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Ecotoxicological risk of microplastics for marine organisms.

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REFERENCES

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LIST OF ORIGINAL PAPERS

This thesis is partly based on the following publications and manuscripts:

a 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. *Environ. Pollut.* 198, 211-222. Research article

b Avio C.G., Gorbi S., 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. Research article

c Avio C.G., Gorbi S., Regoli F. 2016. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. Submitted to *Marine Environmental Research*. Review

* Avio C.G., Gorbi S., Cardelli L., Regoli F. Presence, composition and distribution of microplastics in marine vertebrates and invertebrates; the case study of Giglio island (Norther Tyrrhenian Sea, Italy). In Preparation. Research article

** Avio C.G., Gorbi S., Berlino M.., Regoli F. Presence, composition and distribution of microplastics in marine vertebrates and invertebrates with commercial interest from the Adriatic Sea. In Preparation. Research article

*** Suaria G., Mineo A., Avio C.G, Lattin G., Magaldi M.G., Belmonte G., Regoli F., Aliani S. The Mediterranean Plastic Soup: accumulation of micro-sized synthetic polymers in Mediterranean surface waters. In preparation. Research article

1. INTRODUCTION

1.1 Plastics and microplastic

Plastic is a generic term for man-made polymers that are most often prepared by polymerization process of monomers from oil or gas. In addition, some polymers can be manufactured from other sources as coal, natural gas, cellulose or latex from trees. During polymerization process various chemicals, are added as additives to provide properties, to improve malleability or resistance to combustion and environmental forces (Sears J.K & Darby J.R 1982). The benefits of plastics, including durability and resistance to degradation, are well known and now several authors call Modern age "the age of Plastics": almost everything is made of plastic, and it is impossible to image the human progress without this material.

Plastic production has increased dramatically worldwide over the last 60 years, passing from 0.5 million tons/yr^{-1} in 1960 to 299 million tons in 2013 (Plastic Europe 2014/2015). At now, Europe ranks second in the global plastics material production (20% of the total; 57 million tons of plastics was produced in 2012), and the European plastic industry give direct employment to over 1.45 million people, generating about 26.3 billion euro for public finance and welfare (from tax and social security costs). Packaging is the largest application sector for the plastic industry and represent 39.6% of the total plastics demand. Building and construction is the second one with 20.3%, followed by automotive (8.5%), electrical and electronic (5.6%), agriculture (4.3%). Other sectors such as appliances, household and consumer products, furniture and medical products, and tools for fishing activity comprise a total of 21.7% of the European plastics demand.

It is possible to classify the plastics in five principal families including: 1) Polyethylene (PE) including low density (PE-LD), linear low density (PE-LLD) and high-density polyethylene (PE-HD); 2) Polypropylene (PP); 3) Polyvinyl chloride (PVC); 4) Polystyrene (PS); 5) Polyethylene terephthalate (PET); 6) Polyamide (Nylon).

Despite the many advantages of plastic, plastic litter can pose a serious threat to the environment. Around 50% of the plastic produced is used in low value products designed for disposable single-use (Hopewell et al., 2009). The chemically inert nature of plastic makes it highly durable, a property that is often desirable. However, at the same time this becomes a challenge when products are not properly disposed or recycled, and end up as litter. The longevity of plastic in field is difficult to predict although the majority of polymers can persist for at least decades. It has been estimated that between 60 and 80% of the word's litter is in form of plastic (Derraik 2002), almost the 10% of the annual plastic production ends up into the oceans, and plastic debris accumulation has been reported as a global scale phenomenon for the marine environments, including polar areas and abyssal regions (Barnes *et al*., 2009).

The main origins of plastic into the oceans derive from maritime activities (commercial fishing and illegal dumping), and land-based sources (river, storm water runoff, wastewater, in land litter blown to the sea and the litter people leave behind on beaches) (Ryan et al., 2009).

In this complex scenario, a great scientific interest is being directed toward microplastics, i.e. fragments with a grain size lower than 5 mm (Barnes et al., 2009) that occur worldwide including Antarctica (Zarfl and Matthies, 2010). These particles are called primary or secondary in relation to their source (Barnes et al., 2009).

Primary microplastics are manufactured ex novo for many consumer products (Maynard, 2006), and are introduced directly into the oceans via runoff. These micron-sized plastic particles are typically used as exfoliants in cosmetic formulations (Figure 1.1) (Gregory, 1996; Fendall and Sewell, 2009), in industrial abrasives and in synthetic 'sandblasting' media (beads of acrylic plastics and polyester), but can also be generated in ship-breaking industry (Reddy and Shaik, 2006).

Figure 1.1. Some personal care products with plastics microbeads. In some states of USA, these kinds of exfoliants were banned.

Secondary microplastics (representing the majority) are generated from degradation and fragmentation of mesoplastics and larger fragments (Gregory and Andrady, 2003). Degradation is a chemical change that drastically reduces the average molecular weight of the polymer and its mechanical integrity. Extensively degraded plastics become brittle enough to fall apart into powdery fragments and microplastics, typically not visible to the naked eye. Degradation of plastics is generally due to different processes like photo and thermal degradation, hydrolysis, mechanical fragmentation and biodegradation mediated by microbial activity (Singh and Sharma 2008). When this process goes onto completion and all the organic carbon in the polymer is converted, it is

referred to as complete mineralization (Andrady, 1994, 1998; Eubeler et al., 2009). For most polymers, a complete conversion of the breakdown products into CO2, water and inorganic molecules is extremely low (Lithner et al,2011) and all these processes in marine environment are extremely slow.

However, compared to beaches where temperature raise 40°C in Summer, the rates of weathering are slower in sea-surface and very slow in marine sediments where lower temperature and fouling effects retard the process dramatically (Andrady 2011).

A great variety of synthetic polymers (i.e. LDPE, HDPE and PP) could absorb solar UV radiation and undergo photolytic, photo-oxidative, and thermo-oxidative reactions that result in the degradation (Gugumus, 1993; Andrady,1997).

Coupled with physical abrasion such as wave action and sand grinding, exposure of plastic to solar UV radiation would result in photodegradation, embrittlement and fragmentation. Once initiated, the degradation can also proceed thermooxidatively for some time without the need for further exposure to UV radiation (Andrady, 2011). As long as oxygen is available to the system, the autocatalytic degradation reaction sequence could proceed. Studies show that other types of degradation processes are orders of magnitude slower compared to light-induced oxidation.

Different polymers have a different fate once released in the ocean (Table 1.1).

Polymer	Specific density g/mL
Water	1
Seawater (average)	1.025
PE	0.93-0.98
PP	0.90-0.91
PVC	1.35-1.45
PS	1.04-1.07
NYLON	1.13-.1.5
PET	1.38-1.39

Table 1.1. Specific density of the major plastic polymers. Source: Polymer dictionary by Teiseisha Co, Ltd 1970

The density of plastic material will determine the buoyancy and position in the water column (Table 1.1), thereby influencing the possibility for interaction with biota (Figure 1.2 Wright et al., 2013b). For example, PVC is denser than seawater and will sink, while PE and PP have lowed density and will float in water column.

Biofouling, the colonization of organisms on the surface of polymers, will also influence their buoyancy by increasing the weight of plastics, thus accelerating their sinking on bottom sediments (Ye & Andrady 1991; Lobelle & Cunliffe 2011).

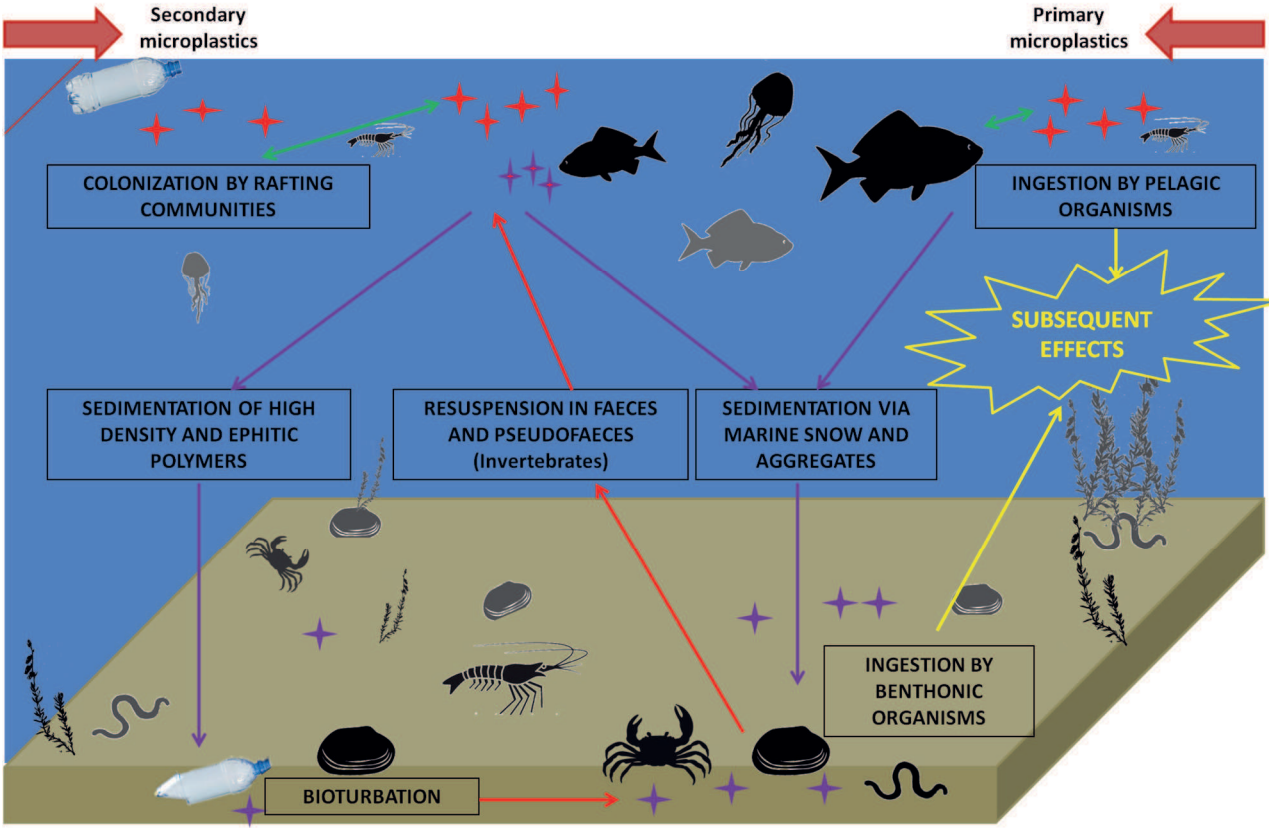


Figure 1.2. Behaviour of microplastics in marine environment, and their relation with biota. Big and soft red arrows represent the input of plastics in marine environment, Red dots represent low densities particles while purple the high densities. Green arrows indicate relation between plastics and organisms that not affect the buoyant properties, while yellow arrows indicate the unset of toxic or harmful effects. Red arrows indicate the resuspension and refloating particles, while purple arrows indicate the sinking particles. Rewied by Wringth et al., 2013b.

The first reports of plastics litter in the oceans go back to early ‘70s, when Carpenter and Smith found macro and microplastics floating in the Sargassum sea (Carpenter et al., 1972; Carpenter and Smith, 1972). At that time, this research drew minimal attention of the scientific community. In the following decades, with accumulation of data on ecological consequences of such debris, the topic received increased research interest (Figure 1.3). Today a significant number of marine species are known to be affected by plastic contamination, with severe consequences such as starvation due to ingestion, entanglement and sub-lethal effects. In 1997 Laist and collaborators counted over 267 species of marine organisms negatively affected by plastic pollution: 43% of all marine mammals, 86% of sea turtles and 44% of all sea bird species; in 2012 the Convention of Biological Diversity reported at least 663 species affected by plastic pollution.

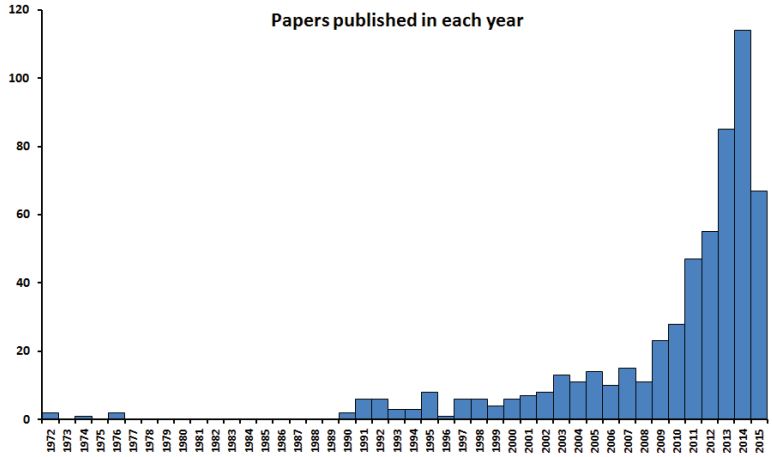


Figure 1.3. Number of papers published for years from ISIweb. Research in the ISI web was conducted using marine debris as Topics keyword.

According to last report from UNEP "Year Book and Valuing Plastic", conservative estimates of the overall economic damage of plastics to marine ecosystems stand at $13 billion each year (Nairobi, 2014). The multiple risks that plastics pose to marine life prompted their inclusion in some international legislation and marine protection projects, like the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA). Also the EU included litter as an emerging form of environmental contamination within The Marine Strategy Framework Directive (MSFD, 2008/56/EC), launched in 2008 with the main goal of achieving Good Environmental Status (GES) of European marine waters by 2020. Among the eleven qualitative descriptors which were defined, marine litter (Descriptor 10), was recognized as an imposrtant typology of marine pollution. All EU member States were required to undertake an initial evaluation on the characteristics of litter in the marine and coastal environment, to monitor trends and dynamics of litter accumulation, as well as potential interactions with marine life (Galgani et al., 2010, 2013).

In this scenario, a better knowledge on the presence and levels of plastics in marine environment, their behaviors and effects on marine organisms (at all level of biological organization) has become a research priority, and a fundamental step toward a more integrated ecological risk assessment.

1.1.1 Distribution, behaviors and occurrence of microplastics in marine environment

Plastic pollution is ubiquitous throughout the marine environment, but estimates of the global abundance and weight of floating plastics are still lacking, particularly for the Southern Hemisphere and more remote regions (Lusher 2015). Some global surveys were conducted in the last 5 years to evaluate the macro and microplastics load in the aquatic environment (Eriksen et al 2014, Cozar et al 2014; Reisser et al., 2013), while only few studies approached their presence in sediments (Van cauwenberghe et al., 2015b) and even less in biota (Lusher et al 2013).

At global scale, several studies identified large-scale convergence zones of plastic debris due to the major ocean currents (Rios et al., 2010). High concentrations of plastics debris were firstly observed in the North Pacific central gyre (Moore et al., 2001) and the term "ocean garbage patches" has been coined (Kaiser 2010; Zhang et al., 2010). Martinez et al. in 2009 described the processes by which debris gets "trapped" in such large-scale oceanographic features using surface circulation estimates in combination with Lagrangian trajectories of debris for the south Pacific subtropical gyre. Law et al. in 2010 defined an accumulation zone in the North Pacific subtropical gyre (25 to 41°N, 130 to 180°W) and estimated a minimum of 21,290 tons of floating plastic debris in this area. At now, a total of 5 ocean gyres (garbage patches) were identified on our planet (North Atlantic, South Atlantic, South Indian, North Pacific and South Pacific), and another patch was predicted in the Barents Sea (Van Sebille et al., 2012).

Indeed ocean gyres are noteworthy areas of debris accumulation, as the rotational pattern of currents cause high concentrations of plastics to be captured and moved toward the center of the region (Karl 1999). Distribution is further influenced by wind mixing, affecting the vertical movements of plastics.

Measurements over time are important in order to know whether the concentrations of microplastics in the ocean are increasing or decreasing. An increasing amount of microplastics is likely to reflect an enhanced amount of plastic litter released into the environment, although, many factors can affect the formation of microplastics.

Temporal data can also be used to measure the abundance of different types of plastic litter (both micro- and macroplastics) over time and to identify main sources, i.e. industrial vs commercial plastic waste, providing useful insights for stakeholders to choose more appropriate actions.

At local scale, Browne et al. in 2010 found spatial patterns of microplastics along an estuarine shoreline (English Channel, UK), where proportionately more microplastics were deposited downwind, in habitats with slow-moving waters. High spatial variability was found in the western

Mediterranean (De Lucia et al., 2014; Collignon et al 2014; Collignon et al., 2012), with higher concentrations of microplastics offshore than coastal, while no distinct spatial pattern was found in a coastal area off South Korea (Song et al., 2014).

Different sediment layers were investigated along the Belgium coast to assess temporal trends of microplastics accumulation in sediments. Different layers of 8 cm which represented a three years period were compared and an increasing trend of microplastics (<1 mm) with time was observed, matching the increasing plastic production worldwide (Claessens et al., 2011).

A similar trend with increasing amounts of macro and microplastic litter is also found in the water phase; plastic debris in the North Pacific Subtropical Gyre in 1999 showed a plastic concentration around 335,000 items km2 (5.1 kg /km2) that was one order of magnitude higher than that measured in the 1980 (Ryan et al., 2009; Moore et al., 2001). The same results come from a long-term study carried out by Thompson and collaborators (2004) in which microplastics were counted from old plankton trawls (samples taken between Aberdeen and Shetland from the 1960s and up to 2000). Results showed microplastics presence in samples from all years and demonstrated an increase by time. On the other hand, in another long-term study (North Atlantic Subtropical Gyre) particles of around 1 mm in size were found in 60% of all plankton net trawls between 1986 and 2008, but no increasing plastic concentration was then seen over time (Law et al., 2010).

Very few long-term surveillance studies have been reported on the occurrence of microplastic, which is a relatively new area within marine research. In addition, a lack of standardization in sampling methodologies makes difficult to compare data between studies; beside different sampling methods also different units of measurement are used, as well as different definition of microplastics. Another factor to consider when interpreting results of microplastic analyses in the water phase, is the hydrophobic nature of polymers that results in an extreme heterogeneous distribution in water column, further complicating comparison of water sample from different times and areas (Ryan et al., 2009).

Despite these technical aspects, last monitoring studies on microplastic distribution and abundance in the oceans revealed the presence microplastics in water column (Lattin et al., 2004), surface waters (Cozar et al., 2014), coastal water (Ng and Oppard 2006) estuaries (Browne et al., 2010), rivers (Sadri and Thompson 2014), beaches (Browne et al 2011), and deep-sea sediments (Van Cauwemberghe et al., 2013b, Woodall et al., 2014; Fischer 2015).

Many studies to date were focused on floating plastics and therefore based on sampling the surface layer and neuston (Collignon et al 2012), up to a few meter depth (Lattin et al., 2004). Concentration of microplastics in water samples ranged from < 1particle/m^3 to several hundreds/m^3 (Table 1.2), but measurements were often inconsistent in terms of sampling methods, units

measured, and further influenced by the sea wheatear condition at the sampling time (Collignon et al., 2014, Lusher et al., 2015, Kukulka et al., 2012). In addition, microplastics can become negatively buoyant in water and sink over time, or alternatively start sinking upon microbial colonization and increase of particles density (Wang et al., 2015).

Location	Average concentration	Average weight (mg/m2)	reference
Northwest Atlantic (coastal)	3 items/m^3		Carpenter et al 1972
Northwest Atlantic (offshore)	67 items/m^3		Colton et al 1974
Northeast Atlantic (Celtic sea)	2.46+-2.43 items/m^3		Lusher et al 2014
Northeast Atlantic (Portuguese coast)	0.002-0.036items/m^3 102000 items/m^3		Frias et al 2014 Noren and Naustvoll 2010
Western Mediteranenan (corsica)	0.116 items/m^2	0.202	Collignon et al 2014
Western Mediteranenan (Sardinia)	0.062 items/m^2		Collignon et al 2012
Western Mediteranenan **Western Mediteranenan (Central)**	130 items/m^2 0.15 items/m^3	58	Faure et al 2013 De Lucia et al 2014
Northeast Pacific (south California)	8 items/m^3		Moore et al 2002
North Pacific (central gyre)	334.3items/m^2	5114	Moore et al 2001
Northeast Pacific	0.004-0.19 items/m^3	0.014-0.209	Doyle et al 2011
North Pacific subtropical gyre **North Pacific subtropical gyre**	0.021-0.448items /m^2 >1000 items/m^2		Goldstein and Goodwin 2013 Law et al 2014
South Pacific subtropical gyre	0.0054 items/m^3		Eriksen et al 2013
Australian coast	0.00085 items/m^3		Reisser et al 2013
East China Sea	0.167+-0.138 items/m^3		Moore et al 2002
Yangtze estuary	4137.3+2461.5 items/m^3		Zhao et al 2014
South Korea coast	13+-11items/m^2		Song et al 2014
Lake Hovgol (Mongolia)	20.26 items/m^2		Free et al 2014

Table 1.2. Comparison of pelagic microplastics measurements in the world's seas.

A recent estimate suggested that more than 250000 tons of plastic are currently floating in the oceans (Eriksen et al., 2014). These data came from 24 expeditions (2007–2013) across all five sub-tropical gyres; costal Australia, Bay of Bengal and the Mediterranean Sea during which surface net tows and visual survey transects of large plastic debris were conducted. Based on the total

number of plastic particles and their weight, researchers calibrated these data, by an oceanographic model of floating debris dispersal corrected for wind-driven vertical mixing, and estimated a minimum of 5.25 trillion particles weighing 268,940 tons.

A recent cruise reported the first quantification of floating plastics in the Mediterranean Sea (Cozar et al., 2015). The average amount of plastics (0.25 item/m^2), and the frequency of occurrence (100% of the sites sampled), were comparable to the accumulation zones described for the five subtropical ocean gyres. Plastic debris in the Mediterranean surface waters was dominated by millimeter-sized fragments, but showed a higher proportion of large plastic objects than that present in oceanic gyres, reflecting the closer connection with pollution sources. The accumulation of floating plastic in the Mediterranean Sea (between 1,000 and 3,000 tons) is likely related to the high human pressure together with the peculiar hydrodynamic features of this semi-enclosed basin, where outflow mainly occurs through a deep-water layer. Given the biological richness and concentration of economic activities in the Mediterranean Sea, the effects of plastic pollution on marine and human life are expected to be particularly frequent in this accumulation region.

In addition to plastic particles in pelagic habitats, the non-floating debris accumulates on the seafloor and in beach sediments, posing risk to the respective communities. In the last two decades, the deposition rate accelerated passing the rate of production, and plastics are now the most common and persistent pollutants in oceans waters and beaches wordwide. The sources of plastic accumulating on the seafloor are variable, depending upon interaction between distance from shore (Galgani et al., 1995), oceanographic and hydrographic processes (Galgani et al., 1996) and human activities such as comercial shipping and leisure craft (Bergmann and Klages 2012). Recently it has been estimated that around the 50% of plastics from municipal waste have higher density than seawater, and that it will readily sink to the seafloor (Engler 2012).

As mentioned above, the spatial distribution and accumulation of plastic litter in the ocean is influenced by hydrography, geomorpholgical factors (Galgani et al., 2000), prevailing winds and anthropogenic activities (Ramirez-Llodra et al., 2013). Indeed there are many transport mechanisms of microplastics from the shallow water to the deep sea. Colonization by organisms, adherence to phytoplankton and the aggregation with organic debris and small particles in the form of marine snow will eventually enhance settling (see Figure 1.2). In addition, a number of oceanographic processes could facilitate the transfer of microplastics to depth, including dense shelf water cascading (Canals et al., 2006), severe coastal storms (Sanchez-Vidal et al., 2012), offshore convection (Stabholz et al., 2013) and saline subduction (Talley LD., 2002). All these processes, induce vertical and horizontal transfers of large volumes of particle loaded waters, including a full spectrum of grain sizes (from sand to clay), as well as litter and contaminants, from shallow ocean

layers and coastal regions to deeper ones (Galgani et al., 1995, 1996), with submarine canyons acting as preferential conduits (Canals et al., 2013). Submarine topographic features may further enhance downwelling flows and increase the retention of microplastics at particular locations such as Taylor columns over seamounts (White et al., 2007). Microplastic fragments, more than larger items, are also likely to be influenced by advection and circulation patterns at all ocean levels. Hence, ocean dynamics can explain the accumulation of plastics in the deep sea, suggesting that marine sediments can be an important sink of these materials (Woodall et al., 2014, Van Cauwenberghe et al 2013b).

Hotspots of litter accumulation include shores close to populated areas, particularly beaches (Cocran et al., 2009) but also submarine canyons where litter originating from land accumulates in large quantities (Mordecai et al., 2011). In Europe, the abundance and distribution of litter on the coastline was shown to be influenced mostly by storm events (Van cauwenberghe et al., 2013a; Martin and Sobral 2011).

Until 2012, about 44 studies determined the density of microplastics in sedimentary environments, and in sandy beaches. Altough sampled tidal zone varied among studies (Hidalgo-Ruz et al., 2012), the higest amounts of macrolitter were found in the upper beach zone (Velander and Mocogni 1999). A rewiev of levels of microplastics measured in different beaches is given in Table 1.3 .

Location (Continent)	Particles size	Measured abundance	Reference
Canary islands (Africa)	1 -5 mm	<1->100g/L	Baztan et al., 2014
Hawaii (America)	1 -5 mm	2.08-725.22 items/L	McDermin and McMuller 2004
	250µm-10mm	0.12-3.3% plastic by weight	Carson et al 2011
Brazil (America)	2 -5 mm	60 items/m2	Ivar do Sul et al., 2009
	0.5-1mm	200items/0.01m^2	Costa et al., 2010
	1 -20 mm	100Items/0.01m^2	Costa et al., 2010
Chile (America)	1 - 4.75mm	<1 -850 items/m^2	Hidalgo-Ruz and Thiel 2013
Nova Scotia (America)	0.8 -5 mm	20-80 fiber /10g	Mathalon and Hill 2014
Singapore (Asia)	1.6µm- 5mm	0-4 items/250g dry	Ng and Oppard 2006
India (Asia)	1 - 5mm	10/180 items/m^2	Jayasiri et al., 2013
South korea (Asia)	1-5 mm	8205items/m^2	Lee et al., 2013
	1-5 mm	27606 items/m^2	
	50µm-5 mm	56-285637 items/m^2	Kim et al., 2015
UK (Europe)	1.6µm - 5mm	0,4 fibres/50ml	Thompson et al.,2004
	1.6µm - 1mm	<1-8items/50ml	Browne et al., 2010
English beach in UK (Europe)	38µm-1mm	0.4-1 fibres/50ml	Browne et al., 2011
North sea beach in UK	38µm-1mm	0.2-0.8 fibres/50ml	Browne et al., 2011

(Europe)			
Belgium (Europe)	38µm-1mm	92.8items/kr dry	Claessens et al., 2011
Portugal (Europe)	1.2µm - 5mm	133.3items/m^2	Martin and Sobral 2011
Germany (Europe)	<1mm	1.3-2.3 items/kg dry	Dekiff et al., 2014
Germany Urban beach	1-15 mm	5000/7000 items/m^3	Ballent et al., 2012
Germany rural beach	1-15mm	150-700 items/m^3	Ballent et al., 2012
Greece (Europe)	1-4 mm	67- 1177 items/m^2	Kaberi et al., 2013
Slovenia (Europe)	0.25-5 mm	177.8items/kg dry	Laglbauer et al., 2014

Table 1.3. Microplastics occurrence in Beaches worldwide (modified from Luscher, 2015).

With the increase of sea floor explorations, benthic litter is progressively being revealed to be more widespread than previously assumed (Galgani et al., 2000; Ramirez-Llodra et al., 2013 Mordecai et al., 2011; Anastasopoulou et al., 2013).

Van Cauwenberghe and collaborators in 2013b found plastic particles sized in the micrometer range in deep-sea sediments collected at four locations (three site in Atlantic and one in Mediterranean) between -1100 to -5000 m. In 2014 Woodall et al. confirmed that deep-sea sediments are a likely sink for microplastics. Microplastics, in the form of fibers, were up to four orders of magnitude more abundant (per unit volume) in deep-sea sediments from the Atlantic Ocean, Mediterranean Sea and Indian Ocean than in contaminated sea-surface waters, showing evidences for a large and hitherto unknown repository of microplastics. Also sub marine canyons appear an important sink zone as reported by several authors (Moredecai et al., 2011; Van Cauwemberghe et al., 2013b; Tubau et al., 2015) (Table 1.4).

Location (Continent)	**Particles size**	**Measured abundance**	**Reference**
Florida (America)	250µm - 4 mm	116-25 items/L	Graham and Thompson 2009
Maine (America)	250µm - 4 mm	105 items/L	Graham and Thompson 2009
NW Pacific (Asia)	300µm - 5mm	60-2020 items/m^2	Fischer et al., 2015
UK (Europe)	1.6µm - 5mm	5.6 fibre/50ml	Thompson et al., 2004
Sweden (Europe)	2µm - 5mm	2-32 items/100ml	Noren 2007
Belgium Harbour (Europe)	38µm - 1 mm	166.7 items/kg dry	Claessens et al., 2011
Belgium continental shelf (Europe)	38µm - 1 mm	97.2 items/kg dry	Claessens et al., 2011
Italy (Europe)	0.7µm-1mm	672-2175 items/kg dry	Vianello et al., 2013
Slvenia (Europe)	5µm-1mm	0.5items/cm^2	Van Cauwemberghe et al., 2013
European deep sediment	2-3 mm	13.4/50ml	Woodall et al., 2014

Table 1.4. Microplastics occurrence in marine sediment worldwide. Data consider studies from the subtidal zone to deep sea sediments

The distribution of litter in European seas, from the shelves to deep basins indicated that litter density in submarine canyons was significantly higher than in all other physiographic settings (Pham et al., 2014); litter density on seamounts, mounds and banks was similar to the densities

found on the continental slopes, while a lower level was found for continental shelves and ocean ridges. For Mediterranean sites, no significant differences were observed in litter density between the three different physiographic settings, but in deep basins it was slightly higher compared to continental slopes and submarine canyons (Figure 1.4).

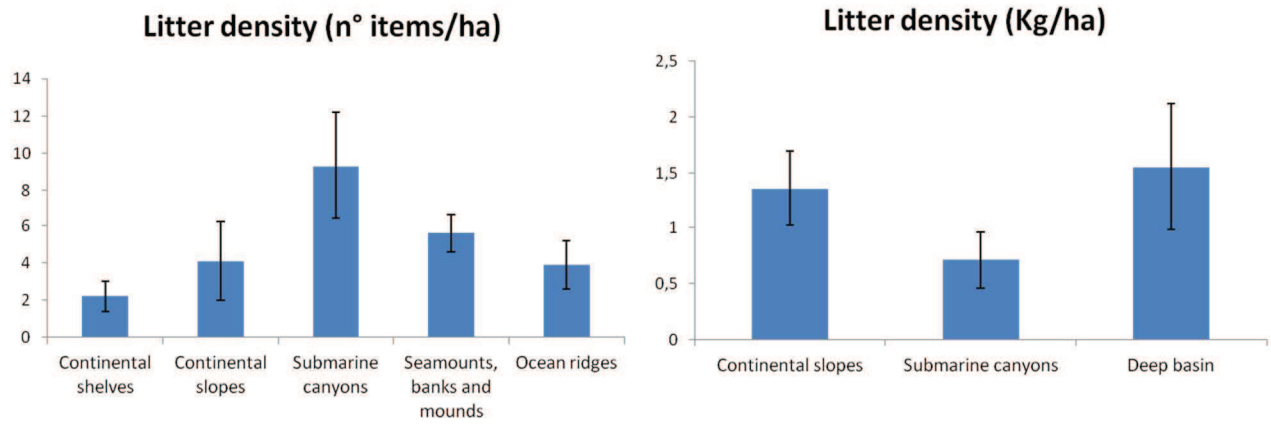


Figure 1.4. Mean litter density (±S.E). On the left histogram show number of items/ha and on the right histogram show Kg of litter/ha across different physiographic setting in European waters (from Pham et al., 2014).

Until last years (2013/2014), there had been no direct study on microplastics in either the Arctic or Antarctica areas. In 2010 Zarfl and Matthies, calculating the plastic flux into the Arctic Ocean concluded that plastic transport levels to the Arctic should be negligible and that plastics are not a likely vector for organic pollutants to the Arctic. However, Obbard et al. (2014) analyzing ice cores collected from remote locations in the Arctic Ocean, observed levels of microplastics (range: 38–234 particles/m3) two orders of magnitude greater than those previously reported in the Pacific gyre (Goldstein et al. 2012). Macroplastics have been identified floating in surface waters of Antarctica, but trawls for micro- plastics did not catch any particle (Barnes et al. 2010). Dietary studies of birds from the Canadian Arctic have reported ingested plastics (Mallory 2006; Provencher et al., 2010), and macroplastics were observed on the deep Arctic seafloor (Bergmann and Klages 2012). This indirect evidence suggests that microplastics have already entered Polar Regions. A modeling study even suggests the presence or formation of a sixth garbage patch in the Barents Sea (van Sebille et al., 2012). All these evidences support the hypotesis that polar sea ice can represent a global sink of man-made particulates including plastics. In this respect, the potential for substantial quantities of legacy microplastic contamination to be released to the ocean as the ice melts therefore needs to be evaluated, as well as the toxicological effects of plastics on marine life (Obbard et al., 2014).

It is now crucial to establish consistent methodologies to allow robust temporal and spatial comparisons, to address how abundance and composition vary with depth, location, topography and

habitat, and apply these data to the already complex oceanographic transport models available for some oceans, which have successfully been used to predict surface plastic accumulation (Law et al., 2010). In addition, the elucidation of the physical and toxicological effects of microplastics is also required.

1.1.2. Occurrence of microplastics in marine organisms

In 2012, negative interactions between debris and marine organisms have been reported for 663 different species (CBD, 2012), representing a 40% increase since the last review (Laist, 1997). Entanglement in and ingestion by large organisms (Table 1.5) can be fatal but can also have a range of sub lethal consequences, compromising the ability to capture and digest food, sense of hunger, escape from predators, decreasing body condition and compromising locomotion, including migration. Reports showed that marine mammals, seabirds, turtles, fishes are the most affected species (Laist 1997; Derraik 2002; Allsopp et al., 2006). About 15% of these were included in the IUCN Red list assuming particular concern (CBD, 2012) and for certain species almost all individuals contain plastic; for example 96% of north sea fulmars have been observed to contain at least one piece of plastic in the stomach. Surely, it can be expected that even more species are currently affected by plastic waste since plastic production has since then increased. The knowledge on the presence of plastics in small fish and invertebrates is still limited due to the difficulty to extract smaller particles (Cole et al., 2014). The next table (Table 1.5-1.6), shows the occurrence of plastics in marine organisms. Despite the importance of such data, it is important to highlight that they 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.

Taxonomic group	Total number of know species	Number of species with entanglement records	Number of species with ingestion records
Marine Mammal	115	52 (45%)	30 (26%)
Seabirds	312	67 (21%)	119 (38%)
Sea turtles	7	7 (100%)	6 (86%)

Table 1.5. Number of species (belonging to large marine organisms) with records of entanglement and ingestion documented in CBD 2012, the number reported here and the total number of species identified worldwide. The percentage of the total number of known species that are affected by entanglement and ingestion is given in brackets. Sources CBD (2012).

Ingestion is the most likely interaction with microplastics for many organisms in benthic and pelagic ecosystems. In some species, feeding mechanisms do not allow to discriminate between preys and anthropogenic items (Moore et al., 2001), and organisms may do feed, partially or totally, on microplastics, instead of typical diet (Moore 2008). If there is a predominance of microplastic particles associated with planktonic prey items, organisms could be unable to differentiate or

prevent ingestion. A number of studies have reported microplastics from the stomachs and intestines of marine organisms, including fish, invertebrates, birds, fish, turtles and mammals (Table 1.6). Watts et al. (2014) showed that shore crabs (*Carcinus maenas*) will not only ingest microplastics along with food (evidence in the foregut), but also contain plastics into the gill cavity due to ventilation mechanisms (Browne et al., 2008).

Although one third of examined gooseneck barnacle *(Lepas* spp.) contained microplastics in stomachs, no adverse effect was reported for these filter feeders (Goldstein and Goodwin 2013). Interestingly, the stomachs of mass stranded Humboldt squids (*Dosidicus gigas*) contained plastic pellets (Braid et al., 2012). This large predatory cephalopod usually feeds at depth between 200 and 700 m. The route of uptake is unclear; the squid may have fed directly on sunken pellets, or on organisms with pellets in their digestive system.

Studies on microplastic ingestion by benthic invertebrates in the field are less common than laboratory studies. Murray and Cowie (2011) identified fibers of monofilament plastics that could derive from fibers of trawls and fragments of plastic bags in the intestines of the commercially valuable Norway lobster (*Nephrops norvegicus*). These results indicated that normal digestive processes do not eliminate the filaments as they cannot pass through the gastric system. Norway lobsters have various feeding modes, including scavenging and predation, and are not adapted to cut flexible filamentous materials (Murray and Cowie 2011). The identification of microplastics in organisms that are caught for commercial purposes and subsequently consumed whole (including guts) highlights potential implications for human health. Field-caught brown shrimps (*Crangon crangon*) (Lusher, 2015) and farmed bivalves (De Witte et al., 2014; Van Cauwenberghe and Janssen 2014) had microplastics in their digestive system. Invertebrates could thus be used as indicator species for environmental contamination by microplastics. Additional studies are required to understand the flux of microplastic within benthic sediments, the interactions between different species of benthic infauna feeding in/or manipulating the sediment, such as bivalves and worms; benthic infauna could ingest and/or excrete microplastics, and the individuals or their fecal pellets may in turn be ingested by secondary consumers, thus affecting higher trophic levels.

Some of the earliest studies noting ingestion of microplastics by wild-caught fish include coastal species from the USA (Carpenter et al. 1972) and the U.K. (Kartar et al. 1973, 1976). More recent studies from the North Pacific Central Gyre reported microplastic (fibres, fragments and films) in mesopelagic fish (Boerger et al. 2010; Davison and Asch 2011; Choy and Drazen 2013). Estuarine environments and their inhabitants are also prone to plastic contamination, which is hardly surprising given the riverine input (e.g. Morritt et al., 2014). Affected estuarine fish, include catfish, *Ariid*ae, (23% of individuals examined) and estuarine drums, *Scianenidae*, (7.9% of

individuals examined), which spend their entire life cycle in estuaries (Possatto et al., 2011; Dantas et al., 2012). Similarly, 13.4% of bottom-feeding fish (Gerreidae) from a tropical estuary in northeast Brazil contained microplastics in their stomachs (Ramos et al., 2012). The authors suggested that ingestion occurred during suction feeding on biofilm.

Lusher et al. (2013) reported microplastic polymers from 10 fish species from the English Channel. Among the 504 examined fish, 37 % had ingested a variety of microplastics, the most common being polyamide and the semi-synthetic material rayon. Similarly, Boerger et al. (2010) recorded microplastics in 35% planktivorous fish examined from the NPCG (94 % of which were plastic fragments). Fish from the northern North Sea ingested microplastics at significantly lower levels (1.2%) compared to those from the southern North Sea (5.4 %) (Foekema et al., 2013).

Romeo and collaborators in 2015 analyzing the stomach content of three different species of large pelagic organisms (*Xiphias gladius, Thunnus thynnus, Thunnus alalunga*) found microplastics in the 18,5% of collected organisms, the first evidence for Mediterranean top predators.

All the cited studies suggest direct ingestion as the prime route of exposure, either targeted as food or mistaken for prey items. Dos Santos and Jobling (1992) showed that microplastic beads (2 mm) were excreted quickly, by Atlantic cod, following ingestion, whereas larger beads (5 mm) were maintained for prolonged periods, suggesting that larger items of plastic might pose a greater risk following ingestion.

Absorption and ingestion of microplastics by organisms from the primary trophic level, e.g. phytoplankton and zooplankton, could be a pathway for transfer into the food chain (Bhattacharya et al. 2010). Many species of zooplankton undergo diurnal migrations, possibly acting as vectors of microplastics to greater depths and its inhabitants, either through predation or through the production of fecal pellets sinking to the seafloor (Wright et al. 2013b). Only a few studies deal with the potential for microplastics to be transferred between trophic levels following ingestion. Field observations highlighted the presence of microplastics in the scat of fur seals (*Arctocephalu*s spp.) and Eriksson and Burton (2003) suggested that microplastics had initially been ingested by the fur seals' prey, the plankton feeding Mycophiids. In feeding experiments, Farrell and Nelson (2013) identified in the gut and haemolymph of the shore crab (*Carcinus maenas*) microplastic which had previously been ingested by blue mussels (*Mytilus edulis*). There was large variability in the number of microspheres in tissues samples, and the results have to be treated with caution as the number of individuals was low and the exposure levels exceeded those from the field. Similarly, Nephrops-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), again implying potential trophic transfer. As mentioned above, microplastics were also detected in several organisms and in

particular invertebrates, which are consumed in toto by humans, raising concerns for trophic transfer to humans and human exposure (Galloway 2015).

Species	Numbers studied	Percentage with plastic (%)	Mean items/ individual (±SD)	Type and size ingested (mm)	Location	References
Phylum Mollusca						
Humbolt squid	30	26.7	Max=11	Nurdles 3-5 mm	Canada	Braid etal 2012
Blue mussels	45	/	3.7 per 10g mussels	Fibres 300-1000µm	Netherland	De Witte et al., 2014
Mytilus edulis	36	/	0.36± 0.07/g	5-25µm	Germany	Van Cauwemberghe and janssen 2014
Mytilus edulis	/	/	0.2±0.3	20-90µm	Belgium	Van Cauwemberghe et al., 2015
Crassostrea gigas	11	/	0.47±0.16/ g	5-2µm	Atlantic Oceans	Van Cauwemberghe and janssen 2014
Phylum Crustacea						
Lepas spp.	385	33.5	1-30	1.41	North Pacific	Goldstein and Goodwin 2013
Nephrops norvegicus	120	83	/	/	Clyde Sea	Murray and Cowie 2011
Crangon crangon	110	/	11.5 fibres/10g	95% fibres 5% films 300-1000µm	Belgium	Devriese et al 2014
Phylum Chaetognata						
Parasagitta elegans	1	100	/	0.1-3mm PS	USA	Carpenter et al 1972
Phhylum Anelida						
Arenicola martina	/	/	1.2±2.8	15-100µm	Belgium	Van Cauwemberghe et al., 2015
Phylum Arthopoda						
Euphasia pacifica	413	/	/	816 ± 108 µm	Northeast Pacific	Desforges et al., 2015
Neocalanus cristatus	960	/	/	556 ± 149 µm	Northeast Pacific	Desforges et al., 2015
Phylum Actinopterigii						
Menidia menidia	9	33	/	0.1-3 mm PS	USA	Carpenter et al 1972

Alepisaurus ferox	144	24	2.7±2	68.3 ± 91.1	North Pacific	Choy and Drazen 2013
Alepisaurus ferox	192	24,5	/	3.1-723.9	North Pacific	Jesley et al., 2013
Cololabis saira	52	35*	3.2±3.05	1-2.79	North Pacific	Boerger et al 2010
Clupea harengus	2	100	1	0.1-3 mm PS	USA	Carpenter et al 1972
Clupea harengus	566	2	1-4	0.5-3	North Sea	Foekema et al., 2013
Stolephorus commersonnii	16	37.5	/	1.14-2.5	India	Kripa et al.,2014
Pollachius virens	1	100	1	0.1-3 mm PS	USA	Carpenter et al 1972
Ciliata mustela	113	0-10	/	1mm PS	UK	Kartare 1976
Merlangius merlangus)	105	6	1-3	1.7 ±1.5	North Sea	Foekema et al., 2013
Merlangius merlangus	50	32	1.75 ±1.4	2.2 ±2.3	English channel	Lusher et al., 2013
Melanogrammus aeglefinus	97	6	1	0.7± 0.3	North Sea	Foekema et al., 2013
Gadus morhua	80	13	1-2	1.2±1.2	North Sea	Foekema et al., 2013
Micromesistius poutassou	27	51.9	2.07±0.9	2±2.4	English channel	Lusher et al., 2013
Trisopterus minutus	50	40	1.95 ±1.2	2.2±2.2	English channel	Lusher et al., 2013
Lampris sp. (big eye)	115	29	2.3±1.6	49.1±71.1	North Pacific	Choy and Drazen 2013
Lampris sp. (small eye)	24	5	5.8±3.9	48.8±34.5	North Pacific	Choy and Drazen 2013
Hygophum reinhardtii	45	35*	1.3±0.71	1-2.79	North Pacific	Boerger et al 2010
Loweina interrupta	28	35*	1	1-2.79	North Pacific	Boerger et al 2010
Myctophum aurolaternatum	460	35*	6±8.9	1-2.79	North Pacific	Boerger et al 2010
Symbolophorus californiensis	78	35*	7.2±8.39	1-2.79	North Pacific	Boerger et al 2010
Diaphus anderseni)	13	15.4	1	/	North Pacific	Davidson and Asch 2011
Diaphus fulgens	7	28.6	1	/	North Pacific	Davidson and Asch 2011
Diaphus phillipsi	1	100	1	Longest 0.5	North Pacific	Davidson and Asch 2011
Lobianchia gemellarii	3	33.3	1	/	North Pacific	Davidson and Asch 2011
Myctophum nitidulum	25	16	1.5	Longest 5.46	North Pacific	Davidson and Asch 2011

(Morone americana	12	33	/	0.1-3 mm PS	USA	Carpenter et al 1972
Tautogolabrus adspersus	6	<83	/	0.1-3 mm PS	USA	Carpenter et al 1972
Pomatoschistus minutus	200	0-25	/	1 mm PS	Estuary, UK	Kartar et al 1976
Stellifer brasiliensis	330	9.2	0.33-0.83	<1	Brazil	Dantas et al., 2012
Stellifer stellifer	239	6.9	0.33-0.83	<1	Brazil	Dantas et al., 2012
Eugerres brasilianus	240	16.3	1-5	1-5	Brazil	Ramos et al 2012
Eucinostomus melanopterus	11	9.2	1-5	1-5	Brazil	Ramos et al 2012
Diapterus rhombeus	45	11.1	1-5	1-5	Brazil	Ramos et al 2012
Trachurus trachurus	100	1	1.0	1-5	North Sea	Foekema et al., 2013
Trachurus trachurus	56	28.6	1.5 ± 0.7	2.2 ±2.2	English channel	Lusher et al., 2013
Seriola lalandi	19	105	1	05-10	North Pacific	Gassel et al., 2013
Callionymus lyra)	50	38	1.79 ± 0.9	2.2 ±2.2	English channel	Lusher et al., 2013
Cepola macrophthalma)	62	32.3	2.15 ±2	2 ±1.9	English channel	Lusher et al., 2013
Pseudopleuronectes americanus	95	2.1	/	0.1-3 mm PS	USA	Carpenter et al 1972
Platichthys flesus	/	/	/	1 mm PS	Estuary, UK	Kartar et al 1976
Platichthys flesus	1090	0-207	/	1 mm PS	Estuary, UK	Kartar et al 1976
Buglossidium luteum	50	26	1.23±0.4	1.9±1.8	English channel	Lusher et al., 2013
Microchirus variegatus	51	23.5	1.58±0.8	2.2±2.2	English channel	Lusher et al., 2013
Myoxocephalus aenaeus	47	4.2	/	0.1-3 mm PS	USA	Carpenter et al 1972
Prionotus evolans	1	100	1	0.1-3 mm PS	USA	Carpenter et al 1972
Liparis liparis liparis	220	0-25	/	1 mm PS	Estuary, UK	Kartar et al 1976
Chelidonichthys cuculus	66	51.5	1.94±1.3	2.1±2.1	English channel	Lusher et al., 2013
Cathorops spixii	60	18.3	0.4	1-4	Estuary, Brazil	Posatto et al., 2011
Cathorops spp.	60	33.3	0.55	1-4	Estuary, Brazil	Posatto et al., 2011
Sciades herzbergii)	62	17.7	0.25	1-4	Estuary, Brazil	Posatto et al., 2011

Astronesthes indopacificus	7	35*	1	1-2.79	North Pacific	Boerger et al.,2010
Sternoptyx diaphana	4	25	1	Longest 1.58	North Pacific	Davidson and Asch 2011
Sternoptyx pseudobscura	6	16.7	1	Longest 4.75	North Pacific	Davidson and Asch 2011
Idiacanthus antrostomus)	4	25	1	Longest 05	North Pacific	Davidson and Asch 2011
Zeus faber	46	47.6	2.65±2.5	2.2±2.2	English channel	Lusher et al., 2013
Xiphias gladius	56	12.5	/	3.69-55.4	Mediterranean	Romeo et al., 2015
Thunnus thynnus	36	32.4	/	0.63-164.5	Mediterranean	Romeo et al., 2015
Thunnus alalunga	31	12.9	/	3.60-58.52	Mediterranean	Romeo et al., 2015
Phylum Elasmobranchs						
Pteroplatytrygon violacea	2	50	1.3±02 items/ind *	5-60	Ionian sea	Anastasopoulou et al 2013
Etmopterus spinax	16	6.3	1.3±02 items/ind *	5-60	Ionian sea	Anastasopoulou et al 2013
Galeus melastomus	741	3.2	1.3±02 items/ind *	5-60	Ionian sea	Anastasopoulou et al 2013
Squalus blainville	75	1.3	1.3±02 items/ind *	5-60	Ionian sea	Anastasopoulou et al 2013

* represent the average of the study

Table 1.6. Occurrence of microplastics in different species of marine organisms. In table are reported the percentage of analyze organisms with plastics, Mean number of particles per individual, type and size of ingested items, location of collection (From Luscher, 2015).

1.2 Hazards of microplastic

1.2.1 Microplastics as vector of alien species

The introduction of vast quantities of plastics debris into the ocean environment over the past half century has massively increased the amount of rafting material and consequently increased the opportunity for the dispersal of marine organisms. This represent a potential mechanism for alien invasions of new habitats (Barnes 2002; Barnes and Milner 2005; Andrady 2011)

At now, there are 32 reports representing 270 species (85 taxa); 3 studies specifically distinguished the presence of 5 invasive species. It is however, likely that this is possibility is still under-estimated due to the limited numbers of reports, and because not all the studies identified organisms to species level.

Organisms ranging from algae to reptiles (i.e. iguanas) have been observed to raft on plastics material, but the most common species on plastic waste (Table 1.7) include barnacles, polychaete worms, bryozoans, hydroids and mollusks (Barnes 2002).

Taxonomic group	Number of taxa	Numbers of papers
Bivalves	1	1
Bryozoans	1	1
Cephalopods	1	1
Cnidaria	3	2
Crustaceans	14	13
Echinoderms	6	3
Fish	10	1
Gastropods	1	1
Pelagic insect	1	1
Polychaetes	2	1
Porifera	1	1
Seagrass and Algae	2	1
Unknow	47	1

Table 1.7. Taxonomic groups and number of taxa founded on plastic debris.

In 1972, two papers firstly reported the occurrence of organisms (diatoms, hydroids, and bacteria) on small plastics (0.1–5 mm long) collected by plankton nets (Carpenter and Smith 1972; Carpenter et al., 1972), and additional studies on microplastic fouling emerged in the 2000's (Carson et al., 2011; Zettler et al., 2013; Goldstein et al., 2013). Zettler et al. (2013) conducted the

first comprehensive characterization of epi-plastic microbial communities, which they coined the "Plastisphere". These authors used scanning electron microscopy (SEM) and next-generation sequencing to analyze three polyethylene and three polypropylene plastic pieces (approx. 2–20 mm long) from offshore waters of the North Atlantic. This pioneer study revealed a unique, diverse, and complex microbial community with approximates 8600 differential OTUs from bacteria, diatoms, cyanobacterias and predatory ciliate, confirming that plastic debris may act as a vector for the transport and dispersal of these species in the oceans (Zettler et al., 2013).

In another study, Reisser et al.,(2014) used scanning electron microscopy (SEM) to examine organisms on the surface of 68 small marine plastics (length range = 1.7–24.3 mm, median = 3.2 mm) from inshore and offshore waters around the Australian continent. Authors found many new taxa associated with millimeter-sized marine plastics and imaged a variety of marine plastic shapes and surface textures resulting from the interaction of polymers with environments and organisms. Diatoms were the most diverse group of plastic colonizers, represented by 14 genera. They also recorded 'epiplastic' coccolithophores (7 genera), bryozoans, barnacles (*Lepas* spp.), a dinoflagellate (*Ceratium*), an isopod (Asellota), a marine worm, marine insect eggs (*Halobates* sp.), as well as rounded, elongated, and spiral cells putatively identified as bacteria, cyanobacteria, and fungi (Figure 1.5). Furthermore, they observed a variety of plastic surface microtextures, including pits and grooves conforming to the shape of microorganisms, suggesting that biota may play an important role in plastic degradation (Reisser et al., 2014).

All these studies highlights how anthropogenic millimeter-sized polymers have created a new pelagic habitat for microorganisms and invertebrates. The ecological ramifications of this phenomenon for marine organism dispersal, ocean productivity, and biotransfer of plastic-associated pollutants, remains to be elucidated opening new field of researcher.

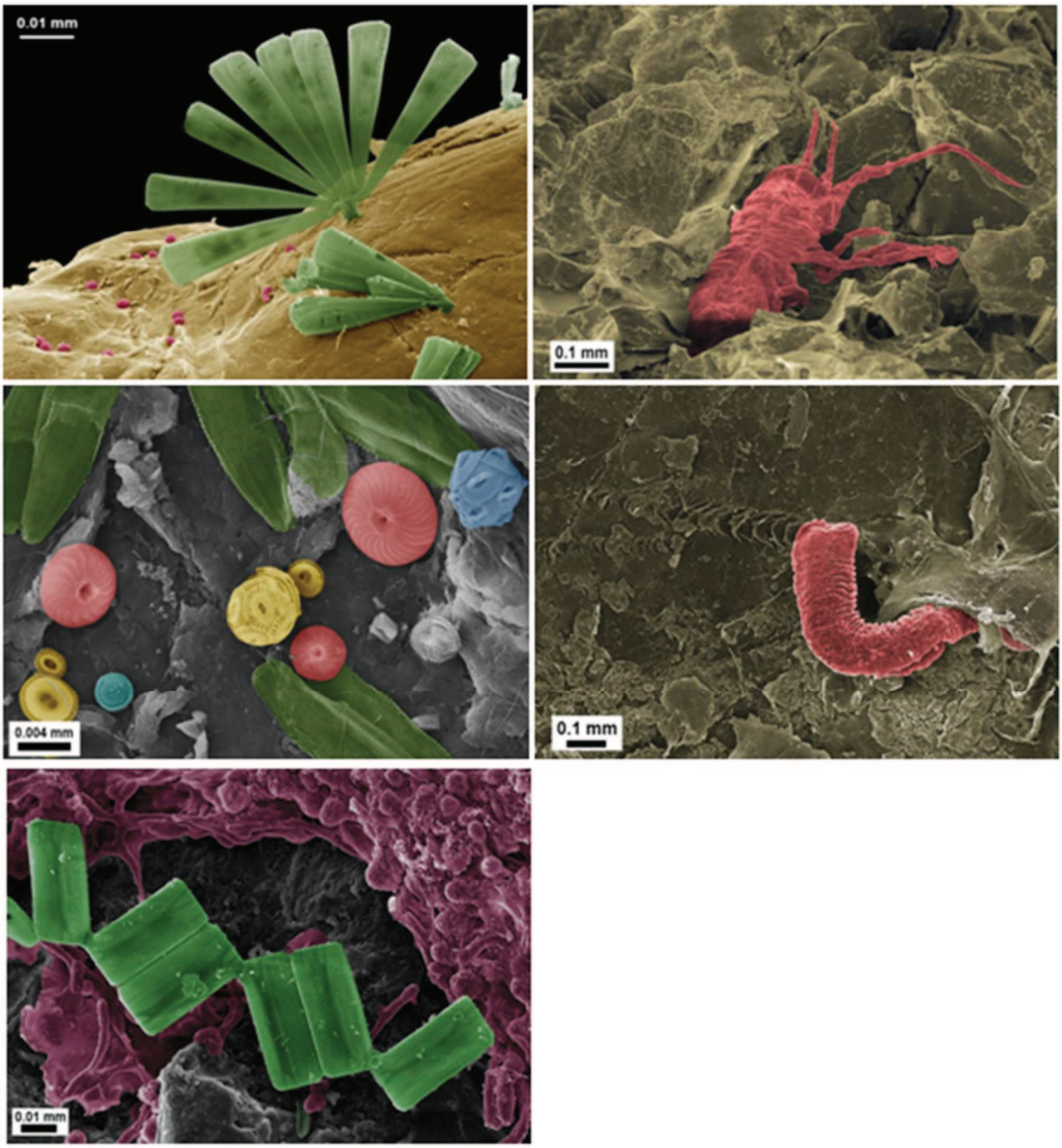


Figure 1.5 This is a false colour SEM image of art of the surface of a 5mm long plastic from waters off eastern Tasmania, Australia. From the top on the left can be observed Diatoms (green) and potential plastic-eater microbes (purple), a marine isopod, Coccolithopores and diatoms on microplastics surface, a marine worm on PE fragment and again some diatoms and microbes. Copyright by Jlia Reisser and Jeremy Shaw.

1.2.2 Microplastics and chemicals

Toxicity associated to both meso or microplastics, can be generally attributed to one or more of the following factors:

1) Reactivity of some intermediates derived from partial degradation of plastics or from residuals monomers.

2) The chemical pollutants present in sea water are slowly absorbed and concentrated in the microplastic fragments. Plastics debris does 'clean' the sea water of the dissolved pollutant chemicals, which, once plastic are ingested, become bioavailable to the organisms (Endo et al., 2005).

Although plastics are typically considered as biochemically inert (Roy et al., 2011; Teuten et al., 2009), plastic additives, often termed ''plasticisers'', are incorporated into plastics during manufacture to change their properties or extend the life of the plastic by providing resistance to heat (e.g. polybrominated diphenyl ethers), oxidative damage (e.g. nonylphenol) and microbial degradation (e.g. triclosan) (Browne et al., 2007; Thompson et al., 2009). These additives are of environmental concern since they both extend the degradation times of plastic and may, in addition, leach out, introducing potentially hazardous chemicals to biota (Barnes et al., 2009; Lithner et al., 2011; Talsness et al., 2009).

Incomplete polymerization during the formation of plastics allows additives to migrate away from the synthetic matrix of plastic; the degree to which these additives leach from plastics is dependent on the pore size of the polymer matrix, the size and properties of the additive and environmental conditions (e.g. weathering; Moore, 2008; Ng and Obbard, 2006; Teuten et al., 2009). Phthalates are emollients that soften plastics by reducing the affinity between molecular chains within the synthetic polymer matrix (Oehlmann et al., 2009; Talsness et al., 2009). In PVC, phthalates can constitute up to 50% of the plastic's weight (Oehlmann et al., 2009). Meanwhile, Bisphenol A is a constituent monomer in polycarbonate which is widely used in food and beverage containers. Neither compound is persistent, but their instability within plastic products facilitates leaching and their high prevalence in aquatic environments has been widely reported, particularly in landfill leachates (vom Saal and Myers, 2008).

Alkylphenols, bisphenol A and polybrominated diphenyl ethers (PBDEs), commonly used as plastic additives, have been found at high concentrations in plastic fragments. In these plastic fragments, alkylphenols were identified at concentrations up to 3940 ng/g, concentrations of bisphenol A were up to 35 ng/g with outliers up to 700 ng/g, whereas concentrations of PBDEs were found to be between 0.1 and 400 ng/g with outliers up to 9900 ng/g (Hirai et al., 2011;

Rochman et al., 2014). These plastic additives were discovered on plastic fragments found both at remote and urban beaches as well as in the open ocean (Hirai et al., 2011).

Due to the large surface-area-to-volume ratio of microplastics, marine biota may be directly exposed to leached additives after microplastics are ingested. Such additives may interfer with biologically important processes, potentially resulting in endocrine disruption, which in turn can impact mobility, reproduction, development, and carcinogenesis (Barnes et al., 2009; Lithner et al., 2009, 2011). Commonly used additives, including polybrominated diphenyl ethers, phthalates and bisphenol A, are known for being endocrine-disrupting chemicals as they can mimic, compete with or disrupt the synthesis of endogenous hormones (Talsness et al., 2009). Hormonal imbalance can cause permanent morphological damages in organisms particularly in the developmental stages, or sexual disruption in adults. Phthalates have been associated with a range of molecular and biological effects in aquatic organisms, including genotoxic damage (micronuclei and apoptosis in mussel haemocytes), inhibited locomotion in invertebrates and intersex conditions in fish (Oehlmann et al., 2009). Bisphenol A is both an estrogen agonist and an androgen antagonist that can differentially affect reproduction and development depending on its concentration and the organism affected. Bisphenol A can also be acutely toxic to both crustaceans and insects (Cole et al., 2011). Chronic and widespread exposure of human populations to Bisphenol A has further been associated with various health effects, including heart disease, diabetes and alterations in circulating hormone levels (Galloway et al., 2010; Lang et al., 2008). Endocrine disruption in adult fish (japanese medaka) exposed to PE particles was suggested by a marked down-regulation in the expression profile of two genes related to endocrine function Chg H and Vtg I (Rochman et al., 2014); exposed males showed a decrement of choriogenin while in females the down-regulation involved also vitellogenin gene.

Although it has been shown that plasticizers can induce negative biological effects within the ng/l–µg/l range, indeed there has been relatively little research into the chronic effects of these additives in long-term exposures to aquatic species (Oehlmann et al., 2009). A list of additives used in plastics industry is given in table 1.8.

Additives	Comment	Examples of hazardous additives
Stabilizers **Halogen** **Antioxidant** **Ultraviolet Absorbers** **Biological Preservatives**	Added between 0.1 to 10.09% by weight. Phenolic antioxidants are used in low amounts and phosphites in high	Arsenic compounds Organis tin compounds Triclosan Barium-Cadmium Zinc Epoxy-Phosphite Bisphenol A Cadmium compounds Lead compounds Nonylphenol compounds Octylphenol
Curing agents	0.1-2% w/w typically Peroxides and other cross linkers, catalysts, accelerators	4,4' diaminophenylmethane (MDA) 2,2' dichloro-4,4' methylnedianiline (MOCA) Formaldehyde
Colorant **Inorganic** **Organic**	Most often inorganic pigments	Titanium dioxide Cadmium Compounds Chromium Compounds Lead Compounds Coballte (II) diactate
Coupling agents	Often low concentration. Combine the ingredients toghether. Act as a bridge between polymer and the filler	
Processing Aids **Lubrificants** **Other Processing Aids** **Flow Controls**	Lubrificants: calcium, zinc, and lead sterates, petroleum and polyethylene waxes, and fatty ester and amides. Other processing aids; plasticol viscosity depressant, mold release agents, antiblocking agents, antifog agents	

Table 1.8. Typologies of additives commonly used in plastics producing process (from Nerland et al 2014).

Ingestion of microplastics by biota, represents an additional concern for their potential to transfer concentrated POPs to the organisms (Bowmer and Kershaw, 2010). The hydrophobicity of organic xenobiotics and the large surfaces of floating polymers facilitate the adsorption of these chemicals on microplastics at concentrations orders of magnitude higher than those detected in seawater (Ogata et al., 2009). Adsorption is not only a physical behavior but also a kind of chemical behavior. While the physical adsorption mainly depends on the great specific surface area and Van der Waals' force, the chemical adsorption is mainly due to the great affinity of organic pollutants for the hydrophobic surface of plastic compared to seawater. The polymer properties that can influence the sorption of chemicals include surface area (Teuten et al., 2007), diffusivity (Mato et al., 2001; Pascall et al., 2005; Karapanagioti and Klontza, 2008), and crystallinity (Mato et al., 2001; Karapanagioti and Klontza, 2008).

Little is known on the effects of plastics weathering on the sorption of contaminants. Mato et al. (2001) supposed three potential reasons for eroded plastics to exhibit different distribution behavior than the virgin plastics. The first is the increase in surface area due to polymer weathering which would increase the effective diffusivity. Photo-oxidation develops cross-linking, chain scissions, oxygen-containing moieties such as carbonyl groups in PE polymers, and ultimately leads to visible cracking on the surface (Severini et al., 1987; Satoto et al., 1997). Secondly, while an increase in the surface area due to fine cracks could enhance sorption, an increased polarity by the reaction with oxygen could decrease affinity for hydrophobic compounds (Endo et al., 2005). Besides, weathered plastic debris can be thought to have had a long residence time in the marine environment and thus might have sorbed larger amounts (Mato et al., 2002; Lohmann et al., 2011). Last but not least, the organisms attached to the plastic debris, including diatoms, hydroids, filamentous algae and tarry residues (Endo et al., 2005), could act as additional sorbents for hydrophobic contaminants: when fouled with life (Zettler et al., 2013), an increase in the surface area and changes in the surface properties, allow concentrations of chemical contaminants to increase over time via sorption and/or bioaccumulation by biofilms.

The possibility for plastic particles to adsorb chemical pollutants from the surrounding environment has been also characterized in laboratory conditions. Different particles polymers, like polyvinyl chloride, polyethylene, polypropylene, polystyrene, were shown to have a high sorption capacity for DDTs, polycyclic aromatic hydrocarbons (PAHs), hexachlorocyclohexanes and chlorinated benzenes (Bakir et al., 2012; Lee et al., 2014).

Consistent with these studies, several persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, PAHs and dioxins have been

detected in plastic pellets stranded on different beaches of the world (Endo et al., 2005; Ogata et al., 2009; Hirai et al. 2011; Heshett et al., 2012) as shown in table 1.9.

Several types of contaminants have been discovered on plastic debris, but a systematic overview of such compounds is still lacking. Some of these compounds can be related to the plastic production process, while other chemicals have no clear association with plastic production process, but are related to environmental pollution sources, such as industrial activities and oil seeps.

Polycyclic aromatic hydrocarbons (PAHs) on beached pellets and plastic fragments showed a mainly petrogenic signature, with total PAH concentrations up to 45,000 ng/g (Hirai et al., 2011; Antunes et al., 2013; Mizukawa et al., 2013). Polychlorinated biphenyls (PCBs) and organochloride pesticides (OCPs), mainly linked to legacy pollution, were found to have concentrations up to 450 ng/g and 200 ng/g, both in plastic fragments as beached pellets respectively (Hirai et al., 2011; Karapanagioti et al., 2011; Antunes et al., 2013; Mizukawa et al., 2013).

Metals also show a strong adsorption capacity to plastic with measured concentrations up to 300 µg/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 uptake with higher adsorption capacities in beached than virgin plastics (Turner and Holmes 2015), due to an increased polarity by weathering processes (Mato et al., 2001). Environmental conditions are also crucial factors for the adsorption of metals onto plastics polymer, i.e. the pH can increase the adsorption of certain metals (Turner and Holmes 2015).

Despite the importance of microplastics in adsorption and transport of hydrophobic pollutants, it is still unclear whether they also represent a potential source of chemical exposure within marine food webs. Various evidences, including the use of a thermodynamic approach and of models simulating physiological conditions in the gut, suggested that both adsorbed pollutants and chemical additives of plastics might be released to organisms (Gouin et al., 2011; Tanaka et al., 2013; Bakir et al., 2014a) as well as a direct observation of the bioavailability of adsorbed chemical is still lacking.

Contaminant	Range of concentration (ng/g plastic)	References
PAHs	1-24364	Ogata et al., 2009, Hirai et al., 2011 Fisner et al., 2013
PCB	1-5000	Ogata et al., 2009,29, Mato et al., 2001, Hirai et al., 2011; Rios et al., 2007; Fisner et al., 2013; 121-123
DDT	0.16- >1000	Mato et al., 2001 Teuten et al., 2009 Colabuono et al., 2010 Rios et al., 2007 Karapanagioti et al., 2011 Ryan et al 2012
PBDEs	0.3-9909	Teuten et al., 2009; Hirai et al., 2011
PBDE 209	0.1-9970	Hirai et al., 2011
Hexachlorocyclohexane isomer	<2-36	Ogata et al., 2009
Chlordanes	4.29 -14.4	Colabuono et al., 2010
Cyclodienes	2.41 - 50.9	Colabuono et al., 2010
Mirex	6.48 - 14.6	Colabuono et al., 2010
Hopanes	2000-6000	Mizukawa et al, 2013
Aliphatic Hdrocarbons	1.1 - 8600	Rios et al., 2007
Hexachlorobenzene	12.4-17.5	Colabuono et al., 2010
Nonylphenols	0.7 -3.936	Mato et al., 2001; Hirai et al., 2011; Teuten et al, 2007
Octyphelos	0.1- 154	Teuten et al., 2009; Hirai et al., 2011;
Bisphenol A	0.2-730	Hirai et al., 2011; Teuten et al, 2007
PFCs	0011-0.116	Llorca et al, 2014

Table 1.9. Reported contaminant concentrations in plastic particles collected in the marine environment (From Nerland et al., 2014).

1.2.3 Microplastics and biological effects

Until a few years ago there was more attention on ecological impacts of macrodebris in respect to microplastics (Browne et al., 2015), but in the last years toxicological effects of microplastics aree drawing researchers attention. While in field organisms it is difficult to separate the toxicity of plastics from that of other stressors, laboratory studies permit to predict and assess the toxicological risk of microplastics to marine organisms.

Several laboratory trials were conducted to assess the ecotoxicological risk of microplastics. Microplastics can enter the very base of the marine food web via absorption. Such was observed when charged nano-polystyrene beads were absorbed into the cellulose of a marine alga (*Scenedesmus* spp.), with inhibition of photosynthesis and onset of oxidative stress (Bhattacharya et al. 2010). Microplastics can affect the function and health of marine zooplankton (Cole et al., 2013; Lee et al., 2013), where decreased feeding was observed following the ingestion of polystyrene beads (Cole et al., 2013). Furthermore, adult females and nauplius larvae of the copepod (*Tigriopus japonicus*) survived acute exposure, but higher mortality rates were observed following a two-generation chronic toxicity test (12.5 µg mL−1) (Lee et al., 2013).

A number of benthic invertebrates has been studied under laboratory conditions to investigate the uptake and consequences of microplastic ingestion (Table 1.11). Laboratory feeding and retention trials have focused on direct exposure of invertebrates to microplastic particles (Cole et al., 2011; Wright et al., 2013a).

Exposure studies demonstrated that benthic invertebrates including lugworms (*Arenicola marina*), amphipods (*Orchestia gammarellus*) and blue mussels (*Mytilus edulis*) feed directly on microplastics (Thompson et al., 2004; Wegner et al., 2012), and the deposit-feeding sea cucumbers selectively ingested microplastic particles (Graham and Thompson 2009). Although microplastic uptake was recorded for a number of species, some organisms appear to reject microplastics before digestion and excrete microplastics after digestion. Pseudofaeces production is a form of rejection before digestion but requires additional energetic cost. Furthermore, prolonged pseudofaeces production could lead to starvation (Wegner et al., 2012). On the other hand, polychaete worms, sea cucumbers and sea urchins are able to excrete unwanted materials through their intestinal tract without suffering obvious harm (Thompson et al., 2004; Graham and Thompson 2009; Kaposi et al., 2014). Adverse effects of microplastic ingestion were reported for lugworms: weight loss was positively correlated with concentration of spiked sediments (40–1300 µm polystyrene) (Besseling et al., 2013). Similarly, Wright et al. (2013b) recorded significantly reduced feeding activity and significantly decreased energy reserves in lugworm exposed to 5 % un-plasticised polyvinyl chloride (U-PVC).

Several studies have raised concern for microplastic retention and transfer between organisms' tissues. In mussels, *Mytilus edulis*, plastic particles (3 and 9.6 µm) were accumulated in digestive tissues and translocated to haemolymph after three days (Browne et al., 2008). In the same organisms, the uptake of microplastics caused notable histological changes in digestive cells with strong inflammatory responses, formation of granulocytomas and lysosomal destabilization that increased with exposure time (Von Moos et al., 2012). Ingestion of microplastics has been demonstrated in various marine organisms with different feeding strategies; this phenomenon may negatively influence both the feeding activity and nutritional value of a plankton-based diet, particularly in those species, which cannot discriminate the food source (Moore et al., 2001; Browne et al., 2008).

Regarding fish, only one study was conducted to evaluate the toxic effects induced by microplastics alone and in co presence of pyrene. Authors showed an important decrease in the activity of AchE, an important enzyme in the neurotransmission signaling (Oliveira et al., 2013).

It should be noted, however, that while these studies succeeded in determining the pathways of microplastics in organisms, the exposure concentrations used to achieve this goal exceeded those expected in the field, such that the results have to be treated with care. Certainly, microplastics can modulate and influence several biological pathways as reported in Table 1.10.

Levels of organization	linkage	Reference
Subatomic------> Macromolecular	nm sizied PS added to lungs of rats caused stress and increased concentration of protein.	Browne et al 2001
	nm-sized PS added to sea urchin embryos cased modulation of genes involved in apoptotic pathway,	Della Torre et al 2014
Macromolecular----->Molecular assemblies	Lugworms ingesting µm sized PVC showed increased oxidative stress with fewer antioxidant	Browne et al 2013
	Lugworms exposed to µmsized uPVC showed inflammation and energy depletion	Wright et al 2013
	Japanese medaka exposed to µm-sixed PE showed early sign of endocrine disruption	Rochman et al 2014
	Common goby exposed to µm-sized PE particles showed AcHE activity decreased.	Oliveira et al 2013 Luis et al., 2015
Molecular assemblies-------> Organelle	Polymethyl methacrilate in mice damaged DNA resulting in increased numbers of cellular micronuclei	Zhang et al 2008
Organelle----->Cells	Cell of mice that take up nmsized PS suffer apoptosis, necrosis and reduced proliferation	Frohlich et al 2009
Cells------>Tissue	Mussels exposed to µmsized PE produced more granulomas in gut-tissue than in normal	Van Moos et al 2012
Tissue----->organs	Mice exposed to µmsized Polymethyl methacrilatecaused their bone to breakdown	Clohisy et al 2006 Pearl et al 2011
Organs system---->Organism	Lugworms ingesting µmsized PVC with triclosan reduced their feeding and suffered mortality	Browne et al 2013
	Marine copepod exposed to µmsized PS reduced their feeing and fecundity	Cole et al 2015

Table 1.10. Example of demonstrated linkage among levels of biological organization for effects of plastic debris in organisms (From Browne et al 2015 modified and implemented)

Organism	Size of ingested particles	Exposure dose	Effects	Reference
Phylum Chlorophyta				
Scenedesnus spp.	20nm	1.6-40 mg/ml	Adsorption, ROS increased, Photosyntesis affected	Bhattacharya et al., 2010
Phylum Haptophyta				
Isochrysis galbana	2µm PS	90000/ml	no	Long et al., 2014
Phylum Dinophyta				
Heterocapsa triquetra	2µm PS	90000/ml	no	Long et al., 2014
Phylum Cryptophyta				
Rhodomonas salina	2µm PS	90000/ml	no	Long et al., 2014
Phylum Ochrophyta				
Chaetoceros neograciis	2µm PS	90000/ml	no	Long et al., 2014
Phylum Ciliophora				
Strombidium sulcatum	0.41-10µm	5-10% of bacteria concentration	Ingestion	Christaki et al., 1998
Tintinnopsis lobiancoi	10µm PS	1000-2000-10000/ml	Ingestion	Setala et al., 2014
Phylum Rotifera				
Synchaeta spp.	10µm PS	2000/ml	Ingestion	Setala et al., 2014
Phylum Annellida				
Arenicola marina	20-2000µm	1.5g/L	Ingestion	Thompson et al., 2004
Arenicola marina	130µm uPVC	0-5% by weight	Ingestion, Reduced feeding, increased phagocytosis, reduced available energy reserves, lower lipid reserves	Wright et al.,2013
Arenicola marina	400-1300µm PVC	0, 1,10,100 g/L	Ingestion, reduced feeding, weight loss	Basseling et al., 2013
Arenicola marina	230µm PVC	1500g of sediment mixture	Ingestion, oxidative stress	Brovne et al, 2013
Galeolaria caespitosa	3 and 10 µm PS	635,2240, 300beads/ml	Ingestion, size selection, egestion	Cole et al., 2013
Marenzellaria spp.	10µm PS	2000/ml	Ingestion	Setala et al., 2014
Phylum Mollusca				
Mytilus edulis	30nm PS	0, 0.1, 0.2,0.3 g/l	Ingestion,reduced filtering	Wegner et al., 2012
Mytilus edulis	0-80µm HDPE	2.5g/l	Ingestion, retention in digestive tract, trasferred to emolymph system, immune response	Von Moos et al., 2012
Mytilus edulis	0.5µm	50µl/ 400ml	Ingestion	Farrel and Nelson 2013
Mytilus edulis	3-9.6µm	0.51g/l	Ingestion, retention in digestive tract, trasferred to emolymph system	Browne et al., 2008

Mytilus edulis	10-30µm PS	310000-865000/ml	Ingestion	Claessens et al., 2013
Mytilus trossuslus	10µm PS	/	Ingestion	Ward et al., 2003
Placopecten magellanicus	10, 15, 16,18,20 µm	1.05/ml	Ingestion	Brillian and MacDonald 2000-2002
Crassostrea virginica	10µm PS	1000/ml	Ingestion	Ward and Kach 2009
Crassostrea gigas	2-6µm PS	1800/ml 200/ml	Increased filtration and assimilation, reduced gamete quality (sperm mobility, oocyte number and size fecundation yield),	Sussarellu et al 2014 CONFERENCE PAPER
Phylum Echinodermata				
Apostichopus californicus	10-20µ PS	2.4/µl	Ingestion	Hart 1991
Thyonella gemmata and Holothuria grisea	0.25-15mm PVC shavings and resin pellet	10g PVC 60g resin pellet	Selective ingestion	Graham and Thompson 2009
Hoothuria floridana and Cucumaria frondosa	0.25-15mm nylon line	2g/600ml sand	Selective ingestion	Graham and Thompson 2009
Tripneustes gratilla	32-35µm PE	1, 10, 100,300/ml	Ingestion	Kaposi et al., 2014
Dendraster excenticus	10-20µm PS	2.4/µl	Ingestion	Hart 1991
Strongylocntrotus spp	10-20µm PS	2.4/µl	Ingestion	Hart 1991
Ophiopholis aculeata	10-20µm PS	2.4/µl	Ingestion	Hart 1991
Dermasterias imbricata	10-20µm PS	2.4/µl	Ingestion	Hart 1991
Phylum Arthropoda				
Semibalanus balanoides	20-2000µm	1g/l	Ingestion	Thompson et al., 2004
Tigriopus Japonicus	0.05µm PS 0.5µm PS 6µm PS	91000000000/ml 91000000/ml 520000/ml	Ingestion, mortality, decrease fecundity	Lee et al., 2013
Acartia tonsa	10-70µm	3000-4000 beads/ml	Ingestion,size selection	Wilson 1973
Acartia spp.	10µm PS	2000/ml	Ingestion	Setala et al., 2014
Eurytemora affinis	10µm PS	1000, 2000, 10000/ml	Ingestion	Setala et al., 2014
Limnocalanus macrurus	10µm PS	1000, 2000, 10000/ml	Ingestion	Setala et al., 2014
Temora longicornis	20µm PS	100/ml	Ingestion 10.7+-2.5 beads/ind	Cole et al 2014a
Calanus helgolandicus	20µm PS	75/ml	Ingestion	Cole et al 2014b
Orchestia gammarellus	20-2000µm	1g/ individual (n=150)	Ingestion	Thompson et al., 2004
Talitrus saltador	10-45µm	10% weight food	Ingestion	Ugolini et al 2013

Allorchestes compressa	11-700µm	0.1g	ingestion	Chua et al., 2014
Neomysis integer	10µm PS	2000, 10000 sphere/ml	Ingestion	Setala et al., 2014
Mysis relicta	10µm PS	2000, 10000 sphere/ml	Ingestion	Setala et al., 2014
Carcinus maenas	8-10µm PS	40000/l ventilation 1000000/l feeding	Ingestion	Watts et al., 2014
Nephrops norvegicus	5 mm PP fibre	10 fibres/cm fish	Ingestion	Murray and Cowie 2011
Nephrops norvegicus	500-600µm PE	150mg in gelatin food	Ingestion	Devriese et al., 2014
Bosmina coregoni	10µm PS	2000, 10000 sphere/ml	Ingestion	Setala et al., 2014
Phylum Chordata				
Pomatoschistus microps	1-5µm PE	18.4, 184 µg/l	Ingestion, decreased energy, inhibited AchE activity	Oliveira et al., 2013
Gadus morhua	3mm LDPE	Ground up as 10% of diet	Liver toxicity, phatology, hepatic stress	Rochman et al., 2013
Oryzias latipes	PE pellets	/	Altered gene expression, dcreased choriogenin regulation in males and dcrase vitellogenin and choriogenin in females	Rochman et al., 2014
Dicentrarchus labrax	10-45µm PE	0-105/g of food	Ingestion, effect on survival of larvae, gastric obstruction	Mazurais et al., 2014

Table 1.11. List of laboratory exposure of microplastics to marine organisms (From Lusher 2015 modified and implemented)

2. AIMS OF THE STUDY

Microplastics represent a growing environmental concern and their potential capability to adsorb different classes of pollutants, represent a still unexplored source of exposure for aquatic organisms. Although several organisms can ingest microplastics, a clear evidence of their potential adverse effects and presence in wild organisms is still lacking. In these respect, the overall objective of this thesis was to evaluate the ecotoxicological potential and presence of microplastics in Mediterranean organisms and to provide new insights on the ecological risks.

The thesis is articulated in four Chapters, each describing a specific aim. A brief explanation of the mains objectives and organization of the thesis are given below.

1. Chapter 1 describes the capacity of microplastics to adsorb organic and inorganic compounds from the environment evaluated through both laboratory and filed experiments.
2. Chapter 2 reports the experiments to assess pollutant bioavailability from microplastic and ecotoxicological effects induced by both virgin and contaminated particles in marine organisms.
3. Chapter 3 describes a newly developed methodology to extract, quantify and characterize microplastics in tissues of marine organisms.
4. Chapter 4 provide the first filed characterization on the microplastics presence and composition in several organisms caught from the Mediterranean Sea.

3. Ability of microplastics to adsorb organic and inorganic compounds. [a]

In this chapter, the capability of microplastics to adsorb organic and inorganic compounds from the surrounding seawater was assessed to evaluate the potential risk of microplastics as chemicals vectors. In this regard, two studies were conducted; i) macroplastic and microplastic items were collected from three local beaches, and levels of PAHs adsorbed into polymers were compared with those on virgin plastic objects; ii) in laboratory conditions, the adsorbing kinetics of pyrene and cadmium onto polyethylene and polystyrene microplastics were evaluated, with the aims to test the potential of such microplastics as carriers of PAHs.

Abstract

Plastic debris is a relative new typology of marine pollution, and microplastics originated from fragmentation of large debris, are found worldwide. Since, microplastics may represent a still unexplored source of exposure for aquatic organisms, in this study the chemical characterization was carried out on virgin macroplastics, and compared with both beached macroplastics and microplastics. Field obtained results were integrated with the assessment in laboratory condition of the adsorbing kinetics of pyrene or cadmium onto polyethylene (PE) and polystyrene (PS) microplastics.

The overall results confirmed the marked capability of microplastics to adsorb PAHs and metals. Level of PAHs found in different typologies of plastic were the lowest in virgin macroplastics and the highest in beached microplastics, highlighting their enhancement with the "ageing" of polymer. In laboratory conditions the concentration of pyrene and cadmium on microplastics particles increased with a clear time and dose-dependent trend of adsorption on both the tested polymers, PE and PS.

[a] Results of this chapter was partially published in Avio C.G., et al., 2015a..

3.1. Introduction

The occurrence of microplastics fragments in the marine environment was established by several studies, documenting their presence from sea surface to the bottom, from offshore to beach zone, and from the poles to the equators (Derraik 2002).

Ingestion of microplastics has been demonstrated in various marine organisms with different feeding strategies; this phenomenon may negatively influence both the feeding activity and nutritional value of a plankton-based diet, particularly in those species which can not discriminate the food source (Moore *et al*., 2001; Browne et al., 2008).

Recent evidences also suggest the potential role of microplastics as vectors of chemical pollutants, either used as additives during the polymer synthesis, or adsorbed directly from seawater (Rios et al., 2007; *Teuten et al.,* 2009; Engler 2012).

Plastics contaminated by persistent organic pollutants (POPs) are found globally from coastal areas to the remote habitats of the subtropical gyres (Hirai et al., 2011). The hydrophobicity of organic xenobiotics and the large surfaces of floating polymers facilitate the adsorption of these chemicals on microplastics at concentrations orders of magnitude higher than those detected in seawater (Ogata *et al*., 2009), increasing their concentration even up to the order of 10^6 (Mato et al., 2001). In addition, notwithstanding the organic fraction of soils and sediments was traditionally considered to be the most important form of sorbent in the environment, some studies have shown the importance of plastics (Mato et al., 2001; Ng and Obbard 2006; Rios et al., 2007), that can accumulate and concentrate pollutants at hundreds fold greater than those in sediments (Teuten et al., 2009).

POPs such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDTs), can be absorbed onto microplastic and reported concentrations of persistent organic pollutants in marine plastic pellets range from 1 to 10,000 ng/g plastic pellet worldwide (Ogata et al., 2009; Hirai et al., 2011). For polychlorinated biphenyls (PCBs), concentrations of 4−980 ng/g plastic pellet (Mato et al., 2001; Rios et al., 2007) were found worldwide and 169 −324 ng/g plastic pellet (Ogata et al., 2009; Kershaw et al., 2011) in the North Sea. The possibility for plastic particles to adsorb chemical pollutants from the surrounding environment has been also characterized in laboratory conditions. Different particles polymers, like polyvinyl chloride, polyethylene, polypropylene, polystyrene, were shown to have a high sorption capacity for DDTs, PAHs, hexachlorocyclohexanes and chlorinated benzenes (Bakir et al., 2012; Lee et al., 2014).

As for organic compounds, also metals were found on plastic debris. Uptake of Ag, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn, Al, Mn, Fe, Mo, Sb, Sn and U by plastics have been recently reported (Ashton et al., 2010; Holmes et al., 2012; Tien and Chen, 2013; Rochman et al., 2014; Turner and Holmes, 2015).

In this regard, the first part of the thesis describes the ability of microplastics to adsorb xenobiotic compounds assessed both in laboratory and in field conditions.

In the field, macro and microplastics were sampled from three local beaches in order to evaluate differences in chemical load and to estimate the effective risks of microplastics as chemical vectors; levels of adsorbed PAHs were further compared with the virgin objects, bought from a supermarket. In laboratory conditions, the ability of two polymers widely diffused in the marine environment, PE and PS, was characterized in terms of adsorbing capacity of pyrene and cadmium dosed at different concentrations.

3.2. Materials and methods

2.1 Macro and Micro plastics collection and extraction

During the winter 2013 macro and microplastics were sampled from 3 different Italian beaches (Falconara, Torrette and Numana) located along the Marche's coast. Site 1 (Falconara), is located near a refinery and is heavily influenced by periodic storms and river input, producing a high degree of sediment mobilization. The Site 2 (Torrette) is located near the harbor of Ancona, while Site 3 (Numana), is about 30Km Southern within a protected area (Figure 3.1).

In each site, three squares of sediments were collected from the high tide line, where a big amount of debris was visually present. Sampling was performed by using squares 0.5 x 0.5 m, apart from each other 1.5 m. Big plastics objects were directly collected using metal tweezers and subsequently the top 2 cm of sand were picked from each square area, (Martinz Sobral 2011). In the laboratory, samples were introduced into a glass beacker with a hypersaline solution of NaCl (1.2 g/cm^3), stirred vigorously for 30 minutes and decanted for the same time. The supernatant with floating plastic particles was recovered, and this procedure repeated 2 times to allow a better extraction (Thompson et al., 2004). The water was then filtered in a vacuum filtration unit with a nitrate cellulose micro filter (pore of 10 µm).

Filters containing recovered microplastics were observed under a stereomicroscope to identify and collect microplastics. Particles were sorted, weighted and stored until chemical analyses.

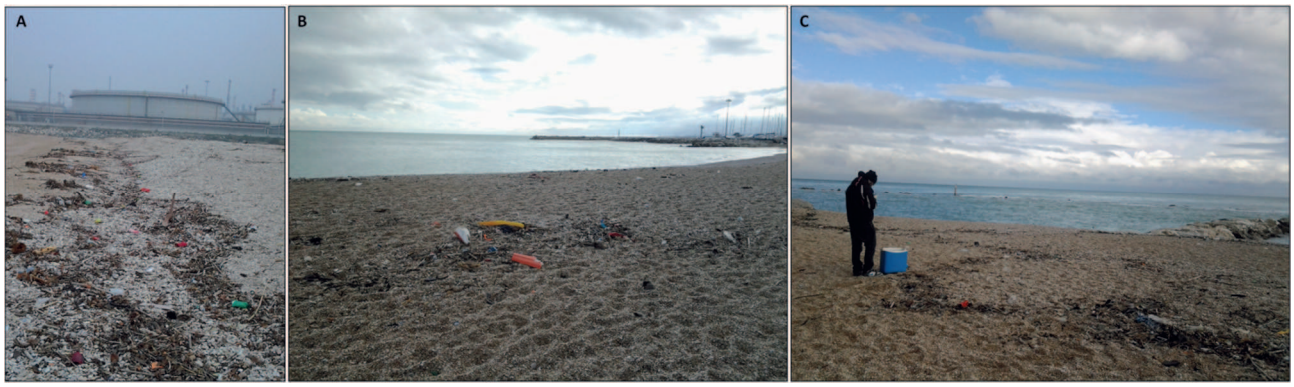


Figure 3.1. Pictures of sampled beaches. A represent Site 1 (Falconara), B represent Site 2 (Torrette) and C represent the the beach of Site 3 (Numana). In each site were sampled three square 0.5*0.5m of sediment from the high tide line.

2.2 Virgin plastic material

Polyethylene (PE) and polystyrene (PS) powders were obtained from a private plastic company. Particles were size-sorted in a 1000-100 µm group used for characterization of the pyrene and cadmium adsorbing capacity.

Virgin macroplastics corresponding as possible to the beached collected objects were bought from a local Supermarket: chemical similarity of the polymers was assessed by FT-IR analysis.

2.3 FT-IR analysis

On both virgin and beached macroplastics, FT-IR analysis was performed in order to compare the same typology of polymers. Analyses were performed using a Cary 660 FT-IR spectrometer (Agilent) equipped with ATR (GladiATR Diamond Crystal Plate, Piketechnologies) which allowed the characterization of microparticles greater than 0.5 mm. Following background scans, 128 scans were performed and CO_2 interference (adsorption at approx 2300-2400 cm^{-1}) was removed for clarity; for each particle, scans were performed with resolution of 4 cm^{-1}. Agilent Resolution Pro v5.2 software was used for the output spectra and the identification of polymers was performed by comparison with a library of standard spectra. Only objects with molecular identity have been investigated.

2.4 *Experimental design for adsorbing kinetics*

The adsorption of pyrene to PE and PS was assessed by mixing solutions of microplastics (20 g/L in seawater) with pyrene dosed at final concentrations of 0.5 µg/L (low, L), 5 µg/L (medium, M) and 50 µg/L (high, H). While the L and M treatments are environmentally realistic for pyrene, the H dose is uncommon but still possible, i.e. after heavy oil spill or in highly

contaminated sewage (Neff 2002). The mixing solutions were maintained in continuously rotating 50 ml glass tubes for 6 days; water was changed and pyrene re-dosed after 3 days. Levels of pyrene adsorbed on polymers were measured after three and six days of treatment.

The adsorption of cadmium to PE and PS was assessed by mixing solutions of microplastics (20 g/L in seawater) with cadmium dosed at final concentrations of 5 µg/L (low, L) and 15 µg/L (medium, M). Also in this case, the two treatments are environmentally realistic (Neff 2002). The mixing solutions were maintained in continuously rotating 50 ml glass tubes for 6 days (Figure 3.2); water was changed and cadmium re-dosed after 3 days. Levels of cadmium adsorbed on polymers were measured after three and six days of treatment.

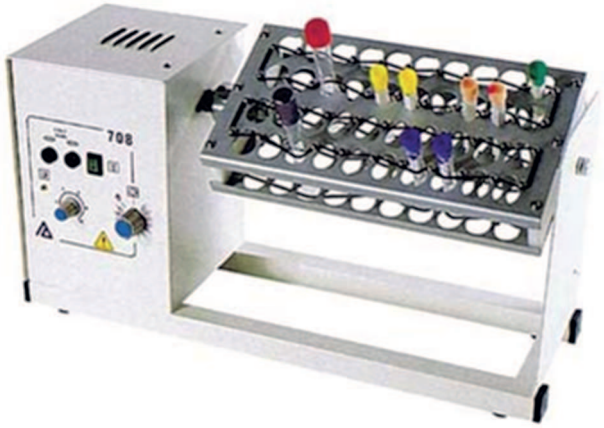


Figure 3.2. Rotation system adopted for the studies of adsorbing kinetics.

2.5 *Chemical analyses of PAHs and Cadmium*

Pyrene adsorbed on laboratory exposed microplastics (PE and PS) and PAHs in virgin, beached macroplastics and in beached microplastics were determined after extraction of samples in 0.5 M potassium hydroxide and methanol (1:10 w:v) with microwave at 55°C for 15 min (Benedetti et al., 2014). After centrifugation for 5 min at 1000 × g, the methanolic solutions were concentrated in speedvac and 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, and HPLC analyses were carried out with water–acetonitrile gradient and fluorimetric detection. Pyrene was identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). Quality assurance and quality control were tested by 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.

Concentrations of Cadmium in PE and PS were assessed by atomic absorption spectrophotometry as previously described (Regoli et al., 2004)). Samples were dried at 60°C until

constant weight was reached and digested under pressure with nitric acid and hydrogen peroxide (7:1) in a microwave digestor system (Mars CEM, CEM Corporation, Matthews, NC, USA). Cadmium, were analyzed by flame and flame less atomization (SpectrAA 220FS and SpectrAA-240Z Zeeman,Varian, Mulgrave, VIC, Australia). Quality assurance and quality control was assessed by processing blank samples and reference standard materials. Concentrations obtained for standard reference materials were always within the 95% confidence interval of certified values. All values are expressed ng/g dry weight (d.w.).

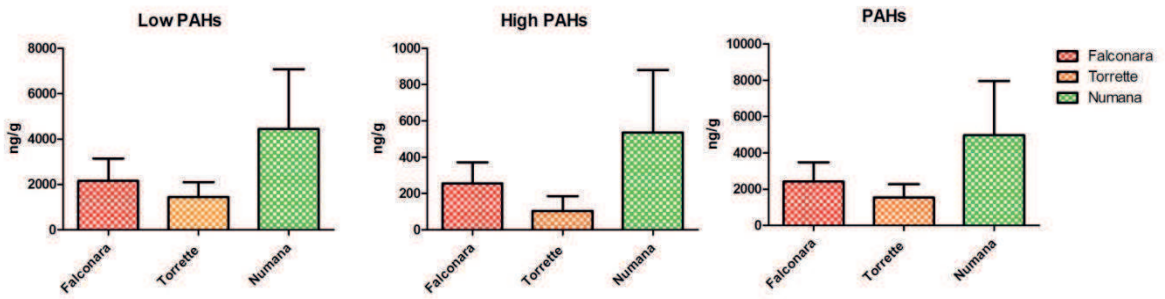
3. Results

3.1 PAHs in virgin macroplastics, beached macroplastics and beached microplastics

PAHs adsorbed on microplastics extracted from the three different beaches were ranged between 273 and 10900ng/g. No statistical differences were founded between locations both for high and low molecular weight hydrocarbons despite mean values of PAHs, were higher in microplastics collected from Numana (Figure 3.3). Overall results showed a high heterogeneity between congeners with a greater contribution of low molecular weight compounds sorbed onto microplastic.

The comparison of PAHs adsorbed on beached microplastics with beached macroplastics (collected at the same time and in the same location of microplastics), and the virgin objects are shown in Figure3.3. Similar level of PAHs were measured on macroplastics, independently from their age (virgin and beached), while significantly higher values of PAHs were highlighted on microplastics, with concentrations 6 times higher than those of macroplastics (Figure 3.3).

Among various congeners of analyzed PAHs, 1- Methyl-Naphtalene, 2-Methyl-Naphtalene, phenantrene, Benzo-a-pyrene and Benzo-b-fluoranthene were the principal compounds with higher level on microplastics than on macroplastics (data not shown).



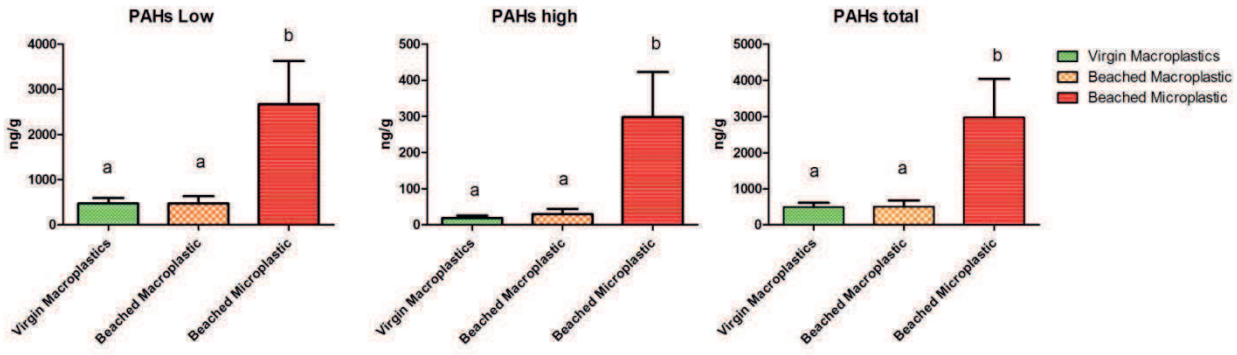


Figure 3.3. On the top, Low molecular weight, High and total level of PAHs founded in microplastics from the three beaches. Down, the comparison of PAHs levels between virgin macroplastics, beached macroplastics and beached microplastics

3.2 Adsorbing kinetics

Microplastics showed an elevated capability to adsorb pyrene with a dose- and time-dependent trend (Figure 3.4). After 6 days of M treatment, concentrations of adsorbed pyrene were 145 ± 17 and 126 ± 17 ng/g on PE and PS microplastics respectively, with an accumulation factor of 29 and 25.2 calculated as the ratio to nominal levels dosed in seawater. Concentrations of adsorbed pyrene were even greater after H dose experiment (305 ± 89 and 244 ± 52 ng/g for PE and PS) but the accumulation factors were 6.1 and 4.8 for the two polymers.

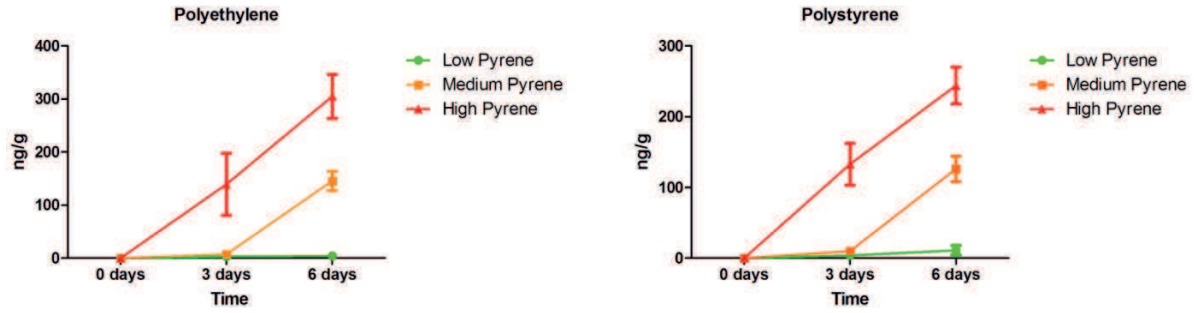


Figure 3.4. Time-course of pyrene adsorption to microplastics particles (polyethylene and polystyrene). Different colored lines indicate nominal doses of pyrene: red line 50 µg/L, orange line 5 µg/L, green line 0.5 µg/L. Data are expressed as ng/g dry weight (mean values ± standard deviation, n=5).

For cadmium (Figure 3.5) both polymers showed a marked ability to adsorb this metal. After 6 days of M treatment, levels of adsorbed cadmium were 104 ± 27 and 109 ± 38 ng/g on PE and PS respectively, with an accumulation factor of 20 and 21.8 calculated as the ratio to nominal levels dosed in seawater, similar to that obtained with pyrene. After 6 days of L treatment, levels of adsorbed cadmium were 49 ± 12 and 49 ± 15 ng/g on PE and PS respectively.

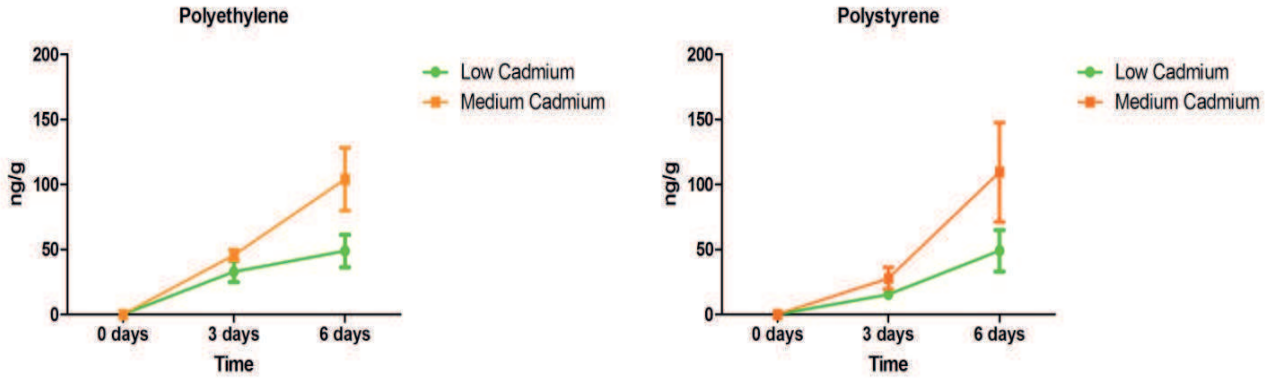


Figure 3.5. Time-course of cadmium adsorption to microplastics particles (polyethylene and polystyrene). Different colored lines indicate nominal doses of Cd: orange line 5 µg/L, green line 0.5 µg/L. Data are expressed as ng/g dry weight (mean values ± standard deviation, n=5).

4. Discussion

The present investigation aimed to provide new insights on the potential role of microplastics as a source of chemical exposure and ecotoxicological challenge to marine organisms. A growing concern is being raised for the possibility of these polymers to adsorb environmental pollutants, and our results clearly confirmed such hypothesis.

The preliminary investigation carried out during the winter to evaluate the PAHs levels onto microplastics particles collected along three local beaches did not show differences between site, and levels of PAHs ranged from 160 to10000 ng/g. This strong heterogeneity is in agreement with results from other studies worldwide, and underlines the high variability that can occur between different typologies and ageing plastics.

Comparing levels of PAHs found in microplastics with those measured in beached macroplastics, a strong and statistically significant difference was observed both for low and high molecular weight hydrocarbons. These results confirm and underline the higher potential risk of microplastics compared to macrodebris to act as vector of pollutants. Levels of PAHs in virgin and beached macroplastics did not show any difference, allowing to assume a limited adsorption ability of macrodebris.

Using environmentally realistic levels of dissolved pyrene, the concentrations on exposed microplastics markedly increased with a time- and dose- dependent trend. Worthy to note, the comparison of various experimental conditions did not reveal a linear relationship with levels of pyrene dosed in seawater, since the greatest adsorption efficiency was obtained for the Moderate treatment (5 µg/L). Adsorption of pyrene did not particularly differ between PS and PE, and chemical values measured on both polymers were comparable to those previously reported in

plastic pellets from beaches and industrial sites in California, Hawaii and Greece (Rios et al., 2007; Karapanagioti et al., 2011). A similar trend of adsorption was observed for cadmium, with no differences between polymers, highlighting that PE and PS have same adsorbing behaviors for PAHs and metals.

These data support the potential of microplastics in trapping and transport marine pollutants, as already suggested by previous studies on equilibrium kinetics and partition coefficients of several hydrophobic chemicals on various typologies of plastic polymers (Zarfl and Matthies, 2010; Bakir et al., 2012; Lee et al., 2014); a transport model for persistent organic pollutants by microplastics has been recently proposed also for estuarine conditions, demonstrating a relatively little effect of salinity compared to chemical concentration in water, plastic density and particle residence time in estuaries (Bakir et al., 2014b). Further research are needed to evaluate the possibility of those adsorbed pollutants to be released in organisms after the microplastics ingestion and thus their bioavailability.

4. Pollutants bioavailability and toxicological risk from microplastics to marine organisms. [a]

In this part of the study, the effects of short term exposure to microplastics (both virgin and pyrene contaminated) in mussels, *Mytilus galloprovincialis,* and fish, *Mugil cephalus,* were studied, through a multidisciplinary approach. An exhaustive investigation was performed in exposed mussels, while lower number of parameter were analyzed on exposed fish, in which the possibly of microplastics to penetrate the hepatic tissue after the ingestion was evaluated (discussed in the next chapter).

On exposed mussels, the study on tissue localization of microplastics was integrated with measurement of pyrene bioaccumulation and a wide battery of cellular biomarkers, in order to detect the early onset of adverse effects. Such analyzed responses included immunological parameters, lysosomal membrane stability, peroxisomal proliferation, antioxidant defences and oxidative stress biomarkers, neurotoxic effects and onset of genotoxicity; in addition for the first time, effects of microplastics were also investigated at the transcriptomic level through a new *M. galloprovincialis* DNA microarray platform, to better elucidate pathways and molecular mechanisms of action (MOA). Data on pyrene bioaccumulation and biomarker responses have been elaborated within a quantitative Weight Of Evidence, WOE model (Sediqualsoft), providing specific hazard indices and an overall risk evaluation (Piva et al., 2011).

A standardized method (see the next chapter) was used to assess microplastics presence in gastrointestinal tract and liver of exposed fish. Results (see the next chapter) were integrated with chemical analyses and the evaluation of some biomarker to evaluate the early onset of genotoxicity, modification of biotransformation pathways, bile metabolites and neurotoxicity.

[a] Results of this chapter was partially published in Avio C.Get al., 2015a..

Abstract

Microplastics represent a growing environmental concern for the oceans due to their potential of adsorbing chemical pollutants, thus representing a still unexplored source of exposure for aquatic organisms. In this study polyethylene (PE) and polystyrene (PS) microplastics were characterized for their capability to transfer pyrene to mussels *Mytilus galloprovincialis*. Results of the tissue localization revealed microplastics in haemolymph, gills and especially digestive tissues where a marked accumulation of pyrene was also observed. Cellular effects were measured as immunological responses, lysosomal compartment, peroxisomal proliferation, antioxidant system, neurotoxic effects, onset of genotoxicity; gene expression profile was also analysed through a new DNA microarray platform. The study provided the evidence that microplastics adsorb PAHs, emphasizing an elevated bioavailability of these chemicals after the ingestion, and the responsiveness of several molecular and cellular pathways to microplastics. Some preliminary analyses on accumulation, tissue transfer and early biomarker response were also carried out on fish *Mugil cephalus* exposed to both virgin and contaminated PE and PS.

1. Introduction

The global production of plastic dramatically increased in the last decades, from 0.5 million tons/yr^{-1} in 1960 to 280 million tons in 2012 (Plastic Europe 2012). Almost 10% of the annual production ends up into the oceans, and plastic debris accumulation has been reported as a global scale phenomenon for the marine environments, including polar areas and abyssal regions (Barnes *et al*., 2009).

Adverse effects of plastics have been documented in terms of entanglement and physical damages to locomotory, respiratory or digestive appendages in marine mammals, turtles, seabirds and crustaceans (Andrady 2011). In addition, since plastics degrade very slowly, they also act as floating substrates for several organisms, and thus contribute to long-range transport of alien species, representing an additional risk to local biodiversity (Andrady 2011).

In the recent years, a great scientific interest is being directed toward microplastics, i.e. fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derive from macroscopic debris after chemical physical and biological fragmentation (Barnes *et al*, 2009).

Ingestion of microplastics has been demonstrated in various marine organisms with different feeding strategies; this phenomenon may negatively influence both the feeding activity and nutritional value of a plankton-based diet, particularly in those species which cannot discriminate the food source (Moore *et al*., 2001; Browne *et al*., 2008).

Recent evidences also suggest the potential role of microplastics as vectors of chemical pollutants, either used as additives during the polymer synthesis, or adsorbed directly from seawater (Rios *et al*., 2007; Teuten *et a*l., 2009; Engler 2012). The hydrophobicity of organic xenobiotics and the large surfaces of floating polymers facilitate the adsorption of these chemicals on microplastics at concentrations orders of magnitude higher than those detected in seawater (Ogata *et al*., 2009). The possibility for plastic particles to adsorb chemical pollutants from the surrounding environment has been also characterized in laboratory conditions. Different particles polymers, like polyvinyl chloride, polyethylene, polypropylene, polystyrene, were shown to have a high sorption capacity for DDTs, PAHs, hexachlorocyclohexanes and chlorinated benzenes (Bakir *et al*., 2012; Lee *et al*., 2014). Consistent with these studies, several persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, polycyclic aromatic hydrocarbons (PAHs) and dioxins have been detected in plastic pellets stranded on different beaches of the world (Endo *et al*., 2005; Ogata *et al*., 2009; Hirai *et al* 2011; Heshett *et al.,* 2012).

Despite the importance of microplastics in adsorption and transport of hydrophobic pollutants, it is still unclear whether they also represent a potential source of chemical exposure

within marine food webs. Various evidences, including the use of a thermodynamic approach and of models simulating physiological conditions in the gut, suggested that both adsorbed pollutants and chemical additives of plastics might be released to organisms (Gouin *et al.,* 2011; Tanaka *et al*., 2013; Bakir *et al*., 2014a).

In laboratory conditions, microplastics have been shown to be ingested by amphipods, barnacles, and lugworms (Thompson *et al*., 2004); in mussels*, Mytilus edulis*, plastic particles (3-9.6 µm) were accumulated in digestive tissues and translocated to haemolymph (Browne *et al.,* 2008). In the same organisms, the uptake of microplastics caused notable histological changes in digestive cells with strong inflammatory responses, formation of granulocytomas and lysosomal destabilization which increased with exposure time (Von Moos *et al*., 2012). Oliveira et al., in 2013 through an exposure of common goby to microplastics both alone and with pyrene, reported some modulation in bioaccumulation and biotraformation pathway of pyrene, a decrease of energy and a clear inhibition of AchE activity.

To further assess the possible risk of microplastics as environmental contaminants, the present investigation aimed at a multidisciplinary approach to characterize the chemical adsorption of hydrophobic pollutants, as well as bioaccumulation, chemical release and onset of potential health effects in the filter feeding mussels *Mytilus galloprovincialis*. Two different polymers, polyethylene (PE) and polystyrene (PS) were exposed to various doses of pyrene, selected as one of the more commonly represented PAHs adsorbed on plastic marine debris (Rios *et al.,* 2007); virgin and contaminated PE and PS were then used in a trophic transfer experiment with mussels. Tissue localization of microplastics was integrated with measurement of pyrene bioaccumulation and a wide battery of cellular biomarkers to detect the early onset of adverse effects. Such analyzed responses included immunological parameters, lysosomal membrane stability, peroxisomal proliferation, antioxidant defences and oxidative stress biomarkers, neurotoxic effects and onset of genotoxicity; for the first time, effects of microplastics were also investigated at the transcriptomic level through a new *M. galloprovincialis* DNA microarray platform, to better elucidate pathways and molecular mechanisms of action (MOA).

Data on pyrene bioaccumulation and biomarker responses have been elaborated within a quantitative Weight Of Evidence, WOE model (Sediqualsoft) which has been validated to integrate and differently weight various typologies of data, or lines of evidence (LOEs), providing specific hazard indices and an overall risk evaluation (Piva *et al*., 2011). The Sediqualsoft model has been previously applied to different multidisciplinary studies for the characterization and classification of risk from industrial and harbour sediments, the assessment of environmental hazards in coastal

areas exposed to natural seepage or the recent Costa Concordia wreck at Giglio Island (Piva *et al*., 2011; Benedetti *et al*., 2012, 2014; Regoli *et al*., 2014).

Fishes, *Mugil cephalus*, were exposed to both virgin and contaminated PE and PS particles to assess the presence of microplastics in gastrointestinal tract and liver of exposed organisms. Results of tissues examination (see the next chapter) were integrated with chemical analysis and the evaluation of some biomarkers to evaluate the early onset of genotoxicity, modification in the activity of cytochrome P450, the presence of biliary metabolites and neurotoxicity.

The overall results of this study were expected to increase our knowledge on the potential toxicological risk of microplastics in the marine environment.

2. Materials and methods

2.1 *Experimental design for mussels*

Polyethylene (PE) and polystyrene (PS) powders were obtained from a private plastic company. Particles were size-sorted in <100 µm group for the exposure of mussels to virgin and contaminated polymers while particles between 100µm and 1mm were used for fish exposure.

For the exposure of mussels to microplastics, specimens of *Mytilus galloprovincialis* (5 ± 1 cm shell length) were obtained from a local farm (Numana, Ancona) and acclimatized for 10 days to laboratory conditions with aerated seawater, at 18 ± 1 °C and 35 ‰ salinity. Contaminated plastics were prepared by maintaining a solution of <100µm microplastics (20 g/L in seawater) with pyrene dosed at final concentration of 5 µg/L in rotating conditions for 6 days. A total of 150 organisms were distributed into fifteen 6L glass-beakers and exposed to virgin or contaminated plastics for 7 days with three replicates for each of the 5 following treatments: Control (CNTR), Polyethylene (PE), Polystyrene (PS), Pyrene-treated Polyethylene (PE-PYR), Pyrene-treated Polystyrene (PS-PYR). Water was changed daily and both virgin and pyrene-treated particles re-dosed at a nominal concentration of 1.5 g/L. No mortality of mussels was observed during the experiments. After the exposure period, haemolymph, digestive glands and gills were rapidly removed from 30 specimens for each treatment, pooled in 10 samples (each with tissues of 3 specimens), frozen in liquid nitrogen and maintained at -80°C for chemical, biochemical and histochemical analyses; for haemolymph samples, an aliquot was also immediately processed for lysosomal neutral red retention time assay (NRRT), phagocytosis activity, and DNA damage, and another aliquot fixed in Carnoy's solution (3:1 ethanol, acetic acid) for the microscopic evaluation of granulocytes and chromosomal alteration. Four additional pools, each with digestive glands of three specimens, were prepared from CNTR, PS and PS-PYR groups for DNA microarray analysis.

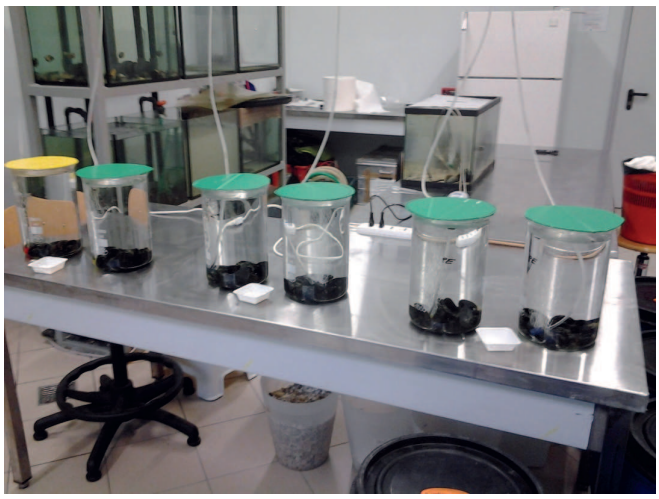


Figure 4.1. Photo of mussels exposure system.

2.2 Chemical analyses of pyrene exposed organisms

Pyrene accumulated in mussels tissue (gills and digestive glands) and fish (liver and gills) was determined after extraction of samples in 0.5 M potassium hydroxide and methanol (1:10 w:v) with microwave at 55°C for 15 min (Benedetti *et al*., 2014). After centrifugation for 5 min at 1000 × g, the methanolic solutions were concentrated in speedvac and 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, and HPLC analyses were carried out with water–acetonitrile gradient and fluorimetric detection. Pyrene was identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). Quality assurance and quality control were tested by 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 pyrene expressed as ng/g dry weight (d.w.).

2.3 Biological analyses in mussel tissues

Presence and histological localization of plastic particles were evaluated in cryostatic sections (8 µm thick) of gills and digestive glands, and on haemolymph smears. After staining with haematoxilyn and eosin, slides were observed with 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 (Gorbi *et al*., 2013, Benedetti *et al*., 2014). Detailed procedures for all presented biomarkers have been described elsewhere (Regoli and Winston, 1998; Bocchetti et al., 2008; Gorbi et al., 2013; Benedetti et al., 2014).

For the analysis of granulocyte–hyalinocytes ratio, aliquots of haemolymph were dispersed on glass slides fixed in Beker's fixative (+2.5% NaCl), stained with May Grunwald Giemsa and observed with a light microscope (Gorbi et al., 2013).

Phagocytosis capacity assay was microscopically evaluated in haemolymph incubated for 2 h with Fluorescein-labelled Zymosan A bioparticles (Invitrogen), added at 10:1 target:haemocyte ratio; phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particle (Gorbi et al., 2013).

For the Neutral Red Retention Time (NRRT) assay, haemocytes were incubated with a Neutral Red solution and microscopically examined at 15 min intervals, to determine the time at

which 50% of cells had lost to the cytosol the dye previously taken up by lysosomes (Gorbi et al., 2013).

The activity of peroxisomal Acyl-CoA oxidase (AOX) was measured in mussels digestive glands with a coupled assay following the production of H_2O_2 by the oxidation of dichlorofluorescein-diacetate in the presence of an exogenous horseradish peroxidase (Bocchetti et al., 2008).

Acetylcholinesterase activity (AChE) was spectrophotometrically assayed in mussels hemolymph and gills using the Ellman's reaction (Bocchetti et al., 2008).

Antioxidants defenses were measured in mussels digestive glands following standardized assay conditions, at a constant temperature of 18 ± 1°C (Bocchetti et al., 2008). Catalase was determined by the decrease in absorbance due to H_2O_2 consumption; glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where β-nicotinamide adenine dinucleotide (NADPH) is consumed by glutathione reductase to convert the oxidized glutathione (GSSG) to its reduced form; glutathione S-transferases (GST) were determined following the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate; glutathione reductase (GR) activity was measured by the oxidation of NADPH during the reduction of GSSG; levels of total glutathione were enzymatically assayed after acidic deproteinization with sulphosalicilic acid.

The Total Oxyradical Scavenging Capacity (TOSC) was measured in mussels digestive glands by the capability of cellular antioxidants to inhibit the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas in the presence of different forms of oxyradicals, like peroxyl radicals (ROO·) and hydroxyl radicals (HO·) which are artificially generated at constant rate (Regoli and Winston, 1998). Ethylene formation was determined by gas-chromatographic analyses and TOSC values were quantified from the equation: TOSC = 100-(∫SA/ ∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (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 and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard (Regoli and Winston, 1998).

Lysosomal membrane stability was measured in mussel cryostat sections (10 μm) by determining the time required to artificially labilize the membranes at pH 4.5 (Gorbi et al., 2013). The acid hydrolase B-N acethylhexosaminidase was tested using naphthol AS-BI-N-actyl-B-D glucosamide as substrate, and the maximum staining intensity was assessed under light microscopy at different incubation times (0, 3, 5, 10, 20, 25, 30, 40 min). Lipofuscin content in mussels digestive gland was determined on cryostat sections (10 μm thick) stained by Schmrol reaction,

while neutral lipids were determined with the Oil Red O (ORO) method (Gorbi et al., 2013). For both lipofuscin and neutral lipids, five measurements were made on digestive tubules of each section (two sections for mussel, 10 mussels for sampling site). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

The content of malondialdehyde (MDA) was measured in homogenates of mussels digestive glands derivatized with 1-metyl-2-phenylindole and spectrophotometrically determined after calibration against a malondialdehyde standard curve (Bocchetti et al., 2008).

The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the Comet assay, and at chromosomal level by the micronucleus test. The comet assay was carried out on mussels haemocytes included in 1% normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (Benedetti et al., 2014); 100 randomly selected "nucleoids" per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51), and the captured images (Image-Pro-Plus package) were analyzed by the Comet Score software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

The micronucleus (MN) frequency was measured in mussels haemocytes rapidly washed in saline buffer, fixed in Carnoy's solution (3:1 methanol: acetic acid), dispersed on glass slides and stained with the fluorescent dye 4',6-diamidino- 2-phenylindole at 100 ng ml^{-1}. 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 (Benedetti et al., 2014).

2.4 Mytilus galloprovincialis oligonucleotide microarray

To increase *M. galloprovincialis* transcriptomic information, Roche 454 sequencing of hemocytes, oocytes, larval stages (trochophores, veliger, pediveliger, settled larvae, one moth seed) and adult tissues (gills, foot, mantle, muscle, digestive gland, gonads) have been performed. A total of 1,325,571 reads have been obtained and assembled through MIRA3 (default parameters). Assembly produced a total of 103,995 transcripts. A preliminary annotation was obtained for 43,218 contigs (41.5%) through Blast similarity searches conducted against several protein databases (e-value 1 E-5). Additional information about cDNA libraries preparation, sequencing and transcriptome characterization will be provide in a dedicated paper discussing *M. galloprovincialis* larval development (Novoa et al. in preparation).

All databases used for the annotation step were considered for DNA microarray platform design. Putative sense-strand orientation was inferred from the matching protein-coding gene in reference data bases. For 1,803 contigs that showed ambiguous orientation two probes with opposite orientations (sense and antisense) were designed. For the remaining 41,415 contigs with putatively unambigous orientation a single (sense) probe was designed. Since the microarray format could accommodate approximately 60,000 probes, the longer (bp) non-annotated contigs were included in the microarray design. In total 7,488 non-annotated contigs were added, and for each of them, two probes with opposite orientation (sense and antisense) were designed. Probe design was carried out using the Agilent eArray interface (https://earray.chem.agilent.com/earray/), which applies proprietary prediction algorithms to design 60-mer probes. A total of 59,971 out of 59,997 probes were successfully obtained, representing 50,680 different *M. galloprovincialis* contigs.

2.5 Labelling, microarray hybridization and data acquisition

For each sample 100 ng of total RNA was linearly amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling to monitor microarray analysis work-flow. Labeled cRNA was purified with QiagenRNAeasy Mini Kit, and sample concentration and specific activity (pmolCy3/µgcRNA) were measured in a NanoDrop® ND-1000 spectrophotometer. A total of 600 ng of labeled cRNA was prepared for fragmentation by adding 5µl 10X Blocking Agent and 1µl of 25X Fragmentation Buffer, heated at 60°C for 30 min, and finally diluted by addition with 25µl 2X GE Hybridization buffer. A volume of 40µl of hybridization solution was then dispensed in the gasket slide and added to the microarray slide (each slide containing eight arrays). Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

Hybridized slides were scanned at 2µm resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together and data was extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software version 10.7.3.1. The software returns a series of spot quality measures in order to evaluate the quality and the reliability of spot intensity estimates.

2.6 Microarray data processing and analysis

Statistical analyses were performed on 52.988 out of 59.971 probes with signal higher than the background in 8 out of 11 analysed samples. The TIGR Multi Experiment Viewer 4.5.1 statistical software (TMeV; Saeed *et al*., 2003) was used to perform T-test statistics (p-value<0.01; 200 permutations) comparing CNTR to both PS and PS-PYR groups. The resulting T-test genes lists were then filtered and only probes with fold change (FC) > 1.5 have been considered as differentially expressed genes (DEGs). A more systematic functional interpretation of differentially transcribed genes was obtained through an enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Huang *et al*, 2009). Since these databases contain functional annotation data for a limited number of species, transcripts of *M. galloprovincialis* were matched to *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10-5). The choice of *D. rerio* allowed the assignment of a putative homologue to a larger number of *M. galloprovincialis* transcripts, and was previously demonstrated a useful option for *Ruditapes philippinarum* functional analyses (Milan *et al*., 2011). A functional annotation was obtained for genes differentially expressed in each T-test pairwise comparison, setting DAVID for gene count=2 and ease=0.1.

2.7 Experimental design for fish exposure.

A total of 50 acclimatized mullets *Mugil cephalus* (26.3 ± 2.1 cm length) were randomly assigned and maintained for 7 days in one of five 80 l glass-tanks (10 fish per tank) corresponding to control tank (CNTR), polyethylene treatment (PE) and polystyrene treatment (PS), pyrene-contaminated polyethylene (PE-PYR), pyrene-contaminated polystyrene (PS-PYR). Particles, with size between 0.1 and 1mm, were given at a nominal concentration of 0.03375 g/L, corresponding to nearly 2500 particles/L. The exposure concentration is not representative of a realistic environmental conditions, although nearly fifty times lower than those previously used in other experimental studies (i.e. Thompson et al., in 2004; Van Moos et al., 2012; Avio et al., 2015). For indications on the exposure conditions and guideline to prevent external contamination see section Material and Methods in the next chapter.

Fish were daily fed and sacrificed at the end of the experiment; kidney, livers and gastrointestinal tracts rapidly removed, frozen in liquid nitrogen and maintained at -80°C until the analyses while a blood aliquot immediately processed for the COMET assay to evaluate the DNA fragmentation. The gallbladder was removed intact and centrifuged (2350×*g* for 2 min) to release the bile fluid. Although bile metabolites are very stable, samples were frozen in liquid nitrogen and

stored at −80 °C. Liver tissue was used to analyzed pyrene bioaccumulation and the activity of cytochrome P450. Lastly, activity of acetylcholinesterase was evaluated in kidneys of exposed fish to evaluate the onset of neurotoxicity

2.8 Biochemical analyses in fish

The analysis of 7-ethoxyresorufina O-deethylase (EROD) was carried out according to Regoli et al., 2003 in the S9 fraction of liver homogenized (1:5) in 100mM K-phospate buffer pH 7.5, containing 0.15 M KCl and 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 12,000g for 15 min, the resulting supernatants (S9) were immediately used for determination of enzyme activities. Incubations were carried out at 30 °C in a final volume of 1 ml containing 100 mM K-phosphate buffer pH 7,5, 4 μM 7-ethoxyresorufin and 0,25 mM NADPH for 5 min, before addition of 2 ml acetone to stop the reaction. Incubation mixtures as described above, but stopped at time zero, were used as blank values and subtracted from the sample fluorescence. Fluorimetric analyses (535/585 nm) were quantified by reference to resorufin standards (0.02–1 μM).

Presence of aromatic metabolites in the bile was determined by the fixed fluorescence (FF) spectrofluorimetric assay (Gorbi et al., 2005). Bile samples were diluted in 48% ethanol between 1:2000 and 1:8000 to obtain linear responses of fluorescent readings. Excitation/emission wavelengths were 341/383 nm for pyrene-type metabolites, using 1-OH-pyrene as reference standards (Gorbi et al., 2005). Results were expressed as metabolites-type ng or μg/ml of bile.

Acetylcholinesterase (AChE) activity was analyzed in brains 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 DNA integrity was evaluated at molecular level as single strand breaks (SB) by the Comet assay, and at chromosomal level by the micronucleus test. The comet assay was carried out on blood cells immediately diluted in Ca^{2+}/Mg^{2+}-free buffer, centrifuged, resuspended in 0.6% low-melting-point agarose, and included in 1% normal-melting-point agarose on glass slides (Frenzilli et al., 2001). Slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10, 4 °C in the dark for 90 min), and the DNA was unwound in 75 mM NaOH, 10 mM EDTA (pH 13) before the electrophoresis (1 V/cm for 10 min). After neutralization and fixation, slides were stained with SYBR Green 1 (Molecular Probes), and 100 randomly selected cells per slide and two replicates per sample were observed under a fluorescence microscope (400 × magnification; Olympus BX-51), and the captured images (Image-Pro-Plus

package) were analyzed by the Comet Score software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

2.8 Statistical analyses and risk assessment

Statistical analyses were performed with the statistical R-software (R. development Core Team, 2010). Adsorption of pyrene to microplastics was tested by analysis of variance (ANOVA) according to typology of polymer (PE-PS), time of exposure (3–6 d), dose of pyrene (L, M, H); bioaccumulation of pyrene and biomarker responses in exposed mussels were also compared by one-way ANOVA and post-hoc comparison (Bonferroni) was used to discriminate between means of values. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary. For biomarkers data, multivariate principal component analysis (PCA) was combined to hierarchical clustering of the PCA patterns which visualize the relationships among the different treatments and organize the samples into groups of homogeneous observations (Husson et al., 2010). The proposed methodology is available in the HCPC (Hierarchical Clustering on Principal Components) function of the FactoMineR package (Lê et al., 2008).

Results on bioaccumulation of pyrene and biomarkers responses in mussels exposed to virgin and contaminated microplastics were further elaborated within a classical Weight Of Evidence WOE approach, using a previously developed quantitative and software-assisted model (Sediqualsoft). According to WOE principles, different typologies of data are initially evaluated with appropriate criteria to provide synthetic indices of hazard for each of considered line of evidence, before their final integration in a quantitative WOE evaluation (Piva et al., 2011). 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,Benedetti et al., 2014 and Regoli et al., 2014).

Briefly, the bioavailability hazard was calculated from the initial calculation of a weighted Ratio to Reference (RTRw), reflecting the magnitude of pyrene accumulation in tissues of exposed organisms, corrected for both the statistical significance of the difference compared to controls and the typology of chemical. Depending on the magnitude of such variations, the model assigns the hazard to 1 of 5 classes: from Absent to Slight if the calculated increase of pyrene tissue concentration is lower than 2.6 folds compared to control organisms, Moderate between 2.6 and 6.5 folds, Major between 6.5 and 13 folds, Severe if greater than 13 folds (Piva et al., 2011 and Benedetti et al., 2012).

For the evaluation of biomarkers results, the model contains a large selection of responses among those more widely used by scientific community in different bioindicator organisms (Piva et al., 2011); according to species and tissue, each biomarker, has a "weight" based on the relevance of biological endpoint, and a "threshold" for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold (Absent or Slight), calculates the average for those with an effect up to two-fold compared to the threshold (Moderate) and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva et al., 2011):

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

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva *et al*., 2011).

The hazard indices elaborated from bioavailability and biomarkers are normalized to a common scale and finally integrated within a classical weight of evidence approach which assigned the level of risk to 1 of 5 classes from Absent to Severe (Piva *et al*., 2011).

3. Results

3.1 Mussels exposure

Concentrations of pyrene on contaminated microplastics used for the laboratory experiments with mussels (< 100 µm) were in the range of 200-260 ng/g for both PE and PS. After 7 days of exposure, a significant increase of pyrene was observed in gills, and a much more marked bioaccumulation occurred in digestive glands, with tissue levels even greater than those measured directly on contaminated polymers (Figure 4.2).

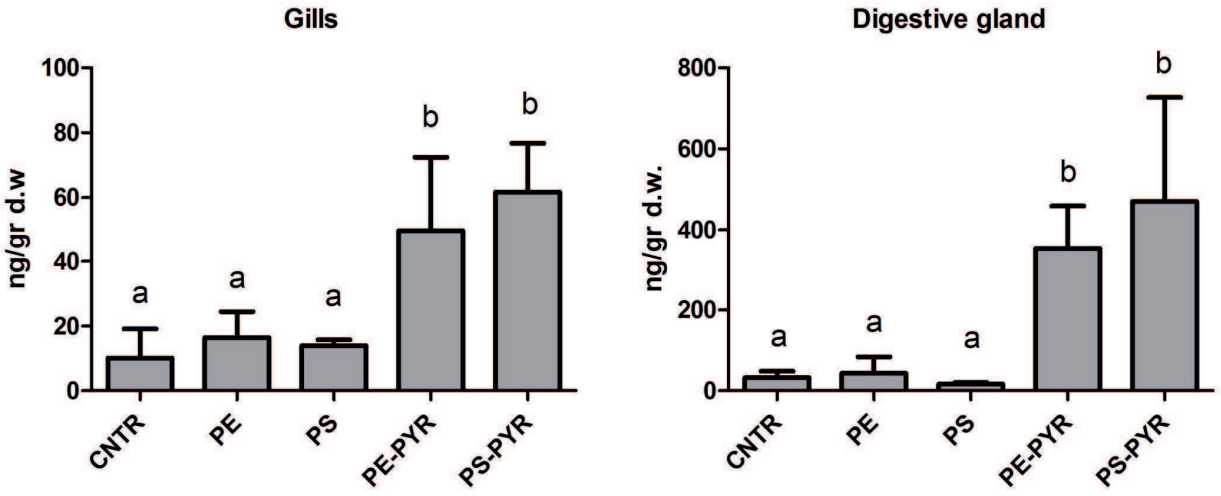


Figure 4.2. Concentrations of pyrene in gills and digestive glands of mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Data are expressed as ng/g dry weight (mean values ± standard deviation, n=5); different letters indicate significant differences between groups of means (post-hoc comparison).

Histological analyses of treated mussels revealed the presence of microparticles in haemolymph, gills and, especially, in digestive glands where numerous aggregates could be observed in the intestinal lumen, epithelium, and tubules (Figure 4.3). No qualitative differences in tissue localization were evident between organisms treated with the two polymers (PE, PS), both as virgin or contaminated particles.

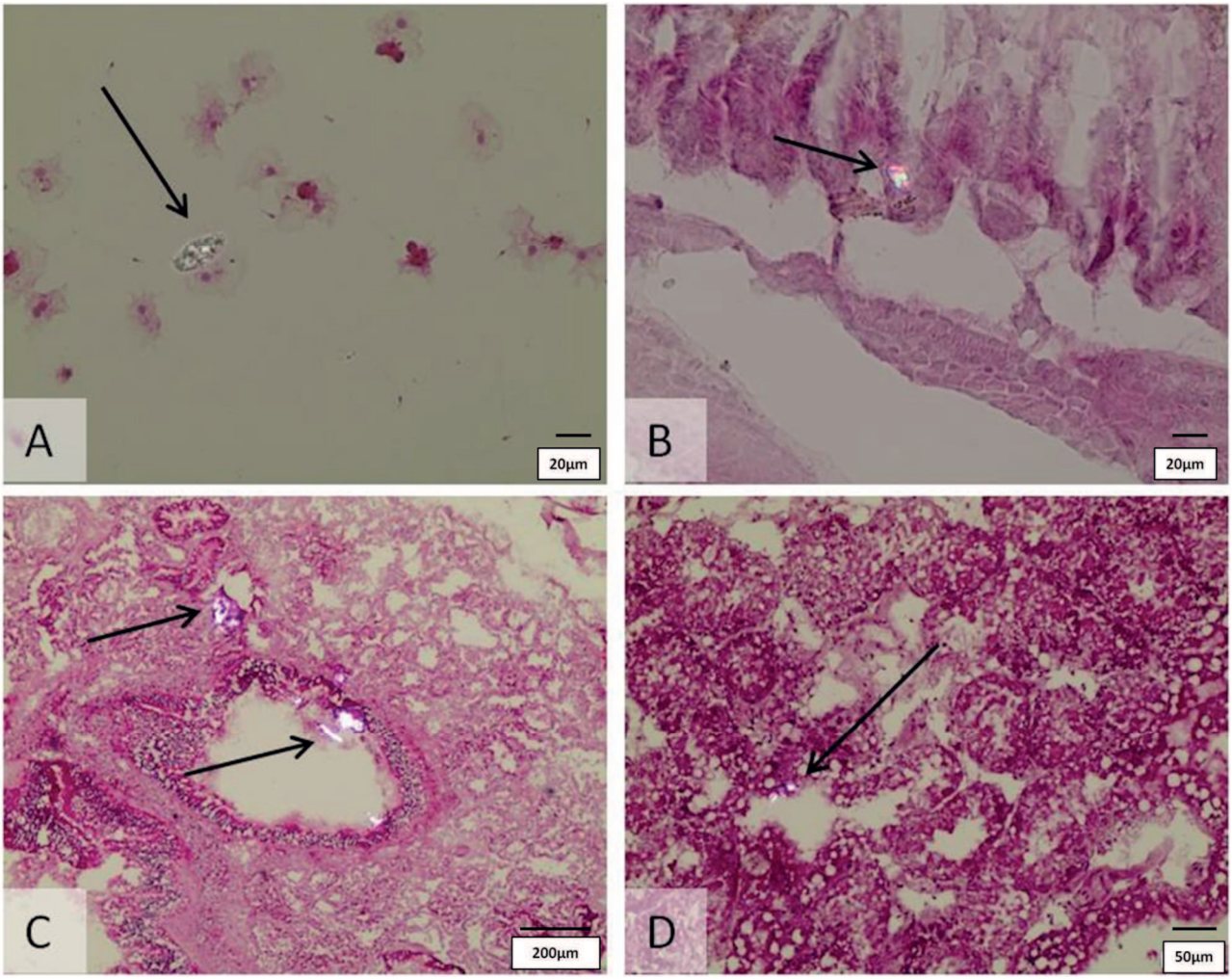


Figure 4.3. Polarized-light microscopy images showing the presence of plastic particles in haemolymph (A), gills (B), gut lumen and epithelium (C), digestive tubules (D).

Among immunological responses of haemocytes, phagocytosis activity did not exhibit particular variations, while a significant decrement of granulocytes versus hyalinocytes type cells was observed in mussels exposed to virgin polymers and to pyrene-contaminated PE; lysosomal membrane stability decreased in all the treatment groups (Figure 4.4).

The comet assay indicated a significant enhancement of DNA strand breaks in haemocytes of mussels treated with virgin microplastics, while nuclear anomalies were higher in all the treatments with either virgin or contaminated polymers; the frequency of micronuclei significantly increased only in specimens exposed to pyrene-treated PS (Figure 4.4). Acetylcholinesterase did not vary in haemolymph and decreased in gills of mussels exposed to both virgin and contaminated microplastics, while activity of AOX was not influenced in any of experimental treatments (Figure 4.4).

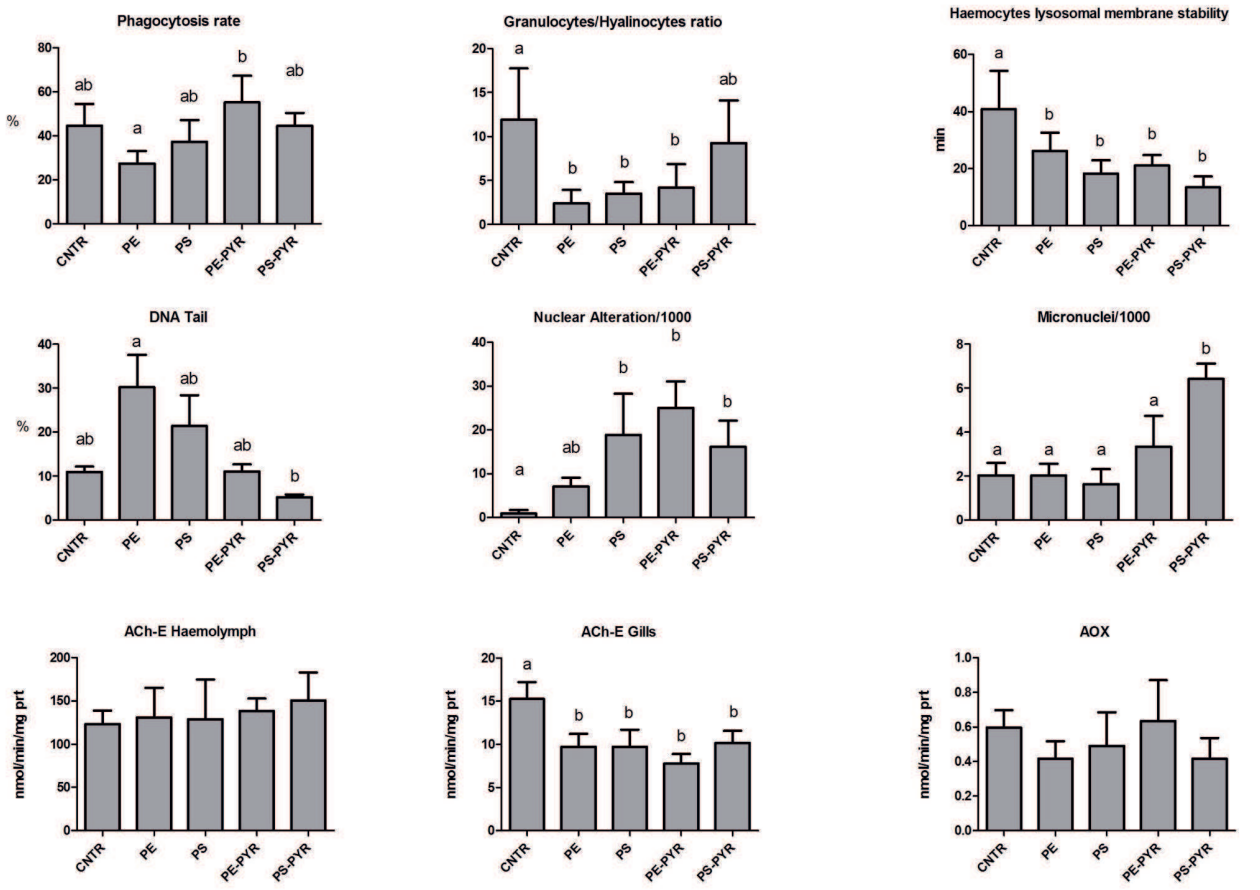


Figure 4.4. Immunological, genotoxic, cholinesterasic and peroxisomal biomarkers in mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Data are expressed as mean values ± standard deviation or standard error of mean for the % of DNA in tail, n=5; different letters indicate significant differences between groups of means (post-hoc comparison).

Antioxidant defenses did not reveal variations in the levels of glutathione and activities of glutathione reductase, glutathione S-transferases, and sum of Se-dependent and Se-independent glutathione peroxidases (Figure 4.5). A significant inhibition was observed in all the treatments for Se-dependent glutathione peroxidases and catalase, the overall significance of those effects being reflected in slight variations of the Total Oxyradical Scavenging Capacity toward both peroxyl and hydroxyl radicals (Figure 4.5).

The moderate pro-oxidant challenge induced by microplastics on mussels was supported by the lack of relevant variations for malondialdehyde, lipofuscin and neutral lipids in digestive tissues; lysosomal integrity appeared more sensitive, and decreased after exposure to both virgin and contaminated microplastics (Figure 4.5).

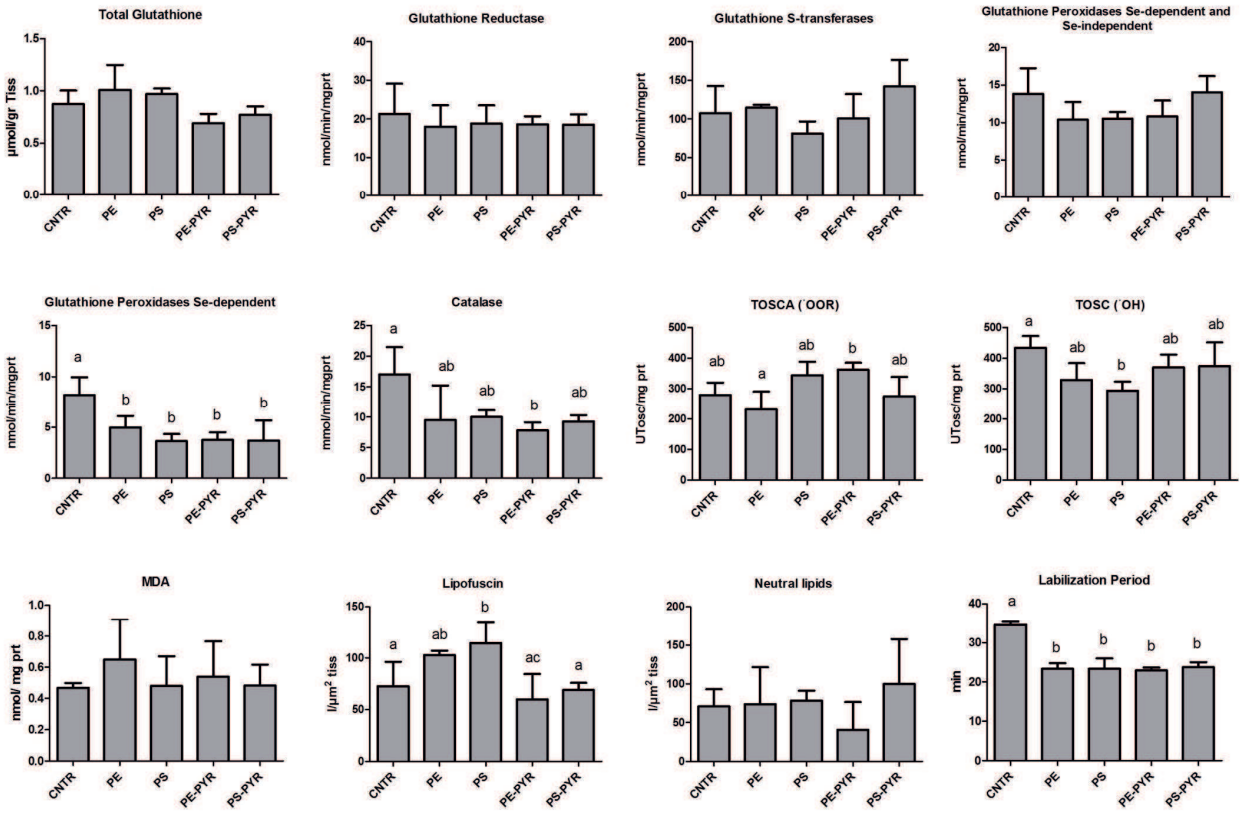


Figure 4.5 Antioxidant defenses, total oxyradical scavenging capacity (TOSCA) toward peroxyl (·OOR) and hydroxyl (·OH) radicals, malondialdehyde, lipofuscin, neutral lipids and lysosomal membrane stability in digestive gland of mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Data are expressed as mean values ± standard deviation, n=5; different letters indicate significant differences between groups of means (post-hoc comparison).

The principal component analysis (PCA) carried out on the whole set of biomarkers produced a two dimensional pattern explaining 72 % of total variance (Figure 4..6). The plot score indicated a clear separation between control mussels and those exposed to either virgin or pyrene-treated microplastics; the parameters determining the separation along to the PC1 axis were lysosomal membrane stability in haemocytes and digestive glands, AChE in gills and some antioxidant responses (catalase, glutathione reductase, Se-dependent glutathione peroxidase, TOSC-HO·). On the other side, genotoxic effects (DNA strand breaks, nuclear anomalies, micronuclei), phagocytosis, AChE in haemolymph and levels of glutathione determined the separation along the PC2 axis between mussels exposed to virgin compared to pyrene-contaminated microplastics (Figure 4.6); the typology of polymer (PE vs PS) did not appear to influence the observed responses.

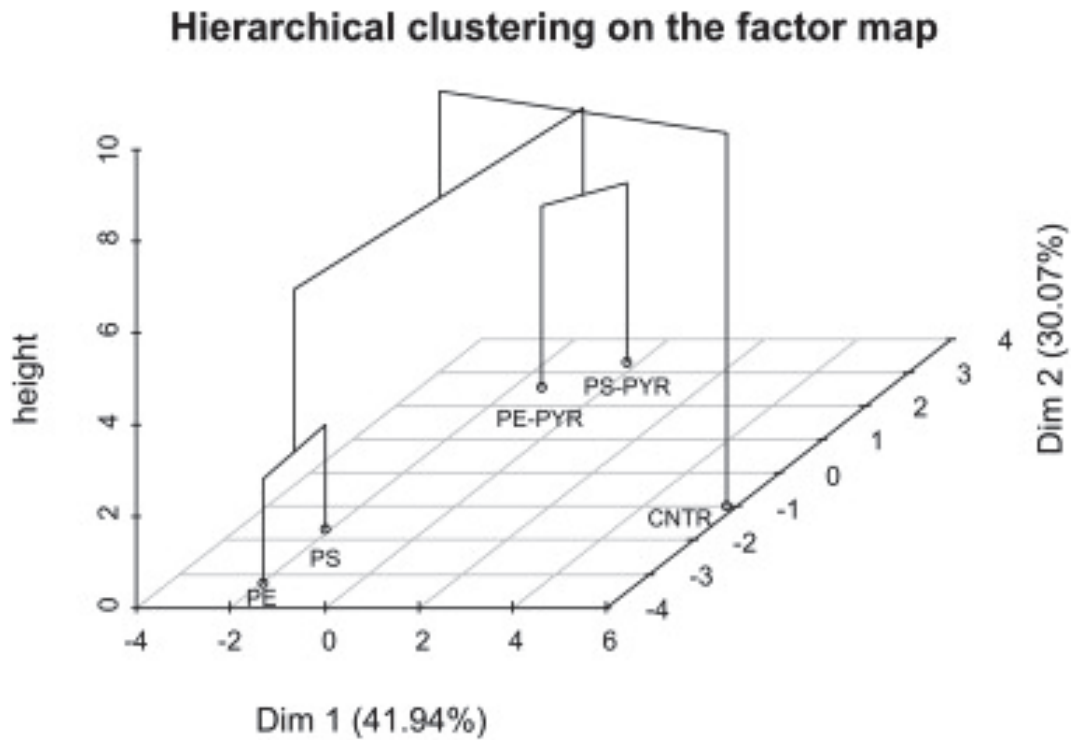


Figure 4.6. Hierarchical clustering analysis on the factor map, carried out on the all set of biological parameters.

The analysis of transcriptional responses revealed a total of 2.143 and 1.320 differentially expressed genes (DEGs, $p<0.01$; FC>1.5) in response to PS and PS-PYR exposures, respectively. Among these, 280 transcripts were significantly affected after both exposures (Figure 4.7), but the majority of transcripts appeared specifically modulated within each treatment (1.863 in PS and 1.040 in PS-PYR). Functional annotation and enrichment analysis was applied to DEGs to highlight the most significantly affected Biological Processes (BP), Molecular Functions (MF), Cellular Component (CC) and KEGG pathways (KP).

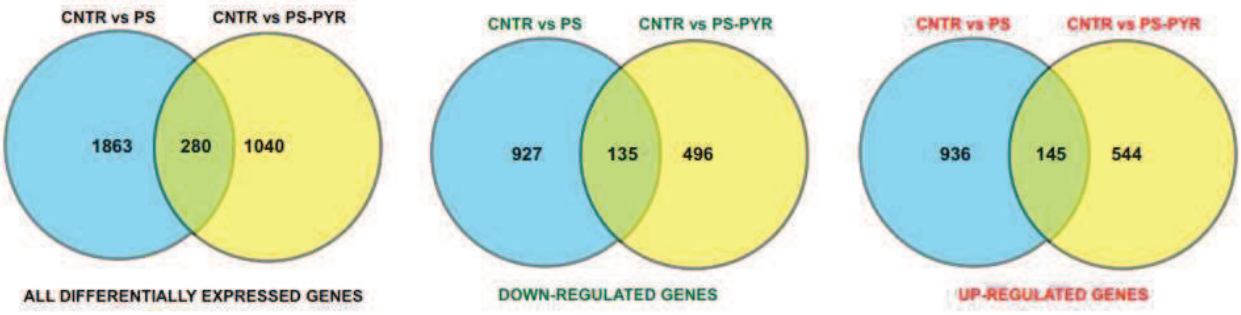


Figure 4.7. Venn diagrams representing differentially expressed transcripts (total, down-regulated and up-regulated) in the comparisons CNTR vs PS and CNTR vs PS-PYR.

Some of the most interesting enriched KEGG pathways/GO terms are reported in Table 4.1: Lysosome (with 16 and 15 DEGs in PS and PS-PYR exposed mussels respectively), Coated membrane (9 and 6 DEGs), Endosome (6 and 3 DEGs), NOD-like receptor signalling pathway (4

and 7 DEGs), Response to bacterium (5 and 3 DEGs), Apoptosis (7 and 8 DEGs), Regulation of programmed cell death (5 and 8 DEGs), Citrate cycle (8 and 3 DEGs) and Arachidonic acid metabolism (5 and 3 DEGs).

Beside the above mentioned GO terms and KEGG pathways, mussels exposed to PS and PS-PYR showed the modulation of several genes involved in DNA repair (i.e. *growth arrest and DNA-damage-inducible protein, GADD45A* and *GADD45G*; *excision repair cross-complementing rodent repair deficiency, complementation group ERCC*; *aprataxin, APTX*), detoxification (i.e. *Glutathione S-transferase pi, GSTP1* and *GSTP2*; *glutathione S-transferase M*, *GSTMU*; *sulfotransferase family 4A, member 1,* S*ULT4A1*) and oxidative processes (i.e. *glutathione peroxidase, GPX2* and *GPX3*; *superoxide dismutase mitochondrial,* SOD2).

CNTRvsPS			CNTRvsPS-PYR		
GO_TERM/KEGG	N° DEGs	GENE NAME	GO_TERM/KEGG	N° DEGs	GENE NAME
dre04142:Lysosome	16	MAN2B1, AGA, CTSLA, CTNS, PSAP, NPC1, CTSC, ATP6V0B, GGA1, LGMN, CLTCA, CLTA, CTSBA, CTSD, AP1S2, CTSBB	dre04142:Lysosome*	15	CD164, CTSLA, AGA, CTNS CD63, GLB1 GGA1, LGMN AP3S2, CTSZ CTSBB, AP1S2 PSAP, LDLR, CTSBA
GO:0048475~coated membrane	9	COPA, COPE, SEC23B, GGA1 COPB2, CLTCA CLTA, COPB1, AP1S2	GO:0048475~coated membrane*	6	LDLR , AP2S1, GGA1, AP3S2, COPB1, AP1S2
GO:0005768~endosome	6	CHMP2BB, CHMP4B, CHMP1A, SNX5 RAB5C,TMEM55B	GO:0005768~endosome	3	CHMP1A, CHMP6B, VPS29
dre04621:NOD-like receptor signaling pathway	4	XIAP, SGUT1, BIRC2, BIRC7	dre04621:NOD-like receptor signaling pathway*	7	HSP90B1, NFKBIAB, XIAP TRAF6, BIRC2, BIRC7
GO:0009617~response to bacterium	5	PGLYRP6, PGLYRP2, CTSD, RHOGB, PCNA	GO:0009617~response to bacterium	3	RHOGB, MYD88, TRAF6
dre04210:Apoptosis	7	DFFB, BAXA BIRC2, BIRC7, CASP3A, PRKAR2AA, XIAP	dre04210:Apoptosis*	8	CASP9, NFKBIAB, BCL2L1, MYD88 XIAP,PRKAR2AA, BIRC2, BIRC7
GO:0043067~regulation of programmed cell death	5	CASP3A, BAXA, TRAF3, BIRC2, CASP2	GO:0043067~regulation of programmed cell death*	8	BIRC2, BCL2L1, CRADD, CASP2, CASP9, CCT3, MCL1B, TRAF6
dre00020:Citrate cycle (TCA cycle)	8	DLDH, PCK1, DLAT, MDH1AA, PCK2, IDH3G, ACO1, SDHB	dre00020:Citrate cycle (TCA cycle)	3	DLST, PCK2, IDH3G
dre00590:Arachidonic acid metabolism	5	PLA2G1B, CYP2P9, CPLA2, CYP2U1, ALOX5A	dre00590:Arachidonic acid metabolism	3	TBXAS1PLA2G1B GPX3

Table 4.1. Lists of the main enriched GO terms/KEGG pathways. Numbers and "gene name" of differentially expressed genes (DEGs) in each comparisons/terms are also reported. Down- and up- regulated transcripts in exposed groups are reported in green and red, respectively. Gene names reported in black indicate transcripts represented by multiple probes showing opposite responses.

The overall results on bioaccumulation and biomarker response were finally elaborated within a Weight Of Evidence approach to assess the biological risk of virgin and contaminated microplastics. The proposed WOE model classified as Severe the hazard for pyrene bioavailability from both PE and PS contaminated polymers (Table 2); on the other hand, the significance of biomarkers responses corresponded to a hazard ranging from Slight to Moderate in various treatments, typically higher for PS compared to PE, and for contaminated compared to virgin polymers (Table 2). The final WOE integration summarized as Slight and Moderate the risk from virgin PE or PS respectively, Major or Severe for the contaminated microplastics (Table 4.2).

Treatments	LOE2 (HQ and Class of hazard)	LOE3 (HQ and Class of hazard)	WOE	
PE	-	1.87 Slight	SLIGHT	
PS	-	4.10 Moderate	MODERATE	
PE-PYR	13.01 Severe	6.44 Moderate	MAJOR	
PS-PYR	14.69 Severe	7.20 Moderate	SEVERE	

Table 4.2 Weight Of Evidence classification of bioaccumulation (LOE2) and biomarkers (LOE3) data, and integrated WOE risk in mussels exposed to virgin or pyrene-contaminated microplastics. The quantitative Hazard Quotients (HQ) for individual LOEs and the assigned classes of hazard or WOE risk are given. Treatments: PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene.

3.2 Fish exposure

Chemical analyses of exposed fish showed did not show any evidence of pyrene bioaccumulation neither in gills nor in liver: in the latter tissue, fish exposed to microplastics (both virgin and contaminated) showed level of pyrene approximately 30% lower than in control fish (Figure 4.8).

Exposure to microplastics did not cause the induction of hepatic EROD activity which, similarity to pyrene accumulations was even lowered by exposure to microplastics, both virgin and contaminated (Figure 4.8).

Levels of pyrene metabolites increased in bile of fish exposed to contaminated microplastics both for PE and PS treatment, with lower and not significant effect in virgin plastics treatments.

A higher frequency of DNA strand breaks was observed in all exposed organisms and results appeared significantly affected by co-presence of adsorbed pyrene.

AchE activity did not show any alteration in relation to the exposure treatment, while significant increase of DNA was observed in all exposed groups (Figure4.8).

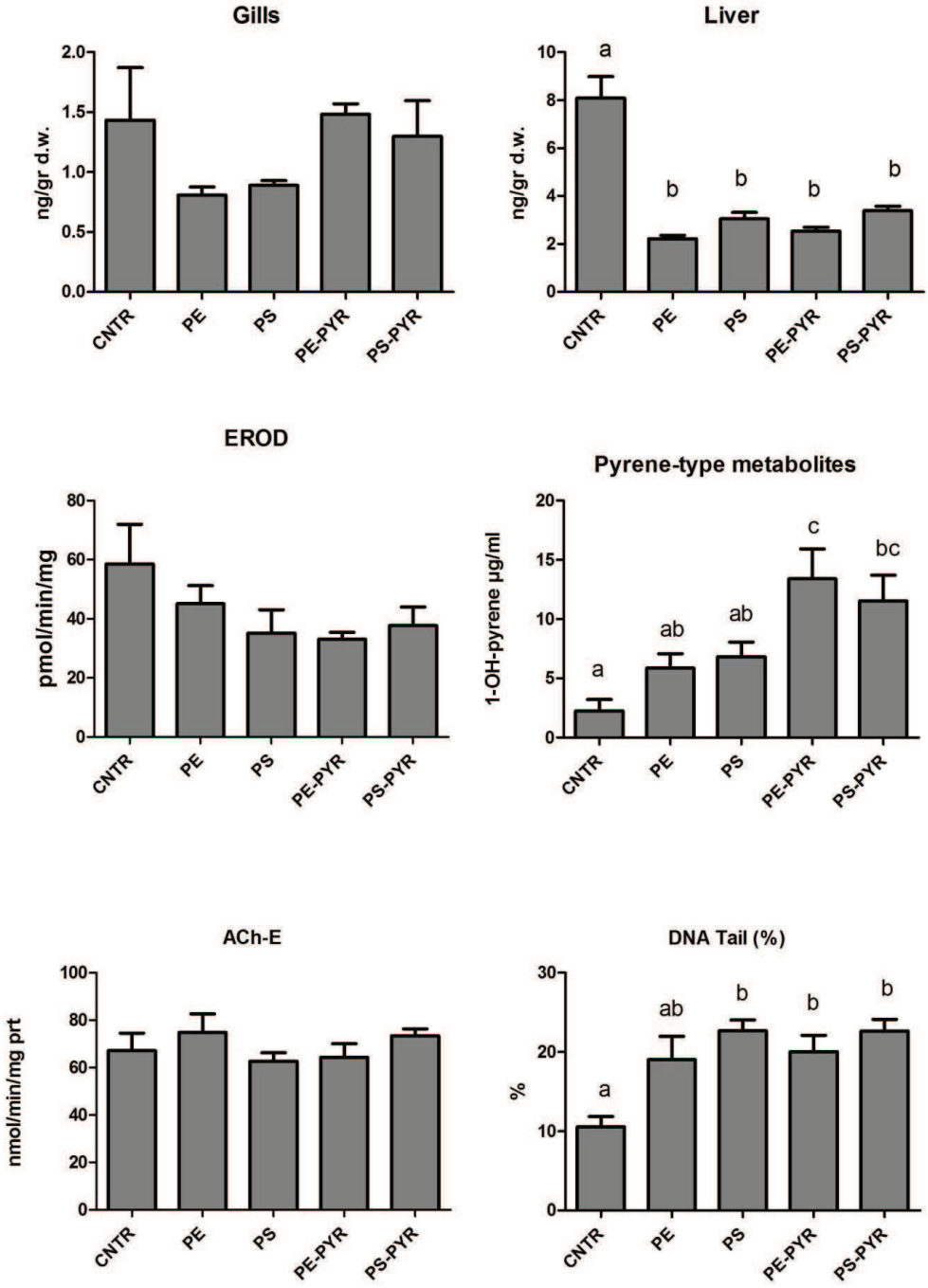


Figure 4.8 .Pyrene level on gills and livers of exposed fish, EROD activity, Bile metabolites (Pyrene), cholinesterase activity and genotoxic effects of various microplastics treatments to exposed fish: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-

PYR= pyrene-contaminated polystyrene. Data are expressed as mean values ± standard deviation or standard error of mean for the % of DNA in tail, n=5; different letters indicate significant differences between groups of means (post-hoc comparison).

4. Discussion

The present investigation aimed to provide new insights on the potential role of microplastics as a source of chemical exposure and ecotoxicological challenge to marine organisms. A growing concern is being raised for the possibility of these polymers to adsorb environmental pollutants, and our results clearly confirmed such hypothesis. Data reported in the previous Chapter, support the potential of microplastics in trapping and transport marine pollutants, as already suggested by previous studies on equilibrium kinetics and partition coefficients of several hydrophobic chemicals on various typologies of plastic polymers (Zarfl and Matthies, 2010; Bakir *et al*., 2012; Lee *et al*., 2014); a transport model for persistent organic pollutants by microplastics has been recently proposed also for estuarine conditions, demonstrating a relatively little effect of salinity compared to chemical concentration in water, plastic density and particle residence time in estuaries (Bakir *et al*., 2014b).

Several species have been shown to ingest and accumulate microplastics, and the ecological impact of this phenomenon would be greatly influenced by the desorption of toxic chemicals. The release of additives or adsorbed chemicals from plastics to organisms has been suggested (Engler, 2012; Tanaka *et al*., 2013; Bakir *et al*., 2014a), but a clear demonstration is still lacking because organisms in field conditions can accumulate the same classes of chemicals from other sources. Our experimental results provided the first clear evidence that pyrene adsorbed on contaminated microplastics was transferred and concentrated in mussels tissues, particularly in digestive glands, confirming an elevated desorption rate of chemicals under physiological gut conditions (Teuten *et al*., 2009; Bakir *et al*., 2014a). Despite the analyses of pyrene in mussels might have been partly influenced by the presence of still un-excreted, contaminated particles, concentrations measured in digestive tissues were up to 3 folds higher than those present on polymers; considering their contribution in weight of ingested microplastics as negligible compared to that of analyzed tissues, it is evident that the amount of pyrene in digestive glands reflects a desorption and bioconcentration process from microplastics to tissues.

The histological analyses qualitatively supported the bioaccumulation data, with observation of particles in the digestive tissues of exposed mussels and, to a lower extent, in gills and haemolymph. Uptake and tissue distribution of microplastics have already been described in the blue mussels *M. edulis* after laboratory exposures to high density polyethylene and polystyrene

(Browne *et al*., 2008; Von Moos *et al*., 2012). A first site of particles uptake occurred at the gill surface, mediated by microvilli activity and endocytosis, while a second pathway occurred via ciliae movement in the stomach, intestine and digestive tubules, followed by accumulation within the lysosomal compartment (Von Moos *et al*., 2012). Polystyrene particles smaller than 9.6 or 3.0 µm were shown to translocate from the gut cavity to the haemolymph and inside the haemocytes (Browne *et al*., 2008). Despite our observations were not quantitatively assessed, they almost reflect the above mechanisms of uptake, with more conspicuous aggregates within intestinal lumen and digestive tissues, and occurrence of particles in branchial epithelial cells and in haemolymph; the lack of microplastics within the haemocytes may be the consequence of a dimensional difference of plastic particles used in our experimental conditions.

Transcriptional profiles and a large battery of biochemical and cellular biomarkers were analyzed in this study to characterize the ecotoxicological potential of both virgin and contaminated microplastics. Significant effects were observed on haemocytes which are responsible for inflammatory and immunological responses. The haemolymph of mussels contains two basic typologies of cells, i.e. the granulocytes involved in phagocytosis and encapsulation of foreign material, and the hyalinocytes with characteristics of undifferentiated cells and responsible for coagulation reactions (Carball *et al*., 1997). Within haemocytes, the lysosomal compartment is particularly important for the immune responses through the degradation and elimination of pathogens and particles, coupled with production of ROS and nitric oxide as typical reactions during microbial attack (Canesi *et al.,* 2002). In our study, exposed mussels exhibited limited variations of phagocytosis, a strong shift of the haemocytic cell population and a significant reduction of lysosomal membrane stability. The lower granulocytes/hyalinocytes ratio did not probably reflect a decrease of granulocytes, which would have affected the phagocytic activity, but rather a sharp increase in hyalinocytes potential precursors of granulocytes. Similar effects were observed in organisms exposed to virgin or contaminated microplastics, suggesting that those immunological responses were mostly induced by the physical ingestion of the particles, more than the chemical toxicity of adsorbed pyrene. The lower lysosomal membrane stability of haemocytes could be reasonably related to the over-production of prooxidant reactive oxygen species involved in the immune responses, as recently observed also toward nanoparticles (Jovanovic and Palic, 2012). Similarly to our results, the ingestion and translocation of polystyrene did not cause measurable changes in the viability and phagocytic activity of haemocytes in *M. edulis* (Browne *et al.,* 2008), while inflammatory responses and lysosomal membrane destabilization occurred as a cellular host response to high density polyethylene microplastics (Van Moos *et al*., 2012).

Exposure to microplastics determined the onset of some genotoxic effects in mussels haemocytes, with different responses between various treatments. While strand breaks were higher in organisms exposed to virgin PE, nuclear alterations appeared more consistently distributed among all the treatments, resulting in an increased frequency of micronuclei after the exposure to pyrene-contaminated PS. This pattern of genotoxic effects allows to hypothesize that DNA strand breaks represent the first form of damage caused by the enhanced production of reactive oxygen species in response to microplastics: a more elevated prooxidant challenge caused by PS compared to PE or by pyrene-contaminated compared to virgin polymers would determine an irreversible loss of DNA integrity (i.e. nuclear alterations), leading to enhanced frequency of micronuclei in the worst condition. In this respect, oxyradical production was shown to modulate immune responses, lysosomal dysfunction and pre-apoptotic processes in haemocytes of mussels exposed to TiO2 nanoparticles (Barmo *et al*., 2013), while genotoxic proprieties of PAHs have been widely reported to produce chromosomal alterations (Benedetti *et al*., 2012).

The activity of AChE was not affected in haemocytes of exposed mussels, but a significant reduction was observed in gills. The levels of AChE were almost comparable among different treatments with both typologies of either virgin or contaminated polymers, thus suggesting a generalized biological reactivity of plastic particles on branchial epithelia. The ability of microplastics to depress AChE was recently described also in juveniles of the common goby *Pomatoschistus microps* exposed to polyethylene microspheres, dosed alone or in combination with pyrene (Oliveira *et al*., 2013); despite mechanisms of action still remain to be elucidated, our results support the hypothesis that anticholinesterasic effects of microplastics should be taken in adequate consideration due to the abundance of these particles in the marine environment and the role of AChE in neurotransmission of fundamental physiological processes (Oliveira *et al.,* 2013).

In our study, accumulation of microplastics caused a significant inhibition of lysosomal latency period also within digestive tissues, confirming the destabilization of these organelles reported in *M. edulis* upon fusion with endocytotic vacuoles containing microplastic particles (Von Moos *et al*., 2012). On the other hand, lysosomal membranes are also particularly sensitive to reactive oxygen species which can be generated throughout a complex network of direct reactions and indirect mechanisms (Regoli and Giuliani, 2014); in this respect, our results indicated that short-term exposures to microplastics do not induce major conditions of oxidative stress, as revealed by the lack of oxidative damages like lipofuscin, malondialdehyde and neutral lipids accumulation, in agreement with previous data on mussels and fish (Von Moos *et al.*, 2012; Oliveira *et al*., 2013). Nonetheless, we could observe variations of some antioxidant enzymes which are more sensitive biomarkers in revealing the early onset of a prooxidant challenge (Regoli and

Giuliani, 2014); among these, a significant inhibition occurred for catalase and Se-dependent glutathione peroxidases, both involved in the removal of hydrogen peroxide which represents the main precursor of hydroxyl radical in aquatic organisms (Regoli and Giuliani, 2014). These enzymes have been widely described as highly responsive even at low levels of environmental disturbance, frequently showing contrasting trends of variations with both inductions and/or inhibitions depending on time and intensity of exposure (Regoli and Giuliani, 2014). Glutathione peroxidases are mainly responsible for eliminating metabolically produced H_2O_2, while catalase act as defense mechanism also toward exogenously generated hydrogen peroxide: the contemporary variation of both these classes of enzymes might thus suggest different mechanisms and cellular sources for H_2O_2 formation in tissues exposed to microplastics.

The overall evaluation of biomarker results by multivariate PCA provided a clear separation between control and microplastics exposed mussels indicating that the majority of observed biological variations (immunological, lysosomal, cholinesterasic and antioxidant effects) were not influenced by the typology of polymer (PE vs PS) or contamination: only genotoxic responses further separated virgin from pyrene-contaminated polymers. The relatively limited impact of pyrene adsorbed on microplastics might suggest that energy resources were primarily directed to activate mechanisms of defense toward the physical rather than the chemical stressor; on the other hand, presence of microplastics was shown to delay the pyrene-induced lethal effects in *P. microps*, thus acting as a transitory mechanism of protection toward chemical toxicity (Oliveira *et al*., 2013).

Transcriptional profiles revealed differences in the total number and specificity of DEGs in digestive glands of mussels exposed to PS and PS-PYR. Nonetheless, in analogy with biomarker results, both the treatments confirmed the modulation and enrichment of KEGG pathways involved in lysosomal metabolism and immunological functions, as primary responses to either virgin or contaminated microplastics. The up-regulation of several genes coding for lysosomal enzymes, putative coating proteins and endosome indicates a coordinated increase of this cellular defense pathway following microplastics accumulation. The synthesis and maturation of lysosomal enzymes occur in the endoplasmic reticulum/trans-Golgi system and the following trafficking of such proteins is regulated by specific recognition mechanisms and packaging into clathrin-coated vesicles for their transport to late endosomes (Bonifacino and Traub, 2003). The over-expression of several proteins involved in endosomes maturation, endocytic trafficking and lysosomal degradation, suggests increased microplastics uptake via endocytosis and their endolysosomal degradation (Bucci *et al.,* 2000). Cathepsins, *clathrin heavy and light chain*, sorting nexins are only some of the several DEGs involved in “lysosome”, “coated membrane”, and “endosome” pathways.

Interestingly, the NOD-like receptor signaling pathway was enriched in mussels exposed to PS-PYR. NOD-like receptors (NLRs) are specific families of pattern recognition receptors, playing key roles in innate immune response, such as in the regulation of inflammatory and apoptotic responses. They act as intracellular sensors which recognize both pathogenic patterns entering the cell via phagocytosis, and damage-associated molecules produced during cellular stress and activating the non-infectious inflammatory response. Within NOD-like receptor signaling pathway, PS-PYR exposed mussels exhibited the enhanced transcription of genes putatively involved in signal transduction and auto-modulation of the stress response NF-kB (i.e. TRAF6 and IκBα) responding to several stimuli such as cytokines, bacterial antigens and reactive oxygen species (Regoli and Giuliani, 2014). Noteworthy, several responses involved in innate immune system, such as putative peptidoglycan recognition proteins (PGRPs), were up-regulated in mussels exposed to virgin PS.

Transcriptional analyses supported cellular biomarkers also regarding the modulation of antioxidant defences, detoxification enzymes and responses to genotoxic effects in mussels exposed to virgin or contaminated microplastics. The up-regulation of putative *GPX2*, *GSTP1*, *GSTP2* and down-regulation of putative SOD2 were observed in PS-exposed mussels, while *GPX3*, *GSTMU* and *SULT4A1* were differentially expressed after exposure to both PS and PS-PYR. On the other hand, the onset of DNA damage in mussels exposed to PS and PS-PYR could be related to the up-regulation of *GADD45A* and *GADD45G* which have a pivotal role in control of cell cycle checkpoint, DNA repair process and cellular responses to a variety of DNA-damaging agents (Fornace *et al.*, 1992). An increased transcription of GADD45 was also reported in the Manila clam *Ruditapes philippinarum* exposed to ibuprofen (Milan *et al.* 2013), corroborating its involvement in counteracting genotoxic stress in bivalves species. In addition, mussels exposed to PS-PYR, showing a significant enhancement of micronuclei frequency, were also characterized by the up-regulation of *ERCC1*, *ERCC2* and *APTX* which are required for the repair of DNA lesions and mainly involved in nucleotide excision repair, single-strand break, double-strand break and base excision repair.

Several probes representing genes coding for putative *baculoviral IAP repeat-containing 2 (BIRC2*), *baculoviral IAP repeat-containing 7* (*BIRC7*) and *X-linked inhibitor of apoptosis (XIAP*) were differentially expressed in both comparisons. The Inhibitors of Apoptosis (IAP) are a highly conserved family of functionally and structurally related proteins acting as endogenous inhibitors of apoptosis by direct binding to caspases (Liston *et al.*, 2003). Both PS and PS-PYR treatments showed up-regulation of BIRC7, while BIRC2 and XIAP were represented by several probes showing opposite gene expression profiles: these findings could be explained either by alternatively spliced transcript variants encoding isoforms with different anti-apoptotic properties, or by the

difficult classification of this gene family. However, the down-regulation of *BCL-2 associated X protein* (*BAX*) and up-regulation of *B-cell CLL/lymphoma 2* (*BCL2*) in PS and PS-PYR respectively, further corroborate an increased anti-apoptotic activity in exposed mussels: overexpression of *BCL2* prevents cells from undergoing apoptosis by controlling the mitochondrial membrane permeability, while *BAX* (down-regulated in PS) promotes apoptosis by antagonizing the *BCL2* and facilitating the release of cytochrome c and other pro-apoptotic factors from mitochondria, leading to caspases activation (Liston *et al*., 2003). The observed down-regulation of caspase 3 and caspase 9 represents an additional support to the hypothesis of a negative regulation of apoptosis in microplastics-exposed groups.

Substantial differences were observed in the transcriptional profile of the energetic metabolism after exposures to virgin or contaminated microplastics. While the "citrate cycle TCA" KEGG pathway was enriched only after PS treatment and mostly characterized by up-regulated genes, mussels exposed to PS-PYR exhibited the down-regulation of a few DEGs. Whether this apparently lower activation of energetic metabolism by PS-PYR may represent a mechanism of protection still remains to be elucidated. Similarly, the enrichment of Arachidonic acid metabolism in PS-exposed mussels, represented by down-regulated *phospholipase A2* and cytochrome P450 (CYP2 family genes), will deserve future studies.

The elaboration of all the bioaccumulation and biomarker data was performed by the Sediqualsoft model which, based on a weight of evidence integration, provided quantitative hazard indices, and a final classification of the risk induced by either virgin or contaminated microplastics in exposed mussels. The level of pyrene bioaccumulation was classified as Severe while the biomarker hazard ranged in various treatments from Slight to Moderate, based on the biological importance of measured endpoints (weight), and the number or magnitude of variations compared to specific thresholds. The overall risk was thus summarized as Slight for virgin polymers, Major or Severe for pyrene-contaminated PE or PS respectively. Despite in the present study only 2 lines of evidence were elaborated within the model, nonetheless it supported the use of WOE approach to classify the risk of microplastics in the marine environment through the integration of multiple indicators (physical, chemical, biological), as required by actual European Directives.

Regarding fish exposure, no bioaccumulation of pyrene was found in gills and livers of exposure fish. This results can be due to the lower concentration of plastics dosed in the exposure compared to the mussels experiment. In fact, while the levels of pyrene adsorbed on microplastics were the same in the two exposures, different level of microplastics were dosed, 1.5g/L of plastics for mussels and 0.0335 g/L for fish. This difference determined a presence of approximately 350

ng/L of pyrene for exposed mussels, versus approximately 7.5 ng/L for fish: a similar level was probably too low to cause any appreciable bioaccumulation of pyrene.

No effects on modulation of EROD activity was found in exposed organisms, despite a certain increase of pyrene metabolites in all exposed organisms. The different responses of two biomarkers involved in the biotraformation pathways could be explained by the capability of microplastics to decrease metabolism and reduce the feeding activity as observed by several authors (i.e. Cole et al., 2015). In this respect, also fish exposed to virgin microplastics revealed a similar increase of pyrene metabolites. As proposed by Gorbi et al. (2005), a significant increase of bile metabolites concentration could be due to a slower metabolism and reduced feeding activity which affect the possibility to empty gallbladder.

No effects were reported in AchE activity, in contrast with evidences observed by Oliveira et al. in 2013, describing a strong inhibition of this enzyme in the common goby exposed to microplastics alone and in co-presence of pyrene. On the other hand, higher level of DNA fragmentation were found in all exposed organisms, with exacerbated effects in organisms exposed to contaminated microplastic compared to virgin ones: similar effects on DNA organization were also obtained in mussels, further corroborating the hypothesis that microplastics enhance pro-oxidant challenge and consequent onset of oxidative damages.

In conclusion, this study confirmed that microplastics can efficiently adsorb organic contaminants from the marine environment, providing the first experimental evidence for the following transfer and bioaccumulation of such chemicals in mussel tissues. Both virgin and contaminated microplastics induced different forms of biological responses at transcriptional and cellular levels highlighting the potential risk of these emerging contaminants, especially under conditions of long-term, chronic exposure. Further studies are needed to better understand both mechanistic pathways modulating the onset and persistence of biological effects, as well as the natural exposure conditions in terms of presence, concentration and magnitude of chemical load in microplastics in the marine environment.

5. Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. [b]

In this chapter a new extraction method for microplastics in fish tissues was developed and validated. Information about the presence of microplastics in marine organisms is still lacking probably due to the difficulty to observe these small particles from the big amount of organic matter.

In this respect, the extraction yield of some already published methods was tested and a new one protocol was developed. The new method gave the best extraction yield and was validated for different size classes of microplastics, to evaluate size limit of the technique. The application to extract, quantify and evaluate the ingestion and compartmentalization of microplastics in exposed fish confirmed the efficiency of the proposed method; allowing to demonstrate for the first time, a passage of microplastics from the intestine to the liver.

Abstract

The presence of microplastics in the marine environment has raised scientific interest during the last decade. Several organisms can ingest microplastics with potentially adverse effects on the digestive tract, respiratory system and locomotory appendages. However, a clear evidence of tissue accumulation and transfer of such microparticles in wild organisms is still lacking, partially hampered by technical difficulties in isolation and characterization protocols from biological samples. In this work, we compared the efficacy of some existing approaches and we optimized a new protocol allowing an extraction yield of microplastics from fish tissues ranging between 78% and 98%, depending on the polymer size. FT-IR analyses confirmed that the extraction procedure did not affect the particles characteristics. The method was further validated on the fish mullet, *Mugil cephalus*, exposed under laboratory conditions to polystyrene and polyethylene; the particles were isolated and quantified in stomach and liver, and their presence in the hepatic tissue was confirmed by histological analyses. The overall results confirmed the newly developed method as a reliable approach to detect and quantify microplastics in the marine biota.

[b] Results of this chapter was published in Avio C.G., et al.,2015b.

1. Introduction

Plastic pollution in the oceans has been recognized as a world phenomenon with nearly 300 million tons of debris floating at sea surface, or accumulating on seafloor and shorelines from polar regions to the equator (Boerger et al., 2010; Browne et al., 2011; Eriksen et al., 2014; Suaria and Aliani, 2014).

In the recent years, scientific interest has been directed also toward microplastics, i.e. plastic fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derived from chemical, physical and biological degradation of larger plastic debris (Barnes et al., 2009; Wright et al., 2013).

Laboratory experiments have shown that microplastics can be ingested by different marine organisms, including polychaetes, crustacean, bivalves and echinoderms (Browne et al., 2008; Gregory, 2009; Graham and Thompson, 2009; Kach and Ward, 2008; Thompson et al., 2004; Von Moos et al., 2012; Van Cauwenberghe et al., 2015b). Due to their hydrophobic properties, microplastics can also adsorb several classes of organic pollutants (Teuten et al., 2007), which may be transferred to organisms and enter the marine food-webs (Teuten et al., 2009; Farrell and Nelson, 2013, Setala et al., 2014); experimental evidence has been recently obtained for the transfer of pyrene from microplastics to mussels (Avio et al., 2015). The consequences of microplastics ingestion may affect the feeding activity, respiratory functions, reproductive output, and also modulate several molecular and cellular pathways (Gregory, 2009; Cole et al., 2015; Avio et al., 2015).

The multiple risks that microplastics pose to marine life prompted their inclusion in some international legislation and marine protection projects, like the European Marine Strategy Framework Directive (MSFD) and the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA). In this scenario, a better knowledge on the presence and characterization of microplastics in marine food webs has become a research priority, and a fundamental step toward a more integrated ecological risk assessment.

Environmental studies on microplastics are often hampered by the limited availability of standardized protocols and technical difficulties in the extraction and characterization of these particles from marine samples. Suitable methodologies have been recently reviewed for sediments and seawater indicating the density separation as the most common approach (Hidalgo-Ruz et al. 2012; Van Cauwenberghe et al., 2015b). More complex is the extraction and quantification of microplastics from organisms, being fragments easily masked within biological material and tissues. In addition, microplastics comprise a very heterogeneous assemblage of pieces that vary in

size, shape, color, specific density, chemical composition and other characteristics which should be considered during the development of appropriate methods for their extraction and characterization.

Some recent protocols tested the extraction of microplastics from marine invertebrates after a pre-digestion of organic matter (Claessens et al., 2013); the comparison of various acid, basic or oxidizing treatments revealed that pH- sensitive polymers can be dissolved or partially degraded by certain acids, thus affecting both the estimation and the characterization of the polymers by FT-IR (Claessens et al., 2013). The enzymatic digestion of organic matter with proteinase k resulted as a reliable method to extract microplastics from zooplanktons samples (Cole et al., 2014), but not a cost-effective approach for larger organisms.

The methodological difficulties in isolation protocols can partly explain why, to date, only a few studies specifically addressed the occurrence of microplastics in wild fish populations. Larger plastic fragments (1-5 mm) have been detected in various Pacific and Atlantic species (Choy and Drazen, 2013, Davison and Asch, 2011, Boerger et al., 2010, Pinnegard, 2009, Laist, 1997), while plastic particles <1 mm have been observed for the first time in demersal and pelagic fish from the English channel (Lusher et al., 2013), and in wild gobids, *Gobio gobio*, from French rivers (Sanchez et al., 2014). All these studies were based on a direct visual sorting of the fish stomach contents, without testing the efficiency of the separation methodology. The destruction of organic material with 10% KOH has been recently applied on intestines of fish from the North Sea, following a 2-3 weeks period of alkaline digestion (Foekema et al., 2013): plastic particles were found in 2.6% of examined fish, with typically one item per fish, ranging in size from 0.04 to 4.8 mm (Foekema et al., 2013).

The aim of the present work was to propose a reliable technique for the isolation of microplastics from marine organisms, preventing any aggressive procedure, and thus allowing the further characterization of the polymers by FT-IR or Raman spectroscopy analysis. In this respect, isolated gastrointestinal tracts of fish were spiked with known amounts of previously characterized microplastics polymers: the extraction yields were then assessed for different methods, including some published protocols and a new one that combined various steps of the available techniques. Tested protocols allowed to extract particles generally within 24 hr; in this respect, we did not include the protocol of Foekema et al. (2013) requiring up to 3 weeks of alkaline digestion.

Possible dimensional limits of the newly developed procedure were tested toward microplastics of different size classes, from 5 mm to less than 0.1 mm, while FT-IR analyses were used to evaluate the integrity of polymers structure. The method has been further validated to analyze accumulation and transfer of microplastics in the gastrointestinal tract and liver of mullets, *Mugil cephalus,* exposed in laboratory conditions to polyethylene and polystyrene polymers. This species

was selected as an experimental model due to its commercial importance and wide distribution in the Mediterranean Sea; being an omnivorous fish, it is also potentially exposed to pollutants and microplastics ingestion, and previous studies demonstrated its utility for ecotoxicological studies (Gorbi et al., 2005; Whitfield et al., 2012).

The results of this study were expected to provide a practical contribution toward the standardization of appropriate procedures for isolating microplastics from marine organisms.

2. Material and methods

2.1. Polyethylene and polystyrene particles preparation

A stock of polyethylene and polystyrene powder was obtained from a private plastic company and sorted in four different grain size classes (5-1 mm; 1-0.5 mm; 0.5-0.1 mm; 0.1-0.01 mm). The particles were used to test various extraction protocols and for the laboratory exposure of the mullets *M. cephalus*.

2.2. Maintenance and acclimation of fish in laboratory conditions

Mullets, *M. cephalus*, were obtained from a local aquaculture and maintained in laboratory conditions with filtered and aerated seawater, at 18±1 °C, salinity 37 for at least two weeks before exposures. Fish were daily fed with a specific grower feed (by Aller-Aqua, Aller Thalassa 2 mm, crude protein 50%, crude fat 15%); this commercial pellet was analyzed and confirmed to be microplastics free (data not shown). All the maintenance and experimental procedures were in accordance with requirements of the Ethical Committee of the Polytechnic University of Marche for scientific activities with marine organisms.

2.3. Protocols for plastic extraction

The efficacy to extract microplastics from fish tissues was evaluated for six different protocols, including five already published and a newly developed one; these procedures were compared in terms of percentage recovery of known amounts of previously characterized particles "spiked" in gastrointestinal tract of laboratory-acclimatized fish.

To this aim, 60 acclimatized mullets (length 24.6 ± 2.7cm) were euthanized by submerging the fish in ice before cervical transection; gastrointestinal tracts (from the esophagus to the anal sphincter) were removed, homogenized and spiked with microplastics (35 g of tissues were added and accurately mixed with 5 polyethylene and 5 polystyrene particles, 1-0.5 mm grain size). Samples were processed as outlined below, using 10 gastrointestinal tracts for each protocol.

Protocol 1, consisted in the direct visual sorting of the stomach content according to Choy and Drazen (2013). Particles were counted, photographed and measured with a steromicroscope (Optika SZM-D, equipped with a DinoEye Camera AM-423X and Dino Capture 2.0 software).

Protocol 2, previously proposed for microplastics determination in sediments (Thompson et al. 2004) was slightly modified for fish samples. Briefly, after the addition of plastic particles, the gastrointestinal tracts were homogenized in ultraturrax and 1L of hypersaline NaCl solution (1.2 g/cm^3) was added to each samples: quite surprisingly this treatment did not result in the expected density gradient separation, at least within 24 hr, thus preventing further filtration and isolation of spiked particles. Settling or resuspension of different components may require a much longer period of decantation, making this approach less attractive and time consuming for fish samples.

For extraction protocols 3, 4, 5 and 6, after the addition of microplastics, the gastrointestinal tracts were desiccated (55°C overnight) and carefully ground in a mortar before the following steps.

Protocol 3, already proposed by Claessens et al. (2011) for sediments is based on a density gradient separation. Shortly, 500 ml of NaCl hypersaline solution (1.2 g/cm^3) were added to each sample and stirred for 10 min in a glass beaker. The obtained solution was then allowed to settle for 20 min before the supernatant was filtered under vacuum on a cellulose nitrate membrane (8 µm pore size), which was then washed with deionized water, dried and microscopically observed for microplastics count.

Protocol 4 contains an oxidation treatment, which was suggested to eliminate the biogenic organic matter in sediments before the microplastic extraction (Nuelle et al. 2014). In this work, microplastics-spiked and desiccated samples were transferred in a conical flask and added to 20 ml of 30% stabilized hydrogen peroxide (H_2O_2) at 55°C for seven days. The resulting mixture was then diluted 1:10 in deionized water, before the filtration and microscopical observation as described above.

Protocol 5 was previously reported for extraction of microplastics from mussel tissues after an acidic digestion (Claessens et al. 2013). According to this procedure, samples were added to 20 ml of 22.5 M nitric acid and digested overnight at room temperature, followed by 30 min of boiling (100°C). The resulting mixture was then diluted 1:10 with warm deionized water, before filtration and microscopical observation.

Protocol 6 is a new procedure obtained integrating, with slight modifications, some steps of the previously discussed extraction methods. Each sample was added to 250 ml NaCl hypersaline solution (1.2g/cm^3), stirred and decanted for 10 min; the filtration step was carried out twice in order to obtain a better extraction performance. The membranes with retained materials were then

transferred in a petri dish with a 15% H_2O_2 solution for the partial digestion of residual organic matter and allowed to dry in oven (50°C, overnight), before the microscopical observation.

To prevent any accidental external contamination of microplastics, deionized water and hypersaline solution were always pre-filtered (0.45µm filter); all the materials used for dissection and during different steps of extraction and analysis were rigorously cleaned with filtered deionized water and air dried. In addition all sample processing was performed in a clean air flow cabinet to exclude external contamination from fibers which might represent a major source of contamination (Foekema et al., 2013).

2.4 Effect of microplastics size and FT-IR validation

The efficacy of the new protocol was tested to evaluate the extraction yields toward microplastics with different grain sizes. The gastrointestinal tracts of 20 mullets (27.2 ± 1.9 cm length) were dissected and homogenized; 35 g of each sample were added with a total of 16 previously characterized plastic particles, equally distributed among 2 polymers (polyethylene and polystyrene) of 4 size classes (5-1 mm; 1-0.5 mm; 0.5-0.1 mm; 0.1-0.01 mm). Microplastics were extracted, microscopically observed and measured according to the previous procedure (Protocol 6).

As an additional control of the procedure and to evaluate a possible degradation of the polymers occurring during the isolation phases, polyethylene and polystyrene particles were analyzed by FT-IR (see below) before and after the extraction process.

2.5 Laboratory exposure and recovery

A total of 30 acclimatized mullets (25.1 ± 2.6 cm length) were randomly assigned and maintained for 7 days in one of three 80 l glass-tanks (10 fish per tank) corresponding to control tank (CNTR), polyethylene treatment (PE) and polyethylene treatment (PS). Particles, with size between 0.1 and 1mm, were given at a nominal concentration of 0.03375 g/L, corresponding to nearly 2500 particles/L. The exposure concentration is not representative of a realistic environmental condition, although nearly fifty times lower than those previously used in other experimental studies (i.e. Thompson et al., in 2004; Van Moos et al., 2012; Avio et al., 2015). However, considering the aim of our experiments, it was considered as appropriate to validate the new extraction procedure in *in-vivo* exposed fish, and to explore the possibility that ingested microplastics may be transferred from gastrointestinal tract to liver.

During the exposure period, water was fully removed and replaced daily with new water and microplastics re-dosed at the initial nominal concentration. Stratification of particles at the surface of the tanks was prevented by water mixing with aerator and pump movement (Koralia nano,

HYDOR); particles distribution in tanks was visually inspected twice a day for all the exposure period.

Fish were daily fed and sacrificed at the end of the experiment as previously reported; livers and gastrointestinal tracts rapidly removed, frozen in liquid nitrogen and maintained at -80°C until the analyses. Microplastics were determined in 10 gastrointestinal tracts and 5 livers for each treatment following the newly developed procedure (protocol 6). The remaining 5 livers for each treatment were histologically analyzed for identification and localization of microparticles: cryostatic sections (10 µm thick) were stained with haematoxilyn/eosin and observed at polarized light microscopy (Avio et al., 2015).

2.7 FT-IR analyses

Analyses were performed using a Cary 660 FT-IR spectrometer (Agilent) equipped with ATR (GladiATR Diamond Crystal Plate, Piketechnologies) which allowed the characterization of microparticles greater than 0.5 mm. Following background scans, 128 scans were performed and CO_2 interference (adsorption at approx 2300-2400 cm^{-1}) was removed for clarity; for each particle, scans were performed with resolution of 4 cm^{-1}. Agilent Resolution Pro v5.2 software was used for the output spectra and the identification of polymers was performed by comparison with a library of standard spectra.

2.8 Statistical analysis

Analysis of variance (ANOVA) was applied to test differences between various extraction protocols, typology and size of polymers, accumulation in different tissues of exposed fish. Level of significance was set at $p < 0.05$ and when differences were detected post-hoc comparison (Bonferroni) was used to discriminate between means of values. Homogeneity and normality of data were tested using Bartlett's test or Shapiro-Wilk's test respectively, and any deviation was corrected by *log* transformation. Statistical analyses were done using GraphPad Prism® software.

3. Results

Figure 5.1 A reports the extraction yields of the 6 protocols applied to gastrointestinal tracts spiked with polyethylene and polystyrene particles. The newly developed procedure (protocol 6)

revealed the highest efficiency, with an average extraction yield of 95% ± 2 (mean% ± S.E.) toward the two polymers. The application of protocol 1 (direct visual sorting), protocol 3 (density gradient on dry samples), protocol 4 (oxidation treatment) and protocol 5 (acid digestion) showed an extraction yield of respectively 57% ± 12, 73% ± 5, 70% ± 3, 4% ± 3, with no significant differences between polyethylene and polystyrene particles; the application of protocol 5 indicated the acid digestion as too aggressive, resulting in the melting of microplastics and thus obtaining a low percentage of extraction yield. The protocol 2 (density gradient on fresh samples) did not allow any separation and particles extraction.

The efficiency of protocol 6 toward particles of different size confirmed similar results toward the 2 polymers (Figure 5.1B): the extraction yield was higher than 90% for the dimensional classes 5-1 mm, 1-0.5 mm 0.5-0.1 mm, while nearly 80% for microplastics smaller than 0.1 mm.

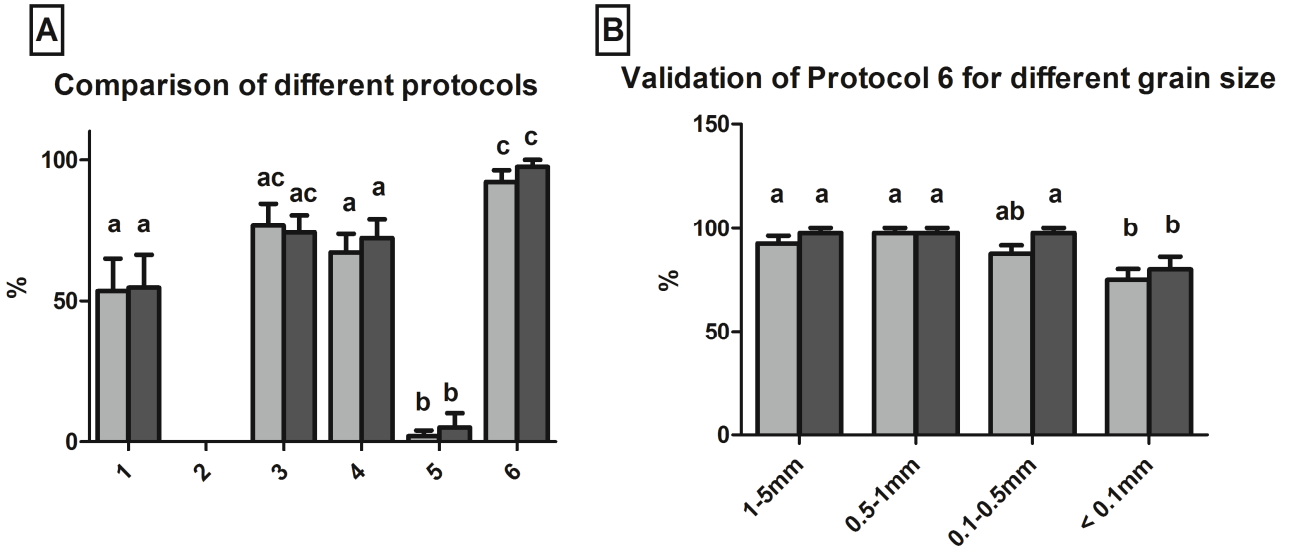


Figure 5.1. (A) Extraction yields of 6 different protocols (see Materials and Methods for details). Values are expressed as percentage recovery of particles from spiked gastrointestinal tracts (± SEM, n = 10); (B) Extraction yield for particles with different size classes (mean yield % ± SEM, n=20). Grey and black bars represent polyethylene and polystyrene particles. Different letters indicate significant differences between groups of means (Bonferroni post-hoc comparison); protocol 2 did not allow any separation and was not included in the statistical analysis.

FT-IR spectra obtained for particles analyzed before and after the new extraction procedure were absolutely comparable (Figure 5.2), with a similarity of approximately 93% for polyethylene profiles, and greater than 87% for polystyrene.

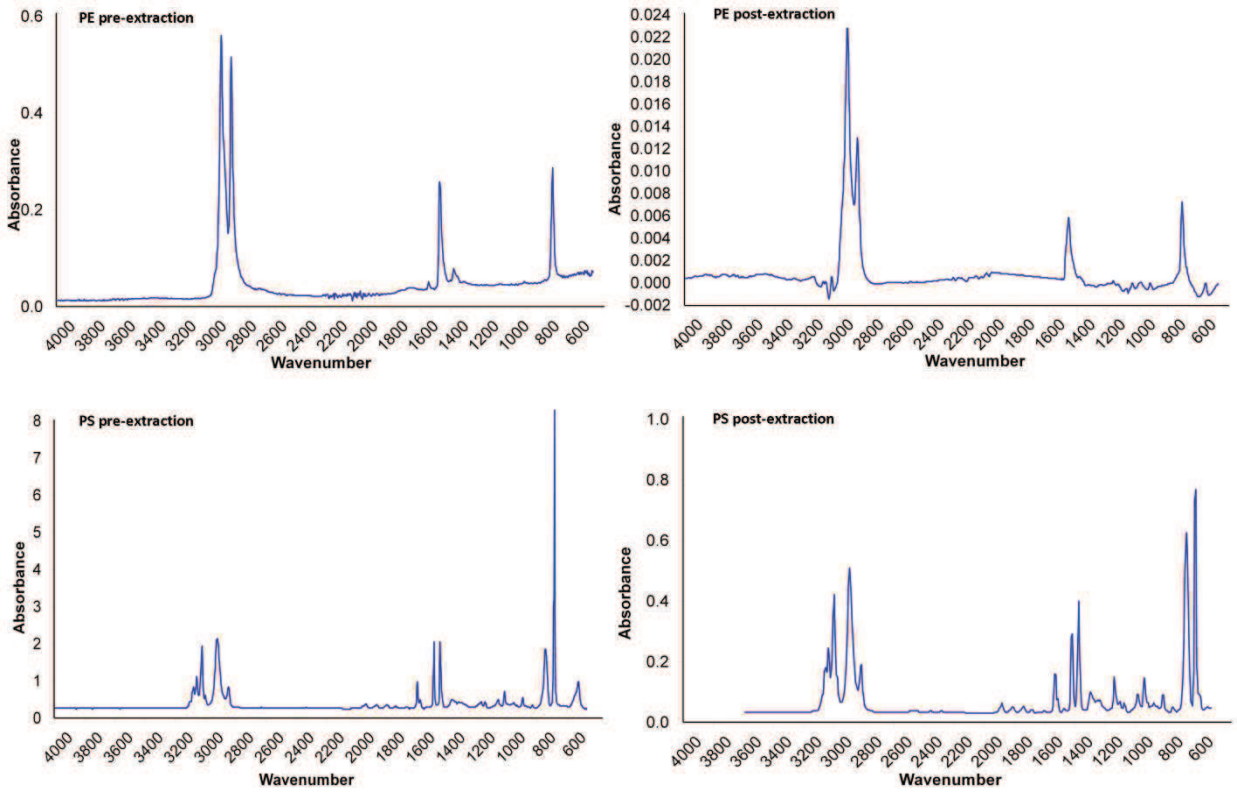


Figure 5.2. FT-IR spectra of polyethylene (PE) and polystyrene (PS) particles before and after the extraction procedure. spectra matched at 93% for PE, at 87% for PS

The newly developed procedure (protocol 6) was applied to extract microplastics from mullets *M. cephalus* exposed under laboratory conditions. An elevated accumulation of polyethylene and polystyrene particles was revealed in gastrointestinal tracts (Figure 5.3), and the absence of microplastics was confirmed in fish maintained in control conditions. Interestingly, the number of measured items was significantly higher in fish exposed to polystyrene compared to those isolated in polyethylene treated fish (Figure 5.3 A). Microplastics were also extracted from liver tissues (Figure 5.3 B), with average numbers of particles approximately 2 orders of magnitude lower than in corresponding gastrointestinal tracts, and without significant differences according to the polymer type.

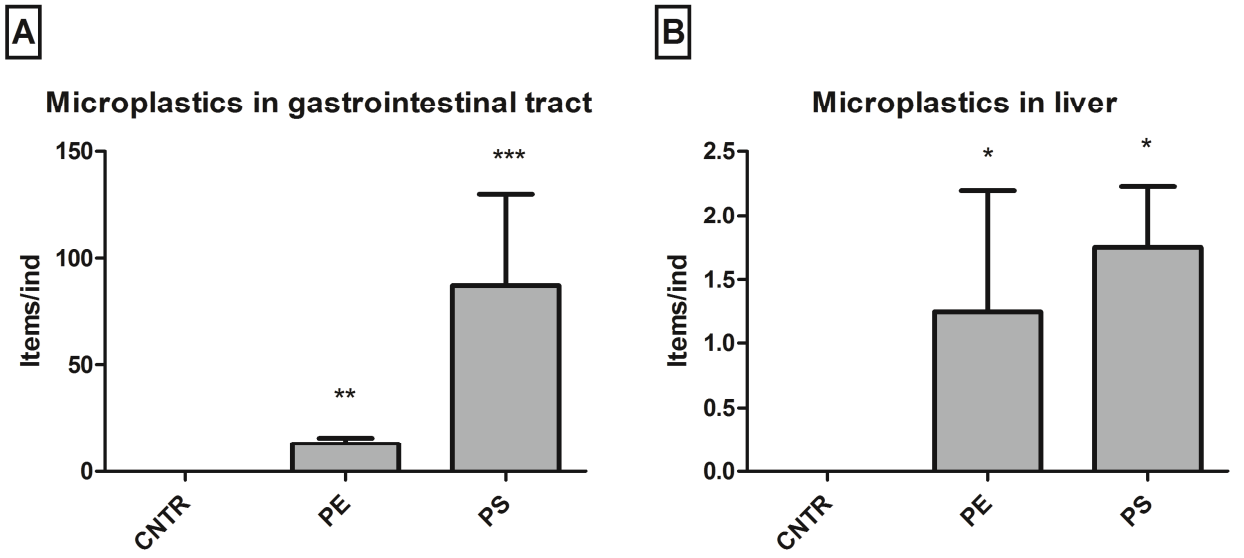


Figure 5.3. Microplastics particles extracted in gastrointestinal tract (A) and liver (B) of exposed fish. CNTR= control; PE = polyethylene treatment; PS = polystyrene treatment. Data are expressed as number of items/individual (mean values ± SEM); asterisks indicate different levels of significance compared the control group (Bonferroni post-hoc comparison).

Histological analyses of liver slices confirmed the presence of both polyethylene and polystyrene particles in the liver (with size ranging between 0.2 and 0.6 mm), randomly localized among the hepatic cells (Figure 5.4).

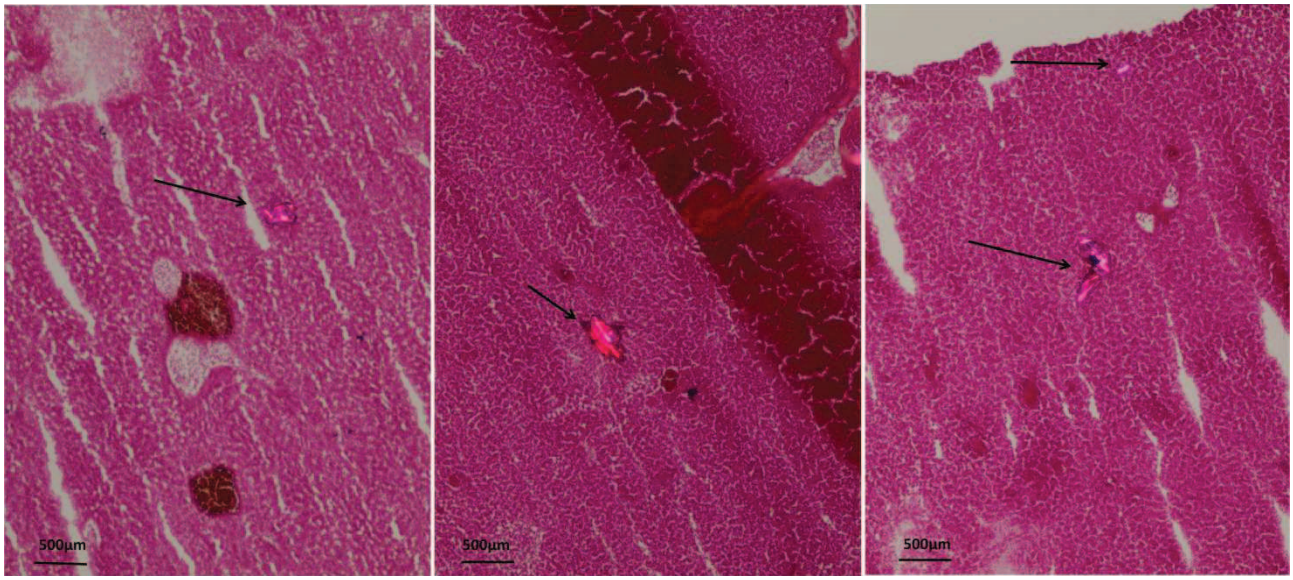


Figure 5.4. Polarized-light microscopy images (X 100 magnification) showing the presence of plastic particles in liver of exposed fishes. Black arrow indicate microplastic particles inside the liver tissue. Scale bar: 500 µm

4. Discussion

The present investigation was aimed to validate a new methodological protocol for microplastics detection in fish tissues, providing also a preliminary insight on their capability to cross intestinal barrier of exposed fish.

The presence of microplastics is being worldwide documented in water-column and sediment samples, and their presence has also been reported in different taxa including planktonic species, invertebrates, fish and cetaceans (Browne *et al*., 2011; Hidalgo-Ruz et al. 2012; Foekema et al., 2013; Lusher et al, 2013, 2015; Fossi et al., 2014; Van Cauwenberghe et al., 2014, 2015a), Laboratory studies further confirmed that microparticles can be ingested by a range of marine biota, including shellfish and fish for human consumption, with a potential impairment of various cellular, metabolic or physiological pathways (Browne *et al.,* 2008; Von Moos et al., 2012; Wegner et al., 2012; Avio et al., 2015).

Considering the growing interest on the impact of microplastics in the marine environment (Galgani, *et al*., 2013), the optimization of protocols to extract and characterize these particles in marine biota is an urgent scientific priority, required for quantitative and comparative assessments. In the present study, different protocols were tested for their quantitative yield in gastrointestinal tracts of fish spiked under controlled conditions with defined amount and typologies of microplastics.

The direct visual sorting of the fresh stomach content (protocol 1) resulted in an extraction yield slightly lower than 60% for both polyethylene and polystyrene, thus indicating a potential underestimation of results obtained with this approach. The elevated content of organic matter and other particles present in the fish stomach can hide the microplastics, interfering with their microscopic visualization; in addition, the application of this protocol on wild specimens would not allow to identify and count the microplastics contained within an ingested prey.

The density separation with an hypersaline solution was derived by a successful approach to extract microplastics from sediments (Thompson et al., 2004). However, this procedure applied to fresh gastrointestinal tracts spiked with microplastics (protocol 2) did not allow any separation of the sample; consequently the filtration was not performed and it was not possible to obtain any extraction yield. For all the other protocols tested in this work (3-6), spiked samples were desiccated and ground in a mortar to facilitate the following microplastics extraction. In this respect, the density gradient on dried, spiked samples (protocol 3) resulted in an efficient separation and filtration, with a final extraction yield higher than 70%.

Digestion of organic matter by either oxidant or acidic treatments has been suggested to facilitate and improve the extraction of microplastics from sediments and/or organisms (Claessens

et al., 2013; Cole et al., 2014; Van Cauwenberghe et al., 2014, 2015b). In this study, the 7 d digestion of dried samples in 30% H_2O_2 (protocol 4), allowed to extract almost 70% of spiked particles; this relatively low recovery could be partly ascribed to a dense foam formed after the addition of hydrogen peroxide, which hampered the filtration and further processing of samples.

Acidic digestion has also been shown as a useful treatment to remove biological material from marine samples, thus facilitating isolation of plastic particles. In this respect, the extraction yield in marine organisms treated with nitric acid was approximately 98 and 94% for 30 µm and 10µm polystyrene spheres, and 98% for 100x400 µm nylon fibers (Claessens et al., 2013). However this procedure has also been suggested to, at least partially, degrade those polymers with a low pH tolerance, e.g. polyammide, polystyrene, polyethylene (Cole at al., 2014). This possibility was confirmed in the present work, where the acidic extraction of spiked gastrointestinal tracts (protocol 5), caused a marked dissolution of both polyethylene and polystyrene tested particles, resulting in an extraction yield of approximately 4%: at the end of the digestion, plastic particles appeared to be melted and fused together, suggesting that this method could be not appropriate to extract all typologies of plastic polymers.

The combination of density gradient separation and oxidant treatment (protocol 6) appeared the most efficient procedure with an extraction yield of approximately 95% for both the polymers. Density separation was confirmed as a fundamental step to eliminate sediment particles and other debris potentially present in the gastrointestinal tract of fish; the higher extraction efficiency compared to protocol 3, highlighted the utility of the hydrogen peroxide treatment which, digesting the residual organic matter retained in the membrane, resulted in an easier plastic detection during the microscopic analysis. At this stage we did not consider the method by Foekema et al. (2013) which requires up to 3 weeks of alkaline digestion of samples; nonetheless a more focused comparison with the newly developed procedure might be addressed in a future investigation.

To evaluate if methodological steps described for protocol 6 may attack the polymers structure thus preventing their further characterization, polyethylene and polystyrene particles were analyzed by FT-IR before and after the extraction procedure. Obtained results showed only a minimal modification of polymers spectra, confirming that microplastics were efficiently extracted without any damage to the polymers. Considering the dimensional classes of particles to extract, the proposed protocol showed an extraction yield higher than 90% for microplastics ranging between 5 and 0.1 mm, and approximately 80% for those smaller than 0.1 mm; future implementation of the method should be directed to improve the recovery at the lowest dimensional limits and, ideally toward nanoplastics.

The validated protocol was applied on mullets, *M. cephalus,* exposed in laboratory conditions to polyethylene and polystyrene microplastics. The exposure concentration (0.03375 g/L) was lower than those used with mussels by Thomson et al., 2004 (1.5 g/L), Van Moos et al., 2012 (2.5 g/L) and Avio et al., 2015 (1.5 g/L) but, due to the limited knowledge on microplastics levels in seawater and the lack of standardized units, the ecological relevance of this treatment is difficult to be defined. Nonetheless, plastic particles were efficiently extracted from gastrointestinal tracts of exposed fish with a statistically significant higher number of polystyrene compared to polyethylene items. The different densities of these polymers (1.04 and 0.92 g/cm^3 respectively) might have partially affected the behavior of microplastics during the exposure. Despite water and particles mixing, polyethylene has a higher tendency to re-float at the surface, while polystyrene remained more uniformly distributed into the water column, possibly enhancing the probability to be ingested by fish actively swimming in the tank volume immediately after the addition of pellets. A similar experimental evidence confirm that physico-chemical properties of polymers have important implications for field studies, influencing the ingestion and accumulation in organisms with different trophic strategy and habit.

Extraction of microplastics from laboratory exposed fish revealed a small but significant presence of particles also inside to the hepatic tissue, a result further confirmed by histological analyses. This is the first evidence for a marine vertebrate that microplastics can be translocated from gastrointestinal tract to another tissue; a similar phenomenon, already observed in marine mussels, corroborates the potential risk of accumulation and trophic transfer of microparticles along marine food webs (von Moos et al., 2012; Avio et al., 2015).

In conclusion, this study provided a new protocol for the extraction of microplastics from fish, with a recovery yield generally higher than 90%. Future studies are required to assess their presence in natural organisms in order to better assess the distribution of microplastics along food webs, and their capacity to penetrate the edible tissues, with potentials concerns also for human consumers.

6. Presence, distribution and characterization of microplastics in Mediterranean organisms.[b, *,**]

This chapter describes the application of the newly developed extraction protocol (see the previous chapter) to assess the presence, composition and characteristics (size, shape, typology) of microplastics in Mediterranean organisms. For an easier reading, the sections Material and Methods and Results were divided in 3 subsections while a unique Introduction and Discussion is given.

A preliminary investigation was carried out to evaluate the effectiveness of the method on field organisms. In this regard, around 120 commercial fish were analyzed to assess the presence of microplastics in the stomach content.

In the second part of the study, more attention was given on microplastics presence in commercial fish and invertebrates collected from two sites of Adriatic Sea, to better understand the interaction between various typologies of polymers and species features. About 140 fish were collected from areas along the coast of Marche, and in each site four fish and one stomatophods species were collected, among those largely used for human consumption.

Lastly, the scenario of the Costa Concordia wreck was chosen because affected by the presence of huge engineering activities that led to its refloating and towing away. The surveyed area provided an interesting scenario to study the possibility to use marine organisms as indicators of microplastic pollution. In these regard, mussels were translocated at two different depth (-5 and - 45m) and in three different seasons (spring and winter 2013, summer 2014) at different distance from the wreck; similarly benthic fish were collected in proximity of the wreck and in control site during the summer 2014. This study offered important evidences and suggestions to use such marine organisms for monitoring anthropogenic pollution of microplastics in the marine environment.

[b]. Results of this study were partially published in Avio C.G., et al 2015b; and will be published in another two papers (*,**) that are in preparation.

Abstract

The presence of microplastics in the marine environment has raised scientific interest particularly during the last decade. Several organisms can ingest microplastics with potentially adverse effects on the digestive tract, respiratory system and locomotory appendages. However, a clear evidence of tissue accumulation and transfer of such microparticles in wild organisms is still lacking, partially hampered by technical difficulties in isolation and characterization protocols from biological samples.

In this work, the presence, distribution and characterization of microplastics was assessed in several commercial species, including both vertebrates and invertebrates from different Mediterranean areas; Central-North of the Adriatic sea, Central Adriatic and Northern Tyrrhenian Sea, respectively. A recently validated extraction protocol was applied to extract microplastics from gastrointestinal tracts of fish and soft tissue of invertebrates. Extracted particles were characterized in term of size, shape and typology.

The overall results showed that microplastics items were mostly represented by fragments and lines, while polyethylene, polystyrene and nylon were the dominant polymers. All the analyzed species showed a variable presence of microplastics in their tissue. No marked differences were highlighted in the number of ingested items per specimens when comparing organisms from the same area, although slightly higher numbers were observed in Tyrrhenian organisms compared to Adriatic ones. On the other hand, organisms sampled in more impacted sites showed a higher presence and heterogeneity of microplastics, suggesting a possible relation between these particles and human activities. Invertebrates typically exhibited a lower frequency of microplastics in soft tissue in respect to the stomach of fish, but with a higher potential of particles transfer to human consumers. Pelagic organisms showed higher numbers of ingested items, but at the same time, a lower occurrence of specimens positive to plastics ingestion compared to benthic specie: those results suggest a different distribution of microplastics particles in the marine environment, with higher levels of floating particles in water columns but distributed in patches in the sea surface, while a lower number but a more homogeneous distribution of particles in marine sediments.

In conclusion these studies, provided new insights on the presence, distribution and typology of microplastics in commercial organisms, representing an important baseline assessment on the level of this kind of contamination in Mediterranean biota.

1. Introduction

Plastic pollution in the oceans has been recognized as a worldwide phenomenon with nearly 300 million tons of debris floating at sea surface, or accumulating on seafloor and shorelines from Polar Regions to the equator (Boerger et al., 2010; Browne et al., 2011; Eriksen et al., 2014; Suaria and Aliani, 2014). In the recent years, scientific interest has been directed also toward microplastics, i.e. plastic fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derived from chemical, physical and biological degradation of larger plastic debris (Barnes et al., 2009; Wright et al., 2013).

The presence of microplastics has been documented in water-column and sediment samples, and their presence has also been reported in different taxa including planktonic species, invertebrates, fish and cetaceans (Browne *et al*., 2011; Hidalgo-Ruz et al. 2012; Foekema et al., 2013; Lusher et al., 2013, 2015; Fossi et al., 2014; Van Cauwenberghe et al., 2014, 2015a). Because of their small dimensions, microplastics have a size range similar to plankton and other suspended particles, making them available to a wide array of marine organisms (Wright et al., 2013).

Several filter feeding invertebrates can ingest microplastics, by filtration and sorting of particulate matter. The uptake mechanisms in these organisms depend on a combination of parameters (i.e. size, shape and density of the plastic particle) that determine the position of these particles in the water column, and hence the availability to be ingested by animals. In laboratory conditions, microplastics have been shown to be ingested by several invertebrates (exhibiting different feeding strategies): amphipods (detrivores), sea cucumbers (deposit and suspension feeders), barnacles and mussels (filter feeders) (Thompson et al., 2004; Graham and Thompson, 2009; van Moos et al., 2012; Avio et al., 2015). Once ingested by mussels, microplastics have the potential to be translocated from the digestive tract to the circulatory system of the organisms. In *Mytilus edulis*, plastic particles (3-9.6µm) were accumulated in digestive system (more specifically the digestive tubules) and in the haemocytes cells (Browne et al., 2008). In the same organisms, the uptake of microplastics caused notable histological changes in digestive cells with strong inflammatory responses, formation of granulocytomas and lysosomal destabilization which increased with exposure time (Von Moos et al., 2012). The same uptake mechanisms were observed in *Mytilus galloprovincialis* exposed to polyethylene and polystyrene microspheres (<100µm) that were translocated to digestive gland and circulatory systems (Avio et al., 2015). Moreover, it is well known that microplastic ingestion may affect the feeding activity, respiratory functions, reproductive output, and also modulate several molecular and cellular pathways (Gregory, 2009; Cole et al., 2015; Avio et al., 2015).

Also marine vertebrates can ingest microplastics (Lusher er al., 2013 and 2015; Romeo et al., 2015; Avio et al., 2015 b). In general, the uptake in fish can be direct or from secondary ingestion through preys (trophic transfer). Laboratory studies showed that *Mugil chephalus* is able to ingest PE and PS particles (diameter between 0.1 and 1mm), that can penetrate not only in the intestine but also be transferred to liver tissue of exposed organisms (Avio et al., 2015 b).

To date, only few studiesin field condition reported that filter feeding organisms (Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014, van Cawenberghe 2015), or commercial fish sampled in various environment (Lusher er al., 2013; Romeo et al., 2015;) ingest and accumulate significant amounts of microplastics. For this reason, an evaluation on the occurrence of microplastics in wild organisms used as sentinel species is a research priority, in order to provide new insights on microplastic pollution monitoring strategies, risk for human health and relation to human activities (Browne *et al.,* 2011; Van cawemberghe et al., 2014).

In this respect, a preliminary field assessment study was carried out to investigate the occurrence, typology and characteristics of microplastics in wild fish species from the Central- North Adriatic Sea, selecting species with different trophic guilds and ecological characteristics. Subsequently, a more standardized study was carried out in order to evaluate differences in the quantity and typology of ingested microplastics from fish and invertebrates collected in different sites along the Marche coastline. Finally, considering the situation at the Giglio Island, affected by the huge engineering project related to the removal of the Costa Concordia wreck, the impact of microplastics pollution was assess, using both native fish and transplanted mussels.

The overall results of this study were expected to provide a practical contribution toward the standardization of appropriate procedures for isolating microplastics from marine organisms, providing both new insights on the presence, distribution and typology of these particles in commercial organisms of Mediterranean Sea, and useful information on the utility of marine species as indicators of microplastics pollution in the marine ecosystem.

2. Material and methods

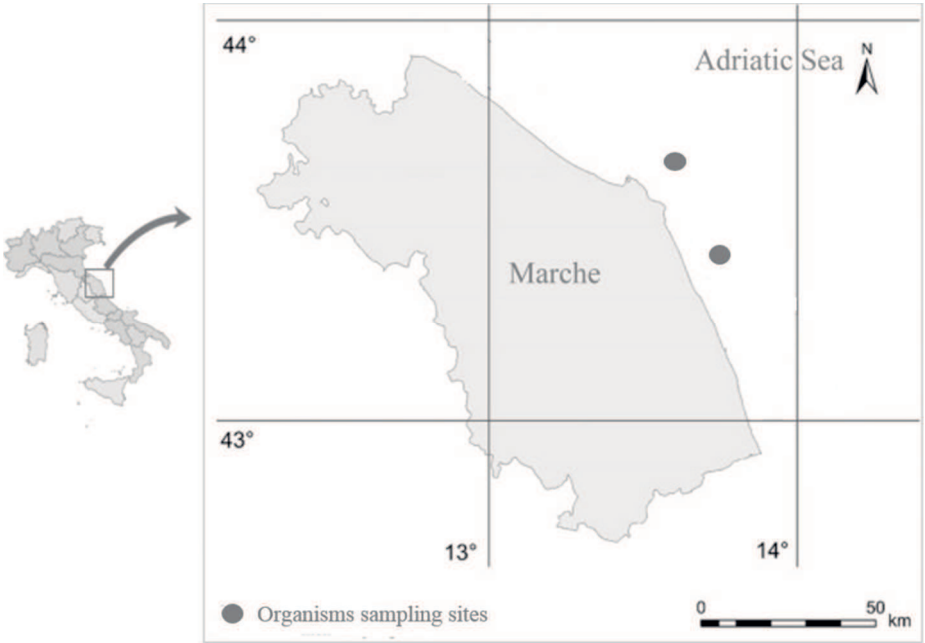
2.1 Preliminary investigation

A total of 125 fish representative of five commercial species, were sampled from the Central and North Adriatic Sea in March 2014. Collected species included *Sardina pilchardus* as a typically pelagic fish, *Squalus acanthias* and *Merlucius merlucius* as bentho-pelagic, *Mullus barbatus* and *Chelidonichthys lucernus* as two closely benthonic species; number and characteristics of analyzed fish are given in Table 6.1

2.2 Sampling areas and experimental design for Adriatic organisms

Adriatic organisms were collected in the same day from two sites located along the Marche coast, Ancona and Civitanova. Ancona is characterized by intense shipping, touristic and fishing activity, while Civitanova has a smaller harbor with more limited maritime activities (Map 6.1).

From each site, fish and stomatopods were collected around 2 nautical miles from the coast using common gillnets. For each site four fish species were selected with different trophic guilds and ecological characteristics, and one species of stomatopods (*S. mantis*). Fish species included *Sardina pilchardus,* and *Sgomber sgomber* as a typically pelagic fish, *Merluccius merluccius* as bentho-pelagic and *Mullus barbatus* as closely benthonic specie. Number and characteristics of analyzed organisms are given in Table 6.2.



Map 6.1. Localization of sampling sites along the coast of Marche. Grey dots indicate sites of collection; Ancona at northern, while Civitanova is located southern.

2.3 Sampling areas and experimental design for organisms at Costa Concordia wreck.

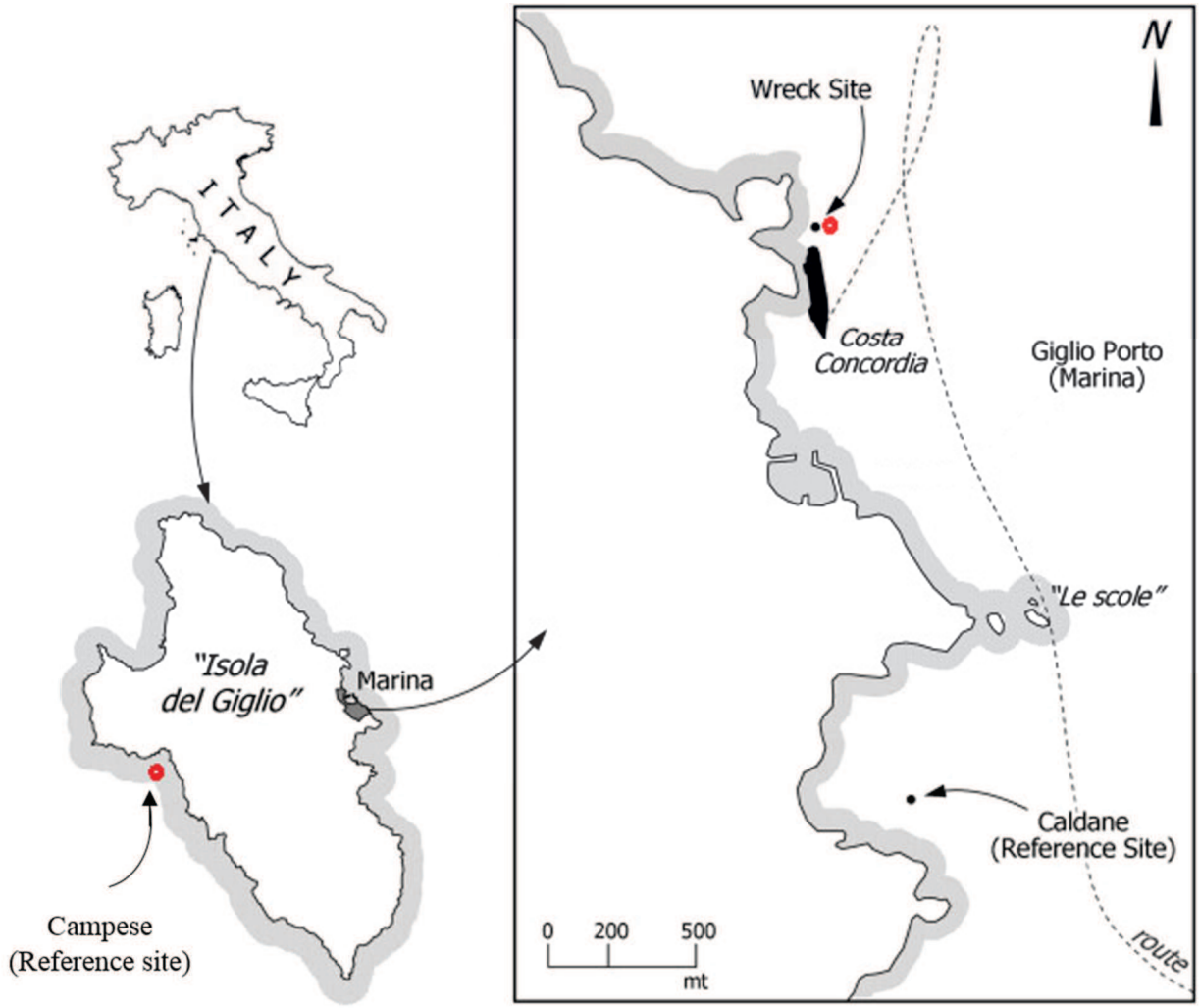
The study area, Giglio Island (Tuscany, Italy), was characterized by a complex framework of activities immediately activated to remove fuels and oil, to monitor the possible impact and to remove the ship of the Costa Concordia that collided with a submerged rock on the night of 13 January 2012. A coincidence of winds and currents prevented the ship from sinking in the deep waters, and the partially submerged wreck laid on a rock edge at the entrance of Giglio harbor. For these reasons a big number of employee and boats arrived at the island: during the Parbuckling project, an estimated number of 25 vessels and crafts were on site with more than 474 workers (see at: www.theparbucklingproject.com) significantly increasing the human population on the island. Browne et al. (2011) demonstrated that there is a significant relationship between MP abundance and human density. Considering also the numerous maritime activities on the seafloor, the studied area represent a perfect scenario to evaluate MP abundance in respect to such human activities.

A total of 40 fish representative of different commercial species, trophic guilds and ecological characteristics, were sampled from two location of Giglio island. Two fishing grounds were selected, respectively north of Costa Concordia wreck in proximity of the stern (42°22′04.80″ N, 10°55′16.80″ E) and a reference site located in front of Campese beach (42°37′06.36" N, 10°86′81.37″ E), located on the opposite side of island in respect to the wreck (Map 6.2).

In short, fishes were sampled at the same time, during night hours, in the summer 2014 (September) from the two selected areas using common gillnets (with mesh of 50 mm), at a depth between 30 and 45 m. Collected species included *Scorpaena sp., Uranoscopus scaber and Phycis phycis* as a typically benthonic fish, and different species of *Sparidae* family (*Spondyliosoma cantharus*, *Pagellus erythrinus* and *Diplodus sargus)* as bentho-pelagic species; number and characteristics of analyzed fish are given in Table 6.3. Gastrointestinal tracts were collected and frozen at -20°C, until the analyses.

Mussels, *M. galloprovincialis* (5.5 ± 0.5 cm shell length), were sampled from the reference site of Portonovo (Ancona, Adriatic sea) at a depth of 8 m, immediately transported to Giglio Island and deployed within 24h. Two caging sites were selected, respectively north of Costa Concordia wreck in in the same site used for fish investigation and in a reference site located in front of the Caldane beach, approximately 2 nautical miles south of the wreck (42°20′42.00″ N, 10°55′27.00″ E). To evaluate possible differences along the water column, at each site mussels were caged at 2 different depths, approximately at 1.5 m from both the surface and the bottom (30–45m). Translocation experiments have been carried out on winter 2013 (T1), spring 2014 (T2) and summer 2014 (T3): organisms have been recovered after 4–6 weeks from the deployment.

At the end of translocation periods, for every site and depth, 10 specimens, were dissected and stored at −20 °C for microplastic analyses. General data on analyzed mussels are given in Table 6.4.



Map 6.2. Localization of mussel's translocation sites (dark dot) and localization of fish collection sites (red dots). Wreck site (WRECK) is located close to the Costa Concordia wreck, while reference site (CTRL) is located at the Caldane beach chosen for mussel's translocation and in front of Campese beach for fish.

2.4 Extraction methods and polymer characterization

Gastrointestinal tracts of fish and soft tissue of invertebrates were collected and frozen at -20°C, until the analyses. Each sample was processed with the newly developed procedure (protocol 6 in the previous chapter), and extracted particles were microscopically observed, photographed, measured at their largest cross section through an ocular micrometer, and categorized according to both size classes (5-1 mm; 1-0.5 mm; 0.5-0.1 mm; <0.1 mm) and shapes (fragments, film, pellet, line). Textile fibers were found only occasionally and excluded from the analysis because they could represent airborne contamination from clothing during the sampling or processing (Foekema et al., 2013; Cozar et al., 2014). Particles were also characterized by FT-IR spectrometry using a Cary 660 FT-IR

spectrometer (Agilent) equipped with ATR (GladiATR Diamond Crystal Plate, Piketechnologies) which allowed the characterization of microparticles greater than 0.5 mm. Following background scans, 128 scans were performed and CO_2 interference (adsorption at approx 2300-2400 cm^{-1}) was removed for clarity; for each particle, scans were performed with resolution of 4 cm^{-1}. Agilent Resolution Pro v5.2 software was used for the output spectra and the identification of polymers was performed by comparison with a library of standard spectra. Only polymers matching reference spectra for more than 60% were accepted, in line with suggestion of Lusher et al. (2013).

2.5 Statistical analysis

One way analysis of variance (ANOVA) was applied to test differences in term of ingested items between species and, for mussels, between season and depths of collection. Beside, student t-test (unpaired) was used to detect significant differences between number of ingested items between organisms collected from the two different sites.

Level of significance was set at $p < 0.05$ and post-hoc comparison (Bonferroni) was used to discriminate differences between means of values. Homogeneity and normality of data were tested using Bartlett's test or Shapiro-Wilk's test respectively, and any deviation was corrected by *log* transformation. Statistical analyses were conducted using GraphPad Prism® software.

3. Results

3.1 Preliminary investigation

The extraction of microplastics from wild fish species from North and Central Adriatic Sea highlighted their presence in gastrointestinal tracts of 35 individuals, i.e. 28% of the 125 examined fish. Despite the comparison between species could be partly affected by the different number of analyzed specimens, nonetheless the pelagic *S. pilchardus* showed the lowest percentage of fish containing plastic particles (19%), followed by the bentho-pelagic *S. acanthias* (44% of sampled specimens, Table 1). In the two benthic species *M. barbatus,* and *C. lucernus*, microplastics were found respectively in 64% and 67% of the analyzed stomachs, while all the three specimens of *M. merlucius* contained such items in the stomach (Table 6.1).

The average number of microplastics extracted in positive fish ranged from 1 item/individual in *C. lucernus* to 1.78 ± 0.97 items/individual in *S. pilchardus* (Table 6.1). The shape of the plastic particles isolated in gastrointestinal tracts of all the fish (without species differentiation) was largely dominated by fragments (57%), followed by line (23%), film (11%) and pellet (9%, Fig. 1A-B). The 18% of extracted microplastics exhibited the larger size class (from 5 to 1 mm), 43% was between 1 and 0.5 mm, 23% between 0.5 and 0.1 mm, and 16% lower than 0.1 mm (Fig. 6.1 C). FT-IR analyses indicated that approximately 65 % of analyzed items were polyethylene, 19 % polyethylene terephthalate (PET), 4 % respectively polystyrene, polyvinyl chloride (PVC), Nylon 6/T and polypropylene (Fig. 6.1 D).

Species	Number of stomach examinated	Fish lenght cm (means ± SD)	Stomach wet weight g (means ±SD)	Stomach with microplastics (%)	N° of items in fish with plastics in stomach (means ±SD)
S. pilchardus	99	11.8 ± 1.5	0.78 ± 0.24	19	1.78 ± 0.7
S. acanthias	9	47.6 ±11.2	22.4 ± 11.2	44	1.25 ± 0.5
M. merluccius	3	35.0 ± 1.4	2.4 ± 0.6	100	1.33 ± 0.57
M. barbatus	11	14.7 ± 1.2	1.1 ± 0.5	64	1.57 ± 0.78
C. lucernus	3	25.4 ± 1.2	8.8 ± 2.019	67	1 ± 0

Table 6.1. General data on analyzed specimens of commercial Adriatic fish species, percentage of individuals containing microplastics in the stomach, average number of items in individuals containing microplastics

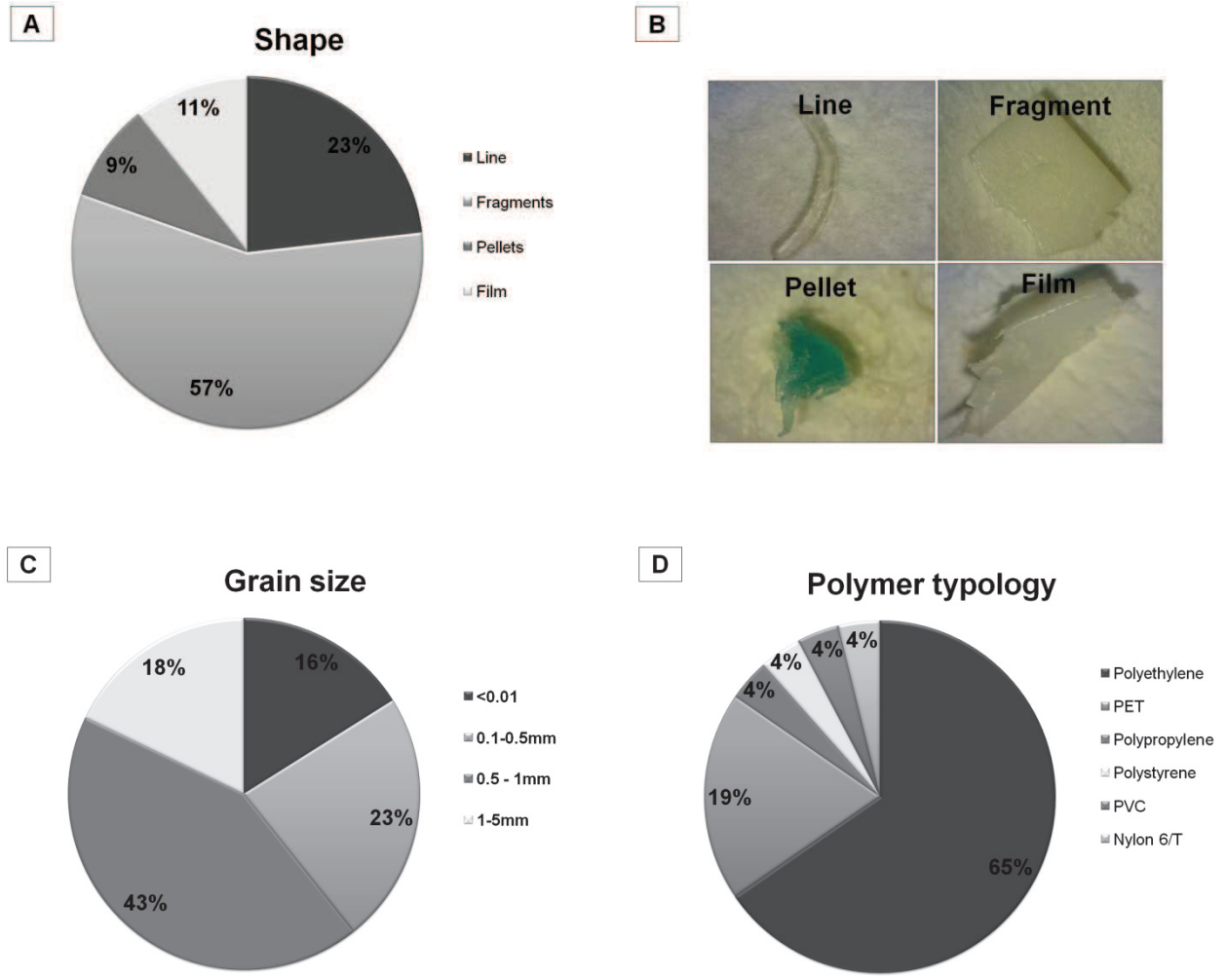


Figure 6.1. Characteristics of microplastics extracted in Adriatic fish species. (A): shape; (B): representative examples of particles; (C): grain size; (D): typology of polymers as indicated by FT-IR. Data in pie graphs are given as percentage distribution.

3.2 Microplastics in Adriatic organisms

The extraction of microplastics in wild organisms from Ancona and Civitanova areas highlighted their presence in gastrointestinal tracts of 63 individuals, i.e. 45% of the 139 examined organisms. The benthonic organisms *S. mantis and M. barbatus* showed a percentage of animals containing plastic particles of 41 and 37% respectively. The bentho-pelagic species, *M. merlucciu,* showed a presence of microplastics in the 48% of sampled specimens, while microplastics were found in 47% and 52% of the pelagic *S. pilchardus and S. scombrus*.

The average number of microplastics extracted in positive fish ranged from 1.2 item/ind. in *M. merluccius* from Civitanova, to 5.16 items/ind. in *S. pilchardus* collected in Ancona (Table 6.2). No statistical differences were found between species, nor between organisms collected from the two investigated sites (Figure 6.2 A - B).

The 25% of extracted microplastics were in the larger size class (from 5 to 1 mm), 35% was between 1 and 0.5 mm, 35% between 0.5 and 0.1 mm, and the 3% lower than 0.1 mm (Fig. 6.3A). Considering differences between species, the benthonic *M. barbatus* and *S. mantis* showed a predominance of particles with grain size between 1 and 0.1 mm while bentho-pelagic and pelagic species showed higher heterogeneity of ingested grain sizes (Figure 6.3 B). Differences in size of ingested polymers in respect to the site of collection are given in Figure 6.3 C. A predominance of items with grain size between 0.5 and 0.1mm was found in organisms from Civitanova, while those from Ancona showered the majority of extracted items between 0.5 and 1 mm. Considering the vertical distribution of analyzed organisms, results indicated a prevalence of items with grain size between 5 and 0.5 mm in pelagic species (*S. pilchardus* and *S. scombrus)*, while in bentho-pelagic and in benthic species was measured a higher ingestion frequency of low size items (Figure 6.3 B).

The shape of the plastic particles isolated in tissues of all the organisms (without species differentiation) was largely dominated by fragments (80%), followed by film (10%), line (6%) and pellet (2%) (Fig. 6.3D). The same pattern was observed in function of species differentiation, except for *S. mantis* that presented a relatively high presence of film and line in soft tissue (Figure 6.3 E). No particular differences were observed between the two investigated site (Figure 6.3 F).

FT-IR analyses indicated that approximately 52 % of analyzed items were polyethylene, 30 % polystyrene, 8.5% polyamide, 2.5 % respectively polypropilene, polyvinyl chloride (PVC), and bioplastic polymers, while 1.2% was PVA polymers (Fig. 6.3 G). Interesting results come from the distribution of polymers in different species and sites of collection (Figure 6.3 H, I). Among the species, the higher heterogeneity in the typology of ingested items was observed in *S. pilchardus* and *M. barbatus* in which at least five different polymers were founded in analyzed tissue; *S. scobrus, M. merluccius* and *S. mantis* had a lower variability of ingested plastics polymers. In addition, also fish collected from Ancona exhibited a higher level of polymer variability was compared to organisms from Civitanova where only PE, PS and Polyamide were present. Last but not least, considering the vertical distribution of collected organisms, a clear pattern of plastics typologies was recognized: fish living in sea surface showed a predominance of low density polymers, while in the benthic organisms different high density plastics were found (i.e. PVC, Polyamide and PVA). In addition, both *S. pilchardus* and *M. barbatus* exhibited some biodegradable polymer, characterized by FT-IR analysis as polycaprolactone.

Overall, the benthic species showed a greater ingestion of high density polymers (i.e. PVC and polyamide) in respect to pelagic ones that had more low density particles in their stomachs (i.e. PS and PP) (Figure 6.3 H).

Site	Species	N. of analyzed organisms	Organisms lenght cm (means ± SD)	Sample wet weight g (means ± SD)	Sample with microplastics (%)	N. of microplastics in organisms with plastic in stomach	N. of microplastic in all analyzed organisms
Ancona	*S. pilchardus*	19	11.32 ± 0.62	0,55 ± 0.10	12 (63%)	3.16 ± 0.80	2 ± 0.61
	S. scombrus	14	22.95 ± 1.94	4.28 ± 1.27	7 (50%)	1.71 ± 0.75	0.85 ± 1.02
	M. merluccius	13	26.26 ± 7.75	7.61 ± 4.88	6 (46,15%)	2 ± 0.63	0.92 ± 1.11
	M. barbatus	16	15.20 ± 3.84	0.80 ± 0.33	6 (37,50%)	2.83 ± 3.54	1.06 ± 2.48
	S. Mantis	6	18.63 ± 0.81	9.22 ± 1.42	3 (50,00%)	1.33 ± 0.57	0.66 ± 0.81
Civitanova	*S. pilchardus*	19	12.13 ± 0.48	0.49 ± 0.13	7 (37%)	2.42 ± 1.71	0.89 ± 1.55
	S. scombrus	7	20.28 ± 3.23	3.49 ± 0.92	4 (57,14%)	1.5 ± 0.57	0.85 ± 0.89
	M. merluccius	18	27.29 ± 9.69	5.35 ± 3.92	10 (55,50%)	1.2 ± 0.42	0.66 ± 0.68
	M. barbatus	19	14.08 ± 1.34	1.33 ± 0.55	7 (36,84%)	1.57 ± 0.78	0.57 ± 0.90
	S.Mantis	6	19.32 ± 1.09	10.65 ± 2.83	2 (33,30%)	1.5 ± 0.70	0.5 ± 0.83

Table 6.2. General data on analyzed specimens of commercial fish species; Site of collection, morphological parameters, percentage of individuals containing microplastics in the stomach, average number of items in individuals containing microplastics.

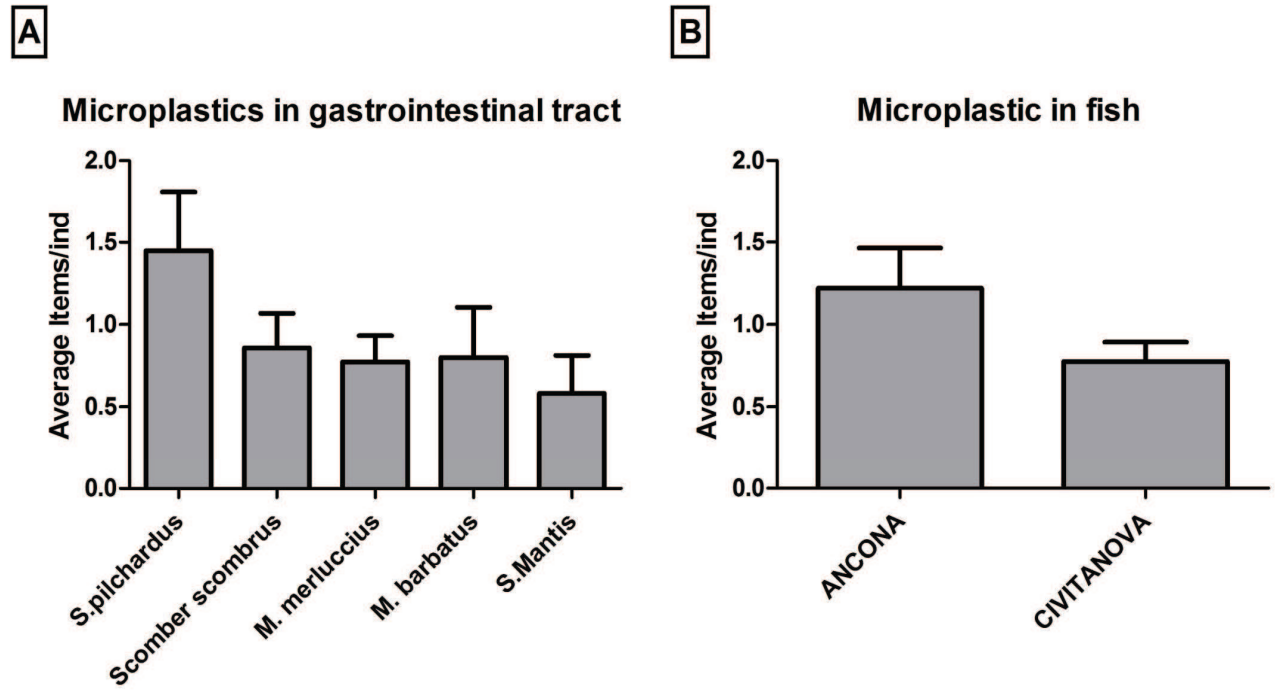


Figure 6.2. A) Microplastics presence in different fish species, B) Difference in microplastics presence between two collection site. Data are expressed as average items per individuals (±S.E).

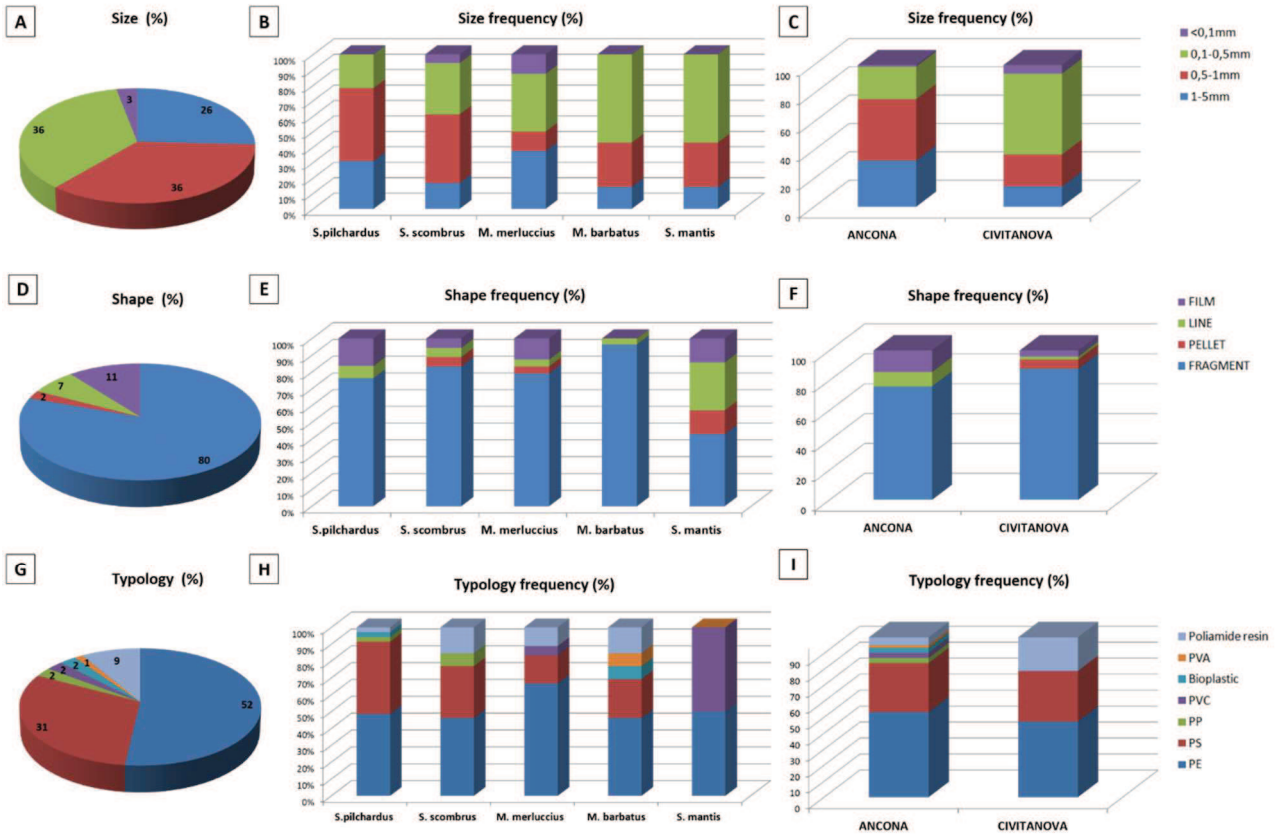


Figure 6.3. Shape, size and typology of extracted microplastics from Adriatic organisms; (A, D, G) average data, (B, E, H) data are given in relation to the species, (C, F, I) in relation to site of collection. In all graphs data are given as percentage of distribution.

3.3 Microplastics in organisms from the Costa Concordia wreck.

The extraction of microplastics from benthic fish at the Giglio Island highlighted their presence in gastrointestinal tracts of 36 individuals, i.e. 90% of the 40 examined fish. Closely benthic species *P. phycis, Scorpaena sp. and U.scaber* showed plastic particles in 77%, 84%, 86% of individuals respectively, while the bentho-pelagic *Sparidae* fam. exhibited microplastics in 100% of analyzed specimens.

The average number of microplastics extracted in positive fish ranged from 2.5 ± 1 (average item/individual ± SEM) in *P. phycis* to 3.5±0.5 items/individual in *Scorpaena sp.* (Figure 6.5 A). Differences in microplastics ingestion between fish species were not found (Figure 6.5 A), while if we compare the area, levels of ingested microplastics were significantly higher in fish sampled close to the wreck (Figure 6.5 B).

The 19% of extracted microplastics exhibited the larger size class (from 5 to 1 mm), 36% was between 1 and 0.5 mm, 35% between 0.5 and 0.1 mm, and the 9% lower than 0.1 mm (Figure 6.6A). Considering the size frequency in each analyzed species, and in the two site of collection (CTRL vs WRECK) no particular trend in size distribution was evident (Figure 6.6 B, C).

The shape of the plastic particles isolated in gastrointestinal tracts of all the fish (without species differentiation) was largely dominated by fragments (58%), followed by line (28%), film (11%) and pellet (5%, Figure 6.6 D). Considering species differentiation, no particular differences were observed between *P. phycis, U. scaber* and *Scopaena* sp. while *Sparidae* sp. showed a prevalence of ingestion from lines and films in respect to fragments and pellets. Again, no particular differences were evident between Control and Wreck site (Figure 6 E, F).

FT-IR analyses indicated that approximately 40% of analyzed items were polyethylene, 28% were polystyrene, 21% were nylon, 4% were respectively polypropylene and bioplastics (i.e polycaprolactone), while 2% was polyamide and PVC (Figure 6.6 G). Interesting evidences come from the analysis of polymer typology in different species, as show in Figure 6.6 H. While in *U. scaber, P. phycis* and *Scopaena* sp. polyethylene, nylon and polystyrene represent important constituents of ingested microplastics, in Sparidae sp. polyethylene particles were not founded (Figure 6.6 H). Lastly, dividing the typology of ingested particles in relation to the site, fish collected from the control area showed a higher heterogeneity between polymer including PE, PS, NYLON, BIOPLASTIC, POLYAMID and PVC, compared to organisms from the Wreck site in which PE, PS, NYLON and PP were found.

The results obtained from the analysis of transplanted mussels, are summarized in Table 6.4.

The extraction of microplastics revealed the presence in the 20.83% of analyzed specimens and, considering only organisms with particles, an average of 1.125 items/individuals. In general, the

variability observed between seasons, depths and sample areas did not highlight any particular trend (Figure 6.7 A).

The average number of microplastics extracted in mussels ranged from 0 (in surface mussels collected in spring near the wreck and in bottom mussels from the control area in winter and spring) to 2.2 ± 0.5 items/individual (in surface mussels collected during the summer in the wreck zone, Table 6.4).

Clustering samples in relation of samples area no differences were observed (Figure 6.7 B), while a significant efect in microplastics presence in mussels tissues was observed in relation to depth (Figure 6.7 C) and season (Figure 6.7 D). Bottom translocated mussels showed a significantly lower level of ingested microplastics compared to those maintained in surface waters, while considering the season, mussels showed lower level of ingested microplastics in spring (T2) and higher in summer (T3).

The characterization of extracted microplastics showed that mussels are able to ingest particles higher than 100µm; in particular 42% of extracted particles have the longest measure between 5-1mm, followed by 0.1-0.5mm (35%), 0.5-1 (20%) while items lower than 0.1mm represent the 3% of extracted microplastics (Figure 6.8 A). Regarding the shape, the 70% of extracted items were lines, while fragments and films represented the 17% and 13% respectively (Figure 8 B). Analyzing the distribution of the shapes in relation to the size of extracted microplastics, it is clear that the biggest particles are mostly represented by lines, while the lower size classes are formed by fragments and film (Figure 6.8 C).

Site	Species	Number of stomach examinated	Fish lenght cm (means ± SD)	Stomach wet weight g (means ±SD)	Stomach with microplastics (%)	Number of microplastics in fish with plastics in stomach (means ±SD)
CTRL (n=22)	*Sparidae*	3	29±12	10±12	100%	2.3±1.15
	P. phycis	5	33±9	26±19	80%	1.75±0.5
	U. scaber	6	25±6	9±7	83%	3.33±1.53
	Scorpaena sp.	8	24±7	8±1.7	62.5%	3.20±3.35
WRECK (n=18)	*Sparidae*	2	33±7	3±0.6	100%	5±0
	P.phycis	3	31±0.5	11±3.6	100%	4±4.24
	U. scaber	3	27±2.4	17±4	67%	5±1.41
	Scorpaena sp.	11	25±4.6	11±6	100%	3.63±1.68

Table 6.3. General data on analyzed specimens of commercial fish species, Site of collection, morphological parameters, percentage of individuals containing microplastics in the stomach, average number of items in individuals containing microplastics.

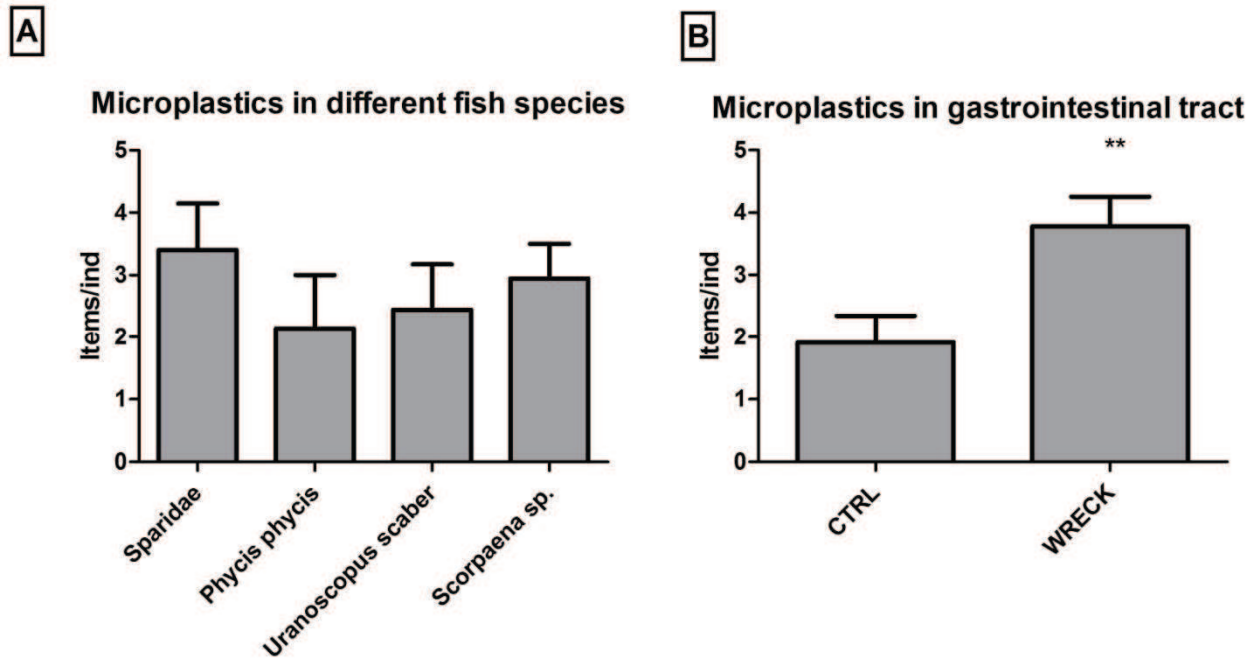


Figure 6.5. A) Microplastics in different fish species (average items/ind. ±SEM);(B) Microplastics presence in fish sampled by the two sites of the Giglio Island (average items/ind. ±SEM) ($p<0.01$; Student t-test).

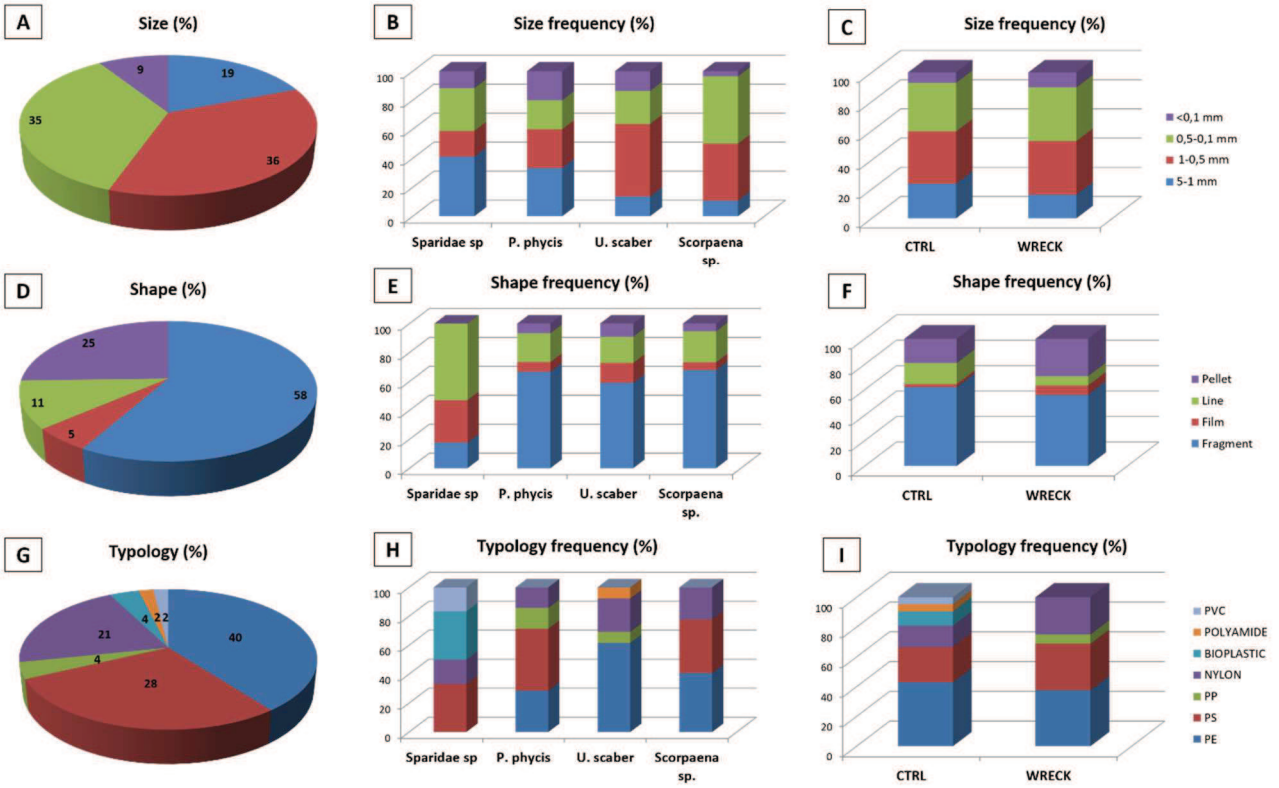


Figure 6.6. Characteristics of microplastics extracted in fish species from the Giglio Island. (A): total grain size frequency, (B) grain size frequency in respect to analyzed species and (C) in respect to site of collection; (D): total shape frequency, (E) shape frequency in respect to fish species and (F) in respect to the site of collection; (G) frequency of polymers typology in total, (H) in respect to fish species and (I) in respect to site of collection. Data are given as percentage distribution.

Table 6.4. General data on analyzed specimens of translocated mussels, percentage of individuals containing microplastics in whole tissue, average number of items in individuals containing microplastics. T1, T2, T3 represent winter 2013, spring 2014 and summer 2014 respectively.

Site	Seasons	Depth	Number of organisms analyzed	Mussel length cm (means ± SD)	Mussels wet weight g (means ±SD)	Mussels with microplastics (%)	Number of microplastics in mussels with plastics in soft tissue(means ±SD)
CTRL (n=60)	T1	Surface	10	5.74±0.3	2.13±0.44	50	2±1.2
		Bottom	10	5.68±0.41	1.85±0.57	0	0
	T2	Surface	10	4.96±0.35	2.78±0.69	10	1±0
		Bottom	10	5.12± 0.41	2.70±0.69	0	0
	T3	Surface	10	5.73±0.66	2.31±0.68	50	1.2±04
		Bottom	10	5.23±0.22	2.43±06	30	1.6±0.57
WRECK (n=60)	T1	Surface	10	5.36±0.47	1.2±0.2	20	1.5±0.7
		Bottom	10	4.89±0.35	2.28±0.59	10	1±0
	T2	Surface	10	5.24±0.43	2.02±0.53	10	1±0
		Bottom	10	5.37±0.53	2.13±0.64	10	1±0
	T3	Surface	10	5.89±0.36	1.93±0.4	50	2.2±1
		Bottom	10	5.49±0.42	1.99±0.2	20	1±0

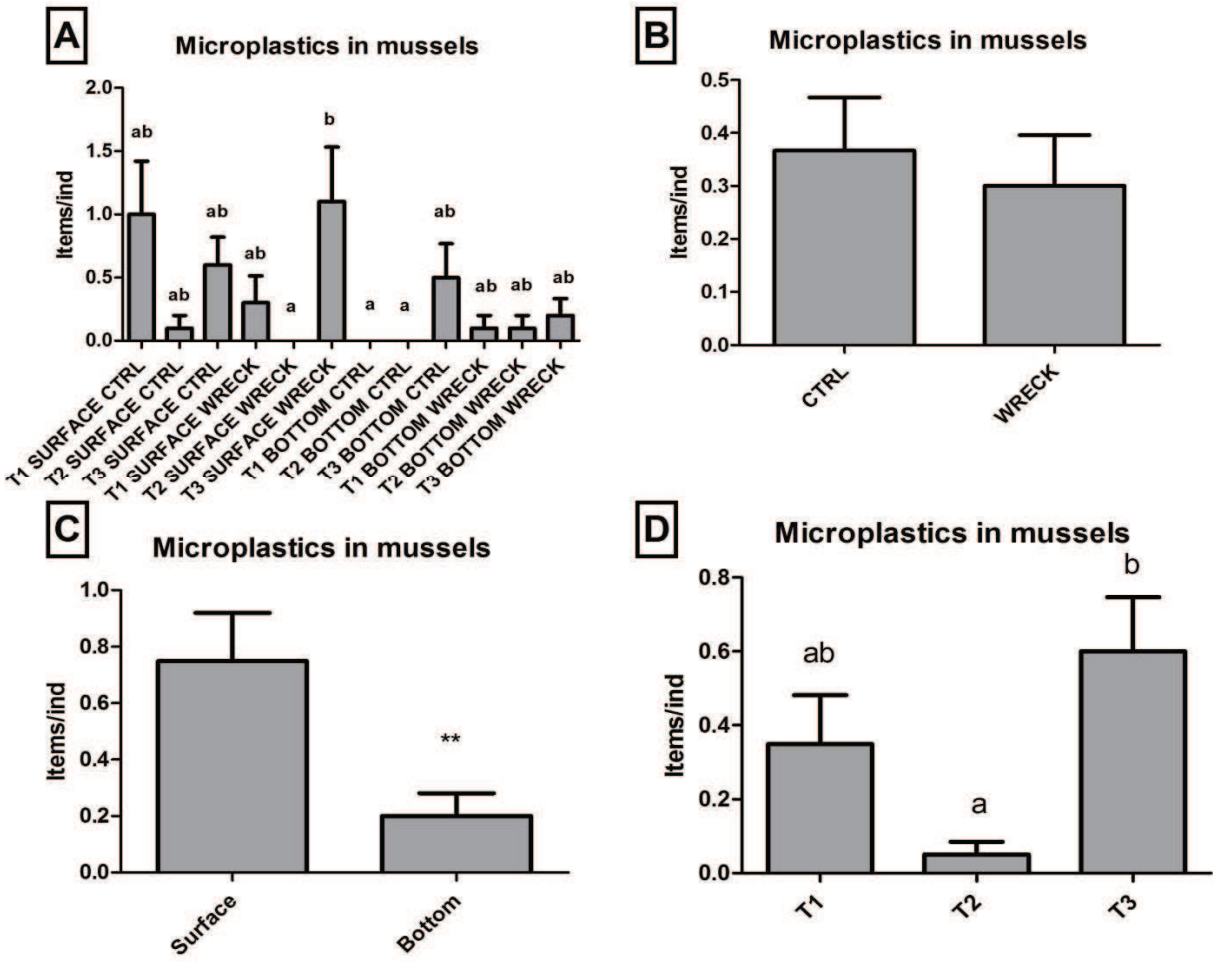


Figure 6.7. A) Microplastics in mussels; B) Microplastics presence in mussels sampled by the two sites of the Giglio Island; C) Microplastics in mussels collected from different depths; D) Microplastics in mussels collected in different seasons

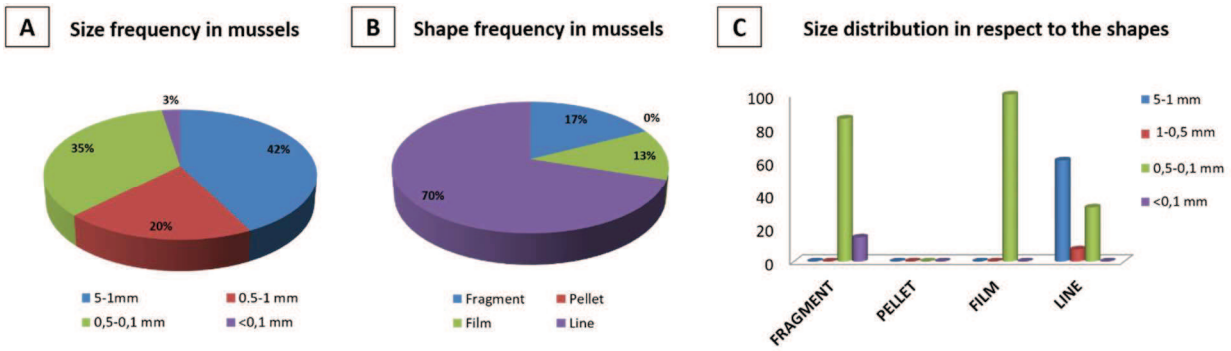


Figure 6.8. Size (A), shape (B) and distribution of different size classes in respect to the shape of extracted organisms (C) in mussels.

4. Discussion

Microplastics are documented worldwide either in water-column and sediment samples, and their presence has also been reported in different taxa including planktonic species, invertebrates, fish and cetaceans (Browne *et al*., 2011; Hidalgo-Ruz et al. 2012; Foekema et al., 2013; Lusher et al, 2013, 2015; Van Cauwenberghe et al., 2014, 2015a). Laboratory studies further confirmed the capability of a wide range of marine biota to ingest particles, including shellfish and fish for human consumption, with a potential impairment of various cellular, metabolic or physiological pathways (Browne *et al.,* 2008; Von Moos et al., 2012; Wegner et al., 2012; Avio et al., 2015).

The present investigation was aimed to provide a preliminary insight on microplastics occurrence in commercial species collected from different site of the Mediterranean Sea. In addition, it attempts to offer new and necessary evidence on the "behaviors" and "interactions" between MPs and marine organisms. Particular attention was given to explain the distribution of different polymer typologies in different species considering various environmental scenarios (anthropic pressure, geographical area, distance to the coast, season and depth) trying to understand practical indications on the interaction and distribution of microplastics and biota in marine environment.

In this respect, the overall results showed the presence of microplastic particles in 38% of the 422 analyzed specimens, irrespective of the species and areas of collection. This percentage is slightly higher than that reported for the English Channel where 36.5% of fishes contained microplastics in their gastrointestinal tracts (Lusher et al., 2012). The presence of small plastic fragments had been demonstrated also in 30% of all planktivorous fish caught in the North Pacific Central Gyre (Boerger *et al.*, 2010), and of catfish collected in a Brazilian estuarine environment (Posatto et al., 2011); a lower occurrence of microplastics was reported in fish collected from southern and northern sectors of the North Sea with respectively 5.4 and 1.2% of specimens containing particles (Foekema et al. 2013). On the other hand, a recent study on large pelagic fish of Mediterranean reported a frequency of microplastics in the 18.2% of analyzed organisms (Romeo et al., 2015) but the number of analyzed organisms was lower than that reported in this chapter (121 vs 422) and authors didn't test the extraction yield of their method.

Within invertebrates, the percentage of *S. manthis* with microplastics in soft tissues (42%) was in the range reported for *N. norvegicus* UK and *Lepas* spp. collected from UK and NPSG where the 83% and the 33.5% of animals contained plastics respectively (Murray and Cowie 2011; Goldstein and Goodwin 2013). The occurrence of particles in tissues of transplanted mussels with was lower (21%) suggesting that the different feeding behaviours (predation vs filtration), or, especially, the shorter time of exposure (4 weeks of translocation in investigated areas) could explain such specific differences in microplastics intake

The number of ingested items, considering only fish with plastics in their stomach, ranged from 1 item/individual in *C. lucernus* analyzed in the preliminary study to 5 items/individual in *Sparidae* sp. and *U. scaber* collected close to the wreck site of the Giglio Island, with an average of 0.98 items/individual considering all analyzed fish and 2.24 items/ind considering only fish with microplastics in their stomach. For invertebrates, the average number of plastics per individual was 0.35 items/ind considering all analyzed organisms and 1.55 items/ind considering only positive specimens. Despite the techniques of extraction are different between the available reports, our evidences seem to be in line with results of Lusher et al. (2013) and Boerger et al. (2010) , reporting an average of 1.9 items/ind and 2.10 items/ind. Lower level of microplastics ingestion were reported in fish of the Portuguese coast and from deep waters of Ionian Sea where authors found an average of 1.4 items/ind. and 1.3 items/individuals respectively (Neves et al., 2015; Anastasopoulou et al., 2013).

In this study, the 44% of the fish had one microplastic items, 32% had 2, 10% had 3 and the 14% had more than 3 particles in gastrointestinal tract. The occurrence of fish with more than 3 particles was higher than reported by several authors; Neves et al. (2015), reported that 67.3% of the fish from the Portuguese coast had one particle, 26.9% had 2, 3.8% had 3, and only one individual had 4 microplastics in the stomach. Moreover, Foekema et al. (2013) reported than the 80% of the fish from North Sea that ingested plastic contained only one particle, suggesting that MPs have a short residence time within the gastrointestinal tract of fish. Evidences of this study suggest that Mediterranean fish actively ingest particles similar or smaller than natural food, highlighting a potential and important secondary plastic intake from their prey.

The overall results seem also to indicate a greater exposure to microplastics for Mediterranean organisms, in agreement with typically high levels of particles recently detected in this basin. In general, results of this study showed more ingested items in Tyrrhenian species compared to the Adriatic ones. However, in our opinion this difference could be speculatively explained in respect to the distance from the coast: Tyrrhenian organisms were collected at Giglio Island very close to the coast, compared to the Adriatic organisms used in this study. A similar hypothesis would be in line with several authors suggesting that microplastics abundance along the coast is much higher than off shore (XXX). Beside, trophic features of the species and/or variations in the trophic state of the habitat could be determined different behavior and hunger of analyzed organisms.

Among the all Adriatic species, *S. pilchardus* showed the highest variability on the average number of items/individual, and percentage of specimens containing microplastics. Recent reports on the microplastics distribution in the water column indicated elevated and variable levels of particles

at the sea surface (Cozar et al., 2014; Eriksen et al., 2014), supporting the possibility for those pelagic specimens to occasionally cross patches of concentrated floating debris.

On the other hand, benthic and bentho-pelagic species showed a relatively lower number of ingested items and a markedly higher percentage of microplastics in gastrointestinal tract (from 37 to 100%) in respect to pelagic organisms. The high frequency of microplastics found in benthic organisms compared to pelagic ones, suggested that microplastic presence may be higher near the seafloor and/or in sediments, or that benthic fish are less selective feeders.

The analysis of grain size distribution in fish showed a predominance of the 1-0.5 mm particles in sampled organisms (35%), the 5-1 mm and 0.5-0.1mm classes had all a similar contribution, each accounting for approximately 28% while smaller class account for the 9%. Interestingly, the analysis of ingested items in mussels, reveal that about 42% of particles were higher than 1mm, despite several authors indicate that the size limit of ingestion for mussels should be around the 500 µm. On the other hand, if we compared the size of items ingested by mussels with their shape, a clear pattern was presented, with a high frequency of lines. Since items are measured for the larger dimension, the dimensional limit for the uptake by mussels for particles like line and fiber should be reviewed.

Plastic particles in gastrointestinal tracts of Mediterranean fish were mostly fragments followed by lines and films with a more limited occurrence for pellets. This pattern was confirmed in Adraitic organisms for both the performed investigations, while for the Giglio Island fish a higher frequency of pellets was observed in respect to line and films. A different composition was reported for fish from the English Channel with 68% of all extracted particles consisting of fibers (Lusher et al 2012). Since fibers were excluded from our count to prevent external contamination (as suggested by Foekema et al., 2013), our results are not fully comparable with those of Lusher et al. (2012); in this respect, it is important to check whether fibers have been considered before comparing results of different studies.

The range of bright colors identified also in smallest extracted particles indicated that the extraction method did not affect the particles in terms of plastic bleaching. FT-IR analyses on microplastics isolated from our samples highlighted the elevated selectivity of the extraction protocol: all the analyzed items were plastic polymers, without any presence of other particles like glass, wood, sand.

Although the density gradient separation used in this study was expected to extract polymers with density ≤ 1.2 g/cm^3 (like acrylic polymers, low density polyethylene, polystyrene, nylon and polypropylene), also higher density particles such as PET or PVC (1.38 g/cm^3) were isolated; the possible attachment of organic matter might have determined a density decrease of these particles and their extraction during density separation.

The most common polymer found in Mediterranean organisms was polyethylene, followed by PET, PS and Nylon. A lower prevalence was observed for PP, PVC, PVA and Polyamide resins. These results suggest plastic bags, bottles and fishing lines as the main sources of microplastics in Mediterranean Sea, confirming polyethylene as the most widespread polymer in oceans (Cozar *et al*., 2014). Moreover, variations in the typology of ingested polymers between organisms collected from different Mediterranean areas might reflect differences in the source of contamination.

In particular, the high presence of nylon particles in fish collected from the Giglio Island could be explain for the huge usage of ropes (often nylon made) in the wreck site, while in Adriatic organisms the high frequency of PS particles cold be related to the use of polystyrene boxes in fishing activities.

The FT-IR characterization revealed the presence of some low density polymers (e.g. polyethylene) in benthic species and of high density particles (e.g. PET) in the pelagic ones. It is known that buoyancy of microplastics can be influenced by factors like fouling which cause their sinking and accumulation in sediments (Wright et al., 2013), while high density particles can be ingested by pelagic or bentho-pelagic organisms through their preys as secondary ingestion (Brandao et al., 2011). Beside, for some polymers it was possible to observe a clear trend of distribution between species; for example in Adriatic organisms PS decreased with the depth of fish collection, while PVC and polyamide particles were more abundant in benthic species compared to bentho-pelagic and pelagic ones.

Of particular interest appeared the results obtained in organisms collected from the Giglio Island during the activities related to refloating of the Costa Concordia wreck. Those activities were previously shown to have had a limited impact in terms of release of chemical pollutants (Regoli et al., 2014) but the results of this study demonstrated a significant increase of microplastics ingestion in fish collected close to the wreck. The origin of such particles (nylon and polypropylene made) can be easily associated to the increased human pressure and intense activities carried out in proximity of the bottom, like the construction of an artificial seafloor, or the impressive platform and anchoring system for the wreck. Also the enhanced turbidity and sediments resuspension caused by those operations, might have favored the deposition of microplastics close to the sediments.

The particularly elevated numbers of plastic items found in fish species close to the wreck (compared to control site, Adriatic organisms and reference data) highlight the usefulness of these organisms as bioindicators for revealing this relatively unconsidered form of anthropogenic pollution. The same results were not obtained with mussels which did not reveal clear and statistically significant differences between organisms transplanted close to the wreck or in the control site.

On the other hand, mussels were more prone in revealing variations according to season and depth of collection. The highest and lowest levels of microplastics found in mussels translocated at the Island during the summer or spring season respectively, can be related to the different presence of touristic activities, while the intermediate values of ingestion observed in winter season can reflect the hydrodynamic condition, with storm events and rainfall enhancing microplastics resuspension from the sediments or input from the inland of the Island. Also the generally low level of microplastics, both in terms of ingested items (0.35 items/ind. vs 0.98 items/ind. for fish) and of percentage of positive organisms to ingestion (23% for mussels vs 43% for vertebrates), indicate that transplanted mussels were more sensitive to environmental factors than anthropogenic activities..

In conclusion, it has been recently pointed out an enormous loss of microplastics from sea surface compared to expected rates of fragmentation (Eriksen et al. in 2014), suggesting the presence of mechanisms removing particles smaller than 4.75 mm; results of this work support the hypothesis that this plastic fraction can be directly ingested by the organisms, stored in the sediments and/or indirectly transferred trough the trophic webs.

Results of the study underline the ubiquitous presence of plastic in the Mediterranean marine biota, including the water surface, column and bottom where fish and invertebrates live and feed. The high frequency of microplastics in Mediterranean fish, represents a further warning signal for the pressure on this basin and marine species, with possible implications also for human health, the consequences of these findings for humans who consume soft tissue of invertebrates containing microplastics are not yet understood. The presence of localized anthropogenic activities was shown to clearly enhance the impact on resident biota, confirming the importance of considering and monitoring microplastics as a true environmental pollution. Future studies are required on a more elevated number of organisms, species and trophic guilds to better assess the distribution of microplastics along food webs, and their capacity to penetrate the edible tissues, with potentials concerns also for human consumers.

7. Conclusion and perspectives

The results of this PhD thesis demonstrated, from one hand, that microplastics have a marked ability to adsorb xenobiotic compounds from the marine environment, due to both chemical properties and elevated surface/volume ratio; from the other, that marine organisms after the ingestion of microplastics, can compartmentalize these particles in different tissues, where adsorbed pollutant became bioavailable and were accumulated. Moreover, following the uptake, both virgin and contaminated microplastics induce several forms of biological responses at transcriptional, biochemical and cellular levels. After the development of an efficient extraction method, microplastics were characterized in a wide array of Mediterranean species suggesting that these organisms are highly exposed to particles ingestion. All these evidences highlight the potential risk of these emerging contaminants for marine environment, highlighting the need of further investigations both on occurrence and toxicological effects in organisms.

The ability of different plastics polymers to adsorb organic and inorganic contaminants was assessed in open field and laboratory condition. Levels of PAHs on virgin plastic objects, and on beached macro and microplastics, increased markedly in particles with high s/v ratio, indicating that the small size of these particles might represent one of the most important factors influencing the adsorption capability. Laboratory experiments confirmed marked adsorption kinetics of pyrene and cadmium on different polymers (PE and PS), highlighting a greater effect of time rather than concentration of exposure for both tested contaminants and investigated polymers.

Pollutants bioavailability and several molecular, biochemical and cellular pathways involved in the early responses to microplastics (both virgin and contaminated) were evaluated in mussels and fish exposed under laboratory conditions. After the ingestion of microparticles, adsorbed contaminants can be transferred from the polymer to the organisms tissue. In mussels, particles were found in gills, digestive gland and haemolymph, while in fish some microplastics could penetrate from the stomach to the liver tissue. Microplastics ingestion induced the modification of several molecular, biochemical and cellular pathways, producing a potential ecotoxicological risk for marine organisms. Mussels exhibited the modulation of several genes and pathways that differed between exposure to virgin or contaminated microplastics. Immunological parameters, genotoxic modification, neurotoxic effects and lysosomal destabilization were the mostly affected parameters by microplastics exposure.

An efficient and sensitive method for extraction and characterization of microplastics from marine organisms was developed and validated, providing an average extraction yield around the 90%; the proposed method did not affect FT-IR characterization of polymer that is, with Raman, the unique tool for confirming the plastic nature of a particles.

With this new protocol microplastics were characterized in 424 Mediterranean organisms including 292 fish and 132 invertebrates, for a total of 12 different species, all with high commercial interest; 38% of analyzed organisms had microplastics in their tissues. Overall, the occurrence of MPs is higher in benthic species, while number of ingested items is typically greater in pelagic ones: these results suggest that marine sediments can be an important sink compartments or that benthonic species are less selective than pelagic ones, but also that near the seawater surface microplastics can have higher densities with a more patched distribution than in sediments. The results obtained at the Giglio Island showed that the release of microplastics in the marine environment can be efficiently monitored in areas impacted by anthropogenic activities (i.e. incidents): fish were more sensitive than translocated mussels as sentinel species, which were useful in revealing seasonal and vertical trends of microplastics ingestions, offering some useful evidence for further monitoring this new form of environmental contamination.

Further studies are still needed to better understand the fate of microplastics in the marine environment, their occurrence and risks for marine organisms. At the same time new techniques should be developed to reduce the input of these materials in the environment (i.e. improving the systems of waste discharge), as well as, offer innovative tools for the collection and reuse of materials already present in the environment.

Abbreviation

AChE - Acetylcholinesterase
AOX – Acyl-CoA oxidase
AP - Alkylphenol
ATR - Attenuated total reflectance
cDNA – Complementary deoxyribonucleic acid
CDNB - 1-chloro-2,4-dinitrobenzene
CYP1A – Cytochrome P450 1A
DDT - Dichlorodiphenyltrichloroethane
DEG - Differentially expressed genes
DNA - Deoxyribonucleic acid
EDTA - Ethylenediaminetetraacetic acid
EROD – 7-ethoxyresorufin O-deethylase
EU – European Union
FC - Fold change
FT-IR - Fourier Trasform InfraRed spectroscopy
GPx - Glutathione peroxidases
GR - Glutathione reductase
GSSG - Oxidized glutathione
GST – Glutathione S-transferase
HCPC - Hierarchical Clustering on Principal Components
HDPE - High density polyethylene
HPLC – High pressure liquid chromatography
HQBM – Hazard Quotient for Biomarker
ICRAM-APAT – Istituto Centrale per la Ricerca scientifica e tecnologica Applicata al mare – Agenzia per la protezione dell'ambiente e per i servizi tecnici
ISPRA – Istituto Superiore per la Protezione Ambientale
LDPE - Low density polyethylene
LOE - lines of evidence
MDA - malondialdehyde
MN - Micronucleus
MOA - Mechanisms of action
mRNA – Messenger ribonucleic acid
MSFD - Marine strategy framework directive
NADPH - β-nicotinamide adenine dinucleotide
NRRT - Neutral Red Retention Time
ORO - Oil Red O
PAH - Polycyclic aromatic hydrocarbon
PBDE - Polybrominated diphenyl ether
PCA - Principal component analysis
PCB – Polychlorinated biphenyls
PE - Polyethylene
PET - Polyethylene terephtalate
PFC - Perfluorinated compound

POPs - Persistent organic pollutants
PP - Polypropylene
PPAR – Peroxisomal proliferator-activated receptor
PS - Polystyrene
PVA - Polyvinyl alcohol
PVC – Polyvinylchloride
PYR - Pyrene
RNA - Ribonucleic acid
TOSC - Total Oxyradical Scavenging Capacity
UK – United Kingdom
USA – United States of America
UV - Ultraviolet
WOE - Weight Of Evidence

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Pollutants bioavailability and toxicological risk from microplastics to marine mussels

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ABSTRACT

Microplastics represent a growing environmental concern for the oceans due to their potential of adsorbing chemical pollutants, thus representing a still unexplored source of exposure for aquatic organisms. In this study polyethylene (PE) and polystyrene (PS) microplastics were shown to adsorb pyrene with a time and dose-dependent relationship. Results also indicated a marked capability of contaminated microplastics to transfer this model PAH to exposed mussels *Mytilus galloprovincialis*; tissue localization of microplastics occurred in haemolymph, gills and especially digestive tissues where a marked accumulation of pyrene was also observed. Cellular effects included alterations of immunological responses, lysosomal compartment, peroxisomal proliferation, antioxidant system, neurotoxic effects, onset of genotoxicity; changes in gene expression profile was also demonstrated through a new DNA microarray platform. The study provided the evidence that microplastics adsorb PAHs, emphasizing an elevated bioavailability of these chemicals after the ingestion, and the toxicological implications due to responsiveness of several molecular and cellular pathways to microplastics.

1. Introduction

The global production of plastic dramatically increased in the last decades, from 0.5 million tons/yr in 1960 to 280 million tons in 2012 (Plastic Europe, 2012). Almost 10% of the annual production ends up into the oceans, and plastic debris accumulation has been reported as a global scale phenomenon for the marine environments, including polar areas and abyssal regions (Barnes et al., 2009).

Adverse effects of plastics have been documented in terms of entanglement and physical damages to locomotory, respiratory or digestive appendages in marine mammals, turtles, seabirds and crustaceans (Andrady, 2011). In addition, since plastics degrade very slowly, they also act as floating substrates for several organisms, and thus contribute to long-range transport of alien species, representing an additional risk to local biodiversity (Andrady, 2011).

In the recent years, a great scientific interest is being directed toward microplastics, i.e. fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derive from macroscopic debris after chemical, physical and biological fragmentation (Barnes et al., 2009).

Ingestion of microplastics has been demonstrated in various marine organisms with different feeding strategies; this phenomenon may negatively influence both the feeding activity and nutritional value of a plankton-based diet, particularly in those species which can not discriminate the food source (Moore et al., 2001; Browne et al., 2008).

Recent evidences also suggest the potential role of microplastics as vectors of chemical pollutants, either used as additives during the polymer synthesis, or adsorbed directly from seawater (Rios et al., 2007; Teuten et al., 2009; Engler, 2012). The hydrophobicity of organic xenobiotics and the large surfaces of floating polymers facilitate the adsorption of these chemicals on microplastics at concentrations orders of magnitude higher than those detected in seawater (Ogata et al., 2009). The possibility for plastic particles to adsorb chemical pollutants from the surrounding environment has

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been also characterized in laboratory conditions. Different particles polymers, like polyvinyl chloride, polyethylene, polypropylene, polystyrene, were shown to have a high sorption capacity for DDTs, polycyclic aromatic hydrocarbons (PAHs), hexachlorocyclohexanes and chlorinated benzenes (Bakir et al., 2012; Lee et al., 2014). Consistent with these studies, several persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, PAHs and dioxins have been detected in plastic pellets stranded on different beaches of the world (Endo et al., 2005; Ogata et al., 2009; Hirai et al. 2011; Heshett et al., 2012).

Despite the importance of microplastics in adsorption and transport of hydrophobic pollutants, it is still unclear whether they also represent a potential source of chemical exposure within marine food webs. Various evidences, including the use of a thermodynamic approach and of models simulating physiological conditions in the gut, suggested that both adsorbed pollutants and chemical additives of plastics might be released to organisms (Gouin et al., 2011; Tanaka et al., 2013; Bakir et al., 2014a).

In laboratory conditions, microplastics have been shown to be ingested by amphipods, barnacles, and lugworms (Thompson et al., 2004); in mussels, *Mytilus edulis*, plastic particles (3–9.6 μm) were accumulated in digestive tissues and translocated to haemolymph (Browne et al., 2008). In the same organisms, the uptake of microplastics caused notable histological changes in digestive cells with strong inflammatory responses, formation of granulocytomas and lysosomal destabilization which increased with exposure time (Von Moos et al., 2012).

To further assess the possible risk of microplastics as environmental contaminants, the present investigation aimed at a multidisciplinary approach to characterize the chemical adsorption of hydrophobic pollutants, as well as bioaccumulation, chemical release and onset of potential health effects in the filter feeding mussels *Mytilus galloprovincialis*. Two different polymers, polyethylene (PE) and polystyrene (PS) were exposed to various doses of pyrene, selected as one of the more commonly represented PAHs adsorbed on plastic marine debris (Rios et al., 2007); virgin and contaminated PE and PS were then used in a trophic transfer experiment with mussels. Tissue localization of microplastics was integrated with measurement of pyrene bioaccumulation and a wide battery of cellular biomarkers to detect the early onset of adverse effects. Such analyzed responses included immunological parameters, lysosomal membrane stability, peroxisomal proliferation, antioxidant defences and oxidative stress biomarkers, neurotoxic effects and onset of genotoxicity; for the first time, effects of microplastics were also investigated at the transcriptomic level through a new *M. galloprovincialis* DNA microarray platform, to better elucidate pathways and molecular mechanisms of action (MOA).

Obtained results have been elaborated with a classical Weight Of Evidence (WOE) approach that combine and differently weight various typologies of data, or lines of evidence (LOEs), providing multidisciplinary characterization of hazard indices and risk evaluation (Chapman et al., 2002; Chapman, 2007). WOE methods are considered as key components of Ecological Risk Assessment (ERA) procedures, according to recent European Directives which require member states to evaluate and classify the ecological status of water bodies integrating different quality elements. Among the available WOE procedures, the Sediqualsoft model elaborates data from sediment chemistry, bioavailability of pollutants and onset of adverse effects at different levels of biological organization (Piva et al., 2011; Benedetti et al., 2012); the computational rules have been successfully validated in filed conditions for the characterization and classification of risk from industrial and harbour sediments, natural hydrocarbon seepage in coastal areas or the recent Costa Concordia wreck at Giglio Island (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014). In this study we have applied the flow-charts and mathematical algorithms developed for elaborating data and summarizing the hazard index for bioavailability and biomarker responses, thus providing a synthetic judgment on the biological relevance of these observed effects.

The overall results of this study were expected to increase our knowledge on the potential toxicological risk of microplastics in the marine environment.

2. Materials and methods

2.1. Experimental design

Polyethylene (PE) and polystyrene (PS) powders were obtained from a private plastic company. Particles were size-sorted in a 1000–100 μm group used for characterization of the pyrene adsorbing capacity, and in a <100 μm group for the exposure of mussels to virgin and contaminated polymers.

The adsorption of pyrene to PE and PS was assessed by mixing solutions of microplastics (20 g/L in seawater) with pyrene dosed at final concentrations of 0.5 μg/L (low, L), 5 μg/L (medium, M) and 50 μg/L (high, H). While the L and M treatments are environmentally realistic for pyrene, the H dose is uncommon but still possible, i.e. after heavy oil spill or in highly contaminated sewage (Neff, 2002). The mixing solutions were maintained in continuously rotating 50 mL glass tubes for 6 days; water was changed and pyrene re-dosed after 3 days. Levels of pyrene adsorbed on polymers were measured after three and six days of treatment.

For the exposure of mussels to microplastics, specimens of *M. galloprovincialis* (5 ± 1 cm shell length) were obtained from a local farm (Numana, Ancona, Central Adriatic Sea) and acclimatized for 10 days to laboratory conditions with aerated seawater, at 18 ± 1 °C and 35‰ salinity. Contaminated plastics were prepared according to the above description, by maintaining a solution of <100 μm microplastics with pyrene (50 μg/L) in rotating conditions for 6 days. A total of 150 organisms were distributed into fifteen 6 L glass-beakers and exposed to virgin or pyrene-contaminated plastics for 7 days with three replicates for each of the 5 following treatments: Control (CNTR), Polyethylene (PE), Polystyrene (PS), Pyrene-treated Polyethylene (PE-PYR), Pyrene-treated Polystyrene (PS-PYR). Water was changed daily and both virgin and pyrene-treated particles re-dosed at a nominal concentration of 1.5 g/L.

No mortality of mussels was observed during the experiments. After the exposure period, haemolymph, digestive glands and gills were rapidly removed from 30 specimens for each treatment, pooled in 10 samples (each with tissues of 3 specimens), frozen in liquid nitrogen and maintained at −80 °C for chemical, biochemical and histochemical analyses; for haemolymph samples, an aliquot was also immediately processed for lysosomal neutral red retention time assay (NRRT), phagocytosis activity, and DNA damage, and another aliquot fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic evaluation of granulocytes and chromosomal alteration. Four additional pools, each with digestive glands of three specimens, were prepared from CNTR, PS and PS-PYR groups for DNA microarray analysis.

2.2. Chemical analyses of pyrene on plastics and exposed mussels

Pyrene adsorbed on microplastics (PE and PS) or accumulated in mussels tissue (gills and digestive glands) was determined after extraction of samples in 0.5 M potassium hydroxide and methanol (1:10 w:v) with microwave at 55 °C for 15 min (Benedetti et al., 2014). After centrifugation for 5 min at 1000 × g, the methanolic solutions were concentrated in speedvac and 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, and HPLC analyses were carried out with water–acetonitrile gradient and fluorimetric detection. Pyrene was identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). Quality assurance and quality control were tested by 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 pyrene expressed as ng/g dry weight (d.w.).

2.3. Biological analyses in mussel tissues

To evaluate the possible presence of plastic particles in different tissues, cryostatic sections (8 μm thick) of gills and digestive glands, and haemolymph smears were histologically examined. After staining with haematoxilyn and eosin, the occurrence and localization of microplastics was assessed through polarized light microscopy.

Standardized protocols were used for measurement of biomarkers in tissues of control and exposed organisms (Gorbi et al., 2013; Benedetti et al., 2014). Detailed methods are given in Supplementary Material 1 for the following measurements: immunological alterations in terms of granolocytes/hyalinocytes ratio, phagocytosis activity and lysosomal membrane stability (NRRT) in haemocytes; neurotoxic responses as acetylcholinesterase (AChE) in haemocytes and gills; cellular and oxidative stress biomarkers in digestive tissues, i.e. acyl-CoA oxidase (AOX), antioxidant defenses (catalase glutathione S-transferases, glutathione peroxidases, glutathione reductase, total glutathione), total oxyradical scavenging capacity (TOSC), lysosomal latency period (LP), malondialdehyde (MDA), lipofuscin, neutral lipids; genotoxic effects in haemolymph in terms of DNA strand breaks, micronuclei frequency (MN) and nuclear alterations (NA).

2.4. Mytilus galloprovincialis oligonucleotide microarray

Gene transcription analyses were performed using an 8 × 60 K Agilent oligo-DNA microarray platform designed within the European project REPROSEED (REsearch project to improve PROduction of SEED of established and emerging bivalve species in European hatcheries). Information about sequencing, assembly, annotation and microarray design are summarized in Supplementary Material 2 and 3. Probe sequences and other details on the microarray platform can be found in the GEO database under accession number GPL18667 (http://www.ncbi.nlm.nih.gov/geo/). Microarrays were synthesized in situ using the Agilent non-contact ink-jet technology including default positive and negative controls.

2.5. Labelling, microarray hybridization and data acquisition

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Full details about labeling, hybridization and data acquisition are reported in Supplementary Material 3.

Normalized gene expression data were deposited in the GEO database under accession number GSE57460.

Due to technical problems during the hybridization step, one of the four pools of the PS exposed mussels was excluded by the gene transcription analyses. Normalization procedures included quantile normalization which always outperformed cyclic loess, and further adjustment by the parametric Combat in R to account for the between-experiments batch effects of the oligonucleotide microarray (Johnson et al., 2007). Normalized data were deposited in GEO archive under accession number GSE57460.

2.6. Microarray data processing and analysis

Statistical analyses were performed on 52.988 out of 59.971 probes with signal higher than the background in 8 out of 11 analysed samples. The TIGR Multi Experiment Viewer 4.5.1 statistical software (TMeV; Saeed et al., 2003) was used to perform T-test statistics (p-value <0.01; 200 permutations) comparing CNTR to both PS and PS-PYR groups. The resulting T-test genes lists were then filtered and only probes with fold change (FC) > 1.5 have been considered as differentially expressed genes (DEGs). A more systematic functional interpretation of differentially transcribed genes was obtained through an enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Huang et al., 2009). Since these databases contain functional annotation data for a limited number of species, transcripts of *M. galloprovincialis* were matched to *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10−5). The choice of *D. rerio* allowed the assignment of a putative homologue to a larger number of *M. galloprovincialis* transcripts (see Supplementary Material 2), and was previously demonstrated a useful option for *Ruditapes philippinarum* functional analyses (Milan et al., 2011). A functional annotation was obtained for genes differentially expressed in each T-test pairwise comparison, setting DAVID for gene count = 2 and ease = 0.1.

2.7. Statistical analyses and toxicological risk assessment

Statistical analyses were performed with the statistical R-software (R. development Core Team, 2010). Adsorption of pyrene to microplastics was tested by analysis of variance (ANOVA) according to typology of polymer (PE-PS), time of exposure (3–6 d), dose of pyrene (L, M, H); bioaccumulation of pyrene and biomarker responses in exposed mussels were also compared by one-way ANOVA and post-hoc comparison (Bonferroni) was used to discriminate between means of values. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary. For biomarkers data, multivariate principal component analysis (PCA) was combined to hierarchical clustering of the PCA patterns which visualize the relationships among the different treatments and organize the samples into groups of homogeneous observations (Husson et al., 2010). The proposed methodology is available in the HCPC (Hierarchical Clustering on Principal Components) function of the FactoMineR package (Lê et al., 2008).

Results on bioaccumulation of pyrene and biomarkers responses in mussels exposed to virgin and contaminated microplastics were further elaborated within a classical Weight Of Evidence WOE approach, using a previously developed quantitative and software-assisted model (Sediqualsoft). According to WOE principles, different typologies of data are initially evaluated with appropriate criteria to provide synthetic indices of hazard for each of considered line of evidence, before their final integration in a quantitative WOE evaluation (Piva et al., 2011). Whole calculations, detailed flowcharts, rationale for weights, thresholds and expert judgments have been fully given elsewhere (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014).

Briefly, the bioavailability hazard was calculated from the initial calculation of a weighted Ratio to Reference (RTRw), reflecting the magnitude of pyrene accumulation in tissues of exposed organisms,

corrected for both the statistical significance of the difference compared to controls and the typology of chemical. Depending on the magnitude of such variations, the model assigns the hazard to 1 of 5 classes: from Absent to Slight if the calculated increase of pyrene tissue concentration is lower than 2.6 folds compared to control organisms, Moderate between 2.6 and 6.5 folds, Major between 6.5 and 13 folds, Severe if greater than 13 folds (Piva et al., 2011; Benedetti et al., 2012).

For the evaluation of biomarkers results, the model contains a large selection of responses among those more widely used by scientific community in different bioindicator organisms (Piva et al., 2011); according to species and tissue, each biomarker, has a "weight" based on the relevance of biological endpoint, and a "threshold" for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold (Absent or Slight), calculates the average for those with an effect up to twofold compared to the threshold (Moderate) and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva et al., 2011):

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

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

The elaborations of results on bioavailability of pyrene and biomarker variations were integrated after normalization of hazard indices to a common scale; the resulting level of toxicological risk was finally assigned to 1 of 5 classes from Absent to Severe (Piva et al., 2011).

3. Results

Microplastics showed an elevated capability to adsorb pyrene with a dose- and time-dependent trend (Fig. 1). After 6 days of M treatment, concentrations of adsorbed pyrene were 145 ± 35 and 126 ± 35 ng/g on PE and PS microplastics respectively, with an accumulation factor of 29 and 25.2 calculated as the ratio to nominal levels dosed in seawater. Concentrations of adsorbed pyrene were even greater after H dose experiment (305 ± 89 and 244 ± 52 ng/g for PE and PS) but the accumulation factors were 6.1 and 4.8 for the two polymers.

Concentrations of pyrene on contaminated microplastics used for the laboratory experiments with mussels (<100 μm) were in the range of 200–260 ng/g for both PE and PS. After 7 days of exposure, a significant increase of pyrene was observed in gills, and a more marked bioaccumulation occurred in digestive glands, with concentrations much greater than those measured directly on contaminated particles (Fig. 2). Tissue levels of pyrene in exposed mussels (up to 470 ng/g) increased by more than 13 folds compared to control specimens, reflecting an hazard index for bioavailability summarized as Severe by the proposed WOE model (Table 1, LOE2).

Histological analyses of treated mussels revealed the presence of microparticles in haemolymph, gills and, especially, in digestive glands where numerous aggregates could be observed in the intestinal lumen, epithelium, and tubules (Fig. 3). No qualitative differences in tissue localization were evident between organisms treated with the two polymers (PE, PS), both as virgin or contaminated particles.

Among immunological responses of haemocytes, phagocytosis activity did not exhibit particular variations, while a decrement of granulocytes versus hyalinocytes type cells was observed in mussels exposed to virgin and to pyrene-contaminated PE; lysosomal membrane stability decreased in almost all the treatment groups (Fig. 4).

The comet assay indicated a significant enhancement of DNA strand breaks in haemocytes of mussels treated with virgin microplastics, while nuclear anomalies were higher in all the treatments with either virgin or contaminated polymers; the frequency of micronuclei significantly increased only in specimens exposed to pyrene-treated PS (Fig. 4). Acetylcholinesterase did not vary in haemolymph and decreased in gills of mussels exposed to both virgin and contaminated microplastics, while activity of AOX was not influenced in any of experimental treatments (Fig. 4).

Antioxidant defenses did not reveal variations in the levels of glutathione and activities of glutathione reductase, glutathione S-transferases, and sum of Se-dependent and Se-independent glutathione peroxidases (Fig. 5). A significant inhibition was observed in all the treatments for Se-dependent glutathione peroxidases and a similar trend appeared also for catalase; the overall significance of those effects was reflected in slight variations of the Total Oxyradical Scavenging Capacity (TOSC) toward both peroxyl

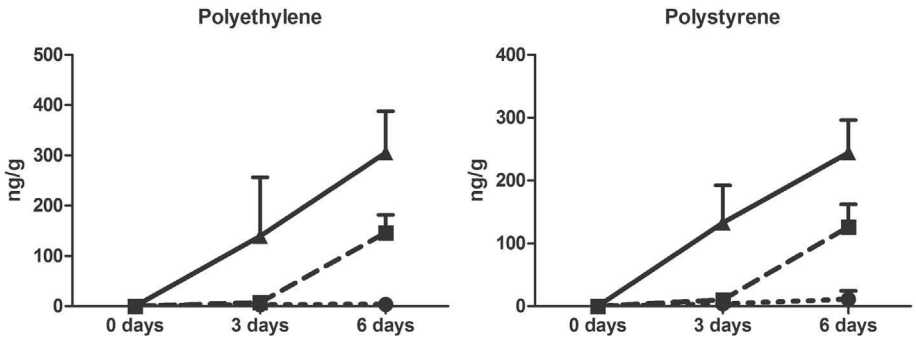

Fig. 1. Time-course of pyrene adsorption to microplastics particles (polyethylene and polystyrene). Different lines indicate nominal doses of pyrene: solid line 50 μg/L, dashed line 5 μg/L, dotted line 0.5 μg/L. Data are expressed as ng/g dry weight (mean values ± standard deviation, n = 5).

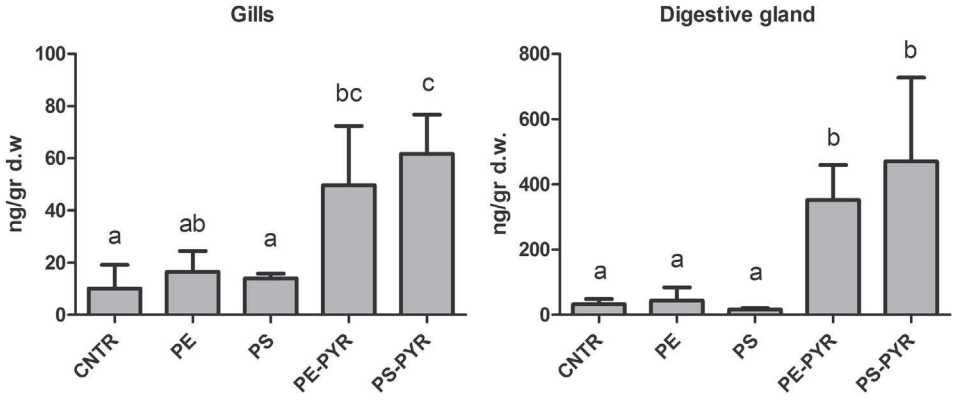

Fig. 2. Concentrations of pyrene in gills and digestive glands of mussels exposed to various microplastics treatments: CNTR = control; PE = virgin polyethylene; PS = virgin polystyrene; PE-PYR = pyrene-contaminated polyethylene; PS-PYR = pyrene-contaminated polystyrene. Data are expressed as ng/g dry weight (mean values ± standard deviation, n = 5); different letters indicate significant differences between groups of means (post-hoc comparison).

and hydroxyl radicals (Fig. 5). The moderate pro-oxidant challenge induced by microplastics on mussels was supported by the lack of relevant variations for malondialdehyde, lipofuscin and neutral lipids in digestive tissues; lysosomal integrity appeared more sensitive, and decreased after exposure to both virgin and contaminated microplastics (Fig. 5).

Considering the magnitude of variations observed for various biomarkers, their statistical significance and the toxicological relevance of each biological endpoint, the WOE model summarized the hazard for cellular responses as ranging from Slight to Moderate, typically higher for PS compared to PE, and for contaminated compared to virgin microplastics (Table 1, LOE3). The combination of hazard indices elaborated for bioavailability and biomarker data resulted in an overall WOE risk classified as Slight or Moderate for virgin PE and PS (reflecting only the cellular effects), Major or Severe for contaminated polymers (integrating both bioaccumulation and cellular perturbations, Table 1, WOE).

The principal component analysis (PCA) carried out on the whole set of biomarkers produced a two dimensional pattern explaining 72% of total variance (Fig. 6). The hierarchical clustering on PCA pattern indicated a clear separation between control and exposed mussels, dividing as homogeneous groups those treated with virgin or pyrene-treated microplastics

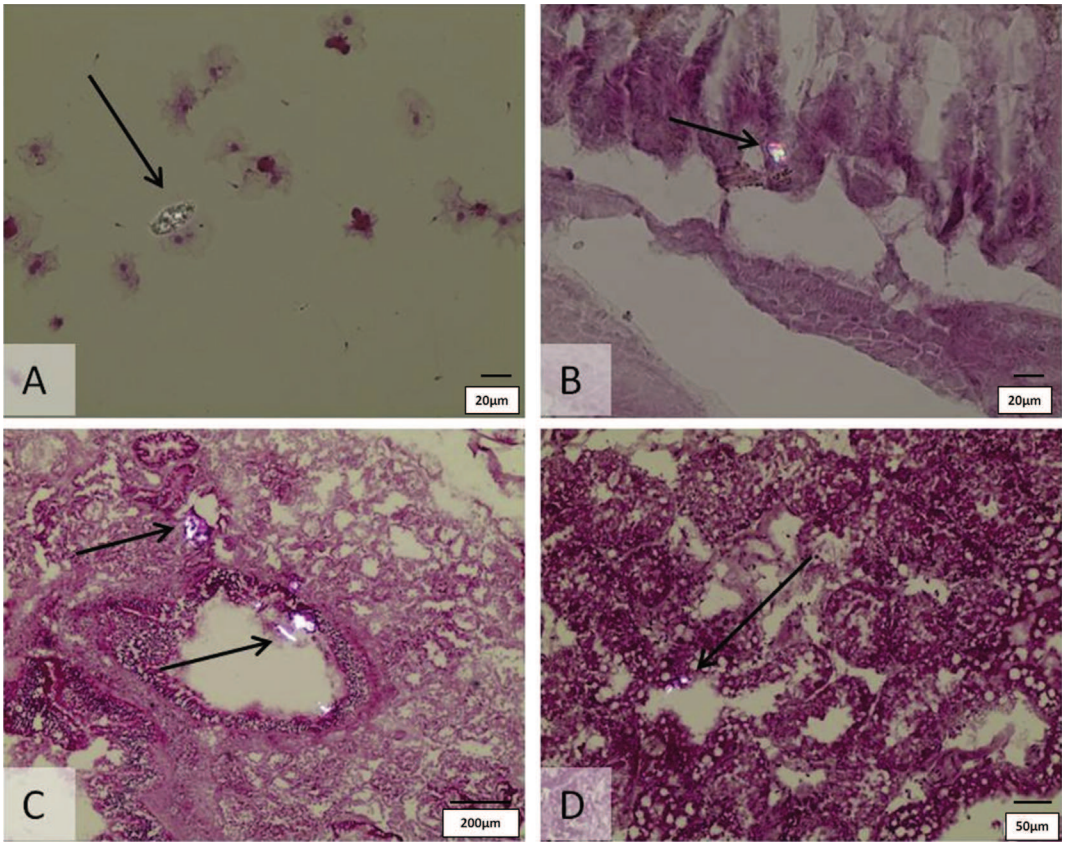

Fig. 3. Polarized-light microscopy images showing the presence of plastic particles in haemolymph (A), gills (B), gut lumen and epithelium (C), digestive tubules (D).

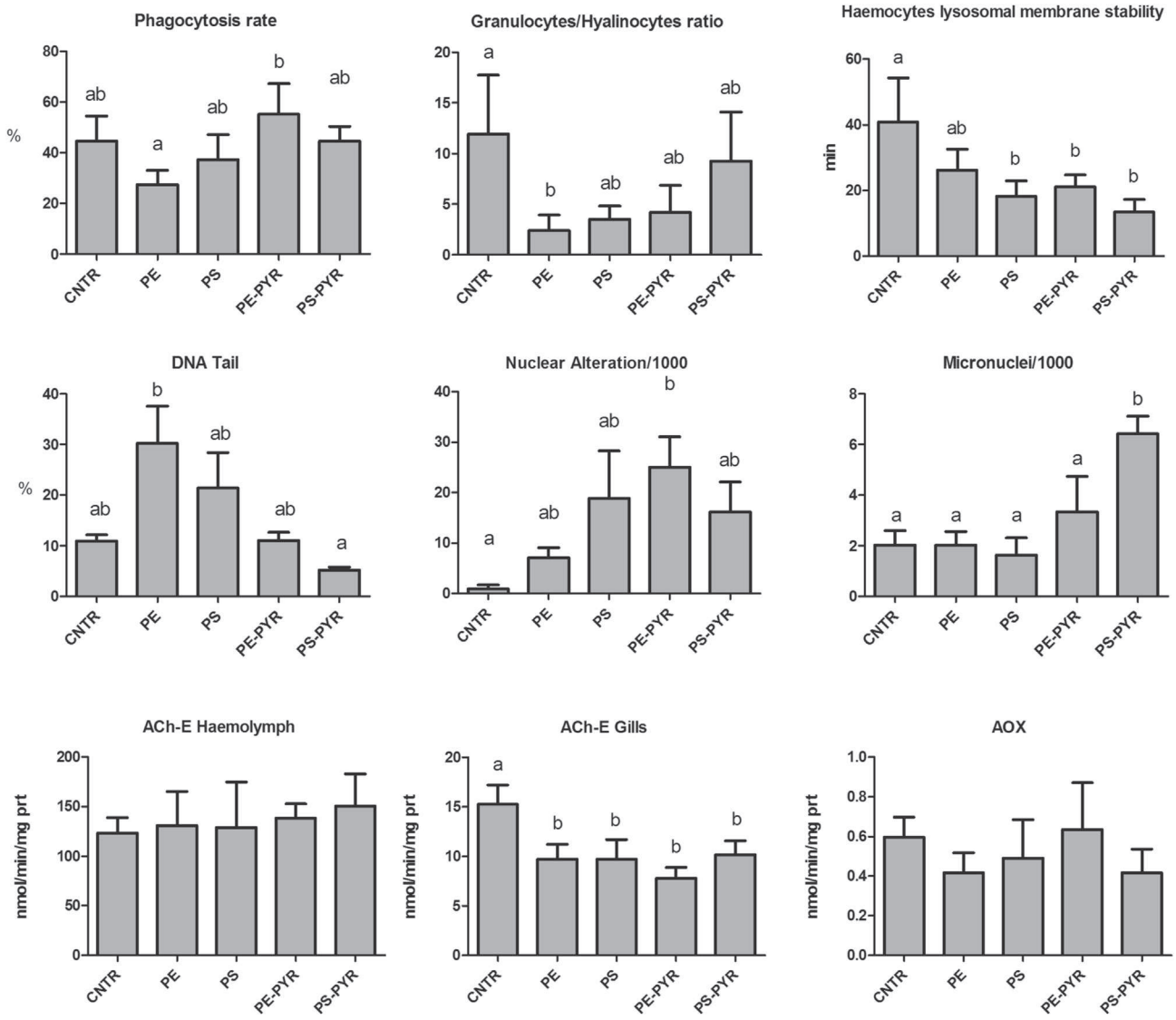

Fig. 4. Immunological, genotoxic, cholinesterasic and peroxisomal biomarkers in mussels exposed to various microplastics treatments: CNTR = control; PE = virgin polyethylene; PS = virgin polystyrene; PE-PYR = pyrene-contaminated polyethylene; PS-PYR = pyrene-contaminated polystyrene. Ach-E: acetylcholinesterase; AOX: Acyl CoA Oxidase. Data are expressed as mean values ± standard deviation, or standard error of mean for % of DNA in tail, n = 5; different letters indicate significant differences between groups of means (post-hoc comparison).

respectively (Fig. 6); the parameters determining the separation along to the PC1 axis were lysosomal membrane stability in haemocytes and digestive glands, AChE in gills and some anti-oxidant responses (catalase, glutathione reductase, Se-dependent glutathione peroxidase, TOSC-HO·). On the other side, genotoxic effects (DNA strand breaks, nuclear anomalies, micronuclei), phagocytosis, AChE in haemolymph and levels of glutathione determined the separation along the PC2 axis between mussels exposed to virgin compared to pyrene-contaminated micro-plastics; the typology of polymer (PE vs PS) did not appear to influence the observed responses.

The analysis of transcriptional responses revealed a total of 2.143 and 1.320 differentially expressed genes (DEGs, $p < 0.01$; FC > 1.5) in response to PS and PS-PYR exposures, respectively (Supplementary Material 4). Among these, 280 transcripts were significantly affected after both exposures (Fig. 7), but the majority of transcripts appeared specifically modulated within each treat-ment (1.863 in PS and 1.040 in PS-PYR). Functional annotation and enrichment analysis was applied to DEGs to highlight the most significantly affected Biological Processes (BP), Molecular Functions (MF), Cellular Component (CC) and KEGG pathways (KP), which are detailed in Supplementary Material 5.

Some of the most interesting enriched KEGG pathways/GO terms are reported in Table 2: Lysosome (with 16 and 15 DEGs in PS and PS-PYR exposed mussels respectively), Coated membrane (9 and 6 DEGs), Endosome (6 and 3 DEGs), NOD-like receptor sig-nalling pathway (4 and 7 DEGs), Response to bacterium (5 and 3 DEGs), Apoptosis (7 and 8 DEGs), Regulation of programmed cell death (5 and 8 DEGs), Citrate cycle (8 and 3 DEGs) and Arachidonic acid metabolism (5 and 3 DEGs).

Beside the above mentioned GO terms and KEGG pathways, mussels exposed to PS and PS-PYR showed the modulation of several genes involved in DNA repair (i.e. *growth arrest and DNA-damage-inducible protein, GADD45A* and *GADD45G*; *excision repair cross-complementing rodent repair deficiency, complementation group ERCC*; *aprataxin, APTX*), detoxification (i.e. *Glutathione S-transferase pi, GSTP1* and *GSTP2*; *glutathione S-transferase M, GSTMU*; *sulfotransferase family 4A, member 1, SULT4A1*) and oxidative

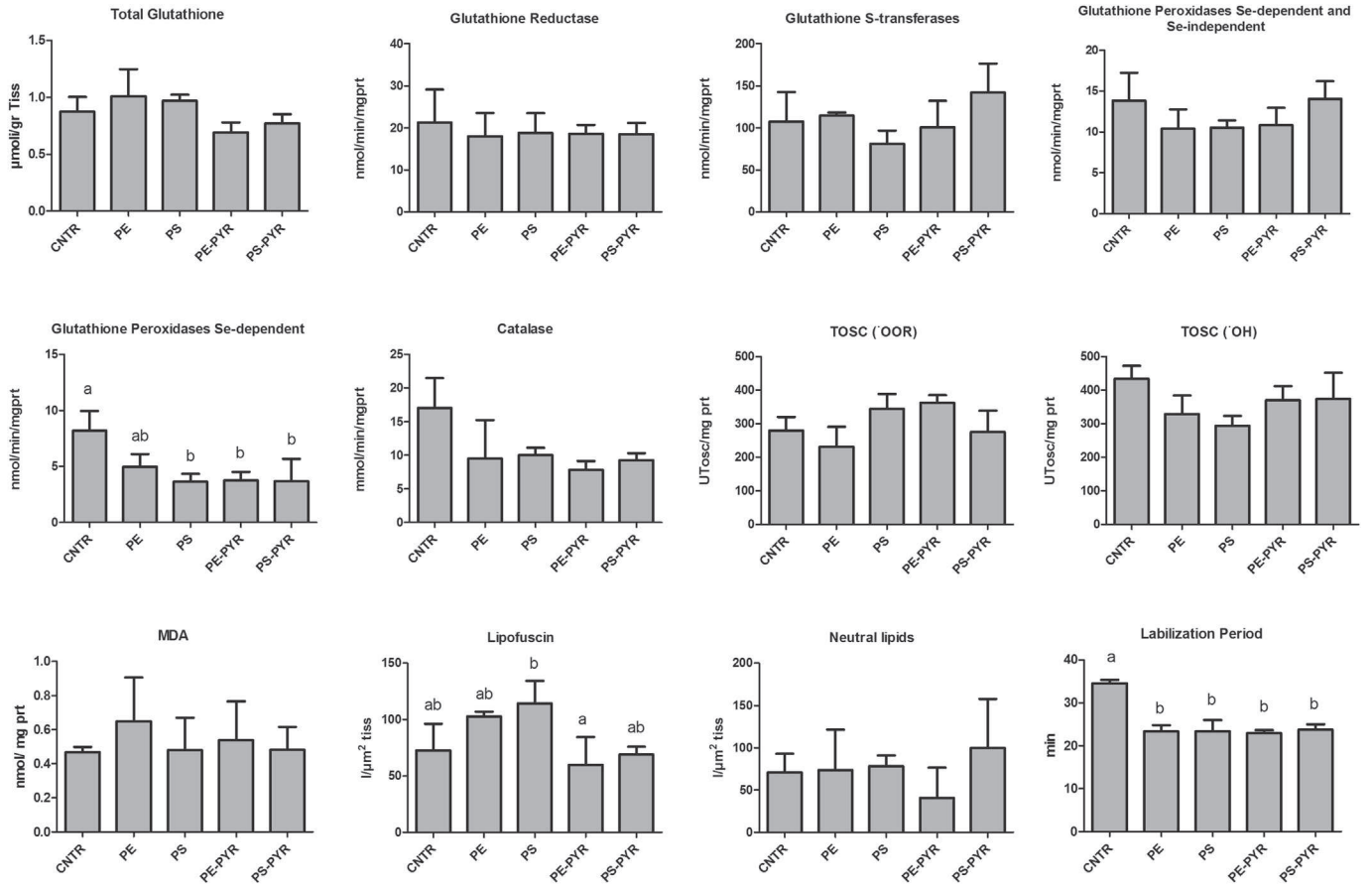

Fig. 5. Antioxidant defenses, total oxyradical scavenging capacity (TOSCA) toward peroxyl (·OOR) and hydroxyl (·OH) radicals, malondialdehyde (MDA), lipofuscin, neutral lipids and lysosomal membrane stability in digestive gland of mussels exposed to various microplastics treatments: CNTR = control; PE = virgin polyethylene; PS = virgin polystyrene; PE-PYR = pyrene-contaminated polyethylene; PS-PYR = pyrene-contaminated polystyrene. Data are expressed as mean values ± standard deviation, n = 5; different letters indicate significant differences between groups of means (post-hoc comparison).

Table 1
Weight of Evidence classification of bioaccumulation (LOE2) and biomarkers (LOE3) data, and integrated WOE risk in mussels exposed to virgin or pyrene-contaminated microplastics. The quantitative Hazard Quotients (HQ) for individual LOEs and the assigned classes of hazard or WOE risk are given. Treatments: PE = virgin polyethylene; PS = virgin polystyrene; PE-PYR = pyrene-contaminated polyethylene; PS-PYR = pyrene-contaminated polystyrene.

Treatments	LOE2 (HQ and Class of hazard)	LOE3 (HQ and Class of hazard)	WOE	
PE	-	1.87 Slight	SLIGHT	
PS	-	4.10 Moderate	MODERATE	
PE-PYR	13.01 Severe	6.44 Moderate	MAJOR	
PS-PYR	14.69 Severe	7.20 Moderate	SEVERE	

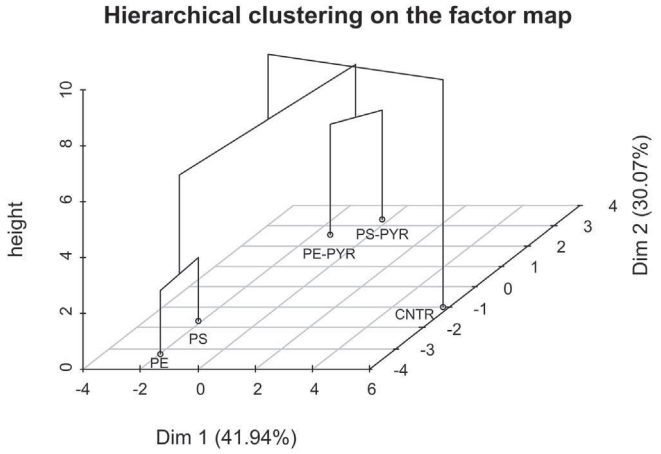

Fig. 6. Multivariate PCA and hierarchical clustering analysis on biomarker data in mussels exposed to various microplastics treatments: CNTR = control; PE = virgin polyethylene; PS = virgin polystyrene; PE-PYR = pyrene-contaminated polyethylene; PS-PYR = pyrene-contaminated polystyrene.

processes (i.e. *glutathione peroxidase, GPX2* and *GPX3*; *superoxide dismutase mitochondrial*, SOD2; see Supplementary Material 4).

4. Discussion

The present investigation aimed to provide new insights on the potential role of microplastics as a source of chemical exposure and ecotoxicological challenge to marine organisms. A growing concern is being raised for the possibility of these polymers to adsorb environmental pollutants, and our results clearly confirmed such hypothesis. Using environmentally realistic levels of dissolved pyrene, the concentrations on exposed microplastics markedly increased with a time- and dose-dependent trend. Worthy to note, the comparison of various experimental conditions did not reveal a linear relationship with levels of pyrene dosed in seawater, since the greatest adsorption efficiency was obtained for the medium treatment (5 μg/L). Adsorption of pyrene did not particularly differ between PS and PE, and chemical values measured on both polymers were comparable to those previously reported in plastic pellets from beaches and industrial sites in California, Hawaii and Greece (Rios et al., 2007; Karapanagioti et al., 2011). These data support the potential of microplastics in trapping and transporting marine pollutants, as already suggested by studies on equilibrium kinetics and partition coefficients of several hydrophobic chemicals on various typologies of plastic polymers (Zarfl and Matthies, 2010; Bakir et al., 2012; Lee et al., 2014); a transport model for persistent organic pollutants by microplastics has been recently proposed also for estuarine conditions, demonstrating a relatively little effect of salinity compared to chemical concentration in water, plastic density and particle residence time in estuaries (Bakir et al., 2014b).

Several species have been shown to ingest and accumulate microplastics, and the ecological impact of this phenomenon would be greatly influenced by the desorption of toxic chemicals. The release of additives or adsorbed chemicals from plastics to organisms has been suggested (Engler, 2012; Tanaka et al., 2013; Bakir et al., 2014a), but a clear demonstration is still lacking because organisms in field conditions can accumulate the same classes of chemicals from other sources. In our experimental conditions, mussels were exposed to microplastics containing adsorbed pyrene at concentrations of 200–260 ng g^{-1}. Despite variable levels of PAHs have been measured worldwide, such values are within the range of pyrene concentrations recently measured in plastic pellets

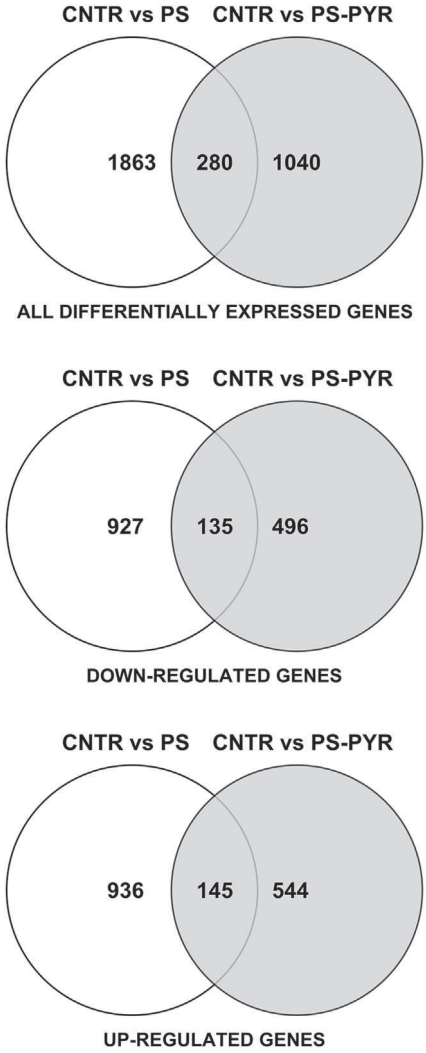

Fig. 7. Venn diagrams representing differentially expressed transcripts (total, down-regulated and up-regulated) in the comparisons CNTR vs PS and CNTR vs PS-PYR.

sampled in differently impacted sites of Portuguese coast (5–530 ng g^{-1}, Mizukawa et al., 2013) and beaches (20–320 ng g^{-1}, Frias et al., 2010). The results obtained with exposed mussels provided the first clear evidence that pyrene adsorbed on contaminated microplastics was transferred to organisms and concentrated in tissues. Despite the analyses might have been partly influenced by the presence of still un-excreted, contaminated particles, this effect can be probably considered as negligible. Average concentrations higher than 50 ng g^{-1} were measured in gill samples and, assuming that all the pyrene was that adsorbed on microplastics, we should expect at least 0.2–0.25 g of particles for each gram of gill tissue, a possibility certainly excluded by histological analyses. The bioaccumulation of pyrene was particularly marked in digestive glands where concentrations of pyrene appeared up to 3 folds higher than those present on contaminated polymers, thus necessarily reflecting a major contribution of the chemical accumulated in tissues to the total content of analyzed pyrene: this results clearly demonstrated an elevated desorption and bioconcentration process of this chemical from microplastics to tissues under physiological gut conditions (Teuten et al., 2009; Bakir et al., 2014a).

The histological analyses qualitatively supported the bioaccumulation data, with observation of particles in the digestive tissues of exposed mussels and, to a lower extent, in gills and

Table 2
Lists of the main enriched GO terms/KEGG pathways. Numbers and "gene name" of differentially expressed genes (DEGs) in each comparisons/terms are also reported. Down- and up-regulated transcripts in exposed groups are reported in green and red, respectively. Gene names reported in black indicate transcripts represented by multiple probes showing opposite responses (Supplementary Material 6). Full names of differentially expressed genes are reported in Supplementary Material 6 and the complete list of enriched KEGG/GO terms in Supplementary Materials 5.

CNTRvsPS			CNTRvsPS-PYR		
GO_TERM/KEGG	**N° DEGs**	**GENE NAME**	**GO_TERM/KEGG**	**N° DEGs**	**GENE NAME**
dre04142:Lysosome	16	*MAN2B1, AGA, CTSLA, CTNS, PSAP, NPC1, CTSC, ATP6V0B, GGA1, LGMN, CLTCA, CLTA, CTSBA, CTSD, AP1S2, CTSBB*	**dre04142:Lysosome***	15	*CD164, CTSLA, AGA, CTNS CD63, GLB1 GGA1, LGMN AP3S2, CTSZ CTSBB, AP1S2 PSAP, LDLR, CTSBA*
GO:0048475~coated membrane	9	*COPA, COPE, SEC23B, GGA1 COPB2, CLTCA CLTA, COPB1, AP1S2*	**GO:0048475~coated membrane***	6	*LDLR , AP2S1, GGA1, AP3S2, COPB1, AP1S2*
GO:0005768~endosome	6	*CHMP2BB, CHMP4B, CHMP1A, SNX5 RAB5C,TMEM55B*	**GO:0005768~endosome**	3	*CHMP1A, CHMP6B, VPS29*
dre04621:NOD-like receptor signaling pathway	4	*XIAP, SGUT1, BIRC2, BIRC7*	**dre04621:NOD-like receptor signaling pathway***	7	*HSP90B1, NFKBIAB, XIAP TRAF6, BIRC2, BIRC7*
GO:0009617~response to bacterium	5	*PGLYRP6, PGLYRP2, CTSD, RHOGB, PCNA*	**GO:0009617~response to bacterium**	3	*RHOGB, MYD88, TRAF6*
dre04210:Apoptosis	7	*DFFB, BAXA BIRC2, BIRC7, CASP3A, PRKAR2AA, XIAP*	**dre04210:Apoptosis***	8	*CASP9, NFKBIAB, BCL2L1, MYD88 XIAP,PRKAR2AA, BIRC2, BIRC7*
GO:0043067~regulation of programmed cell death	5	*CASP3A, BAXA, TRAF3, BIRC2, CASP2*	**GO:0043067~regulation of programmed cell death***	8	*BIRC2, BCL2L1, CRADD, CASP2, CASP9, CCT3, MCL1B, TRAF6*
dre00020:Citrate cycle (TCA cycle)	8	*DLDH, PCK1, DLAT, MDH1AA, PCK2, IDH3G, ACO1, SDHB*	**dre00020:Citrate cycle (TCA cycle)**	3	DLST, PCK2, IDH3G
dre00590:Arachidonic acid metabolism	5	*PLA2G1B, CYP2P9, CPLA2, CYP2U1, ALOX5A*	**dre00590:Arachidonic acid metabolism**	3	*TBXAS1PLA2G1B GPX3*

haemolymph. Uptake and tissue distribution of microplastics has already been described in the blue mussels *M. edulis* after laboratory exposures to high density polyethylene and polystyrene (Browne et al., 2008; Von Moos et al., 2012). In those studies, a first site of particles uptake was shown at the gill surface, mediated by microvilli activity and endocytosis, while a second pathway occurred via ciliae movement in the stomach, intestine and digestive tubules, followed by accumulation within the lysosomal compartment (Von Moos et al., 2012): polystyrene particles smaller than 9.6 or 3.0 μm could translocate from the gut cavity to the haemolymph and inside the haemocytes (Browne et al., 2008). Despite our observations were not quantitatively assessed, they almost reflected the above mechanisms of uptake, with conspicuous aggregates within intestinal lumen and digestive tissues, and more limited occurrence of particles in branchial epithelial cells and in haemolymph; the lack of microplastics within the haemocytes may be the consequence of a dimensional difference of plastic particles used in our experimental conditions.

A large battery of biochemical and cellular biomarkers were analyzed in this study to characterize the ecotoxicological potential of both virgin and contaminated microplastics. Significant immunological effects were observed on haemocytes with a strong shift of the haemocytic cell population, a limited variations of phagocytosis and a significant reduction of lysosomal membrane stability. The lower granulocytes/hyalinocytes ratio did not probably reflect a decrease of granulocytes, which are primarily involved in phagocytic activity, but rather a sharp increase in hyalinocytes, less differentiated cells and potential precursors of granulocytes (Carball et al., 1997). On the other hand, the lower lysosomal membrane stability of haemocytes could be reasonably related to the over-production of prooxidant reactive oxygen species involved in the immune responses, typical during microbial attack and recently observed also toward nanoparticles (Canesi et al., 2002; Jovanovic and Palic, 2012). Similarly to our results, the ingestion and translocation of polystyrene did not cause measurable changes in the viability and phagocytic activity of haemocytes in *M. edulis* (Browne et al., 2008), while inflammatory responses and lysosomal membrane destabilization occurred as a cellular host response to high density polyethylene microplastics (Von Moos et al., 2012). Organisms exposed to virgin or contaminated microplastics exhibited similar effects, suggesting that immunological responses were mostly induced by the physical ingestion of the particles, more than the chemical toxicity of adsorbed pyrene; in this respect, the exposure to irregular particles with potentially sharp surfaces might have contributed to exacerbate these effects compared to the use of microspheres with smooth surfaces (Von Moos et al., 2012).

Exposure to microplastics also determined the onset of various forms of genotoxicity in haemocytes. While strand breaks were higher in organisms exposed to virgin PE, nuclear alterations appeared more consistently distributed among all the treatments, resulting in an increased frequency of micronuclei after the exposure to pyrene-contaminated PS. This pattern of genotoxic effects allows to hypothesize that DNA strand breaks represent the first form of damage caused by the enhanced production of reactive oxygen species in response to microplastics: a more elevated prooxidant challenge caused by PS compared to PE or by pyrene-contaminated compared to virgin polymers would determine an irreversible loss of DNA integrity (i.e. nuclear alterations), leading to enhanced frequency of micronuclei in the worst condition. In this respect, oxyradical production was already shown to modulate immune responses, lysosomal dysfunction and pre-apoptotic processes in haemocytes of mussels exposed to TiO_2 nanoparticles (Barmo et al., 2013), while genotoxic proprieties of PAHs have been widely reported to produce chromosomal alterations (Benedetti et al., 2012).

The activity of AChE was not affected in haemocytes of exposed mussels, but significantly reduced in gills after treatments with both typologies of either virgin or contaminated polymers. The ability of microplastics to depress AChE was recently described also in juveniles of the common goby *Pomatoschistus microps* exposed to polyethylene microspheres, dosed alone or in combination with pyrene (Oliveira et al., 2013); despite mechanisms of action still remain to be elucidated, our results support the hypothesis that anticholinesterasic effects of microplastics should be taken in adequate consideration due to the abundance of these particles in the marine environment and the role of AChE in neurotransmission of fundamental physiological processes (Oliveira et al., 2013).

The marked accumulation of microplastics in digestive tissues caused a significant destabilization of lysosomal compartment as also reported in *M. edulis* upon fusion with endocytotic vacuoles containing microplastic particles (Von Moos et al., 2012). Lysosomal membranes are highly susceptible to oxidative effects of ROS which can be generated throughout a complex network of direct reactions and indirect mechanisms (Regoli and Giuliani, 2014). In this respect, lowered activities were measured for catalase and Se-dependent glutathione peroxidases which are known as particularly sensitive in revealing the early onset of a prooxidant challenge even at low levels of environmental disturbance (Regoli and Giuliani, 2014). These enzymes are both involved in the removal of hydrogen peroxide, the main precursor of hydroxyl radical in aquatic organisms (Regoli and Giuliani, 2014): while glutathione peroxidases are mainly responsible for eliminating metabolically produced H_2O_2, catalase acts as defense mechanism also toward the exogenous source of this molecule. The contemporary variation of these enzymes might thus suggest different mechanisms and cellular pathways for H_2O_2 formation in tissues exposed to microplastics. However, the overall results on oxidative stress biomarkers indicated that short-term exposures to microplastics do not induce major perturbations, as revealed by the limited effects on the total antioxidant capacity and the lack of oxidative damages like lipofuscin, malondialdehyde and neutral lipids accumulation, in agreement with previous data on mussels and fish (Von Moos et al., 2012; Oliveira et al., 2013).

The overall evaluation of biomarker results by multivariate PCA and hierarchical clustering analysis provided a clear separation between control and microplastics exposed mussels indicating that the majority of observed biological variations (immunological, lysosomal, cholinesterasic and antioxidant effects) were not influenced by the typology of polymer (PE vs PS) or contamination: only genotoxic responses further separated virgin from pyrene-contaminated polymers. The relatively limited impact of pyrene adsorbed on microplastics might suggest that energy resources were primarily directed to activate mechanisms of defense toward the physical rather than the chemical stressor; on the other hand, presence of microplastics was shown to delay the pyrene-induced lethal effects in *P. microps*, thus acting as a transitory mechanism of protection toward chemical toxicity (Oliveira et al., 2013).

To better summarize the biological significance of pyrene accumulation and cellular responses in mussels exposed to virgin and contaminated microplastics, these data were elaborated according to the weighted criteria of the Sediqualsoft model. The level of pyrene bioavailability was classified as Severe while the toxicological hazard calculated from biomarkers ranged from Slight to Moderate in various treatments, depending on the number, magnitude and biological importance of measured variations: the combination of chemical and cellular effects summarized as Slight or Moderate the hazard for mussels exposed to virgin polymers, Major or Severe for those exposed to pyrene-contaminated PE or PS respectively.

Transcriptional profiles provided additional insights on molecular mechanisms modulated by microplastics in digestive glands of

mussels. In analogy with cellular biomarker, the enrichment of KEGG pathways involved in lysosomal metabolism and immunological functions, appeared as a primary response to either virgin or contaminated PS. The up-regulation of several genes coding for lysosomal enzymes, putative coating proteins and endosome indicates a coordinated increase of this cellular defense pathway following microplastics accumulation. The synthesis and maturation of lysosomal enzymes occur in the endoplasmic reticulum/trans-Golgi system and the following trafficking of such proteins is regulated by specific recognition mechanisms and packaging into clathrin-coated vesicles for their transport to late endosomes (Bonifacino and Traub, 2003). The over-expression of several proteins involved in endosomes maturation, endocytic trafficking and lysosomal degradation, suggests increased uptake of microplastics via endocytosis and their endolysosomal degradation (Bucci et al., 2000). The complete list of DEGs involved in "lysosome", "coated membrane", and "endosome", i.e. cathepsins, *clathrin heavy and light chain*, sorting nexins, is reported in Supplementary Material 6.

Mussels exposed to microplastics exhibited also the enrichment of the NOD-like receptor signalling pathway, involved in the innate immune defences, such as regulation of inflammatory and apoptotic responses. The NOD-like receptors (NLRs) act as intracellular sensors which recognize both pathogenic patterns entering the cell via phagocytosis, and damage-associated molecules produced during cellular stress and activating the non-infectious inflammatory response. The PS-PYR particles enhanced transcription of genes putatively involved in signal transduction and auto-modulation of the stress response NF-kB (i.e. TRAF6 and IκBα), while virgin PS up-regulated various components of the innate immune system, such as putative peptidoglycan recognition proteins (PGRPs).

Molecular analyses supported cellular biomarkers also regarding the transcriptional modulation of antioxidant defences, detoxification enzymes and responses to genotoxic effects. The up-regulation of putative *GPX2*, *GSTP1*, *GSTP2* and down-regulation of putative SOD2 were observed in PS-exposed mussels, while *GPX3*, *GSTMU* and *SULT4A1* were differentially expressed after exposure to both PS and PS-PYR (Supplementary Material 4). On the other hand, the onset of DNA damage in mussels exposed to PS and PS-PYR could be related to the up-regulation of *GADD45A* and *GADD45G* (Supplementary Material 4) which have a pivotal role in control of cell cycle checkpoint, DNA repair process and cellular responses to a variety of DNA-damaging agents (Fornace et al., 1992); the increased transcription of GADD45 was also reported in the Manila clam *R. philippinarum* exposed to ibuprofen (Milan et al. 2013), corroborating its involvement in counteracting genotoxic stress in bivalves species. In mussels exposed to PS-PYR, the significant enhancement of micronuclei frequency was interestingly paralleled by the up-regulation of *ERCC1*, *ERCC2* and *APTX* which are required for the repair of DNA lesions, being mainly involved in nucleotide excision repair, single-strand break, double-strand break and base excision repair.

Substantial differences were observed in the transcriptional profile of genes related to apoptosis and the citrate cycle TCA. Despite such data may suggest molecular hypotheses on the down-regulation of apoptotic processes and energetic metabolism after exposures to virgin or contaminated microplastics, the functional implications at cellular level still remain to be elucidated.

In conclusion, this study confirmed that microplastics can efficiently adsorb organic contaminants like pyrene from the marine environment, providing the first experimental evidence for the potential transfer and bioaccumulation of this chemical in mussel tissues. Both virgin and contaminated microplastics induced several effects at transcriptional and cellular levels highlighting the potential risk for organisms' health condition, especially under conditions of long-term, chronic exposure. Despite in the present study only bioaccumulation and cellular responses were considered, the clear evidence of a toxicological potential of microplastics supports the WOE approach and the integration of multiple indicators (physical, chemical, biological, ecological), toward a more comprehensive risk assessment analysis, in line with actual European Directives like Marine Strategy Framework Directive. Further studies are needed to better understand the effects of other typologies of chemicals or chemical mixtures adsorbed on microplastics, as well as the natural exposure conditions in terms of presence, concentration and magnitude of chemical load in microplastics in the marine environment.

Data accessibility

The following link has been created to allow review of record GSE57460 when still in private status:http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cxqdcokwnhwxzsf&acc=GSE57460

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2014.12.021.

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Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from Adriatic Sea

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ABSTRACT

The presence of microplastics in the marine environment has raised scientific interest during the last decade. Several organisms can ingest microplastics with potentially adverse effects on the digestive tract, respiratory system and locomotory appendages. However, a clear evidence of tissue accumulation and transfer of such microparticles in wild organisms is still lacking, partially hampered by technical difficulties in isolation and characterization protocols from biological samples. In this work, we compared the efficacy of some existing approaches and we optimized a new protocol allowing an extraction yield of microplastics from fish tissues ranging between 78% and 98%, depending on the polymer size. FT-IR analyses confirmed that the extraction procedure did not affect the particles characteristics. The method was further validated on the fish mullet, *Mugil cephalus*, exposed under laboratory conditions to polystyrene and polyethylene; the particles were isolated and quantified in stomach and liver, and their presence in the hepatic tissue was confirmed also by histological analyses. A preliminary characterization revealed the presence and distribution of microplastics in various fish species collected along the Adriatic Sea. FT-IR analyses indicated polyethylene as the predominant polymer (65%) in the stomach of fish. The overall results confirmed the newly developed method as a reliable approach to detect and quantify microplastics in the marine biota.

1. Introduction

Plastic pollution in the oceans has been recognized as a world phenomenon with nearly 300 million tons of debris floating at sea surface, or accumulating on seafloor and shorelines from polar regions to the equator (Boerger et al., 2010; Browne et al., 2011; Eriksen et al., 2014; Suaria and Aliani, 2014).

In the recent years, scientific interest has been directed also toward microplastics, i.e. plastic fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derived from chemical, physical and biological degradation of larger plastic debris (Barnes et al., 2009; Wright et al., 2013).

Laboratory experiments have shown that microplastics can be ingested by different marine organisms, including polychaetes, crustacean, bivalves and echinoderms (Browne et al., 2008; Gregory, 2009; Graham and Thompson, 2009; Kach and Ward, 2008; Thompson et al., 2004; Von Moos et al., 2012; Van Cauwenberghe et al., 2015b). Due to their hydrophobic properties, microplastics can also adsorb several classes of organic pollutants (Teuten et al., 2007), which may be transferred to organisms and enter the marine food-webs (Teuten et al., 2009; Farrell and Nelson, 2013; Setala et al., 2014); experimental evidence has been recently obtained for the transfer of pyrene from microplastics to mussels (Avio et al., 2015). The consequences of microplastics ingestion may affect the feeding activity, respiratory functions, reproductive output, and also modulate several molecular and cellular pathways (Gregory, 2009; Cole et al., 2015; Avio et al., 2015).

The multiple risks that microplastics pose to marine life prompted their inclusion in some international legislation and marine protection projects, like the European Marine Strategy Framework Directive (MSFD) and the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA). In this scenario, a better knowledge on the presence and

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characterization of microplastics in marine food webs has become a research priority, and a fundamental step toward a more integrated ecological risk assessment.

Environmental studies on microplastics are often hampered by the limited availability of standardized protocols and technical difficulties in the extraction and characterization of these particles from marine samples. Suitable methodologies have been recently reviewed for sediments and seawater indicating the density separation as the most common approach (Hidalgo-Ruz et al., 2012; Van Cauwenberghe et al., 2015b). More complex is the extraction and quantification of microplastics from organisms, being fragments easily masked within biological material and tissues. In addition, microplastics comprise a very heterogeneous assemblage of pieces that vary in size, shape, color, specific density, chemical composition and other characteristics which should be considered during the development of appropriate methods for their extraction and characterization.

Some recent protocols tested the extraction of microplastics from marine invertebrates after a pre-digestion of organic matter (Claessens et al., 2013); the comparison of various acid, basic or oxidizing treatments revealed that pH-sensitive polymers can be dissolved or partially degraded by certain acids, thus affecting both the estimation and the characterization of the polymers by FT-IR (Claessens et al., 2013). The enzymatic digestion of organic matter with proteinase k resulted as a reliable method to extract microplastics from zooplanktons samples (Cole et al., 2014), but not a cost-effective approach for larger organisms.

The methodological difficulties in isolation protocols can partly explain why, to date, only a few studies specifically addressed the occurrence of microplastics in wild fish populations. Larger plastic fragments (1–5 mm) have been detected in various Pacific and Atlantic species (Choy and Drazen, 2013; Davison and Asch, 2011; Boerger et al., 2010; Pinnegard, 2009; Laist, 1997), while plastic particles <1 mm have been observed for the first time in demersal and pelagic fish from the English channel (Lusher et al., 2013), and in wild gobids, *Gobio gobio*, from French rivers (Sanchez et al., 2014). All these studies were based on a direct visual sorting of the fish stomach contents, without testing the efficiency of the separation methodology. The destruction of organic material with 10% KOH has been recently applied on intestines of fish from the North Sea, following a 2–3 weeks period of alkaline digestion (Foekema et al., 2013): plastic particles were found in 2.6% of examined fish, with typically one item per fish, ranging in size from 0.04 to 4.8 mm (Foekema et al., 2013).

The aim of the present work was to propose a reliable technique for the isolation of microplastics from marine organisms, preventing any aggressive procedure, and thus allowing the further characterization of the polymers by FT-IR or Raman spectroscopy analysis. In this respect, isolated gastrointestinal tracts of fish were spiked with known amounts of previously characterized microplastics polymers: the extraction yields were then assessed for different methods, including some published protocols and a new one that combined various steps of the available techniques. Tested protocols allowed to extract particles generally within 24 h; in this respect, we did not include the protocol of Foekema et al. (2013) requiring up to 3 weeks of alkaline digestion.

Possible dimensional limits of the newly developed procedure were tested toward microplastics of different size classes, from 5 mm to less than 0.1 mm, while FT-IR analyses were used to evaluate the integrity of polymers structure. The method has been further validated to analyze accumulation and transfer of microplastics in the gastrointestinal tract and liver of mullets, *Mugil cephalus*, exposed in laboratory conditions to polyethylene and polystyrene polymers. This species was selected as an experimental model due to its commercial importance and wide distribution in the Mediterranean Sea; being an omnivorous fish, it is also potentially exposed to pollutants and microplastics ingestion, and previous studies demonstrated its utility for ecotoxicological studies (Gorbi et al., 2005; Whitfield et al., 2012). Finally, a preliminary field assessment study was carried out to investigate the occurrence, typology and characteristics of microplastics in wild fish species from the Adriatic Sea, with different trophic guilds and ecological characteristics.

The results of this study were expected to provide a practical contribution toward the standardization of appropriate procedures for isolating microplastics from marine organisms, providing new insights on the presence, distribution and typology of these particles in commercial fish species from the Adriatic.

2. Material and methods

2.1. Polyethylene and polystyrene particles preparation

A stock of polyethylene and polystyrene powder was obtained from a private plastic company and sorted in four different grain size classes (5–1 mm; 1–0.5 mm; 0.5–0.1 mm; 0.1–0.01 mm). The particles were used to test various extraction protocols and for the laboratory exposure of the mullets *M. cephalus*.

2.2. Maintenance and acclimation of fish in laboratory conditions

Mullets, *M. cephalus*, were obtained from a local aquaculture and maintained in laboratory conditions with filtered and aerated seawater, at 18 ± 1 °C, salinity 37 for at least two weeks before exposures. Fish were daily fed with a specific grower feed (by Aller-Aqua, Aller Thalassa 2 mm, crude protein 50%, crude fat 15%); this commercial pellet was analyzed and confirmed to be microplastics free (data not shown). All the maintenance and experimental procedures were in accordance with requirements of the Ethical Committee of the Polytechnic University of Marche for scientific activities with marine organisms.

2.3. Protocols for plastic extraction

The efficacy to extract microplastics from fish tissues was evaluated for six different protocols, including five already published and a newly developed one; these procedures were compared in terms of percentage recovery of known amounts of previously characterized particles “spiked” in gastrointestinal tract of laboratory-acclimatized fish.

To this aim, 60 acclimatized mullets (length 24.6 ± 2.7 cm) were euthanized by submerging the fish in ice before cervical transection; gastrointestinal tracts (from the esophagus to the anal sphincter) were removed, homogenized and spiked with microplastics (35 g of tissues were added and accurately mixed with 5 polyethylene and 5 polystyrene particles, 1–0.5 mm grain size). Samples were processed as outlined below, using 10 gastrointestinal tracts for each protocol.

Protocol 1, consisted in the direct visual sorting of the stomach content according to Choy and Drazen (2013). Particles were counted, photographed and measured with a stereomicroscope (Optika SZM-D, equipped with a DinoEye Camera AM-423X and Dino Capture 2.0 software).

Protocol 2, previously proposed for microplastics determination in sediments (Thompson et al., 2004) was slightly modified for fish samples. Briefly, after the addition of plastic particles, the gastrointestinal tracts were homogenized in ultraturrax and 1 L of hypersaline NaCl solution (1.2 g/cm^3) was added to each samples: quite surprisingly this treatment did not result in the expected density gradient separation, at least within 24 h, thus preventing

further filtration and isolation of spiked particles. Settling or resuspension of different components may require a much longer period of decantation, making this approach less attractive and time consuming for fish samples.

For extraction protocols 3, 4, 5 and 6, after the addition of microplastics, the gastrointestinal tracts were desiccated (55 °C overnight) and carefully ground in a mortar before the following steps.

Protocol 3, already proposed by Claessens et al. (2011) for sediments is based on a density gradient separation. Shortly, 500 ml of NaCl hypersaline solution (1.2 g/cm^3) were added to each sample and stirred for 10 min in a glass beaker. The obtained solution was then allowed to settle for 20 min before the supernatant was filtered under vacuum on a cellulose nitrate membrane (8 μm pore size), which was then washed with deionized water, dried and microscopically observed for microplastics count.

Protocol 4 contains an oxidation treatment, which was suggested to eliminate the biogenic organic matter in sediments before the microplastic extraction (Nuelle et al., 2014). In this work, microplastics-spiked and desiccated samples were transferred in a conical flask and added to 20 ml of 30% stabilized hydrogen peroxide (H_2O_2) at 55 °C for seven days. The resulting mixture was then diluted 1:10 in deionized water, before the filtration and microscopical observation as described above.

Protocol 5 was previously reported for extraction of microplastics from mussel tissues after an acidic digestion (Claessens et al., 2013). According to this procedure, samples were added to 20 ml of 22.5 M nitric acid and digested overnight at room temperature, followed by 30 min of boiling (100 °C). The resulting mixture was then diluted 1:10 with warm deionized water, before filtration and microscopical observation.

Protocol 6 is a new procedure obtained integrating, with slight modifications, some steps of the previously discussed extraction methods. Each sample was added to 250 ml NaCl hypersaline solution (1.2 g/cm^3), stirred and decanted for 10 min; the filtration step was carried out twice in order to obtain a better extraction performance. The membranes with retained materials were then transferred in a petri dish with a 15% H_2O_2 solution for the partial digestion of residual organic matter and allowed to dry in oven (50 °C, overnight), before the microscopical observation.

To prevent any accidental external contamination of microplastics, deionized water and hypersaline solution were always prefiltered (0.45 μm filter); all the materials used for dissection and during different steps of extraction and analysis were rigorously cleaned with filtered deionized water and air dried. In addition all sample processing was performed in a clean air flow cabinet to exclude external contamination from fibers which might represent a major source of contamination (Foekema et al., 2013).

2.4. Effect of microplastics size and FT-IR validation

The efficacy of the new protocol was tested to evaluate the extraction yields toward microplastics with different grain sizes. The gastrointestinal tracts of 20 mullets (27.2 ± 1.9 cm length) were dissected and homogenized; 35 g of each sample were added with a total of 16 previously characterized plastic particles, equally distributed among 2 polymers (polyethylene and polystyrene) of 4 size classes (5-1 mm; 1–0.5 mm; 0.5–0.1 mm; 0.1–0.01 mm). Microplastics were extracted, microscopically observed and measured according to the previous procedure (Protocol 6).

As an additional control of the procedure and to evaluate a possible degradation of the polymers occurring during the isolation phases, polyethylene and polystyrene particles were analyzed by FT-IR (see below) before and after the extraction process.

2.5. Laboratory exposure and recovery

A total of 30 acclimatized mullets (25.1 ± 2.6 cm length) were randomly assigned and maintained for 7 days in one of three 80 l glass-tanks (10 fish per tank) corresponding to control tank (CNTR), polyethylene treatment (PE) and polyethylene treatment (PS). Particles, with size between 0.1 and 1 mm, were given at a nominal concentration of 0.03375 g/L, corresponding to nearly 2500 particles/L. The exposure concentration is not representative of a realistic environmental condition, although nearly fifty times lower than those previously used in other experimental studies (i.e. Thompson et al., 2004; Van Moos et al., 2012; Avio et al., 2015). However, considering the aim of our experiments, it was considered as appropriate to validate the new extraction procedure in *in-vivo* exposed fish, and to explore the possibility that ingested microplastics may be transferred from gastrointestinal tract to liver.

During the exposure period, water was fully removed and replaced daily with new water and microplastics re-dosed at the initial nominal concentration. Stratification of particles at the surface of the tanks was prevented by water mixing with aerator and pump movement (Koralia nano, HYDOR); particles distribution in tanks was visually inspected twice a day for all the exposure period.

Fish were daily fed and sacrificed at the end of the experiment as previously reported; livers and gastrointestinal tracts rapidly removed, frozen in liquid nitrogen and maintained at −80 °C until the analyses. Microplastics were determined in 10 gastrointestinal tracts and 5 livers for each treatment following the newly developed procedure (protocol 6). The remaining 5 livers for each treatment were histologically analyzed for identification and localization of microparticles: cryostatic sections (10 μm thick) were stained with haematoxilyn/eosin and observed at polarized light microscopy (Avio et al., 2015).

2.6. Extraction of microplastics in field collected fish

A total of 125 fish representative of five commercial species, were sampled from the Central and North Adriatic Sea in March 2014 (Table 1). Collected species included *Sardina pilchardus* as a typically pelagic fish, *Squalus acanthias* and *Merlucius merlucius* as bentho-pelagic, *Mullus barbatus* and *Chelidonichthys lucernus* as two closely benthonic species; number and characteristics of analyzed fish are given in Table 1.

Gastrointestinal tracts were collected and frozen at −20 °C, until the analyses. Each sample was processed with the newly developed procedure (protocol 6), and extracted particles were microscopically observed, photographed, measured at their largest cross section through an ocular micrometer, and categorized according to both size class (5–1 mm; 1–0.5 mm; 0.5–0.1 mm; <0.1 mm) and the shape (fragments, film, pellet, line). Textile fibers were found only occasionally and excluded from the analysis because they could represent airborne contamination from clothing during the sampling or processing (Foekema et al., 2013; Cozar et al., 2014). Particles were also characterized by FT-IR spectrometry as described below.

2.7. FT-IR analyses

Analyses were performed using a Cary 660 FT-IR spectrometer (Agilent) equipped with ATR (GladiATR Diamond Crystal Plate, Piketechnologies) which allowed the characterization of microparticles greater than 0.5 mm. Following background scans, 128 scans were performed and CO_2 interference (adsorption at approx 2300–2400 cm^{-1}) was removed for clarity; for each particle, scans were performed with resolution of 4 cm^{-1}. Agilent Resolution Pro v5.2 software was used for the output spectra and the identification

Table 1
General data on analyzed specimens of commercial Adriatic fish species, percentage of individuals containing microplastics in the stomach, average number of items in individuals containing microplastics.

Species	Number of stomach examinated	Fish length cm (means ± SD)	Stomach wet weight g (means ± SD)	Stomach with microplastics (%)	Number of microplastics in fish with plastics in stomach (means ± SD)
S. pilchardus	99	11.8 ± 1.5	0.78 ± 0.24	19	1.78 ± 0.7
S. acanthias	9	47.6 ± 11.2	22.4 ± 11.2	44	1.25 ± 0.5
M. merluccius	3	35.0 ± 1.4	2.4 ± 0.6	100	1.33 ± 0.57
M. barbatus	11	14.7 ± 1.2	1.1 ± 0.5	64	1.57 ± 0.78
C. lucernus	3	25.4 ± 1.2	8.8 ± 2.019	67	1 ± 0

of polymers was performed by comparison with a library of standard spectra. Only polymers matching reference spectra for more than 60% were accepted, in line with suggestion of Lusher et al. (2013).

2.8. Statistical analysis

Analysis of variance (ANOVA) was applied to test differences between various extraction protocols, typology and size of polymers, accumulation in different tissues of exposed fish. Level of significance was set at $p < 0.05$ and when differences were detected post-hoc comparison (Bonferroni) was used to discriminate between means of values. Homogeneity and normality of data were tested using Bartlett's test or Shapiro–Wilk's test respectively, and any deviation was corrected by *log* transformation. Statistical analyses were performed with GraphPad Prism®.

3. Results

Fig. 1A reports the extraction yields of the 6 protocols applied to gastrointestinal tracts spiked with polyethylene and polystyrene particles. The newly developed procedure (protocol 6) revealed the highest efficiency, with an average extraction yield of 95% ± 2 (mean% ± SEM) toward the two polymers. The application of protocol 1 (direct visual sorting), protocol 3 (density gradient on dry samples), protocol 4 (oxidation treatment) and protocol 5 (acid digestion) showed an extraction yield of respectively 57% ± 12, 73% ± 5, 70% ± 3, 4% ± 3, with no significant differences between polyethylene and polystyrene particles; the application of protocol 5 indicated the acid digestion as too aggressive, resulting in the melting of microplastics and thus obtaining a low percentage of extraction yield. The protocol 2 (density gradient on fresh samples) did not allow any separation and particles extraction.

The efficiency of protocol 6 toward particles of different size confirmed similar results toward the 2 polymers (Fig. 1B): the extraction yield was higher than 90% for the dimensional classes 5–1 mm, 1–0.5 mm 0.5–0.1 mm, while nearly 80% for microplastics smaller than 0.1 mm.

FT-IR spectra obtained for particles analyzed before and after the new extraction procedure were absolutely comparable (Fig. 2), with a similarity of approximately 93% for polyethylene profiles, and greater than 87% for polystyrene.

The newly developed procedure (protocol 6) was applied to extract microplastics from mullets *M. cephalus* exposed under laboratory conditions. An elevated accumulation of polyethylene and polystyrene particles was revealed in gastrointestinal tracts (Fig. 3), and the absence of microplastics was confirmed in fish maintained in control conditions. Interestingly, the number of measured items was significantly higher in fish exposed to polystyrene compared to those isolated in polyethylene treated fish (Fig. 3A). Microplastics were also extracted from liver tissues (Fig. 3B), with average numbers of particles approximately 2 orders of magnitude lower than in corresponding gastrointestinal tracts, and without significant differences according to the polymer type. Histological analyses of liver slices confirmed the presence of both polyethylene and polystyrene particles in the liver (with size ranging between 0.2 and 0.6 mm), randomly localized among the hepatic cells (Fig. 4).

The extraction of microplastics from wild fish species highlighted their presence in gastrointestinal tracts of 35 individuals, i.e. 28% of the 125 examined fish. Despite the comparison between species could be partly affected by the different number of analyzed specimens, nonetheless the pelagic *S. pilchardus* showed the lowest percentage of fish containing plastic particles (19%), followed by the bentho-pelagic *S. acanthias* (44% of sampled specimens, Table 1). In the two benthic species (*M. barbatus*, and *C. lucernus*), microplastics

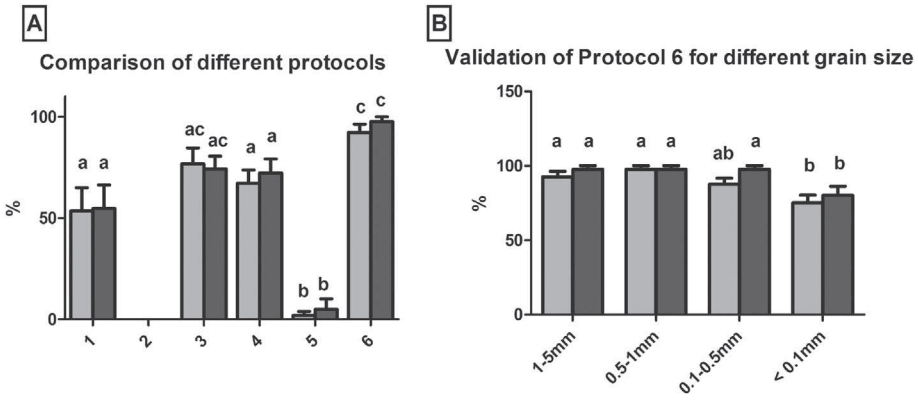

Fig. 1. (A) Extraction yields of 6 different protocols (see Materials and Methods for details). Values are expressed as percentage recovery of particles from spiked gastrointestinal tracts (±SEM, n = 10); (B) Extraction yield for particles with different size classes (mean yield % ± SEM, n = 20). Gray and black bars represent polyethylene and polystyrene particles. Different letters indicate significant differences between groups of means (Bonferroni post-hoc comparison); protocol 2 did not allow any separation and was not included in the statistical analysis.

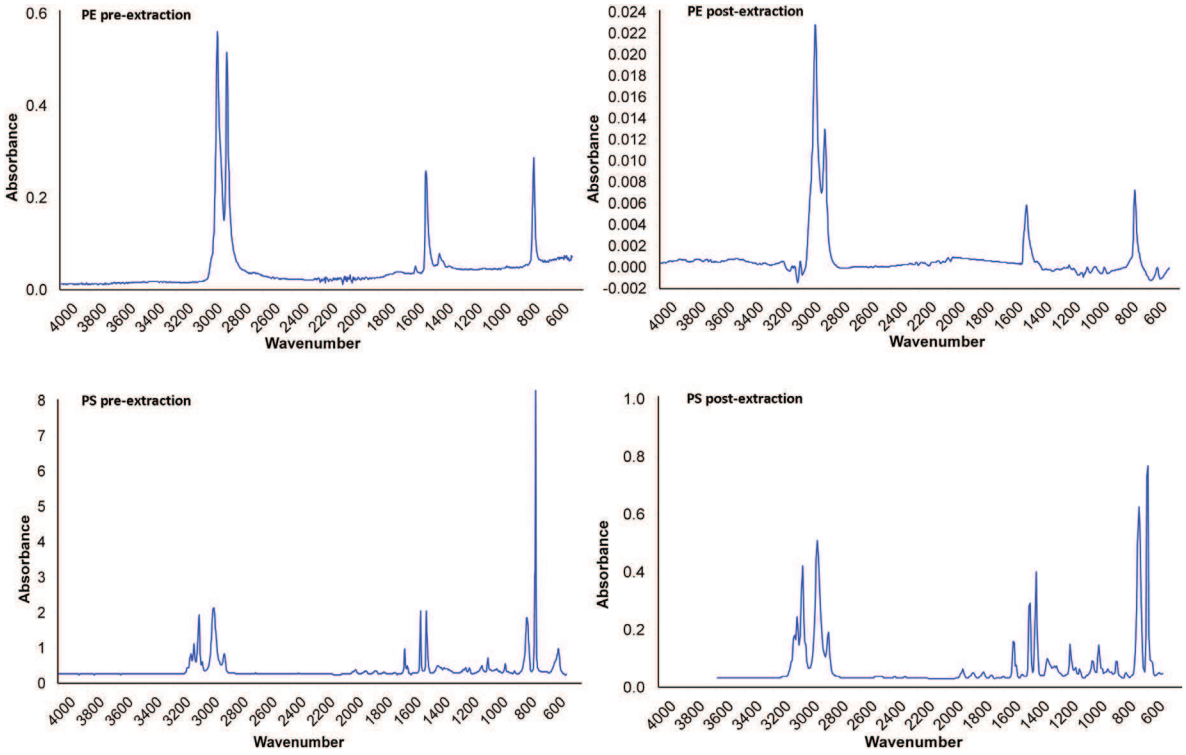

Fig. 2. FT-IR spectra of polyethylene (PE) and polystyrene (PS) particles before and after the extraction procedure. Spectra matched at 93% for PE, at 87% for PS.

were found in 64% and 67% of the analyzed stomachs, while all the three specimens of *M. merlucius* contained such items in the stomach (Table 1).

The average number of microplastics extracted in positive fish ranged from 1 item/individual in *C. lucernus* to 1.78 ± 0.97 items/individual in *S. pilchardus* (Table 1). The shape of the plastic particles isolated in gastrointestinal tracts of all the fish (without species differentiation) was largely dominated by fragments (57%), followed by line (23%), film (11%) and pellet (9%, Fig. 5A–B). The 18% of extracted microplastics exhibited the larger size class (from 5 to 1 mm), 43% was between 1 and 0.5 mm, 23% between 0.5 and 0.1 mm, and the 16% lower than 0.1 mm (Fig. 5C). FT-IR analyses indicated that approximately 65% of analyzed items were polyethylene, 19% polyethylene terephthalate (PET), 4% respectively polystyrene, polyvinyl chloride (PVC), Nylon 6/T and polypropylene (Fig. 5D).

4. Discussion

The present investigation was aimed to validate a new methodological protocol for microplastics detection in fish tissues, providing also a preliminary insight on their occurrence in some commercial species.

The presence of microplastics is being worldwide documented in water-column and sediment samples, and their presence has also been reported in different taxa including planktonic species, invertebrates, fish and cetaceans (Browne et al., 2011; Hidalgo-Ruz et al., 2012; Foekema et al., 2013; Lusher et al., 2013, 2015; Fossi et al., 2014; Van Cauwenberghe et al., 2014, 2015a), Laboratory studies further confirmed that microparticles can be ingested by a range of marine biota, including shellfish and fish for human consumption, with a potential impairment of various cellular, metabolic or physiological pathways (Browne et al., 2008; Von Moos

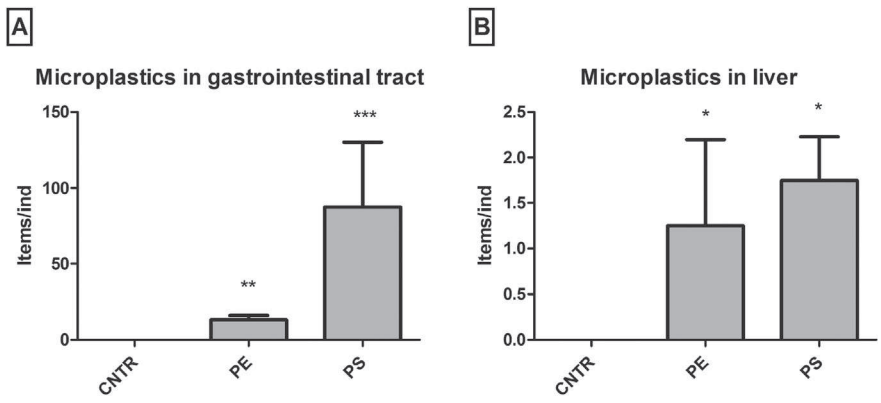

Fig. 3. Microplastics particles extracted in gastrointestinal tract (A) and liver (B) of exposed fish. CNTR = control; PE = polyethylene treatment; PS = polystyrene treatment. Data are expressed as number of items/individual (mean values ± SEM); asterisks indicate different levels of significance compared the control group (Bonferroni post-hoc comparison).

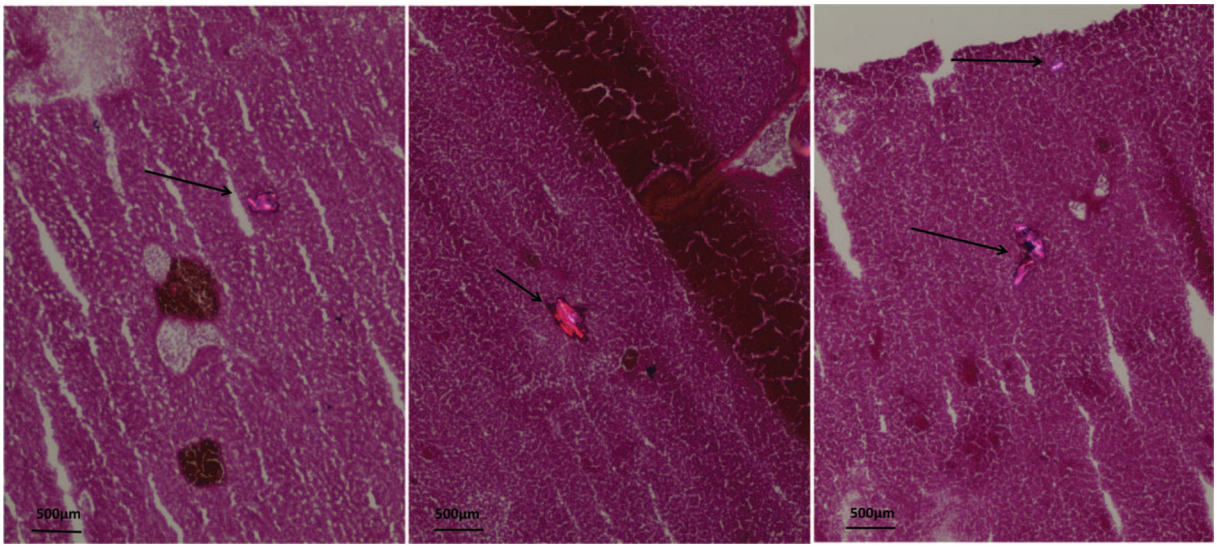

Fig. 4. Polarized-light microscopy images (X 100 magnification) showing the presence of plastic particles in liver of exposed fishes. Black arrow indicates microplastic particles inside the liver tissue. Scale bar: 500 µm.

et al., 2012; Wegner et al., 2012; Avio et al., 2015).

Considering the growing interest on the impact of microplastics in the marine environment (Galgani et al., 2013), the optimization of protocols to extract and characterize these particles in marine biota is an urgent scientific priority, required for quantitative and comparative assessments. In the present study, different protocols were tested for their quantitative yield in gastrointestinal tracts of fish spiked under controlled conditions with defined amount and typologies of microplastics.

The direct visual sorting of the fresh stomach content (protocol 1) resulted in an extraction yield slightly lower than 60% for both polyethylene and polystyrene, thus indicating a potential underestimation of results obtained with this approach. The elevated content of organic matter and other particles present in the fish stomach can hide the microplastics, interfering with their microscopic visualization; in addition, the application of this protocol on

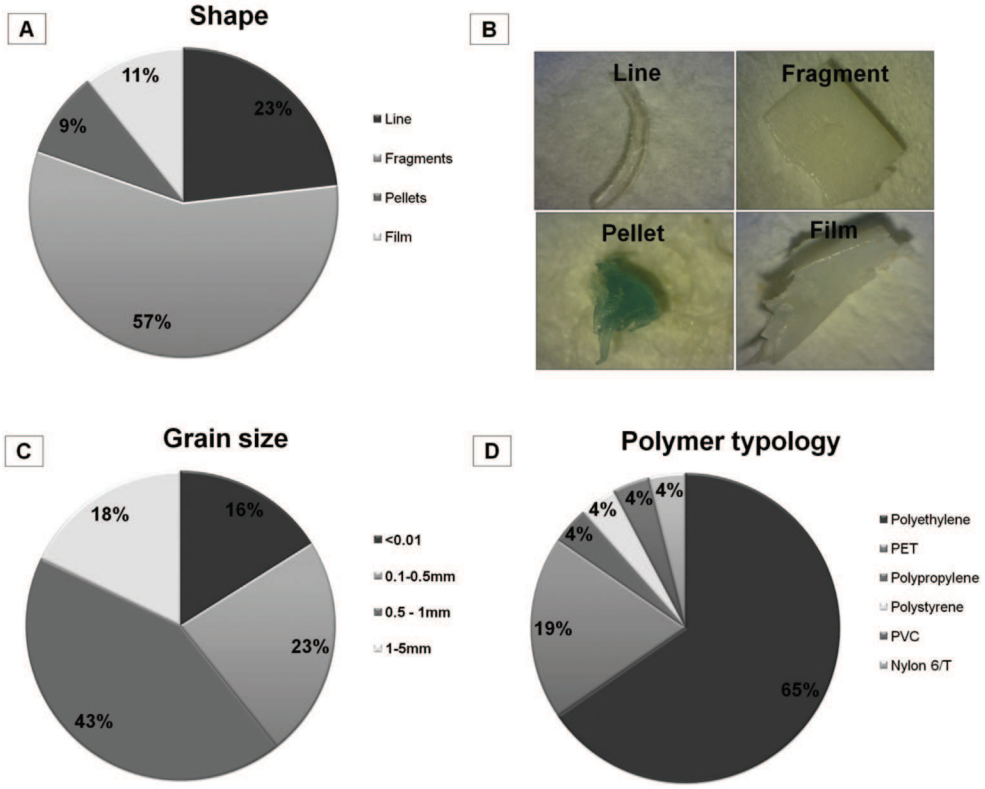

Fig. 5. Characteristics of microplastics extracted in Adriatic fish species. (A): shape; (B): representative examples of particles; (C): grain size; (D): typology of polymers as indicated by FT-IR. Data in pie graphs are given as percentage distribution.

wild specimens would not allow to identify and count the microplastics contained within an ingested prey.

The density separation with a hypersaline solution was derived by a successful approach to extract microplastics from sediments (Thompson et al., 2004). However, this procedure applied to fresh gastrointestinal tracts spiked with microplastics (protocol 2) did not allow any separation of the sample; consequently the filtration was not performed and it was not possible to obtain any extraction yield. For all the other protocols tested in this work (3–6), spiked samples were desiccated and ground in a mortar to facilitate the following microplastics extraction. In this respect, the density gradient on dried, spiked samples (protocol 3) resulted in an efficient separation and filtration, with a final extraction yield higher than 70%.

Digestion of organic matter by either oxidant or acidic treatments has been suggested to facilitate and improve the extraction of microplastics from sediments and/or organisms (Claessens et al., 2013; Cole et al., 2014; Van Cauwenberghe et al., 2014, 2015b). In this study, the 7 d digestion of dried samples in 30% H_2O_2 (protocol 4), allowed to extract almost 70% of spiked particles; this relatively low recovery could be partly ascribed to a dense foam formed after the addition of hydrogen peroxide, which hampered the filtration and further processing of samples.

Acidic digestion has also been shown as a useful treatment to remove biological material from marine samples, thus facilitating isolation of plastic particles. In this respect, the extraction yield in marine organisms treated with nitric acid was approximately 98 and 94% for 30 μm and 10 μm polystyrene spheres, and 98% for 100 × 400 μm nylon fibers (Claessens et al., 2013). However this procedure has also been suggested to, at least partially, degrade those polymers with a low pH tolerance, e.g. polyammide, polystyrene, polyethylene (Cole et al., 2014). This possibility was confirmed in the present work, where the acidic extraction of spiked gastrointestinal tracts (protocol 5), caused a marked dissolution of both polyethylene and polystyrene tested particles, resulting in an extraction yield of approximately 4%: at the end of the digestion, plastic particles appeared to be melted and fused together, suggesting that this method could be not appropriate to extract all typologies of plastic polymers.

The combination of density gradient separation and oxidant treatment (protocol 6) appeared the most efficient procedure with an extraction yield of approximately 95% for both the polymers. Density separation was confirmed as a fundamental step to eliminate sediment particles and other debris potentially present in the gastrointestinal tract of fish; the higher extraction efficiency compared to protocol 3, highlighted the utility of the hydrogen peroxide treatment which, digesting the residual organic matter retained in the membrane, resulted in an easier plastic detection during the microscopic analysis. At this stage we did not consider the method by Foekema et al. (2013) which requires up to 3 weeks of alkaline digestion of samples; nonetheless a more focused comparison with the newly developed procedure might be addressed in a future investigation.

To evaluate if methodological steps described for protocol 6 may attack the polymers structure thus preventing their further characterization, polyethylene and polystyrene particles were analyzed by FT-IR before and after the extraction procedure. Obtained results showed only a minimal modification of polymers spectra, confirming that microplastics were efficiently extracted without any damage to the polymers. Considering the dimensional classes of particles to extract, the proposed protocol showed an extraction yield higher than 90% for microplastics ranging between 5 and 0.1 mm, and approximately 80% for those smaller than 0.1 mm; future implementation of the method should be directed to improve the recovery at the lowest dimensional limits and, ideally toward nanoplastics.

The validated protocol was applied on mullets, *M. cephalus*, exposed in laboratory conditions to polyethylene and polystyrene microplastics. The exposure concentration (0.03375 g/L) was lower than those used with mussels by Thomson et al., 2004 (1.5 g/L), Van Moos et al., 2012 (2.5 g/L) and Avio et al., 2015 (1.5 g/L) but, due to the limited knowledge on microplastics levels in seawater and the lack of standardized units, the ecological relevance of this treatment is difficult to be defined. Nonetheless, plastic particles were efficiently extracted from gastrointestinal tracts of exposed fish with a statistically significant higher number of polystyrene compared to polyethylene items. The different densities of these polymers (1.04 and 0.92 g/cm^3 respectively) might have partially affected the behavior of microplastics during the exposure. Despite water and particles mixing, polyethylene has a higher tendency to re-float at the surface, while polystyrene remained more uniformly distributed into the water column, possibly enhancing the probability to be ingested by fish actively swimming in the tank volume immediately after the addition of pellets. A similar experimental evidence confirm that physic-chemical properties of polymers have important implications for field studies, influencing the ingestion and accumulation in organisms with different trophic strategy and habit.

Extraction of microplastics from laboratory exposed fish revealed a small but significant presence of particles also inside the hepatic tissue, a result further confirmed by histological analyses. This is the first evidence for a marine vertebrate that microplastics can be translocated from gastrointestinal tract to another tissue; a similar phenomenon, already observed in marine mussels, corroborates the potential risk of accumulation and trophic transfer of microparticles along marine food webs (Von Moos et al., 2012; Avio et al., 2015).

In this respect, the last part of this study was a field validation of the extraction protocol, aimed to a preliminary insight on the occurrence and characterization of microplastics in 5 Adriatic fish species. The overall results showed the presence of plastic particles in 28% of the 125 analyzed specimens, irrespective of the species. This percentage is slightly lower than that reported for the English Channel where 36.5% of fishes contained microplastics in their gastrointestinal tracts (Lusher et al., 2012). On the other hand, the presence of small plastic fragments was demonstrated in approximately 30% of all planktivorous fish caught in the North Pacific Central Gyre (Boerger et al., 2010), and of catfish collected in a Brazilian estuarine environment (Posatto et al., 2011); a lower occurrence of microplastics was reported in fish collected from southern and northern sectors of the North Sea with respectively 5.4 and 1.2% of specimens containing particles (Fokoema et al., 2013).

Considering only fish with plastics in the stomach, our study showed an average of particles ranging from 1 item/individual in *C. lucernus* to 1.78 items/individual in *S. pilchardus*; similar levels (1.9 ± 0.1 items/individual) were previously reported by Lusher et al. (2012) who, however, included also textile fibers in their analysis. Among the 5 collected species, the pelagic *S. pilchardus* showed the highest average number of items/individual, but also the lowest percentage of specimens containing microplastics (<20%). Recent reports on the microplastics distribution in the water column indicated elevated and variable levels of particles at the sea surface (Cozar et al., 2014; Eriksen et al., 2014), supporting the possibility for some pelagic specimens to occasionally cross patches of concentrated floating debris. On the other hand, benthic and bentho-pelagic species showed a relatively lower number of items but a markedly higher percentage of fish containing microplastics in the stomach (from >40 to 100%): the more frequent occurrence of particles in benthic compared to pelagic fish,

suggests that plastic occurrence may be high near the seafloor and/or in sediments, or that benthic fish are less selective feeders.

The analysis of grain size distribution showed a predominance of the 1–0.5 mm particles (43%) in sampled fish, while the greater and smaller size classes had all a similar contribution, each accounting for approximately 20%. Plastic particles in gastrointestinal tracts of Adriatic fish were mostly fragments (57%) followed by lines (23%), with a more limited occurrence for films (11%) and pellets (9%); a different composition was reported for fish from the English Channel with 68% of all extracted particles consisting of fibers (Lusher et al., 2013). Since fibers were excluded from our count to prevent external contamination (as suggested by Foekema et al., 2013) our results are not fully comparable with those of Lusher et al. (2013); in this respect, it is important to check whether fibers have been considered before comparing results of different studies.

The range of bright colors identified also in smallest extracted particles indicated that H_2O_2 digestion did not affect the particles in terms of plastic bleaching. FT-IR analyses on microplastics isolated from our samples highlighted the elevated selectivity of the extraction protocol: all the analyzed items were plastic polymers, without any presence of other particles like glass, wood, sand. Although the density gradient separation used in this study was expected to extract polymers with density $\leq$1.2 g/cm^3 (like acrylic polymers, low density polyethylene, polystyrene, nylon and polypropylene), also higher density particles such as PET (1.38 g/cm^3) were isolated; the possible attachment of organic matter might have determined a density decrease of these particles and their extraction during density separation and before H_2O_2 treatment. The most common polymer found in Adriatic fishes was polyethylene (65%) followed by PET (19%), while a lower prevalence was observed for polypropylene, PVC, polystyrene and Nylon polymers. These results suggest plastic bags and bottles as the main sources of microplastics in Adriatic Sea, confirming polyethylene as the most widespread polymer in oceans (Cozar et al., 2014).

Interestingly, the FT-IR characterization revealed the presence of some low density polymers (e.g. polyethylene) in benthic species and of high density particles (e.g. PET) in the pelagic ones. It is known that buoyancy of microplastics can be influenced by factors like fouling which cause their sinking and accumulation in sediments (Wright et al., 2013), while high density particles can be ingested by pelagic or bentho-pelagic organisms through their preys as secondary ingestion (Brandao et al., 2011). It has been recently pointed out an enormous loss of microplastics from sea surface compared to expected rates of fragmentation (Eriksen et al., in 2014), suggesting the presence of mechanisms removing particles smaller than 4.75 mm; results of this work support the hypothesis that this plastic fraction can be directly ingested by the organisms, stored in the sediments and/or indirectly transferred trough the trophic webs.

In conclusion, this study provided a new protocol for the extraction of microplastics from fish, with a recovery yield generally higher than 90%. The field characterization demonstrated the presence of microplastics in Adriatic fish species of commercial interest, supporting a certain transfer of these particles from water column to sediments and benthic species. Future studies are required on a more elevated number of organisms, species and trophic guilds to better assess the distribution of microplastics along food webs, and their capacity to penetrate the edible tissues, with potentials concerns also for human consumers.

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