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The role of biological processes in affecting the dynamics and fate of microplastics in coastal marine systems

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Abstract

Microplastics (MP) are omnipresent contaminants in the marine environment. Ingestion of synthetic particles has been reported for a wide range of marine biota, but to what extent the uptake by organisms affects the dynamics and fate of MP in the marine system has received little attention.

My thesis explored this topic by integrating laboratory tests and experiments, field quantitative surveys of MP distribution and dynamics, and the use of specialised analytical techniques such as Attenuated-Total-Reflectance- (ATR) and imaging- Fourier Transformed Infrared Spectroscopy (FTIR). I used as model study system coastal lagoons, which host extremely valuable and productive ecosystems but are also threatened by a MP contamination from a variety of sources (urban, agricultural, industrial, maritime and touristic activities), and focused on MP transfer among benthic invertebrates with different trophic modes.

I compared different methodologies to extract MP from wild invertebrate specimens, and selected the use of potassium hydroxide (KOH) as the most cost-effective approach. I used this approach to analyse the level of contamination (amount and characteristics) of MP in a range of invertebrate species from salt marshes along the North Adriatic coastline (Italy) and the Shelde estuary (Netherlands). I found that 96% of the analysed specimens (330) did not contain any MP, and that the few contaminated organisms contained mainly fibres. As preliminary environmental analyses showed high levels of environmental MP contamination, I hypothesised that most MP do not accumulate at the tissue level but are rather fast egested, a process that could be slower for fibres compared to fragments due to their elongated shape. Although the percentage of contaminated specimens was too low to draw robust conclusions, results suggested that species traits (feeding modes and localisation into sediments) could influence the species vulnerability to MP uptake, identifying omnivores as the species at greatest risk.

I subsequently used laboratory multi-trophic experiments using the filter-feeding mussel *Mytilus galloprovincialis* and the detritus feeding polychaete *Hediste diversicolor* to test the aforementioned

hypothesis. I found that mussels ingested and rapidly egested small (< 50 μm) MP through faeces and pseudofaeces. Once incorporated into these organic-rich biogenic aggregates MP sank at enhanced rates compared to their theoretical sinking rates values as free particles in the water column, and accumulated to the seafloor. Here their uptake by the detritus-feeding polychaete *Hediste diversicolor* was more than doubled compared to MP in sediments not influenced by mussel presence. I also found that faecal pellets containing MP sank significantly slower compared to faecal pellets without MP, with potential but so far largely unexplored consequences for the transfer of organic carbon from the water column to the seafloor.

I finally analysed temporal variability of these biologically mediated fluxes in the field, using originally designed traps for mussels' biodeposits and advanced automated Imaging-FTIR technique. These measurements confirmed in nature that large amounts of MP are incorporated in mussel biodeposits and conveyed from the water column to the seafloor. This transfer was particularly effective for very small MP (< 25 μm), which were concentrated into organic rich biodeposits. This field study also reported for the first time a notable small-scale heterogeneity in MP fluxes a variability that is generally not captured by traditional monitoring approaches based on the analysis of MP contents in organisms soft tissues.

Overall, results showed that MP are ingested but rapidly egested by marine in vertebrates, which may limit MP transfer via predator-prey interactions but at the same time enhance their transfer via detrital pathways in the sediments. These processes seem to be extremely variable over time, with potential unexplored environmental consequences. This rapid dynamics also limits the conclusions that can be derived from static observations of MP contents in marine organisms, not fully capturing the real levels of potential contaminations by marine species. This emphasises the need to consider such dynamics in future work, and to develop cost-effective methods to measure uptake rates by organisms in natural systems.

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General introduction

Plastics contamination: an emerging environmental threat

Human pressures on the oceans have increased substantially in recent decades. The expansion of coastal and marine activities has adversely affected the marine environment as well as the ecosystem goods and services (Airoldi and Beck, 2007). In addition, coastal and marine anthropogenic activities generate considerable quantities of waste, which has the potential to contaminate the environment and will persist in the sea for years, decades or even centuries. On average three- quarters of all marine litter consists of plastic items that are particularly persistent in the environment (Galgani et al., 2000).

Plastic is a term derived from the Latin “plasticus” which derives from the Greek “plastikos” that describes something able to be molded or fit for molding. Nowadays, we categorize as “plastic” a large and diverse group of synthetic polymers that had their origins in the late 19th century and faced their maximum spread and use in mid-twentieth century (Bergmann et al., 2015). Its versatility has meant that the amount of plastic produced annually has increased rapidly over the last few decades to an estimated 348 million tonnes in 2016 to 355 in 2017 and this total continues to grow about 4 % per year (PlasticsEurope, 2018). Plastics are synthetic organic polymers, derived from the polymerization of oil and gas monomers which are treated and mixed with a wide range of chemicals, usually referred as “plastic additives”, that improve plastic properties (Cole et al., 2011).

Plastic materials can be divided into two categories: thermoplastics and thermosets. The former group of polymers, that includes polyethylene (PE) and polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyester (PES), polyamides (PA), polycarbonates (PC), has reversible properties, as they can be melted when heated and hardened when cooled. The latter group is comprised of polymers such as

polyurethane (PUR), vinyl ester, acrylic and epoxy resins, that change their chemical structure when heated and form plastics that cannot be melted again (PlasticEurope 2017). Due to its low density, durability, excellent barrier properties and relatively low cost, plastics represent the ideal class of materials for a wide range of manufacturing and packaging applications and industrial sectors. Indeed, it has been estimated that between 75 and 80 million tonnes of packaging plastic materials are produced each year worldwide (Andrady, 2011).

In parallel with the increasing global use and production of plastic, their discharge is also increasing, contributing to the growth of urban litter (Andrady and Neal, 2009). While some plastic waste is recycled, the majority ends up in landfill (Lusher et al., 2015). The portion of plastic litter that does not reach landfills will travel around the earth's surface, being transported by wind until it reaches rivers and oceans (Hammer et al., 2012). Thus, through inefficient disposal, a large part of plastic waste enters in the marine environment, where it persists and accumulates (Cole et al., 2011; Gregory, 2013). It was recently estimated that 26% of the total marine plastic litter in the Mediterranean sea originated from vessel–based waste and 69% from land–based sources (Koutsodendris et al., 2008). Once in the aquatic system, plastic litter, due to its durability and buoyancy, can be transported over long distances by oceanic currents (Cole et al., 2016), reaching the most remote areas of the globe including Arctic waters, deep–sea and mid–oceanic gyres (Barnes et al., 2009; Hidalgo-Ruz et al., 2012; Lattin and Moore, 2004; Ryan et al., 2009; Thompson et al., 2010).

The spread of plastic debris in many aquatic compartments increases the habitats and species vulnerability to plastic contamination affecting the normal ecosystem functioning (Andrady, 2011; Moore, 2008). Indeed, the accumulation of plastic litter on the seafloor can inhibit the gas exchange between the overlying waters and the pore waters of the sediments resulting in limited oxygen availability for the benthic compartment (Goldberg, 1997). Also, many

organisms can be entangled in plastic nets, or ingest plastic objects (Laist 1997). Floating plastic debris can also contribute to the transport of microorganisms and larval stages of alien species across different regions, with ultimate potential effects on species composition and ecosystem structure (Aliani and Molcard, 2003).

From plastic to microplastics: sources and fate

Transport and fate of plastics in the ocean is strongly dependent on their physical-chemical properties. Particularly the dimensional range plays a central role in affecting the dynamics of plastic once it reaches the aquatic environment (Hammer et al., 2012). Plastic debris are often categorised in 3 classes: macrodebris (>25 mm), mesodebris (5-25 mm) and microdebris (<5 mm) (GESAMP, 2019). A common example of macrodebris is represented by abandoned or lost fishing nets (commonly named "ghost nets") easy to be transported with currents and tides and prone to trap animals or other debris (Hammer et al., 2012). Mesodebris mainly consists in raw industrial plastic material (pellets or nurdles) that are used by manufactures for remelting and molding into plastic products (Ogata et al., 2009), and due to their small size, are often released into the environment during transport. Microdebris comprises small plastic particles commonly referred to as "microplastics" (hereafter MP), a term was firstly used by Thompson (2004) to name very small plastic items found in the surface waters of rivers and seas and/or in aquatic sediments. The US National Oceanic and Atmospheric Administration (NOAA) (Arthur et al., 2009) defines as MP those particles smaller than 5 mm (to a minimum size of 10-20 μm), even if a standardized size range is jet to be defined. Indeed, different ranges are often considered depending on protocols and techniques used for the extraction and characterisation of the particles. This size inconsistency complicates the data comparisons among different studies and as pointed out by Andrady et al. (2015) a clear size definition is essential to understand the implications and environmental impact of MP

contamination.

Based on their origin we usually categorize as primary MP the synthetic particles industrially produced within the small size range and as secondary MP those originated from fragmentation of larger plastic debris. The primary MP group includes precursors of manufactured plastic products such as virgin industrial pellets or synthetic microbeads present in cleaners and cosmetics (i.e. exfoliates) (Hidalgo-Ruz et al., 2012; Napper and Thompson, 2016). The secondary MP category is represented by small plastic particles (i.e. fragments and fibres) derived from the breakdown and/or degradation of larger plastic items (Arthur et al., 2009; Browne et al., 2011; Cole et al., 2011) due to a combination of mechanical forces, photodegradation, oxidation and biodegradation by microbial activity (Andrady et al., 2015; Cooper and Corcoran, 2010; Sivan, 2011). In addition to fragmentation in the environment, some synthetic items can also fragment during use resulting in MP released in the environment as a consequence of every day use or cleaning (Thompson et al., 2015), such as microfibers that originate as a consequence of washing of clothes (Browne et al., 2011).

MP comprises a wide range of different shapes (e.g. irregular fragments, film, foam, spherical beads, or fibres) that originate from the fragmentation process and different periods of permanence in the environment. For example, a sharp edge indicates the recent break–up of larger pieces into fragments, while smooth edges can be associated with long residence time in the environment (Hidalgo-Ruz et al., 2012).

MP can be very diverse in their physical-chemical features (e.g. shape, size, colour, density and chemical composition), and some of these properties can strongly affect their fate and spatial distribution in the marine environment (Barnes et al., 2009; Cole et al., 2011; Hidalgo-Ruz et al., 2012; Vermeiren et al., 2016; Vianello et al., 2013). As example, by affecting buoyancy and sinking speed MP polymer density can influence their distribution and

permanence time in the water column: polymers denser than seawater, such as PVC, tend to sink and accumulate on the seafloor, while lower density particles, such as PP, tend to float at the water surface (Kukulka et al., 2012; Morét-Ferguson et al., 2010; Van Cauwenberghe and Janssen, 2014). These patterns can be further complicated by a synergistic action of abiotic factors (e.g. water turbulence and currents) and biotic transformations such as those operated by biofouling and bioaggregation, which can influence vertical mixing, transport (Dai et al., 2018) and accumulation of MP in specific geographic areas and compartments (Browne et al., 2011).

Interaction with organisms and effects

One of the major concerns related to the presence of MP in the marine environment is represented by their tendency to adsorb and/or desorb toxic contaminants such as additives or hydrophobic chemical compounds that are adsorbed from the surrounding water (Teuten et al., 2009). Plastic additives, commonly referred as "plasticisers", are chemical compounds (e.g phthalates, bisphenol A) used in manufacturing processes to change and/or improve the plastic properties for example providing malleability, resistance to high temperatures or degradation (Teuten et al., 2009). Plasticizers are not permanently bound to the plastic and can leach out into the aquatic environment or into organisms after ingestion (Hammer et al. 2012).

Synthetic particles can also adsorb from the environment persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (i.e. DDT, DDE). Due to their hydrophobicity POPs, universally occurring in seawater, can easily concentrate in MP at 10^6 orders of magnitude higher than in the sea water (Andrady, 2011). When ingested by marine biota, MP can release plasticisers or POPs conveying these substances in organism tissues and though the marine food webs

(Andrady, 2011; Teuten et al., 2009). This is concerning because, due to their small size and ubiquitous presence, MP can be available for ingestion by a wide range of marine organisms from different compartments and trophic levels including zooplankton, amphipods, polychaetes, barnacles, mussels, echinoderms, crustaceans, fish, seabirds and marine mammals (Browne et al., 2008; Cole et al., 2013; Foekema et al., 2013; Fossi et al., 2012; Graham and Thompson, 2009; Gregory, 2009; Herzke et al., 2016; Lusher et al., 2013, 2015a; Mathalon and Hill, 2014; Thompson et al., 2004).

There are a number of exposure pathways by which organisms can interact with MP. Direct consumption of MP can be prevalent in suspension-feeders and deposit-feeders (Batel et al., 2016; Browne et al., 2008; Cole et al., 2013; Farrell and Nelson, 2013; Van Cauwenberghe et al., 2015; Van Cauwenberghe and Janssen, 2014; Watts et al., 2015, 2014) owing to their inability to differentiate between MP and prey. Predators and detritivores may indirectly ingest plastic while consuming prey or scavenging detrital matter containing MP (Batel et al., 2016; Farrell and Nelson, 2013; Murray and Cowie, 2011; Setälä et al., 2014; Watts et al., 2014). MP can also adhere to external organisms appendages (Cole et al., 2013; Watts et al., 2014) or to micro- and macroalgae being transferred to consumers after ingestion (Gutow et al., 2016; Lagarde et al., 2016). Further, laboratory experiments showed that ingested MP can cause both physical damages (e.g. obstructions, abrasions, inflammation) and physiological stress such as immune response, decreased food consumption, weight loss, decreased growth rate, decreased fecundity and energy depletion (Browne et al., 2008; Capolupo et al., 2018; Cole et al., 2015; Pittura et al., 2018; Rochman et al., 2013). While such laboratory-controlled approach is necessary to identify the toxicological effects of contaminants, including MP, it is difficult to transpose those results to real environmental conditions and MP concentrations. This difficulty can be in part attributed to a lack of standard measure units and methodologies that make the quantification and characterisation of MP in natural

systems challenging and often not comparable among different studies (Löder and Gerdts, 2015).

The United Nations Environment Programme (UNEP) identified plastic pollution as a critical environmental issue (UNEP, 2016). The effctive vulnerability of species, communities and ecosystems, however, stil needs to be comprhensively addressed and quantified. Providing understanding of biological and ecological mechanisms affecting the transfer of MP in natural marine systems and food webs is essential to address these issues, as well as to effectively achieve the 'good environmental status' for marine litter requested by the EU Marine Strategy Framework Directive, 2008/56/EC (Galgani, 2002).

Objectives and thesis structure

My PhD research focused on exploring the role of biological processes in affecting the dynamics and fate of MP in coastal marine systems through the integration of laboratory and field experimentation. First I conducted a methodological study to identify the most suitable approach to extract and characterise MP ingested by organisms. Then, I quantified and characterised MP in different species with different habits and from different coastal areas. Based on the results obtained from this initial field analysis, I carried out laboratory and field experiments to explore how uptake by organisms and egestion via faecal matter affect the transfer of MP between marine compartments and species.

The thesis has been organized in 4 chapters, corresponding to as many stand-alone manuscripts for publication, with possible cross-references.

Chapter I: I focused on identifying the most appropriate method to quantify and characterise MP ingested by organisms. To achieve this scope I firstly reviewed the available protocols to separate MP from organismal soft tissue. Then, I compared an alkaline method with

potassium hydroxyde (KOH) and an enzymatic-based approach for their cost-effectiveness in quantifying MP in wild specimens of invertebrates. I used as model species the wild crab *Carcinus aestuarii,* and also included specific comparative tests on laboratory-prepared synthetic fibres. This chapter has been published in the journal Environmental Pollution (Piarulli et al., 2019).

Chapter II: I coordinated an extensive evaluation of MP contents in invertebrate species from coastal salt marshes from different European brackish systems (the Schelde Estuary and coastal lagoons along the Italian North Adriatic coast). Salt marshes are marine coastal habitat highly valuable from an ecological perspective but also influenced by severe and diverse anthropogenic pressures (Wong et al 2015, Airoldi et al 2016). Surprisingly little attention has been devoted to potential sources of MP contamination. We focused on a variety of species characterised by different ecological traits and life habits, aiming to identify potential biological and ecological features affecting the vulnerability of invertebrate species to MP contamination. This chapter has been accepted for publication by the journal Marine Pollution Bulletin.

Chapter III: I conducted a series of multi-level laboratory experiments to understand the role of biologically-mediated aggregation process in affecting the transfer of MP from the water column to the bottom sediments and across trophic levels (from filter feeders to detritus feeders). I used as model species the Mediterranean mussel *Mytilus galloprovincialis* and the polychaete *Hediste diversicolor,* and tested how the processes could vary in relation to MP with different physical (i.e. size) and chemical (i.e. density) properties. This chapter is a ready-manuscript for submission.

Chapter IV: I designed a novel biodeposits trap and applied it to: i) quantify ingestion/aegestion of MP by the Mediterranean mussel *Mytilus galloprovincialis* under realistic field conditions; ii) estimate the vertical transport of MP from the water column to the sediments conveyed by biodeposits; and iii) explore whether the transport of MP via biodeposits is consistent over time or alternatively temporally variable, which could reflect environmental fluctuations in MP availability or in important abiotic/biotic conditions affecting filtration and egestion rates by mussels, such as seawater temperature or food availability. This chapter is a manuscript in preparation.

All the work for this thesis was done in the framework of the the Joint Programming Initiative Healthy and Productive Seas and Oceans (JPI Oceans) international project Plastox "Direct and indirect ecotoxicological impacts of microplastics on marine organisms" aiming to investigate the ingestion, food web transfer, and ecotoxicological impact of MP, together with persistent organic pollutants (POPs), metals and plastic additives associated with them, on key European marine species and ecosystems (http://www.jpi-oceans.eu/plastox). Part of this work was also conducted through a collaboration with the other JPI Oceans project BASEMAN "Defining the baselines and standards for microplastics analyses in European waters" which aimed to compare and evaluate the approaches from sampling to identification of MP to validate and harmonise the available methods for the quantification and characterisation of MP in natural environments (http://www.jpi-oceans.eu/baseman).

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CHAPTER I

Microplastic in wild populations of the omnivorous crab *Carcinus aestuarii*: A review and a regional-scale test of extraction methods, including microfibres[1]

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Abstract

Microplastic (MP) has become ubiquitous in the marine environment. Its threat to marine organisms has been demonstrated under laboratory conditions, yet studies on wild populations still face methodological difficulties. We reviewed the methods used to separate MP from soft animal tissues and highlighted a lack of standardised methodologies, particularly critical for synthetic microfibres. We further compared enzymatic and a potassium hydroxide (KOH)-based alkaline digestion protocols on wild crabs (*Carcinus aestuarii*) collected from three coastal lagoons in the north Adriatic Sea and on laboratory-prepared synthetic polyester (PES) of different colour and polypropylene (PP). We compared the cost-effectiveness of the two methods, together with the potential for adverse quantitative or qualitative effects on MP that could alter the capability of the polymers to be recognised via microscopic or spectroscopic techniques. Only 5.5% of the 180 examined crabs contained MP in their gastrointestinal tracts, with a notably high quantitative variability between individuals (from 1 to 117 particles per individual). All MP found was exclusively microfibres, mainly PES, with a mean length (± SE) of 0.5 ± 0.03 mm. The two digestion methods provided comparable estimates on wild crabs and did not cause any visible physical or chemical alterations on laboratory-prepared microfibres treated for up to 4 days. KOH solution was faster and cheaper compared to the enzymatic extraction, involving fewer procedural steps and therefore reducing the risk of airborne contamination. With digestion times longer than 4 days, KOH caused morphological alterations of some of the PES microfibres, which did not occur with the enzymatic digestion. This suggests that KOH is effective for the digestion of small marine invertebrates or biological samples for which shorter digestion time is required, while enzymatic extraction should be considered as alternative for larger organisms or sample sizes requiring longer digestion times.

Key Words: Microplastic; Microfibres; Potassium hydroxide; Enzymes; Digestion; Crab

1.1 Introduction

Marine plastic pollution has become a matter of increasing concern because of its ubiquitous presence and worldwide distribution (Carbery et al., 2018), from polar regions to the equator (Barnes et al., 2009). Plastic contamination may represent a threat to wildlife, with considerable economic impacts on fisheries and potential consequences for human health (Van Cauwenberghe and Janssen, 2014). Owing to their small dimensions (<5 mm), microplastic (MP) particles overlap in size range with natural organic matter and plankton, and consequently have been shown to be ingestible by a wide range of marine species from different trophic levels (Wright et al., 2013), including zooplankton, polychaetes, bivalves, crustaceans, demersal and pelagic fish, seabirds, reptiles and mammals (Codina-García et al., 2013; Cole et al., 2013, 2014; Courtene-Jones et al.,2017b; De Witte et al., 2014; Lusher et al., 2013; 2015; Nelms et al., 2018; Tourinho et al., 2010; Van Cauwenberghe et al., 2015; Van Cauwenberghe and Janssen, 2014; Watts et al., 2015). Once ingested, MP can have physical, chemical and biological impacts on biota (Aljaibachi and Callaghan, 2018; Capolupo et al., 2018; Setälä et al., 2014; Straub et al., 2017; Wright et al., 2013) either directly or as a consequence of the associated additives and contaminants (Batel et al., 2018; Mato et al., 2001; Sleight et al., 2017; Teuten et al., 2009).

To date, most studies on the ingestion and transfer of MP have been performed under laboratory conditions, often exposing organisms to non-natural concentrations (Besseling et al., 2013; Cole et al., 2013; Von Moos et al., 2012; Watts et al., 2014). To gain a clear understanding of MP availability to marine organisms, the potential for MP accumulation and transfer through the food webs, and the risks for marine ecosystems and associated ecological processes, it is fundamental to obtain accurate measures of MP distributions in wild populations. Such information is currently limited, particularly in benthic invertebrate food webs. In part, this can be attributed to a lack of harmonisation of unit reports and extraction protocols that makes different studies not comparable. Many scientists and policy institutes highlighted a need of standardisation of methods and unit

measures (Catarino et al., 2017; Panel and Chain, 2016; UNEP, 2009). However, standard methods can be difficult to establish as MP may originate from a wide variety of sources (Andrady, 2011; Barnes et al., 2009; Boucher and Friot, 2017; Browne et al., 2011; Napper and Thompson, 2016; UNEP, 2009), and usually show high heterogeneous physical-chemical properties (e.g. size, shape, colour, density, and chemical composition) (Browne et al., 2008, 2011; Cole et al., 2011; Karlsson et al., 2017; Wright et al., 2013). Moreover, the very long multi-step approach (Iso, 2018) that standard organisation (such as ISO or OECD) usually take to standardise and accept methods makes particularly difficult the selection of a single method to monitor the microplastic contamination in marine organisms. A more feasible action would be to select a range of methods that minimise the risk of over/underestimation of synthetic particles and could be widely use for the same typologies of samples.

Different extraction methods may variously affect the morphology and chemistry of different MP types, making comparison of results challenging (Dehaut et al., 2016). One of the most critical procedural steps is to achieve an appropriate separation of MP from biological tissues without changing the physical-chemical properties of the particles. This is typically done via digestion of the tissue followed by filtration of the digestate. Various methods for tissue digestion are available, including acids such as HCl, HNO_3, $HClO_4$ (Claessens et al., 2013; De Witte et al., 2014), bases such as NaOH, KOH (Claessens et al., 2013; Dehaut et al., 2016; Rochman et al., 2013), hydrogen peroxide (Avio et al., 2015) and enzymes (Catarino et al., 2017; Cole et al., 2014). A recent study by Kühn et al. (2017) suggested that 1 M potassium hydroxide (KOH) offers a good compromise between maximising tissue digestion efficiency and minimising damage to polymeric structures. Although this method has been used in several studies to monitor the occurrence of MP in the gastrointestinal tract of organisms, the effects of this strong base on MP properties remain unclear, particularly for polymers commonly used to produce microfibres (e.g. polyester, polyamide and polyacrylate).

The physical-chemical properties of MP can be extremely variable in coastal and marine areas, significantly influencing their environmental fate and effects. Reliable and robust approaches for extracting and characterising MP are fundamental to understand how they enter the marine environment and their dynamics at community and ecosystem levels. An optimised extraction method should permit an accurate estimate of MP abundance without affecting the key MP physical-chemical properties needed to assess their occurrence and distribution. Currently, there is insufficient information to identify a universal digestion protocol for biological tissues, with the different methods reported in the literature needing more detailed comparison and assessment to identify the most effective solution.

The current study reviews the available knowledge on the effectiveness of different digestion approaches for extracting MP from biological samples and their potential impacts on the physical-chemical properties of MP. The review has a particular focus on field collected organisms and synthetic microfibers. The relatively novel enzymatic and the most established alkaline KOH digestion method resulted particularly promising in terms of effectiveness and low impact on pristine polymers. However, information on the effectiveness and effects of these methods on naturally ingested and weathered MP is still scarce. Thus, using the available literature as a basis, the two digestion procedures were directly compared for their effectiveness in digesting soft tissue, cost and applicability to the study of MP ingested by wild organisms naturally exposed to MP. The selected test species was the omnivorous shore crab *Carcinus aestuarii*. This crab is a common inhabitant of the northern Adriatic coastal lagoons (Mistri et al., 2001). Such estuarine environments can exhibit high MP pollution (Vermeiren et al., 2016) originating from terrestrial run-off, wastewater discharges, aquaculture, fishing, and other anthropogenic sources (Vianello et al., 2013). *C. aestuarii* is a omnivore scavenger and chief predator, feeding on a variety of animal and plant sources (Mistri, 2004), making the species potentially vulnerable to high MP pollution. Studies on the congeneric species *C. maenas* have suggested that ingested MP particles can be

retained for long periods (>2 weeks) by the hair-like setae in the gut, and retention could be even longer for microfibres (Watts et al., 2015). As a result, *C. aestuarii* may also play a critical role in the transfer of MP to higher trophic levels, including high-market-value fish species such as the gilthead seabream *Sparus aurata*, the sea bass *Dicentrarchus labrax*, and the European eel *Anguilla anguilla*, which commonly prey on this crab (Özbek et al., 2012).

We evaluated the occurrence and characteristics of MP in field-collected crabs using the two different methods (enzymatic and alkaline KOH) and assessed any adverse side-effects of each method that could alter the reliability of MP to be recognised or quantified. Since the evaluation of potential adverse effects of the two digestion methods on synthetic microfibres resulted particularly scarce in literature, the physical-chemical effects of the two selected digestion methods on this class of MP were further compared.

1.2 Material and methods

1.2.1 Literature review

A review of the available literature was conducted on the effectiveness of digestion protocols to separate synthetic plastic particles from biological material, mainly represented by soft tissues. Only peer–reviewed articles that explicitly provided information on the effectiveness of the methods and the relative effects on MP morphology and/or chemistry were selected. Literature not accounting for the effects of digestion methods on at least shape, origin, or polymer type were excluded. The search was performed on Web of Science Core Collection, using the following search terms: “Microplastic”, “Digestion”, “Biota” combined using the Boolean operator ‘AND’. The bibliographic research resulted in 56 original peer-reviewed research articles from 1985 to 2018. A total of 11 articles focusing on different digestion methods used to isolate MP particles from both marine and fresh water biota were retained as the basis for the review.

Subsequently two methods, enzymatic and alkaline with KOH, which seemed the most effective in

digesting organismal soft tissue with limited adverse effects on a wide range of polymers were selected and tested on both wild specimens and laboratory trials.

1.2.2 Study area

The northern Adriatic coast of Italy is fringed by a lagoon-river delta system with moderate exposure to wave action and a semi-diurnal micro-tidal regime (Russo et al., 2002). The numerous transitional water bodies and adjoining coasts in this region are intensively employed for multiple activities, with impacts from intensive farming and aquaculture, industry, dense urban centres in the watershed, shipping and tourism (Airoldi et al., 2016). The limited data available suggest considerable MP pollution in these systems (Vianello et al., 2013).

To explore the possible effects of the two digestion methods on a wide range of MP typologies, *C. aestuarii* we sampled from three coastal lagoons with different environmental conditions (hydrodynamics, wave exposure, nutrient loading, sediment granulometry) and varying pressures from potential anthropogenic sources of MP pollution (industries, aquaculture and wastewater effluents). From south to north, the lagoons were:

1. Piallassa Baiona (44°28’26.6’’; 12°14’52.5”E). This lagoon is divided into several ponds connected to the sea by channels, and receives inputs from 6 wastewater channels from urban, agricultural, industrial sewage treatment plants and thermal power plants (Airoldi et al., 2016).

2. Sacca di Bellocchio nature reserve (44°38’01.97’’; 12°15’48.78””E) in the Parco Delta del Po dell’Emilia-Romagna (44°37’39.8’’N, 12°15’55.8’’E). This is a back-barrier lagoon connected to the sea by a channel maintained by dredging (Wong et al., 2015). It is a protected area where the limited human pressures relate mainly to seasonal tourism and recreational activities.

3. Chioggia lagoon (45°13’11.52’’; 12°16’44.45””E). This lagoon is located in the southern part of the Venice Lagoon. It is an area characterised by intensive fishing and aquaculture, which may present a potential source of MP pollution.

1.2.3 Crab sample collection and dissection

Sampling was performed in June 2016. At each lagoon, *C. aestuarii* individuals were collected using seven standard fish traps prepared with fresh fish bait. The traps were deployed partially submerged approximately 10 to 100 meters apart over mudflat areas. Each deployment lasted for two hours. All crabs captured were retained from the traps, stored individually and transported to the laboratory where they were frozen at -20°C.

One hundred and eighty crabs having the same carapace size range (3-5 cm in width) were further dissected to remove the soft gastrointestinal tract tissue. The gastrointestinal tracts were weighed and placed in individual acid-washed and Milli-Q water-rinsed glass beakers for further processing.

1.2.4 Digestion of the soft gastrointestinal tissue

Half of the crabs from each lagoon (n = 30) were treated with the enzymatic procedure and half with the alkaline (KOH) procedure, resulting in a total of 90 crabs being analysed with each method. The enzymatic digestion protocol originally developed by Löder et al. (2017) for the analysis of planktonic samples was simplified (involving less procedural steps and enzymes) for use on the gastrointestinal tract of crabs. The protocol used Biozym F (lipase) and Biozym SE (protease and amylase) produced by Spinnrad® (Bad Segeberg, Germany) combined with a preceding detergent step using sodium dodecyl sulphate (SDS). The enzymes, differently from Löder et al., (2017), were in the form of common laundry detergents, therefore being relatively cheap and with longer durability compared to technical enzymes. Ten mL of the 25% anionic detergent sodium dodecyl sulphate (250 g SDS L^{-1}) (Sigma-Aldrich®) was added to each gastrointestinal tract sample immediately after dissection and samples were left to incubate at 50°C for 24 h. Subsequently, 5 mL of Enzyme F and 5 mL of Enzyme SE (Spinnrad®) were added. Samples were gently shaken and incubated at room temperature for 48 h. Five mL of Milli-Q water was added to samples with a high viscosity allowing for proper filtration of the digestate.

The alkaline digestion was performed using 1 M KOH according to Kühn et al. (2017), with 20 mL of the KOH solution added to each gastrointestinal tract sample. Vials were covered and incubated for 48 h at room temperature without any further manipulation until filtration. Finally, samples treated with both methods were individually vacuum filtered onto nylon filters (mesh size: 20 µm, Ø: 5 cm, PLASTOK®), and dried at room temperature on covered glass petri dishes.

1.2.5 Microplastic quantification and identification

The material retained on each filter was visually inspected under a stereomicroscope (Leica microsystem, ~50X magnification). Particles were manipulated with stainless steel tweezers to identify and thus exclude non-plastic particles such as glass, sand, mineral and shell. Particles exhibiting an obvious cellular structure were excluded as organic material. Any particle visually identified as being potentially made of plastic was characterised (shape, colour, size at the largest cross section), photographed (using a microscope mounted camera Motic BTWB, with Motic 2.0 software). Particles were then retained separately for subsequent Fourier-Transform-Infrared (FTIR) analysis using a Nicolet iN™10MX imaging microscope (Thermo Fisher Scientific, Waltham, MA, USA), fitted with a mercury-cadmium-telluride detector cooled by liquid nitrogen. Measurements were performed using a slide-on Attenuated Total Reflectance (ATR) objective, equipped with a conical germanium crystal, in the range 4,000–675 cm^{-1}, at a spectral resolution of 4 cm^{-1}. OMNIC Picta™ (Thermo Fisher Scientific, Waltham, MA, USA) was applied for a combined manipulation of the spectra dataset. Confirmation of polymer molecular signals in the recorded spectra was achieved by using the internal OMNIC Picta™ database, where similarities in wavenumber position and relative intensities of absorption bands were evaluated and compared.

1.2.6 Contamination prevention and quality control procedures

MP, particularly microfibres, are ubiquitous in indoor environments where they are present in the air and deposited on surfaces. Their presence can lead to contamination of samples and subsequent inaccuracies in results and bias. In addition, the low abundances of MP present within many environmental samples means any particle loss occurring during processing can potentially result in underestimation of MP occurrence and distributions. To counteract these issues the measures listed below were applied at each procedural step from sampling through to final analyses.

1.2.6.1 Sample collection

The use of plastic materials was reduced to a minimum by using metal and glass equipment where possible. All equipment was acidic Milli-Q washed and covered with aluminium foil prior to use. People collecting samples wore natural fibre clothing. All synthetic materials used at this stage were recorded (e.g. colour, polymer type) to enable potential contamination sources to be traced.

1.2.6.2 Sample processing

Sample processing (digestion and filtration) was carried out in a clean laboratory. All laboratory surfaces and floors were vacuum-cleaned and wiped with filtered detergent prior to use. To prevent external contamination, air circulation was minimised and a restricted number of personnel were allowed to enter in the laboratory.

Organisms were rinsed with Milli-Q water prior dissection to remove any residual external plastic particles. Contact with air and plastic surfaces during all laboratory procedures was minimised for samples, instruments and reagents by covering them with milli-Q rinsed aluminium foil before and after use. All instrumentation and equipment, including filters, was cleaned using bio-detergent and rinsed thoroughly with Milli-Q water prior to use. Four air filters were placed in various locations in

the laboratory to monitor potential airborne contamination during sample processing. To validate the effectiveness of the preventive practices and to test for potential differences in contamination risks between the two digestion methods, 4 procedural blanks, treated identically to the samples, were performed for each batch of samples processed. Material retained on air filters and from the procedural blanks was carefully examined following the same procedure as for the biological samples (see Section 1.2.5) to identify any synthetic particles representing external contamination and which should be accounted for using a blank correction.

To limit the risk of losing particles during the extraction procedures, crabs were carefully dissected without breaking the whole gastrointestinal tract, which was immediately placed into a glass beaker. The homogenisation of the samples in the digestion solution was conducted by slowly shaking the digestate only. After filtration of the digestate, each beaker and the filtering apparatus was rinsed 3 times with Milli-Q water to ensure that all particles stuck on the beaker walls were transferred to the filter.

1.2.6.3 Filter inspection

To minimise the risk of contamination during the visual inspection step, filters were exposed to the ambient air for the least amount of time possible. Inspection for microfibres, which are prone to contaminate samples due their ubiquitous presence in indoor air, was carried out first before inspection for other particle shapes (e.g. fragments, films, pellets). To minimise the risk of underestimating the number of plastic particles, two trained people inspected all filters and in the case of doubt regarding composition (synthetic or natural) the item was always selected for analysis by FTIR.

1.2.6.4 Non-synthetic microfibres

A goal of the digestion method comparison study was to provide an estimate for the potential risk of airborne contamination of samples related to the two different approaches. As the measures to prevent MP contamination were successful, the number of non-synthetic anthropogenic microfibres originating from clothing (e.g. laboratory coats), for which no preventative measures were applied, was used as an alternative estimate. Non-synthetic microfibres were counted on both air filters and in procedural blanks. A subsample of the most frequently occurring non-synthetic microfibres was analysed via FTIR to validate their composition and origin.

1.2.7 Digestion methods tested on synthetic microfibres

To determine if the digestion methods used may have influenced the results through physical-chemical alteration of the plastic microfibres during the treatment, additional tests were carried out using laboratory-prepared known microfibres of common synthetic materials found in the marine environment and in the gut of marine organisms: PES of different colours and PP. Microfibres (~5 mm) of each polymer type were cut from known fabrics or other plastic materials and placed in individual 20 mL glass scintillation vials. The microfibres were exposed in triplicate to both extraction methods for a total of 7 days. This long exposure time was chosen to explore whether either method could be applied without any time restrictions, therefore making them relevant for application to organisms needing extended exposures to the digestion agent.

As microfibres treated with KOH showed signs of physical alteration (see Results section), an additional experiment was performed to investigate if the rate of microfibre degradation changes over time (samples analysed after 1, 2, 3, 4, 7 days of exposure).

For all tests, untreated microfibres of each polymer type were used as controls. Microfibres were inspected and photographed under a stereomicroscope before and after the treatments. Any qualitative morphological effects were evaluated following the protocol developed by (Enders et al.,

2017) with L0 corresponding to no change (or unaffected), L1: initial recognizable changes (e.g. colour, morphology), L2: changes and early stage of dissolution (or disintegration), L3: degradation and change of bulk structure, and L4: indicating complete dissolution (or disintegration). Microfibres (controls and treated) were also analysed using FTIR to identify any chemical alteration in the polymer structure due the digestion treatments.

1.2.8 Statistical analyses

Owing to the large number of individual *C. aestuarii* which contained no MP (0 MP), the generated data did not meet the criteria for parametric statistics (Shapiro-Wilk normality test and Fligner-Killeen homogeneity test p-values <0.05). Therefore, the Mann-Whitney-Wilkoxon test was used to compare differences in gastrointestinal MP abundances between crabs digested with the enzymatic (n=90) and alkaline methods (n=90). No blank correction was applied, as air filters and procedural blanks showed no MP contamination (see Results). Differences in external contamination between the two methods were compared by quantifying the non-synthetic anthropogenic microfibres present on the air filters (n=40 and n=36 for enzymatic and KOH air filters, respectively) and procedural blank filters (n=20) with a t-test. These data were normally distributed after log(x+1) transformation and exhibited homogenous variance (Fligner-Killeen P-value > 0.05). For all tests, the level of significance was set to <0.05. All analyses were performed with Rstudio 0.99.903 (R Core Team, 2016).

1.3 RESULTS

1.3.1 Review of the digestion methods

The most critical aspect of digestion methods used to determine MP distributions in biological samples is to ensure an optimal balance between removal of any biological matrix (which could

mask MP identification by e.g. FTIR or Raman spectroscopy) and minimising alteration of MP physical-chemical properties. A review of the existing literature highlighted the common use of strong oxidising agents that can alter the physical-chemical structure of MP, particularly if high temperatures are also employed (Table 1). Acidic methods using nitric or chloric acids (HNO_3, HCl) appear to be the most efficient and effective in molecular cleavage and biogenic material dissolution (Claessens et al., 2013; Karami et al., 2017; Naidoo et al., 2017). However, these acid-based methods can be aggressive and may damage pH-sensitive polymers (Catarino et al., 2017; Enders et al., 2017; Van Cauwenberghe and Janssen, 2014). Oxidising agents such as hydrogen peroxide (H_2O_2) and potassium sulphate (K_2SO_4) have less effect on polymer properties compared to acids (Avio et al., 2015; Ding et al., 2018; Karami et al., 2017), but can result in incomplete soft tissue digestion and production of foam, which may interfere with the subsequent MP identification (Avio et al., 2015).

Some studies report that strong bases can be a valuable compromise between effective digestion of the biological matrix and limited adverse effects on synthetic particles (Budimir et al., 2018; Catarino et al., 2017; Cole et al., 2014; Dehaut et al., 2016; Ding et al., 2018; Enders et al., 2017; Karami et al., 2017; Kühn et al., 2017). Tissues from different species (birds, fish, bivalves) have been successfully digested with the use of 10% or 1 M potassium hydroxide (KOH) in a relatively short time (5-48 h), without any noticeable physical-chemical alterations in most polymer types. Cole et al., (2014) introduced enzymatic digestion with a serine protease (Proteinase K) as a biological method for hydrolysing proteins and breaking down tissues. Enzymatic digestion methods (e.g. with Proteinase K, Corolase 7089, Trypsin), which are widely used in forensic studies, appear to cause no damage to polymers. However, enzymatic-based approaches require long digestion times and multiple procedural steps (Löder et al., 2017), and may also result in incomplete tissue digestion (Karlsson et al., 2017).

Table 1: Overview of reported digestion methods for the isolation of MP from biota. Summarised are (i) the different digestion methods, (ii) the tested particles[a], (iii) the type of the test performed[b], (iv) the procedural duration in hours or days, (v) the efficacy of the digestion, (vi) any effects on the physical structure of MP, (vii) any reported interference with FTIR or Raman identification and (viii) the reference. NP= not performed and NR= not reported by authors.

Method	Test particles[a]	Test[b] & species	Duration	Efficacy	Physical changes	FTIR / Raman	Reference
HNO_3	Pristine spheres (PS), fibres/fragments (nylon), industrially made flakes/fragments (PET, HDPE, PVC); fragments from consumer products (PP, LDPE, HDPE, PS, EPS, ABS, PA, PET, PC, PVC, PMMA, PTFE)	EPR, SPO (*M. edulis* mussel and *C. garepinius*, *A. dussumieri* fish) FO (*A. dussumieri* fish)	2-5 h	Good	EPR = Structural changes on PS, PET, EPS, ABS, PA, PC, PVC, Complete dissolution of nylon SPO = Melting of PE, HDPE, PP and complete dissolution of Nylon FO = None	Altered molecular signals	Catarino et al., 2017 Claessens et al., 2013 Enders et al., 2017 Karami et al., 2017 Naidoo et al., 2017
HNO_3+ H_2O_2 + NaOH	Pristine spheres (PP, PE); fragments from consumer products (PA, PET, ePS, LDPE)	EPR, SPO (various invertebrates)	NR	Good	EPR = Degradation of particles SPO = None	NR	Karlsson et al., 2017
HCl	Pristine fragments (LDPE, HDPE, PP, PS, PET, PVC, PA6, PA66)	SPO (*C. garepinius* fish)	4 d	Good	Melting of PET	Altered molecular signal	Karami et al., 2017
NaClO	Fragments from consumer products (PP, LDPE, HDPE, PS, EPS, ABS, PA, PET, PC, PVC, PMMA, PTFE)	EPR	5 h	Formation of foam	None	Altered molecular signals (ABS and PA)	Enders et al., 2017
VIP1	Fragments from consumer products (PP, LDPE, HDPE, PS, EPS, ABS, PA, PET, PC, PVC, PMMA,	EPR	5 h	Presence of undigested material	None	NR	Enders et al., 2017

	PTFE)						
H_2O_2	Pristine fragments/beads (LDPE, HDPE, PP, PS, PET, PVC, PA6, PA66)	SPO (*C. gariepinus* fish and mullets) FO (*C. farreri* and *M. galloprovincialis* bivalves; *S. pilchardus, S. acanthias, M. merluccius* fish)	4 d	Foamy particles formation	None	Good	Avio et al., 2015 Ding et al., 2018 Karami et al., 2017
NaOH	Spheres (ePS,), line (nylon), fibres (PES), pristine fragments/flakes (CA, HDPE, LDPE, PA-12, PC, PET, PMMA, PP, PS, PSXL, PTFE, PUR, UPVC, ePS, PA6, PVC); fragments from consumer products (PC, PET, HDPE)	EPR, SPO (*M. edulis* mussels; *C. harengus membras* fish) FO (*S. sprattus, G. aculeantus* fish)	1 h-7 d	Good	EPR = Structural changes on PE uPVC, CA and PET, partial disruption of Nylon and loss of PES; SPO (only on PA6) = None	CA not identifiable	Budimir et al., 2018 Catarino et al., 2017 Cole et al., 2014 Dehaut et al., 2016
KOH	Pristine pellets/fragments and from consumer products (LDPE, PP, PET, EVAVA19%, PE-LLD recycled, SAN, PA66, GPPS, PC, PA6, ABS, HDPE, CA, PA-12, PC, PMMA, PS, PSXL, PTFE, PUR, UPVC, ePS);	EPR, SPO (*G. gadus, P. virens,* mussels, *C. gariepinus*, *S. sprattus* fish); FO (mussels, crabs, black seabream; *C. farreri, M. galloprovincialis* bivalves)	5-48 h	Good	EPR, SPO=Structural changes on CA, PET and PVC	Good but slightly altered molecular signals	Dehaut et al., 2016 Enders et al., 2017 Ding et al., 2018 Karami et al., 2017 Kühn et al., 2017
Protease K	Pristine spheres (ePS, PP, PE); line (nylon); fibre (PES); fragments (PE, PA, PET, ePS, LDPE); granules (uPVC)	EPR, FO (plankton; *S. trutta* fish; *M. edulis* mussels)	24-48 h	Presence of undigested material	None (except loss of weight for lighter polymers)	Good	Cole et al., 2014; Karlsson et al., 2017
Corolase 7089	Industrially made fragments and flakes (PET, HDPE, PVC), particles cut from thread	SPO (*M. edulis* mussels) FO (*M. edulis* mussels)	1 h	Good	None	Good	Catarino et al., 2017

	(nylon)						
Trypsin	Fragments from consumer products (PET, HDPE, PVC, PP, PS, PA)	EPR, FO (*M. edulis* mussels)	30 min	Good	None	Good	Courtene-Jones et al., 2017b
SDS + enzymes	Pristine beads (PE, PP)	EPR and FO (Plankton)	10 d	Good	None	Good	Löder et al., 2017

[a]PS = polystyrene; ePS = expanded polystyrene; PES = polyester; PE = polyethylene; HDPE = high density polyethylene; LDPE = low density polyethylene; PA = polyamide; PET = polyethylene terephthalate; PP = polypropylene; CA = cellulose acetate; PVC = polyvinyl chloride; UPVC = unplasticised polyvinyl chloride; PC = polycarbonate; PMMA = polymethylmethacrylate; PTFE = polytetrafluoroethylene; PUR = polyurethane; EVA = ethylene-vinyl acetate; SAN = styrene-acrylonitrile; GPPS = general purpose polystyrene; ABS = acrylonitrile butadiene styrene.

[b]EPR = direct exposure of MP to the chemicals used for the digestion; SPO = test of the digestion method on organisms tissue spiked with MP; FO = extraction of MP from field collected organisms)

1.3.2 Efficiency of contamination prevention procedures

No MP particles were found on the air filters or procedurals blanks. Only natural microfibres of cotton/cellulose (mostly white and blue with a distinctive morphology) were identified by visual inspection and were further confirmed by FTIR spectroscopy.

1.3.3 Microplastics occurrence in C. aestuarii

A total of 201 MP particles were identified in the 180 crabs collected from lagoons in the northern Adriatic Sea, with an average (± SE) of 1.1 ± 0.7 particles per individual. However, only 10 individual crabs contained MP, and there was a high variability in the number of particles among individuals: 8 of the 10 individuals contained only 1 synthetic particle, while 2 individuals had 117 and 76 plastic particles respectively (Figure 1a and Table S2, Supplementary Information). All MP were identified as monofilament microfibres with the exception of one multifilament microfibre. The microfibres ranged in length from 0.03 to 3 mm, with an average length (± SE) of 0.5 ± 0.03 mm (Figure 1b). Most microfibres were red or black in colour (Figure 1c), and the most frequent polymer was PES (99%) (Figure 1d). The characteristics (length, colour, polymer type) of each

individual microfibre, the generated FTIR spectra, and the reference spectra used for identification are reported in Figures S1, S2, S3 and in Table S2 of the Supplementary Information.

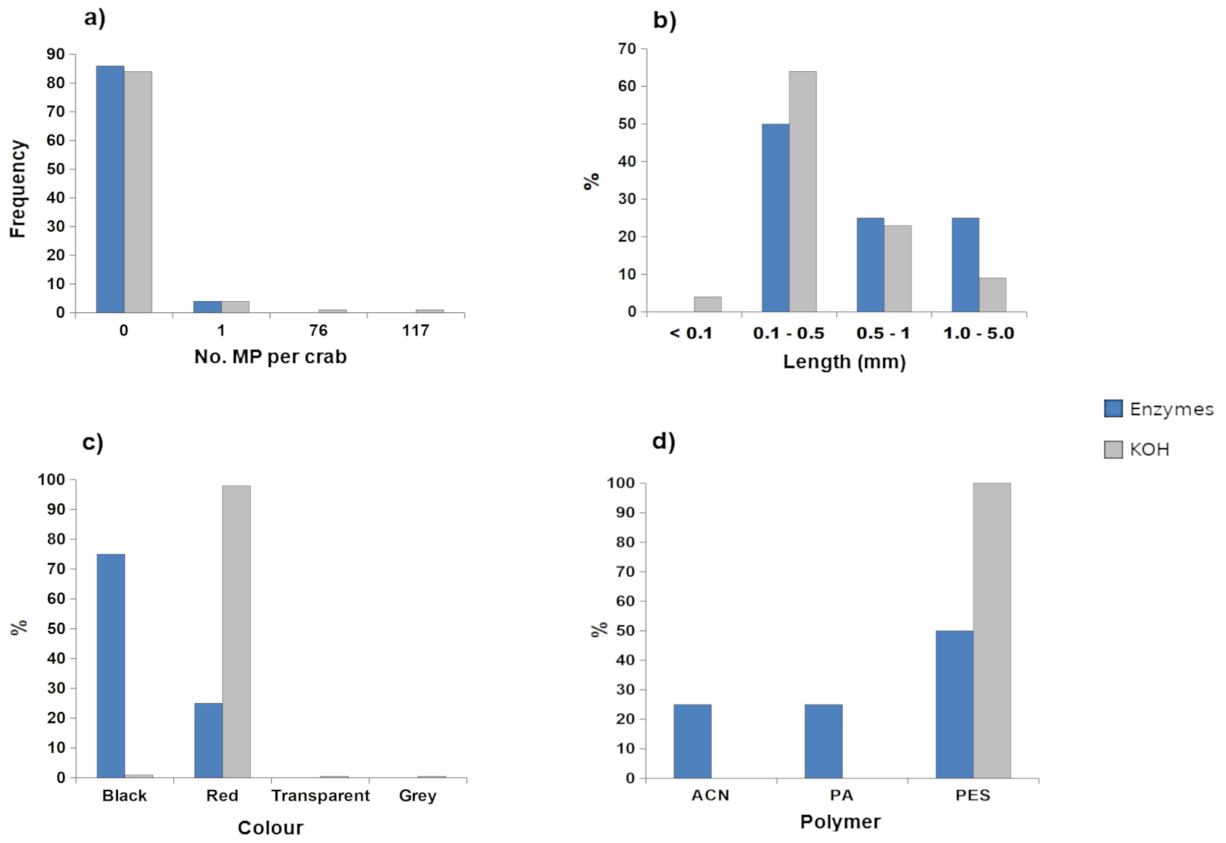

Figure 1. Synthetic particles extracted from crabs (*C. aestuarii*) with the enzymatic and alkaline (KOH) digestion methods. **a)** Number of synthetic particles per crab. **b)** Percentage of synthetic particles per size class. **c)** Percentage of synthetic particles per colour. **d)** Percentage of synthetic particles per polymer type (ACN = acrylonitrile; PA = polyamide; PES = polyester).

1.3.4 Comparison of digestion methods

1.3.4.1 Occurrence of microplastic in field organisms

The number of MP particles extracted from crabs did not differ significantly between the enzymatic and alkaline methods (Mann-Withney-Wilkoxon test p-value= 0.5). Only 4 out of the 90 crabs (4%) digested with the enzymatic method contained MP (Figure 1a) with an average (±SE) of 0.04 (±

0.02) per individual (Table S1, Supplementary Information). All MP particles were microfibres, with most being black in colour (Figure 1c). Only 6 out of the 90 crabs (7%) digested with KOH contained MP (Figure 1a), with an average (±SE) of 2.19 (± 1.5) per organism (Table S2, Supplementary Information). All MP particles were microfibres, with most being red, followed by black, transparent and grey (Figure 1c).

The microfibres extracted from crabs digested with the enzymatic method ranged from 0.19 to 2.55 mm (mean± SE= 0.94 ± 0.5 mm) in length, with 50% (n=2) being 0.1 - 0.5 mm long and the others being 0.5 - 1 mm (25%, n=1) and 1 - 5 mm (25%, n=1) (Figure 1b). The microfibres extracted with KOH ranged had a mean length (± SE) of 0.51 ± 0.03 mm, but exhibited more variation in their length, with 64% (n=126) being 0.1 - 0.5 mm, 23% (n=45) being 0.5 - 1 mm, and small percentage being 1 - 5 mm (9%, n=18)) and < 0.1 mm (4 %, n=8) (Figure 1b). No particles/microfibres from either digestion method presented signs of discoloration or morphological degradation. Figure 2 provides photographic examples of the microfibres extracted with the enzymatic (Figure 2b) and alkaline (Figure 2a) digestion methods. FTIR analysis of the microfibres extracted from crabs digested with enzymes indicated that ~50% (n=2) were PES, 25% (n=1) were polyamide (Nylon) and 25% (n=1) were acrylonitrile (Orlon), while 100% (n=197) of the microfibres isolated from crabs treated with KOH were PES (Figure 1d).

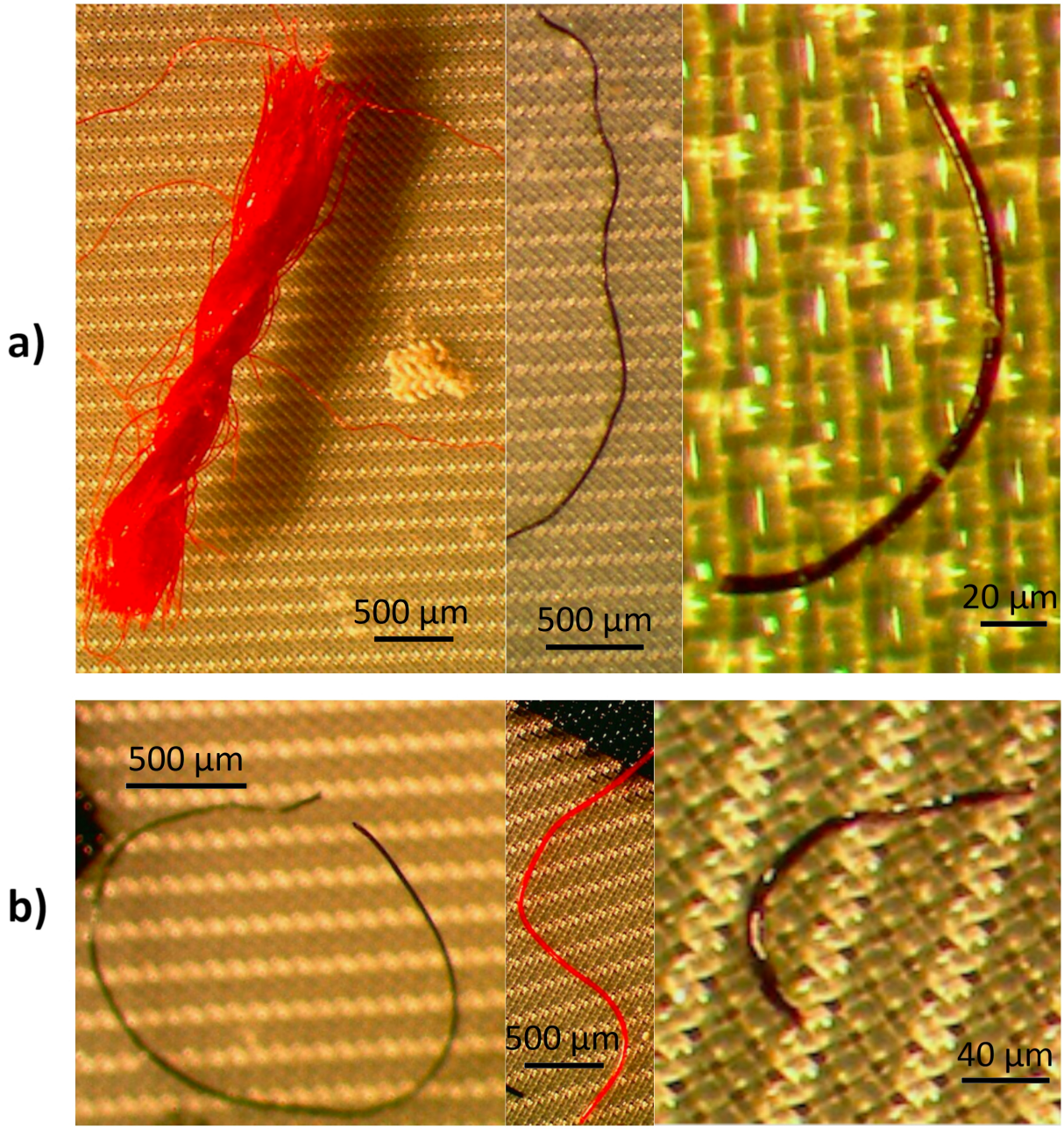

Figure 2. Photographic examples of fibres extracted from the gastrointestinal tracts of crabs (*C. aestuarii*) digested with **a)** the alkaline (KOH) method (polyester), and **b)** the enzymatic method (from left to right: polyamide, polyester, acrylonitrile).

1.3.4.2 Comparison of contamination risks

The quantity of non-synthetic anthropogenic microfibres in the procedural blanks (Figure 3a) and air filters (Figure 3b) did not differ significantly between the enzymatic and alkaline digestion methods (t-test p-values = 0.4 and 0.8, respectively). The average (± SE) number of non-synthetic

anthropogenic microfibres in the procedural blanks and air filters was 3.5 ± 0.5 and 1.5 ± 0.3 for the enzymatic method and 3.1 ± 0.4 and 1.6 ± 0.3 for the alkaline method.

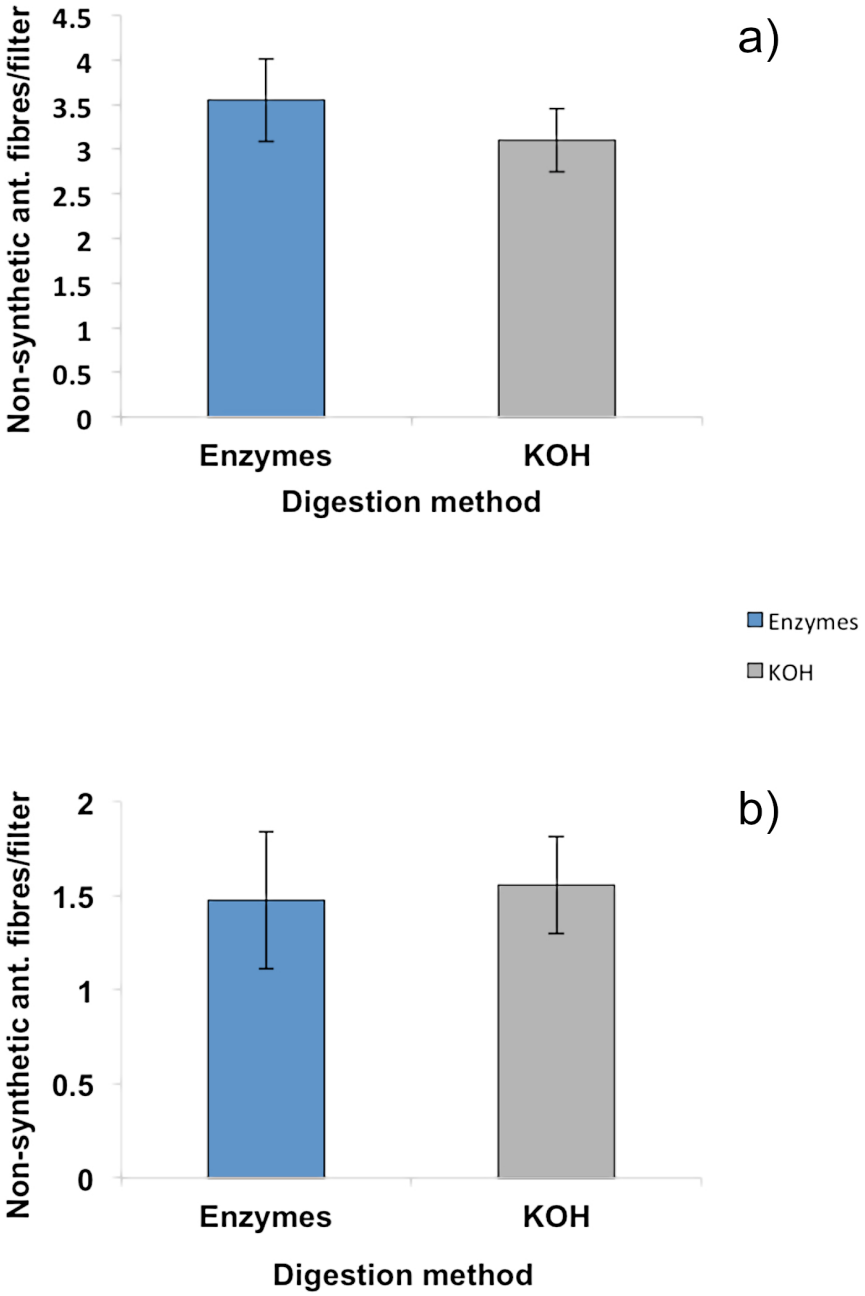

Figure 3. Average (± SE) number of non-synthetic anthropogenic particles in **a)** procedural blanks (n=20) and **b)** air filters (n=40; n=36) associated to either enzymatic or alkaline (KOH) methods.

1.3.4.3 Effects of the digestion procedures on synthetic microfibres

Most of the laboratory-prepared microfibres that were treated with the enzymatic method and the alkaline method did not exhibit any clear structural changes (e.g. colour or shape) or damage (Figure 4a,b). According to the scale of impact developed by Enders et al., (2017), all polymers treated with the enzymatic digestion method were classified as being 'L0 degraded' (i.e. no changes) after a period of 7 days. No alteration (L0) was observed for any type of microfibre exposed to the KOH treatment for up to 4 days. However, one type of PES microfibres presented signs of discoloration and moderate structural changes (classified as L3) after 7 days of exposure to the KOH solution (Figure 4c). None of the microfibres showed any significant change in their FTIR spectra compared to the untreated control materials, irrespective of the digestion method, duration or polymer type (Figures S4-S5, Supplementary Information).

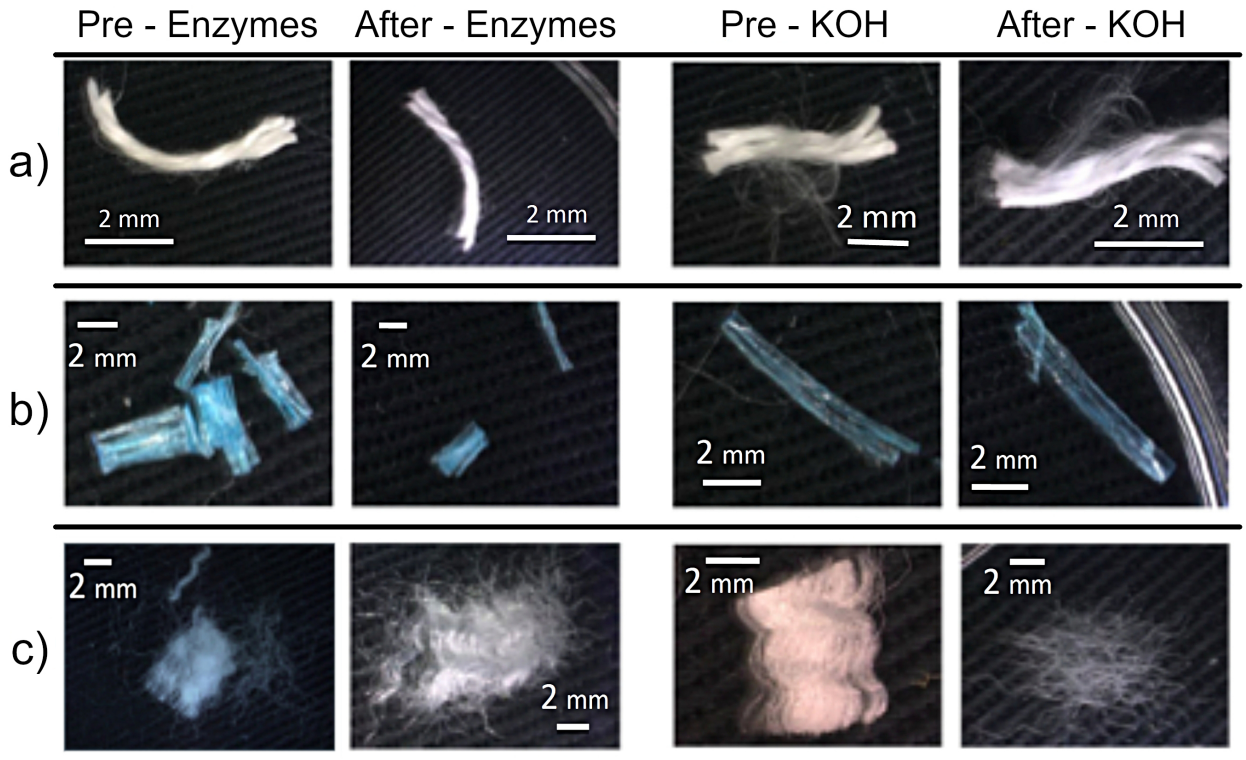

Figure 4. Photographic examples of **a/c)** Polyester (PES) and **b)** Polypropylene (PP) fibres before (Pre-) and after (After-) 7 days of enzymatic (Enzymes) and alkaline (KOH) digestion treatment.

1.4 Discussion

This study represents one the first empirical field assessments of MP occurrence in the gastrointestinal tract of *C. aestuarii.* Extensive field studies on large sample volumes of organisms and environmental samples are still scarce and, in many cases, do not consider microfibres in their quantifications. This is mainly due operational difficulties in applying effective contamination prevention practices on these types of extremely mobile and ubiquitous MP particles. The current study demonstrates that effective contamination prevention procedures can be implemented successfully and allow the reliable extraction and quantification of synthetic microfibres in wild organisms. As a result, future studies should consider implementing equivalent measures so that microfibres, which appear to be a significant proportion of the total environmental MP load, can be quantified.

Interestingly, this study showed that most crabs (96% of 180 organisms) did not contain any MP in their gastrointestinal tract, and where organisms did contain MP they were exclusively microfibres. The average MP content (1.1 ± 0.7 SE microfibres per crab) was within the range of values reported for other coastal invertebrates such as the coastal shrimp *C. crangon* and fish (Devriese et al., 2015; Lusher et al., 2013). However, studies on MP in crabs are scarce, which makes it difficult to make robust comparisons. Furthermore, the MP content was extremely variable among individuals, which is in line with observations made in previous studies on other marine species (Lusher et al., 2013; Nelms et al., 2018). While most crabs did not contain any MP, two individuals had > 50 microfibres in their gastrointestinal tracts. Currently the drivers behind such large variability are unknown, but it is suggested that these variable levels of MP occurrence could reflect differences in uptake and egestion rates among individual crabs (Watts et al., 2015), small-scale patchiness in the distribution and bioavailability of MP particles in the environment (Lourenço et al., 2017) and/or in prey items (Batel et al., 2016; Mattsson et al., 2017; Tosetto et al., 2017). Bour et al., (2018) described a variable occurrence of MP within many species including bivalves which are, together with

polychaetes the main food-source of the predator *C.aestuarii*. However, data regarding small-scale variability at any environmental compartments or trophic levels are still scarce and more studies are needed in this direction.

The observed MP variability may also originate as a consequence of moulting events. During moulting, particularly observed during Summer and Autumn (Chen et al., 2004), crabs experience periods of starvation (Sánchez-Paz et al., 2006), therefore an early moulting event before the sampling could have reduced the uptake of MP for the majority of the examined crabs. Another possible explanation could be that the high quantity of microfibres found in two crabs resulted from the fragmentation of multifilament plastic items during the ingestion and passage through the gastrointestinal tract. In fact, another crab, from the same area, showed a multifilament microfibre with similar properties (length, colour and polymer) of the individual microfibres from the two highly contaminated crabs.

Microfibres constituted 100% of the synthetic particles found in the analysed organisms, confirming that microfibres are the most prevalent synthetic particles in benthic invertebrates (Claessens et al., 2011; Lourenço et al., 2017; Taylor et al., 2016). This could reflect a greater occurrence and bioavailability of microfibres compared to other types of particles (e.g. fragments, films, pellets) in the system. Indeed, microfibres originate from a variety of sources and make up 85% of human-made debris on shorelines around the world (Browne et al., 2011). However, the few available environmental data from the north Adriatic lagoon region where the crabs were sampled in the current study (Vianello et al., 2013) indicate that only a small fraction of MP particles in the sediments are microfibres. Despite appearing to be less abundant than fragments in this ecosystem, the prevalence of microfibres in the gastrointestinal tract of crabs may be due to their physical properties relative to other types of MP. The high aspect ratio of microfibres and their propensity to become entangled with each other and with biological features may lead to longer retention times caused by a slower transit through the digestive system. In contrast, fragments and spheres have

been shown to pass quickly through the gastrointestinal tract and readily excreted (Watts et al., 2015). Further work should explore uptake and egestion rates of MP in relation to both their physical-chemical properties and environmental distribution, to clarify what factors control the distribution of microfibres in the biota (Lourenço et al., 2017).

The length of microfibres found in our analyses were within the same size range of those found by Devriese et al., (2015) in the coastal shrimp *C.crangon* but smaller of those reported in various mediterrean and atlantic fish species (e.g. Avio et al., 2015; Lusher et al., 2013; Nelms et al., 2018). This may depend on the different feeding physiology (e.g. particle selectivity), mobility, marine compartments of the considered species and/or on the different microfibers sources (Devriese et al., 2015). Among all synthetic particles extracted, the dominating polymer type was PES which, together with acrylic and polyamide, is denser than sea water (Courtene-Jones et al., 2017a), thus probably sinking faster into the sediments (Linders et al., 2018).

A slightly greater variability in polymer composition was observed in crabs processed with the enzymatic method than in those digested with KOH, where 100% of particles/fibres were PES. Given the low number of individuals with MP and the very large variability in the number of particles among those few individuals (from 1 to 117 particles per individual), it is difficult to determine if this occurred only by chance or if the KOH digestion process resulted in destruction of certain polymer classes. Moreover, the subsequent laboratory tests with KOH on the prepared microfibres did not result in any noticeable changes to their physical-chemical structure, which is in line with previous results reported by Kühn et al., (2017). Thus, we can probably exclude the occurrence of polymer disruption by KOH in our study.

When considering the outcomes of the methodological review, the laboratory tests confirmed that enzymatic digestion is a gentle digestion method, also applicable when longer (> 4 days) digestions are required (Cole et al., 2014; Courtene-Jones et al., 2017). However, the enzymatic treatment may not result in a complete digestion of biological tissues, making the subsequent visual inspection

more difficult and time consuming (Karlsson et al., 2017). The KOH digestion method did not cause any observable physical-chemical changes to any of the tested polymer microfibres when treated for up to 4 days, consistent with reports from other studies (Dehaut et al., 2016; Enders et al., 2017; Karami et al., 2017; Kühn et al., 2017). After a KOH digestion period of 7 days some of the PES microfibres exhibited slight morphological changes suggesting degradation is occurring, but none of the other microfibre types were affected. This impact of KOH has also been observed by Dehaut et al., (2016) on pristine PES granules particularly characterised as polyethylene terephthalate (PET). The effect of KOH is most unlikely to be a problem when studying MP occurrence in most smaller-sized invertebrates, where the time required for tissue digestion is short (e.g. in the current study < 3 days). However, it suggests the need for caution when long digestion times are required, for example when large quantities of soft tissues are to be digested. Independently of exposure time (up to 7 days), neither digestion method affected the chemical structure of microfibres that prevented ready identification by FTIR.

The careful contamination prevention procedures adopted in our study successfully limited any external plastic contamination of the samples. However, a slightly higher amount of non-synthetic cotton and cellulose microfibres (from laboratory coats and cleaning tissue) were observed in the procedural blanks from the enzymatic digestion procedure. This most likely related to the longer processing time (5 days), a more difficult filtration of the digestate and the higher quantity of steps involved in the procedure. Importantly, this suggests that the enzymatic procedure has a higher potential risk for introducing airborne MP contamination to the samples, in addition to the increased potential for loss and underestimation of the number MP particles.

Overall, the KOH procedure was less time consuming, considerably cheaper, and had a lower contamination risk than the enzymatic digestion method. When using KOH, the digestion of the whole gastrointestinal tract of 90 crabs required only 2 days, fewer procedural steps and a low cost per sample (due to cheaper reagents). In contrast, the enzymatic digestion required 5 days, more

procedural steps and was more time consuming during the filtration step due to the very viscous consistency of the digestate. Even using enzymes from laundry detergents, which are already cheaper than purchasing the pure enzymes usually used for laboratory analyses, the cost per sample was 40 times higher than with the KOH procedure.

1.5 Conclusion

The current study empirically demonstrated a low and variable occurrence of MP particles in the gastrointestinal tract of the shore crab *Carcinus aestuarii* collected from north Adriatic coastal lagoons. While most individuals did not contain any MP, a small number contained extremely high numbers (up to 117) that were identified as synthetic polymer microfibres in all cases. To accurately understand the sources and impacts of MP on biota, it is essential to use extraction and analysis methods that allow quantification and characterisation of microfibres as well as other types of MP. This is important when studies that do report the presence of microfibres often find them to be the most prevalent type of MP debris in environmental samples (water, sediment and biota). Future research should focus on measuring ingestion/egestion rates of different types of MP particles by benthic marine invertebrates, to try and gain a clearer understanding of the highly variable occurrence observed in both the current and other studies. Although both digestion methods tested are applicable for the digestion of biological samples, the KOH method has a lower risk for contamination and is the most cost-effective and efficient, particularly when applied for large-scale monitoring purposes. However, an optimised enzymatic method could represent a better alternative for large invertebrates and fish, for which long (>4 days) digestion times are required.

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Supplementary Information

Table S1: General data on the two batch of crabs *Carcinus aestuarii* analysed with the Enzymatic and Alkaline (KOH) digestion methods. N= Total number of individuals analysed; Mean (± SE) of: the length of carapace, the gastrointestinal tract wet weight after dissection and the number of microplastic per individual for each batch of organisms analysed.

Method	N	Carapace length (cm)	Wet weight (g)	microplastic/individual
Enzymes	90	3.9 ± 0.09	1.4 ± 0.11	0.04 ± 0.02
KOH	90	4.1 ± 0.9	1.5 ± 0.9	2.18 ± 1.54

Table S2: Characteristics (Colour, Length, Polymer type) of fibers extracted with the two digestion methods

Digestion Method	Sample ID	Colour	Length (mm)	Polymer identified with FTIR
Enzymes	BEL2-CR1	Black	0.20	Polyamide
Enzymes	BEL3-CR9	Black	0.19	Acrylonitrile
Enzymes	BEL3-CR5	Red	0.82	Polyester
Enzymes	CHI2-CR2	Black	2.55	Polyester
KOH	(2)BA2-CR3	Red	3.45	Polyester
KOH	(2)BA3CR10-1	Red	0.33	Polyester
KOH	(2)BA3CR10-2	Red	0.57	Polyester
KOH	(2)BA3CR10-3	Red	0.09	Polyester
KOH	(2)BA3CR10-4	Red	0.92	Polyester
KOH	(2)BA3CR10-5	Red	0.87	Polyester
KOH	(2)BA3CR10-6	Red	0.83	Polyester
KOH	(2)BA3CR10-7	Red	0.10	Polyester
KOH	(2)BA3CR10-8	Red	0.13	Polyester
KOH	(2)BA3CR10-9	Red	0.52	Polyester
KOH	(2)BA3CR10-10	Red	0.44	Polyester
KOH	(2)BA3CR10-11	Red	0.31	Polyester
KOH	(2)BA3CR10-12	Red	1.55	Polyester
KOH	(2)BA3CR10-13	Red	0.49	Polyester
KOH	(2)BA3CR10-14	Red	0.41	Polyester
KOH	(2)BA3CR10-15	Red	0.43	Polyester
KOH	(2)BA3CR10-16	Red	0.72	Polyester
KOH	(2)BA3CR10-17	Red	0.44	Polyester
KOH	(2)BA3CR10-18	Red	0.45	Polyester
KOH	(2)BA3CR10-19	Red	0.92	Polyester
KOH	(2)BA3CR10-20	Red	0.76	Polyester
KOH	(2)BA3CR10-21	Red	0.43	Polyester
KOH	(2)BA3CR10-22	Red	0.95	Polyester
KOH	(2)BA3CR10-23	Red	0.59	Polyester
KOH	(2)BA3CR10-24	Red	1.03	Polyester
KOH	(2)BA3CR10-25	Red	0.54	Polyester

KOH	(2)BA3CR10-26	Red	0.40	Polyester
KOH	(2)BA3CR10-27	Red	0.63	Polyester
KOH	(2)BA3CR10-28	Red	0.773	Polyester
KOH	(2)BA3CR10-29	Red	0.35	Polyester
KOH	(2)BA3CR10-30	Red	0.62	Polyester
KOH	(2)BA3CR10-31	Red	0.57	Polyester
KOH	(2)BA3CR10-32	Red	1.02	Polyester
KOH	(2)BA3CR10-33	Red	0.34	Polyester
KOH	(2)BA3CR10-34	Red	0.30	Polyester
KOH	(2)BA3CR10-35	Red	0.20	Polyester
KOH	(2)BA3CR10-36	Red	0.56	Polyester
KOH	(2)BA3CR10-37	Red	0.53	Polyester
KOH	(2)BA3CR10-38	Red	0.47	Polyester
KOH	(2)BA3CR10-39	Red	0.32	Polyester
KOH	(2)BA3CR10-40	Red	0.37	Polyester
KOH	(2)BA3CR10-41	Red	0.12	Polyester
KOH	(2)BA3CR10-42	Red	0.31	Polyester
KOH	(2)BA3CR10-43	Red	0.39	Polyester
KOH	(2)BA3CR10-44	Red	0.20	Polyester
KOH	(2)BA3CR10-45	Red	0.06	Polyester
KOH	(2)BA3CR10-46	Red	0.27	Polyester
KOH	(2)BA3CR10-47	Red	1.04	Polyester
KOH	(2)BA3CR10-48	Red	0.24	Polyester
KOH	(2)BA3CR10-49	Red	0.20	Polyester
KOH	(2)BA3CR10-50	Red	1.45	Polyester
KOH	(2)BA3CR10-51	Red	1.07	Polyester
KOH	(2)BA3CR10-52	Red	0.33	Polyester
KOH	(2)BA3CR10-53	Red	0.35	Polyester
KOH	(2)BA3CR10-54	Red	0.06	Polyester
KOH	(2)BA3CR10-55	Red	0.05	Polyester
KOH	(2)BA3CR10-56	Red	0.06	Polyester
KOH	(2)BA3CR10-57	Red	0.20	Polyester
KOH	(2)BA3CR10-58	Red	0.39	Polyester
KOH	(2)BA3CR10-59	Red	0.41	Polyester
KOH	(2)BA3CR10-60	Red	0.55	Polyester
KOH	(2)BA3CR10-61	Red	0.94	Polyester
KOH	(2)BA3CR10-62	Red	0.14	Polyester
KOH	(2)BA3CR10-63	Red	1.00	Polyester
KOH	(2)BA3CR10-64	Red	0.23	Polyester
KOH	(2)BA3CR10-65	Red	1.08	Polyester
KOH	(2)BA3CR10-66	Red	0.65	Polyester
KOH	(2)BA3CR10-67	Red	0.56	Polyester
KOH	(2)BA3CR10-68	Red	0.44	Polyester
KOH	(2)BA3CR10-69	Red	0.96	Polyester
KOH	(2)BA3CR10-70	Red	1.43	Polyester

KOH	(2)BA3CR10-71	Red	0.20	Polyester
KOH	(2)BA3CR10-72	Red	0.11	Polyester
KOH	(2)BA3CR10-73	Red	0.46	Polyester
KOH	(2)BA3CR10-74	Red	0.24	Polyester
KOH	(2)BA3CR10-75	Red	0.29	Polyester
KOH	(2)BA3CR10-76	Red	0.13	Polyester
KOH	(2)BA3CR10-77	Red	0.10	Polyester
KOH	(2)BA3CR10-78	Red	0.42	Polyester
KOH	(2)BA3CR10-79	Red	0.38	Polyester
KOH	(2)BA3CR10-80	Red	0.40	Polyester
KOH	(2)BA3CR10-81	Red	0.78	Polyester
KOH	(2)BA3CR10-82	Red	0.14	Polyester
KOH	(2)BA3CR10-83	Red	0.60	Polyester
KOH	(2)BA3CR10-84	Red	0.75	Polyester
KOH	(2)BA3CR10-85	Red	0.40	Polyester
KOH	(2)BA3CR10-86	Red	1.13	Polyester
KOH	(2)BA3CR10-87	Red	0.44	Polyester
KOH	(2)BA3CR10-88	Red	0.20	Polyester
KOH	(2)BA3CR10-89	Red	0.26	Polyester
KOH	(2)BA3CR10-90	Red	0.17	Polyester
KOH	(2)BA3CR10-91	Red	0.26	Polyester
KOH	(2)BA3CR10-92	Red	0.76	Polyester
KOH	(2)BA3CR10-93	Red	0.43	Polyester
KOH	(2)BA3CR10-94	Red	0.42	Polyester
KOH	(2)BA3CR10-95	Red	0.96	Polyester
KOH	(2)BA3CR10-96	Red	0.77	Polyester
KOH	(2)BA3CR10-97	Red	0.30	Polyester
KOH	(2)BA3CR10-98	Red	0.18	Polyester
KOH	(2)BA3CR10-99	Red	0.31	Polyester
KOH	(2)BA3CR10-100	Red	0.35	Polyester
KOH	(2)BA3CR10-101	Red	0.45	Polyester
KOH	(2)BA3CR10-102	Red	0.36	Polyester
KOH	(2)BA3CR10-103	Red	0.54	Polyester
KOH	(2)BA3CR10-104	Red	0.54	Polyester
KOH	(2)BA3CR10-105	Red	0.28	Polyester
KOH	(2)BA3CR10-106	Red	0.38	Polyester
KOH	(2)BA3CR10-107	Red	0.27	Polyester
KOH	(2)BA3CR10-108	Red	0.55	Polyester
KOH	(2)BA3CR10-109	Red	0.24	Polyester
KOH	(2)BA3CR10-110	Red	0.03	Polyester
KOH	(2)BA3CR10-111	Red	0.43	Polyester
KOH	(2)BA3CR10-112	Red	0.42	Polyester
KOH	(2)BA3CR10-113	Red	1.05	Polyester
KOH	(2)BA3CR10-114	Red	0.61	Polyester
KOH	(2)BA3CR10-115	Red	0.77	Polyester

KOH	(2)BA3CR10-116	Red	0.66	Polyester
KOH	(2)BA3CR10-117	Red	0.76	Polyester
KOH	(2)BA2CR5-1	Red	0.39	Polyester
KOH	(2)BA2CR5-2	Red	0.43	Polyester
KOH	(2)BA2CR5-3	Red	0.40	Polyester
KOH	(2)BA2CR5-4	Red	0.58	Polyester
KOH	(2)BA2CR5-5	Red	0.30	Polyester
KOH	(2)BA2CR5-6	Red	1.39	Polyester
KOH	(2)BA2CR5-7	Red	0.29	Polyester
KOH	(2)BA2CR5-8	Red	0.37	Polyester
KOH	(2)BA2CR5-9	Red	0.58	Polyester
KOH	(2)BA2CR5-10	Red	0.41	Polyester
KOH	(2)BA2CR5-11	Red	0.51	Polyester
KOH	(2)BA2CR5-12	Red	0.47	Polyester
KOH	(2)BA2CR5-13	Red	0.33	Polyester
KOH	(2)BA2CR5-14	Red	0.39	Polyester
KOH	(2)BA2CR5-15	Red	0.15	Polyester
KOH	(2)BA2CR5-16	Red	0.12	Polyester
KOH	(2)BA2CR5-17	Red	1.33	Polyester
KOH	(2)BA2CR5-18	Red	0.30	Polyester
KOH	(2)BA2CR5-19	Red	0.13	Polyester
KOH	(2)BA2CR5-20	Red	0.33	Polyester
KOH	(2)BA2CR5-21	Red	0.31	Polyester
KOH	(2)BA2CR5-22	Red	0.30	Polyester
KOH	(2)BA2CR5-23	Red	0.29	Polyester
KOH	(2)BA2CR5-24	Red	1.55	Polyester
KOH	(2)BA2CR5-25	Red	0.47	Polyester
KOH	(2)BA2CR5-26	Red	0.32	Polyester
KOH	(2)BA2CR5-27	Red	0.32	Polyester
KOH	(2)BA2CR5-28	Red	0.38	Polyester
KOH	(2)BA2CR5-29	Red	0.36	Polyester
KOH	(2)BA2CR5-30	Red	0.28	Polyester
KOH	(2)BA2CR5-31	Red	0.08	Polyester
KOH	(2)BA2CR5-32	Red	0.28	Polyester
KOH	(2)BA2CR5-33	Red	0.35	Polyester
KOH	(2)BA2CR5-34	Red	0.29	Polyester
KOH	(2)BA2CR5-35	Red	0.33	Polyester
KOH	(2)BA2CR5-36	Red	0.37	Polyester
KOH	(2)BA2CR5-37	Red	0.43	Polyester
KOH	(2)BA2CR5-38	Red	0.57	Polyester
KOH	(2)BA2CR5-39	Red	0.59	Polyester
KOH	(2)BA2CR5-40	Red	0.42	Polyester
KOH	(2)BA2CR5-41	Red	0.59	Polyester
KOH	(2)BA2CR5-42	Red	0.40	Polyester
KOH	(2)BA2CR5-43	Red	0.6	Polyester

KOH	(2)BA2CR5-44	Red	0.44	Polyester
KOH	(2)BA2CR5-45	Red	0.31	Polyester
KOH	(2)BA2CR5-46	Red	0.33	Polyester
KOH	(2)BA2CR5-47	Red	0.27	Polyester
KOH	(2)BA2CR5-48	Red	0.56	Polyester
KOH	(2)BA2CR5-49	Red	0.46	Polyester
KOH	(2)BA2CR5-50	Red	0.58	Polyester
KOH	(2)BA2CR5-51	Red	0.27	Polyester
KOH	(2)BA2CR5-52	Red	0.316	Polyester
KOH	(2)BA2CR5-53	Red	0.367	Polyester
KOH	(2)BA2CR5-54	Red	0.34	Polyester
KOH	(2)BA2CR5-55	Red	0.33	Polyester
KOH	(2)BA2CR5-56	Red	0.28	Polyester
KOH	(2)BA2CR5-57	Red	0.38	Polyester
KOH	(2)BA2CR5-58	Red	0.34	Polyester
KOH	(2)BA2CR5-59	Red	0.29	Polyester
KOH	(2)BA2CR5-60	Red	0.13	Polyester
KOH	(2)BA2CR5-61	Red	0.38	Polyester
KOH	(2)BA2CR5-62	Red	0.36	Polyester
KOH	(2)BA2CR5-63	Red	0.33	Polyester
KOH	(2)BA2CR5-64	Red	0.43	Polyester
KOH	(2)BA2CR5-65	Red	0.13	Polyester
KOH	(2)BA2CR5-66	Red	0.31	Polyester
KOH	(2)BA2CR5-67	Red	0.26	Polyester
KOH	(2)BA2CR5-68	Red	0.36	Polyester
KOH	(2)BA2CR5-69	Red	0.41	Polyester
KOH	(2)BA2CR5-70	Red	0.57	Polyester
KOH	(2)BA2CR5-71	Red	0.42	Polyester
KOH	(2)BA2CR5-72	Red	0.60	Polyester
KOH	(2)BA2CR5-73	Red	0.37	Polyester
KOH	(2)BA2CR5-74	Red	0.30	Polyester
KOH	(2)BA2CR5-75	Red	0.36	Polyester
KOH	(2)BA2-CR5-76	Black	0.41	Polyester
KOH	(2)CHI1-CR5	Transparent	1.81	Polyester
KOH	(2)CHI1-CR8	Black	2.05	Polyester
KOH	(2)CHI3-CR2	Gray	3.25	Polyester

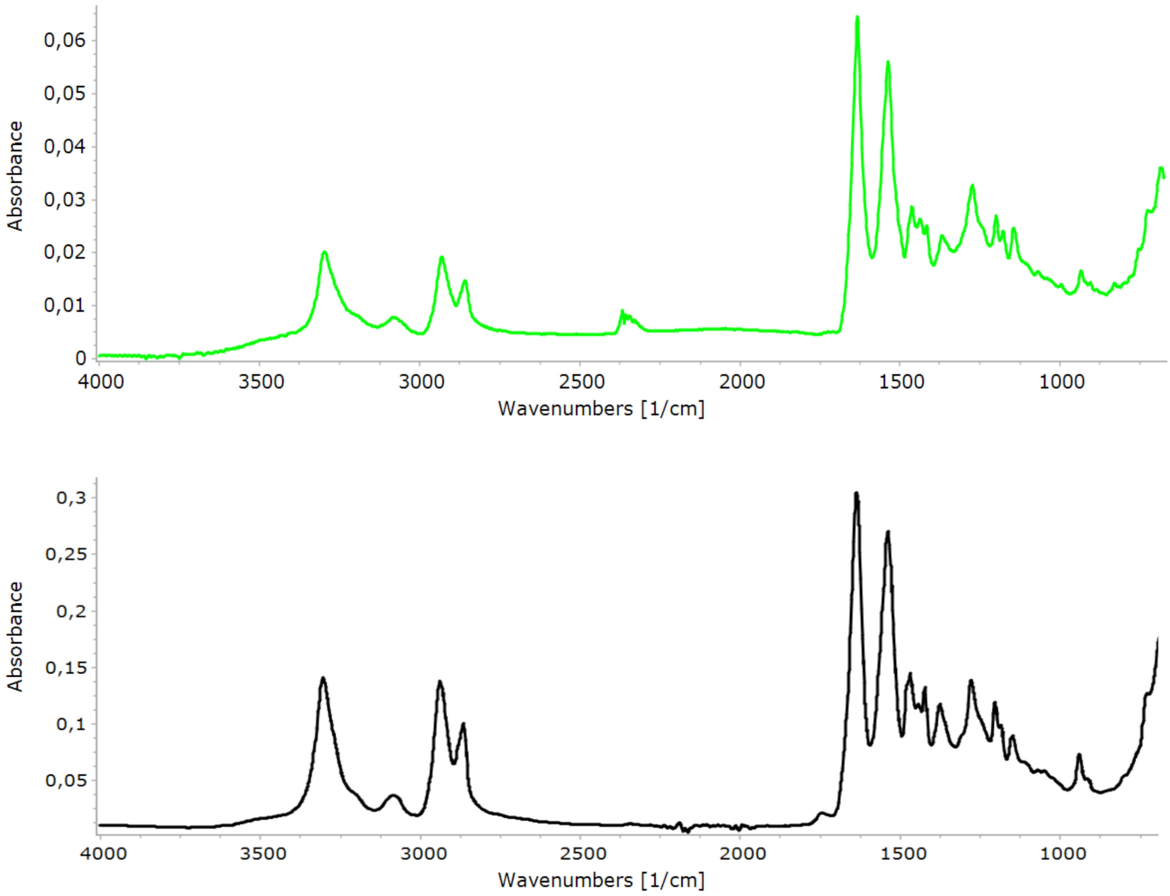

Figure S1: Example of FTIR absorbance spectrum of polyamide (PA) fibre extracted from a wild collected crabs (green) and the PA reference spectrum (black).

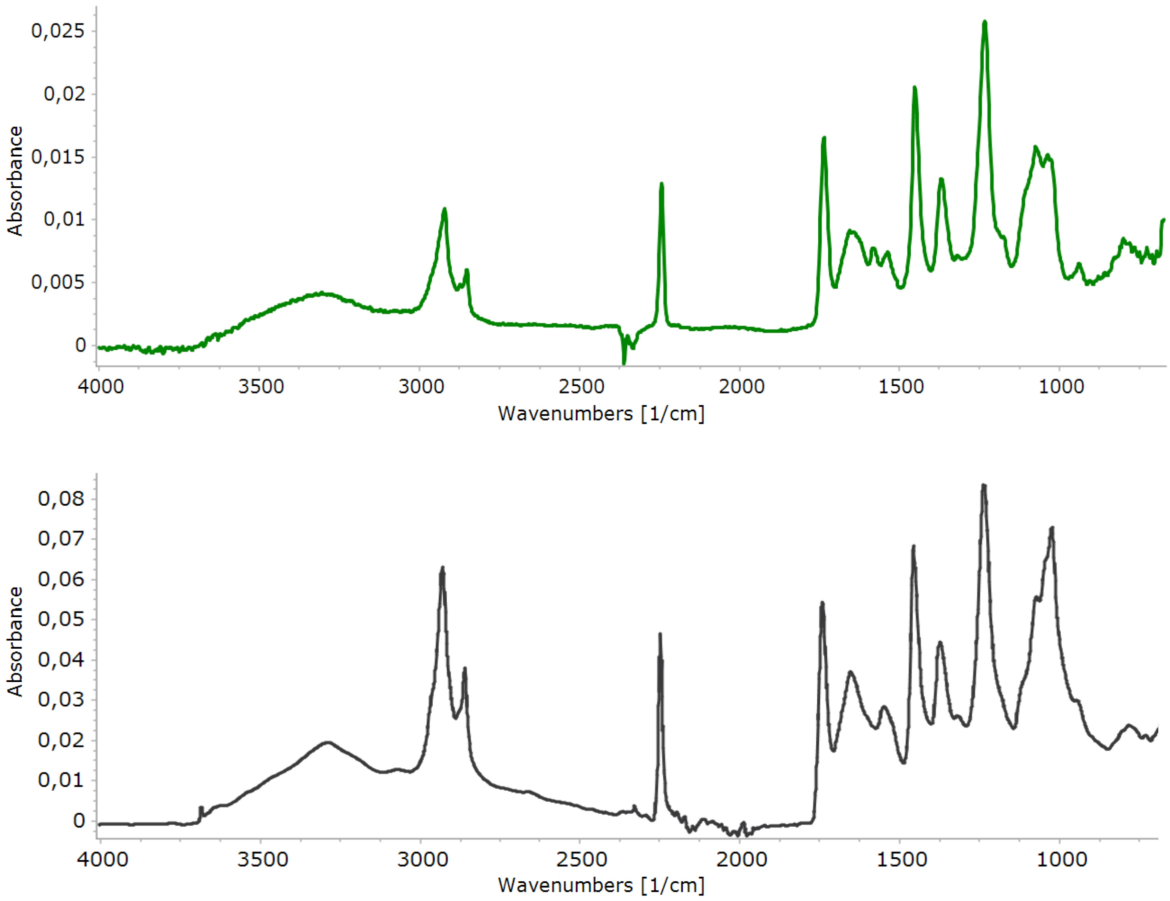

Figure S2: Example of FTIR absorbance spectrum of acrylonitrile (ACN) microfibres extracted from a wild collected crabs (green) digested with enzymes and the ACN reference spectrum (black).

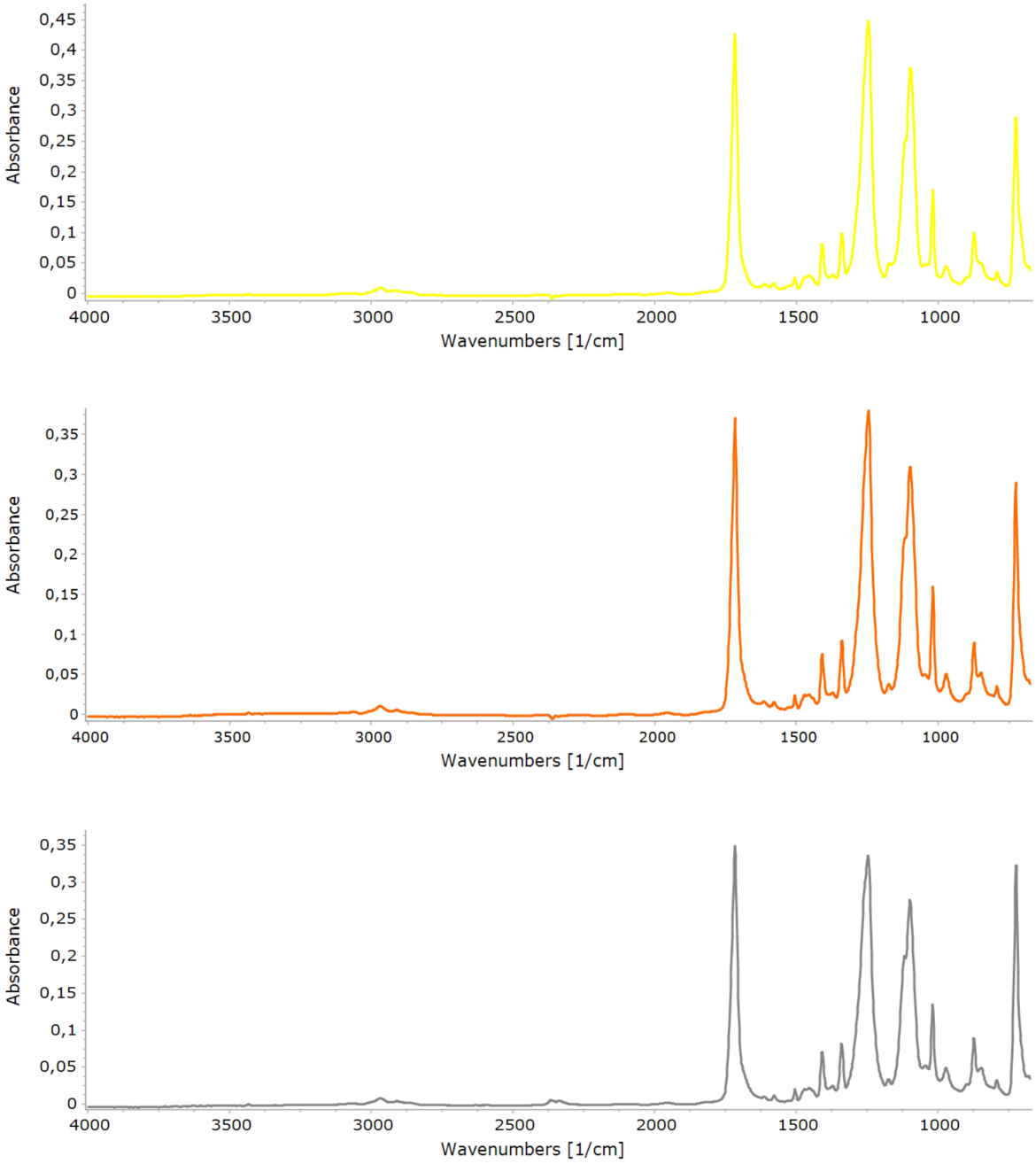

Figure S3: Example of FTIR absorbance spectrum of polyester (PES) microfibres extracted from a wild collected crabs digested with enzymes (yellow), KOH (orange) and the PES reference spectrum (black).

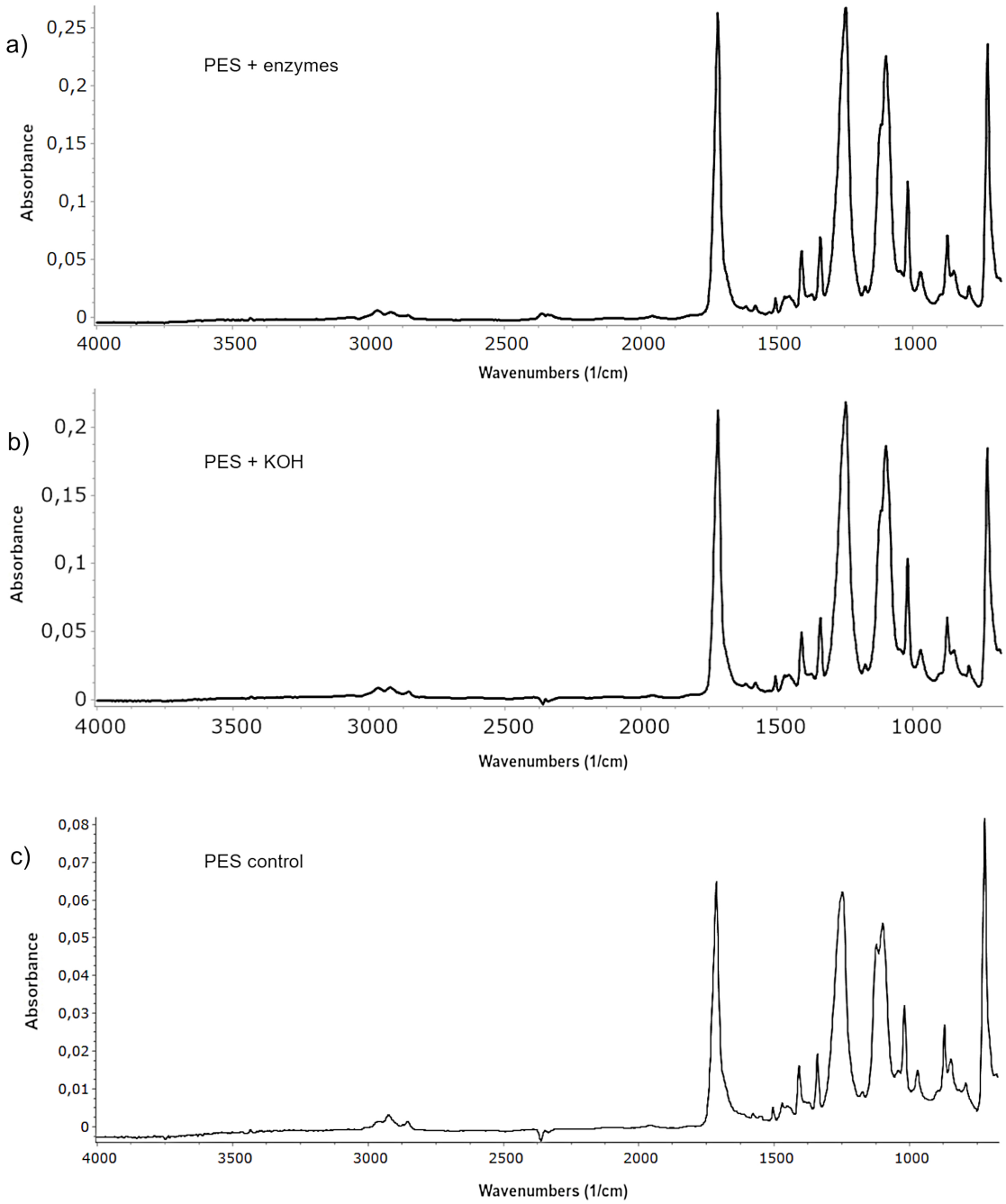

Figure S4: FTIR absorbance spectra of laboratory-prepared fibres of polyester (PES) after over-exposition (7 days) to the enzymatic and alkaline (KOH) digestion methods vs no-treated reference material (control).

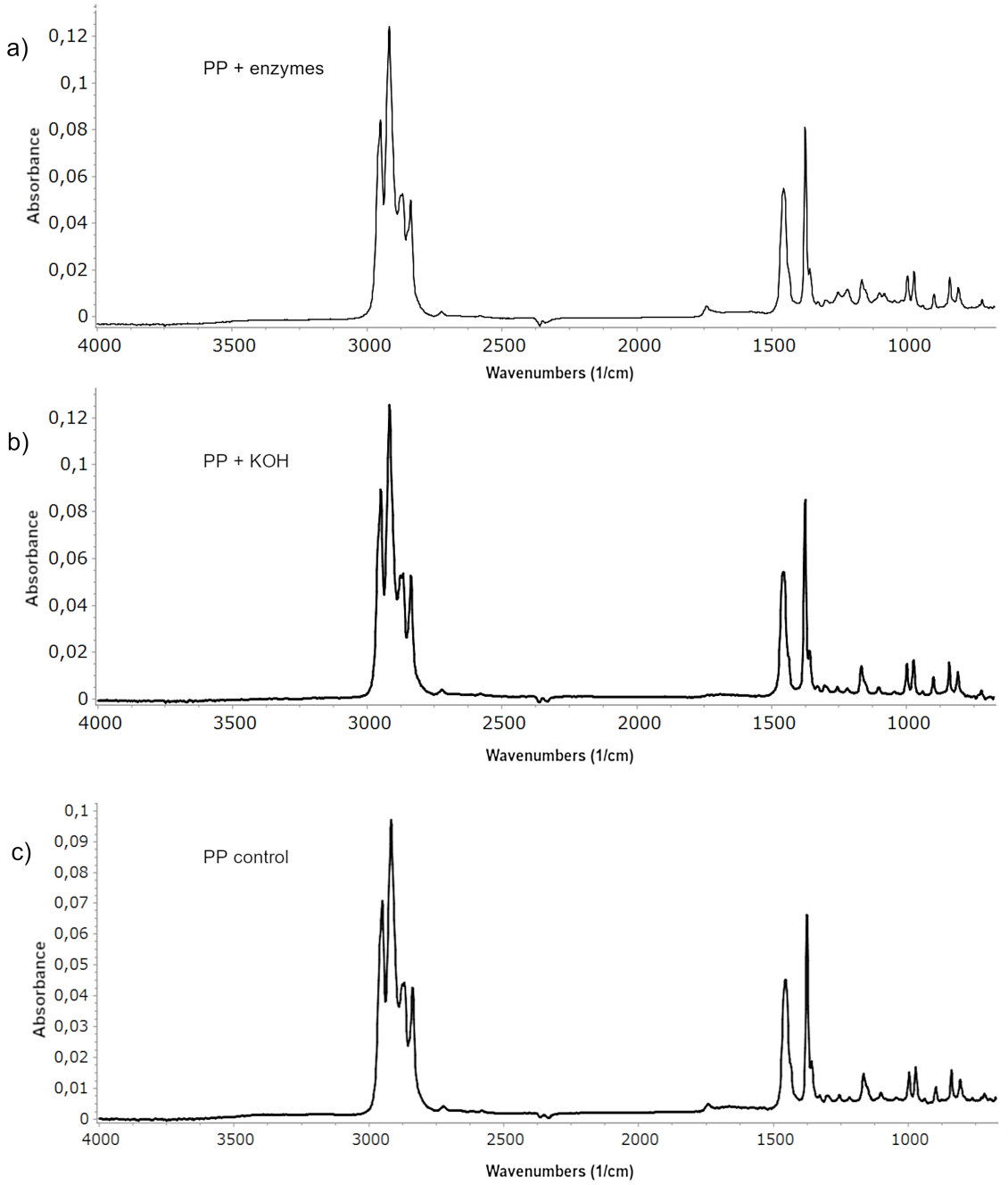

Figure S5: FTIR absorbance spectra of laboratory-prepared fibres of polypropylene (PP) over-exposed (7 days) to the enzymatic and alkaline (KOH) digestion methods vs no-treated reference material (control).

Chapter II

Do different habits affect microplastics contents in organisms? A trait-based analysis on salt-marsh species[1]

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Abstract

Salt marshes in urban watersheds are prone to microplastics (MP) pollution due to their hydrological characteristics and exposure to urban runoff, but little is known about MP distributions in species from these habitats. In the current study, MP occurrence was determined in six benthic invertebrate species from salt marshes along the North Adriatic lagoons (Italy) and the Schelde estuary (Netherlands). The species represented different feeding modes and sediment localisation. 96% of the analysed specimens (330) did not contain any MP, which was consistent across different regions and sites. Suspension and facultative deposit-feeding bivalves exhibited a lower MP occurrence (0.5-3%) relative to omnivores (95%) but contained a much more variable distribution of MP sizes, shapes and polymers. The study provides indications that MP physicochemical properties and species' ecological traits could all influence MP exposure, uptake and retention in benthic organisms inhabiting European salt marsh ecosystems.

.

Key words: Microplastics; Fibres; Suspension-feeders; Deposit-feeders; Omnivores; Salt marsh

2.1 Introduction

Plastic is one of the most used materials in the modern society and the current production level of 348 million tons per year is expected to increase in the future (Plastics Europe, 2018). Poor waste handling practices over recent decades have led to significant release of plastic material into the marine environment, where the amount of plastic litter floating on the ocean surface has been estimated to weigh more than 268,940 tons (Eriksen et al., 2014). Microplastics (MP), commonly defined as small plastic particles <5 mm in size, have been found in all marine habitats and compartments (Carbery et al., 2018), including remote areas of the globe far from human activities (Bergmann et al., 2017; Peeken et al., 2018; Woodall et al., 2014). This widespread contamination has raised global concerns regarding the impact of MP at ecosystem and community levels (Da Costa, 2018).

The ingestion of MP by a broad range of marine species has been confirmed by field and laboratory experiments, with data reported for organisms from different geographical locations and ecosystems (Bour et al., 2018b; Carbery et al., 2018; Codina-García et al., 2013; Cole et al., 2013; Fossi et al., 2012; Lusher et al., 2013; Nelms et al., 2018; Taylor et al., 2016; Van Cauwenberghe et al., 2015; Vandermeersch et al., 2015; Watts et al., 2014; Wright et al., 2013). An increasing number of studies have also demonstrated that MP and their associated additive chemical compounds may elicit harmful effects on organisms, including physical damage, endocrine disruption, and impacts on energy budget, immune system, growth and reproduction (Bour et al., 2018b; Capolupo et al., 2018; Capolupo et al., 2020; Cole et al., 2019; Farrell and Nelson, 2013; Green et al., 2019; Mato et al., 2001; Pittura et al., 2018; Setälä et al., 2014; Teuten et al., 2009; Wright et al., 2013) possibly leading to long-term health impairment (Bour et al., 2018a).

MP can be transferred from lower to higher trophic levels, including humans (Farrell and Nelson, 2013; Gutow et al., 2016; Nelms et al., 2018; Setälä et al., 2014; Schwabl et al., 2019; Van Colen et al., 2020; Watts et al., 2014). Their uptake may be influenced by both the physical and chemical

properties of MP (Watts et al., 2014) and by the different feeding modes of individual species (Scherer et al., 2017). While increasing effort has been given to understanding the potential effects related to MP properties (Gray and Weinstein, 2017; Patterson et al., 2019), current analysis and monitoring of MP transfer and impacts across food webs have largely neglected the potential relationships between species feeding modes or other relevant ecological traits and their susceptibility to MP contamination (Desforges et al., 2015). Understanding the factors affecting the entry of MP into food webs is crucial for predicting which environments or species may be more vulnerable to MP contamination (Gago et al., 2016).

Salt marshes are among the most productive ecosystems on the planet (Lourenço et al., 2017), supporting complex food webs and acting as nursery habitats for profitable fishery and aquaculture species (Silliman et al., 2012; Ysebaert et al., 2011; Zedler and Kercher, 2005). At the same time, salt marshes have historically experienced some of the greatest pressures from human related stressors, including urbanisation and industrial development, inadequate wastewater treatment in the watershed, and intensive aquaculture and maritime activities (Airoldi and Beck, 2011; Silliman and Bertness, 2004). Salt marshes are also depositional areas along river estuaries and have been shown to accumulate a variety of pollutants over long periods (Williams et al., 1994). This combination of factors makes salt marsh species in urban watersheds extremely vulnerable to contamination from MP from a variety of sources (Thompson et al., 2010; Vermeiren et al., 2016; Weinstein et al., 2016). Despite their relevance for food webs ultimately linked to human consumption and the expectation that sediments are likely to be the final sink for many MP (Maes et al., 2017; Simon-Sànchez et al., 2019; Van Cauwenberghe et al., 2013; Vandermeersch et al., 2015), there is a surprising paucity of studies characterising MP properties, distributions and impacts on benthic species living in these ecosystems.

In the current study, MP properties and distributions were characterised in a variety of benthic invertebrate species collected from salt marshes along the Italian North Adriatic coastal lagoons and

the Dutch Schelde estuary. The salt marshes were selected to represent a range of different pressures from potential anthropogenic sources of MP, including shipping, industry, aquaculture and wastewater effluents. Six invertebrate species were selected that represent different feeding modes (suspension-feeder, surface deposit feeder/suspension feeder and omnivores) and sediment localisation (infauna vs epifauna). MP were quantified and characterised in terms of their physicochemical properties (size, shape, and polymer type) and their distributions were compared across the different regions and species traits.

2.2 Materials and methods

2.2.1. Study areas

Benthic invertebrates from salt marsh sediments were sampled from three coastal lagoons on the North Adriatic coast (Italy) and two salt marshes in the Schelde estuary (The Netherlands, Figure S1 in Supplementary Information). Piallassa Baiona (44°28’26.6’’N; 12°14’52.5”E) is a lagoon formed by several ponds connected to the sea by channels and receives input from 6 wastewater channels originating from urban, agricultural and industrial sewage treatment plants and thermal power plants (Airoldi et al., 2016; Lo et al., 2017). Sacca di Bellocchio (44°38’01.97’’N; 12°15’48.78”E) is a back-barrier lagoon connected to the sea by a single channel located in the Parco Delta del Po dell’Emilia-Romagna (Wong et al., 2015). This is a nature reserve where the limited human pressures are mainly related to seasonal tourism and recreational activities. Valle Millecampi (45°13’11.52’’N; 12°16’44.45”E) is located in the southern part of Venice Lagoon, which is an area characterised by intensive fishing and aquaculture activities representing potential MP sources (Vianello et al., 2013). Paulina (51°20’57.1’’N; 3°43’35.4’’E) is located close to the Port of Terneuzen on the southern bank in the polyhaline part of the Schelde estuary, a large macrotidal estuary that drains a very large and heavily populated and industrialised catchment (Ysebaert et al., 2003). Paal (51°21'33.3"N; 4°05'48.4"E) is located on the southern bank in the

mesohaline part of the Schelde estuary near the Belgian-Dutch border, in proximity (<10 km) to the port of Antwerp, which is the second largest sea harbour in Europe.

2.2.2 Field sampling and species

Six invertebrate species were sampled in June 2016: the bivalves *Cerastoderma glaucum* (lagoon cockle), *Limecola balthica* (Baltic clam), *Mytilus galloprovincialis* (Mediterranean mussel) and *Scrobicularia plana* (peppery furrow shell), the polychaete *Hediste diversicolor* (common ragworm) and the crab *Carcinus aestuarii* (Mediterranean green crab). Despite considerable variations in their abundances and distribution across different sites, previous pilot monitoring (unpublished data) identified these species as the most common organisms per trophic level within the selected study areas. Species feeding mode, sediment localisation, region of occurrence and the number of organisms sampled from each region are presented in Table 1. Bivalves and polychaetes were collected from the sediments (sampled between 5-10 cm depth) using stainless steel corers (diameter=15 cm) and washed over a stainless steel sieve (mesh size=1 mm). The crab *C. aestuarii* was collected using standard traps that were left partially submerged at regular intervals (i.e. 10 meters apart) for two hours. After collection, all organisms were immediately stored at -20 °C without any initial treatment or manipulation, prior to processing at a later date.

Table. 1: Sampled species, their localisation in the sediment (infauna vs epifauna), feeding mode, geographical region, sites, number of analysed specimens and number of specimens exhibiting MP contamination. Feeding modes were determined with stable isotope analyses (Figure S2, Supplementary Information) and validated through www.marlin.ac.uk.

Species	Localisation	Feeding mode	Region	Sites	No. collected specimens	No. MP positive specimens
Bivalves						
Cerastoderma glaucum	Infauna	Suspension-feeder	N. Adriatic	P. Baiona	30	0
				S. Bellocchio	30	1
				V. Millecampi	10	0
Mytilus galloprovincialis	Epifauna	Suspension-feeder	N. Adriatic	P. Baiona	10	0
				S.Bellocchio	10	1
				V. Millecampi	10	0
Limecola balthica	Infauna	Surface-deposit feeder/suspension-feeder	Schelde	Paulina	10	3
				Paal	10	1
Scrobicularia plana	Infauna	Surface-deposit feeder/suspension feeder	Schelde	Paulina	10	1
				Paal	10	0
Polychaetes						
Hediste diversicolor	Infauna	Omnivore (Predator and deposit-feeder)	N. Adriatic Schelde	P. Baiona	30	0
				S. Bellochhio	30	0
				V. Millecampi	30	0
				Paulina	10	1
Crabs						
Carcinus aestuarii	Epifauna	Omnivore (Predator and deposit-feeder)	N. Adriatic	P. Baiona	30	3
				S. Bellochhio	30	0
				V. Millecampi	30	3

2.2.3 Macrofauna feeding modes

The feeding modes of the invertebrates from the poly- and mesohaline sediments in the Schelde estuary have been previously well described (Herman et al., 2000; Van Colen et al., 2010; Ysebaert et al., 2003). Both *L. balthica* and *S. plana* are facultative surface deposit feeders, relying on microphytobenthos (MPB) and phytoplankton for their diet, whereas *Hediste diversicolor* is an omnivore species with a strong preference for MPB. Stable isotope analysis was performed on the invertebrates and on suspended (SPOM) as well as bulk sediment (POM) organic matter sampled in the Northern Adriatic to characterise the species' trophic position. Particulate organic matter (POM) was collected from the upper first cm of the sediment from which the macrofauna was removed through sieving (1mm). Suspended POM (SPOM) in the water column was collected from the incoming tide (3 l), from which 0.5 l subsamples were filtered over pre-combusted Whatman GF/F filters (pore size 0.7 μm). Macrofauna was extracted from the sediment using 1 mm sieves, starved for 24 h to allow evacuation of their gut content. Filters and macrofauna were stored frozen at -20 °C, and sediments (~ 30 mg) were dried, ground and homogenised. To remove carbonates, SPOM filters and POM samples were transferred to silver capsules, acidified using HCl (4%) until the bubbling ceased (Carabel et al., 2006), and dried for 24 h at 60 °C. After dissection, the tissue samples (crabs: claw muscle tissue; bivalves: foot muscle tissue; polychaetes: whole organisms) were rinsed with Milli-Q water, transferred to tin capsules and dried in an oven for 24 h at 60 °C. Samples (Table S2 in the Supplementary Information) were sent to the UC Davis Stable Isotope Facility (Davis, California, USA) for stable isotope analysis, which was conducted with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 continuous flow isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). External laboratory standards calibrated against international reference materials were interspersed among the samples. Results are expressed in the δ notation relative to Vienna Pee Dee belemnite for C and atmospheric N_2 for N.

Level of replication for food sources and consumers was 3, except for SPM in Bellochio (n = 2), *C. glaucum* and *M. galloprovincialis* sampled in Baiona (n =4).

2.2.4 MP extraction and characterisation

Prior to dissection, organisms (at least 20 of each species) were rinsed with Milli-Q water and the length of the largest dimension of the shell (bivalves), body (polychaetes) or carapace (crabs) were measured (Table S1, Supplementary Information). Dissection was different across species: for polychaetes the whole organism was used, while for bivalves all the soft tissues were separated from the shell and for crabs, only the gastrointestinal tract was analysed. All isolated tissue samples were pre-weighed (summarised in Table S1, Supplementary Information) and processed with 1 M potassium hydroxide (KOH; 20 mL per sample) following the alkaline digestion protocol described in Piarulli et al. (2019). Glass beakers containing the digestate were covered tightly and incubated for 48 h at room temperature without any further manipulation until filtration. In the final step, the digested samples were individually vacuum filtered onto nylon filters (mesh size: 20 µm, Ø: 5 cm, PLASTOK®) and dried at room temperature in covered glass petri dishes.

Inspection of the filters, MP quantification and MP physicochemical characterisation was performed following the approach described by Piarulli et al. (2019). Briefly, visual inspection of the filters was conducted with a stereomicroscope (WILD M8, Leica microsystems, ~50X magnification) to identify particles that were potentially MP. Particles showing an obvious cellular structure were excluded, as well as glass, sand and shells. Any particles identified visually as potentially being MP were photographed, measured at the largest cross section with the software ImageJ, and characterised for shape (e.g. fibre, fragment, film) and colour. Potential MP particles were then retained separately for subsequent molecular analysis. Fourier-transform infrared spectroscopy (FTIR) analyses were performed by using a Thermo Nicolet iN™10MX microscope, fitted with an MCT detector cooled by liquid nitrogen. At least three measurements for each particle

were performed in attenuated total reflectance (ATR) mode with a germanium crystal (refractive index = 4) operating in contact with the surface of the samples, to evaluate the reproducibility of results for each sample. Spectra were recorded in the range 4000–675 cm^{-1} with a spectral resolution of 4 cm^{-1} and 128 scans. Analyses were performed an objective aperture of 80x80 μm, relative to an investigation area of about 20 μm for each point of analysis. A dedicated software OMNIC Picta (Thermo Fisher Scientific) was used for spectra processing and interpretation: similarities in wavenumber position and relative intensities of absorption bands were evaluated and compared. For each particle analysed, good spectral reproducibility was observed, which allowed correct identification of the polymers.

2.2.5 Quality control

Special attention was given to limiting sample contamination by external MP, with precautions implemented at every step of process following the anti-contamination protocol described in Piarulli et al. (2019). Briefly, MP extraction was performed in a clean laboratory where all surfaces were pre-cleaned with acidified Milli-Q water prior to processing the samples. Plastic equipment was entirely replaced with metal and glass alternatives, which were rinsed with Milli-Q water before use. Organisms were rinsed with Milli-Q water prior to dissection to remove any residual external MP. Contact with air and plastic surfaces during all laboratory procedures was minimised for samples, instruments and reagents by covering them with Milli-Q rinsed aluminium foil before and after use. After filtration, membranes were kept covered in glass petri dishes that had previously been rinsed with Milli-Q water. The KOH digestion solution was prepared using Milli-Q and was pre-filtered on 0.2 μm glass microfibre filters (Whatman®). The use of cotton clothes and lab-coats was mandatory to access the clean laboratory.

To validate the effectiveness of the contamination prevention approach, 4 procedural blanks (sample-free KOH solution) treated identically to the samples and 4 air filters were used for each

batch of samples processed. Material retained on the air filters and in the procedural blanks was carefully examined following the same procedure as for the biological samples (see Section 2.2.4) to identify any MP representing external contamination that should be accounted for in the blank correction. No MP were found on the air filters or in the procedurals blanks, with only natural cotton and cellulose microfibres identified by FTIR spectroscopy. As a result, there was no need to implement any form of blank correction of the MP data for the biological samples.

2.2.6 *Statistical analyses*

MP occurrence data per organism did not satisfy the assumptions for parametric statistics (normality and homogeneity of variances, tested with Shapiro-Wilk and Fligner-Killeen tests, respectively) and showed very different group distributions. Therefore, separate Welch's ANOVA analyses were used to compare the numbers of MP per specimen across species, feeding modes and sediment localisation. The level of significance for the rejection of the null-hypothesis was set at a p-value < 0.05. Non-metric multi-dimensional scaling (nMDS) based on Bray-Curtis similarities was performed as a multivariate ordination analysis to visualise patterns of MP distribution (shape, size, and polymer type) among MP-contaminated organisms with different ecological traits (Clarke and Gorley, 2015). All statistical analyses were performed with R studio 0.99.903 (R Core Team, 2016), except for the nMDS, which was performed with PRIMER 7 (Clarke and Gorley, 2015).

2.3 Results

2.3.1 Microplastics in organisms

A total of 330 specimens, representing different species, feeding modes (validated with the stable isotope analyses as shown in Figure S2, Supplementary Information), sediment localisation and region of origin (Adriatic Sea and Schelde estuary) were analysed (Table 1). Although MP were

detected in organisms from all 6 species, the majority of specimens did not contain any MP (n=316, 96%). In the small number of positive specimens (n=14, 4%), a total of 207 individual MP particles were identified (Table 2), of which 95% (n=197) were recovered from crabs (*C. aestuarii*), 3% (n=6) from *L. balthica* and 0.5% (n=1) from each of *C. glaucum, M. galloprovincialis*, *S. plana* and *H. diversicolor*. No significant differences in contamination levels were observed between species, feeding modes, sediment localisation and geographic regions (Welch's ANOVA p-values=0.27, 0.07, 0.16 and 0.25 respectively), as the few contaminated specimens mostly contained only 1 or 2 MP per organism (Figure 1). However, 2 individual crabs were found to be highly contaminated with 76 and 117 MP, respectively.

The size of the extracted MP ranged from 0.03 to 4 mm and was distributed in the following categories: <0.1 mm (n=9, 4%), 0.1-0.5 mm (n=127, 62%), 0.5-1 mm (n=50, 24%), 1-5 mm (n=21, 10%). The majority of the extracted MP were microfibres (n=204, 98.5%), followed by fragments (n=2, 1%) and foam (n=1, 0.5%). The most common polymer was polyester (PES) (n=202, 98%), followed by low-density polyethylene (LDPE) (n=3, 1.5%), polypropylene (PP, n=1, 0.25%) and polyacrylonitrile (PAN, n=1, 0.25%). The distribution of MP shape, size and polymer type is summarised in Table 2.

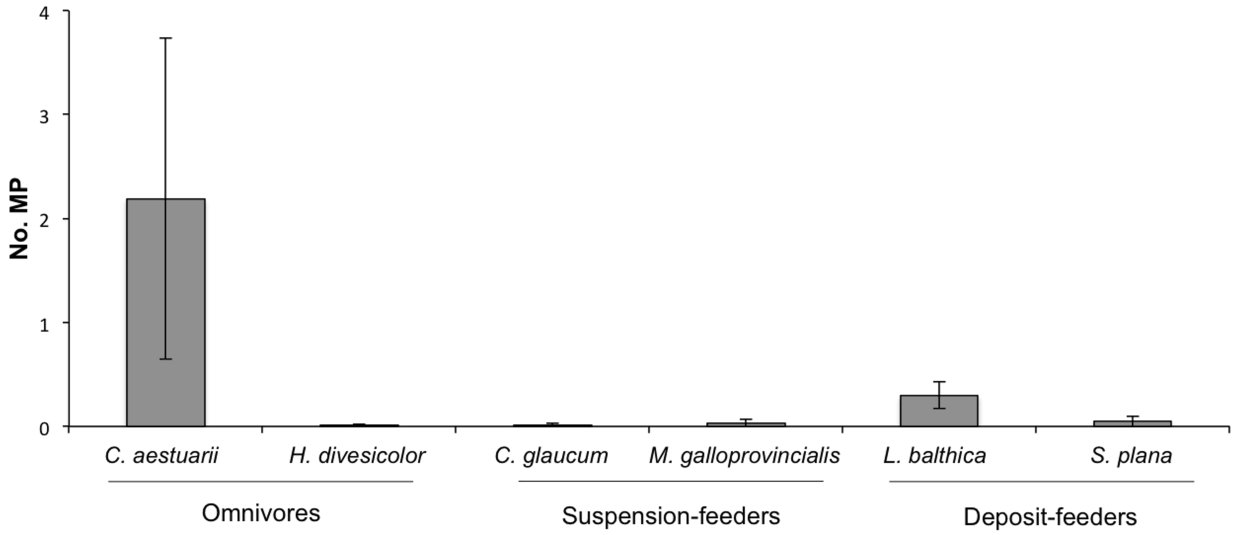

Figure 1: Average number of MP found per each of the six sampled species, where error bars represent the standard error (SE).

Table 2: Number and properties of MP found in sampled species. MP contamination is given as the average number of MP (± 1 SE) per species (including all analysed specimens for the same species). Properties of MP detected are shown as shape, minimum size (Min size), maximum size (Max size) and polymer type. PP=polypropylene; PES=polyester; LDPE= low density polyethylene; PAN=polyacrylonitrile.

Species	% MP positive specimens	No. MP	MP Shape	Min* size (mm)	Max* size (mm)	Polymer
C. glaucum	1.4	0.01 (±0.01)	Fragment	3	3	PP
M. galloprovincialis	3.3	0.03 (±0.03)	Fibre	1.2	1.2	PES
L. balthica	10	0.25 (±0.12)	Fragment, Foam, Fibre	0.06	0.9	PES, LDPE, PAN
S. plana	5	0.05 (±0.05)	Fibre	1.8	1.8	PAN
H. diversicolor	1	0.1 (±0.1)	Fibre	0.4	0.4	PES
C. aestuarii	6.6	2.19 (±1.54)	Fibre, Multifilament fibre	0.03	3.5	PES

* Minimum and maximum sizes of MP represent the length measured at the largest cross section.

A bi-dimensional separation of the MP composition in terms of size, shape and polymer type was observed between organisms with different feeding modes and sediment localisations (Figures 2a, b). Omnivores (*C. aestuarii* and *H. diversicolor*) grouped very close to each other, while bivalves, representing suspension-feeders (*M. galloprovincialis and C. glaucum*) and facultative deposit-feeders (*L. balthica and S. plana*), displayed a major dispersion. These patterns were driven by specific MP typologies being associated with the different groups.

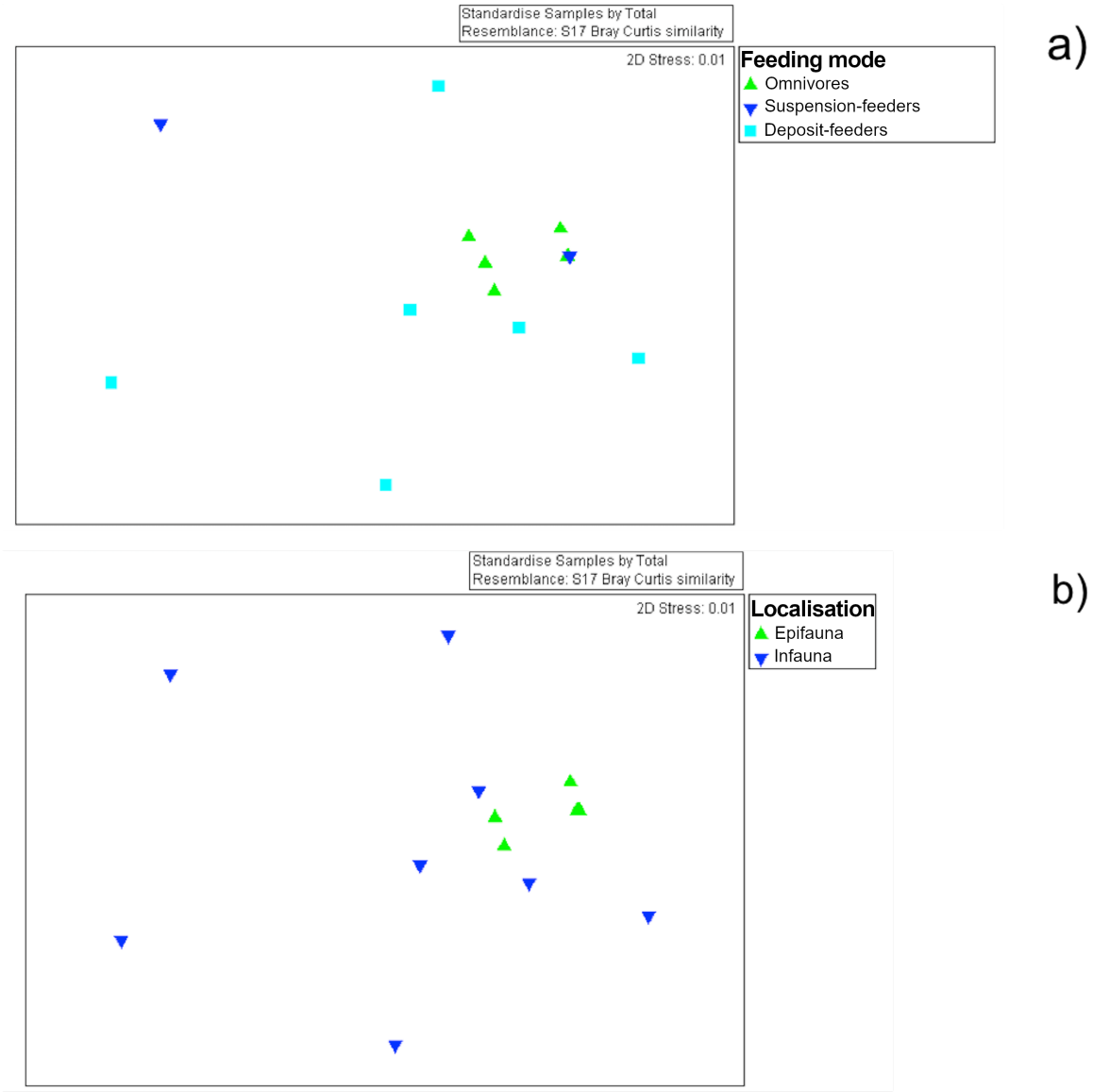

Figure 2: Non-metric multidimensional scaling (nMDS) ordination plot based on the shape, size and polymer type of MP found in contaminated organisms. Symbols and colours represent different **a)** feeding modes and **b)** sediment localisation.

Omnivores and epifaunal species contained exclusively PES microfibres, the majority (n=126, 63%) ranging between 0.1 and 0.5 mm in length. In contrast, the suspension and facultative deposit feeders (accounting for the majority of infaunal species) presented a more varied MP composition, comprising fibres, fragments and foam of different polymer types (Figure 3 a, b, c). The suspension feeders exhibited MP comprised of PES (50%) and LDPE (50%) ranging in length between 1 and 5 mm, while the facultative deposit feeders exhibited the broadest range of MP sizes and polymeric compositions. This included a higher abundance of MP <0.1 mm (29 % of total MP) than observed in both omnivores (4 %) and suspension-feeders (0 %), as well as the presence of PAN and PP MP that were not found in organisms with other feeding modes (Figure 3 b, c).

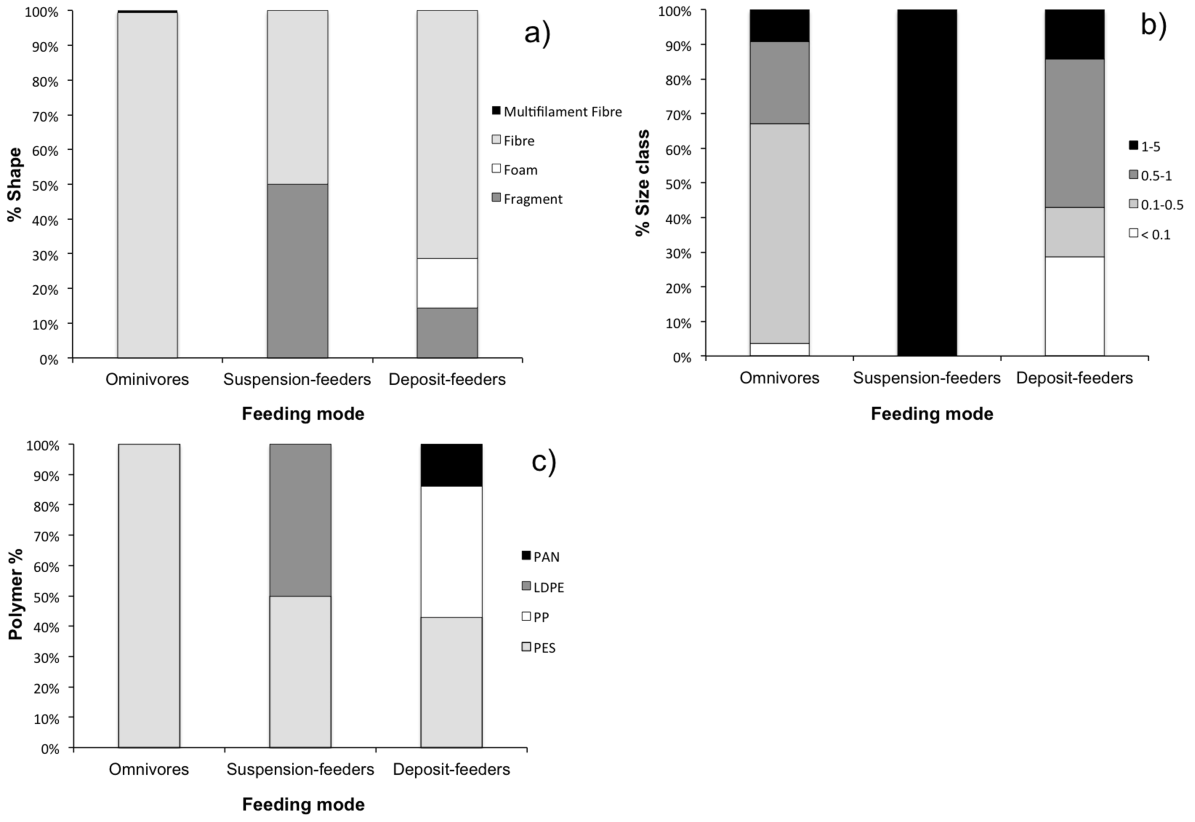

Figure 3: Distribution of MP found in contaminated omnivores, suspension-feeders and deposit-feeders plotted as a function of **a)** shape, **b)** size class (mm) and **c)** polymer type. PAN = polyacrylonitrile; LDPE = low density polyethylene; PP = polypropylene; PES = polyester.

The MP distribution and composition also differed between organisms with different sediment localisations (Figure 4 a, b, c). The epifauna (*M. galloprovincialis*, *C. aestuarii*) contained only PES microfibres, most of which (n=125, 63%) ranged from 0.1 to 0.5 mm. In contrast, the infauna (*C. glaucum*, *L. balthica*, *S. plana*, *H. diversicolor*) also contained fragments (n=1, 22%) and foam (n=1, 11%) in addition to fibres (n=6, 63%), with the polymer distribution being PAN (n=1, 11%), PP (n=1, 11%), LDPE (n=3, 33%) and PES (n=4, 45%). The infauna also exhibited a more variable distribution of size classes, with 22% (n=2) of MP being <0.1 mm, 22% being 0.1 to 0.5 mm (n=2), 33% (n=3) being 0.5 to 1 mm and 22% (n=2) being 1 to 5 mm.

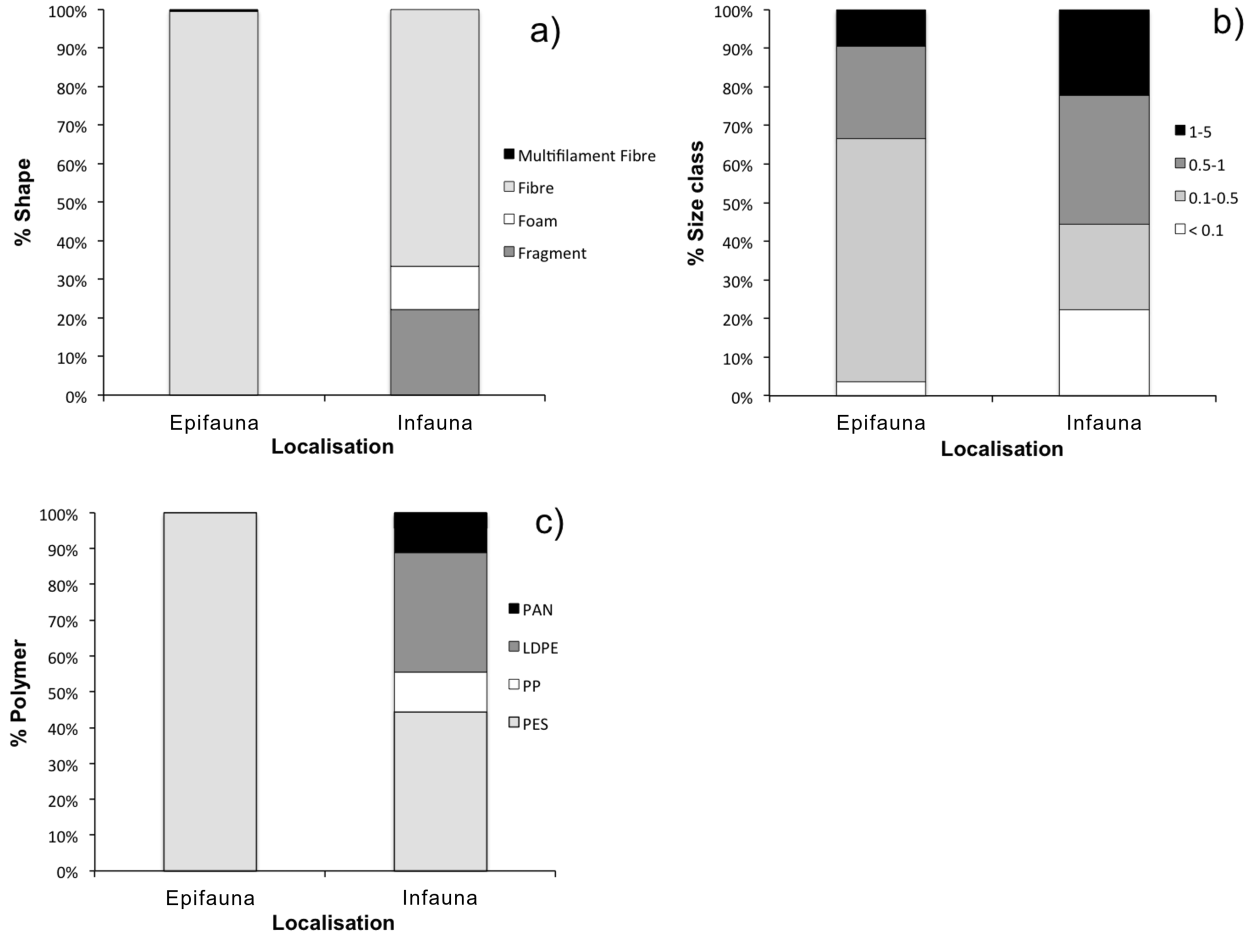

Figure 4: Distribution of MP found in contaminated epifauna and infauna plotted as a function of **a)** shape, **b)** size class (mm) and **c)** polymer type. PAN = polyacrylonitrile; LDPE = low density polyethylene; PP = polypropylene; PES = polyester.

2.4 Discussion

The current study represents one of the first to quantify, characterise and compare MP occurrence in invertebrate species from salt marsh habitats. An extensive number of specimens (330) representing 6 different species were collected and analysed, and the observed levels of MP contamination were then related to their different feeding modes and sediment localisation in an attempt to identify biological and ecological aspects that may affect the vulnerability of individual species and/or groups of species to MP pollution. Unfortunately, the amount of MP data for salt marshes, and other types of wetlands, is very scarce compared to other marine systems (recently reviewed by Kamyab et al., 2018), making it difficult to make robust comparisons with other studies.

Of 330 organisms analysed, 96% (n=316) did not contain any MP and the few positive specimens contained mostly (99%) microfibres. This was consistent among the selected study regions irrespective of their very different geographical characteristics and the nature of their anthropogenic impacts. Very low levels of MP occurrence, as well as a predominance of microfibres, have also been reported in bivalves and crustaceans from other marine systems (Bour et al., 2018; Claessens et al., 2011; Devriese et al., 2015; Lourenço et al., 2017; Neves et al., 2015; Rochman et al., 2015; Taylor et al., 2016; Van Cauwenberghe et al., 2015; Vandermeersch et al., 2015). This common finding across multiple studies suggests scenarios where (i) there are low environmental concentrations of MP combined with a predominance of microfibres for the MP that are present, or (ii) most MP types are either too large to be selected as a food source (Bock and Miller, 1999) or rapidly egested after uptake (Gutow et al., 2016; Piarulli & Airoldi in preparation, Chapter III of this thesis; Vroom et al., 2017; Ward et al., 2019)]

An on-going study to quantify and characterise MP in the sediments of Bellocchio lagoon, the least impacted site in the current study with few direct sources of MP, suggests a high occurrence of MP (ca. 942 MP kg^{-1} of dry sediment), most of which (88%) are fragments (Piarulli et al., in preparation). The first scenario with low MP exposure concentrations and a predominance of

microfibres appears, therefore, unlikely as also by considering that even with high level of MP in sediments only one *C. glaucum* and *M.galloprovincialis* indivisuals and none of the crabs sampled in Bellocchio Lagoon showed any MP contamination. In contrast, it is more likely that MP ≥20 μm, the lowest detectable size in field collected organisms, are too large to be accumulated into the tissues of organisms and are rapidly egested (Ward et al., 2019; Piarulli & Airoldi, in preparation, Chapter III of this thesis). Furthermore, the typically rapid clearance mechanisms in invertebrate species (Van Cauwenberghe et al., 2015) could be accelerated by the physiological stress typically induced by the collection and handling of the organisms during sampling (Lusher et al., 2017). It is therefore plausible that the MP commonly found in organisms may only represent a small fraction of the total quantity of MP they ingest and egest over time.

This may also explain the predominance of microfibres found in most invertebrate species and confirmed in the current study. Due to their elongated shape, microfibres tend to entangle (Kolandhasamy et al., 2018; Murray and Cowie, 2011; Watts et al., 2015) and can remain trapped in the gastrointestinal tract and/or gills (Kolandhasamy et al., 2018). This can lead to longer retention times in the body compared to fragments and microbeads that are usually egested rapidly in faecal pellets (Watts et al., 2015). An increased residence time and the potential for microfibres to become entangled may also increase the likelihood of them causing blockages with adverse health effects on organisms (Watts et al., 2015). Furthermore, multifilament fibres, such as those found in one of the crabs, could fragment into the individual microfibre components during their passage through the gastric mill and due to the churning mechanism of the stomach's cardia, as previously observed by Watts et al. (2015) for the congeneric species *C. maenas*. This also helps to explain the very high microfibre content observed for only 2 of the crab specimens (76 and 117 microfibres, respectively) while the majority of crabs (88 specimens) contained from 0 to a maximum of 1 MP.

The drivers behind such variability are currently unknown, and could reflect both differences in uptake and egestion rates among individual crabs (Watts et al., 2015), and small-scale patchiness in

the distribution and bioavailability of MP particles in the environment (Lourenço et al., 2017) and/or in prey items (Batel et al., 2016; Mattsson et al., 2017; Tosetto et al., 2017). Alternatively, the observed MP variability may also originate as a consequence of asynchronous moulting events during which crabs experience periods of starvation (Sánchez-Paz et al., 2006), leading potential enhanced egestion of MP. With increasing evidence from laboratory studies that properties such as size and shape influence MP residence time in an organism (Ward et al., 2019; Piarulli & Airoldi in preparation, Chapter III of this thesis) the ability to measure the rates of MP uptake in natural conditions is crucial for understanding the potential physiological impacts caused not only by MP, but also by the additives and persistent organic pollutants (POPs) that can be desorbed during transit through an organism.

Despite the generally low levels of MP contamination observed in the current study, some trends emerged in MP distribution between different life habits of species. For example, omnivores appeared to retain only microfibres in their digestive system, sometimes in very high quantities, while suspension-feeding and facultative deposit-feeding bivalves generally contained a mixture of microfibres, fragments and foam MP in lower quantities (maximum 2 MP per specimen). It is therefore important to consider how variations in MP exposure related to the different feeding modes might influence what type of MP is ultimately ingested. Filter-feeding bivalves can capture natural and anthropogenic particles representing a wide range of sizes (Ward and Shumway, 2004), which can be efficiently sorted and discarded to avoid physical damage by well-developed mechanisms that select and eliminate non-food particles as pseudofaeces within approximately 1 hour after ingestion (Farrell and Nelson, 2013; Ward and Shumway, 2004). In the current study, the majority of MP present in bivalves (both suspension and deposit feeders) were >0.1 mm and potentially large enough to get stuck in the digestive system without being efficiently removed. Conversely, omnivores such as *C. aestuarii* and *H. diversicolor* can ingest MP via consumption of contaminated prey items as well as via direct ingestion from the environment (De Witte et al.,

2014). This could result in exposure to multiple sources of MP, especially those MP (e.g. microfibres) that are more readily retained both in the organisms and their prey items.

Differences in the MP composition present in the test organisms, particularly in terms of shape and polymer type, were also observed in relation to the sediment localisation of the benthic invertebrates. While epifaunal species contained only PES microfibres, infaunal species also presented PP and LDPE fragments and foam. This could relate to the tendency for microfibres to sink more slowly than other MP shapes (Porter et al., 2018), meaning they can be more easily captured by organisms living at the water-sediment interface. Even though PP and LDPE fragments and foam are less dense than sea water (Avio et al., 2017), they have been observed to sink faster than microfibres due to their higher volume and weight (Porter et al., 2018). As a result, they could be deposited to sediments at enhanced rates and become bioavailable for infaunal species. It is also possible that the elongated shape of the microfibres reduces their potential for rapid incorporation into the sediment relative to MP that are more spherical in nature, meaning they remain for longer periods on or near the sediment surface, increasing the chances of re-suspension.

The overall MP frequency and quantity observed in organisms in the current study was too low to allow a direct comparison of the MP distribution between species from the sampled geographic regions or sites. However, the consistent MP typologies (PES microfibres) and low levels of MP in organisms collected from both study areas indicate that invertebrates do not accumulate MP, with the exception of microfibres, which may have a slower transit in the gastrointestinal tract. This longer residence time also increases the potential for microfibres to be transferred from prey to predators. The knowledge developed in the current study can help to direct future targeted trait-based research efforts on MP ingested by marine organisms. For the time being, however, there remains a relatively limited understanding of the residence time of different types (e.g. size, shape) of MP in marine invertebrates, with even less known about possible differences in ingestion rates under field conditions compared to laboratory studies. Further field and laboratory research is

therefore needed to validate or refute the above hypotheses and provide a fundamental mechanistic understanding of the entry and fate of MP in trophic webs.

2.5 Conclusion

The current study provides indications that MP characteristics (size, shape, polymer type) and species' ecological traits (feeding modes and sediment localisation) influence MP uptake and retention in organisms inhabiting European salt marsh ecosystems. This can lead to variable risks of adverse physical and/or physiological effects of MP and their possible transfer through the food web. The findings also indicate that the 'snap-shot' approaches currently used for studying and monitoring MP in marine biota are not the best indicator of MP contamination and impacts. For monitoring purposes and to meet the objectives of the European Marine Strategy Framework Directive, it is suggested that MP measurements in biota must be integrated with direct measurements of MP in sediments and surface waters. Furthermore, the development of cost-effective methods that can quantify MP passing through the gastrointestinal tract of organisms in field conditions should be a research priority to facilitate an improved assessment of any potential harmful effects associated with MP contamination.

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Supplementary Information

Table S1: Organisms longest dimension (bivalves=shells, crabs=carapace, polychaetes=body) and grams of soft tissue (wet weight=ww) analysed. All data are reported as mean (± 1 SE, number of replicates as from Table 1).

Species	Longest dimension (cm)	Amount of tissue (g)	MP/g of tissue
Carcinus aestuarii	4.1±0.1	0.9±0.1	2.6±2.1
Hediste divesicolor	5.9±0.2	0.9±0.09	0.01±0.00
Cerastoderma glaucum	1.9±0.07	1±0.1	0.01±0.01
Mytilus galloprovincialis	3.2±0.1	1±0.1	0.00±0.001
Limecola balthica	1.2±0.4	2.3±0.5	0.3±0.3
Scrobacularia plana	2.7±1.4	2.2±0.5	0.01±0.01

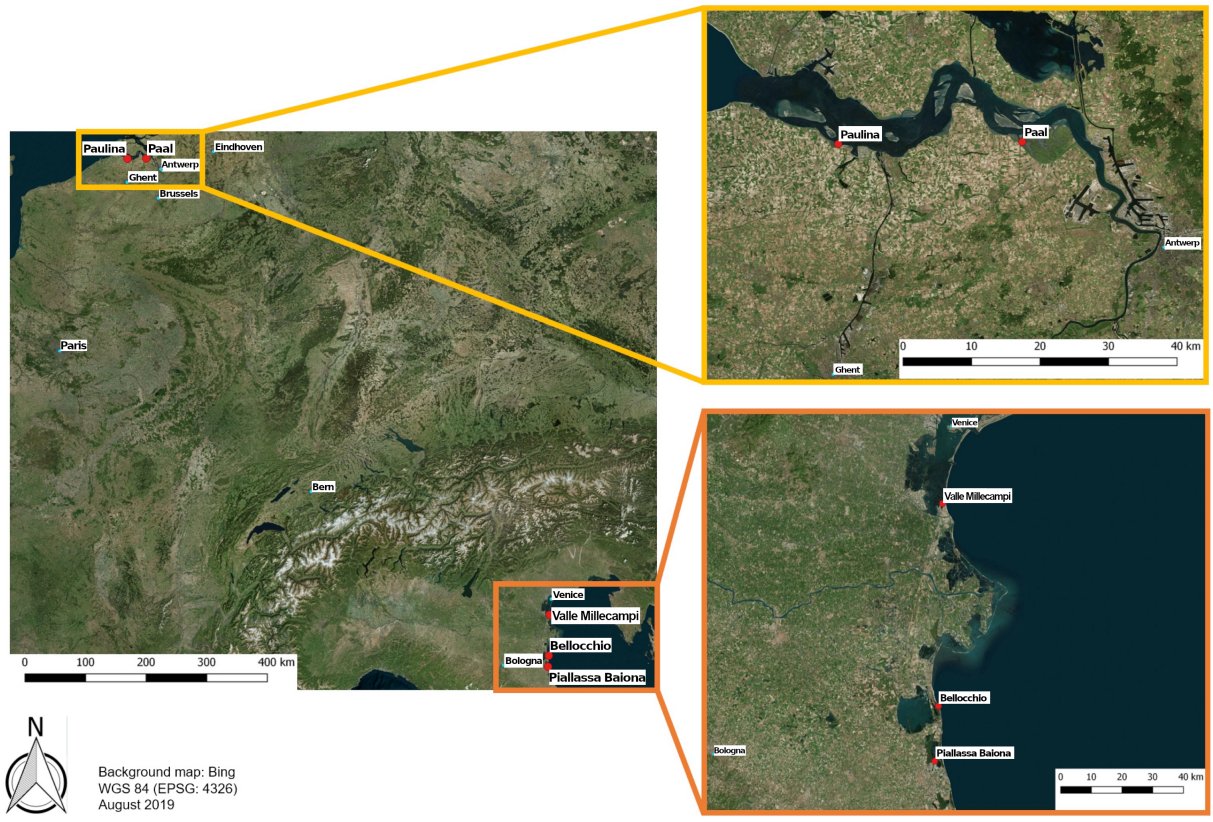

Figure S1: Sampling regions and sites of North Adriatic lagoons (orange) and Schelde estuary (yellow).

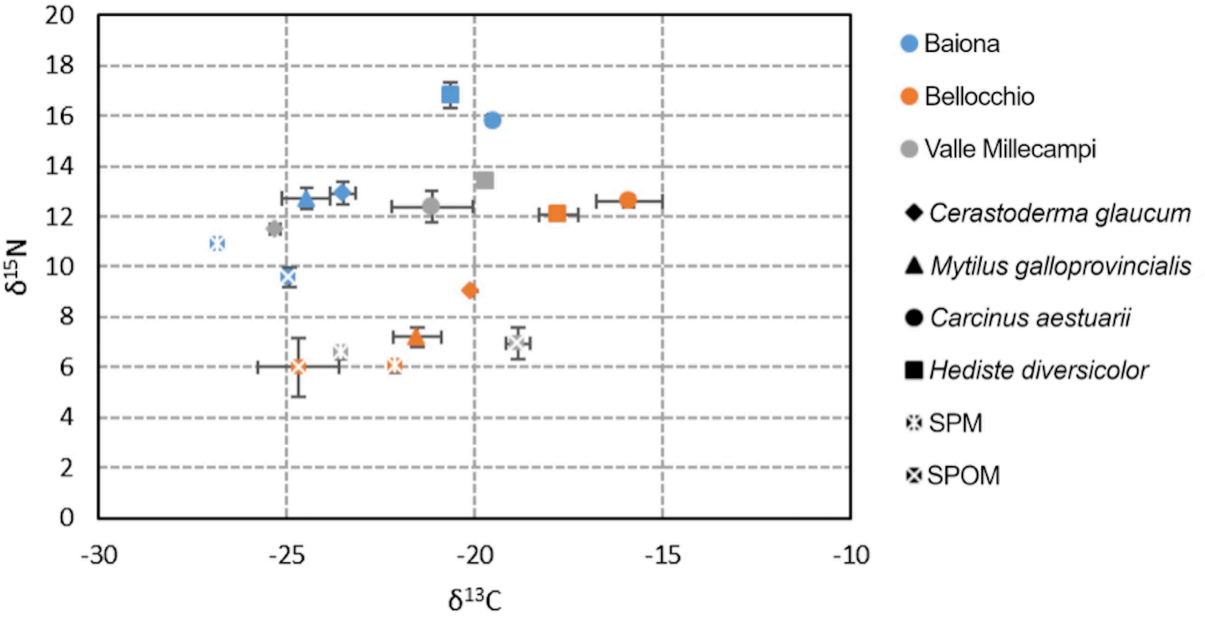

Figure S2: Biplot of $\delta^{13}C$ and $\delta^{15}N$ isotope values (‰) of the primary food sources: suspended particulate matter (SPM), sediment particulate organic matter (SPOM) and 4 infaunal consumer species of the Baiona, Bellochio, and Valle millecampi lagoons. Data are means ± 1SE.

Chapter III

Bivalve-mediated transfer of microplastics across marine compartments and trophic levels

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Abstract

Microplastics (MP) are omnipresent contaminants in the marine environment. They are ingested by a wide range of marine biota, but to what extent this uptake by organisms affects the dynamics and fate of MP in the marine system has received less attention. Using a multi-level laboratory experiment, we showed that the filter-feeding bivalve *Mytilus galloprovincialis* removed MP from the water column by incorporating them into biodeposits (faeces and pseudofaeces). This effect was particularly pronounced for the smallest MP fraction (= 41 μm size) and significantly increased the sinking rate of MP from the water column to the bottom, while at the same time decreasing the speed of deposition of the faecal pellets. The incorporation of MP into mussels' biodeposits also more than doubled their subsequent uptake by the benthic detritus-feeder polychaete *Hediste diversicolor*. We discuss how this bivalve-mediated incorporation in biodeposits can influence MP transfer across marine compartments and trophic levels, and their ultimate distribution in the environment.

***Key words*:** Microplastics; Trophic transfer; Vertical flux; Mussels; Biodeposits

3.1 Introduction

Microplastics (plastic particles < 5 mm in size, hereafter MP) are omnipresent and pervasive pollutants (Taylor et al., 2016), having been recorded in a variety of aquatic ecosystems (Ivleva et al., 2017). Many studies reported the ingestion of MP by marine biota (Katnelson, 2015) across multiple levels of the trophic chain, and showed the potential for MP to be transferred from prey to predators (direct trophic transfer) (Batel et al., 2016; Farrell and Nelson, 2013; Nelms et al., 2018; Setälä et al., 2014; Watts et al., 2015). Once ingested, MP can have adverse biological effects through both physical and chemical mechanisms (Hermsen et al., 2018) and can also potentially act as a vector for the transfer of contaminants across the food webs, with also possible implications for human health (Batel et al., 2018; Diepens and Koelmans, 2018; Mato et al., 2001; Teuten et al., 2009).

In recent years, the use of new procedures and tools (e.g. Fourier Transformed Infrared spectroscopy and Raman spectroscopy) allowed a more precise quantification and characterisation of MP ingested by wild specimens (Lusher et al., 2017). These studies demonstrated that the number of MP found in organisms (particularly in invertebrate species) is usually low (< 1 MP/organism) (Carbery et al., 2018; Desforges et al., 2015; Devriese et al., 2015; Neves et al., 2015; Van Cauwenberghe and Janssen, 2014, Piarulli et al., 2019). This low MP content could reflect either low levels of local environmental contamination or little retention of MP by the organisms due to egestion (Cole et al., 2016; Piarulli et al., 2019; Ward et al., 2019). Should the latter process be common, direct MP trophic transfer via predator-prey interactions could be relatively limited compared to previous hypotheses (Chagnon et al., 2018; Nelms et al., 2018; Setälä et al., 2014). Alternative uptake by detritivorous organisms of MP concentrated into organic-rich marine aggregates (Clark et al., 2016) could play a much larger and so far underestimated role (Au et al., 2017; Watts et al., 2014).

Biogenic aggregates such as marine snow, phythodetritus and faecal matter are ubiquitous and abundant in the marine system (Turner, 2015) and act as a biological pump for the vertical transport of carbon and nutrients from the water surface to the seafloor (Fowler and Knauer, 1986; Turner and Holmes, 2011). The incorporation of MP in marine aggregates was first proposed by Teuten et al. (2009) and Moore (2008). More recently Porter et al. (2018) hypothesised that the incorporation of MP into marine snow could increase the sinking rates of buoyant MP, and enhance their bioavailability to benthic organisms. At the same time, MP could also significantly alter the sinking rates of marine aggregates (Cole et al., 2016) affecting the transfer of particulate organic matter (POM) from the water column to the seafloor (Wieczorek et al., 2019). The ecological relevance and generality of these processes, as well as the role played by specific physical-chemical properties of the MP (e.g. size and density) is yet to be empirically explored.

The present study investigates the role of filter-feeding organisms in mediating vertical fluxes of MP from the water column to the sediments and the subsequent uptake by benthic detritivores. Two widespread species were used as model organisms, the filter-feeding mussel *Mytilus galloprovincialis* and the omnivorous polychaete *Hediste diversicolor*.

M. galloprovincialis is a dominant component of the marine benthos, endemic to and widely distributed in the Mediterranean Sea (Barsotti & Meluzzi 1968). It is very common in harbours and open waters, and often a dominant inhabitant on intertidal and shallow subtidal hard substrates, both natural and artificial (Bacchiocchi & Airoldi 2003). It is also largely aquacultured, using specialized floating rafts, buoys and lines, all of which serve as recruitment surface for juveniles (Carl et al 2012). As other mussels *M. galloprovincialis* has high filtration rates, and captures natural and anthropogenic particles of a wide range of sizes, but has also developed selection mechanisms to eliminate those particles not constituting an energy source (Ward and Shumway, 2004; Galimany et al., 2011). As such they largely contribute to the flux of organic matter from the water surface to the

benthic compartment through their continuous conversion of seston into faeces and pseudofaeces supporting a biodeposition-based food web (Zúniga et al., 2014).

Exposure studies on mussels indicated the potential for nano (1-100 nm)- and small (1-5 μm) micro- plastics to be metabolized, and in some cases also accumulated, at tissue and cell levels (Browne et al., 2008; Von Moos et al., 2012). Conversely, larger (≥20 μm) MP are often excreted after ca. 1 hour via faeces and pseudofaeces (Farrell and Nelson, 2013). This fast uptake/egestion of MP could facilitate MP transfer to the seafloor and associated benthic detritivores.

Hediste diversicolor is one of the most common polychaetes in the northern hemisphere sedimentary environments and is often used as indicator species for a variety of contaminants (Giangrande et al., 2005). This species has been described as carnivore and/or scavenger and detritus-feeder (Riisgird, 1989). Its multiple and various feeding strategies make *H. diversicolor* a key player in structuring soft-bottom communities (Rönn et al., 1988), and a potential recipient of MP through various trophic pathways. Studies have shown that mussel faeces is a high-quality food source for this species of polychaete, with potential application for mitigation of adverse effects on benthic environments in connection with mussel-farming (Bergstrom et al 2019). Furthermore, polychaetes are prey items to a variety of other organisms, with potential application in integrated aquaculture (Jansen et al., 2019), potentially representing a transfer route of MP and associated chemical contaminants to higher trophic levels.

Given the ecological and economic importance of sedimentary environments (Levin et al., 2001), it is fundamental to understand the dynamics and transfer of MP in these systems.

In the present work we experimentally tested whether and in what way *M. galloprovincialis* can affect the vertical transport, sinking rate and accumulation of MP in the benthic compartment through their incorporation in faeces and pseudofaeces (cumulatively referred in the text as biodeposits). Based on previous research performed on other species and marine compartments (Cole et al., 2016; Porter et al., 2018; Wieczorek et al., 2019) we hypothesised that such

mechanisms would be influenced by the size and chemical composition of MP, having a greater magnitude of effect on small sized and/or low density MP compared to large sized and/or high density MP. Further, we also tested whether the incorporation of MP into biodeposits can facilitate their subsequent ingestion by *H. diversicolor,* thereby potentially facilitating their transfer to higher trophic levels.

3.2 Materials and methods

3.2.1 Specimens collection and preparation

All the experiments were conducted during June and July 2018. Specimens of the mussel *M. galloprovincialis* were collected from the artificial jetties, seawalls or floating pontoons at the marina in Ravenna, Italy (44°29'32.6"N, 12°17'15.2"E, see Airoldi et al. (2016) for a detailed description of the sampling site) before the start of each experiment. The mussels were collected with a stainless steel wall-scraper and transported to the laboratory in a cool container within 30 minutes. Individuals 4-7 cm in size (shell length mean ± SE: 5.6 ± 0.1 cm) were selected, scrubbed and rinsed to remove any epibiota and possible adhering MP. The mussels were depurated for 24 hours in aerated aquaria to allow egestion of any previously ingested MP and to adapt to experimental conditions (Van Cauwenberghe et al., 2015; Ward et al., 2019).

To isolate the active filtration effects of live mussels from any potential physical influence of the shells empty mussel shells were used as controls. These were collected from an intertidal area in close proximity to the mussels' sampling site. The shells were carefully rinsed with Milli-Q water and scrubbed to remove any external MP, following which valves were partially joined together using professional water- proof glue, creating the physical shape of live mussels.

Unlike mussels, it was difficult to unequivocally identify and collect in the field enough *Hediste diversicolor* polychaetes of similar size and ontogenetic state. Therefore, we bought commercial

specimens of *H. diversicolor* from an aquaculture facility in Venice lagoon, Italy (LESCACHEPESCA SRL, http://www.lescachepesca.eu). The polychaetes were transferred to the laboratory for 24 hours of depuration (Hentschel, 1998; Van Cauwenberghe et al., 2015) in aerated aquaria with filtered seawater to remove any potential contamination from previously ingested or externally attached MP. The seawater used in all the experiments was collected from the marina in Ravenna and filtered through nylon filters (20 μm mesh, Ø: 47 mm, PLASTOK®) to remove any MP or natural particulate matter > 20 μm.

3.2.2 Effects of mussels on MP vertical transfer

The contribution of mussels to the vertical transfer of MP of different sizes was tested using an experimental set up consisting of 16 rectangular aquaria (5 l vol; 200 x 200 x 400 mm), randomly assigned (n= 4, Figure 1) to an orthogonal treatment combination of mussels vs. empty shells and exposure to MP_{SMALL} (41 μm) vs MP_{LARGE} (129 μm).

Aquaria were maintained at a constant temperature of 25°C, and portable aerators were used to aerate the water and generate a steady mixing effect. We used Separate groups of either 10 individuals of *M. galloprovincialis* or 10 'double-valve' control shells, suspended with a net 15 cm below the water surface in Mussels or Control aquaria, espectively (Figure 1). This set up mimicked realistic environmental conditions of the Mediterranean sea, where *M. galloprovincialis* growths naturally on a variety of shallow floating substrates or is farmed using floating structures (Zúniga et al 2014).

For the MP treatments commercially available polyamide (PA) MP fragments (Environmental Tracing Systems, UK) (Table S1 in Supplementary Information) were used. The MP dimensional range was chosen to favour the egestion of the particles by mussels (Ward et al., 2019) and to use a size detectable range in field samples. The two synthetic particles' sizes were easily recognisable as they were of different colours (pink for MP_{SMALL} and white for MP_{LARGE}) and fluorescence (red

fluorescence for MP_{SMALL} and blue fluorescence for MP_{LARGE}). Fragments were used, as opposed to the commonly used spheres, due to the higher prevalence of this MP morphology in the marine environment (Suaria et al., 2016). PA was chosen as polymer because it is slightly denser than the seawater (1.15 g cm^{-3}), thus less susceptible to resuspension after deposition than lower density polymers but not so dense as to immediately fall to the bottom, being therefore available for filtration by mussels. Before use, each MP amount to be added to the aquaria was individually preconditioned into 50 ml falcon tubes containing pre-filtered seawater and incubated for 48 h at room temperature (20°C) with a natural light regime to encourage a natural biofilm formation as described by Wieczorek et al. (2019). The tubes were manually shaken just prior to addition to the respective treatment aquaria to resuspend all MP particles.

A mass concentration of 0.2 mg L^{-1} (corresponding to 1 mg of MP per aquarium) was used for both size classes of MP, corresponding to ~600 MP L^{-1} for the small class, and ~300 MP L^{-1} for the large class. These concentrations are in the range of those reported from highly polluted coastal waters (Collignon et al., 2012; Doyle et al., 2011; Goldstein et al., 2014; Lattin and Moore, 2004; Moore et al., 2001).

After a 48 h of exposure to MP the aquaria aeration was stopped, the mussels or control empty shells were removed, and suspended and deposited MP were quantified. The aquarium water (supernatant) was slowly pumped out with a portable pump for liquids (D-mail ®, IT), filtered (20 µm mesh; Ø: 9 cm; PLASTOK) using vacuum filtration, and the filter used for determining the residual suspended MP fraction. The final 10 mm of water, with any sedimented material including biodeposits, was filtered separately to quantify the deposited MP fraction. The removed alive mussels were immediately frozen at -20°C, and a random subsample of 5 individuals from each aquarium (n=8 aquaria with alive mussels, n=40 total number of mussels) was processed to quantify the MP potentially retained in the soft tissue after ingestion. Each mussel was dissected to separate the soft tissue from the shell and weighed (wet weigh) before being digested enzymatically in 100

ml glass beakers, following the protocol described by Piarulli et al. (2019). Briefly, after 24 h incubation in 10 ml of 25% SDS (SIGMA – ALDRICH ®) at 50°C, 5 ml of lipase and 5 ml of protease/amylase (Biozym F and Biozym SE respectively; Spinnrad®, Bad Segeberg, Germany) were added. All samples were gently manually shaken and incubated at room temperature for 96 h, followed by vacuum filtration (20 μm mesh, Ø: 9 cm, PLASTOK®).

The filters of each MP fraction (retained in mussels tissue, suspended in the supernatant, and deposited) were positioned in covered glass petri dishes and dried at room temperature in a glass dryer for 1 week. MP were easily recognised and quantified due to their distinctive colour and fluorescence. MP counts of the dried filters were firstly performed under a Stereomicroscope (WILD M8, Leica microsystems) at 50X magnification, and subsequently validated with also a UV light source (λ 365 nm) (Montserrat et al., 2009) placed to the same microscope in the dark. MP counts were then expressed in distinct units based on the nature of their respective fraction: the ingested as MP ind^{-1}, the suspended as MP L^{-1} and the deposited as MP cm^{-2}.

The effect of mussels on the amount of MP in the deposited and suspended fractions was tested separately for the two MP sizes, due to the different initial particle concentrations for the small and large size classes. A Student's t-test was used after checking for normality and homogeneity with Shapiro−Wilk and Bartlett's tests respectively and log (x+1) data transformation if required to meet the assumption for paramentric statistics (Table S3 in Supplementary Information). Statistical analyses were performed with R studio (v. 0.99.903, R Core Team, 2016), and significance was set at $p\text{-value} < 0.05$.

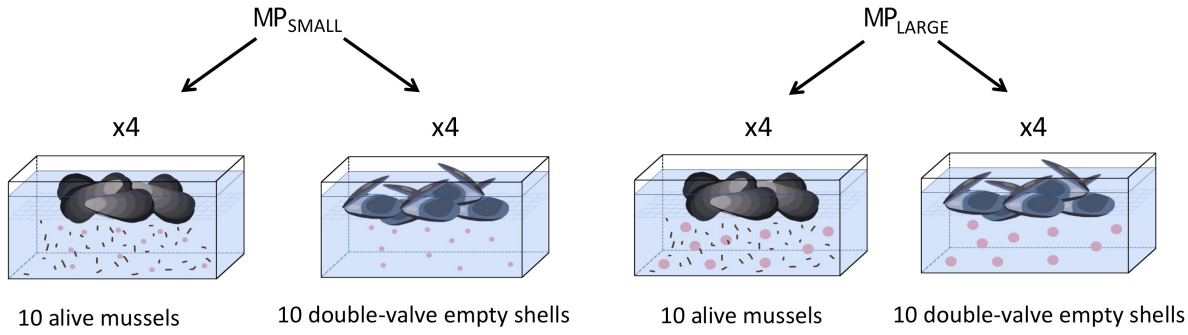

Figure 1: Experimental set-up. There were 4 aquaria for each orthogonal combination of alive mussels vs control empty shells and 41 (MP_{SMALL}) vs 129 µm (MP_{LARGE}) polyamide fragments.

3.2.3 Effects of MP incorporation on sinking rates of mussels' faecal pellets

This experiment tested whether the ingestion of MP by mussels and their subsequent egestion into faecal pellets (hereafter FP) can affect the sinking rates of FP through the water column, and if the degree of such effects varies as a function of MP size or density. Three different polymer types and sizes of MP were used: 41 µm PA fragments (PA_{SMALL}), 129 µm PA fragments (PA_{LARGE}), and polypropylene (PP) fragments of a median gran size of 127 µm (Table S1, Supplementary Information), where PP has a lower density (0.92 g cm^{-3}) compared to PA. The PP fragments were produced by milling PP pellets (Goodfellow Cambridge Ltd. UK) with a pin mill (Alpine C160, Messer group GmbH, DE) into irregular fragments that were subsequently sieved with decreasing mesh size (from 500 to 100).

Mussels' FP incorporating different MP types (FP with 1 MP were selected) were obtained by using 3 groups of 10 mussels in 3 separate aquaria. Each group was exposed to contamination from only one type of the 3 tested MP (concentration 0.2 mg L^{-1}), as described in section 2.2.2 A fourth set of mussels was maintained in filtered seawater in a separate aquarium without MP to produce MP-uncontaminated FP. After 48 h of exposure, FP were removed individually using a Pasteur pipet, and inspected under a stereomicroscope microscope (WILD M8, Leica microsystems) to check for their integrity and for the presence of incorporated MP. MP used in the experiment were recognised

due to their distinctive colour and/or fluorescence. A total of 20 integer FP was selected for each of the 4 treatments (PA_{SMALL}, PA_{LARGE}, PP and no MP). FP were photographed using a mounted camera (Motic BTWB, USA) and images were analysed using imageJ software (Schneider et al., 2012) to determine dimensions and to calculate the equivalent cylindrical volumes and density (Table S2 in Supplementary Information) of each faecal pellet using the Stoke's law modified for use with cylindrical shapes (Komar et al., 1981).

The sinking rates of the 4 FP treatments were assessed adapting the established method originally developed by Smayda et al., (1969) to measure the sinking rates of zooplankton FP. The FP were individually transferred with a Pasteur pipet to a 2 L measuring cylinder filled with 20 μm filtered seawater (25°C, 35 psu). FP were let to sink for the first 10 cm to achieve a constant velocity, and then their descent speed was measured over the remaining 10 cm.

Differences in the sinking rates of the 4 groups of FP were tested using one-way ANOVA (n=20 replicated FP per treatment). The data were log (x+1) transformed to meet the criteria for parametric statistics checked with Shapiro and Bartlett's tests (Table S4 in Supplementary Information). Tukey post-hoc test was used for pairwise comparison of the 4 groups.

The measured sinking rates of the MP incorporated into the FP were further compared with their theoretical sinking rates when free in the water column. Free MP were too small to enable the visual measurement of their sinking rates, therefore their velocity was estimated by using Stoke's law as described by Bach et al. (2012) and Porter et al. (2018), taking into account the physical properties (sizes and densities, Table S1 in Supplementary Information) of the three different MP types (PA_{SMALL}, PA_{LARGE}, and PP) as well as the water temperature and salinity.

3.2.4 Mussel-mediated trophic transfer of MP to polychaetes

To examine whether MP enriched biodeposits produced by mussels can facilitate the uptake of MP by detritivorous polychaetes, a modified version of the previous experimental set-up (described in

section 2.2.2) was used (Figure 2). Eight aquaria containing 5 L of filtered seawater and a 20 mm bottom layer of sand were maintained at 25 °C. Four of the aquaria contained groups of 10 *M. galloprovincialis* and the other four contained groups of 10 control empty shells. Based on observations from the previous experiments regarding size selectivity of MP by mussels, MP_{SMALL} fragments (41 μm; conc. 0.2 mg L^{-1}) were chosen as the size class, and prepared in the same manner as described in section 3.2.2 to establish a biofilm formation prior to use.

Following 48 hours of MP exposure, all mussels and control shell groups were removed and replaced by groups of *H. diversicolor* (n=5 per group). After 24 hours, the groups of polychaetes were transferred to aerated 500 ml beakers filled with 0.2 μm pre-filtered artificial seawater for 8 hours of depuration to allow the elimination of ingested material, based on the results by Bock et al. (1999) the depuration time was kept shorter than 12 h to avoid the re-ingestion of the previously egested MP. The MP retention in polychaetes soft tissue has been estimated about 0.5% (Van Cauwenberghe et al., 2015) therefore the MP egested by polychaetes was considered the most representative indirect measure of the MP uptake (Löder and Gerdts, 2015).

Once depurated, polychaetes were removed from the beakers and the depuration water filtered (mesh size: 20 μm, Ø: 9 cm, PLASTOK®). The filters were dried at room temperature in covered glass petri dishes placed in a glass dryer, and inspected for MP presence under the stereomicroscope. Each group of polychaetes was considered as a single population unit, thereby MP data were expressed as total number of particles per aquarium. Differences in the amount of uptaken (and egested) MP by polychaetes in the presence or absence of mussels and their biodeposits were compared using a t-test without any data transformation and after checking for normality and homogeneity of data (Table S7 in Supplementary Information).

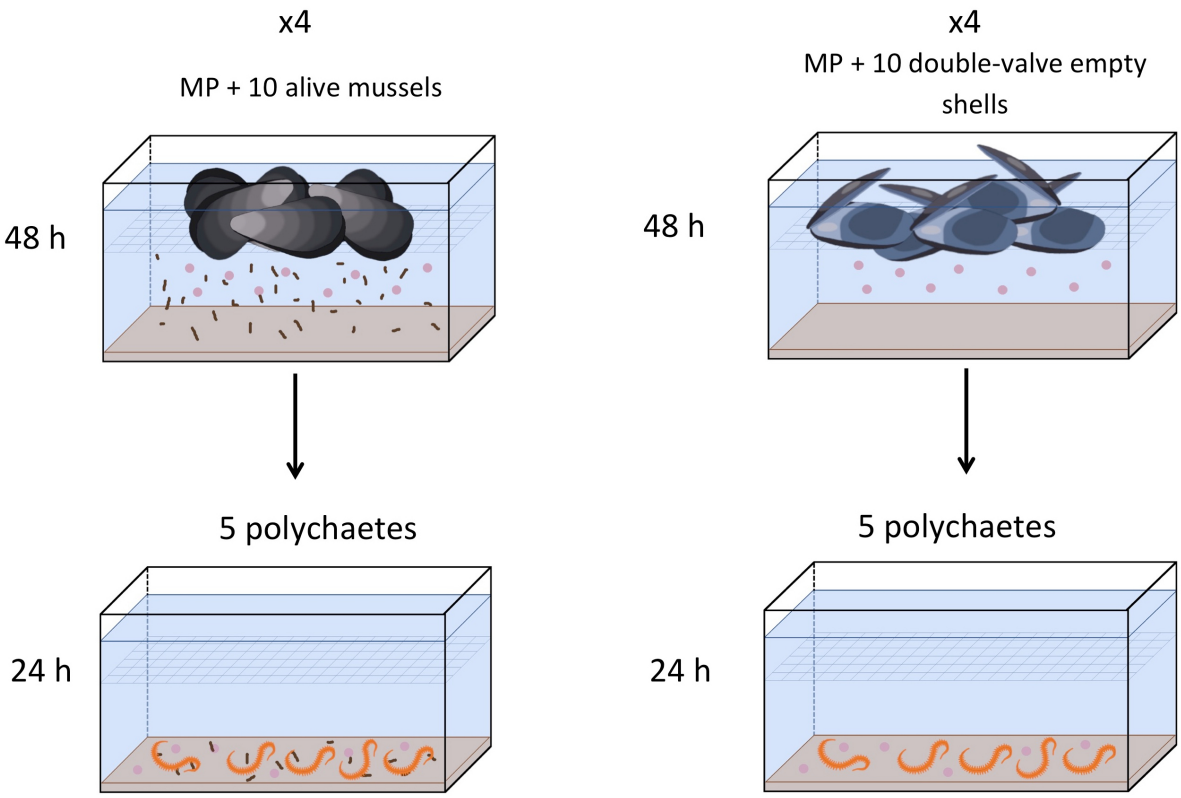

Figure 2: Experimental set-up. Groups of 5 polychaetes were exposed to contamination by MP (41 µm PA fragments) in aquaria previously treated with either alive mussels or control empty shells.

3.3 Results

3.3.1 Effects of mussels on MP vertical transfer

After 48 h, the MP_{SMALL} in live mussel treatments showed a 15% increase in deposited MP compared to control treatments (Fig 3c, Table S3 in Supplementary Information), while no significant differences were detected for MP_{LARGE} (Figure 3d and Table S3 in Supplementary Information). Suspended MP (Figure 3a,b) was significantly less on conclusion of the exposure time in live mussel treatments than in controls for both MP sizes (Table S3 in Supplementary Information).

Occurrence of MP was observed in 15% (2 individuals with 1 MP and 1 with 3 MP) and 20% (4 individuals with 1 MP each) of the analysed mussels exposed to MP_{SMALL} and MP_{LARGE} respectively.

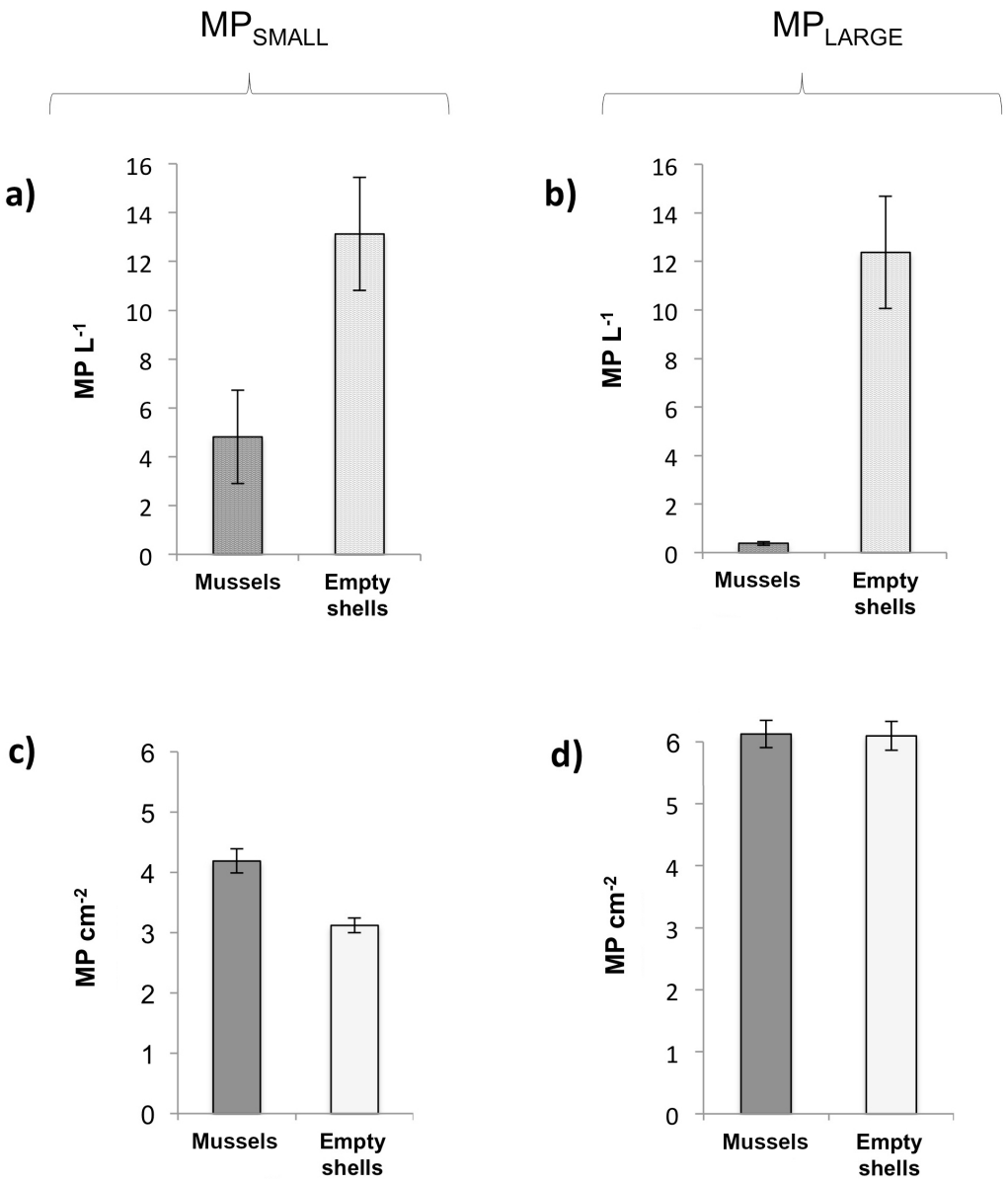

Figure 3: Concentrations and densities (mean ± 1SE, n=4) of two sizes of MP (41 µm MP_{SMALL} and 129 µm MP_{LARGE}) in suspension (**a**, **b**) and deposited (**c**, **d**) in treatments containing either live mussels (dark bars) or empty shells (light bars).

3.3.2 Effects of MP incorporation on sinking rates of mussels' faecal pellets

Ingestion by mussels and incorporation in FP (Figure 4a) accelerated MP sinking rates by over 3 orders of magnitude compared to the calculated theoretical sinking rates of free MP in the water, irrespective of MP dimensions and/or polymer type (Tables S1 and S2 in Supplementary Information). Averaged (± 1 SE) sinking rates of FP contaminated with PA_{SMALL}, PA_{LARGE} and PP

were 422 ± 61, 393 ± 34 and 352 ± 35 m day^{-1} respectively, while theoretical sinking rates of the associated reference free particles were 0.003, 0.03 and 0.03 m day^{-1} respectively.

All FP contaminated by MP exhibited significantly lower sinking rates compared to uncontaminated FP (Tables S5 and S6 in Supplementary Information). This effect was more pronounced with increasing size and decreasing density of the MP (Figure 4b). The differences among MP types were, however, quantitatively limited and were not detected as significant (Table S6 in Supplementary Information).

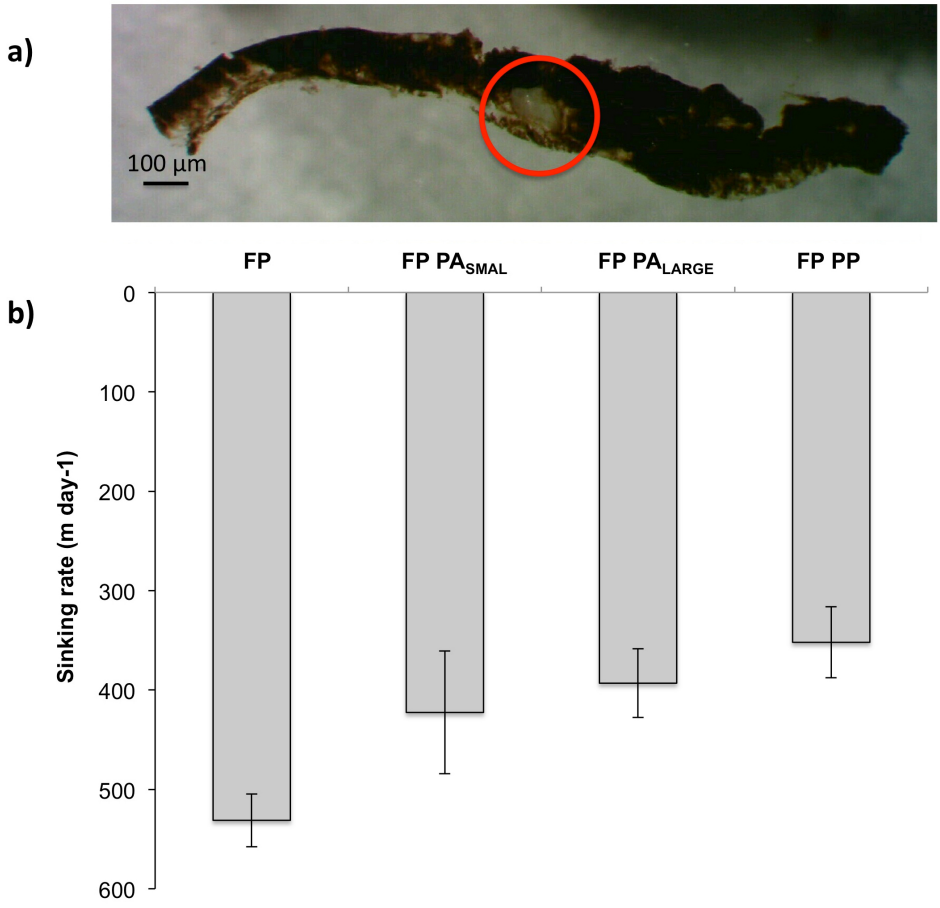

Figure 4: a) Photographic example of faecal pellet (FP) with incorporated MP (PP, red circle); b) Average (± 1SE) sinking rates of FP without MP vs FP incorporating different types of MP (PA_{SMALL}, PA_{LARGE} or PP)

3.3.3 Mussel-mediated trophic transfer of MP to polychaetes

After 24 hours, polychaetes exposed to biodeposits in sediments in the live mussel treatments ingested significantly more MP (40% more, Table S7 in Supplementary Information) than those in empty shell controls. On average, the population of polychaetes exposed to the live mussels treatment ingested 57 ± 9 MP per unit, while those from the control treatments ingested 24 ± 6 MP per unit (Figure 5).

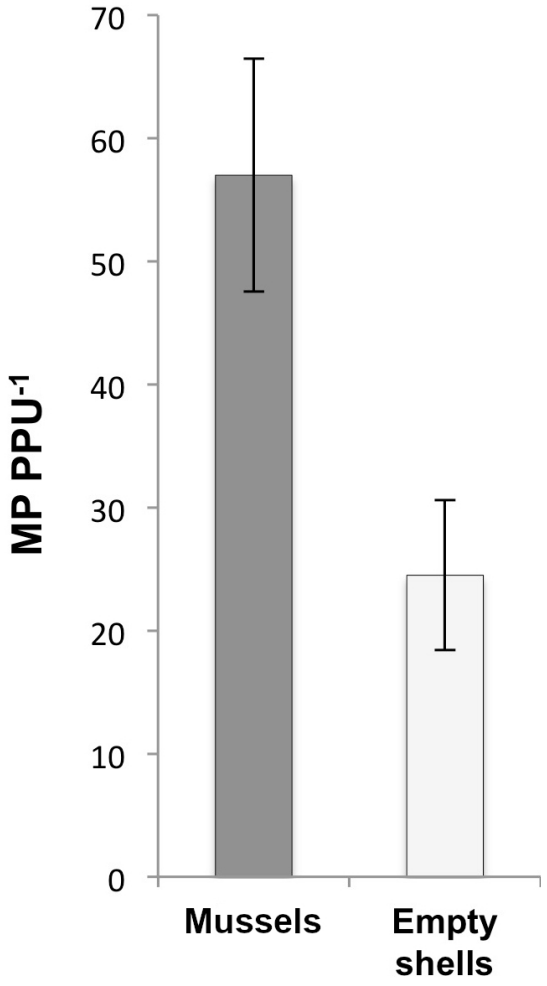

Figure 5: Average (± 1SE, n=4) number of MP ingested by population units of 5 polychaetes (PPU) after exposure to the presence of live mussels or to control empty shells.

3.4 Discussion

Approximately half of the MP introduced to the marine environment is buoyant, (Kooi et al., 2016) and tends to stay in suspension in the water column (Kershaw, 2015).

The present study showed that mussels facilitated and speeded up the vertical transfer of MP by filtering the synthetic particles out of the water column and accumulating them at the base of the aquaria into biodeposits that were subsequently easily ingested by detritus feeding polychaetes.

As hypothesised, this mussel-mediated vertical transfer was particularly relevant for small MP (41 μm), which was 15% more abundant at the bottom of treatments containing live mussels compared to controls. This is consistent with the known particle size selection range for *M. galloprovincialis*, which tends to be < 50 μm (Defossez and Hawkins, 1997; Pittura et al., 2018). The effect of mussels was also observed for large MP (129 μm), but this was limited to the supernatant indicating that mussels had a sequestrating effect also on larger MP fraction but this effect was too small to be clearly quantified at the bottom. Even if large MP due to their higher dimensions tended to be less selected as a potential food source by mussels (Ward and Shumway, 2004; Ward et al., 2019), it is possible that under chronic exposure to MP in natural systems the action of mussels could result in enhanced MP-transfer from the water column to the benthic compartment through egestion (Ward et al., 2019) also for larger synthetic particles which can be difficult to be avoided over time. Ultimately, most MP are likely to reach the seafloor (Van Cauwenberghe et al., 2015), as demonstrated by the levels of MP contamination recorded in sediments (e.g. Lorenz et al., 2019; Vianello et al., 2013), however, biogenic aggregation in faecal pellets and accelerated sinking by mussels (or other benthic filter-feeders) could result in fine-scale heterogeneity of MP distribution in marine sediments, creating concentrated hot-spots of small and highly bioavailable MP. To our knowledge so far this possible biologically-mediated small-scale heterogeneity in MP distribution at the sediment level has never been explored, if proved, it would have important implications both for the ecology of benthic systems and for the management of MP in sediments.

Besides concentrating small MP on the bottom, mussels also significantly removed suspended MP from the water surface and enhanced their sinking rate through the water column. Consistently with the findings by Porter et al. (2018), in the present work all the MP incorporated into FP sank 3-4 orders of magnitude faster than the same MP as free particles. Although our sinking rates calculations were made in a static experimental system, thereby not fully representing natural conditions, the magnitude of the differences suggests that even under environmental conditions, where oceanographic attenuation (e.g. resuspension) as well as other factors that can alter the MP density (e.g. biofouling) may occur, MP incorporation into FP would decidedly change their fate in the water column.

In turn, the incorporation of MP significantly decreased the sinking rates of FP irrespective of polymer type and/or particle size, similarly to Long et al. (2015) who estimated the effects of MP presence in phythoplankton aggregates. Given the central role that bivalves' faecal matter has in mediating the flux of nutrients and carbon cycling across benthic and pelagic systems (Hewitt et al., 2001; Robert et al., 2013) the observed decelerated sinking rates of FP caused by the presence of MP is of concern. There is a potential for the lowering of nutrient regeneration and availability in the benthic compartment, with unknown consequences at the community and ecosystem levels. Wieczorek et al. (2019), who also observed a longer persistency in the water column of MP-contaminated FP from salps, suggested that MP presence could lower the efficiency of the biologic pump, enhancing water turbidity due to the fragmentation of faecal matter by bacterial activity, with the consequent release of CO_2 directly into the water column.

The incorporation of MP into biodeposits also significantly enhanced the uptake of synthetic particles by polychaetes, demonstrating that MP incorporation into marine aggregates constitutes a relevant pathway for the transfer of MP between organisms with different feeding modes and placed at different trophic levels. *H. diversicolor*, known for its adaptable feeding modes, including detritivory, ingested more than twice as much MP when exposed to mussel biodeposits containing

egested MP. This difference does not simply reflect a larger availability of MP in the bottom sediments due to the accelerated sinking from mussels. Indeed, the first experiment showed that mussels increased MP abundance by only 15% compared to controls, while polychaetes ingested 41% more MP in the presence of mussels. This suggests that the effect of mussels is not only quantitative, but also qualitative. Faeces and pseudofaeces are nutritious (Johannes and Satomi, 1966) and readily consumed by a variety of suspension- and deposit-feeding organisms (Tenore, 1988), thereby aggregation in biodeposits could make MP more bioavailable, facilitating their transfer to consumers. It is likely that ingestion and egestion could also structurally and/or chemically change the MP, for example via fragmentation up to the nanoscale (Dawson et al., 2018) or modification of the biofilm community (Kesy et al., 2016), making the MP more suitable for ingestion by organisms.

It has been observed that nanoplastics (particularly those between 40 and 50 nm) can irreversibly accumulate in cells and tissues of organisms (Bergmann et al., 2015) with significant potential for direct trophic transfer. Conversely, our experiments with MP > 20 μm, which is within the size range detectable in field samples, indicate that very fast MP ingestion-egestion rates both by mussels and polychaetes, and little retention in the soft tissue (< 20% contaminated mussels) as also previously indicated by Ward et al. (2019) for mussels and Mazurais et al. (2015) for fish larvae. This evidence allows the conclusion that direct trophic transfer of MP ≥ 20 μm from contaminated prey to predators could be less relevant than previously hypothesised (Gutow et al., 2016). It, however, does not exclude the existence of a potential chemical risk of transfer of hazardous substances associated to MP, such as persistent organic pollutants and/or plastic additives (e.g. phthalates). These pollutants can be adsorbed by plastics from the seawater (Rios et al., 2010), and possibly released after uptake by organisms and accumulated in cells and tissue, with a potential for transfer via predator-prey interactions, bioaccumulation at upper trophic levels (Batel et al., 2016) and severe physiological effects (Batel et al., 2018; Pittura et al., 2018). The direct quantification of

ingestion-egestion rates of MP and associated contaminants is currently operationally challenging (Lanctôt et al., 2018) and developing cost-effective methods to quantify the real integrated impact over time of MP on organisms at environmental concentrations is a research priority.

3.5 Conclusion

Overall, this study represents an important step to understand the role of biologically-mediated processes on the fate of MP in the marine environments. We empirically showed for the first time that incorporation in biodeposits by mussels facilitates the transport of MP from the water column to the sediments and between species at different trophic levels. We presented a multifaceted scenario. On one side faecal pellets egested by mussels can accelerate the vertical transport and aggregation of MP to the sediments, making MP less available to pelagic and mesopelagic species but increasing their bioavailability to benthic detritivores. On the other side, the decelerated vertical fluxes of contaminated faecal pellets could unbalance the flux of nutrients from the water surface to the seafloor. Further work is urged to characterise the relevant spatial and temporal scales of these processes, and the so far largely unexplored consequences at the ecosystem level.

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Supplementary Information

Table S1: Polymer, size (as median grain size for PP), density, concentration and estimated sinking rates of MP (as free particles) used in the experiments. PA_{SMALL}=41 μm polyamide fragments; PA_{LARGE}=129 μm polyamide fragments; PP= 127 μm polypropylene fragments.

Polymer	Grain size (μm)	Density (g cm^{-3})	Concentration (g L^{-1})	Sinking rate (m day^{-1})
PA_{SMALL}	41	1.15	0.2	0.003
PA_{LARGE}	129	1.15	0.2	0.03
PP	127	0.92	0.2	0.03

Table S2: Dimensions, equivalent cylindrical volume, and density of the faecal pellets (FP) that were used to quantify the effects of incorporated MP on FPs' sinking rates (Section 3.2.3). Data are presented as mean±1SE (n=20). PP= 127 μm polypropylene fragments; PA_{LARGE}=129 μm polyamide fragments; PA_{SMALL}=41 μm polyamide fragments; No MP= FP without MP incorporated.

FP group	Diameter (x10^{-3} cm)	Length (x 10^{-3} cm)	Volume (x 10^{-3} cm^3)	Density (g cm^{-3})	Sinking rate (m day^{-1})
PP	60±3	390±18	1.28±0.1	1.56±0.1	351.9±35
PA_{LARGE}	70±4	540±66	1.28±0.4	1.02±0	393±34
PA_{SMALL}	70±4	490±49	1.28±0.4	1.02±0	422.5±61.6
No MP (Control FP)	60±4	570±51	1.28±0.4	2.54±0.3	530.9±26.6

Table S3: Summary of tests to check the data for the use of parametric statistics (normality with Shapiro's test and homogeneity with Bartlett's test) and t-test to compare concentrations and densities of MP (41 μm MP_{SMALL} and 129 μm MP_{LARGE}) in suspension and deposited in treatments containing either live mussels or empty shells (see section 3.2.2 for a complete experimental description). Transf.= data transformation. Significance was set at p-value < 0.05.

Fraction	MP	Treatment	Transf.	Shapiro's p-value	Bartlett's p-value	t-test
Supernatant	MP_{SMALL}	Mussels	Log(x+1)	0.19	0.35	df=4.68 p-value=0.03
		Empty shells	Log (x+1)	0.6		
	MP_{LARGE}	Mussels	Log (x+1)	0.2	0.08	df=3.55 p-value=0.0004
		Empty shells	Log (x+1)	0.98		
Deposited	MP_{SMALL}	Mussels	None	0.44	0.44	df=4.98 p-value=0.006
		Empty shells	None	0.2		
	MP_{LARGE}	Mussels	None	0.76	0.91	df=5.98 p-value=0.92
		Empty shells	None	0.7		

Table S4: Summary of tests to check for normality (Shapiro's test) and homogeneity of data (Bartlett's test) of the experiment described in section 3.2.3. FP=faecal pellets; Transf.= data transformation. Significance was set at p-value < 0.05.

FP group	Transf.	Shapiro's test p-value	Bartlett's test p-value
PP	Log(x+1)	0.12	0.17
PA_{LARGE}		0.17	
PA_{SMALL}		0.2	
No MP (Control FP)		0.07	

Table S5: Summary of the ANOVA test to compare the sinking rates of faecal pellets (FP) incorporating different MP (see sections 3.2.3 and 3.3.2 for a complete experiment description and results). Significance was set at p-value < 0.05.

	Df	SS	MS	F	P-value
FP groups	3	7.02	2.34	13.18	< 0.0001
Residuals	76	13.5	0.17		

Table S6: Summary of the *a posteriori* pairwise comparison with Tukey's test to explore differences in sinking rates of faecal pellets (FP) incorporating different MP. (see sections 3.2.3 and 3.3.2 for a complete experiment description and results). Significance was set at p-value < 0.05.

FP groups pairwise comparison	Tukey post-hoc test's p-value
No MP vs PA_{LARGE}	< 0.0001
PP vs PA_{LARGE}	0.84
PA_{SMALL} vs PA_{LARGE}	0.99
PP vs No MP	0.0001
PA_{SMALL} vs No MP	< 0.0001
PA_{SMALL} VS PP	0.79

Table S7: Summary of tests to check the data for the use of parametric statistics (normality with Shapiro's test and homogeneity with Bartlett's test) and t-test to MP ingested by population units of 5 polychaetes (PPU) after exposure to the presence of live mussels or to control empty shells (see section 3.2.4 and 3.3.3 for a complete experiment description and results).

PPU exposure	Shapiro test's p-value	Bartlett test's p-value	t-test
Mussels	0.81		df=5.11
Empty shells	0.93	0.07	p-value=0.03

CHAPTER IV

Temporal dynamics and small-scale patchiness of mussel-mediated fluxes of microplastics

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Abstract

Filter-feeding bivalves such as mussels are known for modulating benthic–pelagic coupling and depositional processes of organic material in the marine environment, but to what extent the uptake by these organisms affects the temporal dynamics and fate of microplastics (MP) in the marine system has not yet empirically explored. Using specifically designed field traps we quantified the capability of mussels (*Mytilus galloprovincialis*) to mediate the vertical fluxes of MP from the water column to the sediments by incorporation into biodeposits. We further explored how these MP fluxes vary over space and time, and compared whether the observed variability paralleled variations in MP contents in mussels' soft tissues. *M. galloprovincialis* filtered and accumulated MP, particularly those in the small (11-25 μm) and low dense (polypropylene and polyethylene) range. These mussel-mediated fluxes were very variable over time, with a peak in MP abundances that was reached in January. Conversely MP deposited in the absence of mussels was more constant

over time. MP found in mussel tissues were similar in size to those found in biodeposits, but showed a different polymeric composition and marked temporal fluctuations. We also observed a notable small-scale spatial heterogeneity in MP accumulation among nearby biodeposit traps. This small-scale variability is hardly considered in ecological studies of MP, and further work is recommended to explore its consequences at the ecosystem level as well as its underlying drivers.

Key words: Microplastics, Transfer, Vertical flux, Biodeposits, Filter-feeders**,** Mussels

4.1 Introduction

The amount of plastics floating on the ocean surface has been estimated to weight more than 268,940 tons (Eriksen et al., 2014) causing a global environmental concern. While threats caused by macroplastic (items > 25 mm) debris on marine fauna are more clear, less obvious but equally concerning are plastic particles below 5 mm (Arthur et al., 2009) commonly referred to as microplastics (hereafter MP). MP have been found in all the marine compartments (see for review Carbery et al. 2018) and habitats also including very remote areas of the globe far from human activities (Bergmann et al., 2017; Peeken et al., 2018; Woodall et al., 2014). However, there is still limited information on the dynamics, fate and impact of MP at ecosystem and community level (Da Costa, 2018). This, in part, can be attributed to the lack of studies aiming to evaluate spatial and temporal patterns of plastic contamination in natural systems. Often comparisons are confounded by both short- and long-term temporal factors that affect accumulation of MP in natural systems (Underwood et al., 2017). Also, very limited studies use the different characteristics of MP (e.g., shape, size and polymeric composition) in multivariate analyses to identify spatial or temporal patterns (Underwood et al., 2017).

In the marine system contamination by synthethic macro- and micro-debris has been observed to be

typically very patchy and unpredictable, both within and among different marine habitats (Browne et al., 2015), this can be due to the occurrence of multiple environmental factors but also due to the transformations of MP occurring in the marine system, that can change their physical-chemical properties and fate (Porter et al., 2018). For example studies by Kaiser et al. (2017) and Kooi et al. (2017) showed how processes such as biofouling and fragmentation of MP can alter MP buoyancy leading to sedimentation of the particles on the seafloor. A key transport route for the transport of MP from the water surface to the benthos is through marine aggregates which includes marine snow, phytodetritus and faecal matter (Turner, 2015). The incorporation of MP into marine aggregates was first proposed by Teuten et al. (2009) and Moore, (2008) and further explored by Porter et al. (2018) and Zhao et al. (2018) . It has been recently demonstrated that filter-feeding bivalves such as mussels can play a key role in producing and transferring MP from the water column to the seafloor (Piarulli et al., Chapter X this thesis). Indeed, bivalves during their filtration can capture natural and anthropogenic particles of various sizes and have efficient selection mechanisms to eliminate those particles that do not constitute an energy source (Ward and Shumway, 2004). Browne et al. (2008) showed that the blue mussel *Mytilus edulis* is capable to metabolize and also accumulate small MP (1-10 μm) in tissue and cells. Conversely, larger MP are usually egested in few hours via their faeces and pseudofaeces (referred together as biodeposits) (Farrell and Nelson, 2013). This mechanism of ingestion-egestion produces MP enriched biodeposits, potentially creating hot-spots of highly bioavailable MP for benthic detritus-feeding organisms (Piarulli et al., Chapter X this thesis). However, the contribution of biodeposits to the vertical transport of MP in field conditions remains relatively unexplored, as well as any associated spatial and temporal variability.

In the present study the Mediterrean mussel *Mytilus galloprovincialis,* a dominant component of the marine benthos, endemic and widely distributed in the Mediterrean Sea (Barsotti & Meluzzi 1968) and growing on a variety of natural and artificial hard substrata (Bacchiocchi & Airoldi 2003) was

used as model organism. Mussels as well as other shellfish farmed for human consumption often growth on floating artificial structure in coastal waters such as floating rafts, buoys and lines, all of which serve as recruitment surface for juveniles (Carl et al., 2012), therefore they represent a key organisms for the transport of particulate organic matter from the water column to the seafloor (Norkko et al., 2001).

We designed a novel biodeposits trap and applied it in field conditions to quantify the vertical transport of MP conveyed by mussel biodeposits. We tested whether this mussel-mediated fluxes of MP differed by natural vertical fluxes of MP by comparing the deposition of MP in the traps in the presence or absence of mussels. We further explored the temporal variability in these fluxes by using replicated traps deployed at 4 times of the year.

4.2 Material and methods

4.2.1 Study area and field set-up

The work was carried out in the Ravenna harbour, a semi-enclosed area with high levels of anthropogenic activities (for an extensive description of the study area refer to Airoldi et al., 2016). This is a confined area (44°29'32.6''N; 12°17'15.2''E) with limited water circulation and mixing with the open sea, which makes it a potential hot-spot for microplastics contamination and accumulation.

The contribution of *Mytilus galloprovincialis* to the vertical transport of MP was quantified by using conical traps specifically designed to collect mussels' biodeposits (Figure 1). Groups of 60 mussels, collected from artificial jetties and seawalls in the study area, were suspended with a net in each of 4 replicated traps. To compare the amount and properties (shape, size and polymers) of MP sank due to mussels' filtration to those MP sank without the action of mussels 4 additional replicated control traps were used. These control traps contained 60 double-valve empty mussels

suspended similarly to the live mussels, thereby mimicking the physical obstruction of the shells without the filtration action.

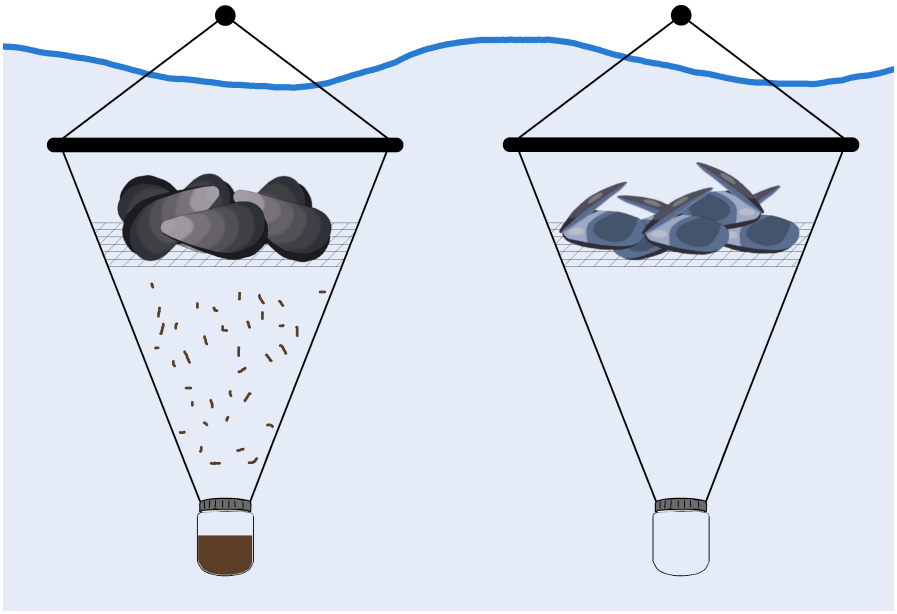

Figure 1: Conical traps to collect deposited matter (DM) with alive mussels (left) and empty shells (right). There were 4 replicated traps per treatment for each of 4 sampling periods (June 2018, October 2018, January 2019 and March 2019).

The conical traps and the nets containing the mussels were made of polytetrafluoroethylene (PTFE). This plastic polymer was preferred over others as the FTIR techniques used in this study to identify and characterise MP do not take into account this polymer (Lorenz et al., 2019), thus any contamination from the traps would not influence the results. The traps were cone-shaped (70 cm high and 40 cm wide at the upper end) to allow the collection of biodeposits into 250 ml jars attached to the narrow end of the cone. Three series of 4 holes (2.5 cm diameter) allowed to maintain the correct water-flow through the trap during the experiment to keep the mussels healthy as in free natural conditions. The 8 traps (4 with alive mussels and 4 with empty shells) were suspended in the field from a jetty (Figure 2a, b, c), with their mouths submerged about 30 cm below the water surface (Figures 1 and 2). The traps were exposed for intervals of 7 days repeated

every two months (June 2018, October 2018, January 2019 and March 2019), to quantify any temporal variations in the amounts and properties of settled MP. After exposure, all jars from the traps were collected, transported to the laboratory and frozen at -20°C an before further processing for MP extraction, quantification and characterisation. Further, 30 mussels at random from each alive mussel unit were collected for subsequent analysis of MP contents, aiming to explore possible relationships between MP found into biodeposits and those retained by mussels. Each mussel was individually wrapped with Milli-Q rinsed aluminium foil, transported to the laboratory and frozen at -20°C.

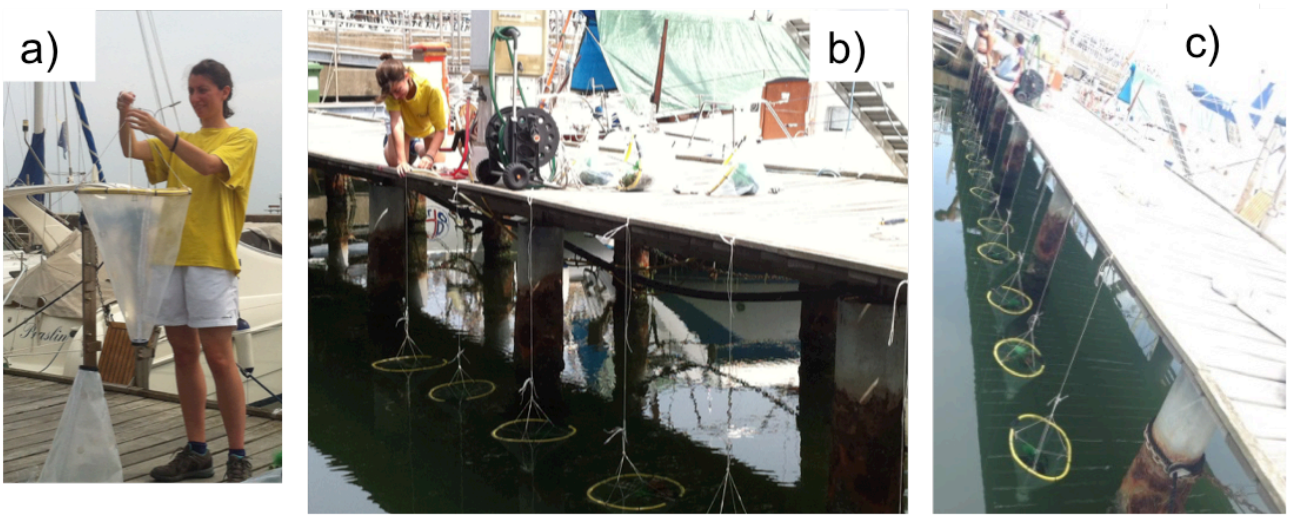

Figure 2: Photographic examples of **a)** a conical trap without the jar during the preparation phase; **b)** and **c)** the conical traps deployed for the experiment.

4.2.2 Sample processing

A total of 30 out of 32 collected jars (n=4 control or mussel collector for each of 4 time points) were successfully processed and analysed while 2 samples from March were lost during the collection phase.

To isolate and characterise the MP accumulated in the jars, the deposited material (hereafter DM) was vacuum filtered through a pre-weighted 10 µm stainless steel filter (Haver & Boecker, 59302

OELDE, Germany) to eliminate the water fraction and concentrate the particulate matter. Very large natural items such as shells or vegetative residuals were manually removed with tweezers during the filtration phase, and rinsed with milli-Q water to allow any MP attached to the items to fall back into the sample. More than one filter per sample was used in the presence of large amounts of DM. The filters were dried at room temperature for 24 hours and then weighted with a precision balance (at precision of 0.01, Sartorius Laboratory) to evaluate the amount of material on the filter before subsequent digestion.

Digestion steps involved an oxidative treatment with Fenton's reagent, which was applied following a modified protocol from Hurley et al. (2018) and further implemented and validated by Song et al., (in prep). Each filter with the concentrated sample was transferred in 100 ml glass beakers and 20 ml of the reaction catalyst iron sulphate ($FeSO_4$) was added followed by a slow addition of 40 ml of 30% hydrogen peroxide (H_2O_2). This was added drop by drop with a glass pipet over 10 minutes, to favour the oxidative reaction without increasing the reaction temperature above 50 °C to avoid polymer alteration and disruption. The solution was left acting for additional 10 minutes to allow the completion of the oxidative digestion. Each digestate was filtered again through a 10 μm stainless steel filter (one or more per sample) and any ferrous sulphate granules formed during the reaction were dissolved by adding from 1 to 5 ml of 5% chloridric acid (HCl).

The 30 mussels subsampled from each unit were separated from their shells and an amount of 50 g of randomly selected mussels soft tissue from each unit were digested with 200 ml of 10% potassium hydroxide (KOH) solution for 24 hours at 50°C, which allowed the complete digestion of soft tissues. The digestate was filtered through a 10 μm stainless steel filter.

The material retained onto the filters was transferred into 100 ml glass separator funnels filled with high-density (1.7 g cm^{-3}) zinc chloride ($ZnCl_2$) solution to allow the density separation of the lighter material (including also MP) from the other undigested natural compounds (e.g. sand). After three to seven days, the denser material settled to the bottom and was removed. The upper phase of the

density separation treatment, containing the lighter material including MP, was vacuum filtered through 10 μm stainless steel filters to remove the ZnCl2. The retained material was transferred into 100 ml glass bottles by rinsing the filter with Milli-Q.

To allow the subsequent MP identification and characterisation, samples were size fractionated, as the FTIR approaches varied for different MP size categories. All the purified samples were filtered over a 500 μm stainless steel mesh (Haver & Boecker OHG). The material retained on the mesh was thoroughly rinsed with Milli-Q and stored separately in 100 ml glass bottles for further analyses. This step divided the sample into two size fractions potentially containing particles of either ≥ 500 μm and < 500 μm, respectively.

4.2.3 Microplastic identification and quantification

The > 500 μm fraction of DM was rinsed into a beaker and manually sorted in a Bogorov chamber under a stereo microscope (Olympus SZX16, Olympus) at 100 and 320x magnification. All particles suspected to be MP were transferred into glass petri dishes, photographed under the microscope (Olympus DP26 Digital Camera, Olympus) and measured (length at their longest dimension) using image analysis software (cellSens, Olympus).

The putative MP, preventively cleaned with 99% ethanol to remove any biofilm formation, were individually identified by Fourier Transformed Infrared Spectroscopy with an Attenuated Total Reflectance objective (ATR-FTIR, Bruker Optik GmbH). The IR spectra were collected in the spectral range of 400 and 4000 cm^{-1} and compared against a reference library developed and presented in Primpke et al. (2018). Particles with a match of at least 700 (out of 1000) were counted as MP. If the match ranged between 600 and 700 the spectra were manually compared to database spectra and evaluated based on expert knowledge, as suggested by other studies (Kroon et al., 2018; Lorenz et al., 2019; Lusher et al., 2013).

For FTIR measurement of the samples' fraction < 500 μm it was necessary to transfer the purified

samples onto aluminium oxide filters (Anodisc, 0.2 mm, Whatman GmbH). A FlowCam (Fluid Imaging Technologies) was used to determine a suitable subsample volume that could be transferred onto the filter area without overloading it (Bergmann et al., 2017). Based on the FlowCam assessments, aliquots ranging from 1.2 to 100% of the total purified samples volumes were concentrated onto a 13 mm diameter filter area, using a vacuum filtration unit.

The loaded filters were transferred into covered glass petri dishes and dried for 48 h at 30 °C.

Particles of the smaller size fraction were analysed using a μ-FTIR microscope (Hyperion 3000 coupled to a Tensor 27 spectrometer, Bruker Optik GmbH) equipped with a 15x objective and a focal plane array (FPA) detector as described in Lorenz et al. (2019). Filters were placed on a custom made sample holder and covered with a BaF2 window (25 mm diameter, 2 mm in thickness, Korth Kristalle, Germany) that allowed the focus both of fragments and fibres (Primpke et al., 2019). Samples were then measured in the wavelength range of 3600–1250 cm^{-1} with a pixel size of 11.1 x 11.1 mm per measured spectra. The FTIR imaging data were automatically analysed by following Primpke et al.. (2019, 2017) and by comparing the obtained spectra with the database developed by Primpke et al. (2018). Based on the identified spectra a subsequent image analysis (Lorenz et al., 2019; Primpke et al., 2019), provided particle numbers, size classes and polymer types of all identified MP.

4.2.4 Anti-contamination and quality control procedures

Specific precaution procedures were applied at all the steps of the sample processing following an integrated protocol between Lorenz et al. (2019) and Piarulli et al. (2019) to avoid external plastic contamination. All the equipment was made of metal or glass, and if plastic was necessary polyvinyl chloride (PVC) or polytetrafluorethylene (PTFE) were preferred over other polymers.

All the processing steps, from sample purification to FTIR analyses, were performed in clean laboratories where the use of a cotton laboratory coat was mandatory and with minimised external

air circulation. Every laboratory involved in the samples processing had a dustbox (DB1000, G4 prefiltration, HEPA-H14 final filtration, Q=950 m^3 h^{-1}, Möcklinghoff Lufttechnik, Gelsenkirchen, Germany) installed to avoid plastic fibres airborne contamination.

All the process involving the direct exposure and handling of the samples were carried out under a laminar flow cabinet (ScanLaf Fortuna 1800, LaboGene, Lillerød, Denmark). All the equipment was milli-Q rinsed before the use and all the laboratories surfaces were regularly cleaned. All chemicals involved in the processing steps were filtered before usage over 0.2 µm (GTTP, polycarbonate) filters to remove external particles.

Contact with air and plastic surfaces during all the laboratory procedures was minimised for samples, instruments and reagents by covering them with milli-Q rinsed aluminium foil before and after use. After filtrations membranes were kept covered in glass petri dishes, previously rinsed with milli-Q water. Samples were processed in batches of 4, for each batch a procedural blank consisting of the sample-free processing reagents was run in parallel of the samples. In total 12 blanks (n=4 for biodeposits, DPM and mussels samples respectively) were processed. After the FTIR analyses, the final MP results were corrected taking into account their accompanying blank (blank correction).

4.2.5 Statistical analyses

The MP counts of each analysed Anodisc were extrapolated based on the respective sample analysed proportion (Lorenz et al., 2019) and corrected for contamination found in the corresponding procedural blank. The total amount of MP in both size fractions for all samples typologies was combined and results were presented as mean (±SE) of MP per trap unit (MP $unit^{-1}$) or percentages of (size class or polymers) species. Both MP fragments and fibres were individually categorised in a priori defined size classes (µm): =11, 11-25, 25-50, 50-100, 100-500 and 500-5000 for fragments and 25-11, 25-50, 50-100, 100-500, 500-1000, 1000-5000 for fibres.

MP quantities and relative (size class and polymer) species composition did not respect the assumption (normality and homogeneity) for parametric statistics, investigated with Shapiro-Wilk and Bartlett's test respectively with R studio 0.99.903 (R Core Team, 2016), and resulted highly skewed, thus they were square root transformed (Sokal and Rohlf, 1995) prior to subsequent analyses. Two-way permutational univariate and multivariate analyses (PERMANOVA, Anderson and Walsh (2013) were applied to test whether the MP accumulated in DM significantly differed in number or (size class and polymer) species composition between traps with mussels or control shells (fixed factor) and/or times (random factor).

PERMANOVA univariate analyses on MP quantities, considering fragments and fibres together, was based on Euclidean distances while multivariate analyses on (size class and polymer) species composition, carried out separately for fragments and fibres, were based on Hellinger distance which is the recommended as similarity measure for ordination of species abundance data and does not put high weights on rare species (Legendre and Gallagher, 2001).

When PERMANOVA resulted significant a pairwise post-hoc test was performed to identify the source of significance among levels of factors. Principal coordinate analysis (PCO), preceded by PERMDISP analysis to test for the homogeneity of the multivariate dispersion (Clarke and Gorley, 2015), was applied as multivariate ordination analysis to visualize patterns of MP composition among different samples and factors.

Further, univariate PERMANOVA was subsequently used to investigate which (polymer) species mainly contributed to the observed patterns. All the univariate and multivariate analyses were performed with 9999 permutations and the significance level was set at $p < 0.05$ with PRIMER 7 & PERMANOVA (Clarke and Gorley, 2015).

4.3 Results

4.3.1 Variability of microplastic content in deposited matter

The amount of MP in DM showed marked variability both among replicates (Table S1 Supplementary Information) and sampling times. The maximum amount (one order of magnitude higher than the other times) of deposited MP in presence of mussels was registered in January (mean± SE= 1139 ± 884 MP unit^{-1}) followed in decreasing order by June (196 ± 79 MP unit^{-1}), October (173 ± 126 MP unit^{-1}) and March (47 ± 37 MP unit^{-1}). Conversely, in the absence of mussels the amounts of MP in DM was less variable among times, with June as the most MP contaminated time (138 ± 69 MP unit^{-1}) followed by January and March with very similar MP amounts (113 ± 52 and 104 ± 36 MP unit^{-1}) and October (39 ± 25 MP unit^{-1}). Similar trends were found by considering fragments and fibres separately (Figure S1 supplementary Information). The amount of MP was considerably higher in DM in presence of mussels (Figure 3) during October and January, but these differences were probably masked by the high variability in MP contents between replicates (p-value > 0.05, Table S2 Supplementary Information).

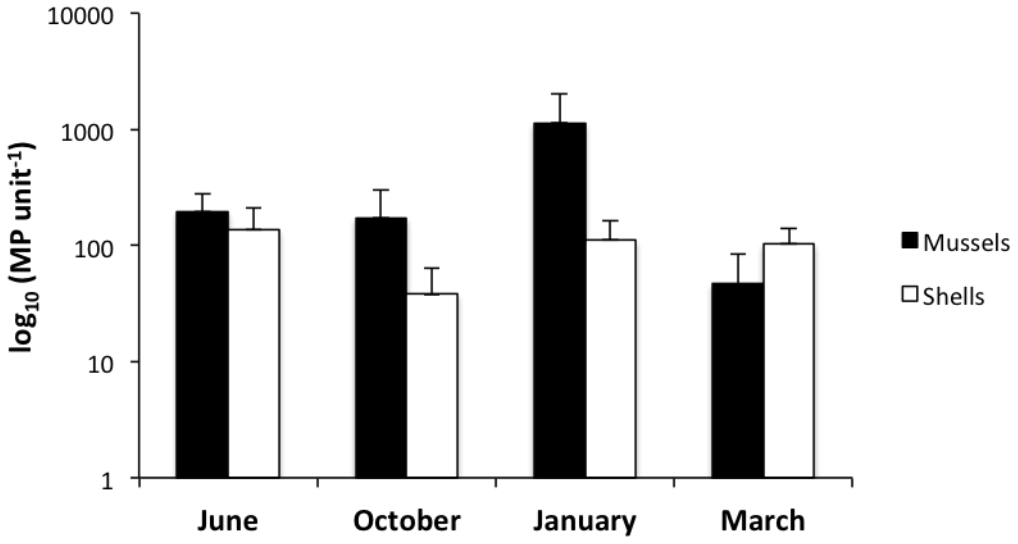

Figure 3: Temporal variation of MP (mean ± 1 SE, n=4 or n=3 for March samples) in DM in the presence of mussels or control shells. Data are represented in $\log_{10}$ scale to allow direct comparison among months.

In presence of mussels 90 to 98% MP was < 50 microns, with almost no MP larger than 100 microns, while in the absence of mussels 10 to 20% of MP was > 50 microns wih 5 to 10% > 100 microns (Figure 4a, b, c, d).

Most MP fragments (Figure 4 a, b) in DM were < 25 µm (76%). particularly the size class between 11-25 µm significantly contributed to differentiate the MP in presence of mussels from those in presence of empty shells (p-value= 0.008 Table S3 in Supplementary Information and Figure 5). Fibres, resulted mainly between 25 µm and 100 µm both in presence or absence of mussels (Figure 4c and Table S4 in Supplementary Information,) but a clear higher abundance of small fibres (<50 µm) was found in October. Overall no statistically significant temporal variation was found both for fragments and fibres (Tables S3, S4 Supplementary Information).

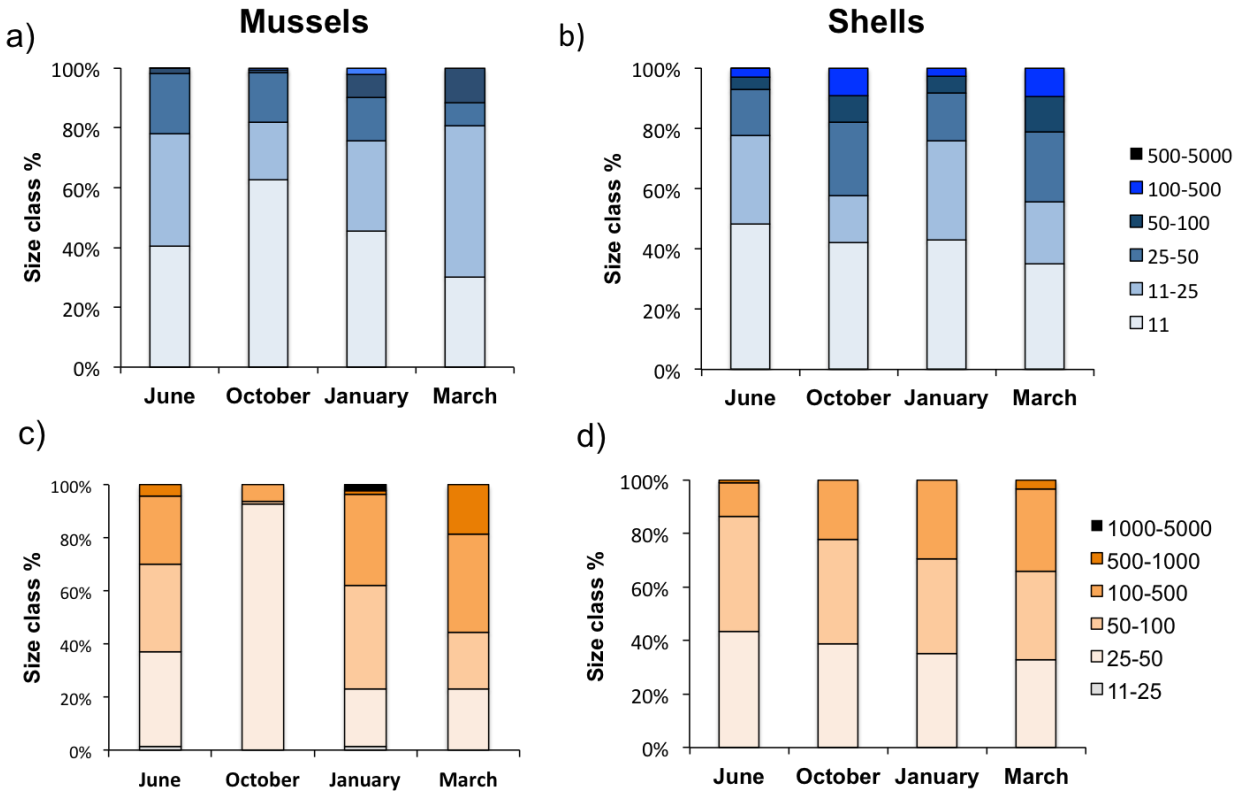

Figure 4: Temporal distribution of the relative MP fragments (**a, b**) and fibres (**c, d**) size class (µm) composition in detritus in presence of mussels (**a, c**) and control shells **(b, d)** .

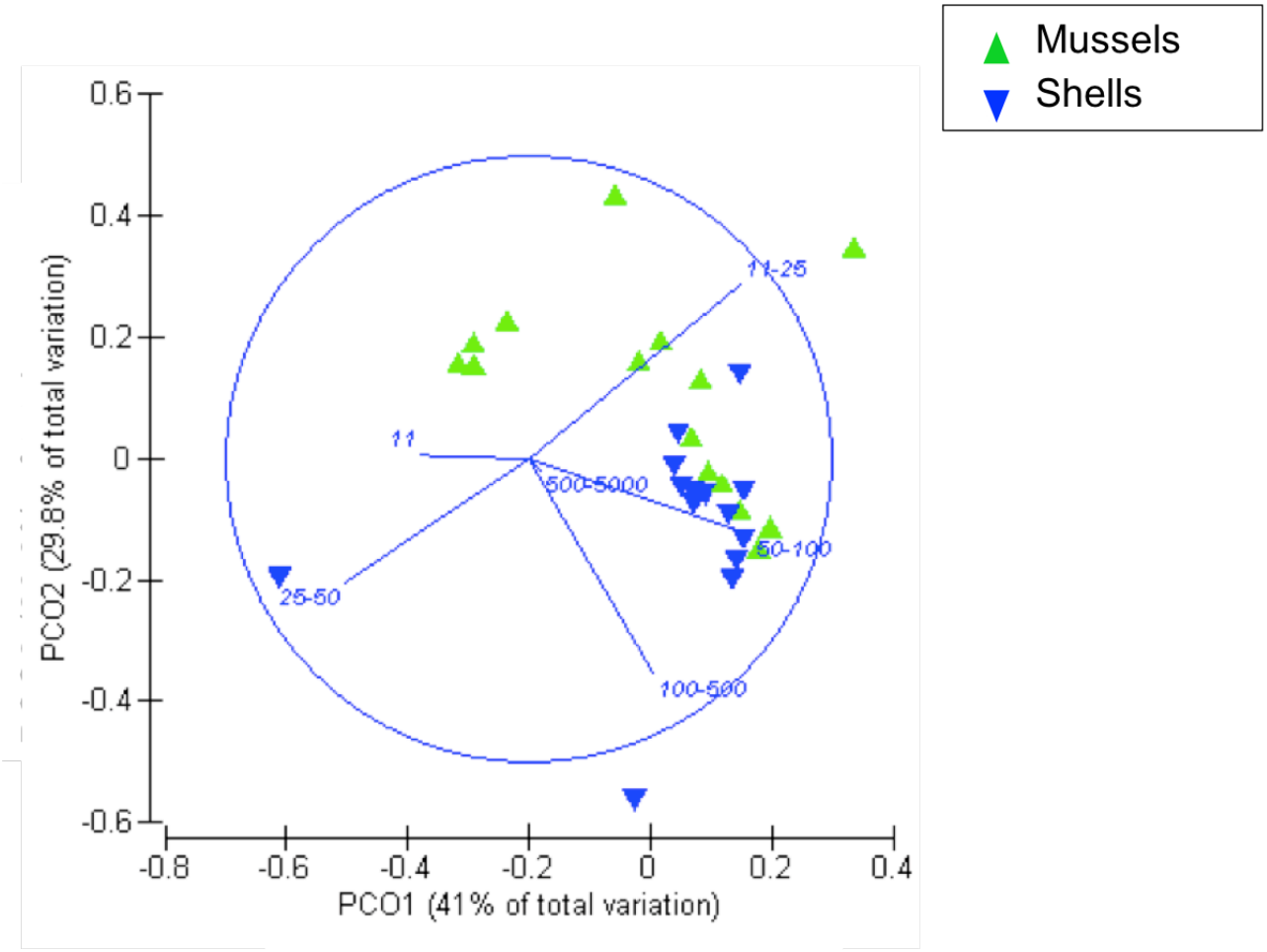

Figure 5: Principal Coordinate analysis ordination plot based on the relative size class composition of MP fragments in DM in presence of mussels (green) or control shells (blue). Similarities between samples were calculated based on Hellinger distance. The PCO1 and PCO2 axes report the percentage of the total variation explained by each axis.

Seventeen and 11 polymers in total (see Table S11 in Supplementary Information for a complete list of the identified polymers) were identified for fragments and fibres respectively. The most represented polymers were: polypropylene (PP), polyethylene (PE), polyamide (PA), polyester (PES), acrylates/polyurethane (PUR)/varnish, and rubber type 3 (Figure 7a, b, c, d). Nitrile rubber was present only in the fragments fraction, while ethylene vinyl acetate (EVA) was present only in the fibres fraction counting the 10% in presence of mussels in October (Figure. 7c).

Overall, PP was the prevailing polymer and its significantly higher abundance (PERMANOVA p-value=0.02) in presence of mussels (55.8%, n=417.6) than in presence of control shells (32.9%, n=63) was responsible for the differentiation in Figure 6 between fibres in DM in presence of

mussels and control shells (Table S6 in Supplementary Information). No different polymeric composition was found for fragments (Table S5 in Supplementary Information).

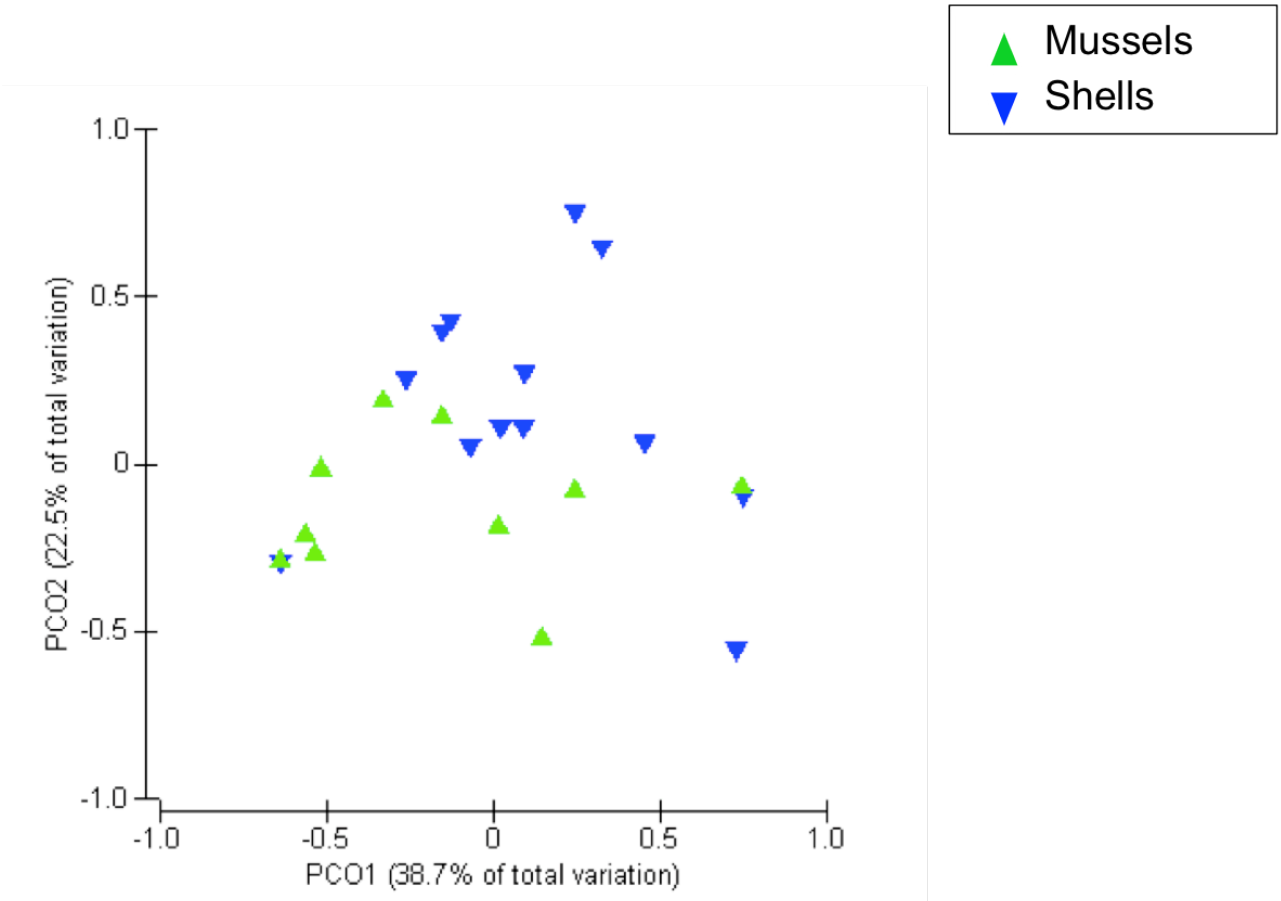

Figure 6: Ordination plot from Principal Coordinate analysis based on the relative polymer composition of MP fibres in detritus in the presence of mussels (green) or control shells (blue). Similarities between samples were calculated based on Hellinger distance. The PCO1 and PCO2 axes report the percentage of the total variation explained by each axis.

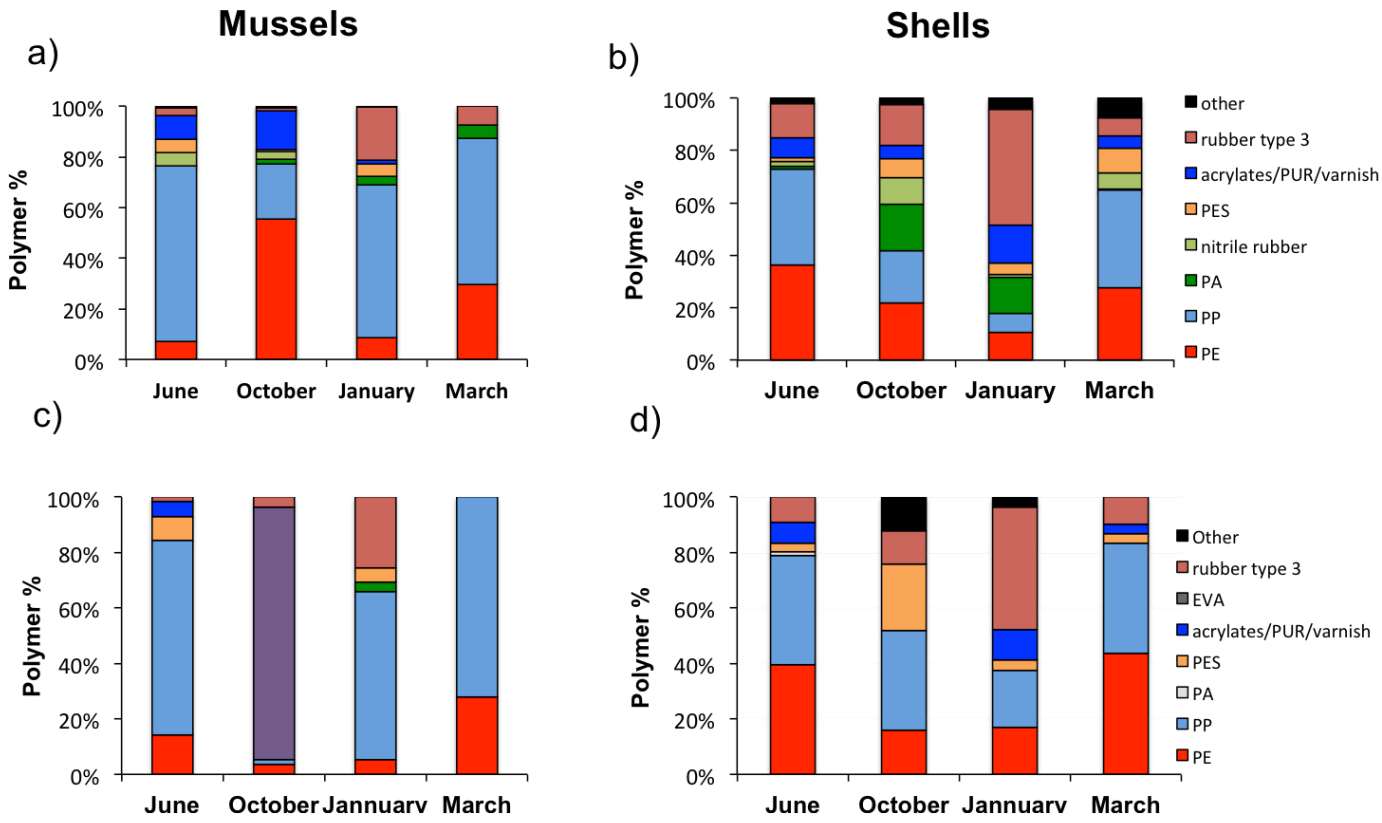

Figure 7: Temporal distribution of the relative MP fragments (**a, b)** and fibres (**c, d)** polymer composition in detritus in presence of mussels (**a, c)** and control shells **(b, d)**.

The relative polymer distribution did not result statistically significant different (Tables S5, S6 in Supplementary Information) across times (Figure 7) although PP and PE in presence of mussels showed a decrease in October with an increase of PE and EVA for fragments and fibres respectively (Figure 7 a, c) .

4.3.2 Microplastics in mussels' soft tissue

The relative abundances of MP fragments and fibres in the soft tissue of mussels was consistent with that found in detritus in presence of mussels and a high variability in MP contents among traps was found. (Table S1 in Supplementary Information). The total amount of fragments (92%, n=2376) was considerable higher than fibres (8%, n=195). The 94% (n=2224) of fragments and 83% (n=161) of fibres was < 25 µm and between 25 and 50 µm respectively (Figure 8 a,b). No

significant temporal variations were observed in the MP size composition of both fragments and fibres (Table S7, S8 in Supplementary Information), although a general increase in MP dimensions was observed from June 2018 to March 2019 (Figure 8a,,b).

The polymeric composition of MP fragments and fibres showed a significant temporal variation (Table S9, S10 in Supplementary Information). Indeed, PP significantly increased its contribution from June to March and conversely PA which, decreased from June to March when it completely disappeared (Figure 8c,d).

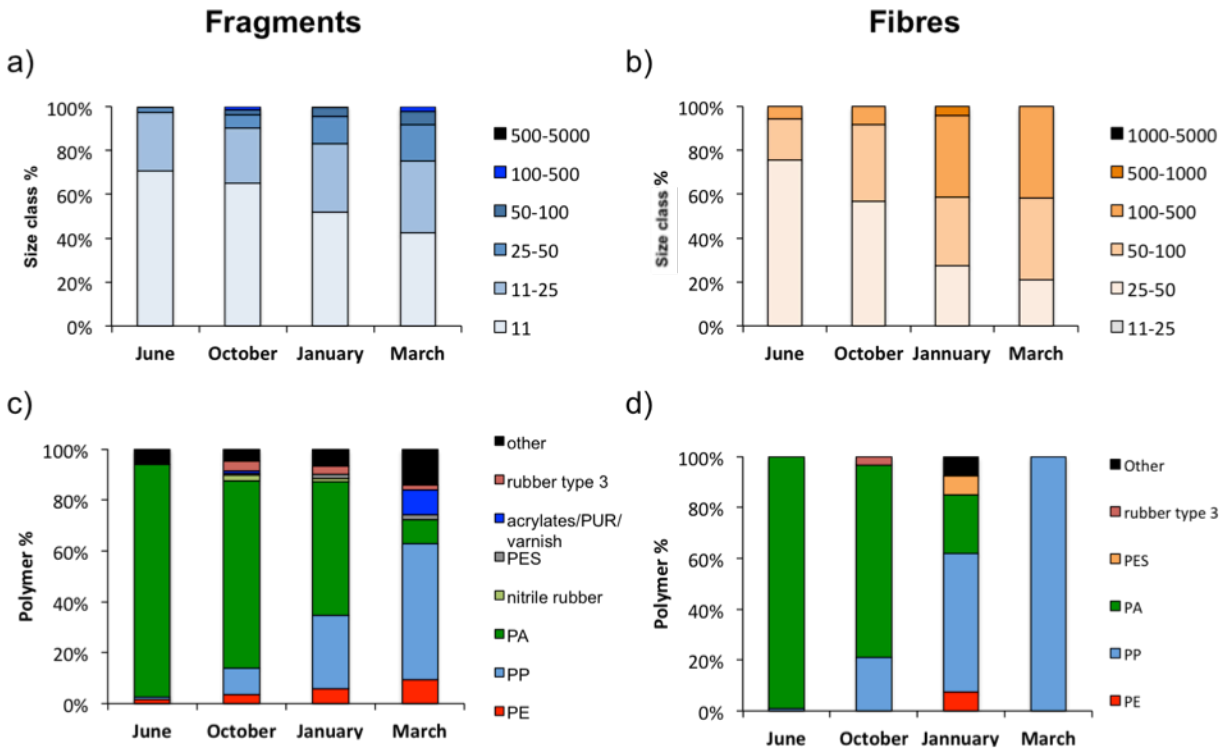

Figure 8: Temporal distribution of MP fragments (**a, c)** and fibres (**b, d)** size class (**a, b)** and polymer composition (**c, d)** in mussels soft tissue.

4.4 Discussion

This study provides one of the first empirical assessments of the role played by filter-feeders in affecting the temporal dynamics and fate of MP in natural environments. We integrated field measurements and effective sample analyses which allowed to quantify and characterise MP fragments and fibres from 11 to 5000 μm in size in both the DM and in mussels soft tissues from replicated collectors deployed at different times of the year. We found that in the wild mussels exposed to "natural" MP pool successfully sequestrated MP of small dimensions (11-25 μm) and low-density polymers (mainly PP and PE) and accumulated in their biodeposits, which is consistent to previous outcomes from laboratory experiments (Piarulli & Airoldi, in preparation, Chapter III of this thesis). .

Variable MP quantities were found in the DM from replicates field traps in the presence of both mussels and control shells, indicating a small-scale spatial heterogeneity in MP fluxes and accumulation. This high variability is very common in natural environments (Underwood et al., 2017), but to our knowledge, it has not been described in previous MP research, and no work has focused on the drivers behind it. This could be in part attributed for a lack of replication in MP research due to the high costs and time consuming analytical procedures (Underwood et al., 2017) that should be promptly solved with the development of cost-effective methods for monitoring MP in environmental samples.

The high small-scale heterogeneity partially masked some of the differences in abundance of MP deposited in presence of mussels and control shells at different times. Some trends, however, were observed and described. Larger temporal fluctuations were identified in MP abundances in DM in presence of mussels than in control shells, particularly in October and January when the presence of mussels allowed a depositiom of MP 4 times higher than in control traps indicating that active uptake followed by egestion by mussels clearly enhance the deposition of MP that tend to be accumulated into biodeposits. The peak of MP abundance in presence of mussels was recorded in

Jannuary, this could be related to i) higher local environmental availability of MP in that period or 2) to an increased filtering action by mussels. The constant level of MP in controls allows rejecting the first hypothesis, suggesting that this peak is more likely to be explained by an increase of mussel ingestion-egestion activity. In fact, during the winter period mussels tend to enhance the filtration rates and egestion of particles not constituting an energy source (potentially including also MP) due to an increase of suspended particles in the water column accompanied by a decrease of phytoplankton availability (Widdows et al., 1979).

Fragments were the dominant MP shape both in the presence or absence of mussels at all sampling times, and significantly differed in their size composition in the presence or absence of alive mussels. The MP fragment composition deposited in the presence of mussels was mainly represented by particles between 11 and 25 μm, while MP fragments sank in control treatments with shells were more variable, with a greater contribution of MP between 50 and 100 μm that were less than 1% in the presence of mussels. This finding is in line with the known size selection range for the congeneric species *M. edulis*, that tends to ingest particles < 50 μm (Defossez and Hawkins, 1997). This is also copnsistent with previous laboratory observations of mussel-mediated MP transfer of MP < 50 μm from the water column to the bottom (Piarulli & Airoldi, in preparation, Chapter III of this thesis),

Mussels can realistically act as vectors for small MP in the marine environment enhancing their sink and accumulation on the seafloor, therefore potentially affecting the spatial distribution of MP in natural conditions (Piarulli et al., in preparation, Chapter III of this thesis). High presence of bivalves, e.g. in aquaculture plants, may result in the creation of hot-spots of small and concentrated MP incorporated into faecal matter. While this raises attention about the potential effects of detritus feeding organisms in these areas, it also offers opportunities for remediation by removing MP from the water column and accumulating it into sediments that can me more easily treasted.

Interestingly, while many studies identified fibres as the dominant MP shape in organisms,

particularly in invertebrates species (Claessens et al., 2011; Lourenço et al., 2017; Piarulli et al., accepted manuscript; Taylor et al., 2016), in this study we found that fibers (ranging in size between 25 and 500 μm) constituted only 12.1 % and 21.7% in detritus in presence of mussels or control shells, respectively, and even less in mussels' soft tissues comprising only 7.6% of the total MP. This observation suggests that when the majority of fibres is found it is more likely due to their propensity to become entangled with each other and with biological features that lead to longer retention times caused by a slower transit through the organisms digestive system rather than higher availability of fibres than fragments in the environment (Piarulli et al., 2019; Watts et al., 2015). The lower environmental availability of fibres than fragments was also showed by Vianello et al. (2013) who found that only the 10% of MP found in sediments of the Venice Lagoon (North Adriatic area) was constituted by fibres. This further proves that MP usually found in organisms may not adequately represent the real MP exposure of natural specimens to MP contamination neither in terms of quantities nor typologies.

The polymeric composition of fragments resulted similar in the presence or absence of mussels while it was significantly different for fibres, that were characterised by high quantities of PP in the presence of mussels. It is likely that the incorporation of PP fibres in biodeposits by mussels enhanced the sinking rates of the very buoyant PP fibres that have the tendency to sink slower as free particles, similary to Porter et al. (2018) who reported that incorporation of PP fibres into marine snow increase their sinking rates about 658 m day^{-1}.

The high contribution of low density polymers (e.g PP and PE), was consistent with other studies (e.g. Lorenz et al., 2019; Zhao et al., 2018) analysing similar compartments and depths. The fact that low density polymers are more abundant in detritus in the presence of mussels adds evidence that the MP-transfer operated by mussels through the production of MP-enriched biodeposits significantly enhances the accumulation of low density polymers in the sediments, thus altering their fate into the water column with further implication at the benthic community level. Low

density polymers such as PP and PE are less chemically stable with lower capacity to withstand stress than for example polystyrene (PS) or PVC (Odien, 2004). Therefore, ingestion-egestion process could also fragment these MP up to the nanoscale (Dawson et al., 2018) making these synthetic particles not only prone to be consumed by benthic species (Zhao et al., 2018) but also to be more easily accumulated in tissue and cells (Bergmann et al., 2015) and transferred to upper trophic levels via direct predator-prey interactions potentially also causing adverse physiological effects on cosumers (Pittura et al., 2018).

The polymeric composition in DM did not show any drastic variation over time both in the presence of mussels or control shells. This could be due to the fact that our experimental environment is a semi enclosed area not strongly influenced by oceanographic variations such as currents or wind that can alter the levels of MP contamination over time. Conversely, stable polymeric composition can be related to local anthropogenic activities (Airoldi et al., 2016) acting all over the year.

By analysing MP in mussels soft tissue we observed that MP in biodeposits corresponded to those egested by mussels. Indeed, sizes and shapes of synthetic particles in mussels were representative of those found into the biodeposits with a dominance of MP fragments between 11 and 25 μm. Te polymeric composition in mussels soft tissue was however larger that in the biodeposits. PA was almost absent in biodeposits but constituted 81.1% and 77% of total MP in mussels soft tissue and showed a clear decrease from June to March. This qualitative temporal variation not oserved in the biodeposits may reflect the different nature of the two MP measurements. Indeed, the quantification of MP in organisms is a static measure, which takes into account only a “snap-shot” of the MP ingested. Conversely, the MP in biodeposits is a dynamic measure of MP ingested and egested over time. It is, therefore, essential for future experiments and monitoring studies to consider such different outcomes in function of the chosen method.

4.5 Conclusion

Overall, this study represents the first field empirical evidence of the central role played by filter-feeder bivalves in affecting the dynamic and fate of MP in the marine environments over time. The differences in MP sizes and polymers, identified in the presence of mussels and in control shells indicated that selective accumulation in biodeposits of certain types of plastic particles occurs in natural conditions. It was demonstrated that bivalve-mediated MP transfer represents an environmentally relevant pathway for the transport of small and low dense MP (< 25 μm) from the water column to the benthic compartment and potentially influencing the MP spatial distribution in sediments at local level. The MP-transfer operated by mussels could be particularly relevant in winter months when the amount of no-food suspended particles is higher resulting in enhanced ingestion-egestion rates and thus, faeces and pseudofaeces production. We also reported a notable small-scale heterogeneity in MP accumulation among nearby biodeposit traps. This small-scale variability is hardly considered in ecological studies of MP, and further work is recommended to explore its consequences at the ecosystem levels as well as its biological and environmental drivers.

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Supplementary Information

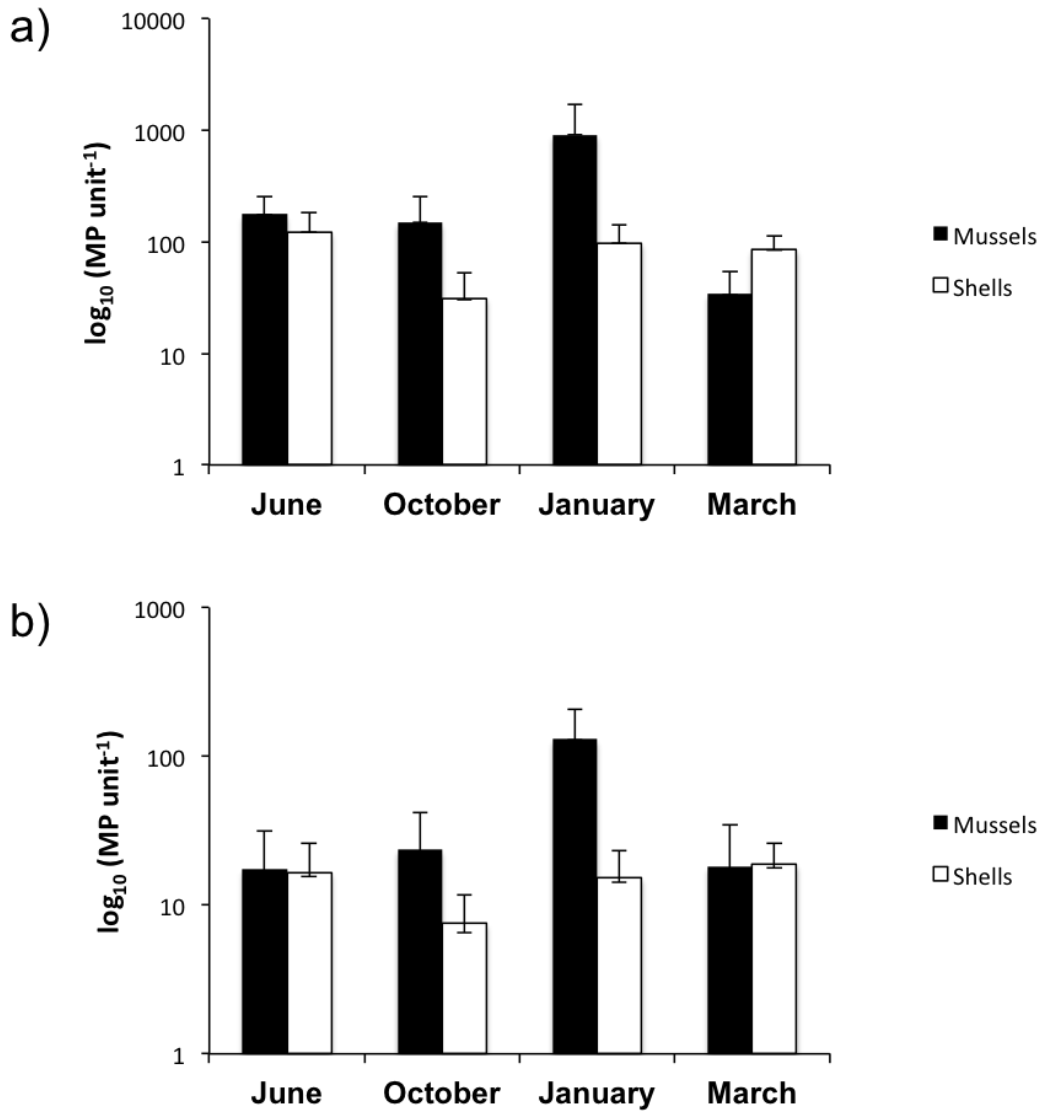

Figure S1: Temporal variation of MP **a)** fragments and **b)** fibres MP (mean ± 1 SE, n=4 for June, October and January or n=3 for March samples) in detritus in presence of mussels (black bars) or control shells (white bars). Data are represented in $\log_{10}$ scale to allow direct comparison among months.

Table S1: Total volume of samples after purification, analysed subsample volume and estimated total MP, only fragments and fibres per sample (MP unit^{-1}). B=detritus in presence of mussels; C=detritus in presence of control shells; M=mussels soft tissue.

Samples	Sample volume (ml)	Portion used for FTIR (%)	No. MP per total volume	No. fragments per total volume	No. fibres per total volume
Bio_B1_June	54.00	100	249.63	190.63	59.00
Bio_B2_June	95.00	100	90.56	79.56	11.00
Bio_B3_June	150.00	2	394.74	394.74	0.00
Bio_B4_June	55.00	100	50.96	50.96	0.00
Bio_C1_June	112.00	100	71.63	63.63	8.00
Bio_C2_June	112.00	100	67.48	60.48	7.00
Bio_C3_June	50.00	100	64.96	58.96	6.00
Bio_C4_June	80.00	100	347.92	302.92	45.00
Bio_B1_October	90.04	17.8	38.19	38.19	0.00
Bio_B2_October	134.00	66.4	67.06	59.53	7.53
Bio_B3_October	79.00	1.3	551.90	472.90	79.00
Bio_B4_October	37.00	91.9	38.08	30.46	7.62
Bio_C1_October	17.00	82.4	11.16	8.73	2.43
Bio_C2_October	13.00	76.9	2.60	2.60	0.00
Bio_C3_October	58.00	55.2	113.80	95.68	18.13
Bio_C4_October	59.20	10.5	27.55	18.00	9.55
Bio_B1_January	54.00	29.6	3779.95	3422.73	357.23
Bio_B2_January	32.18	16.1	363.07	273.78	89.29
Bio_B3_January	43.47	19.5	392.24	325.52	66.72
Bio_B4_January	33.00	90.9	21.40	15.90	5.50
Bio_C1_January	35.00	57.1	173.22	155.72	17.50
Bio_C2_January	23.00	87	29.82	24.07	5.75
Bio_C3_January	13.00	76.9	21.29	19.99	1.30
Bio_C4_January	25.00	88	227.15	190.79	36.36

Bio_B1_March	30.00	46.7	10.86	8.71	2.14
Bio_B2_March	87.00	29.9	21.77	18.42	3.35
Bio_B3_March	49.33	14.9	156.52	89.22	67.30
Bio_C1_March	24.50	87.8	168.12	138.49	29.63
Bio_C2_March	43.00	20.9	44.78	40.00	4.78
Bio_C3_March	29.00	13.8	98.50	76.75	21.75
Bio_M1_June	30.00	90	50.00	44.44	5.56
Bio_M2_June	48.00	28.1	203.12	192.43	10.69
Bio_M3_June	19.00	18.3	922.70	862.64	60.06
Bio_M4_June	40.00	12.5	552.00	528.00	24.00
Bio_M1_October	38.00	65.8	85.12	71.44	13.68
Bio_M2_October	36.50	91.8	162.34	151.45	10.90
Bio_M3_October	43.00	93	63.43	55.90	7.53
Bio_M4_October	40.00	25	56.00	56.00	0.00
Bio_M1_January	33.00	48.5	130.85	116.41	14.44
Bio_M2_January	35.00	57.1	172.10	147.60	24.50
Bio_M3_January	54.00	18.5	101.45	90.65	10.80
Bio_M4_January	30.00	90	6.63	4.41	2.22
Bio_M1_March	26.50	88.7	20.19	17.93	2.26
Bio_M2_March	27.00	13	1.63	1.63	0.00
Bio_M3_March	23.50	87.2	28.64	21.76	6.88
Bio_M4_March	20.00	55	15.59	13.77	1.82

Table S2: PERMANOVA test of differences in MP quantities in detritus in presence or absence of alive mussels (Mussel: 2 levels, fixed), among times (Time: 4 levels, random), their interaction (Mussel x Time) (square root-transformed abundances data, Euclidean distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Mussel	1	190.95	190.95	1.2728	0.3588	9914
Time	3	532.45	177.48	1.6796	0.1585	9958
Mussel x Time	3	450.49	150.16	1.4211	0.2333	9957
Res	23	2430.4	105.67			
Total	30	3655.7				

Table S3: PERMANOVA test of differences in MP fragments (size) species structure in detritus in presence or absence of alive mussels (Mussel: 2 levels, fixed), among times (Time: 4 levels, random), their interaction (Mussel x Time) (square root-transformed relative (size) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Mussel	1	0.38803	0.38803	3.8293	0.0083	9952
Time	3	0.52721	0.17574	1.7342	0.0794	9940
Mussel x Time	3	0.48495	0.16165	1.5952	0.1118	9927
Res	22	2.2293	0.10133			
Total	29	3.5702				

Table S4: PERMANOVA test of differences in MP fibres (size) species structure in detritus in presence or absence of alive mussels (Mussel: 2 levels, fixed), among times (Time: 4 levels, random), their interaction (Mussel x Time) (square root-transformed relative (size) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Mussel	1	0.06722	0.06722	0.91984	0.4776	9965
Time	3	1.5764	0.52548	1.928	0.0546	9921
MusselxTime	3	0.21082	0.07027	0.25783	0.9938	9917
Res	18	4.9061	0.27256			
Total	25	6.8101				

Table S5: PERMANOVA test of differences in MP fragments (polymer) species structure in detritus in presence or absence of alive mussels (Mussel: 2 levels, fixed), among times (Time: 4 levels, random), their interaction (Mussel x Time) (square root-transformed relative (polymer) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Mussel	1	0.17084	0.17084	0.91984	0.55684	9937
Time	3	1.2531	0.4177	1.928	1.3565	9912
MusselxTime	3	0.92038	0.30679	0.25783	0.99633	9900
Res	22	6.7744	0.30793			
Total	29	9.1336				

Table S6: PERMANOVA test of differences in MP Fibres (polymer) species structure in detritus in presence or absence of alive mussels (Mussel: 2 levels, fixed), among times (Time: 4 levels, random), their interaction (Mussel x Time) (square root-transformed relative (polymer) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Mussel	1	1.2105	1.2105	3.1545	0.0353	9961
Time	3	1.9935	0.66451	1.3373	1.2005	9928
MusselxTime	3	1.1434	0.38114	0.76706	0.7081	9922
Res	17	8.4472	0.49689			
Total	24	12.873				

Table S7: PERMANOVA test of differences in MP fragments (size) species structure in mussels' soft tissue among times (Time: 4 levels, random), (square root-transformed relative (size) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Time	3	0.40778	0.13593	1.0751	0.4024	9894
Res	12	1.5172	0.12644			
Total	15	1.925				

Table S8: PERMANOVA test of differences in MP fibres (size) species structure in mussels' soft tissue among times (Time: 4 levels, random), (square root-transformed relative (size) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Time	3	0.7609	0.58697	2.0771	0.0806	9761
Res	10	2.8259	0.28259			
Total	13	4.5868				

Table S9: PERMANOVA test of differences in MP fragments (polymer) species structure in mussels' soft tissue among times (Time: 4 levels, random), (square root-transformed relative (polymer) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Time	3	2.0221	0.67404	3.5433	0.0001	9860
Res	10	1.9023	0.19023			
Total	13	3.9244				

Table S10: PERMANOVA test of differences in MP fibres (polymer) species structure in mussels' soft tissue among times (Time: 4 levels, random), (square root-transformed relative (polymer) species abundances data, Hellinger distance coefficient).

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Time	3	2.921	0.9807	4.2613	0.0001	9614
Res	10	2.3014	0.23014			
Total	13	5.2435				

Table S11: List of polymers and respective symbols found in DM and mussels soft tissue.

Polymer	Symbol
polyethylene	PE
polyethylene oxidized	PE-oxydised
polyethylene-chlorinated	PE-chlorinated
polyproplyene	PP
polystryene	PS
polyamide	PA
polyvinylchloride	PVC
cellulose chemical modified	none
nitrile rubber	none
polyester	PES
acrylates/polyurethanes/varnish/	PUR
polyetheretherketon	none
polychloroprene	none
ethylene-vinyl-acetate	EVA
polyoxymethylene	none
acrylonitrile-butadiene	none
rubber type 1	none
rubber type 2	none
rubber type 3	none

Main findings, conclusions and future perspectives

My thesis addressed pressing and open questions related to the dynamics and fate of MP in natural marine systems, which is a crucial step to predict their ecological effects as well as to identify management actions to minimise their negative impacts on the marine environment. The primary objectives included: **1)** reliably assessing the occurrence of MP in benthic invertebrates in the wild; **2)** identifying possible relationships between MP occurrence in benthic organisms and species biological/ecological traits; **3)** identifying how ingestion/egestion of MP by benthic filter feeders, such as mussels, affects their transfer through marine compartments and food webs; and **4)** to describe the temporal variability of these effects in natural conditions.

The **chapter I** revised the literature on digestion techniques, and qualitatively and quantitatively compared two different methods (alkaline with KOH and an enzymatic-based approach) to digest soft-tissues of benthic marine invertebrates. I found that KOH-based digestion represents the most cost-effective alternative to separate MP from invertebrates' soft tissues. Both enzymatic and KOH tested protocols resulted applicable for the digestion of organismal soft tissue, but the digestion with KOH was faster and cheaper, involving less procedural steps and therefore, owning reduced risk of external airborne plastic contamination. Conversely to the general hypothesis that stronger digestion agents could lead to physical-chemical alteration of synthetic polymers preventing their further recognition in biological samples (Claessens et al., 2013; Cole et al., 2014; Dehaut et al., 2016), KOH affected neither the microscopic nor spectroscopic characterisation of MP extracted from wild organisms or of laboratory-prepared synthetic microfibers. These results offer robust standard operation protocols for future routine monitoring studies on the ecological and environmental risk related to MP contamination of marine invertebrates.

The **chapter II** focused on quantifying and characterising MP occurrence in invertebrate species, from salt marshes along the North Adriatic lagoons (Italy) and the Schelde estuary (Netherlands). We specifically included species with different feeding modes and life habits, to explore whether

their levels of MP contamination could be related to these traits. We generally found a low presence of MP in invertebrates from both regions, and the few MP found were mostly PES microfibers. We interpreted this results as a consequence of the rapid egestion of most MP (between 20 μm and 5 mm) with the exception of fibres that due to their elongate shape could be retained longer in the gastrointestinal tract (Watts et al., 2015). Although the number of MP found was too small to draw robust conclusions about the effects of feeding modes, we found that omnivores generally had higher amounts of MP compared to species with suspension or detritus habits and key MP characteristics particularly shape have the potential to influence MP uptake and retention in organisms.

The laboratory experiments at the core of **chapter III** confirmed that most MP are fast egested via faeces or pseudofaeces, which may limit their transfer via predator-prey interactions while enhancing alternative trophic pathways via uptake by detritus feeders. The incorporation of MP into organic-rich biodeposits accelerated the rate of transfer of MP from the water column to the sediments and more than doubled their subsequent uptake by the detritus feeding polychaete *Hediste diversicolor*. The experiments also identified negative effects of MP on the vertical flux of faecal material, which significantly decelerated when faecal pellets contained MP compared to uncontaminated conditions. Overall these bivalve-mediated processes could have relevant effects on MP distribution and dynamics in marine systems, with so far largely unexplored effects at the ecosystem level.

Chapter IV focused on exploring for the first time temporal scales of natural variation in bivalve-mediated fluxes of MP by using an original trap for mussels' biodeposits. These measurements confirmed in nature what already described experimentally in the laboratory in Chapter III, which is that large amounts of MP are incorporated in mussel biodeposits and conveyed from the water column to the seafloor. The use of the advanced automated Imaging-FTIR technique allowed a very precise quantification and characterisation of synthetic particles, which showed that the transfer

operated by mussels is particularly effective for very small MP (< 25 μm). This small MP fraction would normally float in the water column but due to selective mussel ingestion they become concentrated into organic rich biodeposits. Most importantly this field study reported for the first time a notable small-scale heterogeneity in MP fluxes and accumulation among nearby biodeposit traps. This small-scale variability is hardly considered in ecological studies of MP, and further work is recommended to explore its consequences at the ecosystem levels as well as its underlying drivers. We also found that the high uptake rates of MP measured with the traps and their variations over space and time are not captured by traditional techniques that quantify MP contamination via MP contents in organisms soft tissues.

Overall this thesis provided evidence that MP within the size range detectable in field studies (≥ 10-20 μm), are ingested and rapidly egested by many invertebrate species. This mechanism not only limits the potential for direct transfer of MP via predation, but tends to concentrate MP in biodeposits easily available for uptake by detritus-feeding benthos. This outcome has also important methodological implications, as traditional monitoring approaches focus on quantifying MP found at any time in the organisms gastrointestinal tracts or soft tissues. These "snap-shot" measurements may not fully represent the real vulnerability of wild specimens to MP contamination. Indeed while MP do not accumulate in the organisms' bodies and are rapidly egested, they still can desorbe hazardous chemicals (additives and/or POPs) during the passage, which can subsequently biomagnify through the food web (Batel et al., 2018; Pittura et al., 2018). To date, the direct evaluation of MP uptake mechanisms is operationally challenging and requires the development of new cost-effective technologies and approaches (Lanctôt et al., 2018) to measure the integrated impact over time of MP in natural systems. Traditional MP assessment approaches also do not capture the reported notable fine-scale spatial heterogeneity of MP distribution.. The so far largely overlooked implications of this small-scale patchiness in MP distribution could be relevant, and

require urgent research.

From another perspective, the fact that certain groups of organisms such as bivalves, which filter large quantities of water per unit of time (Ward and Shumway, 2004), and are able to sequester MP from the water column suggests that such organisms could potentially be used to design specific remediation plans and local actions to reduce the MP contamination in coastal areas. There is growing recognition and awareness that nature can help provide cost-effective, flexible solutions to specific societal challenges, using the services offered by natural ecosystems in a smart, 'engineered' way (Cohen et al., 2016). Nature-based solutions to microplastics contaminations have not been considered so far, and should be explored in future work.

The results of this thesis provide an effective contribution and a step forward to comprehensively understand the impact of MP contamination in natural environments. The relative importance of MP as an environmental stressor is difficult to estimate in comparison to other factors such as eutrophication, hypoxia, global warming. One issue is that currently available methods to identify and quantify MP are limited by the size of the particles and do not allow the quantification of particles < 10-20 μm. Therefore the abundance and predicted ecological effects of MP are systematically underestimated. Additionally, there is a complete lack of studies that consider MP in a multiple stressors scenario. It is now imperative to determine the contribution of MP contamination in the context of global changes and to develop innovative solutions to MP management based on solid ecological knowledge.

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