UNIVERSITÀ
POLITECNICA
DELLE MARCHE

PhD Course in Life and Environmental Sciences

Curriculum Civil And Environmental Protection

ECOTOXICOLOGICAL APPROACH AS A TOOL TO SUGGEST STRATEGIES FOR RISK MANAGEMENT OF MICROPLASTICS IN THE MARINE ENVIRONMENT

Academic Tutor:
Prof. Francesco Regoli

Company Tutor:
Dott. Marco Dionisio

PhD Candidate:
Dr. Lucia Pittura

XXXI Cycle

2015-2018

TABLE OF CONTENT

1 INTRODUCTION

1.1 The plastic as a global environmental issue

Since the large scale production of plastic began in the 1950s, plastics have become a part of our everyday life with very many benefits for society. Despite many technological advances, however, growing plastic production, single use plastic utilization and low-recycling rates are creating an increasingly waste stream that is outstripping our capacity for waste management. If current production and waste management trends continue, roughly 12,000 million metric tons (Mt) of plastic debris will be in the natural environment by 2050 (Geyer et al., 2017), anyway, different impacts of plastic pollution are already visible on ecosystems and organisms.

Plastics are a group of synthetic organic polymers mainly synthesized from fossil fuels such as natural gas, oil or coal, although biomass sources can also be used as a feedstock to produce bioplastics, which, however, are not necessarily biodegradable (UNEP, 2016). Polymers may consist of repeating identical monomers units (homopolymers) or different sub-units in various possible sequences (copolymers). Among homopolymers, 6 main classes dominate the markets of plastic materials, accounting for 80% of globally production: polyethylene (PE, high and low density), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS, including expanded EPS), polyurethane (PUR) and polyethylene terephthalate (PET) (PlasticsEurope, 2018). Copolymerization, on the other hand, provides a convenient method of combining desirable properties of two or more materials in the same polymer (Cowie, 2013), for example ABS is a thermopolymer with versatile applications taking advantages of the thermal and chemical strength of acrylonitrile, the duttility of butadiene and the brilliance, easy processing and low cost of styrene (Piergiovanni and Limbo, 2010). Moreover, today, the market pressure is so high that producers of plastics need to provide better and more economic materials with superior combinations of properties as a replacement for the traditional polymers (Parameswaranpillai et al., 2015). So, exploiting over 70 years of research and progress in synthetic chemistry, existing polymers are mixing to create new plastics with different physical properties (i.e. blends and alloys) (Hope and Folkes, 1993; Ruzette and Leibler, 2005). Plastic polymers are rarely used in products alone (virgin resin pellet) and are normally mixed with additive chemicals during the manufacturing process to further enhance its performance (GESAMP, 2015). There are several thousand such additives in use, with different plastics requiring different formulations dependent on their use (Lithner et al., 2011). Organic fillers, such as silica, can impart strength, flame retardants such as polybrominated diphenyl ethers (PBDEs) can improve fire resistance, and plasticisers like phthalates can be used to impart flexibility. Colorants can also be used to enhance the appearance of the material. In addition, alkylphenol ethoxylates are used as antioxidants and organotins as stabilising agents (Lithner et al., 2011).

As a result, thousands of heterogeneous and novel plastic materials have been produced and continuously synthesized to meet the needs of a wide variety of market sectors or of specific applications (Piergiovanni and Limbo, 2010) in the field of packaging, building and construction, automotive, electrical and electronic, textile, agriculture, sports, health and safety and others (PlasticsEurope, 2018). Only in the European Union there are approximately 30,000 different polymer materials registered for use (Horton et al., 2017). Basing on the global analysis of all-mass produced plastics performed by Geyer et al., (2017), the total amount of products manufactured from 1950 through 2015 was 8300 Mt, that include polymer resins, synthetic fibers (polyester, polyamide and acrylic (PP&A)), and additives. Data on fibers and additives production are not readily available and have typically been omitted until the cited study. Fibers are, instead, important components to consider in order to understand sources and fate of plastic in the environment and additives are an environmental concern since they both extend the degradation times of plastic in the environment and may, in addition, leach out, introducing potentially hazardous chemicals with the related risk for wild-life and human health (Cole et al., 2011).

Plastics can enter the environment at all stages of their production-use-disposal cycle, although this is especially prevalent at disposal due to inadequate waste management and inappropriate discarding (UNEP, 2016). Once plastics have entered the environment their durability, which gives them such an advantage as materials, makes them persistent, pervasive and accumulating global pollutants that resist biodegradation (Andrady, 2015). As a result, apart from the proportion of plastic that has been incinerated, it can be argued that all of the plastic waste that has ever been produced is still somewhere in the environment today.
Plastic litter has been found in terrestrial (Rillig et al., 2017; de Souza Machado et al., 2018; Ng et al., 2018; Bläsing and Amelung, 2018), freshwater, such as lakes and rivers (Lambert and Wagner, 2018; Driedge et al., 2015; Horton et al., 2017; Blettler et al., 2018; Wu et al., 2018; Khan et al., 2018), estuarine, coastal and marine environments (Barnes et al., 2009; Law, 2017; Willis et al., 2017; Krelling et al., 2017; Gago et al., 2018) around the world, especially in areas of high anthropogenic influence such as urban, agricultural or fishing areas (Horton et al., 2017). However, plastics have been found even in remote sites of the world including deep-sea sediments (Van Cauwenberghe et al., 2013; Woodall et al., 2014), submarine canyons (Pham et al., 2014) and encapsulated in Arctic sea ice (Obbard et al., 2014), far from any potential land-based source. It has even been observed in some locations that plastic debris can fuse together, becoming associated with volcanic rocks, sediment and organic materials forming 'plastiglomerates', solid rock-like substances, that have the potential to become preserved in the fossil record (Horton et al., 2017). Plastic is so ubiquitous that it has been suggested as a stratigraphic indicator of Anthropocene (Corcoran et al., 2014; Zalasiewicz et al., 2016). In addition, it has to consider that many plastic materials that enter the environment will not remain stationary. Instead, they will be transported between environmental compartments (e.g. from land to rivers and the sea, and from rivers and sea to land during flooding, storm events or tidal surges), with varying residence times in each (Lambert and Wagner, 2018), that depend on many factors, including human behaviors, such as littering or recycling, plastics characteristics such as density, shape and size, weather and environmental topography and hydrology (Horton et al., 2017).

1.2 Plastic in the marine environment

The marine environment represents the major natural environment affected by plastic pollution: it is estimated that between 4.8 and 12.7 Mt of plastic debris enter the oceans annually (Jambeck et al., 2015).
The release of plastics into the marine environment occurs through a variety of pathways, that include deliberate or accidental direct inputs from land-based (e.g. mismanage of household, agriculture and industrial solid wastes, shoreline littering, coastal tourism) or sea-based sources (e.g. fisheries, aquaculture, commercial shipping and offshore industries, tourist cruises, recreational users) and indirect inputs from land via rivers, drainage, sewage systems, atmosphere (UNEP, 2016).
Once plastic materials have entered the ocean they can be distributed throughout the coastline, the surface waters, the water column, the seabed and biota: actually, they are identified in all of such compartments, representing a global ocean phenomenon (Barnes et al., 2009). The study on plastic distribution in marine environment is important to better understand its environmental fate (Goldstein et al., 2013) and to evaluate which organisms should be more affected by their exposure (Andrady, 2017).
Under the influence of oceanic circulation, floating items tend to accumulate in defined "hot spots" areas where they are retained over long time periods (Law et al., 2017) as observed in large-scale subtropical ocean gyres (i.e. North Atlantic, South Atlantic, North Pacific and South Pacific, South Indian) defined "garbage patches" by Kaiser et al., 2010). In these areas, convergent surface currents can accumulate debris in concentrations many orders of magnitude larger than in other regions of the world ocean, reaching densities as high as 890,000 pieces/km^2 (Eriksen et al., 2014). Moreover, in enclosed seas, such as the Mediterranean, surface water is retained for long periods because of limited exchange with the North Atlantic (Collignon et al., 2012). In this area, the average density of plastic (1 item per 4 m^2) as well as its frequency of occurrence,

are comparable to those found in accumulation zones described for the five subtropical ocean gyres (Kaiser, 2010), resulting in accumulation of floating plastic between 1,000 and 3,000 tons (Cózar et al., 2015).
As a result of long distance transport by surface currents, plastics can be also driven towards remote regions, such as the Poles or ocean islands having low population densities or even inhabited. A citizen-science study recently reported the presence of plastics on six beaches of the Svalbard Archipelago (Bergmann et al., 2017a). Analyses of ice cores collected across the Arctic Circle pointed to a considerable abundance of plastics into the sea ice, ranging from 38 to 234 particles/m^3, that are several orders of magnitude greater than those reported in waters of the North Pacific Subtropical Gyre (0.12 particles/m^3) (Obbard et al., 2014). In contrast, recent measurements of plastic debris in the Arctic surface waters produced by Cózar et al., (2017) have shown low to moderate concentrations ranged between 0 and 27,000 items/km^2, with none finding evidence of accumulation of the floating plastic on a large scale previously hypothesized (Cózar et al., 2014; Lusher et al., 2015; Bergmann et al., 2016). Conversely, plastic is confined in the Greenland and Barents seas with concentrations up to 320,000 items/km^2, driven by the Thermohaline Circulation, that actively advects warm surface water from low to high latitudes across the North Atlantic Ocean to the Arctic (Kuhlbrodt et al., 2012). Therefore, the Northeastern Atlantic sector of the Arctic Ocean can be characterized as the single, dominant high accumulation zone for floating plastic debris in this area, confirming the long-term predictions provided by ocean circulation models in 2013 (van Sebille et al., 2012). Floating litter items are also present in the Antarctic Ocean, although densities are low and concentrations have been only estimated as 2-3 orders of magnitude smaller than areas of other oceans (Barnes et al., 2009; Isobe et al., 2017).
An increase in beached debris was recorded on pristine and isolated islands that mirrors the long-term accumulation and the increased abundance of debris in our oceans (Ryan et al., 2009; Eriksen et al., 2014; Monteiro et al., 2018). In addition, most of these islands are closed to oceanic plastic accumulation zones, acting as important sinks for some of the waste accumulated in these areas (Lavers and Bond, 2017). For example, on the surface of the beaches of Henderson Island, located on the western boundary of the South Pacific Gyre, the density of debris was the highest reported anywhere in the world, up to 671.6 items/m^2 and approximately 68% of these was buried <10 cm in the sediment (Lavers and Bond, 2017). Similarly, the Hawaiian Islands lie on the southern edge of the North Pacific sub-tropical gyre and are particularly susceptible to receiving floating debris: beaches in the outer islands contained around 1.2 kg of plastic fragments/m^3 sediment (McDermid and McMullen, 2004). This is similar to patterns found on Easter Island, which adjoins the higher concentrations found in the sub-tropical gyres in the southern Pacific (Hidalgo-Ruz and Thiel, 2013). The result is a strong impact on the high biodiversity, for which remote islands have been recognized, requiring actions for their conservation (Monteiro et al., 2018).
Moreover, plastic litter can be transported out of surface waters, downward within water column, until the seafloor as ultimate destination (Goldstein et al., 2013; Eriksen et al., 2014; Galgani et al., 2015). Several factors potentially influence the vertical distribution of plastics, such as the wind-induced mixing (Kukulka et al., 2012), the incorporation into marine aggregates (Long et al., 2015), the biofouling (Fazey and Ryan, 2016), the incorporation into faecal matter (Cole et al., 2016), the relative density, size and shape of materials (Reisser et al., 2015). For example, for plastic denser than seawater, it was shown that the shape strongly defines the character of the settling, along with the near-bottom current velocity magnitude. On the other hand, the surface currents direction, windage and turbulence are the main processes conditioning transport of buoyant pieces (Bagaev et al., 2017). A model developed by Kukulka et al. (2012) predicted that the largest decrease in plastic concentration occurs over the first meters of the water column. This was confirmed by Reisser et al. (2015) during a study in the North Atlantic Gyre: actually, the authors observed that plastic numerical concentration (pieces/m^3) decays at a lower rate than plastic mass concentration (mg/m^3), as smaller plastics are more susceptible to vertical transport. At now, studies on the distribution of plastics throughout the ocean's water column are still scarce and the processes influencing it, as well as, the rate at which sinking occur are poorly understood (Kukulka et al. 2012; Goldstein et al., 2013; Eriksen et al., 2014;

van Sebille et al., 2015, Cózar et al., 2014; Bagaev et al., 2017, 2018; Kanhai et al., 2018). Instead, since most of the marine organisms inhabit sub-surface waters (Kanhai et al., 2018), it is necessary to improve researches on vertical mixing of buoyant plastics for predict interactions with neustonic and pelagic species of the world's oceans (Reisser et al., 2015).

Plastic debris accumulate also on the seabed which have the potential to highly impact benthic habitats and their biota (Galgani et al., 2015). With low light and low temperature in this environment there is little to enhance the degradation of plastic debris and so its fate is to be covered and buried in deep sea sediment slowly over time (Woodall et al., 2014). Deposition was documented in all seas and oceans with large amounts of plastics reported (Galgani et al., 2015), as an example, syntethic fibres was up to four orders of magnitude more abundant (per unit volume) in deep-sea sediments from the Atlantic Ocean, Mediterranean Sea and SW Indian Ocean than in contaminated sea surface waters (Woodall et al. 2014). Accumulation in the seafloor remains, anyway, uncommon in remote areas such as Antarctica and studies focus on continental shelves, because research into the deeper seafloor, which forms about half the planet's surface, is restricted by sampling difficulties and cost (Barnes et al., 2009). The geographic distribution of debris on the ocean floor is strongly influenced by hydrodynamics, geomorphology and proximity of human activities (Pham et al., 2014). The Mediterranean Sea supports particularly high densities of litter on the seafloor, owing to the combination of a densely populated coastline, shipping in coastal waters, negligible tidal flow and limited water exchange (Galgani et al., 2015). Submarine depressions or channels and smaller scale structures (holes, rocks, geological barriers, etc.) favour sedimentation and increase the retention, thus representing areas with higher densities of plastic items (Barnes et al., 2009). Relatively high levels of fishing-related debris were found on ocean ridges and seamounts of European Seas, reflecting the intensive fishing activities in those areas (Pham et al. 2014). Among the litter collected from the seabed in four Aegean fishing grounds, the 67- 91% is represented by plastics (Papadopoulou et al., 2016). In estuaries, large rivers are responsible for substantial input of debris to the seabed and they can also transport waste far offshore because of their high flow rate and strong currents (Galgani et al., 2000).

Therefore, the abundance and spreading of plastic litter show considerable spatial and temporal variability in the oceans, based on its physico-chemical properties and the environmental conditions (Galgani et al., 2015). For this reason, plastic behavior in marine environment have to be considered in a dynamic and changing perspective and research have to move for develop more robust temporal and spatial comparisons, that allow to address how abundance and composition of plastics vary with depth, location, topography and habitat (Avio et al., 2016).

1.3 Macro and micro components of plastic marine debris

Plastic marine debris has been reported in sizes ranging from meters (or even kilometers in the case of abandoned, lost or discarded fishing gear) to microns in all the oceans compartments (UNEP, 2016).

The terms macroplastics and microplastics (MPs) are widely used, however they do not have generally agreed-upon definition (Law, 2017). Macroplastics conventionally comprise objectives readily visible, sometimes classified in mega-debris (>100 mm), macro-debris (>20 mm), meso-debris (20–5 mm) or often the term refers simply to debris larger than microplastics (Barnes et al., 2009). Microplastics are particles, varying in shapes and colours, most commonly defined as smaller than 5 mm (Arthur et al., 2009), but some authors claim it would be more intuitive to follow the International System of Units definition of "micro" and thus define the maximum size of microplastics as 1 mm (Browne, 2015; Thompson, 2015). There is a lack of clarity also when considering the lower size limit: as pragmatic approach, microplastics are the particles retained by plankton nets or sieves with variable mesh sizes during sampling of matrices for analysis of such particles (Andrady, 2017). This inconsistency is particularly problematic when comparing data referring to

microplastics, making it increasingly important to create a scientific standard (Claessens et al., 2011; Costa et al., 2010).
Anyway, microplastics can be broadly categorized as primary and secondary, basing on sources (i.e. where they originate) and pathways by which they enter habitats (Browne, 2015) (Figure 1.1).

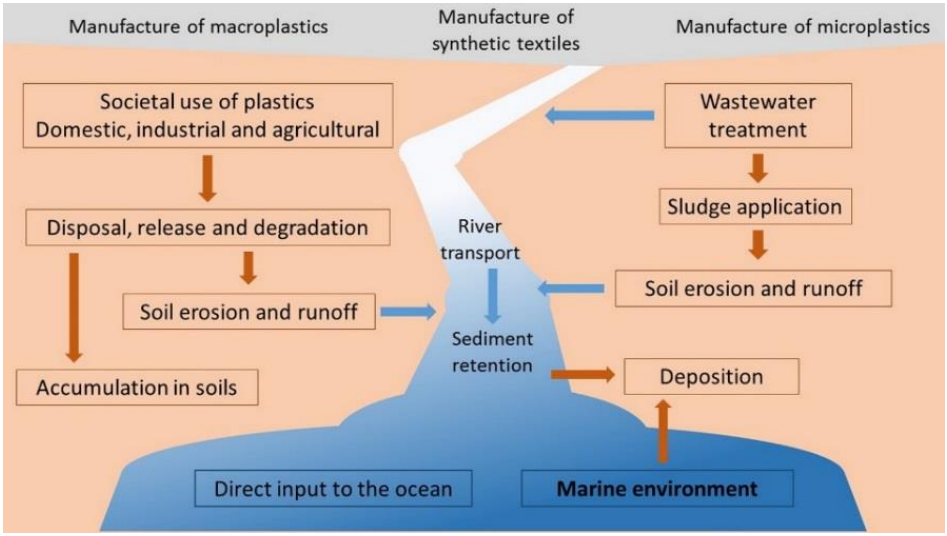


Figure 1.1 *Conceptual diagram of microplastic sources and flows throughout and between anthropogenic, terrestrial, freshwater and marine environment (Horton et al., 2017).*

Primary microplastics are specifically manufactured in the micrometer size range to be directly used in a wide range of applications. Plastic pellets (cylindrical granules around 5 mm diameter) and powders (less than 0.5 mm) are used as a feedstock for the production of larger items (Thompson, 2015) and the presence of these pellets in the marine environment (also known as nurdles or mermaids tears) has been widely reported as a consequence of industrial spillage (Redford et al., 1997; Thompson et al., 2009; Costa et al., 2010; Antunes et al., 2013). Small plastic particles typically around 0.25 mm are also widely used as an abrasive scrubber from industrial and domestic cleaning products (Browne et al., 2007). For instance, surfaces of buildings, machinery and boats can be cleaned using blasting melamine, acrylic, polystyrene, or polyester microplastic with other types of granules (e.g. sand) (Cole et al., 2011). Although “media blasting” has been suspected of being a source of microplastic to habitats there has been no scientific work to the quantity of particles emitted into or found within the environment through this source (Browne, 2015). More work has been done for microplastics used as physical abrasives in personal care products, like hand cleansers, toothpastes and facial scrubs (also known as microbeads) (Zitko and Hanlon 1991; Gregory 1996; Fendall and Sewell, 2009; Napper et al., 2015; Cheung and Fok, 2016). Around 93% of the microbeads used in cosmetics are polyethylene (PE), but they can also be made of PP, PE, PET, polymethyl methacrylate (PMMA) and nylon (Napper et al., 2015). Microplastics used in cleaning products can enter marine habitats waterways via drainage systems (Derraik, 2002), runoff and storm water (Browne, 2015). Despite the capability of some sewage treatment works to remove up to 99.9% microplastic particles from wastewater (dependent on the processes employed by the treatment plant), the sheer number of particles entering the system may still allow a significant number to bypass filtration systems and be released into the environment with effluent or with sludges for applications to land (Horton et al., 2017). It is estimated that between 15 and 31% of all of the plastic in the oceans could originate from primary sources (Boucher and Friot, 2017).
Secondary microplastics describe tiny plastic fragments derived from the breakdown of larger plastic debris, both at sea and on land, by abrasion, wave-action and turbulence (Ryan et al., 2009; Thompson et al., 2004). Besides, more subtle processes of degradation such as the action of light (photolysis), heat and oxygen (thermal-oxidation), water (hydrolysis), organisms (microbial biodegradation) (Browne, 2015), may induce

chemical breakdown of the polymer molecules with consequent change in material properties (Andrady, 2015). With a loss of structural integrity, these particles are increasingly susceptible to fragmentation into progressively smaller pieces (Cole et al., 2011): it is considered that microplastics might further degrade to be nanometer-sized, however nanoplastics are, at now, only hypothesized to exist, because no reliable method has been developed to detect and identify them in the field (Koelmans et al., 2015). However, among the degradation processes, only light-induced oxidation is effective in the ocean environment, just in plastics littered on beaches or floating at the sea surface, even if, for the latter, it is considerably slower (Andrady, 2016). Moreover, floating plastics can be subjected to biofouling, that shields the plastic from the solar radiation and increase its density and hence sinking out of the photic zone, preventing further UV-mediated degradation. In the aphotic (dark) and cold sediment environment no appreciable degradation is expected (Gregory and Andrady, 2003). Slow thermal oxidation of plastics also proceeds in concert with photo-oxidation, especially on beaches (Andrady, 2015). Biodegradation (and even hydrolysis) does occur at sea (Zettler et al., 2013), but the reactions proceed too slowly to result in significant levels of environmental degradation of common plastics under outdoor conditions (Andrady, 2016). Even the bio plastics, that are often seen as a viable replacement for traditional ones, are subjected to partial and slow decomposition under marine conditions and in the absence of terrestrial microbes (Cole et al., 2011). Indeed, their degradable components (e.g. starch and vegetable oils) will decompose but times are prolonged; in addition, an abundance of synthetic polymers, which are generally present in their formulation, will be left behind (Andrady, 2011). Thus, bioplastics too, exactly as traditional plastics, may accumulate in the sea and be a source of microplastics (Thompson et al., 2004).

In addition to fragmentation in the environment, some plastic items can be fragmented and originate microplastics as a consequence of everyday usage or cleaning. This is the case of fibers that are released from textiles and clothing as a consequence of washing (Browne et al., 2011). In fact, it has been shown that laundry washing machines discharge large amounts of microplastics (reaching 1900 fibers in one wash (Browne et al., 2011)) into wastewaters and through wastewater effluents into the aquatic environments. In addition, during wastewater treatment, synthetic fibers are known to contaminate sewage sludge (Zubris and Richards, 2005). It is apparent that a significant number of textile fibers do enter the marine environment, being found in relatively large numbers in shoreline and nearshore sediments close to urban population centers (Browne et al., 2007; Karlsson, 2015). Although microfibers are secondary particles, they will pollute the environment through the same ways of the primary microplastics. In this respect, the fate and transport of these fibers may be more closely aligned with that of primary microplastics, based on similar release routes (Horton et al., 2017). Recently, it has been hypothesized that the atmospheric compartment should not be neglected as a potential source of fibers, after evidences of a significant amount of them in atmospheric fallout (Dris et al., 2016, 2017). These fibers could be transported by wind to the aquatic environment or through the runoff after deposition on land (Free et al., 2014) but more studies are needed to clarify this aspect. It is opportune to point out that only fibers made of petrochemicals are generally considered in the literature as microplastics (Dris et al., 2016). However, Song and co-workers (2015) suggest that fibers made of a mixture of natural and synthetic materials should be also included in the identification of microplastics. In fact, these fibers might also be prevalent in marine and continental environments and could cause physical impacts on organisms. Moreover, harmful additives and dyes can be used when manufacturing these fibers. Further discussions are necessary to identify if artificial fibers (e.g. rayon), which are manufactured by transformation of natural polymers (e.g. cellulose), have to be included in microplastics and thus monitored.

Doubtless, as a collective consequence of these diverse inputs, microplastics are widespread in natural habitats: over the past decades they are documented in huge amount on shoreline, seabed, water column and sea surface worldwide (Wright et al., 2013; Rezania et al., 2018).

Negative impacts on habitats and wildlife have been demonstrated for both macro and microplastics (Avio et al., 2016, Rochman et al., 2016; Galloway et al., 2017; Thiel et al., 2018; Rezania et al., 2018) but differently sized particles are likely to have different effects (Rochman et al., 2016; Thompson, 2015).
For example, marine mammals, seabirds, turtles, fishes are the most affected organisms by entanglement in macro debris, that commonly involve plastic rope and netting and other components of derelict fishing gear but may also be caused by packing or strapping bands (Gall and Thompson, 2015). Entanglement has been reported for 344 species and hazards include bodily harm, such as injury to dermal tissue; interference with growth, potentially causing deformations; restricted movement affecting swimming, feeding, and the ability to escape predators (Law et al., 2017).
Ingestion of plastic debris is widespread and increasing: it has been documented for 233 marine species and the size of the ingested debris is obviously limited by the size of the ingesting organism (Law et al., 2017). Larger litter items, such as potato chip bags and cigarette box wrapping, have been found in the stomachs of large pelagic fish (Jackson et al., 2000) and very large debris items, including 9 m of rope, 4.5 m of hose, two flowerpots, and large amounts of plastic sheeting, were found in the stomach of a stranded sperm whale (de Stephanis et al., 2013). The micro-sized plastics are, on the other hand, more likely to interact with a broader array of marine organisms (Thompson, 2015), indeed, ingestion has been reported from zooplankton to big vertebrates, in pelagic and benthic species, with different feeding strategies (Lusher et al., 2015). Ingested items can produce internal injury, such as a perforated gut, ulcerative lesions, or gastric rupture, potentially leading to death (Law et al., 2017). They have been hypothesized to cause obstruction of the gut and to reduce storage capacity in the stomach (McCauley and Bjorndal, 1999) causing false satiation, that lead to reduced appetite (Day et al., 1985). Moreover, microplastics can accumulate in tissues and may affect organisms at subcellular level, with a potential to impact across successive levels of biological organization (i.e. cells, organs, individual, population, ecosystem) (Galloway et al., 2017).
Animals that ingest plastic debris may also be at risk of contamination by chemicals associated with plastics that are incorporated during manufacture or that are accumulated from contaminated environmental matrices such as sediment or seawater (Avio et al., 2016). Many of these substances are known to be persistent, bioaccumulative, and toxic, with at least 78% of the priority pollutants identified by the US EPA known to be associated with plastic marine debris (Rochman et al., 2013a). In this respect, microplastics can efficiently adsorb organic pollutants from surrounding seawater, because of the hydrophobic nature of these compounds and the high surface/volume ratio of the small particles (Liu et al., 2016); often the concentrations of environmental chemicals on microplastics are orders of magnitude higher than those detected in seawater (Ogata et al., 2009).
Thus, microplastics are nowadays recognized as a serious threat to the marine life, due to the ubiquity in the environment and the potential to affect a range of species, combining a physical disturbance with a chemical challenge (Rochman, 2015).

1.4 Interactions of microplastics with marine organisms

Organisms across many trophic levels interact with microplastics. Microplastics present in the environment (water or sediment) elicit direct exposure of organisms, whereas microplastics which have been previously ingested by prey items may represent an indirect source of contamination to predators through trophic transfer (Lusher et al., 2017a). Organisms might up-take and accumulate microplastics through adherence/adsorption on tissues and consumption/ingestion (Kolandhasamy et al., 2018).
Microplastics bioavailability depends by several biological factors and properties of particles, which, moreover, are constantly changing under environmental conditions (Galloway et al., 2017).
For example, polymers exhibit different densities that affect their buoyancy and behavior on sea surface and within the water column. Planktivores, filter feeders and suspension feeders inhabiting the upper water

column are likely to encounter positively buoyant, low-density plastics, such as PE (specific gravity 0.91-0.94), on the sea surface, while high density plastics such as PET (specific gravity 1.38-1.39) or PVC (specific gravity 1.35-1.45) are expected to sink, thus becoming available for supra-benthic and benthic suspension/deposit feeders as well as detritivores (Paul-Pont et al., 2018). However, it has been demonstrated that once in the sea, microplastics are subjected to the formation of a layer of a complex mixture of organic and inorganic molecules on the surface (defined as an "ecocorona") (Galloway et al., 2017) followed by colonization of micro- and rafting organisms, in addition they tend to form agglomerates by cohesion with other particles and phytoplankton (i.e. hetero-aggregates) (Long et al., 2015). These interactions have been shown to influence MPs vertical distribution, which will inevitably modify their availability for pelagic *vs* benthic organisms (Long et al., 2015). De-fouling in the water column by foraging organisms is, then, a potential pathway for microplastic particles to return to the sea-air interface (Andrady, 2011). The seasonal flocculation of natural particulates into sinking aggregates is an important pathway for energy transfer between pelagic and benthic habitats (Ward and Kach, 2009) and the potential for microplastics to become incorporated into marine aggregates may present a further mode of entry into the food chain. Once ingested, microplastics could sequentially be egested within fecal matter and suspension feeders and detritivores may ingest such egested microplastics (Cole et al., 2016). These cyclic patterns may make microplastics available to organisms occupying different depths of the water column at different times (Wright et al., 2013).

The shape and colour of microplastics too, may potentially contribute to the likelihood of ingestion. Several types of MPs can be distinguished according to their morphology: spheres (beads, pellets, and granules), fibers, lines, films, fragments, and foams (Karami, 2017). Graham and Thompson (2009) revealed that some benthic organisms, like sea cucumber, could preferentially ingest plastic fragments over other shapes. Gray and Weinstein (2017) showed a higher retention time of spheres than fragments in the gut of shrimp whereas in the gills they observed a hierarchy of retention patterns for fragments > spheres > fibers.

Some commercially important fish and their larvae are visual predators, preying on small zooplankton, and may feed on microplastics which most resemble their prey *i.e.* white, tan, blue and yellow plastic (Lusher et al., 2017a). To further support the influence of color on bioavailability, Carpenter et al. (1972) demonstrated that fish from the Niantic Bay area, New England, selectively consumed a white, opaque form of polystyrene spherules, even if, also a crystalline, clear form was present in the coastal waters. Microplastic ingestion due to food resemblance may also be applicable to pelagic invertebrates, which are visual raptorial predators (Wright et al., 2013).

Alternatively, some organisms exert limited selectivity between particles and capture anything of appropriate size (Moore, 2008). Instead, the zooplankton *Acartia longiremis* and *Calanus finmarchicus* showed a preference for aged microplastics over pristine ones, that is probably linked to the formation of a biofilm containing similar microbes to those that copepods feed on in the water column, secreting chemical exudates that enhance chemo-detection and particle attractiveness as food items (Vroom et al., 2017). Savoca et al., (2016) suggested that plastic detection and consumption can be driven by sensory mechanisms: they demonstrated experimentally that marine-seasoned microplastics produce a dimethyl sulfide (DMS) signature that is also a keystone odorant for natural trophic interactions. They further demonstrated a positive relationship between DMS responsiveness and plastic ingestion frequency using procellariiform seabirds as a model taxonomic group. This would imply that some aquatic organisms may also actively search out and ingest microplastic particles (Lusher et al., 2017a).

Possibly, the most likely and, thus, studied interaction is uptake of microplastics through ingestion (Lusher, 2015), but microplastics can adhere to external appendages, including setae, swimming legs and antennules of copepods (Cole et al., 2014, 2015), to gills of crabs and mussels (Watts et al., 2014; Paul-Pont et al., 2016) and they are found also on foot of zooplanktons and mussels (Wegner et al., 2012; Setälä et al., 2016; Watts et al., 2016; Kolandhasamy et al., 2018). Gill can be regarded as one of important feeding organs in many species, foot, however, is not directly related to the feeding process; therefore, Kolandhasamy et al. (2018)

suggested that the consideration of adherence way will increase the estimation of bioavailability of microplastics to organisms, especially to those non-filtering feeders. Gutow et al. (2016) found that the adherence of microplastics to seaweeds would provide a pathway for microplastic from the water to marine benthic herbivores. Similarly, the adherence of microplastics to animals would be a novel way for microplastics to be transferred in food web.

A number of experimental studies have explored the mechanisms of biota-microplastic interactions, since in laboratory settings it is easier to monitor the uptake, movement and distribution of synthetic particles in whole organisms and tissues (e.g. gills, intestinal tract and liver) (Lusher et al., 2017a). Exposure studies have confirmed that a diverse array of marine organisms, across trophic levels, can absorb or consume microplastics. These include seaweeds (Gutow et al. 2016), phytoplanktons (e.g. Davarpanah and Guilhermino, 2015; Zhang et al., 2017; Lyakurwa et al., 2017) protists (Christaki et al., 1998; Lyakurwa et al., 2017), copepods (e.g. Cole et al., 2013, 2015; Sun et al., 2017), annelids (e.g. Besseling et al., 2013; Wright et al., 2013; Van Cauwenberghe et al., 2015), echinoderms (Della Torre et al., 2014; Kaposi et al., 2014; Nobre et al., 2015; Martinez Gomez, 2017), cnidaria (Hall et al., 2015), amphipods (Thompson et al., 2004, Ugolini et al., 2013), decapods (e.g. Watts et al., 2014), isopods (Hämer et al., 2014), molluscs (e.g. Avio et al., 2015a; Paul-Pont et al., 2016; Magni et al., 2018), fish (e.g. Mazurais et al., 2015; Avio et al., 2015b; Pedà et al., 2016 Jovanović et al., 2018), and birds (Tanaka et al., 2013; Savoca et al., 2016). It should be noted however that the exposure concentrations used to achieve this goal exceed those expected in the field, thus, the results should be treated with attention (Phuong et al., 2016).

A number of field studies have documented the occurrence of microplastics in wildlife (reviewed in Lusher, 2015; Avio et al., 2016; Phuong et al., 2016): over 220 different species have been found to consume microplastic debris in a range of habitats, including the sea surface, water column, benthos, estuaries, beaches and aquaculture, as well as the deep sea (GESAMP, 2016; UNEP, 2016). These surveys clearly revealed a high variability of MPs ingestion regardless of the trophic level of the fish or shellfish species concerned; they also tended to indicate a higher frequency of contamination in pelagic feeders and suspension/filter feeders than in other groups (Paul-Pont et al., 2018). The majority of data focus on MPs in commercially targeted species like shellfish (bivalves and crustaceans) and fishes (Lusher et al., 2017a), while more limited are those available for species not intended for human consumption and particularly for benthic/epibenthic organisms (Bour et al., 2018).

Ingestion rates measured on natural zooplankton communities revealed that 83% of Brown shrimps (*Nephrops sp*.) in the north Clyde Sea (Murray and Cowie, 2011), 63% of shrimps (*Crangon crangon*) in the UK, 3% of the copepod *Neocalanus cristatus* and 6% of the euphausids *Euphasia pacifica* in the northeast Pacific consumed plastic debris, most of which were fragments or fibers (Desforges et al., 2014; Devriese et al., 2015). Studies on fish reported between 2 and 40% of individuals to be contaminated, with a mean number of particles detected in the intestinal tract from 1 to 7.2 per individual (Boerger et al., 2010; Foekema et al., 2013; Lusher et al., 2013; Avio et al., 2015b). For mollusks, especially mussels, this MP load varied from 0.2 to 0.5 plastic particles (including fibers) per gram of tissue found in Europe (De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014, Van Cauwenberghe et al., 2015). In the same species analysed in Newfoundland, Canada, microplastics concentration was about 100 fold higher than the levels measured in Europe (Mathalon and Hill, 2014). However, in this last study, ambient particles in laboratory air were very high and blank samples indicated that laboratory contamination could contribute to 25 microplastic particles per g soft tissue. Adjusting for this background, would lead to contents comparable with those found in bivalves from China, that are about 2 particles/g of whole body (Li et al., 2015). Regarding other bivalves: 0.47 ± 0.16 particles/g were found in the soft tissues of oysters (*Crassostrea gigas*) with a decrease of approximately 25% after 3 days of depuration in clean seawater (Van Cauwenberghe and Janssen, 2014); 75% of specimens of *Perna perna* collected in Brazil contained at least one fragments < 5mm (Santana et al., 2016); Davidson and Dudas (2016) did not recorded significant difference in microplastic concentrations

between cultured clams (*Venerupis philippinarum*) (1.7 ± 1.2 particles/g) and wild clams (0.9 ± 0.9 particles/g), fibers represented the dominant microplastic typology.

MPs can be ingested also by marine mammals, for example, they have been found in the stomachs of harbor seals, *Phoca vitulina* (Rebolledo *et al.*, 2013), beaked whales, *Mesoplodon mirus* (Lusher et al., 2015), and baleen whales, *Megaptera novaeangliae* (Besseling et al., 2015). Furthermore microplastics were found in the scats of fur seals, *Arctocephalus* spp (Eriksson and Burton, 2003).

It is important to highlight that studies on microplastics in field organisms were not fully comparable in terms of methods of extraction, size limit detection and sample processing, underlying the need of validated and standardized techniques for the assessment of plastics in marine organisms (Avio et al., 2016).

Beside accumulation in tissues, there is growing concern for the possible trophic transfer of microplastics in aquatic, benthic and pelagic foodwebs, however only a few studies deal with the potential for microplastics to be transferred between trophic levels following ingestion (Phuong et al., 2016). Predatory organisms may indirectly accumulate microplastics during the ingestion of microplastic contaminated prey, which may lead to bioaccumulation at upper trophic levels. Similarly, predators and detritivores may ingest microplastics while scavenging detrital matter containing microplastics (Lusher, 2015). Laboratory experiments have established that green crabs fed with blue mussels containing microplastics accumulated particles in their digestive tract (Watts et al., 2014; Farrel and Nelson, 2013). Similarly, *Nephrops norvegicus*-fed fish which had been seeded with microplastic strands of polypropylene rope, were found to ingest but not to excrete the strands (Murray and Cowie, 2011), further corroborating the potential for trophic transfer. Also, a transfer from green algae (*Scenedesmus spp*.) to the planktonic water flea (Daphnia magna) and then to several species of fish: Crucian carp (*Carassius carassius*), Bleak (*Alburnus alburnus*), Rudd (*Scardinius erythrophthalmus*), Tench (*Tinca tinca*), Northern pike (*Esox lucius*) and Atlantic salmon (*Salmo salar*) has been observed (Cedervall et al., 2012), as well as, transfer of fluorescent polystyrene (PS) microspheres (10 µm) from zooplankton to the mysid shrimp (*Mysis spp.*) (Setälä et al., 2014). During field studies, microplastic particles approximately 1 mm in diameter were recorded in the scat of fur seals and Hooker's sea lions (McMahon et al., 1999). The presence of plastic coincided with otoliths of the myctophid fish *Electrona subaspera*, suggesting a trophic link. Eriksson and Burton (2003) further investigated the transfer of plastic particles in Antarctic fur seals and the authors suggested that microplastics had initially been ingested by the fur seals' prey, the plankton feeding Mycophiids. Contamination of the blubber of the Mediterranean fin whale *Balaenoptera physalus* has been suggested as an indication that microplastic ingestion occurs through indirect consumption via planktonic prey (Fossi et al., 2012).

As mentioned above, microplastics were also detected in species which are consumed by humans and raises concerns about the risk of MPs and associated chemicals for the human health. The majority of studies have documented that largest quantities of microplastics are contained in the gut of marine organisms, an organ that is not generally consumed directly by humans (Galloway, 2015). Similarly, peeling of invertebrates will remove most of the digestive tract, the head and the gills together with microplastics, which apparently are mainly present in these organs: it was observed by Devriese et al., (2015) in the crustacean species *Crangon crangon* (common shrimp) and *Nephrops norvegicus* (Norway lobster). However, exception occurs for most shellfish such as mussels and clams, some echinoderms and several small species of fish which are eaten whole (Lusher et al., 2017a). The amounts of microplastics ingested by humans as a result of consuming seafood are poorly quantified: some estimations reported from 1 particle per day (Vandermeersch et al., 2015) to 30 particles per day (Van Cauwenberghe and Janssen, 2014) depending on seafood consumption habits. Despite that, the overall human health risks posed by microplastics in seafood at present appear to be low, in the worst case exposure scenario too, as demonstrated by the technical paper "Microplastics in fisheries and aquaculture" developed by FAO, IMO, UNEP and GESAMP partnership in 2017. Moreover, no data are available on the potential impact cooking and/or processing seafood at high temperature may have on the toxicity of microplastics in seafood products (Lusher et al., 2017a). Obviously, further studies are

needed to increase understanding of the trophic transfer of MPs within the marine food web and, thus, the potential correlation with human health.

1.5 Biological effects of microplastics in marine organisms

Evidences of up-take of microplastics by a wide array of marine organisms, both in the field and in laboratories, have drawn the attention of ecotoxicologists on toxicity of MPs for biota. Mode of action of microplastics to induced adverse effects should be studied thoroughly on two important aspects: the physical nature and the chemical moiety of such particles (Anbumani and Kakkar, 2018).

Three categories of chemical species are known in plastic, that might be bioavailable to organisms and may present a toxic hazard to them (Andrady, 2017): i) additives mixed with plastics during their manufacture or processing to ensure the functionality of the product (Andrady, 2016); ii) residual monomer, for example, polystyrene found in significant quantities in debris, can contain 0.1–0.6 wt% of styrene monomer and oligomers, that can be carcinogenic and/or mutagenic, as well as, polyvinyl chloride monomer, used in the production of PVC (Lithner et al., 2011), while common plastics found in marine MPs, polyethylene and polypropylene do not have any residual monomer (Garrigós et al., 2004; Andrady, 2016); iii) contaminants adsorbed by the environment. In this respect, persistent organic pollutants (POPs) present in seawater are sorbed very efficiently by MPs; in fact, the equilibrium distribution coefficient K for common POPs in water-plastic systems ranges from 10^3 to 10^5 in favor of the plastic (Teuten et al., 2009), although sorption capacity varies by plastic polymers and considered chemicals (Rochman, 2015). Metals also showed a strong adsorption capacity to plastic with measured levels up to 300 mg/g for Al, Fe, Cu, Pb and Zn and up to 80 ng/g for Cd, Cr, Co, Ni in beached pellets (Holmes et al., 2012). Little is known about mechanisms of metal adsorption which is higher in weathered compared to virgin plastics, due to an increased polarity of particles: environmental conditions like pH, can represent crucial factors for the adsorption of metals onto plastics polymer (Turner and Holmes, 2015).

The result is a complex mixture of chemicals (“cocktail”) associated to microplastics (Rochman, 2013), which might be available to organisms. In fact, indirect evidences, that include the use of a thermodynamic approach and of models simulating physiological conditions in the gut (Gouin et al., 2011; Tanaka et al., 2013; Bakir et al., 2014), suggest that adsorbed pollutants and chemical additives might be released to organisms. This hypothesys has been demonstrated by some laboratory exposures, that gave direct observations of a transfer of chemical compounds from microplastics to tissues of model animals (Browne et al., 2013; Avio et al., 2015a; Paul-Pont et al., 2016).

However, to date, the real capability of microplastics to transfer such compounds to marine biota is under debate, as well as, their relative contribution in respect to other routes of exposure (water, sediments, food web). Actually, recent studies have suggested that given the baseline contamination levels of seawater and marine organisms and the low abundance of microplastics relative to other suspended particles found in oceans (such as organic matter, plankton, detritus etc.), the exposure to organic contaminants via plastic may be negligible compared to natural pathways (Bakir et al., 2016; Beckingham and Ghosh, 2017; Koelmans et al., 2016; Paul-Pont et al., 2018).

Various laboratory exposures have explored the biological effects of MPs in different marine species to better characterize and predict the potential ecotoxicological risk of particles in the marine environment. Among the biological effects, mortality rate, energy budget, loss of weight, feeding activity, embryonic development, predation, biomarker responses and alteration of gene expression have been the most investigated (Figure 1.2).

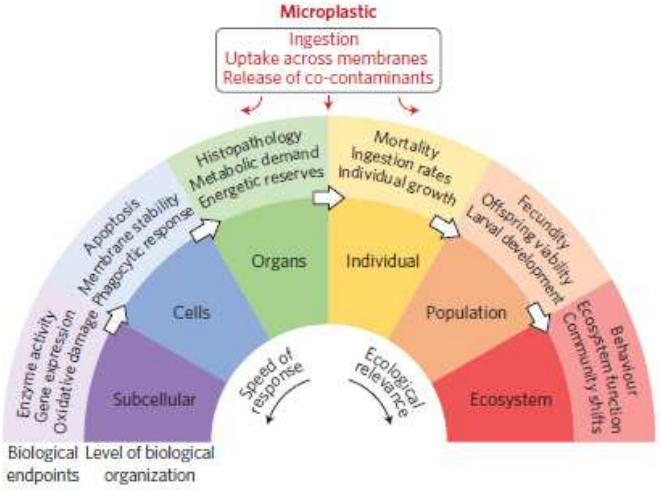


Figure 1.2 *Simplified scheme illustrating potential impacts of exposure to microplastic across successive levels of biological organization (Galloway et al., 2017).*

The plausible mechanism of microplastics in primary producers is thought to be due to the physical adsorption leading to blockage of light and airflow, thereby, impeding photosynthesis (Bhattacharya et al., 2010). Exposure of polyvinyl chloride (PVC) microplastics of 1 µm size on marine microalgae, *Skeletonema costatum*, effectively inhibits 39.7% growth ratio after 96-h exposure (Zhang et al., 2017); the up- take of PS particles by *Rhodomonas baltica* result in loss of motility (Lyakurwa, 2017). As algae play a key role in aquatic food webs, the productivity and resilience of ecosystems could be compromised if high concentrations of plastic particles occur (Wright et al., 2013). At the molecular level, microplastics tend to disrupt the synthesis of rhamnose and xylose sugars of exopolysaccahride biosynthesis pathway (Lagarde et al., 2016), while in invertebrates, microplastics impairs the filtration process that leads to reduced food intake and lethality in crustaceans (Jemec et al., 2016). Rist et al. 2016 observed a significant reduction of filtration behavior, respiration rate and byssus production in the Asian mussel, *Perna viridis* exposed to PVC particles (1-50 µm). Effects of microplastics on the byssus gland secretion eventually affect mussel colonization and subsequent health status (Anbumani and Kakkar, 2018). Upon efficient uptake, PS microplastics exposure leads to reduced carbon biomass and feeding activity and fecundity success in marine copepods (Cole et al., 2013, 2015). Negative effects on reproduction were observed also in oysters exposed to polystyrene microplastics (2 and 6 µm size) with the decrease of oocyte number and sperm velocity (Sussarellu et al., 2016). The echinoderm *Lytechinus variegatus* developed abnormal embryos after 24h of exposure to PE pellets (Nobre et al., 2015). Likewise, Martínez Gomez et al. (2017) evaluated the effects of virgin, aged and leachate of PS and HDPE fluff particles in the sea urchin, *Paracentrotus lividus*. During the 48-h incubation period, fertilization and larval development are impaired to a significant extent. Since the selected microplastic particles failed to aggregate in the exposure media, authors proposed that sea urchin embryotoxicity is attributed to the chemical leachate of the exposed plastic particles. The exposure of brown mussel, *Perna perna* to PP microplastics leachate showed impairs of larval development and embryotoxicity (Gandara e Silva et al., 2016).

Microplastic have been shown to accumulate in the digestive cavity and tubules of bivalve molluscs that could potentially cause blockages and suppressing feeding due to satiation (Wright et al., 2013), moreover they are able to pass the biological membranes, taken up into cells and be translocated between different tissues through the circulatory system (Browne et al., 2008; von Moos et al., 2012, Avio et al., 2015a; Magni et al., 2018). Different studies demonstrated that PS and PE microparticles, both virgin and contaminated by organic contaminants, affect immune and detoxification system in bivalves, produce genotoxicity, induce strong inflammatory responses and enhance the expression profile of genes involved in fundamental physiological process like cell cycle growth arrest, apoptosis, and oxidative stress (Anbumani and Kakkar,

2018). In some of these studies (Avio et al., 2015a; Paul-Pont et al., 2016), the ability of microplastics to play as vector of organic chemicals, like pyrene and fluoranthene, for organisms have been experimentally demonstrated. Similarly, Browne et al. (2013) observed increased accumulation of nonylphenol and triclosan in the presence of polyvinyl chloride (PVC) leading to impaired immune functions, physiological stress, and mortality in the lugworm, *Arenicola marina*.

Concerning fish PE microspheres exposure in common goby, *Pomatoschistus microps*, caused reduction of predatory performance (de Sa et al., 2015) and reduction of acetylcholinesterase activity, an enzyme that induce neuromuscolar transmission, thereby this effect could affect processes related to physiology (growth and reproduction) and behavior (swimming patterns) resulting in dwindled population (Oliveira et al., 2013). In the juveniles of the same species, Fonte et al. (2016) investigated the multiple stressor toxicity (microplastics, cefalexin, and temperature): as the temperature increases from 20 to 25 °C, microplastics-induced mortality is noted with predatory performance inhibition whereas coexposure of microplastics and cefalexin results in reduced predatory performance and acetylcholine esterase inhibition.

Effect of PE microplastics exposure were observed also in *Oryzia latipes* such as downregulation of choriogenin, vitellogenin and estrogen receptor (ERa) mRNA gene expression and abnormal germ cell proliferation. Severe glycogen depletion and fatty vacuolation were also observed (Rochman et al., 2013b). The first reported evidence on the in vitro effects of virgin microplastics are from the findings of Espinosa et al. (2018) in *Sparus aurata* (gilthead seabream) head-kidney leucocytes (HKLs), that showed an upregulation of *nrf2* gene, which control the production of proteins involved in the detoxification and elimination of reactive oxidants, after exposure to PVC and PE microplastics (range size of 40 - 150 µm).

Aside from physical and chemical impacts, microplastics also have a potential role in providing a new hard-substrate habitat for rafting communities, which was previously limited to items such as floating wood, pumice, and sea shells (Wright et al., 2013). A range of species have been found on plastic debris including bryozoans, barnacles, polychaete worms, hydroids and molluscs (Barnes, 2002). Likewise, plastics serve as a floating substrate for bacterial (Lobelle and Cunliffe, 2011) and diatoms colonisation (Carson et al., 2013). Because plastic debris can be transported long distances in the oceans, there is concern that the growing abundance of microplastics could enhance the dispersal of some marine organisms, increasing their range or introducing them to areas from which they were previously absent (Kiessling et al., 2015). Alien invasions can lead to a significant threat to native biodiversity and significant ecological changes (Molnar et al., 2008). However, so far, the establishment of a viable population of an invasive species introduced to a new habitat by plastic debris is yet to be demonstrated and as such the ecological impacts of this rafting effect of ocean plastics are unclear (Browne et al., 2015).

The above studies clearly demonstrated that microplastics should not be considered as biologically inert materials. Growing scientific evidence corroborates the ecotoxicological hazard of microplastics, whether due to a simple mechanical or physical damage induced by these particles, or for a more complex activation of molecular, biochemical and cellular pathways. However, more research is required considering that in the natural environment, organisms may be exposed to microplastics throughout their lifetime as opposed to short experimental durations. Thus, the continual ingestion and accumulation of such particles may incur chronic effects.

1.6 Control and policy making of plastic pollution

Plastic accumulation in the marine environment is recognized as a worldwide growing pollution problem and gives rise to several negative repercussions: from the aesthetic impact of litter and economic costs for beach cleaning, to the detrimental effects on economic sectors and the adverse biological and ecological effects (Avio et al., 2016). According to last conservative estimates from UNEP, impact of plastics would cause an

overall economic damage to marine ecosystems of $13 billion each year, although the true environmental costs are difficult to monetarize (Xanthos and Walker, 2017).

The managements measures to face the problems of plastic litter are, basically, focused on preventive, mitigating, removing and behavior-changing actions. The preventive and behavior-changing measures are particularly important in addressing marine litter at its root (Chen, 2015). The former includes source reduction (e.g. restriction of the use of plastic bags, microbeads in cosmetics and single-use products, or development of more environmentally friendly packaged/produced good through eco-design) in addition to waste reuse, recycling and waste conversion to energy, following the concept of a circular economy.

Behavior-changing schemes aim to encourage people to embrace the notion of waste as a resource and choose the products that generate lower quantities of litter (preventive), dispose of waste in a more environmentally sound way (mitigating) and participate in beach cleanups (removal). Education campaigns and activities raising awareness are examples of such measures (Hartley et al., 2015) (many NGOs are engaged like The 5 Gyres Institute, The Plastic Soup Foundation, the International Coastal Cleanup, Parley for the Oceans and the Ocean Cleanup), as well as, provision of incentives: JPI Oceans launched in 2015 a € 7.5 million call for proposals to increase the knowledge on microplastics in the marine environment (http://www.jpi-oceans.eu/ecological-aspects-microplastics).

Mitigating actions involve various debris disposal and dumping regulations, that has been employed to minimize its adverse impact on the marine environment. Removing measures aim to take away debris already present in the marine environment. Beach cleanups are commonly employed for this but are time-consuming, costly (Newman et al., 2015) and only capture a fraction of the overall debris; some initiatives have employed divers to collect and monitor benthic marine plastics (Donohue et al., 2001; Watson, 2012); in Fishing for Litter initiatives fishers remove all litter items collected during normal fishing operations and deposit them safely on the quayside to then be collected for disposal.

Monitoring marine debris can be also classified as removing measure since it often involves recording information on debris types, amounts and sources concomitantly with their removal. Monitoring is instrumental in devising effective management strategies to prevent specific types of litter from entering the sea. Importantly, long-term monitoring programs enable to assess the effectiveness of legislation and coastal management polices (Rees and Pond, 1995) and have the potential to help management at individual sites and to generate largescale pollution maps (from regional to global) to inform decision makers (Ribic et al. 2010).

Different instruments have been developed at international, regional and national levels to prevent, reduce and manage marine litter, like plastic materials, which comprise conventions, agreements, regulations, strategies, action plans, programs and guidelines (Löhr et al., 2017).

Two major international conventions specifically address marine litter in the ocean: the International Convention for the Prevention of Pollution from Ships (1973), as modified by the Protocol of 1978 (MARPOL 73/78); and the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter 1972 (commonly referred to as the London Convention), then modernized in the London Protocol (1996).

Annex V of MARPOL 73/78 bans ships from dumping plastic at sea and was developed under the auspices of the international Maritime Organization (IMO). However, it is limited to maritime emissions, while 80% of plastic enters the ocean from land (Jambeck et al., 2015). Instead, the London Convention covers the control of dumping of wastes at sea that have been generated on land. It requires the signatories to prohibit dumping of persistent plastics and other non-biodegradable materials into the sea.

In addition, the 1982 United Nations Convention on the Law of the Sea (UNCLOS) sets out the legal framework within which all activities in the oceans and seas must be carried out: although the provisions do not explicitly refer to marine litter, they place a general obligation on states to protect and preserve the marine environment, which can be used in the context of marine litter regulation. (Kershaw et al., 2011) .

The international instruments are then transposed at regional or national level and some example include: the European Union (EU) PRF Directive, the Annex IV of the Helsinki Convention, the United States (US) Marine Plastic Pollution Research and Control Act, the United Kingdom (UK) Merchant Shipping (Prevention of Pollution by Sewage and Garbage from Ships) Regulations 2008 (Chen, 2015).

Among the European regional instruments to protect seas and oceans, the most relevant is the Marine Strategy Framework Directive (MSFD, 2008/56/EC). The Directive calls for all of the EU's marine regions and sub-regions to achieve or maintain "Good Environmental Status" (GES) by 2020. GES is defined by means of 11 qualitative "descriptors": descriptor 10 is relating to marine litter and microplastics are specifically considered. The descriptor will establish baseline quantities, properties, and potential impacts of MPs; it must be noted however, that it was reviewed recently for changes in order to make it simpler and clearer, to introduce minimum standards and to be coherent with other EU legislations (Gago et al., 2016). The MSFD represents the first instance, worldwide, that microplastics in the marine environment have been included in a legislative proposal. In this sense is important to mention that MPs were not included in the Water Framework Directive (WFD), the main EU directive dealing with pollution of river basins.

Other instruments exist and mostly serve as global guidance encouraging regional bodies or countries to follow the actions proposed therein, or as a platform for the states concerned to engage in coordination and cooperation in marine litter issues. The most prominent examples are a series of initiatives developed by the United Nations Environment Program (UNEP), including the Regional Sea Program (RSP), Guidelines on survey and monitoring of marine litter, Guidelines on the use of market-based and economic instruments and the Honolulu Strategy. From the latter two strategies are of particular interest. One focuses on market-based instruments (e.g. levies on new plastic bags) for minimizing waste. A second strategy creates policies, regulations, and legislation to reduce marine debris (e.g. imposing bans on microbeads and/or plastic bag use or production) (Xanthos and Walker, 2017). For example, in Europe the interventions to phase out plastic bags are widespread in respect to other countries (Xanthos and Walker, 2017): in Italy the ban on the sale of non-biodegradable plastic bags has been introduced in 2013 and it became in law in 2017 (L. 123/17). Compared to plastic bags, there have been limited interventions to reduce microbeads, but there has been a rapid proliferation in policies to reduce the use of microbeads (Xanthos and Walker, 2017). USA promulgated "Microbead-Free Waters Act of 2015" that prohibits the manufacture and introduction of cosmetics containing plastic microbeads from cosmetic products. Sweden announced a ban on the sale of cosmetics containing microplastics at the United Nations Ocean Conference at the beginning of June 2017. The ban is to come into effect by 2020. Six other European countries have joined 's initiative: Finland, France, Iceland, Ireland, Luxemburg and Norway. Besides, the European cosmetics and personal care industry (Cosmetics Europe) recommended to its membership to discontinue the use of synthetic, solid plastic particles used for exfoliating and cleansing that are non-biodegradable in the marine environment given the availability of alternative materials. The Cosmetics Europe recommendation, built on voluntary initiatives already taken by individual companies including Italian ones, resulted in a rapid and substantial 82% reduction in the use of plastic microbeads between 2012 and 2015.

In addition, both governmental and non-governmental organizations make use of advice of independent scientific experts on aspects of marine environmental protection (like ICES or GESAMP). For example, a Technical Subgroup on Marine Litter (TSG-ML) was established in 2010 to support Member States in harmonizing monitoring protocols and streamlining monitoring strategies of plastic and microplastics in the framework of the MSFD (Galgani, 2015). In 2016, the GESAMP worked together with FAO, IMO and UNEP to improve the knowledge base on microplastics in the marine environment, with a special focus on microplastics in fisheries and aquaculture, and to provide policy advice on this topic (Lusher et al., 2017a).

It is clear that broad-based policies and other initiatives have to be developed to respond to marine plastic pollution, however it is a complex environmental problem with numerous sources, few easy solutions and still many gaps to fill (Haward, 2018). Therefore, an ever close collaboration is necessary between different

stakeholders such as governments, scientists, non-governmental organizations, industry and members of the public worldwide (Hartley et al., 2018).

2 AIMS OF THE WORK

The overall objective of this thesis was to provide additional evidences of the availability of microplastics for marine organisms, both in field and in laboratory conditions, and to highlight the possible biological disturbance caused by the particles themself and/or by the associated chemicals. In addition, the research activity focused on the presence and fate of microplastics in wastewater treatment facilities, that are considered one of the pathways of microplastics to enter the environment.
This was achieved developing specific studies, which are shown in the below three chapters.

In chapter 3 is presented an investigation on the presence and characteristics of microplastics detected in organisms of ecological and commercial interest of the Adriatic Sea. Despite microplastics contamination in the Mediterranean Sea is of particular concern, information on their bioavailability in the food web of Adriatic basin are still lacking. Furthermore, the survey was conducted in order to detect possible differences between sampling sites, species, feeding behaviour and habitat.

Chapter 4 describes a laboratory experiment, which focused on the capability of microplastics to transfer adsorbed pollutant into tissues of a model marine species and on the onset of biological effects induced by virgin and contaminated microparticles. Considering the complexity of the natural environment, a study in controlled laboratory conditions represents a necessary approach to distinguish the impacts caused by exposure to microplastics alone or in combination with chemicals.

The subject of chapter 5 is a study aimed to quantify and characterize microplastics in wastewaters and sewage sludge from a treatment plant of North Italy. WWTPs have been recognized as both collectors of microplastics from anthropic use and as significant direct inputs of microplastic particles and synthetic fibers in the natural environments, since they are not specifically designed to retain these kinds of micropollutants.

3 CHARACTERIZATION OF MICROPLASTICS IN ORGANISMS OF A TYPICAL FOOD WEB OF THE ADRIATIC SEA

3.1 Introduction

The Mediterranean Sea has been described as one of the areas most affected by plastic pollution in the world (UNEP/MAP, 2016) and the recent review of Deudero and Alomar (2015), on the threat of plastic litter on the marine biodiversity, indicated 134 Mediterranean species, ranging from invertebrate communities to large mammals, having been impacted through ingestion and/or entanglement.

Several conditions provide high potential for plastic accumulation in the Mediterranean Sea: it is a region strongly subjected by massively waste-generating human activities, both on the coast and inland (Galgani et al., 2014), it represents one of world's main shipping routes and receives waters from densely populated watersheds, such as, Nile, Ebro, Po, Rhone (Mistri et al., 2017). Moreover, the Mediterranean Sea has a water residence time of up to a century, because its dynamics is characterized by an inward surface flow of waters from the Atlantic Ocean, with no significant outward flow anywhere along its coastline; the return flow into the Atlantic happens at the subsurface, thus hampering surface floating items from being expelled from the basin and destinating them to accumulating within it (Zambianchi et al., 2017).

Yet, although extensive field studies have documented high concentrations of floating plastics in the Mediterranean (Suaria and Aliani, 2014; Cózar et al., 2015; Mansui et al., 2015; Pedrotti et al., 2016; Suaria et al., 2016; Ruiz-Orejón et al., 2016) no evidence of permanent litter accumulation zone ("garbage patches") has been reported so far, since the variability of the surface currents and the instabilities produced during the year limit stable retention area of plastic debris (Ruiz-Orejon et al., 2016; Suaria et al., 2016). Moreover, a high small-scale variability in plastic abundance and composition emerged by results of that surveys.

Despite this, modeling studies concerning the transport, temporal and spatial distribution of marine litter in the Mediterranean Sea have predicted the Adriatic region as an area of preferential accumulation of plastics (Ruiz-Orejón et al., 2016; Liubartseva et al., 2016, 2018; Zambianchi et al., 2017; Carlson et al., 2017).

In particular, Liubartseva et al. (2016, 2018) showed that the area of highest plastic concentrations (>10 g km^{-2}) corresponds to an elongated band off the Italian coastline narrowing from northwest to southeast, that it is clearly related to the spatial distributions of plastic debris inputs (rivers, cities, and shipping lanes), as well as, being connected with general Adriatic circulation patterns (Gomiero et al., 2018).

Located in the central Mediterranean, the Adriatic Sea is an elongated basin, with its major axis in the NW-SE direction, between Italy and the Balkans. It extends from the Gulf of Venice to the Strait of Otranto, through which it connects to the Ionian Sea (Vlachogianni et al., 2017) and it is characterized by Northern (from Trieste to Ancona), Central (as far as the Gargano promontory) and Southern (limited by the Strait of Otranto) sub-basins (Mannini et al., 2004). The northern section is very shallow and gently sloping, with an average depth of about 35 m, while the central and the southern are on average 140 m deep, with the two Pomo Depressions reaching 260 m (Strafella et al., 2015).

Two main currents dominate the Adriatic circulation: the West Adriatic Current flowing toward South-East along the Western (Italian) coast, and the East Adriatic Current flowing North-East along the eastern (Balkanian) coast. Two main cyclonic gyres occur, one in the northern part and the other in the South. Bora (from North-East) and Sirocco (from South-East) are the major winds blowing over the Adriatic Sea. Two main cyclonic gyres occur, one in the northern part and the other in the South. Bora (from North-East) and Sirocco (from South-East) are the major winds blowing over the Adriatic Sea (Artegiani et al., 1997; Strafella et al., 2015). A great number of rivers along the Italian coast affect the North and Centre: the Po river is the most relevant, while the South Adriatic is characterized by the absence of significant rivers supplies (Mistri et al., 2017).

The Adriatic basin is heavily stressed by many human activities: both urban and industrial areas are concentrated along the coasts as well as many maritime activities (aquaculture, fisheries, shipping). The

western Adriatic coast, possessing the longest beaches in Europe, is home of a thriving tourism industry (Munari et al., 2016). It was estimated that 40% of the marine litter enters the Adriatic basin through rivers; 40% through coastal urban populations; and the remaining 20% is derived from shipping lanes (Liubartseva et al., 2016).

Several works have demonstrated the pervasiveness of plastic pollution in the Adriatic Sea with the micro-component being found in all compartments, as elsewhere in the world: on beaches (Laglbauer et al., 2014; Munari et al., 2016, 2017; Blašković et al., 2017), surface waters (Cózar et al., 2015; Suaria and Aliani, 2014; Gajšt et al., 2016; Suaria et al., 2016; Vianello et al., 2018) and sediments (Vianello et al., 2013; Mistri et al., 2017; Munari et al., 2017).

Very few investigations have, instead, assessed the ingestion of microplastics by Adriatic fauna. Three studies have performed the characterization of microplastics in fish sampled in the Northern and Central Adriatic: Avio et al., (2015b) have choosen 5 target species, including the pelagic *Sardina pilchardus*, the benthopelagic *Squalus acanthias* and *Merlucius merlucius* and the demersal *Mullus barbatus* and *Chelidonichthys lucerna*; Anastasopoulou et al., (2018) analysed 5 demersal fish *Chelon auratus*, *Sparus aurata*, *Solea solea*, *Mullus surmuletus*, *Pagellus erythrinus*, and one pelagic species (*Sardina pilchardus*); Pellini et al. (2018) conducted their survey on the common sole *Solea solea*. Data on the presence of microplastics in Adriatic invertebrates, particularly in natural population of mussels, are available only from the study of Vandermeersch et al., (2015), which collected specimens of *Mytilus galloprovincialis* from Po estuary, finding microparticles for which, polymeric characterization was not performed.

Data on occurrence of microplastics in Adriatic species are urgently needed, considering that evaluating the impact, amount, and composition of microplastics in biota of European seas has become a research priority also in the context of the Marine Strategy Framework Directive (MSFD, 2008/56/EC, Descriptor 10.1.3.) (Bellas et al., 2016). Despite the impact of microplastics on natural population is still not well understood, these particles may have the ability to negative affect organisms' health and to enter and propagate through the marine food web (Bellas et al., 2016), with potentials concerns also for human consumers (Avio et al., 2015b).

The present study aimed to assess the abundance, frequency and typologies of microplastics ingested by different invertebrates and fish at different levels ecological web, collected along the Western Adriatic Sea, in the Northern, Central and Southern basin. In particular, a comprehensive quantification and characterization, in terms of size, shape, and chemical composition of plastic particles contained in organisms was conducted for a comparison between species and to test the hypothesis that site of collection, organism's habitat and feeding mode could influence the occurrence of MPs in biota. This study expected to address the gap in knowledge on the presence of microplastics in Adriatic organisms and to provide valuable information on the extent of the phenomenon at large spatial scale along the Italian coast.

3.2 Material and methods

3.2.1 Sampling sites and target species

Sampling of organisms was carried out between May and September 2016 close to three areas along the Italian coast: i) Venice Lagoon (Chioggia), in the North; ii) Conero Riviera(Ancona) in the Center; iii) Apulian coast (Lecce), in the South Adriatic Sea (Figure 3.1).

A total of 259 specimens of invertebrates (Chioggia, n=73; Ancona, n=85 Lecce, n=101) and 216 of fish (Chioggia, n=77; Ancona, n=72 Lecce, n=67) were collected among 24 different species.

Species have been selected for their ecological relevance, occupying different habitat, covering a range of feeding strategies and including major commercial target in the Adriatic Sea. They comprise bivalves (*Mytilus galloprovincialis* and *Ostrea edulis*), cnidarians (*Actinia spp*. and *Rizostoma pulmo*), crustaceans (*Squilla mantis*, *Penaeus kerathurus*, *Nephrops norvegicus*, *Palaemon spp.*), echinoderms (*Paracentrotus lividus*),

polychaetes (*Sabella spallanzanii*), ctenophores (*Mnemiopsis leydi*) and among fish: *Sardina pilchardus, Scomber scombrus*, *Trachurus trachurus*, *Merluccius merluccius*, *Mullus barbatus*, *Chelidonichtys lucerna*, *Solea solea*, *Sardinella aurita*, *Diplodus vulgaris*, *Pagellus erythrinus*, *Spondylosoma cantharus*, *Trachinus draco*, *Lithognatus mormyrus*.

Table 3.1 and 3.2 show, for invertebrates and fish respectively, the number of collected organisms for each species in each sampling site and their characteristics.

Individuals of *M. galloprovincialis*, *Actinia spp*., *S. spallanzanii, O. edulis* and *P. lividus*, which populate the intertidal zone, were picked up through snorkeling activities; all the others were caught by local fishermen using gillnets at 2 nautical miles from the coast. When possible, the same species have been sampled from each area, otherwise, they were replaced with organisms with similar ecological characteristics.

After sampling, the whole soft tissue of invertebrates and the digestive tract (from buccal cavity to anus) of fish were collected and stored at -20 °C for microplastics analysis.

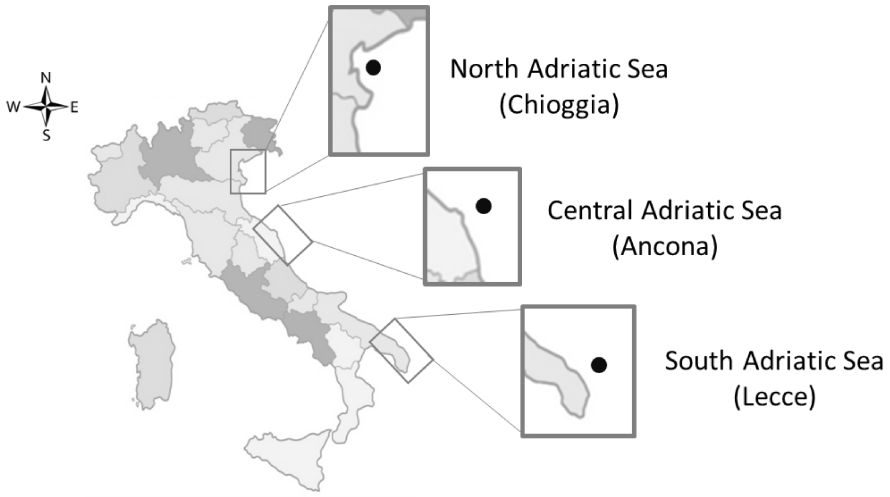


Figure 3.1 *Study areas and sampling site localization.*

Table 3.1 *Species, habitat, feeding mode, number of analysed specimens and morphometric parameters of invertebrates collected in the North (Chioggia), Center (Ancona) and South (Lecce) Adriatic Sea. Morphometric parameters are given as mean ± standard deviation.*

Sampling Site	Species	Habitat	Feeding habits	N° of analysed organisms	Lenght (cm)	Weight of soft tissue (g)
CHIOGGIA	*M. galloprovincialis*	Benthic	Filter feeder	10	5.5 ± 0.5	5.5 ± 1.9
	O. edulis	Benthic	Filter feeder	10	5.3 ± 0.7	1.5 ± 0.7
	S. spallanzani	Benthic	Filter feeder	10	-	14.3 ± 6.9
	Actinia spp.	Benthic	Carnivorous	10	-	6.4 ± 2.7
	S. mantis	Benthic	Carnivorous	10	16.1 ± 1.8	12.8 ± 4.7
	P. kerathurus	Benthic	Planktivorous	10	19.8 ± 0.9	27.5 ± 3.9
	N. norvegicus	Benthic	Detritivorous	10	20.1 ± 1.7	39.9 ± 7.5
	P. lividus	Benthic	Grazer	3	-	11.3 ± 1.3
ANCONA	*M. galloprovincialis*	Benthic	Filter feeder	20	5 ± 1	3.6 ± 1
	O. edulis	Benthic	Filter feeder	10	4.3 ± 0.9	1.9 ± 0.8
	S. spallanzani	Benthic	Filter feeder	8	22 ± 5	3.7 ± 0.4
	Actinia spp.	Benthic	Carnivorous	10	-	12.8 ± 4.4
	S. mantis	Benthic	Carnivorous	10	16.5 ± 1.1	4.9 ± 1.6
	P. kerathurus	Benthic	Planktivorous	10	13.5 ± 0.7	9.5 ± 1.4
	P. lividus	Benthic	Grazer	7	-	13.9 ± 5.9
	M. leydi	Pelagic	Planktivorous	10	-	17.5 ± 5.1
LECCE	*M. galloprovincialis*	Benthic	Filter feeder	18	5.2 ± 0.4	3.7 ± 1.3
	O. edulis	Benthic	Filter feeder	13	5.1 ± 1.4	3.5 ± 2.9
	S. spallanzanii	Benthic	Filter feeder	15	25.4 ± 3.1	5.4 ± 1.8
	Actinia spp.	Benthic	Carnivorous	9	-	21.2 ±1.3
	Palaemon spp.	Pelagic	Carnivorous	21	3.6 ± 0.3	0.2 ± 0.06
	P. lividus	Benthic	Grazer	11	-	10.1 ± 5.6
	R. pulmo	Pelagic	Planktivorous	14	-	185.8 ± 105.9

Table 3.2 *Species, habitat, feeding mode, number of analysed specimens and morphometric parameters of fish collected in the North (Chioggia), Center (Ancona) and South (Lecce) Adriatic Sea. Morphometric parameters are given as mean ± standard deviation.*

Sampling Site	Species	Habitat	Feeding habits	N° of analysed organisms	Lenght (cm)	Weight (g)
CHIOGGIA	*S. pilchardus*	Pelagic	Planktivorous	20	12.6 ± 1.2	18.1 ± 5.7
	S. scombrus	Pelagic	Carnivorous	10	27.8 ± 0.7	226.6 ± 19.8
	T. trachurus	Pelagic	Carnivorous	10	21.5 ± 2.2	86.9 ± 28.9
	M. merluccius	Benthopelagic	Carnivorous	10	20.4 ± 1.4	62.9 ± 15.2
	M. barbatus	Demersal	Carnivorous	10	12.5 ± 0.3	21 ± 1.8
	C. lucerna	Demersal	Carnivorous	7	31.8 ± 2.3	342.9± 74.8
	S. solea	Demersal	Carnivorous	10	28.2 ± 0.8	222.6 ± 23.3
ANCONA	*S. pilchardus*	Pelagic	Planktivorous	13	12.9 ± 0.75	14.9 ± 0.53
	S. scombrus	Pelagic	Carnivorous	10	23.2 ± 0.7	117.6 ± 10
	T. trachurus	Pelagic	Carnivorous	10	19.7 ± 1	58.4 ± 6.9
	M. merluccius	Benthopelagic	Carnivorous	10	17.3 ± 0.8	34 ± 6.5
	M. barbatus	Demersal	Carnivorous	10	13.4 ± 0.7	27 ± 4.2
	C. lucerna	Demersal	Carnivorous	9	20.3 ± 1.6	75.4 ± 15.4
	S. solea	Demersal	Carnivorous	10	17.9 ± 0.5	45 ± 5.2
LECCE	*S. aurita*	Pelagic	Planktivorous	9	25.2 ± 2.3	119.2 ± 21.6
	T. trachurus	Pelagic	Carnivorous	8	24.4 ± 1	121.3 ± 19.4
	D. vulgaris	Benthopelagic	Carnivorous	14	15.3 ± 2.6	71.1 ± 37.8
	P. erythrinus	Benthopelagic	Carnivorous	6	16.1 ± 1.1	60.8 ± 6.8
	S. cantharus	Benthopelagic	Carnivorous	9	16.9 ± 6.7	86.1 ±8.1
	M. barbatus	Demersal	Carnivorous	8	14.6 ±0.8	28.3 ± 4.7
	T. draco	Demersal	Carnivorous	6	23 ± 2.6	77.3 ± 23.6
	L. mormyrus	Demersal	Carnivorous	7	18.8 ± 1.9	78.4 ± 12.6

3.2.2 Method for MPs extraction and polymer characterization

An increasing number of protocols have been developed for separation and identification of microplastics consumed by biota, that, often, makes difficult robust comparisons of findings across different studies. Existing MPs extraction methods from tissues include visual separation, flotation separation, and organic matter digestion (Miller at al., 2017).

Although visual separation is commonly used to separate microplastics from tissue based on physical characteristics or the 'hot needle test', the likelihood of microplastics being trapped within tissues and therefore not detected is high (Miller et al., 2017). As a result, density separation and/or digestion of organic matter are most often employed prior to visual sorting to isolate microplastics (Lusher et al., 2017b).

Although density separation is most commonly applied in studies of water and sediment samples, microplastic particles can be separated from tissues by flotation with saturated salt solutions of high density (Lusher et al., 2017b). Dried sample is usually mixed with concentrated salt solution and agitated (e.g. by stirring, shaking, aeration) for a certain amount of time. Plastic particles float to the surface or stay in suspension while heavy particles such as sand grains settle quickly. Subsequently, microplastics are recovered by filtering the supernatant and examining the resulting material under microscope. Depending on the solution used, different fractions of the range of consumer polymers are targeted: the higher the density of the solution the more polymer types can be extracted (Löeder and Gerdts, 2015). Density separation has been recommended by the MSFD (EU) for Europe and NaCl is suggested because it is inexpensive and non-hazardous compared to more dense saline solutions ($ZnCl_2$, $ZnBr_2$, NaI, $3Na_2WO_4{\cdot}9WO_3{\cdot}H_2O$ and $Li_6(H_2W_{12}O_{40})$), however, the use of NaCl could lead to an underestimation of more dense particles (>1.2 g cm^{-3}) (Bour et al., 2018).

Traditionally, digestion treatments to remove biological materials are conducted using acids (e.g. HNO3, HClO4), oxidizing agents (e.g. H_2O_2, K2S2O8) or strong bases (e.g. NaOH, KOH). However, several plastic polymers can be degraded or damaged by chemical treatments (Miller et al., 2017), thus validation of suitable reagent concentrations and appropriate temperature are required when decided to perform digestion. More promising is the use of enzymes (e.g. lipase, amylase, proteinase, chitinase, cellulase) as a plastic friendly purification step and are less hazardous tecnique to human health (Löeder and Gerdts, 2015). The trade-off is a protracted method, with high time-consuming when considering large-scale field sampling and monitoring (Lusher et al., 2017b).

Due to the challenges in visually identifying microplastics, analytical techniques should be used to confirm the identity of suspected polymeric material. They can include: Fourier Transformed Infra-Red spectrometry (FT-IR); Raman spectrometry and Pyrolysis–Gas Chromatography combined with Mass Spectroscopy (Pyr-GC-MS), which analyses particles using their thermal degradation properties. Alternate analytical methods are: high temperature gel-permeation chromatography (HT-GPC) with IR detection; SEM–EDS and thermoextraction; and, desorption coupled with GC/MS (Eriksen et al., 2013; Dumichen et al., 2015; Hintersteiner et al., 2015). The method employed is often dictated by the equipment available and whilst any chemical characterisation of the polymers recovered is useful, some techniques are more robust than others (Lusher et al., 2017b). The current recommended technique is FTIR, due to the simplicity of analysis and diagnostic spectral information that it provides (Shim et al., 2017).

IR spectroscopy takes advantage of the fact that infrared radiation excites molecular vibrations when interacting with a sample. The excitable vibrations depend on the composition and molecular structure of a substance and are wave-length specific. The energy of the IR radiation that excites a specific vibration will be absorbed to a certain amount, depending on the wave length, which enables the measurement of characteristic IR spectra. Plastic polymers possess highly specific IR spectra with distinct band patterns making IR spectroscopy an optimal technique for the identification of microplastics (Hidalgo-Ruz et al., 2012). The comparison with reference spectra is, then, necessary for polymer identification. Moreover, FTIR

spectroscopy can provide further information on physico-chemical weathering of sampled plastic particles by detecting the intensity of oxidation (Corcoran et al., 2009).
Large particles can be easily analyzed by an FTIR surface technique (attenuated total reflectance (ATR) FTIR spectroscopy), but, if coupled with microscopy (µFTIR), FTIR can be used to identify microplastics with a size around 20 µm (Hidalgo-Ruz et al., 2012; Song et al., 2015). In this context, the use of two measuring modes is feasible: reflectance and transmittance. The reflectance mode bears the disadvantage that measurements of irregularly-shaped microplastics may result in non-interpretable spectra due to refractive error (Harrison et al. 2012). The transmittance mode needs IR transparent filters (e.g. aluminium oxide) and is limited, owing to total absorption patterns, by a certain thickness of the microplastic samples. However, the additional use of micro-ATR objectives in combination with microscopy can circumvent this as IR spectra are collected at the surface of a particle (Löeder and Gerdts, 2015).
In the present study the gastrointestinal tracts of fish and whole soft tissues of invertebrates were processed according to the procedure proposed by Avio et al., (2015). It consists on trituration of dried samples followed by flotation in NaCl hypersaline solution (1.2 g cm^{-3}), settling for 10 min and filtration of the supernatant under vacuum on a sterile cellulose membrane (8 µm pore size). The membranes with retained materials are then transferred in a petri dish with a 15% H2O2 solution for the partial digestion of residual organic matter and allowed to dry in oven (50 °C, overnight), before the visual sorting and the final FT-IR characterization.
The original method was validated and standardized on fish samples spiked with PS and PE microplastics of different sizes; in a comparison with other available methodologies, it showed a recovery yield higher than 90% for particles of dimensions between 5 and 0.1 mm, while nearly 80% for microplastics smaller than 0.1 mm and no effects on polymers characteristics (Avio et al., 2015b). Moreover, the protocol has already been applied to analyse microplastics in several marine organisms collected in the field, showing as the use of NaCl salt can allow to extract also polymers denser than the hypersalin solution, such as Nylon, PVC, PET, PU (Avio et al., 2015b, 2017; Bour et al., 2018).
The visual sorting phase was performed under stereomicroscope: all particles were isolated, photographed, measured at their largest cross section to be assigned in four size classess (5-1mm; 1-0.5 mm; 0.5-0.1 mm; 0.1-0.01 mm) and categorized according to their shape (fragments, film, pellet, line).
According to Viršek et al., (2016), criteria for shape characterization were the following: fragments were considered the irregular shaped particles, like crystals, powder and flakes, rigid, thick, with sharp crooked edges and irregular shape; pellets were particles with spherical shape, like common resin pellets, spherical microbeads and microspheres; films appeared in irregular shapes, thin and flexible and usually transparent in comparison with fragments; lines or filaments were characterized by regular diameter along the particles and not frayed ends; textile fibers, appearing like ribbon, with not regular diameter along the particles and frayed ends.
Isolated particles were then analysed for polymeric identification in ATR mode, using a µFT-IR microscope (Spotlight i200, Perkin Elmer) coupled to a spectrometer (Spectrum Two, Perkin Elmer). Following background scans, 16 scans were performed for each particle, with a resolution of 4 cm^{-1}. Spectrum 10 software was used for the output spectra and the polymer identification was performed by comparison with several libraries of standard spectra. Polymers matching with reference spectra for more than 70% were accepted as microplastics, whilst those with a lower level of certainty (60–70%) were subjected to further visual examination of spectra characteristics before being accepted or rejected (Lusher et al., 2015; Klein et al., 2015).

3.2.3 Contamination control

Great care was taken during this study to minimise microplastic contamination, mainly due to textile fibers. Nitrile gloves and cotton clothing was worn; workbench and equipment were carefully cleaned, lids were

placed over samples wherever possible and all the solutions used (hypersaline solution, deionized water, 15% H_2O_2) have been prefiltered with a porosity filter 0.45 µm. Dissection instruments were soaked in ethanol between samples to avoid cross contamination and each object, used during the extraction phase of the microplastics, was rinsed with deionized water and dried with compressed air.
In addition, a control analysis was carried out every 20 samples, processing the blank samples with pre-filtered water (filter 0.45µm) and subjecting them to the same conditions and to all the steps performed for the actual samples.
Textile fibers were characterized both in blanks, where an average of 3.5 fibers/sample were found, and in organisms, in which the amount of fiber/individual ranged from zero to up 50: the 80% of them has been identified as cotton and wood by µFT-IR analyses. However, it has been decided to exclude textile fibers from results that will be presented below, due to the high variability observed in organisms and to the natural origin of the majority of analysed fibers, and to minimize the risk of their presence, related to air contamination, despite some clean lab procedures have been adopted.

3.2.4 Data analysis

Since data did not satisfy the assumptions required to perform a parametric ANOVA, non-parametric tests were applied to compare the number of MPs extracted per individual considering fish *vs* invertebrates, single species, sampling sites, organism habitat, feeding mode. The Kruskal–Wallis H test for multiple comparisons and the Mann–Whitney U test for pairwise comparisons were used. Level of significance was set at $\alpha < 0.05$. Statistical analyses were conducted using GraphPad® Prism.

3.3 Results

A total of 475 organisms were examined in this study, 259 specimens of invertebrates representative of 11 species and 216 fish representative of 13 species. Microplastics were detected in almost all the investigated species, excepting for *M. galloprovincialis*, *O. edulis, T. trachurus* and *M. barbatus* collected in Chioggia, *M. leydi* and *T. draco* sampled in Lecce and for *Actinia spp.,* irrespective of the three sampling areas (Table 3.3, Table 3.4).
Overall, microplastics were found in 122 of the analysed organisms (i.e. the 25.7%) (Table 3.3, Table 3.4); the number of particles extracted per specimens ranged between 1 and 4, with an average of 1.34 ± 0.61 items/individual. Detected microplastics were mostly constituted by fragments (51%), followed by lines (23%), films (20%) and pellet (6%) (Figure 3.2A). The 63% of plastic particles were less than 0.5 mm and quite equally distributed in the three size classes 0.5-0.1 mm (32%), 0.1-0.01 mm (31%)and 5 to 1 mm (26%) , while the remaining 11% measured between 1 and 0.5 mm (Figure 3.2B). The chemical characterization through µFT-IR identified 19 different polymers where five typologies dominated, accounting for the 80% of the total: polyethylene (PE, 35%), polypropylene (PP, 18%), polystyrene (PS, 10%), polyester (PES, 10%) and polyamide (PA, 7%) (Figure 3.2C-H).
Results were also analysed depending on species (i.e. invertebrates and fish), site of collection (i.e. Chioggia, Ancona, Lecce), organism habitat (i.e. benthic, demersal, benthopelagic, pelagic) and trophic mode (i.e. grazers, filter feeders, detritivorous, planktivorous, carnivorous).
The majority of positive fish and invertebrates contained only 1 (71%) or 2 microparticles (21% of invertebrates and 29% of fish), 3 or 4 items per individual were extracted only from the 3% and 5% of invertebrate species, respectively. On average, concerning invertebrates, values ranged from 1 item/individual in *S. spallanzanii, N. norvegicus, P. lividus* from Chioggia, *O. edulis* from *Ancona* and *P. kerathurus,* from both sites, to 2.5 ± 2.1 in *O. edulis* from Lecce (Table 3.3). Among fish species, 1 plastic particles were found in *S. solea* from Chioggia and Ancona, in *T. trachurus* from Ancona and Lecce, in *D.*

vulgaris, *S. cantharus*, *M. barbatus*, *L. mormyrus* from Lecce, while, *M. merluccius* from Chioggia exhibited the highest value of item/individual with a mean of 1.7 ± 0.6 (Table 3.4).

However, no statistical significant differences were obtained in the number of MPs found per individual between invertebrates and fish considering both the overall specimens (Mann Whitney test, p = 0.8) and in relation to sampling sites (Kruskal-Wallis test, p=0.44) (Table 3.3, Table 3.4).

Significant differences were not obtained even among single invertebrate species (Kruskal-Wallis test, p= 0.47) (Figure 3.3A) and single fish species (Kruskal-Wallis test, p= 0.72) (Figure 3.4A), that were considered without site differentiation.

Concerning the frequency of occurrence of MPs (i.e. percentage of positive organisms, that is individuals containing at least 1 plastic particle): it was similar between the overall invertebrates (24.3%) and fish (27.3%) (Table 3.3, Table 3.4), but, when considering positive organisms in relation to site of collection, a lower percentage was detected in both invertebrates (11%) and fish (16.8%) of Chioggia, than those of Ancona (29.4% and 34.7%) and Lecce (29.7% and 31.3%), where frequencies were comparable (Table 3.3, Table 3.4).

Analysing the frequency of MPs occurrence for single species of invertebrates: the lowest percentage has been recorded in *N. norvegicus* (10%), the highest in *Palaemon spp.* (43%), for the others, the percentage of organisms containing plastic particles ranged from 15% to 30% (Figure 3.3B).

About fish, *T. trachurus* (10%) and *L. mormyrus* (14.3%) were the species with the lowest percentage of organisms positive to microplatics ingestion; the highest frequency was, instead, observed for *P. erythrinus* (66.7%) and *S. cantharus* (55.6%), in the other species microplastics were found in the digestive tract of around the 20%-35% of organisms (Figure 3.4B).

Around the 60% of particles extracted from Northern organisms were lines in invertebrates and films in fish, conversely, roughly the same percentage were represented by fragments in organisms of Central and Southern Adriatic (Figure 3.5A). Microplastics less than 0.1 mm have not been found in none specimens of Chioggia and in fish of Lecce, the upper size class between 5 and 1 mm predominated in invertebrates of Chioggia (80%) and fish of Lecce (71%). Organisms of Ancona have mostly ingested microplastics of the lower size classes (0.1-0.01 mm and 0.5-0.1 mm), accounting together for the 84% of particles extracted from invertebrates and 94% from fish. The majority of microplastics found in invertebrates of Lecce were between 0.5 mm and 0.01 mm in size for a total contribution of 65%, followed by a 22% of microplastics between 5 and 1 mm and a 13% of those in the range 1-0.5 mm (Figure 3.5B). While a high heterogeneity of polymers typologies has been found in organisms of North and South Adriatic Sea, even if, in the latter, PE was the most present in fish (62% of particles), microplastics extracted from species of Center Adriatic were almost exclusively of PE, PP, PS and PA (Figure 3.5C).

Considering characteristics of microplastics in relation to invertebrates without site differentiation (Figure 3.3C-E), it has been highlighted that fragments prevailed in almost all the species, representing the 100% of plastic particles extracted from *R. pulmo*, instead, in *Palaemon spp.* the 91% of detected microplastics were lines (Figure 3.3C). Line was also the shape of the only one item of polystyrene found in *N. norvegicus* that measured 3.2 mm (Figure 3.3C-E). Only in tissues of *P. lividus* all the three typologies of particles shape have been found (Figure 3.3C). Microplastics extracted from *R. pulmo* were less than 0.5 mm, those from *O. edulis* and *P. kerathurus* were less than 1 mm. Also *M. galloprovincialis* and *S. spallanzani* showed mostly the accumulation of particles of that dimensions, with a contribution of approximately 16% of items between 1 and 5 mm. The upper size class of microplastics (5-1 mm) has been found instead at high frequency in *Palaemon spp*. (65%), followed by the size classes 1-0.5 mm (26%) and 0.1-0.01 mm (9%). No plastics between 1 and 0.5 mm were extracted from *P. lividus*, and the same resulted to be the less frequent in *S. mantis*, together with that of 0.5-0.1 mm (11% each), while the lower (0.1-0.01 mm) and higher (5-1 mm) size classes were the most represented in this species (44% and 34%, respectively) (Figure 3.3D). The greater heterogeneity of polymers was identified for microplastics extracted from *S. spallanzanii*: 22.7 % were PP, 18.2% polyvinylchloride (PVC), 13.6% PE, 4.5% PS, polyethylene terephthalate (PET), polyvinyl alcohol (PVA)

and polyisoprene and 9.1% were PA, polyurethane (PU) and silicone. Although in *M. galloprovincialis* different plastic typologies has been found, such as PA (16.7%), ethylene-vinyl acetate (EVA), as both homopolymer and copolymer, polyterpene rubber PP (8.3% each), however most of the particles were of PE (50%). Polyester were found in high abundance in *Palaemon spp.,* accounting for the 73% of the total. *P. kerathurus* showed the lowest heterogeneity of polymers represented by PP and PS (Figure 3.3E).

Considering characteristics of microplastics in relation to fish without site differentiation (Figure3.4C-E): films, fragments and lines were extracted from *S. pilchardus, S. scombrus* and *M. barbatus,* but, while in *S. pilchardus* (42%) and *S. scombrus* (61.5%) fragments were found more frequently than the other shapes and MPs were overall mostly between 0.5 and 0.1 mm in size, in *M. barbatus* lines were the predominant shape type (43%), and 5-1 mm along with 0.1-0.01 mm, the predominant size classes (43% each) (Figure 3.4 C,D). *T. trachurus* exhibited only lines of PE and PES in the same proportion, while only fragments of PE (67%) and PP (33%) were extracted from the gastrointestinal tracts of *D. vulgaris*, in both cases particles had dimension between 5 and 1 mm or 0.5 and 0.1 mm. Microplastics detected in *M. merluccius* were mainly films (54.5%), followed by fragments (27.2%) and pellet (18.3%), this species showed the highest heterogeneity of MPs size and polymers among fish, since all the size classes were represented (5-1 mm and 0.1-0.01 with the 36% of the total, 0.5-0.1 with the 19% and 1-0.5 mm with the 9%) and 6 different polymers where identified (PE, PP, EVA accounting each for around the 18% and PS, PA and a PVC-PVA-PE copolymer accounting each for around 9%). *C. lucerna and S. solea* have ingested only films and fragments smaller than 0.5 mm, that in *C. lucerna* were mostly of PE (83%) and the remaining of polyurethane (PU), while in *S. solea* PE, PVC, PA have been found at same frequency (33.3%). From specimens of *S. aurita* pellets and fragments of PE, EVA and polyacrylates (PAK) were identified and they have been ascribed in the size classes between 5- 1 mm and 0.5-0.1 mm. *P. erythrinus* ingested only larger particles (5-1 mm), represented by fragments (60%) and lines (40%) of PE. Fragment, pellet and lines larger than 0.5 mm have been extracted from *S. cantharus* that were for the 80% of PE and 20% of PP. From organisms of *L. mormyrus* only one plastic fragment of PE (size 0.39 mm) was detected.

In addition, results were elaborated taking into account the habitat (i.e. benthic, demersal, benthopelagic, pelagic) and feeding mode (i.e. grazers, filter feeders, planktivorous, carnivorous) of species (see Table 3.3 and Table 3.4 for these informations on single species).

No significant differences were observed concerning the average number of microplastics extracted per individual, neither between habitats (Kruskal-Wallis test, p=0.99) (Figure 3.6A), nor between trophic groups (Kruskal-Wallis test, p=0.89) (Figure 3.7A).

Despite this, benthopelagic species showed the highest percentage of positive organisms (41%), followed by pelagic species (27.4%), demersal (19.5%) and benthic ones (18%) (Figure 3.6B), worthy to note, demersal organisms are represented by fish species and benthic by invertebrates (Table 3.4 and Table 3.3). No pellets were extracted from demersal specimens; fragments largely predominated in benthic (61%), demersal (53%) and benthopelagic (50%) organisms; in pelagic organisms the difference between the frequency of occurrence for fragments (38%), films (26%) and lines (32%) was less evident, with a minimal contribution of pellets (4%) (Figure 3.6C). Most of the extracted plastic particles were lower than 0.5 mm in size in benthic (69.5%) and demersal (84%) organisms, in the latter, however, no particles within the range 1 and 0.5 mm were detected; in benthopelagic species predominated larger size class 5-1 mm (58%), while no particular differences in the distribution of microplastics in the four size classes were observed for pelagic individuals (Figure 3.6D). More typologies of polymers were extracted from the gastrointestinal tracts of benthic and pelagic species than those from demersal and benthopelagic. However, over the 60% of plastics in benthic organisms were represented by PE (24.2%), PP (27.3%) and PS (13.6%), while, in pelagic specimens the same percentage has been reached by PE and PES (30% each). Instead, roughly the 60% of the total microplastics extracted from demersal (59%) and benthopelagic (57%) species were of PE only, followed by PA in demersal (17.6%) and by PP (19%) in benthopelagic organisms (Figure 3.6E).

Noteworthy about the trophic groups is that grazers and detritivorous are represented by only one species, *P. lividus* and *N. norvegicus* respectively, moreover, from all the analysed individuals of *N. norvegicus* only one PS line of 3.2 mm was extracted. Thus, comparison between feeding mode can be partly affected by this limitation. Nonetheless, grazers showed a 33% of individuals with at least one particles in tissues, filter feeders the 17%, detritivorous the 10%, carnivorous and planktivorous showed the same percentage (around 25%) of positive organisms to plastic ingestion (Figure 3.7B). Unlike carnivorous, planktivorous and grazers species, filter feeders have accumulated more pellets (25%) in respect to the other shape types (Figure 3.7C). No microplastics in the dimensional range 0.5-1 mm have been detected in grazers, for which most of particles have been classified within 0.5 mm and 0.01 mm (80%), as well as, for planktivorous (73%). The 34% of microplastics extracted from filter feeders measured between 5 and 1 mm, roughly the 25% between 1-0.5 mm and 0.5-0.1 mm, the 16% between 0.1-0.01 mm. In carnivorous animals the size class 5-1 mm represented the 36%, followed by those 0.1-0.01 mm (30%), 0.5-0.1 mm (24%) and 1-0.5 mm (10%) (Figure 3.7D). With the exception of grazers and detritivorous organisms, a certain heterogeneity of polymers were registered in relation to trophic strategy, especially in filter feeders and carnivorous groups, however, PE remained the most frequent (43%) (Figure 3.7E).

Table 3.3 *Results of extraction of MPs from invertebrates, including site of collection, species, number and percentage of positive individuals, number of microplastics in individuals (mean ± standard deviation).*

Sampling Site	Species	N° of analysed organisms	N° of positive organism	% of positive organisms	MPs/individual
CHIOGGIA North Adriatic Sea	*M. galloprovincialis*	10	-	-	-
	O. edulis	10	-	-	-
	S. spallanzanii	10	3	30	1 ± 0
	Actinia spp.	10	-	-	-
	S. mantis	10	2	20	2 ± 1.4
	P. kerathurus	10	1	10	1 ± 0
	N. norvegicus	10	1	10	1 ± 0
	P. lividus	3	1	33.3	1 ± 0
All Chioggia species		**73**	**8**	**11**	**1.25 ± 0.71**
ANCONA Center Adriatic Sea	*M. galloprovincialis*	20	4	20	1.5 ± 0.6
	O. edulis	10	3	30	1 ± 0
	S. spallanzanii	8	6	75	1.3 ± 0.5
	Actinia spp.	10	-	-	-
	S. mantis	10	4	40	1.3 ± 0.5
	P. kerathurus	10	5	50	1 ± 0
	P. lividus	7	3	42.8	1.7 ± 0.6
	M. leydi	10	-	-	-
All Ancona species		**85**	**25**	**29.4**	**1.28 ± 0.46**
LECCE South Adriatic Sea	*M. galloprovincialis*	18	5	27.7	1.2 ± 0.4
	O. edulis	13	2	15.4	2.5 ± 2.1
	S. spallanzanii	15	7	46.6	1.7 ± 1.1
	Actinia spp.	9	-	-	-
	Palaemon spp.	21	9	42.8	1.2 ± 0.4
	P. lividus	11	3	27.3	1.3 ± 0.6
	R. pulmo	14	4	28.6	2 ± 1.2
All Lecce species		**101**	**30**	**29.7**	**1.53 ± 0.89**
Overall invertebrates specimens		**259**	**63**	**24.3**	**1.40 ± 0.73**

Table 3.4 *Results of extraction of MPs from fish, including site of collection, species, number and percentage of positive individuals, number of microplastics in individuals (mean ± standard deviation).*

Sampling Site	Species	N° of analysed organisms	N° of positive organism	% of positive organisms	MPs/individual
CHIOGGIA North Adriatic Sea	*S. pilchardus*	20	5	25	1.4 ± 0.5
	S. scombrus	10	3	30	1.3 ± 0.6
	T. trachurus	10	-	-	-
	M. merluccius	10	3	30	1.7 ± 0.6
	M. barbatus	10	-	-	-
	C. lucerna	7	1	14.3	2 ± 0
	S. solea	10	1	10	1 ± 0
All Chioggia species		**77**	**13**	**16.8**	**1.46 ± 0.52**
ANCONA Center Adriatic Sea	*S. pilchardus*	13	4	30.7	1.3 ± 0.5
	S. scombrus	10	7	70	1.3 ± 0.5
	T. trachurus	10	1	10	1 ± 0
	M. merluccius	10	4	40	1.5 ± 0.6
	M. barbatus	10	2	20	2 ± 0
	C. lucerna	9	4	44.4	1.3 ± 0.5
	S. solea	10	3	30	1 ± 0
All Ancona species		**72**	**25**	**34.7**	**1.32 ± 0.48**
LECCE South Adriatic Sea	*S. aurita*	9	3	33.3	1.3 ± 0.6
	T. trachurus	8	1	12.5	1 ± 0
	D. vulgaris	14	4	28.6	1 ± 0
	P. erythrinus	6	4	66.6	1.5 ± 0.6
	S. cantharus	9	5	55.5	1 ± 0
	M. barbatus	8	3	37.5	1 ± 0
	T. draco	6	-	-	-
	L. mormyrus	7	1	14.3	1 ± 0
All Lecce species		**67**	**21**	**31.3**	**1.14 ± 0.36**
Overall fish specimens		**216**	**59**	**27.3**	**1.29±0.46**

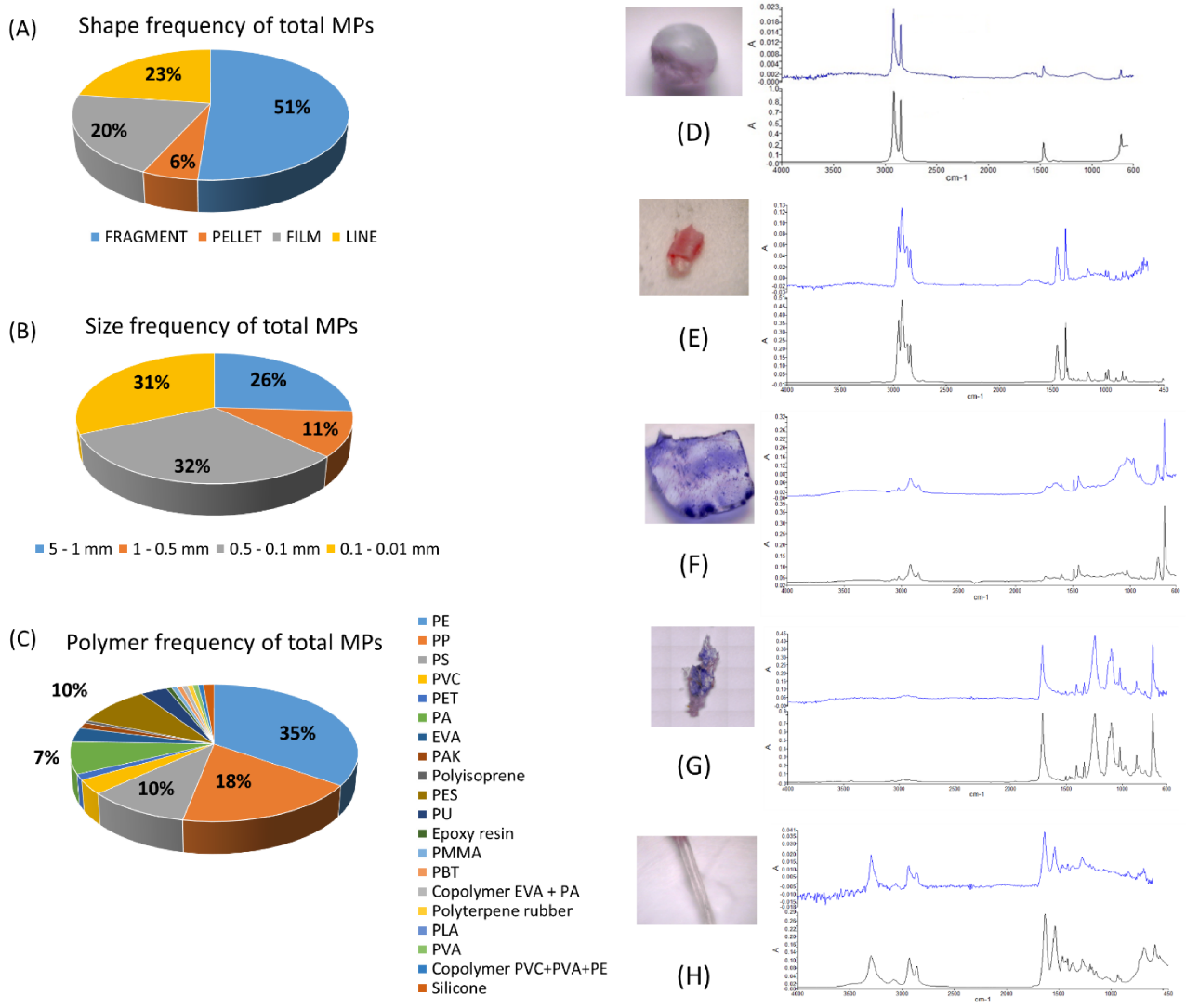


Figure 3.2 *Pie charts showing relative contribution (%) of each shape (A), size (B) and polymers typologies (C) on the total microplastics (n=164) extracted from Adriatic organisms (both invertebrates and fish). Example of particles and IR polymers spectra: (D) pellet of polyethylene; (E) fragment of polypropylene; (F) fragment of polystyrene; (G) film of polyester; (H) line of polyamide.*

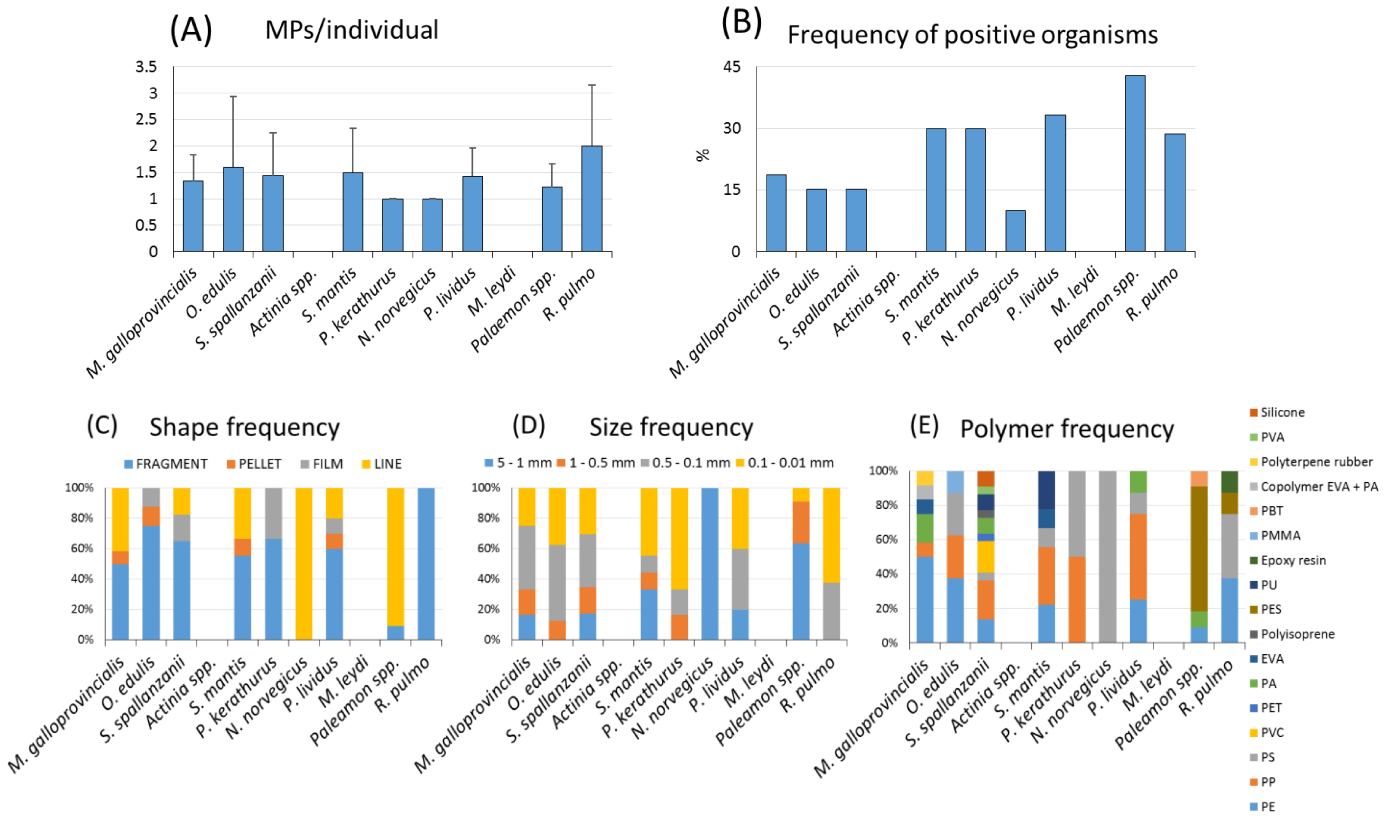


Figure 3.3 *Bar graphs showing the number of microplastics extracted per individual (mean± standard deviation) (A), the frequency of occurrence of microplastics (%) (B) and relative contribution (%) of each shape (C), size (D) and polymers typology (E) on the total microplastics extracted from Adriatic organisms, considering the single invertebrate species.*

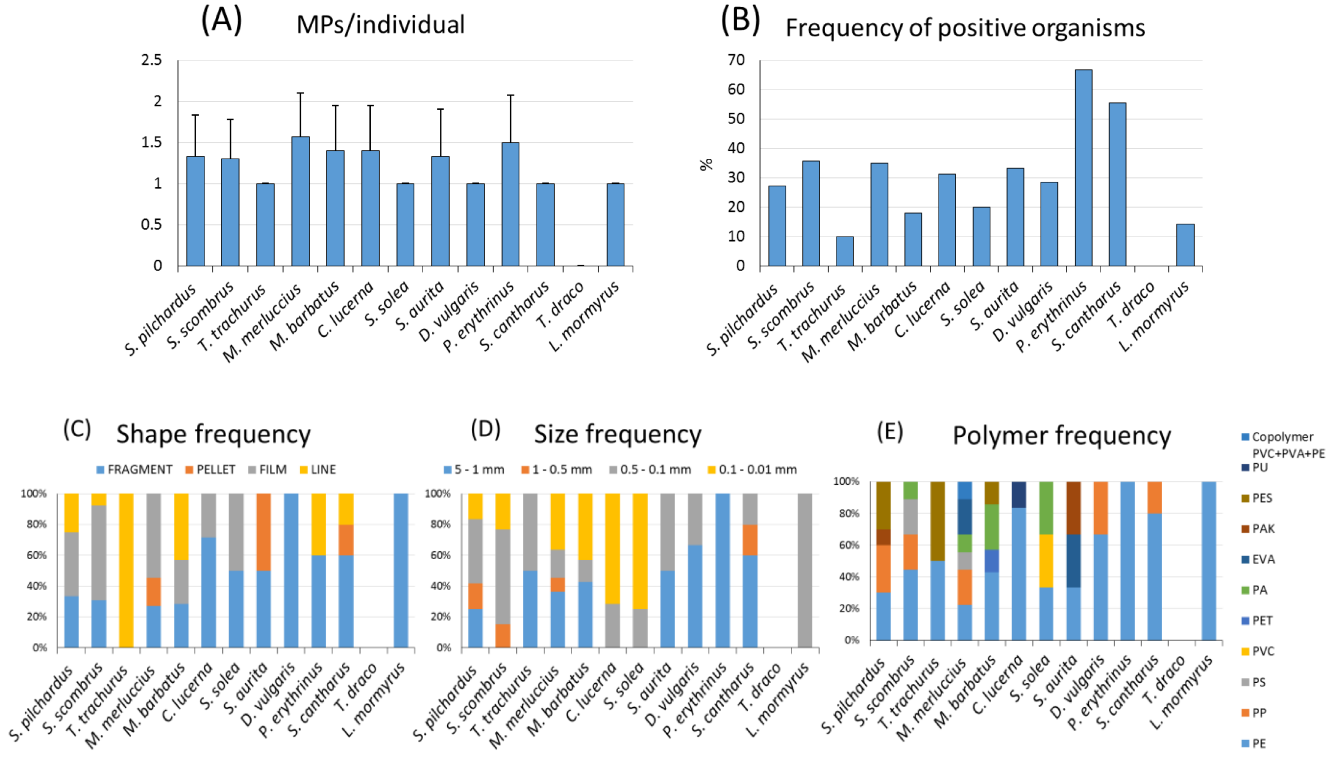


Figure 3.4 *Bar graphs showing the number of microplastics extracted per individual (mean± standard deviation) (A), the frequency of occurrence of microplastics (%) (B) and relative contribution (%) of each shape (C), size (D) and polymers typology (E) on the total microplastics extracted from Adriatic organisms, considering the single fish species.*

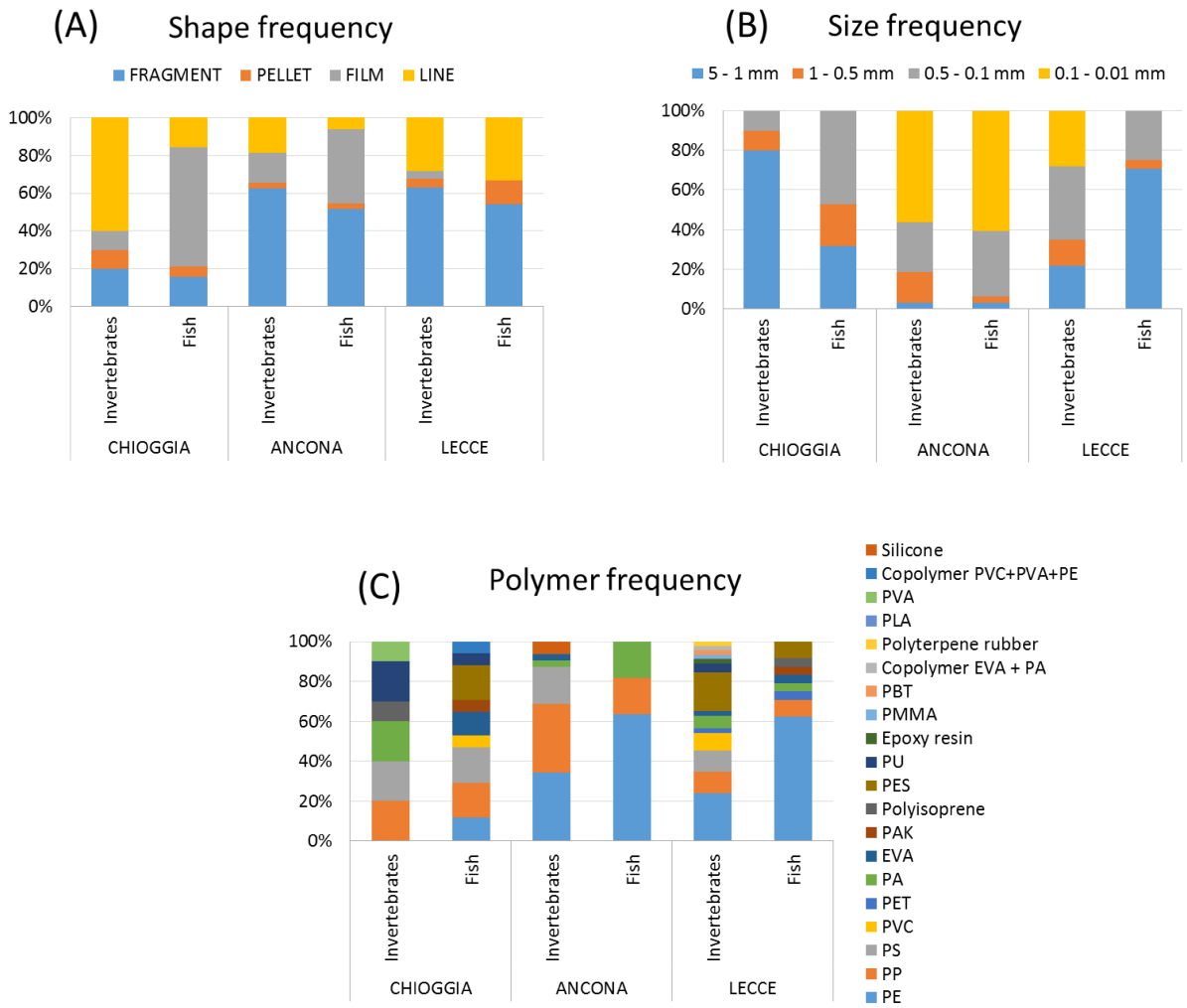


Figure 3.5 *Bar graphs showing relative contribution (%) of each shape (A), size (B) and polymers typology (C) on the total microplastics extracted from Adriatic organisms, considering separately fish and invertebrates for each site of collection (Chioggia, North Adriatic Sea; Ancona, Central Adriatic Sea and Lecce, South Adriatic Sea).*

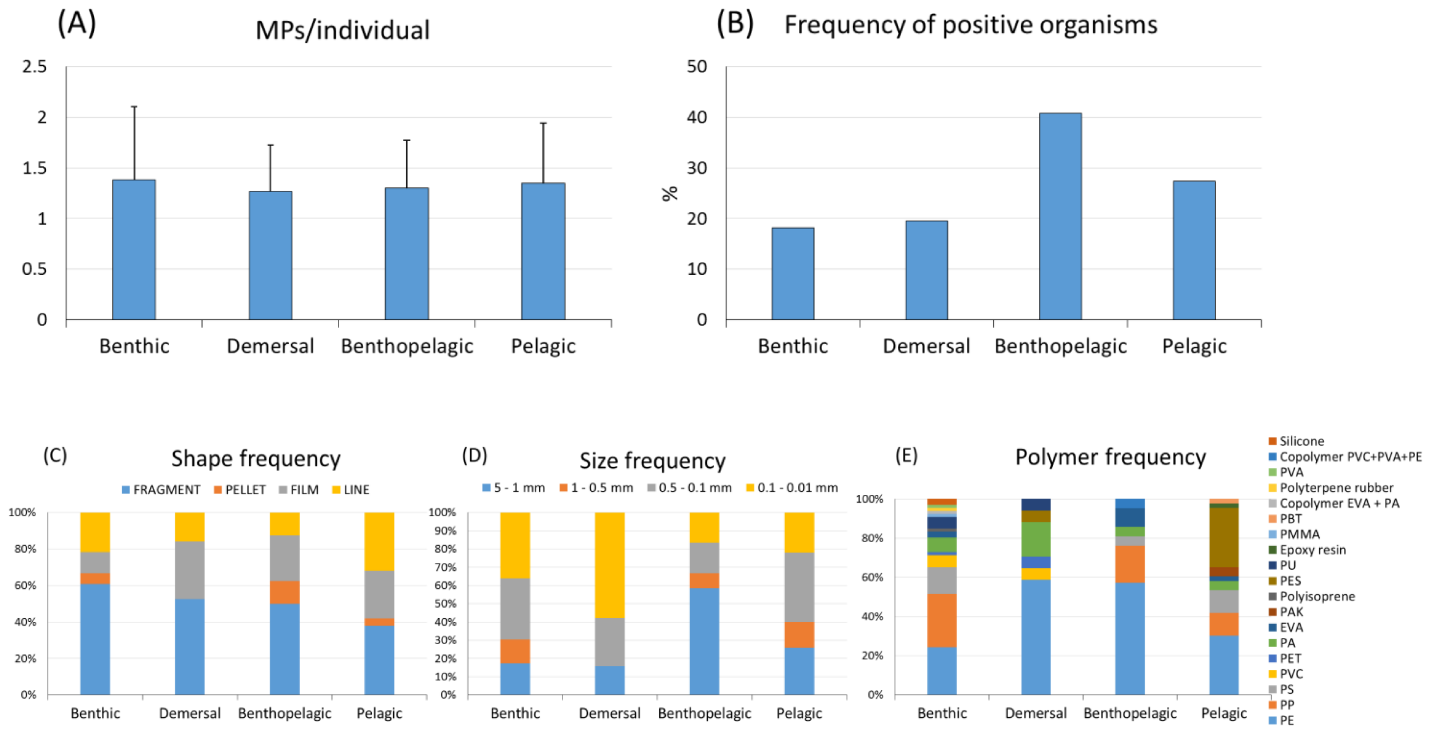


Figura 3.6 *Bar graphs showing the number of microplastics extracted per individual (mean± standard deviation) (A), the frequency of occurrence of microplastics (%) (B) and relative contribution (%) of each shape (C), size (D) and polymers typology (E) on the total microplastics extracted from Adriatic organisms, considering species in relation to habitat which occupy.*

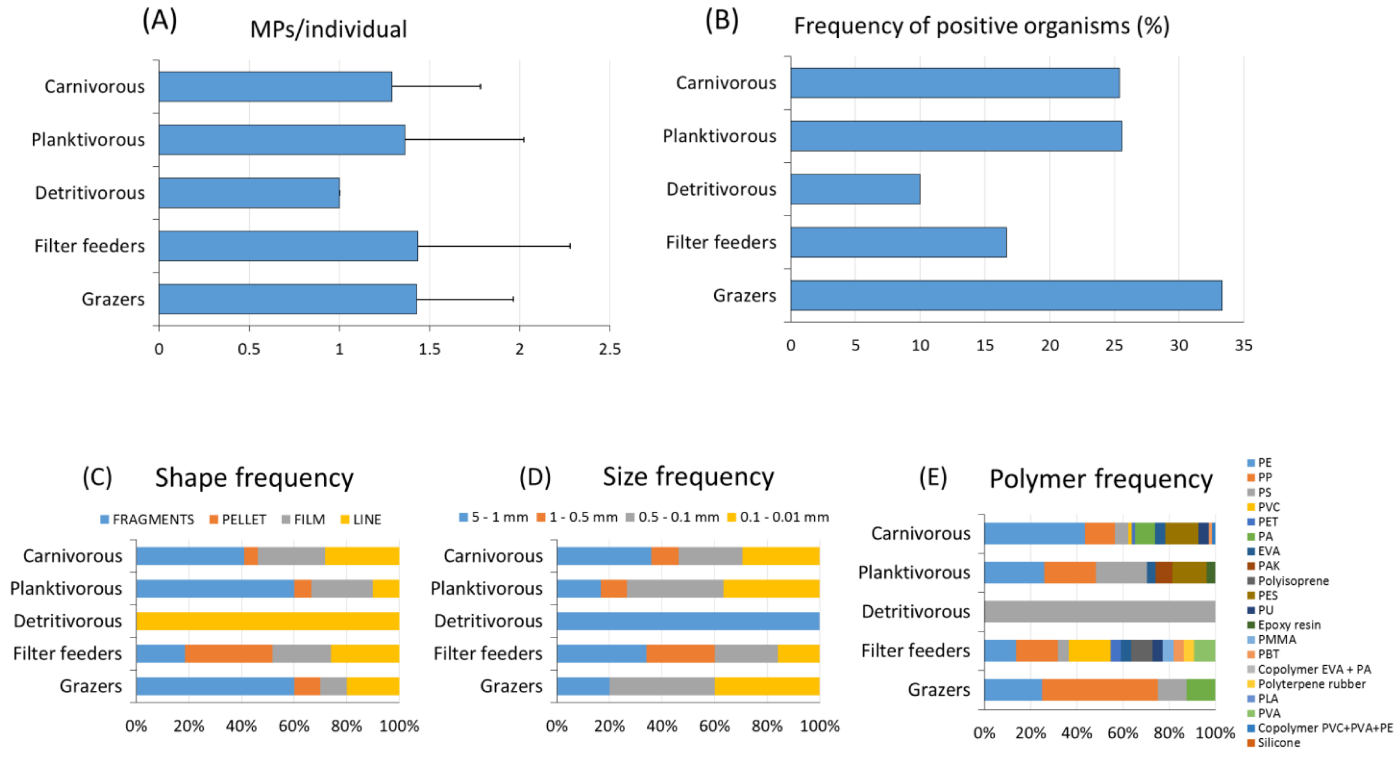


Figura 3.7 *Bar graphs showing the number of microplastics extracted per individual (mean± standard deviation) (A), the frequency of occurrence of microplastics (%) (B) and relative contribution (%) of each shape (C), size (D) and polymers typology (E) on the total microplastics extracted from Adriatic organisms, considering species in relation to feeding mode.*

3.4 Discussion

The present study was aimed to evaluate the ingestion of microplastics in a range of Adriatic organisms collected from a Northern, Central and Southern area along the Italian coast, in order to fill gap in knowledge on the phenomenon, on regional scale. This work was one of the few that have assessed the occurrence of microplastics in natural populations of fish along with in different invertebrate species, including also those not strictly intended for human consumption, such as cnidarians (*Actinia spp*. and *Rizostoma pulmo*), polychaetes (*Sabella spallanzanii*) and ctenophores (*Mnemiopsis leydi*). In spite several reports has been done on the presence of microplastics in the guts and/or tissues of a number of wild marine species, they mostly targeted commercially important mollusks, crustaceans and fish (Lusher et al., 2017a): the potential for humans, as top predators, to ingest microplastics through seafood consumption is plausible and its implications for health need to be considered, but, to date, none real risks have been demonstrated (UNEP, 2016).

The overall results of the study highlighted microplastics ingestion as a widespread phenomenon in the marine food web of the Adriatic Sea, since 21 out of 24 analysed species and around 26% of total organisms showed at least one microplastics in tissues.

Actually, 1 or 2 particles have been mostly found in both fish and invertebrates, as generally reported by previous studies on fish populations of Mediterranean Sea (Anastasopoulou et al., 2013, 2018; Avio et al., 2015b, 2017; Romeo et al., 2015) and of other areas in the world (Boerger et al., 2010; Davison and Asch, 2011; Lusher et al., 2013; Neves et al., 2015; Rummel et al., 2016; Tanaka and Takada, 2016).

In the present study the overall frequency of MPs ingestion for fish (27%) is very close to the 28% reported during a preliminary survey on microplastics content in species collected in the North and Central Adriatic (Avio et al., 2015b) and to the 30% recorded for planktivorous fish from the North Pacific Central Gyres (Boerger et al., 2010), for catfish from a Brazilian estuarine (Possato et al., 2011) and for commercial fish from Indonesia and California areas (Rochman et al., 2015). A much higher percentage of particles occurrence in Adriatic fish has been, instead, observed in the eastern basin (up to 87%) (Anastasopoulou et al., 2018) and in specimens of *Solea solea* (95%) sampled from Trieste to Gargano by Pellini et al., (2018).

More complex is the comparison of results for invertebrates with other studies, because field investigations on the occurrence of MPs in marine invertebrates are still scarce and they include only few species. In addition, a precise quantitative comparison is hampered by the use of different units to express concentrations, which often are given as number of MPs per grams of tissues rather than per individual (Lusher et al., 2017a). Although this urgently requires standardization on the way to express data, the issue can be in part solved, referring to frequency of occurrence rather than abundance of ingested particles.

For example, so far, two studies have analysed the occurrence of microplastics in *N. norvegius*, but Welden and Cowie (2016) gave the results as number of MPs on weight of tissues, not allowing comparison with this work, instead, Murray and Cowie (2011) have recorded a frequency of ingestion of 83% in the Clyde Sea, which is however much higher than the percentage of positive organisms obtained for the same species collected from Chioggia site (10%). It is necessary to consider that in the present study 10 individuals of *N. norvegicus* were analysed versus 120 in the study of Murray and Cowie (2011) and samples size might influence the percentage of positive individuals (Bour et al., 2018).

The lack of significant differences in the number of microplastics ingested per individuals has not allowed to identify any species that can accumulate more plastic than others, nor if site of collection, organism habitat and feeding mode have influenced the abundance of MPs in biota. On the contrary, considering the frequency of ingestion of particles, it was possible to highlight that the likelihood to encounter and ingest microplastics is higher for some species, in particular the benthopelagic ones and for organisms of the Center and South Adriatic compared with those of the Northern site. Some differences have been noticed also in relation to feeding mode of organisms, with the highest percentage of individuals positive to MPs ingestion

recorded in grazers, followed by carnivorous and planktivourous in similar percentage while lower levels were highlighted for filter feeders and detritivorous organisms. However, these results have to be read with caution since the trophic groups are represented by a different number of species and individuals, for example, grazers include only *P. lividus* (n=21) and detritivorous only *N. norvegicus* (n=10) in comparison with the larger group of carnivores (12 species and n=244). As above mentioned, it is plausible that the sample size can influence the percentage of positive organisms and this underlines the importance of increasing the number of individuals and of species with similar ecological characteristics to analyse.

Among invertebrates *Palaemon spp.* was the species that showed the highest frequency of MPs ingestion (43%). To date, only two field studies have been conducted on the presence of microplastics in the most common species of shrimp. Devriese et al., (2015) showed a frequency of ingestion of 63% in individuals of *Crangon crangon* from the Channel Sea and a very similar percentage has been observed by the study of Bour et al., (2018) for *Crangon allmani* (65%). As obtained from the present study, also Bour et al., (2018) found the highest frequency of MPs occurrence exactly in shrimps, in respect to other Norway species. Therefore, shrimps could be considered prime suspects for further investigation in future studies on monitoring of microplastics in the marine environment and also on the process of trophic transfer of these particles (Cole et al., 2013). In fact, these organisms are important food item for a large range of predators and at the same time are opportunistic feeder, consumer of macrofaunal species and juvenile stages of fish, which in turn might have previously consumed plastic, since for some of these preys, MPs ingestion has been demonstrated (Devriese et al., 2015).

Among fish, the benthopelagic *P. erythrinus* and *S. cantharus* showed the highest frequency of MPs occurrence (66.7% and 55.6%). Indeed, the study highlighted that, in general, benthopelagic organisms ingested MPs more frequently than pelagic and, above all, demersal and benthic ones. This tendency has been observed also by Avio et al., (2015b, 2017), which obtained higher percentage of fish with microparticles in benthopelagic Mediterranean species (*S. cantharus* and *M. merluccius*) than in pelagic (*S. pilchardus*) and benthic organisms (*Scorpaena sp*., *Uranoscopus scaber*, *Phycis phycis*, *M. barbatus*, *C. lucerna*). It is quite obvious that organisms moving within the pelagic and benthic habitat are more likely to interact with microplastics that are present in both the compartments. This result underlines the ubiquitous presence of microplastics in the water column and on the seabed and the need to consider the presence of these particles in the marine environment in a dynamic and changing time and spatial perspective, since initially floating particles can sink to sediment and then being remobilized to water column by bioturbation, resuspension or hydrodynamic conditions (Avio et al., 2016).

To explain differences in the frequency of MPs ingestion relating to site of collection it is important to consider that spatial and temporal distributions of plastics in the marine environments depend on input locations and the time-varying intensity of sources, which are highly uncertain. At the same time, ocean currents, waves, and wind control the transport of plastics, redistributing them at sea until they eventually wash ashore or sink (Liubartseva et al., 2016). Following the study of Liubartseva et al., (2016) on distribution and transport of floating plastic debris along the Adriatic Sea, it seems that in the Western coastlines, surface currents drive the accumulation of elevated concentrations of plastics in the Northern and Central basin, on average up to 30 g km^{-2} and 20 g km^{-2}, while lower concentrations of items (up to 3 g km^{-2}), were detected in the Southern Adriatic. This trend does not fully correspond to that observed for frequency of microplastics recorded in biota in the three investigated area, however, it is reasonable to hypothesize possible different dispersion flows by currents due to the smaller dimensions of MPs with respect to large marine litter items. Liubartseva et al., (2016) tried to reconstruct also the sources of plastic debris onto the Adriatic coasts: they revealed that even if more than one-third of the plastics are beached on the coastline from which they originate, complex source–receptor relationships among the various Adriatic subregions exist. For example, rivers runoffs contribute to plastic transport towards areas far from where they directly discharge: it is case of the Central Adriatic that is affected by Po River and of the South Adriatic that receivs runoffs of rivers

entering through the Otranto Strait from the North Ionian Sea. Thus the contribution of rivers runoffs as conduits of plastics in these areas can explain the higher frequency of MPs ingestion recorded in individuals collected from Ancona and Lecce compared with those from Chioggia. Moreover, it could represent the reason why extracted microplastics from Central and Southern organisms are mostly fragments and of the lower size classes (0.5-0.1 mm and 0.1-0.01 mm), in particular those of Ancona, compared with films and pellets of larger size (5-0.5 mm) found in Northern species.

Worthy to note, in the present investigation counts of microplastics did not include textile fibers, thus, results are not fully comparable in terms of both abundance and characteristics of ingested particles, with some of the above mentioned studies reporting also this typology of particles.

For example, the shape characterization of the overall microplastics has highlighted the predominance of fragments (51%) on pellets, lines and films. This result is similar and fully comparable with that reported by Avio et al., (2015b, 2017) on fish of Adriatic and Tyrrhenian Sea, because the authors have excluded fibers in their analyses. Instead, when textile fibers are included, they represents the most abundance type of plastics found in Mediterranean fish (Bellas et al., 2016; Anastasopoulou et al., 2018; Pellini et al., 2018), suggesting the importance to include this class of microplastics in future studies.

Despite shape of microplastics can represent one of the factors that drive their fate in the marine environment (Reisser et al., 2015; Horton et al., 2017) none conclusion can be done by this study over this aspect, since no particular differences in the shape frequency of microplastics has been showed in relation to habitat. This is moreover supported by the fact that species have ingested only fragments, i.e. *R. pulmo*, *L. mormyrus*, *D. vulgaris* belong to different compartments: pelagic, benthopelagic and demersal, respectively.

FT-IR analysis highlighted a certain heterogeneity of polymers ingested by Adriatic organisms, especially in the Northern and Southern areas, in accordance with the innumerable and diversified potential inputs of plastics in the Adriatic basin: mariculture and fisheries, shipping routes, tourism, dense urbanization of the coasts, rivers (Munari et al., 2016). Among the detected polymers, all the Big Six (i.e. polypropylene, polyethylene, polyvinyl chloride, polyurethane, polyethylene terephthalate, polystyrene) accounting for 80% of plastic production in Europe (Paul-Pont et al., 2018) have been found. However, most of microplastics were of PE (35%) and PP (18%), which are presently the polymers predominantly recovered in all environmental compartments (Isobe et al., 2014; Enders et al., 2015; Frère et al., 2017), in accordance with the scale of their global manufacture and use worldwide (Antunes et al., 2013; GESAMP, 2016). Interestingly, benthic species exhibited the highest variety of ingested polymers, that included both denser and lighter plastics, at the same time, polyester, that you would expect to find in the bottom, has been extracted at high frequency (29.5%) from pelagic organisms, confirming that the density of plastics can change due to weathering or other processes in the field (Bour et al., 2018).

In conclusion the present study demonstrated the entrance of microplastics in the marine food web of the Adriatic Sea, providing additional insight on ingestion of particles by a wide range of species and on the factors that might affect their bioavailability in the Adriatic basin. None evidences of biomagnification has been obtained, however a more elevated number of organisms and species with different feeding strategies are required to better assess the distribution of microplastics along the food web, since the trophic transfer has been mentioned as a potential issue. Results of the work have contributed to give new information over the growing need to define the frequency, abundance and characteristics of microplastics ingested by biota, moreover, they can represent the baseline to define the best strategies to adopt in future monitoring studies of these contaminants in the Adriatic Sea.

4 ASSESSMENT OF BIOAVAILABILITY AND BIOLOGICAL EFFECTS OF MICROPLASTICS AND ADSORBED POLLUTANTS

This chapter is based on the following paper:
Pittura L, Avio C. G, Giuliani M. E., d'Errico G, Keiter S. H. , Cormier B., Gorbi S. and Regoli F. (2018). Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels, *Mytilus galloprovincialis*. *Frontiers in Marine Science 5:103*. doi: 10.3389/fmars.2018.00103

4.1 Use of experimental and biomarker approach

Studies on the presence of microplastics in the wildlife demonstrate their availability for the biotic compartment and allow to monitor the abundance, distribution and composition of plastic litter in the marine environment. However, they don't permit to establish nor a direct connection between the ingestion of microplastics and the onset of injurious effects, neither the extent to which such ingestion contributes to the bioaccumulation of environmental chemical pollutants in respect to other pathways (waterborne, sedimentborne or foodborne contamination). It is also necessary to consider that in the field, the bioavailability of microplastics can be affected by many factors (i.e. biofouling and aggregation in marine snow may change MPs buoyancy) (Wright et al., 2013; Porter et al., 2018) and organisms are typically subjected to a variety of other stressors, both natural and anthropogenic (i.e. salinity, pH and temperature variations, mixture of chemical pollutants, eutrophication, hypoxia, altered habitat and hydrologic regimes), that can impact through single, cumulative, or synergistic processes (Adams, 2005; Crain et al., 2008; Hewitt et al., 2016).
Exposure experiments in laboratory are useful to study the biological effects of contaminants with a certain level of control of environmental variables, so, they allow to sort out complexity of the natural environment and to assess the weight of each factor in determining biological disturbance (Paul-Pont et al., 2016). Biological effects can be studied applying sensitive laboratory bioassays based upon responses of biomarkers (van der Oost et al., 2003), that are molecular, biochemical and cellular variations, measured in tissue or body fluid samples or at the level of whole organisms, which provide signals of exposure to xenobiotics and also of a toxic effect (Depledge, 1993; Peakall, 1994). Assessment of such early warning signals may anticipate biological changes at higher levels of organisation like populations, communities and ecosystems, for that, biomarkers are considered as short-term indicators of long-term biological effects (Cajaraville et al., 2000).
In the present study, the bioavailability and the biological effects of MPs and adsorbed pollutant were experimentally investigated under short-term exposure conditions (28 days), using the Mediterranean mussel *Mytilus galloprovincialis* as biological model, low density polyethylene (LDPE) microparticles as representative plastic typology and benzo(a)pyrene (BaP) as organic contaminant (Figure 4.1). Organisms were exposed to both LDPE and BaP alone and to microplastics pre-contaminated with BaP (LDPE-BaP), in order to address both the potential for plastic particles to act as a vector of chemical exposure once ingested and the possible effects caused by single contaminants or their combination. Histological observations were performed to ensure the presence of plastic particles in mussels' tissues, which have been integrated with BaP bioaccumulation analyses and biomarkers measurements, in order to elucidate the role of microplastics in transferring chemical pollutants to organisms and mechanisms of MPs toxicity. Results of this study were expected to provide additional knowledges on the risk of MPs and adsorbed chemicals for marine biota and advance hypotheses on long-term effects on organisms' health status.

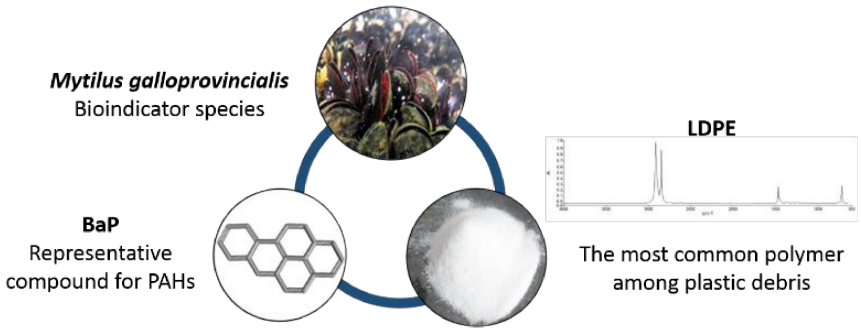


Figure 4.1 *Model organism, microplastics and organic pollutant chosen for the experimental study.*

4.2 Model species, microplastics, organic pollutant and choice of exposure concentrations

M. galloprovincialis was selected for this study as it is widely used as sentinel species in environmental monitoring due to its physiology, behaviour and its broad study in cellular, genetic and biochemical level (Rey-Salgueiro et al., 2017). As filter feeder near the base of the food chain, it is able to accumulate a wide range of contaminants and reflect changes in the contaminant status of the environment (Galloway et al., 2002). Mussels are an important component of benthic assemblages worldwide and are thought to act as ecosystem-engineers via occupation of primary space, filtration, and provision of secondary habitat (Browne et al., 2008). Recently, mussels have been also proposed as possible standard bioindicator for water-born MPs contamination in marine environments (Beyer et al., 2017; Lusher et al., 2017c; Bråte et al., 2018). Actually, the responsiveness of *Mytilus spp* toward microplastics has been documented in the field (Li et al., 2015; Van Cauwenberghe et al., 2014, 2015; Phuong et al., 2018) and in laboratory trials (Browne et al., 2008; von Moos et al., 2012; Avio et al., 2015a; Paul-Pont et al., 2016; Détréé and Gallardo Escárate, 2017, 2018; Capolupo et al., 2018). These organisms are also popular as seafood, indicating a link to the humans' food chain for a potential transfer of contaminants (Bråte et al., 2018; Brandts et al., 2018).

When selecting microplastics in order to assess effects on a model organism, it has to consider typologies that, according to their characteristics, are more likely to be available for that given species. Mussels inhabiting the upper water column, can easily encounter positively buoyant, low-density plastics, such as polyethylene (density 0.91-0.94 g cm^{-3}) (Wright et al., 2013). PE is also, presently, the polymer predominantly recovered in all environmental compartments, in accordance with the scale of their global manufacture and use worldwide (Paul-Pont et al., 2018). Microplastics dimension is an additional factor to take into account when considering exposure experiments. Most studies conventionally choose an MPs size distribution in the same range as that of the test organism' prey (Paul-Pont et al., 2018). Mussels filter suspended particles in seawater up to 500 µm (Goslin, 2003) and studies indicated that they ingest and retain plastic particles up to 80-100 µm after exposure (Ward and Kach, 2009; von Moos et al., 2012; Avio et al., 2015a; Brandts et al., 2018). Microplastics <20 µm have been shown to translocate and pass into cellular membranes, including haemolymph, lysosomal system, digestive tubules (Browne et al., 2008; von Moos et al., 2012), thus, small size particles are more likely to induce larger effects on organisms at the cellular levels (O'Donovan et al., 2018).

In this work, the model microplastic probed was LDPE powder composed of nonuniformly shaped particles ranging from 1 to 40 µm (mean size 20-25 µm), as provided by manufactures (Micro Powders Inc., NY-USA) as the raw material for various industrial applications, mainly related to food packaging, production of reusable bags and agricultural films (Plastic Europe, 2017). Microplastics, both without and with adsorbed BaP, were tested at concentration of 10 mg/L, corresponding to $2.34*10^{7}$ particles/L. These levels are still

high in respect to those reported in the environment (Paul-Pont et al., 2018), although, more similar to values detected in some hotspot areas, such as the waters of Southern North Sea (Dubaish and Liebezeit, 2013) and California Current System (Gilfillan et al., 2009) or the sediments of the Belgian coast (Claessens et al., 2011), of the Artic (Bergmann et al., 2017b) and of the Lagoon of Venice (Vianello et al., 2013). The high dose of microplastics was chosen to provide evidence that if encountered, MPs may be capture, ingested and incorporated by organisms and to elucidate their eco-toxicity after 28 days of exposure.
However, it is worthy to note that data on quantification of MPs in the surface waters is related to particles larger than 333 µm, retained by the manta trawl sampling. Conversely, knowledges of natural levels of microplastics of size similar to those used in this study are poor, despite recent publications demonstrated a high percentage (80%) of MPs 25-50 µm in surface water or sediment, compared with larger sized particles (Enders et al., 2015; Bergmann et al., 2017b) and they estimated an increasingly abundance of smaller MPs, due to the stay of debris in the environment (Erni-Cassola et al., 2017). For these reasons, we might underestimate MPs concentrations in the oceans, as well as the relative importance of small MP fractions in the transfer of hydrophobic organic compounds (e.g. polycyclic aromatic hydrocarbons (PAHs)), especially considering that for the same plastic mass, the surface available for chemicals adsorption is inversely related to the size of plastic debris pieces (Paul-Pont et al., 2018). In addition, the magnitude of this sorption is chemical and polymer dependent; for example, polyethylene and polystyrene have greater affinity for PAHs, than polypropylene (Rochman et al., 2015).
Among the organic contaminants typically found in the marine environment and potentially adsorbed on plastics, the BaP was selected as a representative for PAH compounds. PAHs are widely distributed toxic substances and are among the most water-soluble hydrocarbons, thus allowing them to be accumulated to high concentrations in the tissues of many marine organisms (Wootton et al., 2003). PAHs can enhance the intracellular generation of reactive oxygen species (ROS) with subsequent oxidative damage to macromolecules and some congeners (including BaP) have carcinogenic, mutagenic and teratogenic properties, (Rey Salguerio et al., 2017). Mussels were exposed to environmentally relevant aqueous concentrations of 150 ng/L of BaP (Ren et al., 2015; O'Donovan et al., 2018) that corresponds to 15 µg of compound adsorbed on each gram of LDPE, in the treatment with BaP pre-contaminated microplastics. This concentration approaches levels of total PAHs measured in beached plastic pellet on South America coast of the Atlantic Ocean (Fisner et al., 2013), on Japanese coast of the Pacific Ocean (Teuten et al., 2009) and in pre-production pellets collected from plastic processing facilities in Los Angeles (America) (Rios et al., 2007).

4.3 Adsorption of BaP on LDPE particles

The adsorption procedure of BaP on microplastics was performed in collaboration with the ManTechnology Environment Research Center of Örebro University, Sweden.
Pre-test was conducted on plastics to determine whether there was a background contamination with BaP. Two replicates with 0.25 g of plastic were weighed into 8 mL amber glass vials and extracted three times with 2.5 mL of hexane (≥98%, SupraSolv) with addition of internal standard (500 ng BaP D12 in toluene, Chiron; prepared standard: 10 ng/µL in toluene) by 30 min ultra-sonication. After 10 min of centrifugation upper solvent phase was collected to a new vial. 7.5 ml of the extracted samples were filtrated trough a glass pipet containing fibreglass to eliminate the particles. At that point, the extract was reduced to a volume of 1.5 ml by a nitrogen stream. 500 µL toluene was added and the volume further reduced to 500 µL. GC vials for analysis were filled with 100 ng recovery standard perylene D12 (Chiron) (2 ng/µL in toluene, 50 µL added) and 500 µL of extract transferred from 8 ml vials. In addition, extract vials were rinsed three times with small amount of hexane and added to the sample. The sample volume was reduced to 500 µL using a nitrogen stream. Concentrations of BaP were quantified using a high-resolution GC-MS system (Micromass Autopspec

Ultima), separation on a 30 m (0.25 mm i.d., 25 µm film thickness) DB-5MS column (J&W Scientific, Folsom, USA).

Once the presence of BaP in "virgin" microplastics was excluded, the final plastic material with adsorbed BaP has been prepared. Microplastics were spiked with BaP at concentration of 2,500 µg L^{-1} using 125 g L^{-1} of plastic, weighed into separate 250mL narrowmouth Septa bottles (Thermo scientific) filled with double-deionized water. Bottles were placed on a rotary shaker for 2 days at the lowest speed (20 rpm). The filtration and washing of microplastics was done as described above. Plastic debris were rinsed with double-deionized water and dried by vacuum evaporation. For the determination of the concentrations of BaP on the plastics, chemical analysis were performed in GC-MS system as above, with an internal standard (benzoapyrene-d12). The adsorbed concentration resulted approximately 15 µg BaP/g of LDPE.

4.4 Experimental set-up

Specimens of *Mytilus galloprovincialis* of commercial size (6 ± 1 cm shell length) were purchased on 11th April 2017 from a shellfish farm located off-shore of Ancona, in the Central Adriatic Sea. In laboratory, a stock of approximately 700 organisms were kept in aquaria with artificial seawater at constant aeration and salinity of 37 p.s.u, to facilitate acclimatization at the temperature of 18 ± 1 °C and pH of 8.0 ± 0.5.

The exposure experiment started on 28th April and lasted until 26th May (Figure 4.2). It has been performed in 20L-aquaria, with 60 mussels randomly distributed for each tank in a triplicate design for 4 treatments: control (CTRL); polyethylene microparticles (LDPE); benzoapyrene (BaP); microplastics previously contaminated with benzoapyrene (LDPE-BaP). BaP, virgin and contaminated microplastics were provided according to their respective exposure concentrations and dosed again after each water change; food (a commercial mixture of zooplankton) was supplied 12hours prior, to minimize interactions of microplastics or benzo(a)pyrene with other suspended particles. In addition to an aeration stone, provided to ensure a constant aeration, each exposure aquarium was equipped with a motion pump to avoid microplastics' aggregation on surface or adhesion to walls (Figure 4.2). This reasonable device was already successfully used in other experiments (von Moos et al., 2012; Détrée and Gallardo-Escaráte, 2017; O' Donovan et al., 2018) and also in this study it made possible (and visible) the homogeneous distribution of microplastics throughout the entire tank.

At the end of exposure phase (28 days) and at intermediate times of 7 and 14 days, haemolymph, digestive glands and gills of mussels were recovered and immediately processed or properly stored for the subsequent histological, chemical and biomarkers analyses.

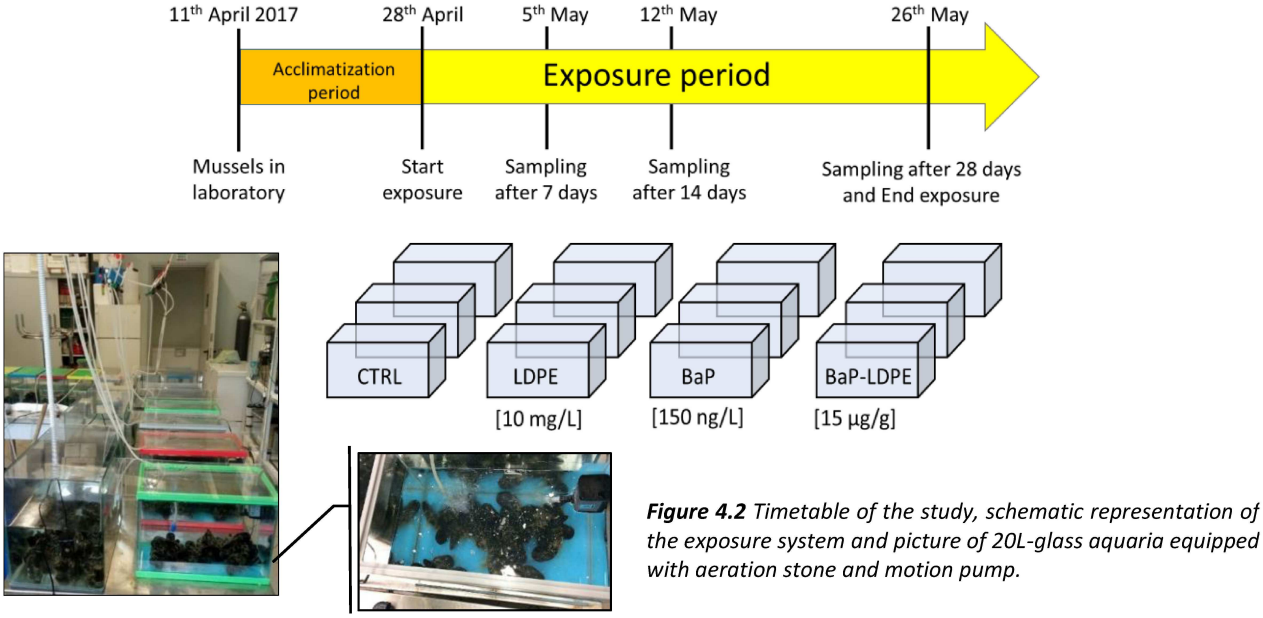


Figure 4.2 *Timetable of the study, schematic representation of the exposure system and picture of 20L-glass aquaria equipped with aeration stone and motion pump.*

4.5 Investigated parameters

A summary of parameters analysed in mussels at each time of exposure, the applied methodologies and the target tissues, is reported in Table 4.1.

The up-take of LDPE microparticles through ingestion, was verified searching for them in that tissues and organs closely related to the ingestion process in mussels, i.e. gills and digestive gland (Kolandhasamy et al., 2018). The presence of microplastics were also investigated in the haemolymph, which represents the circulatory system in bivalve molluscs, passing out the open end or arteries, bathes all organs before returning to the heart by way of sinuses and respiratory structures (i.e.the gills) (Pruzzo et al., 2005). So, as it can transport pollutants throughout exposed organisms (Calisi et al., 2008), as well, it can favour the translocation of microplastics by a tissue to another (Browne et al., 2008).

Chemical analyses are necessary to test the hypothesis of a possible transfer of adsorbed BaP from microplastics to mussels' tissues and evaluate differences in the bioaccumulation of BaP between the exposure to the chemical *via* waterborne and *via* microplastics. Analyses were performed in the digestive gland and gills of mussels: the first has been described as the organ where pollutants accumulate in higher concentrations, whereas the latter are the dominant site of interaction with the environment (Brandts et al., 2018), therefore both being the tissues of most relevant interest for bioaccumulation study.

Various studies have shown that ingestion of both virgin and contaminated microplastics can have detrimental effects on numerous biological processes. For example, reduction of filtration rates (Wegner et al., 2012), decrease in available energy (Van Cauwenberghe et al., 2015), inflammatory responses associated with significant tissues damages (von Moos et al., 2012), neurotoxic and genotoxic effects, and modulation of antioxidant systems (Avio et al., 2015a) occurred in mussels. Effects were also demonstrated at molecular levels, affecting the expression of genes and transcripts involved in cellular stress, immune responses, antioxidant defence, detoxification or metabolism (Avio et al., 2015a; Paul-Pont et al., 2016; Détrèe and Gallardo-Escaráte, 2017). For these reasons, the biological effects of LDPE, BaP and LDPE-BaP were evaluated through the analyses of a set of biomarkers covering a range of responses of systems involved in internal and antioxidant defences in mussels or typically related to stress.

Haemocyte-mediated phagocytosis is the predominant form of internal defence in bivalve molluscs although it is generally suppressed by exposure to a wide spectrum of contaminants (Galloway and Depledge, 2001). The mussel immune response is comprised of an integrated process of phagocytosis and lysosomal degradation and factors inducing dysfunction of these processes may suppress immunocompetence (Nicholson, 2003), with the potential to alter the health of the entire organism (Calisi et al., 2008). Therefore, phagocytic activity of granulocytes, their ratio in respect to hyalinocytes, from whom differentiate for morphology and function, and the stability of lysosomes membrane, were evaluated as biomarkers of immunotoxicity.

Alterations of lysosomes were assessed also in terms of accumulation of neutral lipids in the digestive gland of mussels, where these organelles are involved in the uptake and digestion of food materials as well as in processes of pollutant accumulation and detoxification (Marigómez and Baybay-Villacorta, 2003). Lipid storage disorders, which can lead in lipidosis, are a common response to xenobiotic exposure (Bocchetti and Regoli, 2006), even if they appear to be more strictly linked to organic chemical pollutants (Marigómez and Baybay-Villacorta, 2003).

Lipids represent, as well, a possible target for oxidation by free radicals or reactive oxygen species (ROS), which are continually produce as unwanted bi-products from various endogenous sources or processes, but rates and amounts of ROS production can be enhanced by the presence of a wide range of natural and man-made xenobiotics (Livingstone, 2001). The increase of ROS can, moreover, be turned to other macromolecules, like proteins and DNA. Although several mechanisms have been postulated for prooxidant

contaminant-mediated oxidative effects and genotoxic alterations, various cellular injuries can also increase intracellular ROS generation and alter the oxidative status of organisms (Regoli et al., 2004).
In this work the possible pro-oxidant action of virgin and contaminated microplastics were evaluated in terms of levels of malondialdehyde (MDA) in the digestive glands of mussels, as it is a typical product and thus, indicator, of lipid peroxidation of cellular membrane or oxidation of polyunsaturated fatty acids (van der Oost et al., 2003). MDA is a highly toxic molecule that exerts extremely deleterious effects on cells and tissues, by inducing, in turn, damages to DNA or cellular proteins (Del Rio et al., 2005). In this respect, oxidative damages and oxidative stress conditions can represent the cause but also the consequence of a particular disease state (Regoli et al., 2004). The onset of DNA toxicity was, instead, assessed in the hemolymph by the formation of reversible damages, strand breaks, and generation of less reversible effects, as the micronuclei, small, intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division (Gorbi et al., 2008).
The oxidative damage to key biological molecules is a consequence of an imbalance between pro-oxidant and antioxidant processes (i.e. oxidative stress), that is due to perturbation of antioxidant systems efficiency (Livingstone, 2001). Among the specific enzymes and low molecular weight scavengers involved in antioxidant defences, catalase (CAT), glutathione S-transferase (GSTs), glutathione peroxidases (GPX) and glutathione reductase (GR) along with the levels of glutathione (TGSH), were evaluated in the digestive gland of treated mussels. CAT removes hydrogen peroxide from cells during basal aerobic metabolism or after a pollution-enhanced oxyradical generation (Winston et al., 1990) ; GSTs are a family of detoxification enzymes that act by conjugating GSH to lipid peroxides and xenobiotics; GPxs can be either selenium dependent (Se-GPx) or Se-indipendent forms (total GPx) that protect the cell from oxidative damages by reducing respectively inorganic peroxides (as H_2O_2) to water and organic peroxides (as lipid hydroperoxides) to their respective alcohol; GR is the enzyme that reconverts oxidized glutathione (GSSG) to its reduced form (GSH), which acts both as a scavenger or as a cofactor for previously described enzymes (Regoli and Winston, 1999). Single antioxidants variations are useful in revealing early pro-oxidant challenges but the biological significance of this changes is difficult to summarize. For this reason, analysis of single antioxidant defenses is usually integrated with the analysis of the total oxyradical scavenging capacity (TOSC) toward different ROS, like the peroxyl (ROO•) and hydroxyl (HO•) radicals, thus providing an integrated image of the whole tissue antioxidant status (Regoli and Winston, 1999; Gorbi and Regoli, 2003).
Peroxisome proliferation has been investigated as specific marker of exposure to BaP in terms of induction of Acyl-CoA oxidase (AOX) activity. Peroxisomes are ubiquitous cytoplasmic organelles involved in lipid metabolism and handling of reactive oxygen species, that can proliferate in responses to organic pollutants (Cajaraville et al., 1997). Proliferation involves an increase in peroxisomal volume and numbers, which is usually but not always accompanied by induction of peroxisomal enzymes, particularly those of the fatty acid β-oxidation pathway, such as, indeed, AOX (Cajaraville et al., 2000). Analyses were performed in the digestive gland, an organ that is mainly involved in the acquisition, storage and metabolism of nutrients and also the main site of organic xenobiotic accumulation (Cancio et al., 1999), as above-mentioned. In different laboratory studies, peroxisomes of mussel digestive gland cells have been observed to proliferate in response to organic pollutants such as oil, several PAHs, including BaP, and phtalate plasticizers (Ortiz-Zarragoitia and Cajaraville, 2006).
Acetylcholinesterase (AChE) was evaluated in gills and hemolymph as an enzymatic biomarker of neurotoxicity, as it is responsible for the degradation of the neurotransmitter acetylcholine. Inhibition of its activity is typically related to the exposure to organophosphorus compounds and carbamates pesticides, however there are evidences of modulation induced by some heavy metals (Mora et al., 1999), PAHs (Rank et al., 2007; Cappello et al., 2015) and emerging compounds such as anti-inflammatory drugs (Mezzelani et al., 2016, 2018) and microplastics (Oliveira et al., 2013; Avio et al., 2015a) in mussels. Cholinesterasic effects of microplastics should deserve attention because MPs have been suggested to influence various

physiological and behavioral responses controlled by neurological mechanisms (Oliveira et al., 2013; Mattson et al., 2017; Ribeiro et al., 2017).
In addition to cytological and biochemical analyses, gene-expression biomarkers were applied, i.e. biomarkers based on transcription of stress-, metabolism-, or antioxidant-related genes (Piña et al., 2007).
Gene expression can be an usefull and sensitive endpoint in ecotoxicology for stress identification in organisms (Snell et al., 2003). In fact, a cell responds to alterations induced by xenobiotics by readjusting its metabolism, normally by activating or synthesizing *de novo* specific stress-related proteins, designed to counteract or compensate the damage. As a general rule, the amount of these proteins reflects the relative concentration of the corresponding mRNA (Piña et al., 2007). On the contrary, proteins activity are not always synchronous with variations of corresponding mRNA levels due to time delayed effects and factors controlling gene transcription (Regoli and Giuliani, 2014).
In the study the amount of mRNA levels were quantified in the digestive gland for *cat*, *Se-gpx* and *gst-pi*, that are some genes related to antioxidant system, for *aox1* gene as it is involved in lipid metabolism, and for *hsp70* gene as marker of general cell stress, since it can be modulated by a variety of harmful stimuli, including heat, heavy metals, organic contaminants, injuries, diseases and other stressors (Tedengren et al., 1999). Expression of *cat*, *Se-gpx*, *gst-pi* and *aox1* has been also correlated with their biochemical biomarkers counterparts.

4.5.1 Tracking the uptake of LDPE microparticles

Up take and localization of microplastics were verified by observation of hematoxylin and eosin stained tissues sections under polarized light microscopy (PLM). Analyses were performed in tissues of mussels treated with LDPE and with LDPE-BaP. Haemolymph was extracted from the posterior adductor muscle, immediately smeared onto a glass slides and stained with H&E. Digestive glands and gills were, instead, frozen in liquid nitrogen and stored at - 80 °C prior to be used to obtain H&E stained cryostat sections of 20 µm thickness. Slides of tissues were finally analysed by optical microscopy with polarized light to display microplastics. PLM represents a useful and by now approved tool to investigate the presence and exact location of non-fluorescently labelled MPs, as the model LDPE microplastics are, in stained tissue sections (von Moos et al., 2012; Avio et al., 2015a; Santana et al., 2017). This illumination technique is used to observe anisotropic materials taking advantage of their birefringent properties (Santana et al., 2017). Many plastics are birefringent, as the result of chemical structure of polymers and their crystallinity: when polarised light passes through the tissue sections, it strongly interacts with microplastics and generates a contrast with the background (Lusher et al., 2017b), that allows to highlight the particles in the investigated tissues. Quantification of microplastics by PLM is nevertheless not possible, so in the present study the assessment of uptake and localization of polyethylene microparticles in mussels has been strictly descriptive.

4.5.2 Chemical analyses of BaP bioaccumulation

Benzo(a)pyrene was quantified in digestive glands and gills of mussels previously frozen at -20 °C and stored until need. The analytic procedure is based on methanolic extraction with microwave, solid-phase purification and HPLC analyses with fluorimetric detection (Bocchetti et al., 2008). The BaP was identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). QA/QC was monitored by processing blank and reference standard materials (mussel tissue Standard Reference Material [SRM] 2977, National Institute of Standards and Technology). The water content in tissues was determined and concentrations of BaP were expressed as ng/g dry weight (dw).

4.5.3 Biomarkers assays

For the analysis of immunological responses and DNA fragmentation throught the Comet Assay, haemolymph was withdrawn from the posterior adductor muscle and immediately used to perform the tests. Another aliquot of haemolymph was fixed in Carnoy's solution (3:1, methanol: acetic acid) for the evaluation of micronuclei frequency. The other biochemical and molecular biomarkers were performed on tissues frozen in liquid nitrogen immediately after dissection of organisms and maintained at -80 °C until to performe the analyses. 4 replicates were performed for each analysis.

The lysosomal membrane stability in haemocytes, was evaluated by the Neutral Red Retention Time Assay (NRRT) according to Lowe et al. 1995. The test is based on the use of the cationic probe neutral red which is taken up into cells by membrane diffusion, where it becomes ion trapped within the lysosomal compartment. The end point parameter is the time at which dye loss to the cytosol is evident in 50% of the granular haemocytes. Haemolymph was incubated on a microscope slide with a freshly neutral red working solution for 15 minutes (2 µl/ml filtered seawater from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl sulfoxide). Thereafter, slides were examined systematically at 15 min intervals using light microscopy (40X) until a total time of 120 min.

Phagocyticic capacity assay was performed following previous methods (Gorbi et al., 2013). 50 µl of haemolymph were dispersed onto a glass slides and left to adhere for 15 min for 15 min at 15°C in the dark. Fluorescein-labelled Zymosan A bioparticles (Invitrogen) were added at 10:1 target: haemocyte ratio. After 2 h incubation at 15°C in the dark, uninternalized particles were removed by washing with physiological solution and slides were finally fixed in Beker's fixative (+2.5% NaCl) and mounted in Eukitt. Phagocytosis was expressed as the percentage of cells that internalized at least 3 fluorescent particle (positive cells), observed under a fluorescence microscope, after counting at least 200 cells for each sample.

Granulocytes versus hyalinocytes ratio was assessed on 50 µl of haemolymph dispersed on glass slides, dryed and fixed in Baker's fixative (+2.5% NaCl). The slides were washed with water, stained with H&E and mounted in Eukitt. Observations were carried out with a light microscope (1000X) and the ratio was evaluated after counting almost 200 cells for each sample (Gorbi et al., 2013).

Acetylcholinesterase activity (AChE) was analysed in haemolimph, centrifuged at 3000 xg for 5 min, and in gills, homogenized in 0.1 M Tris–HCl buffer pH 7.2, 0.25 M saccarose and centrifuged at 10 000 *g* for 10 min. Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C , λ = 412 nm, ε = 13.6 mM/cm.

The comet assay was performed according to Machella et al., 2006. Briefly, haemocytes were embedded in 0.6% low-melting agarose (LMA), spread onto microscope slides pre-coated with 1% normal melting agarose (NMA) and covered with a further layer of LMA. Slides were dipped into a lysing solution (NaCl 2.5 M, EDTA 100 mM, Trizma Base 10 mM, 10% DMSO, 1% Triton X-100, pH 10) and kept for 90 min at 4 °C in the dark, in order to solubilise cell membranes and cytoplasm. Successively, slides were treated with alkali (NaOH 75 mM, EDTA 10 mM, pH > 13) for 10 min and placed in a horizontal electrophoresis apparatus. Electrophoresis was performed for 10 min at 1V/cm and 300 mA. After run, slides were neutralized with Tris–HCl (0.4 M, pH 7.4), dehydrated in MetOH, stained with DAPI (at 100 ng/ml) and observed under a fluorescence microscope (400x). Photos of 100 cells were collected for each slide using Image-Pro® Plus 6.2 Analysing Software, while the amount of DNA fragmentation was quantified as the percentage of DNA migrated into the comet tail (tail DNA), using an image analyzer (TriTek Comet Score, Version 1.5).

For the the micronucleus test (MN) haemocytes suspension was dispersed on glass slides and stained with the fluorescent dye 40,6-diamidino-2-phenylindole (DAPI) at 100 ng/ml. For each specimen, 2000 cells with preserved cytoplasm were scored for the presence of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter, on the same optical plan and clearly separated from it (Nigro et al., 2006)

For the analysis Acyl CoA oxidase (AOX) activity samples of digestive gland were homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 x g for 15 min at 4°C. The H2O2 production was measured in a coupled assay (Small et al., 1985) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 µM sodium azide, 0.01% Triton X-100, 1.2 U/ml HRP in a final volume of 1 ml. After a preincubation at 25°C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrates palmitoyl-CoA at final concentrations of 30 µM and 100 µM for acyl-CoA oxidase and readings were carried out against a blank without the substrates at 502 nm.

The content of neutral lipids, were evaluated in cryostat sections (8 µm thick) of digestive glands. Slides were fixed in Beker's fixative (+2.5% NaCl) and washed in 60% isopropilic alcohol solution (Moore, 1988). After that, sections were stained for 20 min in a saturated oil red O solution (1% in isopropyl alcohol 60%), washed in isopropyl alcohol and then in distilled water before mounting in glycerine gelatine. Four measurements were made on digestive tubules of each section Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

For the analyses of antioxidant enzymes, samples of digestive gland were homogenized (1:5 w:v ratio) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ ml bacitracin, 0.008 TIU/ml aprotinin, 1 mg/ml leupeptin, 0.5 mg/ml pepstatin, NaCl 2.5%, and centrifuged at 110000 xg for 70min at 4°C. Measurements were made with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18°C (Bocchetti et al., 2008).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, ε = 0.04 mM-1 cm-1) due to the consumption of hydrogen peroxide, H2O2 (12 mM H2O2 in 100 mM K-phosphate buffer pH 7.0).

Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG (λ = 340 nm, ε = 6.22 mM-1 cm-1). The final assay condition were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 mM NADPH.

Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm (ε = 6.22 mM-1 cm-1) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms. The rate of the blank reaction was subtracted from the total rate.

Glutathione S-transferases (GST) were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH (ε = 9.6 mM-1 cm-1).

Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicilic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37.000 x g for 15 min. The resulting supernatants were enzymatically assayed.

The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM a-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston et al., 1998). Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (•OH) were produced by the Fenton reaction of iron-EDTA (1.8 µM Fe3+, 3.6 µM EDTA) plus ascorbate (180 µM) in 100 mM K-phosphate buffer. Under these conditions the different oxyradicals produced quantitatively similar yields of ethylene in control reactions, thus allowing to compare the relative efficiency of cellular antioxidants toward a quantitatively

similar radical flux (Regoli and Winston, 1999). Ethylene formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 – (∫SA/∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay.

The content of malondialdehyde (MDA) was measured in digestive glands samples homogenized 1:5 w/v in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 × g for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 µl water and an equal volume of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15 000 × g for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/mgPRT (Shaw et al., 2004).

Protein concentrations (PRT) were measured according to Lowry method (1951), using bovine serum albumin (BSA) as standard.

Gene expression levels were analysed in the digestive gland of mussels through real-time RT-qPCR technique by the two-step procedure. Total RNA was purified from tissues using the Hybrid-RTM purification kit (GeneAll®), according to the manufacturer's protocol and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The generated cDNA served as template in the subsequent real-time PCR for the absolute quantification of target genes, which it was carried out by the SYBR green method in a StepOnePlusR Real-Time PCR System (Applied Biosystems) with gene-specific primer pairs (reference table is reported in the attached paper). For each target genes, serial dilutions of known amounts of plasmid containing the amplicon of interest were used as standard. Amplification of the standard dilution series and of the target sequence was carried out in duplicate in the same run. The calibration curve (plot of CT-values/crossing points of different standard dilutions against log of amount of standard) was generated and used to convert CT-values of unknown samples for the determination of the amount of target expressed as mRNA copy number per µg of total RNA.

Table 4.1 *Summary of analysed parameters, applied methodologies and target tissues.*
[BaP]: benzoapyrene concentration; HPLC: High-Performance Liquid Chromatography; H&E: Hematoxylin and Eosin; PLM: Polarized Light Microscopy; LMS: Lysosomal Membrane Stability; NRRT: Neutral Red Retention Time; G/H: granulocytes vs.hyalinocytes type cells ratio; AchE: Acetylcholinesterase; AOX: Acyl-CoA oxidase; CAT: catalase; GR: Glutathione reductase; GST: Glutathione S-transferases; Se-GPX: selenium-dependent glutathione peroxidase; TOSC: total oxyradical scavenging capacity assay (ROO•: peroxyl radical, HO• hydroxyl radical); hsp70: heat shock protein70.

PARAMETERS		METHODOLOGY	TISSUE
UP-TAKE AND LOCALIZATION OF MPs		PLM	Digestive gland Gills Haemolymph
BIOACCUMULATION	[BaP]	HPLC	Digestive gland Gills
IMMUNE DEFENCES	LMS	NRRT assay	Haemolymph
	Phagocytosis	Fluorescence microscopy	
	G/H	Optical microscopy	
LIPID METABOLISM/PEROXIDATION	Neutral Lipid content	Hystochemical analysis	Digestive gland
	Malondialdehyde content	Spectrophotometry	
GENOTOXICITY	DNA fragmentation	Comet assay	Haemolymph
	Nuclear anomalies	Micronucleus test	
SINGLE ANTIOXIDANT DEFENCES	CAT activity and mRNA levels	Spectrophotometry Real time RT-qPCR	Digestive gland
	GR activity	Spectrophotometry	
	GST activity and mRNA levels	Spectrophotometry Real time RT-qPCR	
	Se-GPX activity and mRNA levels	Spectrophotometry Real time RT-qPCR	
	Total GPX activity	Spectrophotometry	
	Total Glutathione levels	Spectrophotometry	
TOTAL ANTIOXIDANT CAPACITY	TOSC ROO• TOSC HO•	Gas cromatography	Digestive gland
PEROXISOMAL PROLIFERATION/ FATTY ACID METABOLISM	AOX activity	Enzyme activity Real time RT-qPCR	Digestive gland
NEUROTOXICITY	AchE activity	Spectrophotometry	Haemolymph Gills
CELL STRESS	hsp70 mRNA levels	Real time RT-qPCR	Digestive gland

4.6 Data analysis

Two-way ANOVA was used to determine possible effects of various treatments, times of exposure and their interaction on BaP bioaccumulation and on each evaluated biomarkers. Significant ANOVA results were analysed by the Newman-Keuls test to compare groups of means, a p-value < 0.05 was considered statistically significant, homogeneity of variance was checked by Cochram C and mathematical transformation was applied if necessary. All tests were performed using the statistical R-software (2010).
Pearson's correlations were performed to highlight the relationship between mRNA levels and activities of catalase, glutathione S-transferases, Se-dependent glutathione peroxidase and acyl-CoA oxidase enzymes; significant relationship was assumed at the 0.05 level.

Principal component analysis (PCA) was applied to biomarkers data in order to discriminate between different exposure conditions.
The results of BaP biovailability and biomarker responses were further elaborated and integrated within a previously developed software-assisted model (Sediqualsoft, Piva et al., 2011), which allows to obtain a quantitative risk evaluation related to the exposure, appling weighted criteria. Briefly, The model calculates a cumulative Hazard Quotient (HQ) for bioavailability basing on the chemical tipology (if "non priority", "priority" or "priority and hazardous" according to EC Directive, 2008/105) and the entity of variations of chemical concentration between control and treated organisms, that is then assigned in one of five classes of hazard: Absent, Slight, Moderate, Major or Severe. The same approach is applied to biomarkers, for whose the HQ is calculated taking into consideration the toxicological importance of the measured endpoint, the variations of each biomarker to a specific threshold, considering the possibility of biphasic responses (induction and inhibition) and the different responsiveness of tissues. The elaborations of results from individual biaccumulation and biomarkers analyses are finally integrated within a classical weight of evidence approach and the level of risk is assigned to 1 of 5 classes of risk from Absent to Severe. Details and conceptual bases of the model are given elsewhere (Piva et al., 2011; Benedetti et al., 2012; Regoli et al., 2014).

4.7 Main results and discussions

Hystological analyses confirmed the ingestion of microplastics by mussels exposed to LDPE and LDPE-BaP and their presence in all the investigated tissues (Figure 4.3). In particular, aggregates of particles were observed within the intestinal lumen and, to a lower extent, inside digestive tubules and in the epithelium; a slight occurrence of MPs was detected also in gills and only few particles were even noticed in the haemolymph and inside the haemocytes. These evidences suggested that the uptake way of microplastics was through ingestion, whose mechanism has been proposed by von Moos et al., (2012): the first site of particle uptake seems to be on the gill surface, mediated by microvilli activity and endocytosis, then microplastics are further uptaken into the stomach, intestine and digestive tubules, via ciliae movement. Instead, the translocation of microplastics *via* the circulatory system, that was clearly demonstrated by some researches (Browne et al., 2008; Farrell and Nelson, 2013; Darmody et al., 2015), can be only hypothesized in this study, because MPs were very rarely seen in the haemolymph. It is important to remeber that despite the tested LDPE microparticles cover different sizes, those compatible with passage in the hemocytes of bivalves (about 5µm) (Paul-Pont et al., 2018) represent the minor component. Therefore, the size of microplastics is a key point to be taken into consideration in studies aiming to demonstrate translocation of MPs in marine organisms.

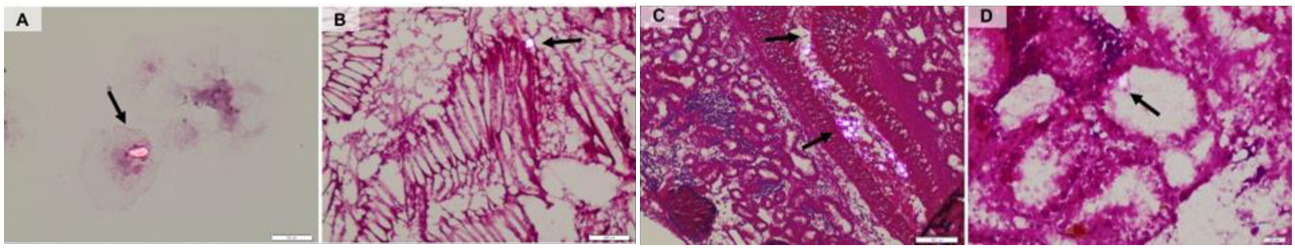


Figure 4.3 *Polarized light microscopy images showing the presence of microplastics (black arrows) in hemolymph (A), gills (B), gut lumen and epithelium (C), digestive tubules (D).*

Results of chemical analyses showed a marked bioaccumulation in both digestive gland and gills of organisms exposed either to BaP alone and to LDPE-BaP (Figure 4.4), demonstrating that the adsorbed chemical was transfer from microplastics to mussels tissues. The possibility that BaP measured in LDPE-BaP treated organisms can reflect the presence of still un-excreted particles more than a real tissue accumulation, can be considered as negligible. The histological analyses can support this assumption because the presence of

microplastics is confirmed in those tissues, but with limited numbers, particularly in gills where only a few and sparse microplastics were observed.

The toxicological importance of bioaccumulation results was further supported by the elaboration of data with weighted criteria, in fact the WOE model summarized the hazard index for bioavailability of BaP as "Severe" for mussels treated with BaP alone, as well as, for organisms exposed to contaminated microplastics at all the exposure periods (Table 4.2), since concentrations increased from 15 to 60 folds in respect to controls. Moreover, a different availability of BaP between the exposure *via* water and *via* microplastics has been highlighted for the investigated tissues (Figure 4.4). Digestive gland showed similar levels of BaP in both the treatments already after 7 days of exposure, then remaining almost constant until the end of the experiment. On the contrary, gills exhibited a rapid bioaccumulation in organisms exposed to BaP alone after 7 days, without further changes over the time, while, in mussels treated with contaminated microplastics, BaP levels significantly increased until 28 days, when values reached those of BaP treatment. A rapid uptake in gills can be explained by the direct contact of this tissue with the chemical dissolved in water (Banni et al., 2017), instead, the slower accumulation from contaminated microplastics may, at least partly, derive from primary desorption of BaP in digestive tissues and a secondary transfer of this chemical to gills.

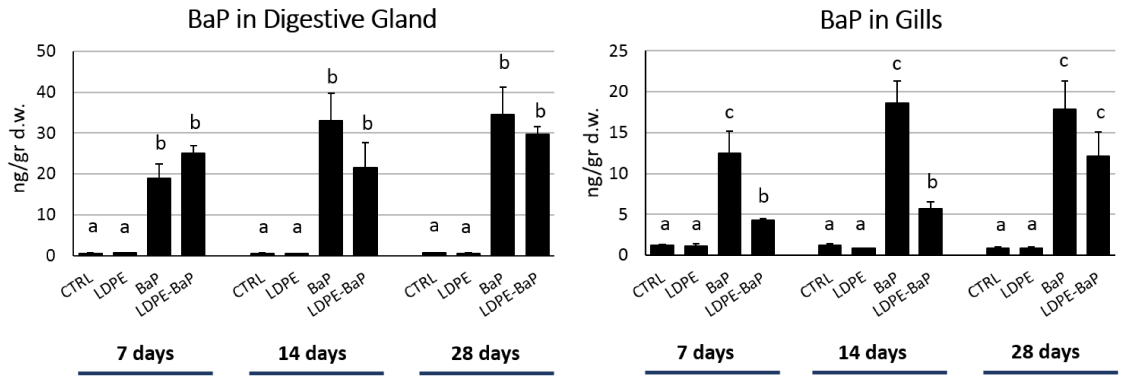


Figure 4.4 *Concentrations of benzo(a)pyrene in digestive glands and gills of mussels exposed for 7, 14 and 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expressed as ng/g of dry weight tissues and given as means values ± standard error (n= 4); different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p <0.05).*

Table 4.2 *Weigh of Evidence (WOE) classification of BaP bioavailability data for each time of exposure and treatments. The assigned classes of hazard are given.*

Time of exposure	Experimental treatment	Class of hazard	Level
7 days	LDPE	Absent	
	BaP	Severe	
	LDPE-BaP	Severe	
14 days	LDPE	Absent	
	BaP	Severe	
	LDPE-BaP	Severe	
28 days	LDPE	Absent	
	BaP	Severe	
	LDPE-BaP	Severe	

Concerning the biological responses, significant effects were observed on the immune system of mussels (Figure 4.5). A strong destabilization of lysosome membrane was measured in organisms exposed to BaP-related treatments after 7 and 14 days, with an enhanced effect of the chemical when it is adsorbed on microplastics, at the initial stage of exposure. BaP and LDPE individually caused an induction of phagocytosis after 7 days, however without a possible additive effect in LDPE-BaP treatments. To notice that, in treatments with LDPE after 7 days, phagocytic efficiency remained elevate despite a decrement of granuolcytes occurred. However, at longer periods (14 and 28 days of exposure), phagocytosis was inhibited by MPs and BaP, both alone and in combination: this might be due to an overload of sequestering capacity of hemocytes by microplastics and to the well-known inhibitory action of PAHs on this function.
The changes of immune parameters observed in this study are not a surprise, given the characteristics of plastic particles that *per sé* can potentially induce a physical stress in hemocytes, further modulated with a chemical challenge in mussels exposed to LDPE-BaP. Moreover, this study joins with other works (Von Moos et al., 2012; Avio et al., 2015a; Paul-Pont et al., 2016; Détréé and Gallardo-Escárate, 2017, 2018) in pointing out how the immune system is an important target of micro-plastics toxicity.

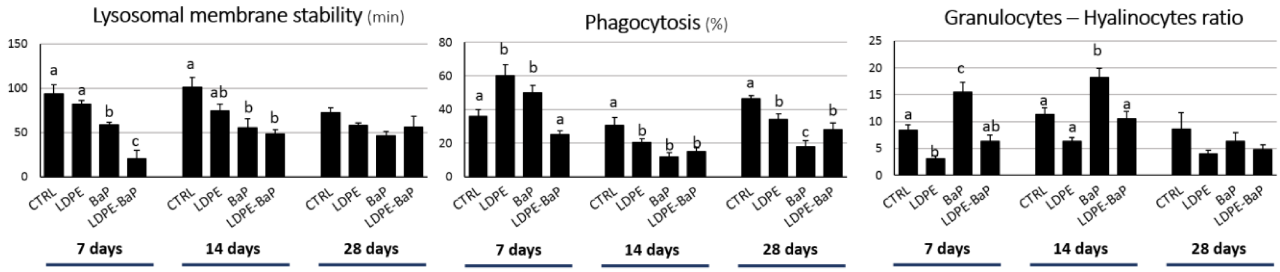


Figure 4.5 *Immunolgical biomarkers in mussels exposed for 7, 14, 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expressed as mean ± standard error (n=4): different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

Despite the impairment of immunological parameters, the exposure to LDPE, BaP and LDPE-BaP didn't reveal significant changes in the oxidative status of mussels. Single antioxidant defences, both at catalitic (Figure 4.6) and transcriptional level (Figure 4.7), showed slight fluctuations mainly related to time of exposure (significant results of two-way ANOVA for times: GPXs with p <0.05; for *Se-gpx* and *gst-pi* with p <0.01; for CAT, *cat* and GR with p <0.001) rather than treatments. For catalase (Pearson correlation coefficient=0.50; p <0.05) and glutathione transferases (Pearson correlation coefficient= 0.40; p <0.05) differences in the activity levels of individual enzymes appeared to reflect differences in corresponding mRNA levels. The lack of pro-oxidant effects of virgin and contaminated microplastics was also supported by an unaltered total antioxidant capacity and scarse responses of biomarkers related to peroxidation processes, lipid metabolism, fatty acid oxidation pathway and DNA damages (Figure 4.8). These results are in contrast with other studies, in which bivalves exposed to microplastics exhibited significant changes of antioxidant defences and signal of oxidative harm (Avio et al., 2015a; Paul-Pont et al., 2016; Détrée and Gallardo-Escárate, 2017; Ribeiro et al., 2017), probably due to the lower levels of particles used in this work.
The absence of significant variations of AchE activity in gills and haemolymph of treated mussels in respect to control groups, suggested that LDPE or LDPE-BaP might not even have neurotoxic properties, at least, to the experimental conditions of this study (Figure 4.9). For example, Avio et al., (2015a) observed an inhibition of AchE in the gills, exposing mussels to PE and PS microplastics both virgin and contaminated with pyrene and at higher concentration (1.5 g/l vs 0.01 g/l of this work).
In spite of the obtained results of biomarkers related to oxidative damages and neurotoxicity, mussels certainly displayed quick responses and a general disturbance, once exposed to virgin and contaminated LDPE, as suggested by immunological responses. In support of this observation, a transient increase of mRNA

levels of hsp70 gene was observed in mussels exposed to LDPE after 14 days, that can reflect a response toward the physical disturbance caused by the ingestion of such particles. Enhanced levels of these proteins are, in fact, a generic biomarker of stress, acting in mussels as a first line of defense to cope with environmental challenges (Franzellitti and Fabbri, 2005; Heindler et al., 2017). The effects of contaminated microplastics were, instead, more similar to those of BaP, with lack of statistical changes and a trend toward lower values of *hsp70*, supporting a limited responsiveness of these proteins to the prevalence of a chemical stress (Figure 4.10).

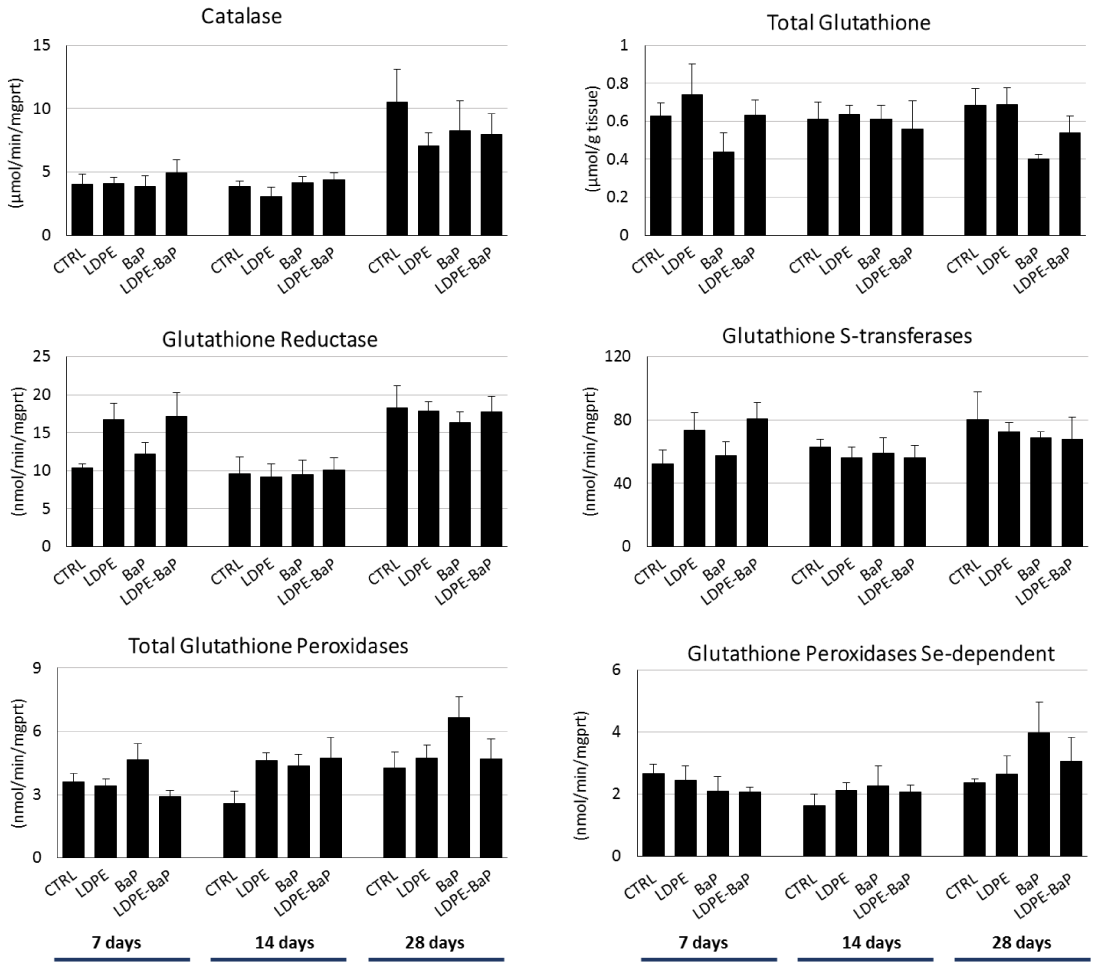


Figure 4.6 *Single antioxidant defences measured in the digestive gland of mussels exposed for 7, 14, 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expressed as mean ± standard error (n=4): different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

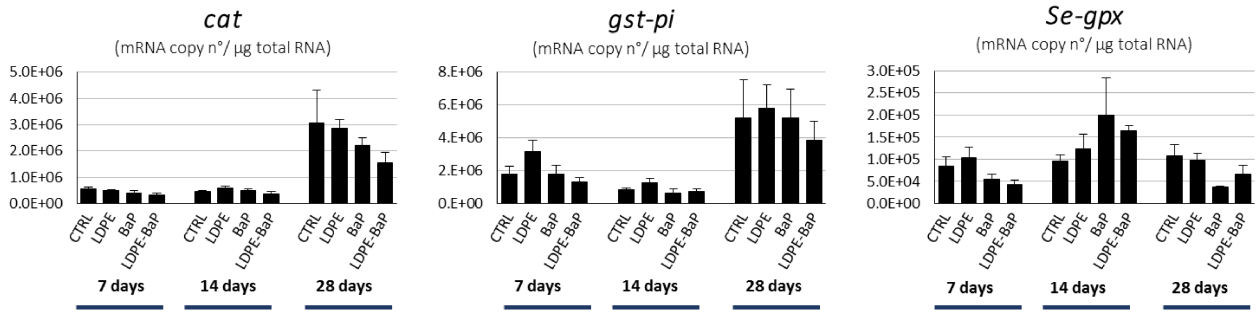


Figure 4.7 *mRNA levels of catalase, glutathione S-transferases, Se-dependent glutathione peroxidase in the digestive gland of mussels exposed for 7, 14, 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expresses as mean ± standard error (n=4); different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

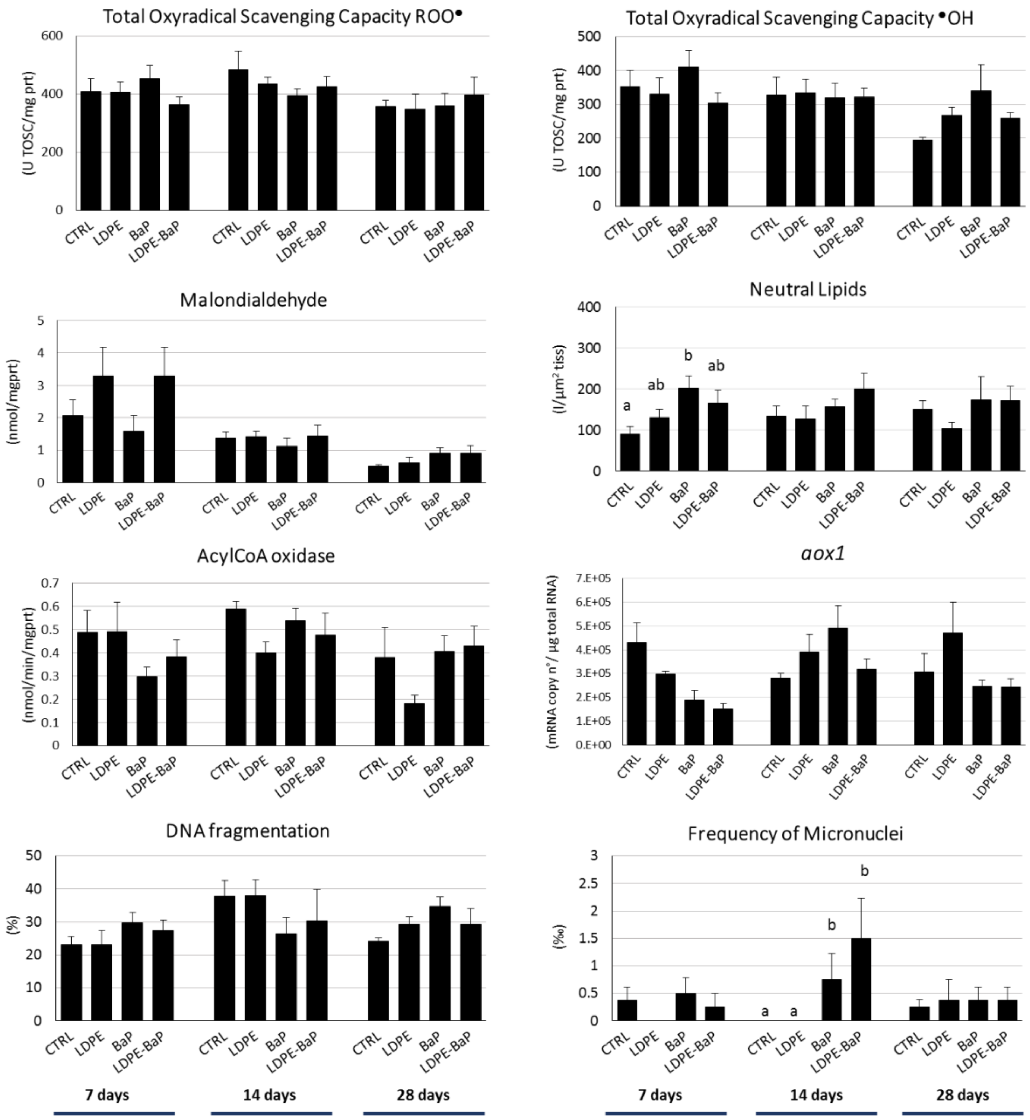


Figure 4.8 *Total antioxidant capacity and biomarkers related to peroxidation processes, lipid metabolism, fatty acid oxidation pathway and DNA damages measured in mussels exposed for 7, 14, 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expressed as mean ± standard error (n=4): different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

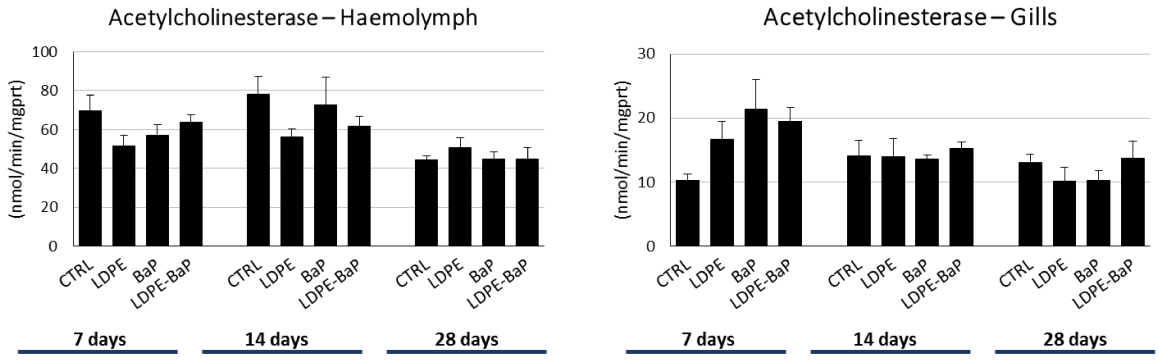


Figure 4.9 *Acetylcholinesterase activity measured in mussels exposed for 7, 14, 28 days to various treatments (CTRL, LDPE, BaP, LDPE-BaP). Data are expressed as mean ± standard error (n=4): different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

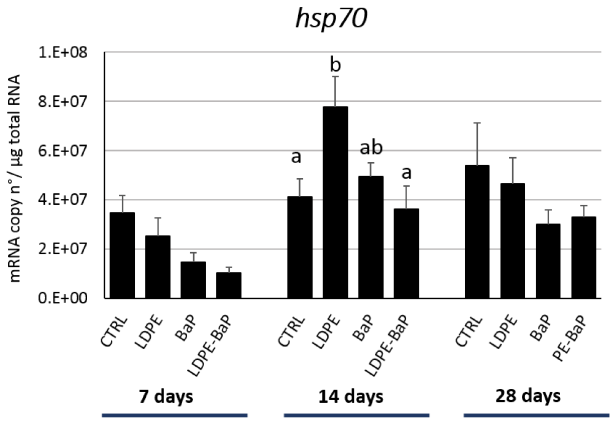


Figure 4.10 *mRNA levels of heat schock protein 70 in the digestive gland of mussels exposed for 7, 14, 28 days to various treatments (CTR, LDPE, BaP, LDPE-BaP). Data are expresses as mean ± standard error (n=4); different letters indicate significant differences between groups of means within the same time of exposure (post-hoc Newman-Keuls comparison, p<0.05).*

The “Slight”/"Moderate" onset of ecotoxicological effects on mussels after 28 days of exposure to virgin and contaminated microplastics, was confirmed by the overall elaboration of biomarkers data in the WOE model (Table 4.3).

Table 4.3 *Weight of Evidence (WOE) classification of biomarkers data for each times of exposures and treatments. The assigned classes of hazard are given.*

Time of exposure	Experimental treatment	Class of hazard	Level
7 days	LDPE	Slight	
	BaP	Slight	
	LDPE-BaP	Slight	
14 days	LDPE	Slight	
	BaP	Moderate	
	LDPE-BaP	Slight	
28 days	LDPE	Slight	
	BaP	Slight	
	LDPE-BaP	Slight	

The final integration of bioaccumulation and biomarker data (Table 4.4), for a quantitative evaluation of the risk related to exposure, has provided, once again, a "Slight" effect for mussels exposed to virgin polyethylene, but the effect has been raised to " Major " for organisms exposed to contaminated particles. It is quite obvious that the "Major" effect was strongly influenced by the marked accumulation of BaP, corroborating the still unexplored possibility of long-term consequences of a release of chemical compounds from microplastics.

Table 4.4 *Weight of Evidence (WOE) integration of bioavailability and biomarkers data for each times of exposures and treatments. The assigned classes of hazard are given.*

Time of exposure	Experimental treatment	Class of hazard	Level
7 days	LDPE	Slight	
	BaP	Major	
	LDPE-BaP	Major	
14 days	LDPE	Slight	
	BaP	Major	
	LDPE-BaP	Major	
28 days	LDPE	Slight	
	BaP	Major	
	LDPE-BaP	Major	

In addition, the results of PCA, carried out on the whole set of biomarkers, have allowed to further discriminate between different exposure conditions (Figure 4.11). A clear separation was obtained between specimens exposed at different treatments for different times, even if, a quite percentage of the total variance remains to be explained. After 7 days (Blu ellipse), LDPE and LDPE-BaP treated mussels separated from the other groups, at 14 days (Red ellipse) mussels treated with BaP and LDPE-BaP were more differentiated, while after 28 days (Green ellipse) the effects of BaP alone became more evident, producing a clear separation between such experimental group and other treatments. It seems that mussels experienced firstly the effects of microplastics, possibly reflecting a physical disturbance of such particles, instead, the combined effects of microplastics with BaP appeared more relevant with time of exposure, while at longer exposure conditions, impact of BaP prevailed on those induced by microplastics and, thus, the chemical stress assumed a major role in biological disturbance.

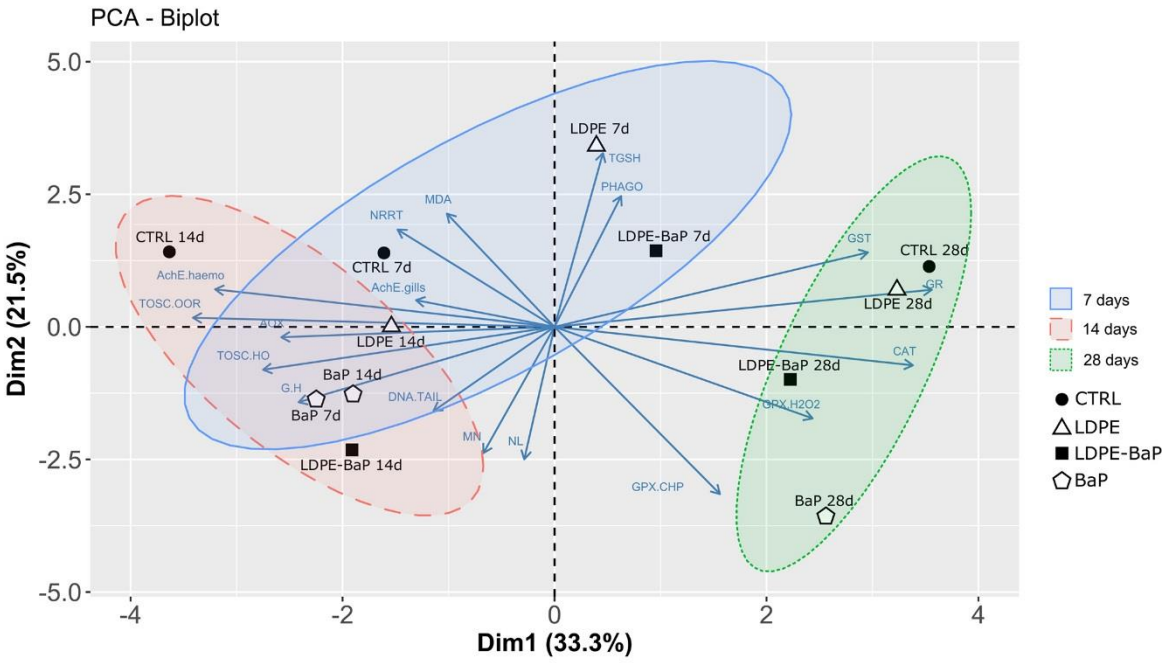


Figure 4.11 *Multivariate PCA analysis on biomarkers data in mussels exposed for 7, 14, 28 days to various treatments.*

4.8 Conclusion

This experimental study has demonstrated that, once ingested by mussels, polyethylene microparticles, can be accumulated in different tissues, with a potential translocation of those of lower size. Moreover, it confirmed that microplastics can transfer adsorbed organic contaminants, like benzo(a)pyrene to mussels tissues, providing an additional experimental evidence on the role of microplastics as source of chemical bioaccumulation. Both virgin and contaminated microplastics did not induce strong ecotoxicological effects after 28 days of exposure, however the clear disturbance observed toward the immune system can be considered as an early signal of possible long term consequences for marine organisms, which in the natural environment are chronically subjected to multiple stressors (i.e. chemicals mixture and climate change), that can, moreover, interact.

In this contest, experimental studies simulating the complexity of realistic scenario are more and more necessary to fill the gaps on toxicity of microplastics, requiring a multidisciplinary and integrated approach, involving expertise of physicists, chemists, biologists and ecotoxicologists.

5 CHARACTERIZATION OF MICROPLASTICS IN A WASTEWATER TREATMENT PLANT OF NORTH ITALY

This chapter is based on the following paper:
Magni S., Binelli A., Pittura L., Avio C.G., Della Torre C., Parenti C.C., Gorbi S., Regoli F. (2019). The fate of microplastics in an Italian Wastewater Treatment Plant. *Science of the Total Environment*, 652, 602-610. https://doi.org/10.1016/j.scitotenv.2018.10.269

5.1 Microplastics in wastewater treatment plants

The global presence of microplastic in the environment has been shown by various studies, however, neither MP concentrations nor their sources or sinks are completely known (Mintening et al., 2017). Wastewater treatment plants (WWTPs) are frequently suspected as significant land-based point sources or conduits of microplastics to marine, freshwater and terrestrial ecosystems (Li et al., 2018), although it is difficult to confirm a direct link between microplastic pollution and the effluents of WWTPs (Carr et al., 2016).
Wastewater includes raw waters from industrial, domestic, agricultural activities and surface and rainwater runoffs, that have a high likelihood to contain macro- and microplastics (Mintening et al., 2017). When wastewater are conveyed to WWTPs to be cleaned up by organic matter and nutrients, large items, such as macroplastics, are removed during the preliminary mechanical treatments, while the used technologies are not specifically designed to retain micropollutants, such as, pharmaceuticals, heavy metals (Binelli et al., 2015, 2014; Magni et al., 2015) and indeed microplastics.
Nevertheless, recent investigations, focused on the removal efficiency of microplastics in WWTPs, demonstrated that these facilities can restrain up to around 99% of the MPs entering through the influents, depending on the processes employed by the treatment plants (Lares et al., 2018; Mintenig et al., 2017; Ziajahromi et al. 2017; Carr et al., 2016; Murphy et al., 2016; Michielssen et al. 2016; Dris et al., 2015; Magnusson and Norén, 2014), contrary to was previously believed (Browne et al., 2011; GESAMP, 2010).
Despite this, when dealing with large volumes of wastewater to treat, even a modest amount of microplastics being released per liter of effluent could result in a sheer number of particles entering daily directly into recipient water bodies (i.e. rivers, lakes, sea) or soils (Mourgkogiannis et al., 2018).
In addition to the discharge of MPs through effluents, WWTPs pose another potential threat for the high percentage of MPs (up to 90%) which settle on the bottom of WWTP tanks, accumulating in the recycled activated sludge (Carr et al., 2016). In fact, sludge is widely re-used in green construction and as fertilizer in fields worldwide (Mahon et al., 2017), representing another route for microplastics accumulation in the aquatic and terrestrial environments, with negative consequences for inhabitant organisms (Anbumani and Kakkar, 2018; de Souza Machado et al., 2018; Horton et al., 2017; Lwanga et al., 2016; Besseling et al., 2013). About this, the potential for MPs to act as a vector of chemical contaminants in the environment and to biota is considerably much more significant in WWTPs (Raju et al,. 2018). Wastewaters transport a diverse range of chemicals from the source to the treatment facility, including flame retardants, endocrine-distrupting compounds, pharmaceuticals, heavy metals, persistent organic pollutants (Carr et al., 2016), that have been detected at high concentrations in many WWTPs effluent samples and in sludge (Yang et al. 2016; Nelson et al., 2011; Ma and Shih 2010; Karvelas et al., 2003; Shareef et al. 2008; Van Beelen, 2007; Ying and Kookana, 2007). Although studies are required to assess the sorption capacity of various MPs in WWTPs context, the possibility of enrichment of that toxic substances on surface of microplastics cannot be excluded.
At now, still few researches have been carried out on microplastics in WWTPs (Prata et al., 2018) and most of studies have mainly focused on the final effluent (Lares et al., 2018). Little work has been undertaken to determine removal efficiencies of WWTP, the stage of the process where microplastics are extracted and the composition of the polymers entering and exiting the treatment facilities (Murphy et al., 2016).

More detailed analysis, on fate and transport pathways of microplastics in wastewater treatment processes, are needed to elucidate the contribuition of WWTPs as sources of accumulation of these contaminants in our environment and the possible related risks for habitats and biota.
In this context, the aim of this study was to evaluate the abundance and physical/chemical characteristics of MPs in one of the main WWTPs of Northern Italy, characterizing these particles in wastewaters at different treatment steps, as well as, in the recycled activated sludge. To our knowledge, this is the first study aimed to identify the fate of MPs through the entire waste treatment process from an Italian WWTP, to evaluate the efficiency of various treatments in removing these particles, and to assess the overall release of such emerging contaminants.

5.2 Matherials and Methods

5.2.1 Characteristics of the WWTP selected for the study and sampling

Wastewater and sludge samples were collected from a municipal WWTP located in the North-Western part of Italy. The plant has a processing capacity of 1,250,000 population equivalents and receives combined sewer, therefore, it collects domestic sewage, industrial waste-water and rainwater runoff all in the same pipe. The plant treats approximately 400,000 m^3 (corresponding to 400,000,000 L) of wastewater every day, that are conveyed into the surrounding farmlands for irrigation and into the existing hydrographic system: since April 2003, when the system was fully operational, it has been estimated a return of 2,143 million m^3 purified water to the rivers and to the Adriatic Sea.
The WWTP adopts the Conventional Activated Sludge (CAS) processes (Figure 5.1), starting with a 30 mm coarse screening, followed by a 3 mm fine screening and a grit and grease removal phase (pre- and primary treatments). The wastewater is then pumped up for biological treatment by activated sludge denitrification and oxidation-nitrification process, followed by sedimentation section (secondary treatments). In order to complete the biological treatment, systems for active sludge and mixed liquor recirculation are also present (recycled activated sludge), and residual sludge is treated by thickening, aerobic stabilization, dewatering and drying to reduce volume and to allow its reuse in the production of compost or for energy recovery. Wastewater is finally filtered through sand filters, disinfected (tertiary treatments) and drained into the above mentioned environmental receptors.
Three stages of the treatment water line were sampled for analysis of microplastics: inlet after coarse screening (IN), after the settler (SET) and outlet (OUT), along with the recycled activated sludge (Figure 5.1). 30 L of surface wastewater were collected from each step using a steel bucket and they were poured to a cascade of three steel sieves with mesh sizes of 5 mm, 2 mm and 63 µm: materials retained on the 2 mm and 63 µm sieves were recovered in glass containers using Milli Q® water. Moreover, 50 mL of activated sludge, corresponding to a concentration of 7.5 g/L dry weight (d.w.), were sampled using a glass beaker.
The sampling campaign has been performed on May of 2017, during dry weather conditions, when the average of inlet flow rate is about 18,000 m^3/h. Sampling was repeated for three days in a week, at the same time, to reduce the variability associated to the weathering and/or to the urban release in drainage systems.

Preliminary sampling tests were carried out before the actual sampling campaign in order to optimize the sampling methods and reduce the incident of microplastic contamination during the in-site operations, samples transfer and laboratory analyses. In this respect, samples were kept covered as much as possible using glass lids; lab coats, cotton clothing and gloves were worn, during samplings and laboratory operations. Work surfaces, equipment and manipulation instruments were cleaned withMilli Q® water and alcohol. All solutions used during samples processing in laboratory were filtered twice on 1.2 µm glass fiber filters to eliminate impurities. In addition, 2 filters were processed as blanks with the same procedure to whom samples were subjected for each processing steps.

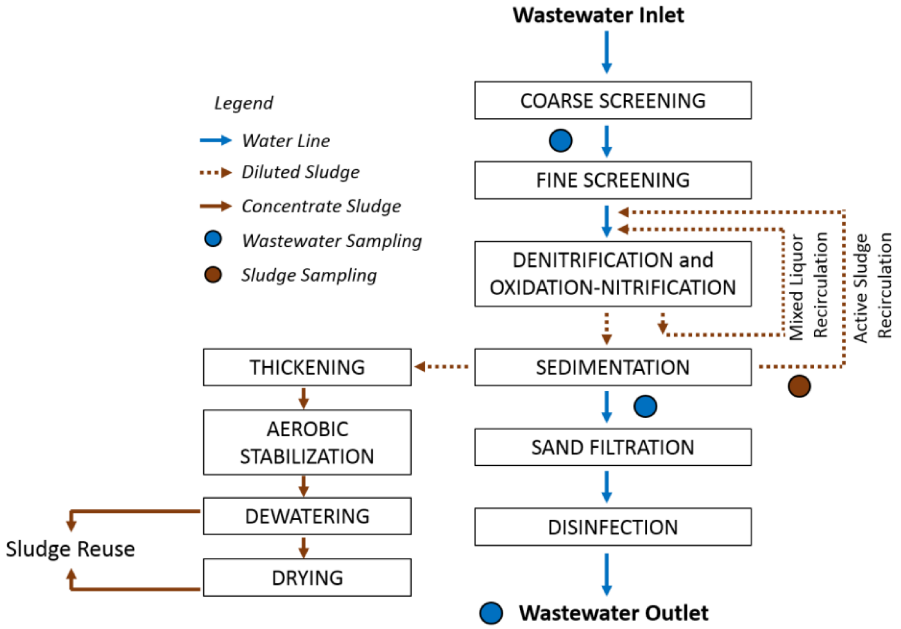


Figure 5.1 *Simplified block diagram of the treatment processes employed by the selected WWTP and identification of the steps for wastewater and sludge sampling.*

5.2.2 Samples processing for MPs extraction and characterization

A density separation, followed by a filtering procedure and an organic matter digestion phase were employed to extract microplastics from collected wastewater and sludge. Specifically, samples were stirred with 500 mL of a saturated NaCl salt solution (density: 1.2 g/cm^3) and left them to decant overnight for filtering the supernatants on 8 µm cellulose nitrate (CN) membranes, using a vacuum pump. Filters were then recovered and treated with a peroxide digestion (15% H_2O_2 solution) for three days, at room temperature, under a laminar flow hood to prevent atmospheric fibers contamination.

The digestion phase significantly reduced the occurrence of non-synthetic particles in the samples, however, due to the complex nature of wastewater and sludge, it did not remove debris sufficiently enough to perform automated µFT-IR analysis for polymer characterization directly on the filters. Therefore, microplastics analysis was initially carried out using a visual identification step: wastewaters and sludge filters were accurately observed under a stereomicroscope and all fibers and suspected microplastic particles were manually collected and transferred onto clean CN membranes. During visual sorting, blanks were left exposed to the air the same amount of time as the samples.

µFT-IR analysis was performed for the individual fibers and particles to verify the visual identification step and to provide information on polymers typologies. Spectra with wavelengths between 600 and 4000 cm^{-1} were acquired in attenuated total reflectance (ATR) mode after 32 scans with a resolution of 4 cm^3 and they were compared with standard polymers libraries. Similarity between acquired samples spectra and the

reference ones was accepted only after visual examination of spectra characteristics and with a *Hit Quality Index* (HQI) ≥ 0.7, as suggested by Klein et al., (2015) and Lusher et al., (2015).
In addition to chemical characterization, particles were categorized according to their shape and measured to be classified into 4 size classes: 5-1 mm, 1-0.5 mm, 0.5-0.1 mm, 0.1-0.01 mm.

5.3 Data Analysis

To evaluate the significant differences (*p <0.05; **p <0.01) about MPs content between the three different treatment steps (IN, SET and OUT), we performed the one-way analysis of variance (one-way ANOVA); each difference, treatment versus treatment, was evaluated using the Fisher LSD post hoc test. For these analyses we used the STATISTICA 7.0 software package.

5.4 Results and Discussion

5.4.1 Quantification of MPs and removal efficiency of treatment processes

The characterization by µFT-IR has confirmed that the 72% of microparticles collected during visual sorting from the IN samples, the 67% from the SET, the 55% from the OUT and the 81% from the sludge were plastics. Instead, the percentage of synthetic fibers, in respect to natural ones, was 34% in the IN, 28% after the SET, 19% in the OUT and 65% in the sludge.
Analyses of blanks highlighted the presence of only natural fibers, thus, the value of 1.6±1.0 cotton fibers has been ignored for the quantification of microplastics in wastewater and sludge samples.
Therefore, the total amount of microplastics (MPs) in the influent (IN), including both microplastic particles (MPPs) and fibers (MPFs) was on average (calculated on the three sampling days) of 2.5 ± 0.3 MPs/L (Figure 5.2A), with a dominance of particles (2.0 ± 0.3 MPPs/L) compared to fibers (0.5 ± 0.1 MPFs/L) (Figure 5.2B). The number of MPs entering in the selected WWTP through the influent (IN) is lower than that obtained by studies on other European facilities. For example, Murphy et al. (2016), have measured 15.7 ± 2.23 MPs/L in the inlet of a Scottish WWTP after coarse screening step; Lares et al., (2018) have obtained on average of 57.6 ± 12.4 MPs/L, analysing the raw waters addressed to a WWTP in Finland. Much higher levels were measured in the influent of another treatment plant in Finland, with high variability of detected microplastics during different sampling days, from 380 to 900 MPs/L (Talvitie et al., 2017a). Raw sewage influents from seven municipal WWTPs in the Netherlands contained a mean plastic particle concentrations of 68-910/L, (Leslie et al., 2017); 15.1 ± 0.89x10^6 MPs/L and 293x10^6 MPs/L have even been found in the inlet flow of a WWTP in Sweden (Magnusson and Norén, 2014) and in France respectively (Dris et al., 2015). Except for the study of Murphy et al., (2016) that highlighted a minor contribution of fibers on the total amount of microplastics (18.5%), similarly to that obtained by this study (20%), the other authors found more synthetic fibers than particles (Magnusson and Norén, 2014; Talvitie et al., 2017a; Lares et al., 2018) or exclusively fibers (Leslie et al., 2017; Dris et al., 2015) in the incoming flows the WWTPs.
The variability of MPs concentration that has been noticed among WWTP inlets of different countries, could be related to the typologies of sewage collected by the facilities, such as, greywater, blackwater or those derived from combined sewer. Considering this latter case, the rainwater runoff entering in the sewage system can produce a "diluting effect" on MPs concentration in the wastewaters. This phenomenon can be particularly present in the Italian WWTPs, because 70% of the national sewage system is made by combined sewages, as it is the case of the selected facility for this study, and rainwater infiltration can reach the 30% of the entire flow rate (Autorità per l'energia elettrica, il gas e il sistema idrico, 2017).
Furthermore, it is important to take into account that different procedures to detect microplastics from wastewater and sludge have been adopted by several studies, including the use of sieves with diverse minimum mesh size, making difficult comparison of results and sometimes polymers identification was performed on subsamples of suspected microplastics (e.g in Lares et al., 2018; Talvitie et al., 2015a) or

chemical analysis is not performed at all (e.g. in Dris et al., 2015), posing the question of a possible underestimation or overestimation of the number of microplastics.

The main aims of the present study are, nevertheless, to highlight the differences in MPs concentrations between different wastewater treatment stages for analyze the efficiency of the selected facility in retaining microplastics and point out the amount and typology of microplastics entering in the environment through the final effluent.

At this regard, quantification of microplastics in the liquid fraction after the sedimentation step (SET) has led to an average of 0.9 ± 0.3 MPs/L and of 0.4 ± 0.1 MPs/L in the final effluent (OUT) (Figure 5.2A). The ratio between particles and fibers remained in favour of microplastic particles, both after the SET (0.6 ± 0.2 MPPs/L *vs* 0.3 ± 0.2 MPFs/L) and in the OUT (0.3 ± 0.1 MPPs/L *vs* 0.10 ± 0.03 MPFs/L) (Figure 5.2B).

Considering that 2.5 ± 0.3 MPs/L have been found in the IN, we obtained a significant effect of treatment steps on MP content in wastewaters (one-way ANOVA result: $F_{2,6} = 50.3$; $p<0.01$), with a significant reduction of concentration between IN and SET (Fisher LSD post-hoc test result: $p<0.01$) and a further decrease of microplastics between SET and OUT, even if it did not result statistically significant ($p=0.07$) (Figure 5.2A). This means that in the treated effluent there was a 84% less microplastics than the raw influent and that the great removal of MPs occurred during the phases of primary and secondary treatment (64%) (Figure 5.2A).

The efficiency of the selected WWTP in retaining microplastics from wastewater (84%) is in the same order of other European facilities, ranging from 72% to 98% (Murphy et al., 2016; Leslie et al., 2017; Lares et al., 2018). Also levels of MPs released with effluent measured in this study is within the range reported for other American and European WWTPs (Lares et al., 2018), with the lower value of 0.005 MPs/L observed in Finnish WWTPs (Talvitie et al., 2017b) and the higher concentration of 91 MPs/L in 7 Dutch WWTPs (Leslie et al., 2017). Moreover, the present study confirmed that primary and secondary treatments are the main responsible of decrease in concentration of microplastics as observed in other studies (Prata et al., 2018). However, also the sand filters at the end of the WWTP contributed to the treatment performance, decreasing by almost 50% the MPs content from the SET (0.9 ± 0.3 MPs/L) to the OUT (0.4 ± 0.1 MPs/L). This is another crucial result in the attempt to define simple and cost-effective treatments to reduce MPs in wastewaters. In this context, Talvitie et al. (2017b) have tested the performance of different final treatment technologies, observing a MP removal of 97% from wastewater after sand filters, however, authors suggested that the result should be further validated in future studies, since the daily washing water is generally carried in counterflow, potentially recirculating also MPs; in addition Phillips (2016) observed a decrease in size of microplastic particles after wastewater tertiary treatments with sand filters.

Doubtless, the performance of WWTPs based on CAS process in retaining MPs, can be further improved with some final-stage wastewater treatment technologies based on mechanical entrapment of particles. For example, Talvitie et al. (2017b) demonstrated a high removal of microplastics by discfilters (98.5%), besides sand filtering. The study of Ziajahromi et al. (2017) indicated that over 90% of microplastics in primary effluent was removed during advanced treatment processes based on ultrafiltration (membrane pore size: 0.002-0.1 µm), as weel as, Mintenig et al., (2017) demonstrated that the installation of a post-filtration unit in a German WWTP has reduced the load of microplastic particles and synthetic fibres substantially (93% and 98% respectively).

However, the highest performance in retaining microplastics, up to 99.9%, were demonstrated for anaerobic membrane bioreactor (AnMBR) technology (Lares et al., 2018; Talvitie et al., 2017b; Michielssen et al., 2016), which couples biological treatment in anaerobic condition and microfiltration (membrane pore size: 0.1-10 µm) or ultrafiltration. AnMBRs have emerged in the field of wastewater treatment processes as one of the best alternatives to CAS systems, due to some enhanced characteristics, including small spatial requirements, higher effluent quality and low sludge production (Gurung et al., 2016). AnMBRs represent a promising solution also to the issue of microplastics contamination, nonetheless, they requires high capital investment (Xiao et al., 2019). Although advanced treatments can remove a high proportion of MPs from wastewater,

WWTPs still have the potential to release a hugh amount of microplastics in the environment, given the large volumes of effluent that are discharged. Indeed, our results highlight that, despite the high MPs removal efficiency of selected WWTP (84%) and the low levels of microplastics per liter measured in the effluent (0.4 ± 0.1 MPs/L), the facility contributes to pollute daily the aquatic ecosystems with the release 160 million of plastics, because it treats an influent flow rate of 400,000,000 L.

The description of the MPs route through the WWTP cannot ignore the recycled activated sludge produced between IN and SET steps. The number of microplastics detected in active sludge during the three sampling days amounted to 113±57 MPs/g sludge dw (Figure 5.3A), that it is comparable to values of 8.2–301.4 MPs/g dw found in the solid fraction of a Dutch WWTP (Leslie et al., 2017) and 186.7 MPs/g dw (Talvitie et al., 2017b) and 170.8±0.287 (Lares et al., 2018) measured in sludge of Finnish facilities. 53% of MPs detected in sludge was recognized as MPPs (59.5 ± 21.6), while plastic fibers are present on average of 53.3 ± 48.9 MPFs/g sludge dw (Figure 5.3B). Considering that the investigated WWTP produces about 30 tons/dw of sludge daily, we can derive an estimate of about 3,400,000,000 MPs accumulating each day in the sewage sludge.

Other studies have measured high concentrations of MPs in WWTP sludge samples (Li et al., 2018; Mahon et al., 2017; Mintening et al., 2017; Bayo et al., 2016; Carr et al., 2016; Lassen et al., 2015; Magnusson and Norén, 2014; Brandsma et al., 2013; Habib et al., 1998), demonstrating that most of the MPs removed from wastewater during primary and secondary treatments, are, actually, laid down in sewage sludge. In fact, it has been shown that grit and grease removal stages can entrap up to 45% of microplastics of the liquid fraction in the primary sedimentation tank (that lacks in the WWTP selected for this study), while final sedimentation retains around 34% of MPs (Prata et al., 2018). Thus around 80% of microlitter can be deposited in the sludge fraction (Prata et al., 2018), that may be applied in fields as a fertilizer leading to terrestrial contamination (Talvitie et al., 2017a; Browne et al., 2011; Zubris and Richards, 2005) and by soils, MPs can be transported into freshwater and coastal marine environment through runoff and storm water (Browne, 2015), partially nullifying the WWTPs activity.

The use of sludge in agriculture is actually banned if they contain high levels of toxic pollutants, as heavy metals, but neither European (EU 86/278/EEC) nor U.S. (Code 503) legislations put limits for MPs (Nizzetto et al., 2016). Moreover, investigation about MPs characteristics in dewatered sewage sludge, that represents the reused solid fraction, is presently lacking (Li et al., 2018), thus a major attention should be towards this final product of wastewater treatment processes. In the meanwhile, applying advanced wastewaters treatments based on reduction of sludge mass (i.e. anaerobic bioreactors) in respect to conventional CAS processes, could be useful to reduce the amount of sludge-based MPs entering into natural environments.

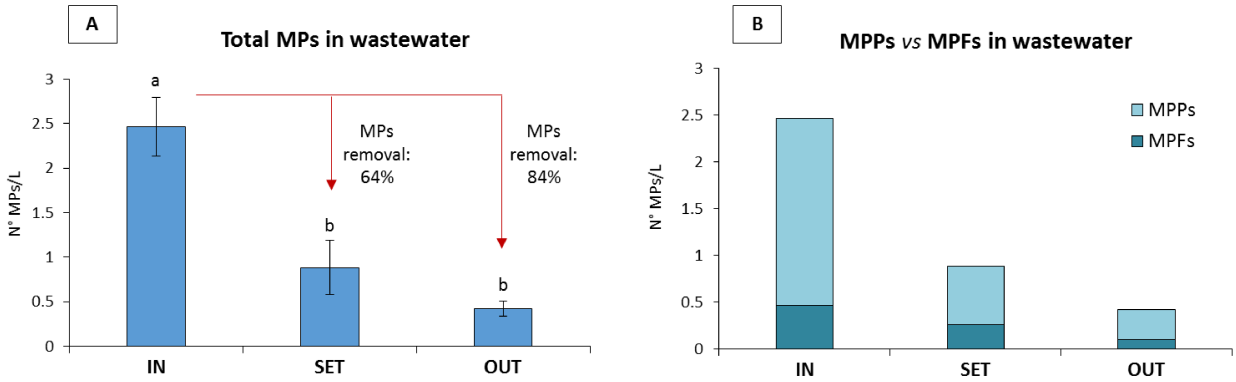


Figure 5.2 (A) *Concentrations of microplastics (MPs/L) in the influent (IN), in the wastewater after the sedimentation step (SET) and in the final effluent (OUT); values are given as mean±standard deviation calculated on three days of sampling; different letters indicate significant differences between groups of mean in the three treatment steps (one-way ANOVA, Fisher LSD post hoc test).* ***(B)*** *Relative contribution of microplastic particles (MPPs) and synthetic fibers (MPFs) to the total amount of MPs in the IN, SET and OUT samples.*

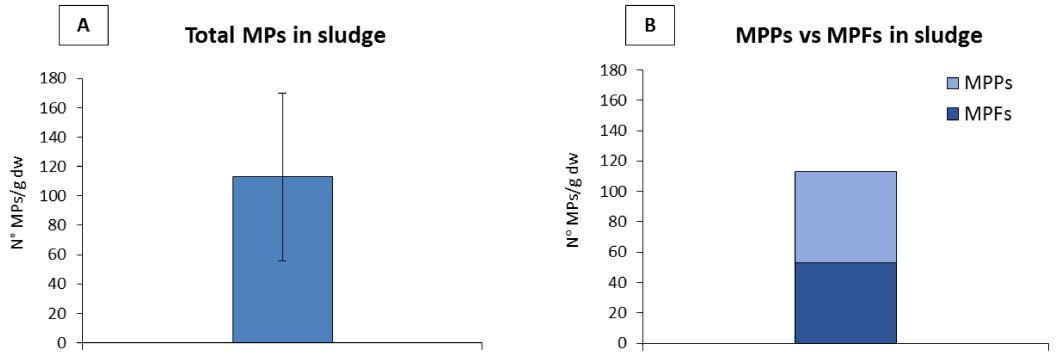


Figure 5.3 (A) *Concentration of microplastics/g dry weight of sludge; values are given as mean±standard deviation calculated on three days of sampling.* ***(B)*** *Relative contribution of microplastic particles (MPPs) and synthetic fibers (MPFs) to the total amount of MPs in the sludge.*

5.4.2 MPs shape, size and polymer typologies

Three types of shape of MPPs were identified, according to criteria expressed in the study of Viršek et al., (2016): fragments, films and lines, these last are distinguished from the MPFs because fibers appear like ribbon, with uneven width and frayed ends (Figure 5.4).

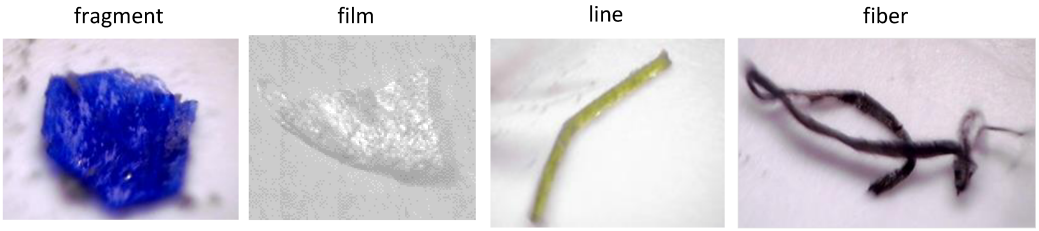


Figure 5.4 *Images of microplastic particles (MPPs) as fragment, film, line and microplastic fiber (MPF) extracted from wastewater and sludge samples.*

In particular, films were the main shape of MPPs (73%) which enter in the plant, followed by fragments (21%) and lines (6%). These ratios change during the wastewater treatments, with more similar percentages of films (36%), fragments (36%) and lines (28%) after the SET, while a greater ratio of lines (41%) and films (38%) followed by fragments (21%) was measured in the WWTP OUT (Figure 5.5). In the sludge 51% of MPPs were films, 34% fragments and 15% lines (Figure 5.5).

The predominant size range of MPPs was 0.5-0.1 mm either in sludge (54%) and in all the wastewater treatment steps, accounting for 36% of the total in the IN, 58% after the SET, 52% in the OUT (Figure 5.5); as we expected, we observed a step by step removal of the biggest particles (5-0.5 mm), corresponding to a total removal of 94% between IN and OUT. Microplastics of size up to 10 µm were also detected in liquid and solid fractions, despite the lower mesh of sieves used during sampling was 63 µm. This is justified by the potential occlusion of the sieve during wastewater filtration and by entrapment of smaller particles in flocs during biological treatment and/or in aggregates with organic and mineral materials that are present in wastewater (Castro et al., 2016; Lares et al., 2018).

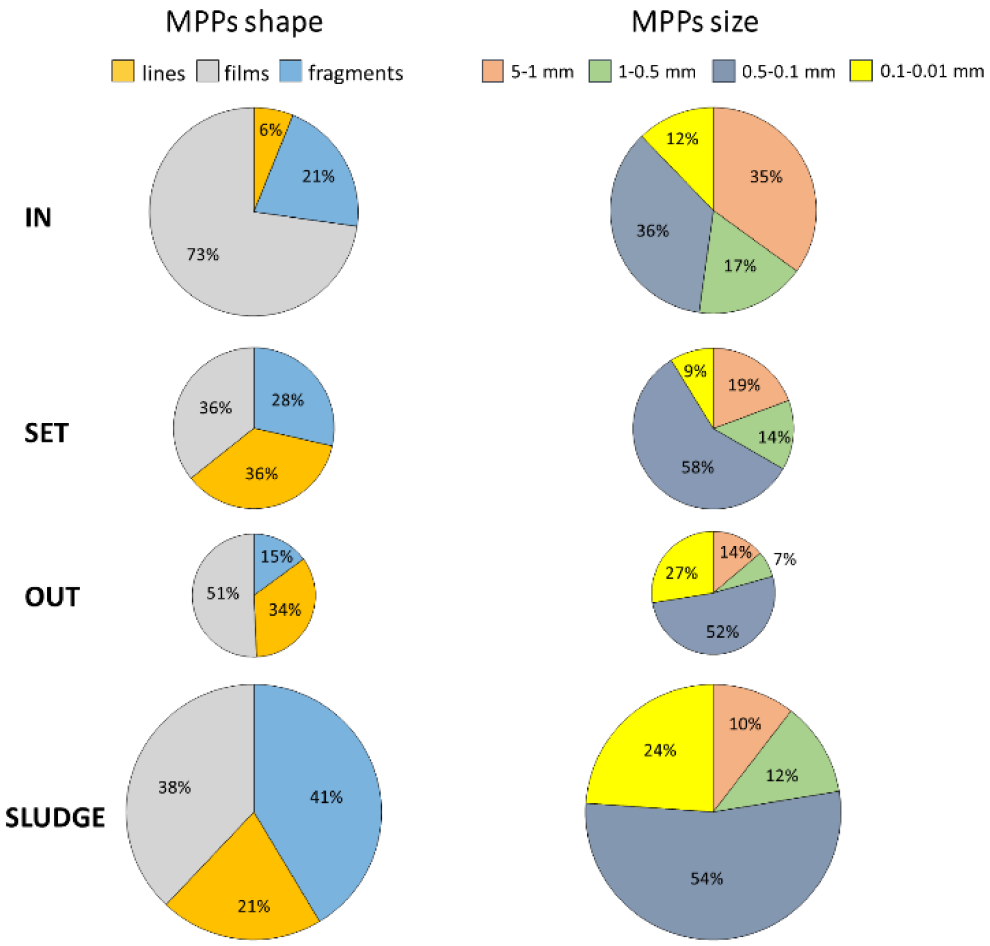


Figure 5.5 *Percentage contribution of shape typologies and size classes on the total amount of microplastic particles (MPPs) extracted from the inlet wastewater (IN, 2.0 ± 0.3 MPPs/L), after the settling step (SET, 0.6 ± 0.2 MPPs/L), in the effluent (OUT, 0.3 ± 0.1 MPPs/L) and in the sludge (59.5 ± 21.6 MPPs/g sludge dw) during the three sampling days.*

Characterization of MPPs by µFT-IR revealed a high eterogeneity of polymers and copolymers (Table 5.1; Figure 5.6). The copolymers, in particular, have been found in higher percentage entering the WWTP, with acrylonitrile-butadiene (or nitrile-butadiene rubber (NBR)) alone accounts for 40% of all MPPs (Figure 5.6A). This rubber is generally used in automotive seals, gaskets and pipes, but also in textiles, where its application to woven and nonwoven fabrics improves the finish and waterproofing properties. Another copolymer usually used in automotive and in the production of pipe seals is ethylene-propylene, that represented the 14% of microparticles in the IN, instead, among homopolymers the most present is polyethylene (17%) (Figure 5.6C), one of the "Big Six" that dominate the markets of plastics (Paul-Pont et al., 2018).

The passage of wastewater through the oxidative tanks and settler led to a decrease of MPPs copolymers after the SET and an increase of polyesters (23%), polyurethane (13%), polypropylene (11%) and polyamide (11%); polyesters further increased in the OUT (35%), along with polyamide (17%) and polyacrylates (7%), used in in personal care products and paints as adhesive agents. In this context, some MP classes, as epoxy resin, polyvinylchloride, polyoxymethylene and styrene-isoprene-styrene, were found only in the OUT wastewaters (Table 5.1). Even if these polymers were detected at a very low percentages (3%), their presence only in the OUT could suggest that the equipment used in WWTP processes might act as a potential direct source of polymers towards the aquatic environment.

This aspect is corroborated by the presence of MPs polymer classes, not detected in the IN wastewaters, also in the sludge, such as polytetrafluorethylene, polystyrene, silicone, styrene-isoprene copolymer (Table 5.1), thus the possible release of plastic materials directly by the WWTP structures should be carefully considered in future assessments of MPs generation and fate. Instead, MPPs of NBR, that have been detected in high abundance in the IN (40%) and much lower percentage in the SET (9%) and OUT (3%), have been found again as the more abundant chemical typologies identified in the sludge (27%), followed by polyethylene (18%) and polyesters (15%) (Table 5.1).

It is clear that in sludge it will be more likely to find plastics of density higher than that of wastewater (1 g/cm³), just like NBR (1.2-1.4 g/cm³) and polyesters (1.4 g/cm³), because the sedimentation process in WWTPs consists, exactly, in the physical separation of solids in suspension, exploiting gravity and their greater density compared to wastewater (Hreiz et al., 2015). However, mechanism of MPs floating/sedimentation can be modulated by coagulation and flocculation techniques adopted in WWTPs to promote separation of solids from the liquid fraction (Bratby, 2006) and by fouling of bacteria during the biological treatments (Carr et al., 2016). These factors can promote the settling of polymers with a density lower or very close to that of water, in fact we found in the sludge, also polyethylene (density: 0.93-0.98 g/cm³), polypropylene (density: 0.9 g/cm³) and polystyrene (density: 1.04-1.07 g/cm³) (Table 5.1).

Synthetic fibers collected from wastewater samples were mainly represented by polyesters, accounting for 83% in the IN, 79% after the SET and 89% in the OUT; the remaining polymers were polyacrylates (12%, 8% and 11% in the three steps) and polyamides (5% and 13% in the IN and after the SET respectively) (Table 5.2). Instead, all the MPFs detected in the sludge were of polyesters (Table 5.2).

The presence of polyester fibers in wastewater and sludge is ascribed to laundry and textile handling activities (Prata et al., 2018). A recent study (Sillanpää and Sainio, 2017) calculated an annual emission of 154,000 polyester MPFs by washing machines with a number of polyester fibers released in the first wash that varies from 2.1×10^5 to 1.3×10^7. The release of MPs from the washing machines will be one of the main challenges in the early future to decrease fibers in domestic wastes. Since the ban of production and use of synthetic clothes would be utopic, considering the pivotal role of non-disposable plastics in our lifestyle, there are already feasible solutions based on the use of filters for MPF retention in the washing machines (Napper and Thompson, 2016), the recourse to the labelling that certify the good practice in the clothes manufacture and the use of laundry soaps, softeners and the washing cycles more conservative.

Table 5.1 *Percentage contribution of polymers (black labels) and copolymers (blue labels) classes on the total amount of microplastic particles (MPPs) extracted from the inlet wastewater (IN), after the settling step (SET), in the effluent (OUT) and in the sludge 'during the three sampling days.*

MPPs Polymer Class	IN (%)	SET (%)	OUT (%)	Sludge (%)
epoxy resin	-	-	3	-
polyacrylates	-	2	7	3
polyamide	2	11	17	6
polyesters	4	23	35	15
polyoxymethylene	-	-	3	-
polytetrafluorethylene	-	-	-	2
polyterpene	2	-	3	-
polyethylene	17	13	10	18
polypropylene	4	11	-	9
polystyrene	-	-	-	5
polyurethane	3	13	7	3
polyvinylchloride	-	-	3	-
silicone	-	-	-	2
acrylonitrile-butadiene	40	9	3	27
acrylonitrile-butadiene-styrene	-	2	-	-
ethylene-acrylate	7	7	3	5
ethylene-propylene	14	-	-	-
ethylene-propylene-diene	2	9	-	5
ethylene-vinylacetate	1	2	-	-
styrene-butadiene-styrene	1	-	-	-
styrene-ethylene-butadiene-styrene	3	-	-	-
styrene-isoprene	-	-	-	2
styrene-isoprene-styrene	-	-	3	-
styrene-vinyltoluene-butylacrylate	1	-	-	-

Table 5.2 *Percentage contribution of polymers classes on the total amount of microplastic fibers (MPFs) extracted from the inlet wastewater (IN), after the settling step (SET), in the effluent (OUT) and in the sludge during the three sampling days.*

MPFs Polymer Class	IN (%)	SET (%)	OUT (%)	Sludge (%)
polyacrylates	12	8	11	-
polyamide	5	13	-	-
polyesters	83	79	89	100

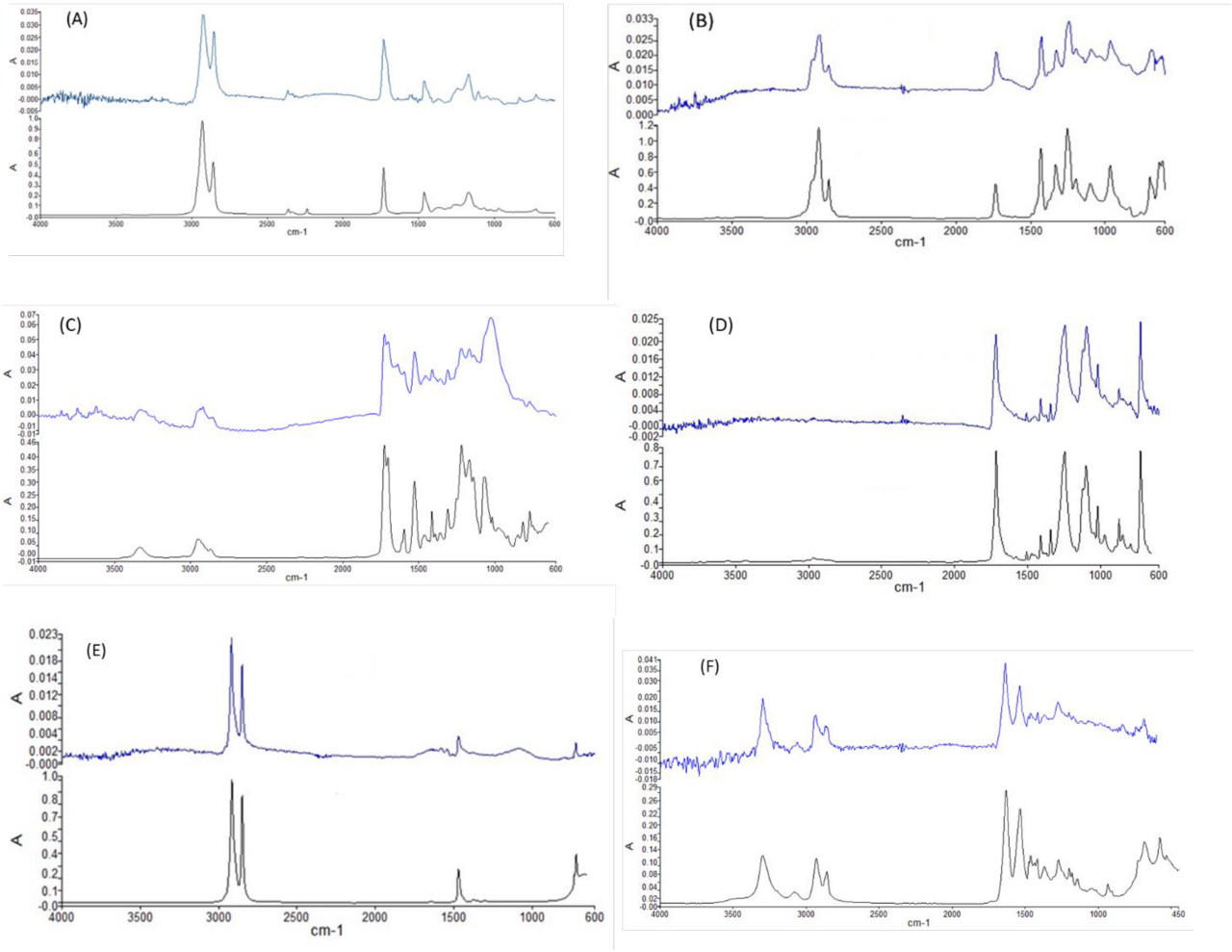


Figure 5.6 *Some IR spectra of detected microplastics in wastewater and sludge. The blue spectrum on the top represents the sample, the below black spectrum is the reference in the standard polymers' libraries. (A) Acrylonitrile butadiene copolymer (NBR); (B) polyvinylchloride; (C) polyurethane; (D) polyester; (E) polyethylene; (F) polyamide.*

5.5 Conclusion

The study highlighted that, despite the high MP removal efficiency of selected WWTP of 84%, its contribution to MPPs and MPFs pollution of surface waters is worrisome, in accordance with results of other European facilities. MPs were removed from wastewaters probably mainly in the grease and sedimentation processes, but also the advanced final stage treatments with sand filters significantly contributed to MP retention.

Unfortunately, MPs were not completely eliminated by the final effluent, confirming the need to refine and improve existing treatments plant processes to manage or eliminate this class of contaminants; in addition, their route towards the sludge provides new elements for regulation of the biosolid disposal in the environment.

Deeper investigations are necessary to further assess the relative importance of WWTPs as pathways for microlitter to the environment. Possible changes in microplastics characteristics during treatment processes should require attention, since, this might affect their behave and, thus, their fate within the WWTPs. Moreover, other sources of microplastics in the facilities should be taken into consideration for future evaluations, like stormwater run-off, atmospheric input and possible in situ degradation or release of plastic items.

6 CONCLUDING REMARKS AND OUTLOOK

The general objective of this PhD thesis was to contribute to the concern of interactions between microplastics and marine organisms, since their ingestion appears to be a widespread and pervasive phenomenon and evidence is also growing for impacts at different levels of biological organization.

The field study demonstrated as the occurrence of microplastics is widespread also in fish and invertebrates of the Adriatic Sea. Despite no differences has been highlighted on the abundance of ingested particles between species, sampling areas, habitat or trophic strategy, a major frequency of ingestion of smaller plastics and fragment-like has been observed in organisms from the Central and Southern sector in respect to those from the Northern one. Results have suggested as hydrodynamic circulation and rivers runoffs can favour the transport and accumulation of plastics in some areas more than others along the Adriatic Sea. The presence of intense and different kind human activities on the coastlines, that represent potential sources of marine litter, can explain the high heterogeneity of polymers extracted from samples, in particular from specimens collected in Chioggia and Lecce sites. Moreover, microplastics ingestion resulted more frequent in benthopelagic species, since they are more likely to interact with particles distributed within the water column and accumulated in sediments. In the study the percentage of positive organisms to microplastics ingestion has allowed to obtained interesting insight, suggesting that frequency of ingestion can be a more appropriate index than the number of ingested items to monitor microplastics in natural population.

The research also provided important insight on the ecotoxicological aspect of microplastics issue. The laboratory exposure of the Mediterranean mussels to both virgin and pre-contaminated particles showed that microplastics can vehicle adsorbed organic contaminants like B(a)P to tissue of organisms. Althought in the natural environment their contribution to the exposure to chemical bioaccumulation is certainly low compared with other sources, nonethless they can cause interaction between chemical and physical challenge. Under short-term exposure conditions of the study, both virgin and contaminated microplastics did not induce marked ecotoxicological effects at molecular and cellular levels, but evidences of the modulation of immune system, that in mussels is involved in the early responses of biological disturbance, along with the bioaccumulation of the organic contaminant after microplastics ingestion, not exclude subtle effects on organisms' health status under chronic exposures. This suggests the need to develop future studies as representative as possible of the real conditions, in order to obtain clearer informations on the effects of microplastics for exposed biota.

The investigation over the fate of microplastics in an Italian wastewater treatment plant confirmed as these facilities represent an important route for plastic particles to enter and contaminate aquatic and terrestrial ecosystems. Despite a step by step reduction on concentration of microplastics was detected during water treatment processes, their final daily release in the environment through the high volume of effluent and sludge fraction remains huge. This evidence draws attention to the need for refine and improve existing processes for minimizing the environmental impact of microplastics, but even more urgent, it is a sustainable management of wastewater flow and sewage sludge and a regulation of the amount of emerging pollutants, like microplastics, in WWTPs.

The overall conclusions of this research activity highlighted the need of further investigations on sources and fate of microplastics in the marine environment, their occurrence in marine food web and risks for organisms. An urgent need exists to extend the geographical scope of studies of microplastic contamination in biota to currently underrepresented areas, and to finalize and adopt standardized methods and quality-assurance protocols for the isolation, identification, and quantification of microplastic contaminants from biological

tissues. Despite the existence of considerable uncertainties and unknowns, there is already a compelling case for urgent actions to identify, control, and, where possible, eliminate key sources of both primary and secondary microplastics before they reach the marine environment, that requires an ever close collaboration among governments, scientists, industry and members of the public worldwide.

7 ACKNOWLEDGMENTS

The PhD research was partly supported by Centro di Assistenza Ecologica (ECOCAE, Ancona) within the Eureka Project. I thank the company and in particular Dr. Marco Dionisio which made possible the fulfillment of this work.

8 REFERENCES

Adams, S. M. (2005). Assessing cause and effect of multiple stressors on marine systems. *Marine Pollution Bulletin*, *51*(8-12), 649-657.

Anastasopoulou, A., Mytilineou, C., Smith, C. J., Papadopoulou, K. N. (2013). Plastic debris ingested by deep-water fish of the Ionian Sea (Eastern Mediterranean). *Deep Sea Research Part I: Oceanographic Research Papers*, 74, 11-13.

Anastasopoulou, A., Viršek, M. K., Varezić, D. B., Digka, N., Fortibuoni, T., Koren, Š., et al. (2018). Assessment on marine litter ingested by fish in the Adriatic and NE Ionian Sea macro-region (Mediterranean). *Marine pollution bulletin*, *133*, 841-851.

Anbumani, S., and Kakkar, P. (2018). Ecotoxicological effects of microplastics on biota: a review. *Environmental Science and Pollution Research*, 1-24.

Andrady, A. L. (2011). Microplastics in the marine environment. *Marine pollution bulletin*, 62(8), 1596-1605.

Andrady, A. L. (2015). Persistence of plastic litter in the oceans. In Marine anthropogenic litter (pp. 57-72). Springer, Cham.

Andrady, A. L. (2017). The plastic in microplastics: a review. *Marine pollution bulletin*, 119(1), 12-22.

Antunes, J. C., Frias, J. G. L., Micaelo, A. C., Sobral, P. (2013). Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. *Estuarine Coastal and Shelf Science*, 130, 62–69.

Artegiani, A., Paschini, E., Russo, A., Bregant, D., Raicich, F., Pinardi, N., (1997). The Adriatic Sea general circulation. Part I: Air-sea interactions and water mass structure. *Journal of Physical Oceanography*, 27, 1492–1514.

Arthur, C., Baker, J. E., Bamford, H. A. (2009). Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington Tacoma, Tacoma, WA, USA.

Avio, C. G., Cardelli, L. R., Gorbi, S., Pellegrini, D., Regoli, F. (2017). Microplastics pollution after the removal of the Costa Concordia wreck: first evidences from a biomonitoring case study. *Environmental pollution*, 227, 207-214.

Avio, C. G., Gorbi, S., and Regoli, F. (2015b). Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. *Marine environmental research*, 111, 18-26.

Avio, C. G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F. (2015a). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution*, 198: 211-222.

Avio, C. G., Gorbi, S., Regoli, F. (2016). Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. *Marine environmental research,* 128, 2-11.

Bagaev, A., Khatmullina, L., Chubarenko, I. (2018). Anthropogenic microlitter in the Baltic Sea water column. *Marine pollution bulletin*, 129(2), 918-923.

Bagaev, A., Mizyuk, A., Khatmullina, L., Isachenko, I., Chubarenko, I. (2017). Anthropogenic fibres in the Baltic Sea water column: Field data, laboratory and numerical testing of their motion. *Science of The Total Environment*, 599, 560-571.

Bakir, A., O'Connor, I. A., Rowland, S. J., Hendriks, A. J., Thompson, R. C. (2016). Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life.

Environmental pollution, 219, 56-65.

Bakir, A., Rowland, S. J., Thompson, R. C. (2014). Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution*, *185*, 16-23.

Banni, M., Sforzini, S., Arlt, V. M., Barranger, A., Dallas, L. J., Oliveri, C., et al. (2017). Assessing the impact of Benzo [a] pyrene on Marine Mussels: Application of a novel targeted low density microarray complementing classical biomarker responses. *PloS one*, 12(6), e0178460.

Barnes, D. K. A. (2002). Invasions by marine life on plastic debris. *Nature*, 416, 808–809.

Barnes, D. K., Galgani, F., Thompson, R. C., Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364(1526), 1985-1998.

Bayo, J., Olmos, S., López-Castellanos, J., & Alcolea, A. (2016). Microplastics and microfibers in the sludge of a municipal wastewater treatment plant. *International Journal of Sustainable Development and Planning*, 11(5), 812-821.

Beckingham, B., Ghosh, U. (2017). Differential bioavailability of polychlorinated biphenyls associated with environmental particles: Microplastic in comparison to wood, coal and biochar. *Environmental Pollution*, 220, 150-158.

Bellas, J., Martínez-Armental, J., Martínez-Cámara, A., Besada, V., Martínez-Gómez, C. (2016). Ingestion of microplastics by demersal fish from the Spanish Atlantic and Mediterranean coasts. *Marine pollution bulletin*, 109(1), 55-60.

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., Ausili, A.,Regoli, F. (2012). A multidisciplinary weight of evidence approach for classifying polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environment international*, 38(1), 17-28.

Bergmann, M., Lutz, B., Tekman, M. B., Gutow, L. (2017a). Citizen scientists reveal: Marine litter pollutes Arctic beaches and affects wild life. *Marine pollution bulletin*, 125(1-2), 535-540.

Bergmann, M., Sandhop, N., Schewe, I., D'Hert, D. (2016). Observations of floating anthropogenic litter in the Barents Sea and Fram Strait, Arctic. *Polar biology*, 39(3), 553-560.

Bergmann, M., Wirzberger, V., Krumpen, T., Lorenz, C., Primpke, S., Tekman, M. B., & Gerdts, G. (2017b). High quantities of microplastic in Arctic deep-sea sediments from the HAUSGARTEN observatory. *Environmental science and technology*, 51(19), 11000-11010.

Besseling, E., Foekema, E. M., Van Franeker, J. A., Leopold, M. F., Kühn, S., Rebolledo, E. B., et al. (2015). Microplastic in a macro filter feeder: humpback whale *Megaptera novaeangliae*. *Marine pollution bulletin*, 95(1), 248-252.

Besseling, E., Wegner, A., Foekema, E.M., Van Den Heuvel-Greve, M.J. Koelmans, A.A. (2013). Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). *Environmental science & technology*, 47(1): 593-600.

Beyer, J., Green, N. W., Brooks, S., Allan, I. J., Ruus, A., Gomes, T., Bråte, I. L. N., Schøyen, M. (2017). Blue mussels (*Mytilus edulis spp.)* as sentinel organisms in coastal pollution monitoring: a review. *Marine environmental research*, 130, 338-365.

Bhattacharya, P., Lin, S., Turner, J. P., Ke, P. C. (2010). Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. *The Journal of Physical Chemistry C*, 114(39), 16556-16561.

Binelli, A., Magni, S., Della Torre, C., Parolini, M., (2015). Toxicity decrease in urban wastewaters treated by a new biofiltration process. *Science of the Total Environment*, 537, 235-242.

Binelli, A., Magni, S., Soave, C., Marazzi, F., Zuccato, E., Castiglioni, S., Parolini, M., Mezzanotte, V., (2014). The biofiltration process by the bivalve D. Polymorpha for the removal of some pharmaceuticals and drugs of abuse from civil wastewaters. *Ecological engineering*, 71, 710-721.

Bläsing, M., and Amelung, W. (2018). Plastics in soil: Analytical methods and possible sources. *Science of the Total Environment*, 612, 422-435.

Blašković, A., Fastelli, P., Čižmek, H., Guerranti, C., Renzi, M. (2017). Plastic litter in sediments from the Croatian marine protected area of the natural park of Telaščica bay (Adriatic Sea). *Marine pollution bulletin*, 114(1), 583-586.

Blettler, M. C., Abrial, E., Khan, F. R., Sivri, N., Espinola, L. A. (2018). Freshwater plastic pollution: Recognizing research biases and identifying knowledge gaps. *Water research*, 416-424.

Bocchetti, R., and Regoli, F. (2006). Seasonal variability of oxidative biomarkers, lysosomal parameters,

metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. *Chemosphere*, 65(6), 913-921.

Bocchetti, R., Lamberti, C. V., Pisanelli, B., Razzetti, E. M., Maggi, C., Catalano, B., Sesta, G., Martuccio, G., Gabellini, M., Regoli, F. (2008). Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, *Tapes philippinarum*, and mussels, *Mytilus galloprovincialis*, from Adriatic sea. *Marine environmental research*, 66(1), 24-26.

Boerger, C. M., Lattin, G. L., Moore, S. L., Moore, C. J. (2010). Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. *Marine pollution bulletin*, 60(12), 2275-2278.

Boucher, J., and Friot, D. (2017). Primary microplastics in the oceans: a global evaluation of sources. Gland, Switzerland: IUCN.

Bour, A., Avio, C. G., Gorbi, S., Regoli, F., Hylland, K. (2018). Presence of microplastics in benthic and epibenthic organisms: Influence of habitat, feeding mode and trophic level. *Environmental Pollution*, 243, 1217-1225.

Brandsma, S.H., Nijssen, P., Van Velzen, M.J., Leslie, H.A., (2013). Microplastics in River Suspended Particulate Matter and Sewage Treatment Plants. Institute for Environmental Studies, VU University Amsterdam, Amsterdam, The Netherlands.

Brandts, I., Teles, M., Gonçalves, A. P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M.A., Soares, A.M.V.M., Tort, L., Oliveira, M. (2018). Effects of nanoplastics on *Mytilus galloprovincialis* after individual and combined exposure with carbamazepine. *Science of The Total Environment*, 643, 775-784.

Bratby, J. (2006). Coagulation and flocculation in water and wastewater treatment. IWA publishing.

Bråte, I. L. N., Hurley, R., Iversen, K., Beyer, J., Thomas, K. V., Steindal, C. C., Green, N. W., Olsen, M., Lusher, A. (2018). *Mytilus spp.* as sentinels for monitoring microplastic pollution in Norwegian coastal waters: A qualitative and quantitative study. *Environmental Pollution*, 243, 383-393.

Browne (2015). Sources and pathways of microplastics to habitats. In *Marine anthropogenic litter* (pp. 229-244). Springer, Cham.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R. (2011). Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environmental science & technology*, 45(21), 9175-9179.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environmental Science and Technology*, 42: 5026-5031.

Browne, M. A., Galloway, T.S., Thompson, R.C. (2007). Microplastic—an emerging contaminant of potential concern? *Integrated Environmental Assessment and Management*, 3, 559–561.

Browne, M. A., Niven, S. J., Galloway, T. S., Rowland, S. J., Thompson, R. C. (2013). Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Current Biology,* 23(23), 2388-2392.

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment*, 247(2-3), 295-311.

Cajaraville, M. P., Orbea, A., Marigómez, I., and Cancio, I. (1997). Peroxisome proliferation in the digestive epithelium of mussels exposed to the water accommodated fraction of three oils. *Comparative Biochemistry and Physiology* C ,117, 233–242.

Calisi, A., Lionetto, M. G., Caricato, R., Giordano, M. E., Schettino, T. (2008). Morphometric alterations in *Mytilus galloprovincialis* granulocytes: a new biomarker. *Environmental Toxicology and Chemistry: An International Journal*, *27*(6), 1435-1441.

Cancio, I., Ibabe, A., Cajaraville, M. P. (1999). Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels Mytilus galloprovincialis and its relationship with the lipid content. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 123(2), 135-144.

Capolupo, M., Franzellitti, S., Valbonesi, P., Lanzas, C. S., Fabbri, E. (2018). Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*. *Environmental Pollution*, 241, 1038-1047.

Cappello, T., Maisano, M., Giannetto, A., Parrino, V., Mauceri, A., Fasulo, S. (2015). Neurotoxicological effects

on marine mussel *Mytilus galloprovincialis* caged at petrochemical contaminated areas (eastern Sicily, Italy): 1H NMR and immunohistochemical assays. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 169, 7-15.

Carlson, D. F., Suaria, G., Aliani, S., Fredj, E., Fortibuoni, T., Griffa, A., Russo, A., Melli, V. (2017). Combining litter observations with a regional ocean model to identify sources and sinks of floating debris in a semi-enclosed basin: the Adriatic Sea. *Frontiers in Marine Science*, 4, 78.

Carpenter, E. J., Anderson, S. J., Harvey, G. R., Miklas, H. P., and Peck, B. B. (1972). Polystyrene spherules in coastal waters. Science, 178, 749–750.

Carr, S. A., Liu, J., Tesoro, A.G., (2016). Transport and fate of microplastic particles in wastewater treatment plants. *Water Research,* 91, 174-182.

Carson, H. S., Nerheim, M. S., Carroll, K. A., Eriksen, M. (2013). The plastic-associated microorganisms of the North Pacific Gyre. *Marine Pollution Bulletin*, 75, 126–132.

Castro, R.O., Silva, M.L., Marques, M.R.C., de Araújo, F.V., (2016). Evaluation of microplastics in Jurujuba Cove, Niter_oi, RJ, Brazil, an area of mussels farming. *Marine Pollution Bulletin*, 110 (1), 555e558.

Cedervall, T., Hansson, L.A., Lard, M., Frohm, B., Linse, S. (2012). Food chain transport of nanoparticles affects behaviour and fat metabolism in fish. *PloS One*, 7(2).

Chen, C. L. (2015). Regulation and management of marine litter. In Marine anthropogenic litter (pp. 395-428). Springer, Cham.

Cheung, P. K., and Fok, L. (2016). Evidence of microbeads from personal care product contaminating the sea. *Marine pollution bulletin*, 109(1), 582-585.

Christaki, U., Dolan, J.R., Pelegri, S., Rassoulzadegan, F. (1998). Consumption of picoplankton-size particles by marine ciliates: effects of physiological state of the ciliate and particle quality. Limnol. *Oceanography*, 43(3): 458-464.

Claessens, M., De Meester, S., Van Landuyt, L., De Clerck, K., & Janssen, C. R. (2011). Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin*, 62(10), 2199-2204.

Cole, M., Lindeque, P. K., Fileman, E., Clark, J., Lewis, C., Halsband, C., Galloway, T. S. (2016). Microplastics alter the properties and sinking rates of zooplankton faecal pellets. *Environmental science & technology*, 50(6), 3239-3246.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., and Galloway, T. S. (2015). The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. *Environmental science & technology*, 49(2), 1130-1137.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T. S. (2013). Microplastic ingestion by zooplankton. *Environmental science & technology*, 47(12), 6646-6655.

Cole, M., Lindeque, P., Halsband, C., Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: a review. *Marine Pollution Buletin*, 62,2588–2597.

Cole, M., Webb, H., Lindeque, P. K., Fileman, E. S., Halsband, C., Galloway, T. S. (2014). Isolation of microplastics in biota-rich seawater samples and marine organisms. *Scientific reports*, 4, 4528.

Collignon, A., Hecq, J. H., Glagani, F., Voisin, P., Collard, F., Goffart, A. (2012). Neustonic microplastic and zooplankton in the North Western Mediterranean Sea. *Marine pollution bulletin*, 64(4), 861-864.

Corcoran, P. L., Biesinger, M. C., Grifi, M. (2009). Plastics and beaches: A degrading relationship. *Marine Pollution Bulletin*, 58(1), 80–84.

Corcoran, P. L., Moore, C. J., Jazvac, K. (2014). An anthropogenic marker horizon in the future rock record. *GSA today*, 24(6), 4-8.

Costa, M., Ivar do Sul, J., Silva-Cavalcanti, J., Araújo, M., Spengler, Â., Tourinho, P. (2010), On the importance of size of plastic fragments and pellets on the strandline: A snapshot of a Brazilian beach. *Environmental Monitoring and Assessment*, 168, 299–304.

Cowie, J. M. G. (Ed.). (2013). Alternating copolymers. Springer Science & Business Media.

Cózar, A., Echevarria, F., Gonzalez-Gordillo, J.I., Irigoien, X., Ubeda, B., Hernandez-Leon, S., Palma, A.t., Navarro, S., Garcia-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., (2014). Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences*, U. S. A. 111, 10239e10244.

Cózar, A., Martí, E., Duarte, C. M., García-de-Lomas, J., Van Sebille, E., Ballatore, T. J., et al. (2017). The Arctic Ocean as a dead end for floating plastics in the North Atlantic branch of the Thermohaline Circulation.

Science advances, 3(4), e1600582.

Cózar, A., Sanz-Martín, M., Martí, E., González-Gordillo, J. I., Ubeda, B., Gálvez, J. Á., Irigoien, X., Duarte, C. M. (2015). Plastic accumulation in the Mediterranean Sea. *PLoS One*, 10(4).

Crain, C. M., Kroeker, K., & Halpern, B. S. (2008). Interactive and cumulative effects of multiple human stressors in marine systems. *Ecology letters*, *11*(12), 1304-1315.

Darmody, G.,Maloy, A. P., Lynch, S. A., Prado-Alvarez,M., Cotterill, J.,Wontner- Smith, T., et al. (2015). Tissue targeting of the European flat oyster, Ostrea edulis, using microencapsulated microbeads as a biological proxy. *Aquaculture International*, 23, 647–659.

Davarpanah, E. and Guilhermino, L. (2015). Single and combined effects of microplastics and copper on the population growth of the marine microalgae Tetraselmis chuii. Estuarine, Coastal Shelf Science, 167: 269-275.

Davidson, K., and Dudas, S. E. (2016). Microplastic ingestion by wild and cultured Manila clams (Venerupis philippinarum) from Baynes Sound, British Columbia. *Archives of environmental contamination and toxicology*, 71(2), 147-156.

Davison, P., and Asch, R. G. (2011). Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. *Marine Ecology Progress Series*, 432, 173-180.

Day, R. H., Wehle, D. H., Coleman, F. C. (1985). Ingestion of plastic pollutants by marine birds. In Proceedings of the Workshop on the Fate and Impact of Marine Debris (Vol. 2, p. 34). US Dept. Commerce.

de Sá, L. C., Luís, L. G., Guilhermino, L. (2015). Effects of microplastics on juveniles of the common goby (Pomatoschistus microps): confusion with prey, reduction of the predatory performance and efficiency, and possible influence of developmental conditions. *Environmental Pollution*, 196, 359-362.

de Souza Machado, A. A., Kloas, W., Zarfl, C., Hempel, S., & Rillig, M. C. (2018). Microplastics as an emerging threat to terrestrial ecosystems. *Global change biology*, 24(4), 1405-1416.

de Stephanis, R., Giménez, J., Carpinelli, E., Gutierrez-Exposito, C., Cañadas, A. (2013). As main meal for sperm whales: Plastics debris. *Marine pollution bulletin*, 69(1-2), 206-214.

Del Rio, D., Stewart, A. J., Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, metabolism and cardiovascular diseases*, 15(4), 316-328.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K.A. Corsi, I. 2014. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *Paracentrotus lividus*. *Environmental science & technology*, 48(20).

Depledge, M. H. (1993). The rational basis for the use of biomarkers as ecotoxicological tools. In Nondestructive Biomarkers in Vertebrates (M. C. Fossi & C. Leonzio, eds), pp. 261-285. Lewis Publishers, Boca Raton, FL, USA.

Derraik, J. G. (2002). The pollution of the marine environment by plastic debris: a review. *Marine pollution bulletin*, 44(9), 842-852.

Détrée, C., and Gallardo-Escárate, C. (2017). Polyethylene microbeads induce transcriptional responses with tissue-dependent patterns in the mussel *Mytilus galloprovincialis*. Journal of Molluscan Studies, 83: 220-225.

Détrée, C., and Gallardo-Escárate, C. (2018). Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel Mytilus galloprovincialis. *Fish & shellfish immunology*, 83,52-60.

Devriese, L. I., van der Meulen, M. D., Maes, T., Bekaert, K., Paul-Pont, I., Frère, L., et al. (2015). Microplastic contamination in brown shrimp (*Crangon crangon*, Linnaeus 1758) from coastal waters of the Southern North Sea and Channel area. *Marine pollution bulletin*, 98(1-2), 179-187.

Donohue, M. J., Boland, R. C., Sramek, C. M., Antonelis, G. A. (2001). Derelict fishing gear in the Northwestern Hawaiian Islands: diving surveys and debris removal in 1999 confirm threat to coral reef ecosystems. *Marine Pollution Bulletin*, 42(12), 1301-1312.

Driedger, A. G., Dürr, H. H., Mitchell, K., Van Cappellen, P. (2015). Plastic debris in the Laurentian Great Lakes: a review. *Journal of Great Lakes Research*, 41(1), 9-19.

Dris, R., Gasperi, J., Mirande, C., Mandin, C., Guerrouache, M., Langlois, V., Tassin, B. (2017). A first overview of textile fibers, including microplastics, in indoor and outdoor environments. *Environmental Pollution*, 221, 453-458.

Dris, R., Gasperi, J., Rocher, V., Saad, M., Renault, N., & Tassin, B. (2015). Microplastic contamination in an urban area: a case study in Greater Paris. *Environmental Chemistry*, *12*(5), 592-599.

Dris, R., Gasperi, J., Saad, M., Mirande, C., Tassin, B. (2016). Synthetic fibers in atmospheric fallout: a source of microplastics in the environment?. *Marine pollution bulletin*, 104(1-2), 290-293.

Dubaish, F., & Liebezeit, G. (2013). Suspended microplastics and black carbon particles in the Jade system, southern North Sea. *Water, Air, & Soil Pollution*, *224*(2), 1352.

Dümichen, E., Barthel, A. K., Braun, U., Bannick, C. G., Brand, K., Jekel, M., Senz, R. (2015). Analysis of polyethylene microplastics in environmental samples, using a thermal decomposition method. *Water research*, 85, 451-457.

Enders, K., Lenz, R., Stedmon, C. A., and Nielsen, T. G. (2015). Abundance, size and polymer composition of marine microplastics >10μm in the Atlantic Ocean and their modelled vertical distribution. Marine Pollution Bulletin, 100, 70–81.

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., et al (2014). Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PloS one*, 9(12), e111913.

Eriksen, M., Mason, S., Wilson, S., Box, C., Zellers, A., Edwards, W., Farley, H., Amato, S. (2013). Microplastic pollution in the surface waters of the Laurentian Great Lakes. *Marine pollution bulletin*, 77(1-2), 177-182.

Eriksson, C., and Burton, H. (2003). Origins and biological accumulation of small plastic particles in fur seals from Macquarie Island. *AMBIO: A Journal of the Human Environment*, 32(6), 380-384.

Erni-Cassola, G., Gibson, M. I., Thompson, R. C., and Christie-Oleza, J. A. (2017). Lost, but found with Nile Red: a novel method for detecting and quantifying small microplastics (1mm to 20μm) in environmental samples. *Environmental science and technology*, 51, 13641–13648.

Espinosa, C., Beltrán, J. M. G., Esteban, M. A., Cuesta, A. (2018). In vitro effects of virgin microplastics on fish head-kidney leucocyte activities. *Environmental Pollution*, 235, 30-38.

Farrell, P., and Nelson, K. (2013). Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environmental Pollution*, 177, 1-3.

Fazey, F. M., and Ryan, P. G. (2016). Biofouling on buoyant marine plastics: An experimental study into the effect of size on surface longevity. *Environmental pollution*, 210, 354-360.

Fendall, L. S., and Sewell, M. A. (2009). Contributing to marine pollution by washing your face: Microplastics in facial cleansers. *Marine Pollution Bulletin*, 58, 1225–1228.

Foekema, E.M., De Gruijter, C., Mergia, M.T., van Franeker, J.A., Murk, A.J., Koelmans, A.A., (2013). Plastic in North Sea fish. *Environmental Science and Technology*, 47, 8818e8824.

Fonte, E., Ferreira, P., Guilhermino, L. (2016). Temperature rise and microplastics interact with the toxicity of the antibiotic cefalexin to juveniles of the common goby (Pomatoschistus microps): Post-exposure predatory behaviour, acetylcholinesterase activity and lipid peroxidation. *Aquatic Toxicology*, 180, 173-185.

Fossi, M. C., Panti, C., Guerranti, C., Coppola, D., Giannetti, M., Marsili, L., Minutoli, R. (2012). Are baleen whales exposed to the threat of microplastics? A case study of the Mediterranean fin whale (*Balaenoptera physalus*). *Marine Pollution Bulletin*, 64(11), 2374-2379.

Franzellitti, S., and Fabbri, E. (2005). Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. *Biochemical and Biophysical Research Communications*, 336, 1157–1163.

Free, C. M., Jensen, O. P., Mason, S. A., Eriksen, M., Williamson, N. J., Boldgiv, B. (2014). High-levels of microplastic pollution in a large, remote, mountain lake. *Marine pollution bulletin*, 85(1), 156-163.

Frère, L., Paul-Pont, I., Rinnert, E., Petton, S., Jaffré, J., Bihannic, I., et al (2017). Influence of environmental and anthropogenic factors on the composition, concentration and spatial distribution of microplastics: a case study of the Bay of Brest (Brittany, France). *Environmental pollution*, 225, 211-222.

Gago, J., Carretero, O., Filgueiras, A. V., Viñas, L. (2018). Synthetic microfibers in the marine environment: A review on their occurrence in seawater and sediments. *Marine pollution bulletin*, 127, 365-376.

Gago, J., Galgani, F., Maes, T., Thompson, R. C. (2016). Microplastics in Seawater: recommendations from the marine strategy framework directive implementation process. *Frontiers in Marine Science*, *3*, 219.

Gajšt, T., Bizjak, T., Palatinus, A., Liubartseva, S., & Kržan, A. (2016). Sea surface microplastics in Slovenian

part of the Northern Adriatic. *Marine pollution bulletin*, 113(1-2), 392-399.
Galgani, F. (2015). Marine Litter Within the European Marine Strategy Framework Directive. In *Marine Productivity: Perturbations and Resilience of Socio-ecosystems* (pp. 93-100). Springer, Cham.
Galgani, F., Claro, F., Depledge, M., Fossi, C. (2014). Monitoring the impact of litter in large vertebrates in the Mediterranean Sea within the European Marine Strategy Framework Directive (MSFD): Constraints, specificities and recommendations. *Marine environmental research*, 100, 3-9.
Galgani, F., Hanke, G., and Maes, T. (2015). Global distribution, composition and abundance of marine litter. In Marine anthropogenic litter (pp. 29-56). Springer, Cham.
Galgani, F., Leaute, J. P., Moguedet, P., Souplet, A., Verin, Y., Carpentier, A., et al. (2000). Litter on the sea floor along European coasts. *Marine pollution bulletin*, 40(6), 516-527.
Gall, S. C., and Thompson, R. C. (2015). The impact of debris on marine life. *Marine pollution bulletin*, 92(1-2), 170-179.
Galloway, T. S. (2015). Micro-and nano-plastics and human health. In Marine anthropogenic litter (pp. 343-366). Springer, Cham.
Galloway, T. S., and Depledge, M. H. (2001). Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology*, 10(1), 5-23.
Galloway, T. S., and Lewis, C. N. (2016). Marine microplastics spell big problems for future generations. *Proceedings of the National Academy of Sciences*, 113(9), 2331-2333.
Galloway, T. S., Cole, M., Lewis, C. (2017). Interactions of microplastic debris throughout the marine ecosystem. *Nature ecology & evolution*, 1(5), 0116.
Galloway, T. S., Millward, N., Browne, M. A., Depledge, M. H. (2002). Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. *Aquatic Toxicology*, 61(3-4), 169-180.
Gandara e Silva, P. P. G., Nobre, C. R., Resaffe, P., Pereira, C. D. S., Gusmão, F. (2016). Leachate from microplastics impairs larval development in brown mussels. *Water research*, 106, 364-370.
Garrigos, M. C., Marin, M. L., Canto, A., Sanchez, A. (2004). Determination of residual styrene monomer in polystyrene granules by gas chromatography–mass spectrometry. *Journal of Chromatography A*, 1061(2), 211-216.
GESAMP (2016). Sources, fate and effects of microplastics in the marine environment: part two of a global assessment (eds Kershaw, P. J. & Rochman, C. M.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP 93, 220 (2016).
GESAMP, (2010). In: Proceedings of the GESAMP International Workshop on Plastic Particles as a Vector in Transporting Persistent, Bio-Accumulating and Toxic Substances in the Oceans. GESAMP Rep. Stud. No. 82, 68pp.Mourgkogiannis, N., Kalavrouziotis, I. K., & Karapanagioti, H. K. (2018). Questionnaire-based survey to managers of 101 wastewater treatment plants in Greece confirms their potential as plastic marine litter sources. *Marine Pollution Bulletin*, *133*, 822-827.
GESAMP, (2015) Sources, fate and effects of microplastics in the marine environment: a global assessment
Geyer, R., Jambeck, J. R., Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science advances*, 3(7), e170078.
Gilfillan, L. R., Ohman, M. D., Doyle, M. J. , Watson, W. (2009). Occurrence of Plastic Micro-debris in the Southern California Current System, vol. 50, California Cooperative Oceanic and Fisheries Investigations Report pp. 123–133.
Goldstein, M. C., Titmus, A. J., Ford, M. (2013). Scales of spatial heterogeneity of plastic marine debris in the northeast Pacific Ocean. *PloS one*, 8(11), e80020.
Gomiero, A., Strafella, P., & Fabi, G. (2018). From Macroplastic to Microplastic Litter: Occurrence, Composition, Source Identification and Interaction with Aquatic Organisms. Experiences from the Adriatic Sea.
Gorbi, S., and Regoli, F. (2003). Total oxyradical scavenging capacity as an index of susceptibility to oxidative stress in marine organisms. *Comments on Toxicology*, 9:5-6, 303-322.
Gorbi, S., Avio, G. C, Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., Bacchiocchi, S., Orletti, R., Graziosi, T., Regoli, F. (2013) Effects of harmful dinoflagellate *Ostreopsis cf. ovata* exposure on immunological, histological and oxidative responses of mussels *Mytilus galloprovincialis*. *Fish and Shellfish Immunology*, 35, 941-950.

Gorbi, S., Lamberti, C. V., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G., Regoli, F. (2008). An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea. *Marine Environmental Research*, 65(1), 34-49.

Gosling, E., (2003). Bivalve Molluscs: Biology, Ecology and Culture, first ed. Wiley-Blackwell.

Gouin, T., Roche, N., Lohmann, R., Hodges, G. (2011). A thermodynamic approach for assessing the environmental exposure of chemicals absorbed to microplastic. *Environmental Science & Technology*, 45(4), 1466-1472.

Graham, E. R., and Thompson, J. T. (2009). Deposit- and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. *Journal of Experimental Marine Biology and Ecology*, 368(1), 22–29.

Gray, A. D., and Weinstein, J. E. (2017). Size-and shape-dependent effects of microplastic particles on adult daggerblade grass shrimp (Palaemonetes pugio). *Environmental toxicology and chemistry*, 36(11), 3074-3080.

Gregory, M. R. (1996). Plastic 'scrubbers' in hand cleansers: a further (and minor) source for marine pollution identified. *Marine Pollution Bulletin*, 32, 867–871.

Gregory, M. R., and Andrady, A. L. (2003). Plastics in the marine environment. In A. L. Andrady (Ed.), Plastics and the Environment (pp. 379–401). Hoboken, NJ: Wiley.

Gurung, K., Ncibi, M.C., Fontmorin, J.M., Särkkä, H., Sillanpää, M., (2016). Incorporating submerged MBR in conventional activated sludge process for municipal wastewater treatment: a feasibility and performance assessment. *Journal of Membrane Science and Technology,* 6 (3).

Gutow, L., Eckerlebe, A., Gimenez, L. & Saborowski, R. (2016). Experimental evaluation of seaweeds as a vector for microplastics into marine food webs. *Environmental Science & Technology*, 50(2): 915-923.

Habib, D., Locke, D.C., Cannone, L.J., (1998). Synthetic fibers as indicators of municipal sewage sludge, sludge products, and sewage treatment plant. *Water Air and Soil Pollution,* 103, 1e8.

Hall, N.M., Berry, K.L.E., Rintoul, L. Hoogenboom, M.O. (2015). Microplastic ingestion by scleractinian corals. *Marine Biology*, 162(3), 725-732.

Hämer, J., Gutow, L., Köhler, A., Saborowski, R. (2014). Fate of microplastics in the marine isopod *Idotea emarginata*. *Environmental science & technology*, 48(22): 13451-13458.

Harrison, J. P., Ojeda, J. J., Romero-Gonzalez, M. E. (2012). The applicability of reflectance micro-Fourier-transform infrared spectroscopy for the detection of synthetic microplastics in marine sediments. *Science of the Total Environment*, 416, 455–463.

Hartley, B. L., Pahl, S., Veiga, J., Vlachogianni, T., Vasconcelos, L., Maes, T., et al. (2018). Exploring public views on marine litter in Europe: Perceived causes, consequences and pathways to change. *Marine pollution bulletin*. 133, 945-955.

Haward, M. (2018). Plastic pollution of the world's seas and oceans as a contemporary challenge in ocean governance. *Nature communications*, 9(1), 667.

Heindler, F. M., Alajmi, F., Huerlimann, R., Zeng, C., Newman, S. J., Vamvounis, G., van Herwerden, L. (2017). Toxic effects of polyethylene terephthalate microparticles and Di (2-ethylhexyl) phthalate on the calanoid copepod, *Parvocalanus crassirostris*. *Ecotoxicology and environmental safety*, 141, 298-305.

Hewitt JE, Ellis JI, Thrush SF (2016). Multiple stressors, nonlinear effects and the implications of climate change impacts on marine coastal ecosystems. *Global Change Biology*, 22, 2665-2675.

Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., Thiel, M. (2012). Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environmental science and technology*, 46(6), 3060-3075.

Hintersteiner, I., Himmelsbach, M., Buchberger, W. W. (2015). Characterization and quantitation of polyolefin microplastics in personal-care products using high-temperature gel-permeation chromatography. *Analytical and bioanalytical chemistry*, 407(4), 1253-1259.

Holmes, L. A., Turner, A., Thompson, R. C. (2012). Adsorption of trace metals to plastic resin pellets in the marine environment. *Environmental Pollution*, 160, 42-48.

Hope P.S., and Folkes M.J. (1993) Introduction. In: Folkes M.J., Hope P.S. (eds) Polymer Blends and Alloys. Springer, Dordrecht.

Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., & Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of the total environment*, *586*, 127-141.

Hreiz, R., Latifi, M. A., & Roche, N. (2015). Optimal design and operation of activated sludge processes: State-

of-the-art. *Chemical Engineering Journal*, 281, 900-920.
Isobe, A., Kubo, K., Tamura, Y., Nakashima, E., Fujii, N. (2014). Selective transport of microplastics and mesoplastics by drifting in coastal waters. *Marine pollution bulletin*, 89(1-2), 324-330.
Isobe, A., Uchiyama-Matsumoto, K., Uchida, K., Tokai, T. (2017). Microplastics in the Southern Ocean. *Marine pollution bulletin*, 114(1), 623-626.
Jackson, G. D., Buxton, N. G., George, M. J. (2000). Diet of the southern opah Lampris immaculatus on the Patagonian Shelf; the significance of the squid Moroteuthis ingens and anthropogenic plastic. *Marine Ecology Progress Series*, 206, 261-271.
Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R., Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, 347(6223), 768-771.
Jemec, A., Horvat, P., Kunej, U., Bele, M., Kržan, A. (2016). Uptake and effects of microplastic textile fibers on freshwater crustacean *Daphnia magna*. *Environmental pollution*, 219, 201-209.
Jovanović, B., Gökdağ, K., Güven, O., Emre, Y., Whitley, E. M., Kideys, A. E. (2018). Virgin microplastics are not causing imminent harm to fish after dietary exposure. *Marine pollution bulletin*, 130, 123-131.
Kaiser, J. (2010). The dirt on ocean garbage patches.
Kanhai L. D., K., Officer, R., Lyashevska, O., Thompson, R. C., & O'Connor, I. (2017). Microplastic abundance, distribution and composition along a latitudinal gradient in the Atlantic Ocean. Marine pollution bulletin, 115(1-2), 307-314.
Kaposi, K.L., Mos, B., Kelaher, B.P., Dworjanyn, S.A. (2014). Ingestion of microplastic has limited impact on a marine larva. *Environmental science & technology*, 48(3).
Karami, A. (2017). Gaps in aquatic toxicological studies of microplastics. *Chemosphere*, 184, 841-848.
Karlsson, T. M., Vethaak, A. D., Almroth, B. C., Ariese, F., van Velzen, M., Hassellöv, M., Leslie, H. A. (2017). Screening for microplastics in sediment, water, marine invertebrates and fish: method development and microplastic accumulation. *Marine pollution bulletin*, 122(1-2), 403-408.
Karvelas M, Katsoyiannis A, Samara C (2003). Occurrence and the fate of heavy metals in the wastewater treatment process. *Chemosphere* 53(10):1201–1210.
Kershaw, P., Katsuhiko, S., Lee, S., Woodring, D. (2011). Plastic debris in the ocean. United Nations Environment Programme.
Khan F.R., Mayoma B.S., Biginagwa F.J., Syberg K. (2018) Microplastics in Inland African Waters: Presence, Sources, and Fate. In Freshwater Microplastics (pp. 1-23). Springer, Cham.
Kiessling, T., Gutow, L., Thiel, M. (2015). Marine litter as a habitat and dispersal vector. In M. Bergmann, L. Gutow, & M. Klages (Eds.), Marine anthropogenic litter (pp. 141–181). Springer, Berlin.
Klein, S., Worch, E., Knepper, T. P., (2015). Occurrence and spatial distribution of microplastics in river shore sediments of the Rhine-Main area in Germany. *Environmental Science* & *Technology*, 49, 6070-6076.
Koelmans, A. A., Bakir, A., Burton, G. A., Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environmental science & technology*, 50(7), 3315-3326.
Koelmans, A. A., Besseling, E., Shim, W. J. (2015) Nanoplastics in the aquatic environment. In M. Bergmann, L. Gutow, & M. Klages (Eds.), Marine anthropogenic litter (pp. 329–344). Springer, Berlin.
Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K., Shi, H. (2018). Adherence of microplastics to soft tissue of mussels: a novel way to uptake microplastics beyond ingestion. *Science of The Total Environment*, 610, 635-640.
Krelling, A. P., Souza, M. M., Williams, A. T., Turra, A. (2017). Transboundary movement of marine litter in an estuarine gradient: Evaluating sources and sinks using hydrodynamic modelling and ground truthing estimates. *Marine pollution bulletin*, 119(1), 48-63.
Kuhlbrodt, T., Smith, R. S., Wang, Z., Gregory, J. M. (2012). The influence of eddy parameterizations on the transport of the Antarctic Circumpolar Current in coupled climate models. *Ocean Modelling*, 52, 1-8.
Kukulka, T., Proskurowski, G., Morét-Ferguson, S., Meyer, D. W., and Law, K. L. (2012). The effect of wind mixing on the vertical distribution of buoyant plastic debris. *Geophysical Research Letters*, *39*(7).
Lagarde, F., Olivier, O., Zanella, M., Daniel, P., Hiard, S., Caruso, A. (2016). Microplastic interactions with freshwater microalgae: hetero-aggregation and changes in plastic density appear strongly dependent on polymer type. *Environmental Pollution*, 215, 331-339.
Laglbauer, B. J., Franco-Santos, R. M., Andreu-Cazenave, M., Brunelli, L., Papadatou, M., Palatinus, A., Grego, M,, Deprez, T. (2014). Macrodebris and microplastics from beaches in Slovenia. *Marine Pollution*

Bulletin, 89(1-2), 356-366.
Lambert, S., and Wagner, M. (2018). Microplastics are contaminants of emerging concern in freshwater environments: an overview. In Freshwater Microplastics (pp. 1-23). Springer, Cham.
Lares, M., Ncibi, M. C., Sillanpää, M., Sillanpää, M., (2018). Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology. *Water Research,* 133, 236-246.
Lassen, C., Hansen, S.F., Magnusson, K., Norén, F., Hartmann, N.I.B., Jensen, P.R., Nielsen, T.G., Brinch, A., (2015). Microplastics-occurrence, Effects and Sources of Releases to the Environment in Denmark. Danish Environmental Protection Agency, Copenhagen, Denmark.
Lavers, J. L., and Bond, A. L. (2017). Exceptional and rapid accumulation of anthropogenic debris on one of the world's most remote and pristine islands. *Proceedings of the National Academy of Sciences*, 114(23), 6052-6055.
Law, K. L. (2017). Plastics in the marine environment. *Annual review of marine science*, 9, 205-229.
Leslie, H. A., Brandsma, S. H., Van Velzen, M. J. M., Vethaak, A. D., (2017). Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota. *Environment International,* 101, 133-142.
Li, J., Yang, D., Li, L., Jabeen, K., Shi, H. (2015). Microplastics in commercial bivalves from China. *Environmental pollutio*n, 207, 190-195.
Li, X., Chen, L., Mei, Q., Dong, B., Dai, X., Ding, G., & Zeng, E. Y. (2018). Microplastics in sewage sludge from the wastewater treatment plants in China. *Water Research*, 142, 75-85.
Lithner, D., Larsson, Å., Dave, G. (2011). Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Science of the Total Environment*, 409(18), 3309-3324.
Liu, L., Fokkink, R., & Koelmans, A. A. (2016). Sorption of polycyclic aromatic hydrocarbons to polystyrene nanoplastic. Environmental toxicology and chemistry, 35(7), 1650-1655.
Liubartseva, S., Coppini, G., Lecci, R., Clementi, E. (2018). Tracking plastics in the Mediterranean: 2D Lagrangian model. *Marine pollution bulletin*, 129(1), 151-162.
Liubartseva, S., Coppini, G., Lecci, R., Creti, S. (2016). Regional approach to modeling the transport of floating plastic debris in the Adriatic Sea. *Marine pollution bulletin*, 103(1-2), 115-127.
Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine pollution bulletin*, 42(8), 656-666.
Lobelle, D.,and Cunliffe, M. (2011). Early microbial biofilm formation on marine plastic debris. *Marine Pollution Bulletin*, 62, 197–200.
Löder, M. G., and Gerdts, G. (2015). Methodology used for the detection and identification of microplastics-A critical appraisal. In Marine anthropogenic litter (pp. 201-227). Springer, Cham.
Löhr, A., Savelli, H., Beunen, R., Kalz, M., Ragas, A., Van Belleghem, F. (2017). Solutions for global marine litter pollution. *Current opinion in environmental sustainability*, 28, 90-99.
Long, M., Moriceau, B., Gallinari, M., Lambert, C., Huvet, A., Raffray, J., & Soudant, P. (2015). Interactions between microplastics and phytoplankton aggregates: Impact on their respective fates. *Marine Chemistry*, *175*, 39-46.
Lowe, D. M., Fossato, V.U., Depledge, M. H. (1995) Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. *Marine Ecology Progress Series*, 129, 189-196.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, 193(1), 265-275.
Lusher, A. (2015). Microplastics in the marine environment: distribution, interactions and effects. In Marine anthropogenic litter (pp. 245-307). Springer, Cham.
Lusher, A. L., Hernandez-Milian, G., O'Brien, J., Berrow, S., O'Connor, I., Officer, R., (2015). Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: the True's beaked whale Mesoplodon mirus. *Environmental Pollution*, 199, 185-191.
Lusher, A. L., Mchugh, M., Thompson, R. C. (2013). Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine pollution bulletin*, 67(1-2), 94-99.
Lusher, A. L., Welden, N. A., Sobral, P., Cole, M. (2017b). Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Analytical Methods*, 9(9), 1346-1360.
Lusher, A., Bråte, I. L. N., Hurley, R., Iversen, K., and Olsen, M. (2017c). Testing of methodology for measuring

microplastics in blue mussels (*Mytilus spp*) and sediments, and recommendations for future monitoring of microplastics (R & D-project).

Lusher, A.L., Hollman, P.C.H., Mendoza-Hill, J.J., (2017a). Microplastics in fisheries and aquaculture: status of knowledge on their occurrence and implications for aquatic organisms and food safety. FAO Fisheries and Aquaculture Technical Paper. No. 615. Rome, FAO. 2017.

Lwanga, E. H., Gertsen, H., Gooren, H., Peters, P., Salánki, T., van der Ploeg, M., Besseling, E., Koelmans, A. A., Geissen, V. (2017). Incorporation of microplastics from litter into burrows of Lumbricus terrestris. *Environmental Pollution*, *220*, 523-531.

Lyakurwa, D. J. (2017). Uptake and effects of microplastic particles in selected marine microalgae species; Oxyrrhis marina and Rhodomonas baltica (Master's thesis, NTNU).

Ma R, Shih K (2010). Perfluorochemicals in wastewater treatment plants and sediments in Hong Kong. *Environmental Pollution*, 158(5):1354–1362.

Machella, N., Battino, M., Pisanelli, B.,Regoli, F. (2006). Influence of the SCGE protocol on the amount of basal DNA damage detected in the Mediterranean mussel, *Mytilus galloprovincialis*. *Environmental and molecular mutagenesis*, 47(8), 579-586.

Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C. C, Bonasoro, F., Binelli, A. (2018). Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). *Science of The Total Environment*, 631, 778-788.

Magni, S., Parolini, M., Soave, C., Marazzi, F., Mezzanotte, V., Binelli, A., (2015). Removal of metallic elements from real wastewater using zebra mussel bio-filtration process. *Journal of Environmental Chemical Engineering,* 3, 915-921.

Magnusson, K., Norén, F., (2014). Screening of microplastic particles in and down-stream a Wastewater Treatment Plant Report. Swedish Environmental Research Institute, Stockholm.

Mahon, A.M., O'Connell, B., Healy, M.G., O'Connor, I., Officer, R., Nash, R., Morrison, L., (2017). Microplastics in sewage sludge: effects of treatment. *Environmental Science and Technology,* 51, 810-818.

Mannini, P., Massa, F., Milone, N. (2004). Adriatic Sea fisheries: outline of some main facts. Interactions Between Aquaculture and Capture Fisheries: A Methodological Perspective. *Studies and Review*s. 78: 124-143.

Mansui, J., Molcard, A., Ourmieres, Y. (2015). Modelling the transport and accumulation of floating marine debris in the Mediterranean basin. *Marine pollution bulletin*, 91(1), 249-257.

Marigómez, I., and Baybay-Villacorta, L. (2003). Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. *Aquatic Toxicology*, 64, 235–257.

Martínez-Gómez, C., León, V. M., Calles, S., Gomáriz-Olcina, M., Vethaak, A. D. (2017). The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. *Marine environmental research*, 130, 69-76.

Mathalon, A. and Hill, P. (2014). Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. *Marine Pollution Bulletin*, 81(1): 69-79.

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L. A., Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific Reports*, 7:11452.

Mazurais, D., Ernande, B., Quazuguel, P., Severe, A., Huelvan, C., Madec, L., Mouchel, O., Soudant, P., Robbens, J., Huvet, A., Zambonino-Infante, J. (2015). Evaluation of the impact of polyethylene microbeads ingestion in European sea bass (*Dicentrarchus labrax*) larvae. *Marine Environmental Research*, 112: 78-85.

McCauley, S. J., & Bjorndal, K. A. (1999). Conservation implications of dietary dilution from debris ingestion: Sublethal effects in post-hatchling loggerhead sea turtles. Conservation biology, 13(4), 925-929.

McDermid, K. J., and McMullen, T. L. (2004). Quantitative analysis of small-plastic debris on beaches in the Hawaiian archipelago. *Marine pollution bulletin*, 48(7-8), 790-794.

McMahon, C. R., Holley, D., Robinson, S. (1999). The diet of itinerant male Hooker's sea lions, Phocarctos hookeri, at sub-Antarctic Macquarie Island. *Wildlife Research*, 26(6), 839-846.

Melli, V., Angiolillo, M., Ronchi, F., Canese, S., Giovanardi, O., Querin, S., Fortibuoni, T. (2017). The first assessment of marine debris in a Site of Community Importance in the north-western Adriatic Sea (Mediterranean Sea). *Marine pollution bulletin*, 114(2), 821-830.

Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Benedetti, M., Milan, M., Bargelloni, L., Regoli, F. (2016).

Transcriptional and cellular effects of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in experimentally exposed mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*, 180, 306-319.

Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Consolandi, G., Milan, M., Bargelloni, L., Regoli, F. (2018). Long-term exposure of *Mytilus galloprovincialis* to diclofenac, Ibuprofen and Ketoprofen: Insights into bioavailability, biomarkers and transcriptomic changes. *Chemosphere*, 198, 238-248.

Michielssen, M.R., Michielssen, E.R., Ni, J., Duhaime, M.B., (2016). Fate of microplastics and other small anthropogenic litter (SAL) in wastewater treatment plants depends on unit processes employed. *Environmental Science*: *Water Research* & *Technology*, 2, 1064-1073.

Miller, M. E., Kroon, F. J., Motti, C. A. (2017). Recovering microplastics from marine samples: A review of current practices. *Marine pollution bulletin*, 123(1-2), 6-18.

Mintenig, S.M., Int-Veen, I., Löder, M.G., Primpke, S., Gerdts, G., (2017). Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. *Water Research,* 108, 365-372.

Mistri, M., Infantini, V., Scoponi, M., Granata, T., Moruzzi, L., Massara, F., De Donati, M., Munari, C. (2017). Small plastic debris in sediments from the Central Adriatic Sea: Types, occurrence and distribution. *Marine pollution bulletin*, 124(1), 435-440.

Molnar, J. L., Gamboa, R. L., Revenga, C., Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment*, 6, 485–492.

Monteiro, R. C., do Sul, J. A. I., Costa, M. F. (2018). Plastic pollution in islands of the Atlantic Ocean. *Environmental Pollution*, 238, 103-110.

Moore, C. J. (2008). Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. *Environmental research*, 108(2), 131-139.

Moore, M.N. (1988) Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cellsto environmental and experimental exposure to xenobiotics. *Marine Ecology Progreess Series*, 46, 81-89.

Mora, P., Michel, X., Narbonne, J. F. (1999). Cholinesterase activity as potential biomarker in two bivalves. *Environmental Toxicology and Pharmacology*, 7(4), 253-260.

Munari, C., Corbau, C., Simeoni, U., Mistri, M. (2016). Marine litter on Mediterranean shores: analysis of composition, spatial distribution and sources in north-western Adriatic beaches. *Waste Management*, 49, 483-490.

Munari, C., Scoponi, M., Mistri, M. (2017). Plastic debris in the Mediterranean Sea: types, occurrence and distribution along Adriatic shorelines. *Waste Management,* 67, 385-391.

Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., (2016). Wastewater treatment works (WwTW) as a source of microplastics in the aquatic environment. *Environmental Science & Technology,* 50, 5800-5808.

Murray, F., and Cowie, P. R. (2011). Plastic contamination in the decapod crustacean Nephrops norvegicus (Linnaeus, 1758). *Marine pollution bulletin*, 62(6), 1207-1217.

Napper, I. E., Bakir, A., Rowland, S. J., Thompson, R. C. (2015). Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. *Marine Pollution Bulletin*, 99(1-2), 178-185.

Napper, I.E., and Thompson, R.C., (2016). Release of synthetic microplastic plastic fibres from domestic washing machines: effects of fabric type and washing conditions. *Marine Pollution Bulletin*, 112, 39-45.

Nelson, E.D., Do, H., Lewis, R.S., Carr, S.A., (2011). Diurnal variability of pharmaceutical, personal care product, estrogen and alkylphenol concentrations in effluent from a tertiary wastewater treatment facility. *Environmental Science* & *Technology*, 45 (4), 1228e1234.

Neves, D., Sobral, P., Ferreira, J. L., Pereira, T. (2015). Ingestion of microplastics by commercial fish off the Portuguese coast. *Marine pollution bulletin*, 101(1), 119-126.

Newman, S., Watkins, E., Farmer, A., ten Brink, P., Schweitzer, J. P. (2015). The economics of marine litter. In Marine anthropogenic litter (pp. 367-394). Springer, Cham.

Ng, E. L., Lwanga, E. H., Eldridge, S. M., Johnston, P., Hu, H. W., Geissen, V., Chen, D. (2018). An overview of microplastic and nanoplastic pollution in agroecosystems. *Science of the Total Environment*, 627, 1377-1388.

Nicholson, S. (2003). Lysosomal membrane stability, phagocytosis and tolerance to emersion in the mussel *Perna viridis* (Bivalvia: Mytilidae) following exposure to acute, sublethal, copper. *Chemosphere*, 52(7), 1147-1151.

Nigro, M., Falleni, A., Barga, I.D., Scarcelli, V., Lucchesi, P., Regoli, F., Frenzilli, G. (2006) Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. *Aquatic Toxicology*, 77, 339-347.

Nizzetto, L., Futter, M., Langaas, S., (2016). Are agricultural soils dumps for microplastics of urban origin? *Environmental Science* & *Technology,* 50, 10777-10779.

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., Turra, A. 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). Marine Pollution Bulletin, 92(1), 99-104.

O'Donovan, S., Mestre, N.C., Abel, S, Fonseca, T. G., Carteny, C.C., Cormier, B., Keiter, S.H., Bebianno, M. J. (2018). Ecotoxicological Effects of Chemical Contaminants Adsorbed to Microplastics in the Clam *Scrobicularia plana*. *Frontiers in Marine Science*, 5:143.

Obbard, R. W., Sadri, S., Wong, Y. Q., Khitun, A. A., Baker, I., Thompson, R. C. (2014). Global warming releases microplastic legacy frozen in Arctic Sea ice. *Earth's Future*, 2(6), 315-320.

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., et al. (2009). International pellet watch: global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Marine pollution bulletin*, 58(10), 1437-1446.

Oliveira, M., Ribeiro, A., Hylland, K., and Guilhermino, L. (2013). Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecological Indicators*, 34, 641–647.

Ortiz-Zarragoitia, M., & Cajaraville, M. P. (2006). Biomarkers of exposure and reproduction-related effects in mussels exposed to endocrine disruptors. *Archives of environmental contamination and toxicology*, 50(3), 361-369.

P.J. Kershaw (Ed.), IMO/FAO/UNESCO-IOC/UNIDO/-WMO/IAEA/UN/UNEP/UNDP. Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection Reports and Studies, GESAMP No. 90 (2015)

Papadopoulou, K. N., Anastasopoulou, A., Mytilineou, C., Smith, C. J., Stamouli, C. (2016). Seabed marine litter, comparison of 4 Aegean trawling grounds.

Parameswaranpillai, J., Thomas, S., Grohens, Y. (2014). Polymer blends: state of the art, new challenges, and opportunities. *Characterization of Polymer Blends*, 1-6.

Pasquini, G., Ronchi, F., Strafella, P., Scarcella, G., Fortibuoni, T. (2016). Seabed litter composition, distribution and sources in the Northern and Central Adriatic Sea (Mediterranean). *Waste management*, 58, 41-51.

Paul-Pont, I., Lacroix, C., Fernández, C. G., Hégaret, H., Lambert, C., Le Goïc, N., et al. (2016). Exposure of marine mussels *Mytilus spp*. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. *Environmental Pollution,* 216, 724–737.

Paul-Pont, I., Tallec, K., Gonzalez Fernandez, C., Lambert, C., Vincent, D., Mazurais, D., Zambonino, J. L., Brotons, G., Lagarde, F., Fabioux, C., Soudant, P., Huvet, A. (2018). Constraints and Priorities for Conducting Experimental Exposures of Marine Organisms to Microplastics. *Frontiers in Marine Science*, 5(252).

Peakall D.W. (1994). Biomarkers: the way forward in environmental assessment. *Toxicology and Ecotoxicology News* 1: 55-60.

Pedà, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T. Maricchiolo, G. (2016). Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: Preliminary results. *Environmental Pollution*, 212: 251-256.

Pellini, G., Gomiero, A., Fortibuoni, T., Ferrà, C., Grati, F., Tassetti, N., Polidori, P., Fabi, G., Scarcella, G. (2018). Characterization of microplastic litter in the gastrointestinal tract of *Solea solea* from the Adriatic Sea. *Environmental Pollution*, 234, 943-952.

Pellini, G., Gomiero, A., Fortibuoni, T., Ferrà, C., Grati, F., Tassetti, N., Polidori, P., Fabi, G., Scarcella, G. (2018). Characterization of microplastic litter in the gastrointestinal tract of Solea solea from the Adriatic Sea. *Environmental Pollution*, 234, 943-952.

Pham, C.K., Ramirez-Llodra, E., Alt, C.H.S., Amaro, T., Bergmann, M., Canals, M., Company, J.B., et al. (2014). Marine litterdistribution and density in European Seas, from the shelves to deep basins. *PLoS One* 9, e95839.

Phillips (2016). Effects of sand filters in wastewater treatment plants on microplastic output. In: 50th Annual

Meeting of The Geological Society of America, Denver, United States, (Abstract retrieved from: https://gsa.confex.com/gsa/2016NC/ webprogram/Paper274766.html).

Phuong, N. N., Zalouk-Vergnoux, A., Kamari, A., Mouneyrac, C., Amiard, F., Poirier, L., Lagarde, F. (2018). Quantification and characterization of microplastics in blue mussels (*Mytilus edulis*): protocol setup and preliminary data on the contamination of the French Atlantic coast. *Environmental Science and Pollution Research*, 25(7), 6135-6144.

Phuong, N. N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C., Lagarde, F. (2016). Is there any consistency between the microplastics found in the field and those used in laboratory experiments?. *Environmental Pollution*, 211, 111-123.

Piergiovanni, L., and Limbo, S. (2010). Food packaging: Materiali, tecnologie e soluzioni. Springer Science & Business Media.

Piña, B., Casado, M., Quirós, L. (2007). Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *TrAC Trends in Analytical Chemistry*, 26(11), 1145-1154.

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., Regoli, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere*, 83(4), 475-485.

Plastics Europe, (2017). Plastics – the Facts 2016. An analysis of European plastics production, demand and waste data. PlasticsEurope – Association of Plastics Manufacturers. Brussels.

PlasticsEurope (2018). Plastics - the Facts 2017, an analysis of European latest plastics production, demand and waste data Plast. Eur. Assoc. Plast. Manuf. Bruss (2018).

Porter, A., Lyons, B. P., Galloway, T. S., Lewis, C. N. (2018). The role of marine snows in microplastic fate and bioavailability. *Environmental Science and Technology*. 52 (12), 7111–7119.

Prata, J.C., 2018. Microplastics in wastewater: State of the knowledge on sources, fate and solutions. *Marine Pollution Bulletin*, 129, 262-265.

Pruzzo, C., Gallo, G., Canesi, L. (2005). Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. *Environmental microbiology*, 7(6), 761-772.

Raju, S., Carbery, M., Kuttykattil, A., Senathirajah, K., Subashchandrabose, S. R., Evans, G., & Thavamani, P. (2018). Transport and fate of microplastics in wastewater treatment plants: implications to environmental health. *Reviews in Environmental Science and Bio/Technology*, 1-17.

Rank, J., Lehtonen, K. K., Strand, J., Laursen, M. (2007). DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis)* in areas close to coastal chemical dumping sites in Denmark. *Aquatic Toxicology*, 84(1), 50-61.

Rebolledo, E. L. B., Van Franeker, J. A., Jansen, O. E., Brasseur, S. M. (2013). Plastic ingestion by harbour seals (Phoca vitulina) in The Netherlands. *Marine pollution bulletin*, 67(1-2), 200-202.

Redford, D. P., Trulli, H. K., Trulli, W. R. (1997). Sources of plastic pellets in the aquatic environment. In J. M. Coe & D. B. Rogers (Eds.), Marine debris: Sources, impacts and solutions (pp. 335–343). New York: Springer.

Rees, G., and Pond, K. (1995). Marine litter monitoring programmes—a review of methods with special reference to national surveys. *Marine Pollution Bulletin*, 30(2), 103-108.

Regoli, F., and Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research 93, 106–117.

Regoli, F., and Winston, G. W. (1999). Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and applied pharmacology*, 156(2), 96-105.

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., Nigro, M. (2004). Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic toxicology*, 68(2), 167-178.

Reisser, J. W., Slat, B., Noble, K. D., Plessis, K. D., Epp, M., Proietti, M. C., et al. (2015). The vertical distribution of buoyant plastics at sea: an observationalstudy in the North Atlantic Gyre.

Ren, X., Pan, L., and Wang, L. (2015). Toxic effects upon exposure to benzo[a] pyrene in juvenile white shrimp *Litopenaeus vannamei*. *Environmental Toxicology and Pharmacology*, 39, 194–207.

Rey-Salgueiro, L., Martínez-Carballo, E., Cid, A., and Simal-Gándara, J. (2017). Determination of kinetic bioconcentration in mussels after short term exposure to polycyclic aromatic hydrocarbons. *Heliyon*

3:e00231.
Rey-Salgueiro, L., Martínez-Carballo, E., Cid, A., Simal-Gándara, J. (2017). Determination of kinetic bioconcentration in mussels after short term exposure to polycyclic aromatic hydrocarbons. *Heliyon*, 3:e00231.
Rezania, S., Park, J., Din, M. F. M., Taib, S. M., Talaiekhozani, A., Yadav, K. K., Kamyab, H. (2018). Microplastics pollution in different aquatic environments and biota: A review of recent studies. *Marine pollution bulletin*, 133, 191-208.
Ribeiro, F., Garcia, A. R., Pereira, B. P., Fonseca, M., Mestre, N. C., Fonseca, T. G., Ilharco, L. M., Bebianno, M. J. (2017). Microplastics effects in *Scrobicularia plana*. *Marine pollution bulletin*, 122(1), 379-391.
Ribic, C. A., Sheavly, S. B., Rugg, D. J., Erdmann, E. S. (2010). Trends and drivers of marine debris on the Atlantic coast of the United States 1997–2007. *Marine Pollution Bulletin*, 60, 1231–1242.
Rillig, M. C., Ingraffia, R., de Souza Machado, A. A. (2017). Microplastic incorporation into soil in agroecosystems. *Frontiers in plant science*, 8, 1805.
Rios, L. M., Moore, C., andJones, P. R. (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. *Marine Pollution Bulletin*, 54(8), 1230-1237.
Rist, S. E., Assidqi, K., Zamani, N. P., Appel, D., Perschke, M., Huhn, M., Lenz, M. (2016). Suspended micro-sized PVC particles impair the performance and decrease survival in the Asian green mussel Perna viridis. *Marine pollution bulletin*, 111(1), 213-220.
Rochman CM, Hoh E, Kurobe T, Teh SJ (2013b) Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific Reports*, 3:3263.
Rochman, C. M. (2013a). Plastics and priority pollutants: a multiple stressor in aquatic habitats. Environmental Science and Technology, 47, 2439−2440.
Rochman, C. M. (2015). The complex mixture, fate and toxicity of chemicals associated with plastic debris in the marine environment, in Marine Anthropogenic Litter, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 117–140.
Rochman, C. M., Browne, M. A., Underwood, A. J., Van Franeker, J. A., Thompson, R. C., Amaral-Zettler, L. A. (2016). The ecological impacts of marine debris: unraveling the demonstrated evidence from what is perceived. *Ecology*, 97(2), 302-312.
Romeo, T., Pietro, B., Pedà, C., Consoli, P., Andaloro, F., Fossi, M. C. (2015). First evidence of presence of plastic debris in stomach of large pelagic fish in the Mediterranean Sea. *Marine pollution bulletin*, 95(1), 358-361.
Ruiz-Orejón, L. F., Sardá, R., & Ramis-Pujol, J. (2018). Now, you see me: High concentrations of floating plastic debris in the coastal waters of the Balearic Islands (Spain). *Marine Pollution Bulletin*, 133, 636-646.
Ruiz-Orejón, L. F., Sardá, R., Ramis-Pujol, J. (2016). Floating plastic debris in the Central and Western Mediterranean Sea. *Marine environmental research*, 120, 136-144.
Rummel, C. D., Löder, M. G., Fricke, N. F., Lang, T., Griebeler, E. M., Janke, M., Gerdts, G. (2016). Plastic ingestion by pelagic and demersal fish from the North Sea and Baltic Sea. *Marine pollution bulletin*, 102(1), 134-141.
Ruzette, A. V., and Leibler, L. (2005). Block copolymers in tomorrow's plastics. Nature materials, 4(1), 19.
Ryan, P.G., Moore, C.J., van Franeker, J.A., Moloney, C.L., (2009). Monitoring the abundance of plastic debris in the marine environment. *Philosophical Transactions of the Royal Society B: Biological Sciences* 364, 1999-2012.
Santana, M. F. M., Ascer, L. G., Custódio, M. R., Moreira, F. T., Turra, A. (2016). Microplastic contamination in natural mussel beds from a Brazilian urbanized coastal region: Rapid evaluation through bioassessment. *Marine pollution bulletin*, 106(1-2), 183-189.
Santana, M. F. M., Moreira, F. T., Turra, A. (2017). Trophic transference of microplastics under a low exposure scenario: insights on the likelihood of particle cascading along marine food-webs. *Marine pollution bulletin*, 121(1-2), 154-159.
Santillo, D., Miller, K., Johnston, P. (2017). Microplastics as contaminants in commercially important seafood species. *Integrated environmental assessment and management*, 13(3), 516-521.
Savoca, M.S., Wohlfeil, M.E., Ebeler, S.E. & Nevitt, G.A. 2016. Marine plastic debris emits a keystone infochemical for olfactory foraging seabirds. *Science Advances*, 2 (11).
Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M. (2014). Ingestion and transfer of microplastics in the planktonic food web. *Environmental pollution*, 185, 77-83.

Setälä, O., Magnusson, K., Lehtiniemi, M., Norén, F. (2016). Distribution and abundance of surface water microlitter in the Baltic Sea: a comparison of two sampling methods. *Marine pollution bulletin*, 110(1), 177-183.

Shareef A, Kookana RS, Kumar A, Tjandraatmadja G (2008). Sources of emerging organic contaminants in domestic wastewater: an assessment based on literature review. CSIRO: water for a healthy country national research flagship.

Shaw, J.P., Large, A.T., Donkin, P., Evans, S.V., Staff, F.J., Livingstone, D.R., Chipman, J.K., Peters, L.D. (2004) Seasonal variation in cytochrome P450 immunopositive protein levels, lipid peroxidation and genetic toxicity in digestive gland of the mussel *Mytilus edulis*. *Aquatic Toxicology*, 67, 325-336.

Shim, W.J., Hong, S.H., Eo, S.E., (2017). Identification methods in microplastic analysis: a review. *Analytical Methods* 9, 1384–1391.

Sillanpää, M., Sainio, P., (2017). Release of polyester and cotton fibers from textiles in machine washings. Environ. *Environmental Science and Pollution Research*, 24, 19313-19321.

Snell, T. W., Brogdon, S. E., Morgan, M. B. (2003). Gene expression profiling in ecotoxicology. *Ecotoxicology*, 12(6), 475-483.

Song, Y. K., Hong, S. H., Jang, M., Han, G. M., Rani, M., Lee, J., Shim, W. J. (2015). A comparison of microscopic and spectroscopic identification methods for analysis of microplastics in environmental samples. *Marine Pollution Bulletin*, 93(1-2), 202-209.

Stergiou, K. I., and Karpouzi, V. S. (2002). Feeding habits and trophic levels of Mediterranean fish. *Reviews in fish biology and fisheries*, 11(3), 217-254.

Strafella, P., Fabi, G., Spagnolo, A., Grati, F., Polidori, P., Punzo, E. et al (2015). Spatial pattern and weight of seabed marine litter in the northern and central Adriatic Sea. Marine pollution bulletin, 91(1), 120-127.

Suaria, G., and Aliani, S. (2014). Floating debris in the Mediterranean Sea. *Marine pollution bulletin*, 86(1-2), 494-504.

Suaria, G., Avio, C. G., Mineo, A., Lattin, G. L., Magaldi, M. G., Belmonte, G., Moore, C.,J., Regoli, F., Aliani, S. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. *Scientific reports*, 6, 37551.

Sun, X., Li, Q., Zhu, M., Liang, J., Zheng, S., Zhao, Y. (2017). Ingestion of microplastics by natural zooplankton groups in the northern South China Sea. *Marine pollution bulletin*, 115(1-2), 217-224.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., et al. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. *Proceedings of the National Academy of Sciences*, *113*(9), 2430-2435.

Takada, H., and Tanaka, K. (2016). Microplastic fragments and microbeads in digestive tracts of planktivorous fish from urban coastal waters. *Scientific reports*, 6, 34351.

Talvitie, J., Mikola, A., Koistinen, A., Setälä, O., (2017b). Solutions to microplastic pollution–removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. *Water Research,* 123, 401-407.

Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., (2017a). How well is microlitter purified from wastewater? - a detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. *Water Research,* 109, 164-172.

Tanaka, K., Takada, H., Yamashita, R., Mizukawa, K., Fukuwaka, M. A., Watanuki, Y. (2013). Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics. *Marine pollution bulletin*, 69(1-2), 219-222.

Tedengren, M., Olsson, B., Bradley, B., Zhou, L. (1999). Heavy metal uptake, physiological response and survival of the blue mussel (*Mytilus edulis*) from marine and brackish waters in relation to the induction of heat-shock protein 70. In Biological, Physical and Geochemical Features of Enclosed and Semi-enclosed Marine Systems (pp. 261-269). Springer, Dordrecht.

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., et al. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society* B 364, 2027–2045.

Thiel, M., Luna-Jorquera, G., Álvarez-Varas, R., Gallardo, C., Hinojosa, I. A., Luna, N., et al. (2018). Impacts of Marine Plastic Pollution From Continental Coasts to Subtropical Gyres—Fish, Seabirds, and Other Vertebrates in the SE Pacific. *Frontiers in Marine Science*, 5(238).

Thompson, R. C. (2015). Microplastics in the marine environment: Sources, consequences and solutions. In Marine anthropogenic litter (pp. 185-200). Springer, Cham.

Thompson, R. C., Moore, C. J., Vom Saal, F. S., Swan, S. H. (2009). Plastics, the environment and human health: current consensus and future trends. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364(1526), 2153-2166.

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W., et al. (2004). Lost at sea: where is all the plastic?. *Science*, 304(5672), 838-838.

Turner, A., and Holmes, L. A. (2015). Adsorption of trace metals by microplastic pellets in fresh water. *Environmental Chemistry*, 12(5), 600-610.

Ugolini, A., Ungherese, G., Ciofini, M., Lapucci, A., Camaiti, M. (2013). Microplastic debris in sandhoppers. Estuarine, *Coastal Shelf Science*, 129: 19-22.

UNEP (2016). Marine Plastic Debris and Microplastics - Global Lessons and Research to Inspire Action and Guide Policy Change. United Nations Environment Programme, Nairobi (2016).

UNEP/MAP (2016). Marine litter assessment in the Mediterranean 2015. United Nations Environment ProgrammeMediterranean Action Plan (UNEP/MAP), 86

Van Beelen ES (2007). Municipal waste water treatment plant (WWTP) effluents a concise overview of the occurrence of organic substances. Association of River Waterworks- RIWA, The Netherlands.

Van Cauwenberghe, L., and Janssen, C. R. (2014). Microplastics in bivalves cultured for human consumption. *Environmental pollution*, 193, 65-70.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M. B., Janssen, C. R. (2015). Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats. *Environmental Pollution*, *199*, 10-17.

Van Cauwenberghe, L., Vanreusel, A., Mees, J., Janssen, C. R. (2013). Microplastic pollution in deep-sea sediments. *Environmental Pollution*, 182, 495-499.

Van der Oost R., Beyer J., Vermeulen N. P. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13(2): 57-149.

Van Sebille, E., England, M. H., Froyland, G. (2012). Origin, dynamics and evolution of ocean garbage patches from observed surface drifters. *Environmental Research Letters*, 7(4), 044040.

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B. D., Van Franeker, J. A., et al. (2015). A global inventory of small floating plastic debris. *Environmental Research Letters*, 10(12), 124006.

Vandermeersch, G., Van Cauwenberghe, L., Janssen, C. R., Marques, A., Granby, K., Fait, G., et al. (2015). A critical view on microplastic quantification in aquatic organisms. *Environmental research*, 143, 46-55.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R,. Sturaro, A., Da Ros, L. (2013) Microplastic particles in sediments of lagoon of Venice, Italy: first observations on occurrence, spatial patterns and identification. *Estuarine, Coastal and Shelf Science*, 130:54–61.

Vianello, A., Da Ros, L., Boldrin, A., Marceta, T., Moschino, V. (2018). First evaluation of floating microplastics in the Northwestern Adriatic Sea. *Environmental Science and Pollution Research*, 25(28), 28546-28561.

Viršek, M. K., Palatinus, A., Koren, Š., Peterlin, M., Horvat, P., Kržan, A. (2016). Protocol for microplastics sampling on the sea surface and sample analysis. *Journal of visualized experiments: JoVE*, (118).

Vlachogianni, Th., Anastasopoulou, A., Fortibuoni, T., Ronchi, F., Zeri, Ch., (2017). Marine Litter Assessment in the Adriatic and Ionian Seas. IPA-Adriatic DeFishGear Project, MIO-ECSDE, HCMR and ISPRA. pp. 168 (ISBN: 978-960-6793-25-7).

Von Moos, N., Burkhardt-Holm, P., and Köhler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* (L.) after an experimental exposure. *Environmental Science and Technology.* 46: 11327-11335.

Vroom, R. J., Koelmans, A. A., Besseling, E., and Halsband, C. (2017). Aging of microplastics promotes their ingestion by marine zooplankton. *Environmental Pollution*, 231, 987-996.

Ward, J. E., and Kach, D. J. (2009). Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Marine environmental research*, *68*(3), 137-142.

Ward, J. E., and Kach, D. J. (2009). Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Marine environmental research*, 68(3), 137-142.

Watson, M. (2012). Marine Debris Along the Florida Keys Reef Tract-Mapping, Analysis and Perception Study.

Watts, A. J., Lewis, C., Goodhead, R. M., Beckett, S. J., Moger, J., Tyler, C. R., Galloway, T. S. (2014). Uptake

and retention of microplastics by the shore crab Carcinus maenas. *Environmental science & technology*, 48(15), 8823-8830.

Watts, A. J., Urbina, M. A., Goodhead, R., Moger, J., Lewis, C., Galloway, T. S. (2016). Effect of microplastic on the gills of the shore crab Carcinus maenas. *Environmental science & technology*, 50(10), 5364-5369.

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., Koelmans, A. A. (2012). Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environmental Toxicology and Chemistry*, 31, 2490–2497.

Willis, K., Hardesty, B. D., Kriwoken, L., Wilcox, C. (2017). Differentiating littering, urban runoff and marine transport as sources of marine debris in coastal and estuarine environments. *Scientific Reports*, 7, 44479.

Winston, G. W., Livingstone, D. R., Lips, F. (1990). Oxygen reduction metabolism by the digestive gland of the common marine mussel, *Mytilus edulis* L. *Journal of Experimental Zoology*, 255(3), 296-308.

Woodall, L.C., Sanchez-Vidal, A., Canals, M., Paterson, G.L.J., Coppock, R., Sleight, V., Calafat, A., Rogers, A.D., Narayanaswamy, B.E. and Thompson, R.C. (2014) 'The deep sea is a major sink for microplastic debris', *Royal Society Open Science*, 1(4).

Wootton, E. C., Dyrynda, E. A., Pipe, R. K., Ratcliffe, N. A. (2003). Comparisons of PAH-induced immunomodulation in three bivalve molluscs. *Aquatic Toxicology*, 65, 13–25.

Wright, S. L., Thompson, R. C., Galloway, T. S. (2013). The physical impacts of microplastics on marine organisms: a review. *Environmental pollution*, 178, 483-492.

Wu C., Zhang K., Xiong X. (2018) Microplastic Pollution in Inland Waters Focusing on Asia. In: Wagner M., Lambert S. (eds) Freshwater Microplastics. The Handbook of Environmental Chemistry, vol 58. Springer, Cham.

Xanthos, D., and Walker, T. R. (2017). International policies to reduce plastic marine pollution from single-use plastics (plastic bags and microbeads): a review. *Marine pollution bulletin*, 118(1-2), 17-26.

Xiao, K., Liang, S., Wang, X., Chen, C., & Huang, X. (2018). Current state and challenges of full-scale membrane bioreactor applications: A critical review. Bioresource technology.

Yang S, Hai FI, Price WE, McDonald J, Khan SJ, Nghiem LD (2016). Occurrence of trace organic contaminants in wastewater sludge and their removals by anaerobic digestion. *Bioresource Technology,* 210 (Supplement C):153–159.

Ying G-G, Kookana RS (2007). Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environment International*, 33(2):199–205.

Zalasiewicz, J., Waters, C. N., do Sul, J. A. I., Corcoran, P. L., Barnosky, A. D., Cearreta, A., et al. (2016). The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. *Anthropocene*, 13, 4-17.

Zambianchi, E., Trani, M., Falco, P. (2017). Lagrangian transport of marine litter in the Mediterranean Sea. Frontiers in *Environmental Science*, 5, 5.

Zettler, E. R., Mincer, T. J., Amaral-Zettler, L. A. (2013). Life in the "plastisphere": Microbial communities on plastic marine debris. *Environmental Science and Technology*, 47, 7137–7146.

Zhang, C., Chen, X., Wang, J., Tan, L. (2017). Toxic effects of microplastic on marine microalgae Skeletonema costatum: interactions between microplastic and algae. *Environmental pollution*, 220, 1282-1288.

Ziajahromi, S., Neale, P. A., Rintoul, L., Leusch, F.D., (2017). Wastewater treatment plants as a pathway for microplastics: development of a new approach to sample wastewater-based microplastics. *Water Research,* 112, 93-99.

Zitko, V., and Hanlon, M. (1991). Another source of pollution by plastics: Skin cleaners with plastic scrubbers. *Marine Pollution Bulletin*, 22, 41–42.

Zubris, K. A. V., & Richards, B. K. (2005). Synthetic fibers as an indicator of land application of sludge. *Environmental Pollution*, 138, 201–211.

LIST OF PAPERS

This thesis is partly based on the following publications:

Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., Gorbi, S., Regoli, F. (2018). Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels, *Mytilus galloprovincialis*. *Frontiers in Marine Science*, 5, 103. https://doi.org/10.3389/fmars.2018.00103

Magni S., Binelli A., Pittura L., Avio C.G., Della Torre C., Parenti C.C., Gorbi S., Regoli F. (2019). The fate of microplastics in an Italian Wastewater Treatment Plant. *Science of the Total Environment*, 652, 602-610. https://doi.org/10.1016/j.scitotenv.2018.10.269

frontiers in Marine Science

ORIGINAL RESEARCH
published: 03 April 2018
doi: 10.3389/fmars.2018.00103

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

Lucia Pittura[1†], *Carlo G. Avio*[1†], *Maria E. Giuliani*[1], *Giuseppe d'Errico*[1], *Steffen H. Keiter*[2], *Bettie Cormier*[2,3], *Stefania Gorbi*[1] *and Francesco Regoli*[1,4*]

[1] *Laboratorio di Ecotossicologia e Chimica ambientale, Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy,* [2] *Man-Technology-Environment Research Centre, School of Science and Technology, Örebro University, Örebro, Sweden,* [3] *UMR Centre National de la Recherche Scientifique 5805 EPOC, University of Bordeaux, Talence, France,* [4] *Consorzio Interuniversitario per le Scienze del Mare, CoNISMa, ULR Ancona, Ancona, Italy*

OPEN ACCESS

Edited by:
Ricardo Beiras, University of Vigo, Spain

Reviewed by:
Laura Guimarães, Centro Interdisciplinar de Pesquisa Marine e Ambiental (CIIMAR), Portugal
Juan Bellas, Instituto Español de Oceanografía (IEO), Spain

***Correspondence:**
Francesco Regoli
f.regoli@univpm.it

[†] *These authors have contributed equally to this work.*

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *14 January 2018*
Accepted: *13 March 2018*
Published: *03 April 2018*

Citation:
Pittura L, Avio CG, Giuliani ME, d'Errico G, Keiter SH, Cormier B, Gorbi S and Regoli F (2018) Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels, Mytilus galloprovincialis. Front. Mar. Sci. 5:103. doi: 10.3389/fmars.2018.00103

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Sorption of Benzo(a)Pyrene on Microplastic Particles

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

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

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

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

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

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

Experimental Design

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

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

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

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

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

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

Chemical Analyses of benzo(a)pyrene

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

Histological and Biochemical Analyses

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

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

Molecular Analyses

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

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

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

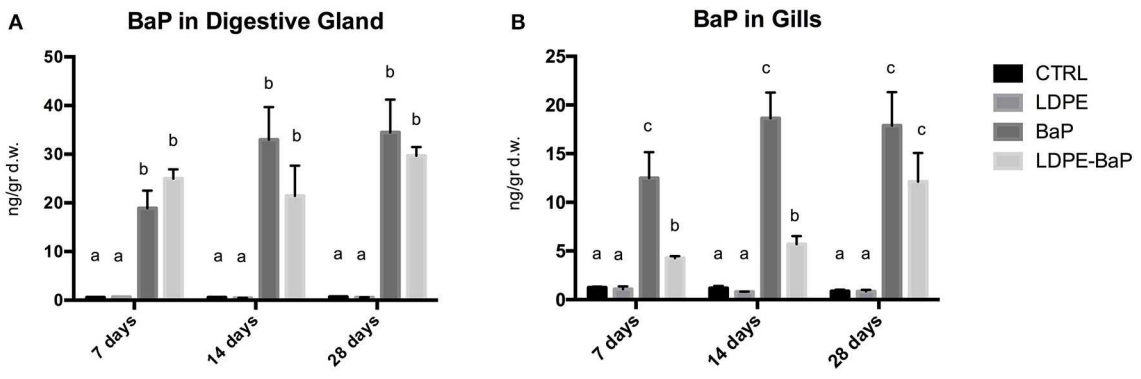


FIGURE 1 | Concentrations of benzo(a)pyrene in digestive glands **(A)** and gills **(B)** of mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). Data are expressed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

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

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

Statistical Analyses and Hazard Indices Evaluation

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

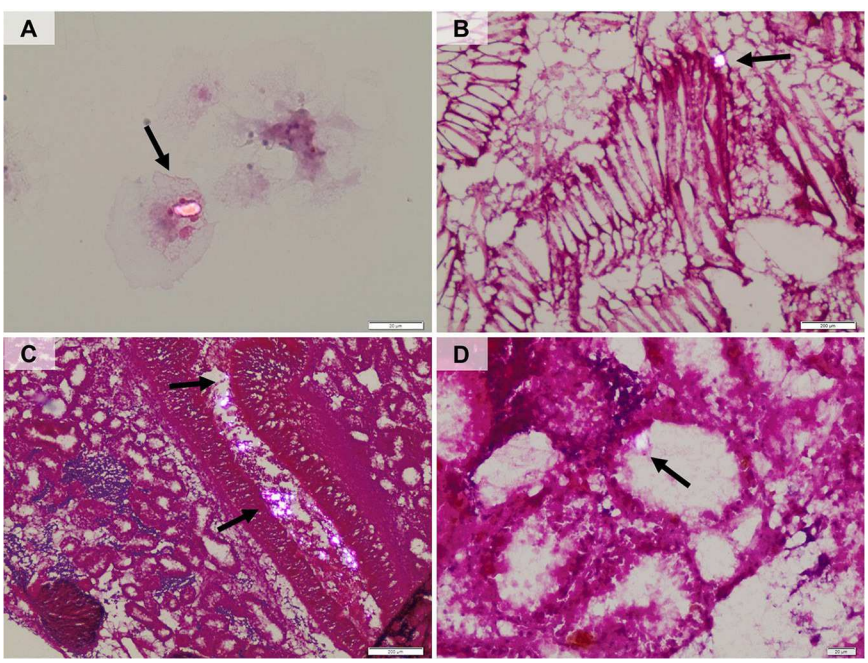


FIGURE 2 | Polarized-light microscopy images showing the presence of microplastic particles in hemolymph **(A)**, gills **(B)**, gut lumen and epithelium **(C)**, digestive tubules **(D)**.

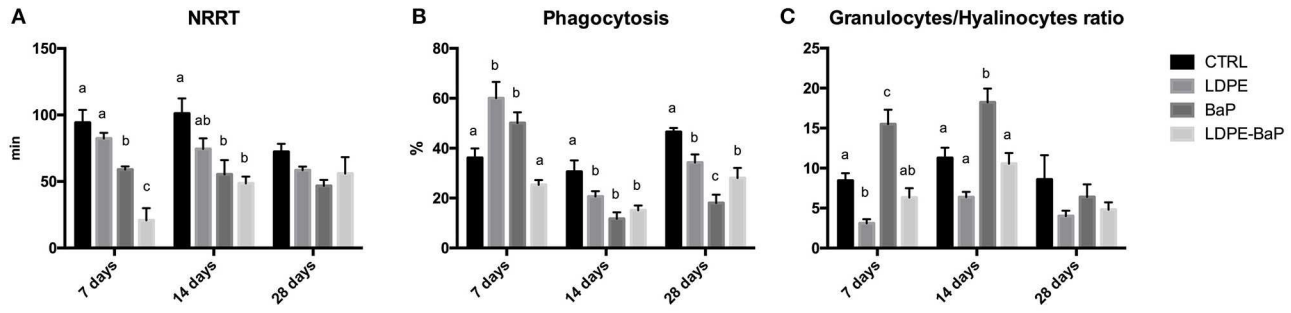


FIGURE 3 | Immunological biomarkers in mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). NRRT: neutral red retention time **(A)**, Phagocytosis **(B)**, Granulocytes/Hyalinocytes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

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

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

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

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

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

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

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

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

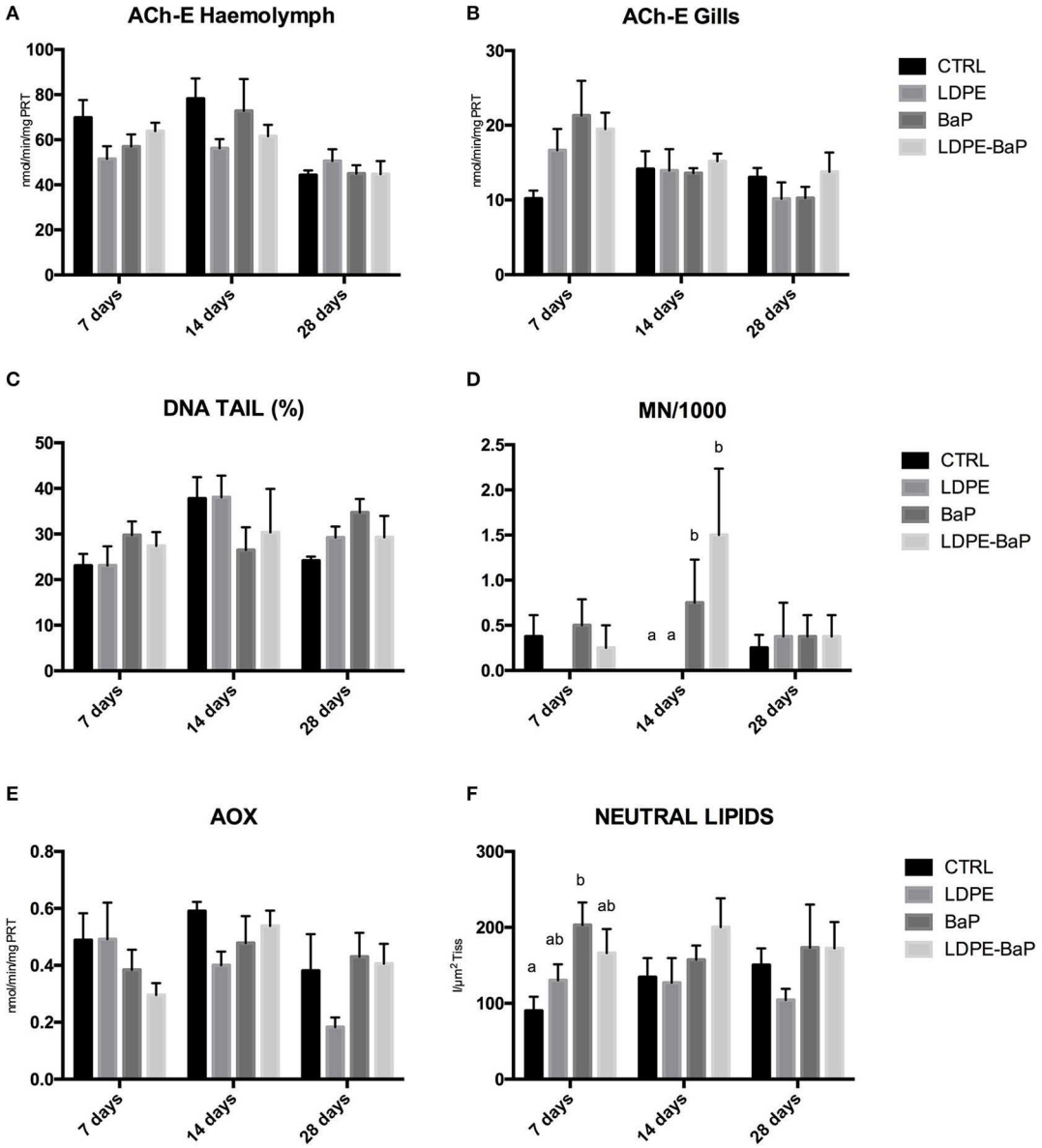


FIGURE 4 | Biomarkers in mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). ACh-E: acetylcholinesterase in haemolymph **(A)** and gills **(B)**; DNA TAIL %: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

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

DISCUSSION

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

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

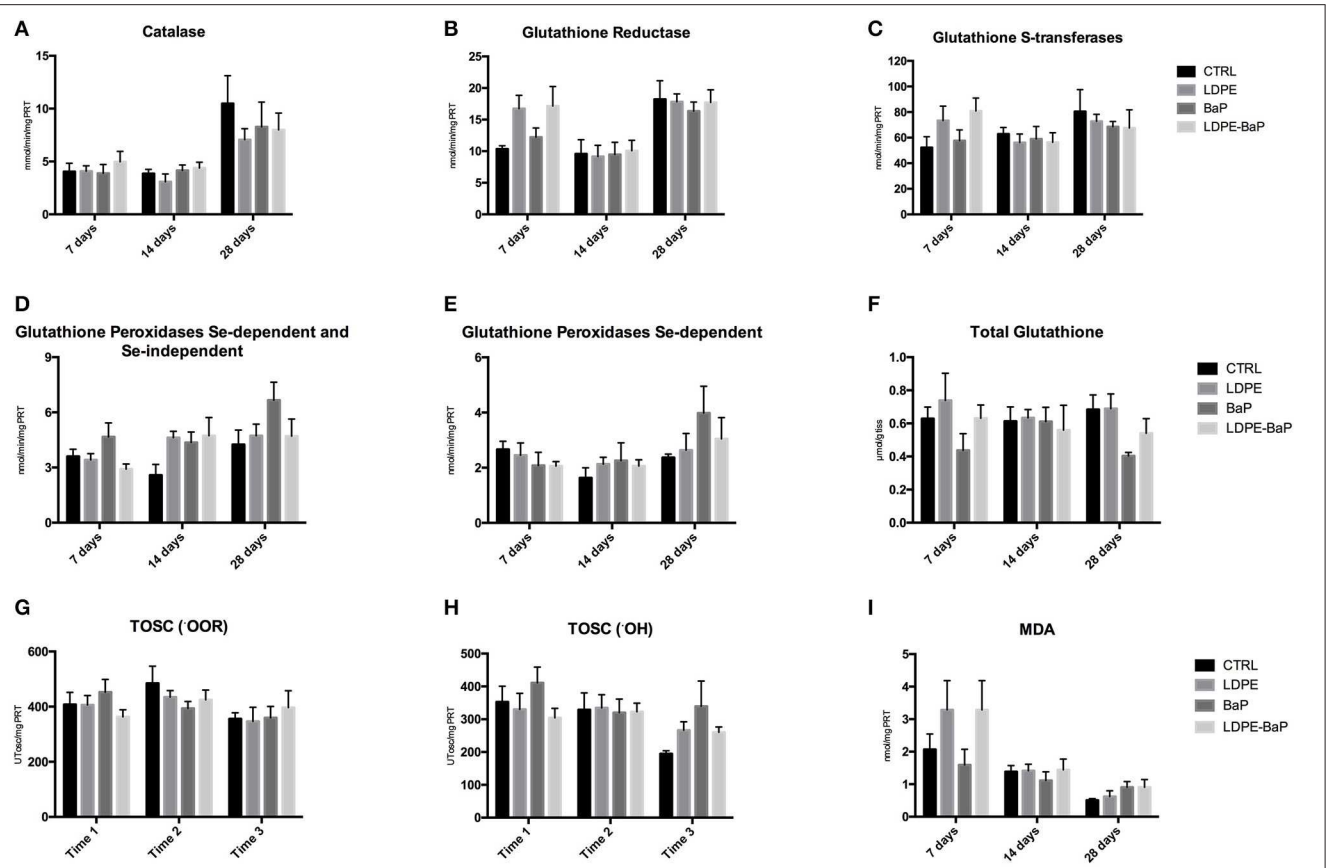


FIGURE 5 | Levels of catalase **(A)**, glutathione reductase **(B)**, glutathione S-transferases **(C)**, sum of Se-dependent and Se-independent glutathione peroxidases **(D)**, Se-dependent glutathione peroxidases **(E)**, total glutathione **(F)**, total oxyradical scavenging capacity (TOSC) toward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

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

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

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

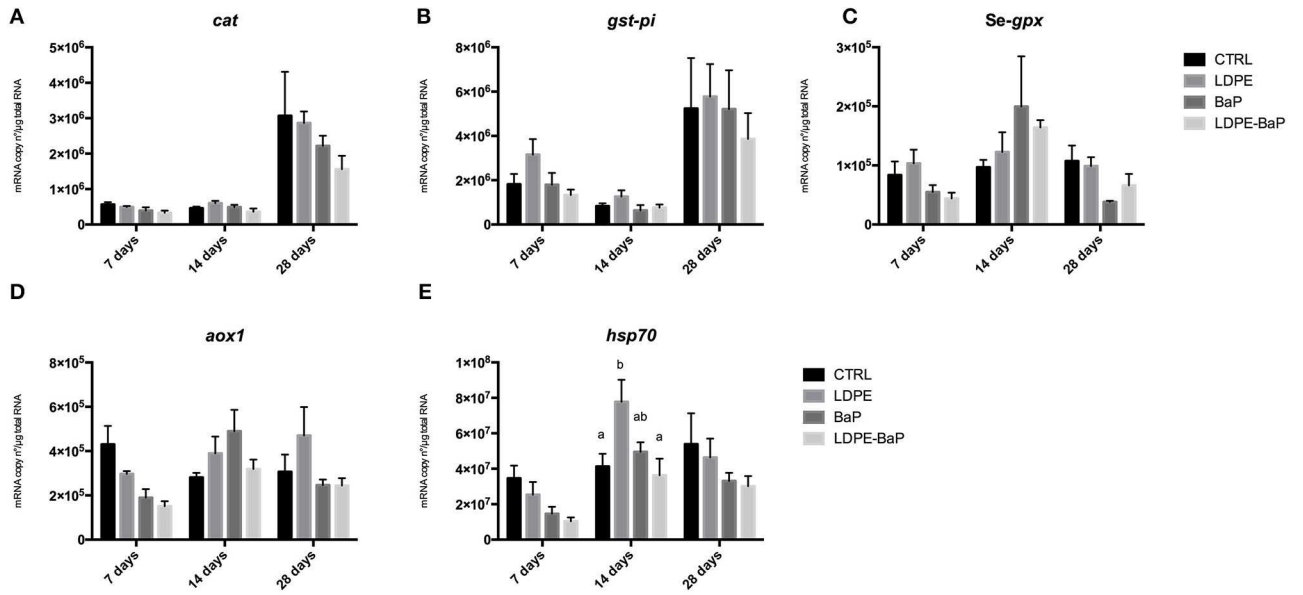


FIGURE 6 | Transcriptional responses in the digestive glands of mussels exposed for 7, 14, and 28 days to various treatments (CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene). *cat*, catalase **(A)**; *gst-pi*, *pi*-class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

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

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

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

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

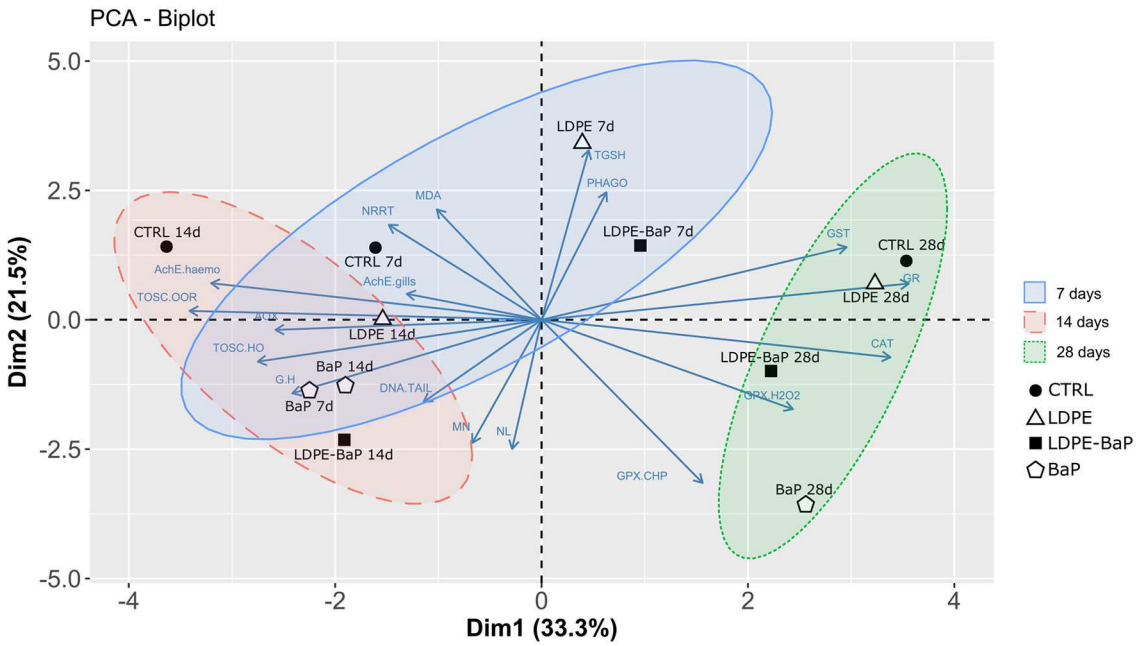


FIGURE 7 | Multivariate PCA analysis on biomarker data in mussels exposed to various microplastics treatments: CTRL, control; LDPE, virgin low density polyethylene; BaP, benzo(a)pyrene alone; LDPE-BaP, benzo(a)pyrene-contaminated polyethylene.

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

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

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

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

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

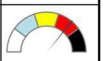
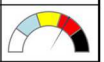
Time	Treatment	Hazard level BIOAVAILABILITY	Hazard level BIOMARKERS	WOE	
7 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP	Severe	Slight	MAJOR	
	BaP	Severe	Slight	MAJOR	
14 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP	Severe	Slight	MAJOR	
	BaP	Severe	Moderate	MAJOR	
28 days	LDPE	Absent	Slight	SLIGHT	
	LDPE-BaP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

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

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

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

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

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

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

ETHICS STATEMENT

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

AUTHORS CONTRIBUTIONS

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

ACKNOWLEDGMENTS

This work has been financially supported within Ephemare Project (by JPI Oceans). Centro Assistenza Ecologica (Ancona) partially funded the Ph.D. fellowship to LP.

REFERENCES

Avio, C. G., Cardelli, L. R., Gorbi, S., Pellegrini, D., and Regoli, F. (2017b). Microplastics pollution after the removal of the costa concordia wreck: first evidences from a biomonitoring case study. *Environ. Pollut*. 227, 207–214. doi: 10.1016/j.envpol.2017.04.066

Avio, C. G., Gorbi, S., and Regoli, F. (2017a). Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. *Mar. Environ. Res.* 128, 2–11. doi: 10.1016/j.marenvres.2016.05.012

Avio, C. G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., et al. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ. Pollut.* 198, 211–222. doi: 10.1016/j.envpol.2014.12.021

Bakir, A., Rowland, S. J., and Thompson, R. C. (2014). Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environ. Pollut.* 185, 16–23. doi: 10.1016/j.envpol.2013.10.007

Banni, M., Sforzini, S., Arlt, V. M., Barranger, A., Dallas, L. J., Oliveri, C., et al. (2017). Assessing the impact of Benzo [a] pyrene on marine mussels: application of a novel targeted low density microarray complementing classical biomarker responses.*PloS ONE* 12:e0178460. doi: 10.1371/journal.pone.0178460

Baršiene, J., Rybakovas, A., Garnaga, G., and Andreikenait,e, L. (2012). Environmental genotoxicity and cytotoxicity studies in mussels before and after an oil spill at the marine oil terminal in the Baltic Sea. *Environ. Monit. Assess.* 184, 2067–2078. doi: 10.1007/s10661-011-2100-0

Bebianno, M. J., Pereira, C. G., Rey, F., Cravo, A., Duarte, D., d'Errico, G., et al. (2015). Integrated approach to assess ecosystem health in harbor areas. *Sci. Total Environ.* 514, 92–107. doi: 10.1016/j.scitotenv.2015.01.050

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., et al. (2012). A multidisciplinary weight of evidence approach for classifying polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environ. Int.* 38, 17–28. doi: 10.1016/j.envint.2011.08.003

Benedetti, M., Gorbi, S., Fattorini, D., d'Errico, G., Piva, F., Pacitti, D., et al (2014). Environmental hazards from natural hydrocarbons seepage: integrated classification of risk from sediment chemistry, bioavailability and biomarkers responses in sentinel species. *Environ. Pollut.* 185, 116–126. doi: 10.1016/j.envpol.2013.10.023

Benedetti, M., Lanzoni, I., Nardi, A., d'Errico, G., Di Carlo, M., Fattorini, D., et al. (2016). Oxidative responsiveness to multiple stressors in the key Antarctic species, *Adamussium colbecki*: interactions between temperature, acidification and cadmium exposure. *Mar. Environ. Res.* 121, 20–30. doi: 10.1016/j.marenvres.2016.03.011

Bilbao, E., Cajaraville, M. P., and Cancio, I. (2009). Cloning and expression pattern of peroxisomal β-oxidation genes palmitoyl-CoA oxidase, multifunctional protein and 3-ketoacyl-CoA thiolase in mussel *Mytilus galloprovincialis* and thicklip grey mullet *Chelon labrosus*. *Gene* 443, 132–142. doi: 10.1016/j.gene.2009.05.008

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., et al. (2008). Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis,* to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. *Aquat. Toxicol.* 89, 257–266. doi: 10.1016/j.aquatox.2008.07.011

Browne, M. A. (2015). "Sources and pathways of microplastics to habitats," in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 229–244.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., and Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environ. Sci. Technol.* 42, 5026–5031. doi: 10.1021/es800249a

Cajaraville, M. P., Orbea, A., Marigómez, I., and Cancio, I. (1997). Peroxisome proliferation in the digestive epithelium of mussels exposed to the water accommodated fraction of three oils. *Comp. Biochem. Physiol. C* 117, 233–242. doi: 10.1016/S0742-8413(97)00057-1

Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Lorusso, L. C., Vergani, L., et al. (2008). Short-term effects of environmentally relevant concentrations of EDC mixtures on *Mytilus galloprovincialis* digestive gland. *Aquat. Toxicol.* 87, 272–279. doi: 10.1016/j.aquatox.2008.02.007

Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Vergani, L., and Gallo, G. (2007). Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas. *Mol. Cell. Endocrinol.* 276, 36–44. doi: 10.1016/j.mce.2007.06.002

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., and Gallo, G. (2012). Bivalve molluscs as a unique target group for nanoparticle toxicity. *Mar. Environ. Res.* 76, 16–21. doi: 10.1016/j.marenvres.2011.06.005

Cellura, C., Toubiana, M., Parrinello, N., and Roch, P. (2006). HSP70 gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micrococcus lysodeikticus*. *Dev. Comp. Immunol.* 30, 984–997. doi: 10.1016/j.dci.2005.12.009

Ching, E. W. K., Siu, W. H. L., Lam, P. K. S., Xu, L. H., Zhang, Y. Y., Richardson, B. J., et al. (2001). DNA adduct formation and DNA strand breaks in green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose- and time-dependent relationships. *Mar. Pollut. Bull.* 42, 603–610. doi: 10.1016/S0025-326X(00)00209-5

Cole, M., Lindeque, P., Halsband, C., and Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: a review. *Mar. Pollut. Bull.* 62, 2588–2597. doi: 10.1016/j.marpolbul.2011.09.025

Cózar, A., Sanz-Martín, M., Mart,í, E., González-Gordillo, J. I., Ubeda, B., Gálvez, J. Á., et al. (2015). Plastic accumulation in the Mediterranean Sea. *PLoS ONE* 10:e0121762. doi: 10.1371/journal.pone.0121762

De Witte, B., Devriese, L., Bekaert, K., Hoffman, S., Vandermeersch, G., Cooreman, K., et al. (2014). Quality assessment of the blue mussel (*Mytilus edulis*): comparison between commercial and wild types. *Mar. Pollut. Bull.* 85, 146–155. doi: 10.1016/j.marpolbul.2014.06.006

Détrée, C., and Gallardo-Escárate, C. (2017). Polyethylene microbeads induce transcriptional responses with tissue-dependent patterns in the mussel *Mytilus galloprovincialis*. *J. Molluscan Stud.* 83, 220–225. doi: 10.1093/mollus/eyx005

Devriese, L. I., van der Meulen, M. D., Maes, T., Bekaert, K., Paul-Pont, I., Frère, L., et al. (2015). Microplastic contamination in brown shrimp (*Crangon crangon*,

Linnaeus 1758) from coastal waters of the Southern North Sea and Channel area. *Mar. Pollut. Bull.* 98, 179–187. doi: 10.1016/j.marpolbul.2015.06.051

Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., et al. (2014). Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PLoS ONE* 9:e111913. doi: 10.1371/journal.pone.0111913

Franzellitti, S., and Fabbri, E. (2005). Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. *Biochem. Biophys. Res. Commun.* 336, 1157–1163. doi: 10.1016/j.bbrc.2005.08.244

Galgani, F., Hanke, G., and Maes, T. (2015). "Global distribution, composition and abundance of marine litter", in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 29–56.

Giannapas, M., Loukas, K., and Stefanos, D. (2012). Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. *Comp. Biochem. Physiol. C* 155, 182–189. doi: 10.1016/j.cbpc.2011.08.001

Gilfillan, L. R., Ohman, M. D., Doyle, M. J., and Watson, W. (2009). Occurrence of plastic micro-debris in the Southern California Current system *Cal. Coop. Ocean. Fish.* 50, 123–133.

Giuliani, M. E., Benedetti, M., Arukwe, A., and Regoli, F. (2013). Transcriptional and cata- lytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. *Aquat. Toxicol.* 134, 120–127. doi: 10.1016/j.aquatox.2013.03.012

Goldstein, M. C., Titmus, A. J., and Ford, M. (2013). Scales of spatial heterogeneity of plastic marine debris in the northeast Pacific Ocean. *PLoS ONE* 8:e80020. doi: 10.1371/journal.pone.0080020

Gorbi, S., Avio, G. C., Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., et al. (2013). Effects of harmful dinoflagellate Ostreopsis cf. ovata exposure on immunological, histological and oxidative responses of mussels *Mytilus galloprovincialis*. *Fish Shellfish Immun.* 35, 941–950. doi: 10.1016/j.fsi.2013.07.003

Gorbi, S., Virno Lamberti, C., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G., et al. (2008). An ecotoxicological protocol with caged mussels *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea. *Mar. Environ. Res.* 65, 34–49. doi: 10.1016/j.marenvres.2007.07.006

Grigorakis, S., Mason, S. A., and Drouillard, K. G. (2017). Determination of the gut retention of plastic microbeads and microfibers in goldfish (*Carassius auratus*). *Chemosphere* 169, 233–238. doi: 10.1016/j.chemosphere.2016.11.055

Grintzalis, K., Christos, D. G., and Stefanos, D. (2012). Total thiol redox status as a potent biomarker of PAH-mediated effects on mussels. *Mar. Environ. Res.* 81, 26–34. doi: 10.1016/j.marenvres.2012.08.004

Hannam, M. L., Bamber, S. D., Galloway, T. S., Moody, A. J., and Jones, M. B. (2010). Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. *Chemosphere* 78, 779–784. doi: 10.1016/j.chemosphere.2009.12.049

Heindler, F. M., Alajmi, F., Huerlimann, R., Zeng, C., Newman, S. J., Vamvounis, G., et al. (2017). Toxic effects of polyethylene terephthalate microparticles and Di (2-ethylhexyl) phthalate on the calanoid copepod, Parvocalanus crassirostris. *Ecotoxicol. Environ. Saf.* 141, 298–305. doi: 10.1016/j.ecoenv.2017.03.029

Karami, A., Groman, D. B., Wilson, S. P., Ismail, P., and Neela, V. K. (2017). Biomarker responses in zebrafish (*Danio rerio*) larvae exposed to pristine low-density polyethylene fragments. *Environ. Pollut.* 223, 466–475. doi: 10.1016/j.envpol.2017.01.047

Koelmans, A. A., Bakir, A., Burton, G. A., and Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environ. Sci. Technol.* 50: 3315–3326. doi: 10.1021/acs.est.5b06069

Kühn, S., Bravo Rebolledo, E. L., and van Franeker, J. A. (2015). "Deleterious effects of litter on marine life," in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 75–116.

Liu, L., Fokkink, R., and Koelmans, A. A. (2016). Sorption of polycyclic aromatic hydrocarbons to polystyrene nanoplastic. *Environ. Toxicol. Chem.* 35, 1650–1655. doi: 10.1002/etc.3311

Livingstone, D. R., and Farrar, S. V. (1984). Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo[a]pyrene metabolism in the common mussel *Mytilus edulis* L. *STOTEN* 39, 209–235.

Lohmann, R. (2017). Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves? *Integr. Environ. Assess. Manag.* 13, 460–465. doi: 10.1002/ieam.1914

Lusher, A. (2015). "Microplastics in the marine environment: distribution, interactions and effects," in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 245–307.

Lusher, A. L., McHugh, M., and Thompson, R. C. (2013). Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Mar. Pollut. Bull.* 67, 94–99. doi: 10.1016/j.marpolbul.2012.11.028

Lusher, A. L., Welden, N. A., Sobral, P., and Cole, M. (2017). Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Anal. Methods* 9, 1346–1360. doi: 10.1039/C6AY02415G

Marigómez, I., and Baybay-Villacorta, L. (2003). Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. *Aquat. Toxicol.* 64, 235–257. doi: 10.1016/S0166-445X(03)00056-0

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L. A., and Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Sci. Rep.* 7:11452. doi: 10.1038/s41598-017-10813-0

Mezzelani, M., Gorbi, S., Da Ros, Z., Fattorini, D., d'Errico, G., Milan, M., et al. (2016). Ecotoxicological potential of non-steroidal anti-inflammatory drugs (NSAIDs) in marine organisms: bioavailability, biomarkers and natural occurrence in *Mytilus galloprovincialis*. *Mar. Environ. Res.* 121, 31–39. doi: 10.1016/j.marenvres.2016.03.005

Moore, C. J., Moore, S. L., Leecaster, M. K., and Weisberg, S. B. (2001). A comparison of plastic and plankton in the north Pacific central gyre. *Mar. Pollut. Bull.* 42, 1297–1300. doi: 10.1016/s0025-326x(01)00114-x

Moore, M. N., Allen, J. I., McVeigh, A., and Shaw, J. (2006). Lysosomal and autophagic reactions as predictive indicators of environmental impact in aquatic animals. *Autophagy* 2, 217–220. doi: 10.4161/auto.2663

Murphy, F., Russell, M., Ewins, C., and Quinn, B. (2017). The uptake of macroplastic & microplastic by demersal & pelagic fish in the Northeast Atlantic around Scotland. *Mar. Pollut. Bull* 122, 353–359. doi: 10.1016/j.marpolbul.2017.06.073

Nardi, A., Mincarelli, L. F., Benedetti, M., Fattorini, D., d'Errico, G., and Regoli, F. (2017). Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. *Chemosphere* 169, 493–502. doi: 10.1016/j.chemosphere.2016.11.093

NOAA (2015). *A NOAA MDP Research Project Focuses on Types and Abundance of Microplastics. Detecting Microplastics in the Marine Environment*. Available online at: http://marinedebris.noaa.gov/research/detecting-microplastics-marine-environment

Oliveira, M., Ribeiro, A., Hylland, K., and Guilhermino, L. (2013). Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecol. Indic.* 34, 641–647. doi: 10.1016/j.ecolind.2013.06.019

Pan, L., Ren, J., and Zheng, D. (2009). Effects of benzo (a) pyrene exposure on the antioxidant enzyme activity of scallop *Chlamys farreri*. *Chinese J. Oceanol. Limnol.* 27, 43–53. doi: 10.1007/s00343-009-0043-x

Paul-Pont, I., Lacroix, C., Fernández, C. G., Hégaret, H., Lambert, C., Le Goïc, N., et al. (2016). Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. *Environ. Pollut.* 216, 724–737. doi: 10.1016/j.envpol.2016.06.039

Pedà, C., Caccamo, L., Fossi, M. C., Gai, F., Andaloro, F., Genovese, L., et al. (2016). Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: preliminary results. *Environ. Pollut.* 212, 251–256. doi: 10.1016/j.envpol.2016.01.083

Petrović, S., Semenči,ć, L., Ozretić, B., and Ozretić, M. (2004). Seasonal variations of physiological and cellular biomarkers and their use in the biomonitoring of north Adriatic coastal waters (Croatia). *Mar. Pollut. Bull.* 49, 713–720. doi: 10.1016/j.marpolbul.2004.05.004

Phuong, N. N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C., et al. (2016). Is there any consistency between the microplastics found in the field and those used in laboratory experiments? *Environ. Pollut.* 211, 111–123. doi: 10.1016/j.envpol.2015.12.035

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., et al. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere* 83, 475–485. doi: 10.1016/j.chemosphere.2010.12.064

Regoli, F. (1992). Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. *Mar. Ecol. Prog. Ser.* 84, 63–69. doi: 10.3354/meps084063

Regoli, F., and Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Mar. Environ. Res.* 93, 106–117. doi: 10.1016/j.marenvres.2013.07.006

Regoli, F., and Winston, G. W. (1998). Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. *Mar. Environ. Res.* 46, 439–442. doi: 10.1016/S0141-1136(97)00119-0

Regoli, F., Pellegrini, D., Cicero, A. M., Nigro, M., Benedetti, M., Gorbi, S., et al. (2014). A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: integrative indices from mussel watch. *Mar. Environ. Res.* 96, 92–104. doi: 10.1016/j.marenvres.2013.09.016

Ren, X., Pan, L., and Wang, L. (2015). Toxic effects upon exposure to benzo [a] pyrene in juvenile white shrimp Litopenaeus vannamei. *Environ. Toxicol. Pharmacol.* 39, 194–207. doi: 10.1016/j.etap.2014.08.006

Rey-Salgueiro, L., Martínez-Carballo, E., Cid, A., and Simal-Gándara, J. (2017). Determination of kinetic bioconcentration in mussels after short term exposure to polycyclic aromatic hydrocarbons. *Heliyon* 3:e00231. doi: 10.1016/j.heliyon.2017.e00231

Ribeiro, F., Garcia, A. R., Pereira, B. P., Fonseca, M., Mestre, N. C., Fonseca, T. G., et al. (2017). Microplastics effects in *Scrobicularia plana*. *Mar. Pollut. Bull.* 122, 379–391. doi: 10.1016/j.marpolbul.2017.06.078

Rochman, C. M. (2015). "The complex mixture, fate and toxicity of chemicals associated with plastic debris in the marine environment," in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 117–140.

Rochman, C. M., Hoh, E., Kurobe, T., and Teh, S. J. (2013). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* 3:3263. doi: 10.1038/srep03263

Santillo, D., Miller, K., and Johnston, P. (2017). Microplastics as contaminants in commercially important seafood species. *Integr. Environ. Assess. Manag.* 13, 516–521. doi: 10.1002/ieam.1909

Suaria, G., Avio, C. G., Mineo, A., Lattin, G. L., Magaldi, M. G., Belmonte, G., et al. (2016). The Mediterranean plastic soup: synthetic polymers in Mediterranean surface waters. *Sci. Rep.* 6:37551. doi: 10.1038/srep37551

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., et al. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. *Proc. Natl. Acad. Sci. U.S.A.* 113, 2430–2435. doi: 10.1073/pnas.1519019113

Syberg, K., Khan, F. R., Selck, H., Palmqvist, A., Banta, G. T., Daley, J., et al (2015). Microplastics: addressing ecological risk through lessons learned. *Environ. Toxicol. Chem.* 34, 945–953. doi: 10.1002/etc.2914

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., et al. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philos. Trans. R. Soc. B* 364, 2027–2045. doi: 10.1098/rstb.2008.0284

Thompson, R. C. (2015). "Microplastics in the marine environment: sources, consequences and solutions," in *Marine Anthropogenic Litter*, eds M. Bergmann, L. Gutow, and M. Klages (London: Springer), 185–200.

Von Moos, N., Burkhardt-Holm, P., and Köhler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ. Sci. Technol.* 46, 11327–11335. doi: 10.1021/es302332w

Wang, W., and Wang, J. (2018). Different partition of polycyclic aromatic hydrocarbon on environmental particulates in freshwater: microplastics in comparison to natural sediment. *Ecotox. Environ. Safe.* 147, 648–655. doi: 10.1016/j.ecoenv.2017.09.029

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., and Koelmans, A. A. (2012). Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environ.Toxicol. Chem.* 31, 2490–2497. doi: 10.1002/etc.1984

Wootton, E. C., Dyrynda, E. A., Pipe, R. K., and Ratcliffe, N. A. (2003). Comparisons of PAH-induced immunomodulation in three bivalve molluscs. *Aquat. Toxicol.* 65, 13–25. doi: 10.1016/S0166-445X(03)00098-5

Wright, S. L., Thompson, R. C., and Galloway, T. S. (2013). The physical impacts of microplastics on marine organisms: a review. *Environ. Pollut.* 178, 483–492. doi: 10.1016/j.envpol.2013.02.031

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

Science of the Total Environment 652 (2019) 602–610

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

The fate of microplastics in an Italian Wastewater Treatment Plant

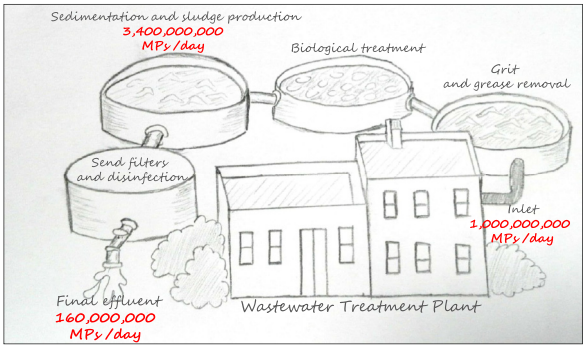
Stefano Magni [a,*], Andrea Binelli [a,*], Lucia Pittura [b], Carlo Giacomo Avio [b], Camilla Della Torre [a], Camilla Carla Parenti [a], Stefania Gorbi [b,c], Francesco Regoli [b,c]

[a] Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy
[b] Department of Life and Environmental Sciences, Polytechnic University of Marche, Via Brecce Bianche, 60131 Ancona, Italy
[c] National Inter-University Consortium for Marine Sciences, CoNISMa, U.L.R., Ancona, Italy

HIGHLIGHTS

- Selected Wastewater Treatment Plant (WWTP) removes the 84% of microplastics (MPs).
- 160,000,000 MPs were released daily by selected WWTP.
- 3,400,000,000 MPs were deposited daily in 30 tons of sludge by selected WWTP.
- WWTPs are a source of MPs in both aquatic and terrestrial environment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 August 2018
Received in revised form 19 October 2018
Accepted 19 October 2018
Available online xxxx

Editor: Damia Barcelo

Keywords:
Microplastics
Wastewater Treatment Plants
Effluents
Sewage sludge
Treatment efficiency

ABSTRACT

The emerged threat of microplastics (MPs) in aquatic ecosystems is posing a new challenges in environmental management, in particular the civil Wastewater Treatment Plants (WWTPs) which can act both as collectors of MPs from anthropic use and as a source to natural environments. In this study, MP fate was investigated in one of the biggest WWTPs of Northern Italy, built at the beginning of the 2000s and which serves a population equivalent of about 1,200,000, by evaluating their presence at the inlet (IN), the removal efficiency after the settler (SET) and at the outlet (OUT), and their transfer to sludge. Samples were collected in three days of a week and plastic debris was characterized in terms of shape, size and polymer composition using the Fourier Transform Infrared Microscope System (μFT-IR). The number of detected MPs was 2.5 ± 0.3 MPs/L in the IN, 0.9 ± 0.3 MPs/L after the SET and 0.4 ± 0.1 MPs/L in the OUT, indicating a total removal efficiency of 84%. However, considering that this WWTP treats about 400,000,000 L wastewaters/day, the potential release of MPs to the receiving aquatic system would be approximately 160,000,000 MPs/day, mainly polyesters (35%) and polyamide (17%). Furthermore, a great amount of MPs removed from wastewater was detected in the recycled activated sludge, with 113 ± 57 MPs/g sludge dry weight, corresponding to about 3,400,000,000 MPs deposited in the 30 tons of sludge daily produced by this WWTP. Given the possible re-use of WWTP sludge in fertilizers for agriculture, our results highlight that WWTPs could represent a potential source of MPs also to agroecosystems.

1. Introduction

Plastic materials have a pivotal role in the modern society and synthetic polymer production increased worldwide in the last decades,

* Corresponding authors.
E-mail addresses: stefano.magni@unimi.it (S. Magni), andrea.binelli@unimi.it (A. Binelli).

https://doi.org/10.1016/j.scitotenv.2018.10.269
0048-9697/

reaching 330 million tons in 2016 (PlasticEurope, 2017). Microplastics (MPs), particles smaller than 5 mm in size, are now recognized as an emerged worldwide issue in both marine and freshwater environments (Cole et al., 2011; Eerkes-Medrano et al., 2015; Avio et al., 2017). Ubiquitary distributed and with degradation periods of hundreds of years (Thompson et al., 2004), MPs are easily ingested and have the potential to accumulate in both biota (Browne et al., 2008; Avio et al., 2015a; Paul-Pont et al., 2016; Magni et al., 2018; Pittura et al., 2018) and aquatic food web (Avio et al., 2017; Carbery et al., 2018; Nelms et al., 2018).

Primary MPs, as plastic pellets, are produced directly with microscopic size to be used in air blasting technology, or as abrasive agents in personal care products (PCPs; Cole et al., 2011); their use in cosmetics has been recently banned or limited in some countries such as US, UK and Canada (Conkle et al., 2018). The large majority of environmental MPs are of secondary origin, deriving from degradation of plastic wastes (Cole et al., 2011) or from synthetic cloth washing (Napper and Thompson, 2016).

Land sources contribute to 80% of global MP pollution (Andrady, 2011), and debris collected from urban areas are expected to be treated by civil Wastewater Treatment Plants (WWTPs; Prata, 2018), with some exceptions during periods of heavy rainfall for areas with combined sewers. However, WWTPs, being designed to remove organic matter and nutrients from wastewaters, are not efficient in the removal of other contaminants as pharmaceuticals, illicit drugs, heavy metals (Binelli et al., 2014, 2015; Magni et al., 2015) and also MPs that might be discharged in the aquatic environment with potential adverse effects on aquatic organisms (Magni et al., 2016, 2017, 2018). In this context, despite the toxic mechanisms of MPs need clarifications, especially on freshwater species, some studies reported the alteration of the oxidative status, neuro- and energy-related enzyme activity modulation, as well as intestinal damage in aquatic organisms after MP exposure (Avio et al., 2015b; Barboza et al., 2018; Lei et al., 2018; Magni et al., 2018).

The presence of MPs has been reported in the outlet of WWTPs in United States (Estahbanati and Fahrenfeld, 2016; Mason et al., 2016; Michielssen et al., 2016; Dyachenko et al., 2017), Australia (Browne et al., 2011; Ziajahromi et al., 2017), Finland (Talvitie et al., 2017a, 2017b; Lares et al., 2018), Germany (Mintenig et al., 2017), Netherlands (Leslie et al., 2017), Sweden (Magnusson and Norén, 2014) and UK (Murphy et al., 2016). Even when the efficiency of MPs removal is very high (72–98%; Murphy et al., 2016; Leslie et al., 2017), due to the great volume of treated wastes, a WWTP with a population equivalent of 650,000 may be responsible for a daily release of 65,000,000 MP debris (Murphy et al., 2016). Consequently, the presence of MPs has been reported in aquatic systems in Europe (Faure et al., 2012; Imhof et al., 2013; Sadri and Thompson, 2014; Wagner et al., 2014; Lechner and Ramler, 2015; Fischer et al., 2016; Guerranti et al., 2017; Imhof et al., 2018; Sighicelli et al., 2018), America, Asia and Africa (Free et al., 2014; Su et al., 2016; Wang et al., 2016, 2017; Anderson et al., 2017; Di and Wang, 2018; Nel et al., 2018).

In addition to the discharge of MPs in inland waters, WWTPs pose another potential threat for the high percentage of MPs (up to >90%) which settle on the bottom of WWTP tanks, accumulating in the recycled activated sludge (Carr et al., 2016). This aspect poses a potential threat also for terrestrial pollution, considering that sewage sludge is widely re-used in agriculture as fertilizer worldwide (Mahon et al., 2017) with a request of 50% of the total sludge production in Europe and America, and approximately 125–850 tons of MPs/million inhabitants added every year in European soils (Nizzetto et al., 2016).

To provide other information regarding MP input in the European inland waters from WWTPs, the aim of our study was to evaluate the abundance and physical/chemical characteristics of MPs in one of the main WWTPs of Northern Italy, characterizing these particles in wastewaters at different treatment steps, as well as in the recycled activated sludge. To our knowledge, this is the first study aimed to identify the fate of MPs through the entire waste treatment process from an Italian WWTP, to evaluate the efficiency of various treatments in removing these particles, and to assess the overall release of such emerging contaminants.

2. Materials and methods

2.1. Wastewater and sludge sampling

The WWTP is located in Northern Italy and it represents one of the biggest station and more recent Italian plants, built at the beginning of the 2000s and receiving waters from combined sewers. It serves about 1,200,000 population equivalent and it is equipped with pre-, primary, secondary and tertiary treatments, articulated in screening, grit and grease removal stages, biological treatment, sedimentation (with recycled activated sludge), sand filter treatment and disinfection. The average inlet flow rate of the plant is of about 400,000,000 L/day (the same value of the outlet flow rate), with an average dry weather flow rate of about 18,000,000 L/h and a maximum flow rate in wet weather conditions of 54,000,000 L/h.

To assess the presence, removal efficiency and release of floating MPs in the plant, wastewaters were sampled at three different treatment steps: inlet (IN), after the settler (SET), and outlet (OUT). In addition, since MPs can settle on the basis of polymer density (ranging from 0.01–0.05 g/cm^3 for the expanded polystyrene, to 2.20–2.30 g/cm^3 for 25% glass filled polytetrafluoroethylene; Crawford and Quinn, 2017), the recycled activated sludge with a concentration of 7.5 g/L dry weight (dw) was sampled. To reduce the intrinsic variability associated to weather conditions and/or possible changes in the urban release, the sampling was repeated for three days in a spring week, without rainfall, at the same time (between 9 and 11 a.m.). In detail, 30 L of surface wastewater were collected every day from each treatment step using a steel bucket; samples were subsequently filtered *in loco* with a suite of steel sieves (ISO 3310-1:2000) with a mesh of 5 mm, 2 mm and 63 μm. Furthermore, 50 mL of recycled activated sludge were collected using a glass beaker.

2.2. MP separation from collected matrices

Each wastewater filtered and collected sludge were put in glass bottles with 500 mL of sodium chloride (NaCl) hypersaline solution (1.2 g/cm^3) to separate MPs from particulate matter, exploiting the density gradient (Thompson et al., 2004). The use of NaCl hypersaline solution for MP separation by sediments, similar to sewage sludge, is recommended by the Marine Strategy Framework Directive (MSFD) and suggested when a huge number of samples needs to be processed. Indeed, this method is cheap, widely available and eco-friendly, despite the extraction performance of high density MPs, as plasticized polyvinylchloride (1.3–1.7 g/cm^3) or polytetrafluoroethylene (2.1–2.2 g/cm^3), could be lower than other synthetic polymers (Crawford and Quinn, 2017).

Samples were stirred and decanted overnight at 4 °C. Supernatants were then filtered on 8 μm cellulose nitrate membrane filters (Sartorius™ 50 mm) using a vacuum pump. In the same filtration apparatus we put 500 mL of Milli Q®, to remove salt crystals, which were then filtered using a vacuum pump across the obtained cellulose membrane filters with collected debris. The organic matter was partially digested by 15% hydrogen peroxide (H_2O_2) for three days at room temperature (RT), maintaining the filters under laminar flow hood to avoid atmospheric contamination by microfibers (Avio et al., 2015a).

2.3. MP quantification and characterization

After the organic matter digestion, wastewaters and sludge filters were visually examined under a stereomicroscope (SZM-D equipped with OPTIKAM B5, Optika). All particles suspected to be plastics or particles whose nature was in doubt, as well as all fibers, were manually

collected, transferred onto a clean filter and then analyzed, that has allowed to distinguish between plastic and *non*-plastic materials. Despite a rigorous visual protocol performed by experienced researchers, an underestimation of smaller particles is possible, especially those between 30 μm and 10 μm that are more difficult to notice. However, visual examination of particles prior their chemical characterization represents a necessary step to isolate particles from filters, on which it is not possible to completely eliminated organic matter and/or mineral components, without involving more destructive or expensive methods. In addition, the validation of the original method on MP-spiked samples revealed an elevated yield of recovery, ranging from 78% to 98%, depending on the particle size (Avio et al., 2015a).

On the basis of the different origin and abundance of microplastic particles (MPPs) compared to microplastic fibers (MPFs - ribbon-like shape with frayed ends; Almroth et al., 2018; Dris et al., 2018), we decided to consider separately these two types of MPs in the presentation and elaboration of results. MPPs were categorized according to the shape (lines - same thickness in all length with sharp ends, films and fragments; Fig. 1) and measured (Optika Vision Lite 2.1, Optika) to be classified into 4 size classes (5–1 mm, 1–0.5 mm, 0.5–0.1 mm, 0.1–0.01 mm).

All the collected MPPs and MPFs on membrane filters were characterized using the Fourier Transform Infrared Microscope System (μFTIR; Spotlight 200i equipped with Spectrum Two, Perkin Elmer) to confirm their plastic nature and to identify polymer typologies. FT-IR spectra of individual MPs were acquired in attenuated total reflectance (ATR) mode (32 scans to produce spectra with wavelengths between 600 and 4000 cm^{-1} and resolution of 4 cm^{-1}), analyzed using Spectrum 10 software and compared with libraries of standard spectra. Similarity of measured sample and reference spectrum was accepted only after visual examination of spectra characteristics and with a *Hit Quality Index* (*HQI*) ≥ 0.7 (Klein et al., 2015; Lusher et al., 2015).

2.4. Contamination prevention during samples processing

To prevent contamination with other MPPs or MPFs, samples were kept covered as much as possible using glass lids; lab coats, cotton clothing and gloves were worn, both during samplings and laboratory operations. Work surfaces, equipment and manipulation instruments were cleaned with Milli Q® water and alcohol, and checked under the microscope before use. All solutions were filtered twice on 1.2 μm glass fiber

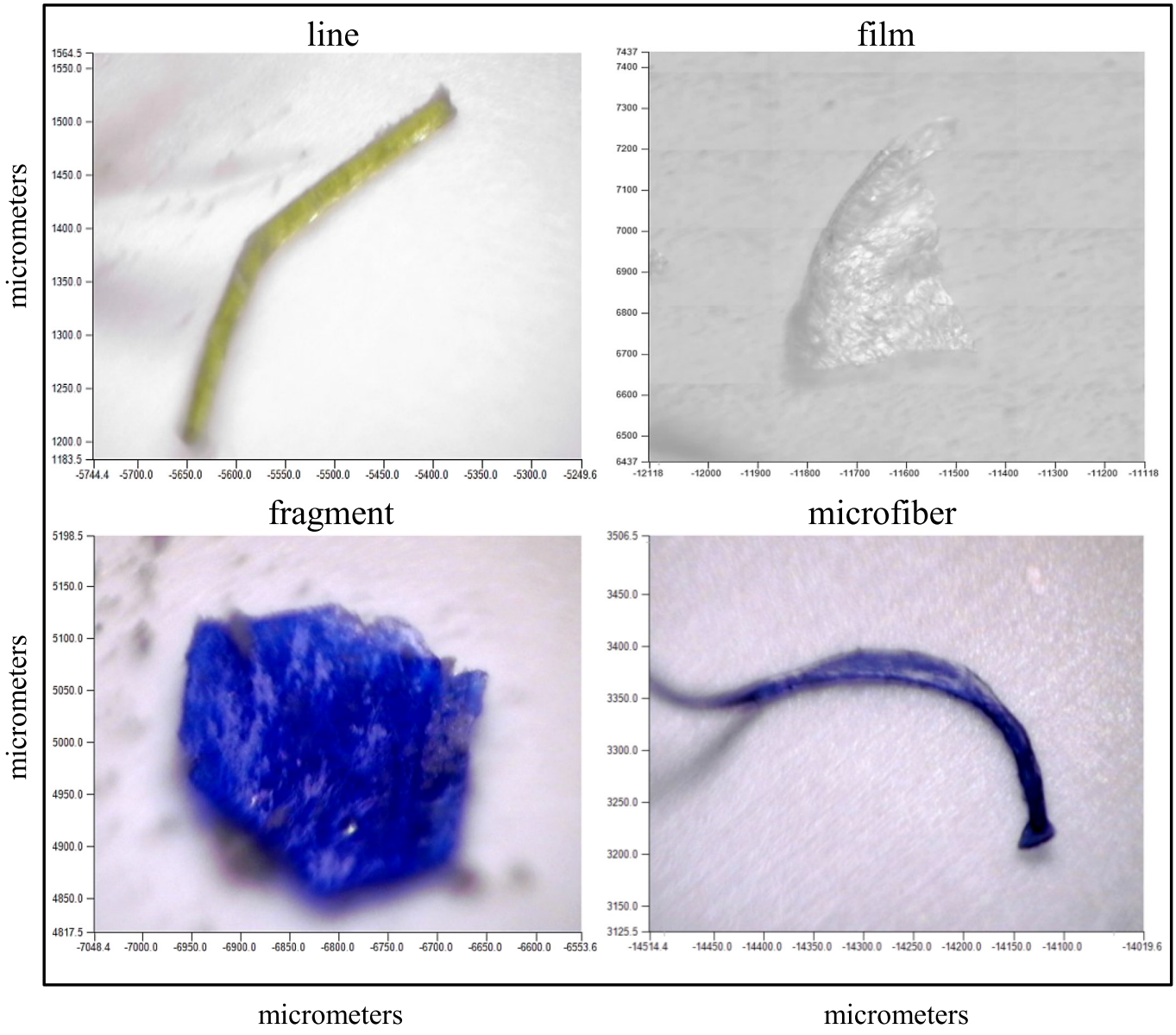


Fig. 1. MPPs (line, film and fragment) and MPF (microfiber) extracted from both wastewaters and sludge and observed at μFT-IR.

filters (Whatman® GF/C 47 mm) to eliminate impurities. In addition, 2 filters were processed as blanks with the same procedure for each experimental condition; during visual sorting, blanks were left exposed to the air the same amount of time as the samples. Despite Simon et al. (2018) presented a mass-based assessment of MPs, a similar approach (requiring the precise determination of both minor and major dimensions of irregularly shaped MPs) was considered not reliable in this study. We thus preferred to express our data as MPs/L, similarly many other studies (*e.g.* Magnusson and Norén, 2014; Talvitie et al., 2015, 2017b; Murphy et al., 2016; Leslie et al., 2017; Mintenig et al., 2017; Lares et al., 2018).

2.5. Statistical approach

To evaluate the significant differences (*$p < 0.05$; **$p < 0.01$) about MP content between the three different treatment steps (IN, SET and OUT), we performed the one-way analysis of variance (one-way ANOVA); each difference, treatment *versus* treatment, was evaluated using the Fisher LSD post hoc test. For these analyses we used the STATISTICA 7.0 software package.

3. Results

3.1. Contamination control

Analyses of blanks showed 1.6 ± 1.0 (mean value ± standard deviation; SD) microfibers of cotton/filter (only natural microfibers were detected in the blanks), corresponding to 30 L of wastewater and 50 mL of sludge; no MPPs were detected in the blanks. These values are much lower than 10% of the overall microfibers average throughout all samples, indicating a good contamination control as suggested by Lusher et al. (2015).

3.2. MPs in wastewaters

The characterization by μFT-IR has shown that, of all the particles collected during the visual sorting, the 72% from the IN, the 67% from the SET and the 55% from the OUT, were plastics. The number, shape (lines, films, fragments), size (mm), polymer composition (example of spectra in Fig. S1, Supplementary material) and library matching score (*HQI*) of individual MPPs detected in each of the three sampling days are reported in Table S1 (Supplementary material). The same results are summarized below as mean value ± SD of the three days.

MPP quantification showed a value of 2.0 ± 0.3 MPPs/L in the IN wastewaters, reduced to 0.6 ± 0.2 MPPs/L after the SET and 0.3 ± 0.1 MPPs/L in the OUT (Fig. 2A); similarly, MPFs decreased from 0.5 ± 0.1 MPFs/L in the IN, to 0.3 ± 0.2 MPFs/L after the SET and 0.10 ± 0.03 MPFs/L in the OUT (Fig. 2A), for a total amount of detected MPs of 2.5 ± 0.3 MPs/L in the IN, 0.9 ± 0.3 MPs/L after the SET and 0.4 ± 0.1 MPs/L in the OUT (Fig. 2A, B). In this context, we observed a significant effect of treatment steps on MP content in wastewaters ($F_{2,6} = 50.3$; $p < 0.01$), with a significant difference in MP concentration between IN and SET ($p < 0.01$) and IN and OUT ($p < 0.01$; Fig. 2B); no significant difference has been observed between SET and OUT ($p = 0.07$; Fig. 2B).

The total percentage of MP decrease between IN and OUT was 84%, with the greater removal (64%) occurring among IN and SET (Fig. 2A). Since this WWTP treats an average of 400,000,000 L of wastewaters/day, the daily inlet and release in surface waters would correspond to about 1,000,000,000 MPs and 160,000,000 MPs respectively. Films were the main shape of MPPs (73%) which enter in the plant (Fig. 3A), followed by fragments (21%) and lines (6%). These ratios change during the wastewater treatments, with more similar percentages of films (36%), fragments (36%) and lines (28%) after the SET, while a greater ratio of lines (41%) and films (38%) followed by fragments (21%) was measured in the WWTP OUT (Fig. 3A). The predominant size range of MPPs was 0.5–0.1 mm in all samples, accounting for 36% of total

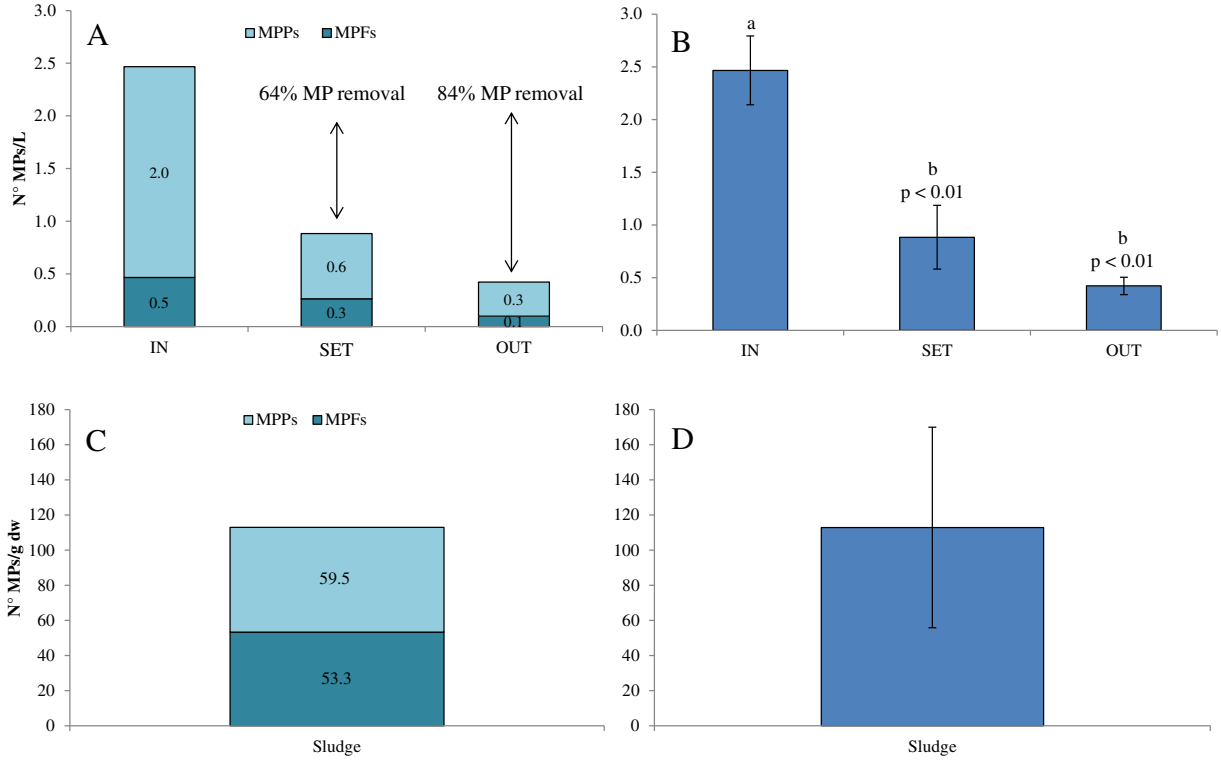


Fig. 2. Mean number of MPPs/L and MPFs/L (A) with the relative total MPs/L amount (B; mean ± SD) in the three different steps of wastewater treatment (IN, SET and OUT); letters indicate the significant differences regarding the MP content in the three different treatment steps (one-way ANOVA, Fisher LSD post hoc test). (C) Mean number of MPPs/g dw and MPFs/g dw in sludge with the relative total MPs/g dw amount (D; mean ± SD).

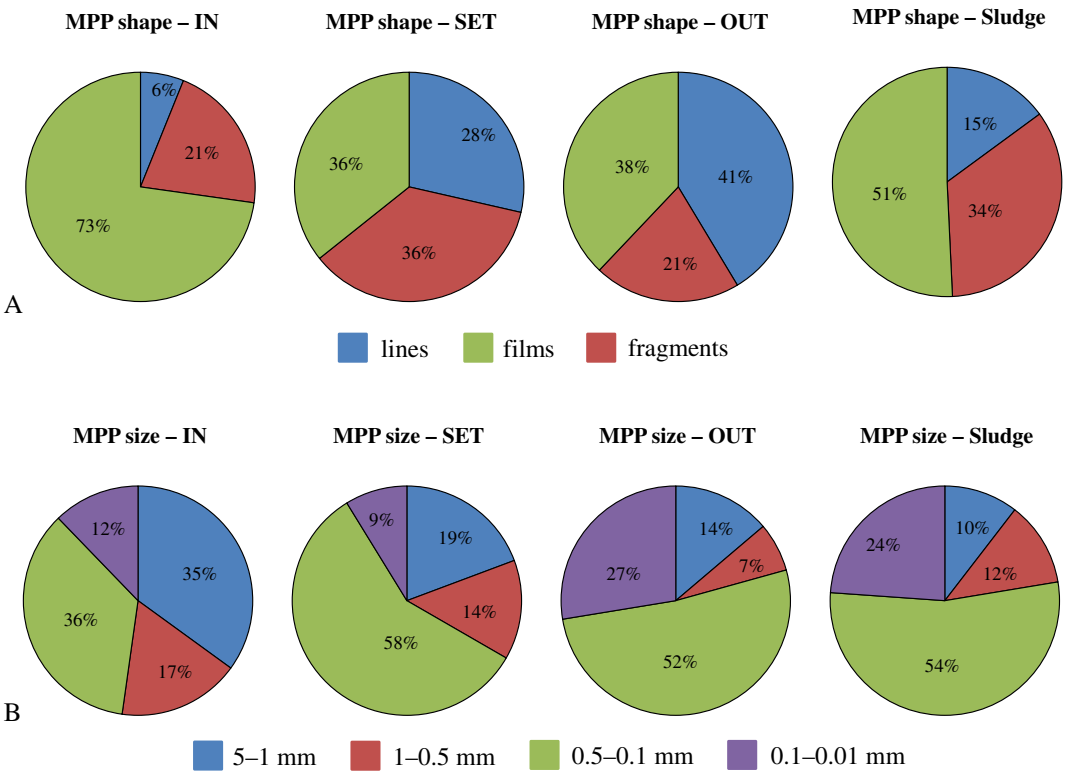


Fig. 3. Percentage of shapes (A; lines, films and fragments) and sizes (B; 5–1 mm, 1–0.5 mm, 0.5–0.1 mm, 0.1–0.01 mm) of detected MPPs in both wastewaters (IN, SET and OUT) and sludge.

particles in the IN, 58% after the SET and 52% in the OUT (Fig. 3B). In this regard, we observed a MP removal, on size basis, of 94% for 5–1 mm and 1–0.05 mm MPs, 77% for 0.5–0.1 mm MPs and 65% for 0.1–0.01 mm MPs.

Table 1 shows that the main MPP classes in the IN were represented by the co-polymer of acrylonitrile-butadiene (40%), followed by polyethylene (17%) and ethylene-propylene (14%). The other two typologies of wastewaters revealed a high concentration of polyesters (23%),

Table 1
Polymer (black labels) and co-polymer (red labels) classes of detected MPPs in both wastewaters (IN, SET and OUT) and sludge expressed as percentage (%), number of MPPs/volume of sampled wastewater (30 L) or sludge (50 mL) and number of MPPs/L of wastewater and MPPs/g dw of sludge. The results are presented as mean value of the three days of sampling (see Table S1, Supplementary materials).

MPP polymer class	IN %	SET %	OUT %	Sludge %	IN MPPs/30L	SET MPPs/30L	OUT MPPs/30L	Sludge MPPs/50mL	IN MPPs/L	SET MPPs/L	OUT MPPs/L	Sludge MPPs/g
epoxy resin	–	–	3	–	–	–	0.33	–	–	–	0.01	–
polyacrylates	–	2	7	3	–	0.33	0.67	0.67	–	0.01	0.02	1.79
polyamide	2	11	17	6	1.33	2.00	1.67	1.33	0.04	0.07	0.06	3.55
polyesters	4	23	35	15	2.67	4.33	3.30	3.33	0.09	0.14	0.11	8.88
polyoxymethylene	–	–	3	–	–	–	0.33	–	–	–	0.01	–
polytetrafluorethylene	–	–	–	2	–	–	–	0.33	–	–	–	0.88
polyterpene	2	–	3	–	1.00	–	0.33	–	0.03	–	0.01	–
polyethylene	17	13	10	18	10.00	2.33	1.00	4.00	0.33	0.08	0.03	10.67
polypropylene	4	11	–	9	2.33	2.00	–	2.33	0.08	0.07	–	6.21
polystyrene	–	–	–	5	–	–	–	1.00	–	–	–	2.67
polyurethane	3	13	7	3	2.00	2.33	0.67	0.67	0.07	0.08	0.02	1.79
polyvinylchloride	–	–	3	–	–	–	0.33	–	–	–	0.01	–
silicone	–	–	–	2	–	–	–	0.33	–	–	–	0.88
acrylonitrile–butadiene	40	9	3	27	24.00	1.67	0.33	6.00	0.80	0.06	0.01	16.00
acrylonitrile–butadiene–styrene	–	2	–	–	–	0.33	–	–	–	0.01	–	–
ethylene–acrylate	7	7	3	5	4.33	1.33	0.33	1.00	0.14	0.04	0.01	2.67
ethylene–propylene	14	–	–	–	8.33	–	–	–	0.28	–	–	–
ethylene–propylene–diene	2	9	–	5	1.33	1.67	–	1.00	0.04	0.06	–	2.67
ethylene–vinylacetate	1	2	–	–	0.33	0.33	–	–	0.01	0.01	–	–
styrene–butadiene–styrene	1	–	–	–	0.67	–	–	–	0.02	–	–	–
styrene–ethylene–butadiene–styrene	3	–	–	–	1.67	–	–	–	0.06	–	–	–
styrene–isoprene	–	–	–	2	–	–	–	0.33	–	–	–	0.88
styrene–isoprene–styrene	–	–	3	–	–	–	0.33	–	–	–	0.01	–
styrene–vinyltoluene–butylacrylate	1	–	–	–	0.33	–	–	–	0.01	–	–	–

polyethylene (13%), polyurethane (13%), polyamide (11%), and polypropylene (11%) after the SET, while the main polymers in the OUT were polyesters (35%), polyamide (17%) and polyethylene (10%).

Regarding microfibers, the ratio of natural microfibers *versus* MPFs was 66 and 34% in the IN, 72 and 28% after the SET, 81 and 19% in the OUT (Table S2, Supplementary material). Natural microfibers were mainly made of cotton (Table S2, Supplementary material) and were excluded from the values of MPFs and MPs. Among MPFs, polyesters represented the main polymer class, accounting for 83% of synthetic polymers in the IN, 79% after the SET and 89% in OUT; remaining polymers were polyacrylates (12%, 8% and 11% in the three steps) and polyamide (5% and 13% in the IN and after the SET respectively; Table 2).

3.3. MPs in recycled activated sludge

The number, shape, size and polymer composition (example of spectra in Fig. S1, Supplementary material) of MPPs detected in active sludge during the three sampling days are individually given in Table S1 (Supplementary material) and presented below as average value ± SD. Among all the particles collected in the visual sorting phase, the 81% resulted to be plastics after μFT-IR characterization.

The number of observed MPs was 59.5 ± 21.6 MPPs/g sludge dw, and 53.3 ± 48.9 MPFs/g sludge dw (Fig. 2C), accounting for a total value of 113 ± 57 MPs/g sludge dw (Fig. 2C, D). Considering that the investigated WWTP produces about 30 tons/dw of sludge daily, we can derive an estimate of about 3,400,000,000 MPs accumulating each day in the sewage sludge.

As reported in Fig. 3A, shapes of MPPs were films (51%), fragments (34%) and lines (15%), while the main size class was 0.5–0.1 mm (54%; Fig. 3B). Co-polymers of acrylonitrile-butadiene were the more abundant chemical typologies detected in the sludge (27%), followed by polyethylene (18%) and polyesters (15%, Table 1). Looking at the distribution of polymers among different shapes, acrylonitrile-butadiene represented 32% of films, 26% of fragments and 10% of lines; for this last shape, the main polymers were polyesters (60%), while fragments were mainly constituted by polyethylene (35%; Table 3).

The 65% of the total microfibers collected in the sewage sludge was synthetic and represented only by polyesters (Table 2); the remaining 35% of microfibers were of natural origin (cotton, Table S2, Supplementary material), and excluded from the count of MPFs and MPs.

Table 3
Percentage of shapes with the relative polymer (black labels) and co-polymer (red labels) classes of detected MPPs in sludge. The results are presented as mean value of the three days of sampling (see Table S1, Supplementary materials).

MPP shape	MPP polymer class	Detection (%)
Line polymer class (15%)	polyamide	20
	polyesters	60
	silicone	10
	acrylonitrile–butadiene	10
Film polymer class (51%)	polyacrylates	6
	polyamide	6
	polyesters	6
	polytetrafluorethylene	3
	polyethylene	14
	polypropylene	15
	polystyrene	3
	polyurethane	3
	acrylonitrile–butadiene	32
	ethylene–acrylate	6
	ethylene–propylene–diene	6
Fragment polymer class (34%)	polyesters	9
	polyethylene	35
	polypropylene	5
	polystyrene	9
	polyurethane	4
	acrylonitrile–butadiene	26
	ethylene–acrylate	4
	ethylene–propylene–diene	4
	styrene–isoprene	4

4. Discussion

The pivotal result of this study was to demonstrate that millions of MPs both in the OUT and sewage sludge can be released in aquatic and terrestrial ecosystems being used for different purposes such as irrigation and fertilization. These evidences confirm the role of WWTPs as collector of MPs from anthropic use towards natural environment, as recently observed in other WWTPs around the world (Prata, 2018).

Analyzing step by step the route of MPs through the WWTP, the mean value of MPs found in the IN (2.5 ± 0.3 MPs/L) was a much lower than those recently reported at the IN of other European WWTPs in UK (15.7 ± 5.2 MPs/L; Murphy et al., 2016, sampling performed with a "lower size limit" -LSL- of wastewater filtration at 65 μm, the same size of sieve used in this study) and Finland (57.6 ± 12.4 MPs/L; Lares et al., 2018; LSL of 250 μm). Since the MPs found in civil wastewaters derive primarily from PCPs and synthetic textiles (Prata, 2018), different habits, weather and season conditions can contribute to the variability of MP concentration in WWTP inlets in different countries. Another explanation to this difference could be the intentional infiltration of waters, *e.g.* groundwaters, that enter in the sewage system thus diluting the MP concentration in the wastewaters. This phenomenon is particularly present in Italy where this infiltration can reach the 30% of the entire flow rate: in addition, >70% of the national sewage system is made by combined sewages that collect domestic wastes with rainwater runoff and industrial wastewaters all in the same pipe (Autorità per l'energia elettrica, il gas e il sistema idrico, 2017). On the basis of these evidences, it is important to take into account that the observed differences in MP concentration can also be related to the use of different MP detection methods, highlighting the importance to establish common standardized protocol(s) for MP monitoring, as well as a uniform unit to express MP abundance to facilitate the data comparison between different sampling sites.

One of the most interesting results of this study was the abundance of co-polymers entering in the WWTP, in particular acrylonitrile-butadiene (40% of total MPs; Table 1). This is called nitrile-butadiene rubber (NBR), it is generally used in automotive seals, gaskets and pipes, but also in textiles, where its application to woven and *non*-woven fabrics improves the finish and waterproofing properties. Characterization of WWTP IN also revealed polyethylene (17%), one of the most common plastic material, and co-polymer of ethylene-propylene (14%), mainly used in automobile parts and in the production of pipe

Table 2
Polymer classes of detected MPFs in both wastewaters (IN, SET and OUT) and sludge expressed as percentage (%), number of MPFs/volume of sampled wastewater (30 L) or sludge (50 mL) and number of MPPs/L of wastewater and MPPs/g dw of sludge. The results are presented as mean value of the three days of sampling (see Table S2, Supplementary materials).

MPF polymer class	IN	SET	OUT	Sludge	IN	SET	OUT	Sludge	IN	SET	OUT	Sludge
	%	%	%	%	MPPs/30 L	MPPs/30 L	MPPs/30 L	MPPs/50 mL	MPPs/L	MPPs/L	MPPs/L	MPPs/g
Polyacrylates	12	8	11	–	1.67	0.67	0.33	–	0.06	0.02	0.01	–
Polyamide	5	13	–	–	0.67	1.00	–	–	0.02	0.03	–	–
Polyesters	83	79	89	100	11.67	6.33	2.67	20.00	0.39	0.21	0.09	53.33

seals (Table 1). To complete the description of MPs entering in the WWTP, it is important to consider that 83% of MPFs were polyesters (Table 2), probably released by synthetic cloth washing (Prata, 2018), since a 6 kg wash load release about 700,000 MPFs (Napper and Thompson, 2016).

The passage through the oxidative tanks and settler changed the polymer ratio percentage of MPPs after the SET with a decrease of co-polymers and an increase of polyesters (23%), polyurethane (13%), polypropylene (11%) and polyamide (11%); polyesters further increased in the OUT (35%), along with polyamide (17%) and polyacrylates (7%), used in PCPs and paints as adhesive agents. In this context, some MP classes, as epoxy resin (3%), polyvinylchloride (3%), polyoxymethylene (3%) and styrene-isoprene-styrene (3%), were found only in the OUT wastewaters (Table 1). These results could suggest the equipment used in WWTP processes as a potential direct source of polymers towards the aquatic environment, which should thus be carefully considered in future assessments of MP generation and fate. However, this aspect need clarifications considering that the abovementioned MP classes were detected only in the OUT and at a very low concentration of 0.01 MPs/L (Table 1). In the OUT, the MP removal efficiency from wastewater was of 84%, with a 94% of removal between IN and OUT of 5–1 mm and 1–0.5 mm MPs, 77% for 0.5–0.1 mm MPs and 65% with 0.1–0.01 mm MPs. After such removal efficiency, a mean value of 0.4 ± 0.1 MPs/L was still detected, corresponding to 160,000,000 MPs released daily in the receiving water-body. In this context, the large release of MPs in surface waters by WWTPs could provoke adverse effects to aquatic species, considering that under laboratory conditions a mixture of 16,000,000 MPs *per* tank (4,000,000 MPs/L) induced a significant alteration of dopamine level on freshwater mussel *Dreissena polymorpha* (Magni et al., 2018). The release of MPs observed in this study is within the range reported for other American and European WWTPs (see Table 3 in Lares et al., 2018), with the lower value of 0.005 MPs/L observed in Finnish WWTPs (Talvitie et al., 2017b) and the higher concentration of 91 MPs/L in 7 Dutch WWTPs (Leslie et al., 2017). Relating the quantity of MPs in the OUT with the population equivalent of the selected WWTP (1,200,000), we calculated a release of 133 MPs/equivalent inhabitant (*per capita*), comparable with that reported by Murphy et al. (2016) of 100 MPs/equivalent inhabitant. More in detail, it would seem that the LSL of wastewater filtration influences the quantity of detected MPs in the effluents of various European WWTPs: 0.00825 MPs/L were found in Swedish WWTP with a LSL of 300 μm (Magnusson and Norén, 2014), 1.05 MPs/L in Finnish WWTP with a LSL of 250 μm (Lares et al., 2018), 0.25 MPs/L in Scottish WWTP with a LSL of 65 μm (Murphy et al., 2016), 0.1–10.05 MPs/L in German WWTPs with a LSL of 20 μm (Mintenig et al., 2017), 0.005–13.5 MPs/L in Finnish WWTPs with a LSL of 20 μm (Talvitie et al., 2015, 2017b), and, lastly, from 9 to 91 MPs/L in Dutch WWTPs with a LSL of 0.7 μm (Leslie et al., 2017).

Also the MP removal efficiency of 84% observed in this study is in the same order of other European WWTPs, ranging from 72% to 98% (Murphy et al., 2016; Leslie et al., 2017; Lares et al., 2018). Since the main removal of MPs occurred in the first steps of treatment (64% of MP retention from IN and SET), the grease removal and sedimentation processes are confirmed the pivotal steps involved in the reduction of floating and settling of MPs from wastewaters respectively (Murphy et al., 2016). However, also the sand filters at the end of the WWTP contributed to the treatment performance, decreasing by almost 50% the MP content from the SET (0.9 ± 0.3 MPs/L) to the OUT (0.4 ± 0.1 MPs/L). This is another crucial result in the attempt to define simple and cost-effective treatments to reduce MPs in wastewaters. In this context, Talvitie et al. (2017b) tested the performance of different final stage technologies, observing a MP removal of 97% after sand filters, and a higher activity for membrane bioreactor (99.9%). The great performance of sand filters should, however, be further validated in future studies, since the daily washing water is generally carried in counterflow, potentially recirculating also MPs.

The description of the MP route through the WWTP cannot ignore the recycled activated sludge produced between IN and SET. The MPs density is not the only factor driving their sedimentation, since also low-density polymers were found in the sludge, as shown for polystyrene (density from 0.01 to 1.05 g/cm^3; Crawford and Quinn, 2017). Fouling by bacteria and other physical/chemical processes can modulate the mechanism of MP floating/sedimentation. In particular, for granular-like MPs, the sedimentation process could be explained by the Stokes law, considering the regime of wastewater flow (laminar or turbulent) around the particles, described by the Reynolds number (Re); for larger MPs with high Re, the shape seems the main factor influencing the sedimentation (Khatmullina and Isachenko, 2017). Also flocculation phenomena are not negligible in wastewaters, explaining the high presence in the collected sludge of low density polymers and MPs without granular sizes; on the other hand, the aggregation, coupled with the potential occlusion of 63 μm mesh sieve during wastewater filtration, can justify the detection of MPs with 0.1–0.01 mm size. The value of 113 ± 57 MPs/g dw (3,400,000,000 MPs/day) observed in the recycled activated sludge is comparable to other European WWTP sludges, with 8.2–301.4 MP/g dw (Leslie et al., 2017), 186.7 MP/g dw (Talvitie et al., 2017a) and 4.2–15.4 MP/g dw (Mahon et al., 2017) in Dutch, Finnish and Irish WWTPs respectively. Worthy to note, 47% of MPs detected in sludge were characterized by MPFs (53.3 ± 48.9 MPFs/g dw). A recent study (Sillanpää and Sainio, 2017) calculated an annual emission of 154,000 polyester MPFs by washing machines with a number of polyester MPFs released in the first wash that varies from 2.1×10^5 to 1.3×10^7. The release of MPs from the washing machines will be one of the main challenges in the early future to decrease fibers in domestic wastes. Since the ban of production and use of synthetic clothes would be utopic, considering the pivotal role of *non*-disposable plastics in our lifestyle, there are already feasible solutions based on the use of filters for MPF retention in the washing machines (Napper and Thompson, 2016), the recourse to the labelling that certify the good practice in the clothes manufacture and the use of laundry soaps, softeners and the washing cycles more conservative. As previously observed in wastewater samples, also the sludge revealed the presence of MPs polymer classes not detected in the IN wastewaters, reinforcing the hypothesis of the possible release of some polymers, as polystyrene, directly by WWTP structures.

On the basis of these results, we observed a *surplus* of 2,560,000,000 MP/day from the final concentration balance: [(160,000,000 MP/day release by final effluent + 3,400,000,000 MP/day deposited in the sludge) − 1,000,000,000 MPs/-day in the inlet]; this difference could be related to the intrinsic variability of wastewaters, to the relatively low volume (30 L) of filtered wastewaters, or to other unaccounted sources like fragmentation of MPs in smaller particles or environmental deposition of MPFs from air.

The sludge is re-used in agriculture in many countries as fertilizer, and detection of MPs poses a potential threat for terrestrial environments. The use of sludge in agriculture is actually banned if they contain high levels of toxic pollutants, as heavy metals, but neither European (EU 86/278/EEC) nor U.S. (Code 503) legislations put limits for MPs (Nizzetto et al., 2016). Considering the adverse effects reported for MPs on earth worms (Huerta Lwanga et al., 2016), degradation from micro- to nanoplastics, and their leaching to groundwater (Hurley and Nizzetto, 2018), results obtained in this study highlight the need of future evaluations of economic and ecological costs of sludge fate. An additional problem associated to release of MPs in the soils could be their re-translocation in freshwater and marine environment, partially nullifying the WWTP activity.

5. Conclusions

Our results highlight that, despite the high MP removal efficiency of selected WWTP of 84%, its contribution to MPP and MPF pollution of

freshwaters is worrisome, in accordance with results of other European WWTPs. MPs were removed from wastewaters probably mainly in the grease and sedimentation processes, but also the advanced final stage treatments with sand filters significantly contributed to MP retention. Unfortunately, MPs were not completely eliminated by the final effluent, considering that 160,000,000 MPs are released daily in freshwaters by selected WWTP, and their route towards the sludge, a matrix often re-used in agriculture in which we calculated a daily deposition of 3,400,000,000 MPs, provides new elements for regulation of the biosolid disposal in the environment. Future studies are necessary to deeper investigate the distribution, removal and release of MPs by WWTPs in the aquatic environment, considering that the links among physical/chemical behavior of these pollutants and efficiency of various treatment steps still remain to be fully elucidated.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.269.

Acknowledgements

This study was supported by AquaLab Foundation and partially financed by Cariplo Foundation (grant number: 2017-2191). We thank also the Centro Assistenza Ecologica (Ancona) who partially funded the Ph.D. fellowship to Lucia Pittura.

References

Almroth, B.M.C., Åström, L., Roslund, S., Petersson, H., Johansson, M., Persson, N.K., 2018. Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment. Environ. Sci. Pollut. Res. 25, 1191–1199.

Anderson, P.J., Warrack, S., Langen, V., Challis, J.K., Hanson, M.L., Rennie, M.D., 2017. Microplastic contamination in Lake Winnipeg, Canada. Environ. Pollut. 225, 223–231.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605.

Autorità per l'energia elettrica, il gas e il sistema idrico, 2017. Relazione annuale sullo stato dei servizi e sull'attività svolta. Volume I Stato dei Servizi, p. 285.

Avio, C.G., Gorbi, S., Regoli, F., 2015a. Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. Mar. Environ. Res. 111, 18–26.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'Errico, G., Pauletto, G., Bargelloni, L., Regoli, F., 2015b. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211–222.

Avio, C.G., Gorbi, S., Regoli, F., 2017. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. Mar. Environ. Res. 128, 2–11.

Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., Guilhermino, L., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquat. Toxicol. 195, 49–57.

Binelli, A., Magni, S., Soave, C., Marazzi, F., Zuccato, E., Castiglioni, S., Parolini, M., Mezzanotte, V., 2014. The biofiltration process by the bivalve *D. Polymorpha* for the removal of some pharmaceuticals and drugs of abuse from civil wastewaters. Ecol. Eng. 71, 710–721.

Binelli, A., Magni, S., Della Torre, C., Parolini, M., 2015. Toxicity decrease in urban wastewaters treated by a new biofiltration process. Sci. Total Environ. 537, 235–242.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environ. Sci. Technol. 42, 5026–5031.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines worldwide: sources and sinks. Environ. Sci. Technol. 45, 9175–9179.

Carbery, M., O'Connor, W., Palanisami, T., 2018. Trophic transfer of microplastics and mixed contaminants in the marine food web and implications for human health. Environ. Int. 115, 400–409.

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res. 91, 174–182.

Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597.

Conkle, J.L., Del Valle, C.D.B., Turner, J.W., 2018. Are we underestimating microplastic contamination in aquatic environments? Environ. Manag. 61, 1–8.

Crawford, C.B., Quinn, B., 2017. Microplastic Pollutants. 1st edition. Elsevier Limited.

Di, M., Wang, J., 2018. Microplastics in surface waters and sediments of the Three Gorges Reservoir, China. Sci. Total Environ. 616–617, 1620–1627.

Dris, R., Gasperi, J., Rocher, V., Tassin, B., 2018. Synthetic and non-synthetic anthropogenic fibers in a river under the impact of Paris Megacity: sampling methodological aspects and flux estimations. Sci. Total Environ. 618, 157–164.

Dyachenko, A., Mitchell, J., Arsem, N., 2017. Extraction and identification of microplastic particles from secondary wastewater treatment plant (WWTP) effluent. Anal. Methods 9, 1412–1418.

Eerkes-Medrano, D., Thompson, R.C., Aldridge, D.C., 2015. Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water Res. 75, 63–82.

Estahbanati, S., Fahrenfeld, N.L., 2016. Influence of wastewater treatment plant discharges on microplastic concentrations in surface water. Chemosphere 162, 277–284.

Faure, F., Corbaz, M., Baecher, H., de Alencastro, L., 2012. Pollution due to plastics and microplastics in Lake Geneva and in the Mediterranean Sea. Arch. Sci. 65, 157–164.

Fischer, E.K., Paglialonga, L., Czech, E., Tamminga, M., 2016. Microplastic pollution in lakes and lake shoreline sediments - a case study on Lake Bolsena and Lake Chiusi (central Italy). Environ. Pollut. 213, 648–657.

Free, C.M., Jensen, O.P., Mason, S.A., Eriksen, M., Williamson, N.J., Boldgiv, B., 2014. High-levels of microplastic pollution in a large, remote, mountain lake. Mar. Pollut. Bull. 85, 156–163.

Guerranti, C., Cannas, S., Scopetani, C., Fastelli, P., Cincinelli, A., Renzi, M., 2017. Plastic litter in aquatic environments of Maremma Regional Park (Tyrrhenian Sea, Italy): contribution by the Ombrone River and levels in marine sediments. Mar. Pollut. Bull. 117, 366–370.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., van der Ploeg, M., Besseling, E., Koelmans, A.A., Geissen, V., 2016. Microplastics in the terrestrial ecosystem: implications for *Lumbricus terrestris* (Oligochaeta, Lumbricidae). Environ. Sci. Technol. 50, 2685–2691.

Hurley, R.R., Nizzetto, L., 2018. Fate and occurrence of micro (nano) plastics in soils: knowledge gaps and possible risks. Curr. Opin. Environ. Sci. Health 1, 6–11.

Imhof, H.K., Ivleva, N.P., Schmid, J., Niessner, R., Laforsch, C., 2013. Contamination of beach sediments of a subalpine lake with microplastic particles. Curr. Biol. 23, R867–R868.

Imhof, H.K., Wiesheu, A.C., Anger, P.M., Niessner, R., Ivleva, N.P., Laforsch, C., 2018. Variation in plastic abundance at different lake beach zones - a case study. Sci. Total Environ. 613–614, 530–537.

Khatmullina, L., Isachenko, I., 2017. Settling velocity of microplastic particles of regular shapes. Mar. Pollut. Bull. 114, 871–880.

Klein, S., Worch, E., Knepper, T.P., 2015. Occurrence and spatial distribution of microplastics in river shore sediments of the Rhine-Main area in Germany. Environ. Sci. Technol. 49, 6070–6076.

Lares, M., Ncibi, M.C., Sillanpää, M., Sillanpää, M., 2018. Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology. Water Res. 133, 236–246.

Lechner, A., Ramler, D., 2015. The discharge of certain amounts of industrial microplastic from a production plant into the River Danube is permitted by the Austrian legislation. Environ. Pollut. 200, 159–160.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619, 1–8.

Leslie, H.A., Brandsma, S.H., Van Velzen, M.J.M., Vethaak, A.D., 2017. Microplastics en route: field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota. Environ. Int. 101, 133–142.

Lusher, A.L., Hernandez-Milian, G., O'Brien, J., Berrow, S., O'Connor, I., Officer, R., 2015. Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: the True's beaked whale *Mesoplodon mirus*. Environ. Pollut. 199, 185–191.

Magni, S., Parolini, M., Soave, C., Marazzi, F., Mezzanotte, V., Binelli, A., 2015. Removal of metallic elements from real wastewater using zebra mussel bio-filtration process. J. Environ. Chem. Eng. 3, 915–921.

Magni, S., Parolini, M., Binelli, A., 2016. Sublethal effects induced by morphine to the freshwater biological model *Dreissena polymorpha*. Environ. Toxicol. 31, 58–67.

Magni, S., Parolini, M., Della Torre, C., de Oliveira, L.F., Catani, M., Guzzinati, R., Cavazzini, A., Binelli, A., 2017. Multi-biomarker investigation to assess toxicity induced by two antidepressants on *Dreissena polymorpha*. Sci. Total Environ. 578, 452–459.

Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C.C., Bonasoro, F., Binelli, A., 2018. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). Sci. Total Environ. 631, 778–788.

Magnusson, K., Norén, F., 2014. Screening of Microplastic Particles in and Down-Stream a Wastewater Treatment Plant Report. Swedish Environmental Research Institute, Stockholm.

Mahon, A.M., O'Connell, B., Healy, M.G., O'Connor, I., Officer, R., Nash, R., Morrison, L., 2017. Microplastics in sewage sludge: effects of treatment. Environ. Sci. Technol. 51, 810–818.

Mason, S.A., Garneau, D., Sutton, R., Chu, Y., Ehmann, K., Barnes, J., Fink, P., Papazissimos, D., Rogers, D.L., 2016. Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent. Environ. Pollut. 218, 1045–1054.

Michielssen, M.R., Michielssen, E.R., Ni, J., Duhaime, M.B., 2016. Fate of microplastics and other small anthropogenic litter (SAL) in wastewater treatment plants depends on unit processes employed. Environ. Sci. Water Res. Technol. 2, 1064–1073.

Mintenig, S.M., Int-Veen, I., Löder, M.G., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. Water Res. 108, 365–372.

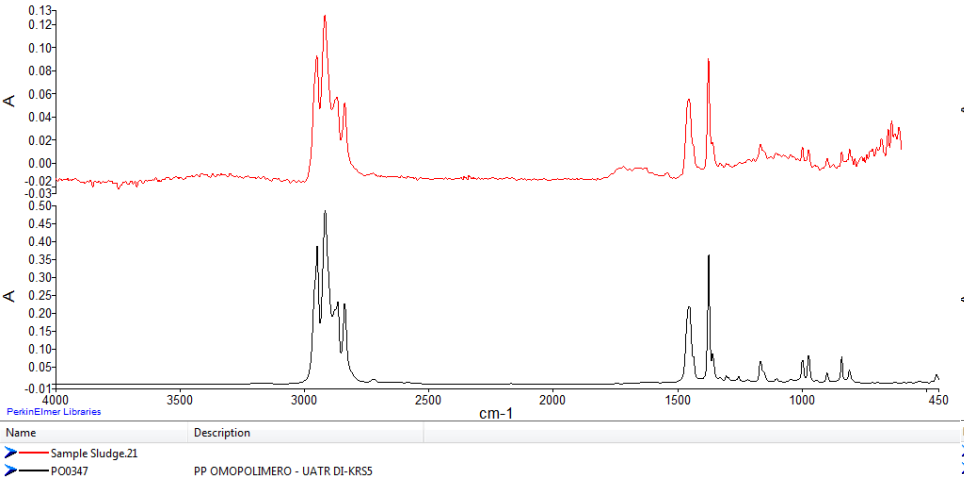
Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater treatment works (WwTW) as a source of microplastics in the aquatic environment. Environ. Sci. Technol. 50, 5800–5808.

Napper, I.E., Thompson, R.C., 2016. Release of synthetic microplastic plastic fibres from domestic washing machines: effects of fabric type and washing conditions. Mar. Pollut. Bull. 112, 39–45.

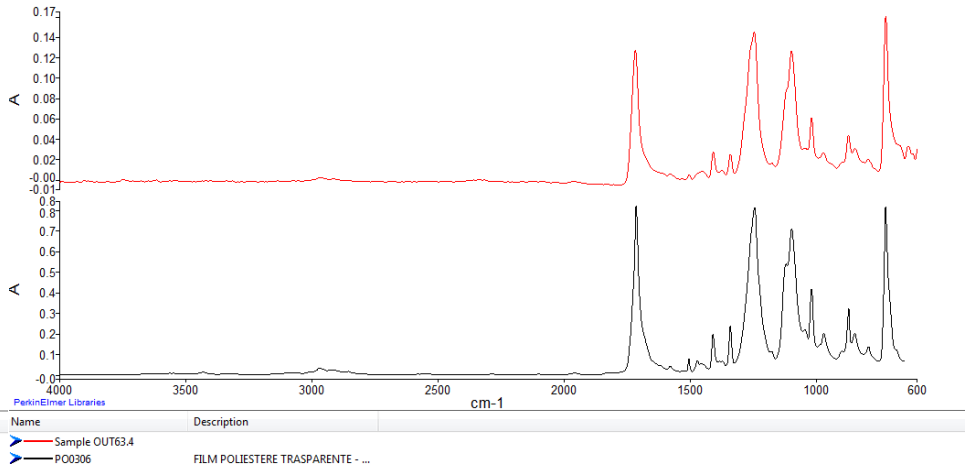
Nel, H.A., Dalu, T., Wasserman, R.J., 2018. Sinks and sources: assessing microplastic abundance in river sediment and deposit feeders in an Austral temperate urban river system. Sci. Total Environ. 612, 950–956.

Nelms, S.E., Galloway, T.S., Godley, B.J., Jarvis, D.S., Lindeque, P.K., 2018. Investigating microplastic trophic transfer in marine top predators. Environ. Pollut. 238, 999–1007.

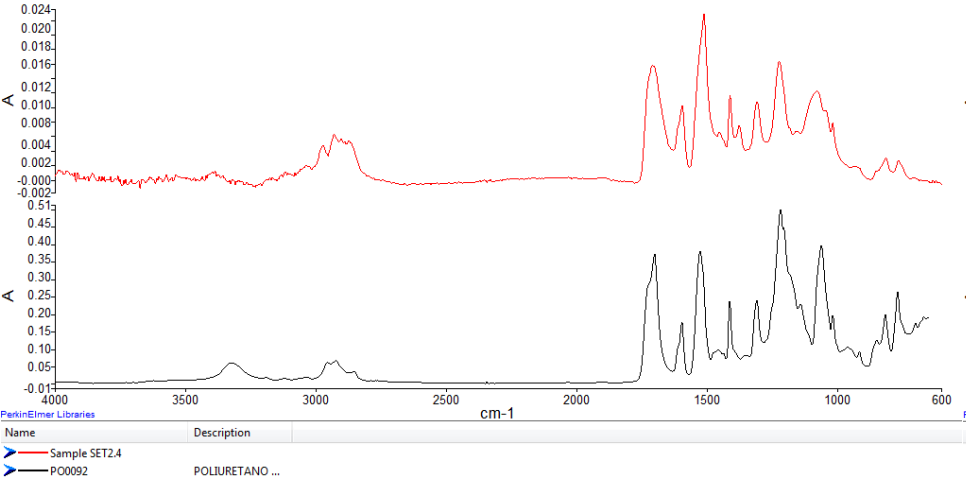
Nizzetto, L., Futter, M., Langaas, S., 2016. Are agricultural soils dumps for microplastics of urban origin? Environ. Sci. Technol. 50, 10777–10779.
Paul-Pont, I., Lacroix, C., González Fernández, C., Hégaret, H., Lambert, C., Le Goïc, N., Frère, L., Cassone, A.L., Sussarellu, R., Fabioux, C., Guyomarch, J., Albentosa, M., Huvet, A., Soudant, P., 2016. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. Environ. Pollut. 216, 724–737.
Pittura, L., Avio, C.G., Giuliani, M.E., D'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. Front. Mar. Sci. 5, 103.
PlasticsEurope, 2017. An Analysis of European Plastics Production, Demand and Waste Data. http://www.plasticseurope.org.
Prata, J.C., 2018. Microplastics in wastewater: state of the knowledge on sources, fate and solutions. Mar. Pollut. Bull. 129, 262–265.
Sadri, S.S., Thompson, R.C., 2014. On the quantity and composition of floating plastic debris entering and leaving the Tamar Estuary, Southwest England. Mar. Pollut. Bull. 81, 55–60.
Sighicelli, M., Pietrelli, L., Lecce, F., Iannilli, V., Falconieri, M., Coscia, L., Di Vito, S., Nuglio, S., Zampetti, G., 2018. Microplastic pollution in the surface waters of Italian Subalpine Lakes. Environ. Pollut. 236, 645–651.
Sillanpää, M., Sainio, P., 2017. Release of polyester and cotton fibers from textiles in machine washings. Environ. Sci. Pollut. Res. 24, 19313–19321.
Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying Focal Plane Array (FPA)-based Fourier Transform Infrared (FT-IR) imaging. Water Res. 142 (1–9).
Su, L., Xue, Y., Li, L., Yang, D., Kolandhasamy, P., Li, D., Shi, H., 2016. Microplastics in Taihu Lake, China. Environ. Pollut. 216, 711–719.
Talvitie, J., Heinonen, M., Pääkkönen, J., Vahtera, E., Mikola, A., Setälä, O., Vahala, R., 2015. Do wastewater treatment plants act as a potential point source of microplastics? Preliminary study in the coastal Gulf of Finland, Baltic Sea. Water Sci. Technol. 72, 1495–1504.
Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., 2017a. How well is microlitter purified from wastewater? - a detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res. 109, 164–172.
Talvitie, J., Mikola, A., Koistinen, A., Setälä, O., 2017b. Solutions to microplastic pollution–removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. Water Res. 123, 401–407.
Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle, D., Russell, A.E., 2004. Lost at sea: where is all the plastic? Science 304, 838.
Wagner, M., Scherer, C., Alvarez-Munõz, D., Brennholt, N., Bourrain, X., Buchinger, S., Fries, E., Grosbois, C., Klasmeier, J., Marti, T., Rodriguez-Mozaz, S., Urbatzka, R., Vethaak, A., Winther-Nielsen, M., Reifferscheid, G., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Environ. Sci. Eur. 26, 12.
Wang, J., Peng, J., Tan, Z., Gao, Y., Zhan, Z., Chen, Q., Cai, L., 2016. Microplastics in the surface sediments from the Beijiang River littoral zone: composition, abundance, surface textures and interaction with heavy metals. Chemosphere 171, 248–258.
Wang, W., Ndungu, A.W., Li, Z., Wang, J., 2017. Microplastics pollution in inland freshwaters of China: a case study in urban surface waters of Wuhan, China. Sci. Total Environ. 575, 1369–1374.
Ziajahromi, S., Neale, P.A., Rintoul, L., Leusch, F.D., 2017. Wastewater treatment plants as a pathway for microplastics: development of a new approach to sample wastewater-based microplastics. Water Res. 112, 93–99.



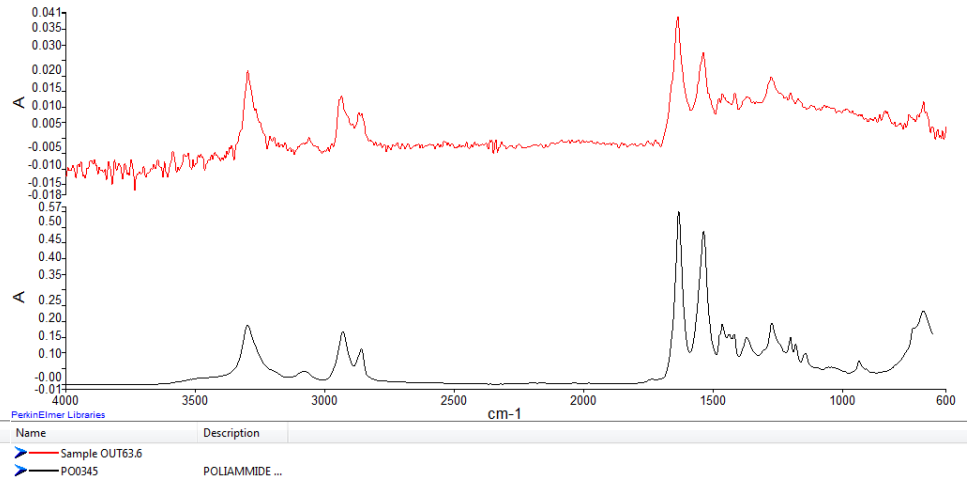
PP



PEST



PU



PA

Figure S1: Some spectra of detected MPs (PP - polypropylene, PEST - polyester, PU - polyurethane, PA - polyamide).

Tratment step	Sampling day	Shape	Size (mm)	Polymer class	Abbreviation	Library search score (*HQI*)
IN	First	film	3.26	acrylonitrile-butadiene	NBR	0.80
		film	1.47	polyethylene	PE	0.90
		film	0.23	polyethylene	PE	0.76
		film	0.13	ethylene-propylene-diene	EPDM	0.73
		film	2.87	polyethylene	PE	0.95
		film	2.81	acrylonitrile-butadiene	NBR	0.83
		film	0.39	ethylene-propylene	EPR	0.88
		fragment	2.23	polyethylene	PE	0.72
		film	3.80	acrylonitrile-butadiene	NBR	0.83
		film	2.34	acrylonitrile-butadiene	NBR	0.73
		fragment	0.11	acrylonitrile-butadiene	NBR	0.87
		fragment	0.29	polypropylene	PP	0.96
		fragment	0.09	styrene-ethylene-butadiene-styrene	SEBS	0.95
		film	1.09	ethylene-propylene-diene	EPDM	0.76
		film	0.46	polyterpene	-	0.93
		film	4.71	ethylene-acrylate	-	0.89
		fragment	2.84	ethylene-propylene-diene	EPDM	0.84
		film	0.45	acrylonitrile-butadiene	NBR	0.84
		film	0.65	polyethylene	PE	0.77
		fragment	2.83	polyethylene	PE	0.79
		film	0.95	acrylonitrile-butadiene	NBR	0.80
		line	4.11	polyester	PEST	0.97
		line	0.29	polyester	PEST	0.92
		line	0.68	polyester	PEST	0.94
		film	0.57	polyethylene	PE	0.75
		film	2.95	ethylene-propylene	EPR	0.80
		line	0.35	polyester	PEST	0.78
		line	0.17	polyester	PEST	0.80
		fragment	0.10	polyethylene	PE	0.90
		film	0.19	acrylonitrile-butadiene	NBR	0.65
		film	0.14	acrylonitrile-butadiene	NBR	0.81
		film	2.88	ethylene-propylene	EPR	0.82
		film	0.31	ethylene-propylene	EPR	0.77
		film	0.22	acrylonitrile-butadiene	NBR	0.86
		film	0.70	ethylene-propylene	EPR	0.75
		film	4.00	acrylonitrile-butadiene	NBR	0.85
		film	1.18	acrylonitrile-butadiene	NBR	0.76
		film	1.38	acrylonitrile-butadiene	NBR	0.85
		fragment	1.60	polyurethane	PU	0.70
		line	1.57	polypropylene	PP	0.96
		line	3.92	polypropylene	PP	0.97
		film	3.48	ethylene-propylene	EPR	0.82
		film	2.21	acrylonitrile-butadiene	NBR	0.84
		film	2.01	ethylene-propylene	EPR	0.74
		fragment	0.26	polyterpene	-	0.72
		fragment	0.56	polypropylene	PP	0.96
		fragment	0.16	ethylene-acrylate	-	0.89
		fragment	1.26	acrylonitrile-butadiene	NBR	0.90
		film	0.21	acrylonitrile-butadiene	NBR	0.76
		line	0.23	polyester	PEST	0.83
		film	0.20	acrylonitrile-butadiene	NBR	0.79
	Second	film	4.27	acrylonitrile-butadiene	NBR	0.79
		film	4.38	acrylonitrile-butadiene	NBR	0.81
		film	4.79	acrylonitrile-butadiene	NBR	0.77
		film	4.91	polyethylene	PE	0.76
		film	1.23	polyethylene	PE	0.83
		fragment	1.09	polyethylene	PE	0.88
		fragment	0.29	polyurethane	PU	0.82
		fragment	0.25	polyurethane	PU	0.79
		film	0.06	polyethylene	PE	0.96
		film	0.08	ethylene-vinylacetate	EVA	0.85
		line	0.42	acrylonitrile-butadiene	NBR	0.82
		film	1.48	acrylonitrile-butadiene	NBR	0.82
		film	1.05	acrylonitrile-butadiene	NBR	0.79
		film	1.35	acrylonitrile-butadiene	NBR	0.81
		film	0.34	polyethylene	PE	0.90
		film	0.11	acrylonitrile-butadiene	NBR	0.78
		film	0.26	acrylonitrile-butadiene	NBR	0.79
		film	0.36	acrylonitrile-butadiene	NBR	0.82
		film	0.55	ethylene-propylene	EPR	0.81
		film	0.32	ethylene-propylene	EPR	0.77
		film	0.08	acrylonitrile-butadiene	NBR	0.83
		film	0.59	ethylene-acrylate	-	0.85
		film	1.59	ethylene-acrylate	-	0.81
		film	0.20	acrylonitrile-butadiene	NBR	0.83
		film	0.37	polyethylene	PE	0.80
		fragment	0.36	polyethylene	PE	0.80
		film	0.51	acrylonitrile-butadiene	NBR	0.82
		film	0.51	acrylonitrile-butadiene	NBR	0.80
		film	0.43	acrylonitrile-butadiene	NBR	0.77
		line	0.28	acrylonitrile-butadiene	NBR	0.72
		film	0.11	styrene-ethylene-butadiene-styrene	SEBS	0.83
		fragment	0.10	acrylonitrile-butadiene	NBR	0.78
		fragment	0.09	styrene-ethylene-butadiene-styrene	SEBS	0.76
		film	0.14	polyester	PEST	0.92
		film	0.03	styrene-ethylene-butadiene-styrene	SEBS	0.76
		fragment	0.08	polyurethane	PU	0.76
		fragment	0.10	polyurethane	PU	0.70

	film	0.07	styrene-butadiene-styrene	SBS	0.81
	film	0.63	ethylene-propylene	EPR	0.86
	film	0.30	polyamide	PA	0.75
	film	0.93	ethylene-acrylate	-	0.91
	film	2.62	polyethylene	PE	0.96
	film	2.83	acrylonitrile-butadiene	NBR	0.78
	film	0.65	ethylene-acrylate	-	0.82
	film	0.58	ethylene-acrylate	-	0.92
	film	1.03	ethylene-acrylate	-	0.77
	film	0.37	acrylonitrile-butadiene	NBR	0.84
	film	0.35	acrylonitrile-butadiene	NBR	0.82
	film	0.10	acrylonitrile-butadiene	NBR	0.79
	film	1.48	ethylene-propylene	EPR	0.83
	film	0.50	acrylonitrile-butadiene	NBR	0.79
	film	0.70	acrylonitrile-butadiene	NBR	0.78
	film	0.39	ethylene-propylene	EPR	0.73
	film	1.23	ethylene-propylene	EPR	0.74
	film	4.16	acrylonitrile-butadiene	NBR	0.79
	film	0.68	polyethylene	PE	0.79
	film	1.17	acrylonitrile-butadiene	NBR	0.80
	line	1.36	polyamide	PA	0.60
	fragment	0.17	acrylonitrile-butadiene	NBR	0.80
	film	0.10	acrylonitrile-butadiene	NBR	0.73
	film	0.09	acrylonitrile-butadiene	NBR	0.81
	film	0.12	polyurethane	PU	0.77
	film	0.39	ethylene-propylene	EPR	0.85
	film	0.16	acrylonitrile-butadiene	NBR	0.77
	film	0.58	ethylene-propylene	EPR	0.85
	film	2.43	ethylene-propylene	EPR	0.81
	film	1.35	acrylonitrile-butadiene	NBR	0.83
	fragment	0.69	acrylonitrile-butadiene	NBR	0.78
	film	0.75	acrylonitrile-butadiene	NBR	0.84
	film	0.98	ethylene-acrylate	-	0.81
	film	0.42	polypropylene	PP	0.96
Third	fragment	0.02	polyethylene	PE	0.42
	fragment	0.06	polyterpene	-	0.78
	film	0.55	styrene-butadiene-styrene	SBS	0.81
	film	1.15	acrylonitrile-butadiene	NBR	0.77
	film	2.48	polyethylene	PE	0.79
	film	1.40	polyethylene	PE	0.79
	film	2.04	acrylonitrile-butadiene	NBR	0.83
	film	2.27	polyethylene	PE	0.90
	film	0.71	polyamide	PA	0.79
	film	0.28	polyamide	PA	0.71
	film	3.66	acrylonitrile-butadiene	NBR	0.71
	film	3.41	acrylonitrile-butadiene	NBR	0.73
	film	5.00	acrylonitrile-butadiene	NBR	0.72
	film	4.47	acrylonitrile-butadiene	NBR	0.70
	film	2.47	ethylene-propylene	EPR	0.72
	film	2.89	ethylene-propylene	EPR	0.73
	film	1.41	acrylonitrile-butadiene	NBR	0.77
	film	0.21	acrylonitrile-butadiene	NBR	0.80
	film	0.24	acrylonitrile-butadiene	NBR	0.82
	film	0.28	acrylonitrile-butadiene	NBR	0.82
	film	0.26	acrylonitrile-butadiene	NBR	0.82
	film	0.14	ethylene-propylene	EPR	0.82
	fragment	0.33	ethylene-propylene	EPR	0.73
	film	3.56	acrylonitrile-butadiene	NBR	0.76
	film	0.71	ethylene-acrylate	-	0.75
	film	1.20	polyethylene	PE	0.94
	film	2.56	ethylene-acrylate	-	0.74
	film	1.32	acrylonitrile-butadiene	NBR	0.72
	film	1.69	acrylonitrile-butadiene	NBR	0.81
	film	0.28	acrylonitrile-butadiene	NBR	0.78
	film	0.85	acrylonitrile-butadiene	NBR	0.79
	film	0.61	ethylene-propylene	EPR	0.74
	film	0.23	ethylene-propylene	EPR	0.82
	film	0.71	ethylene-propylene	EPR	0.79
	fragment	0.08	ethylene-propylene-diene	EPDM	0.77
	film	0.03	ethylene-propylene	EPR	0.75
	fragment	0.16	polyethylene	PE	0.89
	film	0.29	polyethylene	PE	0.70
	fragment	0.28	polyethylene	PE	0.70
	fragment	0.28	acrylonitrile-butadiene	NBR	0.76
	fragment	0.23	acrylonitrile-butadiene	NBR	0.72
	film	0.61	polyethylene	PE	0.89
	film	0.64	acrylonitrile-butadiene	NBR	0.84
	film	0.60	acrylonitrile-butadiene	NBR	0.78
	film	0.89	ethylene-acrylate	-	0.76
	film	0.19	ethylene-acrylate	-	0.74
	film	0.18	acrylonitrile-butadiene	NBR	0.81
	fragment	0.22	polypropylene	PP	0.85
	film	1.37	polyethylene	PE	0.70
	fragment	0.13	ethylene-propylene	EPR	0.79
	film	0.44	acrylonitrile-butadiene	NBR	0.82
	fragment	0.12	polyester	PEST	0.76
	film	0.94	polyethylene	PE	0.89
	fragment	0.39	styrene-ethylene-butadiene-styrene	SEBS	0.76
	fragment	0.08	polypropylene	PP	0.94

		fragment	0.10	styrene-vinyltoluene-butylacrylate	-	0.79
		fragment	0.08	acrylonitrile-butadiene	NBR	0.81
		fragment	0.08	polyethylene	PE	0.70
SET	First	fragment	0.09	polyethylene	PE	0.80
		fragment	0.14	ethylene-propylene-diene	EPDM	0.70
		film	0.12	polypropylene	PP	0.87
		film	1.53	ethylene-acrylate	-	0.75
		line	1.37	polypropylene	PP	0.86
		line	2.27	polytrimethyleneterephthalate	PTT	0.83
		film	0.51	polyamide	PA	0.72
		film	0.98	polyester	PEST	0.85
		line	0.53	polyamide	PA	0.84
		fragment	0.54	polyester	PEST	0.73
		fragment	0.48	ethylene-propylene-diene	EPDM	0.82
		fragment	0.12	acrylonitrile-butadiene	NBR	0.73
		fragment	0.18	acrylonitrile-butadiene	NBR	0.87
		fragment	0.15	acrylonitrile-butadiene	NBR	0.73
		film	0.35	acrylonitrile-butadiene	NBR	0.78
		fragment	0.50	polyuretane	PU	0.80
		fragment	0.21	polyethylene	PE	0.95
		line	0.18	polyester	PEST	0.97
		line	0.36	polyester	PEST	0.95
		film	2.96	ethylene-acrylate	-	0.86
		film	0.50	polypropylene	PP	0.93
	Second	line	0.34	polyester	PEST	0.98
		fragment	0.16	polyurethane	PU	0.83
		film	0.06	polyurethane	PU	0.78
		film	0.08	polyethylene	PE	0.90
		fragment	0.38	polyurethane	PU	0.83
		fragment	0.33	polyethylene	PE	0.97
		film	0.20	polyethylene	PE	0.75
		fragment	0.14	ethylene-acrylate	-	0.70
		fragment	0.33	ethylene-propylene-diene	EPDM	0.86
		fragment	0.12	ethylene-propylene-diene	EPDM	0.70
		line	2.42	ethylene-vinylacetate	EVA	0.71
		line	0.95	polyester	PEST	0.94
		film	0.08	polyester	PEST	0.96
		fragment	0.12	polypropylene	PP	0.95
		film	0.12	polyurethane	PU	0.72
		line	0.34	polyester	PEST	0.93
		film	0.19	ethylene-acrylate	-	0.72
		fragment	0.14	polypropylene	PP	0.98
		line	1.10	polyester	PEST	0.92
		fragment	0.45	polyurethane	PU	0.74
		fragment	0.36	polyurethane	PU	0.80
	Third	line	3.00	polyamide	PA	0.80
		line	2.45	polyamide	PA	0.76
		line	0.25	polyester	PEST	0.91
		film	0.15	polyethylene	PE	0.72
		film	0.24	polypropylene	PP	0.88
		line	0.46	polyester	PEST	0.98
		film	0.26	acrylonitrile-butadiene-styrene	ABS	0.74
		line	1.01	ethylene-propylene-diene	EPDM	0.70
		line	1.24	polyamide	PA	0.99
		film	0.15	polyacrylate	PAK	0.74
		fragment	0.05	acrylonitrile-butadiene	NBR	0.87
		film	0.15	polyethylene	PE	0.94
		film	2.38	polyamide	PA	0.75
OUT	First	film	0.09	polyvinylchloride	PVC	0.88
		film	0.08	ethylene-acrylate	-	0.89
		line	0.68	polyamide	PA	0.88
		film	0.13	styrene-isoprene-styrene	SIS	0.71
		line	0.16	polyester	PEST	0.80
		line	1.10	polyester	PEST	0.96
		fragment	0.09	polyethylene	PE	0.90
		line	4.14	polycyclohexanedimethanolterephthalate	PCT	0.94
		line	1.76	polyester	PEST	0.89
		film	0.18	polybutylmetacrylate	PBMA	0.97
		fragment	0.23	polymethylmethacrylate	PMMA	0.90
	Second	film	0.21	polyoxymethylene	POM	0.97
		line	0.68	polyterpene	-	0.81
		fragment	0.17	polyurethane	PU	0.86
		fragment	0.23	epoxy resin	-	0.84
		fragment	0.06	acrylonitrile-butadiene	NBR	0.70
		fragment	0.14	polyurethane	PU	0.81
		line	0.12	polyester	PEST	0.89
		film	0.12	polyamide	PA	0.83
		line	0.18	polyester	PEST	0.79
		line	0.23	polyamide	PA	0.65
	Third	line	0.18	polyester	PEST	0.85
		film	0.14	polyethylene	PE	0.70
		film	0.07	polyethylene	PE	0.71
		film	0.09	polyamide	PA	0.70
		film	0.07	polyester	PEST	0.98
		line	0.41	polyester	PEST	0.81
		film	0.08	polyamide	PA	0.84
		line	1.44	polyester	PEST	0.83
Sludge	First	film	1.55	acrylonitrile-butadiene	NBR	0.71
		film	1.30	polypropylene	PP	0.83

	film	0.57	polypropylene	PP	0.97
	film	4.40	ethylene-acrylate	-	0.88
	film	0.57	polyethylene	PE	0.75
	film	0.50	polyethylene	PE	0.87
	film	2.19	acrylonitrile-butadiene	NBR	0.79
	film	3.45	polyamide	PA	0.85
	film	1.22	polyethylene	PE	0.93
	fragment	0.30	ethylene-acrylate	-	0.73
	film	0.60	polyester	PEST	0.94
	fragment	0.13	styrene-isoprene	SIR	0.71
	line	0.91	silicone	-	0.78
	fragment	0.22	acrylonitrile-butadiene	NBR	0.77
	fragment	0.14	polyethylene	PE	0.81
Second	line	0.20	polyester	PEST	0.86
	fragment	0.10	polyethylene	PE	0.95
	fragment	0.14	polyester	PEST	0.89
	film	0.14	polytetrafluorethylene	PTFE	0.98
	film	0.08	polyurethane	PU	0.70
	line	0.49	polyester	PEST	0.70
	fragment	0.12	polyethylene	PE	0.96
	film	0.23	acrylonitrile-butadiene	NBR	0.78
	film	0.30	polyethylene	PE	0.87
	fragment	0.08	acrylonitrile-butadiene	NBR	0.83
	fragment	0.04	polypropylene	PP	0.76
	fragment	0.19	acrylonitrile-butadiene	NBR	0.77
	film	0.10	ethylene-propylene-diene	EPDM	0.79
	fragment	0.27	acrylonitrile-butadiene	NBR	0.84
	fragment	0.29	polyester	PEST	0.94
	film	0.68	acrylonitrile-butadiene	NBR	0.70
	film	0.42	acrylonitrile-butadiene	NBR	0.72
	film	0.14	polypropylene	PP	0.88
	fragment	0.07	polystyrene	PS	0.75
	fragment	0.05	polyethylene	PE	0.86
	line	0.26	polyester	PEST	0.95
	fragment	0.08	polystyrene	PS	0.96
	fragment	0.03	polyethylene	PE	0.76
	fragment	0.09	acrylonitrile-butadiene	NBR	0.72
	film	0.03	polystyrene	PS	0.74
	line	0.26	acrylonitrile-butadiene	NBR	0.84
	film	0.15	polyethylene	PE	0.75
	film	0.11	polypropylene	PP	0.80
	film	0.18	ethylene-propylene-diene	EPDM	0.72
	film	0.09	polyacrylate	PAK	0.70
	fragment	0.10	ethylene-propylene-diene	EPDM	0.79
Third	film	0.41	ethylene-acrylate	-	0.85
	film	0.46	acrylonitrile-butadiene	NBR	0.79
	film	0.84	acrylonitrile-butadiene	NBR	0.83
	line	0.47	polyamide	PA	0.90
	film	0.06	polypropylene	PP	0.72
	line	0.11	polyester	PEST	0.88
	fragment	0.08	polyethylene	PE	0.72
	film	0.13	polyester	PEST	0.95
	line	0.43	polyester	PEST	0.85
	fragment	0.26	polyurethane	PU	0.88
	fragment	0.05	polyethylene	PE	0.70
	fragment	0.13	polypropylene	PP	0.95
	film	0.13	polyacrylate	PAK	0.82
	line	0.68	polyester	PEST	0.98
	film	0.47	polyamide	PA	0.70
	line	0.32	polyamide	PA	0.71
	fragment	0.35	acrylonitrile-butadiene	NBR	0.75
	film	0.22	acrylonitrile-butadiene	NBR	0.76
	film	2.40	acrylonitrile-butadiene	NBR	0.78
	film	0.38	acrylonitrile-butadiene	NBR	0.75
	film	0.34	acrylonitrile-butadiene	NBR	0.79

Table S1: Detected MPPs in 30 L (90 L in the three different days of sampling) of wastewater (inlet - IN, after the settler - SET and outlet - OUT) and in 50 mL (150 mL in the three different days of sampling) of sludge.

		Number of detected natural microfibers				Number of detected MPFs		
	Sampling	cotton	viscose	cashmere	flax	polyesters	polyacrylates	polyamide
	First							
IN		16				11	3	2
SET		30	1			11	2	
OUT		9		1		4		
Sludge		3				4		
	Second							
IN		25			2	12	2	
SET		18				4		3
OUT		19				3		
Sludge		10				16		
	Third							
IN		35			2	12		
SET		14				4		
OUT		9				1	1	
Sludge		19				40		

Table S2: Detected microfibers (natural microfibers and MPFs) in 30 L (90 L in the three different days of sampling) of wastewater (inlet - IN, after the settler - SET and outlet - OUT) and in 50 mL (150 mL in the three different days of sampling) of sludge. Natural microfibers of cotton, viscose, cashmere and flax were excluded from the MPF count.