its higher density, once it was not detected in any of the other studied species.

MP ingestion can be a threat to aquatic organisms because, depending on the animal size, MP can be small enough to be expelled along with feces or, if larger, can be retained in the organism causing a false sense of satiety (Butterworth et al., 2012; Woods et al., 2018), while synthetic fibers can get tangled and create agglomerates, blocking organs and therefore hindering or preventing food ingestion (Derraik, 2002). Though there is no evidence of effects in wild aquatic species, laboratory studies reported inflammatory responses upon plastic ingestion in mussels (Von Moos et al., 2012) and neurotoxicity and oxidative damage in fish (Barboza et al., 2020), as well as in other aquatic species as reviewed by Barboza et al. (2018).

It is also important to refer that the comparison of results between different studies is difficult, due to the heterogeneity of the number of individuals analyzed, variability of laboratory procedures and MP extraction and identification methods, and the inconsistency of the reporting units used in results. There is a huge effort being made by the scientific community for standardization of protocols regarding MP studies, which will make future analysis and comparisons more efficient.

The presence of MP in the five species analyzed confirms the current and comprehensive contamination of the marine environment. Despite the knowledge regarding the levels of microplastics in the water and sediments from the coast of Portugal (Frias et al., 2014, 2016; Antunes et al., 2018; Rodrigues et al., 2020), there is still limited information regarding the distribution of microplastics in inland waters and sediments (such as estuarine areas) like those analyzed in the present study. This information would be important for assessing if the levels of microplastics found in the studied species reflect the concentrations found in the environment.

Microplastics entering the marine food webs may affect important seafood species. The results obtained should raise concern regarding bioaccumulation and possible human health risks associated with the consumption of MP contaminated fish and shellfish (Li et al., 2019). Selecting suitable species for monitoring microplastics pollution is an essential step toward achieving the good environmental status aimed by the Marine Strategy Framework Directive (MSFD). The suitable monitoring species listed for the Mediterranean Sea and the Northeast Atlantic, such as the sea turtle *Caretta caretta* and the sea bird *Fulmarus glacialis*, respectively, are very rare in Portuguese coastal waters, making it necessary to find and select suitable species for monitoring (IPMA, 2018). Despite the need for more research, this work provides baseline data from five species representing different habitats and feeding strategies with the potential to be used for monitoring microplastics. In addition, these species are ecologically and economically important and can be found in several locations along the Portuguese coast.

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for this animal study because the vertebrates (fish samples) were fished by local fishermen for commercial purposes and were no longer alive when made available for this study. The remaining animal samples were bivalves and polychaetes—non-higher invertebrates.

AUTHOR CONTRIBUTIONS

PS conceptualised the study; JP and VD collected the samples and performed the experiment; JA performed the micro-FTIR analysis; FB introduced and taught the laboratory procedures; JP and JA drafted the manuscript; JP, JA, FB, and PS contributed to improve the manuscript.

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Combined Approaches to Predict Microplastic Emissions Within an Urbanized Estuary (Warnow, Southwestern Baltic Sea)

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Microplastic river emissions are known to be one of the major sources for marine microplastic pollution. Especially urbanized estuaries localized at the land-sea interface and subjected to microplastic emissions from various sources exhibit a high microplastic discharge potential to adjacent coasts. To adapt effective measures against microplastic emissions a more detailed knowledge on the importance of various microplastic sources is necessary. As field data is scarce we combined different approaches to assess microplastic emissions into the Warnow estuary, southwestern Baltic Sea. Resulting microplastic emission estimates are based on *in-situ* measurements for the catchment emissions, whereas for the remaining microplastic sources within the estuary literature data on microplastic abundances, and various parameters were used (e.g. demographical, hydrological, geographical). The evaluation of the different emission scenarios revealed that the majority of microplastic is likely discharged by the Warnow river catchment (49.4%) and the separated city stormwater system (43.1%) into the estuary, followed by combined sewer discharges (6.1%). Wastewater treatment plant emissions exhibit the lowest percentage (1.4%). Our approach to estimate anti-fouling paint particles emissions from leisure and commercial shipping activities was associated with highest uncertainties. However, our results indicate the importance of this source highlighting the necessity for future research on the topic. Based on our assumptions for microplastic retention within the estuary, we estimate a potential annual emission of 152–291 billion microplastics (majority within the size class 10–100 µm) to the Baltic Sea. Considering all uncertainties of the different applied approaches, we could assess the importance of various microplastic sources which can be used by authorities to prioritize and establish emission reduction measures. Additionally, the study provides parameters for microplastic emission estimates that can be transferred from our model system to other urbanized Baltic estuaries.

Keywords: microplastic, warnow estuary, baltic sea, sewer system, emission estimates, combined sewer overflow, anti-fouling paint particles

INTRODUCTION

Since the first description of small plastic particles in the marine environment (Colton et al., 1974; Morris and Hamilton, 1974), assessments of "microplastic" (MP) contamination revealed its ubiquitous distribution within aquatic ecosystems (Burns and Boxall, 2018; Geilfus et al., 2019). Concentrations of MPs span several orders of magnitude ranging from less than one to several hundreds of thousands of MPs per cubic meter in sea water (Wu et al., 2019). In sediments over ten thousand MPs per kilogram dry weight (DW) have been reported (Cunningham and Sigwart, 2019). Especially semi-enclosed seas with a large catchment area and limited water exchange through narrow straits, such as the Baltic Sea, are subjected to anthropogenic pressures deriving mainly from land-based activities like MP litter. Due to the omnipresence and longevity of MP litter and its potential adverse effects on organisms and human health (Sharma and Chatterjee, 2017; Wang et al., 2019) it has become an integral part of regulations for the protection of the marine environment (Directive of the European parliament, 2008; HELCOM, 2008; Resolution, 2020). The most relevant within the European Union, the Marine Strategy Framework Directive (MSDF, 2008/56/EC), requires its member states to achieve a good environmental status (GES) with marine litter representing one of the qualitative descriptors (D10) for the GES. For Baltic Sea member states, the directive is implemented within the Baltic Sea Action Plan (BSAP) of the Helsinki Convention (HELCOM). Its Regional Action Plan on Marine Litter (RAP ML) requires further "to establish an overview of the importance of the different sources of primary and secondary microplastics" (RL6, HELCOM, 2015) and calls for actions addressing sources of MPs, (i.e. RL4).

Rivers have already been recognized as a major input source of MP litter into the ocean (van Wijnen et al., 2019). Especially urbanized estuaries depict a potential hotspot for MP emissions to coastal waters with measured MP concentrations in urban surface waters reaching 100 items per liter (Leslie et al., 2017). Here a multitude of MP emission sources congregate within a confined space, such as recreational activities, shipping, wastewater emissions, tributaries, and leisure boat facilities. To implement targeted prevention measures against marine MP litter a detailed overview and assessment of the importance of various MP emission sources within urban estuaries is necessary. Concerning southwestern Baltic estuaries, the Warnow estuary, with a high degree of urbanization and industrialization, depicts a suitable model system.

Considering land-based MP emissions, major point sources are municipal and industrial wastewaters (Baresel and Olshammar, 2019; Bellasi et al., 2020). Due to high discharge volumes of wastewater treatment plants (WWTPs) they are considered as significant source even though most plants obtain a MP removal efficiency of >90% (with up to 99.9%) (Prata, 2018; Cristaldi et al., 2020; Uddin et al., 2020). An even higher share of MP emissions has been reported for combined sewer overflows (CSOs) in combined systems (Baresel and Olshammar, 2019) as well as stormwater in separated systems (Liu et al., 2019). Paint particles from buildings, structures, and shipping are thought to be another relevant source of MPs within urban estuaries (Sundt et al., 2014; Soroldoni et al., 2018). Here it must be differentiated between anticorrosive and antifouling paint particles (AFPPs) with the latter possibly possessing a higher relevance due to the self-polishing behavior of AFPPs (Watermann and Eklund, 2019). For example, in the Warnow estuary, a medium of 86.5 MP per kilogram DW and a maximum of 379 (±28) MPs per kilogram DW was measured within subtidal sediments with paint particles generally as common as other microplastics (Enders et al., 2019).

Nevertheless, data on MP inputs from diverse sources and abundances within any river mouth is still scarce (Li et al., 2018; Rezania et al., 2018). Besides challenges on collection of environmental MP samples, cost-effective and standardized operational monitoring methods are currently lacking. An alternative approach to estimate MP emissions is the development of emission scenarios based on available literature data, expert knowledge and via indirect parameters. For example near shore population density, gross domestic product, and mismanaged plastic waste were already utilized as predictors for plastic litter emissions on a global scale (Sherman and van Sebille, 2016; Schmidt et al., 2017; Isobe et al., 2019). Regarding MPs, Siegfried et al. (2017) estimated MP fluxes from European rivers to the sea by combining information on MP litter point sources, sewage management, and plastic retention during river transport. Discharge of MPs by urban waters to the Baltic Sea was further assessed utilizing exemplary sewer systems and a model city (Bollmann et al., 2019). Nevertheless, a spatially resolved differentiation of MP emission sources within an urbanized Baltic estuary is currently missing to the best of our knowledge. Due to its relatively small and well-defined area the Warnow estuary provides an ideal model system for which a comprehensive approach can be applied.

To support authorities in establishing an effective monitoring as well as in implementing mitigation measures, we assessed the importance of different MP sources exemplarily for the Warnow estuary. We first localized major MP point sources, here defined as sources within the estuary where a spatially explicit localization is possible. Secondly, a combination of different approaches was used to estimate MP emissions from the city sewer system and the river catchment as well as to assess AFPP emissions. The specific aims were 1) to establish an overview of the importance of different MP sources within the urbanized Warnow estuary as a model system, 2) to provide a first estimation on annual MP emissions from the estuary to the Baltic Sea, and 3) to assess the suitability of utilized parameters to support the development of simplified indicators for estuarine MP emissions into the Baltic Sea.

MATERIALS AND METHODS

Study System

The Warnow estuary (**Figure 1**), Mecklenburg-Western Pomerania, Germany, has a length of 13 km with a mean water depth of 5.6 m and a maximum depth of 15 m within the shipping channel up to the area of Breitling (Lange et al.,

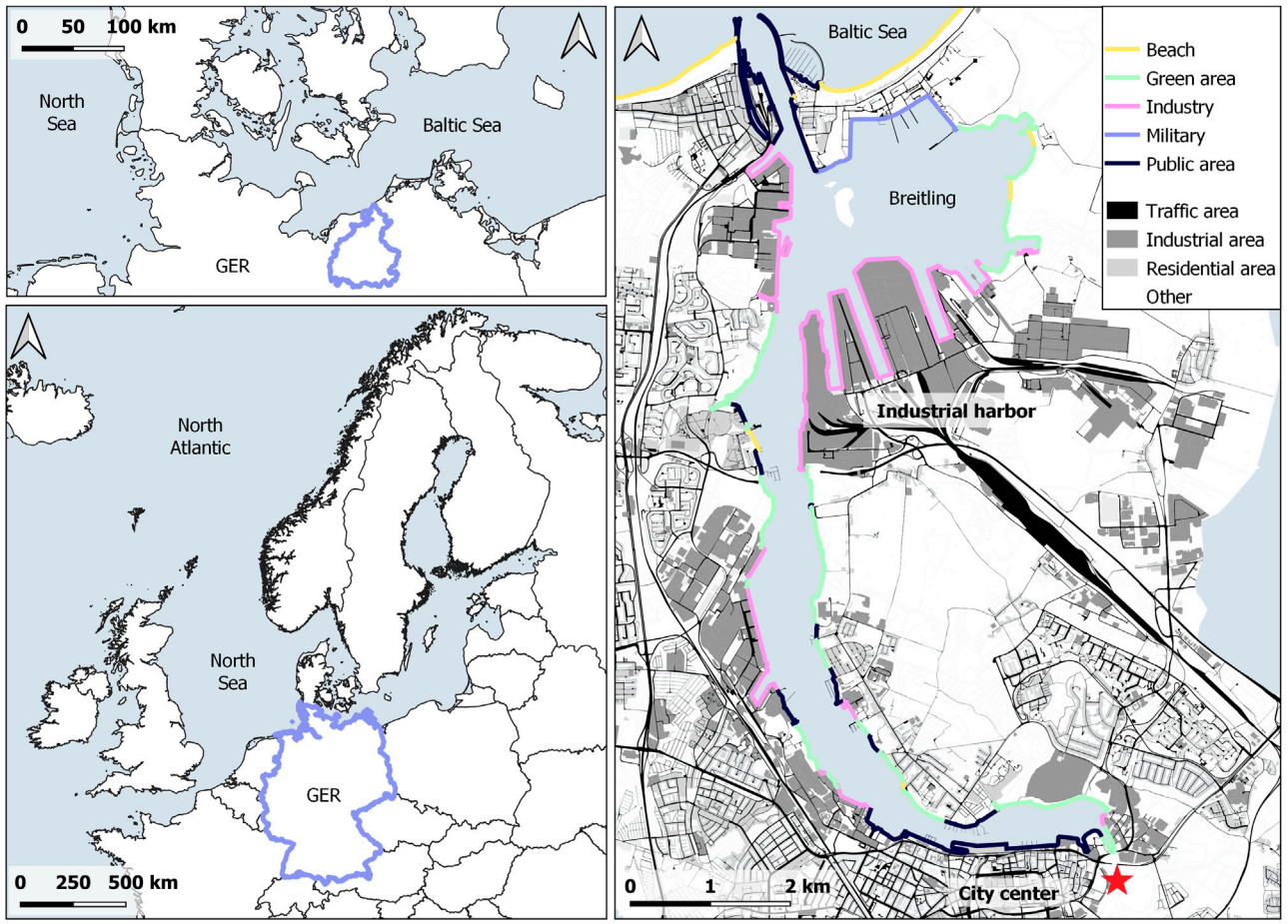

FIGURE 1 | Map of the Warnow catchment (blue line **upper left**) located in northwestern Germany **(lower left)** and the Warnow estuary **(right)** with land use sections around the estuary. The position of the weir where the Warnow river is discharging into the estuary is indicated by a red star. "Other" includes unsealed areas such as forests, agri- and horticulture. Source: Hanse-und Universitätsstadt Rostock (CC BY 4.0).

2020). The estuary holds a water volume of 49.6 million m^3 within an area of 12 km^2 (Bachor, 2005) and exhibits a water exchange time of around 30 days. The average water budget of the estuary is dominated by intruding Baltic Sea water with 1,180 km^3/a in comparison to 440 km^3/a freshwater inflow and a total outflow to the North Sea of 1,660 km^3/a (Szymczycha et al., 2019). The estuarine exchange flow of the tidal weak estuary is sensitive to wind stress and varying salinities of the adjacent coastal waters, which leads to frequent inversion of the classical estuarine circulation (Lange et al., 2020). The 56.5 km long riverbank of the estuary is mostly artificial (74%), and harbor and shipping lanes occupy 37% of the water surface area (Schernewski et al., 2019). In 2018 around 200 cruise ships arrived and around 19.6 million tons of freight and 3.3 million passengers were handled (Hansestadt Rostock, 2019). Besides, leisure boat facilities are common with a share of 6% of all moorings along the German Baltic Sea coast (Watermann et al., 2014). To maintain and safeguard shipping activities, parts of the estuary are regularly dredged. The maintenance interval of the inner and outer shipping lane (access to industrial harbor) is five to ten years whereby on average 60,000 m^3/a of dredged material is extracted (WSV, 2019). Other areas within the estuary are maintained on demand.

The city of Rostock surrounds the estuary and counts about 209,000 residents within an area of 181.4 km^2 yielding a population density of 1,151 residents per km^2 (Hansestadt Rostock, 2019).

The city area is shaped by a dense network of natural and artificial waterways. About 200 small streams with a total length of 196 km exist within the catchment of the city of Rostock. The city center is connected to a combined sewer system (**Figure 2**) but most of the catchment is connected to a separate system with a 430 km long canal network (KOGGE et al., 2018). The wastewater of Rostock is treated at the central wastewater treatment plant (WWTP). The treatment technology comprises mechanical treatment, an activated sludge followed by a two-step biofilter for post nitrification and denitrification. Taking industrial and commercial wastewater contributions into account the population equivalent (PE) of the WWTP is 400,000, corresponding to a wastewater production of 100 L per PE per day.

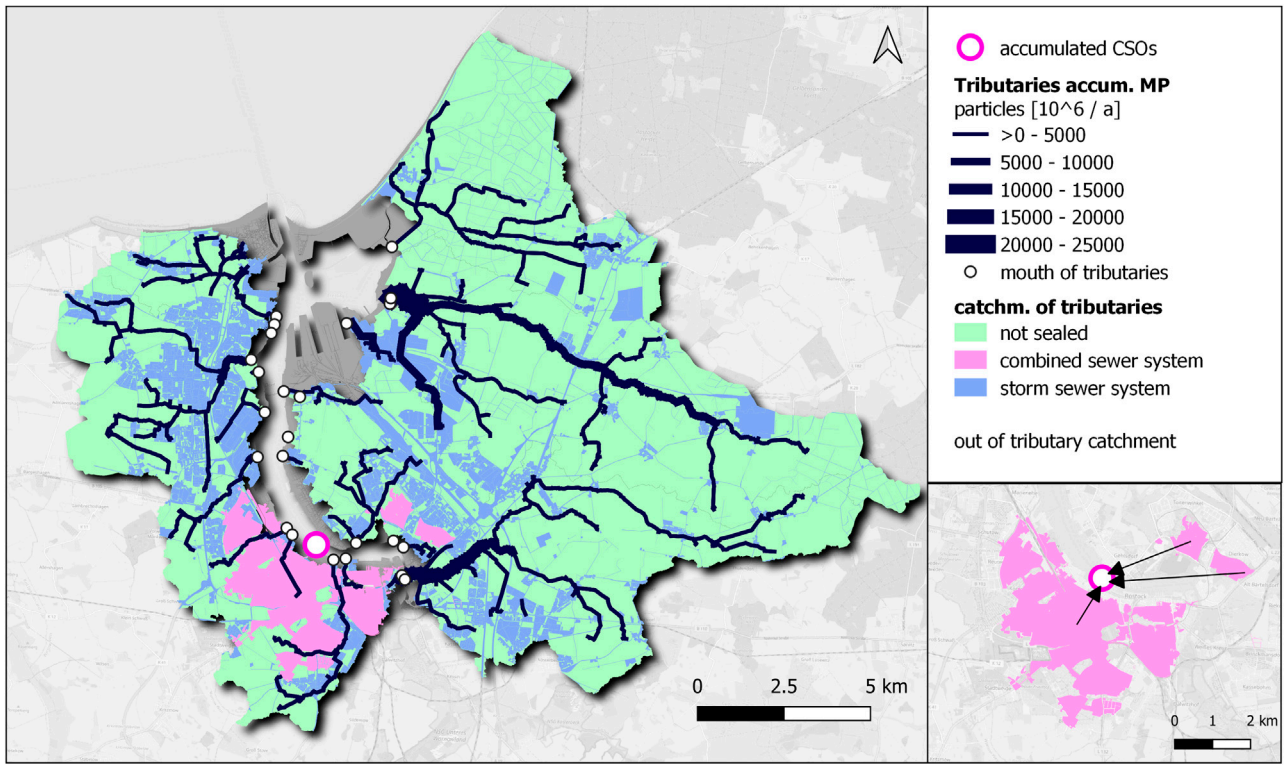

FIGURE 2 | Accumulation of microplastic (MP) emissions from stormwater (blue areas) along tributaries (dark blue lines) within the catchment of the estuary and input of MP emissions from the combined sewer system (magenta areas). Green areas are assumed not to be sealed and include e.g., agriculture, grassland, and parks.

The mean annual rainfall amounts to 621 mm (DWD, 2020) and annually a mean of 16.5 m^3/s of water is discharged from the Warnow river into the estuary. The discharge is regulated by a weir located at the southern part of the city of Rostock (**Figure 1**). The 149 km long Warnow river exhibits a catchment area of 3,280 km^2 with a population density of 60 persons per km^2.

The following described MP sources within the Warnow estuary were located through GIS data (KOGGE et al., 2018; OpenDataHRO, 2019; Wasserbuch, 2019). Despite additional diffuse emissions, e.g., through fragmentation of macroplastics or diffuse aerial inputs, the majority of land-based MP emissions is assumed to be covered according to current knowledge (Galafassi et al., 2019).

In total around 232 point sources for MPs and AFPPs are located within the estuary. Besides the input of the catchment, 24 tributaries discharge into the estuary (**Figure 2**). Among the 167 identified wastewater outlets are the WWTP outlet, 11 combined sewer outlets and 155 stormwater (STW) outlets. Regularly used moorings and leisure boat facilities depict potential emission points for AFPP. The industrial harbor maintains five docks with around 41 moorings. Additionally, five local ferry moorings, four cruise ship moorings as well as fifteen leisure boat facilities are located within the estuary. Due to insufficient data three operating shipyards were neglected for AFPP emission estimates in the current study.

The complex characteristics of MP particles (size, forms, types), the high number of potential sources and spatio-temporal variability of emissions as well as laborious and time-consuming analytical methods currently prevent comprehensive *in-situ* data collections. To provide a comprehensive picture for the Warnow Estuary the additional use of statistical data, literature, and the transfer of knowledge from similar systems was utilized. The different approaches to assess the importance of MP sources and emissions within the estuary are described in the following.

Rostock Sewer System Microplastic Emissions

Wastewater treatment plant

A concentration of 245 (IQR = 913–78) MPs/m^3 (including MPs <100 µm) was applied corresponding to the median of values from WWTPs of various countries with tertiary treatment providing MP analysis for particles <100 µm (**Supplementary Information SI1**). Microplastic emissions were multiplied with monthly discharge data of the WWTP effluent from 2016 to 2018 which was provided by the local WWTP (Nordwasser GmbH).

Combined sewer overflow emissions

Currently, the CSOs are not measured since the discharges and concentrations have to be determined on a high temporal resolution or flow equivalent. Within the scope of this study, the discharge volume was therefore conceptually determined. Assuming that the spillway structures were dimensioned according to the chemical oxygen demand (COD) approach of the ATV-A 128 regulations (DWA, 1992) at the time, the annual

TABLE 1 | Runoff of the annual precipitation of the combined sewerage catchment area. Runoff coefficients (ψ_m), annual rainfall (r), and rainfall runoff ($Q_{R_{CS}}$).

Catchment class	Area [ha]	ψ_m	r [l/m^2]	$Q_{R_{cs}}$ [m^3*10^3/a]
Residential areas	368	0.79	621	1,805
Traffic areas	264	0.86	621	1,410
Industrial areas	95	0.68	621	401
Unsealed areas	433	0.14	621	377
				Σ 3,993

discharge volumes can be calculated. The annual MP emission is in turn the product of the annual discharge volume and the MP concentration. For the calculation of the CSO discharge quantity (**Eq. 1**), the permissible overflow rate corresponding to the DWA 128 regulations (DWA, 1992) is assumed (**Eq. 2**).

$$Q_{\mathrm{CSO}} = e_0 \times Q_{R_{\mathrm{CS}}}, \tag{1}$$

where Q_{CSO} corresponds to the discharge of CSOs (m^3/a), e_0 to the permissible overflow rate, and $Q_{R_{\mathrm{CS}}}$ to the rainfall runoff of the combined sewer (m^3/a).

The permissible overflow rate in relation to the COD was calculated using **Eq. 2** (DWA, 2006). Since the city sewer system is in transition from a combined sewer system to a separated sewer system, the COD concentration of dry weather flow is high. To ensure that a critical COD of 178 mg/L is not exceeded a larger proportion of stormwater is mixed with the wastewater. Hence, the resulting average mixing ratio (m = 11.68:1) between rainwater and wastewater is quite high. Accordingly, the calculated overflow concentration of COD is 178 mg/L (**Supplementary Information SI2**). This results in a permissible overflow rate of 34%.

$$e_0 = \frac{c_R - c_{\mathrm{WWTP}}}{c_e - c_{\mathrm{WWTP}}} = \frac{107\mathrm{mg/L} - 70\mathrm{mg/L}}{178\mathrm{mg/L} - 70\mathrm{mg/L}} \times 100 = 34\%, \tag{2}$$

where c_R is the COD concentration in the storm runoff of the combined sewer (mg/l), c_{WWTP} the COD concentration of the wastewater treatment plant (WWTP) effluent (mg/l), and c_e the COD concentration of the CSOs effluent (mg/l).

The rainfall runoff $Q_{R_{\mathrm{CS}}}$ was calculated as a function of specific runoff coefficients (ψ_m), and area sizes. Runoff coefficients were taken from a long-term simulation study with the Environmental Protection Agency (EPA) Storm Water Management Model (Rossman, 2015) by the Department of Water Management, University of Rostock, Germany. Catchment classes were differentiated for residential areas, traffic areas, industrial areas, and unsealed areas (**Table 1**). The latter including green spaces as well as forests and agricultural and horticultural areas. Based on the total runoff volume of around four million cubic meters per year (**Table 1**), the annual discharge volume of the CSOs was derived using **Eq. 1**.

$$Q_{\mathrm{CSO}} = 34\% \times 3,992,905\mathrm{m}^3/a = 1,357,588\mathrm{m}^3/a, \tag{3}$$

According to the average mixing ratio (m = 11.68:1), the annual rainfall discharge of the CSOs is 1,269,794 m^3 and the annual discharge wastewater of the CSO is 107,065 m^3. The MP quantity (c_{MP}) in **Table 2** was derived from the use-specific concentration (Liu et al., 2019) and outflow fraction which corresponds to the percentage share of the rainfall runoff (**Table 1**). For untreated wastewater the median value of 143 (IQR = 910–68) MPs/l was calculated from data of nine studies (**Supplementary Information SI1**). All considered studies included the analysis of particles <100 µm.

Stormwater emissions

Mean annual MP emissions by stormwater along 24 tributaries were estimated for the riverine inflows below the Mühlendamm weir (**Figure 2**). Sealed and drained areas $A_{E,k}$ within the catchments of tributaries were identified and classified into three land use types in GIS by a spatial intersection with a high-precision land use map. The annual stormwater runoff Q_R of each drained area was represented by the effective rainfall, which is the part of the precipitation that flows superficially to the storm sewer network or stream section. It was calculated based on runoff coefficients shown in **Table 1** (c.f. rational method, DWA, 2006) for each subarea and the long-term average annual rainfall r_a of 621 L/m^2 (DWD, 2020):

$$Q_R = A_{E,k} \times r_a \times \psi_m, \tag{4}$$

where $A_{E,k}$ corresponds to the drained area (m^2), r_a to the mean annual rainfall (l/m^2/a), and ψ_m to the mean annual runoff coefficient. Microplastic emissions B_{MP} were calculated for each drained area using **Eq. 5**:

$$B_{\mathrm{MP}} = c_{\mathrm{MP}} \times Q_R, \tag{5}$$

where Q_R is the stormwater runoff corresponding to the effective precipitation (l/a). Microplastic concentrations c_{MP} of stormwater runoff were derived as mean values from Liu et al. (2019) for each land use type: residential (0.90 MP/l), traffic (0.49 MP/l), and industrial areas (13.16 MP/l).

Estimated MP emissions were allocated to the nearest watercourse section by a spatial join in GIS (**Supplementary Information SI3**). River geometries were available as line sections of 50 m length from the project KOGGE (KOGGE et al., 2018). Each river section is labeled by a unique identification number, which is composed of the classification number and a station number according to the German "LAWA guideline for the labeling of rivers" (WASSER, 2005). The hierarchical structure of the classification number and station number from the estuary to the river head makes it possible to identify river sections located below or above each section. The accumulation of these emissions along the flow path is performed by *Python* scripts in GIS.

Spatial resolved MP emissions of stormwater outlets directly discharging into the estuary are currently not possible due to lacking information regarding the wastewater system (grey area **Figure 2**). Nevertheless, similar calculations of MP runoff, based on available information on the share of industrial (5.3 km^2), residential (3.7 km^2), and traffic (2.4 km^2) areas can be made based on **Eqs. 4, 5**.

River Catchment Microplastic Emissions

The number of MP particles released from the catchment into the estuary was taken from eight *in-situ* collected water surface samples from the Warnow river close to Kessin (N54°03′51.13,

TABLE 2 | Microplastic (MP) emission (B_{MP}) due to combined sewer overflows separated according to the proportion of waste- and rainwater. The outflow fraction corresponds to the percentage share of the rainfall runoff ($Q_{R_{CS}}$) of a catchment class from the total rainfall runoff ($Q_{R_{CS}}$) from **Table 1**. Microplastic concentrations (c_{MP}) for storm- (STW) and wastewater were obtained from the literature (**Supplementary Information SI1**).

Type	Catchment class	Outflow fraction (%)	Overflow [m³/a]	c_{MP} [MPs/l]	B_{MP} [MPs*10⁶/a]
STW	Residential areas	45	574,131	0.9	517
	Traffic areas	35	448,372	0.49	220
	Industrial areas	10	127,576	13.16	1,679
	Unsealed areas	9	119,716	—	—
Sum STW			1,269,794		2,415
Wastewater			107,065	143	15,310
					Σ 17,725

E12°10′15.60), about 3 km upstream the weir, during September and October 2018 and May to March 2019. Sampling was conducted using an encapsulated flow through filtration system as described by Lenz and Labrenz, (2018) with sample volumes between 150 and 600 L. The device was equipped with stainless steel filter cartridges (nominal 10 µm mesh size) in an enclosed setup. The sampling inlet was placed to sample the upper 10 cm of the water column facing the incoming flow. The individual sample volumes and dates can be found in the **Supplementary Table SI4**.

The sample material was further processed in a MP-work adapted laboratory according to Enders et al. (2020). All steps involving exposed sample material were conducted inside a laminar flow bench (S2020 1.8, Thermo Scientific). All chemicals and rinsing water used were filtered through muffled 1.5 µm glass fiber filters (Whatman 934-AH) in a MP-free environment. First, the sample material was recovered from the filter cartridges by soaking in a 15% H_2O_2 solution for 48 h with subsequent rinsing off the particulate matter into ceramic bowls. From there the decision tree proposed by Enders et al. (2020) was followed applying protocol module m1 (freeze drying), m4 (30% H_2O_2 digestion over 24 h), and m5 (simple density separation). In a final vacuum filtration on 10 µm stainless steel meshes the samples were thoroughly rinsed using MP-free ultrapurified water to wash out remaining solutes.

The samples were transferred from the 10 µm stainless steel meshes to 50 ml Erlenmeyer flasks and sent to the IPF Dresden for microspectroscopic analysis. For that purpose, the particle suspensions were filtrated onto 10 × 10 mm silicon filters (Käppler et al., 2015) for the following Raman analysis. The filtration took place in a particle-depleted laboratory under a laminar flow bench using a custom-made glass filtration device with PTFE adapters to host the silicon filters (Brandt et al., 2020). The silicon filters had a pore size of 50 µm with a pitch of 100 µm. The number, type, and color of the MP particles >50 µm of a filtrated sample were determined with a combination of optical particle detection and Raman microspectroscopy using the software GEPARD as described in Brandt et al., 2020.

Microplastic abundances in samples were corrected by MP abundances detected in laboratory and process blanks. Therefore, the mean MP number for each size and polymer type class was calculated and subtracted according to the size and polymer type class from each sample (**Supplementary Information SI4**). The MP data from the two different seasons (spring/autumn) were pooled for calculation of mean annual MP emissions from the catchment. Therefore, measured MP concentrations (C) were plotted against the river discharge (Q) measurements from the sampling day and a linear regression model was fitted to the data (in the following C-Q model; **Supplementary Information SI5**). Subsequently, the C-Q relationship was used to calculate annual MP emissions based on daily discharge data (m^3 per second) from the closest gauging station Rostock/Geinitzbrücke (N54°04′41.17, E12°09′15.16) for the years 2016–2018 (data provided by Staatliches Amt für Landwirtschaft und Umwelt Mittleres Mecklenburg 2019) based on **Eq. 6** (**Supplementary Information SI5**):

$$C = 132.41 + 18.62 \times Q, \tag{6}$$

where C corresponds to the MP concentration (MPs/day) and Q corresponds to the river discharge (m^3/s).

Anti-fouling Paint Particle Emissions

As another potentially significant MP source within urbanized estuaries we assessed anti-fouling paint particle (AFPP) emissions from leisure and commercial shipping activities (**Supplementary Information SI6**). For the leisure boat facility scenario AFPP release from the underwater ship hull of leisure boats were considered. Thereby, 65% of permanent leisure boats (PLANCO, 2004), which are assumed to be at berth for 50% of the time during the whole season (April-October), were considered. Number of berths within leisure boat facilities were obtained from the city of Rostock (OpenDataHRO). Utilizing emission scenarios for active ingredients from AFPPs it is assumed that 2.5 L of anti-fouling paint is applied per leisure boat and year by do-it-yourself maintenance work (OECD, 2005).

To convert paint volume applications to particle numbers a polymer content of 30% was assumed (Watermann and Eklund, 2019). As size distributions of AFPP particles detected in environmental samples are lacking, a basic emission scenario with an idealized AFP particle was applied to get a first impression on spatio-temporal emission patterns within the estuary. Therefore, MP spheres with a density of 1.6 g/cm^3 (Daehne et al., 2017; Enders et al., 2019) and a diameter of 0.5 mm were used for conversion.

Generally, three AFPP emission scenarios were differentiated: emissions during in-service use (ISU), from high-pressure

washing (HPW), and from maintenance and repair work (MR). Emissions during ISU were set to 1% of paint applied (OECD, 2009) of which 50% were assumed to be released while the boats are moored at berth. For the HPW and MR scenario particle emissions were assumed to be equally distributed to soil and water, resulting in a fraction of 0.5 being discharged to the water. At the end of the season in November boats are taken out of the water and often cleaned by HPW where a removal factor of 0.2 of AFPPs was set (OECD, 2005). Only leisure sport facilities offering a slipway were considered for AFPP release through HPW. For MR work during December till March a removal factor of 0.3 and an emission factor of paint application of 0.0125 was adapted (OECD, 2005). Only facilities that store boats in proximity to the water (identified through satellite images) were considered for MR particle emissions.

Oversea ferries, cruise ships, RoRo (Roll-on Roll-off), tanker, and cargo ships were considered for the AFPP emissions during ISU from commercial shipping. A conservative theoretical coverage of 13 m^2/L of AFP was applied. To transfer the AFPP emissions of 1% during ISU to emissions within the estuary, average wetted surface area of different ship types (Miller et al., 2018), data on yearly inruns, and average time at berth for different ship categories (Lorentz and Moldsenhauer, 2014) were utilized (see **Supplementary Information SI6** for calculation).

Comparison of Emission Estimates and Potential Microplastic Discharge to the Baltic Sea

For MP emission estimates of the city sewer system (WWTP, CSO, and STW), particle size was considered as important parameter. We only considered data from studies including the analysis of MPs <100 µm, as MP particle number increases rapidly with decreasing particle size. The MP concentration for raw wastewater and WWTP effluent was obtained as median from several studies, whereas for STW emissions only one study transferable to our system was available to the best of our knowledge (Liu et al., 2019).

The analysis of *in-situ* obtained MP data for the Warnow river did not include MPs between 10 and 50 µm. As fragmentation of MP particles causes the generation of continually smaller MPs, they are expected to follow a power law particle distribution (Kooi et al., 2019). Thus, MP counts from *in-situ* river samples were grouped into size bins of 10 µm and a power law model fitted to the data (**Supplementary Information SI7**). The obtained coefficients (a = 9.231, b = −1.293) were used to predict MP abundance down to 10 µm. Our calculated annual MP emissions by the C-Q model was concurrently corrected by the relative share of 91% of the size class 10–50 µm.

A comparison with AFPP emissions was not considered due to the different data basis, utilizing sales volume of AFP, expert knowledge, and an idealized AFP particle for emission estimates.

To further estimate MP discharge to the Baltic Sea the following considerations and assumptions on MP density, and thus sinking behavior, were made. Microplastic data from *in-situ* measurements and the literature were grouped into two density classes. Low density (LD) MPs with a density <1.0 g/cm^3 included polymers such as polyethylene (PE) and polypropylene (PP). The high density (HD) class, with a density >1.0 g/cm^3, contained polymers such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polyamide etc (**Supplementary Information SI4**).

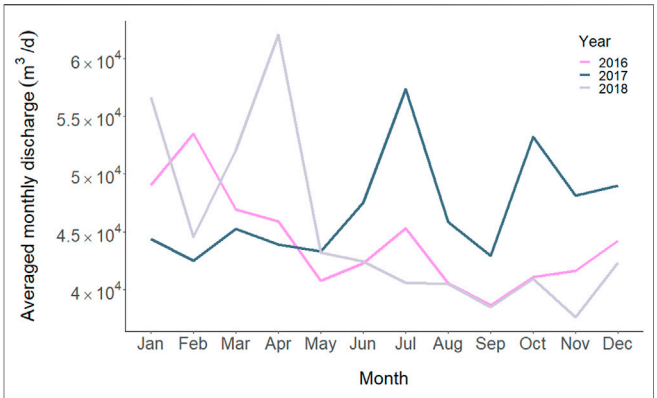

FIGURE 3 | Averaged daily discharge of treated wastewater for the different month at the outlet of the WWTP (data source: Nordwasser GmbH).

For high density AFPPs an accumulation close to their source could be shown for the Warnow estuary (Enders et al., 2019) and hence their retention within the estuary is very likely. In addition, HD-MPs with a higher density than seawater (1.025 g/cm^3) were assumed to sink as processes, such as biofouling or agglomeration (Rummel et al., 2017; Michels et al., 2018), increase MP density and thus the probability of MPs sinking to the riverbed. Besides, for MPs between 1–200 µm retention is reported to strongly increase with density (Besseling et al., 2017).

To provide a conservative estimate on annual MP emissions to the Baltic Sea retention of buoyant LD-MPs was additionally considered. Due to the complex hydrodynamic behavior of the weakly tidal Warnow estuary, measuring discharge and assessing water retention time is difficult. For the saltwater influenced Warnow estuary Rönspieß et al. (2020) reported a retention capacity for particulate phosphorus. Similar calculations for suspended particulate matter (SPM) from the supplementary information of this study (see Suppl. 9_Balance calculations of Rönspieß et al., 2020) results in a retention capacity of 31% for SPM respectively. This retention capacity was further assumed for LD-MPs.

RESULTS

Rostock City Sewer System Microplastic Emissions

On average an annual emission of about 4.1 billion MPs was calculated for the WWTP outlet based on the monthly discharge data of the years 2016–2018 (monthly mean emission of about 11 million MPs).

Similar to the discharge of the catchment, at the WWTP lowest and highest average daily discharge of treated wastewater,

TABLE 3 | Microplastic (MP) emission (B_{MP}) due to stormwater runoff separated according to the catchment class. Accumulated results for the catchment area of the tributaries as well as areas connected to stormwater outlets (grey area **Figure 2**) are shown. Microplastic concentrations (c_{MP}) were obtained from Liu et al. (2019). Runoff coefficients (ψm), annual rainfall (r), and rainfall runoff (Q_R).

Catchment class	Area [ha]	ψ_m	r [l/m^2]	Q_R [m^3*10^3/a]	c_{MP} [MPs/l]	B_{MP} [MPs*10^6/a]
Residential areas	2,510	0.79	621	12,314	0.9	11,082
Traffic areas	2,090	0.86	621	11,162	0.49	5,469
Industrial areas	1,960	0.68	621	8,277	13.16	108,921
Unsealed areas	10,420	0.14	621	9,059	—	—
						Σ 125,477

and thus MP emissions, were calculated for 2018 in November and April respectively (**Figure 3**).

Besides emissions from the WWTP, the rainwater from the combined sewer system pollutes the receiving water with around 2.4 billion particles per year, while around 15.3 billion particles per year are carried into the watercourse by the wastewater (**Table 2**). The total input of the combined sewer system is thus 17.7 billion particles per year.

Based on **Eq. 4** an annual total stormwater runoff of about 41 million m^3/a was calculated (**Table 3**). The 24 tributaries contribute to an additional 91 billion MP particles per year due to runoff from industrial (14.3 km^2), residential (21.4 km^2), and traffic areas (18.5 km^2). The individual contributions of the tributaries range from 1.6 million to 23 billion MPs per year at their mouth into the estuary (white points in **Figure 2**). Around 15% are released close to the weir, 32% at the western and 53% at the eastern sides of the estuary. As industrial areas are supposed to deliver high MP inputs per area according to Liu et al. (2019), especially the eastern tributaries account for high MP emissions in the estimation.

In addition, 34.5 billion MP particles are potentially being directly discharged (grey area **Figure 2**) into the estuary through several stormwater outlets. From the resulting total annual emissions of 125.5 billion MPs, 73% are thus released by the tributaries and 27% through stormwater outlets distributed along the estuary.

River Catchment Microplastic Emissions

For the Warnow river, MP concentrations from 57 to 388 particles/m^3 with a mean of 226.79 ± 119.39 and a median of 281 (IQR: 297–113) particles/m^3 (size range 0.05–5 mm) were measured. Considering the size classes in MP river samples, more than 90% of MPs were <300 µm within the first sampling campaign in autumn 2018. In the second sampling campaign 63% of detected MPs exhibited sizes <300 µm and 30% were between 300–1,000 µm (**Supplementary Information SI4**). Polyolefins (PE and PP) dominated among detected polymer types (72%), followed by polystyrene (13%). Polyethylene terephthalate (8%), polyoxymethylene (4%) and silicone (2%) had a minor share and polymethyl methacrylate and PVC contributed with less than 1% among detected polymer types (**Supplementary Information SI4**).

Applying the C-Q model to daily discharge data for the years 2016–2018, average annual MP emission account for about 13 billion MPs with a daily average of about 36 million MPs being released into the estuary (**Supplementary Information SI5**). Highest average river discharge, and thus calculated MP emissions, was observed in 2017 (18.3 ± 8.4 m^3/s), with high amounts of MPs being released during summer and October till December (**Figure 4**). In the years 2016 and 2018 monthly river discharge follows the generally observed pattern for the Warnow river (Pegelportal, 2020) with accordingly highest MP emissions calculated for January and February, thereafter decreasing until summer and starting to increase in autumn (**Figure 4**).

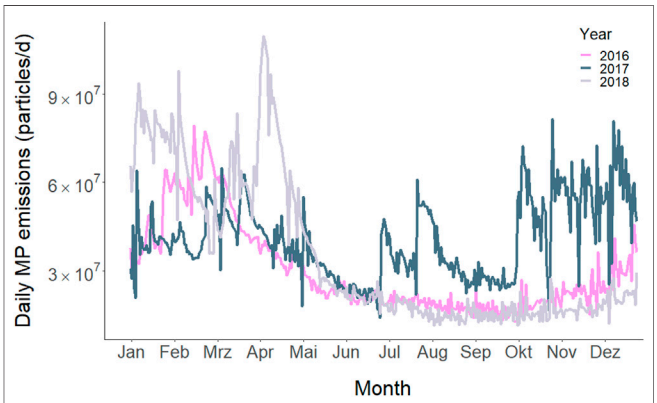

FIGURE 4 | Daily microplastic (MP) emissions by the Warnow river into the estuary calculated based on a linear relationship (F = 11.04, adj. R^2 = 0.6, p = 0.016; y = 132.41 + 18.62*x) of measured MP abundances in water surface (n = 8) and river discharge (data provided by Staatliches Amt für Landwirtschaft und Umwelt Mittleres Mecklenburg 2019).

Anti-fouling Paint Particle Emissions

For the considered 15 leisure boat facilities we estimated an annual emission of about 370 million AFPPs due to ISU, HPW, and MR. Thereof, 3% were released by presumably 1,625 permanent boats due to ISU during April and September. The majority was released by HPW after the season in November (1,274 boats) and due to MR work from November to March (50 and 47% respectively). Thus, areas where HPW is conducted depict potential spatio-temporal hotspots of AFPP release. Based on our assumptions there were no large differences between western and eastern sites of the estuary considering total AFPP emissions (42 and 58% of AFPP emissions; **Supplementary Information SI6**).

Applying the basic emission scenario for AFPP release during ISU from commercial shipping an annual emission of 4.4 billion AFPPs was estimated. Thereof, around 43 and 40% were released

TABLE 4 | Annual microplastic (MP) emissions and percentage share of different sources and density classes. STW: stormwater, CSO: combined sewer overflows, WWTP: wastewater treatment plant, LD: low density MPs (<1.0 g/cm^3), HD: high density MPs (>1 g/cm^3).

Source	Mean		LD		HD	
	[MPs*10^6/a]	[%]	[MPs*10^6/a]	[%]	[MPs*10^6/a]	[%]
Catchment (corrected)	143,965	49.4	103,655	47.2	40,310	56.3
STW	125,500	43.1	101,655	46.3	23,845	33.3
CSO	17,725	6.1	13,471	6.1	4,254	5.9
WWTP	4,064	1.4	935	0.4	3,130	4.4
Σ	291,254	100	219,716	100	71,539	100

TABLE 5 | Descriptive statistics for microplastic concentrations utilized for emission estimates. Data was obtained from the literature for wastewater treatment plant (WWTP) influents, effluents, and stormwater (STW). Microplastic concentration per m^3. IQR: interquartile range, MAD: median absolute deviation, CV_M: coefficient of variability for median. n: number of microplastic measurements.

	WWTP influent (n = 41)	WWTP effluent (n = 10)	STW (n = 7)	Warnow river (n = 8)
Min	1,440	6	490	57
Max	18,285,000	19,000	22,894	388
Median	143,330	245	1,409	281
MAD	192,204	228	1,363	92
CV_M	134	93	97	33

by tanker and cargo ships, 8% by ferries, and 6% by RoRo ships at the industrial port within the area of Breitling. Another 3% of AFPP emissions were released by cruise ships close to Warnemünde. Besides cruise ships, where the peak season is between May and September, a constant release of AFPPs can be assumed during the whole year for all other commercial ship types. For all scenarios it should be kept in mind that results are based on our assumptions and that the applied emission scenarios were calculated for idealized spherical AFPPs of 0.5 mm diameter (density 1.6 g/cm^3).

Comparison of Emission Estimates and Potential Discharge to the Baltic Sea

A total discharge of about 291 billion MPs were estimated to enter the Warnow estuary annually. Thereof about 1.4% are emitted by the WWTP, 6.1% due to CSO emissions, 49.4% by the catchment of the Warnow river, and 43.1% due to stormwater discharges within the city area (**Table 4**).

Microplastic concentrations of WWTP influents (influencing CSO emission estimates), effluents and within STW, obtained as median value considering several studies, show highest variability from 93 to 134% respectively. Microplastic concentrations for the Warnow river catchment, exhibit considerably lower variability (CV_M: 33%; **Table 5**).

Microplastics detected in the Warnow river samples were comprised of 72% LD and 28% HD polymers, with only small differences between sampling campaigns (**Supplementary Information SI4**). Low density polymers are also dominating in MP reports of stormwater runoff and combined sewer overflows. For stormwater runoff LD and HD polymers contribute to 81 and 19% respectively (Liu et al., 2019). Considering the mixing ratio of 11.68:1 for storm- and raw wastewater of the combined sewer system, a share of 76% for LD and 24% for HD polymers was calculated for CSO emissions (**Supplementary Information SI1**). For the MP composition within the effluent at the WWTP a detailed report on a plant with a tertiary treatment in Germany was utilized (Mintenig et al., 2014). Here a higher share of HD polymers (about 77%) was reported, which is in concordance with other reports (Sun et al., 2019).

In total, 75% of MPs released to the estuary belong to the class of LD-MPs and 25% to the class of HD-MPs (**Table 4**). Under the assumption that HD-MPs and 31% of LD-MPs are retained within the estuary (see *Materials and Methods* 2.5), about 152 billion MPs would be released into the Baltic Sea annually.

DISCUSSION

Microplastic Emission Estimates Into the Estuary

Considering all limitations of our emission scenarios, about 291 billion MPs potentially enter the estuary annually. Thereby, the outlet of the WWTP has the lowest percentage of MP emissions (1.4%, **Table 4**), which is in line with previous studies (Simon et al., 2018; Conley et al., 2019). Calculated MP emissions due to CSOs (6.1%) are about five times higher and stormwater runoff from the city area of Rostock exhibits the highest percentage (43.1%) among sewer system emissions. The higher the retention of a compound within a WWTP, the more relevant are discharges due to CSOs and stormwater (Bollmann et al., 2019), which is in line for MPs with a retention by up to 99.9% for plants with tertiary treatment (Prata, 2018). A general seasonal pattern of MP emissions by WWTP outlets could not be detected by a comprehensive study of Conley et al. (2019). Instead, MP concentrations in effluents were found to vary by a factor of 4.8 over the course of the year. In our scenarios MP discharge by stormwater runoff and CSOs is related to precipitation and as a result MP emission for the Warnow estuary would be highest during summer month (**Supplementary Information SI8**). For stormwater runoff and CSOs other factors than precipitation could likewise influence temporal MP emissions. For example, precipitation pattern as well as deposition time before runoff will influence MP runoff from a surface to discharging natural and culture-technical waterways. Besides, particle retention within waterways will differ, which was not accounted for. In our scenarios data from one study reporting MP emissions separated by different land use categories (Liu et al., 2019) was used for MP emissions by stormwater runoff. Here MP emissions per area within the land use categories can vary over time and region. Our utilized MP values are rather conservative, considering reports of on average 7,713 to 111,000 MP/m^3 (>20 μm) in stormwater (Bondelind et al., 2020). Further, tire and road wear particles which can exhibit a significant share were

TABLE 6 | Parameters for microplastic (MP) emission scenarios for the sewer system. WWTP: wastewater treatment plant, CSO: combined sewer overflow, STW: stormwater, WW: wastewater, TRWP: tire and road wear particles. Data sources for utilized MP concentrations are provided within **Supplementary Table SI1**).

Microplastic emission scenario Parameter	Variable	Value Warnow estuary	Unit
Area city		181.4	km^2
Population density city		1,151	Person/km^2
WWTP emissions [MPs/a]			
= annual processed WW [m^3/a] * MPs [MPs/m^3]			
Annual processed WW		16,588,932	m^3/a
MP concentration effluent		245	MPs/m^3
STW emissions [MPs/a]			
= rainfall runoff [l/a] * MPs [MPs/l]			
rainfall runoff [l/a] = drained area [m^2] * mean annual precipitation [l/m^2/a] * runoff coefficient			
Drained area	Traffic area	20.9	km^2
	Industrial area	19.6	km^2
	Residential area	25.1	km^2
	Unsealed area	104.2	km^2
Mean annual precipitation		621	l/m^2
Runoff coefficient	Traffic area	0.86	
	Industrial area	0.68	
	Residential area	0.79	
	Unsealed area	0.14	
MP concentration (*TRWPs not included*)	Traffic area	494	MPs/m^3
	Industrial area	13,164	MPs/m^3
	Residential area	898	MPs/m^3
CSO Emissions [MPs/a]			
= discharge raw WW [m^3/a] * MPs in raw WW [MPs/m^3]			
+ ∑(area specific overflow [m^3/a] * area specific MPs [MPs/m^3])			
discharge raw WW = discharge CSO [m^3/a] * 1/12			
discharge STW = discharge CSO [m^3/a] * 11/12			
discharge CSO [m^3/a] = permissible overflow rate [%] * rainfall runoff [m^3/a]			
area specific overflow [m^3/a] = % share of discharge STW			
Permissiable overflow rate		34	%
Drained area	Traffic area	2.6	km^2
	Industrial area	1.0	km^2
	Residential area	3.7	km^2
	Unsealed area	4.3	km^2
Mixing ratio STW: Raw WW		11.68:1	
MP concentration raw WW		143,000	MPs/m^3

not included in the study by Liu et al. (2019) and are accordingly not represented by our results. When comparing MP emissions among sources the general high variability of MP concentrations has to be taken into account. In our scenarios utilized MP concentrations for raw wastewater (influencing CSO emission estimates) and WWTP effluent were highly variable (CV_M: 134 and 93%) (**Table 5**). But even an increase of 93% of MP emissions for WWTP effluents and 134% for raw wastewater would not notably change the high share of stormwater and river catchment emissions which would still contribute with about 40 and 45% respectively.

Another relevant share of MP is very likely emitted by the catchment of the Warnow river (49.4%; **Table 4**) which discharges about 95% of river water into the estuary (Bachor 2005). The Warnow river discharge varied about 78% within the considered three years with a recorded minimum and maximum values of 0.03 and 60.97 m^3/s respectively. Our *in-situ* measurements only covered river discharge values up to about 15 m^3/s (**Supplementary Information SI5**). Due to high variability of MP concentrations for low flow conditions and a small number of measurements the C-Q relationship is not very robust and needs further validation. But in contrast to precipitation patterns in the city and thus MP discharge through stormwater runoff, highest mean annual discharge of the Warnow river is occurring during winter month (Pegelportal, 2020). An accordingly assumed higher probability of MP emissions agrees with predicted peak MP emissions for the northern hemisphere between January and April (Lebreton et al., 2017).

While our AFPP emission estimates are associated with highest uncertainty and provide qualitative rather than comparable quantitative information, they indicate the importance of this source for the estuary. In-service use (ISU) emissions by commercial shipping for example would result in an annual release of about 4.4 billion AFPPs. Total emissions of AFPP by leisure boats add to another 370 million AFPP according to our scenarios, which represent conservative estimates. Here it needs to be considered that the applied removal and emission factors for AFPPs were provided by experts over a decade ago and that their present-day validity needs further investigation. For comparison, an additional scenario was calculated based on the annual sales

volume of AFP of 794 tons for leisure boats in Germany (and an emission factor of 50% during ISU (Daehne et al., 2017)). Considering the share of berth in the Warnow estuary of 0.79% and transferring this number to our scenario this would amount to about 3.7 billion AFPPs per year (**Supplementary Information SI6**). The example shows the high uncertainties associated with different assumptions. It should further be noted that a single estimate on plastic polymer content of a typical AFPP was used. Common commercial and leisure boat antifouling paint systems span a wide range of compositions, including several polymers in varying amounts as well as non-polymer-based matrices. Nevertheless, our estimates are again conservative considering the applied relatively large particle diameter of 0.5 mm. For example, if adjusting particle diameter to 0.1 mm the total release of AFPPs drastically increases to about 594 billion AFPPs per year. Although our AFPP emission scenarios only provide a very rough first estimation of this source, they are likewise able to provide spatial information on AFPP emissions (**Supplementary Figure SI6**).

Retention of Microplastics and Emission Potential to the Baltic Sea

According to our assumptions an estimated 152 to 291 billion MPs larger than 10 µm (majority <100 µm) are annually discharged from the Warnow estuary to the Baltic Sea.

Currently there is only limited information on MP retention within rivers and no comparable data for estuarine systems to the best of our knowledge. Besides particle characteristics (density, shape, size) and processes altering particle density (Rummel et al., 2017; Michels et al., 2018), the hydrology, morphology, and management of the estuary are important factors for MP retention.

For our study system there are several factors promoting the retention of MPs within the estuary. Deposited and/or sinking polymers are subjected to a predominating upward transport with higher saline bottom water under the general estuarine circulation pattern (Lange et al., 2020). Further river transport inland is prevented by a weir and in the city area close to the weir deposition of particles is promoted by decreased flow velocities (Rönspieß et al., 2020). In addition, floating MPs can be trapped within reed belts or retained due to deposition at riverbanks within the estuary. On the other hand, it has been reported that high amounts of MPs can be mobilized during storm events (Hurley et al., 2018; Hitchcock, 2020). Nevertheless, frequent dredging activities within the estuary to sustain the depth of shipping channels further indicate a net accumulation of particulate matter within the estuary.

Although there is a high probability that the HD-MPs and part of the LD-MPs are retained within the estuary, MPs <10 µm are currently not considered which would additionally contribute to MP emission numbers. Moreover, diffuse emission through for example aerial inputs or fragmentation of macroplastic were not accounted for within the current MP emission scenarios. Thus, the calculated 152 to 291 billion MPs entering the Baltic Sea annually provide a first reasonable estimate.

Besides, comparing our predicted MP emission estimates to the Baltic Sea with literature data yields a rather conservative estimate. For example, Zhao et al. (2019) estimated an annual discharge of 16–20 trillion MPs (>60 µm) from the Changjiang estuary to the ocean. Another study measuring a comparable size class of MPs (>20 µm) in the Nakdong River in South Korea report an annual discharge of 5.4–11 trillion MPs to the ocean (Eo et al., 2019). Dividing the values by the population of the Nakdong catchment results in an emission of 0.5–1.1 million MPs per capita per year which is in a comparable order of magnitude as compared to our calculation (388–746 thousand MPs per capita per year).

Practical Implications of Applied Emission Scenarios

To estimate and compare sewer system related MP emissions from the city area information on land use categories of the drained area in combination with corresponding runoff coefficients, MP concentrations, and amount of precipitation were combined (**Table 6**). With the applied routing-method (**Supplementary Information SI3**) spatially differentiated MP emission estimates by stormwater could be provided. The approach is transferable to other areas, provided that a coherent, hierarchical network of watercourses with associated subcatchments is available.

Generally, precipitation seems to be a suitable proxy for temporal emissions of MPs from impervious surfaces. This is supported by a recent study, reporting a positive relationship between MP concentrations in an estuary and the average rainfall across previous five days (Hitchcock, 2020). For MP emission estimates through CSOs a permissible overflow rate was calculated based on German water management guidelines. In combination with the mixing ratio of discharged stormwater and raw wastewater (mixing ratio 11.68:1) comparable MP emissions could be calculated. Baresel and Olshammar (2019) calculated annual CSO by assuming a fraction of 1.5% of total WWTP inflows and assessed MP emissions by WWTPs and different CSO types. Results showed that weather induced CSO contributed to at least 18% to >50% of MP emissions to the Baltic Sea. Transferring this approach to the sewer system in Rostock would result in about two times higher annual inputs (37 billion MPs per year). In a study additionally considering stormwater MP emissions into the Baltic Sea, CSO emissions had the lowest contribution of MP discharge (Bollmann et al., 2019). And in contrast to our results, high amounts of MP were emitted by treated wastewater (WWTPs). The assumed high amount of treated wastewater discharge (about four times higher than untreated stormwater discharge) and a lower runoff coefficient for impervious surfaces by Bollmann et al. (2019) can explain the discrepancy. Stormwater discharge was about 2.5 times higher than processed wastewater discharge in our study. Moreover, area-specific runoff coefficients were utilized (**Table 6**) which is, considering potential local adaptions, recommended for future studies. Microplastic emissions are

generally controlled by anthropogenic activities (Zhao et al., 2020). As those activities differ among land use, MP emissions should further be differentiated according to specific land use categories. The relevance of unsealed areas is still uncertain and despite the low runoff coefficient, meaning lower MP discharge, those areas contribute to the highest share, (e.g. ~60% in the city area). However, for forst and agricultural soil a higher storage capacity as compared to urban soils has been postulated (Nizzetto et al., 2016) which needs further validation.

For the city area the calculated load of MPs per area would amount to about two billion MPs/km^2/a accounting for traffic, industrial, and residential areas. For the Warnow catchment 3% (98 km^2) of land use is urban area (Bauwe et al., 2019). Assuming a comparable share of catchment classes and population density this would amount to a calculated MP emission of about 200 billion MPs/a. At the weir MP concentration of about 144 billion MPs/a were estimated which is 28% less. This approximation is indeed very rough. But with knowledge on the share of catchment classes of urban areas and more robust data on MP concentration within the according runoff one could approximate retention within rivers.

To estimate river (micro) plastic emissions often mean or median (micro)plastic concentrations in conjunction with mean river discharge have been used (Lebreton et al., 2017; Atwood et al., 2019; Schöneich-Argent et al., 2020). Increased emissions of MPs with increased river discharge has been reported (Wagner et al., 2019) and for the rural Warnow river catchment we observed a significant linear C-Q relationship ($p < 0.05$). Opposed to our results, a C-Q relationship for the rural subcatchment in the study of Wagner et al. (2019) was not detected. The different results can be explained by different MP sampling and analysis techniques which were confined to three polymer types and a lower size limit of 0.5 mm (Wagner et al., 2019). Besides, varying characteristics of rivers and their catchment areas could lead to different results and thus, it needs further investigations on the transferability of C-Q relationships to other systems. A semi-log regression model exhibited a similar model fit (**Supplementary Information SI5**) but additional data covering a wider range of river discharge values would be needed to precisely describe the relationship between MP concentration and river discharge. An exponential or logarithmic increase of MP concentrations with river discharge seems likewise plausible. Higher flow velocities caused by higher discharge or floods can lead to a remobilization of MP within riverbed sediments (Hurley et al., 2018). In addition, increased flow velocities and water levels within a river course can result in remobilization of plastic litter from riverbanks (van Emmerik et al., 2019). High precipitation and following increased runoff from impervious surfaces of urban areas as well as additional wastewater discharges contribute to an increased MP emission into river systems. Yet, our approach accounts for the uncertainty of the Warnow river discharge which was highly variable among month (highest variability in 2018 from 82% in October to 12% in January). Nevertheless, to obtain information on temporal MP emission patterns we support previous suggestions by Wagner et al. (2019) to establish C-Q relationships as proxy to estimate river catchment plastic emissions. Thereby the whole size fraction of MPs as well as a broad range of discharge conditions should be covered.

Finally, combining information from our emission scenarios three potentially high influenced areas by MP emissions could be identified within the estuary (I) the area behind the weir due to emissions from the catchment and loadings of stormwater runoff from large industrial areas (~18% of emissions from all tributaries) (II) the recreational city harbor receiving MPs from the CSO outlets and potential high inputs of AFPPs through several large leisure boat facilities, and (III) the area of Breitling within the northeastern part of the estuary due to high inputs of stormwater (~51% of emissions from all tributaries) and potential MP emissions from the area of the industrial harbor as well as AFPP emissions from the high share of commercial shipping activities in this area (**Figure 1**).

CONCLUSION

Based on our combined approach major MP emissions into the Warnow estuary occur in the following order: stormwater and river catchment > CSO > WWTP. Keeping in mind differences among emission scenarios it still can be assumed that MP emissions by the city sewer system are likely as important as accumulated MP emissions by the catchment of the Warnow river. Furthermore, increased MP emissions from the river catchment probably occur during winter driven by higher water discharge, whereas higher MP emissions from the city sewer system are expected through higher precipitation during summer. Calculated annual MP discharges to the Baltic Sea ranged between 152 and 291 billion MPs whereas most uncertainty results from catchment emission scenarios as well as retention of MP particles within the estuary. A comparison of our MP emission estimates from the city sewer system with existing studies highlights the necessity to consider all components of a sewer system to gain a comprehensive knowledge on the importance of various MP sources and pathways.

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

SP and GS prepared the concept of the paper. All authors made substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.616765/full#supplementary-material.

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Ingestion and Depuration of Microplastics by a Planktivorous Coral Reef Fish, *Pomacentrus amboinensis*

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Microplastics are ubiquitous contaminants in marine environments and organisms. Concerns about potential impacts on marine organisms are usually associated with uptake of microplastics, especially via ingestion. This study used environmentally relevant exposure conditions to investigate microplastic ingestion and depuration kinetics of the planktivorous damselfish, *Pomacentrus amboinensis*. Irregular shaped blue polypropylene (PP) particles (longest length 125–250 μm), and regular shaped blue polyester (PET) fibers (length 600–700 μm) were selected based on physical and chemical characteristics of microplastics commonly reported in the marine environment, including in coral reef ecosystems. Individual adult damselfish were exposed to a single dose of PP particles and PET fibers at concentrations reported for waters of the Great Barrier Reef (i.e., environmentally relevant concentrations, ERC), or future projected higher concentrations (10x ERC, 100x ERC). Measured microplastic concentrations were similar to their nominal values, confirming that PP particles and PET fibers were present at the desired concentrations and available for ingestion by individual damselfish. Throughout the 128-h depuration period, the 88 experimental fish were sampled 2, 4, 8, 16, 32, 64, and 128-h post microplastic exposure and their gastrointestinal tracts (GIT) analyzed for ingested microplastics. While damselfish ingested both experimental microplastics at all concentrations, body burden, and depuration rates of PET fibers were significantly larger and longer, respectively, compared to PP particles. For both microplastic types, exposure to higher concentrations led to an increase in body burden and lower depuration rates. These findings confirm ingestion of PP particles and PET fibers by *P. amboinensis* and demonstrate for the first time the influence of microplastic characteristics and concentrations on body burden and depuration rates. Finally, despite measures put in place to prevent contamination, extraneous microplastics were recovered from experimental fish, highlighting the challenge to completely eliminate contamination in microplastic exposure studies. These results are critical to inform and continuously improve protocols for future microplastics research, and to elucidate patterns of microplastic contamination and associated risks in marine organisms.

Keywords: polypropylene, polyester, particle, fiber, marine environment, uptake, impact, damselfish

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INTRODUCTION

Contamination of the marine environment with microplastics (plastic items 1 μm–5 mm in length) is prevalent, with up to 51 trillion floating microplastics estimated to occur in this environment globally (van Sebille et al., 2015). This staggering amount is also predicted to significantly increase if the global community does not address plastic production, use, reuse, and disposal management (Jambeck et al., 2015; Everaert et al., 2020). Under a "business as usual scenario," concentrations of marine microplastic contamination are estimated to increase up to 10 times by 2100 (Everaert et al., 2020).

Microplastics are frequently found contaminating marine organisms (Halstead et al., 2018; Kroon F. J. et al., 2018; Qu et al., 2018), and may disrupt physiological processes resulting in, for example, cellular stress (Jeong et al., 2016; Espinosa et al., 2017), and energy (Welden and Cowie, 2016; Lo and Chan, 2018) and hormonal (Zhao et al., 2020) imbalances. As a consequence, microplastics and their associated impacts could ultimately affect marine organisms by changing growth, reproduction, and/or mortality of individuals (Liu et al., 2020). Reports of microplastic-related risks for marine organisms have, for the most part, been associated with their uptake, and specifically direct ingestion of microplastic items (GESAMP, 2019). Other pathways, however, such as passive uptake through the gills (Bour et al., 2020a) or via trophic transfer from prey items (Santana et al., 2017; Miller et al., 2020) have been demonstrated in controlled laboratory experiments. Thus, similar to other contaminants (Blanco et al., 2018; Amoroso et al., 2020; Hassell et al., 2020), depuration is a major factor influencing the potential effects of microplastics following ingestion. Microplastic depuration alters the amount of contamination present within the gastrointestinal tract (GIT) of organisms over time, thereby influencing toxicity, vectorization of additives and sorbed chemicals, and the likelihood of trophic transfer (Dawson et al., 2018; Bour et al., 2020b). Hence, a better understanding of microplastic ingestion and depuration kinetics of marine organisms may help elucidate risks posed by this contaminant.

Microplastic ingestion and depuration kinetics have not been rigorously evaluated in marine organisms under controlled exposures, and rarely reflect environmentally relevant exposure characteristics, such as microplastic polymer composition, shape, size, color, and concentration (Lu et al., 2016; Cong et al., 2019; **Table 1**). For example, polystyrene (PS) beads represent the most common microplastic evaluated in controlled ingestion and depuration studies, yet microfibers and irregular microparticles comprising of other polymers, such as polyester (PET), polyethylene (PE), and polypropylene (PP) are more abundant in marine environments (Coyle et al., 2020). Furthermore, experimental microplastic concentrations are generally much higher than reported environmental concentrations (Xu S. et al., 2020). In terms of suitable species, controlled exposure studies specifically examining ingestion and depuration kinetics rarely consider organisms likely exposed to and contaminated with microplastics in the marine environment. For example, ingestion and depuration studies have mainly focused on aquatic invertebrates (Wang X. et al., 2019; Chae and An, 2020; Ehlers et al., 2020), and freshwater fish (Grigorakis et al., 2017; Xiong et al., 2019; Hoang and Felix-Kim, 2020). In contrast, only four ingestion and depuration studies have been conducted on brackish/marine fish (Manabe et al., 2011; Cong et al., 2019; Bour et al., 2020b) despite these being some of the most frequently reported organisms contaminated with microplastics (Lusher, 2015; Kroon F. J. et al., 2018; Jensen et al., 2019). Hence, there is a major shortcoming in the current literature limiting our understanding of microplastic ingestion and depuration kinetics in marine organisms. A more comprehensive exploration of environmentally relevant exposure conditions on a broader range of organisms in the marine environment is warranted (Bour et al., 2020b).

In this controlled exposure study, we investigated microplastic ingestion, body burden and depuration kinetics of the planktivorous damselfish *Pomacentrus amboinensis*. Adults of this species, common to shallow Indo-Pacific coral reefs, actively feed on food particles carried on water currents (McCormick and Weaver, 2012), playing a key role in transferring energy from plankton up the food web (Emslie et al., 2019). Microplastic contamination in adult *P. amboinensis* was common among individuals collected on reefs in the central Great Barrier Reef, Australia (Jensen et al., 2019), making this a relevant species for studies on microplastic impacts resulting from ingestion. Here, adult ambon damselfish were exposed once to environmentally relevant types (irregular shaped blue PP particles and regular shaped blue PET fibers) and concentrations (ERC) of microplastics, based on characteristics or estimations of microplastics found in sea surface waters (Cole et al., 2014; Cozar et al., 2014; Abayomi et al., 2017; Kanhai et al., 2017; Syakti et al., 2017), including at the Great Barrier Reef (Jensen et al., 2019; Everaert et al., 2020), and Lizard Island (Santana et al., unpublished data). Dose response was also assessed by exposing the damselfish to a range of microplastic concentrations (Critchell and Hoogenboom, 2018), including future scenarios of marine microplastic contamination, i.e., 10X ERC and 100X ERC – extending concentrations beyond 2100 predictions (Everaert et al., 2020). To elucidate ingestion and depuration kinetics, ambon damselfish were sampled incrementally at 2, 4, 8, 16, 32, 64, and 128-h post microplastic exposure and GITs analyzed for ingested microplastic body burden. Finally, unintended sample contamination with extraneous microplastics, generally not monitored or reported in the current literature, was determined during the controlled exposure and/or sample processing. Our findings on the residence time of these items within a marine fish and under different exposure scenarios of exposure contribute to improved understanding of the potential ecological risks posed by microplastic contamination in marine environments.

MATERIALS AND METHODS

Study Area

Fish collection and the controlled exposure experiment were conducted at the Australian Museum's Lizard Island Research

TABLE 1 | Summary of microplastic ingestion and depuration studies conducted on aquatic species.

Taxa	Species	Life stage	Environment	Polymer	Shape	Size	Color	Concentration	References
Fish	*Pimephales promelas*	larval	freshwater	PE	S	63–75 μm and 125–150 μm	green	25 mg L^{-1} and 50 mg mps L^{-1} (145,343 mps L^{-1} and 290,686 mps L^{-1} for 63–75 mm mps or 18,367 mps L^{-1} and 36,734 mps L^{-1} for 125–150 mm mps)	Hoang and Felix-Kim (2020)
	Carassius auratus	n/a		PE	Fg, Fb, Fl	3–5 mm, 2–3 mm, and 0.5–2 mm	white, transparent, and cyan	100 mps L^{-1}	Xiong et al. (2019)
		adult		PE, PET	S, Fb	50–500 μm and ≥63 μm	n/a	50 mps food $pellet^{-1}$	Grigorakis et al. (2017)
	Oryzias melastigma	larval and adults	brackish	PS	S	10 μm	green	1×10^5 mps L^{-1} (each 30 larvae)	Cong et al. (2019)
	Oryzias latipes	embryos and larvae		latex	S	50 and 500 nm	n/a	10 mg L^{-1} in embryo culture medium	Manabe et al. (2011)
	Gasterosteus aculeatus	n/a	freshwater and marine	PE, PET	S, Fb	27–32 and 500 μm	blue and black	100,000 mps L^{-1} (1:1 per plastic type)	Bour et al. (2020b)
	Seriolella violacea	juvenile	marine	nylon	tubular	1.2 ± 0.2 mm (length), 1.0 ± 0.1 mm (width)	black, blue, translucent, and yellow	10 food pellets and 2 microplastics	Ory et al. (2018)
	Pomacentrus amboinensis	larval		PS	S	200–300 μm	transparent	167 mps L^{-1} (each 10 larvae)	McCormick et al. (2020)
Snail	*Achatina fulica*	"growing period"	terrestrial	PET	Fb	1257.8 μm (length) and 76.3 μm (width)	n/a	0.01–0.71 g kg^{-1} (dry soil) (6.4% of fodder/lettuce mass rate)	Song et al. (2019)
	Radix balthica	adult	freshwater	PS, polyacrylic wool	Fg, Fb	up to 200 μm; 30 and 2,000 μm	blue and green	15% (fragment or fiber) of available biofilm (4.24 g mps/biofilm)	Ehlers et al. (2020)
Frog	*Xenopus tropicalis*	tadpole	freshwater	PS	S	1 and 10 μm	green	10^3 mps mL^{-1}	Hu et al. (2016)
Zooplankton	*Hyalella azteca*	adult	freshwater	PE, PP	Fg, Fb	10–27 μm and 20–75 μm	blue	0–10^4 mps mL^{-1} (acute) and 0–20 × 10^3 mps mL^{-1} (chronic); 0–90 mps mL^{-1}	Au et al. (2015)
	Daphnia magna	adult	freshwater	PE	S, Fg	10–106 μm and 10–75 μm	white and black	10^{-4}–10 g L^{-1}	Frydkjaer et al. (2017)
		juvenile	freshwater	PS	S	2 μm and 100 nm	n/a	1 mg L^{-1}	Rist et al. (2017)
	Artemia sp.	larval	marine	PS	S	10 μm		10^3 mps mL^{-1}	Wang Y. et al. (2019)
	various	various		PS	S, Fg	15 and 30 μm	green	50–200 microplastics mL^{-1}	Elizalde-Velazquez et al. (2020)
Crab	*Carcinus maenas*	n/a	marine	PS	S	8–10 μm	n/a	n/a (gives nominal concentration of mussel exposure but not concentration in contaminated mussels)	Watts et al. (2014)
Bivalve	*Mytilus galloprovincialis*	adult	marine	PE	S	180–212 μm (203.84 ± 13.76 μm)	n/a	10 mg L^{-1} (2 × 10^3 particles L^{-1})	Chae and An (2020)
		adult	marine	PS	S	2, 6, and 10 μm	yellow-green, red	10 and 1000 mps mL^{-1}	Goncalves et al. (2019)
		adult	marine	HDPE	Fg	up to 22 μm, mean of 4–6 μm	n/a	3 mg L^{-1}	Fernandez and Albentosa (2019a)
		adult	marine	HDPE	Fg	up to 22 μm, mean of 4–6 μm	n/a	3 mg L^{-1}	Fernandez and Albentosa (2019b)
	Mytilus edulis	adult	marine	PET	Fb	459 ± 2.25 μm	pink	up to 30 mps mL^{-1} (0.374% of available seston)	Woods et al. (2018)
		adult	marine	PS	S	49.1 ± 1.3 μm	black	5 mps L^{-1} and 100 mps L^{-1}	Rist et al. (2019)
	Magallana gigas	adult	marine	PS	Fg	100, 250, and 500 μm	orange	60 mps L^{-1} (30 mps per size)	Graham et al. (2019)
Sea urchin	*Tripneustes gratilla*	larval	marine	PE	S	10–45 μm, majority 25–32 μm	green	approx. 500 spheres mL^{-1} (for retention study)	Kaposi et al. (2014)

PE, polyethylene; PS, polystyrene; PET, polyethylene terephthalate (also polyester); PP, polypropylene; HDPE, high density polyethylene; S, sphere; Fg, fragment; Fb, fiber; Fl, film. Sizes reported as one unique size, and as a size range (either min. and max., or average ± standard deviation). Only laboratory studies reporting on microplastic ingestion and depuration kinetics were considered. Note that the number of rows in this table does not correspond to the total number of studies published on the topic because studies reporting on more than one species are included multiple times.

Station (LIRS). Lizard Island (14°40′08″S 145°27′34″E) is a mid-shelf reef located in the northern area of the Great Barrier Reef World Heritage Area (GBR WHA). The reef system is situated approximately 30 km northeast from the Australian continent and 250 km north from the largest city in the region (Cairns; population ∼151,000). Despite its relatively remote location, there is potential for microplastic contamination coral reefs and in reef fish of Lizard Island based on recent studies reporting microplastic contamination from surface waters nearby (Reisser et al., 2013; Hall et al., 2015; Jensen et al., 2019) and from reef fish collected at Lizard Island (Kroon F. J. et al., 2018; Jensen et al., 2019).

Fish Collection and Husbandry

The fish collection and experiment were performed in accordance with relevant institutional and national guidelines and regulations (Great Barrier Reef Marine Park Authority permit G12/35236.1 and James Cook University Animal Ethics Committee Approval Number A2635). In total, 92 adult *P. amboinensis* were captured on SCUBA using fence and dip nets, and temporarily immobilized using a diluted solution containing clove oil (Kroon, 2015). Immediately following collection, four of the 92 fish were individually placed in resealable zip lock plastic bags and euthanized with an overdose of clove oil to establish the background level of microplastics in the study species. These four fish were processed as per laboratory fish (refer to "Quantification of Ingested Microplastics") and GITs analyzed for putative microplastics (refer to "Preventing and Monitoring Contamination"). A sample of these plastic bags was included in the customized contaminant library to monitor unintended sample contamination with extraneous microplastics (refer to "Preventing and Monitoring Contamination"). The remaining 88 fish were transported to LIRS, placed in individual 12 L transparent polystyrene (PS) tanks (34 cm × 20 cm × 21 cm) with PP lids, and given 3–6 days to recover and acclimate prior to microplastic exposure. The use of plastic tanks and lids was due to logistical, financial, and safety risks of shipping glass aquaria via road and sea for experimental use.

The 88 experimental tanks were located in two enclosed and inter-connected laboratory rooms at LIRS, with restricted access throughout the duration of the study. Both rooms and all tanks were thoroughly cleaned with fresh bore water prior the experiment. After cleaning, tanks were air dried overnight, subsequently filled with filtered (50 μm; Puretec®, PP Series Pleated Sediment Cartridge) natural seawater from the Lizard Island lagoon and left in flow-through mode for 48 h prior to introducing the fish. Once individual fish were introduced, tanks were operated in flow-through mode with a complete tank turnover of 1.5 L h^{-1} to ensure good water quality and adequate aeration. The room temperature was maintained at 24°C and fish were subjected to a 12:12 h artificial light:dark cycle. Basic seawater physical (temperature, T; pH, dissolved oxygen, DO) and chemical (ammonia, NH_3; nitrate, NO_3) parameters were monitored with a HACH 40 portable multi-parameter meter (T, 0.1°C; DO, 0.01 mg L^{-1}), Fisherbrand™ strips (pH, 0.1) and Aquasonic test kits (NH_3, 0.1 ppm; NO_3, 5 ppm), respectively. Measurements were taken either directly from the tank (T and DO) or from a subsample taken with a syringe (pH and chemical parameters) every second day during the acclimation, exposure, and depuration periods in at least 30% of tanks using a random number generator each time. During the 128 h depuration period, the random number generator was only applied to those tanks that still contained fish.

Throughout the study, fish were fed with an equivalent of 1.25% of the average adult *P. amboinensis* biomass, adapted from Critchell and Hoogenboom (2018). Food comprised of 125–250 μm irregular shaped commercial food pellets (INVE Aquaculture; proteins min. 55%, lipids min. 9%, and natural fibrous materials max. 1.9%), and post-hatched artemia (500–800 μm) reared from a frozen artemia culture at LIRS. Food items were of similar size dimensions to experimental microplastics (**Figure 1**). Individual fish were considered acclimated when observed consuming the food pellets and artemia.

Experimental Microplastics

The following two experimental microplastics were used: irregular shaped blue secondary PP particles (longest length 125–250 μm), and regular shaped blue secondary PET fibers (length 600–700 μm) (**Figure 1**). Both microplastics were artificially produced at the Australian Institute of Marine Science (AIMS) laboratories in Townsville, QLD, Australia. The PP particles were sourced from 15 mL falcon tube lids (Greiner), milled with a commercial blender (up to 10,000 RPM), and dry sieved through two stainless steel laboratory test sieves (Endecotts) of 125 and 250 μm aperture sizes. The PET fibers were sourced from sewing thread (Gütermann, CA 02776), cut with sterile surgical blades (Paramount, BS EN 27740), and sized using calipers (Kincrome, 1/1000 in). The chemical composition of both PP particles and PET fibers were confirmed by Fourier transform infrared spectroscopy (FTIR) (**Supplementary Information, SI**).

To simulate microplastics found in the marine environment, PP particles, and PET fibers were biofouled at AIMS using a method modified from Kulcsár (2019). Briefly, both experimental microplastics were loosely packed into 85 mL opaque cartridges

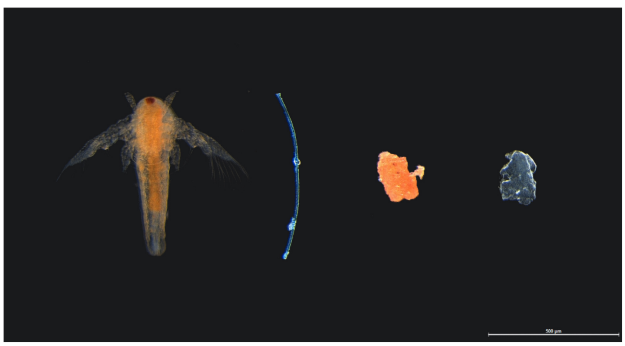

FIGURE 1 | Comparable shapes and sizes of food and experimental microplastics given to adult damselfish *Pomacentrus amboinensis* in a controlled laboratory experiment. Food included orange post-hatched artemia (far left) and orange food pellet (second right). Experimental microplastics included blue polyester fiber (2nd left) and blue polypropylene particle (far right), with photo taken before biofouling of microplastics.

(Telos-Kinesis) (one polymer type per cartridge) each capped with a 263 μm stainless steel mesh on the outflow. Both cartridges were then connected to an overflow from a 2500 L flow-through seawater system inhabited by coral reef organisms including invertebrates (e.g., corals, sea urchins, and sea stars) and fish. To facilitate biofouling of experimental microplastics, cartridges were exposed to natural day:night conditions and ambient temperature for 7 days. Ambient seawater temperature in the cartridges was maintained by immersing both cartridges in a 13 L seawater tank connected to the same flow-through seawater system used to supply seawater for the cartridges. After 7 days, biofouled PP particles and PET fibers were hand picked and individually transferred into 20 mL scintillation vials containing 15 mL filtered (0.45 μm) natural seawater and individual doses of the three concentrations (i.e., ERC, 10x ERC, and 100x ERC) were prepared. ERC was based on monitored (0.04–0.48 m^{-3}) (Jensen et al., 2019) and modeled (0.01–0.02 microplastic/m^2) (Everaert et al., 2020) microplastic concentrations in sea surface of the Great Barrier Reef Region. Nominal microplastic concentrations were manually prepared at 1 PP particle and 1 PET fiber per 12 L [37 (h) × 22 (d) cm] tank (ERC), 10x ERCs at 10 PP particles and 10 PET fibers per 12 L tank, and 100x ERCs at 100 PP particles and 100 PET fibers per 12 L tank (**Table 2**). Prepared microplastic doses were kept at room temperature and exposed to an artificial 12:12 light:dark cycle for a maximum of 15 days prior to use. On the day of the exposure, standard concentrations of food pellets and cultured artemia (see section "Fish Collection and Husbandry") were added to each vial, and to four control vials containing 15 mL filtered (0.45 μm) natural seawater. Each vial was shaken and their entire contents transferred to the designated tank. The vials were rinsed liberally with filtered natural seawater to ensure all contents were transferred.

Experimental Design and Conduct

Individual fish were acclimated for 3–6 days and fed twice daily as described above. Following acclimation, each of the 88 tanks were assigned randomly to one of four treatment groups and seven sampling periods (customized random generator script, RStudio version 1.1.463). Pre-prepared microplastic doses were added to the three exposure treatments: ERC (n = 28 tanks), 10x ERC (n = 28), and 100x ERC (n = 28) (**Table 2**). No experimental microplastics were added to the control group (n = 4). Space restrictions within the laboratory influenced the design of the experiment and the number of controls, precluding an equivalent number of fish for the control replicates, and the use of tanks without fish to monitor for airborne and seawater contaminants.

Prior to exposure with the experimental microplastics, all fish were starved for 24 h. Starvation was imposed to (1) ensure clear GITs prior to experimental procedure, thereby reducing any uncontrolled variability within and between treatments, and (2) increase the likelihood of microplastic ingestion by increasing the desire/need to feed (Grigorakis et al., 2017; Song et al., 2019). On the day of exposure, individual fish received their normal food as well as their randomly assigned and pre-prepared doses of PP particles and PET fibers simultaneously, as described above. During the first 30 s of microplastic exposure, tanks continued to operate in flow-through mode to abet microplastic and food dispersion in the water column. After 30 s, all tanks were switched from flow-through to circulation mode to increase the likelihood of the fish encountering the microplastics. After 2 h exposure, flow-through mode was resumed, and fish were left to depurate for up to a total of 128 h. During depuration, fish were fed as usual – without additional microplastics – twice daily. Excess food and excretions were removed by siphon daily to avoid reingestion of depurated microplastics. Filters were applied to the seawater outflow to prevent the discharge of microplastics (experimental and extraneous) into the Lizard Island coral reef environment.

TABLE 2 | Nominal (1, 10, or 100 particles or fibers 12 L^{-1}) and measured (mean ± standard deviation) concentrations of microplastics in three treatment groups during single exposure of adult damselfish *Pomacentrus amboinensis* to blue polypropylene (PP) particles and polyester (PET) fibers in our controlled laboratory experiment.

Treatment	PP (particle 12 L^{-1})		PET (fiber 12 L^{-1})	
	Nominal concentration	Measured concentration	Nominal concentration	Measured concentration
ERC	1.0	1.0 ± 0.0	1.0	0.7 ± 0.5
10x ERC	10.0	9.3 ± 0.5	10.0	9.0 ± 0.8
100x ERC	100.0	95.0 ± 2.5	100.0	94.3 ± 1.3

ERC, environmentally relevant concentration. Control treatment was not included as no microplastics were added. Microplastic concentrations were measured in nine 12 L control tanks (n = 3 tanks per ERC treatment; seawater without damselfish).

Quantification of Ingested Microplastics

At 2, 4, 8, 16, 32, 64, and 128-h post exposure, four individual fish randomly chosen from each treatment were removed from their tanks and humanely euthanized using ice slurry. Fish were measured (standard and fork length, 0.1 cm, Kincrome, 1/1000 in), weighed (0.01 g, AND EK-410i), and preserved in 70% ethanol (EtOH) for transport and storage. All control fish (n = 4) were sampled at the first sampling time (2 h) to accurately demonstrate if, during the exposure period, fish were exposed to microplastics other than those intentionally offered (i.e., other than the experimental microplastics).

Individual fish were dissected to remove the entire GIT from the top of the esophagus to the rectum. Individual GITs were weighed (wet weight, w.w., Sartorius TE31025, max 3100 g, d 0.01 g) and subjected to alkaline digestion using a method adapted from Karami et al. (2017) to recover PP particles and PET fibers. Briefly, GIT digestion was conducted with 10% potassium hydroxide (KOH; AR, Fisher, CAS No. 1310-58) at 40°C for 48 h in a ratio of 1:20 of GIT wet weight (g) to volume of KOH (mL). Digested GIT solutions were filtered through 77 and 26 μm stainless steel mesh filters (19 mm diameter) (Schlawinsky, 2020), and rinsed with 70% EtOH to remove fat vestiges (Dawson et al., 2020). Microplastics retained on mesh filters were visually identified, counted and photographed using stereomicroscopy (Leica MZ16A, Leica DFC 500, Leica Application Suite LAS 4.4.0). PP particles and PET fibers were

readily distinguishable from other particulates based on the combinations of shape, size and color.

Microplastic Exposure Validation and Spike-Recovery Tests

To validate the nominal concentrations of experimental microplastics, nine individual 12 L tanks without fish were dosed with biofouled and pre-prepared experimental PP and PET doses (ERC $n = 3$, 10x ERC $n = 3$, and 100x ERC $n = 3$) and left for 30 s in flow-through mode. Each tank was then emptied via a drain over a 40 μm nylon mesh to capture microplastics. Experimental microplastics (PP and PET) retained on mesh filters were visually identified and counted using stereomicroscopy (Zeiss SteREO Discovery.V8).

A spike-recovery test was conducted, to (1) account for impacts of the adapted KOH method on the experimental microplastics, and (2) establish recovery rates for spiked PP particles and PET fibers after the KOH digestion. Specifically, biofouled PP particles and PET fibers at the three concentrations of exposure (ERC, 10x ERC, and 100x ERC) were exposed to 1.5 mL of 10% KOH and processed as above. Three replicates were spiked for each treatment (ERC $n = 3$, 10X ERC $n = 3$, 100X ERC $n = 3$). Following digestion at 40°C for 48 h, samples were filtered, rinsed and the 77 and 26 μm stainless steel mesh filters visually assessed using stereomicroscopy. Recovered spiked microplastics were counted to estimate recovery rates for experimental PP particles and PET fibers after the KOH digestion.

Preventing and Monitoring Contamination

A range of measures were implemented to minimize potential microplastic contamination during room preparation, experimental procedure, and sample processing including fish dissection, and GIT digestion and filtration. As previously stated, all experimental tanks were placed in a closed room and isolated from potential air and waterborne microplastic contaminants (as much as possible) using lids and filters. Throughout the study, and whenever used, equipment and tools were sequentially cleaned with reverse osmosis H_2O, Milli-Q H_2O, 70% EtOH, or a combination of these. Cellulose-based cloths were used to wipe surfaces with 70% EtOH. Prior to use, 10% KOH, and 70% EtOH solutions were filtered to 0.45 μm (Millipore® HA filters). Clothing and lab coats worn during microplastic preparation, dosing and sample processing were made from naturally derived materials (e.g., cotton) to eliminate introducing synthetic fibers and specifically non-experimental PETs. All clothing was also delinted using a lint roller (Scotch-Brite®, 3M) prior to sample handling. Nevertheless, the use of plastic material was unavoidable as described above, including consumables such as zip lock bags for fish collection, and plastic gloves and parafilm during sample processing. To control for these sources of plastic items that could be unintentionally introduced into the experiment, we developed a customized contaminant library following Kroon F. et al. (2018) to enable detection of such extraneous microplastic contamination from either the environment, the experimental procedure, or during sample processing. Briefly, samples of plastic gear used during fish collection (e.g., fish net and zip lock bag), experimental procedures (e.g., exposure tanks and lids, seawater pipeline) and sample processing (e.g., spray bottle and gloves) were included in a customized contaminant library (**Supplementary Table 1**), along with any airborne putative microplastic identified from filtered blank processing controls (i.e., Petri dishes with 20 mL of Milli-Q H_2O). Materials from other equipment, such as probes from the HACH 40 portable multi-parameter meter and the cartridge from the Puretec® filter, were not included to avoid damage to expensive and delicate instruments. All items in the contaminant library were photographed for shape and color characterization and analyzed by FTIR for chemical composition.

To determine the occurrence and potential sources of microplastic contamination throughout the study, all putative microplastics (i.e., non-test PP and PET microplastics) identified in fish GITs (including field, control, and exposed fish) and blank processing controls were physically and chemically characterized following Kroon F. et al. (2018) and Kroon F. J. et al. (2018). Items were tentatively identified as microplastics based on key physical parameters (i.e., size, shape, color) commonly used in the literature (Norén, 2007; Hidalgo-Ruz et al., 2012). All putative microplastics were then analyzed by FTIR to confirm polymer composition using either PerkinElmer Spectrum 100 FTIR [1 mm ATR window, pressure gauge = 150, eight scans at 4 cm^{-1} resolution, wavenumber range between 4000 and 600 cm^{-1}, atmospheric (CO_2/H_2O) suppression, atmospheric vapor compensation, and background scans acquired every 10 acquired spectra] or PerkinElmer Spotlight 200i FT-IR microscope [100 μm ATR aperture, pressure gauge = 5%, 32 scans at 4 cm^{-1} resolution, wavenumber range between 4000 and 600 cm^{-1}, atmospheric (CO_2/H_2O) suppression, atmospheric vapor compensation, and background scans acquired for every acquired spectrum]. FTIR spectra were searched against the NICDOCOM IR spectral libraries (Polymers and Additives, Coatings, Fibers, Dyes and Pigments, Petrochemicals; NICODOM Ltd., Czechia) and the matching polymer type assigned. FTIR spectra of microplastics retrieved from fish GITs were then compared with those in the custom-built contaminant library to identify potential microplastic contamination throughout the study, and infer possible sources of contamination (i.e., due to being inadvertently introduced from the environment, the experimental procedure, or during sample processing).

Data Analyses

Based on the size range of the two experimental microplastics and the limited evidence of microplastic translocation into fish tissue after uptake (Lu et al., 2016; Cong et al., 2019), we define microplastic body burden as the amount of microplastics present in the fish GIT. Microplastics body burden was quantified for PP particles and PET fibers separately and per individual fish GIT analyzed.

To determine whether microplastic body burden over time was affected by microplastic type or concentration, we used a

general linear model (GLM, $p < 0.05$) following the equation:

$$Body\ Burden = Type + Concentration + \text{Time} + Weight + Constant \quad (1)$$

where,

Type = PP particles or PET fibers,

Concentration = ERC, 10x ERC, or 100x ERC,

Time = time of collection (or depuration time),

Weight = fish weight (in g).

Fish weight was included as a covariant in the GLM because body weight is a common variable to be considered in toxicokinetic studies (Hendriks and Heikens, 2001; Lebrun et al., 2014; Miller et al., 2016). Based on the GLM model, effects of microplastic type, concentration of exposure, and depuration period were calculated as:

Plastic type:

$$e^{\text{intercept estimate}} / e^{(\text{intercept estimate} - \text{PETvsPP intercept})} \quad (2)$$

Concentration of exposure:

$$e^{(\text{intercept estimate} + \text{ERCvs10xERC intercept})} / e^{(\text{intercept estimate})} \quad (3)$$

$$e^{(\text{intercept estimate} + \text{ERCvs100xERC intercept})} / e^{(\text{intercept estimate})} \quad (4)$$

Depuration period:

$$\left(1 - \left(log_t^{\log(\text{depuration}) \text{ intercept}} / log_2^{\log(\text{depuration}) \text{ intercept}}\right)\right) \quad (5)$$

where, t = depuration period, ranging from 4 to 128-h.

Although fish had been randomly assigned to the four different treatments and seven sampling periods, fish weight was included in the model to account for potential variation in the amount of microplastics ingested and depurated due to fish size. To accommodate overdispersion resulting from high numbers of zero's in the data set, the model followed a negative binomial distribution linked with log function. This data analysis was conducted with RStudio, version 1.1.463. The four control fish were not included in the GLM as these fish were never exposed to the experimental microplastics (see section "Results").

The microplastics body burden as a function of time was used to estimate the depuration rates and elimination half-life of PP particles and PET fibers at the different exposure concentrations. Depuration rate constants were calculated based on the following first-order kinetics model, assuming that the ratio of microplastic elimination is directly proportional to microplastics concentration in the fish (Newman, 2012):

$$C_t = C_0 e^{-k_e t} \quad (6)$$

where,

C_t = amount of microplastics in the fish GIT at a particular time,

C_0 = initial amount of microplastics in the fish GIT,

k_e = elimination rate constant as number of microplastics per h,

t = time of measured concentration (C_t).

From this model, microplastic elimination half-life was calculated following:

$$t_{1/2} = \ln(2)\, k_e \quad (7)$$

where,

$t_{1/2}$ = microplastic elimination half-life,

k_e = elimination rate constant as number of microplastics per h.

Microplastic depuration rates and elimination half-life were calculated using GraphPad, version 8.4.1. Significant differences among depuration rates were compared using one-way ANOVA and Tukey's multiple comparisons tests. Interaction between experimental microplastics and concentration was considered a factor (with six levels; $p < 0.05$).

RESULTS

Basic Water Quality and Fish Condition

Water quality was measured on acclimation days 1, 2, 3, 4, and 5; on the day prior microplastic exposure (starvation day) and on two depuration days (2 and 4), totaling 132 times in randomly assigned tanks. Variability in seawater T (24.6°C ± 0.7), pH (8.0), and DO (8.19 mg L^{-1} ± 0.1) was negligible. Further, concentrations of NH_3 (0 ppm) and NO_3 (≤5 ppm) were consistently below limits considered harmful to marine fish.

Throughout the study, none of the fish presented any signs of stress and no mortality was recorded. Following establishment in their individual tanks, all 88 fish commenced feeding on the first day of the acclimation period and continued to do so throughout the experiment. Following microplastic exposure, no changes in fish behavior were observed across any of the four treatments.

Microplastic Exposure Validation and Spike-Recovery Tests

Measured concentrations of experimental microplastics in seawater across the three experimental treatments were similar to their nominal values (**Table 2**). This confirmed that irregular shaped blue PP particles and regular shaped blue PET fibers were present in the water at the desired concentrations and available for ingestion by individual damselfish.

No discernible changes in the blue color of spiked particles and fibers were observed by stereomicroscopy. Results from the spike-recovery test showed high efficiency for recovering both experimental microplastics after exposure to 10% KOH at 40°C for 48 h (**Table 3**). Specifically, the mean recovery rates for both PP particles and PET fibers were >85% (SD < 10) in all treatments, except for PP particles in the ERC treatment (67% ± 47).

Microplastic Body Burden

Both PP particles and PET fibers were observed in the GITs of exposed fish, confirming ingestion of experimental microplastics by *P. amboinensis* (**Table 4** and **Supplementary Table 2**). PET fibers from the 100x ERC treatment were occasionally found entangled, sometimes with other organic materials (**Figure 2**).

At the 2-h depuration period, all fish exposed to the future projected concentrations of 10x ERC and 100x ERC contained both experimental microplastic types in their GIT (**Figure 3**). The microplastic body burden for these fish represented 25–98% of the total microplastics offered in these treatments. Of fish exposed to the ERC treatment, two contained both microplastic types, one contained a single microplastic type, and one did not contain either of the microplastics offered. Similar trends were observed at the 4-h depuration time point. Both microplastic types offered were present in all fish exposed to the 10x ERC and 100x ERC concentrations, while fish from the ERC treatment contained only one microplastic type each (one a PET fiber; the other a PP particle). While microplastic body burden was similar at the 2 and 4 h depuration periods, it decreased dramatically following 8 h of depuration. At this time, the experimental microplastics were only observed in individuals exposed to the 100x ERC treatment, at a maximum of 5% of the PP particle dose and 20% of the PET fiber dose. In addition, PP were only isolated from one fish, while PET fibers were still present in all four fish sampled at 8 h depuration. After 16 h, PP particles were completely depurated from all fish across all treatments. In contrast, PET fibers were still detected in two individual fish from the 100x ERC treatment up to and including 128 h, albeit at low abundances (<2% of the body burden at 2 h depuration). Based on the fitted GLM (R^2 = 0.86), mean microplastic body burden was influenced by the type of microplastic offered, the exposure concentration and the depuration period ($p < 0.001$), but not by fish weight ($p = 0.49$) (**Table 5**). Overall, mean body burden for PET fibers was 2.2 times higher than for PP particles (**Figure 3**). Within individual treatments, body burden increased as concentration of exposure increased. Fish exposed to the 10x and 100x ERC treatments contained 8.5 and 91.8 times more experimental microplastics than ERC, respectively. Finally, the most significant change in body burden over time occurred within 8 h, when, considering all concentrations of exposure, the number of experimental microplastics in the GIT dropped 83% from the first sampling time (2 h) (**Table 4**).

Microplastic Depuration Rates and Elimination Half-Life

Depuration rates varied from 0.13 microplastics h^{-1} (PET 100x ERC) to 0.52 microplastics h^{-1} (PP ERC) (**Table 6**), and were significantly influenced by microplastic type and concentration offered [Welch's ANOVA W (5, 72.49) = 41.48, $p < 0.0001$] (**Figure 3**). PP depuration rates were significantly faster than those of PET at all concentrations tested ($p < 0.05$) (**Supplementary Table 3**). However, regardless of microplastic type encountered, depuration rates decreased with increasing concentration. Fish from the 100x ERC treatment had significantly slower depuration rates than fish from the two lower ERC treatments ($p < 0.0001$). The shortest elimination half-life was observed for PP particles at the lowest ERC (1.34 h); the longest was for PET fibers at 100x ERC (5.41 h) (**Table 6**).

TABLE 3 | Percentage (mean ± standard deviation) of microplastic recovery rates of from spike-recovery test in three treatment groups during single exposure of adult damselfish *Pomacentrus amboinensis* to blue polypropylene (PP) particles and polyethylene (PET) fibers in our controlled laboratory experiment.

Treatment	PP (% of particle 12 L^{-1})	PET (% of fiber 12 L^{-1})
ERC (*n* = 3)	66.7 ± 47.1	100.0 ± 0.0
10x ERC (*n* = 3)	93.3 ± 9.3	96.7 ± 4.7
100x ERC (*n* = 3)	88.7 ± 4.9	88.7 ± 5.4

Control treatment was not included as no microplastics were added. Spike-recovery test was done in triplicate per treatment group. ERC, environmentally relevant concentration. Spike-recovery test was conducted with experimental microplastics and KOH only but following the protocol used for sample processing.

Monitoring Contamination

No experimental microplastics (PP particles and PET fibers) were found in the GITs of the four control fish, nor in blank processing controls collected during fish dissection, GIT digestion and filtration, confirming that (cross-)contamination over the course of the study was not evident. On the other hand, extraneous putative microplastics were visually identified in the GITs of all field control, experimental control, and experimental fish (n = 92) and likewise in all blank processing controls (n = 7, one per sampling time) despite best efforts to minimize such contamination. In total, 374 putative microplastics were visually identified across all 92 fish. Forty-four putative microplastics were excluded from further analysis as polymer composition could not be determined due to the poor quality of acquired FTIR spectra. Of the remaining 330 extraneous putative microplastics, 67 matched physical and chemical characteristics of items from the contaminant library (i.e., same shape, color and ≥90% spectral match) and were deemed to have originated from sample processing activities (**Figure 4A**). From these items, the majority was airborne contamination (n = 56), the rest originated from clothing, including the lab coat, and was comprised of cellulose-based items (n = 11). No microplastic contamination from the experimental set up was observed (e.g., tanks, pipelines, and filtration system). The other 263 items did not match physical and chemical characteristics of items from the contaminant library, including equipment such as fish tanks and tank lids, and were deemed to come from experimental procedures (i.e., experimental air, water, or fish food). While it is possible that some of these items came with the fish from the environment, the pre-depuration time of 3–6 days should preclude this.

Physical and chemical characterization of these 263 extraneous putative microplastics revealed that 57 were synthetic plastics, 26 were semi-synthetic plastics (**Figure 4B**), and 100 were naturally derived anthropogenic polymers. For example, many fibers were identified as being cellulose-based but were highly colored with uniform shape and/or were intertwined (i.e., anthropogenic but naturally derived). The remaining 80 items were determined to be naturally derived and a natural origin could not be discounted. Three of the four field control fish contained microplastics in their GITs including both synthetic (e.g., 2 PP, 1 PE, and 1 with both PE and PP) and semi-synthetic (e.g., 2 rayon:PET) items (**Figures 4C,D**). In contrast, none of the four laboratory acclimated control fish contained any synthetic items and only one contained a semi-synthetic item (cotton:rayon fiber). Similarly, the majority of laboratory exposed fish were not contaminated with microplastics (n = 56 of 84;

TABLE 4 | Mean microplastic body burden fish^{-1} (absolute number ± standard deviation; n = 4 fish per treatment and depuration time) in three treatment groups (n = 84 tanks) during a single exposure of adult damselfish *Pomacentrus amboinensis* to blue polypropylene (PP) particles and polyester (PET) fibers in a controlled laboratory experiment.

Depuration times (h)			2	4	8	16	32	64	128
	Treatment	**Microplastic type**							
Microplastic body burden fish^{-1} (mean ± standard deviation)	ERC	PP particle	0.8 ± 0.5	0.8 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		PET fiber	0.5 ± 06	0.8 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	10 x ERC	PP particle	6.8 ± 1.3	3.5 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		PET fiber	7.5 ± 2.4	6.8 ± 1.0	0.0 ± 0.0	0.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	100x ERC	PP particle	55.8 ± 15.0	39.5 ± 10.8	1.3 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		PET fiber	58.5 ± 38.5	65.5 ± 18.8	10.5 ± 7.1	3.3 ± 2.1	1.0 ± 2.0	2.5 ± 4.4	0.8 ± 1.0
Microplastic depuration in relation to first sampling time (2 h) (%)			n/a	67.0	83.0	89.0	92.0	94.0	96.0

Proportional decline in microplastic body burden over the 128 h-depuration period, relative to body burden at 2 h depuration. Proportional decline was estimated across the three treatment groups and two experimental microplastic (n = 84 tanks) following the same microplastic exposure as described above. ERC, environmentally relevant concentrations. Control fish treatment was not included as no microplastics were added. Refer to ***Supplementary Table 2*** *for raw data.*

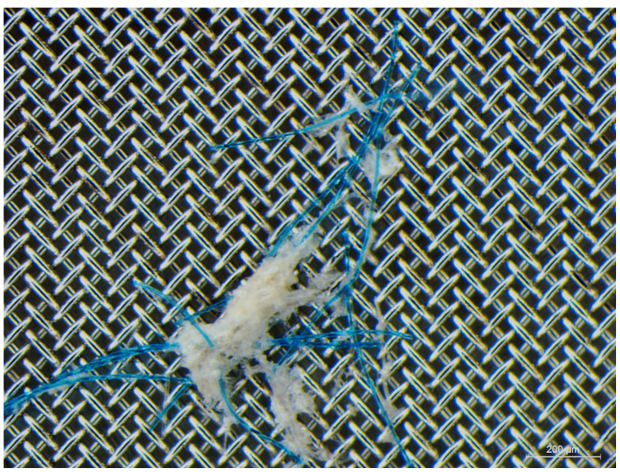

FIGURE 2 | Polyester (PET) fibers, entangled with other ingested materials, recovered from an adult damselfish *Pomacentrus amboinensis*. Gut contents presented were recovered after 2 h of depuration following a single exposure to polypropylene particles and PET fibers at 100x environmentally relevant concentration.

67%). Most of those that were contaminated with extraneous microplastics contained either one (n = 14 fish) or two (n = 9 fish) items, which were predominantly polyacrylate, polyester, and nylon-based synthetic fibers (e.g., cotton:PET, rayon:PET, and rayon:nylon). Nine synthetic items were isolated from a single treated fish, and semi-synthetic items were found in 16 treated fish. Overall, contamination of field, control and treated fish with synthetic and semi-synthetic extraneous items comprised predominantly of fibers (n = 64) rather than particles (n = 19).

DISCUSSION

Ours is one of the first studies to investigate and confirm microplastic ingestion using environmentally relevant exposure conditions in a controlled laboratory experiment (Rochman et al., 2019), and provide strong support for microplastic contamination trends observed in marine organisms collected in the field. Specifically, microplastic ingestion by adult *P. amboinensis,* a planktivorous coral reef fish, occurred regardless of the microplastic polymer type (PP, PET), shape (irregular fragments, regular fibers), size (125–250 μm, 600–700 μm), or exposure concentration (ERC, 10x ERC, 100x ERC). Importantly, our measured microplastic concentrations were similar to their nominal values, confirming that PP particles and PET fibers were present at the desired concentrations and available for ingestion by individual damselfish. Both body burdens and depuration rates differed between the two experimental microplastics, with body burden of PET fibers being 2.2 times greater, and depuration rates of PET fibers being significantly lower than that for PP particles. For both microplastic types, exposure to higher concentrations led to an increase in microplastic body burden and lower depuration rates. These findings confirm ingestion of environmentally relevant PP particles and PET fibers by *P. amboinensis* and demonstrate for the first time the influence of microplastic characteristics and concentration on depuration rates of coral reef fish.

Ingestion of microplastic fragments and fibers has been reported for a variety of marine planktivorous fish species, both in the field (Tanaka and Takada, 2016; Compa et al., 2018; Jensen et al., 2019) and in the laboratory (Critchell and Hoogenboom, 2018; Cong et al., 2019; Xiong et al., 2019). Based on the literature, two mechanisms could explain the pattern of microplastic ingestion observed in our study: (1) fish selectively or non-randomly ingesting microplastics (Mizraji et al., 2017; Ory et al., 2018; Jensen et al., 2019; Bour et al., 2020b), or (2) fish passively or inadvertently ingesting microplastics while feeding (Roch et al., 2020). In our study, we cannot rule out selective feeding as the experimental fish were pre-starved and the biofouled experimental microplastics, likely emitting dimethyl sulfide, may have acted as an attractant as has been reported for seabirds (Savoca et al., 2016), fish (Savoca et al., 2017), and copepods (Procter et al., 2019). Conversely, passive or inadvertent ingestion may have occurred given that exposures were conducted concurrently with normal feeding,

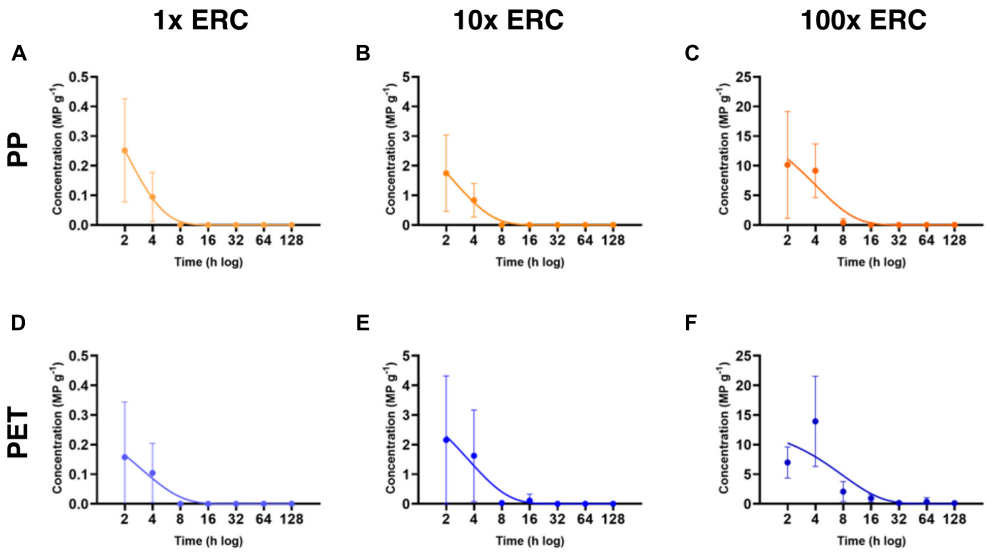

FIGURE 3 | Mean microplastic body burden fish^{-1} (± standard deviation) in three treatment groups (n = 84 tanks) during a single exposure of adult damselfish *Pomacentrus amboinensis* to polypropylene (PP) particles and polyethylene (PET) fibers in a controlled laboratory experiment. Mean body burden is presented for four damselfish collected at each of the seven depuration times (2, 4, 8, 16, 32, 64, and 128 h). ERC, environmentally relevant concentrations. Each graph corresponds to a combination of experimental microplastic and concentration of exposure. **(A)** PP-ERC, **(B)** PP-10x ERC, **(C)** PP-100x ERC, **(D)** PET-ERC, **(E)** PET-10x ERC, and **(F)** PET-100x ERC.

and microplastic body burden appeared proportional to the exposure concentration. Passive or inadvertent microplastic ingestion could also explain the observed variabilities in body burden within the same exposure treatments, such as those ranging from 0 to 100% in the ERC treatment. Highly variable microdebris ingestion, including microplastic ingestion, is commonly reported for fish, both in field (Compa et al., 2018; Kroon F. J. et al., 2018; Garnier et al., 2019; Jensen et al., 2019) and in experimental (Lu et al., 2016; Xiong et al., 2019; Hoang and Felix-Kim, 2020) studies. For example, adult *P. amboinensis* collected in the central GBR WHA contained an average of four microdebris items per individual fish, with a range from zero to 131 items (Jensen et al., 2019). In the laboratory, larvae of the same species also showed high variability in microplastic intake (McCormick et al., 2020). Together, our results and those of previous studies indicate likely but inconsistent ingestion of microplastics by fish, particularly at environmentally relevant levels of exposure. With increasing exposure concentrations, however, variability in microplastic ingestion decreased suggesting that it may become more difficult for fish like *P. amboinensis* to avoid ingesting microplastics inadvertently while feeding.

The depuration rate of experimental microplastics ingested by *P. amboinensis* was relatively short, with most being eliminated within 8 h. At this time point, PP particles, and PET fibers were only observed in the GITs of fish exposed to the 100x ERC treatment. After 16 h, PP particles were no longer observed in any of the fish, and after 128 h only two fish contained a small number of PET fibers. The observed residence time of microplastics in fish GITs suggests temporary microplastic contamination and a low likelihood of accumulation after ingestion. Although microplastics are commonly reported in coastal and marine fish collected in the field, when these studies are more closely evaluated, as in Kroon F. J. et al. (2018), the majority of individual fish examined (i.e., >80%) do not appear to be contaminated. This may indicate that microplastics are transitory in wild fish and may not accumulate at current or future ERCs (Santana et al., 2017). Nonetheless, compared with natural food, which is depurated by *P. amboinensis* typically within 3–5 h and at the most <10 h after ingestion (Marnane and Bellwood, 1997), PP and PET microplastics were substantially slower to traverse the GIT. Whether these differences in depuration rates between natural food and microplastics affect energy acquisition for this species is unknown, but possible in theory. For example, the longer residency time of microplastics compared to natural food items could support the hypothesis that microplastic ingestion can impact organism fitness (Besseling et al., 2013; Cole et al., 2015; Lo and Chan, 2018).

TABLE 5 | Differences in body burden over time due to microplastic type, microplastic concentration or fish weight, following a single exposure of adult damselfish *Pomacentrus amboinensis* to blue polypropylene (PP) particles, and polyester (PET) fibers in a controlled laboratory experiment.

	Estimate	Std. error	*z*-value	*p*-value
Intercept	1.32094	0.42367	3.11800	0.00182
PP	−0.78655	0.22393	−3.51200	0.00044
10x ERC	2.14352	0.40928	5.23700	1.63e-07
100x ERC	4.51990	0.39654	11.39800	<2e-16
Log(depuration)	−1.59942	0.11341	−14.10300	<2e-16
Weight	0.02199	0.03213	0.68400	0.49371

Estimated regression parameters, standard errors (Std. error), z-values and p-values for the general linear model presented in Eq. 1.

TABLE 6 | Microplastics depuration rate (in items h^{-1}) with 95% confidence interval (CI), and elimination half-life (h) of polypropylene (PP) particles, and polyester (PET) fibers in three treatment groups (n = 84 tanks) following a single exposure of adult damselfish *Pomacentrus amboinensis* in a controlled laboratory experiment.

	PP particles			PET fibers		
	ERC	**10x ERC**	**100x ERC**	**ERC**	**10x ERC**	**100x ERC**
Depuration rate (item.h^{-1})	0.52	0.42	0.25	0.33	0.29	0.13
95% CI	0.26 to 1.14	0.21 to 0.87	0.12 to 0.49	0.10 to 0.96	0.10 to 0.72	0.05 to 0.26
Elimination Half-life (h)	1.34	1.64	2.77	2.12	2.4	5.41

ERC, environmentally relevant concentrations.

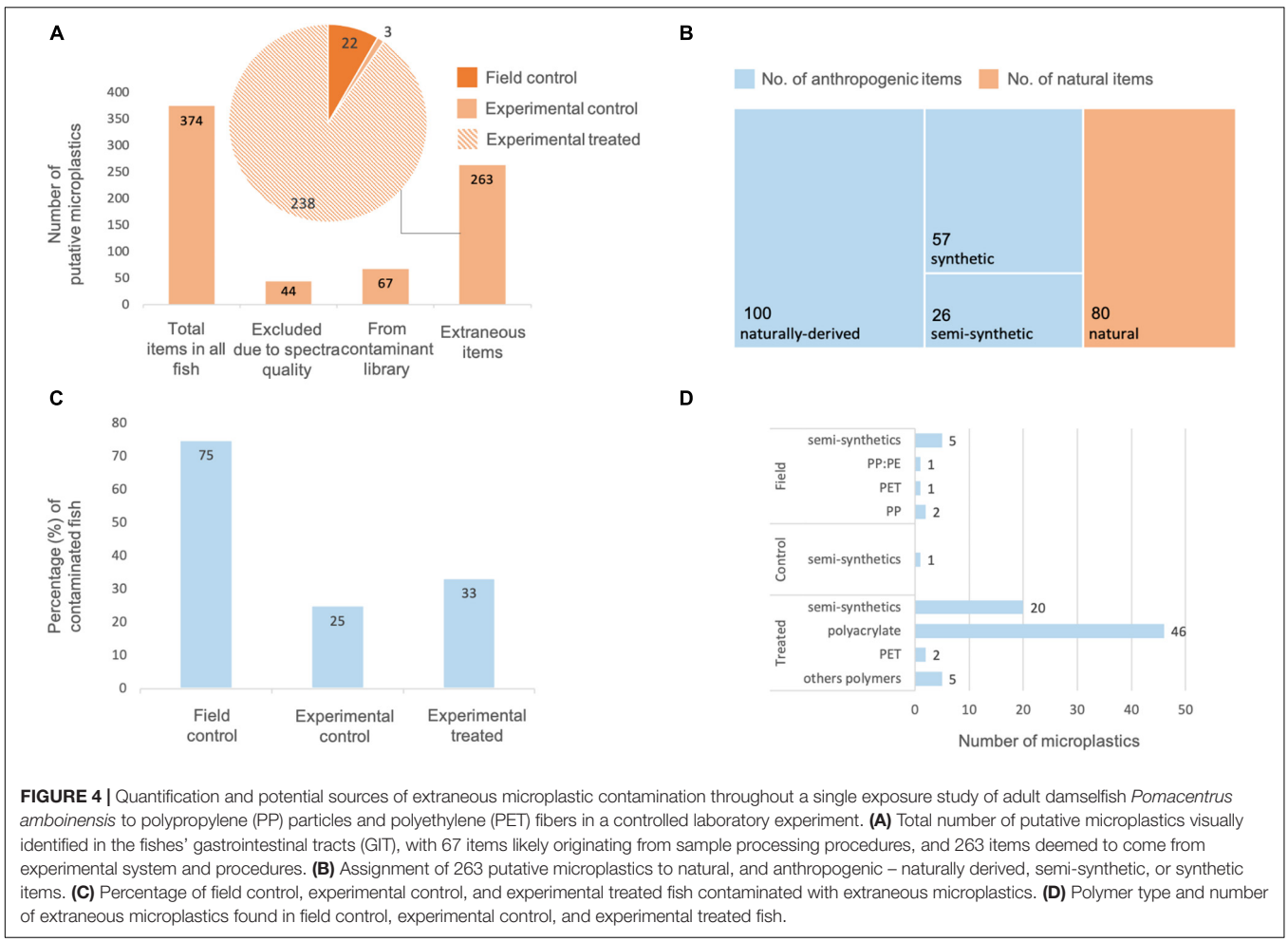

FIGURE 4 | Quantification and potential sources of extraneous microplastic contamination throughout a single exposure study of adult damselfish *Pomacentrus amboinensis* to polypropylene (PP) particles and polyethylene (PET) fibers in a controlled laboratory experiment. **(A)** Total number of putative microplastics visually identified in the fishes' gastrointestinal tracts (GIT), with 67 items likely originating from sample processing procedures, and 263 items deemed to come from experimental system and procedures. **(B)** Assignment of 263 putative microplastics to natural, and anthropogenic – naturally derived, semi-synthetic, or synthetic items. **(C)** Percentage of field control, experimental control, and experimental treated fish contaminated with extraneous microplastics. **(D)** Polymer type and number of extraneous microplastics found in field control, experimental control, and experimental treated fish.

Similar or even shorter microplastic depuration periods have been reported for various aquatic organisms, including fish (Mazurais et al., 2015; Dawson et al., 2018; Xiong et al., 2019). The subtle differences in depuration periods among studies could be related to variations in experimental design, including characteristics of microplastics used, such as polymer type, shape, size, color, and concentration (Xiong et al., 2019). Furthermore, characteristics of the species being investigated (such as feeding habits, trophic levels, morphology, and life stage) may also influence ingestion and depuration rates (Bour et al., 2018; Xu X. et al., 2020). For example, *P. amboinensis* larvae were reported to take up to 14 h to depurate transparent PS microspheres (200–300 μm, 167 microsphere.L^{-1}) ingested during a 1 h exposure experiment (McCormick et al., 2020). In turn, microplastic exposure (i.e., 50 PET fibers of 50–500 μm in length, and 50 PE irregular fragments >63 μm per food pellet, with no color specification) of planktivorous adult goldfish *Carassius auratus* resulted in similar microplastic retention as our fish species (up to 3 of 50 beads and fibers ingested after 6 days), but in slower depuration rate (Grigorakis et al., 2017). To better elucidate patterns of microplastic contamination and associated risks in marine organisms, future depuration studies should compare

and investigate the role of physical and chemical properties of different microplastics, as well as biological and ecological characteristics of the species under investigation.

Our findings show that fish body burden and depuration rates were significantly affected by the experimental microplastic they were exposed to. At all doses, body burdens were lower and depuration rates faster for PP particles compared to PET fibers. Considering that our test microplastics differed in (i) polymer composition, (ii) size, and (ii) shape, any or all of these factors could potentially influence the observed results. We are not aware of any studies comparing microplastic uptake and depuration rates across different polymer compositions (Grigorakis et al., 2017). In contrast, larger microplastics have been observed to be depurated more quickly (Xiong et al., 2019), suggesting that the shape and size of our experimental microplastics may be likely explanations for the observed patterns. The slower depuration of PET fibers observed here corroborates studies that report the prevalence of fibers in wild organisms, including fish, from coastal and marine environments (Kroon F. J. et al., 2018; Jensen et al., 2019; Filgueiras et al., 2020; Xu S. et al., 2020). Moreover, it supports the hypothesis that microplastic fibers may pose a greater risk to marine organisms than microplastic particles (Au et al., 2015; Song et al., 2019), although few studies to date have examined potential biological effects of microplastic fibers. Accumulation and entanglement of fibers in the GIT has been observed in fish (Jensen et al., 2019) and may impact the gut passage time of microplastics in fish and other marine organisms (Welden and Cowie, 2016; Xiong et al., 2019). Xiong et al. (2019) also suggested that the presence of food along with microplastics in the GIT can increase microplastic retention. In the present study, clumps of PET fibers entangled with gut contents were occasionally found in the GIT of exposed fish (**Figure 2**), however, they were not observed consistently enough to confidently state that they contributed to the differences in depuration rates. Another hypothesis is that the GIT morphology may contribute to retention of fibers, potentially influencing fiber gut passage time (Welden and Cowie, 2016); whether this is true for *P. amboinensis* remains to be determined.

For both experimental microplastics, body burdens and depuration rates increased with increasing exposure concentrations. This is opposite to patterns for natural food which moves faster through fish GITs with increasing concentration (German, 2011). This suggests that microplastic ingestion, and potential effects on planktivorous fish like *P. amboinensis*, are strongly influenced by the amount of microplastics present in their environment (Ding et al., 2018; Roch et al., 2020). Thus, higher risk of biological contamination and subsequent effects are likely associated with environments that are more contaminated (Everaert et al., 2020), such as accumulation zones [e.g., gyres (Eriksen et al., 2013) and benthic habitats (Ogata et al., 2009; Barrett et al., 2020)], as well as areas adjacent to highly populated regions or areas of industrial (Li et al., 2020) and commercial fishery (Xue et al., 2020) interest. Similarly, our findings are consistent with predictions that microplastic body burdens will increase in marine fish along with projected increases in marine microplastic contamination (Jambeck et al., 2015; Geyer et al., 2017; Everaert et al., 2020), driven by the estimated 400% rise in annual plastic production by 2100 (Everaert et al., 2020).

Complete elimination of contamination with extraneous microplastics is challenging (Prata et al., 2021) and efforts to do so are rarely quantified and reported on in microplastic exposure studies (e.g., Nanninga et al., 2020; Zhao et al., 2020). Ours is the first to quantify extraneous microplastics in the GITs of individual fish and link them to potential sources (i.e., field, experimental system or sample processing procedures) to discuss potential elimination strategies. Control fish collected and euthanized in the field contained both synthetic and semi-synthetic extraneous items different to those found in experimental control and exposed fish. This corroborates that experimental fish had depurated any microplastics brought in from the field during acclimation. Despite measures put in place to prevent contamination, 67 extraneous putative microplastics were retrieved from experimental control and exposed fish, with 20% of these putative microplastics linked with items used and recovered during sample processing procedures, specifically airborne items and clothing. All clothing, including lab coats, worn during this study were cellulose-based, thus were not considered further. Nevertheless, monitoring it highlighted how important is to avoid synthetic and semi-synthetic clothing while conducting microplastic studies, including experimental procedures. The remaining 80%, primarily polyacrylate and semi-synthetics fibers, could not be linked to specific items and is assumed to have originated from the experimental air, water or fish food. Whether these extraneous microplastics influenced the ingestion and depuration rates of experimental microplastics remains unknown. Our exposure study was conducted in a general use laboratory at a remote field research station, and access to sophisticated filtration systems to clarify incoming air and seawater, was not available. Similar limitations could be faced by many experimental researchers, however, these are not frequently considered or discussed in the microplastic literature (Prata et al., 2021). Our results highlight the need for elimination of contamination sources where possible, and importantly for enhanced data quality control through robust study design with relevant experimental controls and stringent monitoring of background contamination. This is particularly important for microplastic studies using exposure conditions that reflect ERCs.

In summary, this study confirmed that the planktivorous damselfish *P. amboinensis* ingests environmentally relevant types (specifically irregular shaped blue PP particles and PET fibers) and concentrations of microplastics. After a single exposure to experimental microplastics, the majority of PP particles and PET fibers were eliminated from the GIT within 8 h, with most items completely purged by 128 h. Damselfish ingested both experimental microplastics at all concentrations, with body burden of PET fibers being 2.2 times greater, and depuration rates of PET fibers significantly lower than that for PP particles. For both microplastic types, exposure to higher concentrations led to an increase in body burden and lower depuration rates. These results corroborate the higher abundance of microplastic fibers versus particles reported in many wild-caught marine organisms, and highlight

the need for more research on the impacts of microplastic fibers on marine organisms. Our findings also support the hypothesis that environments with higher levels of microplastic contamination (e.g., current "hotspots") pose a greater risk to marine organisms due to increased microplastic ingestion and prolonged depuration rates, and that sporadic exposure to microplastics might have lower microplastic-associated risks. Finally, despite measures put in place to prevent contamination extraneous microplastics were recovered from experimental fish, highlighting the challenge to completely eliminate contamination in microplastic exposure studies. This is of particular concern for experiments examining the impacts of environmentally relevant microplastic exposure. We strongly recommend that controlled exposure studies quantify and report on contamination with extraneous microplastics to inform and continuously improve protocols for future microplastics research.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by the James Cook University Animal Ethics Committee (Approval Number A2635).

AUTHOR CONTRIBUTIONS

MS obtained funding, conceptualized the study, conducted field and laboratory work, analyzed the data, wrote the original draft, and reviewed and edited further versions of the manuscript. AD contributed to study design, analyzed the data, and contributed to reviewing and editing the manuscript. CM contributed to study design and reviewing and editing the manuscript. LH contributed to developing field relevant experimental research at the outset and to reviewing and editing the final manuscript. CL conducted field and laboratory work. FK obtained funding, contributed to study design, conducted field work, and contributed to reviewing and editing the manuscript. 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/fenvs.2021.641135/full#supplementary-material

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