IMPACTS OF MARINE LITTER

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IMPACTS OF MARINE LITTER

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Image: Adam Porter.

According to United Nations, 6.4 million tons of anthropogenic litter end up in the oceans every year. The majority of this litter (up to 83% according to some sources) is plastics. Continental plastic litter enters the ocean largely through storm-water runoff, is dumped on shorelines during recreational activities or directly discharged at sea from ships and trade activities. However, long-term data is scarce and does not show any clear or significant trends with regards to variations in debris quantities. Despite initiatives to reduce marine litter, such as from UNEP, the G20, and G7, the European Marine Strategy Framework Directive and action plans from the regional seas conventions, harm is still to be well understood. Moreover, standardization

of sampling techniques, result units and metrics is still at an incipient stage, and harmonization is needed to achieve reliable monitoring.

The coastal landscape is frequently impacted by marine litter that impairs recreational uses and causes a loss of touristic value. Beyond the aesthetic impact, marine litter also bears potential economic implications to the fishery sector and also strongly affects the marine environment. First, solid particles are ingested by fauna and may remain in the stomach undigested (most of them are excreted). Large differences among taxa, resulting from differences in size and feeding habits, have been described. Second, plastic litter can represent a relevant source of chemical additives, some of them with suspected endocrine disrupting action, that easily leach into the water since they are not bound to the polymeric chains and become available to the estuarine and marine fauna. Third, intentionally or accidentally discarded fishing gear poses special risk for large, air-breathing marine animals, including endangered species, which get entangled in the nets. Conventional plastics are non-biodegradable and they may persist in the environment for hundreds of years but also because of hydrodynamics and exposure to light, they may fragment into small particles readily taken up by marine organisms. Microplastics and nanoplastics are an especially feared component of marine litter and they might play a role as vectors of hydrophobic pollutants (and plastic additives) into the trophic webs, although thermodynamic models and experimental data provide conflicting results and more research is needed on this field. Finally, plastic at sea may transport alien species for long distances or act as substratum for benthic species, providing a support to colonization.

This Research Topic will consider several feature of marine litter impacts from the ecological, ecotoxicological, economical and social impacts. Moreover, it also proposes a discussion forum to better understand all potential harms caused by Marine litter, both to marine organisms as well as to the whole marine/estuarine environment and communities.

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Table of Contents

06 ***Editorial: Impacts of Marine Litter***
Luisa Galgani, Ricardo Beiras, François Galgani, Cristina Panti and Angel Borja

SECTION 1
SOURCES OF MARINE LITTER AND ENVIRONMENTAL DISTRIBUTION AND SINKS: SURVEYS IN WATER, SEDIMENTS, COAST AND BIOTA

10 ***Impacts of Marine Plastic Pollution From Continental Coasts to Subtropical Gyres—Fish, Seabirds, and Other Vertebrates in the SE Pacific***
Martin Thiel, Guillermo Luna-Jorquera, Rocío Álvarez-Varas, Camila Gallardo, Iván A. Hinojosa, Nicolás Luna, Diego Miranda-Urbina, Naiti Morales, Nicolas Ory, Aldo S. Pacheco, Matías Portflitt-Toro and Carlos Zavalaga

26 ***Frequency of Microplastics in Mesopelagic Fishes From the Northwest Atlantic***
Alina M. Wieczorek, Liam Morrison, Peter L. Croot, A. Louise Allcock, Eoin MacLoughlin, Olivier Savard, Hannah Brownlow and Thomas K. Doyle

35 ***Low Abundance of Plastic Fragments in the Surface Waters of the Red Sea***
Elisa Martí, Cecilia Martin, Andrés Cózar and Carlos M. Duarte

43 ***Anthropogenic Litter on Beaches With Different Levels of Development and Use: A Snapshot of a Coast in Pernambuco (Brazil)***
Maria C. B. Araújo, Jacqueline S. Silva-Cavalcanti and Monica F. Costa

53 ***Microplastic Distribution at Different Sediment Depths in an Urban Estuary***
Kathryn A. Willis, Ruth Eriksen, Chris Wilcox and Britta D. Hardesty

SECTION 2
EXPERIMENTAL APPROACHES

61 ***Constraints and Priorities for Conducting Experimental Exposures of Marine Organisms to Microplastics***
Ika Paul-Pont, Kevin Tallec, Carmen Gonzalez-Fernandez, Christophe Lambert, Dorothée Vincent, David Mazurais, José-Luis Zambonino-Infante, Guillaume Brotons, Fabienne Lagarde, Caroline Fabioux, Philippe Soudant and Arnaud Huvet

SECTION 3
IMPACTS ON MARINE ORGANISMS: MACRO-, MEIO-, AND MICRO-FAUNA RELATED TO PLASTICS AND PLASTICS' ASSOCIATED CONTAMINANTS

83 ***Ecotoxicological Effects of Chemical Contaminants Adsorbed to Microplastics in the Clam* Scrobicularia plana**
Sarit O'Donovan, Nélia C. Mestre, Serena Abel, Tainá G. Fonseca, Camilla C. Carteny, Bettie Cormier, Steffen H. Keiter and Maria J. Bebianno

98 ***Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels,* Mytilus galloprovincialis**
Lucia Pittura, Carlo G. Avio, Maria E. Giuliani, Giuseppe d'Errico, Steffen H. Keiter, Bettie Cormier, Stefania Gorbi and Francesco Regoli

113 ***Leachate From Expanded Polystyrene Cups is Toxic to Aquatic Invertebrates (*Ceriodaphnia dubia*)***
Clara Thaysen, Kathleen Stevack, Ralph Ruffolo, David Poirier, Hannah De Frond, Julieta DeVera, Grace Sheng and Chelsea M. Rochman

122 ***Comparative Effects of Ingested PVC Micro Particles With and Without Adsorbed Benzo(a)pyrene vs. Spiked Sediments on the Cellular and Sub Cellular Processes of the Benthic Organism* Hediste diversicolor**
Alessio Gomiero, Pierluigi Strafella, Giulio Pellini, Vera Salvalaggio and Gianna Fabi

134 ***A Review of Plastic-Associated Pressures: Cetaceans of the Mediterranean Sea and Eastern Australian Shearwaters as Case Studies***
Maria Cristina Fossi, Cristina Panti, Matteo Baini and Jennifer L. Lavers

SECTION 4
REGULATIONS

144 ***The Occurrence of Paraffin and Other Petroleum Waxes in the Marine Environment: A Review of the Current Legislative Framework and Shipping Operational Practices***
Giuseppe Suaria, Stefano Aliani, Silvia Merlino and Marinella Abbate

154 ***Governance Solutions to the Tragedy of the Commons That Marine Plastics Have Become***
Joanna Vince and Britta D. Hardesty

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Editorial: Impacts of Marine Litter

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Editorial on the Research Topic

Impacts of Marine Litter

INTRODUCTION: MOTIVATION AND SUBTOPICS

Marine litter is a global environmental concern. Between 61% and 87% of this litter is plastics (Barboza et al., 2019; Tekman et al., 2019). In 2010 alone, the amount of plastics entering the oceans varied between 4.8 million and 12.7 million metric tons (Jambeck et al., 2015), contributing to an estimated total abundance of at least 5 trillion particles (Eriksen et al., 2014). In 2017, 348 million tons of plastics were produced globally (PlasticsEurope, 2018) and in the next two decades, the amount of plastics produced is expected to double (Geyer et al., 2017).

Continental plastic litter enters the ocean largely through storm-water runoff, is dumped on shorelines during recreational activities or directly discharged at sea from ships (Walker et al., 2019). The deep-sea floor is probably the final global sink for most marine litter that is not decomposed or fractionated to the nanosize (Angiolillo, 2019). However, long-term data are scarce and do not show any clear or significant trend with regards to variations in debris quantities (Maes et al., 2018). In many coastal countries, mismanagement of solid waste has caused between 1.7% and 4.6% of the total plastic waste generated to end up in the sea (Hoornweg and Bhada-Tata, 2012; Jambeck et al., 2015). Despite initiatives to monitor and reduce marine litter, such as from United Nations Environmental Programme (UNEP), the G20, and G7, the European Marine Strategy Framework Directive (European Commission, 2008) and action plans from the Regional Seas Conventions (e.g., OSPAR Regional Action Plan for Marine Litter; UNEP/MAP-Barcelona Convention Regional Plan on Marine Litter Management in the Mediterranean), harm is still far from being understood. Moreover, standardization, and harmonization of sampling techniques, result units and metrics is needed to achieve reliable monitoring and assessment, and threshold values of good/not good status are still at an incipient stage, since ecotoxicological information on the effects of the smaller fractions of plastic litter, microplastics and nanoplastics, in the environment and on aquatic organisms is scarce (Gall and Thompson, 2015; De Sá et al., 2018; Ogonowski et al., 2018).

The coastal landscape is frequently impacted by marine litter that impairs recreational uses and causes a loss of touristic value. Beyond the aesthetic impact, marine litter also bears potential economic implications to maritime activities, such as fisheries and the aquaculture sectors (UNEP, 2014). It may also affect the marine environment and the different ecosystem components (Gall and Thompson, 2015; Rochman et al., 2016; Galloway et al., 2017; Barboza et al., 2019). First, solid particles are ingested by fauna; whereas most of the litter will be excreted, some particles may remain in the stomach undigested, including seafood (Rochman et al., 2015). Large differences among taxa, resulting from differences in size and feeding habits, have been described (Kühn et al., 2015; Bour et al., 2018; Fossi et al., 2018). Many studies have focused on selected species such as seabirds or sea turtles which have even been proposed as bioindicators of oceanic plastic pollution (Bonanno and Orlando-Bonaca, 2018; Fossi et al., 2018) because they feed exclusively at sea, they

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show a non-selective surface foraging, and in certain populations most individuals present plastic debris in their stomachs (Wilcox et al., 2015; Domènech et al., 2019). Second, plastic litter can represent a relevant source of chemical additives, some of them with suspected endocrine disrupting action, that easily leach into the water since they are not bound to the polymeric chains and become available to the estuarine and marine fauna (Hermabessiere et al., 2017). Third, intentionally or accidentally discarded fishing gears pose special risks for large, air-breathing marine animals, including endangered species, which get entangled in the nets (Lusher et al., 2018). Conventional plastics are non-biodegradable and they may persist in the environment for hundreds of years but also because of hydrodynamics and exposure to light, they may fragment into small particles readily taken up by marine organisms. Microplastics and nanoplastics are of particular concern: ingested by even the smallest zooplankton species they can transfer hydrophobic pollutants (and plastic additives) into the trophic webs, although thermodynamic models and experimental data provide conflicting results and more research is needed in this field (Beiras et al., 2018; Burns and Boxall, 2018; Ogonowski et al., 2018). Finally, plastic at sea may transport alien species over long distances or act as substratum for vagile and epistratum benthos, providing a support to colonization (Casabianca et al., 2019).

This Research Topic includes, as yet, 13 papers covering broad oceanic aspects of litter: development of methods, distribution in different species and marine areas, ingestion of plastic, toxicity of plastic associated chemicals, and policies dedicated to reduction measures of marine litter. The impacts related to marine litter have been evaluated from ecological, ecotoxicological, economic and social perspectives, identifying four major subtopics for the papers featured:

1. Sources of marine litter and environmental distribution and sinks: surveys in water, sediments, coast and biota
2. Experimental approaches
3. Impacts on marine organisms: macro-, meio-, and micro-fauna related to plastics and plastics' associated contaminants
4. Regulations.

SUMMARY OF THE PAPERS

In this Research Topic, studies considering sources and distributions of marine litter (subtopic 1) span over awide geographic area and different environmental compartments, presenting results from surveys in water, beaches, sediments and biota.

Interactions between species and litter were reviewed for the South East Pacific, indicating the importance of entanglement, microplastic ingestion and the higher sensitivity of sea turtles to marine litter (Thiel et al.). In the North Atlantic, a much higher occurrence of microplastic fragments than previously reported was found in the gut contents of mesopelagic fishes (Wieczorek et al.). Regional surveys suggest that land-based inputs of plastics are to be reconducted to different levels of coastal anthropogenic pressure and population growth. In the Red Sea, low plastic waste was found in surface waters suggesting reduced land-based inputs from the coastal environment (Martí et al.). In Brazil, the nature and predominance of beach litter were directly related to beach users, with less urbanized beaches showing smaller quantities of anthropogenic litter (Araújo et al.). In an urban estuary in Tasmania, the type, distribution and abundance of microplastics observed closely matched data on increasing plastic production, coastal population growth, and proximity to urban water outflows (Willis et al.). Some of the studies highlighted the importance of fibers as part of microplastics with evidence of their non-anthropic origin (Martí et al.; Willis et al.; Wieczorek et al.), but also indicated the need of a careful assessment of the amount of fibers in environmental samples that could be biased by atmospheric contamination while conducting the laboratory analysis (Willis et al.).

Methods were also addressed (subtopic 2) with a review on constraints and priorities for conducting experimental exposures of marine wildlife to microplastics (Paul-Pont et al.).

Impacts of marine litter and marine plastics can be varied and concurrent, arising from entanglement, ingestion, and leaching of plastic-associated contaminants and additives (subtopic 3). In marine fauna, negative effects of marine litter are documented on over 1,400 species and depend on a multiplicity of co-factors that need to be considered when developing management plans for the conservation of ecosystems and biodiversity (Fossi et al.). Five studies describe the release of chemicals and their ecotoxicological effects on marine biota (subtopic 3), including two bivalve species (O'Donovan et al.; Pittura et al.), one crustacean (Thaysen et al.), one annelid species (Gomiero et al.), seabirds and cetaceans (Fossi et al.), indicating that plastics as vectors for organic pollutants may add up to other environmental and anthropic stressors and potentially alter survival rate and reproductive success. Clearly, action modes and toxicological pathways, also considering transcriptional activity, differ according to pollutants. In one freshwater species, significant adverse effects of leachates from expanded polystyrene were detected (Thaysen et al.).

In terms of management (subtopic 4), the role of regulation, public perception and social license to operate in managing waste that enters the ocean were discussed (Vince and Hardesty). More specific case studies on paraffins were also reviewed in the framework of environmental policies, suggesting for regulatory measures (Suaria et al.).

KNOWLEDGE GAPS FILLED, PERSPECTIVES AND CONCLUSIONS

Overall, this Research Topic provides a panel of various aspects of the impacts of marine litter and plastics, and will largely support more research toward a better understanding of any harm caused by any material at all levels of biological organization. Through this platform, this Research Topic also encourages a discussion forum to better understand all potential effects caused by marine litter, both to marine organisms as well as to the whole marine/estuarine environment and communities, and to propose new strategies of intervention, for prevention, mitigation, and monitoring.

Several gaps still need to be covered, from the harmonization of methodological approaches to study marine litter in different environmental compartments (i.e., sea surface, sea floor, water column) to the evaluation of the effects on biota. Moreover, the impact of marine litter and, in particular, microplastics and nanoplastics, on human health is still largely debated and a more focused research needs to be carried out to properly address this issue.

Models on plastic distribution and transfer in all marine compartments are needed to provide reliable estimates of marine fluxes from land to sea. These efforts should be strongly encouraged to better drive globally agreed prevention and mitigation strategies to be adopted and harmonized across countries.

CONTRIBUTION TO THE FIELD

Marine litter is an urgent environmental threat comprising primarily plastic debris. Yet, the global production of plastic is expected to double over the next decades, with envisaged severe impacts across ecosystems and societies. This editorial introduces to the Research Topic "Impacts of Marine Litter", aiming at a better understanding of marine litter impacts from the ecological, ecotoxicological, economic, and social point of view. This Research Topic also aims at proposing new strategies for the prevention, mitigation and monitoring of marine litter. With its 13 published papers, the Research Topic provides new information on the environmental distribution (including sources and sinks) of marine litter across the globe, as well as on the impacts of plastics' associated contaminants to marine micro and macro fauna. It also highlights constraints and priorities for conducting exposure experiments of marine wildlife to plastics, discusses regulation and policy measures for land waste management and litter disposal at sea, and identifies remaining knowledge gaps which should be the focus of future interdisciplinary research and policy interventions.

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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Impacts of Marine Plastic Pollution From Continental Coasts to Subtropical Gyres—Fish, Seabirds, and Other Vertebrates in the SE Pacific

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Anthropogenic Marine Debris (AMD) in the SE Pacific has primarily local origins from land-based sources, including cities (coastal and inland), beach-goers, aquaculture, and fisheries. The low frequency of AMD colonized by oceanic biota (bryozoans, lepadid barnacles) suggests that most litter items from coastal waters of the Humboldt Current System (HCS) are pulled offshore into the South Pacific Subtropical Gyre (SPSG). The highest densities of floating micro- and macroplastics are reported from the SPSG. An extensive survey of photographic records, unpublished data, conference proceedings, and published studies revealed interactions with plastics for 97 species in the SE Pacific, including 20 species of fish, 5 sea turtles, 53 seabirds, and 19 marine mammals. Sea turtles are most affected by interactions with plastics, underlined by the fact that 4 of the 5 species suffer both from entanglement and ingestion. Reports gathered in this review suggest that interactions along the continental coast are mostly via entanglement. High frequencies of microplastic ingestion have been reported from planktivorous fish and seabirds inhabiting the oceanic waters and islands exposed to high densities of microplastics concentrated by oceanic currents in the SPSG. Our review also suggests that some species from the highly productive HCS face the risk of negative interactions with AMD, because food and plastic litter are concentrated in coastal front systems. In order to improve the conservation of marine vertebrates, especially of sea turtles, urgent measures of plastic reduction are needed.

Keywords: anthropogenic marine debris, impacts, biota-litter interactions, entanglement, microplastic ingestion

INTRODUCTION

Marine plastic pollution is generating impacts on marine biota and ecosystems at many different levels (Ryan, 2016). Impacts are reported from a wide range of organisms, including microbiota, invertebrates, and vertebrates (Galloway et al., 2017; Law, 2017). An increasing number of reports document microplastic ingestion by marine invertebrates (Lusher, 2015); certain species also grow on large, floating plastic items, and can be transported to new habitats they had not previously inhabited (Kiessling et al., 2015). Interactions with vertebrates are best known, because vertebrates are larger and therefore more visible and recognizable than small marine invertebrates. Entanglement of seabirds and marine mammals in large plastic litter (nets, ropes, etc.) has been known since the early 1970s (Derraik, 2002). Similarly, ingestion of microplastics by fishes and seabirds is well known since about the same time period (Kenyon and Kridler, 1969; Carpenter et al., 1972; Ryan, 1987), and the number of affected species, such as seabirds (Wilcox et al., 2015), is continuously increasing.

The risk of interactions between marine organisms and plastics is not equal across the oceans. It depends on feeding biology and amount of plastic litter in the environment where the organisms are foraging. For example, seabird species feeding at the sea surface are more susceptible to plastic ingestion than diving species (Ryan, 1987). Species that ingest small microplastics, such as many fishes and surface-foraging seabirds might be at highest risk in areas where microplastics concentrate, such as the subtropical gyres, whereas species ingesting larger plastic items could potentially encounter these closer to the continental coasts where rivers and other human activities spill and accumulate large quantities of plastic litter (Rech et al., 2014, 2015; Di-Méglio and Campana, 2017; Fossi et al., 2017). Indeed, many reports of meso- and macroplastics ingestion include sea turtles and whales stranded on continental shores (Schuyler et al., 2014; Lusher et al., 2018). Similarly, the risk of entanglement for marine vertebrates is likely to be higher in areas with large amounts of derelict fishing gear, such as the North Pacific subtropical convergence zone (Pichel et al., 2007) or coastal areas where ghost nets accumulate (Wilcox et al., 2013).

These considerations suggest that the risk of harmful interactions with marine plastic pollution depends on (a) the biology of the species, and (b) the distribution and abundance of the different plastic types. To examine these predictions, herein we gather reports of interactions with plastic litter for marine vertebrates in the SE Pacific. We compare reports from the highly productive Humboldt Current System (HCS) with those from the oligotrophic open ocean, in particular the Easter Island ecoregion close to the South Pacific Subtropical Gyre (SPSG) accumulation zone (for methodological details see Supplement 1).

PLASTIC LITTER IN THE SOUTH-EAST PACIFIC

Litter Sources and Pathways in the South-East Pacific

In the South Pacific Ocean, anthropogenic marine debris (AMD) originates from the surrounding landmasses and oceanic sources (Thiel et al., 2003, 2013; Kiessling et al., 2017). Especially in the eastern part of the S Pacific, litter pollution comes from land sources (e.g., Hidalgo-Ruz et al., 2018), beach-goers (Bravo et al., 2009), and marine activities including aquaculture (Astudillo et al., 2009; Hinojosa and Thiel, 2009), coastal (Perez-Venegas et al., 2017) and high seas fisheries (Kiessling et al., 2017). Rivers also contribute large amounts of macro- and microplastics (Rech et al., 2014, 2015).

The majority of the litter from land sources is probably trapped in coastal waters or on shores very close to its sources (Hinojosa and Thiel, 2009; Thiel et al., 2011; Rech et al., 2014). The low incidence of marine organisms growing on marine litter stranding on the continental coasts of the SE Pacific suggests that this fouling-free litter has likely passed very little time (if at all) at sea. On the other hand, strong offshore currents within the HCS are thought to move floating plastics quickly to the open ocean, where they become trapped in the SPSG (Martinez et al., 2009).

Floating Macro- and Microplastics in the SE Pacific

Given the multiple sources and transport dynamics of floating AMD in the SE Pacific, the density of macroplastics is high in immediate coastal waters, but rapidly decreases further away from the continental coast (Thiel et al., 2003; Miranda-Urbina et al., 2015). When approaching the center of the SPSG, densities of floating AMD reach very high abundances, as also reflected in recent data taken in two surveys conducted in 2015 and 2016 (**Figure 1**). A total of 477 items of floating AMD were observed across both surveys, the majority corresponding to 2015 when a major area including the Easter Island ecoregion was surveyed (**Figure 1**). Of the total floating AMD, 77% were macroplastics, most of which were large plastic fragments (95.4%). Other items included lines (17.7%), buoys (7.6%), plastic trays (4.9%), and plastic bags and nets (2.5 and 2.2%, respectively) (**Figure 1**). Accounting for the total number of AMD in both years, it seems that the distribution of floating AMD is comparable to patterns previously determined for the SE Pacific (see Miranda-Urbina et al., 2015). Out of the total (477 items), 8% were found in the HCS, only 3% occurred around Juan Fernandez and Desventuradas Islands, while 78 and 11% of all floating AMD were concentrated in the Oceanic and Polynesian sector, respectively. Large numbers of marine litter accumulate on beaches of Rapa Nui (Easter Island) and the uninhabited island Salas and Gómez in the Polynesian sector (Kiessling et al., 2017). Furthermore, marine litter on Salas and Gómez imposes a severe risk of entanglement for several seabird species breeding on the island (Miranda-Urbina et al., 2015).

The distribution of floating microplastics in the SE Pacific shows the typical distribution documented for other ocean basins (Eriksen et al., 2014; Law, 2017), with highest concentrations in the subtropical gyre (Eriksen et al., 2013, 2018). Microplastic densities in the HCS generally remain far below the densities reported from the gyre (**Figure 2**; NO, unpublished data). This distribution pattern is also well reflected in concentrations of small plastics from sandy beaches in the SE Pacific, where beaches along the HCS feature moderate densities, while sandy beaches on Rapa Nui have very high densities (Hidalgo-Ruz and Thiel, 2013). This pattern, with the abundances of microplastics increasing with distance from the continental coast (**Figure 2**),

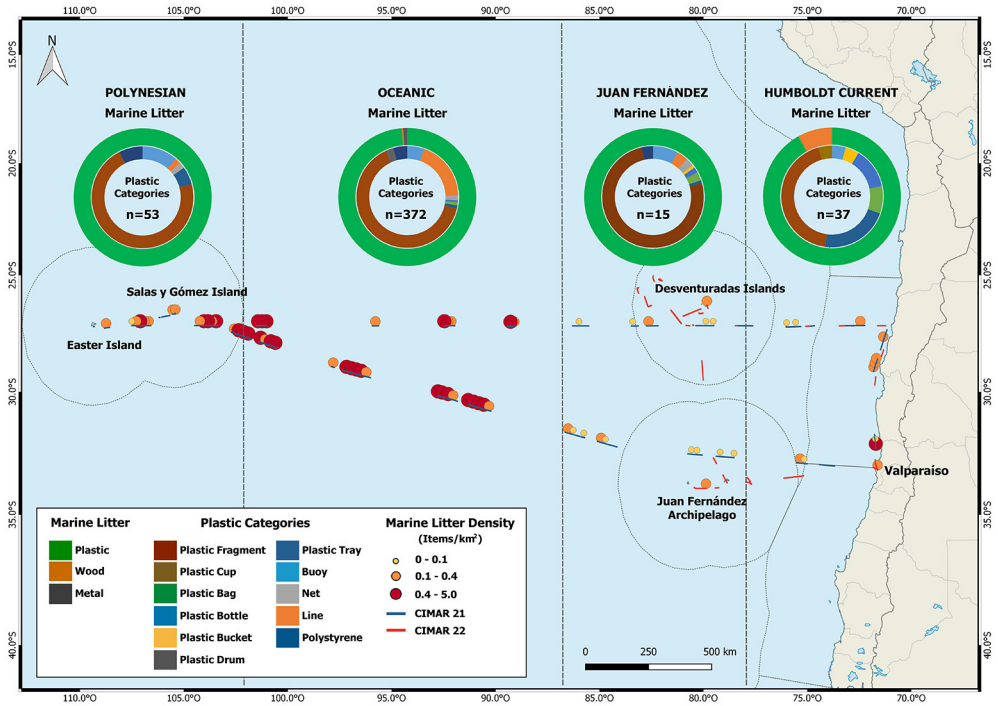

FIGURE 1 | Types and density of marine debris in different sectors of the central SE Pacific, in 2015 (CIMAR 21) and 2016 (CIMAR 22). Open circles at the top indicate the proportions of different plastic types; numbers inside the circles represent the total of items observed in each sector. Dots show the density of marine debris from visual ship surveys (for details see Miranda-Urbina et al., 2015). Thin lines show the Exclusive Economic Zone.

is also suggestive of progressive fragmentation of large plastics during their journey toward the SPSG.

Interactions of Marine Vertebrates From the SE Pacific With Plastic Litter

A large number of marine vertebrate species from the SE Pacific were documented to have interacted with marine litter, including fishes, seabirds, sea turtles, and marine mammals (Thiel et al., 2011; Miranda-Urbina et al., 2015; Ory et al., 2017). In revising and compiling information from diverse sources we found that marine litter affects at least 97 different species (see **Table 1** and text below for details). These reports comprise those of entanglement (including incorporation of plastics in seabird nests) and the ingestion of plastics.

Fishes

The few studies documenting the interaction of microplastics with fishes in the SE Pacific revealed a high incidence of microplastics in planktivorous fish from the coast of Rapa Nui, located within the SPSG: 80% of the examined individuals of the amberstripe scad *Decapterus muroadsi* (Ory et al., 2017) and 14% of the flying fish *Cheilopogon rapanouienesis* (Chagnon et al., unpublished data) had ingested microplastics, many of which were similar in size and color to blue-pigmented planktonic organisms (copepods, crustacean larvae), which are common prey of these fish (**Figure 3B**). These findings suggest that visually-oriented planktivorous fish mostly ingest microplastics resembling their prey. The study of the digestive tracts of 7 planktivorous fish species from the HCS revealed that, overall, few of the fish analyzed (∼2%) had ingested microplastics (Ory et al., 2018). Microplastics were also found more often (23% of all individuals analyzed) in herbivorous fish from Rapa Nui than in bottom-dwelling carnivorous and herbivorous fish species from the HCS (**Table 1**; NO, unpublished data). Such contrasting ingestion of microplastics well reflects the observed gradient of low microplastic densities in surface waters along the SE Pacific continental coast and high densities within the SPSG (Eriksen et al., 2013, 2014, 2018), and in particular along the Rapa Nui coast (Ory et al., 2017; Eriksen et al., 2018).

Apex predators play an important role in the exchange of energy between upper trophic levels in the marine environment (Markaida and Sosa-Nishizaki, 2010). However, little is known about the interaction of these species with AMD (Choy and Drazen, 2013). Plastics have been documented in the stomachs of tuna from Rapa Nui (**Table 1**), but little is known about plastic ingestion by other piscivorous fish from the open ocean. Publications on the diet of piscivorous fishes from the continental coast have not reported plastics (Fariña and Ojeda, 1993; Fariña et al., 2000), which indeed have not been found in the stomach contents of the investigated fish species (Jose Miguel Fariña, personal comment). Similarly, plastic ingestion by oceanic sharks seems to be very rare, estimated to occur in <1% of all analyzed stomachs (Sebastian Klarian, personal comment). Plastics found in sharks were PVC fragments in deep-sea species, and (positively

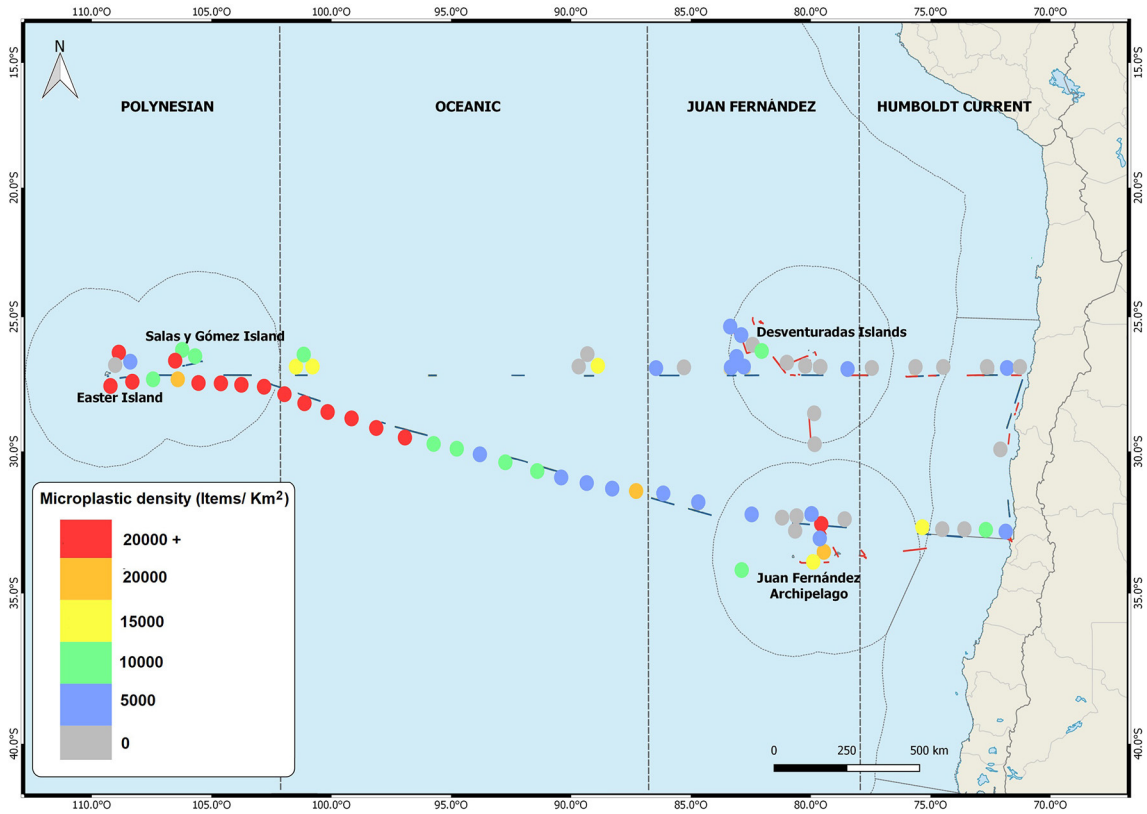

FIGURE 2 | Density of microplastics (0.3–5 mm) in the central SE Pacific, in 2015 (CIMAR 21) and 2016 (CIMAR 22). Data from Eriksen et al. (2013, 2018) and MT, unpublished data. Thin lines show the Exclusive Economic Zone.

buoyant) bottle and pen caps in coastal species (S. Klarian, personal comment; **Figure 3D**).

Reports on entangled fish are scarce, but there are three notable exceptions. In December 2016, an entangled Pacific chub *Kyphosus sandwicensis* was observed in a derelict fishing net found floating in the SPSG (28°23′S, 105°42′W) (**Table 1**). There were several living chubs swimming around the mass of net and ropes, and it is likely that the individual was trapped and died while this raft floated in the gyre. On the coast of Rapa Nui a surgeon fish *Acanthurus leucopareius* was entangled in a fishing line (NL, personal observation). The other case of an entangled fish is of a juvenile Galapagos shark *Carcharhinus galapagensis* that was documented in the waters around Rapa Nui. In June 2017 one specimen (about 160 cm in total length) of *C. galapagensis* was recorded swimming in shore waters with a plastic collar-like debris obstructing its gill region (**Figure 4A**). The animal appeared otherwise healthy, presenting an active feeding behavior. It showed initial tissue damage, which indicates that the collar recently became attached to the animal; similar records have shown that the internal projections of plastic collars have the potential to severely damage the tissue by affecting normal feeding and ventilation (Sazima et al., 2002), followed by body deformations as the animal grows (Wegner and Cartamil, 2012). The plastic ring attached to the animal was identified as the screwing part of a plastic barrel; this type of plastic debris is most likely coming from the industrial fishery that operates in the S Pacific and is an important source of recognizable plastic litter stranding on the shores of Rapa Nui (Kiessling et al., 2017).

Continental coastal species of Chondrichthyes are also known to interact with marine litter. Oviparous species like cat sharks (Scyliorhinidae) and skates from the genus *Sympterygia* lay egg capsules with long tendrils (Oddone and Vooren, 2002, 2008; Hernández et al., 2005; Flammang et al., 2007; Concha et al., 2013). These long tendrils are used to entangle the egg capsule to different substrata in order to maintain the vertical positioning and facilitate oxygen flow (Flammang et al., 2007). Along the central coast of Chile, dense multispecies coils of capsules are commonly found firmly attached to algae and/or plastic debris floating close to the coast or stranded on the shores after storms (MT and NM, personal observations).

Sea Turtles

Sea turtles are exposed to a variety of anthropogenic stressors, including marine plastic pollution, because of their use of diverse habitats, migratory behavior, and complex life histories (Nelms et al., 2016). Indeed, litter ingestion and entanglement in plastic debris have been recognized as serious threats to these species worldwide (Nelms et al., 2016; Clukey et al., 2017; Duncan et al., 2017). Five sea turtle species inhabit the SE Pacific (*Caretta caretta, Chelonia mydas, Dermochelys coriacea,*

TABLE 1 | Reports on entanglement with macroplastics or plastic ingestion by marine vertebrates from open ocean (OO) and continental coastal (CC) (<5 nm from the land) waters of the southeast Pacific, based on literature, or anecdotal reports.

Species	Diet	Environment	Effect		Source
			Ent	Ing	
FISHES					
Acanthurus leucopareius	H	OO	+		Video by Nicolas Luna
Aplodactylus punctatus	H	CC		+	Nicolas Ory, unpubl. data
Auchenionchus microcirrhis	C	CC		+	Mizraji et al., 2017
Brama australis	P	CC		+	Nicolas Ory, unpubl. data
Carcharhinus galapagensis	C	OO	+		Video by Naiti Morales
Cetengraulis mysticetus	P	CC		+	Ory et al., 2018
Cheilodactylus variegatus	C	CC		0	Nicolas Ory, unpubl. data
Cheilopogon rapanouiensis	P	OO		+	Chagnon et al., unpubl. data
Decapterus muroadsi	P	OO		+++	Ory et al., 2017
Engraulis ringens	P	CC		+	Ory et al., 2018
Girella laevifrons	O	CC		+	Mizraji et al., 2017
Graus nigra	C	CC		+	Mizraji et al., 2017
Helcogramoides chilensis	C	CC		+	Mizraji et al., 2017
Kyphosus sandwicensis	H	OO	+		Photo by Tim Kiessling
Kyphosus sandwicensis	H	OO		++	José Abalos, unpubl. data
Odontesthes regia	P	CC		+	Ory et al., 2018
Opisthonema libertate	P	CC		+	Ory et al., 2018
Pinguipes chilensis	C	CC		0	Nicolas Ory, unpubl. data
Prionace glauca	C	OO		+	Photo by Carlos Canales-Cerro
Sardinops sagax	P	CC		0	Ory et al., 2018
Scarthychthys viridis	H	CC		+	Mizraji et al., 2017
Scomber japonicas	P	CC		+	Ory et al., 2018
Sebastes capensis	C	CC		+	Nicolas Ory, unpubl. data
Strangomera bentincki	P	CC		0	Ory et al., 2018
Thunnus albacares	C	OO		+	Chagnon et al., unpubl. data
SEA TURTLES					
Caretta caretta	O	OO	+	0	Duncan et al., 2017; Photo by Camila González
Chelonia mydas	O	CC	+	++	Brito, 2001; IMARPE, 2011; Alemán, 2014; Duncan et al., 2017; Jiménez et al., 2017; Photo by Guerra-Correa et al., 2007
Dermochelys coriacea	O	OO	+	+	Brito, 2001; IMARPE, 2011; Duncan et al., 2017
Eretmochelys imbricata	O	CC	+	+	Brain et al., 2015; Duncan et al., 2017; Photo by Anita Espinoza
Lepidochelys olivacea	O	CC-OO	+	++	Brito, 2001; IMARPE, 2011; Alemán, 2014; Duncan et al., 2017; Photos by Miguel Angel Mansilla, Rubén Alemán
SEABIRDS					
Ardenna griseus	Pis	CC-OO		+	Ainley et al., 1990; Spear et al., 1995
Ardenna pacifica	Pis	OO		+	Ainley et al., 1990; Spear et al., 1995
Ardenna creatopus	Pis	CC-OO		+	Cañoles et al., 2017; Photo by OIKONOS Chile
Cinclodes nigrofumosus	I	CC	+		Photo by Carolina Henríquez
Daption capense	Pis	OO		+	Ainley et al., 1990
Fregata minor	Pis	OO	n+		Miranda-Urbina et al., 2015; Luna Jorquera, unpubl. data
Fregetta grallaria	P	OO		+	Ainley et al., 1990; Spear et al., 1995
Gygis alba	Pis	OO		+	Spear et al., 1995
Haematopus ater	I	CC	+		Photo by Matías Portflitt-Toro
Haematopus palliatus	I	CC	+		Photo by Pedro Valencia
Larosterna inca	Pis	CC	+		Photo by Fernanda Barilari
Larus belcheri	Pis	CC	+		Photo by Ana García
Larus dominicanus	Pis	CC		+	Ludynia et al., 2005
Larus dominicanus	Pis	CC	n+		Thiel et al., 2011; Arce et al., 2014; Photo by Matías Portflitt-Toro, Pedro Valencia, Katherine Muñoz, Angélica Contador, Shannon Montecinos, Jorge Rivera Torres

TABLE 1 | Continued

Species	Diet	Environment	Effect		Source
			Ent	Ing	
Leucophaeus modestus	Pis/I	CC	+		Photo by Matías Portflitt-Toro, Andrés Puiggros
Leucophaeus scoresbii	C/Pis/I	CC	+		Photo by Cristían Larrere
Macronectes sp.	C/Pis/I	OO	+		Photy by Paulo Davalos—Revista Trile
Nesofregetta fuliginosa	P	OO		+	Miranda-Urbina et al., 2015; Luna Jorquera, unpubl. data
Nesofregetta fuliginosa	P	OO	n		Luna Jorquera, unpubl. data
Oceanodroma leucorhoa	P	OO		+	Ainley et al., 1990; Spear et al., 1995
Oceanodroma markhami	P	OO		+	Ainley et al., 1990; García-Godos et al., 2002
Oceanodroma tethys	P	OO		+	Ainley et al., 1990; Spear et al., 1995
Onychoprion fuscatus	P	OO		+	Spear et al., 1995
Pachyptila belcheri	P	OO		+	Ainley et al., 1990
Pachyptila vittata	P	OO		+	Matías Portflitt-Toro, unpubl. data
Pelagodroma marina	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pelecanoides garnotii	P	CC		+	Luna-Jorquera in: Thiel et al., 2011
Pelecanoides urinatrix	P	CC		+	Ryan, 1987
Pelecanus thagus	Pis	CC	n+		Thiel et al., 2011; Arce et al., 2014
Phaethon rubricauda	Pis	OO		+	Guillermo Luna-Jorquera, unpubl. data
Phaethon rubricauda	Pis	OO	n+		Miranda-Urbina et al., 2015
Phalacrocorax atriceps	Pis	CC	n		Photo by Jorge Navarro
Phalacrocorax bougainvillii	Pis	CC	+		Photo by Carlos Vallejos
Phalacrocorax bougainvillii	Pis	CC		+	Carlos Zavalaga, unpubl. data
Phalacrocorax brasilianus	Pis	CC	n+		Thiel et al., 2011; Arce et al., 2014; Photo by Victor Rios
Phalacrocorax gaimardi	Pis	CC	n		Fernández et al., 2011; Photo by Ivan Torres, Paola Araneda, Manuel Segovia
Procellaria aequinoctialis	I	OO		+	Ainley et al., 1990, Matías Portflitt-Toro, unpubl. data
Procelsterna albivitta	Pis	OO		+	Luna Jorquera, unpubl. data
Procelsterna albivitta	Pis	OO	n+		Luna Jorquera, unpubl. data
Pseudobulweria rostrata	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma brevipes	Pis/I	OO		+	Spear et al., 1995
Pterodroma cervicalis	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma defilippiana	Pis/I	OO		+	Ainley et al., 1990
Pterodroma externa	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma leucoptera	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma longirostris	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma nigripenis	I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma pycrofti	I	OO		+	Ainley et al., 1990; Spear et al., 1995
Pterodroma ultima	Pis/I	OO		+	Spear et al., 1995; Luna-Jorquera, unpubl. data
Puffinus bulleri	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995
Puffinus nativitatis	Pis/I	OO		+	Ainley et al., 1990; Spear et al., 1995; Luna-Jorquera, unpubl. data
Rynchops niger	Pis	CC	+		Photo by Pedro Valencia
Spheniscus humboldti	Pis	CC		+	Photo by Matías Portflitt-Toro; Carlos Zavalaga unpubl. data
Spheniscus humboldti	Pis	CC	n		Arce et al., 2014
Stercorarius longicaudus	C/Pis/I	OO		+	Spear et al., 1995
Sternula lorata	Pis	CC	+		Thiel et al., 2011
Sula variegata	Pis	CC	+		Thiel et al., 2011; Photo by Issa Ramos
Thalassarche chrysostoma	Pis/I	OO		+	Cristían Suazo, unpubl. data
Thalassarche chrysostoma	Pis/I	OO	n		Cristían Suazo, unpubl. data
Thalassarche melanophrys	Pis/I	OO		+	Cristían Suazo, unpubl. data
Thalassarche melanophrys	Pis/I	OO	n		Photo by Cristían Suazo
MAMMALS					
Arctocephalus philippi	C	CC	+		Thiel et al., 2011, Photo by Lukas Mekis

(Continued)

TABLE 1 | Continued

Species	Diet	Environment	Effect		Source
			Ent	Ing	
Balaeonoptera bonaerensis	C	CC	+		Campbell et al., 2017, Photo by Aldo S. Pacheco
Balaeonoptera edeni	C	CC	+		Campbell et al., 2017
Balaeonoptera musculus	C	CC	+		Campbell et al., 2017
Cephalorhynchus commersonii	C	CC	+		Aguayo-Lobo, 1999
Cephalorhynchus eutropia	C	CC	+		Aguayo-Lobo, 1999
Delphinus capensis	C	CC	+		Mangel et al., 2013
Eubalaena australis	C	CC	+		Aguayo-Lobo, 1999
Globicephala spp.	C	CC	+		Mangel et al., 2013
Grampus griseus	C	CC-OO		+	Photo by Macararena Bravo
Lagenorhynchus australis	C	CC	+		Aguayo-Lobo, 1999
Lagenorhynchus obscurus	C	CC	+		Mangel et al., 2013
Lissodelphis peronii	C	CC	+		Aguayo-Lobo, 1999
Lontra felina	C	CC	+		Photo by Fernando Olivares, Juan Valqui
Megaptera novaeangliae	C	CC	+		Photo by Aldo S. Pacheco
Otaria byronia	C	CC	+		Photo by Claudio Godoy, Aldo Pacheco, Natalie Pozo, Mauricio Ulloa, ONG Vuelve al Océano
Phocoena spinipinnis	C	CC	+		Mangel et al., 2013
Physeter macrocephalus	C	CC	+		Campbell et al., 2017
Tursiops truncatus	C	CC	+		Mangel et al., 2013

Information based on photographic evidence is given together with the name of the author of the photograph or video; all photographers agreed that this information be included in this table and publication. Abundance categories: + present, ++ common, +++ very frequent. 0 means that the species was examined and no plastic items were found in its stomach; n = seabirds reported with plastics entangled in nests, n+ = individuals and nests with plastic entanglement; please, note that herein we consider incorporation of plastics in seabird nests also as a case of entanglement (see also Supplement 1). The Diet can be: C, Carnivorous; H, Herbivorous; I, Invertivorous; O, Omnivorous; P, Planktivorous; and Pis, Piscivorous.

Eretmochelys imbricata, and *Lepidochelys olivacea*); all are listed from vulnerable to critically endangered on the IUCN Red List (IUCN, 2018) and have documented interactions with marine litter.

The green turtle (*C. mydas*) is the species most commonly mentioned to have ingested plastic items, with a frequency ranging from 28% in the Ecuadorian part of the HCS (Alemán, 2014) to 56 and 91% in Peru (Alfaro-Shigueto et al., 2005; Jiménez et al., 2017). The olive ridley turtle (*L. olivacea*) also has a high incidence of plastic ingestion, reaching up to 43% in Ecuador (Alemán, 2014), but this species has a lower incidence in other parts of the HCS (8%), both in Peru and southern Chile (de Paz et al., 2005; Brito et al., 2007). Furthermore, specific cases of plastic ingestion have been reported for leatherback turtles (*D. coriacea*) from the HCS in southern Peru and central Chile (Brito, 2001; IMARPE, 2011) and a hawksbill turtle (*E. imbricata*) in Rapa Nui (Brain et al., 2015).

Items most commonly found in stomachs or intestines of sea turtles are plastic pieces of intermediate size, including plastic bags, monofilament nylon, rope, and fishing nets (Brito, 2001; Guerra-Correa et al., 2007; IMARPE, 2011; Jiménez et al., 2017; **Figure 3C**). Several authors suggested that plastic ingestion has been the cause of death of stranded turtles in Ecuador and Chile (Brito et al., 2007; Silva et al., 2007; Alemán, 2014).

Even though many studies have focused on evaluating sea turtle bycatch rates (in active fishing gear) in the Pacific Ocean (Wallace et al., 2010), to date almost no reports exist on sea turtle entanglements (derelict fishing gear) in the region (Nelms et al., 2016). In fact, to our knowledge no peer-reviewed articles have informed incidents of entanglements from the SE Pacific. In Rapa Nui, a case of *C. caretta* with fishing line in both anterior flippers caused their amputation and subsequent death a few hours later (RAV, personal observation). In addition, several cases of entanglements of green and olive ridley turtles have been informed from Ecuador (Rubén Alemán, personal communication).

Seabirds

Many different seabird species have been reported to be entangled in marine debris or have ingested plastic (**Table 1**). Interestingly, for most fish-feeding species from the HCS, the incidence of individuals with microplastics in their stomachs is low, although these species face other threats to their conservation (Luna-Jorquera et al., 2012). We found 6 species in which plastic litter has been found in their stomachs, 3 of them (*Pelecanoides garnotii*, *P. urinatrix*, *Phalacrocorax bougainvillii*, and *Spheniscus humboldti*) being true diving species, and one a plunge diver (*Pelecanus thagus)*. One species with relatively high frequency of plastic ingestion is the kelp gull *Larus dominicanus*, which is commonly observed feeding in fishing ports, at garbage containers, and on waste disposal facilities.

In addition to the low number of continental species with plastic in their stomach, it seems that the number of affected individuals per species is relatively low: 10 out of 450 examined individuals (2.2%) *S. humboldti*, 4 out of 103 (3.9%) *Pelecanoides garnotii,* and 12 out of 363 studied pellets (3.3%) of *Phalacrocorax bougainvillii* (CZ, unpublished data). The diet of the Humboldt penguin *S. humboldti* has also been examined in another study, but the authors did not mention any plastic items in the species' stomach contents (Herling et al., 2005). The tendency of low incidence of plastic ingestion in seabirds inhabiting the HCS is supported by several other studies. Jahncke et al. (1997) and García-Godos and Goya (2006) studied the diet of *P. bougainvillii*, *Sula variegata,* and *Pelecanoides garnotii,* and did

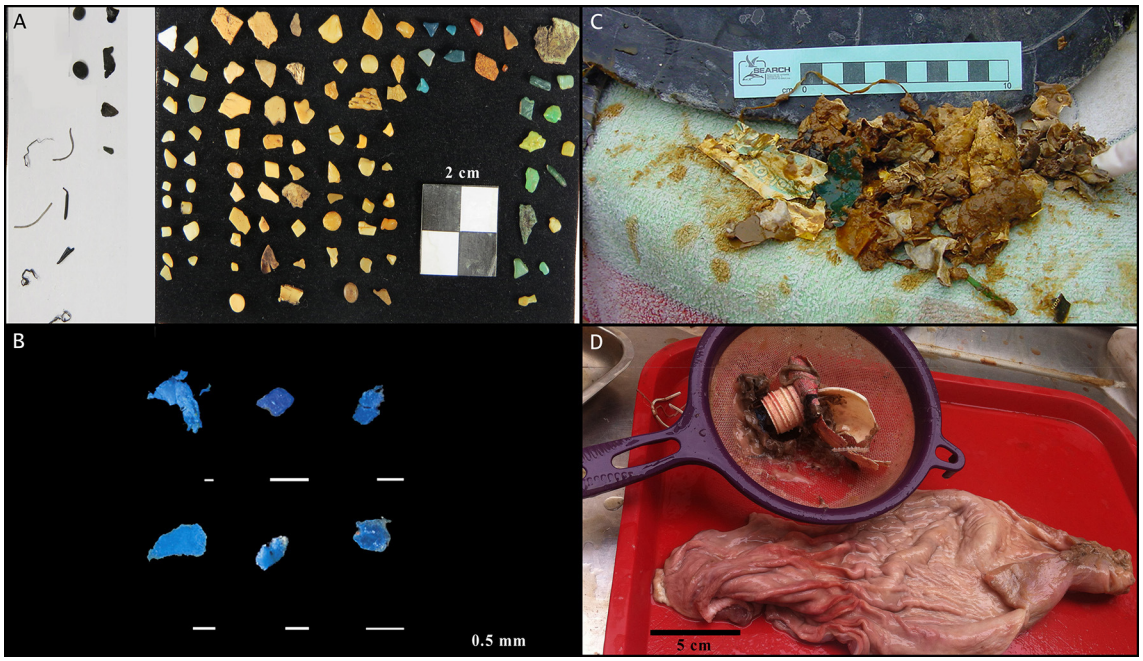

FIGURE 3 | Examples of ingestion of plastic litter by marine species. **(A)** Microplastics collected from the stomach of one Christmas shearwater *Puffinus nativitatis* found dead on Salas y Gómez Island; Image by Matías Portflitt Toro. **(B)** Blue microplastics found in the stomach of amberstripe scads *Decapterus muroadsi*, fished at Rapa Nui; Image by Nicolas Ory. **(C)** Meso- and macroplastics found obstructing the cloacal opening of a green turtle *Chelonia mydas* found near Antofagasta. After carefully taking out these items the turtle recovered and later was liberated by the Wildlife Rescue and Rehabilitation Centre (Centro de Rescate y Rehabilitación de Fauna Silvestre) from Universidad de Antofagasta, Chile; Photo by Guerra-Correa et al. (2007). **(D)** Meso- and macroplastics found in the stomach of a blue shark *Prionace glauca*; Image courtesy of Carlos Canales-Cerro.

not find any plastic artifacts in any of the examined individuals (I. García-Godos, personal communication).

In contrast to continental seabirds, oceanic species are severely affected (see **Table 1**). Of the 37 seabird species from the SE Pacific with reported ingestion of plastic, 31 are oceanic and the majority are Procellariiformes, which retain particles for several months before the particles are evacuated from the digestive system by regurgitation (Terepocki et al., 2017; **Figure 3A**). The high incidence of microplastic ingestion in the species listed in **Table 1** is very likely due to the high concentration of AMD observed in the Oceanic and Polynesian sectors of the SE Pacific (see **Figure 2**). Several harmful effects have been reported due to microplastic ingestion, ranging from stomach ulcers, intestinal obstruction, reduced body condition, and increased contaminant load (Derraik, 2002; Lavers et al., 2014).

Body injuries, severe negative effects on behavior, and even mortality, are typical consequences of seabird entanglement in floating or stranded marine litter. As opposed to the observed tendency of plastic ingestion, entanglement mostly affects continental species. Seventeen seabird species inhabiting the HCS are affected by entanglement, which most of the time occurs during foraging activities at sea, when seabirds are trapped in discarded fishing lines, derelict fishing nets (**Figure 4C**) (Thiel et al., 2011), and single-use plastic bags on beaches (see Supplements 2, 3). We have also observed that lines and rope fragments discarded by aquaculture activities affect coastal species, such as gulls and cormorants (**Figure 4C**) (MPT and GLJ, own unpublished data). Entanglement in derelict fishing nets is also affecting diving waterbirds, including grebes (*Podilymbus podiceps*, Photo PP01 in Supplement 3 by *P. Valencia*, and *Podiceps occipitalis*, Portflitt-Toro et al., 2016). During the winter season, grebes are often observed foraging in nearshore waters in the bays of the Coastal System of Coquimbo, where fishermen use gillnets to capture pelagic fishes.

A handful of seabird species are affected by both entanglement and ingestion (*Larus dominicanus* and *Phalacrocorax bougainvilli*), but the negative effects of marine litter are also reaching the nests of several species. The incidence of anthropogenic marine litter for nest construction is not well known for the SE Pacific, but gulls from the Peruvian coast have been reported to use plastic in their nests (Stucchi and Figueroa, 2006). Our revision revealed that 12 species are using litter for nest construction, comprising both continental and oceanic seabirds; at least 10 of those 12 species are also exposed to organism entanglement and plastic ingestion at sea. Species like cormorants (*Phalacrocorax* spp.), frigatebirds (*Fregata minor*), and albatrosses (*Thalassarche* spp.) are actively selecting and transporting marine litter to their nests, while other species inhabiting the oceanic Salas and Gómez Island are affected by plastic litter accumulated by oceanic currents near their foraging grounds. Abundant incorporation of plastic litter in nests (see **Figure 4D**) calls for further research due to (i) the

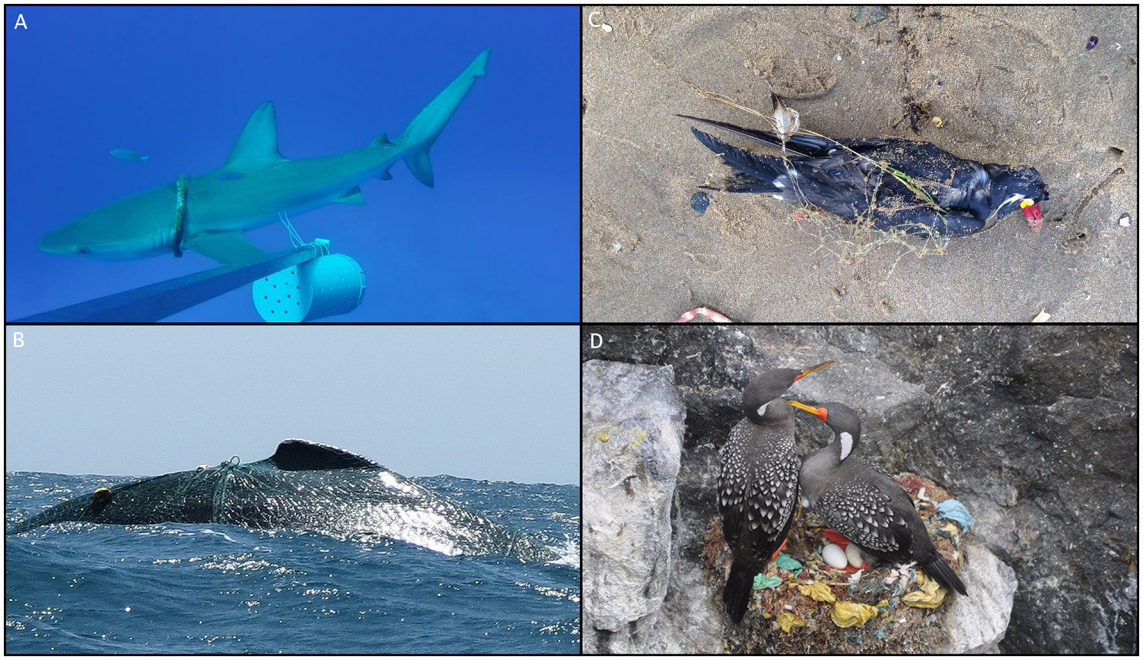

FIGURE 4 | Examples of species entangled with marine plastic litter. **(A)** Galapagos shark *Carcharhinus galapagensis* from coastal waters of Rapa Nui (Easter Island) entangled with a closure ring for plastic drums; Image Naiti Morales. **(B)** Humpback whale *Megaptera novaeangliae* from the Peruvian coast entangled in large fishing net; Image Aldo Pacheco. **(C)** Inca tern *Larosterna inca* found entangled in remains of fishing net near Valparaiso; Image courtesy of Fernanda Barilari. **(D)** Red-legged cormorant *Phalacrocorax gaimardi* from Taltal in nest; Image courtesy of Ivan Torres.

risk of entanglement of adults and chicks, and (ii) the potential effects of anthropogenic litter on the thermal properties of the nest both during nesting and chick-rearing phases.

Marine Mammals

There is no published information regarding the ingestion of marine debris for marine mammals in the SE Pacific. Overall, few studies examined the stomach contents of marine mammals. In a study on the diet of the South American sea lion *Otaria flavescens*, George-Nascimento et al. (1985) did not report any plastic items in stomach contents. Feces from the South American sea otter *Lontra felina* contained a diverse range of prey remains from fishes, crustaceans, and molluscs, but no plastic items were listed (Medina-Vogel et al., 2004; Córdova et al., 2009). A study of four delphinid species captured along the central coast of Peru reported prey items in stomach contents based on fish otoliths and cephalopod beaks, but no marine debris was documented (García-Godos et al., 2007). In southern Chile, an examination of seven stranded false killer whale carcasses reported only empty stomachs (Haro et al., 2015). Similarly, a study on the diet of long-finned pilot whales from southern Chile (Mansilla et al., 2012) found no plastic particles or marine litter (Carlos Olavarría, personal communication). However, the case of a Risso's dolphin *Grampus griseus* with a plastic bag in its mouth (Photo by M. Bravo) in the Coastal System of Coquimbo, in northern-central Chile, shows that plastic ingestion by cetaceans should not be ruled out.

Reports on the entanglement of marine mammals along the SE Pacific are much more common than reports about plastic ingestion. Throughout the HCS, fisheries are very intense at both artisanal and industrial levels (Thiel et al., 2007; Alfaro-Shigueto et al., 2010), and many of the observed entanglements are likely to have occurred with active fishing gear. An important number of pinnipeds, large baleen whales, and Odontocetes in general (dolphins, porpoises, and toothed whales) have been reported entangled in fishing gear throughout neritic and oceanic waters off Peru and Chile (**Table 1**). Entanglements may occur when large gillnets are deployed at the bottom or drifting near the sea surface, depending on the fishery (Aguayo-Lobo, 1999; Alfaro-Shigueto et al., 2010). Marine mammals swim or dive through the mesh and become entangled. Small animals, such as sea otters and sea lions, may die shortly after entanglement, as these species may not have the necessary strength to escape from nets and being air-breathers will consequently drown. This is probably also the case for dolphins such as the dusky dolphin (*Lagenorhynchus obscurus*), bottlenose dolphin (*Tursiops truncatus*), long-beaked common dolphin (*Delphinus capensis*), and the Burmeister porpoise (*Phocoena spinipinnis*) in Peru. In addition to the aforementioned species, in Chile the southern right whale dolphin (*Lissodelphis peronii*), Commerson's dolphin (*Cephalorhynchus commersonii*), Chilean dolphin (*Cephalorhynchus eutropia*), and Peale's dolphin (*Lagenorhynchus australis*) also suffer mortality from entanglements (**Table 1**).

Large baleen whales get into nets but may keep moving, subsequently carrying large pieces of entangled nets on their body. Along the HCS of Peru, the humpback whale *Megaptera novaeangliae* is the most affected large whale species. Campbell et al. (2017) reported that 51% of stranded whales off Peru were humpback whales and all individuals showed evidence of entanglement (**Figure 4B**). Observations made during daily sightings of humpback whales throughout their breeding migration (mid July to late October, Guidino et al., 2014) in northern Peru (∼4°S) suggest that in the HCS, humpback whales frequently get entangled with drifting gillnets (ASP, unpublished data). As the stranding data suggest (Campbell et al., 2017), the fate of entangled whales is often death, unless the net is quickly removed from the whale.

Along the HCS of Chile, artificial coastal structures such as breakwaters and harbors appear to accumulate more marine debris than the natural rocky intertidal shore (Aguilera et al., 2016). This accumulation could be a problem for the marine otter (*Lontra felina*), a small mustelid endemic along the Pacific coast of South America. This species uses natural rocks or artificial constructions as habitat. At the northern-most location of its distributional range in northern Peru, the marine otter has been observed resting in an artificial cave full of marine debris (see Figure 3 in Alfaro-Shigueto et al., 2011). While this case does not constitute a direct interaction with marine plastic litter, the risk for entanglement or ingestion is evident.

Overview of Plastic Ingestion and Entanglement by Marine Vertebrates From the SE Pacific

This first review of interactions with AMD of marine vertebrates from the SE Pacific reported a total of 97 species (**Table 1**). Seabirds represent nearly 55% of the total, followed by fishes with 21%, marine mammals with 19%, and sea turtles with 5%. Considering the number of species per taxonomic group, the type of interaction (ingestion or entanglement), and environment (continental or oceanic), an overall pattern is emerging. For fishes, more species with plastic ingestion were documented along the continental coast than in the open ocean, and few oceanic species become entangled near oceanic islands (Rapa Nui and Salas and Gómez Island). However, it needs to be taken into account that the simple species list presented in **Table 1** only shows whether a species had ingested microplastics or not, which does not allow for inferences on the individual risk of ingestion. For example, in the case of the Peruvian anchoveta *Engraulis ringens* from the HCS, of 116 studied individuals only one (<1%) had one microplastic in its stomach (Ory et al., 2018), whereas of 20 amberstrip scads *Decapterus muroadsi* from Rapa Nui 16 individuals (80%) had ingested on average 2.5 microplastics per individual (Ory et al., 2017), underscoring that the risk of microplastic ingestion is much higher for oceanic planktivorous species than for species from the HCS. In contrast to the general pattern documented for fishes, the incidence of microplastic ingestion in seabirds is much higher for oceanic species than for those from the HCS, but the latter are suffering intense entanglement (**Table 2**). Marine mammals are scarce in oceanic waters, so marine mammals are principally affected by entanglement with AMD floating in the productive waters of the HCS (**Table 2**). Regarding sea turtles, none of the species from the SE Pacific are safe from interactions with AMD, ingestion or entanglement, and thus are equally threatened in coastal and oceanic waters. This overall pattern agrees well with the spatial distribution of micro- and macroplastics in the SE Pacific (see **Figures 1**, **2**). Future systematic studies in the SE Pacific should provide more information about the actual number of species affected, which will also help authorities to improve efforts for efficient solutions.

Other Interactions

Similar to other oceans (Carson, 2013), in the SE Pacific litter is frequently bitten by large marine organisms, which are thought to be vertebrates (Eriksen et al., 2017), but might also originate from large invertebrates with powerful jaws, in particular squids and/or cuttlefish. On Rapa Nui, up to 10% of stranded AMD can have bitemarks (MT, unpublished data).

DISCUSSION

Ingestion of Plastics

The results from this review indicate that microplastic ingestion is uncommon along the Pacific coast of South America. Neither fishes nor seabirds from the continental coast had

TABLE 2 | Number of species of marine vertebrates for which ingestion and entanglement has been documented.

Group	Entanglement			Ingestion			Entang. and Ingest.			Total
	Continental	Oceanic	Continental and Oceanic	Continental	Oceanic	Continental and oceanic	Continental	Oceanic	Continental and oceanic	
Fishes	0	2	0	13	4	0	0	1	0	20[a]
Sea turtles	0	1	0	0	0	0	2	1	1	5
Seabirds	14	2	0	3	24	2	3	5	0	53
Marine mammals	18	0	0	0	0	1	0	0	0	19

*Total refers to the numbers of species that have been reported for plastic ingestion and entanglement (based on the data presented in **Table 1**).*
*[a]This value does not consider four fish species that were examined but in which no plastic was found in their stomachs (see **Table 1**).*

high frequencies of microplastic ingestion, and information on the diet of marine mammals in the SE Pacific is very limited and thus does not allow inference on the risk of microplastic ingestion. An exception from this pattern seems to be the relatively high incidences of plastic ingestion in sea turtles in the HCS, but most of those plastics are of larger sizes and can be characterized as meso- and macroplastics. Also, intertidal habitats, such as beaches, tidepools, estuarine saltmarshes, and especially the seashores in the fjords of southern Chile require future research attention, because intense microplastic pollution may cause localized impacts in species from shore habitats (see e.g., Mizraji et al., 2017).

The low incidences of microplastic ingestion in most marine vertebrates from the HCS could be resulting from low concentrations of microplastics in coastal waters or due to specific foraging behaviors, or a combination of both. A data comparison from the SPSG and from other parts of the world can shed some light on these questions. For example, some planktivorous fish species from the SPSG had a very high frequency of microplastic ingestion (Ory et al., 2017), and other species had ingested microplastics more frequently than any of the planktivorous species from the HCS (Chagnon et al., unpublished data; Ory et al., 2018). For seabirds, the pattern was similar: microplastic ingestion was much more common in oceanic species than in species from the HCS (**Tables 1**, **2**), regardless of feeding types (planktivorous, invertivorous, and piscivorous). This suggests that the observed pattern might not be due to differences in foraging and feeding behaviors, but rather to differences in microplastic abundances. Nevertheless, plastic ingestion was also documented in a number of species from the HCS, which might be consequence of their biology. For example, incidence of plastics in the digestive system of different seabird species is related to their foraging behavior (e.g., surface feeders, pursuit feeders, among others) and the digestive system morphology (i.e., muscular gizzard in petrels) (Ainley et al., 1990; Spear et al., 1995; Roman et al., 2016). This latter aspect causes indigestible items to become trapped in the bird's gut system (Ainley et al., 1990).

Other factors such as the geographic distribution may impact the amount of plastic ingested, as found by Spear et al. (1995), who reported that seabirds foraging predominantly in the North Pacific had higher incidence of ingestion than species from the South Pacific. However, the paper suggested that this pattern may be biased by the lack of studies from the SE Pacific. This is very similar for marine mammals, in particular dolphins and whales. Since the end of whaling in the region during the early 80's, the scientific examination of body parts (including stomachs) of large baleen and sperm whale carcasses have considerably ceased in the region. Scientific treatment of stranded animals is limited due to the difficult logistics needed to examine cases of cetacean strandings in remote areas (e.g., Haro et al., 2015; Häussermann et al., 2017). In this regard, there is an urgent need for the implementation of effective action plans for the scientific treatment of stranded whales and dolphins, if we aim to understand the impacts of the ingestion of marine litter in megafauna. This is particularly important since researchers are adopting non-invasive methods (e.g., stable isotopes, DNA analysis) for both live (Haro et al., 2016) and stranded charismatic animals. At present, it is difficult to understand the magnitude of the plastic ingestion problem for marine mammals in the SE Pacific. However, plastic ingestion by diverse cetacean species has been reported in several coastal areas elsewhere (Baulch and Perry, 2014; Lusher et al., 2018), often with lethal consequences (see Jacobsen et al., 2010; de Stephanis et al., 2013; for cases on sperm whales). Microplastics of several polymer types have been documented for the first time in a humpback whale stranded on the coast of the Netherlands (Besseling et al., 2015). In the SE Pacific, the problem is likely being underestimated.

The relatively high frequency of bitemarks in plastics stranded on Rapa Nui shores indicates that some species directly bite into floating plastics. It is currently not well known which species engage in this behavior and why (Carson, 2013), but most of the bitemarks found on plastics from Rapa Nui resemble those of the green turtle *Chelonia mydas* (Eriksen et al., 2017), a species commonly reported to ingest larger plastic pieces (see above). The potential risk of plastic ingestion as a result of biting into floating plastic litter is reason for concern.

Entanglement

Entanglement reports of fishes are very rare, while they are common for seabirds, marine mammals, and sea turtles. The lack of reports from fishes might be due to the fact that mortality at sea would immediately cause sinking, whereas seabird, mammal, and sea turtle carcasses float at the sea surface; not surprisingly many reports of entanglement come from dead animals (see above).

From all the records of top fish predators interacting with plastic debris around the world, carcharhinid sharks seem to be most at risk of entanglement (Laist, 1997; Sazima et al., 2002; Ceccarelli, 2009), probably due the high abundance and species diversity in this group (Compagno, 1984). Cliff et al. (2002) reported an increase over time in entanglement of a carcharhinid species from South Africa, which furthermore underscores that species from this group are at highest risk of negative interactions with floating litter. Oceanic shark species are also likely to be impacted by plastic debris, but the limited number of studies on oceanic sharks underscores that more research is required to determine the full extent of this problem.

Seabird entanglement is common in the world's oceans (Kühn et al., 2015). Herein entanglement was reported mostly for species from the HCS, and observations included fisheries litter (nets, lines) and consumer plastics (mostly plastic bags). While interactions with fisheries items are likely to happen at sea (e.g., Moore et al., 2009), entanglement with consumer plastics may occur on the shore or at waste disposal facilities, as highlighted by the frequent observations of kelp gulls *L. dominicanus* with plastic bag entanglement (**Table 1**). Our data suggest that entanglement is more common in species from the continental coast than in oceanic species. The fact that Procellariiformes (which are mostly oceanic species) have the lowest frequencies of entanglement (Kühn et al., 2015) seems to support this pattern. However, herein we observed

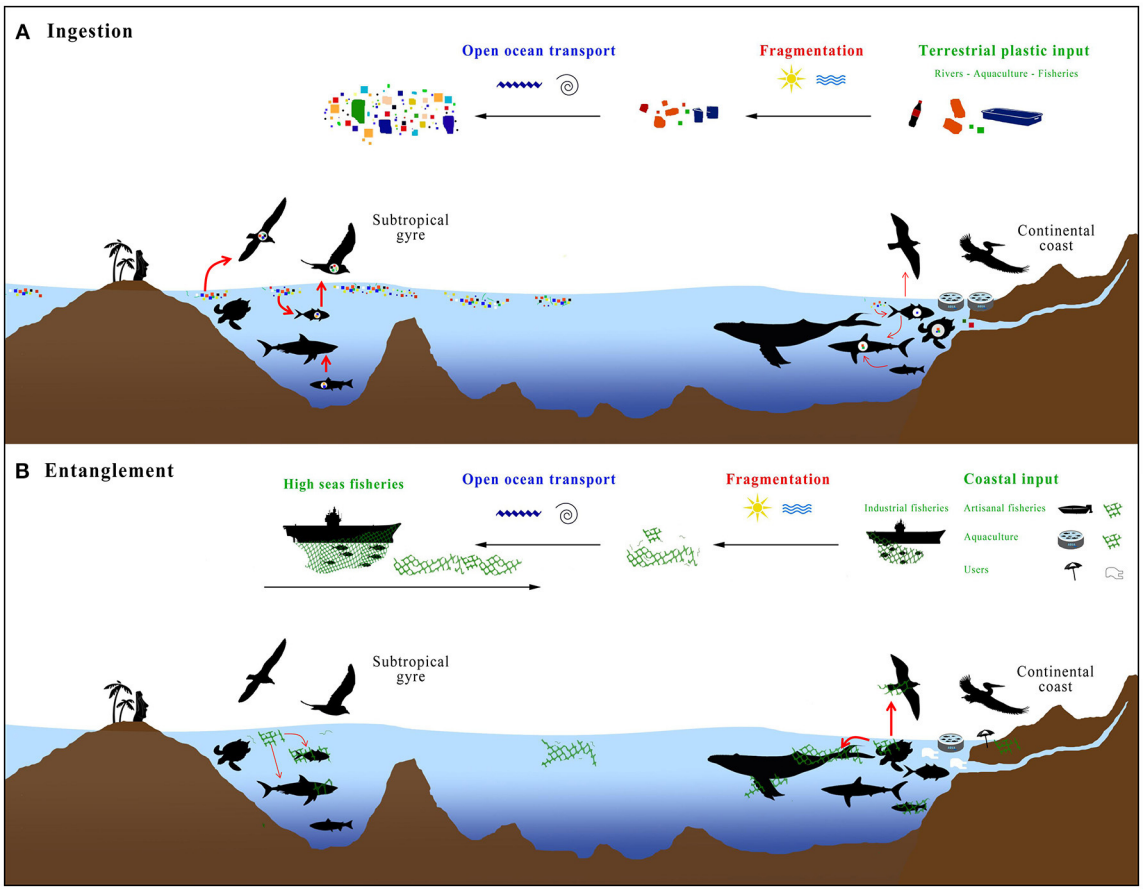

FIGURE 5 | Conceptual model of **(A)** ingestion, and **(B)** entanglement by marine vertebrates with anthropogenic marine plastics, highlighting the litter sources and abiotic processes (upper part of figures) and the interactions with marine invertebrates (bottom part of figures).

plastic litter in the nests of several oceanic species (**Table 1**), which underscores the imminent risk of entanglement for these species.

Although sea turtle entanglement in AMD has been recognized as a cause of mortality globally, there are quantitative knowledge gaps on rates and population implications (Duncan et al., 2017). Recent studies report entanglements across all species, life stages, and ocean basins, with higher vulnerability in pelagic juveniles (Nelms et al., 2016; Duncan et al., 2017). However, reports of entanglement incidents in the scientific literature are scarce and it is likely that many individual cases are never published, and therefore these data may be highly underestimated (Nelms et al., 2016). Duncan et al. (2017) reported that derelict fishing gear contributed globally to the majority of entanglements, while debris from land-based sources contributed to a lesser extent. Entanglements are a greater threat to sea turtles than climate change and direct exploitation, but less of a threat than plastic ingestion and bycatch (active gear) (Duncan et al., 2017).

Incidences of entanglement were reported for many species of marine mammals from the SE Pacific (**Table 1**), mostly with items of fisheries origin. Similar to other areas of the southern hemisphere (e.g., Page et al., 2004), sea lions seem to be most at risk of entanglement with derelict fishing gear as indicated by several independent observations recorded herein (**Table 1**). However, our review also reveals that many whale species from the HCS become entangled in (active?) fishing gear. Some efforts are being conducted to mitigate the entanglement problem. In Peru, the implementation of acoustic alarms (pingers) has proven to have dissuasive effects, thus reducing dolphin entanglements (Mangel et al., 2013). The use of modified long lines in the Patagonian toothfish (*Dissostichus eleginoides*) fishery in southern Chile reduces the interaction of killer and sperm whales with active fishing gear (Moreno et al., 2008). Yet, these efforts are only localized and eventually should be implemented at the whole scale of the respective fisheries.

Differential Risk of Marine Litter Interactions Across the Oceanic Gradient

Several studies highlight that risk of both ingestion and entanglement is highest where main foraging grounds overlap with accumulation areas of floating AMD (Wilcox et al., 2013; Fossi et al., 2017). Our study showed that some species foraging

in the highly productive HCS frequently interact with marine plastics, which at first glance might be surprising given that litter densities are substantially lower than in the open ocean. However, many of these species feed in areas where hydrographic features, e.g., frontal systems or meso-scale gyres, concentrate food and also floating plastics (Pichel et al., 2007). Interestingly, one of the first direct observations of this phenomenon comes from the HCS off the central coast of Chile, where Bourne and Clark (1984) observed planktivorous seabirds feeding in a coastal front that also had concentrated large amounts of floating plastics. The high incidences of entanglement and also plastic ingestion, especially by sea turtles and some seabird species from the HCS, likely occurred in these temporary hotspots. These interactions are common in the productive upwelling systems of the eastern boundary currents (for overview see Scales et al., 2014), and cause high risk for marine vertebrates despite the fact that densities of floating litter are lower than in the subtropical gyres.

In the open ocean, especially in the oligotrophic subtropical gyres, marine productivity is low, and often concentrated above seamounts or near oceanic islands. If these islands are located within the range of the litter accumulation zones of the subtropical gyres, some species are at high risk of negative interactions with floating plastics (**Figure 5**). Our review showed that planktivorous fish and seabirds living on the oceanic islands in the vicinity of the SPSG have high incidences of microplastic ingestion, possibly due to the extraordinarily high densities of floating microplastics in this region (**Figure 5**). The limited number of entanglement reports from this area is likely a combination of lower densities of marine vertebrates in the subtropical gyres (see also Titmus and Hyrenbach, 2011) and the limited number of observers, compared to the continental coasts.

CONCLUSIONS AND OUTLOOK

Herein interactions with marine plastic litter were documented for a total of 97 species of marine vertebrates. The risk of microplastic ingestion seems to be high in nearshore waters (including tidepools), decreases above the continental shelf of the eastern boundary currents, but again reaches very high probabilities in oceanic waters associated with the gyre accumulation zones, especially for fishes and seabirds (**Figure 5**). The current interaction records suggest that marine vertebrate species living in the productive waters of the HCS are at higher risk of facing entanglement than species from the open ocean, albeit several oceanic species have also been observed to be entangled in marine plastics, mostly from high seas fisheries (**Figure 5**).

Further systematic research on the ingestion and entanglement rates in marine vertebrates and their impacts on populations from the SE Pacific is required. Investigations to determine hotspots of marine plastic pollution will also enable prioritizing resources and to focus and steer conservation measures. Detailed stranding data and a centralized regional database are recommendable for a better documentation of negative interactions of marine vertebrates with plastic litter. Education, community involvement, together with effective measures to reduce the amounts of plastic litter entering the ocean, are essential to reduce the impact on marine vertebrates, particularly the highly threatened sea turtles.

ETHICS STATEMENT

The paper is based mostly on previously published information and does not include any sampling and laboratory analysis of marine organisms. Therefore, no ethics approval was required.

AUTHOR CONTRIBUTIONS

MT and GL-J designed the review, coordinated the team, and led the writing. RÁ-V, CG, IH, NL, DM-U, NM, NO, ASP, MP-T, and CZ contributed the observations and helped with the writing. IH and MP-T coordinated the quest for entanglement observations from citizen scientists. CG, DM-U, and MP-T prepared the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00238/full#supplementary-material

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Frequency of Microplastics in Mesopelagic Fishes from the Northwest Atlantic

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Microplastics are a ubiquitous pollutant in our seas today and are known to have detrimental effects on a variety of organisms. Over the past decade numerous studies have documented microplastic ingestion by marine species with more recent investigations focussing on the secondary impacts of microplastic ingestion on ecosystem processes. However, few studies so far have examined microplastic ingestion by mesopelagic fish which are one of the most abundant pelagic groups in our oceans and through their vertical migrations are known to contribute significantly to the rapid transport of carbon and nutrients to the deep sea. Therefore, any ingestion of microplastics by mesopelagic fish may adversely affect this cycling and may aid in transport of microplastics from surface waters to the deep-sea benthos. In this study microplastics were extracted from mesopelagic fish under forensic conditions and analysed for polymer type utilising micro-Fourier Transform Infrared Spectroscopy (micro-FTIR) analysis. Fish specimens were collected from depth (300–600 m) in a warm-core eddy located in the Northwest Atlantic, 1,200 km due east of Newfoundland during April and May 2015. In total, 233 fish gut contents from seven different species of mesopelagic fish were examined. An alkaline dissolution of organic materials from extracted stomach contents was performed and the solution filtered over a 0.7 μm borosilicate filter. Filters were examined for microplastics and a subsample originating from 35 fish was further analysed for polymer type through micro-FTIR analysis. Seventy-three percent of all fish contained plastics in their gut contents with *Gonostoma denudatum* having the highest ingestion rate (100%) followed by *Serrivomer beanii* (93%) and *Lampanyctus macdonaldi* (75%). Overall, we found a much higher occurrence of microplastic fragments, mainly polyethylene fibres, in the gut contents of mesopelagic fish than previously reported. Stomach fullness, species and the depth at which fish were caught at, were found to have no effect on the amount of microplastics found in the gut contents. However, these plastics were similar to those sampled from the surface water. Additionally, using forensic techniques we were able to highlight that fibres are a real concern rather than an artefact of airborne contamination.

Keywords: myctophids, marine litter, micro-FTIR, fibres, eddy, deep sea, biogeochemical cycling, carbon sequestration

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INTRODUCTION

As a consequence of decades of marine litter entering our seas (Ryan, 2015), microplastics have been found in coastal and pelagic environments around the globe with an ever increasing distribution (Barnes et al., 2009; Galgani et al., 2015). Considering the prevalence of microplastics, there is now a substantial amount of research effort investigating their abundance in the gastrointestinal tracts of various organisms. For example, some early studies found 83% of *Nephrops norvegicus* had microplastics in their tracts (Murray and Cowie, 2011) and Lusher et al. (2013) found microplastics in the tracts of 35% of the pelagic and demersal fish species examined. Indeed, Gall and Thompson (2015) have reported that over 690 marine species are impacted by marine litter. More recent studies have moved from quantifying which animals have ingested microplastics to examining the physical and health implications of microplastic ingestion (Rochman et al., 2013; Cole et al., 2014; Pedà et al., 2016). For example, Wright et al. (2013) demonstrated how the ingestion of microplastics by the polychaete *Arenicola marina* (lugworm), an important ecosystem engineer of Northern Europe's intertidal zones, caused inflammation and decreased feeding and ultimately depleted energy reserves. Such studies have prompted researchers to investigate the impact on ecosystem processes. Indeed, Cole et al. (2016) found that microplastics alter the sinking rates of copepod faecal pellets if ingested and in consequence may affect the downward flux of carbon to the ocean floor. With the increasing evidence that microplastics represent an ecosystem and environmental health concern, UNEP and the EU Commission have established bodies and efforts to guide in decision making and legislation (Galgani et al., 2013; UNEP, 2016). Furthermore, several governments have taken legislative steps by introducing a ban on microbeads in cosmetics and detergents by 2020 (Sutherland et al., 2017).

Despite this substantial increase in studies investigating the ingestion of microplastics and their associated impacts, there are still important taxa playing key roles in ecosystem functioning that have not been well-studied. Mesopelagic fish inhabiting the disphotic zone of the pelagic realm (200–1,000 m depth) from the Arctic to the Antarctic (Gjøsaeter and Kawaguchi, 1980) are one of these understudied groups. Many species are known to undergo diurnal vertical migrations by residing at depth during the day before migrating to the surface at night to feed (Gjøsaeter and Kawaguchi, 1980). Smaller mesopelagic fish such as *Myctophum punctatum* and *Benthosema glaciale* feed by filtering zooplankton, predominantly copepods, euphausiids, amphipods, eggs, and larvae over their gill rakers (Scotto di Carlo et al., 1982; Roe and Badcock, 1984). Larger mesopelagic fish such as *Stomias boa* and *Serrivomer beanii* also actively target decapods and fish using their anterior vertebrae and branchial apparatus to swallow larger prey (Roe and Badcock, 1984; Bauchot, 1986). Thus, mesopelagic fish are exposed to microplastics either through the direct consumption of a microplastic mistakenly identified as prey item, or indirectly, through the consumption of a prey item (e.g., copepod or euphausiid) that had already consumed microplastics.

As mesopelagic fish undergo large vertical migrations, they are known to play a key role in the cycling of carbon and nutrients to the deep ocean (Radchenko, 2007; Davison et al., 2013). For instance, Radchenko (2007) has shown that such species in the Bering Sea transport 15,000 tonnes of carbon daily to the deep ocean. Therefore, the ingestion of microplastics by mesopelagic fish may disrupt carbon cycling and aid in the transport of microplastics to deeper waters, as suggested by Lusher et al. (2016).

The importance of mesopelagic fish was recently further highlighted in studies by Kaartvedt et al. (2012) and Irigoien et al. (2014) who found that the mesopelagic fish biomass in the global oceans may have previously been underestimated by at least one order of magnitude due to avoidance behaviour and mesh extrusion. Because they make up such a large biomass in the pelagic realm they provide an important food source for a variety of predatory fish and marine mammals which, through trophic transfer from their mesopelagic fish prey, may suffer from the impacts of microplastics and associated toxins (Lusher et al., 2016). Some of the species preying on mesopelagic fish such as tuna and swordfish (Scott and Tibbo, 1968; Varela et al., 2013) are commercially important food sources and thus toxins and microplastics transferred to these species may also pose a danger to human health. To date mesopelagic fish have not been exploited as a human food source due to the high levels of wax esters in their tissue (Gjøsaeter and Kawaguchi, 1980). This may change in the near future as the demand for fish protein increases and new policies (e.g., Blue Growth Strategy by the European Union) encourage sustainable exploitation of potential resources (St. John et al., 2016). Furthermore, the food safety issues concerned with microplastics and the associated toxin exposure through the consumption of commercially exploited fish have recently been outlined in an extensive report by the Food and Agriculture Organization of the United Nations drawing attention to the potential threat of microplastics to human health (Lusher et al., 2017).

However, to date, only a few studies have investigated microplastic ingestion by mesopelagic fish: one in the North Atlantic (Lusher et al., 2016) and two in the North Pacific Gyre region (Boerger et al., 2010; Davison and Ash, 2011). Since then, new and improved methodologies for microplastic extraction have been developed with an emphasis on ultra-clean techniques in order to prevent airborne contamination (Wesch et al., 2017).

This study set out to quantify microplastic ingestion by mesopelagic fish from an eddy region in the Northwest Atlantic, known to be a hot spot for mesopelagic fish (McKelvie, 1985; Fennell and Rose, 2015) and potentially microplastics (Yu et al., 2018). Specifically, this study investigated whether: (1) species, stomach fullness, and the depth at which fish were caught at had an effect on the amount of microplastics found in the gut contents of mesopelagic fish, and (2) how the type, shape, and size of microplastics found in the gut contents compared to those found in the surface waters. Importantly, we applied strict measures to prevent microplastic contamination during extraction and identified microplastic type using micro-FTIR spectroscopy.

METHODS

Sample Collection

Mesopelagic fish samples were collected during a Northern Atlantic crossing (*CE*15007) from Galway, Ireland to St. John's, Newfoundland aboard the RV *Celtic Explorer* between the 20th of April and the 5th of May 2015. In total, eight 30-min pelagic trawls were carried out during daylight hours at a towing speed of 4 knots (**Figure 1**). The opening of the net was fitted with a Scanmar depth sensor to enable three trawls to be conducted in the upper mesopelagic zone between 300 and 350 m (shallow) and five in the lower mesopelagic zone between 500 and 650 m (deep). Once hauled aboard, a random subsample of 35 intact mesopelagic fish was taken from each trawl.

Furthermore, surface water samples were taken during each trawl by utilising the ship's underway water pumping system with its intake located at 3 m depth. The intake water initially passed through a 1 mm mesh and was then pumped into the lab facilities, where the underway hose was positioned to allow water to pass through a 180 µm plankton sieve. Sieved particles were then washed down with 0.2 µm filtered ultrapure water into cylindrical aluminium containers (5 cm Ø) which were then folded over at the top. The flow rate of water through the underway pump was measured at 10 L min^{-1} and the volume of water filtered was estimated using the flow rate and duration of each trawl. Fish and water samples were stored in Ziploc® bags and immediately transferred into a −20°C freezer and stored there until the vessel returned to Galway on the 15th of May 2015 when samples were transferred to a −20°C freezer at the National University Ireland, Galway.

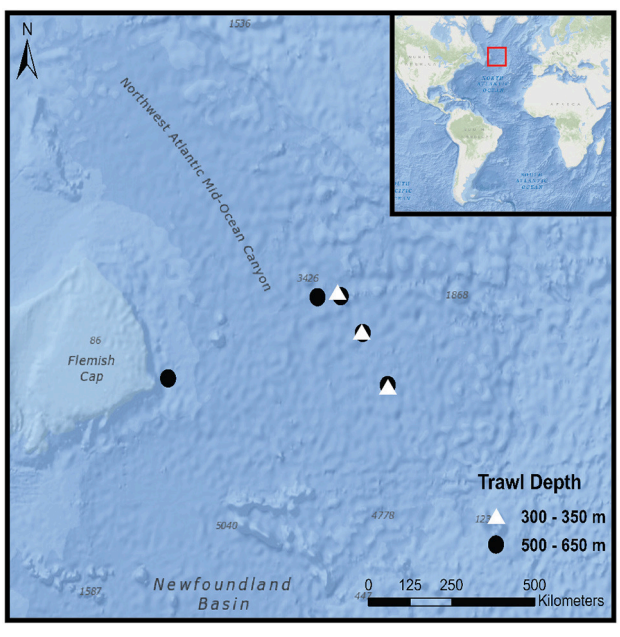

FIGURE 1 | Trawl locations during CE15007 survey aboard the RV *Celtic Explorer*; red rectangular box in the inset corresponds to outer figure margin.

Ethics Statement

Fish were taken dead from midwater trawls carried out to ground truth the backscatter from a Simrad EK60 scientific echo sounder investigating the deep scattering layer in the Northwest Atlantic, and are thus exempt from ethical approval, dealing with regulated animals, that is live vertebrates and higher invertebrates.

Sample Processing and Polymer Analysis

Samples were processed between September 2015 and June 2016. Fish samples were defrosted ∼3 h prior to processing and identified by counting number of dorsal, pectoral, and anal fin rays and number of gill rakers as well as prominent features such as photophores and barbels (Marine Species Identification Portal, 2015). Fish which displayed visible physical damage to their digestive tract were excluded from analysis. The standard length (to the nearest millimetre) of each fish was recorded. Fish were rinsed with 0.2 µm filtered MilliQ™ water (18.2 MΩ cm^{-1}) (Millipore, Bedford, USA) and weighed (to the nearest 0.0001 g) before being transferred into a borosilicate container located inside a laminar flow hood (AirClean600®: ISO class 5) where part of their alimentary tract, the oesophagus to the duodenum, was extracted. The extracted alimentary tract was then opened and the gut contents emptied into 20 ml borosilicate scintillation vials and the alimentary tract lining thoroughly washed with 0.2 µm filtered MilliQ™. The removed alimentary tract and the dissected fish were then weighed (to the nearest 0.0001 g) to obtain gut contents weight. Vials containing gut contents were filled with MilliQ™ water and sodium hydroxide (Certified analytical reagent for analysis, Fisher Scientific, UK) to give a 1 M concentration and subsequently incubated at room temperature for 24 h, following an effective and cost efficient microplastic extraction protocol outlined by Cole et al. (2014). Water samples were processed in a similar fashion whereby the frozen contents of the aluminium containers were emptied into glass scintillation vials and organic materials digested also using a 1 M solution of sodium hydroxide solution over 24 h.

After incubation, vial contents were filtered over borosilicate filters (42 mm Ø, 0.7 µm mesh) using a vacuum pump and Büchner flask; filters were then rinsed with 200 ml of 0.2 µm filtered MilliQ™ water to rinse sodium hydroxide from the filter and retained particles. Filters were kept in borosilicate glass petri dishes, covered with a lid and examined for microplastics using an Olympus SZX16 stereo microscope (Olympus, SZX16) with a digital camera attached (Olympus, DP17). Once all potential microplastics were identified on the filter, the glass lid was removed and potential plastics were examined and manually manipulated to confirm polymer characteristics (brittleness, softness, transparency). Plastic particle colours and sizes were recorded (to the nearest µm) using CellSense Standard software package (Olympus, version 1.2). Two microplastic fibres were gold coated (Emitech K550, Quorum Technologies Ltd., West Sussex, United Kingdom) and subjected to scanning electron microscopy (SEM) in secondary electron mode using a Hitachi model S-4700 (Hitachinaka, Japan). The analyses were performed at an acceleration voltage of 20 kv, an emission current (Ic) of 10 µA and a working distance of 12 mm (Morrison et al., 2009).

Five individuals of each species were randomly selected and microplastics originating from their gut contents, as well as those originating from one randomly selected surface water sample, were further analysed for polymer identification using micro-Fourier-transform infrared spectroscopy (micro-FTIR). The absorbance for each polymer was obtained using a Perkin Elmer Spotlight 200i FT-IR Microscopy System (Perkin Elmer, USA) at 100 μm^{-1} resolution with spectra collected over the wavenumber range of 7,800–600 cm^{-1}.

Contamination Prevention

The extraction of microplastics and subsequent examination of the filters was performed in compliance with the most recent findings in microplastic contamination prevention methodologies (Woodall et al., 2015; Wesch et al., 2017). All equipment used was pre-rinsed with 0.2 µm filtered MilliQ™ water and all clothing worn during laboratory work was of non-polymer nature. Furthermore, samples and filters were not at any time air exposed and always kept under a clean air laminar flow hood (HEPA filter, class ISO5) or maintained within covered borosilicate petri dishes. During dissections and filtrations on each day a wet filter (blank) was kept in a borosilicate petri dish inside the laminar flow hood for control purposes. After filtration of all samples on each day the filter was then also assembled within the Büchner flask and 200 ml of 0.2 filtered MilliQ™ water were filtered through it and the filter was later assessed for microplastics for quality assurance purposes.

Data Analysis

A stomach fullness index (FI) was calculated for each fish by dividing the weight of the gut content by the weight of the fish.

To test whether stomach fullness had any effect on microplastics being present or not in the alimentary tract of the fish, a Mann-Whitney-*U*-test (as the distribution of FI was non-parametric) was carried out using R (R Development Core Team, 2017) and compared the median stomach fullness value for fish that had microplastics with those that did not.

As the microplastic count data were non-parametric, a Kruskal–Wallis test (using R) was used to test whether there was any difference in the abundance of microplastics between the seven different species. A Mann–Whitney-*U*-test (using R), was used to test whether more microplastics were identified from fish found in shallow compared with those found in deep waters.

RESULTS

A total of 280 fish was captured of which 233 were examined for the presence of microplastics in their gut contents. The most common species amongst the subsampled fish were the spotted lantern fish *M. punctatum* (with 86 individuals, or 37% of catch), the glacier lantern fish *B. glaciale* (69 indiv., 29%) and the white-spotted lantern fish *Diaphus rafinesquii* (34 indiv., 15%). The remaining species were the Rakery beaconlamp *Lampanyctus macdonaldi* (16 indiv., 7%), the stout sawpalate *S. beanii* (14 indiv., 6%), the scaly dragonfish *S. boa* (9 indiv., 4%) and *Gonostoma denudatum* (5 indiv., 2%). Where information on sexual maturity size exists (*M. punctatum, B. glaciale, D. rafinesquii, G. denudatum*), every sampled fish was assessed as being sexually mature.

Overall 73% of fish contained plastics in their stomachs with *G. denudatum* having the highest frequency of occurrence (100%), followed by *S. beanii* (93%) and *L. macdonaldi* (75%) (**Table 1**). In total, 452 microplastic fragments were extracted from the fish gut contents, with an average of 1.8 microplastic fragments per fish. The highest average number of microplastics in the gut contents was recorded in *S. beanii* (2.36), followed by *M. punctatum* (2.28), and *G. denudatum* (2.2) (**Table 1**).

There was no significant difference between the median stomach fullness indices of fishes which had microplastics in their stomachs and those that did not ($W = 5253$, $P = 0.976$). Furthermore, there was no significant difference in median microplastic counts among the seven different species ($H = 10.904$, d.f. $= 6$, $P = 0.091$), nor between fish caught in shallower and deeper waters ($U = 5877$, $P = 0.389$).

In total, 341 particles were found in the surface water samples (8 samples totalling 2,400 L of surface water) resulting in an estimated concentration of 14 microplastic fragments per 100 litres of water. Plastics identified from fish guts were very similar to those found in the surface waters (**Figure 2**). Ninety-eight percent of microplastics identified from the fish and 99% of those identified from the water samples were classed as fibres with the remainder being flattened fragments of plastics. Recorded

TABLE 1 | Fish species, numbers and length examined for microplastic ingestion and associated microplastic abundances in gut contents.

Species	No. fish dissected	Average length (mm) (±*SD*)	No. of fish with MPs	% of fish with MPs	Average MPs in fish
Myctophum punctatum	86	67.86 ± 7.49	64	74.42	2.28
Benthosema glaciale	69	57.93 ± 5.80	47	68.12	1.46
Diaphus rafinesquii	34	75.15 ± 8.25	24	70.59	1.15
Lampanyctus macdonaldi	16	243.34 ± 221.15	12	75.00	1.75
Serrivomer beanii	14	496.76 ± 258.95	13	92.86	2.36
Stomias boa	9	70.31 ± 58.99	6	66.67	1.33
Gonostoma denudatum	5	17.84 ± 4.00	5	100.00	2.20
Total	233	–	171	73.39	1.80

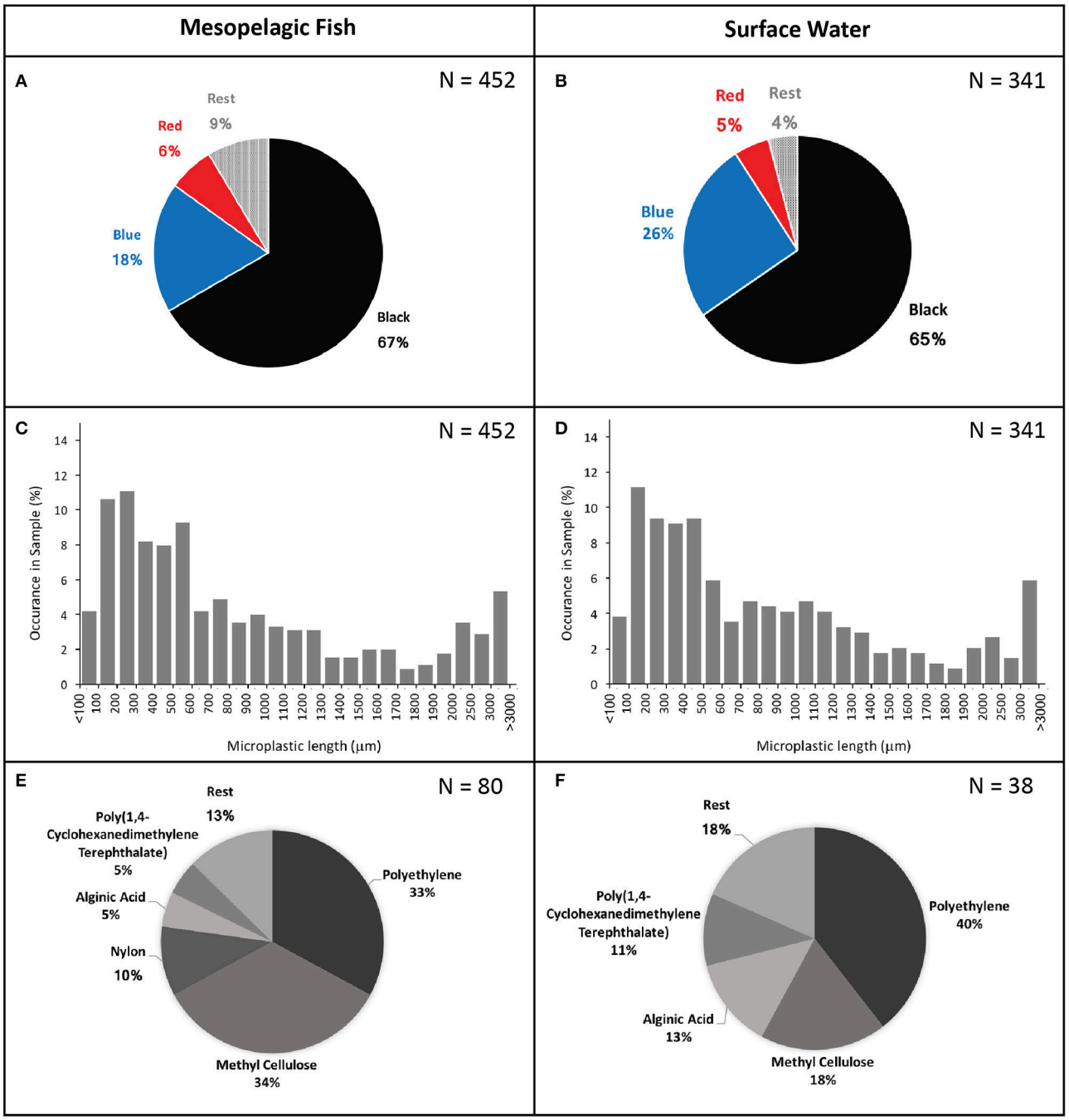

FIGURE 2 | Microplastic colours **(A,B)**, length **(C,D),** and type **(E,F)** found in mesopelagic fish gut contents (left) and surface waters (right).

microplastic colours included black, grey, blue, green, purple, red, yellow, and white. Black and blue were by far the most common colours, followed by red, making up 67, 18, and 6% and 65, 26, and 5% of sampled plastics from fish guts and surface waters, respectively. Polymers of other colours only made up a minor fraction of the particles in both cases (**Figure 2**). Likewise, sizes of extracted microplastics were very similar between those found in fish (mean ± *SD*: 969 ± 1,048) and in surface waters (mean ± *SD*: 985 ± 1,101). The smallest recorded polymer fragment had a length of 42 µm and the largest a length of 8,150 µm.

Micro-FTIR analysis was successfully carried out for 118 of the 191 microplastic fragments originating from 35 fish and from one surface water sample. The 73 particles which could not be assessed for their polymer nature either fractured when pressure was applied by the diamond of the micro-FTIR machine or did not show a significant fit with any of the materials within the FTIR spectra library and thus were excluded from analysis.

Polymers identified from fish and water samples were of similar polymer nature with the majority being polyethylene, followed by methyl cellulose and a relatively small proportion were identified as alginic acids. An exception was nylon, which comprised 10% of the particles found in the fish guts, but was not identified amongst the particles extracted from the surface water (**Figure 2**). Scanning electron microscopy images of two fibres extracted from fish gut contents had visible signs of polymer fracturing (**Figure 3**).

No microplastics were found on the filters used as blanks to ensure no airborne contamination or any contamination from the filtration equipment and procedure.

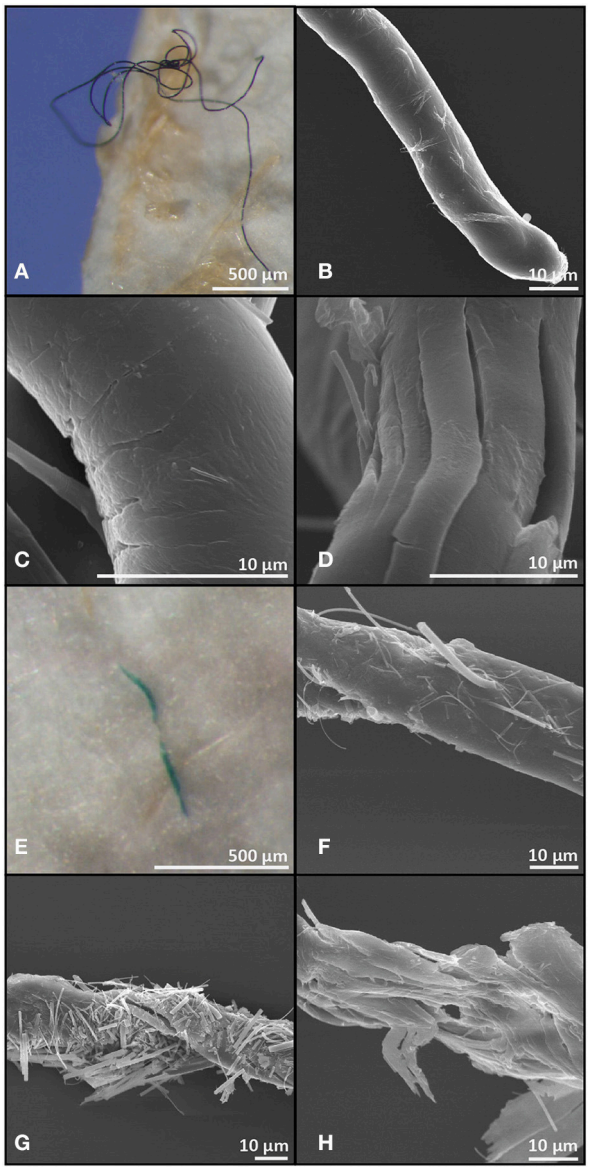

FIGURE 3 | Light microscopy and scanning electron microscopy images of a black **(A–D)** and a green **(E–H)** microplastic fibre recovered from gut contents of *Myctophum punctatum*.

DISCUSSION

Using forensic methods, this study assessed microplastic frequency of occurrence in mesopelagic fish gut contents from a warm-core eddy in the Northwest Atlantic. We detected a significantly higher occurrence rate of 73% in contrast to previous studies reporting occurrence rates of 11% in the North Atlantic and 9 and 35% in the North Pacific Gyre regions (Boerger et al., 2010; Davison and Ash, 2011). There are several reasons which may explain our much higher frequency of occurrence. Firstly, there are no standardised methods for the extraction of microplastics from gastrointestinal tracts of fish and so different research teams have used different protocols such as visual sorting of gut contents (Boerger et al., 2010), staining of organic materials (Davison and Ash, 2011), and extraction by the use of alkaline dissolution (Lusher et al., 2016). In the latter, the authors used a similar approach to this study but used a more concentrated caustic solution (1.8 vs. 1 M) and incubated samples for a longer time period (14 days vs. 1 day). Cole et al. (2014) assessed many different extraction methodologies and found that the hydrolysis of organic compounds using a caustic solution was an efficient and viable method. However, they noted that extractions using higher concentrations and longer incubation times than recommended damaged and discoloured pH sensitive polymers such as nylon, uPVC, and polyethylene and thus these may have been underestimated previously. Furthermore, we used fine-meshed borosilicate filters in contrast to Lusher et al. (2016) who used a 250 µm filter. As a result, our study detected much smaller microplastics (down to 42 µm) which made up 20% of all detected microplastics.

Another potential explanation for differences among reported microplastic occurrence rates may be due to differences in the abundances of microplastics found in the study areas. Samples for this study were collected around a warm core eddy ∼1,200 km off the Newfoundland coast which is known to be an area of enhanced biomass for all trophic levels (Dufois et al., 2016), including mesopelagic fish (McKelvie, 1985; Fennell and Rose, 2015), and may also potentially aggregate microplastics (Yu et al., 2018). Surface water samples collected within this study indeed showed 10 times higher concentrations of microplastics than reported for other regions of the Atlantic (Lusher et al., 2014) where Lusher et al. (2016) collected their samples. The other two studies collected samples at the edge region of the North Pacific Gyre, which while potentially having slightly higher concentrations of plastics, were still not located close to the centre of the gyre, known to be a hot spot for microplastics (Eriksen et al., 2014). At this point it is also important to consider how mesopelagic fish may be exposed to microplastics. All of the seven investigated species migrate to the surface at night to feed and therefore ingestion could happen through the direct consumption of microplastics mistaken as prey items or through trophic transfer from their prey species. Indeed the most common prey of mesopelagic fish are copepods, euphausiids, amphipods, larvae, and decapods and all have been reported to ingest microplastics (Carpenter et al., 1972; Setälä et al., 2014; Desforges and Ross, 2015). Lusher et al. (2016) previously excluded trophic transfer as a likely route of exposure as the

average size of microplastics they found in the gut contents was 1.9 mm. This was considered too large to be ingested by their prey species but they also noted that their study only targeted plastic particles over 250 μm in size. In this study the average size of identified microplastics from fish guts was 970 μm with 20% of all plastics being smaller than 250 μm and thus trophic transfer from planktivorous prey species may indeed be a likely route of exposure. In addition to size, it is also worth noting that the colour of any microplastics is unlikely to play an important role in the ingestion of microplastics by mesopelagic fish as the colours of the microplastics identified from the fish gut contents were similar to those identified from the surface waters.

Lastly, different microplastic abundances in the gut contents may be caused by some mesopelagic species being more selective or impacted than others. For example, it is well known that some bird species are more prone to microplastic and marine litter ingestion than others (e.g., petrels: Van Franeker and Bell, 1988). However, our study found no differences in microplastic occurrence rates between the seven mesopelagic fish species examined. Neither did depth seem to explain any variation in microplastic abundances amongst individuals caught at different depths. Therefore, we can conclude that the notably higher occurrence rates reported within this study are likely due to the differences in microplastic extraction methods as well as the fact that the present study was carried out in a hot spot for mesopelagic fish and microplastics alike. While this study reports one of the highest abundances of microplastics in the gut contents of fish, other studies have reported similar results for different species, particularly in polluted areas. For example, Tanaka and Takada (2016) report a 77% encounter rate of microplastics in Japanese anchovies (*Engraulis japonicus*) sampled from Tokyo Bay and Nadal et al. (2016) found microplastics to occur in 68% of seabream (*Boops boops*) sampled around the Balearic Islands. It is also noteworthy that while Lusher et al. (2016) and Davison and Ash (2011) reported a lower average microplastic count per individual fish of 0.13 and 0.11, respectively, Boerger et al. (2010) indeed found the average microplastic count per fish to be 2.1, higher than observed by us (1.8).

Such high numbers of microplastics in the gut contents of mesopelagic fish is of great concern. Microplastics have previously been shown to adversely impact invertebrate species such as lugworms, causing weight loss, reduced feeding activity and inflammation (Besseling et al., 2013; Wright et al., 2013), and detrimental effects on the intestinal functioning of seabass (*Dicentrarchus labrax*) have also been noticed (Pedà et al., 2016). Furthermore, there is growing concern about the effect of chemical pollutants sorbed to microplastics. For example, Mato et al. (2001) reported up to 10^6 higher concentrations of PCBs on polypropylene pellets than in the surrounding sea water and recently, it has been shown that Japanese rice fish (*Oryzias latipes*) and rainbow fish (*Melanotaenia fluviatilis*) readily accumulate chemical pollutants from ingested microplastics (Rochman et al., 2013; Wardrop et al., 2016).

The ingestion of microplastics by mesopelagic fish may also have secondary implications for other species as well as the entire ecosystem. Mesopelagic fish are now known to make up a substantial biomass in the pelagic realm (Kaartvedt et al., 2012) and provide an important food source for many large predators such as dolphins, seals, and tuna as well as sea birds (Cherel et al., 2008; Danielsen et al., 2010; Spitz et al., 2010; Varela et al., 2013). These taxa consume large amounts of mesopelagic fish and consequently ingest the microplastics within them. More importantly, due to trophic transfer, predators of mesopelagic fish may also bioaccumulate chemical pollutants absorbed from ingested microplastics. As some of the species preying on mesopelagic fish are commercial exploited fish the transfer of microplastics and biaccumulated toxins may also pose a threat to human health (Lusher et al., 2017).

Mesopelagic fish are also responsible for a significant amount of carbon and nutrient cycling (Radchenko, 2007). Organic material released as faeces or from dead and decaying organisms, sink very slowly from the upper surface to the deep ocean. A large proportion of this organic material is recycled by other organisms and re-released before it can reach the ocean floor. Mesopelagic fish however, undergo diurnal migrations, quickly travelling long distances from the epipelagic layer where they feed, to the deeper ocean where they deposit their faeces. Therefore, they play a key role in speeding up the downward flux of carbon and nutrients to deeper depth and circumvent recycling by other organisms (Irigoien et al., 2014). As discussed above, we are now aware that microplastic ingestion can have substantial effects on fish health and in particular digestive functions. Therefore, reported microplastic abundances in the fish gut contents may have implications for the cycling of carbon and nutrients by these species. Moreover, as suggested by Lusher et al. (2016), mesopelagic fish may aid in the downward transport of microplastics to the deep-sea benthos and cause potential harm to organisms in this habitat.

In terms of our methods, the applied protocols have successfully extracted very small plastic particles. However, the micro-FTIR spectroscopy analysis identified a large proportion of the analysed microplastics as methyl cellulose and alginic acids. This, while seeming unusual at first, is very likely a consequence of insufficient cleaning of the microplastics after extraction. Methyl cellulose is produced synthetically by heating cellulose with a caustic solution. As we used a caustic solution to hydrolyse organic materials some of the sodium hydroxide seemed to have remained on the plastic particles and potentially skewed the absorbance spectrum. Similarly, alginic acids are likely to be a reading of an outer biofilm coating of the microplastic particles which had not been removed during extraction. For future studies we strongly recommend a more thorough cleaning of plastic particles with filtered, ultrapure water. Furthermore, it is interesting to note that despite taking a forensic approach during the extraction of microplastics (Wesch et al., 2017) we noted a large amount of fibres (98%) amongst the sampled microplastics. This is in agreement with other findings (Lusher et al., 2013, 2016; Neves et al., 2015; Rochman et al., 2015; Bellas et al., 2016). However, fibres are often considered to be a contaminant of airborne nature and are sometimes excluded from analysis (Foekema et al., 2013; Rummel et al., 2016; Tanaka and Takada, 2016). As we did not observe any fibres on the filters used as blanks, we argue that fibres do indeed make up a large proportion of microplastics and are not of

airborne nature. In support of this, Rochman et al. (2015) found high numbers of fibres in fish sampled from USA fish markets, but not in those sampled from Indonesian fish markets. The authors suggest that this is due to the large amount of waste water effluents carrying synthetic fibres from washing machines as such machines are more common in developed areas. In fact, the microplastics we identified from the fish gut contents closely overlapped in colour, size, shape, and type with those sampled from the surface water (**Figure 2**) and we can thus assume that types of microplastics sampled from organisms are a reflection of those found in the environment they inhabit.

While large gyres have been a major focus of microplastic research, this study together with that of Yu et al. (2018) show that mesoscale features such as eddies may also be a hot spot for microplastics and should be further investigated. Furthermore, future studies quantifying microplastic ingestion by predatory fish species should also consider to sampling their putative prey to investigate trophic transfer of microplastics.

In conclusion, this study reports the highest ingestion rates of microplastics in the gastrointestinal tracts of mesopelagic fish. This has important consequences for the health of pelagic ecosystems and biogeochemical cycling in general. Additionally, using forensic techniques, we provide more evidence that fibres are found throughout our oceans rather than being an artefact of airborne contamination.

AUTHOR CONTRIBUTIONS

TD and EM conceptualised the study; EM and AA (chief scientist) participated in the research cruise and collected samples; TD and AW designed the experiments; AW and HB performed the experiments; HB and LM took SEM images; AW, OS, and LM carried out micro-FTIR analysis; AW analysed the data; AW and TD wrote the manuscript with contributions from PC, LM, AA, and EM.

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Low Abundance of Plastic Fragments in the Surface Waters of the Red Sea

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The floating plastic debris along the Arabian coast of the Red Sea was sampled by using surface-trawling plankton nets. A total of 120 sampling sites were spread out over the near-shore waters along 1,500 km of coastline during seven cruises performed during 2016 and 2017. Plastic debris, dominated by millimeter-sized pieces, was constituted mostly of fragments of rigid objects (73%) followed by pieces of films (17%), fishing lines (6%), and foam (4%). These fragments were mainly made up by polyethylene (69%) and polypropylene (21%). Fibers, likely released from synthetic textiles, were ubiquitous and abundant, although were analyzed independently due to the risk of including non-plastic fibers and airborne contamination of samples in spite of the precautions taken. The plastic concentrations (excluding possible plastic fibers) contrasts with those found in other semi-closed seas, such as the neighboring Mediterranean. They were relatively low all over the Red Sea (<50,000 items km^{-2}; mean ± SD = 3,546 ± 8,154 plastic item km^{-2}, 1.1 ± 3.0 g km^{-2}) showing no clear spatial relationship with the distribution of coastal population. Results suggests a low plastic waste input from land as the most plausible explanation for this relative shortage of plastic in the surface waters of the Red Sea; however, the additional intervention of particular processes of surface plastic removal by fish or the filtering activity of the extensive coral reefs along the coastline cannot be discarded. In addition, our study highlights the relevance of determining specific regional conversion rates of mismanaged plastic waste to marine debris, accounting for the role of near-shore activities (e.g., beach tourism, recreational navigation), in order to estimate plastic waste inputs into the ocean.

Keywords: plastic fragments, Red Sea, Mediterranean Sea, Côte d'Azur, surface waters

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INTRODUCTION

Efforts to assess the global distribution of marine floating plastic debris over the last years are now allowing for a comprehensive depiction of global patterns. High plastic accumulation zones were hypothesized, and confirmed, in each of the five subtropical gyres (Law et al., 2010; Goldstein, 2012; Cózar et al., 2014), and semi-enclosed systems, such as the Mediterranean (Cózar et al., 2015; Ruiz-Orejón et al., 2016; Suaria et al., 2016) and the Arctic Ocean (Cózar et al., 2017), which has been equated to a "polar Mediterranean" because of being surrounded by continents and linked to global ocean circulation through terminal surface currents (Aagaard et al., 1985; Østerhus et al., 2005). However, extensive areas remain yet unexplored, particularly semi-enclosed basins highly susceptible to accumulate plastic because of the limited hydrodynamic capacity to transfer the

inputs they receive from land or import through ocean circulation into the open ocean. This is the case for the Red Sea, spreading over 438,000 km² in one of the world's warmest regions.

The Red Sea is characterized by low annual rainfall, the absence of permanent rivers in its catchment area and high evaporation. In the north, the artificial canal in the Gulf of Suez opens an important shipping route connecting the Mediterranean to the Red Sea. In the south, the Red Sea connects to the Indian Ocean through the strait of Bab el Mandab, importing the Indian Ocean into the Red Sea at the surface and exporting hypersaline Red Sea water at depth (Smeed, 2004). Maritime traffic is intense along the Red Sea, one of the major shipping routes for oil tankers and merchant vessels in the ocean (Gladstone et al., 1999). The shoreline is sparsely populated, with Jeddah (Kingdom of Saudi Arabia) being the only human settlement above 150,000 inhabitants along the extensive Saudi coast spanning the entire Eastern margin of the Red Sea. The Red Sea is largely a coastal ocean, with a deep (up to 2,800 m) central zone and extensive shallow areas supporting mangroves, seagrass beds and one of the most extensive coral reef systems in the world (Bruckner et al., 2012; **Figure 1**).

The Red Sea basin, like the Mediterranean, shows an inverse estuarine circulation driven by high evaporation in the basin, with the water exchange with the Indian Ocean defined by a surface inflow of low-salinity water over a deeper outflowing high-salinity water layer (Cessi et al., 2014). This dominant two-layer transport is replaced between June and September by a three-layer exchange comprised of a shallow surface outflow, an intermediate intrusion of the relatively fresh and cold Gulf of Aden water and a deep hypersaline outflow (Sofianos, 2002). This hydrodynamic pattern implies a limited capacity to export floating debris from the Red Sea and a more likely import from the Indian Ocean, suggesting that the Red Sea may act as a trap for floating plastic pollution, as reported for the Mediterranean Sea (Cózar et al., 2015). On the other hand, the Mediterranean Sea and the Red Sea show also relevant dissimilarities in relation to coastal population levels, touristic activity or biological communities, which may affect the input and loads of plastic pollution.

Here, we assessed, for the first time, the load and distribution of floating plastic litter across the Red Sea, with a focus on the Eastern half, encompassing the Economic Exclusive Zone (EZZ) of Saudi Arabia. Our aim was to examine the occurrence, composition and distribution of plastic debris in the surface waters and, by using other world seas and particularly the Mediterranean Sea as reference, to characterize its pollution level and possible sources.

MATERIALS AND METHODS

A total of 120 samples were collected during 7 cruises on board KAUST R/V *Thuwal* covering 1,500 km of the Arabian coastline of the Red Sea. During 2016, 95 samples were collected during five cruises from February 17th to December 1st and 25 additional samples were collected during two cruises from March 1st to April 6th 2017 (Table S1). Plastic debris was sampled following standard procedures, similar to those used in the assessment of plastic pollution in the Mediterranean Sea (Cózar et al., 2015), by using a manta trawl (0.5 × 0.15-m mouth, 150-μm mesh). The net was towed at the top 10 cm of the sea surface with an average speed of 2.5 knots during 15–30 min. The material collected by the net was mixed with 0.2-mm-filtered seawater and floating plastic debris was carefully picked out from the water surface with the aid of a stereoscopic microscope in the laboratory. This examination was repeated to ensure the detection of the smallest particles, isolating separately plastic items and fibers. Plastic items were extracted from the seawater samples and washed and dried at room temperature, whereas the materials identified as possible plastic fibers found in the samples were counted and analyzed independently. Rigorous precaution was taken during the sample processing, using cleaned instruments and blanks from sampling operations until the end of the sorting at the laboratory.

Plastic items were counted, photographed and processed to measure each item using ImageJ Software (http://imagej.nih.gov). They were classified into four size intervals: 0.2–0.5, 0.5–1, 1–5, and 5–500 mm (width of the net mouth). To render plastic counts per bin independent of the width of the bin, the abundance of plastic items for each bin was normalized by dividing them by the size interval width (Cózar et al., 2014). In addition, plastic items were classified according to aspect and consistency in rigid fragments (hard pieces from broken objects); films (bags, wrappings, or pieces of them); fishing lines (including those released from nets); foamed plastic; pellets (raw form of plastic) and beads (possibly derived from products for cleansing and personal care).

To confirm the plastic nature of the material collected in the examinations, Fourier transform infrared spectrometry (Nicolet 10 FT-IR for particles $\geq$250 μm and Nicolet 6700 μFT-IR coupled with a microscope for particles <250μm) was applied to a random subset of items ($n = 198$). Thirty-two scans at medium resolution were performed per item. Wave number ranged between 4,000 and 650 cm^{-1} and the spectra were verified using the OMNIC Spectra Library. Unfortunately, this could not be applied to items identified as possible fibers, as this FT-IR testing of this particular type of debris requires a Diamond Compression cell, which was not available. Hence, plastic fibers are reported separately, as their identification has not been confirmed and remains putative.

The area sampled in each net tow was estimated from the product of trawling length (from GPS positions) and width of the net. Given the effect of wind mixing on the vertical distribution of buoyant plastic debris, the wind correction proposed by Kukulka et al. (2012) was applied to the net tows carried out with average friction velocity in water (u^*) > 0.6 (30.8% of the tows). Differences in wind speed among the seven surveys were statistically tested with the non-parametric Kruskal-Wallis test.

The population within the 50-km coastal strip of the Red Sea was estimated from gridded global population data from the 2008 LandScan data set (http://web.ornl.gov/sci/landscan/datasets/LS2008.ris). The linear distance from

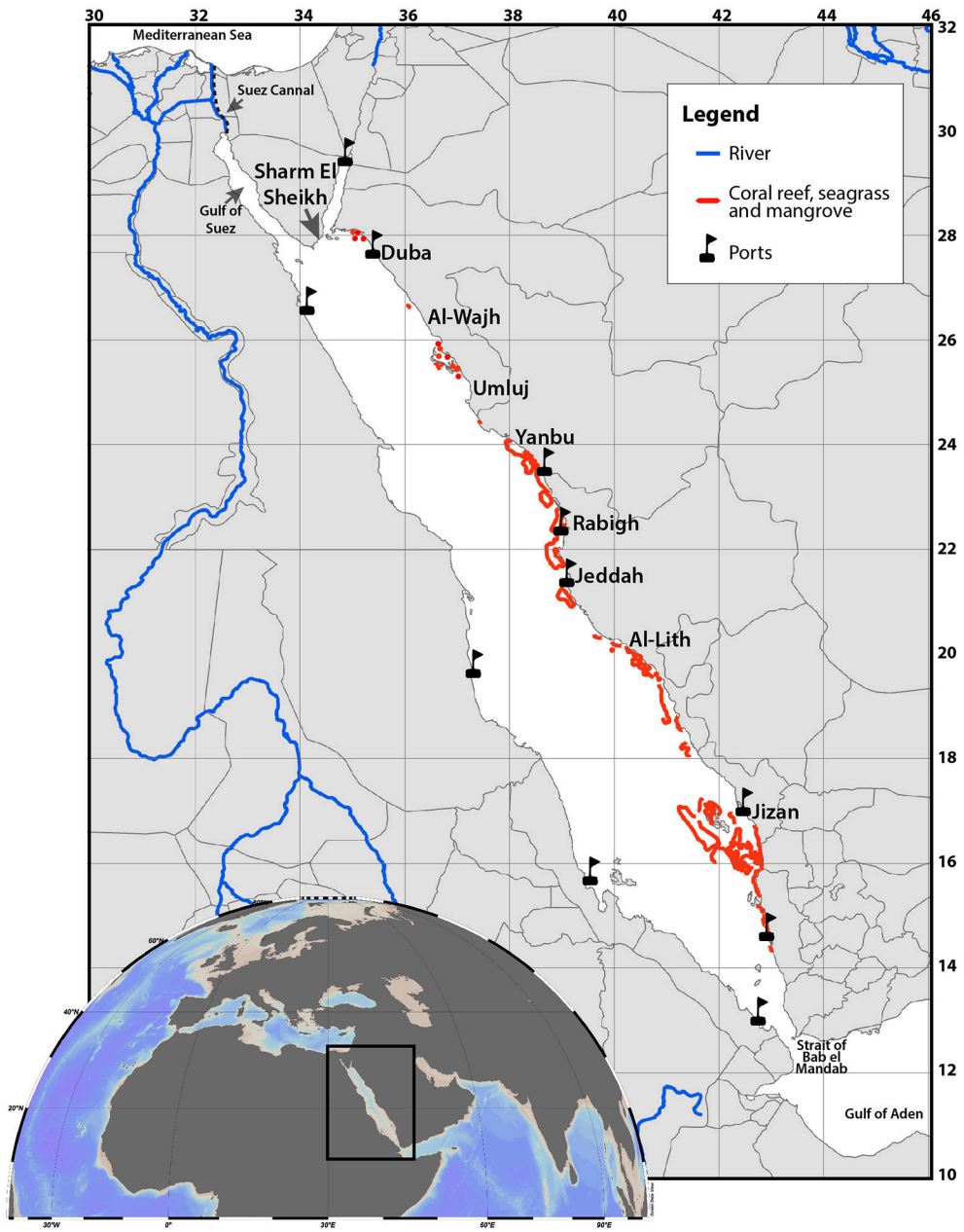

FIGURE 1 | Geographic location of the study area. Blue lines show rivers, red lines indicate coral reefs, seagrass beds and mangroves, and black flags are major ports (Adapted from Natural Earth and Bruckner et al., 2012).

Sharm El Sheikh, at the northernmost part of the Red Sea, was used as a reference to analyze the distribution of plastic and population along the Red Sea. Data on floating plastic abundance and population was aggregated into 29 bins of 50-km length each along the north-south transect (1,400 km long). The relationship between these variables was explored using Spearman's correlation in the R statistical package.

Plastic concentrations in the Red Sea and Mediterranean Sea were compared using a non-parametric Mann–Whitney *U*-test. Likewise, plastic concentration in relation to the distance to land was analyzed using different distance bins and compared to plastic abundances measured in the Côte d'Azur (French Mediterranean coast) (Pedrotti et al., 2016). To allow this comparison, sampling sites in the Red Sea ($n = 120$, distance from land: 0.17–151 km) and the Mediterranean Sea ($n = 70$, 0.3–340 km) were grouped into four logarithmic bins of distance to shore (0.1–1, 1–10, 10–100, and 100–1,000 km). A non-parametric Kruskal-Wallis test was used to test for significant differences in floating plastic fragment abundance between distance bins.

RESULTS

Plastic fragments were found in 60.8 % of the samples, with a total of 298 plastic items (excluding fibers) found in the 120 stations sampled during the seven cruises carried out across the Red Sea. The concentrations of plastic fragments were generally low, ranging from 0 to 50,393 item km^{-2} (**Figure 2A**), with an average ± SD concentration of 3,546 ± 8,154 plastic item km^{-2} (median = 1,030 items km^{-2}, cf. **Table 1** for concentration per unit volume). The mass concentration of plastic fragments ranged from 0 to 22 g km^{-2} (median = 0.03 g km^{-2}, mean ± SD = 1.1 ± 3.0 g km^{-2}). The highest plastic fragment concentration was sampled around 300 km South of Sharm El Sheikh (**Figure 2D**).

Possible plastic fibers were found in all samples. However, despite all precautions, blank controls showed between 0 and 3 possible plastic fibers, highlighting the high risk of contamination of samples by fibers. After subtracting the fibers in the blank controls from that in the corresponding samples, the concentration of possible plastic fibers ranged between 9 and 222,838 fibers km^{-2} (median = 25,110 fibers km^{-2}, mean ± SD = 32,703 ± 30,208 fibers km^{-2}) (**Figure 2B**). These items were the most abundant and prevalent (**Figure 2C**) although they were not combined with the estimate of the load of plastic debris in the Red Sea due to uncertainties as to the plastic nature of these fibers, which could not be verified. Nevertheless, because of the low mass of the fibers (about 0.002 mg $fiber^{-1}$ assuming an average plastic density, 1.6 g cm^{-3}, Hidalgo-Ruz et al., 2012), its contribution to the total plastic mass would be minimal (<7%) even if all possible plastic fibers would be included.

Coastal population is relatively low along the Arabian coast, except for the human settlement of Jeddah, accounting for almost 74% of the total coastal population in the East coast of the Red Sea (**Figure 2D**), but we found no clear relationship between plastic concentration and population, either for plastic fragments ($r = -0.19$, $p = 0.30$, $n = 29$) or possible plastic fibers ($r = 0.34$, $p = 0.06$, $n = 29$).

In relation to plastic categories, rigid fragments were the most abundant items (73%), followed by pieces of films (17%), fishing lines (6%), and foam (4%) (**Figure 3A**). Neither pellets nor beads were found in the samples. Thus, we will refer to these plastic categories (pieces of rigid objects, films, fishing lines, and foam) as plastic fragments, in contrast to fibers. Fourier Transform infrared (FT-IR) micro spectroscopy confirmed the plastic identity of all particles analyzed. Most of the plastic items were polyethylene (PE, 69%), followed by polypropylene (PP, 21%), polystyrene (PS, 4%), polyvinyl chloride (PVC, 3%) and polyurethane (PU, 1%), cellophane (CP, 1 %) and polyamide (PA, 1%) (**Figure 3B**). The predominance of PE and PP, accounting for 90% of the total plastic, is in close agreement with the dominance of these materials in floating plastic fragments reported for the Mediterranean Sea (Suaria et al., 2016).

The mean length of the plastic items was 2.08 ± 2.74 mm, ranging from 0.26 to 29.67 mm. The low number of plastic items collected only allowed the use of four size bins to analyse the plastic size distribution, in contrast with narrower size classes allowed by the larger number of items obtained in previous studies (Cózar et al., 2014, 2015, 2017). Once the plastic abundances were normalized by the width of the size bins, the highest abundance appeared in the size range between 0.5 and 1 mm (55.1%) (**Figure 3C**).

Kruskal-Wallis tests indicated that there were statistically significant differences in wind speeds between the surveys likely due to seasonal variability between months of sampling (Kruskal-Wallis test, $p < 0.001$). However, there were no statistically significant differences in abundance of plastic fragments nor possible plastic fibers between the seven surveys (Kruskal-Wallis test, $p = 0.19$ for plastic items and $p = 0.48$ for fibers).

The comparison of plastic fragment concentrations in the Mediterranean Sea and Red Sea showed significant differences (Mann-Whitney, $p < 0.001$), with the concentration in the Red Sea being two orders of magnitude lower than that in the Mediterranean Sea (**Figure 4**). However, we did not observe significant variation in plastic abundance in the Red Sea between water strips to land (Kruskal-Wallis test, $p = 0.4$).

DISCUSSION

The concentration of plastic debris found in the surface waters of the Red Sea is the lowest thus far reported for a coastal sea (**Table 1**). The average concentration of plastic items (excluding fibers) in the Eastern Red Sea was 1.08 g km^{-2}, two orders of magnitude below the concentrations measured using comparable sampling methods in in the Mediterranean Sea (range 423–672 g km^{-2}, Cózar et al., 2015; Ruiz-Orejón et al., 2016; Suaria et al., 2016), and comparable to that in the areas with the lowest plastic loads in the global open ocean (Cózar et al., 2014; **Table 1**).

There are two reasons, not mutually exclusive, that may explain such low abundance of floating plastic fragments in the semi-enclosed Red Sea, (1) low inputs of plastic waste into the Red Sea; and/or (2) fast removal rates of plastic debris from the surface. The average number of inhabitants per linear kilometer settled on Arabian coast is 4,100 km^{-1}, whereas the Côte d'Azur (in the French Mediterranean coast) supports 10,300 inhabitants per linear kilometer (http://web.ornl.gov/sci/landscan/), which is consistent with the difference in plastic load between these two areas (**Figure 4**). Indeed, the lack of a significant correlation between the concentration of floating plastic fragments and population along the Red Sea is in contrast with the correlation found by Pedrotti et al. (2016) in the Côte d'Azur, where there was a more even distribution of population, with several large coastal cities, such as Toulon, Nice, Monaco, or Genova.

In addition, the lack of rivers discharging in the Red Sea may also lead to low inputs, as rivers are major conduits for plastic inputs into the ocean (Lebreton et al., 2017). However, waste management remains an unresolved problem in the Red Sea region (Gladstone et al., 1999). Using the estimates of mismanaged plastic waste per person for Saudi Arabia and France by Jambeck et al. (2015), the total amount of mismanaged plastic waste was estimated to be higher in the Red Sea, at

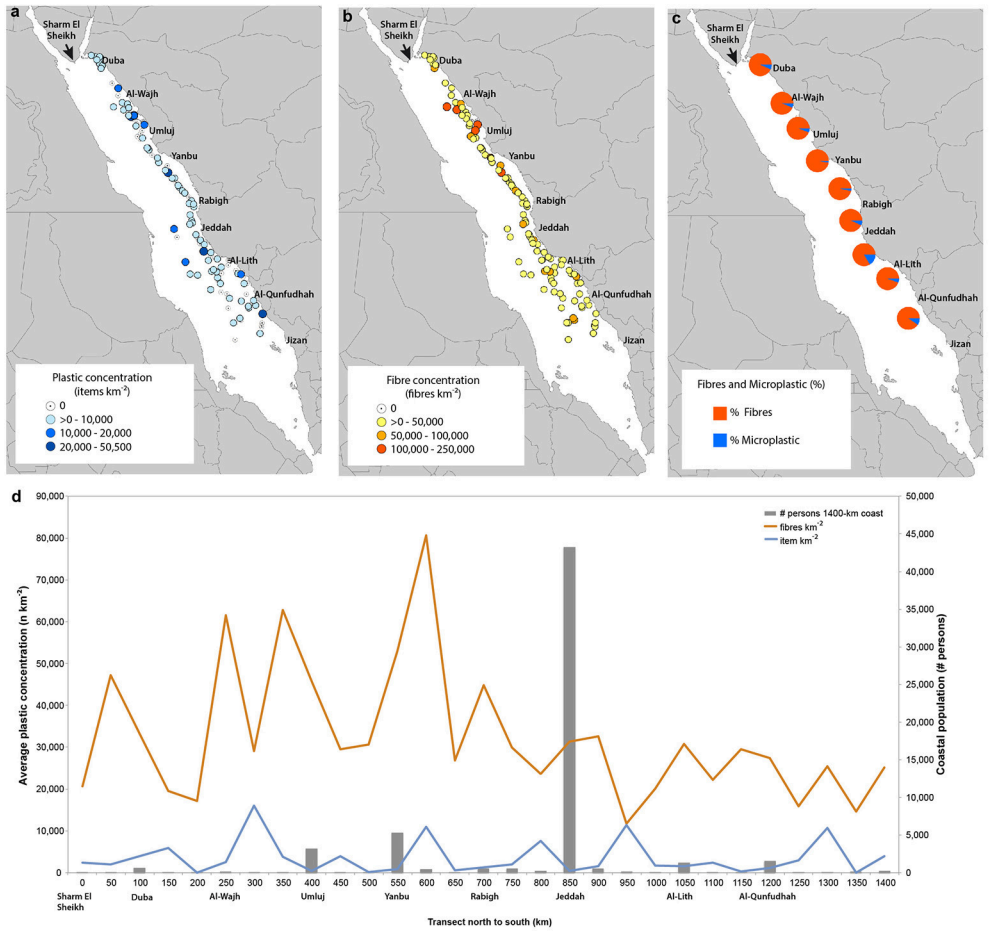

FIGURE 2 | Concentration of plastic fragments **(A)** and possible plastic fibers **(B)** in the 120 sampling sites. Average percentage of plastic fragments and possible plastic fibers along the Red Sea transect **(C)**. Coastal population (gray bars), plastic concentrations (blue line), and possible plastic fibers (orange line) in relation to the linear distance from Sharm El Sheikh (0 km), at the northernmost part of the Red Sea **(D)**.

23.7 tons km^{-1} $year^{-1}$ for the Arabian coast, than for the Côte d'Azur, with 14.4 tons km^{-1} $year^{-1}$, which contrasts with the concentrations of floating plastic debris measured in the waters. The explanation for this mismatch could be related to important differences in the transfer of mismanaged plastic waste from land to sea and/or a higher loss rate of floating plastic. First, the efficiency of this transfer in the Arabian coast and the Côte d'Azur may differ because of the absence of rivers in the Red Sea, which possibly accounts for part of the differences found in floating plastic pollution. In addition, near-shore activities, such as beach tourism, cruises and recreational navigation are far more intense in the Côte d'Azur, which experiences a large increase in summer population and waste generation on the coasts, than in the Arabian coast of the Red Sea, where coastal tourism is virtually absent. The monitoring of beach debris in the Mediterranean Balearic Islands demonstrated the importance of near-shore activities associated with summer coastal tourism in the abundance and composition of the waste stranded on beaches (Martinez-Ribes et al., 2007), which has high probability to become marine floating debris.

Provided the lack of rivers in the Red Sea, wind could play a major role in transporting plastic waste from land into the sea, which would be most effective in delivering fibers and light film-type objects, such as wrappings and bags. Indeed, this typology of light debris was relatively abundant in Red Sea surface waters. The concentration of possible plastic fibers was comparable to that reported for other ocean regions (Setälä et al., 2016), with fibers representing, numerically, 92%, on average of the total number of possible plastic items (**Figure 2C**). The typology of the floating plastic fragments (hard pieces, films, lines, and foam) showed a proportion of 17% of films in abundance, which is in the upper range of the proportion of film reported in assessment of floating plastic litter in the Mediterranean Sea (range from 1 to 6%, Cózar et al., 2015; Suaria et al., 2016; Van der Hal et al., 2017).

Surface losses of plastic may be related to multiple processes. Shore deposition, nano-fragmentation, ingestion by marine

TABLE 1 | Comparison of floating plastic fragment concentrations (listed by increasing number) obtained in previous studies performed in the open ocean and other semi-closed seas and those derived here for the Red Sea.

	Average abundance (items km^{-2})	Average abundance (items m^{-3})	Maximum abundance (items km^{-2})	Average weight (g km^{-2})	*N*	Source
Red Sea	3,546	0.04	50,393	1.1	120	This study
Open Ocean: non-accumulation zones	7,894	0.05	190,000	21	629	Cózar et al., 2017
Baltic Sea	14,667	0.09	48,000	ND	12	Setälä et al., 2016
Greenland and Barents Seas	80,796	0.54	317,000	99	17	Cózar et al., 2017
Mediterranean Sea	147,500	0.59	1,164,403	579	71	Ruiz-Orejón et al., 2016
Open Ocean: subtropical accumulation zones	155,389	1.04	1,260,000	407	275	Cózar et al., 2017
Mediterranean Sea	195,510	1.96	576,555	ND	33	Pedrotti et al., 2016
Mediterranean Sea	243,853	0.49	1,224,084	423	39	Cózar et al., 2015
Mediterranean Sea	382,400	0.76	4,321,120	672	74	Suaria et al., 2016
Mediterranean Sea	1,518,340	7.68	64,812,600	ND	17	Van der Hal et al., 2017
East Asian Seas	1,720,000	3.70	ND	ND	56	Isobe et al., 2015

All plastic concentrations exclude fibers.

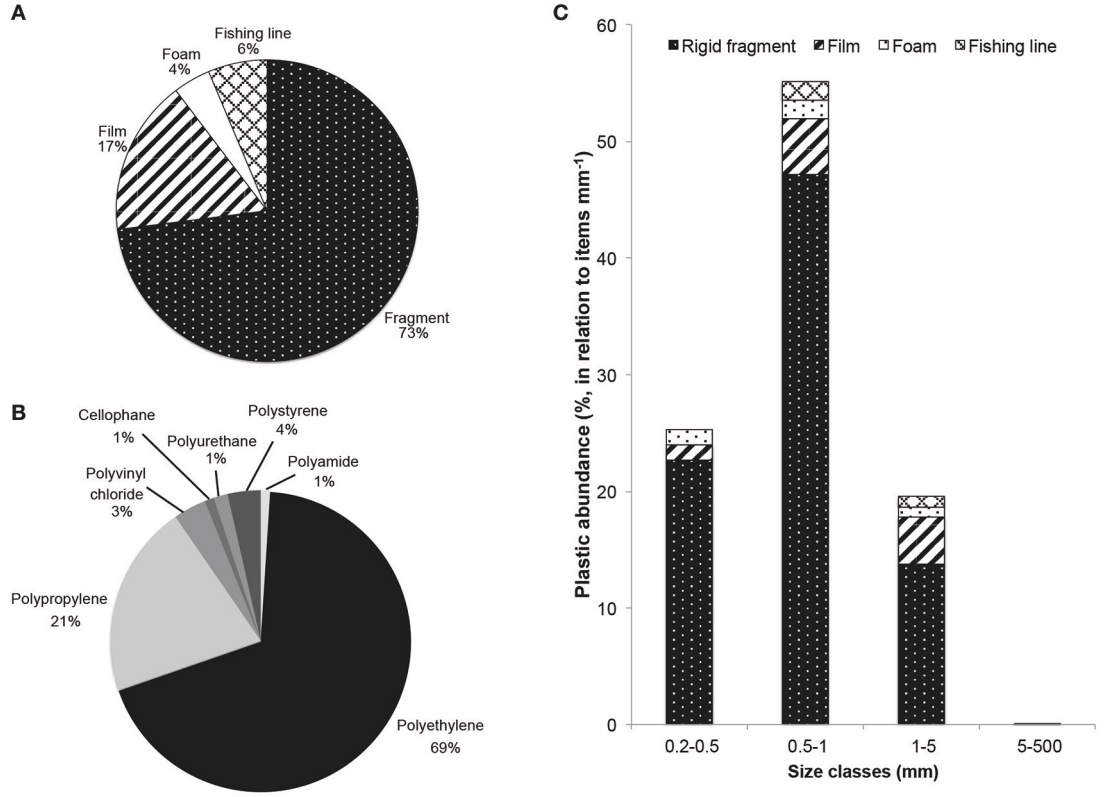

FIGURE 3 | Frequency of occurrence of plastic items by category **(A)** and type **(B)**, and relative abundance of plastic items per size classes and category **(C)**. Please note that fibers are excluded because just fragments of rigid objects, films, fishing lines, and foam could be tested through FT-IR spectroscopy.

organism, or sinking by aggregation or ballasting due to epiphytic growth are some of the general pathways suggested as conduits of plastic debris from the surface waters to coast (Cózar et al., 2014). Here, we limit our discussion on the surface losses of plastic to suggest some processes that could operate with particular intensity in the Red Sea. Firstly, the presence of mangroves and extensive coral reefs is an additional feature that differentiates Arabian coast of the Red Sea from that of the Mediterranean.

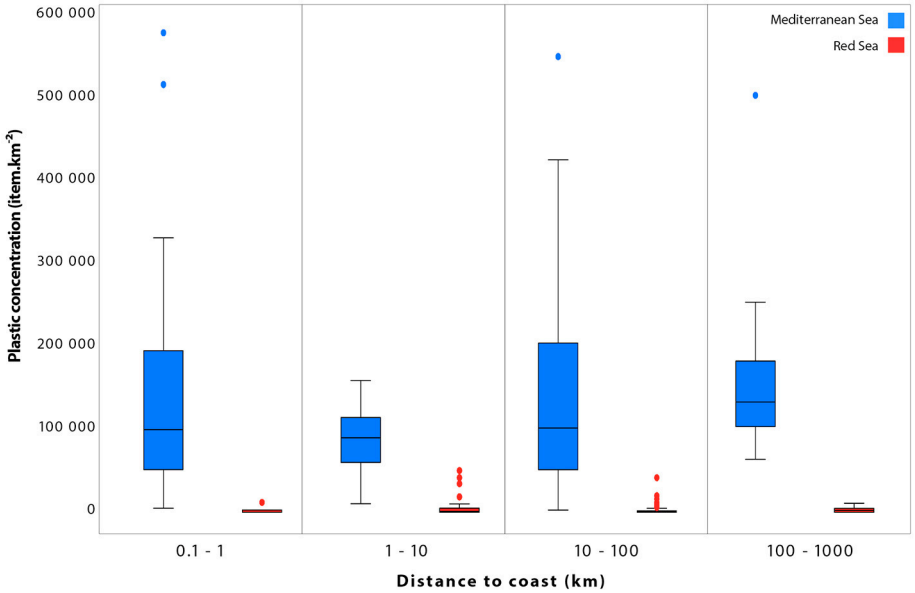

FIGURE 4 | Comparison between floating plastic concentrations in Mediterranean (blue) and Red Sea (red) for different water strips from land (0.1–1, 1–10, 10–100, and 100–1,000 km). Data from the Mediterranean are described in Pedrotti et al. (2016). The Mediterranean water strips close to land (<10 km to land) mainly accounts for the Côte d'Azur in France. Black lines into the boxes indicate median values, boxes indicate first and third quartiles, and whiskers indicate the 90 and 10th percentiles.

Mangroves have been reported to act as traps for microplastics in Brazil and Singapore (Ivar do Sul et al., 2014; Nor and Obbard, 2014). Therefore, mangroves could be trapping part of the waste transported by the occasional rainfall events as well as marine debris stranded on the coasts during storm events. Although we do not have quantitative information on plastic waste in Red Sea mangroves, macro-debris was commonly observed in these coastal ecosystems during our surveys. The extensive coral reefs along the coast of the Red Sea (**Figure 1**), which act as large filters of particulate materials, may also act as important microplastic sinks. Indeed, numerous coral-reef inhabitants, including the corals themselves, efficiently capture bacteria, phytoplankton, zooplankton and small-sized detritus in the water advected over the reefs (Tranter and George, 1969; Yahel et al., 1998; Houlbreque and Ferrier-Pages, 2009). Although the studies of plastic ingestion in corals are yet very scarce, researchers recently demonstrated that scleractinian corals do capture and ingest microplastics, from 0.1 to 2 mm in length (Hall et al., 2015), and from 0.125 to 1 mm (Allen et al., 2017); matching the size range of plastic debris reported in our assessment. Mesopelagic fish assemblages have also been reported to be relevant feeders of plastic fragments in the size interval from 0.5 to 5 mm (Boerger et al., 2010; Davison and Asch, 2011; Foekema et al., 2013), and are especially abundant in the Red Sea, with nocturnal feeding migration to the surface particularly active in comparison with other world regions (Dypvik and Kaartvedt, 2013). Other important consumers may include seabirds and turtles, which are abundant in the Red Sea. The size distribution of floating plastic debris found in the Red Sea, with a drop in abundance toward the smallest sizes, is consistent with the hypothesis of an efficient biological removal of small plastic particles (Cózar et al., 2014), but the role of Red Sea biota, including mangroves, coral ecosystems, mesopelagic fish and other consumers, as sinks for floating plastic debris need yet to be assessed.

Here we show a remarkable low concentration in abundance and weight of plastic fragments in the surface waters of the Red Sea, orders of magnitude below that reported for other semi-enclosed seas, such as the Mediterranean Sea. Whereas, this may be partially attributable to sporadic surface runoff into the Red Sea and low levels of coastal development and tourism, the poor waste management in the region suggests relatively high inputs. Hence, the exceedingly low floating plastic concentrations in the Red Sea also suggest intense biological removal as an important process in the mass balance conducive to the low loads of plastic fragments that characterize Red Sea waters.

AUTHOR CONTRIBUTIONS

EM, CM, AC, and CD performed research; EM and CM collected and processed the samples; EM, CM, AC, and CD analyzed data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2017.00333/full#supplementary-material

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Anthropogenic Litter on Beaches With Different Levels of Development and Use: A Snapshot of a Coast in Pernambuco (Brazil)

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This research aimed to evaluate anthropogenic litter found on beaches with different levels of development and use along the coast of Pernambuco (northeast Brazil) to determine patterns in composition and origin. The study was conducted in January 2013 at nine beaches classified into three groups according to the level of urbanization. At each beach, three sections measuring 100 m long and 1 m wide were defined along the high-tide waterline where all items of anthropogenic litter larger than 2 cm were visually counted and classified. The sections were separated by intervals of 100 m. Within each 100 m section, random stretches of 10 m were selected where all the visible plastic fragments with sizes between 0.5 and 2 cm were collected and bagged for subsequent counting. Sources of anthropogenic litter were divided into three categories, namely beach users, land-based (houses/residences), mixed, and fisheries. A total of 12,815 items were found on the nine beaches within the three transects on each beach (one survey per beach), with densities (items/m^2) of 2.3, 5.7, and 6.3 for groups 1, 2, and 3, respectively. The most-represented items of anthropogenic litter in the evaluated samples were plastic, food scraps, and wood (wooden skewers). With respect to items composed of plastic, the majority were cigarette butts (45%). Additionally, cigarette butts made up 26% of all anthropogenic litter samples collected. A larger amount of fragments smaller than 2 cm occurred in all beaches; for this size fraction, the densities were 0.6, 0.5, and 0.76 items/m^2 for groups 1, 2, and 3, respectively, which were 1.5, 2.5, and 1.65 times higher, respectively, compared to those of fragments larger than 2 cm.The beaches with lower levels of urbanization also had smaller quantities of anthropogenic litter. Items related to beach users were predominant for most of the beaches. The confirmation that beach users are primarily responsible for the generation of anthropogenic litter may contribute to the development of strategies to reduce the problem, such as installing bins and distribution containers for anthropogenic litter collection and designing educational campaigns for beach users.

Keywords: coastal environments, marine pollution, marine litter, plastic litter, beach users

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INTRODUCTION

Beaches are used by different social groups with diverse interests. The level of use of each beach varies based on several factors (Tudor and Williams, 2006; Oh et al., 2010). The different levels of beach usage are mainly determined by factors such as proximity to urban centers and ease of access (Paula et al., 2013), infrastructure availability (Silva et al., 2013), and frequency of routine cleaning of the area (Pendleton et al., 2001; Nelson and Botterill, 2002; Tran et al., 2002; Tudor and Williams, 2006). The rapid growth in coastal development, stimulated by tourism and residential expansion, often results in dense population and infrastructure that can cause resource degradation and pollution (Lithgow et al., 2014; Botero et al., 2015). Beach quality assessments are often related to user perception, and aesthetic values, such as hygiene and cleanliness, are their main concerns (Lozoya et al., 2014; Botero et al., 2015) despite current environmental conditions (UNEP, 2005, 2009, 2016; Scisciolo et al., 2016).

Brazil is endowed with high tourism potential owing to its 8,500 km coastline, along which the population is concentrated (IBGE, 2011). Therefore, Brazilians are regular visitors to beaches and enjoy the low cost of this type of entertainment and the mild climate of most states. Zuanazzi (2016) evaluated the floating population in 11 coastal municipalities of Rio Grande do Sul during the summer period, and found that the total amount of people increases from 207 thousand to more than 500 thousand. In some cases, the population increase is over 400%.

The occurrence of anthropogenic litter in coastal areas (populated or not) has been reported globally by numerous researchers, and is highlighted as a growing threat (Debrot et al., 1999; Coe and Rogers, 2000; Araújo and Costa, 2007a; Moore, 2008; Galgani et al., 2014; Scisciolo et al., 2016). Anthropogenic litter deposited on beaches usually has several sources. As such, it may have originated from rivers that flow into the area, from users, or from the sea itself through currents and wind (Ivar do Sul and Costa, 2007; Moore, 2008).

Although the occurrence of anthropogenic litter on beaches can be related to several factors, such as location and morphology of the beach, the presence of rivers and streams, and winds, the contribution of users is proven and has been reported in numerous studies (Santos et al., 2003; Araújo and Costa, 2007a; Silva et al., 2008, 2015, 2016; Silva-Cavalcanti et al., 2009; Dias Filho et al., 2011; Scisciolo et al., 2016; Wilson and Verlis, 2017). Urban beaches are typically littered with cigarette butts, food wrappers, cups, plastic straws, and beverage cans (Williams and Simmons, 1997, 1999; Araújo and Costa, 2006; Silva et al., 2008; UNEP, 2009, 2016; Ivar do Sul and Costa, 2013; Ivar do Sul et al., 2014; Leite et al., 2014). The presence of a large number of users is also responsible for greater commercial exploitation of beaches. Commercial activity has been developed in these environments, especially related to the sale of food and beverages. This activity forms a constant source of anthropogenic litter that is often discarded on the beach itself (Araújo et al., 2012).

The intense use of natural environments such as beaches is almost always accompanied by irregular anthropogenic litter disposal (Santos et al., 2003; Araújo and Costa, 2007a; Silva et al., 2008, 2016; Silva-Cavalcanti et al., 2009; Vieira et al., 2011; Scisciolo et al., 2016; Wilson and Verlis, 2017). Especially in urban beaches, the accumulation levels of solid residues are related to the arrival of visitors. Marshall et al. (2014) stated that the proximity of an urban environment to a beach is of particular importance as a source of anthropogenic pressure on the beach.

Anthropogenic litter may be responsible for economic, social, and environmental damage, such as expenditure incurred by public agencies to clean beaches instead of using these funds for other areas in need (Araújo and Costa, 2006), loss of aesthetic value and tourism potential of the sites (Araújo and Costa, 2007a; Silva-Cavalcanti et al., 2013), pollution by pathogenic agents (Zuza-Alves et al., 2016), and damage to marine biota by accidental ingestion and entanglement, which can cause choking, injury, illness, and death of marine organisms (Moore, 2008; Attademo et al., 2015; Mendes et al., 2015). Along with other forms of extremely harmful pollution, plastics present in anthropogenic litter are one of the biggest concerns for the ocean in terms of marine pollution because of their intrinsic properties, such as low density (which facilitates their fluctuation and consequent dispersion), persistence, cumulative build-up over time, and widespread use (Moore, 2008; Corcoran et al., 2009; Thompson et al., 2009; Scisciolo et al., 2016). To determine the source of anthropogenic litter, researchers must know their location and use, assess the quantity and composition of anthropogenic litter, and relate these data to the environmental and socioeconomic characteristics of the area. Identification of the sources of anthropogenic litter is fundamental for planning strategic actions to minimize the problem (Pasquini et al., 2016).

This research aimed to evaluate anthropogenic litter found on beaches with different levels of development and use along the coast of Pernambuco (a state in northeast Brazil) in order to determine patterns in composition and origin and to answer the following questions. (i) Is there a relationship between beach use/development and anthropogenic litter? (ii) Apart from the larger items, is there a large number of small plastic fragments, which pose particular threats?

MATERIALS AND METHODS

This study was conducted in January 2013 at nine beaches located along the coast of Pernambuco (**Figure 1**), which were classified into three groups according to the level of urbanization (**Table 1**), namely Low (Forte Orange, Maracaípe, and Carneiros), Medium (Porto de Galinhas, Maria Farinha, and Campas), and High (Bairro Novo, Boa Viagem, and Piedade).

The beach groups differed from each other with respect to the level of urbanization, use, and environmental conditions (**Table 1**).

At each beach, three sections (replicates) measuring 100 × 1 m of the strandline (maximum level reached by the tide and where anthropogenic litter was deposited) were sampled based on Silva-Cavalcanti et al. (2009, 2013) and Jayasiri et al. (2013). The sections were separated by intervals of 100 m. In each section, all items of anthropogenic litter larger than 2 cm were visually counted and classified according to their composition (plastic,

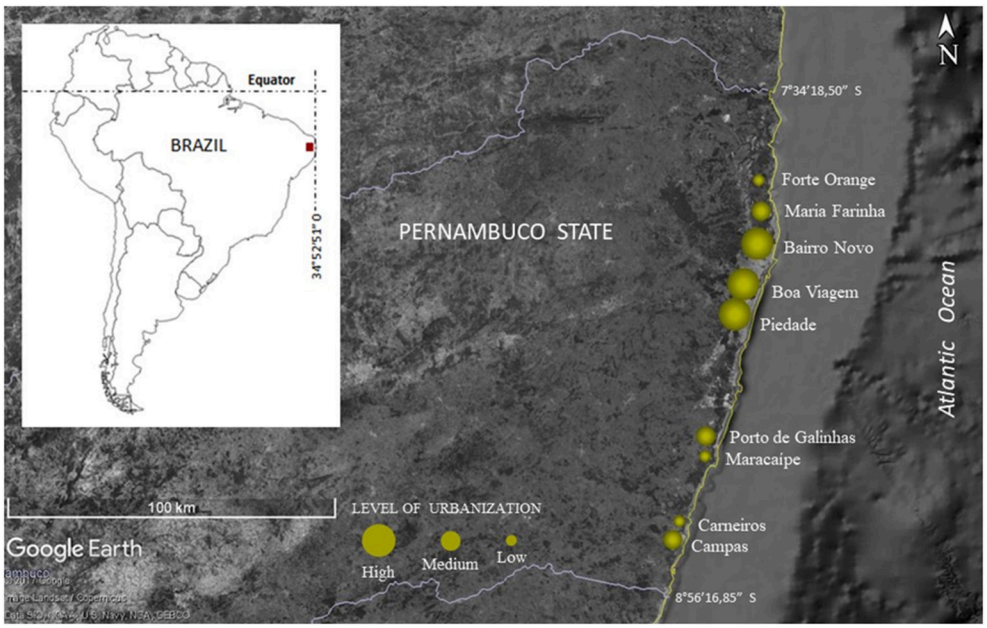

FIGURE 1 | Location of the analyzed beaches and level of urbanization.

TABLE 1 | Characteristics of the studied beaches based on type of use and occupation of land, which determined the environmental conditions of the studied area (Araújo and Costa, 2008).

Group	Level of urbanization	Beaches	Characterization of urban occupation and use	Environmental conditions
1	Low	Forte Orange Maracaípe Carneiros	Nearby small-sized urban centers. Few residences Low use and largely confined to periods of high season (December to March). Low informal commercial activity.	Foreshore and backshore preserved. Many stretches of preserved vegetation.
2	Medium	Maria Farinha Porto de Galinhas Campas	Close to medium-sized urban centers with predominance of homes (<2 floors) and hotels. High use during periods of high season, and lower use in other seasons. Moderate informal commercial activity.	Reduction of foreshore and backshore in some stretches. Reduction of native vegetation areas.
3	High	Bairro Novo Boa Viagem Piedade	Urban beaches Beachfront almost completely verticalized. High use during the whole year. High informal commercial activity.	Fore shore and backshore reduced. Lack of native vegetation. Erosion

glass, metal, paper, wood, and food/organic). Categories were used to identify the most likely sources of anthropogenic litter based on Silva-Iñiguez and Fischer (2003). The categories were beach users, land-based (houses/residences), mixed, and fisheries (**Table 2**). The category of mixed source included anthropogenic litter of unclear origin (e.g., disposable diapers could be either from beach users or from land-based sources). Cigarette butts were also of uncertain origin because they can be discarded by users on beaches or in urban centers, and can reach the beaches through transportation by urban runoff (Armitage and Rooseboom, 2000; Becherucci and Pon, 2014; Williams et al., 2016).

Fragments larger than 2 cm were sampled following the same sample design of the aforementioned categories (100 × 1 m sections). For comparison, a random 10 m stretch of all sections was subsampled for all plastic fragments (0.5–2 cm).

An analysis of variance (ANOVA) was performed to determine the possible differences in the total number of litter items when compared between beach groups. The amount of beach-user related items was then compared between beaches

TABLE 2 | Classification of anthropogenic litter items according to the most likely source (Silva-Iñiguez and Fischer, 2003).

Sources	Items
Beach users	Straws, lollypop sticks, wooden skewers, water bottles, plates, cups, and cutlery made from plastic, food wrappers made from bio-orientated metalized polypropylene, hydrogen peroxide and sunscreen bottles, rubber sandals, cans and metal beverage lids, food scraps, food containers (such as take-away, and discardable plates and trays)
Land-based	Medicine packaging/containers, cotton swabs, bottles of cleaning products and personal hygiene products, food containers (such as margarine and cereal bags), cardboard.
Mixed	PET bottles, bottle rings/caps, plastic lids, disposable diapers, cigarette butts, condoms, maxipads, corks, plastic bags and wrappers, toys, lighters, pens, syringes and needles, Tetra Pak packages.
Fisheries	Nylon (monofilament lines, cables and ropes polyfilament) and tape, nets, styrofoam, fishing lines, glow/light sticks.

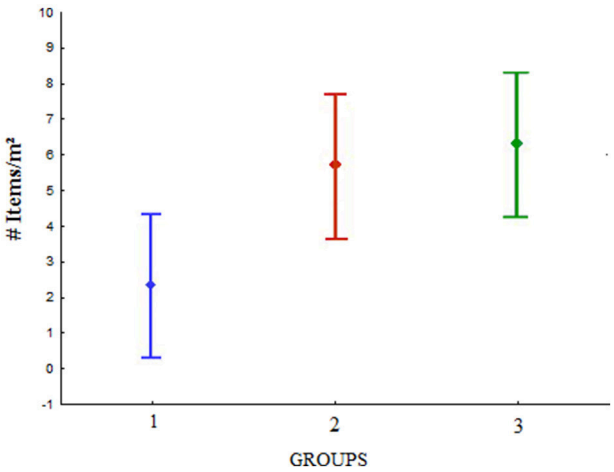

FIGURE 2 | Mean value and standard deviation of total items for the evaluated groups (1, 2, and 3).

in each group. The premises of normality and homoscedasticity were assumed. Where the ANOVA indicated a significant difference, a Tukey test followed by an HSD test was used to determine which beaches and items were significantly different at the 0.05 level of probability. These analyses were performed using STATISTICA software (Box et al., 2005; Silva-Cavalcanti et al., 2009).

RESULTS

A total of 12,815 items were found on the nine beaches within the three transects on each beach (one survey per beach), with densities (items/m^2) of 2.3, 5.7, and 6.3 for groups 1, 2, and 3, respectively. Although the same types of waste occurred on all beaches, the mean amount varied, especially among groups, and increased according to the level of urbanization when considering the total per group (16.3, 39.8, and 43.9% for groups 1, 2, and 3, respectively) (**Figure 2**).

However, when only the beaches were considered, the largest amounts of anthropogenic litter occurred at Porto de Galinhas (group 2) and Bairro Novo (group 3) beaches, containing 25.4 and 21.4% of litter, respectively. The lowest values were found on Carneiros and Maracaípe beaches (group 1), with 3.3% and 3.1% of litter, respectively (**Figure 3**). Forte Orange and Bairro Novo presented the largest amounts of residues (60 and 49%, respectively) within their respective groups.

The most-represented items of anthropogenic litter in the evaluated samples were plastic, food/organic, and wood (wooden skewers), which constituted 57.3, 31.4, and 8.5% of the total samples, respectively. Other items accounted for less than 3% of the samples.

With respect to items composed of plastic, the majority were cigarette butts (45%); cigarette butts made up 26% of all anthropogenic litter samples collected.

Possible Sources of Anthropogenic Litter

Items related to beach users were predominant for seven beaches, and were the majority for all the beaches in groups 2 and 3 (**Table 3**, **Figure 4**).

Porto de Galinhas showed a higher value of litter from beach users than that of the other beaches with higher levels of urbanization (**Table 4**). The results demonstrated that Porto de Galinhas beach was significantly different from the other beaches in group 2 ($p < 0.05$), as well as those in group 1 ($p < 0.05$). However, group 3 beaches ($p < 0.05$) were similar to Porto de Galinhas beach ($p > 0.05$) (**Tables 3**, **4**).

In the category of beach-user related litter, 5 types of items (food scraps, wooden skewers, plastic straws, metallized plastic packaging, and lollypop sticks) made up 89.15% of all items of this source. The largest component was food scraps, which comprised 55.66% of all beach-user related litter (**Figure 5**).

Regarding the land-based source, Forte Orange beach obtained the highest values of items connected to this source, followed by Bairro Novo. Maracaípe and Porto de Galinhas had the lowest amounts. Within the mixed-source category, Porto de Galinhas and Bairro Novo beaches had the highest mean number of items, while Carneiros and Maracaípe beaches had the lowest amounts.

For the fisheries source category, Porto de Galinhas, Campas, and Maracaipe had the highest mean number of items. On the

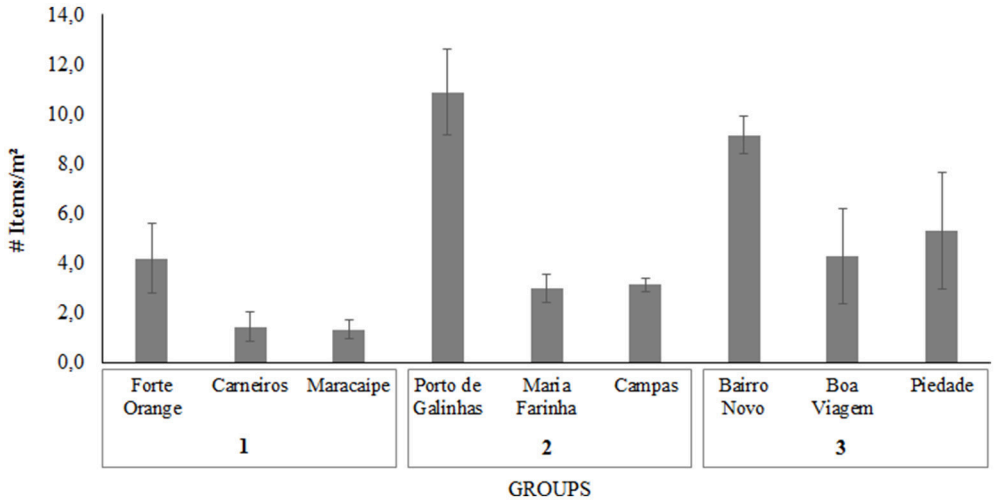

FIGURE 3 | Mean value and standard deviation of total items for each evaluated beach.

TABLE 3 | Mean value of items according to their possible source for all evaluated beaches.

		Sources			
Group	**Beach**	**Beach users** $\bar{X} \pm$ S.D.	**Land based** $\bar{X} \pm$ S.D.	**Mixed** $\bar{X} \pm$ S.D.	**Fisheries** $\bar{X} \pm$ S.D.
1	Forte Orange	153.3 ± 69.5	36.7 ± 33.8	170.3 ± 49.6	9.3 ± 7.5
	Maracaípe	47.7 ± 15.2	0.7 ± 1.1	57.0 ± 20.8	12.0 ± 7.5
	Carneiros	78.7 ± 36.1	4.7 ± 1.5	33.3 ± 20.5	11.3 ± 6.7
2	Maria Farinha	164.7 ± 27.8	7.7 ± 1.1	101.7 ± 25.5	4.3 ± 1.5
	Porto de Galinhas	637.0 ± 147.0	2.7 ± 1.1	404.3 ± 57.5	16.3 ± 4.7
	Campas	172.0 ± 32.4	7.0 ± 2.6	95.7 ± 4.7	16.3 ± 8.5
3	Bairro Novo	572.3 ± 116.2	9.0 ± 4.6	287.7 ± 80.4	4.3 ± 2.0
	Boa Viagem	259.0 ± 110.0	5.3 ± 4.0	138.7 ± 75.7	0.0 ± 0.0
	Piedade	332.0 ± 175.1	7.0 ± 3.6	161.3 ± 63.5	1.7 ± 0.6

other hand, Boa Viagem and Piedade beaches had the lowest amounts (**Table 3**).

Evaluation of Plastic Fragments

Fragments larger than 2 cm corresponded to 4.47% of the total items, with 0.40, 0.20, and 0.46 items/m^2 for groups 1, 2, and 3, respectively. The largest amounts of fragments for both sizes were found in Forte Orange and Bairro Novo (**Figure 6**). A larger amount of fragments smaller than 2 cm occurred in all beaches; for this size fraction, the densities were 0.6, 0.5, and 0.76 items/m^2 for groups 1, 2, and 3, respectively, which were 1.5, 2.5, and 1.65 times higher, respectively, compared to those of fragments larger than 2 cm.

DISCUSSION

The level of urbanization had an influence on anthropogenic litter abundance, with the amount of anthropogenic litter increasing among the three beach groups. Leite et al. (2014) showed that there was a significant relationship between the proximity to an urban center and the contamination of the studied beaches. Hardesty et al. (2016) also found high debris densities near cities. Becherucci et al. (2017) evaluated the presence of litter on beaches at two locations on the Argentine coast, and noted that the largest proportion of litter occurred on beaches with greater use; according to the results, the amount and composition of the litter were reflections of the recreational use of the beaches. However, Porto de Galinhas presented the largest amount among all beaches, although it belonged to group 2 (medium level of urbanization). Two factors may have contributed to the large amount of anthropogenic litter in Porto de Galinhas. First, this beach is a scenic attraction and the main tourist destination in Pernambuco, with a larger concentration of users than the two other beaches with the same urbanization level, especially during the summer season (November to February). Second, the foreshore and backshore

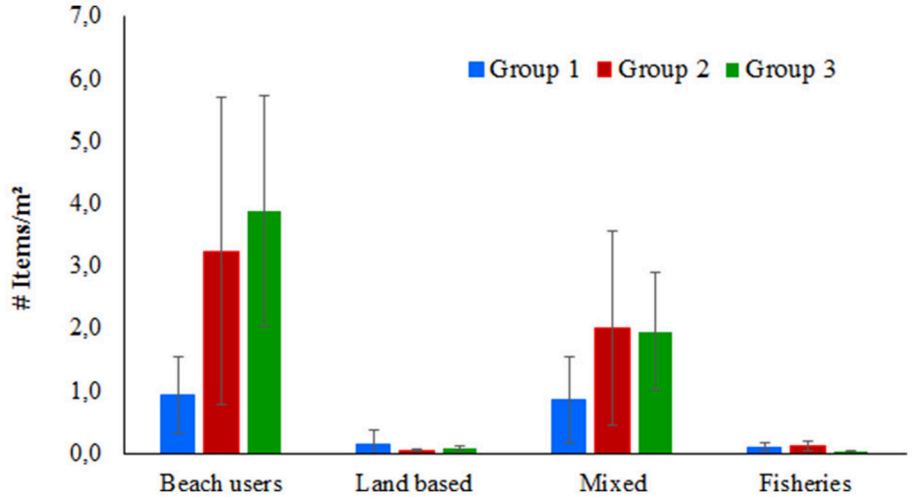

FIGURE 4 | Mean value of items according to their possible source evaluated in the three beach groups.

TABLE 4 | Summary of ANOVA analyses for the total number of items, development beach groups, and number of beach users.

Source of variation	Interaction	*F*-value
Total number of items x Groups	* 1 2 3* 1* 2 3	4.67
TOTAL OF USERS SOURCE ITEMS x GROUPS		
Group 1	* FO* CAR MARAC	69.887
Group 2	* PG* MF CAM	
Group 3	NS	

Tukey's HSD test post hoc comparison determined the differences in the total items on beaches in each group. FO is Forte beach, CAR is Carneiro beach, MARAC is Maracaípe beach, MF is Maria Farinha, PG is Porto deGalinhas, CAM is Campas beach, and NS is no significance.

$p \leq 0.05$ NS-no significant. Underline means NO difference.

are reduced and are completely occupied by users, thereby favoring an accumulation of anthropogenic litter that is worsened by a poor city cleaning system.

Some factors may have contributed to the large amounts of litter in Forte Orange and Bairro Novo compared with the other beaches in their respective groups. Forte Orange is located on the mouth of an estuary, and thus receives a greater contribution of anthropogenic litter deposited by the tide and waves. When anthropogenic litter is improperly disposed of near watercourse environments, there is a high probability of anthropogenic litter being transported to coastal environments (Araújo and Costa, 2007b). Carneiros is a more isolated beach with access limitations due to the presence of a large number of private lands that prevent the entrance of vehicles and users. Maracaípe is a beach without the protection of beach rocks and with waves that favor surf; thus, it is mainly used by surfers. Therefore, on both beaches (Carneiros and Maracaípe) the amount of users is lower, which reduces the amount of litter generated. Although Bairro Novo also has a high concentration of users, it is distinct from the other beaches in its group (within large cities) because it lacks an efficient system of street cleaning to remove the large amount of anthropogenic litter produced by its users. In contrast, the Boa Viagem and Piedade beaches rely on a system of street cleaning that removes most of the anthropogenic litter. For example, in Boa Viagem, 60 men clean the pavement and the sand strip three times per day by collecting litter (manually and by sweeping), which is then bagged and taken away. At night, sand cleaning is conducted by two tractors with sieves that remove litter up to 20 cm deep. Twenty tons of waste are removed from the beach daily (https://www.recife.pe.gov.br/pr/servicospublicos/emlurb/praiaviva.php).

In relation to the most likely sources of anthropogenic litter assessed, the confirmation that users are primarily responsible for the generation of anthropogenic litter may contribute to the development of actions and strategies aimed at reducing the problem, such as installing bins, installing distribution containers for anthropogenic litter collection, and implementing educational campaigns aimed at beach users.

Although food scraps (the most abundant items related to beach users) degrade faster than other types of anthropogenic litter, they are ideal substrates for the proliferation of pathogenic microorganisms (Zuza-Alves et al., 2016) and serve as a food source for many disease-spreading animals, such as insects, rats, and pigeons (Araújo et al., 2012).

Porto de Galinhas and Campas possess large expanses of sandstone reefs that have a high diversity of organisms (Ferreira et al., 2004; Frédou et al., 2009; Barradas et al., 2010). Thus, these beaches are extensively used for artisanal fishing, which may explain the greater number of items related to fisheries. The

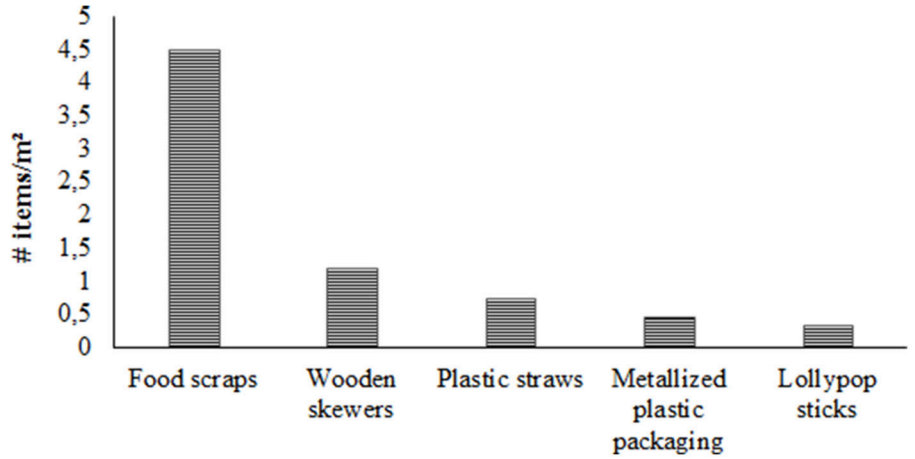

FIGURE 5 | Most abundant beach-user related litter items.

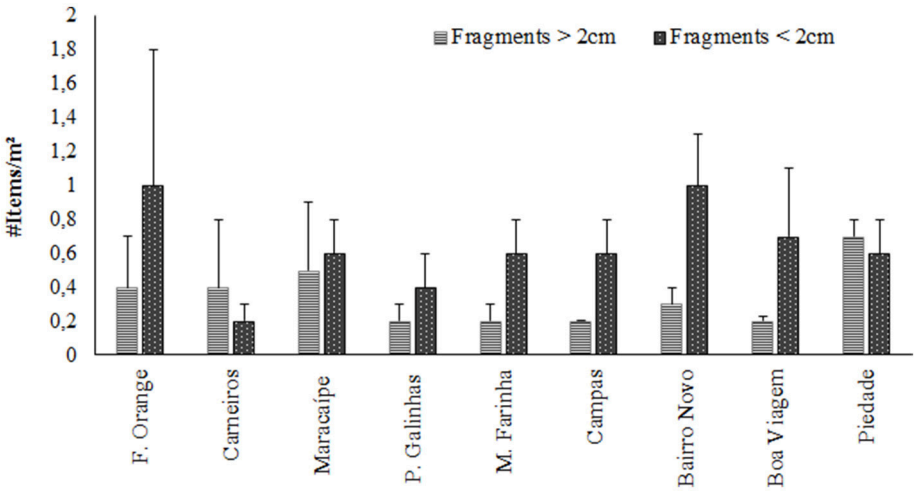

FIGURE 6 | Mean number and standard deviation of plastic fragments found on the strandline at the evaluated beaches.

presence of these residues in Maracaípe was probably due to its proximity to Porto de Galinhas.

For all beaches, the occurrence of residues unrelated to beach users was likely a consequence of the lack of effective anthropogenic litter management conducted by the municipalities.

With respect to the composition of the litter, plastic was the most abundant material. The high presence of plastic on all evaluated beaches is a pattern that occurs in numerous places in Brazil and other countries (UNEP, 2005, 2009, 2016; Araújo and Costa, 2007a; Ivar do Sul and Costa, 2007, 2013; Moore et al., 2011; Vieira et al., 2011).

Cigarette butts were also abundant, there by demonstrating that this item can be used as a marker of anthropogenic litter pollution level in highly urbanized and/or heavily used beaches. Santos et al. (2005) evaluated the relationship between beach users and anthropogenic litter and quantified the input of tourism-related anthropogenic litter by users with different socio-economic attributes in the southern Brazilian coast. Despite interviewing people that do not usually admit to littering on the beach, they observed that smokers usually leave their cigarette butts in the sand without much concern. Worldwide, the majority of sandy public beaches in tourist areas are also littered with cigarette butts (Novotny and Slaughter, 2014; Scisciolo et al., 2016; Becherucci et al., 2017). According to the International Coastal Cleanup program, which was conducted in 2016, 504,583 volunteers collected 13,840,398 items, of which 1,863,838 were cigarette butts (Ocean Conservancy, 2017)[1]. The small size and coloration of these items facilitate mixing in with the sand, thereby making it difficult for garbage disposal workers to gather

[1]Available online at: http://www.oceanconservancy.org/our-work/international-coastal-cleanup/2016-ocean-trash-index.html (Accessed November 13, 2017).

them, which leads to adverse effects on the environment (Ariza et al., 2008; Ariza and Leatherman, 2012; Leite et al., 2014).

The presence of small fragments (<2 cm) in large quantities demonstrated that larger items undergo fragmentation into smaller pieces. Items smaller than 2 cm can still suffer successive breakage and become increasingly smaller items.This fact is potentially impactful, mainly because the small size favors a nearly imperceptible accumulation in the environment; consequently, these fragments persist in the environment for indefinite periods. The size of the residue is directly related to its hazard to animals; the smaller the fragments are, the greater the risk of accidental ingestion or confusing them as food (Galloway, 2015; Lusher et al., 2015).

When ingested, fragments can cause obstruction in the digestive system of the animal or give it a feeling of satiety, which will then reduce its search for food and eventually cause malnutrition and death. There are numerous reports of animals that contained plastic fragments or whole items inside their digestive tracts (Bugoni et al., 2001; Copello and Quintana, 2003; Ivar do Sul and Costa, 2007; Moore, 2008; Possatto et al., 2011; Galloway, 2015; Lönnstedt and Eklöv, 2016; Vendel et al., 2017).

Beach cleaning is costly because it is time-consuming as well as economically expensive. According to Mouat et al. (2010), municipalities throughout the northeast Atlantic region continue to face high costs associated with the removal of anthropogenic litter. UK municipalities spend approximately €18 million each year removing anthropogenic litter, which represents a 37% increase in cost over the past 10 years. Similarly, removing anthropogenic litter costs municipalities in the Netherlands and Belgium approximately €10.4 million per year. Clearly, costs increase with sample area. Therefore, the collection effort must be planned with the goal of saving both economic and human resources.

Planning should establish the minimum effort required to collect data in order to produce satisfactory results, so that the methodology can be repeated with clear and economical ways to manage research activity as well as other research. The choice of representative items (such as cigarette butts) to determine the level of beach pollution can reduce the sampling effort by enabling diagnosis for more extensive areas.

The human component, including attitude toward the environment, is critical for effective anthropogenic litter management. Public awareness and encouraging changes in attitudes related to anthropogenic litter management are essential components in efforts to mitigate the presence of anthropogenic litter in marine and coastal environments.

AUTHOR CONTRIBUTIONS

MA contributed with study planning and execution, manuscript writting and work coordination. JS-C contributed with field work, samples treatment and statistical analysis. MC made comments to the text and contributed with figures and tables.

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Microplastic Distribution at Different Sediment Depths in an Urban Estuary

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As plastic production increases, so to do the threats from plastic pollution. Microplastics (defined as plastics <5 mm) are a subset of marine debris about which we know less than we do of larger debris items, though they are potentially ubiquitous in the marine environment. To quantify the distribution and change in microplastic densities through time, we sampled sediment cores from an estuary in Tasmania, Australia. We hypothesized that the type, distribution and abundance of microplastics observed would be associated with increasing plastic production, coastal population growth, and proximity to urban water outflows and local hydrodynamics. Sediments ranging from the year 1744 to 2004 were sub-sampled from each core. We observed microplastics in every sample, with greater plastic frequencies found in the upper (more recent) sediments. This time trend of microplastic accumulation matched that of global plastic production and coastal population growth. We observed that fibers were the most abundant type of microplastic in our samples. These fibers were present in sediments that settled prior to the presence of plastics in the environment. We propose a simple statistical model to estimate the level of contamination in our samples. We suggest that the current trend in the literature suggesting very high loads of fibers, particularly in remote locations such as the deep seafloor, may be largely due to contamination.

Keywords: core samples, estuary, microplastics, plastic fibers, sediment

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INTRODUCTION

Microplastics (plastics <5 mm, Arthur et al., 2008) have been observed throughout bottom sediments of marine and freshwater environments, including sediments in rivers (Casta-eda et al., 2014), estuaries (Thompson et al., 2004; Sruthy and Ramasamy, 2017), lagoons (Vianello et al., 2013), lakes (Corcoran et al., 2015), seas (Zobkov and Esiukova, 2017), and deep sea trenches (Van Cauwenberghe et al., 2013; Woodall et al., 2014; Fischer et al., 2015). Microplastics are commonly found in the environment in three forms; fragments which form from mechanical and biological fragmentation of larger plastic items (ter Halle et al., 2016), microbeads which are manufactured as abrasives in cosmetics and air-blasting (Fendall and Sewell, 2009; Mason et al., 2016), and microfibers from sources such as synthetic fabrics and ropes (Browne et al., 2011). Studies have shown multiple damaging effects of microplastics in the environment, including adsorption of toxic organic contaminants (Endo et al., 2005; Teuten et al., 2007; Rochman et al., 2013), ingestion by animals with implications for human consumption (Van Cauwenberghe and Janssen, 2014; Rochman et al., 2015) and changing the heat transfer and water movement of sediment (Carson et al., 2011).

Microplastics have been observed in benthic environments since the late 1970s (Gregory, 1977; Shiber, 1979) and sediments are suggested to be a long-term sink for microplastics (Morét-Ferguson et al., 2010; Cózar et al., 2014). Benthos is an important feeding environment for many marine species (Anderson and Lovvorn, 2008; MacDonald et al., 2012; Gittman and Keller, 2013) and a recent study has shown marine benthic species ingest microplastics (Courtene-Jones et al., 2017). Knowledge of the harmful effects of microplastics on benthic marine species and communities is growing (Green, 2016; Galloway et al., 2017), however, little is known of past microplastic accumulation in benthic environments.

Several studies have examined microplastics in sediments (Claessens et al., 2011; Corcoran et al., 2015; Klein et al., 2015). For example, Claessens et al. (2011) quantified the number of microplastics in 16 year old sediments collected at the intertidal and high water mark. However, there have been no evaluations of microplastic contamination in deep sediments with known age cores, allowing analysis of deposition rates and concentrations. To address this knowledge gap, we compared sediment cores taken from an urbanized estuary and asked the following questions: Do microplastic densities change in time (with known age of sediment cores)? If microplastics are present, do we detect different densities as we move further from areas of higher human population densities?

MATERIALS AND METHODS

Sampling Location

Core samples were taken from Elwick Bay and Dogshear Point in the Derwent Estuary, Tasmania, Australia (**Figure 1**). The area is classed as urban/light industrial and the shoreline is partially reclaimed land. Surrounding land use includes residential areas, a horse race course and stables, low lying recreational areas, a high school and an entertainment estate. A major highway also runs adjacent along both shores. Two urban rivulets, Humphreys and Barossa, which exit at Elwick Bay, pass through urban areas in their lower reaches. Both have gross pollutant traps that trap anthropogenic debris that is larger than 10–15 cm in diameter. Sedimentation rates in Elwick Bay range from 0.4 to 0.5 cm per year, to a higher rate of 0.7 cm per year during a major flood or erosional event (Townsend and Seen, 2012).

Cores were collected in November 2004 as outlined in Townsend and Seen (2012). Core A was taken from the middle region of Elwick Bay around 3 km upstream of a zinc refinery (Townsend and Seen, 2012). Core B was taken off Dogshear Point, 3 km north-east of core A. Each core was sliced into 2 cm sections and stored in sealed containers. Core samples were stored refrigerated upright until analysis. A total of seven, 2 cm section samples from each core were processed and analyzed. Samples were systematically selected at different depth intervals of the core, with a maximum sample depth of 104 cm. This allowed us to analyse the samples in a temporal fashion, as deeper samples are from older sediments.

Aging Sediment Core Samples

A duplicate of core A was aged using lead isotopes (Townsend and Seen, 2012). Using the sedimentation rates and ages calculated in Townsend and Seen (2012) the age of sediment sections analyzed in this study were inferred. Sediments between 0 and 10 cm took 21 years to settle at a rate of 0.48 ± 0.05 cm/yr^{-1} with sediments at 10 cm aged at 1983 ± 2. Sediments between 10 and 30 cm settled at a rate of 0.69 cm/yr^{-1} with sediments at 30 cm aged at 1954 ± 5. Sediments between 30 and 50 cm took 46 years to settle at a rate of 0.43 ± 0.07 cm/yr^{-1} with sediments at 50 cm aged at 1908 (Townsend and Seen, 2012).

Laboratory Analysis

Sample processing was adapted from Reeves et al. (2016). In brief, samples were processed using a stepwise approach include sieving, organic material digestion, density separation, centrifuging, and filtration to separate microplastics from the bulk sediment. All laboratory work was conducted under a vacuum hood and exposed samples and equipment were covered with foil to prevent contamination from airborne microplastics. Natural fiber clothing and laboratory coats were worn throughout the analysis to reduce microplastic contamination from synthetic clothing.

Sieving

Each sample was placed in a beaker with deionized water and agitated with a metal spatula to disassociate large clumps of sediment. The contents of the beaker were then poured through a sieve stack. Sieves were stacked sequentially according to mesh size, with the largest mesh size at the top. Each sieve was rinsed with deionised water and left to dry.

Organic Material Digestion

Each dried sample was placed in a beaker with 20 ml of 30% hydrogen peroxide, 20 ml of 0.05 M iron (II) solution and a magnetic stir bar. The sediment solution was left at room temperature for 5 min, then was placed on a heating magnetic stirrer and heated to 75°C for 30 min. If organic material was visible after 30 min, 20 ml of hydrogen peroxide was added every 15 min and stirring/heating continued until all visible organic material was digested.

Density Separation, Centrifuging, and Filtration

Each digested sample was placed in a 50 ml centrifuge tube. Large sediment samples were split evenly between two tubes. Sodium iodide (NaI) solution (density 1.6–1.8 g.ml) was added to each tube until 30 ml of NaI was overlaying the sediment sample. Each tube was capped, shaken manually for 20 s and then placed in a benchtop centrifuge for 5 min at 3,500 revolutions per minute. Tubes were removed gently to minimize sediment re-suspension. The top 10 ml of supernatant NaI solution was poured off into a glass Büchner vacuum apparatus fitted with a 1.2 μm polycarbonate membrane filter. The remaining sediment in the tube was topped up with NaI, so 30 ml was overlaying the sediment. The sediment then underwent the previously described treatment of manual shaking, centrifuging, and filtering of supernatant, twice. A

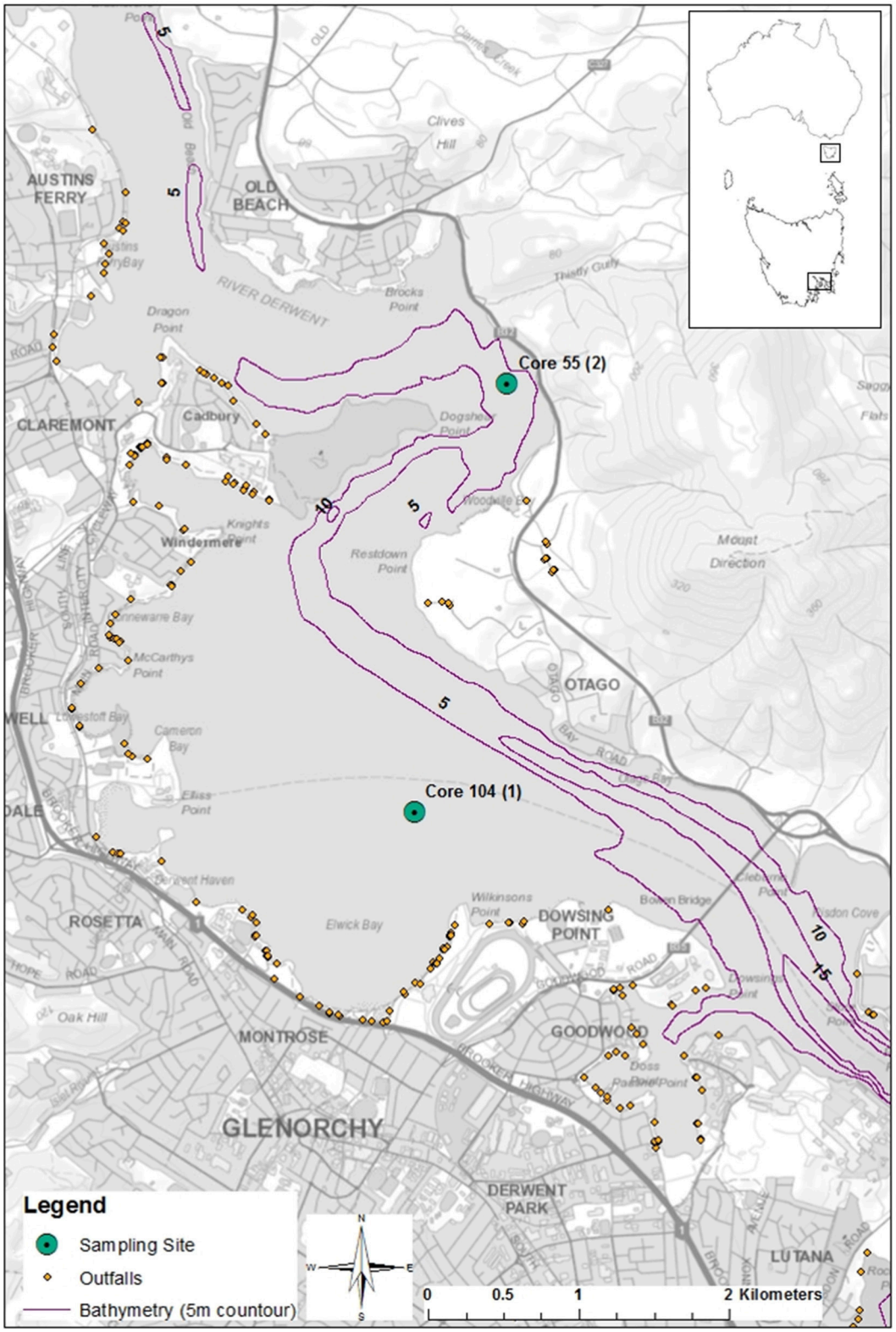

FIGURE 1 | Core sample locations in the Derwent Estuary, Tasmania, Australia. River flows north to south. Generated by Kathryn Willis using ArcGIS, [Desktop version 10.2], (https://www.arcgis.com/features/index.html). Bathymetry from Lucieer (2007), SeaMap Tasmania Bathymetric Data. Tasmanian Aquaculture and Fisheries Institute. Data accessed at http://metadata.imas.utas.edu.au/geonetwork/srv/eng/metadata.show?uuid=fa2dbc70-44ab-11dc-8cd0-00188b4c0af8 on 27/04/2017.

total of three supernatants, per sample, were poured into the vacuum apparatus and filtered. To ensure all possible separated microplastics were poured on the filter paper, on the last round of treatment (i.e., the third round), the total supernatant NaI solution was poured into the vacuum apparatus. The Büchner funnel and 1.2 µm filter were then rinsed with deionised water to capture any microplastics that may have adhered to the glass during filtration. All filtered samples were stored individually in sealed petri-dishes until microscope analysis.

Microscope Analysis

Filtered samples were analyzed for microplastic content using a stereomicroscope (magnification × 40). Each sample was divided into seven sorting sections using a fine-point metal probe. Samples were observed for 15 min using a constant magnification setting. Following the "Guidelines for Microscope Inspection" (Masura et al., 2015) an object was identified as plastic if it held shape or stretched when rubbed/pressed with a metal probe. Organic material would break a part under the above treatment. Positively identified microplastics were sorted into four categories according to their shape and texture (**Table 1**).

Deposition Trend

We tested whether the rate of micro fiber deposition observed in the cores correlated with plastic production using linear regression. We used the time trend in global plastic production values (PlasticsEurope, 2016) as a proxy for the relative time trend in production in the Derwent estuary. We used an exponential mode l to fit to the data available on global production to estimate the proportional change on an annual basis since plastic went into commercial production in the mid 1900s.

We adjusted our trend estimate for contamination by assuming the contamination rate was constant with respect to depth of the core slice. Based on this assumption the intercept term of the linear regression of microfiber concentration on plastic production change is an estimate of the contamination rate in the samples. The concentration of fibers in sediment slices from before 1950 serves as a second estimate of the contamination rate, as these sediments were deposited prior to the availability of plastic in the environment. The slope of the relationship between microfiber concentration and plastic production gives an estimate of the proportional increase in plastic in sediments, per unit of increase in production, which can be interpreted as the leakage rate from production.

We only used micro fiber values in the analysis as micro fibers made up nearly 90% of all microplastics in the cores.

TABLE 1 | List of features used to identify different microplastic categories.

Microplastic category	Shape	Texture	Color
Fiber	String-like with irregular bends	Soft, malleable	Any
Sheet	Thin, flat	Soft, malleable	Often clear, black, or translucent blue
Fragment	Thin, flat	Hard, rigid	Often black or red
Bead	Spherical, smooth	Hard	Often black or brown

RESULTS

Microplastics were observed in every sediment sample of both cores ($N = 14$). A total of 211 microplastics were observed in core A ($N = 7$), and 252 microplastics were observed in core B ($N = 7$). The mean weight of dry sediment samples was 63.36 g for core A and 52.36 g for core B with a mean of 2.43 plastic fragments per gram of sediment for core A and 4.2 plastic fragments per gram of sediment for core B. Microplastics mainly occurred as fibers (87% of total items observed), followed by sheet (9%), fragment (3%), and beads (1%) (**Figure 2**). A control sample, i.e., conducting the laboratory method without a sediment sample, presented a maximum of eight microplastic fibers (range = 0–8).

Size Classes of Microplastics Detected

In both cores, the frequency of microplastics increased with a decrease in size class. In core A, 137 microplastics were observed in size class 63 µm, 49 in size class 100 µm, 24 in size class 1 mm, and 1 in size class 4 mm (**Figure 3**). In core B, 132 microplastics were observed in size class 63 µm, 93 in size class 100 µm, and 27 in size class 1 mm (**Figure 3**). In core A an unusually high number of microplastics were observed in sample 7.

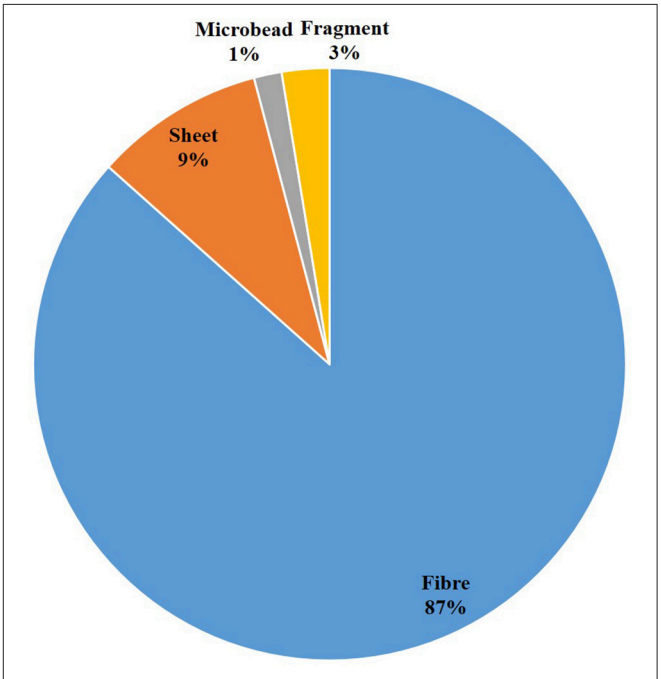

FIGURE 2 | The percentage of microplastic types observed in core samples A and B.

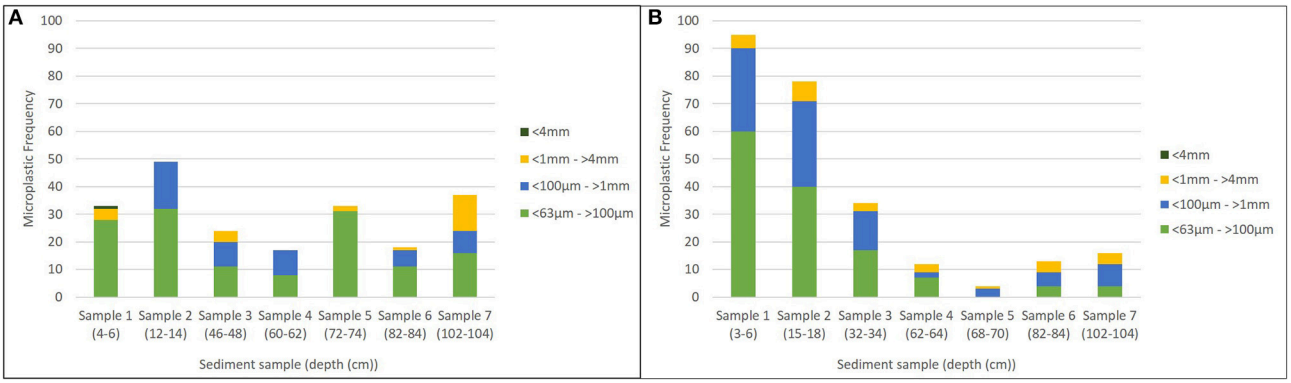

FIGURE 3 | The number of microplastics in each size class for core samples **(A,B)**.

Depth

In each core, we found more microplastics in the upper layer of sediments (more recent) than in the deeper (older) sediments. In core A, 38.9% of microplastics were observed in sample 1 and sample 2 depth classes, whereas only 26.1% of all microplastics were observed in the deeper layers (samples 6 and 7). In core B, we found 68.7% of microplastics in the upper, more recent layers (sample 1 and sample 2) whereas only 11.5% of microplastics were observed in the deeper layers (samples 6 and 7). Smaller microplastics were more common in shallower samples (i.e., there were more microplastics in the smallest size class (<63 µm−100 µm) in sample 1 than in sample 7 (**Figure 3**). In core A, 20.4% of all 63 µm microplastics were observed in sample 1 whereas only 11.7% in sample 7. In core B, 45.5% of all 63 µm microplastics were observed in sample 1 whereas only 3.0% in sample 7.

Deposition Trend

The number of micro fibers was higher in the shallower (younger) sediment samples. The rate of micro fiber deposition in the sediment correlated strongly with the expected change in production in the study region, based on the annual global plastic production (**Figure 4**; **Table 2**). The predicted accumulation model was a better model than the null (AIC: Predicted = 101, Null = 112). As global plastic production increased, the number of micro fibers deposited in sediments significantly increased ($p = <0.05$).

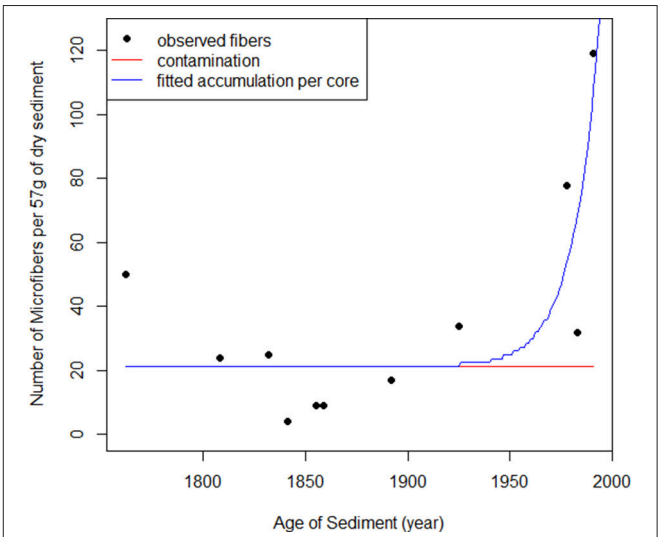

FIGURE 4 | The number of observed micro fibers in the sediment cores through time (black dots) (y = 0.6793x + 21.1055) compared to the predicted accumulation of micro fibers in the sediment through time (blue line) (y = 1.208x + 21.294). The predicted values are estimated from the global plastic production values. The red line is the constant level of micro fiber contamination throughout time (y = 21.294). As plastic production did not start until the 1950s, all observed micro fibers pre-1950s are considered contamination.

DISCUSSION

We observed more microplastics in the upper sediments. In core B there were six times more microplastics present in the top 15 cm (1976 and younger) than in the bottom 22 cm (1799 and older). In core A there were one and a half times the number of microplastics in the top 10 cm (1983 and younger) than in the bottom 22 cm (1799 and older). This observation is likely due to the exposure of upper sediments to a higher proportion of microplastics settling out from the water column. The production of plastics has increased from 100 million tons per year in 1993 to 322 million tons per year in 2015 (PlasticsEurope, 2008, 2016).

The environments for our sediment cores differed; core B was taken 150 m off shore from a relatively high energy position in a deep channel, whereas core A was taken 1,000 m off shore in a large shallow bay with low energy and more rapid sedimentation (**Figure 1**). Despite the normal low energy dynamics of site core A, it is occasionally subject to the passage of large floods. This scours the embayment with fresh water and greatly changes the potential sources of microplastic contamination for short periods of time. These differences in site conditions could explain the higher concentration of plastics at site B. The site is not subject to large scouring events, thus any deposition is likely

TABLE 2 | Results from the model of predicted accumulation of micro fibers in sediment through time.

Model	Terms	Estimate	*P*-value	AIC
Predicted Accumulation	Number of fibers	21.2944	0.01329	101.0587
	Plastic production estimate	1.2084	0.00142	
Null	Number of fibers	36.45	0.00578	112.1359

to remain in place. In addition, the site is much closer to sewage and stormwater outfall drains, which we found to be sources of plastic pollution in previous work (Willis et al., 2017). This result is similar to those found in other studies, which found higher numbers of plastics at sites closer to storm drain outfalls (Duckett and Repaci, 2015; Horton et al., 2017).

Most microplastics observed were fibers (87%). This phenomenon has been observed in other sediment analyses (MONAS, 2014; Woodall et al., 2015; Zobkov and Esiukova, 2017). Browne (2015) suggests microplastic fibers found in marine habitats may be derived from sewage as consequence of washing clothes. Furthermore, up to 1,770 microplastics have been reporting to leave a waste water treatment plant in effluent water per hour (Magnusson and Norén, 2014). As many outflows enter the Derwent Estuary, they are a probable source for the large quantity of microfibers observed in the samples. Outfalls as a source of micro fibers in the marine environment also indicates the dispersion from source to point of deposition is relatively local.

The strong correlation between the observed micro fibers and the predicted change in plastic production (**Figure 4**) indicates there is a clear temporal trend in micro fiber deposition in sediments (**Table 2**). This suggests that as plastic production increases, microplastic pollution is increasing proportionately. This is likely due to an increase in plastic leakage from the waste stream, as has been reported elsewhere (Thompson et al., 2009). The increase in coastal populations has been observed to increase the amount of pollution entering waterways (Jambeck et al., 2015; Lebreton et al., 2017). For example, 40% of Tasmania's population lives around the margins of the Derwent Estuary, with the population doubling between 1950 and 2015 (Carver, 1954; Coughanowr et al., 2015). Hence, older deeper sediments were exposed to an environment with markedly lower population and plastic production rate and less opportunity for plastic contamination than the younger, shallower sediments. This pattern was also observed on Belgian beaches where plastic pollution in sediments had tripled over 20 years (Claessens et al., 2011).

Our results demonstrate micro fibers are present in marine sediments that settled pre-plastic production. Microplastics have been recorded in marine sediments since the 1970s (Gregory, 1977; Shiber, 1979). We expected to only observe microplastics in sediments shallower than 30 cm, as the sediments were aged to be younger than 1954 $\pm$ 5 years (Townsend and Seen, 2012). However, we observed micro fibers down to 104 cm. Sediment accumulation rates in Elwick Bay range from 0.4 to 0.7 cm per year (Townsend and Seen, 2012). Hence, sediment at 104 cm would have settled between 149 and 260 years ago (i.e., in 1855 to 1744). Observing micro fibers down to 104 cm does not indicate that microplastics have been settling in sediment for 260 years. Rather it raises concerns that the sediment cores were exposed to micro fiber contamination either during collection or analysis. We calculated the mean frequency of fibers in our samples pre-1950s (i.e., plastic production) to estimate the fraction of fibers from both field and laboratory contamination, which yielded an estimate of up to 17.2 fibers (~60%) per sediment sample due to contamination, which is in line with the estimate of 21 fibers per sample, based on the intercept term in our linear regression (**Figure 4**). As the exact date that plastic production commenced in the Derwent Estuary is not known the values from the predicted model will give a better estimation of past microplastic levels in the sediment of the Derwent Estuary.

The fine structure of microplastics could enable them to move deeper into the older sediment via mixing due to bioturbation or water flows e.g., storm/flood events and direct transport by animals. This downward transport should affect all microplastic types, however, we only found micro fibers in the older sediment. The high number of fibers observed in the laboratory blanks indicates that the preventative contamination measures employed during laboratory analysis were ineffective. As we were unable to conduct our microscope analysis under a fume hood, airborne fibers could be one point of sample contamination. It is also possible that contamination occurred during the field collection and slicing of the cores. The cores were not originally collected for microplastic analysis. Hence, methods to prevent microplastic contamination were not a component of the field sampling program. We suspect contamination from researcher's clothing and equipment (i.e., synthetic fibers from rope fragmentation; Thompson et al., 2004) or from airborne microplastics (Dris et al., 2015) as the most likely contamination sources. This points to a major issue with the common opportunistic use of sediment samples to look for microplastics in deep sea sediments and other places. The opportunistic nature of sampling questions whether adequate procedures to prevent microplastic contamination were undertaken. Samples in this study and others should be interpreted with contamination in mind as the results showed 20 micro fibers per 50 g of sediment can be due to contamination alone.

Considerably fewer sheet, fragment, and bead microplastics were observed than fibers (13% for these three categories combined). These non-fiber plastics were all observed in the upper sediment layers which suggests there was no contamination issue for these materials. The lower counts of non-fiber microplastics could indicate these microplastics are not a common contaminant in estuaries. However, observer bias is also a likely explanation. Microfibers may be more conspicuous under a microscope due to their unique irregular bent filament shape and commonly settling on top of the other filtered particles (i.e., sediment granules, undigested biological matter, diatom shells). Sheet, fragment, and bead microplastics may be harder to detect as their shapes were more similar to those of undigested plant material and sediment granules. Hence, they could be underrepresented in counts from sediment cores.

We found size class 63 μm had the highest count of microplastics in every sample but one. Eighty-five percent of sediments in Elwick Bay are <63 μm (Koehnken and Eriksen, 2004), reflecting a highly organically enriched depositional area. As both sediments and microplastics are a similar size, it can be inferred that the same forces act on sediment accumulations and microplastics accumulation (Vianello et al., 2013). The high frequency of 63 μm microplastics in sediments is quite different to the size distribution observed from net trawls of the ocean surface. In net trawls, small microplastics (i.e., 100 to 63 μm) are not observed even though they are likely present on the surface (Law et al., 2010; Eriksen et al., 2013). This discrepancy in microplastic sizes may be largely due to the difference in sample analysis (e.g., visual versus microscope identification) (van Sebille et al., 2015) as it would be unlikely or impossible to observe microplastics between 100 and 63 μm solely scanning with the naked eye.

CONCLUDING REMARKS

Our study shows that sediments are a useful record of past and present plastic leakage from the waste stream into the marine environment. This is not unlike samples from other parts of the marine ecosystems, including the water column (van Sebille et al., 2015), seabirds (Wilcox et al., 2015), and turtles (Schuyler et al., 2014). Microplastics were present in all samples, even in sediments dated from pre-plastic production. Based on our estimates, current microplastic concentrations in sediment are 115 microplastics, and are increasing at an accelerated rate of 1.208 microplastics per year. Generally speaking, however, the frequency of plastics corresponded with the increase in plastic production and coastal populations. The presence of microplastics in the older sediments indicates there was possible contamination during sampling or laboratory analysis, which is an element that must be carefully considered when estimating microplastics loads and their presumed ubiquity in the marine environment.

AUTHOR CONTRIBUTIONS

BDH and CW: designed the study; KAW: completed laboratory work and wrote original draft, with contributions by other authors; BDH and RE: provided technical advice and support for laboratory work; RE: collected the samples; CW: completed data manipulation, statistical analyses, and provided technical expertise on manuscript.

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Constraints and Priorities for Conducting Experimental Exposures of Marine Organisms to Microplastics

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Marine plastic pollution is a major environmental issue. Given their ubiquitous nature and small dimensions, ingestion of microplastic (MP) and nanoplastic (NP) particles and their subsequent impact on marine life are a growing concern worldwide. Transfers along the trophic chain, including possible translocation, for which the hazards are less understood, are also a major preoccupation. Effects of MP ingestion have been studied on animals through laboratory exposure, showing impacts on feeding activity, reserve depletion and inflammatory responses, with consequences for fitness, notably reproduction. However, most experimental studies have used doses of manufactured virgin microspheres that may not be environmentally realistic. As for most ecotoxicological issues, the environmental relevance of laboratory exposure experiments has recently been debated. Here we review constraints and priorities for conducting experimental exposures of marine wildlife to microplastics based on the literature, feedback from peer reviewers and knowledge gained from our experience. Priorities are suggested taking into account the complexity of microplastics in terms of (i) aggregation status, surface properties and interactions with organic and inorganic materials, (ii) diversity of encountered particles types and concentrations, (iii) particle bioavailability and distribution in experimental tanks to achieve reproducibility and repeatability in estimating effects, and (iv) strict experimental procedures to verify the existence of genuine translocation. Relevant integrative approaches encompass a wide spectrum of methods from -omics to ecophysiological approaches, including modeling, are discussed to provide novel insights on the impacts of MP/NP on marine ecosystems from a long-term perspective. Knowledge obtained in this way would inform stakeholders in such a way as to help them mitigate impacts of the micro- and nano-plastic legacy.

Keywords: microplastics, nanoplastics, experimental exposure, impacts, marine organisms, physiology

INTRODUCTION

Production of plastics is constantly increasing to sustain our broadening uses in daily life (Thompson et al., 2009). As a direct consequence, plastic waste in the environment is a growing problem (e.g., Barnes et al., 2009); plastic waste entering the oceans was calculated for 2010 at 4-12 million tons *per* year and is predicted to increase by an order of magnitude by 2025 in the absence of waste management improvements (Jambeck et al., 2015). Once in the environment, plastic debris fragments into smaller particles such as microplastics (MP; <5 mm, NOAA, 2008), and presumably nanoplastics (NP; defined as particles <100 nm or <1,000 nm according to Galloway et al. (2017) and Gigault et al. (2018), respectively) whose presence in the Atlantic gyre has been recently suggested (Ter Halle et al., 2017). MP can also be produced as such, mainly in the form of microbeads used in cosmetics, synthetic fibers discharged with washing waters, and industrial abrasives. Microplastics have been reported in the environment worldwide, from surface waters to deep-sea sediments (Eriksen et al., 2013; Vianello et al., 2013; Wright et al., 2013b; Cózar et al., 2014; Lusher et al., 2015), even in areas far from human activities such as in polar waters (Lusher et al., 2015; Cózar et al., 2017; Munari et al., 2017; Obbard, 2018). All environmental matrices appear contaminated: surface waters (Moore et al., 2001; Eriksen et al., 2013), the water column (Lattin et al., 2004; Ng and Obbard, 2006), sediments (Van Cauwenberghe et al., 2013; Vianello et al., 2013) and biota (Murray and Cowie, 2011; Fossi et al., 2012; Lusher et al., 2013; Devriese et al., 2015; **Figure 1**).

Given their ubiquitous nature and small dimensions (Cózar et al., 2014), their ingestion by and subsequent impact on marine life-including transfer of biological or chemical contaminants-is a growing concern (e.g., Wright et al., 2013a). This is especially true when considering transfer along the trophic chain and possible translocation, for which the hazards are less well understood (GESAMP, 2016; **Figure 1**).

Overall, experimental studies focusing on the effects of MP on marine organisms have increased sharply over the past few years (**Figure 2**). Substantial effects have been reported on feeding activity, reserve depletion, impairment of oxidative balance and the immune system, and inflammatory responses, with impacts on animal fitness, notably reproduction (e.g., Wright et al., 2013a; Paul-Pont et al., 2016; Rochman et al., 2016; Sussarellu et al., 2016; Galloway et al., 2017). Effects may scale up to the community level, as suggested by recent publications demonstrating modifications in nutrient cycles and benthic assemblage structures upon exposure to MP (Green, 2016; Green et al., 2016), as well as increasing disease risk for coral communities (Lamb et al., 2018).

However, most previous experimental studies used unrealistic scenarios with mostly high doses of manufactured virgin microspheres that may not be representative of the variety of microplastics found in the environment. The difficulty of performing laboratory experiments to assess MP toxicity lies in the fact that MP consist of a complex, dynamic mixture of polymers and additives, to which organic material and contaminants can successively bind, along with microorganisms, influencing their density, surface charge, bioavailability and toxicity (Galloway et al., 2017). As a consequence, the environmental relevance of laboratory exposure experiments has recently been debated and challenged (Lenz et al., 2016; Rochman, 2016). In order to meet decision-makers' expectations, it is critical/essential to consider MP shape, interaction with organic matter, and the biological and chemical loads of microplastics, as well as using exposure doses as close as possible to environmentally realistic concentrations (Huvet et al., 2016; Karami, 2017). Based on the literature, feedback from peer reviewers and knowledge gained from our experience, we make a review of the constraints and priorities for experimental exposures of marine organisms to MP and NP. Having defined the main specific features of MP and NP that need to be taken into account in laboratory studies, we identify the major limitations that could be avoided in such studies, and discuss some ways to improve how MP and NP are handled in laboratory experiments. Finally, we cover the use of mesocosm experiments, which advantageously combine a similar level of control to that offered by laboratory experiments with some of the complexity of natural ecosystems. Such a combination may benefit from integrative analytical approaches ranging from -omics to ecophysiological approaches, including modeling, in order to objectively assess the complex and dynamic impacts of MP and NP on marine ecosystems.

WHICH PARTICLES SHOULD BE USED IN LABORATORY EXPERIMENTS?

Polymer Nature

Among the "Big Six" [polypropylene (PP), high- and low-density polyethylene (HDPE and LDPE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET), and polystyrene (PS)], which account for 80% of plastic production in Europe (PlasticsEurope, 2016) and are the most frequently reported plastics in marine environments (Browne et al., 2010; Karapanagioti et al., 2011; Vianello et al., 2013), polyethylene (PE) and PP are presently the polymers predominantly recovered in all environmental compartments (Isobe et al., 2014; Enders et al., 2015; Frère et al., 2017), in accordance with the scale of their global manufacture and use worldwide (Antunes et al., 2013; GESAMP, 2016). Assuming that the chemical nature of these polymers can modulate their impact, it appears relevant to test the big six first, both separately and in complex mixtures. However, experiments may also consider geographical differences especially in estuarine and coastal ecosystems, e.g., polyester is generally dominant in fibers collected at sea but it can be locally nylon due to local activities. Biodegradable polymers would deserve attention in the near future. Exposure experiments in the micro- and nano-ranges mainly use PS and, to a lesser extent, PE microbeads (reviewed by Phuong et al., 2016) due to the lack of commercial micro- and nano-beads made of other polymers. Previous studies testing the influence of polymer type are difficult to interpret as the detailed chemical compositions (including all additives among

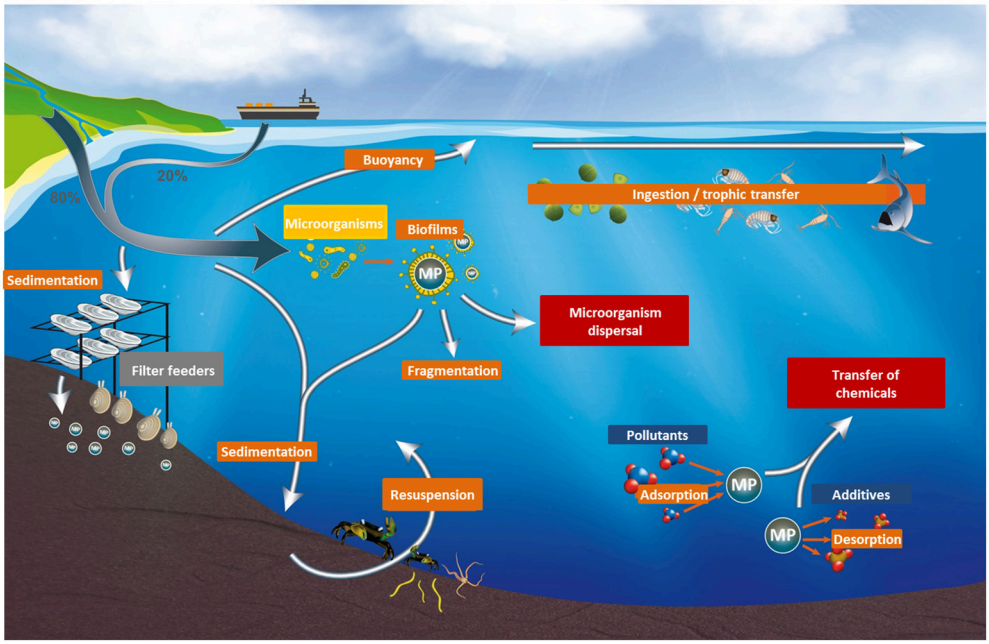

FIGURE 1 | Fate of microplastics in the marine environment.

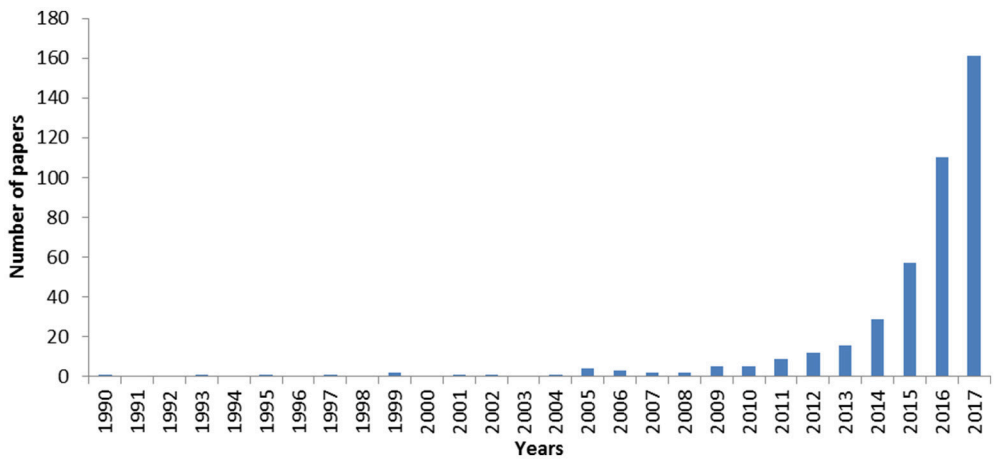

FIGURE 2 | Number of published scientific papers related to the assessment of the microplastic effects on aquatic organisms through lab experiments over time. Source: Web of Science; Period: 1975-2017; Keywords: (microplastic* OR "micro-plastic*" OR nanoplastic* OR "nano-plastic*" OR "plastic particle*) AND (impact* OR exposure OR experimental OR lab* OR tank) AND (ocean* OR sea OR seas OR marin* OR seawater* OR water OR aquatic).

which some may be hazardous) are never given because manufacturers unwilling to provide them (e.g., Green et al., 2016). It therefore remains difficult to compare the effects of polymers based on their monomer chemistry when part of the other unidentified constituents may be as toxic if not more so than the monomers themselves (Hermabessiere et al., 2017). To address this issue and helping deciphering the respective influence of the physical (mechanical) and chemical toxicities of MP, the production of laboratory model polymers without additives or with controlled introduction of the most common additives would clarify the toxicity of MP according to their polymer nature. Other important aspects (such as morphology, weathering of MP) would be more hardly accounted for using laboratory produced model particles because natural weathering and organic coating in environmental conditions are complex processes that are difficult to mimic in laboratory. With the current level of knowledge, laboratory particles can be classified by size, by polymer nature, can be artificially weathered or even colonized by microorganisms.

Besides these aspects, polymers exhibit different densities that affect their buoyancy, behavior and bioavailability to marine organisms. As reported by Wright et al. (2013b), pelagic filter/suspension feeders and plankton feeders are more likely to encounter positively buoyant, low-density plastics such as PE (density 0.91–0.94) while high density plastics such as PET (density 1.38) are expected to sink, thus becoming available for supra-benthic and benthic suspension/deposit feeders as well as detritivores. Therefore, focusing on the most produced polymers according to their bioavailability for a given species in a given area is a key point to consider when assessing microplastic effects on model organisms. For instance, wild bivalves living close to a harbor may not be exposed to the same MP (paints, PE fragments) as those cultivated in high-density farming areas (PP rope fibers, PS foam fragments). Therefore, to improve experiment relevance, in-situ measurements of the types and forms of MP in a given area should be envisaged even though it may be very costly and time-consuming. Nevertheless, such data should be used with caution, as up to day traditional sampling methods (e.g., manta trawl) do not take into account vertical distribution and small size particles.

Shape

Several types of MP can be distinguished according to their morphology: spheres (beads, pellets, and granules) that are produced as such (primary MP); and fibers (filaments and lines), films, fragments, and foams (Free et al., 2014; Karami, 2017), which are produced from the fragmentation of larger plastic debris (secondary MP). Accumulation of MP of different shapes, such as planar, granular, fragments or fibers, has been shown in different organisms (Lusher et al., 2013; De Witte et al., 2014; Devriese et al., 2015; Li et al., 2015). Few studies have examined the relative contributions of different MP sources in aquatic environments. A first detailed estimation of the different sources of MP in Norway showed that the sources of primary MP (between 3.7 and 15.5%) are of minor importance compared with secondary MP sources (between 84.5 and 96.3%) (Sundt et al., 2014). If these estimates are correct and valid at a wider environmental scale, then studies using primary MP (such as microbeads) are disproportionally frequent compared with those using secondary MP. This imbalance has occurred because microbeads are the most easily commercially-available product to use in tests, mostly with embedded fluorescent labeling or dyes for easier detection (Yokota et al., 2017). Even though uniformly sized and shaped analytical grade commercial microbeads are useful tools for establishing the basic patterns of ingestion and organism responses to MP exposure, their exclusive use in laboratory experiments may lead to a biased representation relative to the full range of microplastics found in water bodies (Free et al., 2014; Mazurais et al., 2015; **Figure 3**). Indeed, Graham and Thompson (2009) revealed that some benthic organisms like sea cucumber could preferentially ingest plastic fragments over other shapes. Recently, Gray and Weinstein (2017) revealed that the ingestion, residence time and toxicity of particles in shrimp depended on particle shape and size. For instance, they showed a higher retention time of spheres than fragments in the gut, whereas in the gills they observed a hierarchy of retention patterns for fragments > spheres > fibers. Moreover, fragments and fibers increased the mortality rate of both shrimp and daphnia relative to beads (Gray and Weinstein, 2017; Ziajahromi et al., 2017) while no apparent post-ingestion effects were observed on brine shrimp (*Artemia* sp.) exposed to 10 × 40 µm nylon fibers (Cole, 2016). Given the lack of data regarding impacts of fibrous particles on organisms compared with their spherical counterparts, it appears necessary to assess their persistence in the environment to better understand their potential impacts.

FIGURE 3 | Microplastics of various colors, shapes and sizes collected at sea.

The difficulty of conducting experiments with secondary MP lies in the fact that they are by definition more difficult to collect or produce, especially regarding the need to obtain sufficient amounts of relevant sizes. They are also more difficult to monitor over the course of laboratory exposures. These forms are indeed irregular and, with the exception of nylon tubes (Cole, 2016), most often non-standard in shape and size, making it difficult to design reproducible ecotoxicology studies. Furthermore, their composition, original source, and traceability are often impossible to determine consistently (Kedzierski, 2017).

Despite these limitations, using microfibers and fragments and not only beads are of interest. A recent method using a cryotome and Nile Red staining was developed to produce labeled nylon microfibers (40-100 µm length and 10–28 µm width) (Cole, 2016). To obtain realistic fragments, the best practical method is to mill plastic objects from everyday life to obtain MP of various sizes and shapes and/or use MP directly sampled at sea (Graham and Thompson, 2009; Ogonowski et al., 2016).

Several approaches can be tested for comparison purposes and depending on the monomer nature: (i) physical degradation (milling) of commercial pellets in order to obtain fragments and (ii) milling of micro- and nano-thick films before and after photo-degradation, as plastics become more brittle after UV exposure. Samples are obtained in the form of a powder containing a wide range of particle size and shape, which would be expected to better mimic real particles in the environment. Such a powder would require dissolution in an appropriate solvent, which will also require testing for intrinsic toxicity before use (as done with Tween in Ostroumov, 2003; Paul-Pont et al., 2016). Because it is difficult to fine tune such milling, sieving may be necessary to remove an unwanted fraction, especially the smallest sizes such as NP, or to produce specific size classes according to the ingestion capacity of the studied animals. The first major pre-requisite to perform laboratory experiments using laboratory-generated fragments and fibers is to accurately determine size distribution, shape and behavior (buoyancy and aggregation) in seawater. Some of these parameters (e.g., size and/or shape) can be monitored using microscopy coupled with image analysis software or an electronic particle counter mostly employed for phytoplankton counts (Huvet et al., 2015). More complete but unaffordable techniques are Atomic Force Microscopy (or laser granulometry) and 3D-optical profilometry (morphology and surface properties), scanning and transmission electron microscopy (shape and size), and diffusion light scattering or x-rays for NP and small MP (aggregation, charge). A major drawback of these techniques is that they can be quite time consuming. It is noteworthy that milling large plastic objects to obtain enough micro-fragments of appropriate and precise size range is still very challenging and requires significant methodological improvements.

Size

In the natural environment, marine organisms encounter plastic pieces with a wide size range from nanometers to meters (e.g., Mattsson et al., 2015; Galloway et al., 2017; Ter Halle et al., 2017). Particle size controls the probability of consumption and thus potential adverse effects (Wright et al., 2013b). In the literature, different approaches have been tested to study MP ingestion, trophic transfer and impacts: (i) exposure to a specific size (Farrell and Nelson, 2013; Setälä et al., 2014; Watts et al., 2016); (ii) exposure to several sizes separately (Lee et al., 2013; Cole and Galloway, 2015; Jeong et al., 2016, 2017); and (iii) exposure to a mixture of different sizes (Von Moos et al., 2012; Avio et al., 2015; Green, 2016; reviewed by Phuong et al., 2016). All approaches offer different advantages. Using a broad range of sizes helps to determine the size range of particles that organisms can ingest or interact with. The experiment performed by Erni-Cassola et al. (2017) with a mixture of MP from a few micrometers to 5 mm, sampled from surface sea waters, is particularly relevant in this respect. However, ensuring homogeneous distribution of particles across experimental conditions may be difficult to achieve and reproduce. Most studies conventionally chose an MP size distribution in the same range as that of the test organisms' preys. For example, particles from 7 to 30 μm appeared to be preferentially ingested by several zooplankton groups (decapods larvae, copepods and chaetognaths) although this varied according to species (Cole et al., 2013). Adult Pacific oysters *Crassostrea gigas* preferentially ingested 6-μm spheres over 2 μm ones with a 5-fold difference (Sussarellu et al., 2016). The size range of ingested particles also depends on biological stage. Indeed, the diameter of the mouth of a juvenile oyster was estimated at around 80 μm, and even smaller during the larval stages (Cole and Galloway, 2015), thus limiting the size of particles ingested.

Although MP ingestion is certainly the most studied entry process, it is not the only mechanism of entry, especially when considering nanoplastics (NP). Besides the usual digestive ingestion, respiration processes of organisms such as fish can also favor MP-NP entry. Effects were also reported to be size-dependent, especially for nanometric sizes, due to the physico-chemical properties of NP (Lee et al., 2013; Jeong et al., 2016, 2017; Mattsson et al., 2017). MP ingestion is often reported to cause problems in the digestive tract (satiation, clogging, inflammations, ulcers, etc.) with impacts expected on energy balance (Sussarellu et al., 2016), whilst potential translocation of NP could induce different adverse effects within organs and on various physiological functions (Kashiwada, 2006; Wright et al., 2013b; Mattsson et al., 2015, 2017). Mixing particles of different sizes clearly produces a more realistic scenario (Von Moos et al., 2012; Mazurais et al., 2015) and should be favored in laboratory exposures as long as the particle size distribution is well characterized prior to the experiment. Measuring the preferentially ingested size by analysis of gut and fecal content using histology, digestible fluorescent coating (Karakolis et al., 2018), microscopy and/or cytometry tools would also help to identify the most bioavailable and, putatively, the most toxic fraction according to species, physiological state and biological stage. It would also allow the assessment of potential digestive fragmentation processes that could reduce MP to NP, as recently demonstrated for Antarctic krill *Euphausia superba* (Dawson et al., 2018).

Concentrations

The need of elucidating eco-toxicity of MP is the main justification for testing concentrations of MP far above those found in marine waters (Pittura et al., 2018). Applying environmentally relevant microplastic doses is still a challenge for decision support (Huvet et al., 2016; Lenz et al., 2016; Rochman, 2016) especially when considering the lack of consistent field quantifications of MP as small as those used in most experimental studies (Filella, 2015). Environmental data on MP contamination in surface water and sediment are numerous (**Table 1**) and are compiled in the freely available Litter Database "Litterbase"(http://litterbase.awi.de/litter)[1] Among reported values, some of the highest have been found in the southern North Sea (1,700,000 items m^{-3}; ∼ 8.5 mg L^{-1}) for debris >80 μm (Dubaish and Liebezeit, 2013); in South Korea (15,560 items m^{-3}; ∼ 0.0778 mg L^{-1}) for debris >333 μm (Song et al., 2014), and in a Swedish bay (2,400

[1]Litterbase. *Distribution of litter types in different realms*. Http://litterbase.awi.de/litter. Accessed January 7,2018

TABLE 1 | Environmental concentrations of microplastics in surface water (SW) and sediment (Sed).

Compartments	Study sites	Concentrations in original units	Estimated concentrations ($mg.L^{-1}$ for SW and mg/kg^{-1} for Sed.)	Size (μm)	References
Surface water	North pacific gyre	30,169 $g.km^{-2}$	0.0002	>333 μm	Moore et al., 2001
	South pacific gyre	369,342 $items.km^{-2}$	0.0732	<1 −4.75 mm>	Eriksen et al., 2013
	South pacific gyre	969,777 $items.km^{-2}$	0.511	<1–4.75 mm>	Eriksen et al., 2013
	California (US)	0.009 $g.m^{-3}$	0.009	>333 μm	Moore et al., 2002
	California (US)	2.44 $mg.m^{-3}$	0.00244	>333 μm	Lattin et al., 2004
	NE pacific	0.209 $mg.m^{-3}$	0.0003	>500 μm	Doyle et al., 2011
	NW mediterranean	2.28 $mg.m^{-2}$	0.0228	>333 μm	Collignon et al., 2012
	N Atlantic gyre	2.67 $mg.m^{-3}$	0.00267	>150 μm	Reisser et al., 2015
	South Korea	15,560 $items.m^{-3}$	0.0778	>333 μm	Song et al., 2014
	Swedish coasts	2,400 $items.m^{-3}$	0.012	>80 μm	Norén, 2007
	European coasts	501 $items.m^{-3}$	0.002505	<10–1,000 μm >	Enders et al., 2015
	Great Lakes USA	32 $items.m^{-3}$	0.00016	>333 μm	Baldwin et al., 2016
	West Med/Adriatic	10,432.36 $g.km^{-2}$	0.026081	>700 μm	Suaria et al., 2016
	Southern North Sea	1,700,000 $items.m^{-3}$	8.5	>80 μm	Dubaish and Liebezeit, 2013
	Yangtze Estuarie (China)	10,200 $items.m^{-3}$	0.051	>333 μm	Zhao et al., 2014
	NE Pacific	9,200 $items.m^{-3}$	0.046	>333 μm	Desforges et al., 2014
	Qatar	3 $items.m^{-3}$	0.000015	>120 μm	Castillo et al., 2016
	Bohai Sea (China)	1.23 $items.m^{-3}$	0.00000615	>330 μm	Zhang, W. et al., 2017
	Three Gorges Reservoir China	12,611 $item.m^{-3}$	0.063055	>48 μm	Di and Wang, 2018
Sediment	Belgium	390 $items.kg^{-}1$	7.21	>38 μm	Claessens et al., 2011
	Artic	6,595 $items.kg^{-}1$	32.975	<1–275 μm >	Bergmann et al., 2017
	Urban breach (Brazil)	313 $items.kg^{-}1$	1.565	<0.5–20 mm >	Costa et al., 2010
	Kalinigrad (Russia	36.3 $items.kg^{-}1$	0.1815	<0.5–20 mm >	Esiukova, 2017
	Beach Bohai Sea (China)	163.3 $items.kg^{-}1$	0.8165	N/A	Yu et al., 2016
	Three Gorges Reservoir (China)	300 $items.kg^{-}1$	1.5	>48 μm	Di and Wang, 2018
	Lagoon of Venice (Italy)	2,175 $items.kg^{-}1$	10.875	>32 μm	Vianello et al., 2013
	India	N/A	81	N/A	Reddy et al., 2006

Data are expressed in original units and were transformed in mass concentrations ($mg.L^{-1}$ or $mg.kg^{-1}$) using methods described by Besseling et al. (2014a) (trawling depth of 0.1 m and an average weight of MP equate to 5 μg).

items m^{-3}; ∼0.012 mg L^{-1}) for debris >80 μm (Norén, 2007; **Table 1**). The MP (2 and 6 μm) mass concentration used for exposure in Sussarellu et al. (2016) and Paul-Pont et al. (2016), for instance, was in the range of the highest estimated field concentration obtained from manta trawl sampling: >333 μm. However, the corresponding number of particles per volume was on average 1,000 times higher than the highest estimate of 1.7 MP mL^{-1} for particles >80 μm obtained by Dubaish and Liebezeit (2013). However, small MP were recently demonstrated to be increasingly abundant following a power-law increase (by a factor of ∼2.2) with a decreasing particle size in sea surface samples (Erni-Cassola et al., 2017). Similarly, recent publications demonstrated a high percentage (80%) of small MP (25–50 μm, for which few data are available) in surface water or sediment, compared with large sized particles (Enders et al., 2015; Bergmann et al., 2017). Applying the power-law increase factor calculated by Erni-Cassola et al. (2017) to the >80 μm plastic debris collected by Dubaish and Liebezeit (2013) gives an estimated average of 1,000 MP mL^{-1} for particles whose size is centered around 4 μm, and therefore in the range of those used in Sussarellu et al. (2016) and Paul-Pont et al. (2016), but still several orders of magnitude higher than the estimate of 1.4 MP mL^{-1} based on (Norén, 2007) data. If such a high concentration for small MP was confirmed in local aquatic hotspots, the environmental relevance of "high" concentration previously used in most laboratory experiments should be carefully revised. This

is especially true for benthic species, as MP concentration at the sediment–water interface is estimated to be high, reaching up to 16.9 mg L^{-1} (Besseling et al., 2014a,b). Plastic contamination is indeed estimated from surface layer sampling, although small MP seem to have a lower residence time than larger debris in this compartment (Enders et al., 2015). This partly explains the lower contamination level of surface water compared to sediment, especially at the water–sediment interface (Vianello et al., 2013; Martin et al., 2017). For still smaller particles, the detection and quantification of the nano-fraction in natural environments remains a challenge. Promising studies are on their way, with a recent first demonstration of nano-sized putative plastic particles (comprised between 1 and 999 nm) in natural seawater samples collected in the North Atlantic Subtropical Gyre (Ter Halle et al., 2017), and the very recent high contamination levels discovered in Artic (Peeken et al., 2018). Combination of analytical methods such as asymmetric field flow fractionation (A4F), dynamic light scattering (DLS) and pyrolysis gas chromatography coupled with mass spectrometry (Pyr-GC/MS) may allow the determination of nano-sized particle concentrations in natural environments in the near future; the next challenge will be quantification to determine the level of contamination by small microplastics faced by marine organisms. Meanwhile, until data on NP and the smallest MP become available, the use of dose–response experiments like those performed by Jeong et al. (2016, 2017) is of interest. Indeed, although effects of MP may not be considered as "dose-dependent" in natural conditions, this approach could still provide relevant insights on toxicity thresholds for a given contaminant and organism.

WEATHERING IMPLICATIONS

Modification of Surface Properties

Once they have entered the environment, all plastic fragments or pellets undergo aging, a term used to encompass all changes in polymer properties over a given period of time. These changes can independently and/or simultaneously affect polymer composition, and modify the physical integrity of the particle and its surface properties (White, 2006). When such changes occur after a long time in the natural environment, the term used is "natural" aging or weathering. Weathering of polymers in the marine environment leads to polymer degradation through the addition of a number of complex processes: organic matter coating of their surface, photo-oxidation, hydrolysis, mechanical abrasion, additive release and pollutant adsorption, micro-organism colonization, and possibly biodegradation. Surface coverage by a complex mixture of organic and inorganic molecules (defined as an "ecocorona"; Galloway et al., 2017) is the first modification of surface properties that occurs when MP and NP are introduced into natural seawater, as this brings them immediately into contact with a more complex medium containing natural colloids, inorganic (e.g., poly-ions and minerals) and organic matter (e.g., mixtures of polysaccharides, proteins, lipids, and nucleic acids, Keller et al., 2010). The nature of the ecocorona will have a greater influence on smaller than larger particles due to their higher surface to volume ratio (Lin et al., 2014; Mattsson et al., 2015) and may change plastic surface characteristics (Canesi and Corsi, 2016), their identity, and the way they interact with organisms. For instance, an ecocorona around polystyrene NP created by proteins released by *Daphnia magna* caused heightened uptake, retention, and toxicity of NP (Nasser and Lynch, 2016). Ecocorona formation and colonization by microorganisms occur rapidly, at the scale of hours to days (see section Biofouling and Hetero-Aggregation), while other weathering mechanisms such as additive leakage and photo-oxidation are longer term processes (taking months or years; **Figure 4**). Moreover, complex interactions can occur between processes, e.g., the presence of a biofilm on the polymer surface can hinder photo-oxidation, but degradation and fragmentation of polymers increases their active surface available for colonization (Rummel et al., 2017). Hence, over their stay in the environment, pieces of plastic debris will exhibit various physical and chemical properties as well as biological modifications (**Figure 4**). Their geometries, surface properties and chemical composition will be permanently altered (Andrady, 1990; Rajakumar et al., 2009) and these changes could strongly modify their behavior, bioavailability, and ultimately their toxicity.

The results of very long weathering times on polymer fragments in the marine environment have recently been described by Ter Halle et al. (2017), based on samples from the North Atlantic Gyre. These authors showed that even the most robust polymers such as PE were severely damaged during weathering, with a decrease in molar mass and degradation of polymer chains, especially for micro-sized fragments (<1 mm). Regarding macroplastics, a significant decrease of the native functional groups of PET bottles was reported from approximately 15 years spent in marine environment (Ioakeimidis et al., 2016). Most exposure studies are conducted using pristine polymers. However, comparing pristine and weathered PE pellets over a few days to a few months in the environment (Rochman et al., 2013b, 2014; Nobre et al., 2015; Bråte et al., 2018) has revealed different effects on exposed organisms. Indeed, in one case, fish were more impaired when exposed to weathered pellets than new ones, possibly as a consequence of organic compound adsorption on the polymers during their "life" in marine waters (Rochman et al., 2013b). In contrast, Nobre et al. (2015) reported lower toxicity of stranded pellets than pristine ones, suggesting that polymers release part of their additives during weathering. These discrepancies are not surprising as it is assumed that the effects of environmental weathering of plastic particles will differ depending on both polymer and additive compositions, as well as on the environmental conditions during weathering. For instance, PE, PP, and PS pellets exposed to UV radiation showed different degradation states according to the environment (air > ultrapure water > synthetic seawater) as well as discrepancies in terms of surface functional groups and textures according to polymer type, as observed by FTIR, Raman, and SEM (Cai et al., 2018). Further experiments should be conducted to determine the importance of plastic weathering in impact studies.

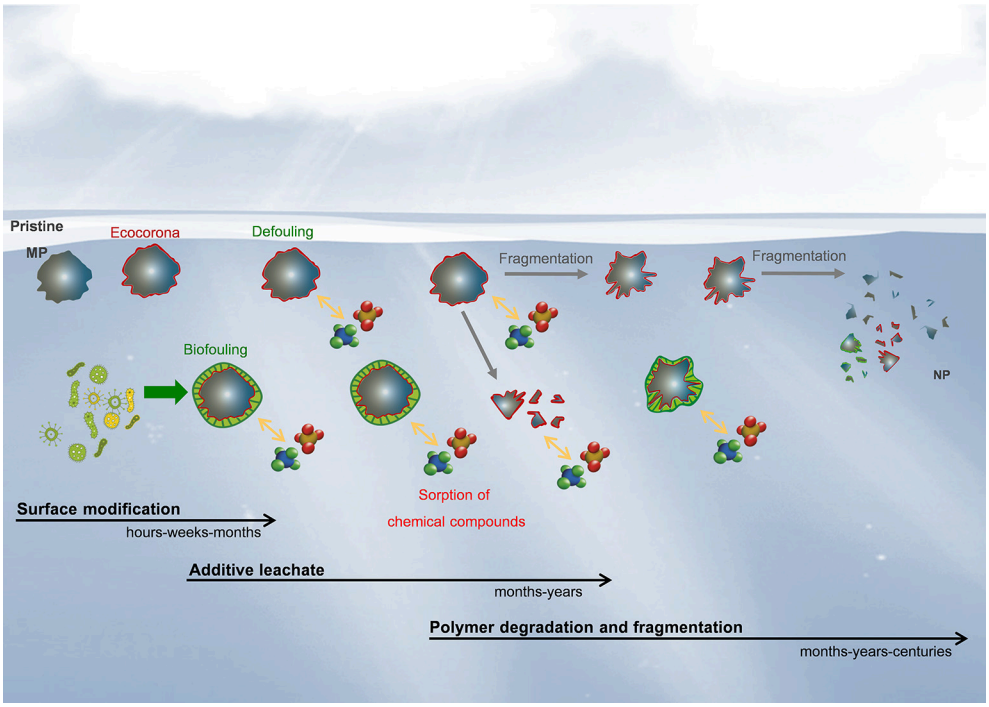

FIGURE 4 | Hypothetical evolution of the physicochemical and biological modifications of microplastics and nanoplastics released in aquatic environments.

To increase the environmental relevance of laboratory exposures, MP weathered in natural or artificial environments (from several days to weeks) can be prepared from pristine plastics to allow ecocorona formation and modification of chemical, physical and biological surface properties of the particles as detailed in the following sections.

Particle Charges in Seawater

MP and NP charge and aggregation have been little reviewed compared with their other aspects. In the absence of sedimentation, sub-micron particles move freely due to Brownian motion in water and finally aggregate (coagulate or flocculate) over time. This behavior depends highly on the particle surface charges and on the nature of the ions in the medium. In the absence of surface charges, hydrophobic particles are not thermodynamically stable and aggregate very easily, forming agglomerates. However, when particles interact with molecules present in seawater they can gain surface charges (Keller et al., 2010). Interestingly, this interaction differs according to plastic characteristics such as composition and size, as noted by Fotopoulou and Karapanagioti (2015) who studied the surface alteration of beached polyethylene (PE) and polypropylene (PP) plastic pellets compared with virgin pellets. The beached PE pellets mostly had a highly eroded surface and a negative charge, while virgin plastics and beached PP pellets had a neutral charge. In recent years, particle charge has been considered important in the field of plastics due to increasing production and use of small plastic particles (micro- and nano-sized) in various consumer products (Leslie, 2012). Materials that are insoluble in water when they are neutralized, can become soluble if they are charged since electrostatic repulsions fight against attractive van der Waals forces, as explained by classic DLVO theory (named after its authors Derjaguin, Landau, Verwey, and Overbeek; Derjaguin, 1941; Verwey et al., 1999). This is well illustrated for NP, considering hydrophobic dispersions stabilized with surface charges in water. Most commercially-available nano-beads are made of polystyrene (PS) and commonly have surface functionalization, with anionic (COOH) or cationic (NH_2) groups displaying negative and positive charges, respectively, to give them stability (Casado et al., 2013). Nevertheless, once resuspended in seawater, the charge can change following complex molecular mechanisms due to interaction of the surface groups with high number of ions (Hofmeister, 1888; Cole and Galloway, 2015). In the rich environment of marine salts, specific ions might have specific chemical interactions with surface groups and thus neutralize particle charges (Hofmeister, 1888). In addition, Afshinnia et al. (2018) reported that natural colloids from the medium (e.g., humic substances) could influence the charge of the particles, suppressing the positive charge and enhancing the negative one, depending on both the point zero charge (pzc) of the particles and the pH of the solution. An ecocorona can favor the adhesion of the particles to each other or instead reduce their flocculation in accordance with new surface affinities (Yu et al., 2017). MP and NP charge is an important factor since surface properties play a notable role in determining its effects on organisms. Several studies have revealed a higher impact of positively charged nano-polystyrene particles on different

marine organisms: Crustacea (Bergami et al., 2016, 2017; Nasser and Lynch, 2016) Bivalvia (Balbi et al., 2017) Equinodea (Della Torre et al., 2014), and Chlorophyceae (Bergami et al., 2017). This may be due to the interaction between the positive charge and biological membranes, which generally contain at least a small fraction of negatively charged lipids among larger numbers of neutral or zwitterionic ones (Rossi et al., 2014). Aggregation and charge of NP employed in experiments should be monitored to avoid confounding effects and misinterpretation.

Chemical Aspects

MP can be associated with many chemical agents such as hydrophobic organic chemicals (HOC) and additives (Oehlmann et al., 2009; Teuten et al., 2009). This is of concern as these contaminants can be noxious for wildlife and cause effects such as endocrine-disruption (Talsness et al., 2009; Teuten et al., 2009; Manikkam et al., 2013). In natural environments, sorption of HOC to plastic waste occurs as a result of their hydrophobic characteristics and the magnitude of this sorption is chemical and polymer dependent (Rochman et al., 2013a; Bakir et al., 2014). Evaluating risks associated with MP as vectors of HOC has been widely done in laboratory exposure experiments and an increase in HOC bioaccumulation in organism tissues has often been reported after exposure to contaminated MP (Teuten et al., 2007; Besseling et al., 2013; Rochman et al., 2013b; Avio et al., 2015), although not systematically (Paul-Pont et al., 2016). *In vitro* studies demonstrated that MP transfer through the digestive tract can enhance leaching of HOC due to changes in pH and temperature (Bakir et al., 2014). However, to our knowledge, most experimental studies carried out so far did not take into account (i) the potential bias due to their unrealistically high MP concentrations, and (ii) the role of other suspended particles (detritus, colloids, bacteria, phytoplankton, organic matter, food, etc.) capable of transferring HOC in higher amounts because of their higher abundance in marine ecosystems compared with MP (Bakir et al., 2016; Herzke et al., 2016; Koelmans et al., 2016; Paul-Pont et al., 2016; Besseling et al., 2017). Koelmans et al. (2016) extensively reviewed this question using field, laboratory and modeling data and concluded that, given the currently known low concentration of large MP ($>333\,\mu m$) in the oceans, exposure to HOC via plastic is likely to be negligible compared with other natural pathways. However, a possible shift from mechanical to chemical toxicity through the release of additives and HOC according to time of exposure (from 7 up to 28 days), was recently hypothesized (Pittura et al., 2018). Caution must be taken however as (i) marine plastic litter is expected to increase over coming decades and the concentrations above which an effect can be seen on HOC bioaccumulation could be exceeded locally; and (ii) little is known regarding the NP and small MP fraction in the oceans ($<10\,\mu m$; see section Concentrations), which could mean that we are currently underestimating MP concentrations in the oceans. The relative importance of such NP and small MP fractions in the transfer of HOC might still be underestimated, especially considering that for the same plastic mass, the surface available for HOC adsorption is inversely related to the size of plastic debris pieces (Velzeboer et al., 2014). Finally, the presence of an ecocorona is also expected to influence the plastic–HOC interaction by modifying HOC sorption processes (Koelmans et al., 2009) and oxidation, leading to the potential production of metabolites that may be more toxic than the original compound. There is a need to further testing to what extent organic matter/ecocorona limits or, on the contrary, favors HOC adsorption and detrimental impacts (Galloway et al., 2017).

Far fewer studies have focused on plastic additives or plastic leachates than on HOC, although plastic additives are widely used throughout manufacturing to improve plastic properties (flame retardants, plasticizers, stabilizers, antioxidants, etc.) at very high concentrations ranging from 7% (non-fibrous plastic; Geyer et al., 2017) to 60% (PVC; Net et al., 2015) of the plastic polymer mass. Transfer and toxicity of plastic additives to marine organisms upon plastic ingestion has been demonstrated both in laboratory experiments and field studies (Browne et al., 2013; Rochman et al., 2013b). However, Koelmans et al. (2014) demonstrated via modeling approaches that only a limited transfer of nonylphenol and bisphenol A occurred from MP to both lugworm and North Sea cod, compared with aqueous environmental concentrations of these additives. Indeed, leaching of plastic additives from plastic debris to surrounding seawater may occur rapidly and concentrations of the major additives (phthalates, bisphenol A, polybrominated diphenyl ethers, and nonylphenols) ranging from pg L^{-1} to $\mu g\ L^{-1}$ have been recorded in natural environments (Hermabessiere et al., 2017). Many studies have reported significant toxicity of plastic leachates (obtained from various plastic debris) on aquatic organisms such as fish, bivalves and crustaceans (reviewed in Hermabessiere et al., 2017). More recently, Martínez-Gómez et al. (2017) focused on the commercially-available particles often used in laboratory experiments, reporting significant toxicity of virgin and aged PS and HPDE MP as well as their leachates on the fertilization success and embryonic development of sea urchin. Interestingly, plastic leachates were found to have higher embryo-toxicity than the virgin and aged materials themselves. Most commercial brands offer particles that are supposed to be free of additives or residual monomers; however, most of the time no technical specifications are available from the supplier. For instance, a chemical analysis performed on virgin micro-PS (Polysciences) revealed putative endocrine disruptors such as bibenzyl and 1(2H)naphthalenone,3,4,dihydro4phenyl (Sussarellu et al., 2016). Overall, such studies suggest that commercial microplastics frequently used as model materials in laboratory experiments may leach unknown chemicals such as additives or residual toxic monomers. With the aim of understanding mechanical effects of MP, washing commercial MP in seawater is relevant to allow the leaching of unwanted adsorbed toxic compounds before performing exposure experiments. Alternatively, studies focusing on comparing additive-free MP vs. MP loaded with known amounts of specific additives are of interest to ascertain both physical and chemical toxicities resulting from MP ingestion.

Biofouling and Hetero-Aggregation

Besides contaminants, microorganisms, and rafting organisms can rapidly develop at the surface of plastic debris on the

ecocorona layer (Galloway et al., 2017) within the first few hours (Datta et al., 2016). Yokota et al. (2017) reported that, regardless of their size, positively charged plastics promote microorganism adhesion. Recent studies using high-throughput sequencing have demonstrated that MP-associated microorganism assemblages (including bacteria, microalgae, protozoans and fungi; no information for viruses is yet available) are distinct from those present in the surrounding media or other particulate organic and inorganic matter (McCormick et al., 2014; Amaral-Zettler et al., 2015; De Tender et al., 2015). These specific plastic-associated communities were recently termed the "plastisphere" by Zettler et al. (2013).

Despite an increased research interest in the characterization of the communities forming biofilms on MP, very little is known regarding the reciprocal effects of these biofilms on MP fate (reviewed in Rummel et al., 2017). Although most experimental studies investigating MP impacts on marine biota have used manufactured virgin MP without taking into account the absence/presence of a biofilm on the particles and/or the formation of hetero-aggregates, a few exceptions included these aspects (Green, 2016; Martínez-Gómez et al., 2017). A biofilm can lead to the cohesion of plastic particles with microorganisms which can be defined as hetero-aggregation. This shortcoming needs to be carefully addressed, as hetero-aggregate formation has been shown to influence MP vertical distribution in the water column (Campos et al., 2013; Long et al., 2015), which will inevitably modify the availability of MP for pelagic vs. benthic organisms (Long et al., 2017; Yokota et al., 2017). Modifications of size, shape and surface properties on biofilm/hetero-aggregate formation may also affect the ingestion of MP by zooplankton and filter feeders. For instance, 3-week-aged microbeads were preferred over pristine ones by females of *Acartia longiremis* as well as by juvenile copepodites and adult *Calanus finmarchicus* (Vroom et al., 2017). The preference for aged MP was suggested to be linked to the formation of a biofilm containing similar microbes to those that copepods feed on in the water column, secreting chemical exudates that enhance chemo-detection and particle attractiveness as food items. Also, ingestion of nano-PS (100 nm) by mussels was increased when they were incorporated into hetero-aggregates rather than remaining as free particles, which were probably too small to be efficiently retained by the gills (Ward and Kach, 2009). Conversely, large hetero-aggregates may not be efficiently ingested, possibly explaining the accumulation of MP on the deep-sea floor in the absence of major grazing processes (Van Cauwenberghe et al., 2013). In addition, biofilm formation can (i) alter the diffusion of HOC from or into the particles as previously shown for passive samplers, usually made of PE, and glass beads (Wicke et al., 2008; Harman et al., 2009), and (ii) facilitate the metabolism of HOC leading to the degradation of bio-degradable contaminants including PAH, heavy metals, and pharmaceutical compounds and/or the production of metabolites of greater toxicity (Sowada et al., 2017; Tiwari et al., 2017). Therefore, this aspect should be taken into account in laboratory studies focusing on the role of MP in the transfer of contaminants into marine organisms/ecosystems.

Finally, a few recent studies investigated the role of MP microbial assemblages as vectors for pathogens *in vitro* (Foulon et al., 2016) and *in vivo* after passage through the gut of lugworms (Kesy et al., 2016) and mussels (Kesy et al., 2017). Preliminary results suggest a minor role of the particle itself and a greater importance of the presence of primary colonizers influencing chemotactic attraction to the particle surface. Furthermore, a laboratory trial attempted to clarify the role of microplastics as possible vectors of pathogenic vibrios in oysters (Cassone et al., 2014), but methodological limitations prevented us from drawing any conclusion as it was impossible to work out the origin of the detected infection in the oysters, i.e., to distinguish between the *Vibrio aestuarianus* attached to the plastic beads and the free *V. aestuarianus* bacteria still present in the seawater medium at low concentrations.

Finally, using mostly secondary microplastics collected at sea that have undergone physicochemical and biological modifications related to weathering processes in the environment may appear at first sight as a nice environmentally relevant approach. However in such experimental design, it may be difficult or even impossible to disentangle the potential observed toxicity as some may be due to partially unknown molecules or microorganisms at the particle surface (**Figure 5**). If experiments are performed using particles not collected from the environment (i.e., home-made or commercially-available MP), washing and weathering pristine microplastics in natural seawater before performing exposure experiments will allow (i) the modification of MP surface properties including modification of surface charge, formation of a natural ecocorona and biofilm colonization; and (ii) the leaching of unwanted adsorbed toxic compounds in order to get closer to realistic environmental conditions (**Figures 5**, **6**).

ACHIEVING REPRODUCIBLE AND ENVIRONMENTALLY RELEVANT EXPOSURE OF MARINE ORGANISMS

Phytoplankton

Only a few studies have explored the interactions of MP and NP with phytoplankton and related biological impacts (Bhattacharya et al., 2010; Davarpanah and Guilhermino, 2015; Lagarde et al., 2016; Sjollema et al., 2016; Long et al., 2017; Zhang, C. et al., 2017). Most of these studies were limited to experimental approaches and monospecific cultures. Although the tested concentrations of MP were high, ranging from 4 mg to 2 g L^{-1}, overall effects on growth and physiological parameters were low or absent. Significant impact on growth rate was reported for the marine flagellate *Dunaliella tertiolecta* exposed to 250 mg L^{-1} of 50 µm micro-PS (Sjollema et al., 2016). Exposure to micro-PVC (average 1 µm) at a concentration of 50 mg L^{-1} led to a significant reduction of *Skeletonema costatum* growth and photosynthetic activity (Zhang, C. et al., 2017). These pioneering studies produced some conflicting results and do not allow us to confidently exclude that environmental concentrations of MP/NP could negatively affect freshwater and marine phytoplankton in aquatic environments. While the ecological relevance of laboratory experiments is arguable because they are far from reflecting the complexity of the marine

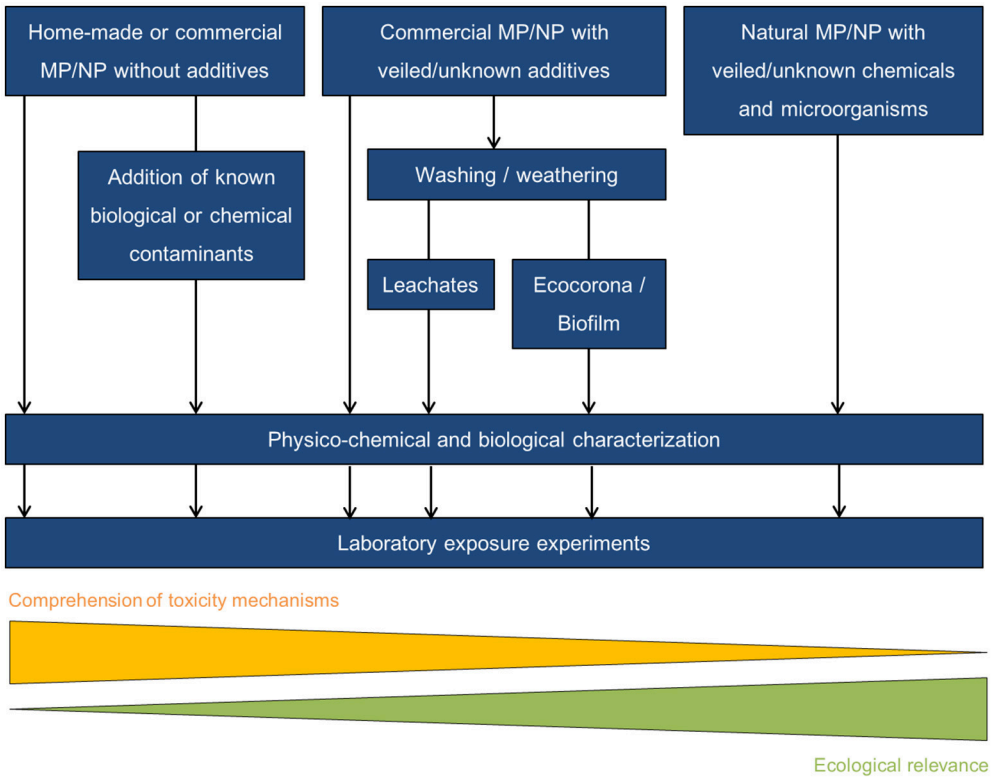

FIGURE 5 | Ways of performing laboratory exposures.

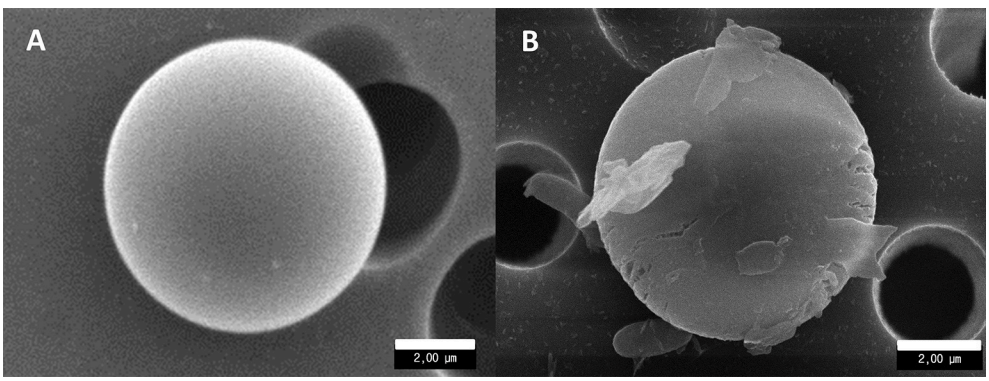

FIGURE 6 | Scanning electron microscopy observations of 5-µm PMMA beads in pristine condition **(A)** and showing signs of erosion, presence of an organic corona and attached bacteria **(B)**.

environment, they may still contribute to exploring the effect of MP/NP pollution on phytoplankton. However, biases, artifacts, and experimental flaws need to be borne in mind.

Prior to considering the effects of MP/NP on phytoplankton *per se*, it is of primary importance to first assess how MP/NP and phytoplankton/microalgae interact with each other. Indeed, MP/NP and microalgae interactions depend on particle physico-chemical characteristics (including size, shape, and charge) and on the species and physiological status of the microalgae (Lagarde et al., 2016; Long et al., 2017). For instance, hetero-aggregation of 2-µm PS with microalgal cells appeared more frequently with diatoms than with *Prymnesiophycea* or dinoflagellates (Long et al., 2017). This study also revealed that formation of hetero-aggregates is favored during the stationary phase of microalgal culture, probably in relation to an increase in cell stickiness, exopolysaccharide (EPS) production, and/or bacterial aggregates that changed with the age of the culture (Long et al., 2017). However, as in most of the studies cited above,

absence of EPS measurement is a major flaw when considering the influence of the ecocorona on MP and NP behavior (see section Modification of Surface Properties).

The other forgotten player in phytoplankton–MP/NP interactions is the bacterial community associated with microalgal culture (such cultures are rarely, if ever, axenic), which varies in concentration and species assemblage according to microalga species, origin, culture condition and age. It is of high interest (as for all kinds of experiments using phytoplankton culture) to quantify bacterial concentration and describe their general characteristics using microscopy and cytometry tools. If some significant influence of the bacterial community is suspected, additional characterization by molecular tools should be considered.

Also, as for any experimental exposure of organisms to MP/NP, it is paramount to quantify the bioavailability and distribution of MP in the experimental systems. MP distribution in different media (e.g., suspended, floating, adsorbed to experimental containers, trapped in organic aggregates, or adsorbed on microalgae or bacteria) must be assessed for each species and experiment to obtain accurate values of the actual MP concentration to which the microalgae are exposed. Long et al. (2017) clearly showed that micro-PS may attach to glassware, form homo-aggregates and hetero-aggregates with phytoplankton cells, residual organic matter, and/or bacterial exudates. Unfortunately, these control measurements, which are tedious and time consuming, need to be performed for every MP/NP tested as their distribution and behavior is expected to change according to polymer nature, size, shape, charge, biofilm and additive content.

Zooplankton, Fish, and Shellfish

There is abundant recent literature examining the presence of MP in different organisms including zooplankton, fish, and shellfish species in different natural marine environments (e.g., Desforges et al., 2014; De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014; Devriese et al., 2015; Rummel et al., 2016; Wesch et al., 2016; Güven et al., 2017; reviewed in Phuong et al., 2016). These surveys clearly revealed a high variability of MP ingestion regardless of the trophic level of the fish or shellfish species concerned; they also tended to indicate a higher frequency of contamination in pelagic feeders and suspension/filter feeders than in other groups. Ingestion rates measured on natural zooplankton communities revealed that 83% of Brown shrimps assessed (*Nephrops* sp.) in the north Clyde Sea (Murray and Cowie, 2011), 63% of shrimps (*Crangon crangon*) in the UK and 3% of the copepod *Neocalanus cristatus* and 6% of the euphausids *Euphasia pacifica* in the northeast Pacific consumed plastic debris, most of which were fragments or fibers (Desforges et al., 2014; Devriese et al., 2015). Studies on fish reported between 2 and 40% of individuals to be contaminated, with a mean number of particles from 1 to 7.2 per individual (Boerger et al., 2010; Foekema et al., 2013; Lusher et al., 2013). For mollusks, especially mussels, this MP load varied from 0.2 to 0.5 plastic particles per gram of tissue (De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014) while for zooplankton contamination generally increased with the size of the organisms and ranged from mean value of 0.026 ± 0.05 for copepods (Desforges et al., 2014) to 1.23 ± 0.99 particles ind^{-1} for shrimps (Devriese et al., 2015). One practical consequence of these observations is the fact that bioassays for studying MP impacts on both zooplankton and fish could be implemented according to taxonomic group, species developmental stage and trophic level. Mode of feeding is an additional factor to take into account when considering exposure experiments with fish and jellyfish (e.g., ctenophores and cnidarians). The buoyant or non-buoyant nature of the targeted MP will also strongly dictate the species chosen to assess the effects of MP and the mode of exposure (bathing vs. trophic pathway).

Bathing is certainly the most prevalent exposition mode used in MP bioassays, probably because it is technically the easiest to implement even though the equal probability of encounter must be verified for each exposed organism by using a dedicated homogenization system (e.g., water current in a kreisel-like incubator or rotation of incubated bottles) and particle counting (using flow cytometry for instance) throughout the entire tanks to ensure homogeneous distribution. Bathing makes it possible to study a broad range of external and/or internal MP effects on zooplankton, fish, and shellfish (Wright et al., 2013b) by both contact (particularly via particle adherence to the carapace/skin/ectoderm, feeding and swimming appendages/tentacles, and gills) and ingestion and its potential effects (e.g., clogging or accumulation in the digestive tract; Bergami et al., 2016). In such assays, it is logical to use either buoyant MP, which are tested free in presence of live prey (microalgae, Artemia, copepods) or feed pellets. In zooplankton studies, ingestion rates are often derived from gut content (i.e., a snapshot of the number of particles eaten at a given time) or from analysis of microplastic-laden fecal pellets. Karakolis et al. (2018) recently highlighted a number of biases linked to gut content and fecal pellet analyses of MP, encouraging the use of digestible coatings (protein/fluorophore) for accurate estimation of MP intake. Biased estimates of MP intake can originate from MP that pass through the digestive tract without being emitted as fecal pellets, or that undergo digestive fragmentation as NP (Dawson et al., 2018); the same holds for MP that adhere to organisms or fecal pellets and that could be erroneously taken into account though not ingested. Finally, operator-dependent errors in counts and measures can strongly limit experimental efficacy. MP coating is an innovative method that needs to be investigated in future experiments, regarding possible combination of coating/fluorophore, types of plastics and experimental conditions.

While most zooplankton are too small to develop true vision (i.e., the capability to form an image) and rely on chemosensing/mechanosensing to detect prey, predatory organisms like fish could confuse suspended MP with potential food/prey, although particle color should be considered since it may modulate the capacity of predator fish to discriminate them from food or preys (Carlos de Sá et al., 2015).

To specifically study the effects of MP ingestion by fish, trophic transfer experiments should be implemented either with artificial feeds or live food. Live fish preys containing MP could be obtained with several mesozooplanktonic organisms ($>200\,\mu m$)

depending on targeted fish species or stages. Setälä et al. (2014) described a simple procedure incubating MP with live preys in bottles placed on a plankton wheel (1 rpm). Importantly, the incubation time that allows the maximal incorporation of MP into the live preys needs to be determined for each species-prey-plastic combination tested by scoring the number of ingested MP by microscopic observation. MP-contaminated live preys could then be given to fish larvae or adults according to their usual feeding protocol. MP could be incorporated into aquafeed pellets, by manual insertion under the microscope as described by Grigorakis et al. (2017) in goldfish, in order to ensure that fish would ingest a precise amount of MP. Such a strategy is very useful for learning about gut retention specificities according to plastic types and shapes. However, it may not be applicable to long-term experiments as the preparation procedure of such aquafeed is time-consuming and may not be realistically manageable. An easier strategy is to incorporate MP at known ratio within aquafeed pellets by aqueous mixing with feed pellets or ingredients (Mazurais et al., 2015; Pedà et al., 2016); then, after gentle air-drying, the feeds can be easily sieved to obtain a precise size range depending on the fish species studied.

For filter/suspension feeders, the way in which MP-NP may interact with phytoplankton is expected to have consequences for MP-NP trophic transfer. MP are likely to form hetero-aggregates when incubated in natural seawater and could possibly modify ingestion by filter/suspension feeders in both natural environment and experimental conditions (Long et al., 2015; Vroom et al., 2017; section Biofouling and Hetero-Aggregation). Therefore, integrating trophic pathway is of great interest to assess particle ingestion and toxicity in laboratory exposures handling filter/suspension feeders. Green (2016) reduced the buoyancy of neutral MP (high density polyethylene and polylactic acid) by mixing them with cultures of *Isochrysis galbana* 3 days prior to exposure in order to make them bioavailable to the flat oyster *Ostrea edulis*. Although such studies remain scarce, they highlight the role of phytoplankton as a potential vector for MP-NP trophic transfer in marine food webs via a more realistic expected scenario.

The post-ingestion process generally ends with microplastic-laden fecal pellets exhibiting reduced sinking rates compared to those derived from a natural food diet, particularly when loaded with PS particles (e.g., Cole et al., 2016). Regarding zooplankton grazers, particularly copepods, coprophagy of MP, i.e., ingestion of entire fecal pellets loaded with MP can lead to a second MP recycling that could induce additional impacts on organisms. Both coprorexhy (fecal pellet fragmentation into smaller pieces) and coprochaly (disruption of the fecal pellet peritrophic membrane) will cause a partial dispersal of the fecal pellet content and particularly in the release of MP into the incubation medium. While working with acknowledged coprophagous/coprochalous copepod species (e.g., *Calanus helgolandicus*, *Oithona similis*, *Acartia tonsa,* and *Temora longicornis*), incubation should be shortened to avoid fecal pellet production and secondary exposure to MP. Otherwise, primary or secondary (i.e., via fecal pellets) ingestion of MP and their impacts will remain difficult to distinguish. In any case, for exposure experiments using either large (fish, shellfish or jellyfish) or small (zooplankton or shellfish larval stages) water volumes, collection of non-ingested food and feces on a 1-μm filter is absolutely essential for all experimental MP exposures in order to prevent any dissemination of microbeads in waste water effluents (Mazurais et al., 2015; Paul-Pont et al., 2016; Sussarellu et al., 2016). Peer reviewers must request an explanation of the treatment procedures used for experimental water effluents. For NP, it is still technically difficult to prevent particle escape with experimental outflow, making it impossible to carry out experiments with running seawater and therefore producing a large water volume to treat. Until the necessary technology to prevent accidental release becomes available, NP exposures should be restricted to small water volumes that can be treated (burned) by specialized companies.

EVIDENCE AND UNCERTAINTIES ON THE TRANSLOCATION OF MICROPLASTICS IN MARINE ORGANISMS

Although it has often been reported that spherical MP particles are rapidly egested (e.g., Mazurais et al., 2015; Cole et al., 2016), the possibility that small microspheres or microparticles of irregular shape could be transferred into tissues of marine organisms other than the digestive tract and then through the food web to humans (Wright et al., 2013b; Setälä et al., 2014; Grigorakis et al., 2017) raises some concerns. When organisms are exposed to micro- or nanoparticles, some of these could pass through the epithelia and enter the circulatory system and sometimes the tissues. This phenomenon is called "translocation." Two entry routes are possible for aquatic organisms: (i) by passive diffusion through the epithelia in direct contact with the external environment (skin, gills or mantle) during water filtration or respiration processes; and/or (ii) by transfer through the digestive epithelium after ingestion. Once epithelial barriers have been passed, particles may be distributed to other tissues via the circulatory system, and potentially pass through cell membranes, including the nuclear membrane. Translocation efficiency primarily depends on particle size, but also on shape, nature, charge, concentration, and the organism concerned (Gratton et al., 2008; Lunov et al., 2011; Bannunah et al., 2014). In recent years, several studies have focused on or discussed the translocation of plastic microparticles within marine organisms (Supplementary file 1), and this remains a topical issue. Observation of translocation is very challenging, and the route of MP entry is not yet identified. There are key points that need to be taken into consideration in studies aiming to demonstrate translocation in marine organisms. Below, we show by means of examples the strengths and weaknesses of the different techniques used to date in both experimental and field studies to accurately assess translocation.

Strengths and Weaknesses of Protocols Designed to Study Translocation

The main techniques used up to now to study translocation have been microscopy to visualize MP within tissues and flow cytometry for circulating fluids. Upon exposure of bivalves to

microplastics, it is likely that MP can enter the pallial cavity and adhere to the mantle or gills and possibly embedded in mucus; This possibility is one of the conclusion of Kolandhasamy et al. (2018) working on experimental exposure of mussels to 0.05-5 mm MP: "adherence rather than ingestion led to the accumulation of microplastics in those organs (foot, mantle) which are not involved in ingestion." Considering that bivalve hemolymph is sampled by suction, with a depression effect in the adductor muscle through the pallial cavity and the body, the possibility of hemolymph contamination during sampling could not be eliminated. Furthermore, translocation could not be demonstrated by histological analysis on transversal sections of eight oysters after 2, 5 or 8 weeks of exposure, demonstrating that "*micro-PS particles were only detected in the stomach and intestine*" (Sussarellu et al., 2016). Based on these observations, it seems important to improve protocols aiming to demonstrate translocation of plastic particles in marine organisms.

One of the first and the most frequently cited publications addressing translocation in marine invertebrates is Browne et al. (2008). These authors used the same hemolymph sampling method as described above, being particularly careful to avoid contamination as "*shell water was drained from each mussel prior to hemolymph extraction.*" Nevertheless, the possibility of contamination during sampling cannot be totally eliminated by flow cytometry analysis alone and histological evidence of the presence of microplastics in the non-digestive tissues would help to exclude artefactual contamination. This is why histology and fluorescent microscopy are among the most frequently used techniques in studies on translocation (Supplementary file 1), although strict experimental and analytical protocols must again be used. Darmody et al. (2015) used epifluorescence microscopy to follow the fate of fluorescent styrene-maleic acid (SMA) microbeads (1–2 μm in size) encapsulated in alginate upon ingestion by oysters (*Ostrea edulis*). However, the microscope features (emission and excitation filters) were not compatible with one of the fluorescent probes used, and no histological sections of control oysters were reported, even though autofluorescence of bivalve tissue is a well-known phenomenon (Heaney et al., 2011). When exposed to 0.05 μm beads, the copepod *Paraoithona nana* exhibited "*fluorescence dispersed throughout the body*", which differed from specimens exposed to 0.5 and 6 μm beads, where fluorescence was "*mostly limited to the digestive organs.*" Jeong et al. (2017) stated that this fluorescence pattern "*could be explained by translocation of polystyrene microbeads across the cellular membranes through the digestive organs of* P. nana." although this could not be clearly verified. Reporting the presence of MP inside specific organs, such as the liver as in Collard et al. (2017), should also be avoided when the same study also makes a statement such as the following *"it was unfortunately not possible to precisely localize MPs in the liver because of the conservation and the cryosections preparation which altered the tissue structure."*

Conclusive dedicated techniques would be an asset to reveal translocation phenomena. For instance, an *in vitro* approach applying Ussing chambers should be considered to accurately show transepithelial transport of MP/NP and potential associated effects on passive or active flux across the enterocyte membranes (Hamilton, 2011; Herrmann and Turner, 2016). This would provide proof of translocation and information on the underlying mechanisms. Histology also appears to be one of the most suitable techniques, provided that there are appropriate controls. The collection of samples must be done very carefully, following strict rules to prevent contamination. Among these, the flesh should be rinsed before dissection, as in Browne et al. (2008), to limit the risk of contamination by MP located outside the tissues. A control comprising tissues of unexposed individuals should also be systematically included for epifluorescence microscopy to take into account tissue and/or non-plastic particle autofluorescence. Finally, cryohistology should be used rather than classic histology as solvents and paraffin embedment may impair MP integrity within tissues and their Raman signal when using a micro-Raman on histological slides.

How Does Particle Size Influence Translocation?

Various sizes of microplastic particles (from 0.5 to 280 μm, Supplementary file 1) have been tested in translocation experiments on multiple marine models. The routes of entry differ according to size and thus between MP and NP. The probability of translocation is considered much higher for NP than for MP. Nanoparticles can enter any tissue by endocytosis, phagocytosis (for aggregates) or passive membrane movement (Gustafson et al., 2015). Silver nanoparticles (5–20 mm) detected using transmission electron microscopy (TEM) and electron-dispersive x-ray analysis (EDS), translocated into brain, heart, yolk, and blood of Zebrafish embryos exposed for 72 h to 5–100 $\mu g\ mL^{-1}$, mainly by endocytosis (Asharani et al., 2008). For plastics, translocation has been shown in fish for nanoparticles of polystyrene (39.4, 53, 180 nm) (Kashiwada, 2006; Mattsson et al., 2017; Supplementary file 1). Regarding MP, Lusher et al. indicate that "*Microplastics larger than this (0.5 mm) do not readily pass through the gut wall without pre-existing damage, and the likelihood of translocation into tissues is too low to warrant regular investigation"* (Lusher et al., 2017).

It is likely that MP $< 10\,\mu m$ are compatible with passage through an epithelium since, for example, bivalve hemocytes (about 5 μm) are known to cross the digestive epithelia (Haberkorn et al., 2010; Rolton et al., 2016) using membrane surface recognition elements. Plastic particles would not be recognized by biological systems and their passage would therefore probably be passive or use non-selective transporters and depend on the presence and nature of their eco- or biocorona (Galloway et al., 2017). Such coating mechanisms involving proteins and biomolecules was previously suggested to occur at the surface of nanomaterials in biological fluids, thus influencing their interaction with cells and tissues (Monopoli et al., 2012). The most detailed studies performed are those on mammals, for which the uptake of diverse types of inert microparticles mainly occurs in the digestive track through normal enterocytes and specific M-cells of Peyer's patches (Pappo and Ermak, 1989; Hussain et al., 2001). To the best of our knowledge, however, such structures have not yet been found in fish, shellfish or zooplankton.

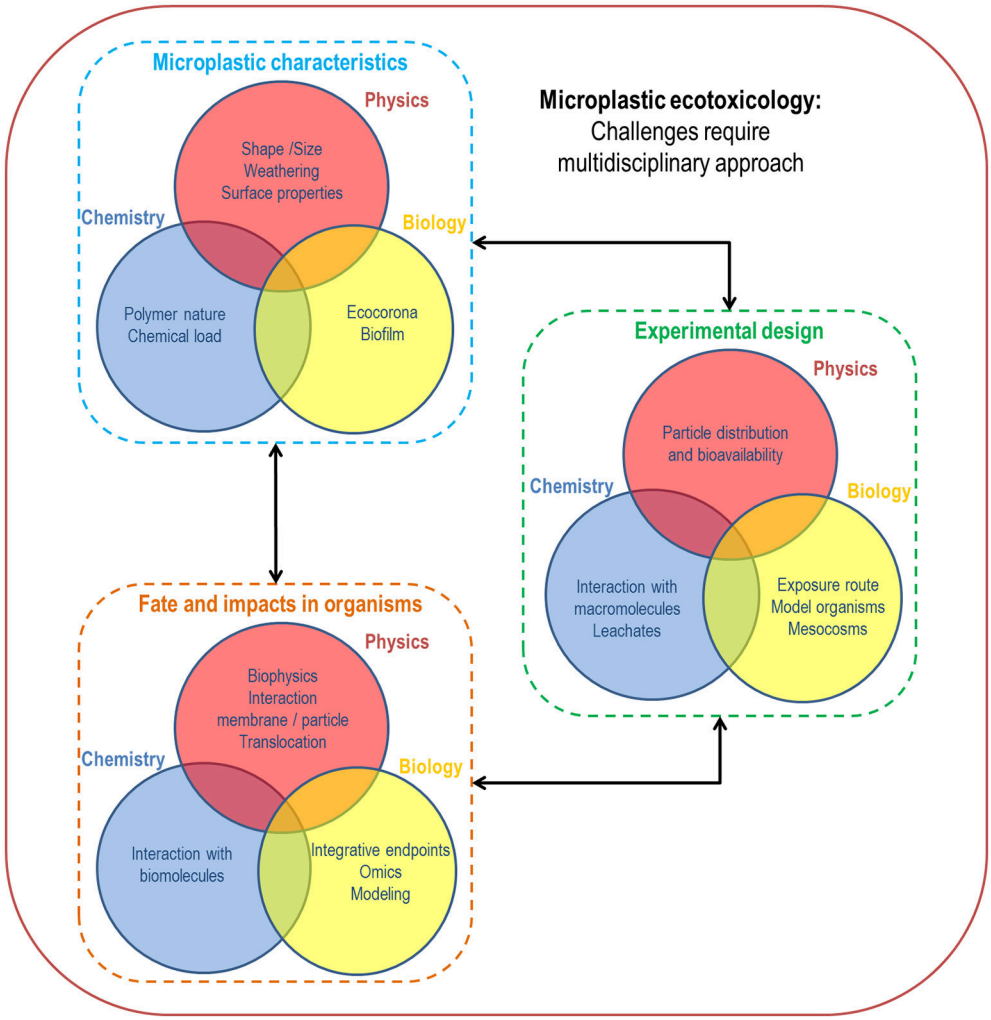

FIGURE 7 | Main challenges in microplastic ecotoxicology requiring multidisciplinary approaches including physics, chemistry and biology.

Future challenges will be to characterize entry mechanisms of MP/NP at the cellular and molecular levels, the largest size of particles able to translocate, and possible effects of form, shape, corona and biofouling. More specifically, three set of actions are required: (i) designing protocols specifically dedicated to translocation studies and preventing contamination; (ii) studying the detailed mechanisms of MP/NP entry through the different epithelia using suitable or innovative approaches and methods (Ussing chambers; electron microscopy; MP radiotracing using isotope-coating technology; Oberhänsli et al., 2017); and (iii) quantifying this phenomenon. Afterwards, to estimate the specific physiological consequences of translocation, it is crucial to assess whether the translocation is "limited" to the circulatory system, whether and how MP/NP interact with biological membranes, and if they can penetrate into cells within which interactions are possible (mitochondria, DNA) inducing stress responses such as ROS production and/or apoptosis.

IS THERE AN IDEAL WAY TO EXPERIMENTALLY STUDY THE EFFECTS OF MP ON MARINE ORGANISMS?

Ecotoxicology is defined as "*the study of harmful effects of chemicals upon ecosystems, which includes effects on individuals and consequent effects at the levels of population and above*" (Walker et al., 2012). One of its first aims is to inform the public (scientists, policy makers, and citizens) on the potential hazards associated with a given contaminant, and how best to protect our environment. As regulatory decisions regarding a given contaminant rely partly on data from the ecotoxicology literature, it is our responsibility to provide high quality data. Recently, a set of recommendations were proposed to design, conduct, analyze and report ecotoxicological studies in the most detailed and transparent manner possible in order to improve their relevance, reproducibility and value (Hanson

et al., 2017; Tincani et al., 2017). These baseline expectations can be fully applied to microplastic studies and concern (i) the characterization of the contaminant and actual exposure in experimental conditions/units, (ii) the experimental design and conditions (replication, randomization), (iii) the characteristics and relevance of test organisms, (iv) the adequate interpretation of the endpoints, (v) the quality of data reporting, (vi) the robustness of statistical analyses, and (vii) the availability of the raw data. Given the variety of microplastic types and mixtures in marine environments, special considerations must be made to properly assess the complexity of MP/NP in natural ecosystems (as detailed in sections Which Particles Should Be Used in Laboratory Experiments? and Weathering Implications), and to choose the most relevant MP and exposure route according to the species habitat and mode of nutrition (as detailed in section Achieving Reproducible and Environmentally Relevant Exposure of Marine Organisms). Another step forward would be the integration of the complex interactions occurring between species in a given ecosystem and how this may influence MP exposure, availability and toxicity, including ingestion, fate in organisms and effects. As field evidence of contaminant-specific adverse effects is almost always impossible to obtain in marine science due to the large open-scale characteristics of marine ecosystems, high variation of physicochemical properties of seawater and co-occurrence of contaminants in impacted areas; mesocosm studies may offer a solution. Indeed, as mesocosms combine the control possible in laboratory experiments with some of the complexity of natural ecosystems, they are a relevant research direction for this field (Sagarin et al., 2016). Long-term exposures in small mesocosms with several sizes of MP (PLA: 0.6–363 μm; HDPE: 0.48–316 μm) showed the destabilization of ecosystem equilibrium, modification of respiration/filtration rates of bivalves, species richness, and offspring recruitment (Green, 2016; Green et al., 2016). However, while mesocosms approach the complexity of environmental scenarios, the understanding of observed effects occurring at different ecological integration levels (molecule, cell, organ, organism, community) calls for multidisciplinary approaches combining ecophysiology, cellular aspects, -omics, and modeling.

"Integrative Biology" concerns levels of integration of life into its environment and integrates the functional and comparative analysis of genomes. It largely originates from the rapid development of new technologies, from genomics and genome sequencing to functional analysis for gene, protein and metabolite networks (e.g., transcriptomics, proteomics, and metabolomics). Based on molecular biology or genomics methods, physiology has gained in precision and has also considerably increased its ability to comprehensively capture functional assemblages for both individual and communities levels and the finest anatomical and cellular elements including when these are disrupted by natural and anthropogenic factors. Since MP and NP may have a wide range of effects on marine organisms depending on their type, shape, size, and the nature of additives, pollutants, and microorganisms they carry, these high-throughput techniques seem particularly well suited to studying the physiological processes and metabolic pathways impaired by complex MP/NP contaminations in mesocosms and laboratory experiments. They can usefully be associated with life-trait endpoints to understand the overall response of organisms, as mentioned for other areas of ecotoxicology (Garcia-Reyero and Perkins, 2011; Jager et al., 2013). Genomic-based endpoints may be more powerful for detecting effects and the presence of stressors such as endocrine disruptors, which are often present below the detection or quantification limits of chemical analysis methods commonly used (GC-MS/MS). Compared to chemical quantification approaches, bioassays are advantageous as they can respond to undetectable trace contaminants and integrate the biological effects of all compounds present, taking into account factors such as bioavailability, synergism, or antagonism. Bioassays coupled with bio-marker analyses are sometimes more powerful than chemical quantification methods for assessing estrogenicity (Kiyama and Wada-Kiyama, 2015). Finally, bioenergetic modeling such as the Dynamic Energy Budget (DEB) theory (Kooijman, 2010) offers a way to describe how an organism acquires and uses energy for physiological functions, maintenance, growth, maturation and migration, in addition to how physiological performance is influenced by environmental variables (Nisbet et al., 2012). The strength of modeling is to help the design of experimental procedures, testing initial hypotheses or, on the contrary, providing explanatory hypotheses to the observed data (e.g., Sussarellu et al., 2016).

CONCLUSION

Many challenges remain to be overcome to adequately address concerns about MP/NP toxicity to marine organisms and ecosystems so as to provide stakeholders with the necessary data to limit the impact of the microplastic legacy. Laboratory exposure experiments must be carefully designed to cover the wide range of MP/NP contaminations in marine environments, and multidisciplinary approaches involving physics, chemistry and biology appear more than ever essential in this field of research (**Figure 7**). Because it is not possible to reasonably address all aspects of MP/NP forms, concentrations, mixtures and chemical and biological characteristics at once in each experiment, the scientific question and objectives behind each set of experiments must be clearly defined in order to adequately prioritize the features of MP/NP that potentially account for confounding effects on the endpoints of exposure evaluations. Even if realistic experiments considering ecosystem scenarios are called for decision support, fundamental studies unraveling origin(s) of MP/NP toxicity remain unavoidable as a large body of basic information is missing. A single study cannot be expected to provide all the answers and effectively capture the synergies and antagonisms of contaminants in marine ecosystems. It will be desirable to compile evidences from multiple sources; the more rigorous studies are, the more relevant a meta-analysis will be to quantitatively assess risks of MP/NP in environments.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00252/full#supplementary-material

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Ecotoxicological Effects of Chemical Contaminants Adsorbed to Microplastics in the Clam *Scrobicularia plana*

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Although microplastics (MPs) are distributed globally in the marine environment, a great deal of unknowns relating to their ecotoxicological effects on the marine biota remains. Due to their lipophilic nature, microplastics have the potential to adsorb persistent organic pollutants present in contaminated regions, which may increase their detrimental impact once assimilated by organisms. This study investigates the ecotoxicological effects of exposure to low-density polyethylene (LDPE) microplastics (11–13 μm), with and without adsorbed contaminants (benzo[a]pyrene—BaP and perfluorooctane sulfonic acid—PFOS), in the peppery furrow shell clam, *Scrobicularia plana*. Environmentally relevant concentrations of contaminants (BaP—16.87 $\pm$ 0.22 μg g^{-1} and PFOS—70.22 $\pm$ 12.41 μg g^{-1}) were adsorbed to microplastics to evaluate the potential role of plastic particles as a source of chemical contamination once ingested. *S. plana* were exposed to microplastics, at a concentration of 1 mg L^{-1}, in a water-sediment exposure setup for 14 days. Clams were sampled at the beginning of the experiment (day 0) and after 3, 7, and 14 days. BaP accumulation, in whole clam tissues, was analyzed. A multi-biomarker assessment was conducted in the gills, digestive gland, and haemolymph of clams to clarify the effects of exposure. This included the quantification of antioxidant (superoxide dismutase, catalase, glutathione peroxidase) and biotransformation (glutathione-S-transferases) enzyme activities, oxidative damage (lipid peroxidation levels), genotoxicity (single and double strand DNA breaks), and neurotoxicity (acetylcholinesterase activity). Results suggest a potential mechanical injury of gills caused by ingestion of microplastics that may also affect the analyzed biomarkers. The digestive gland seems less affected by mechanical damage caused by virgin microplastic exposure, with the MPs-adsorbed BaP and PFOS exerting a negative influence over the assessed biomarkers in this tissue.

Keywords: polyethylene, benzo[a]pyrene, perfluorooctane sulfonic acid, DNA damage, oxidative stress, neurotoxicity, oxidative damage

INTRODUCTION

Advances in plastic production have resulted in more versatile, lightweight, durable, and cheap plastics, which have become incorporated in every part of our day-to-day lives (Andrady and Neal, 2009). Yet, plastic is now a ubiquitous, long lasting source of litter on the planet (Barnes et al., 2009). Since mass production of plastic began in the 1940's, annual production has increased from ∼5 Mt in the 1950's to 322 Mt in 2015 (Plastics-the Facts, 2016). Low density polyethylene (LDPE) is used in the production of reusable bags and agricultural films, with its predominant use in the food packaging industry (Andrady, 2003; Plastics-the Facts, 2016).

Macroplastics (>5 mm) and their effect on the marine environment have been studied for some years (Cole et al., 2011). Due to their large size, being clearly visible they create "eyesores" on the landscape and in the oceans as well as entangling and endangering marine life. Estimates of the amount of plastic currently residing in the oceans varies from 7,000 t (Cózar et al., 2014), to over 250,000 t (Eriksen et al., 2014). In recent years microplastics (MPs; <5 mm) have become an area of concern due to their ubiquitous distribution in the marine environment, occurring in all geographical regions of the Oceans, including the Arctic (Obbard et al., 2014), and Antarctica (Lusher, 2015). The presence of microplastics have been reported in a wide range of marine habitats - beach sediments (Thompson et al., 2004), subtidal and benthic sediments (Barnes et al., 2009), deep-sea sediments (Fischer et al., 2015), the water column, surface waters (Hidalgo-Ruz et al., 2012), near densely populated areas (Barnes, 2005), and even in remote island atolls where no input or production of plastics occurs (McDermid and McMullen, 2004). Properties of plastic polymers, such as density, surface charge and aggregation potential, as well as abiotic (oxidation, weathering, and vertical mixing) and biotic factors (biofouling) all contribute to the ubiquitous distribution of MPs within the oceans (Lusher, 2015). Microplastics have been reported in 61% of Portuguese water samples with higher concentrations in Costa Vincentina (0.036 particles m^{-3}) and Lisbon (0.033 particles m^{-3}) compared to the Algarve (0.014 particles m^{-3}) and Aveiro (0.002 particles m^{-3}) (Frias et al., 2014). Microplastic resin pellets (3–6 mm, 5% > 5 mm) represented 53% of total marine debris collected (1,289 items m^{-2}, 30 g m^{-2}) in another study on the Portuguese coastline, with 98% of marine debris being identified as plastic (Antunes et al., 2013). In both cases, higher microplastic abundances were reported in proximity to urban, industrial, and shipping areas.

MP ingestion, or uptake by other means, has been reported in a variety of marine species including plankton, invertebrates, fish, sea birds, marine mammals, and turtles (e.g., Browne et al., 2008; Moore, 2008; von Moos et al., 2012; Galgani et al., 2014; Avio et al., 2015, 2017; Lusher, 2015; Setälä et al., 2016; Lourenço et al., 2017; Germanov et al., 2018; Kolandhasamy et al., 2018). MPs may enter the base of the food chain through ingestion or adsorption by phytoplankton and/or zooplankton (Lusher, 2015) and trophic transfer has been reported (e.g., Farrell and Nelson, 2013). Commercial marine species which are eaten whole, such as shrimps and bivalves, constitute a potential transfer pathway of MPs from the marine environment to humans (Lusher, 2015; Rochman et al., 2015; Santillo et al., 2017).

MP particle size plays an important role in their biological fate within the marine environment. Impacts on the marine biota may vary across the size spectrum of MPs. Large microplastics (2–5 mm) may take more time to pass from the stomach of organisms, having the potential to be retained in the digestive system. Toxicant adsorption, dependent on polymer type, may occur with increased exposure time to plastics. Feeding and digestion may occur with particles in the upper end of the size spectrum (1–2 mm) (Lusher, 2015). Small marine invertebrates have been shown to actively ingest and egest particles <20 μm (Thompson et al., 2004; Lee et al., 2013). Smaller size microplastics have larger effects on organisms at the cellular level. In the micro- to nanometer range microplastics have been shown to translocate and pass into cellular membranes, including the haemolymph (Browne et al., 2008; Ribeiro et al., 2017), and the lysosomal system (von Moos et al., 2012) of marine invertebrates.

Many additives are added in the production of plastics, which give them various desirable qualities and enhance their performance (Andrady and Neal, 2009). Many of the additives have known or suspected toxicity, containing persistent organic pollutants (POPs), synthetic organic compounds of anthropogenic origin. POPs are chemically stable and do not easily degrade in the environment. Plastics are known to sorb hydrophobic chemical contaminants from the surrounding sea water, and have been shown to concentrate them (Mato et al., 2001; Rios et al., 2007; Barnes et al., 2009; Wu et al., 2016; Zhang et al., 2018). Degradation of MPs to smaller plastic particle sizes adds more surface area to sorb contaminants. The combination of increased surface area due to weathering, long exposure times in the marine environment, and the hydrophobicity of organic xenobiotics may facilitate adsorption of these contaminants to MPs at concentrations significantly higher than those detected in seawater (Ogata et al., 2009; Antunes et al., 2013). Hence, MPs are vehicles for organic pollutants to enter marine organisms (Besseling et al., 2013; Bakir et al., 2014, 2016; Chua et al., 2014; Ziccardi et al., 2016; Hartmann et al., 2017; Rainieri et al., 2018) Under laboratory conditions, various polymer particles have been shown to adsorb chemical pollutants from the surrounding environment, with PE, PVC, PP, and PS displaying high sorption capacity for polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexanes, chlorinated benzenes, musks, pharmaceuticals, personal care products (Bakir et al., 2012; Lee et al., 2014; Wu et al., 2016; Ziccardi et al., 2016; Hartmann et al., 2017; Zhang et al., 2018). Various organic pollutants, including PAHs, polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, and dioxins have been detected in plastic pellets from beaches worldwide (Ogata et al., 2009; Avio et al., 2015). Contamination in, and concentration of pollutants on MPs are of great importance as MPs may be ingested by marine organisms with contaminants having the potential to desorb and accumulate in fatty tissues due to their lipophilic nature, posing a long-term risk to the environment (Mato et al., 2001; Rios et al., 2007; Besseling et al., 2013; Avio et al., 2015; Bakir et al.,

2016; Ziccardi et al., 2016; Batel et al., 2018). Exposure to organic chemicals adsorbed to MPs revealed biomarker responses at cellular and sub-cellular level, such as alterations in oxidative stress, immune and neurological responses, and gene expression profiles (Browne et al., 2013; Oliveira et al., 2013; Rochman et al., 2014; Avio et al., 2015; Paul-Pont et al., 2016). In mussels exposed to PS MPs with adsorbed fluoranthene, an increase in reactive oxygen species production in hemocytes was noted, along with high activities of anti-oxidant and glutathione-related enzymes (Paul-Pont et al., 2016). In another study with mussels exposed to PS and PE with adsorbed pyrene, negative, and greater effects were noted, when comparing with MPs without adsorbed chemicals, inducing changes in immune responses, antioxidant enzyme activities, neurotoxic, and genotoxic effects (Avio et al., 2015).

Known for its pro-carcinogenic properties, benzo[a]pyrene (BaP) (C20H12) is thought to be one of the most toxic PAHs and is classified by the International Agency for Research on Cancer as a group 1 human carcinogen (IARC, 2011; Liu et al., 2015; Châtel et al., 2017). Formed through the incomplete pyrolysis of combustible organic material, the main sources are anthropogenically derived from fossil fuel combustion, waste incineration, and oil spills. PAHs emitted, as soot or gas, to the atmosphere, enter the marine environment through rain and surface run-off (Antunes et al., 2013; Liu et al., 2015; Châtel et al., 2017). BaP is ubiquitously distributed in coastal and marine environment (Antunes et al., 2013; Liu et al., 2015; Châtel et al., 2017) and has been used as a model to investigate the effects and metabolic pathways of PAHs in marine organisms (Liu et al., 2015).

Perfluorooctane sulfonic acid (PFOS) has been classed as an emerging chemical of concern, along with its precursors—perfluoroalkylated acids, and is classified as a POP under the Stockholm Convention (Paul et al., 2009). Environmental contamination of PFOS may occur in two ways, either through direct release to the environment during manufacture and application, or indirectly, through degradation from precursor compounds. Under environmental conditions PFOS does not hydrolyse, photolyze, or biodegrade. Considered a widespread contaminant, PFOS has been recorded globally in seawater, human blood, and in the biota (Giesy and Kannan, 2001; Kannan et al., 2001; Yamashita et al., 2005). The chemical properties of PFOS, including high water solubility and negligible vapor pressure, imply that it will reside in surface waters once released into the environment (Paul et al., 2009). Although limited information is available on the volume of PFOS, and its precursors, released into the environment, empirical oceanographic data estimates that ∼235–1,770 t of PFOS currently reside in oceanic surface waters (Paul et al., 2009). PFOS has been shown to bioaccumulate and biomagnify to higher trophic levels (Giesy and Kannan, 2001). Unlike other POPs, PFOS does not accumulate in fatty tissues, but binds to the protein albumin, mainly present in blood, liver, and eggs. As such the behavior of PFOS within the body is similar to that of fatty acids, with hydrophobic interactions playing a role in bioaccumulation (de Vos et al., 2008).

Scrobicularia plana is an environmentally relevant species to use as a bioindicator for evaluating the health status of coastal and estuarine ecosystems (Mouneyrac et al., 2008), playing a key role in their structure and functioning (Châtel et al., 2017). As burrowing deposit filter feeders, clams can assimilate particles, and associated contaminants, from both the sediments and the water column. Being positioned at the base of the food web, the clam is an important food source for crabs, fish, birds, and increasingly for human consumption (Rodríguez-Rúa et al., 2003; Langston et al., 2005, 2007).

In the present study, a battery of biochemical, cellular, and physiological biomarkers was analyzed in order to characterize the ecotoxicological potential of both virgin and contaminated LDPE microplastics, in the gills, digestive gland, and haemocytes of the peppery furrow shell clam, *S. plana*. Environmentally relevant concentrations of known marine contaminants, benzo[a]pyrene (BaP), and perfluorooctane sulfonic acid (PFOS), were adsorbed to microplastics to address both the potential for plastic particles to act as a vector of chemical exposure once ingested, and evaluate the effect of each respective contaminant. To clarify any effects of exposure, a set of biomarkers were employed, including the quantification of antioxidant (superoxide dismutase—SOD, catalase—CAT, glutathione peroxidase—GPx) and biotransformation (Glutathione-S-transferases—GST) enzyme activities, which play a role in detoxification under conditions of oxidative stress; lipid peroxidation (LPO) levels, indicative of oxidative damage; single and double strand DNA breaks to evaluate genotoxicity; the activity of the enzyme acetylcholinesterase (AChE), involved in neuro- and neuromuscular transmission; and the condition index, evaluated to assess the overall health status of the organisms.

MATERIALS AND METHODS

Preparation and Characterization of Microplastics

Non fluorescent, low-density PE microparticles (0.96 g cm^{-3}), MPP-635G (11–13 μm) were purchased from Micro Powders Inc. (NY-USA). Microplastics were spiked with BaP using 125 g L^{-1} of plastic, weighed into separate 250 mL narrow-mouth Septa bottles (Thermo scientific) filled with double-deionized water. Benzo[a]pyrene, CAS 50-32-8 (purity ≥ 96%) was purchased from Sigma Aldrich, and a concentration of 2,500 μg L^{-1} was used. Bottles were placed on a rotary shaker for two days at the lowest speed (20 rpm). Samples were filtered using a ceramic funnel and glass microfiber filters (1.0 μm, Whatman® glass microfiber filters, GE Healthcare Life Sciences). Samples were rinsed with double-deionized water, dried by vacuum evaporation and extracted in hexane (≥98%, SupraSolv). Extracts were sonicated and centrifuged at 2000 RCF. Extracts were filtrated trough fiberglass and transferred to toluene (purity 96%, SOLVECO). The sample volume was reduced to 500 μL using a nitrogen stream. Concentrations of BaP were quantified using a high-resolution GC-MS system (Micromass Autopspec Ultima),

separation on a 30 m (0.25 mm i.d., 25 μm film thickness) DB-5MS column (J&W Scientific, Folsom, USA). Details about the instrumental method can be found in Larsson et al. (2013). As an internal standard, benzo[a]pyrene-d12 in toluene was added to the vial. Quantification was performed against perylene-d12 recovery standard, dissolved in toluene and purchased from Chiron. Final concentration of BaP adsorbed to LDPE (11–13 μm) MP was 16.87 (±0.22) $\mu g\ g^{-1}$.

For the preparation of PFOS-spiked MPs, 50 g L^{-1} of plastic was weighed into a 1 L polypropylene bottle filled with 500 mL of double-deionized water. Heptadecafluorooctanesulfonic acid potassium salt (CAS 2785-37-3, purity ≥98%) was purchased from Sigma-Aldrich (Germany) and a concentration of 20 mg L^{-1} was used. The bottles were placed on a rotary shaker at the lowest speed (20 rpm) for 7 days. Filtration of the samples was performed using a funnel and glass microfiber filter (1.0 μm, Whatman® glass microfiber filters, GE Healthcare Life Sciences). Samples were rinsed with double-deionized water and dried by vacuum evaporation on a ceramic funnel. MPs were extracted in methanol (>99.9% purity, Fisher Scientific) by ultra-sonication followed by centrifugation (7000 RCF). Extracts were filtrated trough out a polyethylene syringe (Norm-Ject®, 5 mL, ref.4050-000V0) with a filter of 0.2 μm (AcrodiscGHP, 13 mm, 0.2 μm). Recovery standard was added with a mobile phase methanol and ammonium acetate (Fluka, Steinheim, Germany), 40%/60% (v/v). Analysis was performed on an Acquity UPLC system coupled to a Xevo TQ-S quadrupole mass spectrometer (Waters Corporation, Milford, U.S.A.). PFOS were separated on 100 mm Acquity BEH C18 column (2.1, 1.7 mm). Detailed description of LC method and instrumental settings can be found in the supplementary information of the publication by Eriksson et al. (2016). Final concentration of PFOS adsorbed to LDPE (11–13 μm) MP was 70.22 (±12.41) $\mu g\ g^{-1}$.

Experimental Design

Clams (*S. plana*, 4 cm ± 0.5 cm) were collected from Cabanas de Tavira, Ribeira do Almargem, Southern Portugal (N 37°7′59.75″W 7 36′34.95″), transported to the laboratory alive, and acclimatized for 5–7 days in natural seawater, at constant aeration with a photoperiod of 12 h light to 12 h dark. Collection occurred in early February, during the period of sexual inactivity. Post acclimation, clams were transferred to 25 L aquaria containing a proportion of 1:4 of sediment/ seawater. The height of the water column was 18 cm above the 5 cm of sediment layer. Sediment, previously collected from the top 30 cm at the same site, was passed through a 4 mm sieve to remove any macro-organisms and debris, and dried at 65°C for 48 h to remove organic matter, volatile compounds, and water (Maranho et al., 2014). Sediment was rehydrated, to the same original sediment moisture content (%) as when collected, calculated by the difference in the wet weight of a known volume of sediment after reaching constant dry weight at 65°C.

The exposure experiment consisted of 8 aquaria (each containing 85 clams), with 4 treatments (control, virgin LDPE, BaP contaminated LDPE, and PFOS contaminated LDPE) in a duplicate design. All aquaria, excluding the two controls, were exposed to LDPE microplastics (11–13 μm) at a concentration of 1 mg L^{-1} for a duration of 14 days. The exposure to microplastics was conducted by adding microplastics to the water column. The mixing of microplastics in the entire water column was possible (and visible) by the relatively strong aeration supplied to each aquarium.

Water was changed every 72 h, with the subsequent re-application of microplastics. Abiotic parameters; water temperature (19.31 ± 0.21°C), oxygen saturation (95.78 ± 1.77%), salinity (34 ± 1 ppt), and pH (8.05 ± 0.04), were measured using a multiparametric probe (ODEON V3.3.0). Mortality was observed in all aquaria, with the highest being in the control and LDPE treatments (7%), followed by LDPE+PFOS (5%), and LDPE+BaP (3%) treatments. Food was not supplied during the exposure period to minimize interactions of microplastics with other suspended particles. Glass Pasteur pipettes were attached to the end of plastic aeration tubes to avoid plastic contamination.

Individuals were randomly sampled from each aquarium before the addition of microplastics (day 0), and after 3, 7, and 14 days of exposure. Haemolymph was extracted from the posterior adductor muscle of *S. plana* at each sampling time, using a 1.5 mm sterile hypodermic syringe with an attached needle and used for genotoxic analysis. Gills and digestive gland tissues were dissected immediately, flash-frozen in liquid nitrogen and stored at −80°C for later analysis. Whole clams, to be used for chemical analysis, were frozen at −20°C for chemical analysis. Microplastics contents were not analyzed in the clams tissues.

Tissue Chemical Analysis

BaP was quantified in the whole soft tissues of freeze-dried clams using the method previously described by De Witte et al. (2014), with some modifications. 6 clams per treatment and per time (at days 0 and 14) were analyzed. Briefly, the samples were extracted by accelerated solvent extraction (Dionex, ASE350), using a mixture of hexane and acetone 3:1 at 100°C. After two evaporation steps, 5 μL of each sample was injected on a GC (Agilent 7890A) equipped with a PTV-injector (Gertsel, 6495-U). An Agilent 5975C MS-detector with electron impact ionization in single-ion mode was used for detection. As an internal standard, chrysene-d12 in toluene was added to the vial. Quantification was performed against benzo[a]pyrene-d12 recovery standard, dissolved in iso-octane and purchased from LGC-standards. The analysis is accredited by BELAC under the ISO/IEC 17025 standard, with a quantification limit of 1.65 ng g^{-1} dw.

Condition Index

The gravimetric condition index (CI) was assessed in 6 individuals per treatment, at each sampling time (day 0, 3, 7, and 14 of exposure), to determine the physiological status of both control and exposed clams. Tissues were dried at 80°C until a constant dry weight was achieved. The CI was estimated by calculating the percentage (%) of the ratio between dry weight of the soft tissues (g) and the dry weight (g) of the shell (Walne, 1976).

DNA Damage

DNA damage was determined in 6 individuals per treatment and per time (pre-exposure and 14 days exposure) using a slightly modified alkaline comet assay (Singh et al., 1988) as described by Almeida et al. (2013).

Post extraction, haemolymph cells were centrifuged at 3,000 rpm for 3 min at 4°C, and the supernatant removed. The DNA pellet was re-suspended in phosphate buffered saline (PBS) solution and low melting point agarose (LMA 0.65%, dissolved in Kenny's salt solution). DNA cell suspensions were cast on microscope slides previously coated with normal melting point agarose (NMA 0.65%, dissolved in Tris-acetate EDTA). One slide, with two replicate agarose gels embedded with cells, was prepared per sample. Slides were immersed in Lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Sarcosil, 10% Dimethylsulfoxide, 1% Triton X 100, pH 10) at 4°C in the dark for 1 h, enabling cell lysis. Microscope slides were rinsed with ultrapure water (Milli-Q), placed in an electrophoresis chamber, submerged in buffer (300 mM NaOH, 1 mM EDTA, ultrapure water, pH > 13, at 4°C) for 15 min prior to running the current, to allow DNA to unwind. Electrophoresis was run under the following conditions: 25 V, 300 mA, for 5 min. Slides were removed, immersed in neutralization solution (0.4 mM Tris, pH 7.5), rinsed with ultrapure water, and allowed to dry, in the dark at room temperature. Once dry, slides were stained with 4,6-diamidino-2-phenylindole (DAPI). Each well was examined using an optical fluorescence microscope (Axiovert S100), under 400x magnification. 25 photographs of randomly chosen, individual cell nuclei were taken from each well (50 for each slide/sample), using a camera (Sony) attached to the microscope. Photographs were analyzed using the Komet 5.5 image analysis system (Kinetic Imaging Ltd). DNA damage was quantified by measuring the displacement between the genetic material of the cell nucleus ("comet head") and the migrating comet "tail" with both comet tail length and olive tail moment parameters assessed. Results are expresses as mean ± standard deviation (SD).

Antioxidant and Biotransformation Enzyme Activity

Enzyme activities (SOD, CAT, GPx, and GST) were assessed in the gills and digestive gland tissues of 6 individuals per treatment, at each sampling time (0, 3, 7, and 14 days). Tissues were defrosted, weighed and homogenized, on ice, in 5 mL of Tris sucrose buffer (Sucrose 0.5 M, Tris 20 mM, KCL 0.5 M, DTT 1 M, EDTA 1 mM, at pH 7.6). The homogenate was centrifuged at 500 × *g*, at 4°C for 15 min. The supernatant was separated and re-centrifuged at 12,000 × *g*, at 4°C for 45 min. The cytosolic fraction was divided into 5 aliquots, stored in Eppendorf tubes and frozen at −80°C for the determination of SOD, CAT, GPx, and GST activities as well as total protein concentrations. Percentage inhibition in the reduction of cytochrome *c* by the superoxide anion generated by the xanthine/hypoxanthine system, measured at 550 nm (McCord and Fridovich, 1969) was used to determine SOD activity, expressed in Units (U) mg^{-1} of total protein concentration, where 1 U of activity corresponds to the amount of sample required to cause 50% inhibition. CAT activity was determined by measuring the consumption of hydrogen peroxide (H_2O_2) at 240 nm, as described by Greenwald (1987), with results expressed as μmol min^{-1} mg^{-1} of total protein concentration. GPx activity was determined through the reduction in NADPH, in the presence of glutathione reductase (GR) and reduced glutathione (GSH) using a cumene hydroperoxide probe (Lawrence and Burk, 1978). Measured at 340 nm, the decrease in NADPH is directly proportional to GPx activity. Results are expressed as nmol min^{-1} mg^{-1} of total protein concentration. GST activity was determined by the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH) with the resulting increase in absorbance measured at 340 nm (Habig and Jakoby, 1981). Results are expressed in nmol CDNB min^{-1} mg of total protein concentration.

Acetylcholinesterase (AChE) Activity

AChE activity was assessed in the gills of 6 individuals per treatment, at each sampling time (day 0, 3, 7, and 14) following the protocol modified from Ellman's colorimetric method (Ellman et al., 1961). Tissues were defrosted, weighed and homogenized, on ice, in 5 mL of Tris HCL buffer (100 mM, pH 8.0) and 50 μL of Triton—X 100 (0.1%). The homogenate was centrifuged at 12,000 × *g*, at 4°C for 30 min. Acetylthiocholine degradation rate was determined through the increase in 5-mercapto-2-nitrobenzoate, a compound of yellow color produced due to the non-enzymatic reaction of thiocoline with 5,5′-dithio-bis (2- nitrobenzoic acid). Absorbance was read at 405 nm and results expressed in nmol ACTC min^{-1} mg of total protein concentration.

Lipid Peroxidation (LPO)

LPO levels were quantified in gill and digestive gland tissues of 6 clams per treatment, at each sampling time (0, 3, 7, and 14 days of exposure) following the colorimetric method described by Erdelmeier et al. (1998). Tissues were defrosted, weighed and homogenized on ice, in 5 mL of Tris HCL buffer (0.02 M, pH 8.6) and 50 μL of butylated hydroxytoluene solution (BHT). The homogenate was centrifuged at 30,000 × *g*, at 4°C for 45 min. Lipid peroxidation levels were determined through the quantification of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides. Supernatant (200 μL) was mixed with 1-methyl-2-phenylindone diluted in methanol (650 μL), and methanesulfonic acid (150 μL, 15.4 M), and incubated at 45°C for 60 min. Following incubation, the mixture was centrifuged at 15,000 × *g*, at 4°C for 10 min. Absorbance was read at 586 nm and results expressed in nmol of MDA mg^{-1} of total protein concentration.

Total Protein Concentration

Total protein concentrations were determined in the cytosolic fraction of gill and digestive gland tissues, of 6 individuals per treatment and per sampling time (0, 3, 7, and 14 days), post homogenisation, using the Bradford Assay (Bradford, 1976). Concentrations (mg ml^{-1}) were used to normalize enzyme activities and LPO levels.

Statistical Analysis

Analyses were performed using R 3.3.1 software (R Core Team, 2016). Data are expressed as mean ± *SD*. Biomarker results were compared using two-way ANOVA, with polymer type and time as variables. Significant ANOVA results were analyzed using Tukey's HSD test. Chemical concentrations in tissues were compared using the non-parametric Mann–Witney *U*-test. A p-value ≤ 0.05 was considered statistically significant. Principal component analysis (PCA) was used to evaluate the relationship between biomarkers, BaP concentration, and the different treatments along the exposure period per contaminated LDPE MP.

RESULTS

Condition Index

The CI of the organisms' pre-exposure (day 0) was 7.7 ± 2.7%. No significant differences were found between control (7.6 ± 0.4%) and virgin MP treatments (7.7 ± 0.3%) ($p > 0.05$), nor between virgin MP and contaminated MP treatments (LDPE+BaP 7.4 ± 0.5%, LDPE+PFOS 7.4 ± 0.9%, $p > 0.05$). No significant changes were observed between sampling times of the same treatment ($p > 0.05$). Results, expressed as treatment means ± *SD*, indicate that *S. plana* clams remained in good health for the duration of the experiment.

Virgin LDPE MP

Genotoxic effects, analyzed using the alkaline comet assay, for control and virgin LDPE treatments are displayed in **Figure 1**, with results expressed as comet tail length and olive tail moment. No significant differences were found between virgin LDPE and control treatments, nor between different time points of the same treatment, when quantifying DNA damage through the resulting comet tail length or OTM (**Figure 1**) ($p > 0.05$).

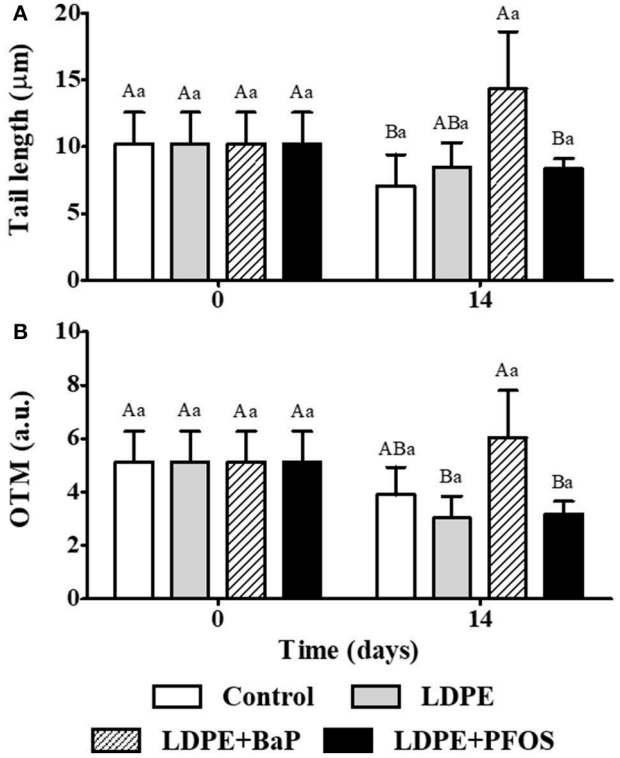

FIGURE 1 | DNA damage (mean ± *SD*) in the haemocytes of *S. plana,* expressed as Tail length (μm) **(A)** and OTM **(B)** for control, virgin LDPE, LDPE+BaP, and LDPE+PFOS treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$). a.u. = arbitrary units.

No significant differences were found between virgin LDPE and control treatments, neither through time, when quantifying SOD activity in the gills of *S. plana* ($p > 0.05$) (**Figure 2A**). A significant difference in digestive gland SOD activity occurred between treatments with the control displaying significantly higher activities than the virgin LDPE after 3 days of exposure ($p < 0.05$). SOD activity in the digestive gland of virgin LDPE treatments remained unchanged over time ($p > 0.05$) (**Figure 3A**).

A tissue specific response is observed in CAT activity, with a 2 to 3-fold higher activity in the digestive glands relative to gills, for both treatments (control and virgin LDPE) (**Figures 2B**, **3B**). Exposure to virgin LDPE caused a significant increase in CAT activity in gill tissues after 3 days, significantly differing to the control at this time ($p < 0.05$) (**Figure 2B**). CAT activity in the gills subsequently decreased at day 7 and 14, with a significant difference occurring between tissues exposed to virgin LDPE after 3 days of exposure and both these times ($p < 0.05$). No significant differences were observed in CAT activity of digestive gland tissues between control and virgin LDPE treatments, nor over time for either treatment ($p > 0.05$) (**Figure 3B**).

GPx activity was highly variable among tissues and treatments, being null in gill tissues at day 3 for all treatments and control at day 7. A significant increase in GPx activity was observed in the gill tissues of virgin LDPE treatment after 14 days of exposure, relative to both pre-exposure and the control at day 14 ($p < 0.05$) (**Figure 2C**). In the digestive gland, a significant decrease in GPx activity in virgin LDPE treatments is observed after 7 days, remaining with similar levels at day 14 ($p < 0.05$), when compared to day 3 (**Figure 3C**).

GST activity increases steadily in gill tissues of organisms exposed to virgin LDPE during the experimental period (**Figure 2D**). Relative to pre-exposure, a significant increase in GST activity in the gills is first observed after 7 days ($p < 0.05$), with a significant difference in activity occurring between day 14 and pre-exposure and day 3 ($p < 0.05$). Significantly higher GST activity in the gills occur in virgin LDPE relative to control after 14 days of exposure ($p < 0.05$) (**Figure 2D**). No significant differences in GST activity in the digestive gland tissues were observed between treatments within the same exposure time, neither through time for each treatment ($p > 0.05$) (**Figure 3D**).

No significant differences were observed in AChE activities of gill tissues between control and virgin LDPE treatments, nor over time for either treatment ($p > 0.05$) (**Figure 2E**).

LPO levels in gill tissues remained stable for both control and virgin LDPE exposed treatments, until day 14 when a significant

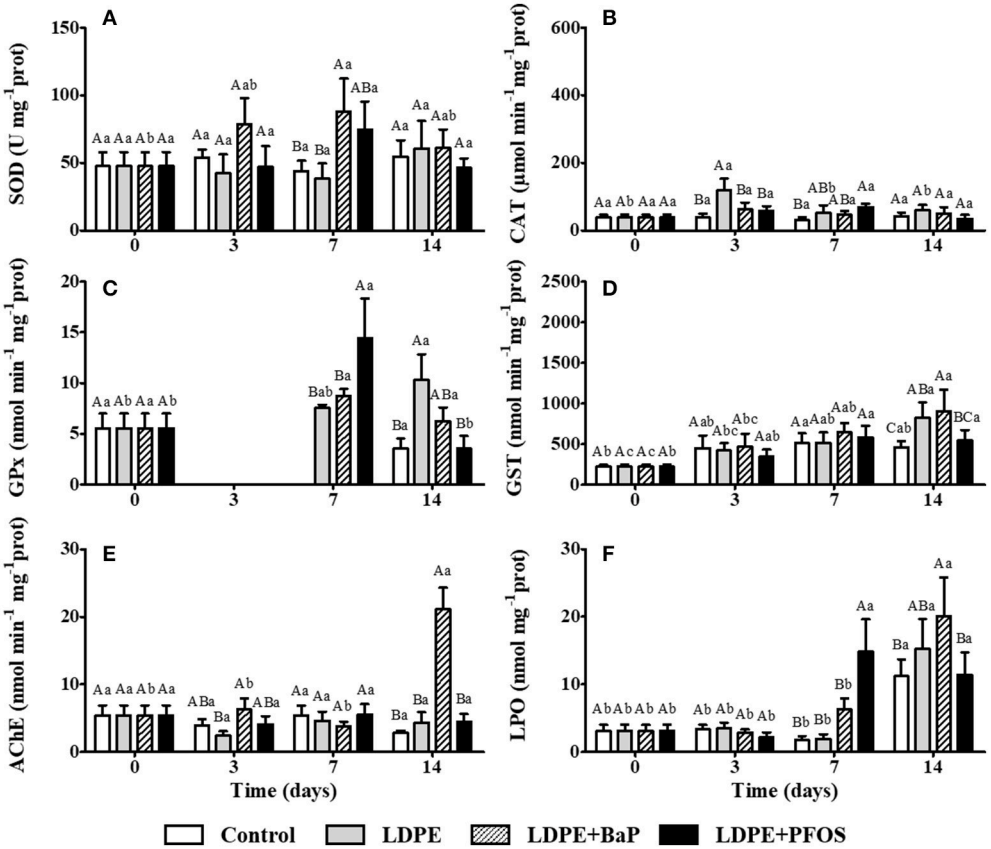

FIGURE 2 | SOD **(A)**, CAT **(B)**, GPx **(C)**, GST **(D)**, AChE **(E)**, and LPO **(F)** activities/levels (mean ± *SD*) in gill tissues of *S. plana* for control, virgin LDPE, LDPE+BaP, and LDPE+PFOS treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

increase occurred, with both increasing simultaneously ($p < 0.05$) (**Figure 2F**). Levels significantly differ at this time for both treatments in respect to all previous times ($p < 0.05$). No significant difference in LPO levels in gill tissues occurred between control and virgin LDPE treatments over the 2-week experimental period ($p > 0.05$) (**Figure 2F**). LPO levels in digestive gland tissues remained stable for the duration of the experiment in virgin LDPE exposed treatments ($p > 0.05$) (**Figure 3E**). Significantly lower LPO levels were observed in digestive gland tissues exposed to virgin LDPE on days 7 and 14 when compared to controls ($p < 0.05$) (**Figure 3E**).

BaP Contaminated LDPE MP

Background BaP contamination (<1.6 ng g^{-1} dw) occurred in whole tissues of clams from all treatments (**Figure 4**), with levels remaining stable over time in both control and virgin LDPE treatments ($p > 0.05$). A significant increase in BaP concentration in whole tissues occurred after 14 days exposure to LDPE+BaP ($p < 0.05$), reaching 7.3 ± 2.0 ng g^{-1} dw (**Figure 4**).

The tail length of haemocyte cells was significantly higher in LDPE+BaP treatments when compared to control after 14 days exposure ($p < 0.05$) (**Figure 1A**). When quantifying DNA damage through OTM, significantly higher OTM values were observed in LDPE+BaP treatments after 14 days of exposure when compared to virgin LDPE ($p < 0.05$) (**Figure 1B**) No significant differences were observed between times for LDPE+BaP treatments for both tail length and OTM ($p > 0.05$).

An increase in SOD activity in the gills of *S. plana* was noted after 3 days of exposure to LDPE+BaP, being significantly higher after 7 days when compared to control and virgin LDPE treatments ($p < 0.05$) (**Figure 2A**). This increase in activity was also significantly higher when compared to time 0 ($p < 0.05$). SOD activity remained stable in digestive gland tissues of LDPE+BaP treatments for the duration of the experiment ($p > 0.05$) (**Figure 3A**).

CAT activity in the gills of organisms exposed to LDPE+BaP remained stable during the experiment ($p > 0.05$) (**Figure 2B**). A significantly higher activity occurs in virgin LDPE when compared to LDPE+BaP treatments after 3 days of exposure ($p < 0.05$). A significant increase in CAT activity in digestive gland tissues of organisms exposed to LDPE+BaP was noted after 14 days exposure, when compared to virgin LDPE ($p < 0.05$) (**Figure 3B**). The increase in CAT activity in digestive gland was also significantly higher in LDPE+BaP treatment after 14 days

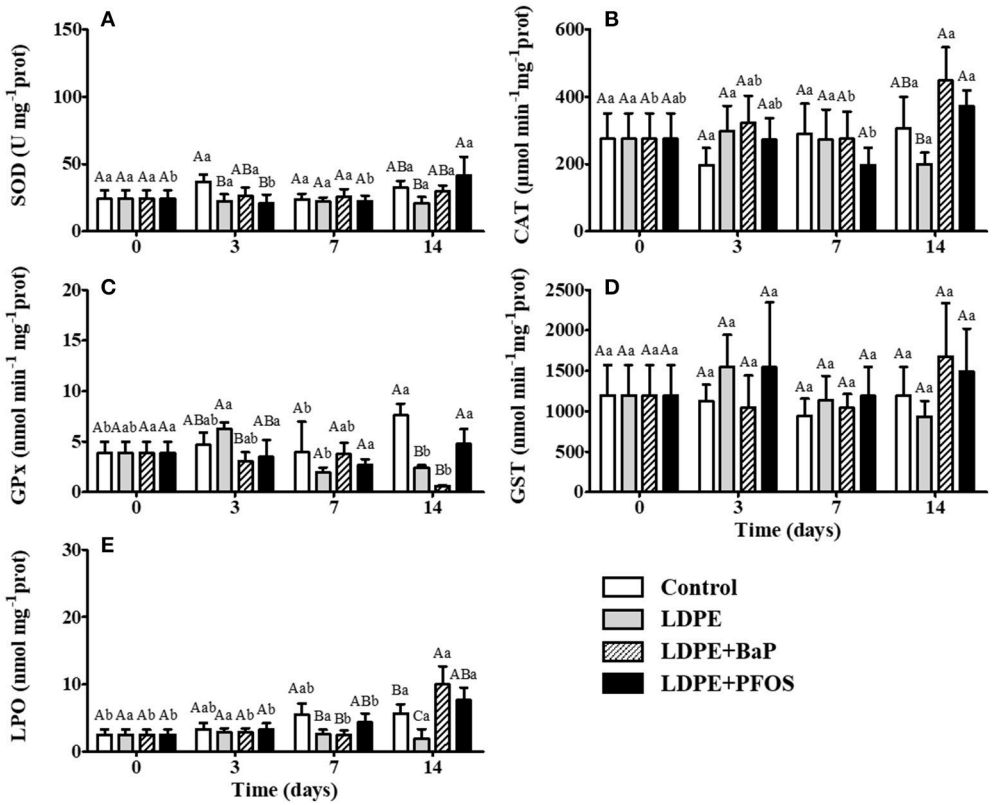

FIGURE 3 | SOD **(A)**, CAT **(B)**, GPx **(C)**, GST **(D)**, and LPO **(E)** activities/levels (mean ± *SD*) in digestive gland tissues of *S. plana* for control, virgin LDPE, LDPE+BaP, and LDPE+PFOS treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

exposure when compared to the start and day 7 of exposure ($p < 0.05$).

Exposure to LDPE+BaP did not induce a significant response in GPx activity in gill tissues through time nor relative to control and virgin LDPE ($p > 0.05$) (**Figure 2C**). A significant decrease in GPx activity in the digestive gland tissues of LDPE+BaP treatments occurs after 14 days exposure, when compared with pre-exposure values and to control at this time ($p < 0.05$) (**Figure 3C**). Significantly lower GPx activities were noted after 3 days exposure in LDPE+BaP when compared to virgin LDPE ($p < 0.05$) (**Figure 3C**).

Exposure to LDPE+BaP induces a steady increase through time in GST activity in gill tissues, with a significant increase observed after 7 and 14 days, when compared to pre-exposure ($p < 0.05$) (**Figure 2D**). After 14 days GST activities in gill tissues are significantly higher in both virgin LDPE and LDPE+BaP treatments than the control ($p < 0.05$). GST activity in the digestive gland tissues remained stable over time in each respective treatment and no significant differences were found between treatment per exposure time ($p > 0.05$) (**Figure 3D**).

A significant increase in AChE activity in LDPE+BaP treatments at day 14 of exposure was noted when compared to other exposure times ($p < 0.05$) (**Figure 2E**). At this time, LDPE+BaP AChE activity is significantly higher when compared with both virgin LDPE and control treatments ($p < 0.05$).

Exposure to LDPE+BaP induced a significant increase in LPO levels after 14 days in both gill and digestive gland tissues ($p < 0.05$) (**Figures 2F**, **3E**). Levels at this time significantly differ to all previous times of LDPE+BaP exposure for both tissues ($p < 0.05$). Significantly higher LPO levels after 14 days occurred between LDPE+BaP when compared to control treatments in the gills ($p < 0.05$) (**Figure 2F**), and between LDPE+BaP and both the control and virgin LDPE treatments in the digestive gland tissues ($p < 0.05$) (**Figure 3E**).

Principal component analysis was applied to BaP concentration and biomarkers data for days 0 and 14, for all treatments (control, virgin LDPE, and LDPE+BaP) (**Figure 5**). The two principal components represent 87.3% of variation within the data, with PC1 accounting for 54.6% and PC2 32.7%. A clear separation exists between the control (T0 and T14) and LDPE+BaP, and also for virgin LDPE treatment, after 14 days exposure. PC1 is negatively determined by treatment LDPE+BaP after 14 days exposure, with the remaining treatments being placed on the positive side of the axis. BaP concentration is positively related with LDPE+BaP treatment T14, as most of the biomarkers analyzed, except GPx. Results obtained for AChE,

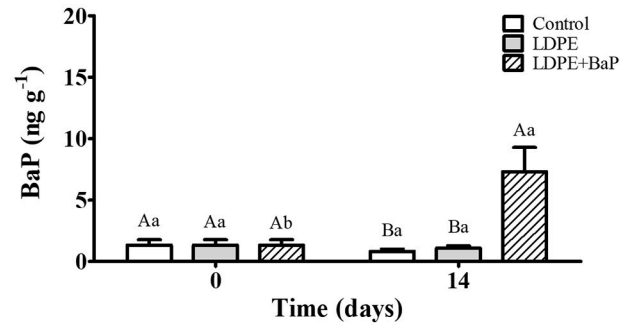

FIGURE 4 | *S. plana* whole tissue BaP concentrations (ng g^{-1}) in control, virgin LDPE, and LDPE+BaP treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

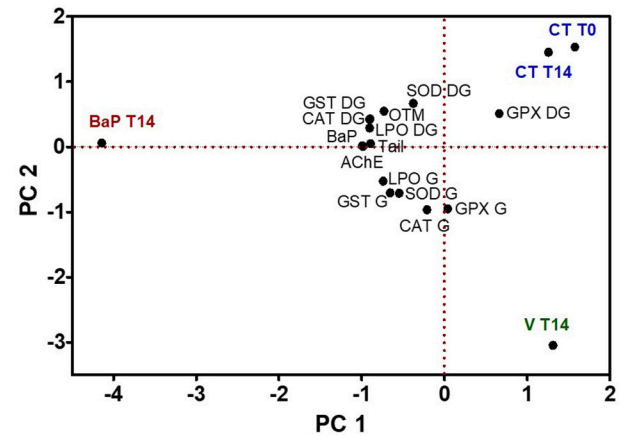

FIGURE 5 | Principle component analysis (PCA) of a battery of biomarkers in the gills (G), digestive glands (DG), and haemocytes (expressed as Tail and OTM) of *S. plana,* in control (CT), virgin LDPE (V), and LDPE+BaP (BaP) treatments, over the duration of the experiment, where T0 = day 1 and T14 = day 14.

tail length, and also for LPO and CAT in the digestive gland are strongly related to the concentration of BaP, determined in whole tissue. A differential tissue response is noted with biomarkers results for digestive gland remaining on the positive side of PC2 axis, while biomarkers data for gills remain on the negative side of PC2. Control treatments appear on the positive side of axis for PC2, while virgin LDPE remains on the negative side of the axis that is positively influenced by most of the biomarkers responses for gills (**Figure 5**).

PFOS Contaminated LDPE MP

No significant differences were observed between control and LDPE+PFOS treatments, nor between virgin LDPE and LDPE+PFOS treatments when quantifying DNA damage through the resulting comet tail length or OTM ($p > 0.05$) (**Figure 1**). LDPE+PFOS treatment after 14 days did not show any significant difference when compared with pre-exposure ($p > 0.05$).

A slight increase in SOD activity in the gill tissues was observed following 7 days exposure to LDPE+PFOS but no significant differences were noted when compared to pre-exposure, neither with control or virgin LDPE treatments ($p > 0.05$) (**Figure 2A**). SOD was significantly induced in digestive gland tissues after 14 days exposure to LDPE+PFOS when compared to previous exposure times ($p < 0.05$), with activity being significantly higher than that of the virgin LDPE treatment at this time ($p < 0.05$) (**Figure 3A**).

CAT activity in the gills of organisms exposed to LDPE+PFOS remained stable during the experiment ($p > 0.05$) (**Figure 2B**). A significant difference in activity occurs between virgin LDPE and LDPE+PFOS treatments in gill tissues after 3 days of exposure, following an increase of activity in tissues exposed to virgin LDPE ($p < 0.05$). After 7 days exposure, a significant difference is observed in CAT activity, of gill tissues, between the control and LDPE+PFOS ($p < 0.05$) (**Figure 2B**). A significant difference in CAT activity in the digestive gland of organisms exposed to LDPE+PFOS occurs between day 7 and 14 of exposure, with higher activities occurring at day 14 ($p < 0.05$), yet activities at these times are comparable to both day 3 and pre-exposure levels ($p > 0.05$) (**Figure 3B**). After 14 days of exposure significantly higher CAT activity levels were found when compared to virgin LDPE ($p < 0.05$) (**Figure 3B**).

GPx activity in gill tissues of virgin LDPE and LDPE+PFOS treatments significantly differ after 7 days exposure, with LDPE+PFOS inducing an increase in activity relative to virgin LDPE ($p < 0.05$) (**Figure 2C**). Activity is significantly reduced in gill tissues for LDPE+PFOS treatment by day 14 when compared to day 7, with GPx activity comparable to pre-exposure levels ($p < 0.05$). A significant difference in GPx activity in the gills occurs between virgin LDPE and LDPE+PFOS at day 14 ($p < 0.05$) (**Figure 2C**). GPx activities remain stable for the duration of the experiment in the digestive glands of organisms exposed to LDPE+PFOS ($p > 0.05$) (**Figure 3C**). On day 14 significantly higher GPx activities are observed in digestive gland tissues of LDPE+PFOS when compared to virgin LDPE ($p < 0.05$) (**Figure 3C**).

When compared to pre-exposure, LDPE+PFOS treatment induces a significant increase in GST activity in gill tissues after 7 days exposure with levels remaining high at day 14 ($p < 0.05$) (**Figure 2D**). GST activities remain stable for the duration of the experiment in the digestive glands of organisms exposed to LDPE+PFOS ($p > 0.05$) (**Figure 3D**). No significant differences are observed in digestive gland tissues between, control, virgin LDPE, and LDPE+PFOS treatments ($p > 0.05$) (**Figure 3D**).

No significant difference in AChE activity in the gills of *S. plana* exposed to LDPE+PFOS occurred during the experimental period ($p > 0.05$) (**Figure 2E**). Likewise, no significant differences were observed in AChE activity between the different treatments at any exposure time ($p > 0.05$) (**Figure 2E**).

LPO levels are comparable in both gill and digestive gland tissues until day 7 of exposure when a > 2-fold increase in LPO levels is observed in the gills of LDPE+PFOS treatments

(**Figures 2F**, **3E**). LPO levels in gill tissues at this time are significantly higher than control and virgin LDPE treatments ($p < 0.05$) (**Figure 2F**). In gills, LPO stabilizes between day 7 and day 14 of exposure to LDPE+PFOS, being significantly higher when compared to both pre-exposure and day 3 ($p < 0.05$) (**Figure 2F**). LPO levels remain stable in digestive gland tissues of LDPE+PFOS treatments until 14 days of exposure when a significant increase is observed ($p < 0.05$) (**Figure 3E**). LPO levels in digestive gland tissues of LDPE+PFOS treatments are significantly higher at this time than in that of virgin LDPE treatments ($p < 0.05$) (**Figure 3E**).

Principal component analysis was applied to all biomarkers data for days 0 and 14, for all treatments (control, virgin LDPE, and LDPE+PFOS) (**Figure 6**). 87.2% of the variation within the data is explained by two principal components, with PC1 accounting for 53.3 and PC2 33.9%. PC1 is positively determined by virgin LDPE treatment after 14 days exposure, with the remaining treatments being placed on the opposite side of the axis. A clear tissue specific biomarkers response is noted given the separation by tissues observed in the PCA, with gills biomarkers placed on the positive side of the PC1 axis and digestive gland biomarkers agglomerated on the negative side of PC1 axis. Gill biomarkers are positively related to virgin LDPE after 14 days of exposure, while digestive gland biomarkers are positively related to LDPE+PFOS after 14 days exposure, along PC1 axis. AchE and DNA damage biomarkers have a similar response among them, and positively related with pre-exposure (CT T0) and the positive side of PC2 axis (**Figure 6**).

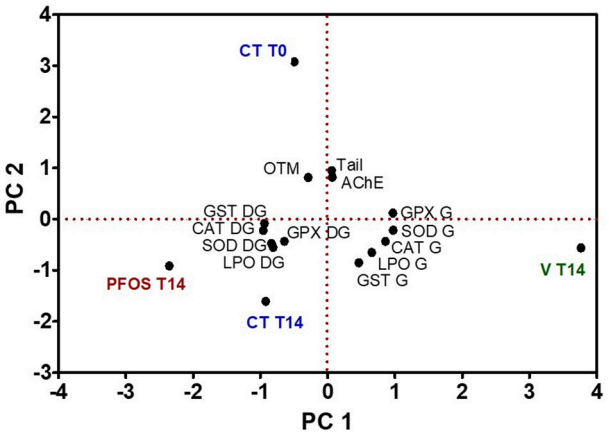

FIGURE 6 | Principle component analysis (PCA) of a battery of biomarkers in the gills (G), digestive glands (DG), and haemocytes (expressed as Tail and OTM) of *S. plana,* in control (CT), virgin LDPE (V), and LDPE+PFOS (PFOS) treatments, over the duration of the experiment, where T0 = day 1 and T14 = day 14.

DISCUSSION

Microplastics are a major contaminant in the marine environment and are bioavailable to benthic organisms. Deposition of low density plastics may be biologically mediated, through the formation of biofilms, through excretion in fecal pellets, or caught in marine snow (Lobelle and Cunliffe, 2011; Zalasiewicz et al., 2016). Deposition of low density plastics may also occur because of density changes resulting from mineral adsorption while in the water column (Corcoran et al., 2015). Little information has been reported on the environmental concentrations of microplastics < 50 μm in size (Ribeiro et al., 2017). As such, the question of an environmentally relevant concentration of microplastics to be used in laboratory studies may also vary. A concentration of 1 mg L^{-1} was decided upon to encompass measured environmental concentrations of MP in both sediment and water compartments of the world's Oceans.

A battery of biomarkers was used to assess the biological effects and toxicity of LDPE microplastics in the gills, digestive glands, and haemocytes of *S. plana*. The potential for microplastics to act as a vector for chemical exposure was investigated by comparing biomarker activities between organisms exposed to virgin LDPE and contaminated LDPE microplastics.

No significant enhancement in DNA strand breaks were detected in the haemocytes of *S. plana* clams exposed to virgin LDPE MP or to LDPE+PFOS MP (**Figure 1**). There is some evidence to suggest that exposure to LDPE+BaP MP induces DNA damage after 14 days, yet the strength of these results is low. The comet assay has been proven to be a robust, sensitive and cost-effective tool for assessing genotoxicity in haemocyte cells of *S. plana* (Petridis et al., 2009). Studies have indicated the genotoxic potential of both virgin polystyrene MP in *S. plana*, and virgin PE MP in *M. galloprovincialis,* through a significant increase in DNA strand breaks in haemocyte cells (Avio et al., 2015; Ribeiro et al., 2017).

Results indicate a time- and tissue-dependent oxidative stress response that varies depending on treatment used and biomarker investigated (**Figures 2**, **3**). Antioxidant defence systems, comprised of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), play a crucial role in maintaining cellular homeostasis by the removal of ROS (Jo et al., 2008).

A significant increase in SOD activity is observed in gill tissues exposed to LDPE+BaP after 7 days when compared with pre-exposure and with virgin MP treatment (**Figure 2A**). Such an effect is only observed in the digestive gland tissues of organisms exposed to LDPE+PFOS after 14 days (**Figure 3A**). An increase in SOD activity is indicative of the first line of defence in protecting tissues against oxidative stress. SOD, found in every living organism that consumes oxygen, catalyzes the partitioning of the superoxide anion radical ($O2\bullet^{-1}$) into hydrogen peroxide (H_2O_2) and water, thus reducing the potential for oxidative damage to occur (Jo et al., 2008). Results in the gill tissues of contaminated MP treatments are comparable to those reported for virgin PS exposure in the gills of *S. plana* by Ribeiro et al. (2017). Exposure to virgin LDPE does not induce the same time dependent increase in SOD activity, in either tissue, as exposure to virgin PS at the same concentration, 1 mg L^{-1} (Ribeiro et al., 2017). As SOD activity remained stable for the duration of the experiment in both gill and digestive gland tissues exposed to virgin MP, and did not significantly differ to that of the control

in the majority of cases, it may be hypothesized that the observed increase results from the toxicity of the adsorbed contaminants.

The extent to which microplastics act as a vector for contaminants once ingested by organisms within the marine environment is still unclear, as in field conditions organisms may accumulate the same chemical contaminants from other sources (Oliveira et al., 2013). Laboratory studies have demonstrated the desorption of contaminants from ingested microplastics, including PCBs from ingested microplastics in shearwater chicks (Teuten et al., 2009), yet it must be noted that in field conditions environmental contaminants will rarely exist solely, but do so as a mixture with varying concentrations. Before guideline values for the environmental risk assessment of plastic debris can be produced, further research is needed into the adsorption and desorption kinetics of common marine contaminants to various plastic polymers, and their effects on the marine biota once ingested. The ecological impact of MP ingestion may be significantly increased by desorption of toxicants. Desorption of chemicals from ingested MP is dependent on the residence time of the particles (Engler, 2012), which in turn is dependent on the particle size (Lusher, 2015). PS MP particles have been shown to accumulate in the digestive gland and gill tissues of *S. plana* in a laboratory experiment by Ribeiro et al. (2017), residing in both tissues for the 1 week depuration period investigated.

A 5-fold higher activity of CAT in the digestive gland when compared to gills was noted in all treatments and times (**Figures 2B**, **3B**). The antioxidant enzyme CAT, prevents cellular damage from ROS by reducing both endo- and exogenous sources of H_2O_2 to H_2O (Oliveira et al., 2009; Solé et al., 2009). CAT induced activity was not observed in gill tissues of the LDPE+BaP MP treatment, but an increase was noted after 7 days of exposure to LDPE+PFOS when compared to control. In the digestive gland an increase in CAT activity was noted after 14 days for both LDPE+BaP and LDPE+PFOS when compared to virgin LDPE. Moreover, a significant induction in CAT activity in gill tissues occurred after 3 days exposure to virgin LDPE MP, with the activity being subsequently reduced over time reaching levels comparable to pre-exposure by day 14. Previous studies have observed an inhibition of CAT activity in response to MP exposure, both in the digestive gland of *S. plana* exposed to PS MP, and in the digestive gland of *M. galloprovincialis* exposed to both virgin PE and PS, and pyrene contaminated PE and PS (Avio et al., 2015; Ribeiro et al., 2017). It may be argued that CAT is not the antioxidant defence mechanism used by *S. plana* in response to PE MP exposure, or that 2 weeks exposure to both virgin LDPE and contaminated LDPE MP, under the experimental conditions observed, is not sufficient to induce a significant response.

A defence mechanism reaction to oxidative stress is suggested by the significant increase in GPx activity in the gills of virgin LDPE MP treatment by day 14 (**Figure 2C**). A significant increase followed by a subsequent decrease in activity is observed in the gills of LDPE+PFOS treatments (**Figure 3C**). Glutathione peroxidases which catalyze the reduction of metabolically produced H_2O_2 to H_2O and O_2, have been proven to be a sensitive biomarker in revealing pro-oxidant challenges, even at low levels of environmental contamination (Jo et al., 2008; Regoli and Giuliani, 2014). Previous studies have shown that an inhibition in GPx may occur in response to elevated toxicity levels. Such inhibition was demonstrated in mussels (*M. galloprovincialis*) exposed to virgin and pyrene contaminated PE and PS MP (Avio et al., 2015). A similar trend was observed in whole tissues of *S. plana* exposed to mercury, with higher mercury levels inhibiting GPx activity (Ahmad et al., 2011). This hypothesis—that an inhibition of GPx activity occurs due to the inability to process excess ROS—may explain the antagonistic effects observed in the fore-mentioned treatments.

A tissue dependent response was observed in regard to GST, with the digestive gland displaying little variation in activity (**Figures 2D**, **3D**). An increase in GST activity in gill tissues is observed in all MP treatments over time, although less pronounced in LDPE+PFOS. Gill tissues, being the major organ involved in filtration, have been demonstrated to be the first site of MP particle uptake in mussels (Browne et al., 2008; von Moos et al., 2012; Ribeiro et al., 2017), with *S. plana* also ingesting particles through the inhalant siphon, which are subsequently transported to the mouth and digestive gland (Hughes, 1969). A similar increase in GST activity in the gills of *S. plana* has been described by Ribeiro et al. (2017) in response to PS MP exposure. GST activity has also been reported to increase in the gills of *M. galloprovincialis* following exposure to the persistent organic pollutant pp'DDE (2,2-bis-(p-chlorphenyl)-1,1-dichlorethylene), a metabolite of DDT (Khessiba et al., 2001). The phase two enzymes, glutathione-S-transferases (GST), play an important role in cellular protection against various xenobiotics and toxic endogenous substances by converting reactive lipophilic molecules into non-reactive water-soluble molecules which can be excreted by the organism (Hoarau et al., 2002). As a significant increase in GST activity in gill tissues of contaminated LDPE MP treatments compared to virgin LDPE MP is not observed, it may be hypothesized that synergistic effects of ingestion and chemical exposure did not occur, indicating that the overall increase in activity with time results from the physical ingestion of MP, rather than the chemical toxicity of the respective contaminants.

It may be hypothesized, from the overall results on oxidative stress biomarkers, that ROS are produced as a result of short term exposure to both virgin LDPE and contaminated microplastics. Such stress may lead to lipid peroxidation, protein denaturation as well as cellular and DNA damage (Jo et al., 2008).

Inhibition of AChE activity did not occur in any of the MP exposed treatments (**Figure 2E**), with LDPE+BaP MP treatments showing a significant increase after 14 days of exposure. In contrast, a reduction in AChE activity in gill tissues of *S. plana* was observed following 2 weeks exposure to polystyrene microplastics at similar concentrations (Ribeiro et al., 2017). Previous studies have indicated that a reduction in AChE activity in the gills of mussels (*M. galloprovincialis*) occurred upon 7 days exposure to polystyrene and polyethylene microplastics, with and without pyrene contamination (Avio et al., 2015). Comparisons between these, and other MP feeding experiments, are confounded by numerous factors: different species used, different polymer concentrations used (1.5 g L^{-1} by Avio et al., 2015 compared to 1 mg L^{-1} in this experiment, a difference of 1500x), different polymer type (when comparing

polyethylene, densities, and sizes differ), different contaminants used and different exposure times. Exposure to polyethylene microplastics, with and without added pyrene have also shown to significantly reduce AChE activity in juveniles of the common goby, *Pomatoschistus microps* (Oliveira et al., 2013). No significant changes occur in AChE activity in either virgin LDPE or LDPE+PFOS MP treatments, suggesting that a neurotoxic potential of LDPE MP without adsorbed contaminants does not exist under the experimental conditions of the present study.

There is a clear effect of lipid peroxidation in the gills of organisms exposed to LDPE+BaP by day 7, when compared to both control and virgin LDPE, with a similar effect in the digestive gland of organisms exposed for 14 days to LDPE+adsorbed chemicals (**Figures 2F**, **3E**). An increase in lipid peroxidation may result from inefficient oxidative stress reduction mechanisms in the processing of excess ROS. This may, in part, explain the inhibition of both CAT and GPx, due to an inability to respond to elevated ROS levels. In contrast to the results of this experiment, levels of oxidative damage were reduced in the gills of *S. plana* exposed to PS MP at similar concentrations, and showed no significant changes over time in digestive gland tissues (Ribeiro et al., 2017). No increase in LPO levels was reported in tissues of the common goby, *P. microps*, following exposure to both PE MP and pyrene contaminated PE MP, at a concentration of 18.4 and 184 $\mu g\ L^{-1}$ (Oliveira et al., 2013).

Results from the PCA for BaP (**Figure 5**), taking into account the biomarkers analyzed, and the determined concentration of BaP in the tissues, reveal that most biomarkers are more positively related with the LDPE+BaP treatment than to virgin LDPE. Still, it appears that gills are more influenced by virgin LDPE than are digestive gland tissues. Similarly, in the PCA for PFOS (**Figure 6**), virgin LDPE is in the opposite side of LDPE+PFOS in the axis for PC1, with gills biomarkers more related to virgin LDPE and digestive gland more related to LDPE+PFOS. This may be a result of potential mechanical injury of gills that may also affect the analyzed biomarkers, but more importantly that digestive glands are apparently less affected by potential mechanical damage cause by microplastics alone but more influenced by the presence of the contaminants.

CONCLUSION

A tissue specific response in oxidative stress was observed due to LDPE MP exposure, with each respective LDPE+BaP and LDPE+PFOS exhibiting varied response per biomarker investigated. Oxidative damage was observed in all microplastics, with adsorbed BaP and PFOS, treatments in both digestive gland and gill tissues. The digestive gland seems less affected by mechanical damage caused by virgin LDPE exposure, with the MP-adsorbed BaP and PFOS exerting a negative influence over the assessed biomarkers in this tissue. It is clear that further research is needed into the ecotoxicological effects of MP in the marine environment with the potential for MP with adsorbed chemicals to desorb once ingested and their subsequent mode of action within organisms being of particular concern.

AUTHOR CONTRIBUTIONS

SO: Performed the experiment and analyses, analyzed the data, and wrote the manuscript; NM and MB: Designed the experiment and contributed to the data analysis and to the writing of the manuscript; SA: Conducted biomarker analyses; TF: Contributed the running of the experiment, analyses, and to the writing of the manuscript; SK and BC: Prepared and characterized the contaminated MPs and contributed to the writing of the manuscript; CC: Performed the chemical analysis and contributed to the writing.

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Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels, *Mytilus galloprovincialis*

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The ubiquitous occurrence of microplastics (MPs) in the marine environment is raising concern for interactions with marine organisms. These particles efficiently adsorb persistent organic pollutants from surrounding environment and, due to the small size, they are easily available for ingestion at all trophic levels. Once ingested, MPs can induce mechanical damage, sub-lethal effects, and various cellular responses, further modulated by possible release of adsorbed chemicals or additives. In this study, ecotoxicological effects of MPs and their interactions with benzo(a)pyrene (BaP), chosen as a model compound for polycyclic aromatic hydrocarbons (PAHs) were investigated in Mediterranean mussels, *Mytilus galloprovincialis*. Organisms were exposed for 4 weeks to 10 mg/L of low-density polyethylene (LDPE) microparticles ($2.34 * 10^7$ particles/L, size range 20–25 μm), both virgin and pre-contaminated with BaP (15 μg/g). Organisms were also exposed for comparison to BaP dosed alone at 150 ng/L, corresponding to the amount adsorbed on microplastics. Tissue localization of microplastics was histologically evaluated; chemical analyses and a wide battery of biomarkers covering molecular, biochemical and cellular levels allowed to evaluate BaP bioaccumulation, alterations of immune system, antioxidant defenses, onset of oxidative stress, peroxisomal proliferation, genotoxicity, and neurotoxicity. Obtained data were elaborated within a quantitative weight of evidence (WOE) model which, using weighted criteria, provided synthetic hazard indices, for both chemical and cellular results, before their integration in a combined index. Microplastics were localized in hemolymph, gills, and especially digestive tissues where a potential transfer of BaP from MPs was also observed. Significant alterations were measured on the immune system, while more limited effects occurred on the oxidative status, neurotoxicity, and genotoxicity, with a different susceptibility of analyzed pathways, depending on tissue, time, and typology

of exposure. Molecular analyses confirmed the general lack of significant transcriptional variations of antioxidant and stress genes. The overall results suggest that microplastics induce a slight cellular toxicity under short-term (28 days) exposure conditions. However, modulation of immune responses, along with bioaccumulation of BaP, pose the still unexplored risk that these particles, under conditions of more chronic exposure (months to years) or interacting with other stressors, may provoke long-term, subtle effects on organisms' health status.

Keywords: microplastics, mussels, bioavailability, biomarkers, immune responses, gene transcription, weighted criteria, hazard index

INTRODUCTION

Microplastics are particles smaller than 5 mm in diameter (NOAA, 2015), now identified as the predominant component of plastic debris in the marine environment (Goldstein et al., 2013; Eriksen et al., 2014). The huge amount of microplastics documented over the past decade (Wright et al., 2013), is partly due to the direct release of micro-debris into the ocean (Browne, 2015), but in larger quantities, it depends on fragmentation of macro- and meso-plastic (Galgani et al., 2015; Thompson, 2015). The small dimensions of microplastics and their ubiquitous presence in marine habitats, are key factors promoting their interactions with organisms (Wright et al., 2013).

Ingestion of microplastics is well-documented for several marine vertebrates and invertebrates, including commercially important species, which differ by trophic level, feeding strategies, and distribution along the water column (Lusher, 2015; Phuong et al., 2016; Avio et al., 2017a; Lusher et al., 2017; Santillo et al., 2017).

Several laboratory experiments have been performed, in recent years, to understand dynamics of particles uptake, bioaccumulation and toxicological mechanisms possibly leading to detrimental effects in a variety of bioindicators organisms (Lusher, 2015; Phuong et al., 2016). Such studies demonstrated that ingested microplastics can be taken up into the cells by endocytosis, retained and even traslocated to different tissues (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015). Several effects have been described in terms of histological alterations, inflammatory reactions, and ecotoxicological responses at cellular, biochemical, and molecular levels, but also in terms of modulations of physiological functions such as respiration, nutrition, reproduction and growth (Avio et al., 2015; Paul-Pont et al., 2016; Pedà et al., 2016; Détrée and Gallardo-Escárate, 2017; Karami et al., 2017).

Harmful consequences of microplastics to marine organisms may also derive from the possible transfer of hazardous chemicals associated to the plastic during manufacturing or adsorbed from the environment (Rochman et al., 2013; Wright et al., 2013). In this respect, microplastics can efficiently concentrate organic pollutants from surrounding seawater, due to the hydrophobic nature of these compounds and to the high surface/volume ratio of the small particles (Liu et al., 2016), with a sorption capacity that varies by plastic polymers and considered chemicals (Rochman, 2015).

Although the ingestion of microplastics does not certainly represent the main route of exposure to organic xenobiotics for aquatic animals, when compared with other environmental sources (i.e., water, sediments, food web) (Koelmans et al., 2016; Lohmann, 2017; Wang and Wang, 2018), plastic particles have the peculiar characteristic to combine a physical stress with a chemical challenge (Rochman, 2015). In this respect, studies addressing the ecotoxicological risk of microplastics in the marine environment, should consider both the individual effects of particles and chemicals, as well as their interactions, possibly causing synergistic, additive, or antagonistic effects (Syberg et al., 2015).

While in field conditions it is virtually impossible to distinguish adverse effects caused by exposure to microplastics, chemicals, or their combined effects, controlled laboratory experiments remain a necessary approach to understand such mechanisms of toxicological action.

In the present study, the contribution of microplastics to benzo(a)pyrene (BaP) bioavailability and the onset of adverse effects caused by pristine and contaminated particles were evaluated at cellular, biochemical, and transcriptional levels, using mussels *Mytilus galloprovincialis*, as biological model. These organisms have high ecological and commercial relevance in the Mediterranean Sea, where microplastics contamination is also of particular concern (Lusher, 2015). Organisms were exposed for 4 weeks to 10 mg/L of virgin low density polyethylene (LDPE) microparticles, one of the most common polymers in floating debris (Cózar et al., 2015; Suaria et al., 2016), BaP chosen as representative compound for polycyclic aromatic hydrocarbons (PAHs), and to BaP pre-treated particles (LDPE-BaP). Selected levels of microplastics are at least two orders of magnitude higher than those observed in the Mediterranean (Suaria et al., 2016) and more similar to those of the Californian Current System (5.33 mg/L, Gilfillan et al., 2009) and of North Pacific Central Gyre (3.02 mg/L, Moore et al., 2001; Sussarellu et al., 2016). The high dose of microplastics was chosen in our study to explore potential long-term mechanism of action of these particles after 28 days of exposure.

Chemical analyses of BaP and histological examinations were performed in digestive glands, gills, and hemolymph to confirm microplastics ingestion, translocation, and bioaccumulation in different tissues. A wide battery of biomarkers was measured at both cellular and transcriptional levels including lysosomal, immunological, and antioxidant responses, markers of neuro and

genotoxicity, peroxisomal proliferation, lipid peroxidation, and oxidative stress. Results were further elaborated and integrated within a weight of evidence (WOE) model which provided a quantitative evaluation of hazard based on the extent of BaP accumulation, as well as on the toxicological relevance and magnitude of variations observed at cellular level. Overall, the study was expected to provide additional insights on potential ecotoxicological risk of microplastics and their role in transferring chemical pollutants to marine biota.

MATERIALS AND METHODS

Sorption of Benzo(a)Pyrene on Microplastic Particles

Low density polyethylene (LDPE) particles (20–25 μm) were purchased from Micro Powders, Inc. (USA), while BaP was obtained from Sigma-Aldrich.

The adsorption of BaP on LDPE was obtained mixing 4 g of LDPE micropowder in 32 ml of double-deionized water, spiked with 80 μl of BaP stock solution (1 μg/μL of BaP in toluene, purity 96%, SOLVECO). After 2 days in continuous rotation at the lowest speed (20 rpm, in 40 ml amber glass vials with Teflon lids), the solution was filtered on glass microfiber filters, rinsed with double-deionized water and dried by vacuum evaporation to obtain contaminated microplastic debris.

To confirm the adsorption of BaP on microplastics, an aliquot of 0.25 g treated-LDPE was extracted in 2.5 mL of hexane, ultra-sonicated for 30 min, and centrifugated for 10 min. Supernatant was reduced to a volume of 1.5 ml using a nitrogen stream; 500 μL toluene were added and the volume further reduced to 500 μL. GC vials were filled with 100 ng recovery standard perylene D12 (Chiron) (2 ng/μL in toluene, 50 μL added) and 500 μL of extract transferred. Concentrations of BaP were quantified using a high-resolution GC-MS system (Micromass Autopspec Ultima), separation on a 30 m (0.25 mm i.d., 25 μm film thickness) DB-5MS column (J&W Scientific, Folsom, USA). Quality assurance/quality control procedures included the internal standard method using labeled standards. Reference microplastic (virgin microplastic) was tested in triplicates, spiked with internal standard solutions before extraction, and spiked with recovery standard before GC/MS-analysis. BaP was quantified by use of five points calibration curves. Relative standard deviation (RSD) of the triplicates was <15%. Quantification standards were analyzed after every 10 or 12 sample. Procedure blanks were included in all batches, the limit of detection (LOD) was defined as mean concentration in blanks +3 times the standard deviations. The absorbed concentration resulted approximately 15 μg BaP/g of LDPE.

TABLE 1 | Primer pair sequences, amplicon size, annealing temperatures, and Genbank accession numbers of genes analyzed in quantitative PCR in the digestive gland of mussels.

Gene	Primer sequences	Amplicon size (bp)	Annealing *T* (°C)	Accession number
cat	Fwd: CGACCAGAGACAACCCACC[a]	132	55	AY743716
	Rev: GCAGTAGTATGCCTGTCCATCC[a]			
Se-gpx	Fwd: AGCCTCTCTCTGAGGAACAACTG	166	55	FL499839
	Rev: TGGTCGAACATGCTCAAGGGC			
gstpi	Fwd: TCCAGTTAGAGGCCGAGCTGA[b]	172	55	AF527010
	Rev: CTGCACCAGTTGGAAACCGTC[b]			
hsp70	Fwd: GGTGGTGAAGACTTTGACAACAG[c]	295	62	AY861684
	Rev: CTAGTTTGGCATCGCGTAGAGC[c]			
aox1	Fwd: ACAGTCGTGCAAAACAGGGAC	153	62	EF525542
	Rev: CTGCTGCTTCAACCAACCTGG			

[a]*Canesi et al., 2007*; [b]*Canesi et al., 2008*; [c]*Cellura et al., 2006*.

Experimental Design

Specimens of *M. galloprovincialis* (6 ± 1 cm shell length) were obtained in March 2017 from a local farm in an unpolluted area of Central Adriatic Sea (Ancona) and acclimated for 15 days to laboratory conditions in glass aquaria with aerated artificial seawater (ASW; Instant Ocean®) at salinity 37 p.s.u. and 18 ± 1°C.

A total of 720 organisms were randomly distributed into twelve 20 L- glass-aquaria and exposed, in triplicates, to one of the following conditions for 4 weeks: (1) control (CTRL); (2) virgin LDPE (10 mg/L corresponding to 2.34 * 10^7 particles/L); (3) BaP alone (150 ng/L); (4) BaP-treated polyethylene (LDPE-BaP) (15 μg BaP/g LDPE). BaP was dissolved in acetone which had a final concentration of 0.0015%, previously shown to have no effects on exposed organisms (Giannapas et al., 2012; Grintzalis et al., 2012; Avio et al., 2015).

The microplastics concentration (10 mg/l) is much lower than those used in previous exposures to mussels (Von Moos et al., 2012; Wegner et al., 2012; Avio et al., 2015), but still higher than the maximum levels detected in the Mediterranean Sea (0.026 mg/L) (Suaria et al., 2016). Although in the range of levels measured in California Current System and North Pacific Central Gyre (Gilfillan et al., 2009; Sussarellu et al., 2016), it was chosen to highlight the possible onset of long-term effects after 28 days of exposure. The administered dose of BaP (150 ng/l) was based on the amount of BaP adsorbed on microplastics and it also represents an environmentally realistic value, lower than those frequently used to assess ecotoxicological effects of BaP in marine invertebrates (Marigómez and Baybay-Villacorta, 2003; Pan et al., 2009; Ren et al., 2015; Banni et al., 2017; Rey-Salgueiro et al., 2017).

Water was daily changed in each tank and virgin, pre-treated microplastics and BaP redosed. Mussels were fed 12 h prior the water change with a commercial mixture of zooplankton (50–300 μm) for filter-feeding organisms, and no mortality was observed during the experiment. To avoid the stratification of particles in the surface of the aquaria, air bubbling and motion pumps were used (Coral®, 250lt/h).

Organisms were collected after 7, 14, and 28 days of exposure. Hemolymph, digestive glands and gills were rapidly removed from 60 specimens (20 from each tank) for each treatment, pooled in 20 samples (each containing tissues of three specimens), frozen in liquid nitrogen and maintained at −80°C for chemical, biochemical, molecular, and histochemical analyses. An aliquot of hemolymph was immediately processed for lysosomal neutral red retention time assay (NRRT), phagocytosis activity, granulocytes/hyalinocytes ratio, and DNA

damage (Comet Assay), while another aliquot was fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic evaluation of micronuclei frequency.

Chemical Analyses of benzo(a)pyrene

Benzo(a)pyrene in mussels digestive glands and gills was analyzed in samples extracted in 0.5 M potassium hydroxide and methanol (1:10 w:v) with microwave at 55°C for 15 min (Benedetti et al., 2014). Centrifugation was performed for 5 min at 1,000 × g, and resulting methanolic solutions, concentrated in speedvac, were finally purified with solid phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient grade acetonitrile, before analyses were performed with water–acetonitrile gradient and fluorimetric detection. Appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix) were used to identify BaP by the retention time. Quality assurance and quality control (QA/QC) included processing blank and reference samples (mussel tissues SRM 2977, NIST); concentrations obtained for the SRM were always within the 95% confidence interval of certified value. The water content in tissues was determined and concentrations of BaP expressed as ng/g dry weight (d.w.).

Histological and Biochemical Analyses

Presence and histological localization of plastic particles were evaluated in cryostatic sections (20 μm thick) of gills and digestive glands, and in hemolymph smears. After staining with Haematoxylin and Eosin, slides were observed through polarized light microscopy. No quantitative assessment was performed and results on microplastics in tissues are thus of descriptive and qualitative nature.

Standardized protocols were used for measurement of biomarkers in tissues of control and exposed organisms (Regoli and Winston, 1998; Bocchetti et al., 2008; Baršiene et al., 2012; Gorbi et al., 2013; Benedetti et al., 2014). Detailed methods have been given elsewhere (Avio et al., 2015) for the following typologies of effects: immunological alterations of hemocytes in terms of lysosomal membrane stability (NRRT), phagocytosis activity and granulocytes/hyalinocytes ratio (G/H ratio); neurotoxic responses in hemocytes and gills measured as enzymatic activity of acetylcholinesterase (AChE); cellular and oxidative stress biomarkers in digestive tissues, i.e., acyl-CoA oxidase (AOX), antioxidant defenses (catalase glutathione S-transferases, glutathione peroxidases, glutathione reductase, glutathione), total oxyradical scavenging capacity (TOSC), content of malondialdehyde (MDA), and neutral lipids (NL); genotoxic effects in hemolymph measured as DNA strand breaks and micronuclei frequency (MN).

Molecular Analyses

Transcriptional responses were measured in digestive glands for some antioxidant and stress genes including catalase (*cat*), glutathione peroxidase Se-dependent isoform (*Se-gpx*), glutathione S-transferase pi-isoform (*gstpi*), acyl CoA oxidase 1 (*aox1*), heat shock protein 70 (*hsp70*). Selected genes reflect at molecular level some of the responses also measured at the functional, catalytic level, and they are all typical responses to cellular stress.

For mRNA isolation and cDNA synthesis, total RNA was purified from tissues using the Hybrid-R™ purification kit (GeneAll®), according to the manufacturer's protocol. Total RNA concentrations were measured by Nano-Drop ND-1000 UV-Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was verified on agarose-formaldehyde gel. Total cDNA was generated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) from 1 μg of total RNA for each sample using combined oligo(dT) and random hexamer primers (iScript cDNA Synthesis Kit, Bio-Rad).

Absolute quantitative real-time PCRs (qPCRs) were performed with gene-specific primer pairs (**Table 1**) and mRNA levels of individual target genes were quantified through the SYBR green method in StepOnePlus® Real-Time PCR System (Applied Biosystems). Each 15 μl DNA amplification reaction contained 7.5 μl of SYBR Select Master Mix (Life Technologies), 5 μl of total cDNA (synthesized as described

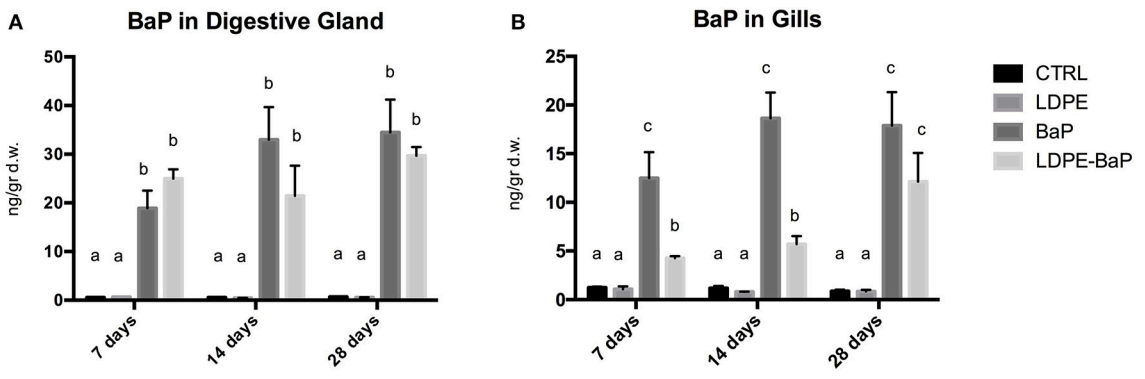


FIGURE 1 | Concentrations of benzo(a)pyrene in digestive glands **(A)** and gills **(B)** of mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). Data are expressed as ng/g dry weight (mean values ± standard error, $n = 4$); different letters indicate significant differences between groups of means within the same time of exposure (*post-hoc* Newman-Keuls comparison).

above and diluted 1:5), and 200 nM of each forward and reverse primers. The real-time PCR program included an enzyme activation step at 95°C (2 min) and 40 cycles each composed by 15 s at 95°C, 15 s at the annealing temperature (**Table 1**), and 1 min at 72 °C. The absence of a specific amplifications was checked by including negative controls lacking cDNA template and by a melting analysis (1 min at 95°C, 10 s at 65°C, and fluorescence detection at increasing temperature between 65 and 95°C).

For each target gene, serial dilutions of known amounts of plasmid containing the amplicon of interest were used as standards. Samples and standards were run in duplicate in the same run. A calibration curve was built by plotting cycle threshold (Ct)-values vs. log copy numbers. Ct-values of unknown samples were converted into mRNA copy number by interpolating the standard plot. Obtained data from the same experimental group ($n = 4$) were averaged and expressed as mRNA copy number per μg of total RNA.

Statistical Analyses and Hazard Indices Evaluation

Analysis of variance (Two-way ANOVA) was used to evaluate the effects of various treatments, time of exposure and their interactions on investigated parameters. Combined effects of microplastics and BaP were further assessed by post-hoc comparisons (Newman-Keuls) between LDPE, BaP, and LDPE-BaP. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary. Multivariate statistical analyses (principal component analysis, PCA) were applied to biomarkers data in order to discriminate between different exposure conditions; a threshold factor loading of 0.6 was used as cut-off value.

A quantitative and software-assisted WOE model (Sediqualsoft) was applied to elaborate results of BaP bioavailability and biomarkers analyses and to summarize specific hazard indices. Whole calculations, detailed flow-charts, rationale for weights, thresholds, and expert judgments have been fully given elsewhere (Piva et al., 2011; Benedetti et al., 2012) and successfully applied to several multidisciplinary studies (Piva et al., 2011; Benedetti et al., 2012, 2014, 2016; Regoli et al., 2014; Avio et al., 2015; Bebianno et al., 2015; Mezzelani et al., 2016; Nardi et al., 2017).

Briefly, the elaboration of Hazard Quozient for bioavailability (HQ_{BA}) was calculated by the increase of BaP tissue concentration in exposed organisms in respect to controls, corrected for the significance of the difference and assigned

TABLE 2 | Results of two-way analysis of variance for the biological responses in mussels, *M. galloprovincialis*, exposed to different treatments (LDPE, BaP, and LDPE-BaP) for different times (7, 14, and 28 days).

	Treatment			Time			Interaction		
	dF	*F*	*p value*	*dF*	*F*	*p value*	dF	*F*	*P value*
BaP in digestive gland	3	50.72	$P < 0.001$	2	1.892	ns			
BaP in gill	3	41.52	$P < 0.001$	2	3.379	$P < 0.05$	6	1.482	ns
Neutral Red Retention Time	3	20.55	$P < 0.001$	2	2.100	ns			
Phagocytosis activity	3	16.02	$P < 0.001$	2	46.19	$P < 0.001$	6	11.32	$P < 0.001$
G/H ratio	3	19.76	$P < 0.001$	2	15.02	$P < 0.001$	6	3.176	$P < 0.05$
Acetylcholinesterase in hemolymph	3	1.482	ns	2	10.30	$P < 0.001$			
Acetylcholinesterase in gills	3	1.417	ns	2	4.702	$P < 0.05$			
Micronuclei	3	3.365	$P < 0.05$	2	3.267	$P < 0.05$	6	1.621	ns
DNA TAIL	3	0.136	ns	2	2.695	ns			
Acyl CoA oxidase	3	1.311	ns	2	3.621	$P < 0.05$			
Neutral lipis	3	3.197	$P < 0.05$	2	0.056	ns			
Catalase	3	0.632	ns	2	15.75	$P < 0.001$			
Glutathione S-transferases	3	0.270	ns	2	2.003	ns			
Glutathione reductase	3	1.117	ns	2	16.16	$P < 0.001$			
Glutathione peroxidases total	3	3.419	$P < 0.05$	2	4.722	$P < 0.05$			ns
Glutathione peroxidases Se-dip	3	0.628	ns	2	3.943	$P < 0.05$			
Total glutathione	3	2.376	ns	2	0.108	ns			
TOSC OH	3	1.490	ns	2	4.269	$P < 0.05$			
TOSC ROO	3	0.165	ns	2	2.870	ns			
Malondialdehyde	3	1.553	ns	2	16.51	$P < 0.001$			
catalase	3	1.539	ns	2	32.30	$P < 0.001$			
Se-dependent glutathione peroxidases	3	0.156	ns	2	6.975	$P < 0.01$			
glutathione S-transferases pi class	3	0.909	ns	2	16.03	$P < 0.01$			
acyl CoA oxidase	3	2.724	ns	2	2.505	ns			
heat shock protein 70	3	4.620	$P < 0.01$	2	12.16	$P < 0.001$			ns

DNA TAIL, single DNA strand breaks; TOSC, total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (•OH) radical; Df (degrees of freedom). F- and P-value are reported.

to one of five classes of effect, Absent (no increase compared to control concentrations), Slight (up to 2.6-folds increase), Moderate (up to 6.5-folds increase), Major (up to 13-folds increase), Severe (more than 13-folds increase, Piva et al., 2011).

For elaboration of biomarkers results, each response has a weight based on its toxicological relevance (from 1 to 3), and a specific threshold defining changes of biological relevance which consider the possibility of biphasic responses and the different responsiveness among tissues (Piva et al., 2011). Each biomarker variation is compared to its specific threshold (effect), corrected for the weight of the response and the statistical significance of the difference in comparison to control values. The Hazard Quotient for biomarkers (HQ_{BM}) is calculated without considering the contribution of responses with an effect <1 (lower than threshold), the average for those with an effect up to 2-folds compared to the threshold and the summation (Σ) for the responses more than 22-folds greater than the respective threshold (Piva et al., 2011):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right)$$

The level of cumulative HQ_{BM} is summarized in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

The hazard indices elaborated for bioavailability and biomarker results are normalized to a common scale and finally integrated within a classical WOE approach which assigns one of five classes of risk, from Absent to Severe (Piva et al., 2011).

RESULTS

Chemical analyses revealed a marked bioaccumulation in mussels exposed to either BaP alone or LDPE-BaP, in both digestive gland and gills (**Figures 1A,B**, **Table 2**). After 7 days of exposure, levels of BaP in the digestive glands were significantly enhanced, then remaining almost constant until the end of exposure and without significant differences as a function of time in organisms exposed to contaminated microplastics or to BaP alone (**Figure 1A**, **Table 2**). Gills exhibited rapid accumulation of BaP in organisms exposed to the chemical alone where the elevated concentration measured after 7 days did not further change (**Figure 1B**). On the other hand, in gills of mussels treated with contaminated microplastics, BaP levels significantly increased until the end of exposure at 28 days when values were similar to those of BaP treatment (**Figure 1B**, **Table 2**).

Histological analyses revealed the presence of microparticles in hemolymph, gills and digestive glands and no qualitative differences were observed between organisms treated with virgin LDPE or contaminated LDPE-BaP, as well as between different times of exposure (7, 14, and 28 days). Particles were observed inside hemocytic cells (**Figure 2A**), in the lamellae of gills (**Figure 2B**) and in digestive glands, where numerous aggregates could be observed in the intestinal lumen (**Figure 2C**) and, to a lower extent, inside the digestive tubules (**Figure 2D**) and in the intestinal epithelium.

Immunological responses of hemocytes exhibited statistically significant variations (**Figures 3–C**, **Table 2**). A significant

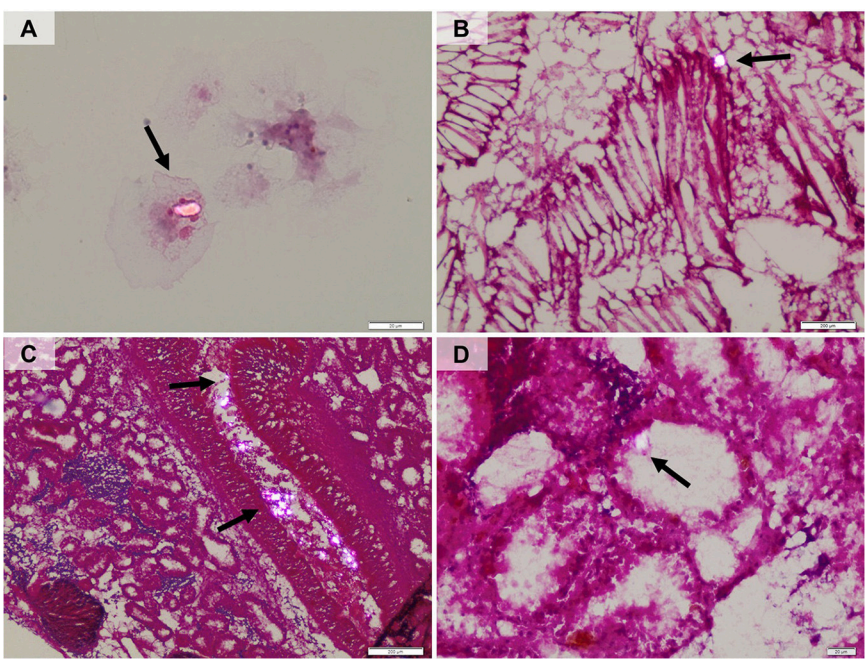


FIGURE 2 | Polarized-light microscopy images showing the presence of microplastic particles in hemolymph **(A)**, gills **(B)**, gut lumen and epithelium **(C)**, digestive tubules **(D)**.

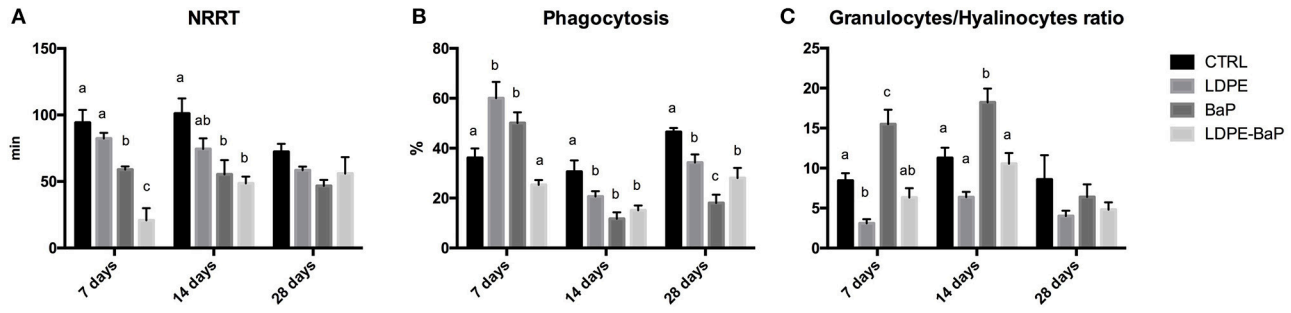


FIGURE 3 | Immunological biomarkers in mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). NRRT: neutral red retention time **(A)**, Phagocytosis **(B)**, Granulocytes/Hyalinocytes ratio **(C)**. Data are expressed as mean values ± standard error, $n = 4$; different letters indicate significant differences between groups of means within the same time of exposure (*post-hoc* Newman-Keuls comparison).

destabilization of lysosomal membrane stability was observed in mussels exposed to various treatments (**Figure 3A**, **Table 2**); post-hoc comparison revealed a marked effect of BaP and LDPE-BaP after 7 and 14 days of exposure, while no differences were obtained among different treatments after 28 days (**Figure 3A**). Phagocytosis exhibited significant changes as a function of treatment and time, with a temporary increase after 7 days in mussels exposed to virgin polymer and to BaP alone, while a significant decrease appeared at longer times in all experimental conditions (**Figure 3B**, **Table 2**). Granulocytes-hyalinocytes ratio was significantly affected by treatment with marked increase caused by with BaP after 7 and 14 days, while no effects were observed in mussels exposed to both virgin and contaminated LDPE (**Figure 3C**, **Table 2**): after 28 days no differences were observed between exposed and control groups (**Figure 3C**).

Acetylcholinesterase showed significant effects as a function of time with a slight decrease in hemolymph and a slight increase in gills after 7 days of exposure to all the treatments (**Figures 4A,B**, **Table 2**): no significant variations were observed between different treatments (**Table 2**).

DNA strand breaks in hemocytes were always comparable for various treatments and times of exposure (**Figure 4C**, **Table 2**), while micronuclei showed a significant increase in mussels exposed to BaP and BaP contaminated LDPE after 14 days of exposure (**Figure 4D**, **Table 2**).

Peroxisomal AOX did not significantly vary in any treatments, although a clear trend of inhibition was observed over time in mussels exposed to LDPE (**Figure 4E**, **Table 2**). A slight increase of neutral lipids was observed in mussels exposed to BaP and BaP contaminated microplastics particularly after 7 days (**Figure 4F**, **Table 2**).

Antioxidant defenses revealed minor fluctuations caused by various treatments, with only a slightly higher oxidative pressure after 28 days of exposure to BaP (**Figures 5A–F**, **Table 2**). The limited pro-oxidant challenge was further supported by MDA, showing a moderate increase only after 7 days in mussels exposed to LDPE and BaP (**Figure 5I**), and by general lack of variations for TOSC toward both peroxyl and hydroxyl radicals (**Figures 5G,H**, **Table 2**).

The results on molecular analyses confirmed the absence of statistically significant differences between treatments on mRNA levels of antioxidants *cat*, *gst-pi*, *Se-gpx,* and of *aox1* (**Figures 6A–D**, **Table 2**). Generally higher transcriptional levels were measured for *cat* and *gst-pi* in mussels after 28 days independently on exposure treatment, while fluctuating levels of *Se-gpx* mRNA were observed in mussels treated with BaP and with LDPE-BaP (**Figures 6A-C**, **Table 2**). Transcriptional levels of *hsp70* appeared downregulated by various treatments after 7 days, while a significant increase was observed in organisms exposed to LDPE for 14 days (**Figure 6E**).

The PCA carried out on the whole set of biomarkers produced a two-dimensional pattern explaining 54% of total variance (**Figure 7**). Although a quite large percentage remained to be explained, obtained results indicated a clear separation between specimens exposed at different treatments for different times. After 7 days (Blu ellipse), LDPE and LDPE-BaP treated mussels separated from the other groups, at 14 days (Red ellipse) mussels treated with BaP and LDPE-BaP were more differentiated, while after 28 days (Green ellipse) the effects of BaP alone became more evident, producing a clear separation between such experimental group and other treatments (**Figure 7**). The parameters determining the separation along the PC1 axis were related to immune system responses (G/H ratio), neurotoxic effects (AchE), and antioxidant system (catalase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase Se-dep, TOSC •OH and ROO•), and AOX. On the other side, genotoxic effects (micronuclei), neutral lipids (NL), total glutathione (TGSH), total glutathione peroxidases (GPX_CHP), and phagocytosis activity determined the separation along the PC2 axis.

Elaboration of data with weighted criteria summarized as Severe the hazard index for bioavailability in mussels exposed to BaP or BaP contaminated LDPE at all exposure periods (**Figure 8**). On the other hand, based on the magnitude of variations exhibited by various biomarkers, their statistical significance of such differences and the toxicological relevance of each biological endpoint, the model summarized the hazard for cellular responses as Slight for organisms exposed to BaP, virgin, and contaminated LDPE, and Moderate only for organisms

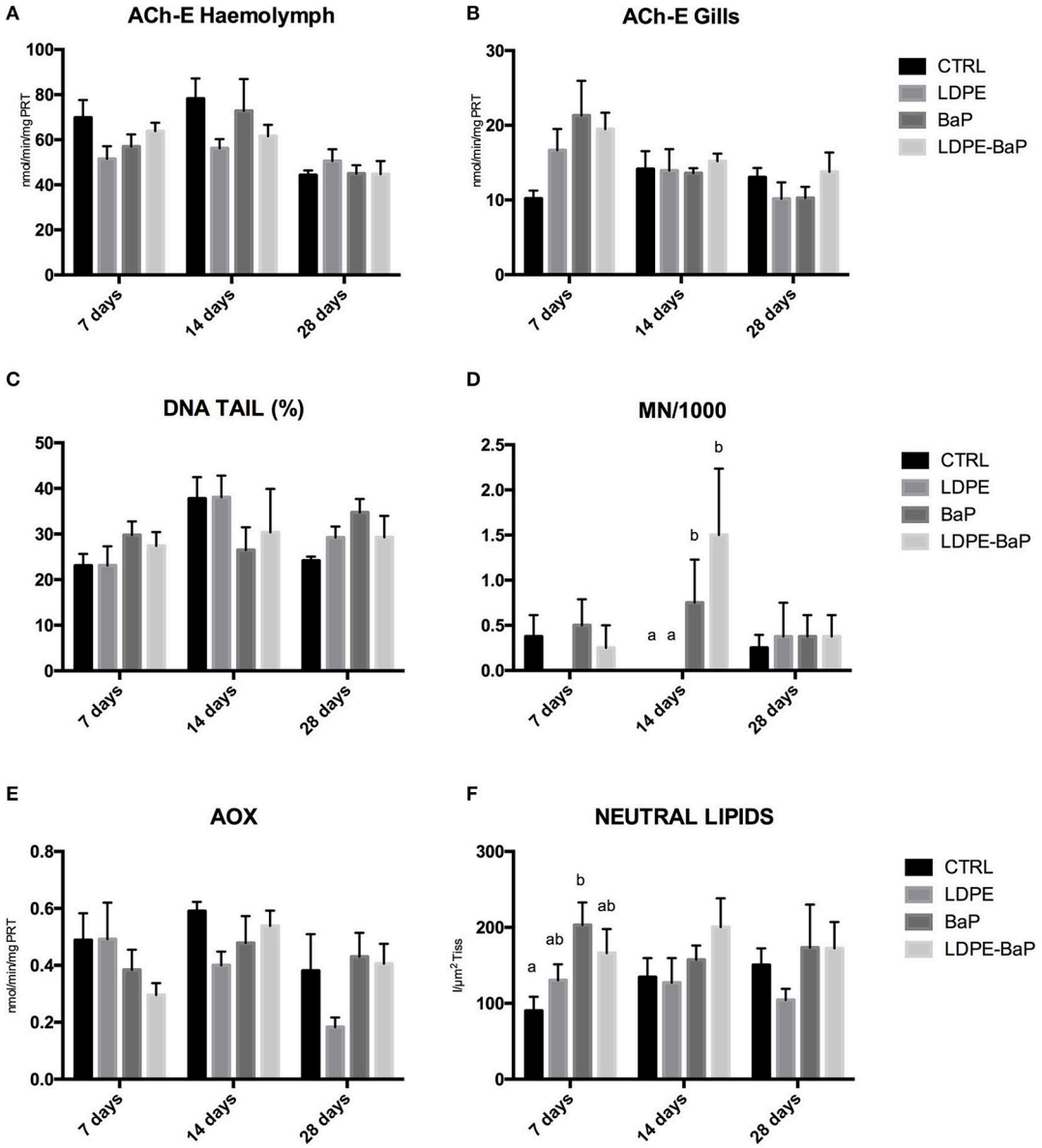


FIGURE 4 | Biomarkers in mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). ACh-E: acetylcholinesterase in haemolymph **(A)** and gills **(B)**; DNA TAIL %: fragmentation of DNA **(C)**; MN/1000: frequency of micronuclei **(D)**; AOX: Acyl CoA Oxidase **(E)**; Neutral Lipids **(F)**. Data are expressed as mean values ± standard error, $n = 4$; different letters indicate significant differences between groups of means within the same time of exposure (*post-hoc* Newman-Keuls comparison).

exposed to BaP after 14 days (**Figure 8**). The integration of hazard indices elaborated for bioavailability and biomarker data resulted in a combined WOE effect classified as Slight for mussels exposed to virgin LDPE and Major for those treated with both contaminated LDPE and BaP alone, without variations at different times of exposure.

DISCUSSION

The increase of plastics and microplastics in marine ecosystems has raised concern on their impact to marine organisms, and several species have been shown to ingest these particles under experimental and wild conditions (Cole et al., 2011; Lusher et al., 2013; De Witte et al., 2014; Avio et al., 2015, 2017b; Devriese et al., 2015; Paul-Pont et al., 2016; Sussarellu et al., 2016; Murphy et al., 2017). The capability of microplastics to efficiently adsorb chemical pollutants from the environment (Avio et al., 2017a) poses an additional risk although there is not yet clear evidence that microplastics ingestion has adverse consequences on the health status of marine species, especially under long term conditions.

In this respect, the present study was aimed to provide new insights on the capability of microplastics to transfer adsorbed

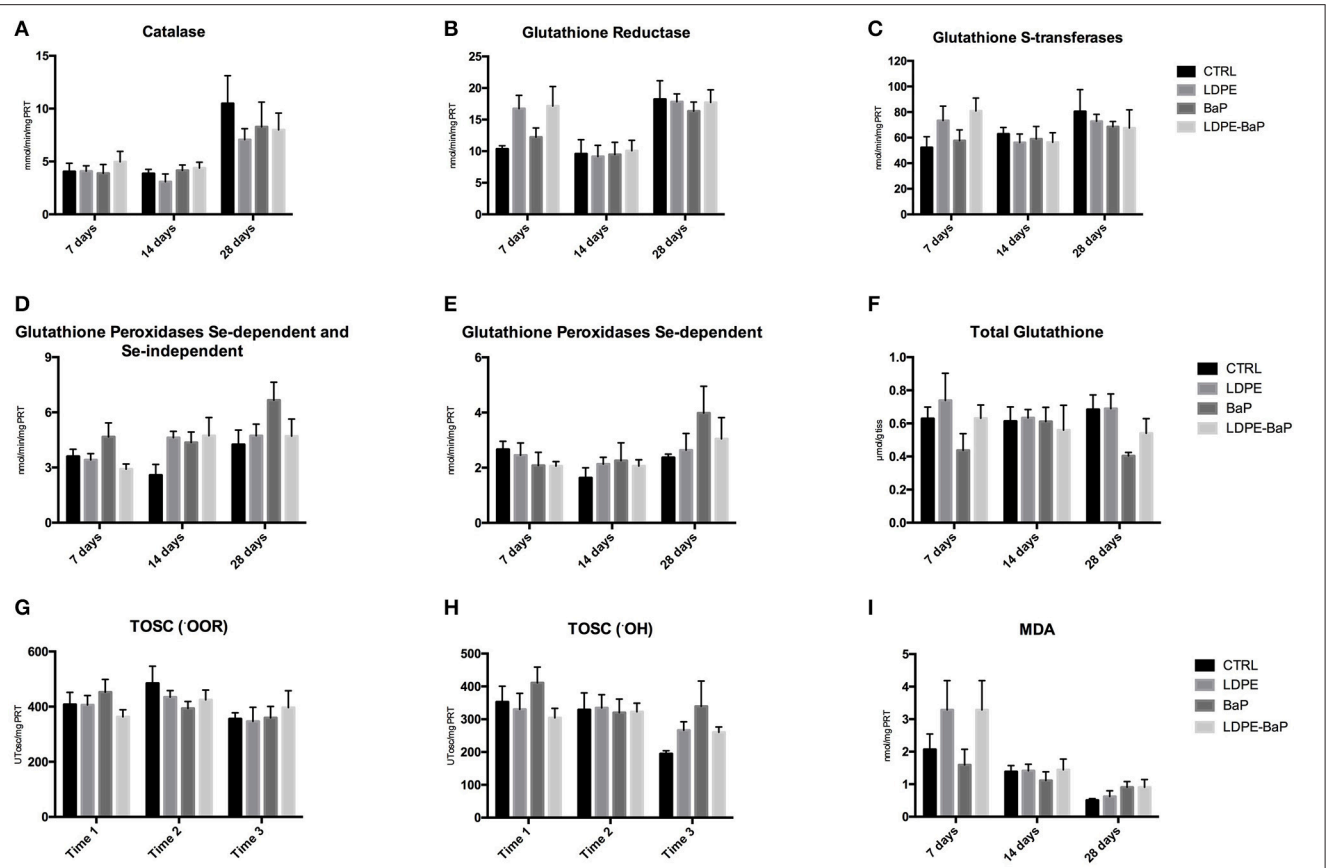


FIGURE 5 | Levels of catalase **(A)**, glutathione reductase **(B)**, glutathione S-transferases **(C)**, sum of Se-dependent and Se-independent glutathione peroxidases **(D)**, Se-dependent glutathione peroxidases **(E)**, total glutathione **(F)**, total oxyradical scavenging capacity (TOSC) toward peroxyl (•OOR) radicals **(G)**, total oxyradical scavenging capacity (TOSC) toward hydroxyl (•OH) radicals **(H)**, malondialdehyde (MDA) **(I)** in mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). Data are expressed as mean values ± standard error, $n = 4$; different letters indicate significant differences between groups of means within the same time of exposure (*post-hoc* Newman-Keuls comparison).

pollutant to organisms after ingestion and to evaluate potential ecotoxicological effects of virgin and contaminated microplastics, using the Mediterranean mussel *M. galloprovincialis* as model marine organism. Although the selected level of microplastics (10 mg/L) appears higher than environmental data, it is worthy to note that a direct comparison between experimental and field values is not necessarily appropriate. Reported seawater concentrations are typically referred to microplastics >200 µm, while natural levels are still unknown for smaller particles, like those used in the present study (20–25 µm), which represent the size range preferentially ingested by filter feeding organisms. Considering the need to characterize the ecotoxicological potential of such biologically relevant microplastics, at the present state of knowledge, concentrations of few mg/L are still in an ecologically relevant range to evaluate in laboratory conditions the disturbance of cellular pathways, possibly involved in long-term responses to small microplastics.

Our results revealed that microplastics can act as efficient vehicles of chemical pollutants. Bioaccumulation analyses showed a marked and rapid enhancement of BaP concentrations in digestive gland of mussels exposed to LDPE-BaP, reaching a steady state after 7 days and values comparable to those observed in BaP treated mussels. This result corroborates the hypothesis of a marked release of BaP from microplastics and an elevated bioconcentration process in tissues under physiological gut conditions, as previously suggested by other authors (Teuten et al., 2009; Bakir et al., 2014; Avio et al., 2015). A slightly different trend was observed for bioaccumulation of BaP in gills: LDPE-BaP treated mussels exhibited only a moderate increase during the initial phases of exposure, reaching tissue concentrations similar to those observed in BaP exposed mussels only after 28 days. While a rapid uptake in gills can be explained by the direct contact of this tissue with the chemical dissolved in water (Banni et al., 2017), the slower accumulation from contaminated microplastics may, at least partly derive from primary desorption of BaP in digestive tissues and a secondary transfer of this chemical to gills.

The possibility that BaP measured in LDPE-BaP treated organisms can reflect the presence of still un-excreted particles more than a real tissue accumulation, can be considered as negligible. Concentrations higher than 15 and 30 ng/g were measured in gills and digestive glands, respectively; assuming

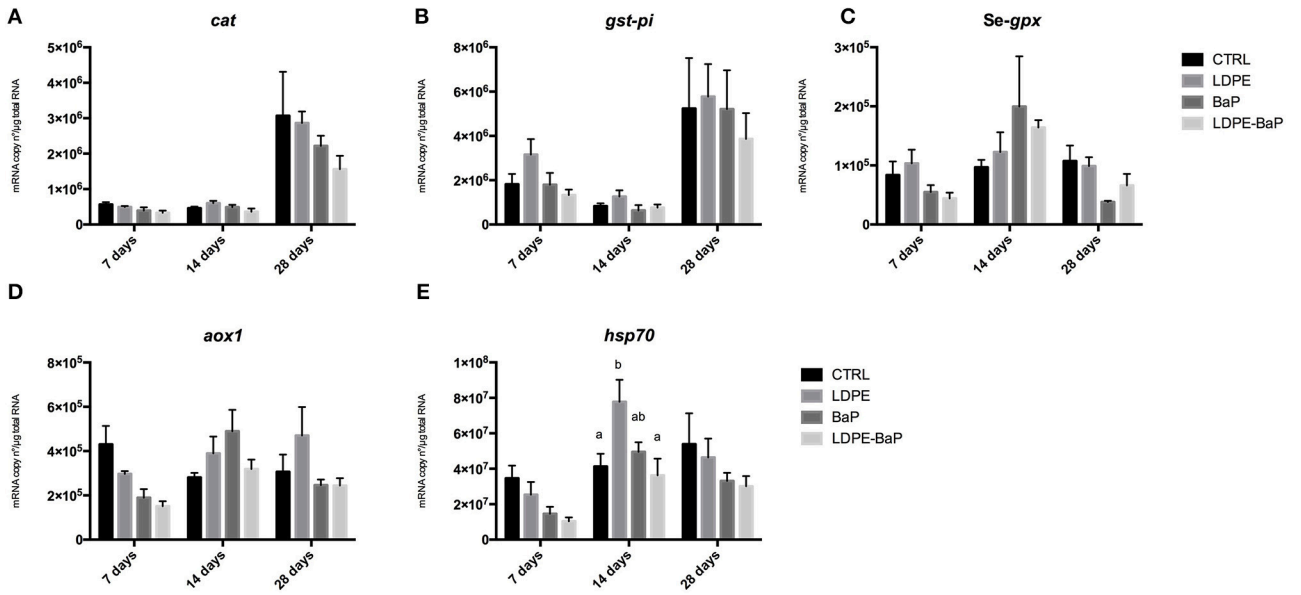


FIGURE 6 | Transcriptional responses in the digestive glands of mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). *cat*, catalase **(A)**; *gst-pi*, *pi*-class glutathione S-transferase **(B)**; *Se-gpx*, selenium-dependent glutathione peroxidase **(C)**; *aox1*, acyl-CoA oxidase, isoform 1 **(D)**; *hsp70*, heat shock protein 70 **(E)**. Data are expressed as mean values ± standard error, $n = 4$; different letters indicate significant differences between groups of means within the same time of exposure (*post-hoc* Newman-Keuls comparison).

that all the measured BaP was still adsorbed on microplastics, we should expect at least 1 mg of particles for each gram of gill tissue (corresponding to $2.34 * 10^5$ particles), and at least 2 mg ($4.68 * 10^5$ particles) for each gram of digestive gland. A similar assumption is excluded by histological analyses that confirmed the presence of particles in those tissues, but with much more limited numbers, particularly in gills where only a few and sparse microplastics were observed.

Uptake and tissue distribution of microplastics has already been investigated in marine bivalves such as the mussels *Mytilus edulis* and *M. galloprovincialis* exposed to virgin and contaminated polyethylene and polystyrene (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015). Although these studies used extremely high concentrations of microplastics (up to three order of magnitude greater than in the present work), they were important in demonstrating the initial uptake of particles at the gill's surface through microvilli activity and endocytosis, while via ciliae movement in the stomach, intestine and digestive tubules are responsible for a second pathway mediated by accumulation within the lysosomal compartment (Von Moos et al., 2012). Our observations almost reflected the above mechanisms of uptake, with aggregates of particles observed within intestinal lumen and digestive tissues, lower occurrence in gills, and some particles noticed also inside hemocytes, as previously documented in other experiments (Browne et al., 2008; Von Moos et al., 2012). Histological analyses were of qualitative nature, but no marked differences in the amount of microparticles were visible for various treatments and times of exposure, thus supporting a short retention time of such particles in mussels, as reported in fish exposed to microbeads (Grigorakis et al., 2017).

Significant immunological effects were observed on hemocytes lysosomal membrane stability, phagocytosis, and granulocytes/hyalinocytes ratio. The impairment of immune system has already been measured in marine organisms exposed to microplastics by several authors (Von Moos et al., 2012; Avio et al., 2015; Paul-Pont et al., 2016). Lysosomes, beside representing major sites for intracellular sequestration and detoxification of xenobiotics, have been also demonstrated as sensitive organelles toward micro- and nano-plastics (Regoli, 1992; Petrović et al., 2004; Moore et al., 2006; Canesi et al., 2012; Avio et al., 2015; Nardi et al., 2017). The destabilization of lysosomal membrane caused by LDPE or BaP alone, was synergistically enhanced in mussels exposed to LDPE-BaP, particularly after 7 days and, to a lower extent, 14 days of exposure. Effects of various treatments were observed also for phagocytosis which initially increased in mussels exposed to LDPE and BaP, while decreasing at longer periods as a consequence of BaP, virgin, and contaminated LDPE: similar effects might be due to an overload of sequestering capacity of hemocytes by microplastics, and to the well-known inhibitory action of PAHs on this function (Wootton et al., 2003; Hannam et al., 2010). Interestingly, LDPE and LDPE-BaP did not affect the granulocytes/hyalinocytes ratio that was statistically increased only by BaP until 14 days. The changes of immune parameters observed in this study are not a surprise given the characteristics of plastic particles, and the physical stress that potentially induce in hemocytes, further modulated with a chemical challenge in mussels exposed to LDPE-BaP.

Our results did not reveal significant effects on AChE activity neither in hemolymph nor in gills, although both the

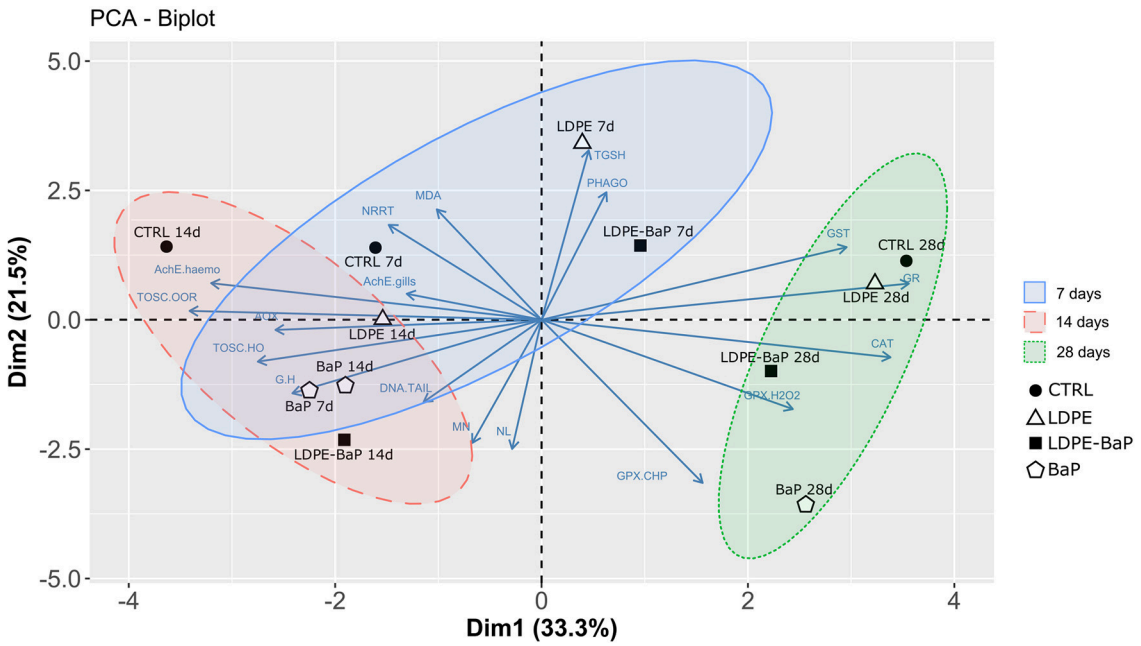


FIGURE 7 | Multivariate PCA analysis on biomarker data in mussels exposed to various microplastics treatments: CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene.

tissues exhibited after 7 days a clear trend toward reduced or enhanced values, respectively. The only moderate and temporary modulation of AChE may reflect the low exposure period. However, cholinesterasic effects of microplastics still deserve scientific attention due to the abundance of these particles in the marine environment and their suggested role in influencing various physiological and behavioral responses controlled by neurological mechanisms (Oliveira et al., 2013; Avio et al., 2015; Mattsson et al., 2017; Ribeiro et al., 2017).

No variations were measured on levels of DNA strand breaks in organisms exposed to microplastics (both virgin and contaminated) or to BaP. A high DNA fragmentation had been previously measured in mussels exposed to polyethylene microplastics (Avio et al., 2015), but the more elevated amount of particles used in those treatments (1.5 vs. 0.01 g/L of this study) can explain the different results. Similarly, the lack of DNA fragmentation in BaP treated mussels might reflect the low experimental concentration as compared to those frequently used for assessing ecotoxicological effects of BaP in mussels (Pan et al., 2009; Banni et al., 2017): in this respect, no formation of DNA adducts or strand breaks was observed in mussels exposed to 300 ng/L of BaP for 24 days (Ching et al., 2001).

Some authors have suggested that microplastics ingestion can potentially cause pseudo-satiety in mussels, thus lowering fatty acids metabolization (Kühn et al., 2015). The AOX, one of the enzymes involved in fatty acid oxidation (Cajaraville et al., 1997; Bilbao et al., 2009) did not show significant effects neither at catalytic nor at transcriptional levels. Content of neutral lipids tended to increase in mussels exposed to BaP and LDPE-BaP, confirming a typical effect of this chemical in inducing lipidosis in digestive gland of mussels (Livingstone and Farrar, 1984; Gorbi et al., 2008).

Treatments with virgin and contaminated microplastics did not affect the oxidative status of mussels, and only minor fluctuations of a few enzymes (glutathione S-transferases and glutathione reductase) were observed, without clear trends as a function of treatment or time of exposure. Responses of antioxidant system were investigated also at molecular level, since transcriptional changes might be more sensitive than enzymatic biomarkers, despite more useful in revealing "exposure" rather than functional "effects" at cellular level (Giuliani et al., 2013; Regoli and Giuliani, 2014). Also these analyses exhibited minor and not significant variations, allowing to exclude an oxidative challenge, as further supported by the lack of effects on the total antioxidant capacity and peroxidation processes in mussels exposed to virgin and contaminated LDPE. The lower levels of particles used in this study, might explain the different results on oxidative effects in comparison to other studies in which mussels exposed to microplastics exhibited significant changes of antioxidant defenses (Avio et al., 2015; Paul-Pont et al., 2016; Détrée and Gallardo-Escárate, 2017; Ribeiro et al., 2017).

A transient upregulation of *hsp70* was observed only after 14 days in mussels exposed to virgin LDPE, suggesting a response toward the physical disturbance caused by the ingestion of such particles. Enhanced levels of these proteins are a generic biomarker of stress, acting in mussels as a first line of defense to cope with environmental challenges (Franzellitti and Fabbri, 2005; Heindler et al., 2017). The effects of contaminated microplastics were more similar to those of BaP, with lack of statistical changes and a trend toward lower values of *hsp70*,

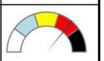
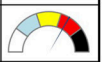
Time	Treatment	Hazard level BIOAVAILABILITY	Hazard level BIOMARKERS	WOE	
7 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP	Severe	Slight	MAJOR	
	BaP	Severe	Slight	MAJOR	
14 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP	Severe	Slight	MAJOR	
	BaP	Severe	Moderate	MAJOR	
28 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP	Severe	Slight	MAJOR	
	BaP	Severe	Slight	MAJOR	

FIGURE 8 | Weighted elaboration of bioaccumulation and biomarkers data in mussels exposed for 7, 14, 28 days to LDPE, BaP, and LDPE-BaP. The assigned classes of hazard are given. Treatments: LDPE, virgin low density polyethylene; BaP, Benzo(a)pyrene alone; LDPE-BaP, Benzo(a)pyrene-contaminated polyethylene.

supporting a limited responsiveness of these proteins to the prevalence of a chemical stress.

The overall evaluation of biomarker results by multivariate PCA provided a clear separation between times and typologies of exposure, highlighting a shift from a physical to a chemical stress. After 7 days, the main effects were those induced by microplastics (possibly reflecting a physical challenge), followed at 14 days by those combined of microplastics with BaP, while at longer exposure conditions effects of BaP prevailed on those induced by microplastics (chemical impact). The multivariate analysis indicated that the majority of observed immunological, lysosomal, and cholinesterasic effects were influenced by polymer (LDPE), while genotoxicity and antioxidant defenses were mostly related to BaP. The impact of LDPE-BaP appeared more biologically relevant with time of exposure, suggesting that energy resources were initially directed to activate primary mechanisms of defense toward the physical stress of particles, while later the chemical stress assumed the major role in biological disturbance. A similar delay of chemical-induced toxic effects was previously observed in fish *Pomatoschistus microps* exposed to microplastics and organic compounds, where these particles acted as a transitory mechanism of protection toward chemical insult (Oliveira et al., 2013).

The overall data were elaborated according to the weighted criteria of the Sediqualsoft model to synthesize the biological significance of bioaccumulation results and cellular responses in mussels exposed to virgin and contaminated microplastics. The bioavailability of BaP was classified as Severe for both the chemical dosed alone and for LDPE-BaP, since concentrations increased from 15- to 60-folds in tissues of exposed mussels compared to controls. On the other hand, the toxicological hazard calculated from the number, magnitude and biological importance of biomarkers was typically Slight for all the treatments, raising to Moderate only in BaP exposed mussels after 14 days. The combination of chemical and cellular hazards provided a WOE index Slight for mussels exposed to virgin LDPE, and Major for those exposed to BaP and LDPE-BaP for all the periods. Considering the similarity of biological effects observed after 28 days, it is quite obvious that the final evaluation of the risk caused by virgin and contaminated LDPE was greatly influenced by the marked accumulation of BaP, further corroborating the still unexplored possibility of indirect, long-term consequences of released chemicals.

In conclusion, this study confirmed that microplastics can transfer adsorbed organic contaminants like BaP to tissues of marine organisms, providing an additional experimental evidence to the role of these particles as source of chemical bioaccumulation. Both virgin and contaminated microplastics did not induce marked ecotoxicological effects at molecular and cellular levels after 28 days of exposure. However, the observed susceptibility of the immune system, the accumulation of BaP and the probable shift from physical to chemical challenge, suggest that the toxicological risk of microplastics for marine organisms is probably low, but not negligible. Additional studies are needed to elucidate conditions of chronic exposure

and whether interactions of particles with other stressors may provoke long term, subtle effects on organisms' health status.

ETHICS STATEMENT

The study was exempt from the above requirements because they do not apply to invertebrates which were used in this study.

AUTHORS CONTRIBUTIONS

LP, CA, SG, and FR: Conceived the study; SK and BC: Prepared the contaminated microplastics; LP and CA: Performed the experiments; LP, CA, and MG: Made laboratory analyses, GdE: Statistical and weighted elaboration of data; LP, CA, SG, and FR: Wrote the manuscript, FR: Edited and reviewed the final version of the manuscript which all the authors approved before submission.

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Leachate From Expanded Polystyrene Cups Is Toxic to Aquatic Invertebrates (*Ceriodaphnia dubia*)

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Expanded polystyrene (EPS) products and their associated chemicals (e.g., styrenes) are widespread in the marine environment. As a consequence, bans on their use for single-use packaging materials are being proposed in several municipalities. To better understand how science can inform decision-making, we looked at the available scientific literature about contamination and effects and conducted experiments to measure chemical leachate from polystyrene products and toxicity from the leachate. We conducted leaching experiments with common food matrices (water, soup broth, gravy, black coffee and coffee with cream and sugar) at relevant temperatures (70 and 95°C) that are consumed in or with several polystyrene products (coffee cup lids, polystyrene stir sticks, polystyrene spoons, EPS cups, EPS bowls, and EPS takeout containers). We analyzed each sample for styrene, ethylbenzene, toluene, benzene, meta- and para- xylene, isopropylbenzene, and isopropyltoluene—chemicals associated with polystyrene products. To determine whether the leachates are toxic, we conducted chronic toxicity tests, measuring survival and reproductive output in *Ceriodaphnia dubia*. Toxicity tests included nine treatments: seven concentrations of ethylbenzene, EPS cup leachate and a negative control. Overall, we found that temperature has a significant effect on leaching. We only detected leachates in trials conducted at higher temperature −95°C. Ethylbenzene was the only target analyte with final concentrations above the method limit of detection, and was present in the greatest concentrations in EPS and with soup broth. Measurable concentrations of ethylbenzene in the leachate ranged from 1.3 to 3.4 μg/L. In toxicity tests, the calculated LC50 for ethylbenzene was 14 mg/L and the calculated LC20 was 210 μg/L. For the treatment exposed to the EPS cup leachate, mortality was 40%—four times greater than the negative control. Finally, there was no significant difference ($p = 0.17$) between reproductive output for any treatment with ethylbenzene, but there was a significant reduction ($p = 0.01$) in reproductive output for the treatment exposed to the EPS leachate compared to the negative control. Thus, although the target analyte ethylbenzene was not toxic at concentrations detected in the leachate, significant adverse effects were detected in the whole EPS cup leachate sample.

Keywords: polystyrene, leachate, toxicity, plastic debris, ethylbenzene

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INTRODUCTION

Plastic debris has become an issue of concern for marine and freshwater habitats globally (Kershaw and Rochman, 2015; Löhr et al., 2017). Plastic items of many types, whole and fragmented, are found on beaches (Browne et al., 2015), floating on the surfaces of oceans (van Sebille et al., 2015) and lakes (Eriksen et al., 2013), in the deep sea (Woodall et al., 2014), and in a great diversity of wildlife (Gall and Thompson, 2015). There are many solutions that have been proposed for reducing plastic emissions into the environment. Some of these solutions are at the local scale (Xanthos and Walker, 2017), while others aim to tackle the problem internationally (Borrelle et al., 2017; Löhr et al., 2017).

In general, there is no one-size-fits-all solution for mitigating plastic debris, and thus many solutions working in tandem are likely necessary. These can include innovation of more sustainable plastic products, new and improved waste management infrastructure, a global fund to help pay for development of new infrastructure and sustainable technologies, educational campaigns, clean-ups, and product bans (Borrelle et al., 2017). Single-use plastic product bans have become a popular solution, as single-use items are some of the most commonly found plastic litter items on beaches (e.g., bottle caps, plastic bags, plastic bottles, expanded polystyrene (EPS) takeout containers, straws) (Ocean Conservancy, 2017). For some single-use plastic items (e.g., plastic bags and microbeads in personal care products), bans are consistently being proposed and passed around the world (Xanthos and Walker, 2017). EPS (often referred to by the general public as Styrofoam™) is another item that is now on the table for a ban in several municipalities (http://www.surfrider.org/pages/polystyrene-ordinances). To better understand how scientific evidence could inform such legislation, we looked at the available scientific literature to review the evidence about contamination and effect. We also performed experiments of our own to measure chemical leachate from polystyrene products that come into contact with food and to measure the toxicity of the leachate.

In regards to contamination, EPS is commonly reported as one of the top items of debris recovered from shorelines and beaches worldwide (Garrity and Levings, 1993; Bravo et al., 2009; Lee et al., 2013; Ocean Conservancy, 2017), including in Antarctica (Convey et al., 2002). It has also been found on the surface of the open ocean (Morét-Ferguson et al., 2010) and on the seafloor (Keller et al., 2010). Widespread contamination has resulted in EPS being found in the gut contents of marine invertebrate and vertebrate wildlife (Boerger et al., 2010; Schuyler et al., 2014; Jang et al., 2016). In addition to physical EPS material, styrenes, the monomeric building blocks of the polymer, are found in ocean water and sediments globally (Kwon et al., 2015, 2017). Because polystyrene plastic is thought to be one of the only sources of styrenes to the environment, the contamination is expected to be from polystyrene weathering and leaching in the oceans (Kwon et al., 2017). Furthermore, in some parts of the world EPS has been cited as a source of other chemicals to the environment (Rani et al., 2015; Jang et al., 2017) and wildlife (Jang et al., 2016). In Asia, hexabromocyclododecanes (HBCDs) have been detected in EPS buoys and other consumer products (Rani et al., 2014). This contamination is thought to originate from the recycling of EPS materials with added flame retardants into other materials, namely materials that do not come into contact with food. Still, HBCDs were detected in some EPS products used for food packaging (Rani et al., 2014). These findings could have consequences to humans when they use the products and/or wildlife if the EPS products become marine debris and leach HBCD. The same research group found that sediments near aquaculture farms using EPS buoys have relatively higher concentrations of HBCD compared to other sites (Al-Odaini et al., 2015) and mussels living on EPS buoys have EPS fragments and greater concentrations of HBCD in their tissues than mussels that live on other materials (Jang et al., 2016). These studies suggest that HBCD from EPS can leach into environmental matrices, including wildlife. In general, there is no doubt that polystyrene and its associated chemicals contaminate oceans (Kwon et al., 2015; Jang et al., 2016).

There is concern that polystyrene may be more harmful than other plastic types because it is composed of relatively hazardous chemicals (Lithner et al., 2011). Because polystyrene microspheres are one of the only types of microplastics available from scientific companies, several studies have conducted laboratory toxicity tests with polystyrene. These laboratory studies suggest polystyrene microspheres can impact organisms. Here, only studies using more environmentally relevant concentrations are highlighted. Laboratory studies demonstrate that polystyrene microplastic can impact feeding behavior (Besseling et al., 2012; Cole et al., 2015), cause weight loss (Besseling et al., 2012), and affect reproduction (Cole et al., 2015; Sussarellu et al., 2016) in invertebrate species. These studies used microplastic particles, and thus whether these effects are from the physical plastic particle or chemical leachate is not known. Other studies measured effects using only chemicals related to polystyrene. A study testing toxicity of leachate from several plastic materials at room temperature found no toxicity from the treatment using a polystyrene cup (Bejgarn et al., 2015). In *Daphnia magna*, LC50 values for 48 h toxicity tests are reported as 23 mg/L for styrene, 75 mg/L for ethylbenzene, 200 mg/L for benzene and 310 mg/L for toluene (LeBlanc, 1980). Acute toxicity tests using fathead minnow determined LC50s of 10 mg/L for styrenes (Cushman et al., 1997). For styrenes, these concentrations are several orders of magnitude greater than those found in nature (Kwon et al., 2017).

The leaching of styrenes and other associated chemicals is one of the reasons why humans are more concerned with polystyrene relative to other plastic types. Under certain conditions, EPS leaches styrene and benzene, chemicals that have known toxic properties (Gibbs and Mulligan, 1997; Erickson, 2011; Andersen et al., 2017; Niaz et al., 2017). There is concern that EPS may cause harm if it leaches chemicals into the environment and/or into our food (Sanagi et al., 2008; Rani et al., 2014). The World Health Organization (WHO) lists the maximum permissible limit at 20 parts per billion (ppb) for styrene (World Health Organization, 2004). The amount that styrene leaches from polystyrene into food and drinks varies in the literature (from about 1 to 300 ppb), and several studies perform leaching experiments with

different conditions, using various foods and/or solvents (Tawfik and Huyghebaert, 1998), varying time periods, and varying temperatures (Ahmad and Bajahlan, 2007; Sanagi et al., 2008). To try to understand exposure concentrations that may be realistic to human exposure, we chose to do our own leaching trials.

Our primary objectives were to better understand how chemicals leach from polystyrene products that come into contact with food and whether there is toxicity from the leachate. We conducted leaching experiments with common food matrices that are consumed in polystyrene packaging at relevant temperatures to test the hypothesis that polystyrene products leach styrenes and related chemicals (i.e., ethylbenzene, toluene, benzene, meta- and para- xylene, isopropylbenzene, and isopropyltoluene) (Ahmad and Bajahlan, 2007) into food consumed by humans. To test the hypothesis that such leachates may be toxic, we conducted toxicity experiments, measuring mortality and reproductive output in a standardized test species, *Ceriodaphnia dubia*. Aside from being a standardized test species, *C. dubia* also plays an important role in the food webs of freshwater habitats globally.

MATERIALS AND METHODS

Leaching Experiments

Leaching experiments were conducted with several products made from polystyrene, three of which were EPS and three of which were non-expanded. Polystyrene products included coffee cup lids, stir sticks, spoons, EPS cups, EPS bowls, and EPS takeout containers. All products were either purchased from local grocery stores in Toronto, Ontario or donated from local coffee shops and restaurants. Where the material of the product was uncertain, a HORIBA XploRA Raman spectrometer was used to confirm polymer type.

Liquids and foods were chosen to be similar to what would be expected to be used for each product. This included leaching tests with water, instant coffee, instant coffee with cream (10% lipid) and sugar, instant chicken soup broth, and instant gravy. Treatments included coffee in a paper cup with a polystyrene lid, coffee with cream and sugar in a clean glass beaker with a polystyrene stir stick, soup broth in a clean glass beaker with a polystyrene spoon, water, coffee and coffee with cream and sugar in an EPS cup, soup broth in an EPS bowl, and instant gravy in an EPS takeout container. All treatments used 250 mL of liquid except for the paper cup with the polystyrene lid (200 mL of coffee), the EPS takeout container (50 mL of gravy), and the EPS cup with water (200 mL). Leaching trials lasted 30 min—roughly the length of time we might expect a person to have food or drink in a polystyrene product. For the paper cup with the polystyrene lid, the cup was tipped every 2 min to simulate drinking and allow the liquid to come in contact with the lid.

For leaching experiments, we ran three separate trials using temperatures that are realistic to hot food and drink −70 and 95°C (Brown and Diller, 2008; **Table 1**). For Trial 1, all food and liquid matrices were prepared with 70°C water and were in contact with polystyrene products for 30 min. All liquid and food matrices were prepared, added to the polystyrene product and allowed to sit uncovered (except for the polystyrene lid) for 30 min. Each treatment was run in triplicate ($n = 3$; See **Table 1** for more detail). For Trial 2, all treatments were identical to Trial 1, except for one treatment where soup broth was prepared at 95°C for an EPS bowl and another treatment where an EPS bowl was microwaved for 3 min, reaching 95°C, and then allowed to sit outside the microwave uncovered for a subsequent 27 min (**Table 1**). Each treatment was run individually ($n = 1$). For Trial 3, all treatments were leached at 95°C for 30 min and covered with a petri dish. To simulate a "worst-case" scenario, an EPS cup was torn into pieces and placed in a glass flask with water that was kept at 95°C for the full 30 min by boiling it on a hot plate (**Table 1**). Each treatment was run in triplicate ($n = 3$). Over the 30 min period, 70°C liquids cooled to roughly 30 and 95°C liquids to 55°C. Directly after the 30 min leaching period, the leachate from each sample was transferred to a clean glass vial with no headspace and stored overnight at 4°C.

The following day, leachates were prepared and analyzed for seven volatile compounds (styrene, benzene, toluene, ethylbenzene, meta- and para- xylene, isopropylbenzene, and isopropyltoluene) using gas chromatography coupled with mass spectrometry (GC-MS). For Trial 1, all samples were analyzed using Headspace coupled to a GC-MS. For Trials 2 and 3, all samples were analyzed using Purge and Trap with GC-MS.

Chemical standards used for analysis were purchased from Sigma Aldrich. All samples were spiked with 5 μL of surrogate standard (Fluorobenzene, d8-toluene, bromofluorobenzene).

To analyze all samples in Trial 1, we used a Tekmar HT3 Headspace sampler coupled to an Agilent 7890A gas chromatograph with an Agilent 5975C (MSD) mass spectrometer with ultrapure grade (helium) carrier gas. 10 mL of sample was introduced to the Tekmar HT3 and a 2 mL sample from the headspace was injected into a J&W DB-VRX 20 m × 0.18 mm × 1.0 μm film column in split mode (50:1). The oven program started at 35°C, held for 4 min, increased by 14°C per minute until 100°C, increased by 20°C per minute until 220°C and then held for 2.72 min. The Agilent 5975 (MSD) was operating in full scan mode (mass range 34–350). Target analytes were quantified using the extracted ion and confirmed using retention times and the ratio of confirmation ions. Concentrations were determined using external calibration with surrogate standards. The limit of detection for this analysis was 25 ng/mL.

To analyze all samples in Trials 2 and 3, a Tekmar Atomx Purge and Trap system with a Vocarb 3000 coupled with a Thermo Trace gas chromatograph and DSQII mass spectrometer with ultrapure grade (helium) carrier gas was used. 20 mL of sample was purged directly in soil mode on the Atomx Purge and Trap concentrator and subsequently injected into a J&W DB-VRX 20 m × 0.18 mm × 1.0 μm film column in split mode (60:1). The oven program was the same as described above for Trial 1. The Thermo DSQII (MSD) was operating in full scan mode (mass range 34–350). Target analytes were quantified using the extracted ion and confirmed using retention time. The ratio of confirmation ions and concentrations were determined using external calibration with surrogate standards. The limit of detection for purge and trap analysis was approximately 1.25 ng/mL.

TABLE 1 | Detailed information about all treatments in leaching experiments.

Trial #	PS product	Food/Liquid matrix	Temperature (°C)	Length of time (min)	Covered (Y/N)	Microwaved (Y/N)	Instrument for chemical Analysis
1	PS lid	Coffee	70	30	Y	N	Headspace-GC-MS
1	PS stir stick	Coffee + cream and sugar	70	30	N	N	Headspace-GC-MS
1	PS spoon	Soup broth	70	30	N	N	Headspace-GC-MS
1	EPS cup	Coffee	70	30	N	N	Headspace-GC-MS
1	EPS cup	Coffee + cream and sugar	70	30	N	N	Headspace-GC-MS
1	EPS bowl	Soup broth	70	30	N	N	Headspace-GC-MS
1	EPS takeout container	Instant gravy	70	30	N	N	Headspace-GC-MS
2	PS lid	Coffee	70	30	Y	N	Purge and trap with GC-MS
2	PS stir stick	Coffee	70	30	N	N	Purge and trap with GC-MS
2	PS spoon	Soup broth	70	30	N	N	Purge and trap with GC-MS
2	EPS cup	Coffee	70	30	N	N	Purge and trap with GC-MS
2	EPS bowl	Soup broth	70	30	N	N	Purge and trap with GC-MS
2	EPS bowl	Soup broth	95	30	N	N	Purge and trap with GC-MS
2	EPS bowl	Soup broth	95	30	N	Y	Purge and trap with GC-MS
3	PS lid	Coffee	95	30	Y	N	Purge and trap with GC-MS
3	PS stir stick	Coffee	95	30	Y	N	Purge and trap with GC-MS
3	PS spoon	Soup broth	95	30	Y	N	Purge and trap with GC-MS
3	EPS cup	Coffee	95	30	Y	N	Purge and trap with GC-MS
3	EPS cup	Water	95	30	Y	N	Purge and trap with GC-MS
3	EPS bowl	Soup broth	95	30	Y	N	Purge and trap with GC-MS

All glassware was cleaned and baked at 250°C for 12 h prior to use. Laboratory blanks were prepared for each sample matrix (e.g., hot water, coffee, and broth) using a clean glass beaker and no polystyrene product. Target analytes detected in laboratory blanks were not subtracted from the concentrations detected in all samples. See Tables S1 and S2 for concentrations of all target analytes in the laboratory blanks from Trial 2 and 3, respectively. Concentrations in laboratory blanks for Trial 1 are not listed because all samples were below the limit of detection. Spiked matrix blanks were also extracted and run with each sequence of samples to determine recovery. In spiked matrix blank samples, recoveries of the seven target analytes ranged from 29 to 120% across all matrices for headspace-GC-MS and 67–154% across all matrices for purge and trap with GC-MS (see Tables S3–S5 for detailed recoveries).

Leachate Toxicity Tests With *C. dubia*

Testing was conducted following the Environment Canada and Climate Change standard method for assessing survival and reproduction in the freshwater cladoceran *C. dubia* (EPS 1/RM/21; ECCC, 2007). Test solutions included different concentrations of ethylbenzene and leachate from the same EPS cups used in the leaching experiments described above.

Ethylbenzene was purchased from BDH Ltd. (99% purity) and used to make stock solutions. Stock solutions for EPS cups were prepared by placing 20 torn cups in 5 L of laboratory dilution water (dechlorinated City of Toronto tap water) in a stainless-steel stockpot and boiling for 30 min. Leachate was prepared on day 0 (test initiation), and stored in amber glass bottles with minimal headspace for use in water changes on each day of toxicity testing. Stock solutions of ethylbenzene were prepared each day of the test by spiking 6 uL into 1 L of lab dilution water and used for dilutions to make test concentrations. Because the solubility of ethylbenzene in water is 0.015 g/100 mL (20°C), no carrier solvent was used. Stock solutions were stored in glass vials with minimal headspace and used for dilutions to make test concentrations. Nominal test concentrations for ethylbenzene included 5.2, 2.6, 1.3, 0.7, 0.32, 0.16, and 0.08 mg/L. For the 5.2 mg/L ethylbenzene solution and the leachate from the EPS cup, actual concentrations were measured in solution at the start (day 0) and at day 8 using the same methods as above for leachates in Trials 2 and 3 (i.e., using purge and trap with GC-MS), except in water mode with a 10-mL purge. Because this method is slightly more sensitive, the limit of detection is 0.2 ug/L. At day 8, solutions were measured at the start and end of the 24 h period (i.e., to measure the decayed concentration). Measured concentrations of ethylbenzene in the 5.2 mg/L stock solution were 2.3 mg/L at day 0 and 4.8 mg/L at day 8. We note that the concentration on day 0 was much lower than expected. On this day only, it took a few hours before putting the test animals in the solution. On all other days, this only took a few minutes. Because the measured concentration at day 8 was what we expected, we are fairly confident that exposure concentrations in the toxicity test were similar to what we expected on all other days of the procedure. The measured concentration for the decayed solution was 0.2 mg/L, decaying as much as 96% over the 24 h period between renewal of the test solution. This is likely due to the volatility of ethylbenzene, which helps explain our lower concentration of stock solution on day 0. Measured concentrations in the leachate of the EPS

cup were consistently below the limit of detection for toluene, meta- and para- xylene, isopropylbenzene, and isopropyltoluene. For styrene, concentrations in the starting solution were 0.6 μg/L at day 0 and 0.8 μg/L at day 8. The measured concentration of styrene in the decayed solution was below detection. For benzene, concentrations were 0.2 μg/L (at the detection limit) at day 0 and below the detection limit at day 8. The measured concentration of benzene in the decayed solution was also below the detection limit. For ethylbenzene, concentrations were 2.4 μg/L at day 0 and 2.1 μg/L at day 8. The measured concentration of ethylbenzene in the decayed solution was below the detection limit.

C. dubia were a single genetic stock cultured at the Ontario Ministry of the Environment and Climate Change. *C. dubia* are cultured at 25 ± 2°C temperature under a 16 h light/8 h dark photoperiod. Individuals are fed daily 0.5 mL unicellular green algae (Pseudokirchneriella subcapitata) and 0.01 mL of YCT (yeast/cerophyll/trout chow mix) (ECCC, 2007). Organisms used for testing met the culture health criteria of no ephipia, brood mortality did not exceed 20%, and produced broods of at least 15 neonates per female in the 7 days prior to test initiation. Water used in culturing and testing was City of Toronto tap water dechlorinated by activated carbon beds, and spiked with Selenium (3 μg/L) (Winner, 1989).

For each of the nine treatments (i.e., seven concentrations of ethylbenzene, EPS cup leachate, and a negative control) there were ten replicates ($n = 10$). Animals were exposed for 8 days. Each individual replicate consisted of a test volume of 15 mL and one female daphnid. Solutions were renewed daily. *C. dubia* were fed daily during the test following the same diet and ration as above. Water quality parameters pH, conductivity, dissolved oxygen (DO), and temperature were measured daily. In all treatments except for the EPS leachate, pH ranged from 8.2 to 8.5, conductivity from 270 to 353 μS/cm, DO from 7.6 to 9 mg/L and temperature from 21.5 to 22.8°C. In the EPS leachate, pH ranged from 8.1 to 9.9, conductivity from 229 to 305 μS/cm, DO from 4.6 to 8.5 mg/L and temperature from 21.7 to 22.6°C. Animals were acclimated to the experimental system for a 24 h period before commencement of the experiment. Each day, mortality of the first-generation individual daphnid and the number of live neonates produced each day were recorded. Overall, mortality, total brood size per individual and time to first brood were measured. For the test to be valid, we required 80% survival and at least 15 young per female on average for control animals over the 8-day test period.

Test results were analyzed statistically to determine the LC50 and LC20 for ethylbenzene and to test the hypothesis that ethylbenzene and EPS cup leachate would alter total brood size. LC50 and LC20 values and their 95% confidence limits were determined using the Probit Analysis method and calculated in a Probit Analysis calculator developed by Dr. Alpha Raj (Finney, 1952). Using GMAV (EICC, University of Sydney), a 1-factor ANOVA tested for differences in total brood size among ethylbenzene treatments ($n = 10$, $\alpha = 0.05$) with fixed factor treatment (eight levels: 5.2, 2.6, 1.3, 0.7, 0.32, 0.16, 0.08, and 0 mg/L). We assured that our data was normally distributed via histograms. We did not run statistical tests for normality because ANOVAs are not very sensitive to moderate deviations from normality (Underwood, 1997). A Cochran's C-test (1951) showed homogeneity of variances ($\alpha = 0.05$). A 2-tailed equal variances *t*-test analyzed differences in total brood size between the control and the EPS cup leachate treatments ($n = 10$, $\alpha = 0.05$) using SYSTAT 12 (SYSTAT Software, Chicago, IL).

RESULTS

Leachates From Polystyrene Products

For the leaching experiments in Trial 1 (**Table 1**), all products were exposed to food matrices at 70°C, uncovered, for 30 min. After the leaching experiments, all matrices were analyzed via headspace-GC-MS. For all seven target analytes, concentrations were below the limit of detection (25 μg/L). Because this limit of detection is relatively high, we decided to repeat the experiments and analyze the leachates using a more sensitive instrument with a lower detection limit (1.25 μg/L).

For the leaching experiments in Trials 2 and 3 (**Table 1**), we analyzed all samples using purge and trap with GC-MS. Experiments in Trial 2 were run with no replication to see if any of the target analytes could be detected. Because of the sensitivity of this instrument, we omitted samples with cream or gravy (i.e., relatively high lipid content) to keep the instrument from becoming too contaminated. In addition to running each polystyrene product with 70°C coffee or soup broth, we included two samples with soup broth at higher temperature in an EPS bowl. One sample was microwaved in the EPS bowl for 3 min to 95°C and allowed to sit out for 27 more minutes. The other was boiled to 95°C and the hot broth was poured into the EPS bowl and allowed to sit for 30 min. Across all samples run at 70°C, all target analytes were below the limit of detection or at trace levels that were similar to the concentration in the blank (see Table S1 for all data from Trial 2). For the two samples run at 95°C, ethylbenzene was the only target analyte above the limit of detection and that was not detected in the blanks. Ethylbenzene concentrations were similar between the two hot samples, with concentrations of 3.2 μg/L in the microwaved EPS bowl and 3.4 μg/L in the non-microwaved EPS bowl. This suggests that the higher heat is the cause of the higher concentrations of ethylbenzene in the leachate.

Trial 3 was conducted to repeat our test in Trial 2 with replication ($n = 3$) and to conduct all leaching trials at a higher temperature −95°C (**Table 1**). All of the same treatments in Trial 2, with the exception of the microwaved EPS bowl, were replicated in Trial 3 at 95°C. In addition, we added one more treatment with EPS in water maintained at 95°C for the full 30 min by boiling it on a hot plate. For this treatment one EPS cup per replicate was torn into pieces and placed in the flask of boiling water for the full 30 min. Again, some target analytes were detected at trace levels in some samples, but were similar to the concentration in the blank (see Table S2 for all data from Trial 3). Similar to Trial 2, ethylbenzene was the only target analyte that was above the limit of detection and not detected in the blanks. Ethylbenzene was detected in all three replicates of boiling water with EPS at concentrations of 1.5,

1.6, and 1.5 μg/L, of coffee with EPS at 1.3, 1.4, and 1.4 μg/L, and of broth with EPS at 1.6, 1.8, and 2.6 μg/L. In general, EPS leaches more than the other polystyrene products tested and soup broth induces greater leaching than hot coffee or water.

Toxicity in *C. dubia*

Across all treatments, there was no obvious response curve. This may be due to the high volatility of ethylbenzene. Higher concentrations did not always lead to a greater response. Total mortality ranged from 10 to 70% (**Table 2**; see Table S6 for mortality data). There was no difference in mortality between the control and the lowest two concentrations of ethylbenzene (0.16 and 0.08 mg/L), with all three having 10% mortality. One ethylbenzene treatment, 0.65 mg/L, had 20% mortality. Mortality in the EPS cup leachate and in the 0.325, 1.3, and 5.2 mg/L ethylbenzene treatments suffered 40% mortality—four times more than the control and two times above the acceptability criteria in this chronic test. The highest mortality was in the 2.6 mg/L ethylbenzene treatment, at 70% mortality. For ethylbenzene, the calculated LC50 was 14 mg/L (95% confidence interval 3.5–61 mg/L) and the calculated LC20 was 0.21 mg/L (95% confidence interval 0.05–0.9 mg/L).

Across all treatments, the average time to first brood ranged from 4.2 to 5.9 days (**Table 2**; see Table S7 for all reproductive data). Time to first brood ranged from 4.2 to 4.9 days for all treatments, except the 0.325 mg/L and EPS cup leachate treatments. For these two treatments, time to first brood was 5.7 ± 1.4 and 5.9 ± 1.2 days respectively—roughly an entire day later than the control treatment (4.8 ± 1 days).

Across all treatments, average total brood size ranged from 5 to 15 offspring. For total number of offspring, there was no significant difference between ethylbenzene treatments ($p = 0.17$; **Figure 1**). There was a significant difference in total number of offspring between the EPS cup leachate and the control treatments ($p = 0.01$), with the total brood size of *C. dubia* exposed to EPS cup leachate being significantly smaller than of *C. dubia* in the control treatment (**Figure 2**). Total average brood size for *C. dubia* in the control treatment was 15 ± 9 offspring, whereas total average brood size for *C. dubia* in the EPS cup leachate treatment was 5 ± 5 offspring.

TABLE 2 | Chronic toxicity data for ethylbenzene and EPS leachate in *C. dubia*.

Chemical	Treatment	Chronic parameter				
	Concentration (mg/L)	Adult mortality (%)	Avg. # of offspring	StDev	Time to first brood (days)	StDev
Ethylbenzene	0	10	15	9	4.8	1
	5.2	40	9	10	4.2	0.5
	2.6	70	6	10	4.7	0.6
	1.3	40	12	10	4.9	1.1
	0.65	20	15	12	4.4	1.1
	0.325	40	6	7	5.7	1.4
	0.162	10	12	6	4.8	0.8
	0.0812	10	10	7	4.4	0.7
EPS leachate	Control	10	15	9	4.8	1
	1 EPS cup/250 mL water	40	5	5	5.9	1.2

DISCUSSION

Here, we tested whether polystyrene products leach chemicals into food and drink matrices under realistic exposure scenarios and whether their leachates led to toxicity in freshwater zooplankton.

Low Levels of Volatile Compounds Leach From Polystyrene Products During Use

We only detected chemical leachates in trials conducted at 95°C, and the only chemical that was confidently detected in leachates was ethylbenzene. Ethylbenzene was present in concentrations ranging from 1.3 to 3.4 μg/L. In leaching trials, the highest concentrations were in the soup broth. In general, this suggests that temperature has a significant effect on the amount that chemicals will leach from polystyrene products, a trend that has been demonstrated in other studies (Tawfik and Huyghebaert, 1998; Ahmad and Bajahlan, 2007; Sanagi et al., 2008). It also suggests that matrices with lipids (chicken soup broth) cause greater leaching or better retain volatile leachates than matrices without lipids (water and coffee). This trend has also been found in a previous study (Tawfik and Huyghebaert, 1998). In addition, our results suggest that EPS leaches more than non-expanded polystyrene products, such as polystyrene cutlery and coffee cup lids.

Here, we aimed to conduct leaching experiments under scenarios that are realistic to how each product is used for eating and drinking. Temperatures used in this study ranged from 70 to 95°C (Brown and Diller, 2008), and products were not exposed to leachate for more than 30 min. Under these conditions, leachate concentrations for styrene and ethylbenzene were below the limits accepted by the WHO: 20 ppb for styrene and 300 ppb for ethylbenzene (World Health Organization, 2004). Concentrations of ethylbenzene in our experiments were two orders of magnitude lower than the limit deemed acceptable by World Health Organization (2004). Other studies that use realistic leaching conditions have found concentrations that do raise concerns for human health. Sanagi et al. (2008) found styrene concentrations ranging from 45 to 293 ppb in water under leaching conditions of 24–80°C for 30 min in a polystyrene cup. Tawfik and Huyghebaert (1998) found styrene concentrations of 24 ppb in whole milk under conditions at 40°C for 24 h and in ice-cream under conditions at −10°C for 30 days in polystyrene cups.

In this study, we targeted a suite of volatile chemicals that have been demonstrated to be associated with polystyrene and/or EPS in previous studies. As with any chemical analysis, there may be other chemicals present in these polystyrene products that we did not target. For example, Rani et al. (2014) detected flame

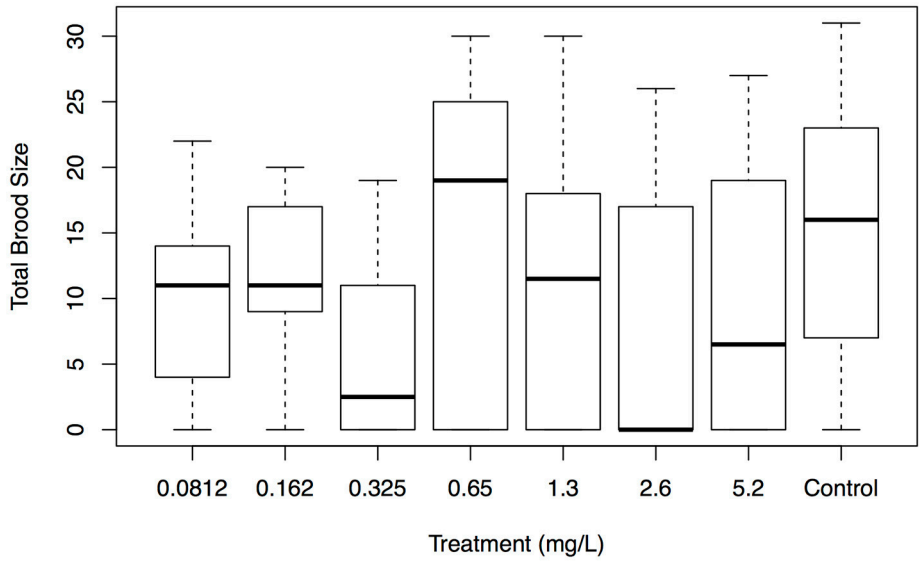

FIGURE 1 | Total brood size of *C. dubia* exposed to different concentrations of ethylbenzene and the negative control. The box and whisker plot displays a five-number summary of the total brood size data for each treatment with ethylbenzene from lowest to highest concentration (mg/L) with the negative control on the right. The bar in the middle of each box represents the median, the top, and bottom of the box the lower and upper quartiles (25 and 75%) and the whiskers the minimum and maximum values.

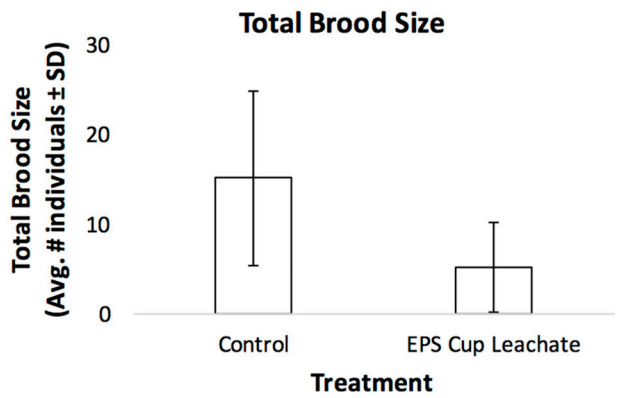

FIGURE 2 | Total brood size of *C. dubia* exposed to the EPS cup leachate and the negative control. Each bar represents the mean total brood size of each treatment and the error bars represent the standard deviation.

retardants in polystyrene products at concentrations ranging from 24 to 199 ng/g (Rani et al., 2014).

Toxicity of the Leachate From EPS Food Containers

Because ethylbenzene was the only chemical detected at quantifiable levels in our leaching experiments, we focused on ethylbenzene for our toxicity tests. In addition, because EPS seemed to leach more than other products, we included a treatment that consisted of the whole leachate from an EPS cup. This treatment was included to determine whether there may be any toxicity due to chemicals we did not target for analysis.

For toxicity tests using several concentrations of ethylbenzene, higher concentrations did not always lead to greater effects (**Table 2**). This may have been due to the fact that ethylbenzene is a volatile chemical, and thus concentrations in the vials were variable based on the fast decay rates we observed. Here, the calculated LC50 was 14 mg/L and the calculated LC20 was 210 μg/L. These concentrations are several orders of magnitude greater than the ethylbenzene measured in our leaching trials. We also did not observe a significant difference in reproductive output among all treatments with ethylbenzene. These results suggest that the leachates from all of our leaching trials are not toxic. However, the results from the treatment with the EPS cup suggest otherwise.

The mortality observed in the treatment exposed to the EPS cup leachate was 40%, which is four times greater than the negative control. Moreover, the time to first brood was >1 day later than the control and we observed a significant reduction in reproductive output. The average total brood in the EPS treatment was three times less than that of the control. Such reproductive effects have the potential to lead to population level effects. Similar effects, demonstrating reduced reproduction in oysters (Sussarellu et al., 2016) and marine species of zooplankton (Cole et al., 2015) exposed to polystyrene, have also been observed.

Although we observed significant toxicity in *C. dubia* that were exposed to the EPS leachate, we do not know what led to the observed effects. One possible explanation is the high pH measured in the test solution at various time points. Another possible explanation is a chemical or combination of chemicals that we did not target in our analyses. Our results highlight the importance of measuring toxicity from the whole sample vs. simply measuring toxicity with one targeted chemical at a

time. A whole sample provides a more holistic outlook on what types of effects we might observe in the real-world. Future studies should aim to conduct whole leachate toxicity tests using more products, under different scenarios and measuring more diverse effects. Different scenarios might include comparing leachates under different temperatures and in marine vs. freshwater.

Implications for Policy

When planning legislation many factors need to be considered and all informed by scientific evidence. It is important to consider implications for human health, wildlife, and sustainability. Here, we focused on implications for human health by measuring leaching and implications for wildlife by measuring toxicity in a freshwater invertebrate. In regards to human health, the results from our leaching experiments do not suggest that polystyrene is unsafe for humans. However, our results contradict those from other studies which do measure chemical leachates above safe limits (Tawfik and Huyghebaert, 1998; Sanagi et al., 2008). Thus, more evidence is necessary. For wildlife, our results, and those of others (Cole et al., 2015; Sussarellu et al., 2016), suggest that increasing accumulations of polystyrene in marine and freshwater environments could lead to population-level effects in invertebrate species. In regard to sustainability, data should be compiled from cradle-to-grave to determine how sustainability metrics for polystyrene and EPS compare to other material types.

AUTHOR CONTRIBUTIONS

CR, CT, and RR designed the leaching experiments. CR, DP, and KS designed the toxicity experiments. CT conducted the leaching experiments. KS, DP, and HD conducted the toxicity experiments. Chemistry was run and analyzed by RR, CT, JD, and GS. Data were statistically analyzed by CR. The initial draft of the manuscript was written by CR and CT. All authors contributed to all drafts of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00071/full#supplementary-material

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Comparative Effects of Ingested PVC Micro Particles With and Without Adsorbed Benzo(a)pyrene vs. Spiked Sediments on the Cellular and Sub Cellular Processes of the Benthic Organism *Hediste diversicolor*

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Plastic micro litter represents an emerging contaminant as well as a multiple stress agent in aquatic environments. Microplastics are found even in the remote areas of the world. Together with their occurrence in all environmental compartments, there is a growing concern about their potential to adsorb pollutants co-occurring in the environment. At present, little is known about this source of exposure for aquatic organisms in the benthic environment. Exposure conditions were set up to mimick the contribution of microplastics through different exposure routes. Potential biological effects resulting from these exposures were investigated in the model organism *Hediste diversicolor*, an annelid worm. Cellular effects including alterations of immunological responses, lysosomal compartment changes, mitochondrial activity, oxyradical production and onset of genotoxicity were assessed in coelomocytes while temporary and permanent effects of oxidative stress were also performed at tissue level. In this study polyvinylchloride (PVC) microparticles were shown to adsorb benzo(a)pyrene with a time and dose-dependent relationship. The elevated bioavailability of the model pollutant after ingestion induced a clear pattern of biological responses. Toxicity mainly targeted impairment of cellular functioning and genotoxicity in *H. diversicolor* coelomocytes, while permanent effects of oxidative stress were observed at tissue level. Coelomocytes responded fast and with a higher degree of sensitivity to the adverse stimuli. The results showed that microplastic particles in sediments may play a significant role as vectors for organic pollutants. The highest adverse responses were observed in those *H. diversicolor* exposed to sediments spiked with PVC particles pre-incubated with B[a]P when compared against sediments spiked with B[a]P and plastic microparticles separately.

Keywords: microplastics, trojan horse effect, coelomocytes, *hediste diversicolor*, exposure assessment

INTRODUCTION

Along with environmental stressors such as global warming, ocean acidification, and habitat destruction, pollution causes undesirable changes in marine ecosystems. In particular, plastic particles accidentally or deliberately released as a result of anthropogenic activities represent a growing environmental concern for aquatic environments. Marine debris, particularly the fraction composed of plastic fragments at micrometric size, is considered a multiple stressor in aquatic habitats. Firstly it acts as physical stressor congesting digestive traits and ducts hampering the functioning of key organs i.e., gill, stomach, liver, and kidney in vertebrate and invertebrate marine species (Wright et al., 2013; Kühn et al., 2015; Clark et al., 2016; Pellini et al., 2018). Secondly, micrometric scaled plastic fragments can act as chemical reservoirs and potential vectors for the widespread dispersal of hydrophobic pollutants co-occurring in the aquatic environment. Polymers with densities higher than seawater (e.g., PVC) tend to sink and accumulate in seafloor sediments, making organisms within the benthic community more vulnerable to organic pollutants, as they may be adsorbed from both the water column and the sediment. Ingestion of particles has been documented in vertebrate and invertebrate marine organisms (Gregory, 2009; Davison and Asch, 2011; Carson, 2013; Hall et al., 2015). After the ingestion, the ingestion processes, dominated shifts in pH, temperature and redox conditions, microplastic particles can potentially release the adsorbed environmental pollutants therefore enhancing both their bioavailability and biological adverse effects to biota. This route of exposure for aquatic organisms, the so called "Trojan horse" effect, remains under-investigated with limited data concerning such phenomena (Syberg et al., 2015; Paul-Pont et al., 2016; Gaspar, 2017).

In our study the possible contribution of plastic particles toward increasing dispersion, bioavailability and associated adverse biological effects of an organic pollutant molecule model benzo(a)pyrene (B[a]P) was investigated in a zoobenthic model organism, the ragworm *Hediste diversicolor*. B[a]P was chosen as a representative of the polycyclic aromatic hydrocarbon (PAH) class of compounds. PAHs are widely distributed toxic compounds with, in some cases, carcinogenic, mutagenic, and teratogenic effects, as is the case for B[a]P (IARC, 1987). In aquatic environments, most PAHs primarily accumulate in the sediment as a result of their chemical and physical characteristics. Bottom-dwelling deposit-feeding organisms such as polychaetes are potentially exposed to PAHs, both directly through dermal contact with interstitial water or particulate matter and indirectly through the gut, from food and particle ingestion (Jørgensen et al., 2008). Furthermore, polychaetes can be also exposed by the water phase and during high tide, they can be exposed by the water compartment. For this reason, they are a good candidate species for toxicological studies addressing different exposure routes.

A number of different exposure scenarios were set up mimicking the rising occurrence of plastic micro litter in an environment already subjected to organic pollution contamination. An extended suite of biomarkers was selected to compare the biological responses observed both in cells extracted from the coelomic fluid and at tissue level and to unveil microplastics driven toxicity mechanisms at organism, cellular and sub-cellular level.On coelomic fluid cells the phagocytosis assay (PhC) and the lysozyme activity responses (Lz) were measured to investigate possible impairments of the immune system. On the other hand, the mitochondrial activity (MtO) and the lysosomal membrane stability (LMS) were estimated to assess general information about cellular functioning; while the oxyradical production assay (ROx) was estimated as index of oxidative stress. Finally DNA fragmentation and micronuclei frequency (Mn) were assessed to investigate genotoxic effects in exposed organisms. In the meantime, lipid peroxidation (LPO) and the activity of catalase (CAT) were investigated at tissue level as marker of oxidative stress in treated organisms. Obtained biological responses were compared with as the body burden accumulation of B[a]P.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were of analytical grade and were used as received without any further purification. Methanol (purity: $< 99.5\%$), B[a]P (purity: $< 99.5\%$) and Hank's artificial seawater salts mixture was procured from Sigma-Aldrich. Polyvinyl chloride (PVC) powder of $250.0 \pm 2.5\,\mu m$ size was obtained from Goodfellow Ltd (Cambridge, UK). Reagents for artificial sea water (ASW) preparation were of purity $> 99.5\%$ from Sigma Aldrich. Bi-distilled water used in this study was obtained from a MilliQ apparatus (Millipore, Milan, Italy) according to the manufacturer's guidelines.

Test Design

Laboratory assays followed the ASTM guidelines (ASTM, 2013) for conducting sediment tests with annelids with some minor adaptations. A set of five replicate test aquaria (10 l glass beakers) each containing eight organisms were used per treatment. Ragworms were first put individually in beakersto avoid possible cannibalism and then transferred on aquaria for the exposure test,., Each aquaria contained some glass tubes with 1 cm diameter, placed in the sediment surface as possible temporary shelter for the organisms. Approximately 3 kg of sediments ($\approx$ 8-cm layer of sediment) were carefully placed, at the bottom of the beakers. Artificial seawater medium (ASW) of 34 g l^{-1} salinity (ASTM, 2013) was added yielding a sediment: overlying water depth ratio of 1:3 (w:v). Organisms were exposed at $20.0 \pm 1.0^{\circ}C$ and under a 16 h light: 8 h dark cycle. Food was added during the assays at 1% of body weight and 2/3 of the water was carefully siphoned out from the water surface and replaced every week. Water parameters (salinity, pH, dissolved oxygen) were monitored throughout the exposure period.

A series a chronic 28 days experiments were carried out by exposing *H. diversicolor* to uncontaminated sediments (C), sediments spiked with B[a]P (S-B[a]P), sediments spiked only with virgin PVC at 200 particles/kg of sediment (LC-MPS) and 2,000 particles/kg (HC-MPS) as well as similar concentrations for B[a]P spiked microplastic particles (B[a]P-LC; B[a]P-HC). The

lower bead concentration was set to match with environmentally realistic levels (Claessens et al., 2013; Hanvey et al., 2017), the 10 times higher level was chosen to address potential acute exposure effects and does not represent environmental conditions.

Ragworm Collection

Specimens of *H. diversicolor* were purchased from a bait dealer (Lescachepesca.eu, Italy). Only individuals in good condition and similar size-class length (12.0 ± 2.0 cm) were selected for testing. Organisms were left for 7 days in ASW and in the dark at 20.0 ± 2.0°C to acclimate and depurate. During the acclimatization period individuals were fed *ad libitum* every 2–3 days with commercial fish food (48.6% protein and 7.7% fat). 5 gr of food was extracted in triplicate and analyzed for microplastic content. No plastic particles were detected in the analyzed samples.

Sediment Collection and Characterization

A total of 100 kg of natural sediments were collected using a stainless-steel box corer in October 2013 from an off-shore site (coordinates) considered distant from major anthropogenic sources of hydrocarbons and micro plastic particles. Collected material was used to prepare the control and spiked sediments. Sediments were homogenized and native animals removed before taking a subsample to determine: salinity and organic matter content (OM, Schumacher, 2002). Particle size distribution was determined using a LS 13-320 laser particle-sizing instrument (Beckmann Coulter, Milan, Italy) and the total content of polyaromatic hydrocarbons assessed according to the methods of Durou et al. (2007). Furthermore, microplastic content and characterization, based on a density separation method according to Nuelle et al. (2014) and a detection method according to Vianello et al. (2013), was carried out. A negligible (2.0 ± 0.9 particles/kg) amount of polyethylene fragments in a range of 30–40 μm were detected ([2]). This sandy-mud sediment contained 1.5% of organic carbon, 82.4% of sand, 13.9% of clay and 3.7% of silt.

Spiking Preparation

The adsorption of B[a]P to PVC particles was performed by mixing solutions of microplastics (∼10 g/L in ASW) with B[a]P at an environmentally realistic concentration of 5 μg/L (Bihari et al., 2007; Maria and Bebianno, 2011; Avio et al., 2015). The incubating solutions were kept in 50 ml glass tubes under continuous rotation within a Gallenkamp chamber for 7 days at room temperature in darkness. PVC adsorption efficiency was checked at the end of the incubation process by spinning down spiked plastic beads (5,000 g, 10 min at RT), collecting the pellet, sub sampling it three times and placing sub samples in a GF/A fiberglass filter. Samples were flushed with 5 ml of ASW and adsorbed B[a]P extracted by 10 ml of methanol (purity< 99,9%) using a microwave assisted technique. Desorbed B[a]P in methanol was preconcentrated by a Rotavapor and quantified by an Agilent 1100 series HPLC-DAD system (**Figure 1**—SEM). B[a]P was subsequently identified by the retention time of standard solution of CRM48743 Supelco mix.

The stock of the spiked microplastic beads were stored at−20 C prior to exposure tests.

To realize S-B[a]P testing conditions, sediments were contaminated by spiking with B[a]P (>99% dissolved in acetone, HPLC-grade, Sigma-Aldrich, Italy). To insure a comparable exposing dose among all tested exposure routes, ∼16 kg of fresh sediments were spiked with 80 μg of B[a]P considering a concentration of 5 μg/L previously adopted for PVC particle incubation as well as a standard rate of sediment: overlying water of 1:3 (w/v) set within the exposure tests. Spiked sediments were gently mixed with a stainless-steel spoon for 20 min and allowed to equilibrate for a week at RT in dark conditions in a well-ventilated fume hood. For the uncontaminated treatment, fresh sand was only spiked with acetone. Final B[a]P concentrations obtained with this procedure were 4.58 ± 0.51 μg kg^{-1} (ww) while sediments analyzed before the spiking exercise did not contain detectable levels of B[a]P (< 1,0 μg kg^{-1}).

To realize S-B[a]P testing conditions, previously spiked sediments were equilibrated with filtered seawater for 48 h prior to the addition of microplastic beads and test organisms. In the meantime, to realize LC-MPS, HC-MPS, B[a]P-LC, and B[a]P-HC testing conditions, B[a]P spiked particles and virgin particles at the two different concentrations were added on top of each of the aquaria used for the different experimental conditions were re-equilibrated natural sediments were previously placed.

The number of particles to be added to the aquaria was calculated using the concentration of a mixture of either virgin or B[a]P spiked beads mixed with 5 ml of ASW. The working mixtures were sub-sampled 10 times, collecting 100 μl working solution, diluting it 1:1,000 (v:v) in Isoton II Diluent (Beckmann, Milan, Italy) and finally analyzing each of the subsamples by a Multisizer II Coulter Counter coupled with a 1,000 μm aperture size tube (Beckmann, Milan, Italy). Spiking solutions with concentration of 50.0 ± 6.0 beads / ml were then prepared. Beads were allowed to sediment for 3 days, mimicking the naturally occurring deposition of microplastic from the water column, prior to turning on the aeration and placement of test organisms. Aeration was provided using a 1 ml glass pipette suspended ∼5 cm above the sediment surface, with test organisms added to the vessel 2 h later. While adding the organisms, aeration was stopped for 10 min to allow them to settle. A set of randomly placed glass tubes and pipettes were added to the aquaria as shelters for the organisms.

Chemical Analyses

The body burden of B[a]P in exposed organisms was estimated according to Durou et al. (2007) on three individuals collected from each replicate of each testing condition, while the accumulation of MPS was estimated on an individual per replicate of each testing condition according to a combined enzymatic and alkali driven extraction followed by a μFTIR analysis. Briefly, single individuals were incubated with 50 ml protease from *Bacillus* sp (P3111, Sigma, Italy) and PBS-buffer at pH 9 mixture (1:5). The sample was sonicated for 5 min and incubated for 24 h at 45°C and then filtered through a 20 μm stainless steel sieve (Setacci Giuliani Spa, Italy). The residue was washed off with MilliQ water pre-filtered through a 0,45 μm filter, collected in a clean 100 ml beaker and further treated with 50 ml Chitinase from *Trichoderma* (C8241, Sigma, Italy) and

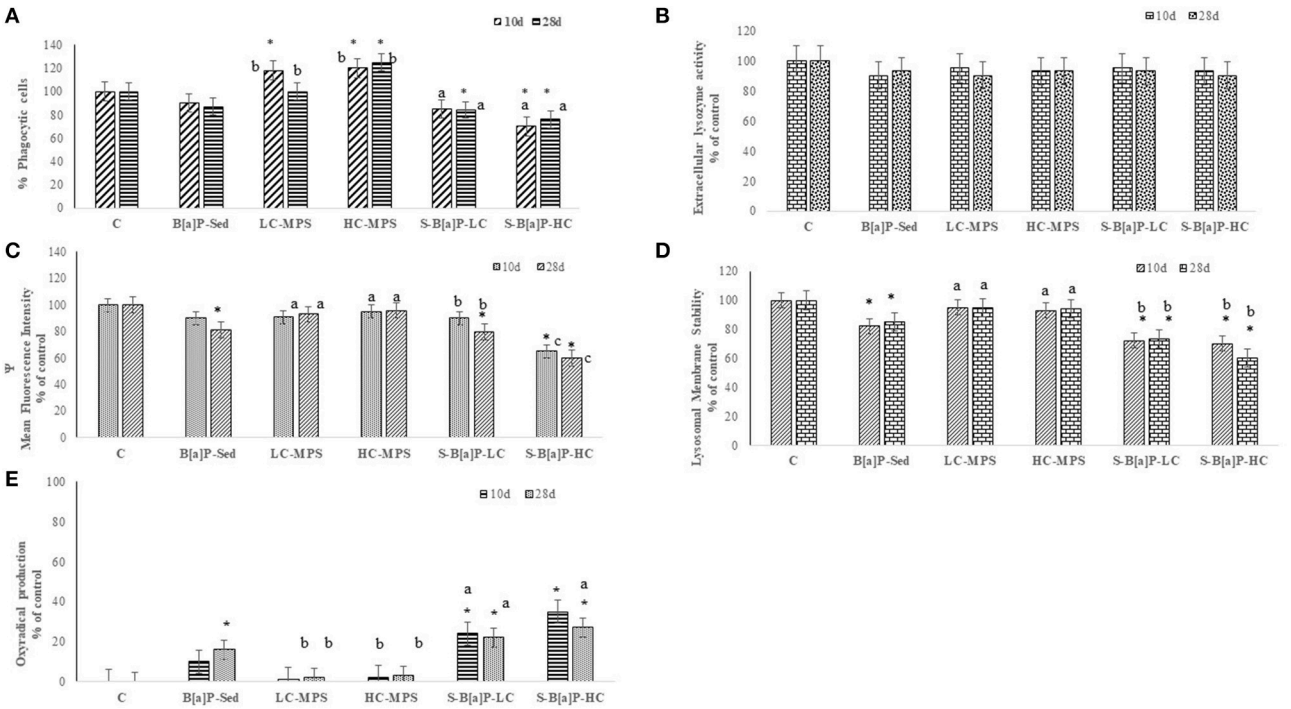

FIGURE 1 | Biological responses in coelomocytes of *H. diversicolor* exposed to uncontaminated sediments (C), D sediments spiked with 1 mg/L B[a]P (S-B[a]P), sediments spiked with 200 and 2000 particles/kg virgin PVC (LC-MPS, HC-MPS) and sediments with B[a]P spiked microplastic particles (B[a]P-LC: 200 particles/kg.; B[a]P-HC: 2000 particles/kg). **(A)** Response of the phagocytic activity, **(B)** Extracellular lysozyme release, **(C)** mitochondrial activity, **(D)** Lysosomal membrane stability, (E) Oxyradical production. *$p < 0.05$. Data are expressed as mean values ± standard deviation, $n = 6$. Different letters indicate significant differences between groups of means (*post-hoc* comparison).

PBS-buffer at pH 7 mixture (1:10). The sample was sonicated for 5 min, incubated 24 h at 30°C and finally filtered through a 20 μm stainless steel sieve. The residue was transferred to a 50 ml beaker and incubated with KOH 10% 12 h at 50°C. The alkali degraded sample was then diluted 1:1 with MilliQ water and filtered through a 47 mm Anodisc filter with pore size of 0.2 μm (Whatman, Germany) and submitted to μFTIR according to Vianello et al. (2013). Four individuals collected from each testing condition involving PVC beads were analyzed.

Biological Analyses

Endpoints were measured after 10 and 28 days of exposure to the tested conditions, being the measurements after 10 days of exposure a good intermediate sampling point useful to follow up the development of the stress syndrome in the investigated organisms. Ragworms were removed from exposure and individually maintained in ASW to void gut content in preparation for MPS accumulation assessment as well as biological and chemical analyses. After ∼10 h, ragworms were submitted to biological analyses or snap-frozen and stored at −40°C until chemical analyses.

Mortality was recorded as a high-level endpoint test following the exposure period. All sub lethal biological responses were conducted on four individuals per replicate in each of the treatment and sampling time.

Phagocytosis, lysozyme activity, mitochondrial activity, LMS, oxyradical production, Comet assay, and micronuclei frequency were assessed on the coelomic fluid cells. Coelomic cells were obtained using a non-invasive extrusion method according to Eyambe et al. (1991) with some modifications. Polychaetes were individually placed in 15 ml glass tubes containing 10 ml of 3% ethanol and 1,0 mg/ml EDTA in Hanks' balanced salt solution (H6648, Sigma Italy). The obtained cell suspension was centrifuged at 200 g at 4°C for 5 min to recover the cells; water was discarded and the pellet gentle resuspended in 2 ml ASW. In detail, phagocytosis analysis was assessed by quantifying cellular intake of Neutral Red-stained Zymosan according to Ciacci et al. (2012). 100 uL of cell suspension was incubated with stained Zymosan with 20 to 40 cells from each exposure condition then examined using image analysis by ImageJ release 1.51 K shareware software (NiH, USA).

Lysosomal enzyme release was evaluated by measuring lysozyme activity in the extracellular medium according to Chu and La Peyre (1989). Lysozyme activity in coelomic cell suspensions obtained from organisms exposed to the different conditions was determined spectrophotometrically at 450 nm utilizing *M. lysodeikticus* and referenced against results of organisms exposed to natural sediment (Ciacci et al., 2012).

Mitochondrial activity in coelomocytes was assessed by the mitochondrial membrane potential estimation thorough the tetramethylrhodamine ethyl ester perchlorate (TMRE, ex/em: 488/580 nm) selective fluorescent dye (Scaduto and Grotyohann, 1999). A commercially available kit (AB113852, Abcam, Italy) was used. Coelomic cells were incubated with 50 nM TMRE for

15 min in a 2 ml Eppendorf tube, centrifuged 500 g at RT °C for 2 min and placed on a glass slide. Fluorescent emission was captured using an inverted microscope coupled with fluorescent emission (Axiovert 135, Carl Zeiss, Italy). From 30 to 60 cells were image analyzed from each exposure condition.

To assess oxyradical production (ROx) the cell-permeant 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, C400, Thermofisher, Italy) was used as an indicator for reactive oxygen species formation in coelomocytes. 100 uL of cell suspension were incubated in humid chambers with 1 μM H2DCFDA in ASW for 15 min at RT in dark conditions following the general recommendation supplied with the manufacturer's protocol. LMS was evaluated by the Neutral Red Retention time assay as described by Catalano et al. (2012) with minor modifications. Briefly, 50 μl of cell suspension was placed on a polylysinated glass slide in a dark humidity chamber. 10 μl of neutral red working solution (50 μg/mL) was added to the slide. Cells were observed under a light inverted microscope every 10 min and pictures from several fields taken to visualize a minimum of 80–100 cells. Observation was stopped when the number of cells showing leakage of neutral red in the cell's cytosol exceeded 50% of the total. This time was taken as the neutral red retention time.

DNA fragmentation (Comet assay) was estimated according to Buffett et al. (2014). Analyses were performed on 50 cells per where 2 slides per individual were prepared, 4 individuals per treatment. After staining with DAPI (50 ng/ mL), nuclei were individually observed under a fluorescence microscope (500x). The level of DNA damage was expressed as % Tail DNA.

Micronuclei frequency (Mn), lipid peroxidation (LPO), and enzymatic antioxidant catalase (CAT) activity were assessed according to Catalano et al. (2012).

Briefly, for Mn assessment aliquots of ∼1,000 coelomocytes were collected from each individual and fixed in Carnoy's solution with 4 individuals per treatment evaluated. Fixed cells were distributed on glass slides and stained with DAPI. For each specimen, 200 coelomocytes were scored to determine micronuclei formation.

LPO was assayed on intestinal cross sections (7 μm) of individual ragworm, obtained using a Leica cryostat. Cryo-sections were stained by the Schorml's reaction according to Moore (1988) and lipid peroxidation end-products quantified by image analysis (ImageJ 1.51 K, NiH, USA).

CAT was measured using enzyme-specific spectrophotometric assays at constant temperature. The enzymatic activity was assayed by absorbance at 240 nm, using 10 mM hydrogen peroxide as substrate. For enzymatic assays, protein quantification was performed according to Lowry et al. (1951) using the single enzyme homogenates.

Data Analysis

Data were preliminary checked to meet the assumptions of normality and homogeneity of variances prior to analysis. Changes in the biochemical responses as well as in the accumulation pattern of microplastics and B[a]P were evaluated by analysis of variance using Newman–Keuls test for *post-hoc* comparisons (α: 0.05; p below to 0.05 expressed as < 0.05). When not normally distributed, data were analyzed using the Kruskal–Wallis non-parametric test. Statistical analyses were performed by SPSS 11.0 for Windows. To facilitate the visualization of correlations among all performed treatments as well as to rank samples into groups of homogeneous observations, a principal component analysis (PCA) of all chemical and biological results, was combined to a hierarchical clustering (HCA, Avio et al., 2015). Factorial analysis and hierarchical clustering are complementary tools for exploring data. However, HCA presents some advantages as removing the last factors of a factorial analysis it also removes noise and makes the clustering more robust (Ivosev et al., 2008). Hierarchical clustering analysis was performed in R (https://cran.r-project.org/). The degree of similarity of each pair of patterns was computed using Euclidean distance on normalized sub lethal stress index responses.

RESULTS

No significant increments of mortality rate were observed among the treatments indicating that all observed biological responses were at the sub lethal level.

Among all investigated sub-lethal responses, the efficiency of the immune system was tested thorough Zymosan-stimulated phagocytic activity on ragworm's coelomic cells (**Figure 1A**). Results are expressed as % of response referred to the control (assumed as 100%). Coelomocytes of both organisms exposed to sediments spiked with virgin microplastics beads (LC-MPS; HC-MPS), and sediments spiked with B[a]P pre-incubated plastic beads (B[a]P-LC; B[a]P-HC) showed significantly different immune system responses with respect to control cells ($p < 0.05$). However, a different pattern of responses was observed among tested exposure conditions. Polychaetes exposed to sediments spiked with virgin microplastics showed an increased induction of immune system activity (LC-MPS: + 20%; HC-MPS: + 20–25% respect to control). On the contrary, coelomocytes of annelids exposed to sediments spiked with different concentrations of B[a]P pre-incubated plastic beads (B[a]P-LC; B[a]P-HC) showed a suppression of immune system activity (− 13–30%). No clear sampling time and microbeads concentrations correlations were observed among the reported biological responses. On the other hand, none of the tested exposure conditions stimulated lysozyme release with respect to controls (**Figure 1B**).

The effects on mitochondrial activity in haemocytes extracted from polychaetes submitted to the different exposure conditions were evaluated utilizing specific fluorescent dyes for mitochondrial membrane potential (TMRE) and the results are reported in **Figure 1C**. The results indicate that sediments spiked with B[a]P (−19% respect to control) as well as sediments spiked with plastic beads pre-incubated with B[a]P (B[a]P-LC; B[a]P-HC) induced a clear suppression of mitochondrial activity (− 10–30%; $p < 0.05$). Furthermore, a significant correlation among the biological responses and microplastic concentration was observed ($p < 0.05$) thus suggesting the potential role of the plastic beads to extend the toxicity of B[a]P. On the other hand, where statistically significant, the extent of the

observed biological responses was directly correlated to both the concentration of the plastic particles and the exposure time ($p < 0.05$).

S-B[a]P, B[a]P-LC ,and B[a]P-HC exposure conditions also induced a negative effect on the LMS of polychaete coelomocytes (**Figure 1D**). Among all treatments, significantly higher disruption levels were reported for treatments dealing with plastic beads pre-spiked with B[a]P when compared to those where the organic pollutant molecule was administrated directly to the sediments and the control ($p < 0.05$). Furthermore, no significant LMS alterations were observed in treatments containing only virgin microbeads. as well as no clear correlation among the observed biological responses, the concentration of administrated B[a]P spiked microbeads and the sampling time.

The effects of oxidative stress in coelomocytes were estimated as a function of oxyradical production (**Figure 1E**). Highest levels were observed in B[a]P-HC (+ 27–35% respect to control, $p < 0.05$) treatments followed by B[a]P-LC (+ 22–24%, $p < 0.05$) while coelomocytes exposed to sediments spiked with B[a]P showed a significant increase in oxyradical production only after 28 days of exposure. Organisms incubated with virgin microplastics did not show any significant difference in oxyradical production in respect to control organisms. Further statistical analysis identified a significant correlation between effects and the concentration of B[a]P-spiked plastic particles, though there was no connection among the observed biological responses and sampling time.

The potential of plastic particles to increase dispersion and effects of genotoxic pollutants such as B[a]P was investigated by both scoring the increments of micronuclei formation and DNA strand breaks in coelomocytes. As expected, all treatments involving the polyaromatic molecule induced a significant increase of micronuclei frequency with respect to control organisms (**Figure 2A**; $p < 0.05$). Among all exposure conditions, the highest Mn values were scored in organisms submitted to B[a]P-HC treatments (4–5‰). These values did not differ to those recorded in organisms exposed to B[a]P-LC (3–4‰) but they were significantly higher than those observed in organisms exposed to S-B[a]P conditions (2–3‰, $p < 0,05$). A similar pattern of response was observed while analyzing the results of DNA fragmentation (**Figure 2B**). B[a]P-HC exposure conditions induced the strongest effects on DNA fragmentation with 6% tail in scored cells; followed by B[a]P-LC (5%) and S-B[a]P (3–4%). No significant biological effects were observed in treatments where sediments were spiked with virgin microplastic alone. No clear correlation between the distribution pattern of the biological responses and the sampling time was observed; while on the contrary, a significant direct correlation between the DNA strand breaks analysis and the B[a]P spiked microbeads levels was observed ($p < 0.05$).

The effects of lipid peroxidation as well as the efficiency of the antioxidant system against the pro-oxidant effect of B[a]P activity were investigated at tissue level in *H. diversicolor*. Results of lipofuscin content and the activities of catalase are shown in **Figure 3**.

As expected, only exposures involving B[a]P induced a significant increase in lipofuscins with respect to control organisms (**Figure 3A**, $p < 0.05$). Interestingly, significantly increases only occurred after 28 days of exposure. The highest values were recorded within treatment B[a]P-HC (+38%, respect to control), followed by B[a]P-LC (+28%) and S-B[a]P (+12%). *Post-hoc* analysis further discriminated two separate groups of results, with biological responses obtained within B[a]P-LC and B[a]P-HC treatments statistically different to those recorded following S-B[a]P exposure conditions ($p < 0.05$).

In a similar way, catalase activity was triggered by exposures to low and high concentrations of B[a]P spiked microbeads ($p < 0.05$). However, different from the distribution of the LPO response, significant increments of this anti-oxidant enzyme were limited to samples collected after 10 days of exposure. On the other hand, due to the large standard variation no significant time related responses were observed across all treatment conditions even if CAT activity values were generally lower in samples collected after 28 days of exposure.

Through different routes, polychaetes were exposed to similar doses of B[a]P. The resulting body burden in the whole body of organisms sampled 10 and 28 days after the beginning of the experiments is presented in **Figure 4**.

Accumulation patterns ranged from below the detection limit (0,1 ng/g ww) to 3.35 ± 0.40 ng/g ww., were observed in

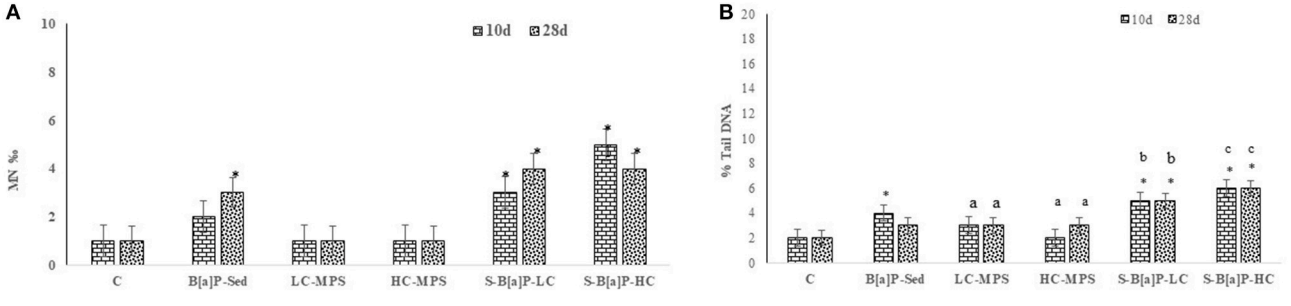

FIGURE 2 | Genotoxicity assessment in coelomocytes of *H. diversicolor* exposed to uncontaminated sediments (C), D sediments spiked with 1 mg/L B[a]P (S-B[a]P), sediments spiked with 200 and 2000 particles/kg virgin PVC (LC-MPS, HC-MPS) and sediments with B[a]P spiked microplastic particles (B[a]P-LC: 200 particles/kg.; B[a]P-HC: 2000 particles/kg). **(A)** Microunuceli frequency, **(B)** DNA strand breaks. *$p < 0.05$. Data are expressed as mean values ± standard deviation, $n = 4$. Different letters indicate significant differences between groups of means (*post-hoc* comparison).

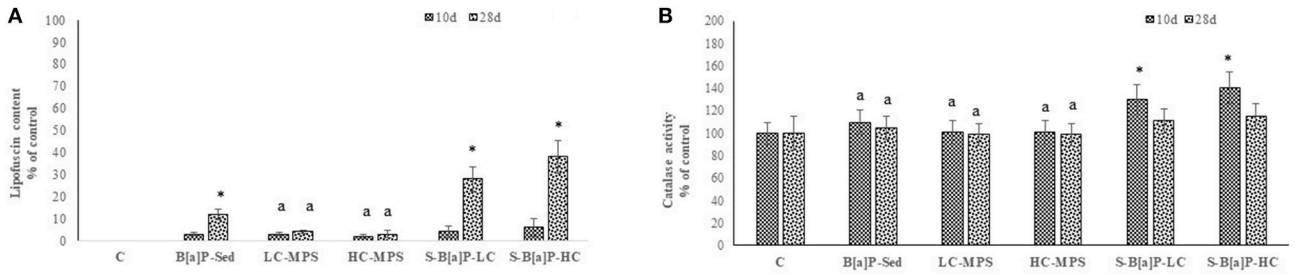

FIGURE 3 | Biological responses in tissues of *H. diversicolor* exposed to uncontaminated sediments (C), D sediments spiked with 1 mg/L B[a]P (S-B[a]P), sediments spiked with 200 and 2000 particles/kg virgin PVC (LC-MPS, HC-MPS) and sediments with B[a]P spiked microplastic particles (B[a]P-LC: 200 particles/kg.; B[a]P-HC: 2000 particles/kg). **(A)** Lipofuscin content, **(B)** Catalase activity. *$p < 0.05$. Data are expressed as mean values ± standard deviation, $n = 6$. Different letters indicate significant differences between groups of means (*post-hoc* comparison).

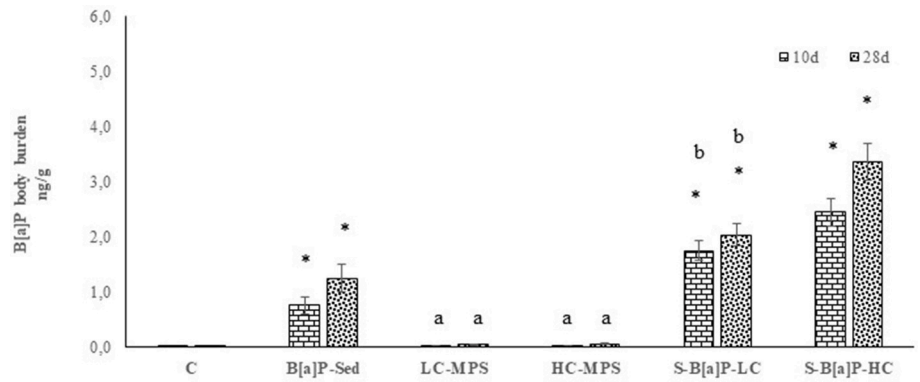

FIGURE 4 | Benzo(a)pyrene body burden accumulation in whole body of *H. diversicolor* exposed to uncontaminated sediments (C), D sediments spiked with 1 mg/L B[a]P (S-B[a]P), sediments spiked with 200 and 2000 particles/kg virgin PVC (LC-MPS, HC-MPS) and sediments with B[a]P spiked microplastic particles (B[a]P-LC: 200 particles/kg.; B[a]P-HC: 2000 particles/kg). Data are expressed as mean values ± standard deviation, n = 6. Different letters indicate significant differences between groups of means (*post-hoc* comparison).

organisms exposed to marine sediments with a high loading of B[a]P-spiked plastic particles (2,000 particles/Kg of sediment). Intermediate levels of accumulation ranging from 1.75 to 2.03 ng/g ww., were shown by organisms exposed to 200 particles/Kg of sediments (B[a]P-LC), with organisms treated with B[a]P spiked sediments (S-B[a]P) recording 0.76–1.75 ng/g ww. Organisms treated with low (200 particles/Kg of sediment) and high (2,000 particles/Kg of sediment) levels of virgin plastic beads did not show any accumulation pattern as B[a]P levels were always below the detection limit.

Furthermore, the accumulation of MPS in tissues of organisms was investigated at the end of the exposure and purging processes. Very few, from zero to six, plastic particles were detected in tissues of organisms exposed to all treatments dealing with plastics beads (data not presented).

Data were further analyzed to investigate possible correlations among sampling time and levels of B[a]P-spiked plastic particles among all testing conditions. Three defined groups were identified by the analysis: S-B[a]P, B[a]P-LC and B[a]P-HC ($p < 0.05$). Furthermore, a direct correlation between B[a]p tissue's accumulation and the levels of B[a]P-spiked particles; while in the meantime, a direct correlation with sampling time turned to be significant only for S-B[a]P and B[a]P-HC ($p < 0.05$).

All biological and chemical analyses were combined to perform a PCA and Hierarchical Clustering (**Figure 5**). The PCA produced a two-dimensional pattern explaining 73% of total variance. Parameters like ROx, LPO, LMS, B[a]P accumulation, DNA strand breaks and Mn mainly contributed in the discriminatory power of the first dimension (46% of explained variance. On the other hand, CAT, PhC, MtO, and Lz determined the separation along the second dimension contributing to explain the 27% of the variance.

The hierarchical clustering within the PCA pattern indicated a clear separation among control, B[a]P treated (cluster 1) and virgin plastics exposed ragworms (cluster 2). Furthermore, within cluster 1 a sub-separation among B[a]P spiked sediments and sediments spiked with B[a]P pre-incubated plastic particles was observed.

DISCUSSION

Several recent studies have confirmed that plastic micro fragments may act as vectors for organic pollutant dispersion in aquatic environments (Rios et al., 2010; da Costa et al., 2016; Lohmann, 2017). Further research has shown that plastic debris sorb, concentrate and transport POPs in the marine

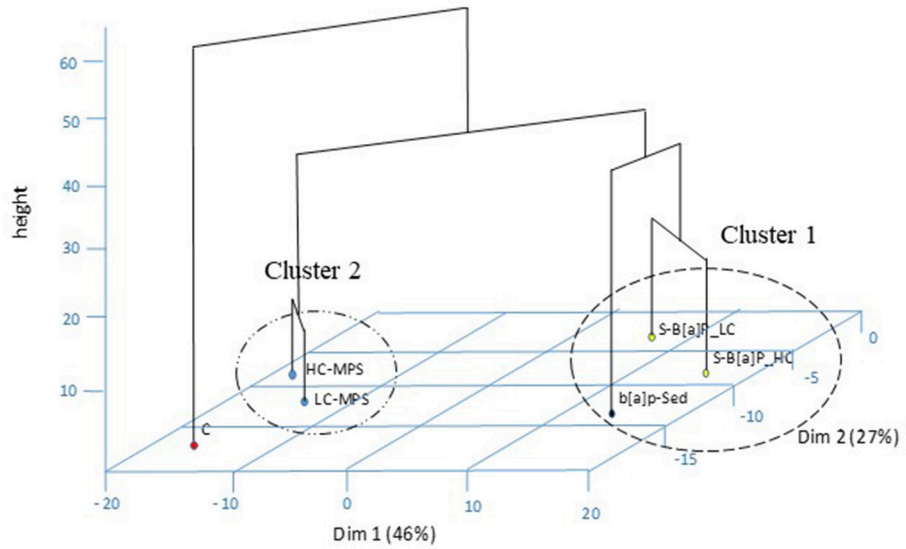

FIGURE 5 | Principal Component Analysis and hierarchical clustering on chemical and biological data in ragworms exposed to various microplastics treatments: uncontaminated sediments (C), D sediments spiked with 1 mg/L B[a]P (S-B[a]P), sediments spiked with 200 and 2000 particles/kg virgin PVC (LC-MPS, HC-MPS) and sediments with B[a]P spiked microplastic particles (B[a]P-LC: 200 particles/kg.; B[a]P-HC: 2000 particles/kg).

environment that can subsequently be ingested by marine organisms (Teuten et al., 2009; Zarfl and Matthies, 2010; Browne et al., 2013; Schirinzi et al., 2016). However, little is known about pollutant's desorption rate during digestive processes hence chemicals' potential ability to accumulate in tissue and organs and disrupt key ecophysiological processes in organism of ecological relevance (Bakir et al., 2014). In our study we focused on the benthic marine environment as sediments acts as relevant repository compartments for a broad range of organic and inorganic pollutants as well as final destination of large amounts of different plastic polymers. The present investigation aimed at unveiling the contribution of ingesting microplastic vs. sediments as vectors for pollutants to the tissues as well as its derived ecotoxicological implications to aquatic organisms. The ragworm *H. diversicolor* was selected as candidate model organism as its biological responses to several different toxicants such as PaHs, heavy metals, surfactants, pharmaceuticals, engineered nanoparticles, etc., have been extensively investigated in the past (Catalano et al., 2012; Browne et al., 2013; Buffet et al., 2014a,b; Mouneyrac et al., 2014) and thus supporting its use both in laboratory and environmental monitoring worldwide (Mouneyrac et al., 2003; Durou et al., 2007; Gomes et al., 2013). Early studies with PAHs spiked sediments using Arenicola marina pointed out that the solubilisation of organic chemicals is significantly increased in the presence of digestive fluids compared to seawater alone, thus increasing their bioavailability (Voparil and Mayer, 2000). (Rios et al., 2007; Karapanagioti et al., 2011). The observed behavior supports the potential of plastic micro litter in trapping and facilitating the distribution of pollutants in aquatic environments, as previously addressed by several studies aiming at calculating the partition coefficients of organic chemicals on various typologies of plastic polymers (Zarfl and Matthies, 2010; Bakir et al., 2012; Heskett et al., 2012; Mizukawa et al., 2013; Lee et al., 2014; Avio et al., 2015; Kedzierski et al., 2018). On the other hand, the results of the body burden analysis on ragworms exposed to the different testing conditions clearly show a direct time- and dose-dependent accumulation of B[a]P in tissues of organisms treated with pre-incubated PVC microbeads. Furthermore, the highest accumulation peaks were observed within exposures where the organic molecule model was carried by plastic beads respect to those where the chemical was carried by the sediments. To exclude any possible contribution of un-excreted contaminated particles in the bioaccumulation assessment, the residual occurrence on MPS on tissues of investigated individuals were performed allowing to exclude any significant contribution. Therefore, the obtained results with exposed ragworms provided the clear evidence that B[a]P adsorbed on contaminated microplastics can be transferred to organisms under physiological gut conditions and concentrated in tissues. Similar conclusions were drawn by Browne et al. (2013) after exposing the annelid *A. marina* to sand with PVC microplastics pre-sorbed with nonylphenol and phenanthrene.. The observed desorption realized during digestive processes has ecotoxicological implications since it has been speculated that the bioconcentrated chemicals may induce adverse biological effects in aquatic organisms potentially able to unpair key ecophysiological process (Oliveira et al., 2013; Bakir et al., 2014; Luís et al., 2015). To determine whether plastic microparticles are capable of transporting levels of pollutants able to disrupt functions of ragworms, we used established bioassays for mortality, immune system efficiency estimation, index of sub cellular functioning and oxidative stress assessment. At the environmentally realistic levels of dissolved benzo(a)pyrene no mortality was reported within all testing condition, leading to

classify any potential biological effect at sub lethal level. Similar results were reported by Browne et al. (2013) in *A. marina* exposed to nonylphenol or phenanthrene. On the other hand, at sub lethal level both treatments with sediments spiked with virgin plastics as well as those treated with benzo(a)pyrene showed a biphasic effect on phagocytosis activities of NR-conjugated particles. Since phagocytosis is frequently used as a proxy for immunocompetence in aquatic organisms (Ellis et al., 2011), data on coelomocytes phagocytic activity may reproduce the global impact on the immune system. A significant stimulation in exposures dealing with virgin plastic was observed; while in the meantime an opposite inhibition of the phagocytotic activity was reported in coelomocytes of individuals treated with sediments spiked with B[a]P pre-incubated plastic particles. Since natural sediments used in the present study were not autoclaved to preserve at best their chemical and physical properties, this could have promoted a biofilm formation in virgin particles (Zettler et al., 2013). Such phenomena already reported in marine environments by Lobelle and Cunliffe (2011) could have facilitate the transport and the exposure of bacteria and other microorganisms already naturally occurring in the sediment triggering the immune system. On the other hand, B[a]P transferred by microplastics clearly inhibited the phagocytosis activities of coelomocytes of exposed ragworms. Similar conclusions are drawn by Gopalakrishnan et al. (2011) in hemocytes of the gastropod abalone *H. diversicolor*, by Liu et al. (2014) in hemocytes of the clam *V. philippinarum* as well as by Danion et al. (2011) in hemocytes of sea bass *D. labrax* exposed to B[a]P. According to these authors the immunosuppressive effects of B[a]P could be explained by the substantial disruption of sub cellular processes and organelle's functioning of the immune system's cells in the exposed organism. On the other hand, none of the exposure conditions induced a significant release of lysozyme thus supporting some previously reported conclusions that in hemocytes of invertebrates, the assessment of a sole parameter cannot be considered as entirely illustrative of immunocompetence (Ciacci et al., 2012). On the contrary, all treatments involving B[a]P clearly affected mitochondrial activity of coelomocytes. Such response was not surprising as several authors already reported a similar reduction of mitochondrial functionality in oyster's (*P. martensii*) coelomocytes, Hep 3B human cell lines and fish haemocytes exposed to polyaromatic hydrocarbons (Yang et al., 2011; Chen et al., 2016). More interesting, even if the reduction of the mitochondrial activity was similar in organisms exposed to both benzo(a)pyrene spiked sediments and sediments spiked low levels of plastic particles pre-incubated with benzo(a)pyrene, higher alterations were reported in organisms exposed to higher levels of pre-incubated particles. Such trend demonstrates the substantial direct-like contribution of microplastics levels in facilitating the pollutants translocation to marine organisms, hence inducing increments of the exposure levels promoting the alteration of key organelles functioning. A similar trend was also observed while assessing the LMS. LMS is a regularly used sub lethal stress index acting as non-specific indicator of the adverse effects of pollutants in aquatic organisms (Viarengo et al., 2007). On the other hand, the capability of benzo(a)pyrene to alter the stability of the lysosomal membranes has been previously reported for unicellular, vertebrate and invertebrate marine organisms (Marigómez and Villacorta, 2003; Moore et al., 2006; Giannapas et al., 2012; Gomiero et al., 2012) including *H. diversicolor* (Catalano et al., 2012). Similar to the results of mitochondrial activity assessment, highest levels of LMS were observed in testing conditions where polychaete were exposed to high loadings of pre-spiked microplastics. On the other hand, the lower LMS of coelomocytes could further explain the results of the immune system as in ragworms' haemocytes the reduction of lysosomal stability is closely linked with both the impaired cellular immunity and to the over-production of prooxidant reactive oxygen species. Indeed, a pattern of biological responses similar to the results of the LMS was reported for the oxyradical production. Lysosomal membranes are highly vulnerable to oxidative stress induced by reactive oxygen species throughout a complex pattern of direct and indirect mechanisms (Regoli and Giuliani, 2014). On the other hand, early studies report that benzo(a)pyrene and more in general all polyaromatic hydrocarbons are oxidative stress promoters in marine organisms (Bouraoui et al., 2009, 2015). High values of oxyradical production were observed in coelomocytes of organisms treated with sediments spiked with high concentrations of benzo(a)pyrene pre-incubated PVC particles respect to those exposed to both low loadings of the same particles and benzo(a)pyrene spiked sediments. The oxidative stress was also evaluated at tissue level in exposed *H. diversicolor* and compared to the results observed on coelomocytes aiming at benchmarking the sensitivity, accuracy, and rapidity of the responses in floating cells vs. tissue's cells. Lipofuscins content in tissue cryosections provides an integrated thorough time indication of the membrane's lipid peroxidation levels and of oxidative stress. Oxyradical species reacts with membrane lipids thorough a sequence of lipid peroxidation reactions which produce non-degradable end-products which are continuously incorporated into the lysosomes, where they accumulate as insoluble molecules (Viarengo et al., 2007). In this study, differently to what reported for the oxyradical production in coelomocytes, only organisms exposed to pre-spiked plastic particles showed a significant accumulation of lipofuscins respect to control organisms. On the other hand, lower lipofuscins increments were observed in organisms exposed to sediments spiked directly with B(a)P. This pointing out a relatively lower contribution of the bioconcentration processes thorough ragworms' dwelling activity and skin contact respect to exposures driven by pre-spiked particles. Interestingly, lipofuscins accumulation levels turned to be significant only at the end of the experiments after 28 days of exposure while levels were homogeneously distributed among all treatments during the mid-term sampling, after 10 days of exposure showing therefore a delay of the response. On the contrary, a faster, within 10 days of exposure response, was observed while assessing the catalase activity in annelid's tissues. Catalase is a well-recognized enzyme protecting vertebrate and invertebrate organism against the pro-oxidant activity of organic chemicals like B(a)P (Regoli, 2000; Bouraoui et al., 2009). Significant increments were only observed in exposures involving pre-spiked PVC plastic particles, while no effects were observed in organism exposed to spiked sediments.

Such outcomes provide important indications about the exposure routes further supporting the major contribution of plastic particles in the bioavailability and indirectly the toxicity of the adopted organic molecule. Estimated enzymatic activities turned to be homogeneously distributed in organisms sampled at the end of the exposure experiments. As catalase together with other antioxidant enzymes are often reported having a bell-shaped trend (Viarengo et al., 2007; Gomiero et al., 2011). Therefore, a possible explanation is that effectiveness of antioxidant defenses was most likely overwhelmed by either the duration or the intensity of B[a]P pre-spiked PVC particles were adopted.

Exposure to B(a)P also determined the occurrence of several forms of genotoxicity in haemocytes of exposed ragworms. Treatments with virgin plastics did not induced any significant increment of DNA damage while significant alterations were reported within B(a)P administrated treatments. Benzo(a)pyrene like all polycyclic aromatic hydrocarbons is a well-known DNA strand breaks and micronuclei formation. Previous laboratory exposures with 0.1 and 0,5 mg/L B(a)P significantly induced DNA strand breaks and micronuclei formation in *H. diversicolor* (Catalano et al., 2012). Similar results were reported also by Sforzini et al. (2012) after exposing earthworms to 0.1, 10, and 50 ppm of B(a)P. In our study, while magnitude of the strand breaks was comparable in organisms exposed to B(a)P spiked sediments and organisms exposed to sediments mixed with low concentrations of pre-spiked microplastics, significantly higher values were reported in ragworms exposed to high concentrations of pre-spiked microplastics. On the other hand, nuclear alterations appeared more steadily distributed among all the treatments, resulting in a progressive increment of micronuclei frequency after the exposure to benzo(a)pyrene-contaminated PVC microparticles. This pattern of genotoxic effects allows to hypothesize that DNA strand breaks represent the first form of damage caused by the pro-oxidant properties of B(a)P. A more elevated prooxidant challenge caused by both sediments and PVC contaminated particles compared to virgin polymers would determine an irreversible loss of DNA integrity, leading to enhanced frequency of micronuclei in the most severe exposure conditions. On this context, oxyradical production was already shown to adversely modulate immune responses, lysosomal dysfunction, mitochondrial activity disruption in haemocytes of marine organisms exposed to polyaromatic molecules (Catalano et al., 2012; Browne et al., 2013; Avio et al., 2015). The overall evaluation of biological response and chemical analyses were performed by hierarchical clustering analysis. Such PCA based analysis provided a clear separation between organisms treated as control of exposed to sediments spiked with virgin plastics, sediments directly spiked with B(a)P and sediments spiked with pre-incubated PVC. A fist separation showed two distinctive clusters one dominated by virgin PVC particles and one dominated by B(a)P treatments. A second separation in the B(a)P cluster divided data in two sub-cluster: one represented by results of exposures with sediments spiked with pre-incubated PVC and the other grouping data from sediments spiked directly with the organic molecule model. Such separation shows as B[a]P adsorbed on contaminated microplastics can be more easily transferred to organisms under physiological gut conditions, concentrated in tissues and elicit biological than pollutants adsorbed in sediments and translocated to organism by passive bioconcentration phenomena.

CONCLUSIONS

In conclusion, the present study provides evidence that microplastic (PVC) particles adsorb the organic contaminant benzo(a)pyrene from seawater and can assist in transferring this compound to the representative benthic species (*H. diversicolor*), enhancing its rate of bioaccumulation. Compared to responses at tissue level, coelomocytes of *H. diversicolor* responded faster to B(a)P in a sensitive manner, helping to both understand the complexity of the induced stress syndrome and unveil the related toxicodynamic. Further endpoint tests of high ecological meaning not investigated within in the present study i.e., the potential impact on burrowing behavior could be considered in future investigations. The use of *H.diversicolor* for effective ecotoxicological research, both in the laboratory and field, is supported by the presented research.

ETHICS STATEMENT

The use of annelids in laboratory experiments did not require the approval of the ethical committee according to the Italian regulations.

AUTHOR CONTRIBUTIONS

AG and PS: designed the outline of the study; PS and VS: were involved in the exposure setup; PS and GP: were involved in field collection of sediments; VS and GP: conducted statistical analyses; AG: performed chemical and biological analyses supported by VS; AG and GF: wrote the manuscript with input from all other authors.

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A Review of Plastic-Associated Pressures: Cetaceans of the Mediterranean Sea and Eastern Australian Shearwaters as Case Studies

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Impacts of debris on marine fauna occur throughout the marine ecosystems, with adverse impacts documented on over 1,400 species; impacts can be divided into those arising from entanglement, and those from ingestion. Ingestion of, and entanglement in, debris has been documented in over 60% of all cetacean species. Seabirds are also impacted by debris predominately through entanglement and ingestion, with the number of species negatively impacted increasing from 138 to 174 over the past two decades. In the marine environment, cetaceans and seabirds are widely regarded as reliable sentinels due to their position near the top of the marine food web, conspicuous nature, and reliance on marine resources; for this reason, this paper is focused on seabirds and cetaceans as sentinels of ocean change. In particular, two case studies are considered in relation to different levels of environmental anthropogenic impact: the cetaceans of the Mediterranean Sea and seabirds of eastern Australia. Here we describe two recent studies used to diagnose the toxicological stress related to debris-associated pressures in cetaceans and seabirds. These studies highlight the diversity and scale of impacts being felt by marine species and the role these organisms can play in our society as charismatic sentinels of ocean health. Seabirds and marine mammals are exposed, in these key areas, to a variety of adversities that potentially decrease their survival or reproductive success. These include weather, food shortages, predators, competitors, parasites, disease, and human-induced effects and plastic pollution. Each factor affects seabirds and marine mammals in a different way, but more importantly, factors can also interact and create impacts far greater than any one factor alone. The Australian and Mediterranean case studies presented here emphasize the need to consider multiple sources of mortality when developing management plans for the conservation of vulnerable species.

Keywords: anthropogenic impacts, apex predator, cumulative pressures, marine debris, plastic pollution, seabirds, cetaceans

WILDLIFE AND PLASTIC INTERACTION: THE CASE STUDIES OF CETACEANS AND SEABIRDS

Records of interactions between anthropogenic marine debris (hereafter simply "debris") and wildlife have been increasing rapidly in recent decades. In the marine environment alone, the number of species reported to be affected by debris increased by more than 159% during 1995–2015 (from 267 to 693 species; Laist, 1997; Gall and Thompson, 2015) and has since doubled in only 2 years to around 1,465 species (http://litterbase.awi.de/interaction_detail; date accessed: 17 April 2018). The ingestion of debris, one of the most common interaction, can occur either directly when an animal mistakes an item for prey (Donnelly-Greenan et al., 2014; Lavers and Bond, 2016), indirectly through the consumption of prey that contain debris (Setälä et al., 2014; Rochman et al., 2017), or through off-loading of debris from adults to young through regurgitation (Carey, 2011).

Ingestion of debris can contribute to false feelings of satiation, blockages of the digestive track, reduction of fat stores and body condition, and can ultimately lead to death (van Franeker and Law, 2015). Worryingly, plastic items contain chemical additives such as UV stabilizers and flame retardants which are compounded at the time of manufacture (Rani et al., 2015), or adsorb to the surface of items once in the marine environment (Mato et al., 2001; Rochman et al., 2013). Such chemicals include persistent organic pollutants (POPs), like dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), and heavy metals like lead and cadmium (Massos and Turner, 2017), many of which are known neurotoxins or endocrine disruptors (Sussarellu et al., 2016). Once ingested, debris items can act as a vector, or pathway, for potentially toxic chemicals to bioaccumulate across all levels of aquatic food webs (Lavers et al., 2014; Bakir et al., 2016; Gutow et al., 2016). These contaminants become bioavailable through leaching into digestive fluids and transferring to the tissues (Tanaka et al., 2015). Biomagnification is also a concern, particularly for high-trophic predators such as seabirds, sharks, and whales (Santana et al., 2017).

In light of the increasing pressure and diversity of factors faced by marine wildlife in recent years, the main objective of this paper was to review the growing threat posed by marine plastics and associated chemicals on two charismatic groups of marine organisms: the cetaceans of the Mediterranean Sea and seabirds of eastern Australia. These two case studies were selected as the regions are geographically distinct, yet the species considered are all top predators that experience similar threats (e.g., plastic debris). Additionally, the species included in this paper are often considered umbrella species, providing valuable insights for other marine life that inhabit these threatened ecosystems.

CETACEANS AND SEA BIRDS AS SENTINELS OF OCEAN HEALTH

Indicator species or "sentinels" have been used as a tool to communicate the health of ecosystems for decades (Zacharias and Roff, 2001), and when used correctly, they can synthesize large quantities of information on pollution, fish abundance, and other natural and anthropogenic changes (Cairns, 1988; Burger and Gochfeld, 2004). In the marine environment, cetaceans and seabirds are widely regarded as reliable sentinels due to their position near the top of the marine food web, conspicuous nature, and reliance on marine resources (Furness, 1997; Durant et al., 2009; Schwacke et al., 2013; Fossi and Panti, 2017).

Sentinel species with physiology and/or diets similar to those of humans, such as cetaceans, may provide an early indication of potential adverse health effects and insight into the toxic mechanisms of a given hazardous agent (Schwacke et al., 2013). Multiple stress factors stemming from the bioaccumulation of anthropogenic contaminants combined with infectious diseases, invasive species, food depletion, and climate change pose potential hazards to both marine mammal and seabird populations worldwide (Jenssen, 2005; Poloczanska et al., 2013; Dirzo et al., 2014; BirdLife International National Audubon Society, 2015). For this reason, attention is focusing on seabirds and cetaceans as charismatic sentinels of ocean change.

Cetaceans, in particular, have similar mammalian physiology to humans and are long-lived, top predators, so they can be effective indicators for chronic or slow developing pathologies that are more difficult to detect in human populations exposed to lower levels of the same hazard (Bossart, 2011). In the past, cetaceans were not generally considered to be useful sentinel species because of their protected status and the difficulty of obtaining tissue samples. However, after several large-scale mortality events of marine mammals worldwide, concern from the scientific community has led to the establishment of a global biomonitoring programs to collect data to help elucidate temporal and geographic trends, including for plastic pollution (IWC, 2013). To this end, marine mammal tissue banks and marine mammal stranding networks were established worldwide. They have proven to be very useful tools for evaluating temporal and geographic trends of environmental exposure to contaminants, biotoxins, pathogens and recently plastic debris, using standardized collection, banking, and analysis techniques for marine mammal tissues (Schwacke et al., 2013). However, an alternative option to monitoring the health status of marine mammals, also related to the impact of plastic additives, is the relatively non-invasive method of sampling skin biopsies from free-ranging animals (Fossi and Marsili, 1997; Fossi and Panti, 2017).

The following section describes two recent studies which diagnose the toxicological stress related to plastic-associated pressures in cetaceans and seabirds (plastic pollution, bioaccumulation of anthropogenic contaminants combined with infectious diseases, food depletion, and climate change). These studies, originating from environments exhibiting contrasting levels of anthropogenic pressure, highlight the diversity and scale of impacts being felt by marine species and the role these organisms can play in our society as charismatic sentinels of ocean health.

IMPACT OF MARINE DEBRIS ON CETACEANS

Impacts of debris on marine fauna occur throughout the marine ecosystems, with adverse impacts documented on over 800 species (Gall and Thompson, 2015; Kühn et al., 2015). For marine mammals, impacts can be divided into those arising from entanglement, which can result in injury, drowning or strangulation, and those from ingestion, with pathology ranging from no discernible impact through to blockage of the digestive tract, suffocation and starvation (Sheavly and Register, 2007). Sub-lethal effects may compromise feeding and associated malnutrition, disease and reduced reproduction, growth and longevity (Moore et al., 2013). New data suggests when the dimension of the items ingested by marine fauna range from millimeter to nanometer in size (i.e., micro- debris 1 μm-5 mm and nano-plastics <1 μm, GESAMP, 2016), this can lead to inflammation, damage of the tissues at the cellular level, or altered molecular pathways (Mattsson et al., 2015, 2017; Pedà et al., 2016) . Baulch and Perry (2014) and Kühn et al. (2015) reviewed the data on plastic ingestion and entanglement rates available for cetaceans, showing an increase in the number of cases being reported over the last five decades. A total of 130 papers/documents were published from 1965 to January 2018, 44 on entanglement and 86 on ingestion of debris by cetaceans. Only 2 out of the 13 cetacean families analyzed have not interacted with debris, and ingestion appears to be the most common, occurring in over 58% of all cetacean species, including species employing a variety of feeding techniques throughout the water column (Fossi et al., 2018a; **Figure 1**).

In contrast, entanglement events have only been documented in ∼30% of cetacean species (**Figure 1**). The majority of entanglements for cetaceans are in ghost or active fishing gear (Baulch and Perry, 2014). Cetaceans tend be entangled around their neck, flippers and flukes (Moore et al., 2013; van der Hoop et al., 2014).

However, for the ingestion of debris, the number of records does not reflect the magnitude of the issue, due to low detection rate and difficulty in retrieving and analyzing specimens. Sixty-three percent of the 89 species of cetaceans (excluding the possibly extinct *Lipotes vexillifer* according to Committee on Taxonomy 2017) have been reported to be affected by debris. Items ingested are most commonly plastic (46% of all items ingested) and range in size from small fragments (<5 mm, Besseling et al., 2015; Lusher et al., 2018) to large sheets of plastic and netting over one meter long (Jacobsen et al., 2010; de Stephanis et al., 2013). However, globally, the paucity and homogeneity of data prevented a robust identification of whether, at a species level, there are certain cetacean species particularly prone to ingesting debris. This is mainly due to the difficulties in performing such analysis in these species and the lack of harmonized and standard protocols (e.g., many entanglement events or cases of debris ingestion are not reported). Seventy per cent of the documents analyzed were published after 2000, although only in the last few years were standardized protocols applied, and this can affect the reliability of the results reported.

Ingestion of Microplastics by Cetaceans

The study of microplastic ingestion by cetaceans is a challenging task, due to the difficulty in obtaining accurate samples during necropsies and analyzing large volumes (e.g., from large cetaceans). Few studies have directly identified microplastics in the digestive tracts of stranded cetaceans. Applying standard protocols for the detection and identification of microplastics in the digestive tract (Lusher et al., 2015), microplastics were found throughout the stomach/intestine of seven odontontocetes species: *Ziphius cavirostris*, *Delphinus delphis*, *Stenella coeruleaolba*, *Phocoena phocoena*, *Orcinus orca*, and *Tursiops truncatus* (Lusher et al., 2018; van Franeker et al., 2018). Only one study on Mysticetes, a stranded humpback whale (*Megaptera novaeangliae*), recorded the presence of microplastic in its intestines, including fragments, and threads (Besseling et al., 2015).

There are multiple possible routes of microplastic uptake, including direct ingestion from the water column while feeding, inhalation at the air-water interface, or via trophic transfer from prey items (IWC, 2013). Uptake of microplastics has been demonstrated in zooplankton species such as copepods and euphasiids (Kühn et al., 2015; Fossi et al., 2018b), which are some of the main prey of baleen whales and may thus be a source of secondary transfer of debris to cetaceans.

MARINE DEBRIS IMPACT ON MEDITERRANEAN CETACEANS: THE CASE STUDY OF THE MEDITERRANEAN FIN WHALE

The Mediterranean Sea is one of most affected areas by debris in the world: 115,000–1,050,000 particles/km^2 are estimated to float in the Mediterranean Sea (Fossi et al., 2012; UNEP/MAP, 2015; Suaria et al., 2016). Plastics and other polymer materials are the most common types of marine debris, representing some 80% of debris found on sea surface (Fossi et al., 2017). As larger pieces of plastic debris fragment into smaller pieces, the abundance of microplastics in marine habitats increases. Despite the recent advances made within the framework of the Barcelona Convention Regional Plan for Marine Litter Management in the Mediterranean and the EU Marine Strategy Framework Directive (Descriptor 10), there is still a long way ahead to tackle debris in the Mediterranean and reduce the risks posed to Mediterranean marine wildlife.

Recent studies suggest that debris, including micro-plastics and chemical additives (e.g., phthalates), tend to accumulate in pelagic areas in the Mediterranean (Panti et al., 2015; Pedrotti et al., 2016), indicating a potential overlap between debris accumulation areas and endangered species' feeding grounds (**Figure 2**; Fossi et al., 2016). This fact highlights the potential risks posed to endangered, threatened and endemic species of Mediterranean biodiversity. In one of the most biodiverse area of the Mediterranean Sea, the Pelagos Sanctuary, cetaceans coexist with high human pressure and are subject to a considerable amount of plastic debris, including microplastics (Collignon et al., 2014; Cózar et al., 2014).

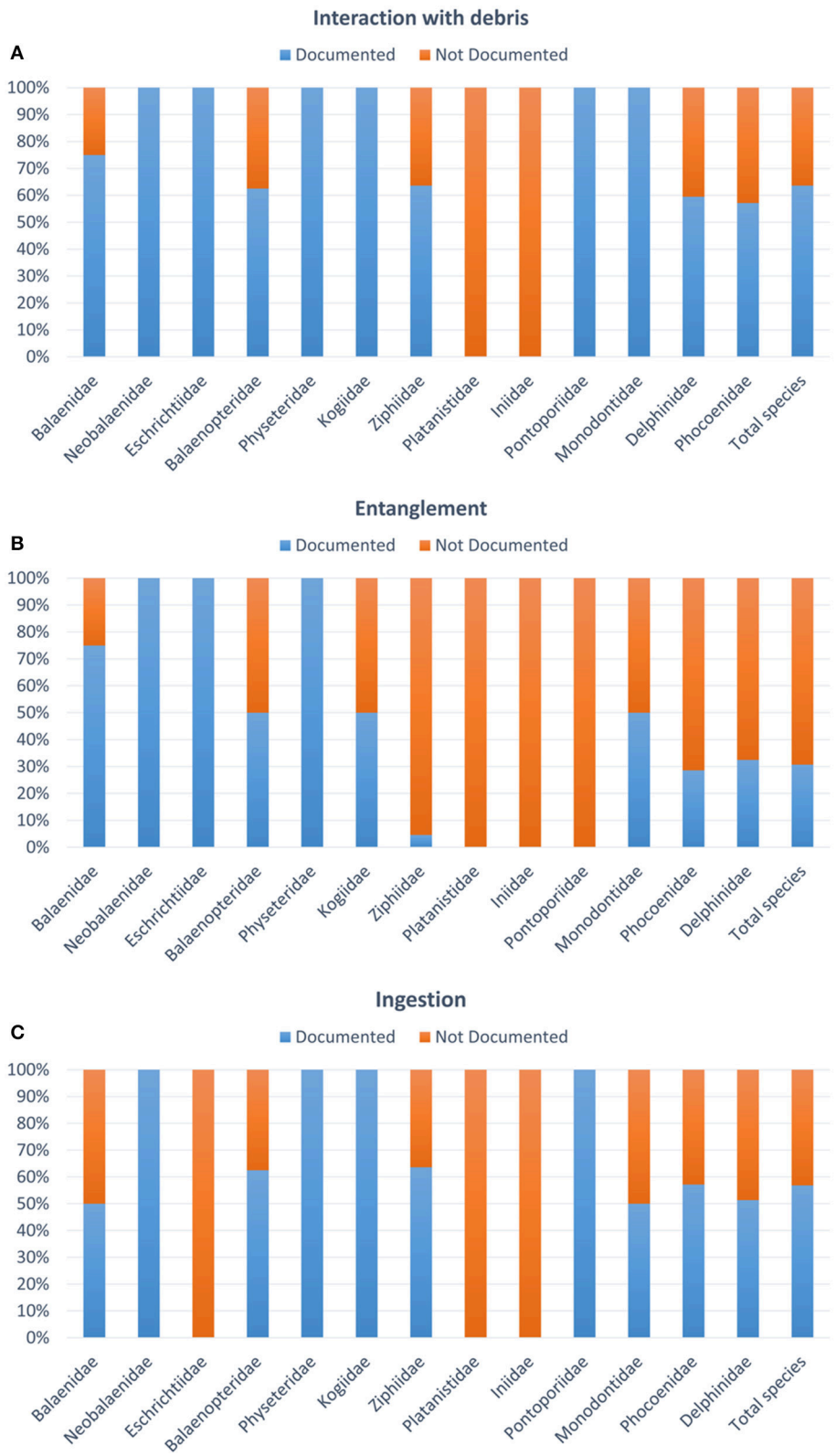

FIGURE 1 | Percentage of studied species for debris in relation to the total number of species per family in the order Cetaceans. **(A)** Interaction with debris including both entanglement and ingestion; **(B)** percentage of species with documented/not documented entanglement; **(C)** percentage of species with documented/not documented ingestion.

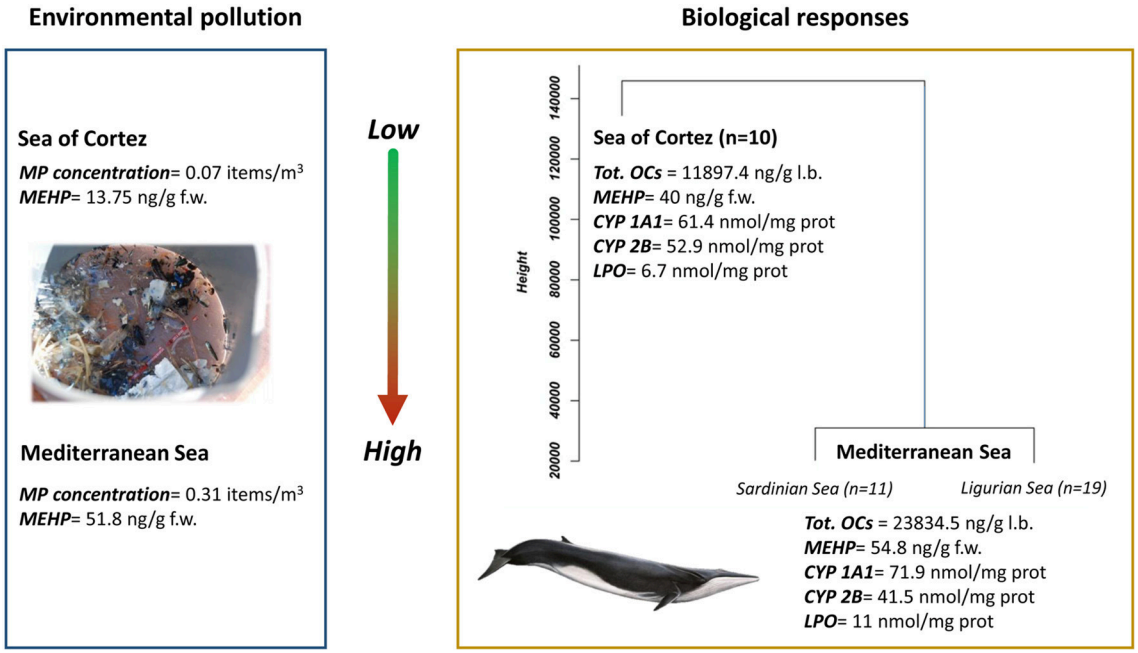

FIGURE 2 | Comparison between microplastic concentration and phthalates in zooplankton samples and the cluster dendrogram resulting from the analysis of biological parameters (mean values) measured on fin whale skin biopsies from the Sea of Cortez (Mexico) and Mediterranean Sea (Sardinian and Ligurian Sea). MP, microplastic; MEHP, mono-(2-ethylhexyl) phthalate; CYP1A1, cythocrome P450 1A1; CYP2B, cythocrome P450 2B; LPO, lipid peroxidation; OCs: organochlorine compounds (modified from Fossi et al., 2016).

Fin whales (*Balaenoptera physalus*) forage on the dense aggregations of krill in the water column and near the surface, engulfing an average of 71 m^3 of water per mouthful (Goldbogen et al., 2007). These whales are potentially exposed to the ingestion of debris as a result of their filter-feeding activity. The Mediterranean fin whale has therefore been estimated to potentially consume more than 3,000 microplastic particles per day, along with associated persistent, bioaccumulative and toxic (PBT) chemicals (Fossi et al., 2014). Using phthalates (a common plastic additive that leaches from plastic debris) as a tracer of microplastic uptake, Fossi et al. (2014) found that concentrations of the phthalate metabolite and organochlorines were markedly higher in the fin whale compared to another filter-feeders living in the same area, the basking shark (*Cetorhinus maximus*). The authors attributed this to a difference in the total plankton consumed daily and excretory activity of the fin whales, in particular, the potential excretion of such contaminants through the gills in fish vs. bioaccumulation in adipose tissue in cetaceans. Particularly high levels of microplastics have been documented in the Ligurian Sea, the summer feeding ground of the Mediterranean fin whale, in the same order of magnitude as the North Pacific Gyre (Cózar et al., 2015).

The interactions between cetaceans and micro-debris items has also been investigated in free-ranging fin whales, comparing populations living in two semi-enclosed basins, the Mediterranean Sea and the Sea of Cortez (Gulf of California) (Fossi et al., 2016). Fin whales are resident both in the Mediterranean and the Sea of Cortez. As a result, fin whales are exposed to a high potential risk of micro-debris ingestion in their feeding grounds due to the ingestion of contaminated prey and the direct ingestion of floating debris items. This species can therefore function as a critical indicator of the microplastic contamination across an entire basin (Fossi and Panti, 2017). In this case study, a considerably higher abundance of micro-debris and plastic additives were demonstrated in zooplankton samples from the Pelagos Sanctuary of the Mediterranean Sea compared to samples from the Sea of Cortez.

Given the abundance of plastics in the Mediterranean environment (Fossi et al., 2016), high concentrations of PBT chemicals, and biomarker responses detected in the biopsies of Mediterranean whales compared to whales inhabiting the Sea of Cortez, the exposure of Mediterranean whales to micro-debris because of direct ingestion and consumption of contaminated prey appears to pose a major threat to the health of fin whales in this region. The temporal and regional ecotoxicological differences support the hypothesis that the fin whale is as a large-scale indicator of the impact of microplastics and related contaminants in pelagic environments, as well as a sentinel of the integrity of the marine food chain on the basin scale.

In a recent paper, Fossi et al. (2017) investigated the possible overlap between micro-debris, meso-debris (from 5 to 25 mm) and macro-debris (>25 mm) accumulation areas and the fin whale feeding grounds in the pelagic Specially Protected Area of Mediterranean Importance, the Pelagos Sanctuary. Models of ocean circulation and potential fin whale habitat were merged to compare debris accumulation with the presence of whales. Field data on the abundance of micro-, meso-, and macro-debris, and on the presence of cetaceans were collected simultaneously. The resulting data were compared, as a multi-layer, with the simulated distribution of plastic concentration and the whale habitat model.

Field and model observations on marine debris distribution and accumulation areas overlapped the fin whale feeding habitat, paving the way for a risk assessment of fin whale exposure to microplastics. The approaches used in this paper, and by Darmon et al. (2017) for sea turtles predict where species will be the most affected by plastic debris, enabling the identification of sensitive areas for species-specific ingestion to be defined, and providing a basis for the mapping of areas to be protected. Based on data or outputs from models on both macro- or micro-plastics, and species distribution, from plankton to large vertebrates, the same approach could be largely used to predict areas where the risk of ingestion occurs and the possible consequences on biodiversity.

IMPACT OF MARINE DEBRIS ON SEABIRDS

Seabirds are also impacted by debris through entanglement and ingestion, with the number of species negatively impacted increasing from 138 to 174 over the past two decades (Laist, 1997; Gall and Thompson, 2015). The considerable threat plastic poses to marine biodiversity has led to it being recognized as a problem at international, national and regional levels (e.g., Australian Threat Abatement Plan for the Impacts of Marine Debris on Vertebrate Marine Life, 2009). The pervasive and ubiquitous nature of plastic debris has also led to the recommendation that plastic be listed as hazardous waste (Rochman et al., 2013). In Australia, at least seven species of pelagic seabird (Family Diomedeidae and Procellariidae) have either a significant proportion of their breeding population, or important foraging areas, located in this region. Of these, six (86%) species are known to ingest plastic debris (Ryan, 1987; Carey, 2011; Verlis et al., 2013), one of which—the Flesh-footed Shearwater (*Ardenna carneipes*)—is perhaps the most heavily impacted seabird, globally (Lavers et al., 2014).

THE CASE STUDY OF MARINE DEBRIS AND ASSOCIATED CHEMICALS IN FLESH-FOOTED SHEARWATERS

For some seabird species, foraging within debris accumulation zones (e.g., the North Pacific subtropical gyre near Hawaii) has been shown to positively influence the volume of debris consumed by these birds (Young et al., 2009), suggesting individuals that forage in more pristine areas may experience lower risk of debris-associated impacts. This pattern has also been observed for fin whales (Fossi et al., 2016). However, an exception to this is the Flesh-footed Shearwater.

Ingestion of Plastic by Flesh-Footed Shearwaters

The world's largest population of Flesh-footed Shearwaters (∼16,000 pairs; Reid et al., 2013) breeds on the remote, UNESCO World Heritage listed Lord Howe Island, New South Wales (31.5°S, 159.1°E) with adult birds foraging almost exclusively in the central Tasman Sea off eastern Australia (Reid, 2010). These birds exhibit some of the highest debris ingestion rates of any marine vertebrate (90% of birds contain an average of 17 pieces weighing ∼3 g; Lavers et al., 2014), which suggests there may be significant quantities of debris floating within the east Australian marine environment. However, compared to the North Pacific Gyre which is estimated to contain ∼334,200 items km^2 (Moore et al., 2001), the Tasman Sea is relatively pristine with only 248–3,711 items km^2 recorded during recent surveys (Rudduck et al., 2017).

The ingestion of debris by Flesh-footed Shearwaters, and associated exposure to chemicals, are now thought to contribute to the ongoing decline of this species (Lavers et al., 2014; Lavers, 2015). Increased quantities of ingested debris have been linked with higher concentrations of metals in shearwater fledglings (80–90 days old), and have also been shown to significantly reduce fledgling body mass and wing length, which is thought to lower juvenile survival by ∼11% (Lavers et al., 2014). Recent high resolution images generated using an X-ray fluorescent microprobe (XFM) have highlighted the distribution and uptake of elements, such as arsenic, in shearwater feathers that may have originated from ingested debris items (Howell et al., 2012, 2017). Examination of plastic items from these same birds using XMF suggests the surface is enriched with potentially hazardous elements, including arsenic and mercury, likely adsorbed from the surrounding aquatic environment (Howell et al., 2014). Once ingested, these plastic items may leach contaminants into an animal's blood stream (Tanaka et al., 2015). Preliminary data also suggest shearwater fledglings which are fed debris by parent birds may be exposed to increased concentrations of PCBs (Lewis, 2016).

Young seabirds cannot regurgitate ingested plastics for the majority of the nestling period (∼12 weeks in the Procellaridae), during which time they are at greater risk from the effects of ingested debris than adults (Carey, 2011). While adult birds offload the majority of debris items to their chicks during the breeding season, individuals may still be exposed to chemicals, either through their prey or while foraging and collecting items at-sea (i.e., short-term exposure). In Flesh-footed Shearwaters, the mean concentration of mercury in feathers (6.04 ± 4.00 ppm; Bond and Lavers, 2011) from adult birds exceeds the hypothesized toxic effect level (5 ppm; Burger, 1993) and are among the highest values recorded for any seabird.

Entanglement Records of Flesh-Footed Shearwaters

Flesh-footed Shearwaters are frequently caught in commercial and recreational fishing debris, including nets and line (Abraham et al., 2010; Reid et al., 2012, 2013). These types of interactions are typically documented as fisheries by-catch (incidental take) as the gear is in active use or recently discarded (i.e., the bird was cut free with gear still attached). Entanglements in fishing gear that was discarded long-ago (i.e., mortality attributed to marine debris, not by-catch) and other types of debris (e.g., balloons,

plastic bags) are more difficult to quantify, and are therefore underestimated, as the data are based on anecdotal accounts spread over a wide geographic area. Published records of Flesh-footed Shearwaters entangled in marine debris do not exist, but have been documented by the public (**Figure 3**).

AUSTRALIAN CETACEANS AND MEDITERRANEAN SEABIRDS

The two regions investigated in this review face similar threats, however the Mediterranean has benefitted from substantially more research on cetaceans, probably due to being surrounded by more countries/people and involving several different research institutions. In contrast, only a handful of studies are available on cetacean-debris interactions in Australia, with most data derived secondarily from studies focused on diet. Only one report documented the ingestion of small pieces of plastic material by sperm whales (Evans and Hindell, 2004). The Australian Government's Threat Abatement Plan (TAP) for marine debris documented significant numbers of cetacean entanglements in Australian waters between 1998 and 2008, unfortunately these interactions were attributed to fishing nets of unknown status (active or derelict) (Ceccarelli, 2009). As a result, it is not always clear whether these interactions should be classified as by-catch (fishing gear was active at the time of the interaction) or marine debris (inactive gear).

Remarkably, only one study has been published on seabird-plastic interactions in the Mediterranean basin which suggests 70–94% of shearwaters, 13–50% of gulls, 13% of Northern Gannets *Morus bassanus*, and 50% of Great Skua *Catharacta skua* contain plastic debris (Codina-García et al., 2013).

Seabirds and cetaceans may have a key role to informing society about the health of the oceans. In the Mediterranean Sea, cetaceans have provided valuable data as well as in Australia data on seabirds is strong, but the data should be enhanced by considering also seabirds and cetaceans in the two areas, respectively. This lack of data, underline the need to further target research efforts in the areas to understand the magnitude of the issue of plastic pollution on seabirds and cetaceans in the Australian and Mediterranean waters.

FIGURE 3 | Adult Flesh-footed Shearwater entangled in a mylar foil balloon, Coogee Beach, New South Wales, 7 April 2018 (photo credit: Marina DeBris).

THE "MULTIPLE-STRESS" CONCEPT

Seabirds and marine mammals are exposed to a variety of adversities that potentially decrease their survival or reproductive success. These include weather, food shortages, predators, competitors, parasites, disease, and human-induced effects (Fair and Becker, 2000; Weimerskirch, 2002). Each factor affects seabirds and marine mammals in a different way, but more importantly, factors can also interact and create impacts far greater than any one factor alone (Burger and Gochfield, 1994; Dirzo et al., 2014). For example, the cumulative impact of mortality from hunting, oiling, predation, and habitat destruction is reducing the ability of many sub-Arctic seabird populations to grow, leaving populations with little room to buffer against increased mortality in poor years (Piatt and Naslund, 1995; Wiese et al., 2004; Lavers et al., 2008). In remote parts of the Southern Ocean, other seabirds don't appear to be experiencing any less pressure. For example, the highly constrained foraging range of penguins (compared to the Procellariiformes) means the health of these birds mirror local conditions. Recent data suggest pollution, habitat loss, fishing, and climate change are all critical threats to penguin populations (Finger et al., 2015; Trathan et al., 2015).

These same anthropogenic pressures are potentially affecting the population stability of cetaceans and other large marine organisms in the Mediterranean Sea where areas with high species diversity overlap with areas that suffer cumulative anthropogenic threats (Coll et al., 2012). Analyzing persistent bioaccumulative and toxic chemicals (e.g., DDT, PCBs), diagnostic markers of exposure to anthropogenic contaminants (e.g., protein and gene expression levels of cytochrome P450) and the genetic variation using microsatellite markers, a statistical model revealed that, among three different subpopulations of striped dolphin, an association between genetic diversity and toxicological stress exists, confirming genetic variability is linked to resilience (Panti et al., 2011; Fossi et al., 2013). Dolphins with lower heterozygosis exhibited significantly higher contaminant loads (50% originated from the Pelagos Sanctuary). Application of the model provided an outline of the toxicological status of striped dolphin populations and represented a potential tool for the monitoring and conservation of cetacean biodiversity and their habitats. These results underline that in areas where several anthropogenic activities place pressure on populations, top predators are exposed to multiple stressors including plastic pollution, and these species may function as useful sentinels of the consequences for the food chain and human health.

Another example of cumulative stress in Mediterranean cetaceans comes from a recent mass stranding of seven sperm whales along the Adriatic coast (Mazzariol et al., 2011). Necropsy suggested a plethora of different pressure may have caused the mass stranding. Sperm whales presented lymphoid cell depletion acute opportunistic bacterial infections of the respiratory tract *T. gondii* was detected within a wide range of tissue. Concerning the chemical analysis the total hepatic and renal mercury concentrations, were higher than those measured in sperm whales involved in other mass strandings and also the levels of PCBs and DDT metabolites in tissue samples from of all the

seven whales. The analysis of the stomach content highlight the presence of different parasites and debris, including fishing gear and hooks, rope, and several plastic items (Mazzariol et al., 2011).

CONCLUSIONS

The Australian and Mediterranean case studies presented here emphasize the need to consider other species and multiple sources of mortality (by-catch, ship strikes, zoonosis, etc.) when developing management plans for the conservation of ecosystems and biodiversity. For marine debris, there is currently a lack of evidence of impact at the level of the population (Rochman et al., 2016) for all but a handful of species (an exception is Lavers et al., 2014 where ingestion of debris is thought to reduce juvenile survival by ∼11%). Across species, if factors driving populations trends are not identified or are ignored, effort may be concentrated on sources of mortality which are not the most crucial for the population, resulting in ineffective mitigation. While most anthropogenic pressures on the marine environment are increasing in scope and severity, for some species, the removal of certain threats has been offset by increased pressure from another emerging on the scene. This appears to be the case for Australia's Flesh-footed Shearwater, with populations continuing to decline despite significant reductions in domestic bycatch (Reid et al., 2012). The impacts of lastic debris and associated chemicals are now thought to be driving population trends, at least on Lord Howe Island (Lavers et al., 2014). This outcome highlights the importance of continually re-assessing parameters of highest importance when managing wild species (Bottrill et al., 2009).

AUTHOR CONTRIBUTIONS

MF coordinated the manuscript, put together the different scientists and wrote the manuscript. CP wrote, commented on and edited the manuscript. MB wrote, commented on and made the figures. JL wrote and proof-edited the paper.

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The Occurrence of Paraffin and Other Petroleum Waxes in the Marine Environment: A Review of the Current Legislative Framework and Shipping Operational Practices

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Among the various materials that make up marine debris, lumps of petroleum waxes such as paraffin and microcrystalline wax, are regularly found on beaches worldwide, although not included in the current definition of marine litter. Ingestion by marine organisms is occasionally documented in the scientific literature and mass beaching events are frequently reported along the European coasts, with obvious detrimental consequences to the local communities that have to manage the clean-up and disposal of this substance. According to Annex II of the MARPOL regulation, petroleum waxes are classified as "high viscosity, solidifying, and persistent floating products," whose discharge at sea of tank-washing residues is strictly regulated, but currently permitted within certain limits. Starting from the description of a large stranding event occurred along the Italian coasts in 2017, we review the existing knowledge and regulatory framework and urge the relevant authorities to address this issue, showing that wax pollution is creating evident damages to the European coastal municipalities. Pending further investigations on the potential hazard that this kind of pollution is posing to marine ecosystems, we suggest a careful and more stringent revision of the policies regulating discharges of these products at sea.

Keywords: marine litter, paraffin wax, policy, MARPOL, annex II, pollution, petroleum waxes, microcrystalline wax

1. INTRODUCTION

The global production of industrial waxes currently amounts to 4.79 million tons, with a market size valued at 6.7 billion USD and an expected annual growth of 1.5–2%, driven mainly by increasing demand for single-use packaging applications (Wei, 2012; Grand View Research, Inc., 2017). The market is mainly segmented into bio-based, synthetic, and fossil-based waxes. Fossil-based waxes comprise mineral waxes (such as montan wax derived from coal and ozokerite) and petroleum waxes (petrolatum, paraffin, and microcrystalline waxes), also known as hydrocarbon waxes. Other types of industrial wax include synthetic waxes produced by a series of chemical reactions (e.g., through the Fischer-Tropsch process, or using alpha-olefin and polyethylene waxes), waxes of animal origin (e.g., beeswax, chinese wax, tallow, lanolin) and vegetable waxes, such as candelilla,

carnauba, castor, and soy wax (Bennett, 1963; Casadei et al., 2010). Petroleum waxes are by far the most important in terms of volume produced and economic impact, accounting for 85–90% of the global wax consumption, although demand for synthetic and vegetable waxes has been growing steadily in recent years (Kline & Company, Inc., 2010).

Petroleum waxes are crude oil derivatives primarily consisting of a mixture of hydrocarbons with typical melting points comprised between 35 and 95°C (Buchler and Graves, 1927; Mansoori et al., 2004). They appear as creamy white to dark yellow or pale brown water-insoluble substances, generally solid at room temperature but highly viscous at moderate temperatures (Moore & Munger Marketing Inc., 1995). Most producers offer three distinct types of petroleum waxes: paraffin waxes, which are characterized by large, well formed crystals; microcrystalline waxes (also known as microwax), which have higher melting points and smaller irregular crystals and petrolatum, also known as petroleum jelly or jelly wax (Warth, 1956; Petersson et al., 2008). Paraffin waxes are typically obtained as a by-product during the production of lubricating oils and mainly consist of saturated long-chain hydrocarbons, ranging from C_{18} to C_{60}, and predominantly greater than C_{25} (Cottom, 2000). Microcrystalline waxes instead, are produced by de-oiling petrolatum, as part of the petroleum refining process and contain a much higher percentage of branched and naphthenic hydrocarbons, in addition to normal alkanes (Srivastava et al., 1993; Mansoori et al., 2004). They have a higher molecular weight and are generally darker, more viscous, denser, tackier and more elastic than pure paraffins (Cottom, 2000). Most of the petroleum waxes sold commercially however, are a mixture of "normal" (straight chained) and "iso" (or branched) alkanes with varying levels of purity. Fully refined waxes have oil contents < 0.5–0.75%. Semi-refined waxes have up to 1.5–3% oil, while scale and slack waxes have even more oil, up to a common maximum of 35–40% (Freund et al., 1983; Kumar et al., 2007). These waxes are thermoplastic materials but, due to their relatively low molecular weight, they are normally not considered to be plastics or polymers. Depending on the formulation, on the crude-oil source and on the method and degree of refinement, petroleum waxes can range from being soft enough to be molded by hand to being brittle and hard enough to be carved with rotary tools (Dwivedi et al., 2017). Their high versatility and low reactivity makes them suitable for a myriad of industrial applications. Candles production is by far the most important segment, currently accounting for around 40–50% of the global market revenue (Wei, 2012; Kline & Company, Inc., 2010). Other important applications include coatings for wood, paper, packaging and food products, cosmetics, chewing-gums, crayons, home-care products, pharmaceuticals, polishes, hot-melt adhesives, surf and ski waxes, electrical insulators and tires, plastic and rubber additives—such as plasticizers, binders, flame retardants, and rheology modifiers (Nasser, 1999; Mansoori et al., 2004; Kumar et al., 2005).

Each year, large volumes of fully refined or unrefined (slack) petroleum wax are transported in bulk by tankers and cargo ships around the world (Wei, 2012). To be loaded or discharged in liquid form, certain products must be kept at temperatures above their melting point and to do so, vessels are often equipped with cargo heating coils. After unloading, certain amounts of product will typically remain on the bottom of the cargo tanks or crystallize against the bulkheads and interior equipments, forming the so-called "stripping" residuals. Unrefined crude oils, also contain substantial amounts of paraffin waxes, which being highly viscous, tend to crystallize and adhere to pumps, piping, and tank walls during loading and unloading operations—a phenomenon known as "clingage", causing every year losses of billions of dollars to the petroleum industry (Sanjay et al., 1995; Mansoori et al., 2004). The amount of these residuals is generally in the order of a few hundred liters per tank and is mainly related to the age and design of the ship, as well as to the efficiency of the stripping system and to the position of the suction intakes. Tanks are usually cleaned manually by the crew or automatically by rotary-jet cleaning systems using steam, hot water or chemical solvents (Sea-Mer Asso, 2017). The residuals can then be treated by port reception facilities, or be discharged at sea under certain conditions.

Operational practices are regulated by the Annex II of the International Convention for the Prevention of Pollution from Ships (MARPOL 73/78) issued by the International Maritime Organization (IMO), which contains regulations for the control of pollution by Noxious Liquid Substances (NLS) transported in bulk, defining the standards and principles which must be adopted to discharge harmful substances at sea, as well the standards for controlling such releases. According to the latest version of Annex II, entered into force in 2007, petroleum waxes are classified as "high viscosity and solidifying substances" that fall within the intermediate pollution category Y: *"Noxious Liquid Substances which, if discharged into the sea from tank cleaning or deballasting operations, are deemed to present a hazard to either marine resources or human health or cause harm to amenities or other legitimate uses of the sea and therefore justify a limitation on the quality and quantity of the discharge into the marine environment."* The other two pollution categories are Category X (NLS presenting a major hazard to the marine environment whose discharge at sea is completely prohibited) and Category Z (NLS presenting a minor hazard to the marine environment therefore justifying less stringent discharge regulations).

When unloading category Y high-viscosity or solidifying substances (i.e., with a viscosity equal to or greater than 50 mPa·s at 20°C and/or a melting point greater than or equal to 0°C), MARPOL Annex II provides that the ship tanks should be emptied (stripped) to the maximum extent possible, a tank prewash procedure shall then be applied and the residue/water mixture generated during the prewash shall be discharged to a reception facility at the port of unloading—or to another port provided that it has been confirmed in writing that an adequate facility is available—without the need of achieving any final concentration in the effluents, unlike what happens for category X substances (Regulation 13, Paragraph 7.1.3). Therefore, within the so called "stripping limits"—i.e., between 75 and 300 liters + 50 liters tolerance, depending on the ship's age and category—remaining cargo residues can be legally discharged at sea, provided that the discharge is made below the waterline, en route at a minimum speed of

7 knots and at least 12 nautical miles from the nearest land and in water depths exceeding 25 m. The only exception to this is the Antarctic region where any discharge of NLS or mixtures containing such substances is prohibited. No other region is listed as special area for discharge restrictions under the provisions of Annex II, therefore in particularly sensitive regions such as the Mediterranean Sea, the Arctic Ocean, the North and the Baltic Seas, there is no general ban on the discharge of waxy residuals, contrary to what is foreseen for plastic waste and ship's garbage for instance, under MARPOL Annex V.

2. WAX IN THE MARINE ENVIRONMENT

Currently, there are no reliable estimates on the amount of petroleum waxes being discharged at sea every year. Big pollution events were already reported in the early 1990s along the coasts of Netherlands, Denmark, and Germany (Dahlmann et al., 1994). In some cases, up to 8 tons of paraffin wax were released by a single tank-washing and stranded on a German beach in 1992, while in 1993 a single pollution event was estimated to have killed more than 2,000 birds in the northern part of the Netherlands, although it is not clear if the killing was caused by paraffin wax or palm oil (Dahlmann et al., 1994). During the same year, Scholten (1993) reported that between 10,000 and 20,000 seabirds (mainly guillemots and, to a lesser extent auks and kittiwakes) stranded in the North Sea due to impairment of the bird's feathers protective layer caused by pollution with a refined liquid paraffin, mainly composed of C_{14}–C_{20} alkanes.

As reported in UEG (2014), "complex pollution incidents" are defined as large spills involving at least 30 m^3 of material or alternatively, impacting at least 10 km of coastline. Poorly reported in the scientific literature, these large beaching events are frequently disclosed by local and national newspapers across Europe. A web search revealed for instance that in recent years large strandings of yellow or white waxy materials, often but not always confirmed as petroleum wax, occurred in the Baltic Sea (May 2010), North Yorkshire (May 2017), Northern France (several events in 2016 and two events in July and October 2017), Suffolk and Norfolk (May 2011), Netherlands (Multiple events in 2007, 2015, 2016, and 2017), Italy (2012, 2014, October 2016 and June and November 2017), Denmark (March, June, and August 2017) and 7 more accidents occurred in Germany between 2007 and 2014 according to UEG (2014), with most of these records being in the order of few tonnes of beached wax stranded along tens or hundreds of kilometers of coastline. According to a report released from KIMO (2017), at least 91 incidents occurred between 2012 and 2016 in 5 Northern European countries (Denmark, Germany, The Netherlands, Sweden, and France), costing well over 1.4 million euros to clean up. The German Federal Maritime and Hydrographic Agency (BSH) also reported that paraffin pieces were found in 24 of 33 trawl nets performed in the North Sea, but that no estimates were available about the total amount of paraffin wax currently floating in the North Sea. The only other certain record pertaining to off-shore waters came in 2013, when several fragments of a white paraffinic wax were found in a sample collected in the Southern Adriatic Sea during a survey for floating microplastics (Suaria et al., 2016).

Lumps and pieces of wax are also commonly found during beach litter surveys, despite chemical identification of these materials is rarely provided by the authors. The first record dates back to the 1960's on a Southern Californian beach (Ludwig and Carter, 1961). Since then, the occurrence of wax has been reported from beaches in Panama (Garrity and Levings, 1993), South Korea (Jang et al., 2014), Brazil (Leite et al., 2014), Spain (Williams et al., 2016), Italy (Peirano, A., pers. comm. pertaining to 2017), Portugal (Zhukov, 2017), Bulgaria (Simeonova et al., 2017), South Africa (Lamprecht, 2013), Germany (Liebezeit, 2008), Hawaii (Moore C. J., pers. comm. pertaining to 2006), Russia (Chubarenko et al., 2018), and even from the shores of remote islands such as the Pitcairn archipelago (Benton, 1995), the sub-antarctic Macquarie Island (Slip and Burton, 1989), and Tristan da Cunha, the most remote inhabited island of the world (Ryan, 1987).

Three groups of "Paraffin or wax pieces" (100_108 to 100_111 according to their size) are also included in the OSPAR Beach Litter Monitoring Program under the category "other pollutants" (OSPAR Commission, 2010), even if these items were later omitted from statistical analysis (Schulz et al., 2015, 2017), as "not always easily and consistently identified, and generally not considered as "litter" or "debris" but as chemical pollution" (van Franeker, 2013). Data extracted from the OSPAR Beach Litter Database (freely retrieved from https://www.mcsuk.org/ospar/), show that between 2001 and 2016, paraffin or wax pieces (visually identified as such) were found in 371 out of 2,824 litter surveys performed on 151 different beaches, with a mean estimated abundance—when the wax was present—of 14.6 items per meter of strandline (max 738 items/m). The vast majority of these items were found in the North Sea region, with most records coming from Denmark, Sweden, France, Germany, Belgium, and the Netherlands. Also, a sharp increasing trend seemed to occur during the 16 years survey. Just 8.9% of all the wax observations were related to the first 10 years of monitoring (2001–2010), while over 91% of the wax was found in the last 6 years period (2011–2016), with mean abundances ($\pm$ standard error) going from 0.41 $\pm$ 0.10 items/m (n = 1,159 surveys; max: 80 items/m) to 2.96 $\pm$ 0.64 (n = 1,665 surveys; max: 738 items/m), respectively. Wax lumps were found also in the Arctic (including Iceland and Greenland) with a maximum abundance of 9 items/m reported from a Norwegian beach in the Tromsø Region.

On a global scale, wax is generally outnumbered by the more abundant plastic items, but it can occasionally dominate the composition of beach litter. For instance, on 4 Lithuanian beaches sampled 10 times between 2014 and 2016, paraffin wax (visually determined as such) was reported to be the main polluter, accounting for 63% of all litter items, with values peaking to 70% of the total and to 94% of the micro-litter fraction <5 mm (Haseler et al., 2018). Similarly in the Russian Baltic, Esiukova (2017) showed that maximum contents of (visually distinguished) paraffin wax in sand samples can range from 0.03 to 8.66% of dry mass. Her analysis also showed that wax aggregates can concentrate microplastic items, and that inside wax lumps there are on average 31.1 $\pm$ 18.8 microplastics

per sample or 11,479 ± 10,785 items per kg of wax. As pointed out by the author, these quantities are three orders of magnitude larger than those found in the surrounding beach sediments, indicating that lightweight sticky waxes (especially crooked pieces) collected from the beach wrack lines, can act as effective accumulators of various types of contamination, including microplastics (Esiukova, 2017).

Surprisingly, although China and U.S. are the world leaders in wax production and consumption (Wei, 2012), we could not find any records of wax strandings in these two countries. It should be noted however, that during beach litter surveys—with the notable exception of the OSPAR region—wax residues are often placed in the categories "others" or "miscellaneous," as also recommended by UNEP/IOC monitoring guidelines for beach litter (Cheshire et al., 2009). For this reason, their presence is almost never explicitly mentioned in the scientific literature and their real occurrence on worldwide beaches is largely unknown.

3. BIODEGRADATION, TOXICITY, AND INGESTION BY MARINE ORGANISMS

In laboratory conditions, various degrees of biodegradability of long-chain n- and iso-alkanes, paraffin wax and polyethylene waxes have been demonstrated by various strains of bacteria and fungi (e.g., Hanstveit, 1992; Marino, 1998; Rahman et al., 2003; Kawai et al., 2004; Sood and Lal, 2008; Zahed et al., 2010; M'rassi et al., 2015; Zhang et al., 2016). No information however is currently available on the actual residence time of these substances at sea, as field studies on their actual biodegradation rates in the marine environment have never been performed. Early observations from paraffin-rich wax inclusions in beached oil—likely derived from crude oils deposits during tank cleaning operations—suggested that the half life of this waxy precipitates must be measured in terms of years, and that only few signs of degradation occur after 16 months of exposure in the marine environment (Blumer et al., 1973).

Regardless, once at sea or on the shoreline these substances can interact with marine fauna, with most of the studies concerning ingestion by seabirds. Lumps of wax and paraffin-like materials have been reported in the stomach content of northern fulmars (*Fulmarus glacialis*) from the North Sea (van Franeker et al., 2011) and from the Labrador Sea (Avery-Gomm et al., 2017), as well as in regurgitates from Black Legged Kittiwakes (*Rissa tridactyla*) and Great Cormorants (*Phalacrocorax carbo*) in Ireland (Acampora et al., 2017). Interestingly, a statistically significant increase in the ingestion of wax by Northern fulmars was found to occur from 1982 to the year 2000 in the North Sea, with paraffin-like substances being also the major category in terms of incidence and weight in ingested litter (28% incidence and mean mass of 0.54 ± 3.53 g and 2.2 ± 6.6 items per bird) (van Franeker and Meijboom, 2002). The authors in this case suggested that changes in the occurrence of a substance in the bird's stomachs would be proportional to a change in its abundance at sea. The only other available record of wax ingestion by marine organisms pertains to 2015, when a piece of wax was found in the gastroinstestinal tract of a post-hatchling loggerhead turtle (*Caretta caretta*) stranded lifeless on a South African beach (Ryan et al., 2016). However, as most of the studies are lacking chemical identification of the ingested material, realistic levels of exposure for marine populations are currently unknown.

As already reported by UEG (2014) and EFSA (2013), it is not possible to make a general statement regarding the health risks of petroleum waxes. Since they are widely used in food, packaging, cosmetics, and pharmaceutical products, their safe use for human consumption has been historically supported by a number of chronic and sub-chronic feeding studies in mice, rats, and rabbits, showing that no health hazard is present if the wax meets certain purity requirements (Shubik et al., 1962; Elder, 1984; Ekelman, 1993; EFSA, 2013), event though in later studies, some inflammatory responses and histopathological reactions were observed in certain strains of laboratory rats fed with relatively high doses of paraffin waxes (Smith et al., 1996; Griffis et al., 2010). Whereas, refined paraffins (<0.75% oil content) are generally deemed as not dangerous as they are not known to have hazardous or irritating properties, most industrial waxes have a lower level of purity and their polyaromatic hydrocarbon (PAH) content—mainly originating from the paraffin extraction process—can cause irritation to the skin and eyes (Shubik et al., 1962; Lijinsky et al., 1963; Ekelman, 1993; UEG, 2014). As a matter of fact, varying concentrations of different PAHs have been measured in petrolatum (Lijinsky et al., 1963), microcrystalline wax (Mazee et al., 1966; EFSA, 2013), paraffin wax (Mazee et al., 1966; Lau et al., 1997), and in an industrial wax washed ashore in Germany in 2012, in which case a PAHs content of 18 mg/kg was measured, far exceeding safe exposure levels for children (UEG, 2014). In addition, many of these PAHs, some cleaning agents used in the shipping industry such as perchlorethylene or trichlorethylene, as well as petrolatum and montan wax are all classified as carcinogenic by the European Union (UEG, 2014; Sea-Mer Asso, 2017). Few information however exist about the health risk posed by exposure to unrefined waxes and the ecological impacts in marine ecosystems are currently unknown, as to the best of our knowledge a rigorous environmental impact assessment has never been performed.

4. AN ITALIAN CASE STUDY

This work originates from a large-scale stranding event that took place in the Ligurian Sea (Northern Thyrrenian) between 16 and 19 of June 2017. In this occasion, more than 350 kg of yellow wax lumps were recovered by a special boat after that an aerial survey initially identified several patches of this floating substance along a 5 miles front across the northern side of Elba Island in Italy. As much as double the quantity were later recovered along a 200 km stretch of coast in Tuscany, with mean densities peaking along the water's edge to 15 kg/m^2 and 16,400 fragments/m^2, mainly comprised between 5 and 30 mm in diameter (with the most abundant being <10 mm) (**Figure 1**). In those same days, beaching of the same yellow material were reported from neighboring regions such as Liguria

FIGURE 1 | Pictures of the yellow wax lumps found on the Beach in Migliarino-San Rossore National Park (Tuscany, Italy) in June 2017, later revealed to be a microcrystalline wax. The top-right panel shows the extent of the beaching event which likewise involved hundreds of kilometers of coastline.

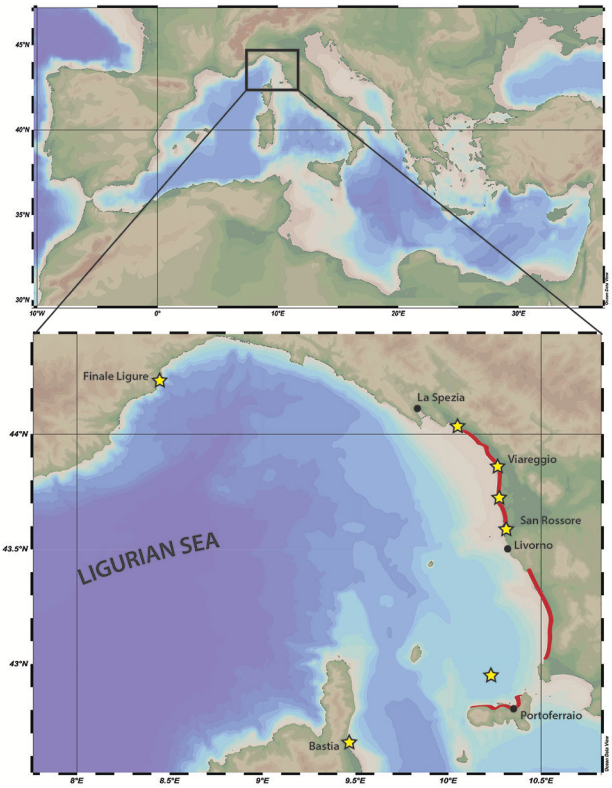

FIGURE 2 | Map showing the location of the pollution event occurred in the Ligurian Sea between 16 and 19 of June 2017. The yellow stars indicate the locations where the wax was initially found and collected, while the red lines indicate the portions of the coastline affected by the spill.

and Corsica (**Figure 2**), demonstrating the rapid dispersion of this substance through wind drift and surface currents. Cleaning was largely paid and organized by the local authorities and private beach owners, as the event occurred during high touristic season. In the same area, a previous beaching event occurred in 2012, when two metric tons of a white paraffinic wax were scooped by draining pumps. In that case, the investigations carried out by the competent authorities ascertained that the material had been discharged from a ship during tank-cleaning operations. Local authorities subsequently reported that the clean-up intervention costed around 20,000 euros to the involved municipalities, highlighting how such events imply considerable economic costs to local businesses and tax-payers which are not indemnified by the polluters and improperly burden the local communities.

We collected and characterized the beached substance through FT-IR analysis (Fourier-Transform Infrared Spectroscopy), which identified the material as microcrystalline wax (CAS Registry Number: 63231-60-7) with a hit quality with reference spectra of 80.6% (**Figure 3**). Further gas-chromatographic determinations made by ARPAT laboratories in Livorno (Regional Agency for the Environmental Protection of Tuscany), classified the substance as a paraffinic or polyethylenic wax and excluded acute toxicity and the presence of volatile organic compounds and inorganic contaminants (heavy metals); such as to exclude its classification as hazardous waste. It was then concluded that the stranded material should be ascribed to urban waste and disposed of accordingly. Cleaning however, was carried out using mechanical equipment such as sieving machines and agricultural harrows, or by hand using wide meshed nets, largely resulting in the burial and displacement of many residuals and leading to a further fragmentation of the material, which is likely to represent an increased availability for local fauna and avifauna. A monitoring activity, part of a larger annual sampling program, involving several beaches in marine protected areas of the italian coast (Merlino et al., 2015), revealed that in September, three months after cleaning of the beach from local authorities, values up to 4,740 items/m^2 were still found in Viareggio beach, with several fragments observed also in the back-shore as well as in the dune areas.

It is worth noting that this accident occurred in the hearth of the Pelagos Sanctuary for Mediterranean Marine Mammals, which is an important feeding ground for baleen whales and the only international high-seas protected area in the world (Notarbartolo-di Sciara et al., 2008). Additionally, large amounts of wax stranded along a portion of coastline comprised within the "Migliarino, San Rossore, Massaciuccoli Natural Park," designated by UNESCO as Biosphere Reserve and in close proximity to the Marine Protected Area of "Secche della Meloria." Being a protected area, this beach is not subject to normal cleaning operations and the wax here has never been removed. During a visit carried out in November 2017, 5 months after the beaching event, substantial quantities of wax were still found on this beach. The fragments in this case were more rounded, suggesting a levigating effect of the sea and waves on the shape of the particles.

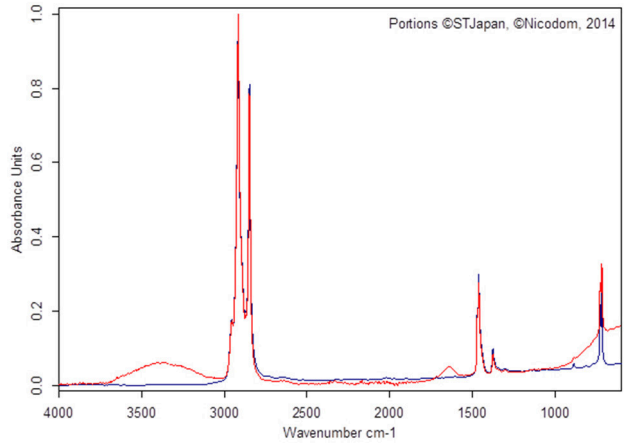

FIGURE 3 | FT-IR spectra of the material collected on the beach in Pisa-San Rossore (in red). Comparison with reference spectra (in blue) confirmed the material as a microcrystalline wax (CAS No. 63231-60-7) with a hit-quality of 80.6%. The analysis was performed on 26/10/2017 with a LUMOS FT-IR Microscope (Bruker Optics Inc.) and processed with OPUS software.

5. REVIEW OF THE POLICY OPTIONS

There is ongoing discussion at the International Maritime Organization (IMO) to strengthen discharge rules for certain liquid chemicals in particular high-viscosity and persistent floating products, like petroleum waxes, and vegetable oils. In 2004 *"paraffins and hydrocarbon waxes"* were reclassified by the GESAMP Working Group on the Evaluation of the Hazards of Harmful Substances carried by ships (EHS 40/9), but their hazard category remained substantially unchanged. More recently, proposals for amendments to MARPOL Annex II have been made to the IMO Marine Environment Protection Committee in May 2015 (MEPC 68). The Committee agreed to include in the agenda of the Pollution Prevention and Response Sub-Committee (PPR) the *"Review of MARPOL Annex II and the IBC code requirements (International Code for the Construction and Equipment of Ships carrying Dangerous Chemicals in Bulk), that have an impact on cargo residues and tank washings of high viscosity, solidifying and persistent floating products and associated definitions and preparation of amendments."* Discussions included the revision of the definition of high-viscosity cargoes to widen its application, increased tank pre-washing and the use of shore reception facilities.

In January 2017, the fourth session of the Pollution Prevention and Response Sub-Committee (PPR 4) requested GESAMP/EHS and the working group on the Evaluation of Safety and Pollution Hazards (ESPH 23) to handle amendments to Annex II, proposing a target completion date scheduled for 2018. It concurred however about the inclusion of a definition for persistent floaters in the Annex II; the requirements for the discharge of category Y residues and a means of identifying a group of persistent floaters for which a prewash would be required. The group noted that the lack of adequate port reception facilities was an ongoing concern and emphasized that this issue would need to be carefully considered in the development of new amendments to Annex II. PPR 4 also suggested that the most practical way forward would be a mix of the geographical and product-based approaches, i.e., *"The identification of a geographical region of application, based on the known area of impact, and the establishment of a provisional list of products, or groupings of products, based on those substances that were known to have been discharged and had resulted in the impacts on beaches in the North and Baltic Sea coastal States."*

ESPH 23 was held in October 2017 and continued its work in drafting amendments to the Annex II. The debate mainly focused on the definition of persistent floaters, now defined as *"Slick forming substances with density ≤ sea water (1,025 kg/m^3 at 20 °C); vapor pressure ≤ 0.3 kPa; solubility ≤ 0.1% for liquids and ≤ 10% for solids and kinematic viscosity > 10 cSt at 20°C,"* and on the creation of a list of such products which would require specific prewash and carriage requirements. GESAMP/EHS 54 also agreed on a substantial revision of the entries and compositional characteristics of different kind of paraffins in their composite list and to update their risk categories and carriage requirements. One of the greatest problem was in fact the great blur on the commercial names under which paraffins and other petroleum waxes are being transported. Hence, ESPH established that paraffins should now be grouped under four main categories, with the first two categories still ascribed to risk category Y and the latter two being classified in category X:

- n-Alkanes (liquid paraffins $C_{10} - C_{20}$, containing predominantly n-alkanes with up to 5% iso- and cyclo- alkanes and some aromatics below 2%, but with no carcinogenic aromatic compounds).
- Paraffin wax, highly-refined (pharmaceutical or food grade paraffins consisting of n-, iso-, and cyclo- alkanes, up to 0.5% mineral oil and PAHs below 0.1%).
- Paraffin wax, semi-refined (technical quality paraffins consisting of n-, iso-, and cyclo- alkanes with up to 15% aromatic hydrocarbons, up to 5% mineral oil and up to 1% PAHs with <0.1% carcinogens such as benzene).
- Hydrocarbon wax (crude material from the refinery consisting of n-, iso-, and cyclo- alkanes with up to 15% aromatic hydrocarbons and PAHs above 0.1%, i.e., slack wax or petrolatum).

Discussion at ESPH 23 also focused on the definition of the sea areas in which the new prewash requirements would apply, suggesting as examples of potential areas:

- The North West European waters (including the North Sea, the Irish Sea, the Celtic Sea, the English Channel, and part of the North East Atlantic).
- The Baltic Sea.
- The Western European waters (Covering UK, Ireland, Belgium, France, Spain, and Portugal)
- Norwegian waters north of 62°.

Lastly, ESPH suggested that Annex II could be amended to clarify that the use of small amounts of cleaning additives (not containing pollution category X components) would improve

and maximize the removal of high-viscosity cargo residues during prewash operations. These amendments were submitted to the fifth session of the PPR Sub-Committee (PPR 5) which was held in February 2018 and will be further discussed during MEPC 72 in April 2018.

6. ACTIONABLE RECOMMENDATIONS

Whilst acknowledging that IMO is actively working to solve the problem, we feel compelled to raise few points while amendments to Annex II are being drafted, in the attempt of making future policies as effective as possible:

- Wax pollution is global in scope and is certainly not restricted to Northern European countries. If a geographical approach is to be adopted, the sea areas in which the new prewash requirements would apply should be extended to include other sensitive areas such as the Mediterranean Sea, where at least three large strandings occurred in recent years. Nevertheless, once recognized the danger posed by these persistent floaters, a product-based approach would be undoubtedly more appropriate.
- In light of the GESAMP hazard profiles, which take into account bioaccumulation in marine organisms; damage to marine life and habitats; hazard to human health and reduction of amenities such as beach uses and tourist activities (Wells et al., 1999), it would be advisable to reclassify all different kinds of petroleum waxes (including highly refined and liquid paraffins) in the pollution category X, i.e., substances whose discharge at sea is completely prohibited and for which a residual concentration of 0.1% should be met in the prewash effluents—inasmuch as they all possess the same physico-chemical characteristics and pose the same potential risk to the marine environment of slack waxes, semi-refined paraffins and petrolatums, regardless of their aromatic hydrocarbon, and PAHs content.
- Despite the revision of the names for paraffin entries in the GESAMP/EHS Composite List, there is still a great blur on the terminology used. The updated list of paraffin-like products, does not currently mention the term "petroleum waxes," which encompasses many other commonly traded products such as "microcrystalline waxes." The term "hydrocarbon wax" is considered as a synonym for petrolatum or slack wax, while in other contexts it is mainly used as synonym for the more general "petroleum waxes" (see the Introduction section and references therein). This great blur is also reflected in the updated list of carriage requirements, under which the new paraffin-like categories all share the same synonyms. The adoption of a univocal vocabulary and the creation of a less ambiguous classification system are an urgent necessity.
- As reported by Sea-Mer Asso (2017), the amount of beached material often exceeds the limits set by Annex II for stripping residuals, as it was the case for the event occurred along the Italian coasts in 2017. Hence, these episodes almost certainly represent examples of MARPOL violations, which would not be resolved regardless of the proposed amendments. Greater attention perhaps, should be directed by national governments toward the enhancement of adequate port reception facilities, surveillance bodies, and policy enforcement mechanisms.
- According to MARPOL Annex V, shipboard generated garbage, including plastics, domestic wastes, cooking oil, incinerator ashes, operational wastes, and fishing gear are prohibited to be discharged at sea, under no circumstances, inside as well as outside special areas. Given that once in the marine environment, solidifying substances and persistent floaters behaves and possesses the same risk characteristics of plastic waste (i.e., long residence times, potential for ingestion by marine organisms, aesthetically detrimental, progressive fragmentation, etc.), it seems inconsistent that dumping of wax cargo residues is still permitted, while dumping of any other kind of solid waste is already prohibited. In light of this, the inclusion of solidifying products and persistent floaters under the provisions of MARPOL Annex V could be ultimately taken into consideration by the IMO.
- It should be noted that within the EU Marine Strategy Framework Directive (MSFD 2008/56/EC) and especially in light of the achievement of Good Environmental Status (GES) of European waters by 2020, pollution by paraffin and other petroleum waxes is not exhaustively codified. The category "paraffin/wax" is included in the master list of litter categories within GES Descriptor 10 (TSG_ML code G213; Hanke et al., 2013). However, as already highlighted by Galgani et al. (2010), the current definition of marine litter, described as "*Any persistent, manufactured, or processed solid material discarded, disposed of or abandoned in the marine and coastal environment,*" does not include semi-solid remains of mineral and vegetable oils, waxes, and chemicals. In addition, although the establishment of a "*minimum list of elements and/or parameters for assessing GES for acute pollution events: number and extent of petroleum/oil related (hydrocarbons) and analogous oil compounds (paraffin, vegetable oils) slicks*" was recommended by Tornero (2015), petroleum waxes are not explicitly mentioned in GES Descriptor 8.1.2 (Acute pollution events) and associated criteria (e.g. D8C3: "The spatial extent and duration of significant acute pollution events are minimized"), nor they are included in the MSFD list of "Potential chemical contaminants in the marine environment" (Tornero and Hanke, 2016, 2017). Therefore, we suggest to make explicit reference to paraffin and other petroleum waxes in Descriptor 8 and—as policy against marine litter is already coming in place—we recommend to expand the current definition of marine litter, as to include solidifying and persistent floaters within the framework of Descriptor 10, so that the objectives set by recent and future policy actions—such as the monitoring and reduction targets set by the MSFD—will also embrace this particular category of contaminants.

7. CONCLUSIONS

Pollution events by paraffins and other petroleum waxes are posing a significant problem to local municipalities across Europe with new incidents being reported on a regular basis. Clean-up

costs often fall on the local governments and the severity of the problem becomes even more apparent when taking into account the frequent occurrence of these materials at sea. In most cases, these episodes originates from the inadequacy of the current legislative framework, with this contravening the spirit of the MARPOL provisions on the protection of the marine environment. The new version of Annex II which is currently being drafted, will surely improve the situation. But as long as the discharge at sea of these residuals will be a legal practice, the clean-up costs will keep burdening the local communities and there will be no legal tools to ensure fair compensation by the polluters. As strongly highlighted by the IMO Marine Environment Protection Committee (MEPC) in their "Action Plan to tackle the alleged inadequacy of port reception facilities" and in the following EU Directive on port reception facilities (2000/59/EC), one of the main limiting factors is the inadequacy of most reception facilities and the cost for ship's owners to properly dispose of tank washings. We therefore call on national governments to provide adequate shore infrastructures, so that the MARPOL regulations can be successfully implemented. Ultimately, a more effective protection of the marine environment could be eventually achieved—in line with the adoption of the precautionary principle—through the enforcement of more stringent regulations by individual governments, federal or supranational bodies, in order to ensure suitable protection of their territorial waters and those of neighboring regions.

AUTHOR CONTRIBUTIONS

SM and MA: provided early notice of the beaching event, collected samples of the spilled substance, and performed repeated monitoring of the impacted sites; GS: performed the literature search and wrote the first draft of this manuscript; SA: revised the text. All authors accepted it in its final version.

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Governance Solutions to the Tragedy of the Commons That Marine Plastics Have Become

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Plastic pollution has become the new millennium's tragedy of the commons. This is particularly true with the marine debris plastic pollution issue, which has seen significant global interest recently. There is long-standing acknowledgment of the difficulty in managing the commons, with regulations, economic and market based instruments and community-based solutions all having a role to play. We review the global plastic pollution issue in the context of governance and policy, providing examples of successes, opportunities and levers for change. We discuss the role of regulation, public perception and social license to operate (SLO) in managing waste that enters the ocean. We argue that while plastic pollution is a tragedy, there are many opportunities for reduction, management, and changes to the global community's relationship with plastic.

Keywords: marine debris, microplastic, plastic pollution, social license, tragedy of the commons, waste mismanagement

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INTRODUCTION

Since Hardin (1968) published his seminal piece outlining the difficulties with managing the commons, the oceans have been identified as a common pool resource that are susceptible to degradation and over exploitation. In our modern "plastic era" plastic debris in the marine environment has become as much a "commons" and a "tragedy" as is the ocean itself. It is now estimated that 8,300 metric tons of plastic have been produced by humans since the 1950s and if these rates continue, 12,000 metric tons will be in the natural environment by 2050 (Geyer et al., 2017). Plastics have been found in even the most remote parts of the Arctic and Antarctic oceans and microplastics in particular (particles $\leq$ 5 mm in size, see Masura et al., 2015) have been identified in every marine habitat (Ivar do Sul and Costa, 2014).

Plastic pollution, and indeed, littering is not a new phenomenon. Plastics have been used since World War II (Joyner and Frew, 1991), with plastic production growing exponentially for an array of polymer types since the 1950s (Andrady and Neal, 2009). However, plastic pollution did not become a concern to the global community until the 1960s. Similarly to other pollutant problems of the time, it has become increasingly recognized as a potential significant detriment to the health of ocean; similarly to how DDT was identified by Rachel Carson in "Silent Spring" (Carson, 2002; Worm, 2015).

Marine litter has been defined by the United Nations Environment as "any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment. Marine litter consists of items that have been made or used by people and deliberately discarded into the sea or rivers or on beaches; brought indirectly to the sea with rivers, sewage, storm water or winds; accidentally lost, including material lost at sea in bad weather (fishing gear, cargo); or deliberately left by people on beaches and shores)" (Jeftic et al., 2009).

Litter, much of which is plastic, is found in the marine environment and ranges from large industrial containers to plastic bags, drink containers, cigarette butts, plastic fragments, manufactured plastic pellets (often called nurdles) (see Ogata et al., 2009) and numerous other consumer items. This anthropogenic litter, interacts not only with marine megafauna such as seabirds (Spear et al., 1995; Wilcox et al., 2016, others), turtles (Schuyler et al., 2014 and references therein), marine mammals and fish (Davison and Asch, 2011; Choy and Drazen, 2013; Rochman et al., 2015 and others), but also with bivalves, lugworms, oysters and corals (Hall et al., 2015; Van Cauwenberghe et al., 2015). To date, the demonstrated impacts to wildlife have most frequently been documented and reported at the individual organism and sub-organismal levels (Rochman et al., 2016), with experts viewing entanglement, ingestion and chemical contamination as all having the potential for significant (e.g., lethal or sub-lethal) impacts to marine vertebrate fauna (Wilcox et al., 2016).

Microplastics are a specifically identified subset of marine pollution that is of increasing concern. They result from the breakdown of larger plastics and are also manufactured specifically for use in consumer goods (as microplastic beads). These small particles have high surface to volume ratios and can sorb environmental contaminants. Also, they are accessible to a wide array of marine organisms from the smallest (e.g., plankton) to the largest marine fauna (e.g., whales, fish, seabirds, and so on). Furthermore, as people eat filter-feedings marine delicacies such as shrimp, scallops, mussels and sea cucumbers, the relationship to human health and food security becomes an increasing concern (Ivar do Sul and Costa, 2014).

In addition to impacts on biodiversity, anthropogenic debris or litter has implications for aesthetics and economics, which are tightly intertwined (Hardesty et al., 2017). For example, after a heavy rainfall event which resulted in a significant increase in coastal debris loads in South Korea, revenue losses from tourism were estimated at $29–37M USD (Jang et al., 2014). In coastal California, visitors are reported to travel longer distances to avoid beaches with more waste (Leggett et al., 2014), and in Brazil, a recent survey reports that 85% of beachgoers will avoid beaches with high litter loads (>15 pieces per m^2) (Krelling et al., 2017). This is also interesting in light of numerous reports (and anecdotal evidence) that beachgoers themselves can be a contributing source of debris (Santos et al., 2005).

Solutions to managing the tragedy of plastic pollution, as any commons, are multifaceted requiring a mixture of regulation, economic/market and community-based efforts (Feeny et al., 1990; Ostrom et al., 2002; Dietz et al., 2003). They range from local community efforts to global actions (Vince and Hardesty, 2016). Globally, the need to address the plastic problem is increasingly recognized with discussions on marine plastic pollution occurring at international fora such as the World Oceans Summit (2017) and at recent meetings of the top seven and top 20 global economies G7 and G20. Furthermore, a Ministerial Declaration "Toward a Pollution Free Planet" was adopted by consensus by the UN Environment Assembly (2017). While a new legally binding international agreement is urgently needed (Chen, 2015; Vince and Hardesty, 2016; Raubenheimer and McIlgorm, 2017; Worm et al., 2017), it will need to work in context with economic and biodiversity goals.

We discuss solutions to the marine plastic pollution issue, and we describe examples of successes, opportunities and levers for change. These can be achieved in addition to regulatory measures including community's ability to give or withhold social license to operate (SLO) and self-regulatory measures in the private sector (through tools such as corporate or environmental social responsibility policies). We argue that marine plastic pollution is a tragedy of the commons. However, it is a tragedy that can be reversed, and one where communities both local and global can successfully contribute to change.

REGULATORY MEASURES

Global Approaches

Three quarters or more of waste that ends up in the ocean comes from land-based sources (Derraik, 2002; Hardesty et al., 2014; Jambeck et al., 2015). Accordingly, management of this waste needs support not only on the global scale, but also at national and local levels. There is a large gap in international hard law specifically dealing with land based plastic marine pollution. The United Nations Law of the Sea Convention (UNCLOS) Part XII (articles 192–237) is dedicated to the protection and preservation of the marine environment. States are required to take all measures "that are necessary to prevent, reduce and control pollution of the marine environment from any source, using for this purpose the best practicable means at their disposal and in accordance with their capabilities, and they shall endeavor to harmonize their policies in this connection"(Article 194). It also sets out the responsibilities of states and necessary measures they need to undertake to minimize pollution their own and other states' jurisdictions. While UNCLOS recognizes the differences between sea based and land based pollution, it does not address the type of pollutants and technical rules in great detail (Palassis, 2011). States are required to adopt their own laws and regulations that address marine pollution.

In the case of ship-sourced pollution, the International Maritime Organization (IMO) takes responsibility for the 1972 Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (London Dumping Convention) and the 1973 International Convention for the Prevention of Pollution from Ships (MARPOL) (Joyner and Frew, 1991). Annex V of MARPOL (entered into force in 2013 with further revisions in March 2018) is particularly important with regard to anthropogenic debris as it prohibits the disposal of plastics anywhere at sea. Ships are required to dispose of their waste at land based wasted facilities. MARPOL Annex V "requires states to provide reception facilities for garbage at ports and terminals, and to present a list of these facilities to the IMO." Compliance, however, remains a significant issue and states around the world are in varying phases of implementing their domestic policies that reflect this regulation (Ryan, 2015).

To date, soft law has dominated efforts to address plastic marine debris and it has had a discernible influence in some areas. For instance, the UN Conference on the Environment and Development's Agenda 21 encourages integrated, precautionary

and anticipatory marine environmental protection (UN, 1992). It sets out an approach to addressing damaging impacts from air, land and water; recycling; sewerage treatment; and the prevention, reduction and control of ship sourced pollution (Palassis, 2011). The Conference of the Parties to the Convention on Biological Diversity (COP CBD) Scientific and Technical Advisory Panel of the Global Environment Facility adopted Decision XI/18 at the 11th Meeting (2012) which addresses the impacts of marine debris on marine and coastal biodiversity. The Parties also agreed upon a Strategic Plan for Biodiversity (2011–2020) that includes Aichi Biodiversity Targets. Target 8 states that a goal that "by 2020, pollution, including from excess nutrients, has been brought to levels that are not detrimental to ecosystem function and biodiversity." Monitoring measures to assess such targets, however, will need to be established appropriately to assess whether targets are met.

The United Nations Environment Program (UNEP) has also addressed marine pollution through specific guidelines (UNEP, 2009a,b) that include the *Guidelines On the Survey And Monitoring Of Marine Litter* (2009), *Guidelines On The Use Of Market-Based And Economic Instruments* (2009) and *Marine Litter a Global Challenge* (Jeftic et al., 2009). The latter report provides a number of recommendations for the 13 participating Regional Seas programmes including, *inter alia,* the development of a Regional Action Plan or strategy to deal with marine pollution; mitigation should be global but coordinated at the regional level and implemented at the national level; National Plans of Action that draw on existing legislation; and the coordination of UN organizations working on the marine litter problem (see regional approaches, below).

The Honolulu Strategy (UNEP, 2012) was adopted by participants of the Fifth International Marine Debris Conference (5IMDC). The Honolulu Strategy is a volunteer-supported, global strategy to reduce marine debris. Also in 2012, the UNEP Global Partnership of Marine Litter (GPML) was announced. GPML is part of the UN Environment Global Programme of Action for the Protection of the Marine Environment from Land-based Activity (GPA). This global partnership is a coordinating forum for stakeholders at all levels working on marine debris prevention and management. It is generally agreed on a global level that a coordinated effort is required by governments, the private sector and civil society to reduce and prevent plastic pollution entering the ocean (Global Ocean Commission, 2014). This was further highlighted by The G7 (consisting of Canada, France, Germany, Italy, Japan, the United Kingdom and the United States) which released an *Action Plan to Combat Marine Litter* in June 2015. This Action Plan included land and sea-based priorities to reduce marine debris.

Outcomes of the G7 and G20 meetings include Action Plans on Marine Litter and the 2017 UNEP launching of the CleanSeas Campaign (Mendenhall, in press). The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) has supported multiple working groups on various components of plastics and microplastics in the ocean (GESAMP W40 – see http://www.gesamp.org/work/groups/40) which is managed by the United Nations Educational, Scientific and Cultural Organization-Intergovernmental Oceanographic Commission (UNESCO-IOC) and UNEP. The key objective for the current working group (2017–2018 period) is to develop guidelines for the terminology and methodologies for sampling and analysis of macro and micro plastics, which has long been identified as a key gap or challenge.

There has also been a recent focus on global plastic pollution in the Sustainability Development Goals (SDG), in particular, SDG14.1, which focuses on life below water. Substantial efforts have been made to implement these sustainability development goals through the Oceans Conferences held in 2017 and 2018. The 2017 meeting resulted in the creation of the Communities of Ocean Action that included representation from governments, non-government organizations (NGOs) and civil society groups (Haward, 2018). In December 2017, the UN Environment Assembly passed a non-binding resolution on marine litter and microplastics that encouraged member states to "develop integrated and source-to-sea approaches to combat marine litter and microplastics from all sources" and it recognized "that private sector and civil society, including non-governmental organizations, can contribute significantly to prevent and reduce marine litter and microplastics" (resolution UNEP/EA.3/L.20).

There are numerous efforts afoot at international, national and sub-national levels to collate information about existing efforts, to engage institutions, governments, and other bodies to incorporate sustainability measures aimed at reducing plastic pollution. Furthermore, there is an expanding interest in the circular economy of plastic. The circular economy in the plastic context aims to shift from a produce, use, dispose approach to a design, use, re-design/re-use approach. Furthermore, the circular economy encourages supply chain investment opportunities to address marine plastic pollution—before such waste makes it to the ocean (MacArthur et al., 2016; Moss et al., 2017).

A new legally binding global instrument will take time and is complex as it requires agreement from multiple partners with varying capacity, resources and waste management infrastructure capability. It will also benefit from a holistic, integrated approach that combines community and economic/market instruments to help provide solutions to the marine litter issue. In the meantime, the initiatives mentioned above are recognized for providing a broad framework for addressing the plastics pollution issue at the large scale (see **Figure 1**).

Regional Approaches

Regions around the world are also addressing marine plastic pollution at appropriate regional geographic scales. Regional approaches occur between the national and global efforts (as seen on the continuum in **Figure 1**). For example, regional fisheries organizations have provisions to address sea and ship based pollution. The Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) also has a mandatory requirement for fishers in the Southern Ocean to report gear loss to the Scientific Committee (CCAMLR, 2015). The activities that can cause pollution are relatively well monitored in the Southern Ocean. However, not all areas of ocean are well managed and compliance remains an issue. Plastic pollution is a transboundary challenge, and when it occurs in Areas Beyond National Jurisdiction (ABNJ) the issue of responsibility is particularly problematic, particularly when it comes to removal of plastic debris (Vince and Hardesty, 2016).

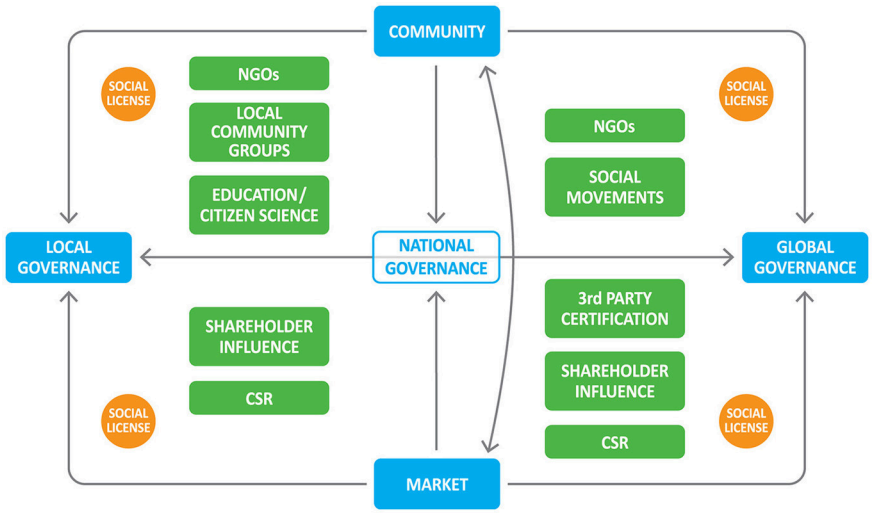

FIGURE 1 | A holistic governance approach to the reduction of plastic marine litter.

Around the world there are a number of regional seas conventions and action plans underway to combat plastic pollution. For example, there is a Regional Action Plan on Marine Litter management (RAPMaLi) for the wider Caribbean Region. This plan addresses litter issues in the wider Caribbean basin, supported by the UN's Caribbean Environment Programme (http://www.cep.unep.org/regional-action-plan-on-marine-litter-management-rapmali-for-the-wider-caribbean-region). Similarly, the Northwest Pacific Action Plan (NOWPAP; www.nowpap.org) contributes to the global action program that aims to protect the marine environment from land-based activities in the Northwest Pacific Region. NOWPAP has developed regional activity centers, including coastal environment assessment and emergency preparedness regional centers to address plastic pollution and other environmental issues within the region.

Within Europe the pollution issue is being addressed through Regional Sea Conventions—the Barcelona Convention, the Bucharest Convention, the HELCOM Convention and the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR). In 1972, for the first time all sources of pollution were recognized through the Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area (the HELCOM Convention) (adopted in 1992). In 2015, a Regional Action Plan for Marine Litter in the Baltic Sea was adopted by nine coastal Baltic Sea states who are signatories of HELCOM (HELCOM, 2015).

The Convention for the Protection of the Marine Environment and Coastal Region of the Mediterranean (the Barcelona Convention) (initiated in 1976 and reviewed in 1995) addresses pollution from land and sea based sources. In 2013, the Mediterranean countries adopted the Regional Plan for Marine Litter Management in the Mediterranean of the Barcelona Convention—the first *legally binding* regional plan for marine litter management at European Regional Seas Level. Its signatories adopted the Mediterranean Action Plan which was one of UNEP's first regions in the Regional Seas Programme. In 2016 UN Environment launched the ambitious Integrated Monitoring and Assessment Programme (IMAP) which aims to enable a " quantitative, integrated analysis of the state of the marine and coastal environment, covering pollution and marine litter, biodiversity, non-indigenous species, coast, and hydrography, based on common regional indicators, targets and Good Environmental Status descriptions." (http://web.unep.org website; accessed 20 May 2018).

The OSPAR Commission also has a Regional Action and implementation plan that focusses on key areas that include *inter alia* port reception facilities, fishing for litter, education and outreach and reduction of single use items (https://www.ospar.org/documents?v = 34422). OSPAR has developed consistent data collection approaches for marine litter monitoring and data reporting for the last several years (https://www.ospar.org/work-areas/eiha/marine-litter). The Black Sea Region, under the auspices of the Bucharest Convention, is the last region which is yet to develop an Action Plan and when implemented, will complete the region's efforts in having regional action plans to combat marine pollution.

The Marine Strategy Framework Directive (MSFD), adopted by European Union (EU) member states in 2008, identifies marine litter as one of the descriptors of Good Environmental Status. The MSFD requires EU Member States to ensure that, by 2020, "properties and quantities of marine litter do not cause harm to the coastal and marine environment." The key measures toward this end—reflected within the European Strategy for Plastics in a Circular Economy—include measures against single use plastics and fishing gear; restrictions related to the use of microplastics in products or measures against microplastics generated during the life cycle of products; measures to reduce marine litter from ships, including fishing vessels and recreational craft (http://ec.europa.eu/environment/marine/good-environmental-status/descriptor-10/index_en.htm).

While we cannot include all regional approaches here, those described above provide relevant examples of significant steps

being undertaken at regional levels around the world. It is relevant to note that Small Island Developing States (SIDS) are also becoming more deeply engaged in the marine pollution issue. This is a significant issue for SIDS, particularly because resources and infrastructure for waste disposal are inadequate in many of these developing countries. The Secretariat of the Pacific Regional Environment Program (SPREP) is now a regional node of marine litter prevention, supported by the GPML. The Pacific Ocean Pollution Prevention Programme (PACOL) Strategy and Work Plans document was released by SPREP and the IMO in 2015. The recommendations will be implemented through a bottom up approach which can be a time-intensive process. We acknowledge that regional oceans governance in the South Pacific is difficult to achieve (Vince et al., 2017) and the tragedy of the commons through plastic pollution adds another layer of complexity to already stretched resources in marine—and waste—management which may be particularly difficult for many small island nations.

National Approaches

While global decision making can direct national incentives, national-level policy actions are the mechanisms for steering action. Numerous nations around the world are addressing plastic pollution in various significant ways that we are unable to cover in depth here. However, efforts in developing countries such as Indonesia, Ghana and Kenya highlight the significance of this issue and its increasing recognition as an issue of concern, and legislation in New Zealand and the United States demonstrate that incentives can prove successful in reducing waste mismanagement.

In 2017, the coordinating Ministry of Maritime Affairs for the Republic of Indonesia, recently cited as one of the countries with the most significant waste mismanagement issues resulting in plastic pollution in the ocean (Jambeck et al., 2015), released Indonesia's Plan of Action on Marine Plastic Debris for 2017–2025. The Plan includes the five components of improving behavioral change, reducing land—and sea-based leakage, reducing plastics production and use, and enhancing funding mechanisms, policy reform and legislation enforcement. Although in its infancy, this Plan has already made an impact on reducing marine litter through community efforts that have been assisted and coordinated by government (Lasut et al., 2018). The Kenya government has recently passed legislation that prohibits the importing, making, or selling plastic bags. Any offenses will be punishable by fines of up to US$40,000 (or up to 4 years in jail, ABC News, 2017). This may be viewed as a success story, and as a story of caution. The government is taking the plastic issue seriously, however, such sanctions can result in adverse effects in the community. The Waste and Environment Association of Kenya have opposed the ban on the basis that it will cost thousands of jobs (Xinhuanet, 2017). What seem like straightforward solutions are often complex, with myriad factors to consider.

In 2002, the government of Bangladesh was the first to ban plastic bags due to flooding caused by blocked stormwater drains (Dauvergne, 2018). Similarly, in 2015 in Ghana, plastic waste blocked drains and caused flooding that resulted in approximately 150 human deaths (Jambeck et al., in press). The initial response was to ban plastics in a similar effort to that undertaken in Kenya, though this has been delayed. Officials of Ghana have recognized that policy responses need to be made through collective decision making with the participation of a range of stakeholders. While a National Plastic Management Policy is being developed, the country is focusing on innovative methods to solve the plastic pollution issue rather than through bans on particular products (see http://www.ghananewsagency.org/science/-ghana-is-not-ready-to-ban-plastics-now$-$126770; http://mesti.gov.gh/mesti-embraces-innovative-use-plastic-waste/). To date, a large number of countries have introduced taxes, bans or restrictions on the use of plastic bags. In some countries, such as the United States of America and Australia, however, legislation has typically been implemented at a state-based level rather than through national approaches (Xanthos and Walker, 2017).

Other national measures to reduce (micro) plastic pollution from entering the ocean's waterways include New Zealand's plastic microbeads ban which is scheduled to come into effect in June 2018. Under section 23 of the Waste Minimisation Act 2008, wash off cosmetics (including body exfoliants and toothpastes and abrasive cleansing products) will be forbidden. In 2015, then-president Barak Obama signed the Microbead-Free Waters Act of 2015 into law within the United States of America. The law bans plastic microbeads in both personal care and cosmetic products and aims to stop the introduction of plastic microbeads into lakes, coastal areas and the ocean. However, the ban did not come to full effect until January 2018 revealing that implementation takes time (Stoett and Vince, 2018). Other countries that have pursued similar microbead bans or restrictions include Canada, Finland, France, Iceland, Ireland, Luxenburg, Norway, Sweden and the UK (Dauvergne, 2018).

COMMUNITY-BASED MEASURES

Governance solutions can also come from communities. Dietz et al. (2003) reported that effective commons governance is achieved when communities communicate effectively. Through communication, communities become involved in strong social networks and increase their social capital. This results in a decrease in monitoring of behavior and increases compliance. They also found that the tragedy of the commons can be overturned not only by regulation and market-based mechanisms but also through adaptive governance strategies (Dietz et al., 2003). Although regulation and market approaches do manage resources successfully, the socio-economic environment contributes to the degree of this success (Feeny et al., 1990). Furthermore, public opinion and good will is a key component to driving change.

Strength to create social change (and SLO) can be found in communities and other non-state actors. For example, there has been a swell in grass root efforts, such as those initiated by the global #breakfreefromplastic movement which was launched in September 2016 (www.breakfreefromplastic.org). Since then, more than 1,000 non-governmental organizations from across the world have joined the movement demanding massive reductions in single-use plastics and to encourage new lasting solutions to plastic pollution. The organizations involved in

the movement share the common values of environmental protection and social justice. This in turn helps guide their work at the community level while representing a global, unified vision through the #breakfreefromplastic campaign. Movement members deliver campaigns that help avoid the plastics crisis from worsening. Some of these campaigns include working with local governments to implement zero waste programs, and exposing the role of companies in promoting and perpetuating the use of non-recyclable packaging (Von Hernandez, pers. comm. 4 Dec 2017). Another example is "Plastic Free July" (http://www.plasticfreejuly.org/). Started by a local government organization (Western Metropolitan Regional Council) in Western Australia in 2011, *Plastic Free July* is a campaign to nudge citizens into changing their consumer behavior in favor of avoiding single-use plastics. Individuals, schools, and organizations pledge to refuse single-use plastic during a single month (or week or day). The focus is on solutions and providing a toolbox in which participants can select appropriate measures for themselves (or their institutions). To date, more than 2 million people have participated in the event, which supports networking, storytelling and collaborative approaches to the challenge of single-use plastics in society (R. Prince-Ruiz, pers. comm., 1 Dec 2017).

There are many situations where communities have the capacity for self-management and it makes administrative and economic sense to include them in decision making in resource management (Feeny et al., 1990). *Bye Bye Plastic Bags* is one example (see http://www.byebyeplasticbags.org/) of a recent, successful community-based campaign aimed at reducing single use plastics, which has been driven by a change in public perception. This social initiative is driven by youth in Bali, Indonesia. Their community-based campaign aims to reduce single use plastic bags and has become a well-known international movement which focuses on education, joint messaging and youth empowerment. Shared governance between the community and the state, along with self-management (or co-management) "can capitalize on the local knowledge and long-term self-interest of users, while providing for coordination with relevant uses and users over a wide geographic scope at potentially lower transaction (rule-enforcement) cost" (Feeny et al., 1990). Large scale co-management, where communities drive the solution and share responsibilities with regulatory bodies, is one of the many means of combatting the plastic marine pollution problem. Such an approach, similar to the 1987 Montreal Protocol, would be a practical option internationally to help resolve this transboundary problem.

Education is also a key to strengthening community support and understanding of the impact of plastic on the marine environment. Supported by the United Nations, the GPML has sponsored numerous projects aimed to increase understanding of losses and movement of debris in the marine environment. These efforts have aimed to increase educational and public awareness through the development of a Massive Online Open Course (MOOC) on marine debris (https://www.ou.nl/documents/40554/72652/MOOC_Marine_Litter_2017_leaflet.pdf/5d520cb2-b334-488e-826b-e19284916935) and to broaden the community engagement with the topic more generally. Around the world there are hundreds of groups that have engaged with the public and with school children around marine litter. Whilst many organizations focus on an advocacy approach, others use the topic as an educational tool, developing content that addresses curriculum requirements (e.g., www.Teachwild.com.au). NOAA, for example, developed a "Turning the Tide on Trash" program in the United States (see www.marinedebris.noaa.gov for details); The University of California at Davis has developed a marine debris lesson, which includes analysis of debris on university campuses; and the Plastic Pollution Coalition (http://www.plasticpollutioncoalition.org/) has developed curriculum content for grades 7–12 that can be used in educational systems across the world. These organizations and dozens more make their content freely available on line. While there are a growing number of groups providing content and making materials available in different countries, a consistent message is the role and value of community engagement around an environmental issue that is relevant for people of all ages, from primary school children to senior citizens (see van der Velde et al., 2017 and references within).

ECONOMIC/MARKET-BASED POLICY INSTRUMENTS AND THE IMPACT OF SOCIAL LICENSE

The traditional form of governance through government and regulation has been unable to solve many of the world's "tragedy of the commons" environmental issues. Regulatory frameworks have often experienced difficulties and challenges with the implementation of sustainable, conservation measures, demonstrating that these regulatory measures alone cannot bring about the required change to effectively stop marine plastic pollution and land-based waste. However, when regulatory and market based incentives are combined, interesting developments can occur in the reduction of plastic use and pollution. A recent analysis highlighted the effectiveness of small incentives in reducing waste mismanagement in Australia and the United States. An incentive of as little as 5–10 cents through container deposit legislation (CDL) or cash for containers was effective in reducing beverage container waste (Schuyler et al., in press). The proportion of beverage containers found in coastal surveys from states with incentives was approximately 40% less than in states without incentives—and was consistent between the two countries (Schuyler et al., in press). Importantly, the reduction in beverage containers was greater in areas with lower socio-economic status, where debris loads are highest, providing strong evidence that incentives are particularly effective where incomes are lower (Schuyler et al., in press). This suggests that putting a price on plastic would likely be effective in terms of material recovery and would reduce loss rates to the environment. We already see this with material such as aluminum, steel and copper, as these materials are valuable and can be sold back into the market.

Market governance solutions are being developed and tested and indeed the economic cost of marine plastic pollution is another factor that needs to be considered as part of this solution

(McIlgorm et al., 2011). For instance, there are efforts to clean up the well- known "Pacific Garbage Patch" located in both the East and West parts of the Pacific (Moore et al., 2001; Kaiser, 2010). However, such clean-up efforts are complex and unlikely to yield desired results. They are also addressing the "end of the pipe" rather than where efforts are likely prove successful (Rochman, 2016; Sherman and Van Sebille, 2016). Moreover the gap in international law addressing areas beyond national jurisdiction complicates the mitigation and removal of marine debris from these ABNJ areas. Finding solutions to removing marine debris or taking responsibility for it within the high seas from a governance and practical perspective adds another layer of complexity.

As a result, alternative tools and approaches, including external third party assessment and certification systems, have been developed to address perceived regulatory failure, including economic and community based management. These approaches (see link between the community and market in **Figure 1**) step outside state-based governance and address market and consumers directly through product certificates and ecolabels (Potts and Haward, 2007). Certification and labeling initiatives encourage industry best practices that influence shareholders and the market (such as sustainability labeling, green labeling, etc.). Moreover, certification and labeling can add another layer of legitimacy for community groups in providing their SLO. The legitimacy of third party certifiers can be removed at any time if the community decides not to accept the standards or organization. Certification schemes can therefore be considered "new markets of governance" through their organizational set up, consultancy services and contractual arrangements (Foley and Hébert, 2013). Although self-regulatory industry measures, policies and standards can also be effective in the plastics pollution issue, the opportunity to use third party certification organization as a regulatory measure has been little explored. Landon-Lane (2018) suggests that a "Plastics Stewardship Council" be enacted, based on the Marine Stewardship Council (MSC) model and we believe that this gap in governance will provide a unique and innovative way to address plastic pollution issues and to identify additional solutions to this problem.

Industry can also obtain social license through Corporate Social Responsibility (CSR) (Gjølberg, 2009). Corporate Social Responsibility is becoming an increasingly important priority for some companies involved in the development, distribution and life cycle of plastics. Although social license and CSR concepts are interrelated and overlap, there are key differences (Parsons and Moffat, 2014). SLO is an intangible, unwritten and impermanent social contract between industry and social groups (Parsons and Moffat, 2014). Through social license, communities and consumers can instigate changes to corporate policies and products (Morrison, 2014). Industry is, however, ultimately in control of its CSR policies and activities. According to Steurer (2013) "new governance and CSR are complementary concepts that both fundamentally reshape the roles of the public and the private sectors in similar directions." CSR can be driven by community support through social license or government regulation (Vince and Hardesty, 2016). Solutions to plastic pollution can be driven by willing industry and their use their CSR policies to gain consumer confidence and to demonstrate their commitment to social and environmental issues. The Australian Packaging Covenant is but one co-regulatory non-government organization that partners government and industry with a goal of helping its industry-based signatories realize CSR opportunities.

The Secretariat of the Convention on Biological Diversity and the Scientific and Technical Advisory Panel - GEF (2012) has stated that "many companies now see packaging and plastics sustainability as part of broader corporate social responsibility, and negative brand image is becoming a major driving force which is being harnessed in the interests of improving packaging materials and technologies." This has been evidenced by a global Declaration for Solutions on Marine Litter which was developed and signed by industry plastics associations in 2011. Their aims include to "contribute to solutions by working in private-public partnerships aimed at preventing marine debris" and to promote science-based policies and enforcement of existing legislation. As of May 2016, 65 members from 34 countries have signed this Declaration and supported 260 projects (https://www.marinelittersolutions.com; accessed 4 Dec 2017). Industry is also being encouraged to take responsibility of the full life-cycle of the products they produce through Extended Producer Responsibility (EPR). This can be voluntarily driven by CSR policies, or in the case of many European nations EPR legislation has been enacted to reduce use and increase the reuse and recycling of plastics (Tibbetts, 2015; Worm et al., 2017). Changes to market and industry through EPR can result in an increase in Sustainable Development and Consumption production methods and address the SD goal 12—Responsible Consumption and Production (see https://www.un.org/sustainabledevelopment/sustainable-consumption-production/).

The flow on effect of this in industry decision making is becoming evident. For example, Selfridges and Company, a UK based high end department store, initiated an intense oceans campaign that highlighted the impacts of single-use plastics on marine fauna. As part of their campaign, they no longer offer plastic bags in their stores, they aim to make communities and cities plastic water bottle free, and have a growing "Project Ocean" which targets consumer awareness with respect to micro-bead free products, sustainable seafood practices, and responsible purchasing. Community acceptance and trust offers stronger and higher levels of social license (Thomson and Boutilier, 2011; Parsons and Moffat, 2014), which may be apparent with the public's positive response to Selfridge's recent campaign.

Local communities in South Korea have used SLO to reduce mismanagement of polystyrene buoys (Lee et al., 2015). In this instance, their broader community views were represented in behavior change workshops with government and key stakeholders. A successful collaboration resulted in changes to national governmental policy—a significant SLO change that is particularly relevant when one considers that polystyrene buoys are the most abundant littered item found on Korea coastal beach surveys, and that they can account for 10% or more of marine debris nationwide (Lee et al., 2015).

As most are aware, the media is a powerful, non-state actor that is capable of steering trust and social license (Vince and Haward, 2017). The media has the ability to influence community views and public perception and can affect how industry is scrutinized (Lester, 2016). Consequently, social license through social media has become a useful tool to bring about change (Boutilier et al., 2012). Recent campaigns (2016, 2017) by Greenpeace to target major industry beverage manufacturers are but one example (https://www.youtube.com/watch?v=Q7Uxaw6YoRw) of the power of media to shape the conversation.

USING SCIENCE TO INFORM THE "PLASTIC TRAGEDY"

Industry has called on science to help drive marine litter policy (Vince and Hardesty, 2016), and indeed, there is a growing body of work on plastic pollution that is being used to inform the discussion and to underpin policy decisions at a multitude of levels. The regional, national and sub-national plans of action acknowledge the importance of evidence-based informational at relevant scales (as described above). Increasingly we see the inclusion of scientific experts in discussions on the threat posed by plastic pollution with bodies such as the Convention on Biological Diversity, the International Whaling Commission and the United Nations Environment. Similar conversations are being held at numerous local, state, national and fora in countries around the world.

Documentation of the ubiquity of plastics interactions between fauna and marine debris has exploded in recent years (see review by Gall and Thompson, 2015), with the number of peer-reviewed publications on the topic having quadrupled in the last few years alone (Dauvergne, 2018). Researchers have even identified what commonplace debris items are expected to have the most significant, deleterious impacts on major marine taxa and where major marine taxa are at the most significant risk (Wilcox et al., 2016). Other work has evaluated the effectiveness of various policies on mismanaged waste (Hardesty et al., 2017; Schuyler et al., in press; Willis et al., in press) visitors' response to local litter loads (Leggett et al., 2014) and the economic costs of marine debris pollution due to major weather events (Jang et al., 2014). Scientists have moved beyond providing evidence alone of the interactions between plastic and the environment, with the field having matured to address questions around what we know vs. what is believed (i.e., demonstrated evidence, opinions and public perception) (Rochman et al., 2015; Hardesty and Wilcox, 2017).

In an ideal world, a holistic approach to the governance of plastic pollution, science would underpin policy decision making so that decisions are based upon best available evidence. Policies, whether local, national, regional or global; communities that grant or withhold social license; and industries that ignore or respond through CSR efforts, all have been steered by scientific evidence that identifies and quantifies the extent of plastic pollution and its impacts on biodiversity, economics and society. However, there remains a gap between the way science is interpreted and translated into policy. This challenge is being continually addressed by researchers in the ocean and coastal science and governance space (Nursey-Bray et al., 2014; Rudd, 2015; Vince and Hardesty, 2016).

Currently, one of the emerging scientific questions that has significant potential to shift the conversation, is whether there is an impact on human health from plastics in the environment. This is of particular concern with respect to the potential for chemical contaminants in seafood consumed by humans—and the question points to a difficult-to-resolve knowledge gap. Applying a risk-based approach to the issue and considering the severity and certainty of particular events may prove particularly useful, particularly as we view through the management and impact lenses (see Hardesty and Wilcox, 2017, Figures 3, 4).

CONCLUSION—A HOLISTIC APPROACH WILL BE MOST EFFECTIVE

Arguably, the conversation about plastics and society is changing. There is an increasing focus on a circular economy approach which focuses on purposeful design to minimize waste, along with repurposing, reusing or recycling products (MacArthur et al., 2016). This is in sharp contrast to the linear economy approach of make, use, discard. A societal shift in the form of a new global social movement advocating awareness of plastic pollution is also emerging (Vince and Stoett, in press). This movement or shift from liner to circular is supported through scientific evidence, educational tools (Hartley et al., in press) and citizen science initiatives that foster a greater understanding of the vast task facing the global community to reduce plastic pollution. The individuals in this social movement are also consumers who by granting or withholding social license can steer policy changes, and more broadly alter societal attitudes and behaviors. The tragedy of the plastic commons is tractable and solvable. It will take a shared public will, effective policies and coordination to work effectively on global, regional, national, local and individual levels. We propose that a new global agreement could prove important in driving change. We also acknowledge that coming to such an agreement will be a difficult and time-consuming process, as there are a multitude of actors, drivers and competing agendas. In the meantime, regional, national and local governance approaches will provide some of the regulatory measures required to reduce plastic losses to the environment. The success of some of these regulatory measures will be reliant upon resources being available to develop and support the essential infrastructure. Industry-based solutions that utilize market/economic based initiatives will also prove useful—if they are environmentally and socially responsible. Profit and CSR policies can be mutually beneficial in driving such change in the market. Presumably, when all of these align, consumers will be supportive.

There is no "silver bullet" or single approach that will effectively resolve this complex environmental and societal challenge. Instead, an ever-changing variety of actions, activities, legislative and cooperative approaches will ultimately help resolve this tragedy of the commons that plastic pollution has become.

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

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