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Impacts of weathered microplastics on bioavailability of pollutants in *Paracentrotus lividus* and *Danio rerio*: molecular and ecotoxicological approaches

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1.1 Abstract

Plastic materials provide countless applications in every sector of human life, from food and health preservation to textiles and electronics industry (Cole et al., 2011;Thompson et al., 2009) which is resulting in huge amount of plastic waste. Recently, it was estimated that plastics account for the 80-90% of the whole marine litter (Derraik, 2002) and over 5 trillion microscopic plastic fragments are floating on the surface of the World Oceans (Eriksen et al., 2014).

The predominant form of marine plastic litter is called “Microplastics (MPs)", terminology by Thompson et al. 2004, used to indicate small plastic fragments, fibers and granules of microscopic size (1 µm to 5 mm in diameter). Being small in size, they are likely to be ingested and accumulate by different classes of organisms (Wright et al., 2013), even can be consumed by algae and bacteria (Mattsson et al., 2015; Bouwmeester et al., 2015). Their presence, therefore, can represent a growing threat to ecosystems and to organisms for which it can represent a hazard (Galloway and Lewis, 2016).

MPs are known to interact with a variety of chemicals in the environment through many sorption mechanisms (Yu et al., 2019). Owing to their ability to sorb xenobiotic chemicals, MPs are regarded as vectors of hazardous contaminants to aquatic organisms (Torres et al., 2021). The study of the interactions and the sorption mechanisms occurring between the sorbent (microplastic polymer) and sorbate (chemical contaminant) are important to improve our understanding of MP-mediated contaminant transfer as well as the impact of MPs on the marine environment, especially considering MPs from natural environment. MPs in natural environments undergo weathering processes due to physico-chemical interactions with environmental stress, including with biota. Over time, these stressors may lead to a progressive degradation of plastic polymers and affect their sorption capacity as well as their behavior in aquatic environments. In this context, it is key to know the bioavailable fraction able to determine a potential ecotoxicity in organisms, and to investigate the role of MPs in transferring bioavailable hazardous chemicals to aquatic biota. A multidisciplinary approach to evaluate the influence of the different factors altering plastic surface as well as to investigate the interactions behind the complex mixtures between toxicants and MPs should be considered. The aim of this chapter is to review the main MPs properties focusing particular reference on the sorption mechanisms of MPs, the factors that influence those mechanisms, the sorption behavior of a wide range of chemical contaminants and toxicity associated to combined exposures to MPs and pollutants. An overview of studies that allow relating toxicity to the sorption to MPs and help understand its importance for the bioavailability of chemical compounds is included. A pilot study carried out to evaluate the sorption, over time, of different metals and BDE-47 on a pristine model MP is described. Finally, the objectives of this thesis are presented.

1.2 Microplastics in the marine environment

Marine litter from non-natural sources includes man-made items that have been produced or used by people and deliberately or not, released into the sea or on beaches. It includes also materials that were transported from land by rivers, drainage or wind and that reach the marine environment. Generally, marine debris consist of metal, glass, paper, fabric or plastic, but of these, plastic is considered the dominant type of debris in the marine environment (GESAMP, 2019). Due to the large production, the durability and versatility, it can therefore be expected that a wide portion of plastics ends up in the environment. The main classes of plastics that are commonly encountered in marine environment are represented by polyethylene PE (high and low density), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyurethane (PUR), and polyvinyl chloride (PVC).

Plastics are increasingly used across the economy, serving as a key enabler for sectors as diverse as packaging, construction, transportation, healthcare and electronics. Plastics have brought massive economic benefits to these sectors, thanks to their combination of low cost, versatility, durability and high strength to-weight ratio (Rouch, 2021). Their high volume usage, specifically for packaging industry, reflect their production (figure 1.1) (Andrady, 2011).

Figure 1.1 Plastics demand by polymer types. Source: Plastic Europe Market Research Group (PEMRG).

As a result of higher plastic demand, worldwide plastic production has largely increased in last decades (figure 1.2) since 1950 up to 2018 (Geyer et al., 2017; Barra et al., 2018) is forecast to double by 2050 to reach about 800 million tonnes annually (Galloway et al., 2015). Less than half of the plastic produced are consigned to landfill or recycled, but the remaining part in many cases can reach marine environment.

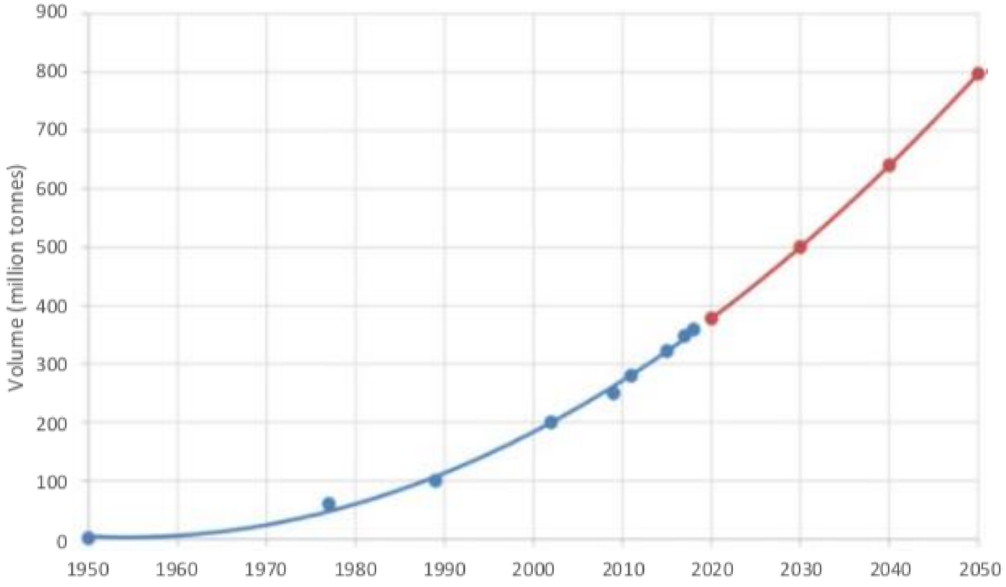


Figure 1.2 Global production of plastics. Blu line indicate production since 1950 to 2018; red line indicate forecast production since 2020 to 2050. Data from: Plastics Europe 2016 and 2019. Source: Rouch, 2021.

1.3 Microplastics distribution

Although initially, scientific attention was focused on macroscopic plastic debris, the presence of plastic microparticles in the marine environment was already described in the early 1970s (Carpenter et al., 1972).The disposal and breakup of consumer products and industrial waste in environmental areas can result in extremely small pieces of plastic debris, called Microplastics (MPs), whose effects on the marine environments have become an emerging issue of international concern. Microplastics are synthetic organic polymer particles with range in size is from a few microns to five millimeters in diameter (Thompson et al., 2004). They have been found in the oceans worldwide (Barnes et al., 2009), including remote regions such as Antarctica, sediments and wastewater effluents around the world (Derraik et al., 2002; Zarfl et al., 2010; Leslie et al., 2013). Considering the high levels of MPs detected into the oceanic convergence zones (Andrady, 2011; Moore et al., 2001), the most of studies have been focused on marine environment. Large areas with high concentrations have been discovered in main oceanic gyres (figure 1.3) (Law et al., 2010; Eriksen et al., 2014; Cózar et al., 2015), especially close to industrial centers and metropolitan areas (Vianello et al., 2013;

Claessens et al., 2011) as well in enclosed or semi-enclosed seas such as the Caribbean and the Mediterranean Sea (de Borrero et al., 2020; Suaria et al., 2016). A study on total floating MPs in the world's oceans estimated until to 35000 million tons with a size below 4.75 mm; differentiating microplastics and macroplastics estimated quantities are at least 5250 trillion plastic pieces weighing over 270.000 million tons and that are currently floating at sea (Ericksen et al., 2014). However, the data available are based on different numerical distribution models, and derived from the observations of just small surface areas of these high-concentration zones. For this reason, these numbers are likely to be an underestimation of the real extent of the plastic pollution due to the constant pollution of the marine environment with vast amounts of plastic debris (Jambeck et al., 2015) and sampling techniques that exclude small plastic fragments (Lindeque et al., 2020).

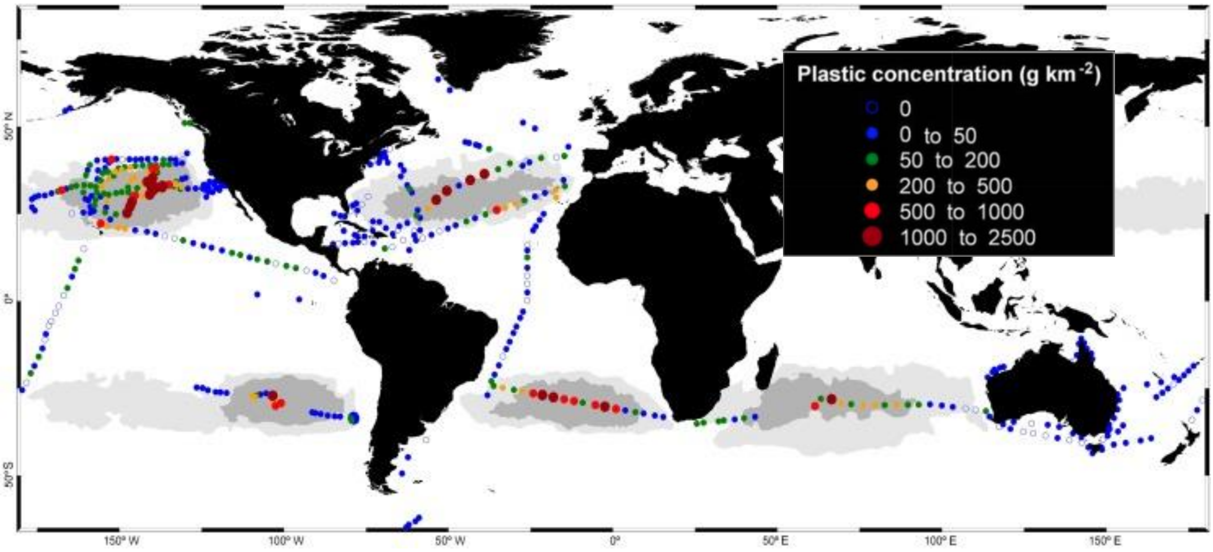


Figure 1.3. Plastic debris concentration in global ocean. Dark and light gray areas representing inner and outer accumulation zones, respectively, indicate five subtropical gyres. Source: Cózar et al., 2015.

1.4 Defining microplastics: nomenclature

It is unclear when the term "microplastic" was first used in relation to marine debris. It was mentioned by Ryan and Moloney (1990) in describing the results of surveys of South African beaches, and in cruise reports of the Sea Education Association in the 1990s and by Thompson et al. 2004, describing the distribution of plastic fragments in seawater. It has since become widely used to describe small plastic particles at first with a size not formally recognized (Gesamp, 2019). The size definition of MPs was defined by National Oceanic and Atmospheric Administration NOAA (Arthur et al., 2009) where it was suggested an upper size limit of 5 mm in order to include a wide range of particles (including visible components of the small plastic spectrum) that could be ingested by biota, and to focus the microplastics discussion on possible ecological effects associated with them. On the other hand, the lack of definition of a lower size limit together with the limitations of sampling and detection techniques, has led to a confusing use of the term "microplastic". Varying from study to study, MPs have been attributed several size-range (see Table 1.1) from a diameter of <10 mm (Graham and Thompson, 2009), 2-6 mm (Derraik, 2002), <2 mm (Ryan et al., 2009) and <1 mm (Browne et al., 2007; Browne et al., 2010; Galloway and Lewis, 2016). For example, Gregory and Andrady (2003), just to differentiate between small particles visible to human eye and those only discernible with use of microscopy, defined "microlitter" as the visible particles that range between 500 µm and ~60 µm, while particle larger than this were called "mesolitter", a more scientifically definitions for plastic particles although not yet formally adopted by the international research community. Others, (Arthur et al., 2009) instead defined the microplastics as particles that range between 5 mm and 333 µm (recognizing 333 µm as a practical lower limit when plankton nets are used for sampling).

This confusion in size-range appears problematic when in order to create a scientific standard for microplastic data, a comparison is not possible (Claessens et al., 2011; Costa et al., 2010). For this reason, on basis of the several nomenclatures used in literature, here we propose a general and clear size-range to define the different type of "microplastics" (figure 1.4). That issue also concerns the nano-scale plastic debris, defined as "nanoplastics" that constitute a very recent area of the environmental sciences. It has not yet been provided a clear definition in size for the term "nanoplastic"; therefore, could be correct to use the general definition used for nanomaterials implying that a plastic particle is said to be "nanoplastics" if its size is <100 nm (Klaine et al., 2012).

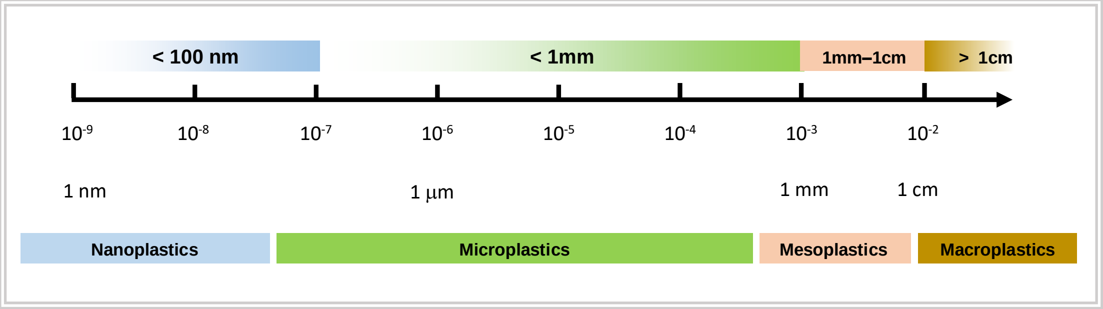


Figure 1.4: Size-range representation of nano- and microplastics based on the nomenclature used in literature (Refs).

Table 1.1: A comparison between plastics nomenclature used in several studies.

Proposed definitions in literature	Year	Size	Nomenclature
Derraik	2002	2 - 6 mm	Microplastic
Gregory and Andrady	2003	67-500 µm	Microplastic
		>500 µm	Mesoplastic
Browne et al.	2007	<1 mm	Microplastic
		<1 µm	Nanoplastic
		>5 mm	Macroplastic
Moore et al.	2008	<5 mm	Microplastic
		>5 mm	Macroplastic
Ryan et al.	2009	<2 mm	Microplastic
		2mm-2cm	Mesoplastic
		>2 cm	Macroplastic
Arthur et al.	2009	<5 mm	Microplastic
Fendall&Sevell	2009	<5 mm	Microplastic
		>5 mm	Macroplastic
Costa et al.	2010	<1 mm	Microplastic
EU Commision	2011	1-100 nm	Nanoplastic
Hidalgo-Ruz et al.	2012	<5 mm	Microplastic
Claeesens et al.	2013	<1 mm	Microplastic
Desforges et al.	2014	1 µm - 5 mm	Microplastic
Kaposi et al.	2014	<1 mm	Microplastic
Hartmann et al.	2015	<1 µm	Nanoplastic
Cole et al.	2015	<1 mm	Microplastic
		<1 µm	Nanoplastic
Nobre et al.	2015	<5 mm	Microplastic
Mattsson et al.	2015	1-100 nm	Nanoplastic
Galloway and Lewis	2016	<1 mm	Microplastic
Bergami et al.	2016	<5 mm	Microplastic
		< 100 nm	Nanoplastic
Huvet et al	2016	< 100 µm	Microplastic
Brun et al.	2017	< 100 nm	Nanoplastic
Chen et al.	2017	<5 mm	Microplastic
		1-100 nm	Nanoplastic
Gambardella et al.	2017	>5 mm	Macroplastic
		1 µm - 5 mm	Microplastic
Karami et al	2017	1 µm - 1 mm	Microplastic
Manfra et al	2017	< 100 nm	Nanoplastic
Messinetti et al	2017	1 µm - 1 mm	Microplastic
Sleight et al	2017	<5 mm	Microplastic
Steer et al	2017	0.1 µm - 5	Microplastic
Capolupo et al	2018	<5 mm	Microplastic
Pitt et al	2018	1-100 nm	Nanoplastic

In view of the definition of nanoparticle (1–100 nm), (Commission European (2011) - Commission recommendation of 18 October 2011 on the definition of nanomaterial), in this thesis the term microplastics is used to describe plastic particles with a size between 100 nm and 1 mm.

1.5 Sources of microplastics

Microplastics found in the environment are a very heterogeneous group of particles differing in size, shape, chemical composition and specific density that originate from a variety of different sources. Depending on their origin, MPs can be defined as primary and secondary. That distinction is based on whether the particles were produced intentionally to be that size (primary) or whether they were degraded from larger plastic items into smaller pieces (secondary) (Cole et al., 2011; GESAMP, 2019; Anderson et al., 2016). Primary microplastics include “scrubbers” products used for air-blasting technology or industrial cleaning products (Derraik, 2002), powders used in molding, microbeads used as exfoliants in personal care products (e.g. hand and facial cleaners or toothpaste (Lassen et al., 2015), plastic particles used in a wide type of industrial process and in medical applications as vector for drugs (Patel et al., 2009). Any of these forms of plastics, if not used in closed systems and disposed properly, has the potential to end up in municipal waste-water and freshwater systems (Doughty and Eriksen, 2014; Anderson et al., 2016) and subsequently reach marine environment. Secondary microplastics are small plastic fragments generated from breakdown and weathering of larger plastic materials present both in sea and on land (Ryan et al., 2009; Thompson et al., 2004).

The inappropriately management of landfill sites together with natural disasters are considered the main routes of entry of plastic items into the marine environment (Desforges et al., 2014; Thompson et al., 2005). Once in the water, plastic debris undergo weathering degradation and progressive fragmentation into smaller particles. Over time, a combination of physical, biological and chemical process determines a reduction of mechanical integrity resulting in fragmentation (Browne et al., 2007). Weathering degradation is probably the most important process for the generation of secondary microplastics in the marine environment. Plastic debris exposed to solar ultraviolet (UV) radiation experience oxidation of the polymer matrix that leads to bond cleavage (Andrady, 2011; Barnes et al., 2009; Browne et al., 2007; Moore 2008; Rios et al., 2007). Over time, exposure to sunlight can result in plastic debris being discolored, weak and embrittle which, due to mechanical forces resulting from abrasion, wave-action, turbulence and biota activity, break into smaller fragments until generating MPs (Fendall and Sewell, 2009; Rios et al., 2007; Ryan et al., 2009; Cole et al., 2011). These reactions might further degrade the microplastic particles generated (Gregory and Andrady, 2003) and possibly progressing to yield plastic fragments in nano-scale (Al - Thawadi, 2020; Lambert and Wagner 2016). The identification of the sources of primary and secondary MPs is enables an assessment of their local distribution in the marine environment and to determine the better management measures to reduce their input. For example, primary MPs, used mainly in cosmetics and care products, can enter in the environment through domestic or industrial drainage systems (Derraik, 2002) and large quantity of them, pass through waste-water treatment plants and the filtration systems (Browne et al., 2007). In this way, the plastic debris that enter in the river system, with a unidirectional flow of freshwater system, are transported into the oceans (Browne et al., 2010). Other sources of plastics include fishing gears, polystyrene box and nylon nets and are the most commonly plastic debris found in marine environment (Andrady, 2011). Microplastics can also stem from plastic products industries and marine traffic, given an accidental loss of plastic products (granules and small pellets used as raw material) during the transport and/or a directly or indirectly outflow from production facilities can lead to the entry of them in marine ecosystem. Finally, tourism along beaches and coastal zones represents a critical source for plastic debris as observed in Huatulco Bay (Southern Mexico), where a higher concentration of MPs was reported in relation to density and activities tourists and also effluents discharged from the hotels and restaurants located along the beaches (Retama et al., 2016).

1.6 Sorption of chemical contaminants to MPs

The high accumulation of MPs in the environments made them not only potential contaminants but also able to act as an extra compartment for chemicals transport and accumulation. MPs are known to interact with a variety of chemicals in the environment and to carry considerable amounts of them (Frias et al., 2010; Fries et al., 2013). These may be categorized into two principal groups. The first include plasticizers, monomers, by-product and other additives which are incorporated into the plastic matrix during the manufacturing process in order to alter the physical plastic properties and give them more benefits and lifetime (Meeker et al., 2009; Lithner et al., 2011). The second group include substances sorbed from the surrounding seawater that may be categorized into organic pollutants and heavy metals. Among the organic pollutants Polycyclic Aromatic Hydrocarbons (PAHs), PolyChlorinated Biphenyls (PCBs), PolyBrominated DiphenylEthers (PBDEs), Persistent Bio-accumulative Toxic substances (PBTs), pesticides, and pharmaceuticals are generally found (Brennecke et al., 2016; Camacho et al., 2019; Li et al., 2018; Wang and Wang, 2018). Heavy metals, such as Cu, Pb, Hg, Cd, and Cr, are also found sorbed on MPs (Duan et al., 2020; Mahfooz et al., 2020; Holmes et al., 2012; Rochman et al., 2014a).

The presence of organic pollutants sorbed to MPs has been reported in particles from different matrices including sediment, seawater and freshwater. Studies focused on determination of these contaminants sorbed to MPs reported very high concentration of them compared to surrounding medium. For example, PCBs, a series of organochlorine compounds used as cooling liquids in electrical appliances, as well pesticides (DDTs) were found to reach concentrations up to 159,67 ng/g and 156,01 ng/g, respectively, on MPs collected from beach sediments in Hong Kong (Lo et al., 2019). High concentrations (125,79 ng/g) of PBDEs, organobrominated compounds used as flame retardants, were found sorbed to MPs collected from seawater of urban Tokyo Bay areas compared to off shore areas. (Yeo et al., 2020).

The sorption of chemical pollutants is generally related both to physical properties and to chemical properties of MPs. The most common polymers found in the environment are typically hydrophobic, such as PS, PE, PP or PET, and the organic pollutants shown a greater affinity for hydrophobic plastic surface if compared to seawater. Thus, given the higher surface area-volume ratio of MPs than larger plastic particles (Teuten et al., 2007), they tend to accumulate on their surface organic and lipophilic pollutants concentrations a hundred times higher than those found in sediments or in seawater (Teuten et al., 2009). For example, PBTs being hydrophobic and having a low water solubility tend to partition out of the water column and onto another environmental matrix with similar hydrophobic properties. Therefore, when PBTs are in contact plastic debris they tend to sorb to this material (Engler, 2012).

Among the adsorbed toxic contaminants on MPs, heavy metals are key contaminants of inorganic nature (Khalid et al., 2018). The main sources of heavy metals in environmental MPs include (1) the production of plastic, during which heavy metals and their compounds are added to polymers to improve properties and performance of polymers (Mao et al., 2020; Cao et al., 2021); and 2) the surrounding environment (Ashton et al., 2010; Holmes et al., 2014). In natural environments, MPs have been confirmed to have a high affinity to heavy metals in aqueous phase (Ashton et al., 2010; Rios et al., 2007; Brennecke et al., 2016). Although, the knowledges about mechanism of metal adsorption on plastics are limited, accumulation of metals on marine plastic debris, may be explained by both the chemical components of the plastic (e.g. catalysts, fillers, plasticizers) and the degradation and fouling of aquatic plastic debris via microbial biofilms and colonization by algae or invertebrates that may generate active sites for the sorption and/or bioaccumulation of metals (Holmes et al. 2012; Tien and Chen 2013). For example, MPs sampled in the Celtis sea, the North Sea, and the English Channel showed a higher concentrations of Sb, Hg and Cr than surrounding environments. Significant high adsorption capacity for Cu and Zn was observed by PVC fragments and PS beads, even though some studies have found differences in the accumulation of metals between plastic types (Brennecke et al., 2016; Rochman et al., 2014a; Gao et al., 2019).

The chemicals added in the production process are relatively stable and are retained physically within plastic polymer. Thus, they have a low tendency to migrate outside of plastic polymer. However, as plastic break into smaller sizes of fragment or pieces via physical abrasion, chemical oxidation or biodegradation (Hurley et al., 2018; Song et al., 2017), there is potential for the constant migration of chemicals along a concentration gradient to the surface of MPs (Browne et al., 2013; Cao et al., 2021). In addition, the production of several plastic types is carried out polymerizing individual monomers that form the backbone of the polymer (Galloway, 2015). This type of process is rarely completed. Thus, unpolymerized residual monomers can migrate off the synthetic matrix plastic to other contact media because of low molecular weight (Lithner et al., 2011). The instability of these compounds, within plastic products facilitates their leaching and the high prevalence in aquatic environments (Vom Saal and Myers, 2008). This release can occur at all lifecycle stages and depends on how a specific chemical interacts with polymer matrix (Lambert et al., 2014) as for example low molecular weight additives weakly fixed in the polymer matrix and that will tend to migrate easily. The hazard potential of leachable chemicals will depend on different factors including concentration in the parent plastic, degree of plastic degradation and crystallinity of polymer chains structure. Based on ecotoxicological risk assessment, defined as a product of the intrinsic hazards of a substance combined with an estimate of its environmental exposure, risks associated with plastics not only come from the material itself but also from sorbed pollutants (Wang et al., 2016). The chemical pollutants sorbed from the surrounding environment are not chemically bound to the surface of the microplastics. Therefore, they are known to leach from the plastic resulting in the release of potentially harmful chemicals for biota (Guart et al., 2011; Koelmans et al., 2014; Thevenon et al., 2014). Ingestion of plastics with sorbed pollutants has been previously suggested as a possible exposure pathway for pollutants to organisms (Fry et al., 1987; Ryan et al., 1988). Some studies suggested the transfer of plastic-derived chemicals from ingested plastics to the tissues of marine-based organisms (Tanaka et al., 2013). For example, greater concentrations of PCBs and PBDEs was found in fish fed with the marine plastic than those fed with the virgin plastic (Rochman et al., 2013a) therefore indicating that plastic debris can be a vector of sorbed pollutants to aquatic organisms. Further studies focused on bioavailability of sorbed pollutants showed an elevated bioavailability of pyrene after MPs ingestion in mussels (Avio et al., 2015). Similarly, heavy metals have been found easily desorbed from the MPs (Wang et al., 2019; Zhou et al., 2019), determining several potential ecological risks to organisms.

1.7 Mechanisms of sorption

Owing their ability to sorb chemical contaminants, MPs can accumulate pollutants from the surrounding environment, increasing their concentration by even several orders of magnitude. This phenomenon is related to one or many sorption mechanisms occurring between the sorbent (plastic polymer) and sorbate (chemical contaminant) (i.e., hydrophobic interactions, electrostatic interactions, Van der Waals forces, hydrogen bonds, and π- π interactions) (Yu et al., 2019).

1) Hydrophobic and electrostatic interactions are the predominant mechanisms for the sorption of many chemical contaminants (Liao and Yang, 2019; Guo et al., 2018; Llorca et al., 2018; Wu et al., 2019). Hydrophobic interactions relate to the attraction between two non-polar molecules. Due to the hydrophobic nature of most environmental MPs (PS, PE, PP), this type of interactions is considered as one of the main mechanisms governing the sorption of hydrophobic organic pollutants to the non-polar MP surface (Hüffer & Hofmann, 2016; Tourinho et al., 2019).

2) Electrostatic interactions represent the main adsorption mechanisms for heavy metal ions (Liao and Yang, 2019) and relate to the attraction between oppositely charged molecules or the repulsion by molecules with the same charge (Tourinho et al., 2019). The pH of medium is reported to influence these type of attractions and specifically the surface electric potential (zeta potential) of plastic material (Xu et al., 2018). When the MP zeta potential, which describes the surface electric potential, is lower than the pH of the medium, their surfaces become negatively charged and more easily attract positively charged chemicals (Liu et al., 2018; Guo et al., 2018). In addition, the polarity of the plastic surface can be altered by its physical and chemical properties as well as by the presence of charged contaminants or additives (Holmes et al., 2012).

3) Hydrogen bonds are weak interactions involving proton donor and acceptor groups (Torres et al., 2021). This mechanism is not predominant, but for some polymer types may significantly enhance their chemical sorption capacity. For example, polar polymers such as PA was found to have a greater sorption capacity for organic compounds (i.e antibiotics and 17ß-Estradiol). Being the presence of hydrophilic groups associated with a lower hydrophobicity, the sorption of the organic chemicals by hydrophobic interactions is reduced. Thus, sorption mechanism is associated to amide group in PA that forms hydrogen bonds with the hydrogen-donor chemicals present (Liu et al., 2019; Li et al., 2018).

4) Finally, other interactions that play a minor role on determining chemical sorption on MPs are Van der Waals forces and π- π interactions. Van der Waals forces are weak interactions occurring between molecules not involving covalent or ionic bonding, while π- π interactions mediate aromatic polymers associations (Müller et al., 2018; Hüffer et al., 2018). For instance, aromatic polymers such as PS showed a higher sorption of PCBs compared to other polymers as result of π- π and hydrophobic interactions that took place simultaneously (Velzeboer et al., 2014).

1.8 Factors influencing chemical contaminants sorption

Physical and chemical properties of microplastics, medium and contaminants

The interaction mechanisms between MPs and chemical contaminants are dependent on the physical and chemical properties of the MPs, contaminants and medium where the sorption occurs (figure 1.5) (Torres et al., 2021; Fred-Ahmadu et al., 2020).

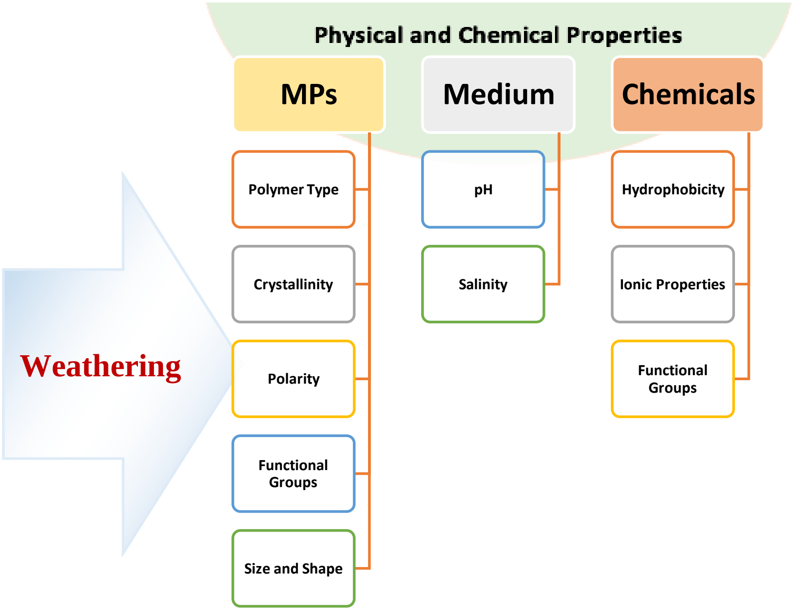


Figure 1.5: The physical and chemical properties influencing chemical contaminants sorption to MPs.

MPs properties

Polymer type: among MPs properties, the polymer type play a key role in determining the different factors (i.e, crystallinity, polarity, and functional groups) affecting the sorption of chemical contaminants to MPs. The type of material in fact determines the interactions occurring on the MP surface (as discussed above), and sorption capacity can vary by several orders of magnitude among materials (Teuten et al., 2007; Brennecke et al., 2016; Velzeboer et al., 2014; Hüffer & Hofmann, 2016). However, the type of MP material should not be the only characteristic to be taken into account, as other characteristics have been found to interfere with the sorption process.

Crystallinity: a major characteristic affecting sorption is the degree of crystallinity of the polymers. Based on alignment of molecular chains, plastic polymers can be more or less, crystalline. Crystalline domains consist of an ordinated and regularly aligned polymer chains structure, while domains characterized by not specific alignment constitute amorphous parts (Torres et al., 2021). The degree of crystallinity can influences physical properties such as density and permeability, which in turn drives their hydration and swelling behavior (Lambert et al., 2017). A higher degree of crystallinity indicates that the polymeric chain is more ordered and therefore a high energy is necessary for sorption of chemicals; while more disordered chains result in a greater proportion of amorphous regions and consequently, in a more easy chemical sorption (Tourinho et al., 2019).

Size and shape: other factors that are significantly related with the sorption process include size and shape. Generally, particles with a higher area/volume ratio, such as small particles or their irregular shape, are expected to have a higher sorption capacity than bigger ones (Wang et al., 2018; Liu et al., 2018; Tourinho et al., 2019). For instance, decreasing PP polymer size from 5 to 0.18 mm increases PCB concentration on its surface area, as it becomes larger with decreasing particle size (Zhan et al., 2016). Similarly, a larger specific plastic surface area resulted in a higher sorption capacity of pyrene compared with plastic having a lesser area and same mass (Wang & Wang, 2018a). However, the influence of MP area in certain cases can be negligible due to other characteristics influencing sorption process. For instance, Xu et al., 2018 reported a higher sorption capacity of tetracycline for PS than PE, even though PE had a higher surface area and smaller size (150 µm) compared with PS (280 µm). In this case, the higher sorption observed was attribute to polar and π-π interactions between tetracycline and PS.

Functional groups and polarity: additionally, the specific functional groups present on MPs play a pivotal role in determining the type and the number of sorption mechanisms described previously as well as in identifying polarity of plastic polymer itself. Overall, when MPs are in a virgin state, they mainly have hydrophobic surfaces and interact with organic pollutants because they possess high, specific surface areas and hydrophobicity. However, once in the environment, MPs undergo weathering process that may modifies their surface functional groups and, consequently, their sorption features as well MPs morphological properties and their either physical (surface area, particle shape, particle size, and density) or chemical (leaching of additives, adsorption of pollutants) properties (Murphy, 2018; Zhao et al., 2020). The several environmental stressors can determine changes in crystallinity of the polymer particles, which become less crystalline and more amorphous (Endo et al., 2005; Yu et al., 2020). On the surface of plastic polymers new functional groups as ketones, esters, carboxylates, and hydroxyl groups may be generated decreasing their hydrophobicity and molecular weight and making them brittle and irregularly shaped (Alimi et al., 2018; Celina et al., 2013). Notably, the shift of sorption plastic surface from hydrophobic to hydrophilic leads to a creation of a dynamic system for contaminants bioavailability. Although on the one hand weathering conditions reduce the potential retention of chemicals (Mato et al., 2001), on the other hand due to separation of the chains and fragmentation in smaller particles, with higher flexibility and lower molecular weight, chemical sorption can increase (Jahnke et al., 2017; Arienzo et al., 2021). Some studies reported that fragmented MPs, as result of a mechanical and chemical degradation, increase on their specific surface area, which influences the sorption capacity of chemical pollutants (Torres et al., 2021; Wang et al., 2019; Li et al., 2019). Moreover, these changes in plastic surface chemistry make it more accessible for several organisms including diatoms, hydroids, filamentous algae and organic residues which provide an additional sorbent surface area. Thus, this fouling material (i.e biofilm) allows concentrations of chemical contaminants to increase over time (Zettler et al., 2013; Oberbeckmann et al., 2015).

Medium properties

pH: pH is among the most significant factors influencing the sorption process. The sorption of tylosin to MPs, for example, is mediated by electrostatic interactions between positive charges of tylosin and negative charges on the polymers that lead to increase in sorption up to pH 7.1 (Guo et al., 2018). However, when the pH exceeds that value, a decrease in tylosin sorption occurs due to decrease of its cation species and to prevail of hydrophobic forces on electrostatic interactions. For metals, the pH influence is metal-dependent (Holmes et al., 2014; Turner & Holmes, 2015). Some studies showed that the adsorption of Cd was weak at acidic pH but increased with increasing pH values (Wang et al., 2019). Indeed, when the pH is > 7, the negatively charged surface of MPs favored adsorption toward Cd with positive charges. However, when the pH is acidic, the decrease in adsorption can be due to the electrostatic repulsion between the positively charged surface and the cationic Cd ions. Whilst, for other metal species such as Cr, sorption decreased with increasing pH, suggesting that Cr speciation produced negative forms like $HCrO^{4-}$ and CrO_2^{4-} (Holmes et al., 2014; Turner & Holmes, 2015).

Salinity: the contribute of salinity of the surrounding aquatic medium varies depending on the chemical contaminants and the sorption behaviors of them. High salinity levels generally alter the aqueous solubility of organic pollutants enhancing their addition to solid phases and leading to their greater sorption capacities (i.e

PAH and PCB) onto MPs (Turner, 2003; Alimi et al., 2018). However, for other organic contaminants such as BDE-47 salinity seem not influence sorption capacity (Wu et al., 2020). Moreover, a contrasting trend concerns the adsorption of heavy metals by MPs. For example, the sorption of Cd, Co, and Ni by PE was found to decline significantly with salinity increasing (Holmes et al., 2014).

Chemical and physical properties of sorbates

Hydrophobicity, ionic property, and functional groups: the chemical and physical properties of sorbates are also equally important in affecting its sorption on MPs (Zhan et al., 2016; Velzeboer et al., 2014). These properties are mainly hydrophobicity, ionic property, and functional groups (Mei et al., 2020; Wang et al., 2020). For organic compounds hydrophobicity and molecular weight play a key role on sorption process. Given their high hydrophobic and lipophilic nature, they can interact with several non-polar surfaces, such as MPs, and suspended organic material and sediments (Rodrigues et al., 2019). The interaction is driven by their octanol-water partition coefficient (K_{ow}) (O'Connor et al., 2016) and diffusive mass molecular diffusion (Hartmann et al., 2017). Notably, there is a positive and significant correlation between the distribution coefficient (lipophilicity) and octanol-water partition coefficient of different sorbates (hydrophobicity) (Hüffer and Hofmann, 2016; Torres et al 2021). Therefore, due to these features, plastics can accumulate POPs up to two-fold when compared to natural sediments and soil, and up to six-fold the levels in seawaters (Wright et al., 2013; Arienzo et al., 2021). For metals, their sorption can be driven by the formation of free cation species able to react with negatively charged region of MPs surface or by generation of organo- metallic complexes in the medium that can interact with the neutral areas of MP surfaces due to hydrophobic interactions (Holmes et al., 2014).

Weathering factors

Plastic debris in the environment undergo weathering processes, which lead to a gradual degradation of the plastic material. Through weathering, MPs become able to bind various organic and inorganic contaminants, which form an "ecocorona" on plastic surfaces, change their density and surface charge and consequently, their ways to interact with the surrounding environment (Galloway et al., 2017). Marine plastic material can undergo to different types of degradation process: biological, thermal, mechanical, and hydrolysis (Browne et al., 2007; Andrady, 2011; Singh and Sharma, 2008). Several synthetic polymers can absorb solar UV radiation and undergo photolytic, photo-oxidative, and thermo- oxidative reactions that result in a degradation and modification of polymer features (Gugumus, 1993; Andrady, 1993). For common polymers present in marine environment such as LDPE, HDPE, PP, and nylon, degradation start with UV-B photo-oxidation followed by thermo-oxidation (Andrady, 2011). These fragments can undergo further degradation (e.g., biological) where carbon in the matrix is converted to carbon dioxide and incorporated into biomass, until complete mineralization which time required is estimated to be on the order of hundreds to thousands of years (Andrady, 1993 and 1998; Eubeler et al., 2009; Barnes et al., 2009). UV degradation is an efficient process in relation to plastics exposed in air or beached. However, when the same plastic items are floating in seawater, degradation is severely delayed due to the deficiency of the solar UV radiation and the low temperature that lead to a rate of degradation in the marine environment slower than in the terrestrial environment (Barnes and Milner, 2005; Ryan et al., 2009; Anderson, 2016).

The effects of weathering of plastics on the sorption of contaminants are not clearly known, but several studies have highlighted that photo and oxidative degradation processes affect the plastic surface by creating new functional groups through reactions with -OH radicals, O, N oxides, and other photo-generated radicals (Chandra and Rustgi, 1998). Photo-oxidation leads to cascade chemical reactions (developing cross-linking, chain scissions, oxygen-containing groups) that, ultimately, cause cracks on plastic surface opening up new surfaces for further degradation processes to occur (Lambert et al., 2013 and 2016). This process on one side increase surface area and consequently enhance plastic adsorption capacity but, on the other hand, an increasing polarity by the reaction with oxygen could decrease affinity for hydrophobic compounds (Endo et al., 2005). Thus, the weathering makes polymers capable of interacting with organic chemicals in the one hand, and increases or decreases their retention on the other hand (Arienzo et al., 2021). Some studies retain that the main effect weathering-induced is related to increased crystallinity that lead to decreased chemicals sorption (Lee et al., 2014). Others reported that polymer fragmentation and crack lead to creation of new surface available to sorption (Rodrigues et al., 2019). To date, the debate on whether weathering process enhances or decreases chemical contaminants sorption to MPs is still open.

Finally, environmental conditions play a very important rule also for metals adsorption, as observed in relation to a higher adsorption capacity for beached plastic pellets in comparison to the virgin counterpart (Holmes et al., 2012; Turner and Holmes, 2015). This condition would seem to be due to an increase polarity determined by weathering process and a biofilm development that leads to metals accumulation (Tien and Chen, 2013).

1.9 The effects of microplastics in the marine environment: a special insight on contaminant bioaccessibility and bioavailability

The potential toxicity of microplastics to marine organisms appears to be attributable to different pathways: microplastics ingestion as they may physically and mechanically affect organism; leakage of additives from plastics; action as vectors for hydrophobic pollutants (Andrady, 2011; Cole et al., 2011; Ross and Morales-Caselles, 2015) or as substrate for organisms. Considering their small size and occurrence in both pelagic and benthic ecosystems, a wide range of marine organisms (Betts, 2008; Thompson et al., 2009) potentially ingests

MPs. Many of these organisms are unable to discriminate between MPs and food particles (Blarer et al., 2016; Iannilli et al., 2019). Specifically, the lower trophic level organisms, such as zooplankton and invertebrates, are particularly vulnerable to ingesting MPs since many of them are indiscriminate feeders and unable to distinguish plastic particles from food (Moore, 2008). The potential for microplastics to cause harm in marine organisms is likely to be governed by the susceptibility of species to ingest and/or interact with them. The degree of impacts seems to be related to the size, shape and number of the ingested particles besides foraging strategies and diet (Solomon, 2016) as suggested by studies in which different individuals with different feeding techniques were selectively ingesting plastic particles (Graham and Thompson, 2009).

The presence of various chemical contaminants on MP surfaces represents an additional risk for biota. Indeed, once ingested, plastic particles can induce toxicity damage from chemicals leaching in addition to mechanical effects such as obstructions, blockages or clogging that hinder the transit of food towards the intestinal tract (Prichard et al., 2016; Tourinho et al., 2010). Several studies have raised the possibility that MPs represent a vector for transferring chemicals to aquatic organisms (Koelmans et al., 2016; Ziccardi et. al, 2016; Khan et al., 2021). In the environment, only a portion of the total quantity of chemical present is potentially available for uptake by organisms. This concept is referred to as the biological availability (or bioavailability) of a chemical that, desorbing from MPs, becomes immediately available to cause harm to a living organism, after passing through the organism's membrane (Casarett and Doull's, 2001; Umeh et al., 2020). While, bioaccessibility describes the fraction of the chemical that desorbs from its matrix (e.g., soil, dust, wood) and is available for absorption and bioaccumulation in the tissue of an organism (Paustenbach et al., 1997). Thus, if MPs are ingested, the contaminants associated with them can desorb from MPs (bioaccessible fraction), and then can penetrate in cells chemically interacting with biological macromolecules (bioavailable fraction). The transfer of these chemicals to animal tissue increase their hazardous potential, since many plastic additives and persistent waterborne chemicals are capable of activating several signal transduction pathways in target tissues and altering metabolic and reproductive endpoints (Galloway et al., 2015; Koelmans et al., 2016; Rochman, 2015). Biota can sorb chemical contaminants transported by MPs by several pathways such as via the skin, gills, gut (Endo et al., 2005). For instance, chemical transfer can already occur by simple attachment of contaminated microplastics on epithelia of zebrafish where harmful effects of toxic substances on early life stages are been observed (Batel et al., 2018). Alternatively, a transfer of contaminants to biota MP-mediated can occur by indirect contact with desorbed contaminants in aqueous phase (Hartmann et al., 2017; Sleight et al., 2017; Batel et al., 2018). Moreover, contaminants on environmental MPs are seldom present as a single chemical but rather a complex mixture of pollutants. In most cases, the simultaneous exposure to several chemicals may results in variable levels of toxicity due to neutralizing, additive or synergistic effects (Ragusa et al., 2017; Wah Chu et al., 2002). To date, there are few studies assessing the adverse effects ascribable to the complex mixture of pollutants associated with microplastics reported liver toxicity, glycogen depletion, tumor predisposition and endocrine disruption in fish (Rochman et al., 2013c). However, the questions on whether components of the mixture influence the uptake of other components have not been comprehensively addressed.

Contaminants bioaccessibility and bioavailability by MPs are critical concepts to predict the adverse effects associated to MPs in the environment. Studies focused on the bioaccessibility of sorbed contaminants on MPs reported a higher bioaccumulation of fluoranthene in mussels exposed to PS-fluoranthene than in organisms exposed to the pollutant alone (Paul-Pont et al., 2016). On the other hand, other studies shown that organic contaminants, such as phenanthrene and fluoranthene, are able to impact marine organisms if they are present in aqueous phase rather sorbed to MPs (Sørensen et al., 2020). Therefore, bioaccessibility and bioavailability of contaminants sorbed to MPs is likely to relate to the physicochemical properties of MPs and contaminants associated to them (Bakir et al., 2012). Biomarkers are often used to evaluate bioavailability of contaminants associated with MPs as well as to assess biochemical, molecular, physiological, and morphological changes (Varò et al., 2019). Changes in expression of specific biomarker genes have been used to investigate bioavailability of phenanthrene and 17α-ethinylestradiol associated with MPs (Sleight et al., 2017) and metals associated to engineered nanomaterials (Henry et al., 2013).

The toxicological impacts on biota concerning MP-associated contaminants reveal quite numerous and concern oxidative stress via free radical production (Pittura et al., 2018), alteration of immunological responses (Tang et al., 2018), modification of gene expression profiles, reproductive toxicity, developmental neurotoxicity, modification endocrine activities in fish and mammals (Rochman et al., 2014b; Brandts et al., 2018).

1.10 Sorption of pollutants on microplastic-associated biofilm

In the last years, interest into evaluate the formation, composition and likely effects of microplastic-associated microbial biofilms was increased. MPs can offer a substrate for microbiota, the so-called "plastisphere", for attachment and settlement (Zettler et al., 2013). Plastisphere microorganisms have been suggested to play several roles in determining MPs and their associated contaminants bioaccessibility. Notably, the presence of biofilm changes the properties of microplastics surface (e.g., size, density, and surface roughness), which may influence the fate of microplastics as well as the sorption capacity of pollutants on microplastics (Chubarenko et al., 2016; Tu et al., 2020; Wang et al., 2020). This dynamic mixture influences density and surface charge of MPs, changing their ability to interact with surrounding environment (Monopoli et al., 2012; Tenzer et al., 2013). For example, it has been demonstrated that a thick ecocorona reduces substantially the amount of UV light (~90%) reaching plastic particles (O'Brine and Thompson 2010) and also reduces plastic hydrophobicity.

That reduction lead to an increase of particles sinking velocity influencing their accessibility for marine organisms that live in other levels of the water column (Li and Yuan, 2002).

Some studies reported that MP-associated biofilm increases the affinity to pollutants than virgin ones (Guan et al., 2020). Therefore, biofilm on MPs surface can affect the transport of chemicals between plastics and water due to its sorptive properties and its ability to metabolize associated chemicals (Rummel et al., 2017). Several studies have investigated the sorption behaviors of metal ions and organic pollutants on microplastics but the question of whether sorption process is influenced by MP-associated biofilm represents the focus of recent studies. The few researches focused on sorption of metal ions on MPs with biofilm indicate that biofilm can enhance the sorption capacity of metal ions.

Electrostatic interaction, ion exchange, surface complexion, and precipitation could mediate sorption of metals on MPs (Guan et al., 2020; Wang et al., 2020). On the other hand, the studies on the sorption of organic pollutants, such as antibiotics, hydrophobic organic contaminants HOCs, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) onto MP-associated biofilm reported contrasting results (Magadini et al., 2020; Jin et al., 2020). Thus, more evidence is needed to illustrate the influence of biofilm on the sorption capacity of organic pollutants onto microplastics.

In addition to the role of MP-associated biofilm to act as vector of pollutants to aquatic environments and to organisms, the presence of biofilm influences the transport and deposition of bacteria (Bozorg et al., 2015). Indeed, likewise contaminated microplastics in marine environment, may be transported over long distances and may determine the transfer of contaminants between ecosystems (Zarfl and Matthies, 2010). Also microbial communities associated with microplastics, consisting of diatoms, coccolithophores, bryozoans, barnacles, dinoflagellates, invertebrate eggs, cyanobacteria, fungi and bacteria can be transported (Zettler et al., 2013; Reisser et al., 2014; De Tender et al., 2015; Eich et al., 2015; Quero and Luna, 2017). Several studies have verified that microorganisms in biofilms can be transferred to the environment (He et al., 2020; Hall-Stoodley et al., 2004). Since it has been reported that biofilm on microplastics contains pathogens, such as *Pseudomonas monteilii, Pseudomonas mendocina, Pseudomonas syringae, Vibrio spp,* and *Acinetobacter* (Zettler e al., 2013; Kirstein et al., 2016) a role as additional vector for the dispersal of pathogens could be hypothesized. However, the investigation on the transfer of microorganisms from microplastics to water is still limited.

1.11 The ecological aspect: potential effects on ecosystems

The lack of knowledge of how impact on individual organisms might lead to population and ecosystem harms has hampered a reclassification as waste hazardous for microplastics (Rochman et al., 2013b). Most studies have been focused on harmful effects of microplastics at cellular and individual level leaving out the effects related to higher levels of biological organizations (Galloway et al., 2017). Actually, there is an indication that MPs may not only affect species at the individual level but these harmful effects could be translated on population structure with potential impacts on ecosystem dynamics (Wright et al., 2013). For example, adverse effects on the photosynthesis capacity of primary producers or on the growth rate of secondary producers, could potentially result in a lower productivity of the whole ecosystem (GESAMP, 2019). Although the ecological impacts caused by micro- and nanoplastics in the marine environment are not clearly understood, responses evaluation at lower levels of biological organization can provide new and useful insights onto relationships between stressors and their effects at ecological level (Browne et al., 2015). One of the most important challenges of ecotoxicology is to understand the impact of a pollutant across all levels of biological organization. To develop an effective risk assessment for microplastics, was developed the adverse outcome pathway (AOP) consisting in a series of measurable key events linked to another event by specific relationships (Ankley et al., 2010). Biochemical or molecular changes at subcellular levels may represent an initiating event of the interaction between a chemical with a biological macromolecule; as consequence, this interaction triggers new key events which may result in adverse outcome (AO) at the individual or population level (Allen et al., 2016; Villeneuve et al., 2014; Ankley et al., 2010; AOP, OECD, 2016). In this succession of events finding the link between ecological and subcellular levels in relation to a specific contaminant, is very hard. Individual behavioral changes represent one the main early warning signs to evaluate effects at ecosystem level since for many animals a behavior change is the first response to environment conditions changes (Weis et al., 2014; Wong et al., 2015). For example, ingested plastic substantially reduce the physiological condition of individuals, but is not likely to cause population-threatening shifts in population sizes (Marn et al., 2020). Indeed, the effect concentrations at population level can be lower than effect concentrations at individual level, although effect concentrations at population level remain higher than ambient microplastic concentrations (Everaert et al., 2022). Therefore, before performing a correct assessment of possible environmental risks caused by microplastics, several data gaps need to be filled in environmental risk assessment procedures have to be adapted.

1.12 Pilot study: sorption of metals and BDE-47 to PS

The present experiment represents a short preliminary study carried out to evaluate the sorption, over the time, of different metals and BDE-47 on a pristine model MP (PS microbeads, 6 µm) purchased from Polyscience (Warrington, PA, USA). Specifically, this study was designed to select the most suitable contaminants for the evaluation of the impacts of weathered MPs on their bioavailability in *Paracentrotus lividus* and *Danio rerio*. The sorption behaviors of different metal ions (Cd, Pb, Zn and Cu) and organic contaminant (BDE-47) were investigated evaluating the concentration of each contaminants sorbed on PS-

MPs. Using filtered and autoclaved sea water, PS contamination was carried out following the experimental protocol used by Avio et al., 2015. Briefly, the sorption of metals to MPs was assessed by mixing solutions of MPs (1 mg L^{-1} in seawater) with each metal ions dosed at final concentration of 500 µg L^{-1}. The high metal concentration was selected in order to facilitate the analysis related to determination of fraction associated with microplastic. The mixing solutions were maintained in continuously rotating 50 mL glass bottles and samples were collected after 14 days for metals determination. Each metal test solution containing MPs was filtered on Whatman filter (pore size 0,45 µm) and filtration solution collected. Then, filters were digested in microwave with 5 mL 67% HNO_3 , 1 mL H_2O_2 and 4 mL distilled water. The concentrations of Cd, Pb, Zn and Cu in the digestion and filtration solutions were determined by inductively coupled plasma-mass spectrometry (ICP-MS). The same exposure protocol of MPs to contaminant have been used for BDE-47 (10 µg L^{-1}), although for the organic compound a different extraction protocol was followed (Rochman et al., 2013a). Then, BDE-47 concentration was measured by gas chromatography–mass spectrometry (GC-MS). Quality assurance and quality control were tested by processing blank samples and blank filter membrane sample.

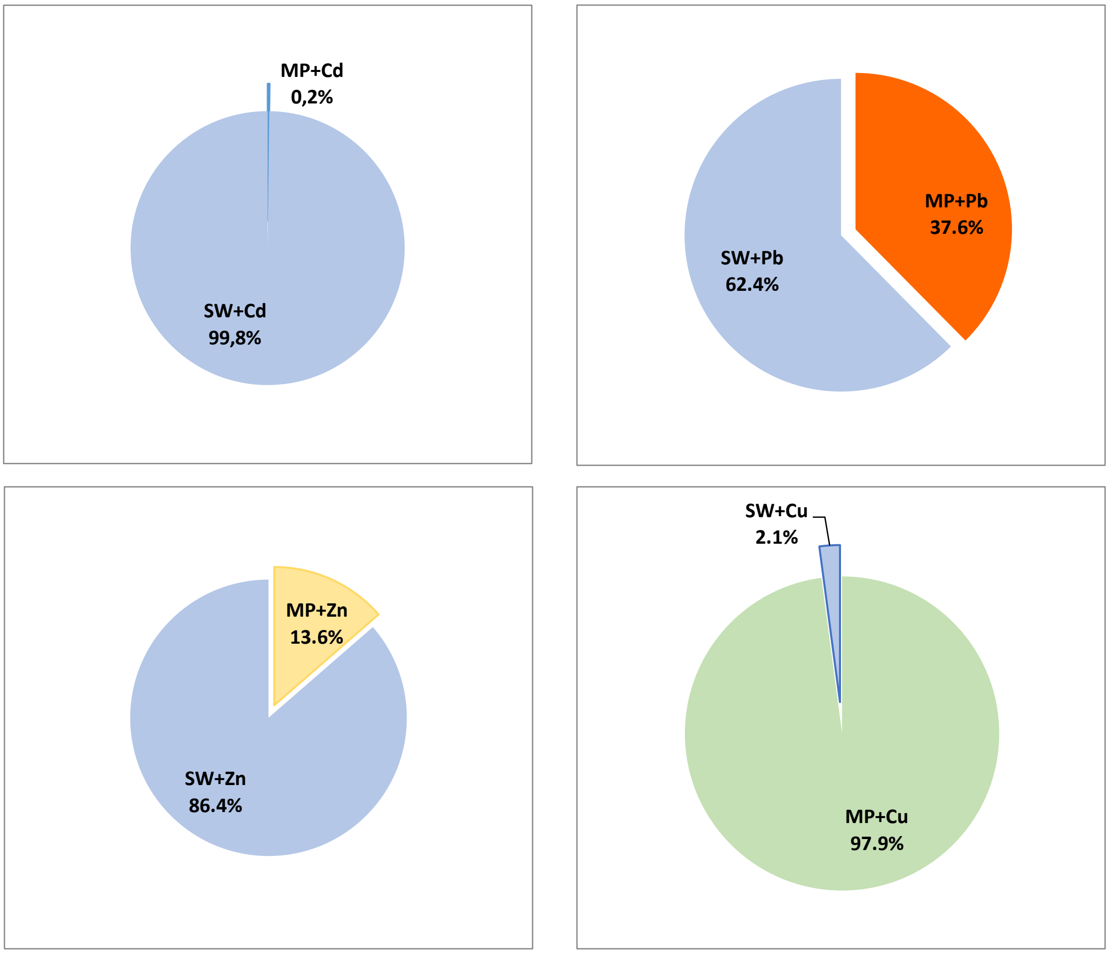


Figure 1.6: Percentages of trace metals Cd, Zn, Cu, Pb (500 µg L^{-1}) sorbed to MPs (1 mg L^{-}1) after 14 days of exposure. SW=seawater; MP= PS microparticles (6 µm).

The results showed that different heavy metals have different adsorption characteristics and for selected MPs the order of sorption was Cu > Pb> Zn> Cd (figure 1.6). Thus, PS showed a sorption capacity for different heavy metals (2,1% for Cu, 37,6% for Pb, 13,6% for Zn, and 0,2% for Cd). That sorption may be mediated by electrostatic interactions occurring between negative charge on PS surface and metal ions positive charge. However, the different metal ions sorption may be related to the physicochemical properties of the different metal ions themselves (Li et al., 2019; Mao et al., 2020).

For BDE-47, an evaluation of its binding kinetics to MPs at different exposure times (over 14 days) was carried out. The results showed that BDE-47 sorption process was quick initially and almost completed in the first 2 days (figure 1.7).

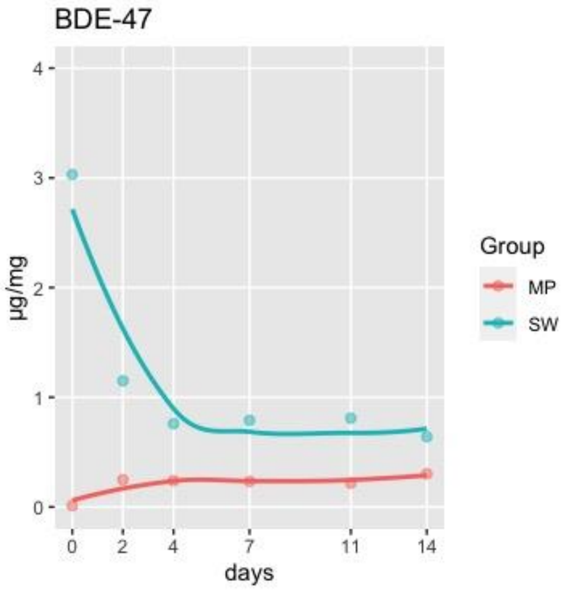


Figure 1.7: Long term exposure test. BDE-47concentration in SW and sorbed to MPs after 14 days of exposure. BDE-47 concentration used for experiment (10 µg L^{-1}); MPs concentration (1 mg L^{-}1). SW=seawater; MP= PS microparticles (6 µm).

Thereafter, as the contact time increased, a plateau value (~0,2 µg/mg) was reached which remained unchanged after 11 days. Thus, since from batch experiments sorption of BDE-47 occurred rapidly in 48 hours after which reaching equilibrium, a short time exposure (48 h) was carried out (figure 1.8). The short time exposure results confirmed the adsorption capacity of MPs for BDE-47. Notably, the sorption of BDE-47 occurred rapidly in 2 hours and reached equilibrium within 6 hours.

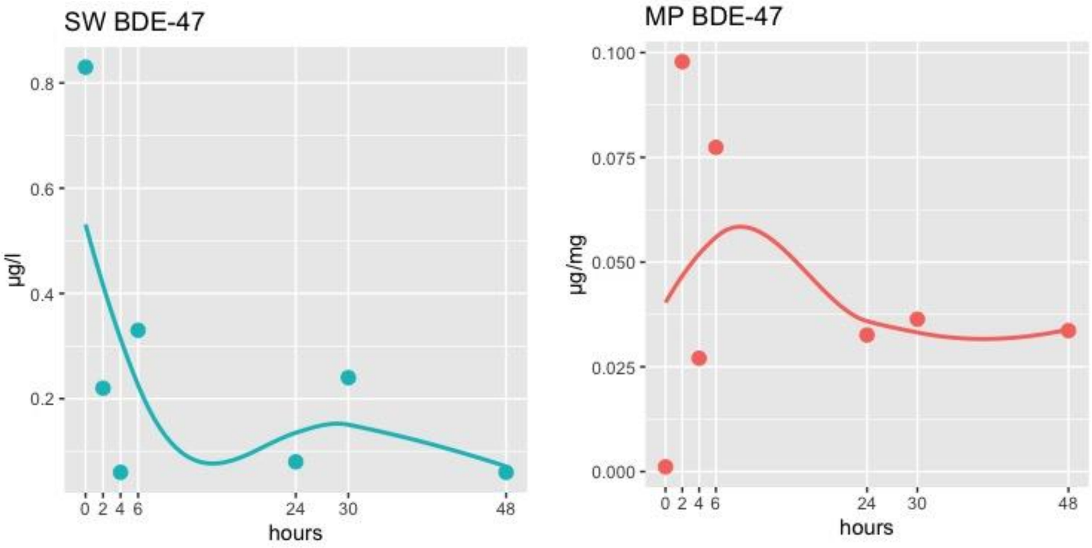


Figure 1.8: Short-term exposure test. BDE-47concentration a) sorbed to MPs and b) in SW after 48 h of exposure. BDE-47 concentration used for experiment (10 µg L^{-1}); MPs concentration (1 mg L^{-}1). SW=seawater; MP= PS microparticles (6 µm). BDE-47 concentration expressed as µg L^{-1}. T0=time 0; T1=2h; T2=4h; T3=6h; T4=24h; T5=30h; T6=48h.

These results show that the adsorption capacity of microplastics increases with an increase in aging and the particle size of microplastics has an effect on its adsorption capacity (Ma et al., 2019). Although the influence of aging on the adsorption of pollutants by microplastics has been reported, a systematic study is needed on the influence of microplastics in different aging stages on pollutants and the influencing characteristics of different kinds of pollutants.

Overall, these preliminary results showed the sorption capacity of MPs for the inorganic and organic chemicals tested. In the present study, to evaluate the influence of weathered microplastics on bioavailability of pollutants to model organisms, Cd, Cu and BDE-47 have been selected. The opposite sorption pattern showed by Cd and Cu prompted us to investigate on how aging process and biofoulant attachment on plastic surface may influence the sorption mechanism and bioaccessibility of these toxic metals. Cu and Cd represent the most common contaminants recorded in polluted areas (Baltas et al., 2017; Martínez-Soto et al., 2016). Thus, co-occurrence of these metals in combination with MPs is highly probable. The low sorption capacity of Cd to pristine MPs observed in preliminary tests highlights that Cd may have high bioaccessibility and consequent on bioavailability for animals present in aqueous phase, compared to other metals showing a higher sorption by MPs (i.e. Cu). Since after entering the environment, plastics polymers undergo aging process and biofoulant attachment that change their physicochemical properties, the interaction with pollutants by MPs in the environment will be more complex (Mao et al., 2020; Guo et al., 2018). Thus, knowing the sorption characteristics of Cd as well as of Cu by MPs and analyzing their environmental behavior when alterations of plastic surface occur, are important factors clarifying the impact of MPs on the sorption of pollutants as well on their bioavailability and toxicity for animals. In addition, since MPs were shown to sorb PBDEs, in the present study congener BDE-47 was selected as hydrophobic organic pollutant to examine the influence of weathered microplastics on its bioavailability. BDE-47 is one of the most abundant PBDE congeners detected in animal and human tissues showing a widespread accumulation and a high concentration in the aquatic environments (Ge et al., 2018; Shao et al., 2018).

1.13 Thesis Objectives

To summarize, the identification of the interactions between MPs and co-contaminants as well as the characterization of mechanisms influencing these interactions represents a key factor for a proper MPs risk assessment. After entering the environment, plastics can go through changes in their physico-chemistry due to several factors (i.e., aging process, residence time and biofilm) which influence sorption capacities of chemical contaminants alter their bioaccessibility. There is a need to investigate the sorption behavior of several contaminants and to assess the multiple interactions of two or more contaminants simultaneously under realistic scenarios. Indeed, although the scientific research regarding the fate and effects of MPs on marine biota is growing, the knowledge gaps related to the complex mixture of contaminants associated on MPs surface and the effects associated to uptake are open. The evaluation of sorbed chemical contaminants bioavailability represents a highly relevant and sensitive tool to investigate the potential role of MPs in the incorporation of different type of environmental contaminants and provides important new information towards understanding sorption phenomena. Finally, it is important to note that a contaminant is not necessarily harmful, since contamination refers simply to the presence of a substance where it should not be, while pollutant is always a harmful substance. In this thesis, the terms are used as synonymous since the organic substances used are pollutants as well as contaminants, while the heavy metals as Cu, although normally present in biotic system being used at high concentrations were considered pollutants.

The present thesis aimed to assess the impacts of weathered microplastics on the bioaccesibility and bioavailability of pollutants to *Paracentrotus lividus* and *Danio rerio* through molecular and ecotoxicological approaches. In particular, the influence of artificially aging and MPs biofilm-associated on sorption of different types of contaminants in Early Life Stages (ELSs) of model invertebrate and vertebrate organisms was evaluated. Specifically, the thesis objectives were:

1. Evaluation of the impacts of biofilm on MP-sorbed co-contaminants bioavailability. Two different model compounds, Cadmium and benzo[a]pyrene, were selected to assess the effect of the presence of biofilm on sorption/desorption of them to High-Density PolyEthylene (HDPE-MPs) in aqueous phase. Differences in sorption of these contaminants to MPs in the presence or absence of an

associated biofilm were assessed using changes in expression of specific biomarker genes (mt2 and *cyp1a*) in early-life stages zebrafish (*Danio rerio*). The experiments on sorption of Cd and B[a]P on MPs (HDPE) through an ecotoxicological approach in addition to a metagenomic characterization of microorganisms constituting biofilm on MPs is presented in Chapter 2.

2. Investigation of the effects of polymer artificially aging on the sorption of different pollutants to Mediterranean sea urchin embryos (*Paracentrotus lividus*). The sorption of two different metal ions (Cd and Cu) and BDE-47 was assessed to analyze the effect of plastic artificial aging (via UV radiation) on their bioavailability to *Paracentrotus lividus* embryos. In addition, two plastic polymer, polyamide 6.6 (PA66) and PS, different in type as well as in size were considered. A chemical approach was used to investigate changes in polymer surface (i.e. particle size, hydrophobicity and polarity) occurred after MPs photoexposure. Changes in expression profile of target genes in embryos *P. lividus* exposed to aged MPs alone or in mixture with different chemical contaminants were analyzed using a molecular approach. Thus, co-contaminants bioavailability was assessed by analysis of mRNA levels of members of *P. lividus* Gene Regulatory Networks (GRN) involved in stress response (*Mt4, hsp60, hsp70*), skeletogenesis (*sm50, p16, msp130*) and endo-mesodermal specification (*foxa, hox11/13b*). The transcriptional profiles emerged sea urchin embryos at 24 hpf (*hours post fertlization*)exposed to artificially aged MPs and MP-sorbed co-contaminants is presented in Chapter 3. Additionally, changes in co- contaminants sorption arise from artificially aging process are also described.

References

Al-Thawadi, S., 2020. Microplastics and Nanoplastics in Aquatic Environments: Challenges and Threats to Aquatic Organisms. Arabian Journal for Science and Engineering, 45, pages 4419–4440. https://doi.org/10.1007/s13369-020-04402-z

Alimi, O. S., Farner Budarz, J., Hernandez, L. M., Tufenkji, N., 2018. Microplastics and nanoplastics in aquatic environments: aggregation, deposition, and enhanced contaminant transport. Environ. Sci. Technol. 52, 1704–1724. https://doi.org/10.1021/acs.est.7b05559

Allen, T. E., Goodman, J. M., Gutsell, S., Russell, P. J., 2016. A history of the molecular initiating event. Chem Res Toxicol. 29:2060–2070. https://doi.org/10.1021/acs.chemrestox.6b00341

Anderson, A.G., Grose, J., Pahl, S., Thompson, R.C., Wyles, K.J., 2016. Microplastics in personal care products: exploring perceptions of environmentalists, beauticians and students. Mar. Pollut. Bull. 113, 454-460. https://doi.org/10.1016/j.marpolbul.2016.10.048

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Andrady, A. L., Pegram, J. E., Song, Y., 1993. Studies on enhanced degradable plastics. II. Weathering of enhanced photodegradable polyethylenes under marine and freshwater floating exposure. J Environ Polym Degrad 1(2):117–126. https://doi.org/10.1007/BF01418205

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., et al. 2010. Adverse out-come pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 2010;29:730–41. https://doi.org/10.1002/etc.34

Adverse Outcome Pathways, Molecular Screening and Toxicogenomics (OECD, 2016).

Arienzo, M., Ferrara, L., Trifuoggi, M., 2021. Research Progress in Transfer, Accumulation and Effects of Microplastics in the Oceans. J. Mar. Sci. Eng., 9, 433. https://doi.org/10.3390/jmse9060642

Arthur, C., Baker, J.E., Bamford, H.A., 2009. In: Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris. Sept 9-11, 2008. NOAA Technical Memorandum NOS-OR&R-30. https://repository.library.noaa.gov/view/noaa/2509

Ashton, K., Holmes, L., Turner, A., 2010. Association of metals with plastic production pellets in the marine environment. Mar. Pollut. Bull., 60, 2050–2055. https://doi.org/10.1016/j.marpolbul.2010.07.014

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to mussels. Environ. Pollut. 198, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021

Bakir, A., Rowland, S.J., Thompson, R.C., 2012. Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar. Pollut. Bull. 64, 2782–2789. https://doi.org/10.1016/j.marpolbul.2012.09.010

Baltas, H., Kiris, E., Sirin, M., 2017. Determination of radioactivity levels and heavy metal concentrations in seawater, sediment and anchovy (Engraulis encrasico- lus) from the Black Sea in Rize, Turkey. Mar. Pollut. Bull. 116, 528e533. https:// doi.org/10.1016/j.marpolbul.2017.01.016

Barnes, D.K.A., Milner, P., 2005. Drifting plastic and its consequences for sessile organism dispersal in the Atlantic Ocean. Mar. Biol. 146, 815-825. https://doi.org/10.1007/s00227-004-1474-8

Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos. Trans. R. Soc. B 364 (1526), 1985-1998. https://doi.org/10.1098/rstb.2008.0205

Barra et al (2018), Plastics and the circular economy, Scientific and Technical Advisory Panel to the Global Environment Facility, Washington, DC. DO: 10.13140/RG.2.2.11515.57128

Batel, A., Borchert, F., Reinwald, H., Erdinger, L., Braunbeck, T., 2018. Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (Danio rerio) gills and zebrafish embryos. Environ. Pollut. 235, 918–930. https://doi.org/10.1016/j.envpol.2018.01.028

Bergami, E., Bocci, E., Vannuccini, M.L., Monopoli, M., Salvati, A., Dawson, K.A., Corsi, I., 2016. Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp Artemia franciscana larvae. Ecotoxicol. Environ. Saf. 123, 18–25. https://doi.org/10.1016/j.ecoenv.2015.09.021

Betts, K., 2008. Why small plastic particles may pose a big problem in the oceans. Environmental Science & Technology, 42 (24). pp 8995-8995. https://doi.org/10.1021/es802970v

Blarer, P., Burkhardt-Holm, P. 2016. Microplastics affect assimilation efficiency in the freshwater amphipod Gammarus fossarum. Environ. Sci. Pollut. Res. 23, 23522–23532. https://doi.org/10.1007/s11356-016-7584-2

Bouwmeester, H., Hollman, P.C.H., Peters, R.J.B., 2015. Potential health impact of environ- mentally released micro- and Nanoplastics in the human food production chain: experiences from Nanotoxicology. Environ. Sci. Technol. 49, 8932–8947. https://doi.org/10.1021/acs.est.5b01090

Bozorg, A., Gates, I. D., Sen, A., 2015. Impact of biofilm on bacterial transport and deposition in porous media. J. Contam. Hydrol., 183, 109−120. https://doi.org/10.1016/j.jconhyd.2015.10.008

Brandts, I., Teles, M., Gonçalves, A.P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M.A., Soares, A.M.V.M., Tort, L., Oliveira M., 2018. Effects of nanoplastics on *Mytilus galloprovincialis* after individual and combined exposure with carbamazepine. Sci. Total Environ. 643, 775–784. https://doi.org/10.1016/j.scitotenv.2018.06.257

Brennecke, D., Duarte, B., Paiva, F., Caçdor, I., Canning-Clode, J., 2016. Microplastics as vector for heavy metal contamination from the marine environment. Estuar. Coast. Shelf Sci. 178, 189–195. https://doi.org/10.1016/j.ecss.2015.12.003

Browne, M.A., Galloway, T., Thompson, R., 2007. Microplastic - an emerging contaminant of potential concern? Integr. Environ. Assess. Manag., 3, 559–561. http://doi.org/10.1002/ieam.5630030412

Browne, M. A., Galloway, T. S. & Thompson, R. C., 2010. Spatial Patterns of Plastic Debris along Estuarine Shorelines. Environmental Science & Technology, 44 (9). pp 3404-3409. https://doi.org/10.1021/es903784e

Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr. Biol. 23, 2388-2392. https://doi.org/10.1016/j.cub.2013.10.012

Browne, M., Thomson, P., Rockloff, M. J. ,Pennycook, G., 2015. Going against the Herd: Psychological and Cultural Factors Underlying the 'Vaccination Confidence Gap'. PLoS ONE 10(9):e0132562. http://doi.org/10.1371/journal.pone.0132562

Brun, N.R., Beenakker, M.M., Hunting, E.R., Ebert, D., Vijver, M.G., 2017. Brood pouch-mediated polystyrene nanoparticle uptake during Daphnia magna embryogenesis. Nanotoxicology 11 (8), 1059–1069. https://doi.org/10.1080/17435390.2017.1391344

Camacho, M., Herrera, A., Gómez, M., Acosta-Dacal, A., Martínez, I., Henríquez-Hernández, L.A., Luzardo, O.P., 2019. Organic pollutants in marine plastic debris from Canary Islands beaches. Sci. Total Environ. 662, 22–31. https://doi.org/10.1016/j. scitotenv.2018.12.422

Cao, Y., Zhao, M., Ma, X., Song, Y., Zuo, S., Li, H., Deng, W., 2021. A critical review on the interactions of microplastics with heavy metals: Mechanism and their combined effect on organisms and humans. Sci Total Environ. Sep 20; 788:147620. https://doi.org/10.1016/j.scitotenv.2021.147620

Capolupo, M., Franzellitti, S., Valbonesi, P., Sanz-Lanzas, C., Fabbri, E., 2018. Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*. Environ. Pollut., Volume 241, Pages 1038-1047, ISSN 0269-7491, https://doi.org/10.1016/j.envpol.2018.06.035

Carpenter, E. J., Smith, K. L. Jr, 1972. Plastics on the Sargasso sea surface. Science 175:1240–1241. DOI: 10.1126/science.175.4027.1240. http://doi.org/10.1126/science.175.4027.1240

Casarett and Doull's. 2001. Toxicology. The Basic Science of Poisons, Sixth Edition. C. D. Klaassen (ed.). New York: McGraw-Hill.

Celina, M. C., 2013. Review of polymer oxidation and its relationship with materials performance and lifetime prediction. Polym. Degrad. Stabil. 98, 2419–2429. https://doi.org/10.1016/j.polymdegradstab.2013.06.024

Chandra R, Rustgi R. 1998. Biodegradable polymers. Prog Polym Sci 23(7):1273–1335. https://doi.org/10.1016/S0079-6700(97)00039-7

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki., M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Science of the Total Environment 584–585, 1022–1031. http://dx.doi.org/10.1016/j.scitotenv.2017.01.156

Chubarenko, I.; Bagaev, A.; Zobkov, M.; Esiukova, E. On some physical and dynamical properties of microplastic particles in marine environment. Mar. Pollut. Bull. 2016, 108, 105−112. https://doi.org/10.1016/j.marpolbul.2016.04.048

Claessens, M., De Meester, S., Van Landuyt, L., De Clerck, K., Janssen, C.R., 2011. Occurrence and distribution of microplastics in marine sediments along the Belgian coast. Mar. Pollut. Bull. 62 (10), 2199–2204. https://doi.org/10.1016/j.marpolbul.2011.06.030

Cole, M., Galloway, T.S., 2015. Ingestion of nanoplastics and microplastics by pacific oyster larvae. Environ. Sci. Technol. 49 (24), 14625–14632. https://doi.org/10.1021/acs.est.5b04099

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

Costa, P.M., et al., 2010. Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]Pyrene. Environ. Pollut. 158 (10), 3338-3346. https://doi.org/10.1016/j.envpol.2010.07.030

Cózar, A., Sanz-Martín, M., Martí, E., González-Gordillo, J.I., Ubeda, B., Gálvez, J.Á., et al., 2015. Plastic Accumulation in the Mediterranean Sea. PLoS ONE 10(4): e0121762. http://doi.org/10.1371/journal.pone.0121762

De Tender, C. A., Devriese, L. I., Haegeman, A., Maes, S., Ruttink, T., Dawyndt, P., 2015. Bacterial community profiling of plastic litter in the Belgian part of the North Sea. Environ. Sci. Technol. 49, 9629–9638 (2015). http://doi.org/10.1021/acs.est.5b01093

Delvalle de Borrero, D., Fábrega Duque, J., Olmos, J., Garcés-Ordóñez, O., Amaral, S. S. G. d., Vezzone, M., de Sá Felizardo, J.P., Meigikos dos Anjos, R., 2020. Distribution of plastic debris in the Pacific and Caribbean beaches of Panama. Air Soil. Water Res., 13. https://doi.org/10.1177/1178622120920268

Derraik, J.G.B., 2002. The pollution of the marine environment by plastic debris: a review. Mar. Pollut. Bull. 44 (9), 842-852. https://doi.org/10.1016/S0025-326X(02)00220-5

Desforges, J.P.W., Galbraith, M., Dangerfield, N., Ross, P.S., 2014. Widespread distribution of microplastics in subsurface seawater in the NE Pacific Ocean. Mar. Pollut. Bull. 79 (1), 94-99. https://doi.org/10.1016/j.marpolbul.2013.12.035

Doughty, R. & Eriksen, M., 2014. Plastic Pollution: The Case for a Ban on Microplastics in Personal Care Products. Tul. Envtl. LJ, 27. pp 277-393. https://journals.tulane.edu

Duan Z., Duan X., Zhao S., Wang X., Wang J., Liu Y., Peng Y., Gong Z., Wang L., 2020. Barrier function of zebrafish embryonic chorions against microplastics and nanoplastics and its impact on embryo development. J. Hazard. Mater., 395:122621. http://doi.org/10.1016/j.jhazmat.2020.122621

Eich, A., Mildenberger, T., Laforsch, C., Weber, M., 2015. Biofilm and diatom succession on polyethylene (PE) and biodegradable plastic bags in two marine habitats: early signs of degradation in the pelagic and benthic zone? PLoS ONE. https://doi.org/10.1371/journal.pone.0137201

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R., Date, T., 2005. Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. Mar.Pollut. Bull. 50, 1103–1114. https://doi.org/10.1016/j.marpolbul.2005.04.030

Engler, R.E., 2012. The complex interaction between marine debris and toxic chemicals in the ocean. Environ. Sci. Technol. 46 (22), 12302-12315. https://doi.org/10.1021/es3027105

Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 tril- lion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9, 1–15. https://doi.org/10.1371/journal.pone.0111913

Eubeler, J. P., Zok, S., Bernhard, M., Knepper, T. P., 2009. Environmental biodegradation of synthetic polymers I. Test methodologies and procedures. TrAC Trends in Analytical Chemistry 28(9): 1057-1072. http://doi.org/10.1016/j.trac.2009.06.007

Everaert, G., Vlaeminck, K., Vandegehuchte, M.B. and Janssen, C.R., 2022. Effects of Microplastic on the Population Dynamics of a Marine Copepod: Insights From a Laboratory Experiment and a Mechanistic Model. Environ Toxicol Chem. Accepted Author Manuscript. https://doi.org/10.1002/etc.5336

Fendall, L. S., Sewell, M. A., 2009. Contributing to marine pollution by washing your face: Microplastics in facial cleansers. Marine Pollution Bulletin, 58 (8). pp 1225-1228. https://doi.org/10.1016/j.marpolbul.2009.04.025

Fred-Ahmadu, O.H., Bhagwat, G., Oluyoye, I., Benson, N.U., Ayejuyo, O.O., Palanisami, T., 2020. Interaction of chemical contaminants with microplastics: principles and per- spectives. Sci. Total Environ. 706, 135978. https://doi.org/10.1016/j. scitotenv.2019.135978

Frias, J.P.G.L., Sobral, P., Ferreira, A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. Mar. Pollut. Bull. 60, 1988–1992. https://doi.org/10.1016/j.marpolbul.2010.07.030

Fries, E., Dekiff, J. H., Willmeyer, J., Nuelle, M.-T., Ebert, M. & Remy, D., 2013. Identification of polymers types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environmental Science: Processes & Impacts, 15 pp 1949-1956. https://doi.org/10.1039/C3EM00214D

Fry, D. M., Fefer, S. I., Sileo, L., 1987. Ingestion of plastic debris by Laysan Albatrosses and Wedge-tailed Shearwaters in the Hawaiian Islands'. Marine Pollution Bulletin, 18 (6, Supplement B). pp 339-343. https://doi.org/10.1016/S0025-326X(87)80022-X

Galloway, T.S., Lewis, C.N., 2016. Marine microplastics spell big problems for future generations. P. Natl. Acad. Sci. USA 113, 2331-2333. https://doi.org/10.1073/pnas.1600715113

Galloway, T. S., 2015 in Marine Anthropogenic Litter (eds Bergmann, M., Gutow, L. & Klages, M.) 343–366 (Springer) http://doi.org/10.1007/978-3-319-16510-3

Galloway, T., Cole, M., Lewis, C., 2017. Interactions of microplastic debris throughout the marine ecosystem. Nat Ecol Evol 1, 0116. https://doi.org/10.1038/s41559-017-0116

Gambardella, C., Morgana, S., Ferrando, S., Bramini, M., Piazza, V., Costa, E., Garaventa, F., Faimali, M., 2017. Effects of polystyrene microbeads in marine planktonic crustaceans. Ecotox. Environ. Saf. 145: 250-257. http://doi.org/10.1016/j.ecoenv.2017.07.036

Gao, F., Li, J., Sun, C., Zhang, L., Jiang, F., Cao, W., Zheng, L., 2019. Study on the capability and characteristics of heavy metals enriched on microplastics in marine environment. Mar. Pollut. Bull. 144, 61–67. https://doi.org/10.1016/j.marpolbul.2019.04.039

Ge, W., Mou, Y., Chai, C., Zhang, Y., Wang, J., Ju, T., Jiang, T., Xia, B., 2018. Polybrominated diphenyl ethers in the dissolved and suspended phases of seawater from Sanggou Bay, east China. Chemosphere 203:253-262. https://doi.org/10.1016/j.chemosphere.2018.03.184

GESAMP, 2019. Guidelines or the monitoring and assessment of plastic litter and microplastics in the ocean. In: Kershaw, P.J., Turra, A., Galgani, F. (Eds.), Rep. Stud. GESAMP 99, p. 130.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3 (7). http://doi.org/10.1126/sciadv.1700782

Graham, E. R., Thompson, J. T., 2009. Deposit- and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. Journal of Experimental Marine Biology and Ecology, 368 (1). pp 22-29. https://doi.org/10.1016/j.jembe.2008.09.007

Gregory, M.R, Andrady, A.L., 2003. Plastics in the marine environment. In: Andrady AL (ed) Plastics and the environment. Wiley, Hoboken, pp 379–401. ISBN 0-471-09520-6

Guan, J., Qi, K., Wang, J., Wang, W., Wang, Z., Lu, N., Qu, J., 2020. Microplastics as an emerging anthropogenic vector of trace metals in freshwater: significance of biofilms and comparison with natural substrates. Water Res., 184, 116205. https://doi.org/10.1016/j.watres.2020.116205

Guart, A., Bono-Blay, F., Borrell, A., Lacorte, S., 2011. Migration of plasticizers phthalates, bisphenol A and alkylphenols from plastic containers and evaluation of risk. Food Addit Contam Part A Chem Anal Control Expo Risk Assess., 28(5):676-85. https://doi.org/10.1080/19440049.2011.555845

Gugumus, F., 1993. Re-evaluation of the stabilization mechanisms of various light stabilizer classes. Polym. Degrad. Stabil. 39 (1), 117e135. https://doi.org/10.1016/0141-3910(93)90131-2

Guo, X.T., Pang, J.W., Chen, S.Y., Jia, H.Z., 2018. Sorption properties of tylosin on four different microplastics. Chemosphere 209, 240–245. https://doi.org/10.1016/j.chemosphere.2018.06.100

Hall-Stoodley, L., Costerton, J. W., Stoodley, P., 2004. Bacterial biofilms: from the Natural environment to infectious diseases. Nat. Rev. Microbiol., 2, 95−108. https://doi.org/10.1038/nrmicro821

Hartmann, N.B., Jensen, K.A., Baun, A., Rasmussen, K., Rauscher, H., Tantra, R., Cupi, D., Gilliland, D., Pianella, F., Riego Sintes, J.M., 2015. Techniques and protocols for dispersing nanoparticle powders in aqueous media—is there a rationale for harmonization? J Toxicol Environ Heal Part B 18:299–326. https://doi.org/10.1080/10937404.2015.1074969

Hartmann, N.B., Rist, S., Bodin, J., Jensen, L.H., Schmidt, S.N., Mayer, P., Meibom, A., Baun, A., 2017. Microplastics as vectors for environmental contaminants: Exploring sorption, desorption, and transfer to biota. Integr. Environ. Assess. Manag., 13, 488–493. https://doi.org/10.1002/ieam.1904

He, L., Rong, H., Wu, D., Li, M., Wang, C., Tong, M., 2020. Influence of biofilm on the transport and deposition behaviors of nano- and micro-plastic particles in quartz sand. Water Research, Volume 178, https://doi.org/10.1016/j.watres.2020.115808

Henry, T.B., Wileman, S.J., Boran, H., Sutton, P., 2013. Association of Hg^{2+} with aqueous $(C_{60})n$ aggregates facilitates increased bioavailability of Hg^{2+} in zebrafish (Danio rerio). Environ. Sci. Technol. 47, 9997-10004. https://doi.org/10.1021/es4015597

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microsplastics in the marine environment: a review of the methods used for identification and quantification. Environ. Sci. Technol. 46 (6), 3060–3075. https://doi.org/10.1021/es2031505

Holmes, L.A., Turner, A., Thompson, R.C., 2012. Adsorption of trace metals to plastic resin pellets in the marine environment. Environ. Pollut. 160:42–48. http://dx.doi.org/10. 1016/j.envpol.2011.08.052

Holmes, L.A., Turner, A., Thompson, R.C., 2014. Interactions between trace metals and plastic production pellets under estuarine conditions. Mar. Chem. 167, 25e32. https://doi.org/10.1016/j.marchem.2014.06.001

Hüffer, T.; Hofmann, T., 2016. Sorption of non-polar organic compounds by microsized plastic particles in aqueous solution. Environ. Pollut., 214, 194–201. http://doi.org/10.1016/j.envpol.2016.04.018

Hüffer, T., Weniger, A.-K., Hofmann, T., 2018. Sorption of organic compounds by aged polystyrene microplastic particles. Environ. Pollut. 236, 218-225. https://doi.org/10.1016/j.envpol.2018.01.022

Hurley, R., Woodward, J., Rothwell, J.J., 2018. Microplastic contamination of river beds significantly reduced by catchment-wide flooding. Nat. Geosci. 11 (4), 251-257. https://doi.org/10.1038/s41561-018-0080-1

Huvet, A., Paul-Pont, I., Fabioux, C., Lambert, C., Suquet, M., Thomas, Y. et al., 2016. Reply to Lenz et al.: Quantifying the smallest microplastics is the challenge for a comprehensive view of their environmental impacts. Proc. Natl. Acad. Sci. U. S. A., 113, Article E4123 https://doi.org/10.1073/pnas.1607221113

Iannilli, V., Pasquali, V., Setini, A., Corami, F., 2019. First evidence of microplastics in- gestion in benthic amphipods from Svalbard. Environ. Res. 179, 108811. https://doi.org/10.1016/j.envres.2019.108811

Jahnke, A., Arp, H.P.H., Escher, B.I., Gewert, B., Gorokhova, E., Kühnel, D., Ogonowski, M., Potthoff, A., Rummel, C., Schmitt-Jansen, M., Toorman, E., MacLeod, M., 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ. Sci. Technol. Lett. 4, 85e90. https://doi.org/10.1021/acs.estlett.7b00008

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science (80-.) 347, 768–771. https://doi.org/10.1126/science.1260352.

Jin, M., Yu, X., Yao, Z., Tao, P., Li, G., Yu, X., Zhao, J., Peng, J., 2020. How biofilms affect the uptake and fate of hydrophobic organic compounds (HOCs) in microplastic: Insights from an In situ study of Xiangshan Bay, China. Water Res., 184, 116118. https://doi.org/10.1016/j.watres.2020.116118

Kaposi, K., Mos, B., Kelaher, B.P., Dworjanyn, S.A., 2014. Ingestion of microplastic has limited impact on a marine larva. Environ. Sci. Technol. 48, 1638–1645. https://doi.org/10.1021/es404295e

Karami, A., Groman, D.B., Wilson, S.P., Ismail, P., Neela, V.K., 2017. Biomarker Responses in Zebrafish (Danio Rerio) Larvae Exposed to Pristine Low-Density Polyethylene Fragments. Environmental Pollution, 223:466-475. http://doi.org/10.1016/j.envpol.2017.01.047

Khalid, N., Hussain, M., Young, H.S., Boyce, B., Aqeel, M., Noman, A., 2018. Effects of road proximity on heavy metal concentrations in soils and common roadside plants in Southern California. Environ. Sci. Pollut. Res. 25, 35257–35265. https://doi.org/10.1007/s11356-018-3218-1

Khan F.R., Patsiou D., Catarino A.I., 2021. Pollutants Bioavailability and Toxicological Risk from Microplastics. In: Rocha-Santos T., Costa M., Mouneyrac C. (eds) Handbook of Microplastics in the Environment. Springer, Cham. https://doi.org/10.1007/978-3-030-10618-8_19-1

Kirstein, I.V., Kirmizi, S., Wichels, A. et al., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar Environ Res 120:1–8. http://doi.org/10.1016/j.marenvres.2016.07.004

Klaine, S. J., Koelmans, A. A., Horne, N., Handy, R. D., Kapustka, L., Nowack, B., et al., 2012. Paradigms to assess the environmental impact of manufactured nanomaterials. Environmental Toxicology and Chemistry, 31, 3–14. http://doi.org/10.1002/etc.733

Koelmans, A. A., Besseling, E., Foekema, E. M., 2014. Leaching of plastic additives to marine organisms. Environmental Pollution, 187, 49–54. http://doi.org/10.1016/j.envpol.2013.12.013

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

Lambert, S., Wagner, M., 2016. Characterisation of nanoplastics during the degradation of polystyrene. Chemosphere 145, 265–268. https://doi.org/10.1016/j.chemosphere.2015.11.078

Lambert, S., Sinclair, C.J., Bradley, E.L., Boxall, A.B.A., 2013. Environmental fate of processed natural rubber latex. Environ Sci Process Imp 15(7):1359–1368. https://doi.org/10.1039/C3EM00192J

Lambert, S., Sinclair, C.J., Boxall, A.B.A., 2014. Occurrence, degradation and effects of polymer-based materials in the environment. Rev Environ Contam Toxicol 227:1–53. https://doi.org/10.1007/978-3-319-01327-5_1

Lambert, S., Scherer, C., Wagner, M., 2017. Ecotoxicity testing of microplastics: Considering the heterogeneity of physicochemical properties. Integr Environ Assess Manag. May;13(3):470-475. https://doi.org/10.1002/ieam.1901

Lassen, C., Hansen, S.F., Magnusson, K., Norén, F., Hartmann, N.B., Jensen, P.R., Nielsen, T.G., Brinch, A., 2015. Microplastics: occurrence, effects and sources of releases to the environment in Denmark, Danish Environmental Protection Agency. http://mst.dk/service/publikationer/publikationsarkiv/2015/nov/rapport-om-mikroplast

Law, K.L., Morét-Ferguson, S., Maximenko, N.A., Proskurowski, G., Peacock, E.E., Hafner, J., Reddy, C.M., 2010. Plastic accumulation in the North Atlantic subtropical gyre. Science 329(5996): 1185–1188. http://doi.org/10.1126/science.1192321

Lee, H., Shim, W. J., Kwon, J. H., 2014. Sorption capacity of plastic debris for hydrophobic organic chemicals. Sci. Total Environ., 470, 1545–1552. https://doi.org/10.1016/j.scitotenv.2013.08.023

Leslie, H. A., Van Velzen, M. J. M., & Vethaak, A. D., 2013. Microplastic survey of the Dutch environment—Novel data set of microplastics in North Sea sediments, treated wastewater effluents and marine biota. IVM Institute for Environmental Studies Final Report R-13/11, Free University, Amsterdam. https://science.vu.nl/en/Images/IVM_report_Microplastic_in_sediment_STP_Biota_2013_tcm296-409860.pdf

Li, X.Y., Yuan, Y., 2002. Collision frequencies of microbial aggregates with small particles by differential sedimentation. Environ Sci Technol., Feb 1;36(3):387-93. http://doi.org/10.1021/es010681d

Li, J., Zhang, K., Zhang, H., 2018. Adsorption of antibiotics on microplastics. Environ. Pollut. 237, 460–467. https://doi.org/10.1016/j.envpol.2018.02.050

Li, X., Mei, Q., Chen, L., Zhang, H., Dong, B., Dai, X., He, C., Zhou, J., 2019. Enhancement in adsorption potential of microplastics in sewage sludge for metal pollutants after the wastewater treatment process. Water Res. 157, 228–237. https://doi.org/10.1016/j.watres.2019.03.069

Liao, Y.L., Yang, J.Y., 2019. Microplastic serves as a potential vector for Cr in an in-vitro human digestive model. Sci. Total Environ. 703, 134805. https://doi.org/10.1016/j.scitotenv.2019.134805

Lindeque, P.K., Cole, M., Coppock, R.L., Lewis, C.N., Miller, R.Z., Watts, A.J.R., Wilson-McNeal, A., Wright, S.L., Galloway, T.S., 2020. Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. Environ. Pollut., 265 (114721). http://doi.org/10.1016/j.envpol.2020.114721

Lithner, D., Larsson, A., & Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Science of the Total Environment, 409, 3309–3324. https://doi.org/10.1016/j.scitotenv.2011.04.038

Liu, X., Zheng, M., Wang, L., Ke, R., Lou, Y., Zhang, X., Dong, X., Zhang, Y., 2018. Sorption behaviors of tris-(2,3-dibromopropyl) isocyanurate and hexabromocyclododecanes on polypropylene microplastics. Mar. Pollut. Bull. 135, 581–586. https://doi.org/10.1016/j.marpolbul.2018.07.061

Liu, X., Xu, J., Zhao, Y., Shi, H., Huang, C.H., 2019. Hydrophobic sorption behaviors of 17Β- estradiol on environmental microplastics. Chemosphere 226, 726–735. https://doi.org/10.1016/j.chemosphere.2019.03.162

Llorca, M., Schirinzi, G., Martínez, M., Barceló, D., Farré, M., 2018. Adsorption of perfluoroalkyl substances on microplastics under environmental conditions. Environ. Pollut. 235, 680-691. https://doi.org/10.1016/j.envpol.2017.12.075

Lo, H.S., Wong, C.Y., Tam, N.F.Y., Cheung, S.G., 2019. Spatial distribution and source iden- tification of hydrophobic organic compounds (HOCs) on sedimentary microplastic in Hong Kong. Chemosphere 219, 418–426. https://doi.org/10.1016/j. chemosphere.2018.12.032

Magadini, D. L., Goes, J. I., Ortiz, S., Lipscomb, J., Pitiranggon, M., Yan, B., 2020. Assessing the sorption of pharmaceuticals to microplastics through in situ experiments in New York City waterways. Sci. Total Environ, 729, 138766. https://doi.org/10.1016/j.scitotenv.2020.138766

Mahfooz, Y., Yasar, A., Guijian, L., Islam, Q.U., Akhtar, A.B.T., Rasheed, R., Irshad, S., Naeem, U., 2020. Critical risk analysis of metals toxicity in wastewater irrigated soil and crops: a study of a semi-arid developing region. Sci. Rep. 10, 12845. https://doi.org/10.1038/s41598-020-69815-0

Manfra, L., Rotini, A., Bergami, E., Grassi, G., Faleri, C., Corsi, I., 2017. Comparative ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer Brachionus plicatilis, Ecotoxicology and Environmental Safety,Volume 145, ISSN 0147-6513, https://doi.org/10.1016/j.ecoenv.2017.07.068

Manzo, S., Buono, S., Cremisini, C., 2010. Cadmium, lead and their mixtures with copper: Paracentrotus lividus embryotoxicity assessment, prediction, and offspring quality evaluation. Ecotoxicology 19, 1209-1223. https://doi.org/10.1007/s10646-010-0506-z

Mao, R., Lang, M., Yu, X., Wu, R., Yang, X., Guo, X., 2020. Aging mechanism of microplastics with UV irradiation and its effects on the adsorption of heavy metals. Journal of Hazardous Materials,Volume 393, 122515, ISSN 0304-3894, https://doi.org/10.1016/j.jhazmat.2020.122515

Marn, N., Jusup, M., Kooijman, SALM, Klanjscek, T., 2020. Quantifying impacts of plastic debris on marine wildlife identifies ecological breakpoints. Ecol Lett 23:1479–1487. https://doi.org/10.1111/ele.13574

Martínez-Soto, M.C., Tovar-Sánchez, A., Sánchez-Quiles, D., Rodellas, V., Garcia- Orellana, J., Basterretxea, G., 2016. Seasonal variation and sources of dissolved trace metals in Maó Harbour, Minorca Island. Sci. Total Environ. 565, 191e199. https://doi.org/10.1016/j.scitotenv.2016.03.244

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., Kaminuma, T., 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. Environ. Sci. Technol. 2001, 35, 318–324. https://doi.org/10.1021/es0010498

Mattsson, K., Ekvall, M.T., Hansson, L.A., Linse, S., Malmendal, A., Cedervall, T., 2015. Altered behavior, physiology, and metabolism in fish exposed to polystyrene nanoparticles. Environ. Sci. Technol. 49, 553-561. https://doi.org/10.1021/es5053655

Meeker, J. D., Sathyanarayana, S., Swan, S. H., 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 364(1526), 2097–2113. https://doi.org/10.1098/rstb.2008.0268

Mei, W., Chen, G., Bao, J., Song, M., Li, Y., Luo, C., 2020. Interactions between microplastics and organic compounds in aquatic environments: a mini review. Sci. Total Environ. 736, 139472. https://doi.org/10.1016/j.scitotenv.2020.139472

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., Pennati, R., 2017. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environ. Pollut. 237, 1080-1087. https://doi.org/10.1016/j.envpol.2017.11.030

Monopoli, M. P., Åberg, C., Salvati, A., and Dawson, K. A., 2012. Biomolecular coronas provide the biological identity of nanosized materials. Nat. Nanotech. 7, 779–786. http://doi.org/10.1038/nnano.2012.207

Moore, C.J., 2008. Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. Plast. World 108, 131e139. http://dx.doi.org/10.1016/j.envres.2008.07.025

Moore, C., Moore, S., Leecaster, M., Weisberg, S., 2001. A comparison of plastic and plankton in the north pacific central gyre. Mar. Pollut. Bull. 42, 1297e1300. http://dx.doi.org/10.1016/S0025-326X(01)00114-X

Müller, A., Becker, R., Dorgerloh, U., Simon, F.G., Braun, U., 2018. The effect of polymer aging on the uptake of fuel aromatics and ethers by microplastics. Environ. Pollut. 240, 639–646. https://doi.org/10.1016/j.envpol.2018.04.127

Murphy, F., and Quinn, B., 2018. The effects of microplastic on freshwater Hydra attenuata feeding, morphology & reproduction. Environ. Pollut. 234, 487–494. http://doi.org/10.1016/j.envpol.2017.11.029

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereora, C.D.S., Turra, A., 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin Lytechinus variegatus (Echinodermata: Echinoidea). Mar. Pollut. Bull. 92, 99–104. https://doi.org/10.1016/j.marpolbul.2014.12.050

O'Brine, T., Thompson, R.C., 2010. Degradation of plastic carrier bags in the marine environment. Mar. Pollut. Bull. 60 (12), 2279–2283. https://doi.org/10.1016/j.marpolbul.2010.08.005

O’Connor, I.A., Golsteijn, L., Hendriks, A.J., 2016. Review of the partitioning of chemicals into different plastics: consequences for the risk assessment of marine plastic debris. Mar Pollut Bull. http://doi.org/10.1016/j.marpolbul.2016.07.021

Oberbeckmann, S., Löder, M. G. J., and Labrenz, M., 2015. Marine microplastic- associated biofilms – a review. Environ. Chem. 12, 551–562. http://doi.org/10.1071/en15069

Patel, M.M., Goyal, B.R., Bhadada, S.V. et al., 2009. Getting into the Brain. CNS Drugs 23, 35–58. https://doi.org/10.2165/0023210-200923010-00003

Paul-Pont, I., Lacroix, C., Fernández, G.C., Hégaret, H., Lambert, C., Le Goïc, N.L., Frére, L., Cassone, A.L., Sussarellu, R., Fabioux, C., et al., 2016. Exposure of marine mussels Mytilus spp. To polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. Environ. Pollut., 216, 724–737. https://doi.org/10.1016/j.envpol.2016.06.039

Paustenbach, D. J., G. M. Bruce, and Chrostowski, P., 1997. Current views on the oral bioavailability of inorganic mercury in soil: implications for health risk assessments. Risk Analysis 17(5):533-544. http://doi.org/10.1111/j.1539-6924.1997.tb00895.x

Pitt, J.A., Kozal, J.S., Jayasundara, N., Massarsky, A., Trevisan, R., Geitner, N., Wiesner, M., Levin, E.D., Di Giulio, R.T., 2018. Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (Danio rerio). Aquat. Toxicol. 194 https://doi.org/10.1016/j.aquatox.2017.11.017

Pittura, L., Avio, C.G., Giuliani, M.E., d'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the mediterranean mussels, Mytilus galloprovincialis. Front. Mar. Sci. 5, 103. https://doi.org/10.3389/fmars.2018.00103

Prichard, P., Granek, E.F., 2016. Effects of pharmaceuticals and personal care products on marine organisms: From single-species studies to an ecosystem-based approach. Environ. Sci. Pollut. Res., 23, 22365–22384. https://doi.org/10.1007/s11356-016-7282-0

Quero, G. M., Luna, G. M., 2017. Surfing and dining on the “plastisphere”: microbial life on plastic marine debris. Adv Oceanogr Limnol 8(2):199–207. https://doi.org/10.4081/aiol.2017.7211

Ragusa, M.A., Costa, S., Cuttitta, A., Gianguzza, F., Nicosia, A., 2017. Coexposure to sulfamethoxazole and cadmium impairs development and attenuates transcriptional response in sea urchin embryo. Chemosphere, 180, 275–284, https://doi.org/10.1016/j.chemosphere.2017.04.030

Reisser, J., Shaw, J., Hallegraeff, G., Proietti, M., Barnes, D. K. A., Thums, M., et al., 2014. Millimeter-Sized Marine Plastics: A New Pelagic Habitat for Microorganisms and Invertebrates. PLoS ONE 9(6): e100289. https://doi.org/10.1371/journal.pone.0100289

Retama, I., Jonathan, M.P., Shruti, V.C., Velumani, S., Sarkar, S.K., Roy, P.D., Rodríguez-Espinosa, P.F., 2016. Microplastics in tourist beaches of Huatulco Bay, Pacific coast of southern Mexico. Marine Pollution Bulletin 113: 530–535. https://doi.org/10.1016/j.marpolbul.2016.08.053

Rios, L.M., Moore, C., Jones, P.R., 2007. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Mar. Pollut. Bull 54 (8), 1230-1237. https://doi.org/10.1016/j.marpolbul.2007.03.022

Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013a. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: Implications for plastic marine debris. Environ. Sci. Technol., 47, 1646–1654. https://doi.org/10.1021/es303700s

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

Rochman, C.M., Manzano, C., Hentschel, B.T., Simonich, S.L., Hoh, E., 2013c. Polystyrene plastic: A source and sink for polycyclic aromatic hydrocarbons in the marine environment. Environ. Sci. Technol., 47, 13976–13984. https://doi.org/10.1021/es403605f

Rochman, C.M., Hentschel, B.T., Teh, S.J., 2014a. Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. PLoS One 9, e85433. https://doi.org/10.1371/journal.pone.0085433

Rochman, C.M., Kurobe, T., Flores, I., Teh, S.J., 2014b. Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci. Total Environ. 493, 656–661. https://doi.org/10.1016/j.scitotenv.2014.06.051

Rochman, C.M., 2015. The complex mixture, fate and toxicity of chemicals associated with plastic debris in the marine environment. In: Bergmann M, Gutow L, Klages M (eds) Marine anthropogenic litter. Springer, Cham, pp 117–140. doi:10.1007/978-3-319-16510-3_5

Rodrigues, J.P., Duarte, A.C., Echeandía, J.S., 2019. Significance of interactions between microplastics and POPs in the marine environment: A critical overview. Trends Analyt. Chem., 111, 252–260. https://doi.org/10.1016/j.trac.2018.11.038

Ross, P.S., Morales-Caselles, C., 2015. Out of sight, but no longer out of mind: microplastics as a global pollutant. Integr. Environ. Assess. Manag. 11 (4), 719-728. https://doi.org/10.1002/ieam.1681

Rouch, D. A., 2021. Plastic future: How to reduce the increasing environmental footprint of plastic packaging. Working Paper No. 11, Clarendon Policy & Strategy Group, Melbourne, Australia.

Rummel, C.D., Jahnke, A., Gorokhova, E., Kühnel, D., Schmitt-Jansen, M., 2017. Im- pacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. Environ. Sci. Technol. Lett. 4 (7), 258-267. https://doi.org/10.1021/acs.estlett.7b00164

Ryan, P. G. and Moloney, C. L., 1990. Plastic and other artefacts on South_african beaches – temporal trends in abundance and composition. South African Journal of Science 86(7-10): 450-452.

Ryan, P.G., Connell, A.D., Gardener, B.D., 1988. Plastic ingestion and PCBs in seabirds: is there a relationship? Mar. Pollut. Bull. 19 (4), 174-176. https://doi.org/10.1016/0025-326X(88)90674-1

Ryan, P.G., Moore, C.J., van Franeker, J.A., Moloney, C.L., 2009. Monitoring the abundance of plastic debris in the marine environment. Philos. Trans. R. Soc. B Biol. Sci. 364 (1526), 1999e2012. https://doi.org/10.1098/rstb.2008.0207

Shao, Y., Han, S., Ma, L., Luo, M., Yang, G., Liu, W., Xu, D., 2018. Polybrominated diphenyl ethers in surface waters around Beijing: Occurrence, distribution and sources, Applied Geochemistry, Volume 98, Pages 58-64, ISSN 0883-2927, https://doi.org/10.1016/j.apgeochem.2018.09.011

Shaw, D.G., Day, R.H., 1994. Colour- and form-dependent loss of plastic microdebris from the North Pacific Ocean. Mar. Pollut. Bull. 28 (1), 39-43. https://doi.org/10.1016/0025-326X(94)90184-8

Singh, B., Sharma, N., 2008. Mechanistic implications of plastic degradation. Polym. Degrad. Stabil. 93 (3), 561-584. https://doi.org/10.1016/j.polymdegradstab.2007.11.008

Sleight, V.A., Bakir, A., Thompson, R.C., Henry, T.B., 2017. Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar. Pollut. Bull. 116, 291e297. https://doi.org/10.1016/j.marpolbul.2016.12.055

Solomon, O.O., and Palanisami, T., 2016. Microplastics in the Marine Environment: Current Status, Assessment Methodologies, Impacts and Solutions. J. Pollut. Eff. Cont. 1-13. DOI: 10.4172/2375-4397.1000161

Song, Y.K., Hong, S.H., Jang, M., Han, G.M., Jung, S.W., Shim, W.J., 2017. Combined effects of UV exposure duration and mechanical abrasion on microplastic fragmentation by polymer type. Environ. Sci. Technol. 51, 4368–4376. https://doi.org/10.1021/acs.est.6b06155

Sørensen, L., Rogers, E., Altin, D., Salaberria, I., . 2020 Booth, A.M. Sorption of PAHs to microplastic and their bioavailability and toxicity to marine copepods under co-exposure conditions. Environ. Pollut, 258, 113844. https://doi.org/10.1016/j.envpol.2019.113844

Steer, M., Cole, M., Thompson, R. C., and Lindeque, P. K. (2017). Microplastic ingestion in fish larvae in the western english channel. Environ. Pollut. 226, 250–259. https://doi.org/10.1016/j.envpol.2017.03.062

Suaria, G., Avio, C.G., Mineo, A., Lattin, G.L., Magaldi, M.G., Belmonte, G., Moore, C.J., Regoli, F., Aliani, S., 2016. The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. Sci. Rep. 6, 37551. https://doi.org/10.1038/srep37551

Tanaka, K., Takada, H., Yamashita, R., Mizukawa, K., Fukuwaka, M.-a. & Watanuki, Y., 2013. Accumulation of plastic- derived chemicals in tissues of seabirds ingesting marine plastics. Marine Pollution Bulletin, 69 (1–2). pp 219-222. https://doi.org/10.1016/j.marpolbul.2012.12.010

Tang, J., Ni, X., Zhou, Z., Wang, L., Lin, S., 2018. Acute microplastic exposure raises stress response and suppresses detoxification and immune capacities in the scleractinian coral Pocillopora damicornis. Environ. Pollut., 243, pp. 66-74, https://doi.org/10.1016/j.envpol.2018.08.045

Tenzer, S., Docter, D., Kuharev, J. et al., 2013. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nature Nanotech 8, 772–781. https://doi.org/10.1038/nnano.2013.181

Teuten, E. L., Rowland, S. J., Galloway, T. S., Thompson, R. C., 2007. Potential for plastics to transport hydrophobic contaminants. Environ. Sci. Technol, 41, 7759–7764. https://doi.org/10.1021/es071737s

Teuten, E.L., Saquing, J.M., Knappe, D.R., Barlaz, M.A., Jonsson, S., Bjorn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., et al., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philos. Trans. R. Soc., 364, 2027–2045. https://doi.org/10.1098/rstb.2008.0284

Thevenon, F., Carroll, C., Sousa, J., 2014. Plastic Debris in the Ocean: Characterization of Marine Plastics and their Environmental Impacts, Situation Analysis Report. Gland, Switzerland, IUCN 52. https://doi.org/10.2305/IUCN.CH.2014.03.en

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., McGonigle, D., Russell, A.E., 2004. Lost at sea: where is all the plastic? Science, 304, 838-838. DOI: 10.1126/science.1094559

Thompson, R., C. Moore, A. Andrady, M. Gregory, H. Takada and S. Weisberg, 2005. New directions in plastic debris. Science 310(5751): 1117-1117. DOI: 10.1126/science.310.5751.1117b

Thompson, R.C., Swan, S.H., Moore, C.J., vom Saal, F.S., 2009. Our plastic age. Phil. Trans. R. Soc. B Biologic. Sci. 364, 2153-2166. https://doi.org/10.1098/rstb.2009.0053

Tien, C.J., Chen, C.S., 2013. Patterns of metal accumulation by natural river biofilms during their growth and seasonal succession. Arch. Environ. Con. Tox. 64 (4), 605-616. https://doi.org/10.1007/s00244-012-9856-2

Torres, F. G., Dioses-Salinas, D. C., Pizarro-Ortega, C. I., De-la-Torre, G. E., 2021. Sorption of chemical contaminants on degradable and non-degradable microplastics: Recent progress and research trends. Science of The Total Environment, Volume 757, 143875, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2020.143875

Tourinho, P.S., Ivar do Sul, J.A., Fillmann, G., 2010. Is marine debris ingestion still a problem for the coastal marine biota of southern Brazil? Mar. Pollut. Bull. 60 (3), 396-401. https://doi.org/10.1016/j.marpolbul.2009.10.013

Tourinho, P.S., Kočí, V., Loureiro, S., van Gestel, C.A.M., 2019. Partitioning of chemical contaminants to microplastics: sorption mechanisms, environmental distribution and effects on toxicity and bioaccumulation. Environ. Pollut. 252, 1246–1256. https://doi.org/10.1016/j.envpol.2019.06.030

Tu, C., Chen, T., Zhou, Q., Liu, Y., Wei, J., Waniek, J. J., et al., 2020. Biofilm formation and its influences on the properties of microplastics as affected by exposure time and depth in the seawater. Sci. Total Environ. 734:139237. https://doi.org/10.1016/j.scitotenv.2020.139237

Turner, A., Holmes, L.A., 2015. Adsorption of trace metals by microplastic pellets in fresh water. Environ. Chem. 12 (5), 600-610. https://doi.org/10.1071/EN14143

Turner, A., 2003. Salting out of chemicals in estuaries: implications for contaminant partitioning and modelling. Sci. Total Environ. 314–316, 599–612. https://doi.org/10.1016/S0048-9697(03)00076-7

Umeh, A.C., Naidu, R., Owojori, O.J., Semple, K.T., 2020. Bioavailability, bioaccessibility of hydrophobic organic contaminants in soils and associated desorption-based measurements. J.J. Ortega-Calvo, J.R. Parsons (Eds.), Bioavailability of Organic Chemicals in Soils and Sediments. Handbook of Environmental Chemistry, 100, Springer, pp. 293-341. https://doi.org/10.1007/698_2020_521

Varó, I., Perini, A., Torreblanca, A., Garcia, Y., Bergami, E., Vannuccini, M. L., Corsi, I., 2019. Time-dependent effects of polystyrene nanoparticles in brine shrimp Artemia franciscana at physiological, biochemical and molecular levels. Science of The Total Environment, Volume 675, Pages 570-580, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2019.04.157

Velzeboer, I., Kwadijk, C. J., A. F., Koelmans, A. A., 2014. Strong sorption of PCBs to nanoplastics, microplastics, carboon nanotubes, and fullerens. Environ. Sci. Technol., 48, 4869−4876. https://doi.org/10.1021/es405721v

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A. & Da Ros, L., 2013. Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence, spatial patterns and identification. Estuarine, Coastal and Shelf Science, 130, pp 54-61. https://doi.org/10.1016/j.ecss.2013.03.022

Villeneuve, D. L., Crump, D., Garcia-Reyero, N. et al., 2014. Adverse outcome pathway (AOP) development I: strategies and principles. Toxicol Sci 142(2):312–320. https://doi.org/10.1093/toxsci/kfu199

vom Saal, F. S., Myers, J. P., 2008. Bisphenol A and risk of metabolic disorders. JAMA. Sep 17;300(11):1353-5. Epub 2008 Sep 16. PMID: 18799451. doi: 10.1001/jama.300.11.1353

Wah Chu, K., Chow, K.L., 2002. Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquat. Toxicol. 2002, 61, 53–64, https://doi.org/10.1016/S0166-445X(02)00017-6

Wang, W., Wang, J., 2018. Comparative evaluation of sorption kinetics and isotherms of pyrene onto microplastics. Chemosphere 193, 567e573. https://doi.org/10.1016/j.chemosphere.2017.11.078

Wang, J., Tan, Z., Peng, J., Qiu, Q., Li, M., 2016. The behaviors of microplastics in the marine environment. Mar. Environ. Res. 113, 7–17. https://doi.org/10.1016/j. marenvres.2015.10.014

Wang, Z., Chen, M., Zhang, L., Wang, K., Yu, X., Zheng, Z., Zheng, R., 2018. Sorption behaviors of phenanthrene on the microplastics identified in a mariculture farm in Xiangshan Bay, southeastern China. Sci. Total Environ. 628–629, 1617–1626. https://doi.org/10.1016/j.scitotenv.2018.02.146

Wang, F., Yang, W., Cheng, P., Zhang, S., Zhang, S., Jiao, W., Sun, Y., 2019. Adsorption characteristics of cadmium onto microplastics from aqueous solutions. Chemosphere, Volume 235, Pages 1073-1080, ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2019.06.196

Wang, Y., Wang, X., Li, Y., Li, J., Wang, F., Xia, X., Zhao, J., 2020. Biofilm alters tetracycline and copper adsorption behaviors onto polyethylene microplastics. Chem. Eng. J., 392, 123808. https://doi.org/10.1016/j.cej.2019.123808

Weis, J. S., 2014. Physiological. Springer, Netherlands, Developmental and Behavioral Effects of Marine Pollution. doi:10.1007/978-94-007-6949-6

Wong, B. B., Candolin, U., 2015. Behavioral responses to changing environments. Behav. Ecol. 26, 665–673. https://doi.org/10.1093/beheco/aru183

Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol., 23, R1031–R1033. https://doi.org/10.1016/j.cub.2013.10.068

Wu, P., Cai, Z., Jin, H., Tang, Y., 2019. Adsorption mechanisms of five bisphenol analogues on PVC microplastics. Sci. Total Environ. 650, 671-678. https://doi.org/10.1016/j.scitotenv.2018.09.049

Xu, B., Liu, F., Brookes, P.C., Xu, J., 2018. Microplastics play a minor role in tetracycline sorption in the presence of dissolved organic matter. Environ. Pollut. 240, 87-94. https://doi.org/10.1016/j.envpol.2018.04.113

Yang, R., Li, Z., Huang, B., Luo, N., Huang, M., Wen, J., Zhang, Q., Zhai, X., Zeng, G., 2018. Effects of Fe(III)-fulvic acid on Cu removal via adsorption versus coprecipitation. Chemosphere 197, 291–298. https://doi.org/10.1016/j.chemosphere.2018.01.042

Yeo, B. G., Takada, H., Yamashita, R., Okazaki, Y., Uchida, K., Tokai, T., Tanaka, K., Trenholm, N., 2020. PCBs and PBDEs in microplastic particles and zooplankton in open water in the Pacific Ocean and around the coast of Japan. Mar Pollut Bull., Feb;151:110806. https://doi.org/10.1016/j.marpolbul.2019.110806

Yu, Y., Yu, Z., Chen, H., Han, Y., Xiang, M., Chen, X., Ma, R., Wang, Z., 2019. Tetrabromobisphenol A: disposition, kinetics and toxicity in animals and humans. Environ. Pollut., 253, pp. 909-917. https://doi.org/10.1016/j.envpol.2019.07.067

Yu, H., Hou, J.H., Dang, Q.L., Cui, D.Y., Xi, B.D., Tan, W.B., 2020. Decrease in bioavailability of soil heavy metals caused by the presence of microplastics varies across aggregate levels. J. Hazard. Mater. 395, 122690. https://doi.org/10.1016/j.jhazmat.2020.122690

Zarfl, C., Matthies, M., 2010. Are marine plastic particles transport vectors for organic pollutants to the Arctic? Mar. Pollut. Bull. 60, 1810−1814. https://doi.org/10.1016/j.marpolbul.2010.05.026

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “plastisphere”: microbial communities on plastic marine debris. Environ. Sci. Technol. 47 (13), 7137-7146. https://doi.org/10.1021/es401288x

Zhan, Z., Wang, J., Peng, J., Xie, Q., Huang, Y., Gao, Y., 2016. Sorption of 3, 3’, 4, 4’- tetrachlorobiphenyl by microplastics: a case study of polypropylene. Mar. Pollut. Bull. 110, 559e563. https://doi.org/10.1016/j.marpolbul.2016.05.036

Zhao, L., Rong, L., Xu, J., Lian, J., Wang, L., Sun, H., 2020. Sorption of five organic compounds by polar and nonpolar microplastics. Chemosphere 257, 127206. https://doi.org/10.1016/j.chemosphere.2020.127206

Zhou, Y., Liu, X., Wang, J., 2019. Characterization of microplastics and the association of heavy metals with microplastics in suburban soil of central China. Sci. Total Environ. 694, 133798. https://doi.org/10.1016/j.scitotenv.2019.133798

Ziccardi, L. M., Edgington, A,, Hentz, K., Kulacki, K. J., Kane, Driscoll, S., 2016. Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. Environ Toxicol Chem., Jul; 35(7):1667-76. PMID: 27093569. https://doi.org/10.1002/etc.3461

Chapter 2: Evaluation of Microplastic-associated Biofilm on bioavailability of Cadmium and Benzo[a]pyrene through analysis of biomarker gene expression in larval zebrafish

2.1 Abstract

The accumulation of Microplastics (MP) in the environment is continuous and persistent, posing a concern for potential adverse effects on biota. Since environmental MPs can carry considerable amounts of diverse substances (co-contaminants), the combined exposure to MPs and other contaminants represents one of the most important aspects. The presence of a biofilm on MPs surface can potentially change co-contaminant bioavailability to organisms. The aim of this study was to investigate if the presence of a biofilm on the surface of MPs (High Density Polyethylene, HDPE) altered the sorption of benzo [a] pyrene (B[a]P) and its bioavailability to zebrafish (*Danio rerio*) larvae (ZF). Since in environmental conditions, organisms are exposed to multiple stressors and toxic chemical mixtures, a mixture of B[a]P with Cadmium (Cd) was also investigated. The bioavailability of B[a]P and Cd were assessed by measuring fold changes in the expression of biomarker gene transcripts for these toxicants (*cyp1a* and *mt2*, respectively) in larval zebrafish. The bioavailability of B[a]P and Cd was reduced by more than 50% when there was biofilm on MPs surface, although a stronger association of B[a]P compared to Cd emerged. A significant reduction and induction of *cyp1a* and *mt2* transcripts ($p<0.001$), respectively, were found in zebrafish larvae exposed to the mixture of co-contaminants with MPs, also when there was a biofilm on their surface. These data indicated that additional interactions and active sites between MPs and B[a]P were provided by biofilm on MPs surface, reducing its bioavailability. A competition mechanism promoting B[a]P sorption and reducing availability of sorption sites for Cd interactions occurred when mixture of contaminants in presence of a MP-associated biofilm was tested. Therefore, when plastic materials were exposed to mixture of Cd and B[a]P, the interactions between them resulted in pattern of bioavailability not overlapping to those arose by single contaminant. Data also indicated that biofilm microbial structure included hydrocarbon-degrading bacteria able to metabolize hydrophobic chemicals and to use them as alternative carbon and energy sources supporting the metabolic biosynthesis pathways. Therefore, a potential microbial role by biofilm into influence benzo[a]pyrene bioavailability to zebrafish larvae was suggested.

2.2 Introduction

Microplastics contamination has gained the public concern, and in some instances have even considered them as a potential “planetary boundary threat” (Galloway and Lewis, 2016; Jahnke et al., 2017). In the aquatic environment, MPs are ubiquitously present in seas and rivers (Horton et al., 2017; Eriksen et al., 2017) offering several and constant routes of exposure for aquatic biota, and potentially to humans via ingestion (Boyle et al., 2020; Senathirajah et al., 2021). It has been documented that MPs contamination reaches remote locations (i.e. Polar Regions) (Peeken et al., 2018; Kesy et al., 2019), in addition to their distribution in beaches, shorelines, lakes (Imhof et al., 2013) and ocean gyres (Browne et al., 2011). Considering that the projections related to the accumulation of plastic debris in the aquatic environment by 2050 are on the order of trillions particles (Eriksen et al., 2014) there is an urgent research need to better understand the adverse effects of MP exposure at all trophic levels. This prevision are due to the increasing use of single-use plastic products, uncontrolled disposal of waste and recycling practices, and that the estimates of human consumption of plastic exceeding 10,000 particles for year (Catarino et al., 2018).

Several studies have demonstrated that MPs induce negative of effects on marine organisms (Wright et al., 2013; Wagner et al., 2014; Eerkes-Medrano et al., 2015). The main negative effects induced by MPs are stress of ingestion derived from physical blockage, leaching of additives from plastic particles, and exposure to co-contaminants associated with MPs (Wright et al., 2013; Anderson et al., 2016; Hermabessiere et al., 2017; Sleight et al., 2017). Although in the last years the scientific community have investigated effects of MPs (Paul-Pont et al., 2018; Cole et al., 2011), the potential toxic effects linked to transport hazardous co-contaminants sorbed on their surface remain poorly understood. Due to their chemical properties, MPs can adsorb persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), either during the manufacturing process or from the environment contributing to MPs hazard effects (Gallo et al., 2018).

Laboratory-based assessments have indicated that plastic-mediated transfer of POPs to the organisms can take place, and that the mechanisms on how MPs interact with substances affects their bioaccessibility by organisms and consequent bioaccumulation and bioavailability (Trevisan et al., 2019). For example, the co-exposure of nanoplastics (50 nm) and Bisphenol A (BPA) enhances BPA accumulation in zebrafish tissues after 3 days of exposure if compared with BPA alone treatment (Chen et al., 2017). In a simulated natural food chain from *Artemia nauplii* to zebrafish, a transfer of MPs spiked with Benzo[a]pyrene (B[a]P) was observed in addition to an induction of *cyp1a* transcription (Batel et al., 2016). A *cyp1a* induction was also detected upon exposure to MPs spiked with oxybenzene (BP3) or B[a]P (Cormier et al., 2019). Other authors reported a higher bioaccumulation of fluoranthene after depuration in mussels exposed to PS-fluoranthene than in organisms exposed to the pollutant alone (Paul-Pont et al., 2016).

Microplastics can physically or chemically interact with metals and influence their uptake, bioaccumulation, and bioavailability to zebrafish (Lu et al., 2018; Zhang et al., 2020; Holmes et al., 2012). For instance, Khan et al., 2015 demonstrated that adult zebrafish exposed to silver (Ag) incubated PE-MPs significantly reduced metal uptake, influenced its localization in zebrafish tissues as well as its toxicity. Lu et al., 2018 found that combined exposure of Cd and MP enhanced the toxicity of Cd as well as induced oxidative damage and inflammation in adult zebrafish tissues.

Although it has been demonstrated that co-exposure of MPs with co-contaminants enhances the toxicity when compared to individual contaminant exposure (Qiao et al., 2019; Alessio et al., 2018; Avio et al., 2015); some studies reported a reduction in bioavailability and toxicity when MPs are mixed with co-contaminants (Sleight et al., 2017; Herzke et al., 2016). For this reason, since the retention of toxicants on MPs represents a key factor to predict their environmental risks, more data is required on the bioavailability of sorbed co-contaminants to organisms, especially in relation to interactive effects derivable from complex mixtures between toxicants (de Zwart and Posthuma, 2005).

In addition to this complex process, several studies have demonstrated that the surface of MPs acts as an anthropogenic substrate for phylogenetically and functionally distinct communities of microorganisms called "biofilm" or "epiplastic community" (Reisser et al., 2014; Zettler et al., 2013). Epiplastic microbiota appears to play a key role in the fate and ecological impacts of plastic pollution and in the last years microbiologists are investigating these communities present on MPs surfaces. For instance, Zettler et al., 2013 using a molecular approach based on next-generation sequencing, shown an exclusive and complex plastics-associated community (i.e. diatoms, ciliates and bacteria) collected from offshore zones of North Atlantic. Although research directed to such study is still limited, knowledge about community structure and function may elucidate the impact of plastic pollution on different environments (Carpenter, 1972; Goldstein et al., 2012; Zettler et al., 2013). This organic layer can act as a reservoir for pollutants, affecting the adsorption of chemicals for organisms ingesting MPs with unpredictable effects on the co-contaminants ecotoxicity (Rummel et al., 2017; Flemming et al., 1995). Due to the biofilm sorption characteristics and ability to degrade organic chemicals (Writer et al., 2011; Wen et al., 2015), the presence of biofilm on microplastic surfaces can influence plastic-mediated transfer of pollutants to organisms. Although several studies have suggested that a contaminant transfer in organism can occur (Chua et al., 2014; Rochman et al., 2014; Browne et al. 2013; Gaylor et al., 2012), it is still unclear how biofilms interact with plastic-associated chemicals and consequently their bioavailability for organisms ingesting MPs (Rummel et al., 2017).

The goal of this study is to assess:

1) the bioavailability of two model contaminants [Benzo[a]pyrene (B[a]P) and Cadmium (Cd), in single substance solutions and in combination] in early-life stage (ELS) of zebrafish (*Danio rerio*);

2) the potential of biofilms to modify fluxes of these contaminants plastic-associated and consequently their bioavailability in zebrafish larvae (ZF);

3) the structure and function microbial community forming biofilm on plastic surfaces used for exposure tests.

To have an indication on the bioavailability of MPs co-contaminants, defined as a measure by which a chemical penetrates cells and chemically reacts with biologically important molecules (Semple et al., 2004), a well-characterized organism-response is required. For instance, changes in expression of specific biomarkers have been used in several studies to assess bioavailability of pollutants or xenobiotics (Souza et al., 2013; Henry et al., 2013; Oliveira et al., 2013), but also associated with MPs, as observed in few studies measuring phenanthrene (Phe) and 17 α-ethynylestradiol (EE2) bioavailability in zebrafish larvae after co-exposure with MPs (Sleight et al., 2017; Chen et al., 2017). In this study, the bioavailability of B[a]P and Cd were assessed by measuring the expression of related biomarker genes such as Cytochrome P450 1A (*cyp1a)* and Metallothionein 2 (*mt2*), respectively.

Zebrafish, selected as exposure model, has been widely used in toxicological studies of MPs. Boyle et al. (2020) found that MPs exposure release additive that become bioavailable in zebrafish through analysis of biomarker gene expression. Batel et al., (2018) showed that MPs could transfer pollutants via ingestion, attachment to epithelia or via the water column to zebrafish based on enzymatic biomarker analysis. Early life stages - embryos, larvae, neonates, juveniles - (ELS) of aquatic species display a higher vulnerability and sensitivity to external challenges and chemical pollutants than adult fishes (Beiras et al., 2018). Therefore, in this study, not free-feeding zebrafish larvae (72 hours post fertilization, hpf) were employed to assess bioavailability of MP-sorbed co-contaminants, focusing attention on potential role of MPs as a vector for contaminants rather than on physical aspect of them.

2.3 Materials and Methods

2.3.1 Experimental fish

Brood stock zebrafish (Wild type line – WIK) were maintained in an aquarium facility at Heriot-Watt University, Edinburgh, UK and fish welfare regulations were followed for all experimental procedures. Adult fish were kept in recirculating tank systems with a 12:12 light/ dark photoperiod and temperature between 27 and 29 °C. The water used in all experiments and for culturing stock fish (also used in the aquarium water) was prepared following OECD guidelines (Test 201; Organization for Economic Co-operation and Development 2011) adding to reverse osmosis purified water reagent grade salts. The final concentrations of salts in the water were: 0.294 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 0.123 mg L^{-1} $MgSO_4$, $7H_2O$, 0.0647 mg L^{-1} $NaHCO_3$, 0.0057 mg L^{-1} KCl and the water was at pH 8.0. Water quality was routinely monitored (e.g. for total ammonia, nitrate and nitrite) and part-refreshed on a twice weekly basis or more frequently if required. Fish were fed newly hatched brine shrimp Artemia spp. and a commercial zebrafish diet (ZM Fish Food, ZM Systems, Winchester, UK), daily. To obtain larvae for experiments, pairs of zebrafish (one male and one female) were separated from stock tanks and gently transferred to 1 L breeding tanks (Mbk Installations Ltd, Nottingham, UK) fitted with a partition to separate the fish, overnight. The following morning, the water in the breeding tanks was refreshed, the partitions were removed, and fish proceeded to spawn. Approximately 60 min later, the adult fish were transferred back to stock tanks and all embryos from multiple spawning pairs were pooled and distributed between Petri dishes at a density of approximately 50 embryos per dish. Zebrafish were left to develop until

72 hour post-fertilization (hpf) and hatched larvae were then used in experiments. The duration of fish exposure was 24 h and all larvae were 96 hpf when they were sacrificed at the end of the experiments. At this stage of development of zebrafish larvae were not able free feeding (aged < 120 hpf), so they have been used without any specific permissions, using only the number necessary for robust statistical analysis.

2.3.2 Microplastics model and preparation of stock solutions

This work was carried out using High-Density Polyethylene (HDPE) microparticles that were sieved to a size range of 250 – 125 µm. HDPE was selected in this study both because is one of the most commonly found polymers in the marine environment (Ajith, N. et al., 2020) and because it has a high sorption capacity for hydrophobic organic chemicals (Lee et al., 2014; Ziccardi et al., 2016). The HDPE concentration was set at 500 mg L^{-1} for all experiments according to previous MP co-contaminants sorption studies (Bakir et al., 2012; Sleight et al., 2017; Boyle et al., 2020). Stock solutions of Benzo[a]pyrene (B[a]P, Sigma-Aldrich, DE) and Cadmium Chloride ($CdCl_2$, Sigma-Aldrich, DE) were prepared before addition to fish water to achieve the target nominal concentrations. The B[a]P stock solution was prepared in dimethyl sulfoxide (DMSO, ≥ 99.5%, Sigma-Aldrich), while Cd stock solution was prepared in ultrapure water (Milli-Q, Merck Millipore, UK) at 50 and a 10 mg L^{-1} concentrations, respectively. The final concentrations of DMSO in testing solutions did not exceeded 0.01% (v/v) (Kais et al., 2013). The concentration of B[a]P (5 µg L^{-1}) was selected on basis of previous data that suggested a 10-fold increase in expression of cyp1a at this concentration after 24 h exposure of zebrafish larvae at 72 hpf. The Cd concentration (0.24 mg L^{-1}) was chosen according to previous studies testing acute molecular response to Cd.

2.3.3 Experimental design

Sorption of B[a]P and Cd on HDPE microplastics (indicated as HDPE-MPs) was assessed by a bioavailability assessment. The experiment carried out in the present study investigated the activity of well-known biomarkers [Cytochrome P450 1A (cyp1a), metallothionein (*mt2*)] for the evaluation of B[a]P and Cd bioavailability, respectively, in larval zebrafish with and without biofilm formation on HDPE-MPs(figure 2.1). Each experiment (both B[a]P alone and in mix with Cd) included three replicate beakers (the experimental replicates, *n=3* treatment^{-1}) with 20 larvae in each replicate. A negative control (no HDPE-MPs, media) and a negative control treatment (media and vehicle (DMSO) solution) were included in the analysis.

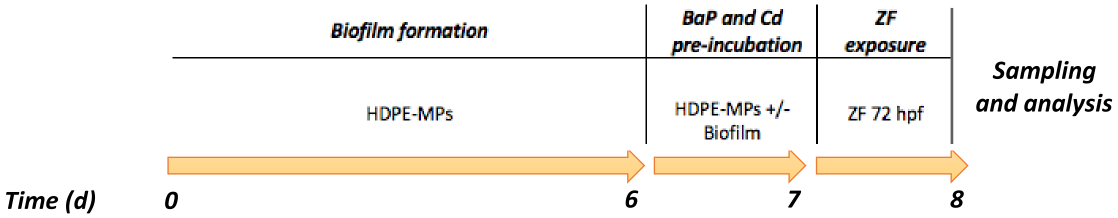


Figure 2.1 Experimental design and timeline of the present study featuring the experimental steps to assess the bioavailability of B[a]P and B[a]P and Cd mixture from HDPE-MPs to zebrafish larvae with and without (+/-, respectively) the presence of a biofilm. Time "*day 0*" indicates the start of biofilm formation which for 6 days. B[a]P and Cd have been equilibrated with HDPE-MPs for 24 h, and finally, zebrafish larvae (ZF at 72 hpf) have been exposed for 24 h to HDPE-MPs. In total the experimental steps took 8 days, at the end of which zebrafish samples were collected.

2.3.4 Biofilm formation

For biofilm formation (figure 2.2), 20 mg of HDPE-MPs were weighted and added directly into each (*n = 11*) clear 20 mL borosilicate vials with screw top containing water (20 mL). The water used for this step, was collected from the water reservoir of zebrafish recirculating system. Prior collection, bioballs (the substrate of the biofilter of the water system) in the box were agitated using an acid washed beaker. Water was collected and filtered two times through two filter with different size (125 µm and 100 µm) to avoid larger detritus and then pipetted into each of the experimental vial. To protect the biofilm forming treatments from light and prevent algal growth, vials were covered with aluminum foil and then incubated at 27 - 29 °C for 6 days. After incubation with media, HDPE-MPs were collected by filtration (100 µm Celltric® filters) and transferred into new vials containing 20 mL of filtered ZF media. Of the vials (*n = 11*) used in the biofilm formation step, *n = 3* vials have been used as control HDPE-MPs with biofilm for the following test exposures and *n = 2* vials have been used for metagenomic analysis to evaluate the taxonomic composition of the biofilm attached on HDPE-MPs (HDPE-MPs + BF). Finally, remaining vials (*n = 6*) have been pre-incubated with co-contaminants.

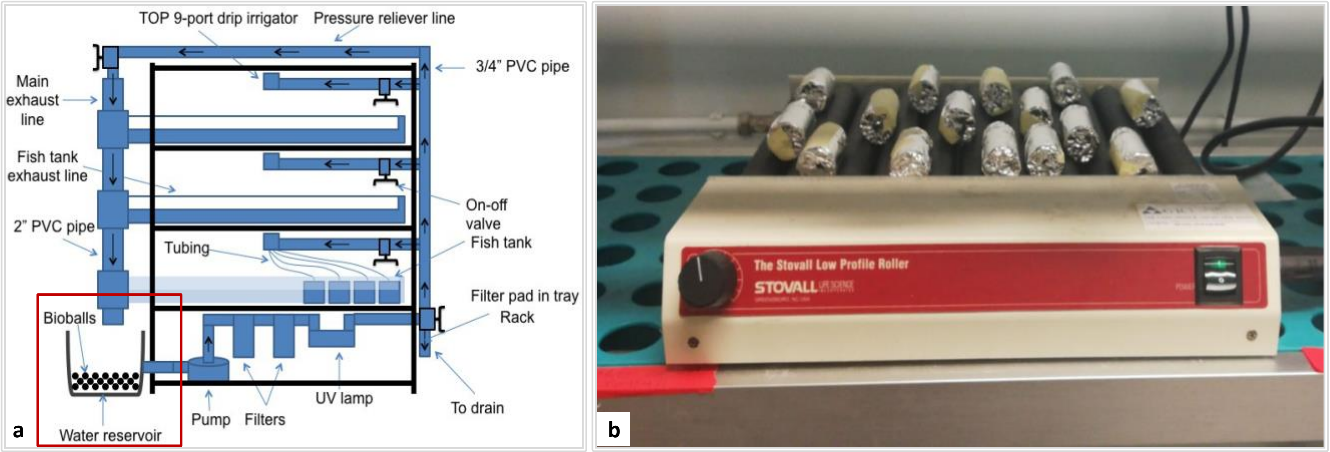


Figure 2.2 Biofilm formation phases: (a) Box from which water has been collect to form biofilm; (b) Vials on the roller covered with aluminum foil in incubation for 6 days

2.3.5 Co-contaminants pre-incubation

B[a]P and Cd have been pre-incubated with HDPE-MPs for 24 h before exposure to zebrafish. Pre-incubation concerned three treatment groups including: 1) HDPE-MPs without biofilm (HDPE-MPs), 2) HDPE-MPs with biofilm (HDPE-MPs + BF, figure 2.3) and 3) positive controls B[a]P, Cd and mixture of them (without HDPE-MPs). The adsorption of contaminants on MPs was obtained weighing 20 mg of HDPE-MPs into each ($n = 6$ for each treatment group – B[a]P and Cd alone and mixture of them) clear borosilicate vials with screw top and adding 20 mL of filtered zebrafish media. For the HDPE-MPs treatment groups (HDPE-MPs + B[a]P and HDPE-MPs + Cd), 4 µl of B[a]P and 960 µl of Cd stock solutions (50 mg L^{-1} of B[a]P in DMSO, ≥ 99.5%, Sigma-Aldrich; 10 mg L^{-1} of Cd) have been solved to reach 10 µg L^{-1} and 0.48 mg L^{-1}, respectively. After pre-incubation (24 h), the concentration has been diluted by half, adding filtered zebrafish media and bringing the volume to 40 mL. The same pre-equilibration procedure has been applied to Cadmium and mixture treatments, including positive controls and HDPE-MPs + BF. All treatments were placed in a roller system for 24 h and then used for the exposure experiments. Finally, after 24 h incubation, from *3 (n)* vials (for each treatment group), aqueous and MPs phases were collected chemical and trace metal analysis.

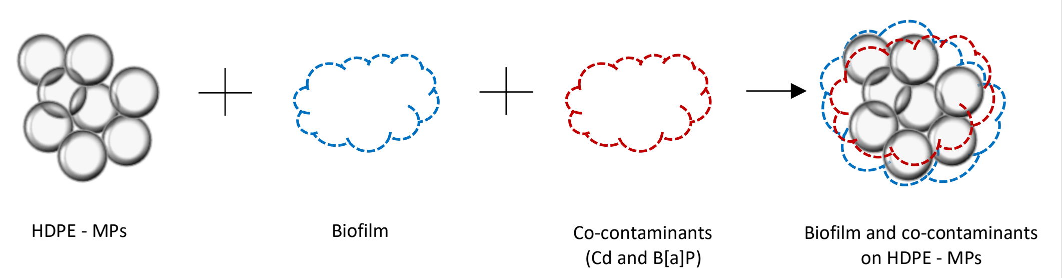


Fig. 2.3 Schematic representation of biofilm and co-contaminants: Cadmium and Benzo[a]pyrene lining HDPE-MPs.

2.3.6 Zebrafish exposure

For each experiment involving B[a]P, Cd and mixture B[a]P/Cd test, pre-incubation solutions (20 mL) were poured into pre-acid washed (5% HNO_3) 50 mL glass beakers. After bringing the volume to 40 mL with filtered zebrafish media, zebrafish larvae at 72 hpf were gently collected from Petri dishes with a transfer pipette and added to the preincubation and control solutions (Table 2.1). Each beaker ($n = 3$ treatment^{-1}) contained 20 larvae. After 24 h of exposure, larvae from each beaker were pooled, placed in a microcentrifuge tube, the exposure media removed with a pipette tip and finally the tubes containing larvae were immediately stored at -80°C until RNA extraction. During all exposure tests, no developmental toxicity associated with HDPE-MPs and co-contaminants, and no mortality were observed.

Exposure group	Contaminant concentration	Treatment type
Fish media		Not Pre-incubation
DMSO	0.01% (v/v)	Not Pre-incubation
HDPE	500 mg L^{-1}	Not Pre-incubation
HDPE+BF		Biofilm formation
Cd	0.24 mg L^{-1}	Pre-incubation
B[a]P	5 µg L^{-1}	Pre-incubation
Cd+B[a]P	0.24 mg L^{-1}+5 µg L^{-1}	Pre-incubation
HDPE+Cd	500 mg L^{-1}+ 0.24 mg L^{-1}	Pre-incubation
HDPE+B[a]P	500 mg L^{-1}+ 5 µg L^{-1}	Pre-incubation
HDPE+Cd+B[a]P	500 mg L^{-1}+0.24 mg L^{-1}+ 5 µg L^{-1}	Pre-incubation
HDPE+BF+Cd	500 mg L^{-1}+ 0.24 mg L^{-1}	Biofilm formation + Pre-incubation
HDPE+BF+B[a]P	500 mg L^{-1}+ 5 µg L^{-1}	Biofilm formation + Pre-incubation
HDPE+BF+Cd+B[a]P	500 mg L^{-1}+0.24 mg L^{-1}+ 5 µg L^{-1}	Biofilm formation + Pre-incubation

Table 2.1: Exposure groups used in this study. Details of concentrations used in single and mixture exposures

2.3.7 Gene expression analysis

Expression of genes that are biomarkers of the bioavailability of organic xenobiotics, *Cytochrome p450 1 a (cyp1a)* and metals, *Metallothionein 2 (mt2)*, were evaluated in zebrafish larvae from two experiments carried out (with B[a]P and mixture of B[a]P/Cd). After sample homogenization with a motor-driven hand homogenizer, total RNA was extracted (RNeasy Mini Plus Kit, Qiagen, UK) from pooled zebrafish larvae (20 larvae in each sample). A treatment with DNase (15 min, Qiagen, UK) was used to eliminate any genomic DNA contamination, and 30 µL of RNase/DNase-free water was added for elution of RNA. The concentration and quality of RNA were evaluated spectrophotometrically through NanoDrop (ND-1000, Thermo Fisher Scientific, UK). Each sample was diluted to 100 ng µL^{-1} total RNA and cDNA was synthesized using 2 µg RNA in 10 µL reactions according to the manufacturer's instructions (Precision nanoScript 2 Reverse Transcriptional Kit, Primer Design, UK). Primers for amplification of zebrafish transcripts of *cytochrome P450 1a (cyp1a), metallothionein 2 (mt2)* and *ß-actin* (Table 2.2) had been previously described according to Sleight et al., 2017; Henry et al., 2009; Henry et al., 2013. Quantitative Polymerase Chain Reaction (qPCR) was carried out in 20 µL of total volume for each real-time PCR reaction. cDNA was diluted with nuclease free water (1:25) and mixed with SYBR Green qPCR mastermix (PrecisionPLUS qPCR MasterMix Primer Design, UK) and 300 nM gene specific primers. Fluorescence was detected over 40 cycles using a PCR machine (StepOne Real-Time PCR System, Applied Biosystems, Warrington, UK) with the following conditions: 2 min of initial enzyme activation at 95°C followed by 40 cycles of denaturation at 95°C for 15 s and primer-specific annealing at 60°C for 1 min. All samples reactions were repeated in triplicate with appropriate no-template controls included in each run and with dissociation analysis performed within the qPCR run to verify the specificity of the primer pair use.

Gene	Accession #	Primer	Nucleotide sequence (5' – 3')	Amplicon (bp)
cyp1a	NM_131879.2	Forward Reverse	AGGACAACATCAGAGACATCACCG GATAGACAACCGCCCAGGACAGAG	174
mt2	NM_001131053.2	Forward Reverse	TGTTCTCAATCTTGTCTGTTTAATG CATCTCGTGATAGTCTTATTTGC	108
ß-actin	NM_131031.1	Forward Reverse	ACA CAG CCA TGG ATG AGG AAA TCG TCA CTC CCT GAT GTC TGG GTC GT	138

Table 2.2: Gene specific primers for cytochrome P450 1a (*cyp1a*), metallothionein 2 (*mt2*) and *ß-actin* reference gene used in early life-stage zebrafish. Reference sequence numbers from NCBI and amplicon length in base pair (bp).

The efficiency (between 90 - 110%) of the reaction was checked through an evaluation of the slope of the standard curve prepared for each gene transcript for which 10-fold serial dilutions of cDNA were tested. Relative quantification of mRNA transcripts was calculated by comparative C_T method that takes into account of normalization of the change in expression of the gene of interest to that an internal housekeeping gene. Since no differences in expression of *ß-actin* was observed between treatment and control, including vehicle (DMSO) control, this gene transcript was therefore considered appropriate as an internal reference for all experiments. Data analysis was performed in normalized values to expression of control treatment groups namely subtracting the C_T value of *cyp1a* or *mt2* from that of *ß-actin* in the same sample (ΔC_T). The $\Delta\Delta C_T$ value was obtained from subtracting the mean ΔC_T of control groups and ΔC_T of each sample in all treatment groups. The gene expression fold change was calculated using the $2^{-\Delta\Delta CT}$ method [Livak et al., 2001].

2.3.8 Metagenomic analysis

DNA from microplastic particles was extracted with the Powersoil DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, United States) according to the manufacturer's recommendations. To prepare the samples for metagenome sequencing library, the total nucleic acid extractions from two biofilm samples was quantified using a Qubit HS (high sensitivity) dsDNA kit. The library preparation was achieved using the Swift Biosciences Accel-NGS 2S Plus DNA Kit, as per the manufacturer's instructions. Using a Covaris S220 ultrasonicator, fragments were sheared to ~ 450 bp, prior to adding barcodes using the Swift Biosciences 2S dual indexing kit. The subsequent libraries were checked for fragment length using a Bioanalyser DNA 7500 chip and quantification was performed using a picrogreen fluorescence assay. Libraries were then standardized to 4 nM as verified by qPCR (Kapa Biosystems Library Quantification kit, Applied Biosciences). An equimolar pool of all samples was generated and sequenced on Illumina HiSeq 2500 rapid runs (10-11 pM: V2 rapid sequencing chemistry), yielding reads of 251 bp paired-end sequences.

The metagenomic sequence analyses was done using Shotgun sequencing reads, which were assessed for quality, and adapters were removed using the Trim Galore package (v0.6.4_dev). Low quality ends were removed from sequences with a phred score lower than 20. Paired reads were merged using PandaSeq (v2.11), using default settings. The resulting countigs were randomly sub-sampled, which resulted in 2.2 million reads per sample. Metagenome merged reads were de-novo assembled by passing the merged reads into megahit (v1.2.9), thus generating meta-contigs. Taxonomic identification of the adapter trimmed reads was performed by comparison to the Maxikraken2 database 38, using the Kraken2 package (v2.0.8_beta) and visualized as Sankey plots using the Pavian Package (Breitwieser, 2016) in R (v1.2.0). Gene annotation of the Meta-contigs was conducted using Prokka (1.13) and these were examined for functional identification using Microbe annotator (v2.0.5) (Seemann, 2014; Ruiz-Perez et al., 2020).

2.3.9 Statistical analysis and data handling

All data presented are means ± standard deviation (SD) and all statistical analysis were performed using R software v. 3.5.0 (R Core Team, 2018). Bioavailability data were tested for normality and homogeneity of variances using Shapiro-Wilk test and Leven's test, respectively. Statistically differences between datasets were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (for a p value < 0.05, differences were considered significant) in order to make multiple comparisons.

2.4 Results

2.4.1 Sorption of B[a]P or Cd on HDPE-MPs and bioavailability in ELS zebrafish

The sorption of contaminants onto all MPs tested in the present study is showed by reduced *cyp1a* gene expression in zebrafish larvae exposed to different conditions test. Compared to co-contaminant exposure without MPs (positive control with only B[a]P – 6.5-fold) mRNA expression of *cyp1a* gene was significantly reduced (up to 2.5-fold) (one-way ANOVA, $p < 0.05$) when HDPE-MPs were present into the medium (figure 2.4 a). Considering change in expression of *cyp1a,* in zebrafish larvae exposed at the same concentration of B[a]P, the presence of MPs reduced bioavailability of B[a]P by up to 60.6%. However, zebrafish larvae exposed to HDPE-MPs with biofilm attached on their surface, shown a reduction of B[a]P bioavailability by up to 75.5% and a *cyp1a* gene expression more significantly reduced (one-way ANOVA, $p < 0.001$), than B[a]P exposure without MPs.

A similar trend was observed for Cd exposure, where the reduction in Cd bioavailability in zebrafish larvae co-exposed to HDPE-MPs and Cd was 25,4%. Here, a significant reduction of mt2 mRNA expression ($p<0.01$) compared to positive control (only Cd without HDPE-MPs) was found (figure 3.4 b). Co-exposure to HDPE-MPs with an associated biofilm and Cd resulted in a decrease of Cd bioavailability, as emerged by the significant reduction of mt2 mRNA expression ($p<0.05$) was found than where there was no biofilm (HDPE-MPs + Cd). For both individual contaminants tested, no significant change in expression of *cyp1a* or *mt2* in zebrafish larvae exposed to HDPE-MP alone or HDPE with biofilm attached was observed ($p>0.05$).

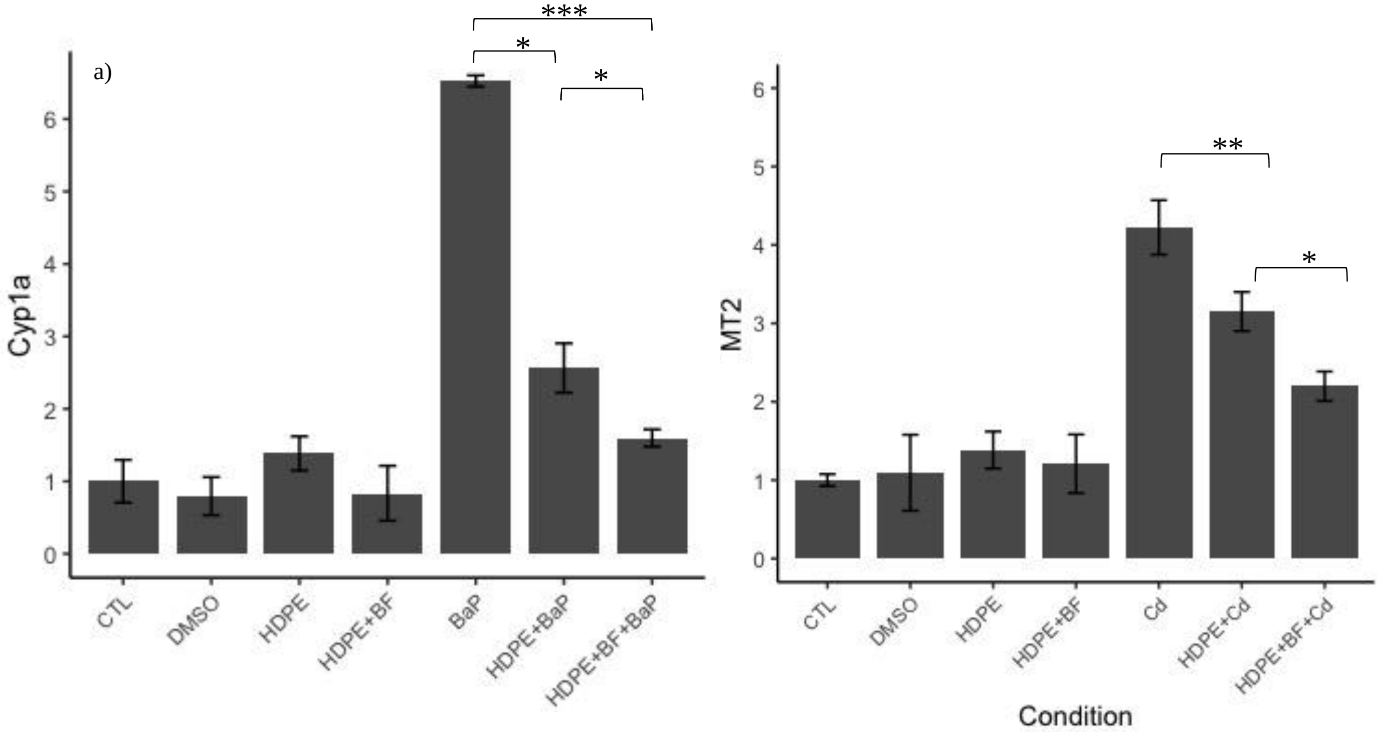


Figure 2.4 Relative fold change (FC) in gene expression of a) *cyp1a* and b) *mt2* in ZF larvae exposed to 5 µg L^{-1} of B[a]P and 0.24 mg L^{-1} of Cadmium, respectively, in water, after 24 h pre-equilibration with 20 mg HDPE-MPs (HDPE+B[a]P or HDPE+Cd), and after 24 h pre-equilibration with 20 mg HDPE-MPs with 6 days biofilm formation (HDPE+BF+B[a]P or HDPE+BF+Cd). Concentration of HDPE-MPs in suspensions was 500 mg L^{-1}. Expression was normalized to control larvae (CTL) consisting of aquarium ZF media (no HDPE-MPs or contaminant was added). Data are presented as means ± SD (n = 3). Significance differences between treatment groups are indicated by asterisk above error bars (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; one-way ANOVAs with Tukey's test)

2.4.2 Sorption of B[a]P and Cd mixture on HDPE-MPs and bioavailability of them in ELS zebrafish

The co-exposure of B[a]P and Cd was evaluated in relation to each single toxicant exposures used as positive controls by assessing the expression levels of biomarker gene transcripts for these toxicants (*cyp1a* and *mt2*, respectively) in larval zebrafish at 72 hpf. However, although *cyp1a* and *mt2* were commonly employed as specific biomarkers of organic xenobiotics (Batel et al., 2016; Sleight et al., 2017) and metals exposures (Henry et al., 2013), respectively, a cross analysis between both specific biomarkers for the mixtures of these contaminants was required. The expression of biomarker genes (cyp1a and mt2) related to each individual chemical treatments (B[a]P and Cd, respectively) were similar to what observed previously (figure 2.4 a and b; figures 2.5 a and b).

Considering *cyp1a* gene expression (figure 2.5 a), a significant induction was observed for both individual treatments with B[a]P and Cd ($p<0.001$). For both individual treatments of B[a]P and Cd, mRNA expression of *cyp1a* gene was significantly reduced in presence of HDPE-MPs (HDPE + B[a]P, $p<0.001$; HDPE + Cd, $p<0.05$ respectively), if compared to positive controls (treatments without HDPE-MPs). Based on changes in expression of *cyp1a,* the presence of HDPE-MPs reduced bioavailability of each contaminant by up to 42% and to 73% for Cd and B[a]P, respectively. However, although a significant reduction of *cyp1a* expression ($p<0.001$) in zebrafish larvae exposed to HDPE-MPs with biofilm was observed for B[a]P (HDPE+ BF + B[a]P) if compared to positive control (only B[a]P), a different result was observed for Cd (HDPE+ BF + Cd), where a significant increase of *cyp1a* expression ($p<0.05$, 2.81-fold) by up to the positive control level was detected in comparison to HDPE with Cd but without biofilm (HDPE+Cd, 1.8-fold). Concerning co-exposure with Cd and B[a]P, a significant reduction of *cyp1a* expression (3.15-fold, $p<0.05$) was observed in comparison to individual treatment with B[a]P (7.2-fold) (figure 2.5a). Unlike the reduction of expression level of *cyp1a* biomarker observed for each contaminant associated with HDPE-MPs (HDPE + Cd and HDPE+ B[a]P), for the mixture (HDPE+Cd+B[a]P) no relevant change in bioavailability was observed, compared to positive control related to mixture without MPs. Finally, a significative difference for treatment groups that differed for presence of biofilm (HDPE+BF+B[a]P+Cd and HDPE+Cd+B[a]P) was emerged. In particular, a significative reduction in *cyp1a* expression ($p<0.001$) was observed for the mixture in presence of biofilm (HDPE+BF+B[a]P+Cd, 1.4-fold) if compared to the same treatment without biofilm (HDPE + B[a]P +Cd, 3.5-fold) or to single exposure with Cd, HDPE-MPs and biofilm (HDPE + BF + Cd, 2.8-fold). Instead, no change in bioavailability was observed from the comparison between single exposure with B[a]P and the mixture Cd/B[a]P in presence of HDPE-MPs and biofilm (HDPE+BF+B[a]P and HDPE+BF+B[a]P+Cd).

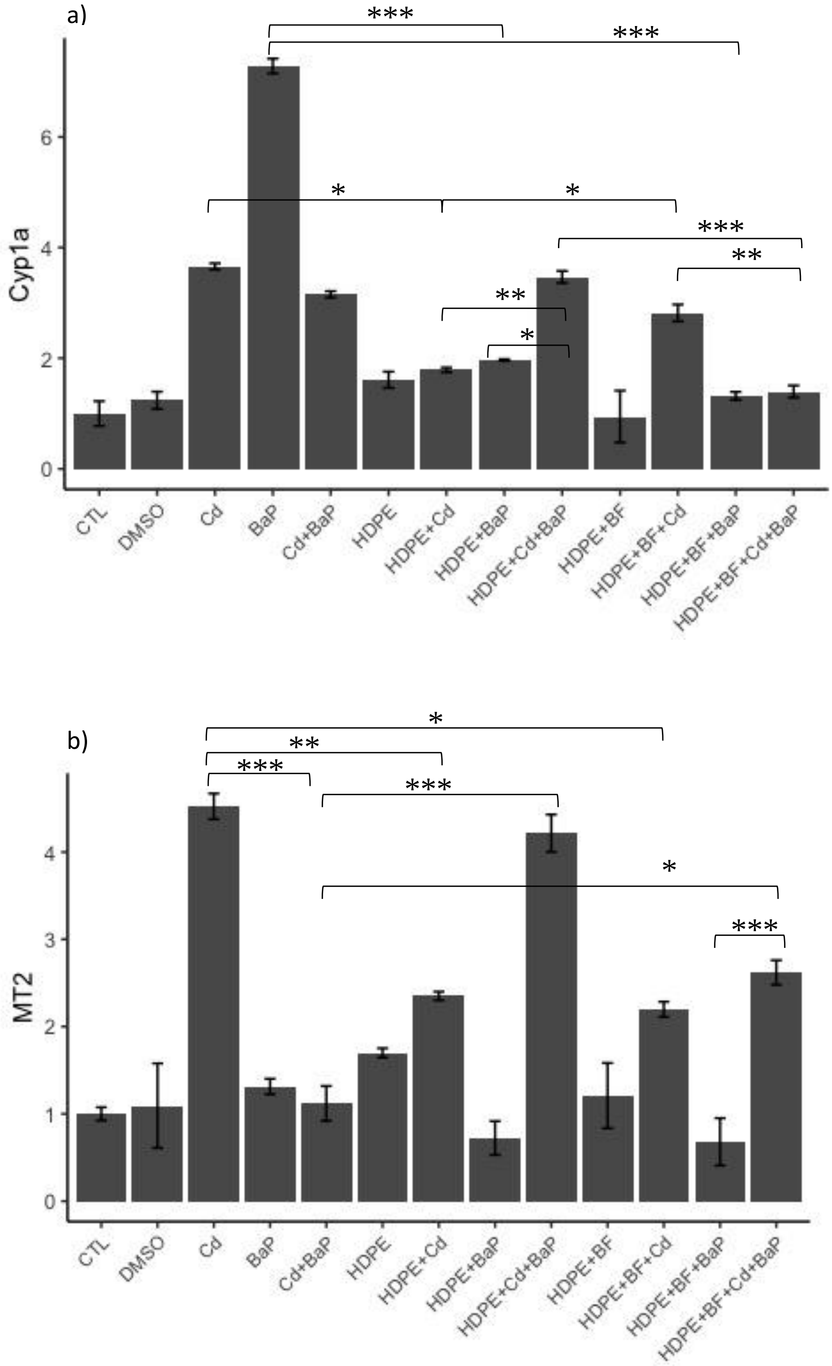


Figure 2.5 Relative fold change (FC) in gene expression of a) *cyp1a* and b) *mt2* in ZF larvae exposed to 5µg L^{-1} of B[a]P or to 0.24 mg L^{-1} of Cd or to a combination of B[a]P and Cd, in media, after 24h pre-equilibration with 20 mg HDPE-MPs (HDPE+B[a]P or HDPE+Cd or HDPE+B[a]P+Cd), and after 24 h pre-equilibration with 20 mg HDPE-MPs with 6 days biofilm attached (HDPE+BF+B[a]P or HDPE+BF+Cd or HDPE+BF+B[a]P+Cd). Concentration of HDPE-MPs in suspensions was 500 mg L^{-1}. Expression was normalized to control larvae (CTL) consisting of aquarium ZF media (no HDPE-MPs or contaminant was added). Data were presented as means ± SD (n = 3). Significance differences between treatment groups are indicated by asterisk above error bars (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; one-way ANOVAs with Tukey's test).

No change in *mt2* mRNA expression was observed in zebrafish larvae exposed to single B[a]P treatment, whilst a strong induction of biomarker (4.5-fold) resulted after Cd treatment (figure 2.5b). However, a significant reduction ($p<0.001$) was found after Cd/B[a]P co-exposure if compared with individual treatment with Cd. For all treatments involving individual exposure to B[a]P (HDPE + B[a]P and HDPE + BF + B[a]P) no differences were detected in comparison to negative and positive control, whilst for co-exposure with B[a]P and Cd in combination with HDPE-MPs (HDPE + Cd + B[a]P), an induction of *mt2* transcript comparable to individual Cd treatment (positive control) was observed.

A significant induction of *mt2* mRNA expression ($p<0.05$) also emerged from Cd/B[a]P treatment in presence of HDPE-MPs and biofilm (HDPE+BF+Cd+B[a]P, 2.6-fold) than positive control of mixture (Cd+B[a]P). However, such induction was found reduced compared that detected after exposure to the mixture of contaminants with HDPE-MPs but without biofilm (HDPE+Cd+B[a]P, 4.2-fold). Finally, from the comparison between treatment groups that differed for Cd presence (such as HDPE+BF+B[a]P and HDPE+BF+Cd+B[a]P), a significant induction was observed ($p<0.001$) (figure 2.5b).

2.4.3 Taxonomic analysis and metabolic profiling of biofilm lining the HDPE-MPs

Taxonomic assignment of shotgun metagenomic merged reads revealed that the biofilm lining the HDPE-MPs (HDPE + BF) was mainly composed by Bacteria (95.6 %), whereas only a small fraction of reads was associated to Eukaryota (2.2 %), Archaea (1.9 %) and Viruses (0.2 %) (figure 2.6 a). The microbial community were composed in majority by the phylum *Proteobacteria* (77 %), followed by *Actinobacteria* (15.8 %), *FCB-Bacteria* (2.2 %), *PVC-Bacteria* (1.8 %), *Nitrospirae* (1.2 %) and *Firmicutes* (1.1 %), which appeared less represented (figure 2.6 b). The phyla with <1% of total reads was grouped as Others (0.6 %), while Unclassified represented only 0.04%. Three classes of *Proteobacteria* (*Alpha, Beta and Gamma*) were identified but among them the order of *Burkholderiales,* belonging to *Betaproteobacteria* class, was the most represented (figure 2.6 c). Whilst, the taxonomic characterization related to *Actinobacteria* phylum highlighted *Actinobacteria* as the most predominant class, with *Corynebacteriales* as the most abundant order (figure 2.6 d). Also Sankey diagram related to the microbial community composition based on relative species abundance for the two samples analyzed, showed that *Betaproteobacteria* and *Actinobacteria* were the most numerically abundant group in the community (figure S1, Appendix A).

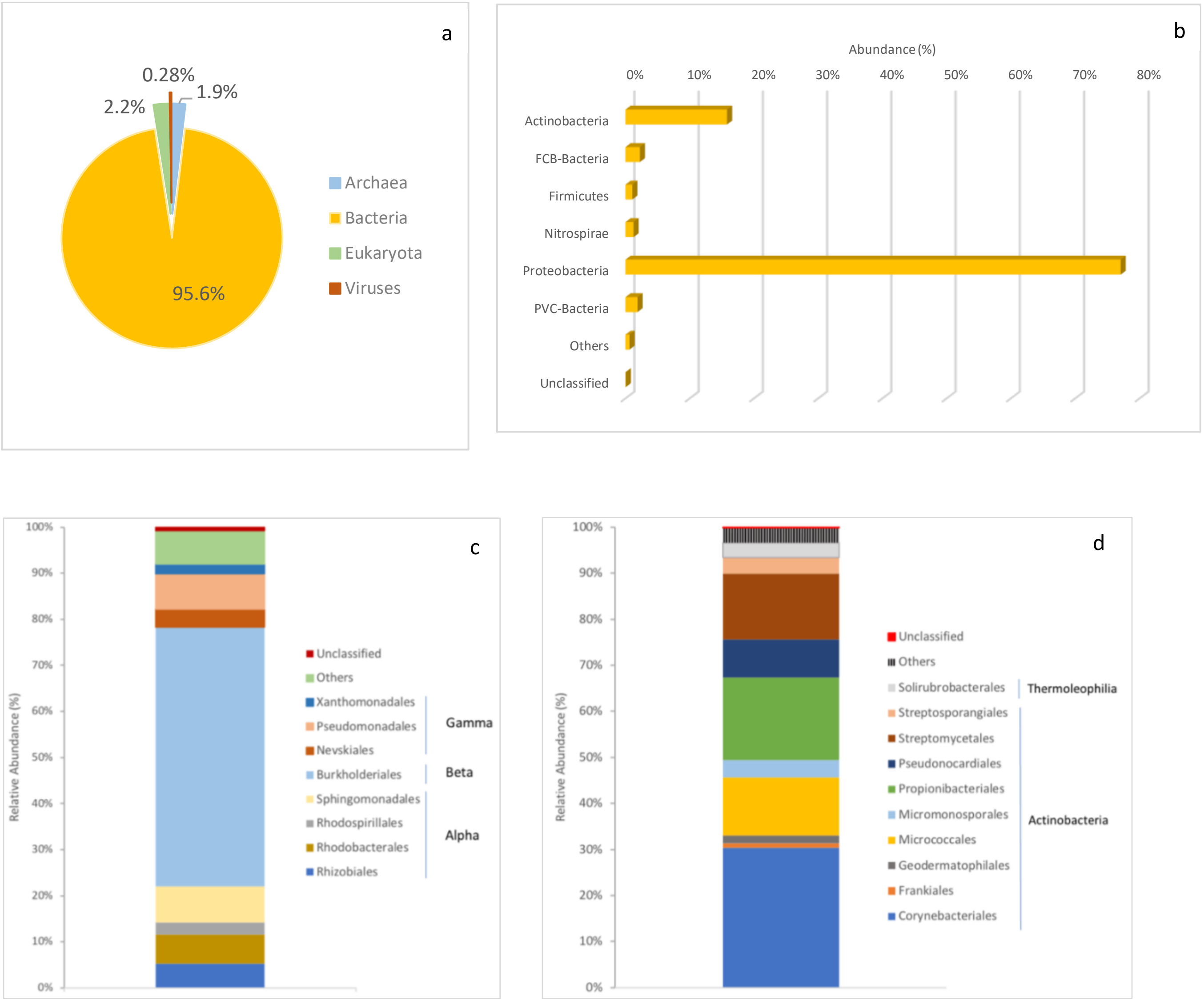


Figure 2.6 Taxonomic profiles of the biofilm lining the HDPE-MPs (HDPE + BF). a) Composition at domain level obtained by shotgun analysis; b) Bacterial composition at phylum level inferred from shotgun analysis. For lower values (<1 %), the sum of the relative percentages of phyla and orders is indicated as "Others". Relative composition related to the predominant phyla *Proteobacteria* c) and *Actinobacteria* d) indicated as Relative Abundance (%) and classified by class and order levels. Only the percentage values more than 1 % of the total reads obtained are considered as specific phyla b) or orders c, d) in the figures

Metabolic pathway prediction through MicrobeAnnotator (figure S2, Appendix A) clustered the majority of reads in the "Biosynthesis of other secondary metabolites, "Drug resistance", "Enediyne biosynthesis", "Macrolide biosynthesis" and "Type II polyketide biosynthesis". Note the high similarity in metabolic pathways exhibited by both genomes analyzed. For the purpose of analyzing the metabolic potential of the microbial community, in the heatmap showing module completeness for specific pathways, the completeness level of all modules recovered is identical or almost identical for the examined genomes (figure S3, Appendix A). Barplots of modules with completeness above 80% grouped by the category (pathway) shown a high similarity in metabolic pathways by genomes analyzed, as not differences were evident due to overall similar metabolic potential encoded.

2.5 Discussion

In this study, short-term B[a]P and Cd sorption to MPs resulted in lower bioavailability of co-contaminants to zebrafish larvae, compared to treatment group of each individual co-contaminant where there were no MPs. Thus, we hypothesize that a sorption process of each contaminant on MPs has been occurred. MPs and their characteristics (i.e. surface topography or surface area charge) are expected to be same for all contaminant exposures. Although zebrafish co-exposure to MPs and co-contaminants resulted in similar bioavailability patterns of B[a]P and Cd , based on gene expression data, the presence of MPs reduced B[a]P and Cd bioavailability by up to 60% and 25%, respectively. Thus, B[a]P appeared more strongly associated with the MPs than Cd. The different bioavailability detected could be ascribable to differences in contaminants physico-chemistry that influence sorption by MPs. In addition, the concentrations of B[a]P and Cd were different (5 µg L^{-1} and 0.24 mg L^{-1}, respectively) and each specific biomarker gene could show differences in response profile (i.e. expression of cyp1a may be more sensitive than expression of mt2). Thus, the greater sorption of B[a]P to MPs indicated by lower relative bioavailability (*cyp1a* expression) may associated to a lower concentration of B[a]P present compared to Cd. The stronger association of B[a]P with MPs compared to Cd may be dependent by polymer and contaminants physico-chemistry properties. Indeed, since most microplastic polymers found in the environment are hydrophobic structure, the hydrophobic interactions between non-polar (or slightly polar) molecules to the non-polar MP surface are considered one of the main mechanisms governing the sorption of hydrophobic organic chemicals to MPs (Wang et al., 2015; Tourinho et al., 2019). On the other hand, although plastics are generally considered inert, medium conditions and/or plastic additives can alter their surface leading to electrostatic interactions (Liao and Yang, 2020). It is known that MP surface can become negatively charged due to the pH of point of zero charge (pHpzc) being lower than most environmental pHs (Liu et al., 2018; Xu et al., 2018; Zhang et al., 2018). As a result, the negative net charges on the MP surface are likely to attract positively charged species (Tourinho et al., 2019). So, overall, electrostatic interactions may have led to Cd association with MPs albeit a predominant effect of hydrophobic interactions on electrostatic interactions could be hypothesized.

A lower bioavailability of co-contaminants to zebrafish larvae resulted after short-term B[a]P and Cd sorption to MPs with an associated biofilm. Different from expectation, B[a]P appeared more strongly associated to HDPE-MPs with biofilm than Cd. Based on previous reports, plastic biofilms in aquatic environment showed maximum metal sorption, and over time, as biofilms continuously accrue to the surface of plastic, the sorption of metals is likely continually enhanced (Tien and Chen, 2013). In addition, the presence of biofilms generally increases the charge, roughness, porosity and hydrophilicity of the plastic surface (Artham et al., 2009). As consequence, the presence of hydrophilic groups that are associated with a lower hydrophobicity, limit the enrichment of organic molecules (i.e, B[a]P) by means of hydrophobic interactions (Liu et al., 2019). Here, biofilm formation occurring in 6 days, was not enough to alter the surface of the plastic physically. However, the stronger sorption to MPs of B[a]P compared to Cd could be related to additionally interactions (such as Van der Waals forces, π-π and hydrogen bonding) promoting the sorption of organic contaminants, besides hydrophobic dominating interaction. This suggests that the bioavailability of co-contaminants sorbed to MPs cannot be predicted by co-contaminant physico-chemistry alone. Our results indicate that the sorption is influenced by biofilm sorptive properties able to promote additional interactions between sorbent (microplastic polymer) and sorbate (chemical contaminant) and to generate additional active sites for sorption of B[a]P that, consequently, reduce its bioavailability.

The presence of several pollutants simultaneously in the aquatic environment can produce interactive effects between toxicants resulting in changes in their bioavailability compared to individual exposure. It has been shown that several pollutants, usually present simultaneously in the aquatic environment, may produce either neutralizing, additive or synergistic effects, thus resulting in variable degrees of toxicity (Di Natale et al., 2019; Ragusa et al., 2017). The short-term interactive results of Cd and B[a]P co-exposure were generally in accordance with previous studies (van der Hurk et al.,2000; Costa et al., 2010; Risso-De Faverney et al. 1999). Simultaneous co-exposure of these contaminants suppressed the induction of *cyp1a* gene expression caused by B[a]P exposure alone (Sorrentino et al., 2005). As in the present study, inhibitory influences on fish MT responses have been recorded by several other investigators after B[a]P and Cd mixed exposure (Sandvick et al., 1997). These results suggest that the co-occurrence of different stressors, which simultaneously trigger different pathways, may result in gene expression profiles of *cyp1a* and *mt2* slightly related to those activated by a single contaminant at a time. Moreover, regulatory mechanisms can act differentially in protecting larvae from increased oxidative stress activated by a single contaminant (Di Natale et al., 2019).

The presence of MPs in the B[a]P and Cd mixed exposure resulted in different patterns of bioavailability between *cyp1a* and *mt2*. The expression of *cyp1a* did not highlight reduction in co-contaminants bioavailability compared to positive control. In contrast, an opposite pattern was observed for both co-contaminants bioavailability by assessment of *mt2* expression. This opposite trend, consisting in mt2 transcript induction, could be most likely be due to the different sorption capacity of the co-contaminants. The chemical fraction analysis related to both contaminants sorbed to HDPE microparticles was not used in this study, due to difficulty in detecting such low concentrations. It was not the objective of this study, to investigate the toxicity nor bioaccumulation of B[a]P, Cd and MPs or mixture toxicity of these substances. However, previous studies have demonstrated that HDPE tended to accumulate lesser concentration of metals (including Cd) than other plastic types (Rochman et al., 2014). Based on bioavailability data of this study, B[a]P may be more strongly associated with the MPs than Cd in mixture exposure. The lower sorption of Cd to MPs indicated by the higher relative bioavailability (*mt2* expression) may be a consequence of a competition mechanism occurred between both co-contaminants. Thus, by competing with metal ions for the adsorbing sites, the organic contaminant (i.e, B[a]P) may contribute to reduce on plastic polymer sorption sites available for Cd interactions. This suggests that short-term co-presence of different contaminants simultaneously may influence their bioavailability to ELS zebrafish. Therefore, unpredictable toxicological effects may occur if long-term impacts are considered.

The bioavailability pattern shown after exposure to Cd and B[a]P mixture in presence of MPs with an associated biofilm, indicated a reduced co-contaminants bioavailability compared to treatment groups without biofilm, as emerged by *cyp1a* and *mt2* gene expression data . A strong sorption of both contaminants by MPs may be suggested given the lower co-contaminants bioavailability detected by reduction in *cyp1a* expression. However, the high *mt2* induction observed may likely to be due to weak association of Cd by MP compared to B[a]P. Thus, in fish larvae co-exposed to mixture of Cd and B[a]P, biofilm presence could promote B[a]P sorption reducing the availability of sorption sites for Cd interactions. As result, a higher Cd bioavailability may have occurred. Additionally, although in individual Cd exposure the presence of an associated biofilm determined a reduction in Cd bioavailability to ELS zebrafish, co-occurrence of both contaminants differentially influenced its bioavailability. Therefore, the data herein presented suggest that when plastic materials are exposed to different mixtures of chemical pollutants the interactions between them may result in patterns of bioavailability not overlapping to those arose by a single contaminant. This means that contaminated MPs might accumulate higher amounts of several contaminants from polluted areas but, due to biofilm presence that over time continuously accrue to the surface of plastic, could become potentially bioavailable to aquatic animals in clear areas. Further analyses with high-precision chemical determination of concentrations of adhering chemicals appear necessary to definitely conclude if contaminants bioavailability through microplastics poses a significant exposure pathway in natural environments. Longer exposure times would be necessary to investigate the long-terms effect of biofilm and bioavailability of these contaminants. Moreover, since different plastic surface may have different sorption capacities for different metal ions (Li et al., 2019), an assessment of interactive effects of B[a]P with other metals on HDPE-MPs matrix could be important.

The characterization of the biofilm lining the HDPE-MPs used in this study, carried out through shotgun metagenome sequencing, showed the massive prevalence of organisms from the group of Bacteria. Consistent with previous studies (Vaksmaa et al., 2021; Basili et al., 2020; Turrini et al., 2020), *Proteobacteria* and *Actinobacteria* are the predominant phyla, both comprising bacteria with high diversity in relation of number of classes and orders. Among the microbial community, we identified several genera previously described to include hydrocarbon-degrading bacteria (HCB), dominantly belonging to the *Burkholderiales* order, some of which were very abundant reaching around 4% of all sequencing reads. These included *Mycolicibacterium*, *Nocardioides*, *Streptomyces*, *Nitrospira*, *Aquabacterium*, *Acidovorax*, *Variovorax*, *Solimonas*, *Perlucidibaca*, *Pseudomonas*, *Polaromonas*, *Limnohabitans*, *Hydrogenophaga*, *Sphingobium* and *Sphingomonas*. HCB use linear, branched, or aromatic hydrocarbons as sole energy, and carbon source and are often found in oil reservoirs, oil seeps, or oil spills (Joye et al., 2014), contaminated sediments (Kimes et al., 2013) and coal beds (Beckmann et al., 2019), but they are also found ubiquitously in the marine environment (Yakimov et al., 2007; Erni-Cassola et al., 2020; Thompson et al., 2020). HCB comprise >175 genera (Prince et al., 2010) and feature enzymes such as mono- and dioxygenases or peroxidases (Brzeszcz and Kaszycki, 2018). In principle, these might be able to attack the primary polymer structure of plastic and/or further degrade daughter products generated through weathering of the primary polymers (Wayman and Niemann, 2021). Previous studies have found HCB on different polymer types (Zettler et al., 2013; Oberbeckmann et al., 2016; Erni-Cassola et al., 2020) as well as higher abundance of them in the early stages of biofilm formation (Dussud et al., 2018; Erni-Cassola et al., 2020). Microbial degradation of PAHs depends on various environmental conditions, such as nutrients, number and kind of the microorganisms, nature as well as chemical property of the PAH being degraded. The identification of abundant hydrocarbon degraders lead to consider their potential role within biofilm into influence benzo[a]pyrene bioavailability to zebrafish larvae. The metabolic predictions inferred from shotgun metagenomic data grouped the metabolic modules with completeness above 80% in biosynthesis pathways, specifically “Biosynthesis of other secondary metabolites, “Drug resistance”, “Enediyne biosynthesis”, “Macrolide biosynthesis” and “Type II polyketide biosynthesis”. Other modules with completeness above 50% highlighted clustered in “aromatic degradation” pathway. Overall, these metabolic information could support the hypothesis that the biofilm microbial composition on HDPE-MPs includes microbial organisms able to metabolize chemical compounds, such as hydrophobic hydrocarbons. Thus, the microbial (Archaea, Bacteria, and Fungi) diversity of biofilm may using them as alternative carbon and energy sources need to support the different biosynthesis pathways.

2.6 Conclusion

Our results indicate that the biofilm associated with MPs differentially influences co-contaminants bioavailability to ELS zebrafish. Short-term B[a]P or Cd sorption to MPs reduced bioavailability of both contaminants. However, combined co-contaminants exposure showed patterns of bioavailability unpredictable and not overlapping to the bioavailability of each contaminant. A stronger MPs association of B[a]P compared to Cd occurred, especially in the presence of an associated biofilm and when mixture of both contaminants was tested. Therefore, it could be hypothesized that biofilm sorptive properties might create additional active sites promoting B[a]P sorption and higher Cd bioavailability to zebrafish larvae. The microbial community forming biofilm may play a pivotal role in affecting co-contaminants bioavailability as a wide range of microorganisms within biofilm are able to degrading hydrophobic organic compounds sorbed by serving as a nutrient source. Metabolic microbial pathways acting within biofilm may also affect the bioavailability pattern observed for organic chemical as the microbial diversity of biofilm are able to metabolize hydrophobic organic compounds by serving as a nutrient source. This demonstrates the high relevance of biofilms for the accumulation and/or removal via metabolization of plastic-associated chemicals, which may affect their bioavailability for consumers ingesting MP. In this study, the exposure scenarios applied highlighted that multilateral and additional factors as mixture of different contaminants, polymer and chemicals properties, and micro-surroundings of the organisms may act into affecting contaminants bioavailability. Here, the effects on bioavailability are attributed only to bioavailable B[a]P and Cd fractions released from the MPs and not the particles themselves, since only early-life stage and not free-feeding zebrafish were considered. This allowed to evaluate the influence of biofilm on co-contaminants bioavailability discarding the possibility to have environmentally unrealistic exposure concentration able to induce any morphological, physiological or developmental disturbances.

Differently from individual exposure, the biomarker expression patterns generated by organisms exposed to mixture of contaminants suggested that the presence of a MP-associated biofilm can affect chemicals bioavailability in a contaminant-dependent manner. At the same time, since biofilm, accruing to the surface of plastics can change their physicochemical properties, desorption process may occur over time differentially influencing co-contaminants bioavailability with unpredictable ecotoxicological effects at long-term. Overall, these factors may lead to faster uptake and release of chemicals into polymeric phase through the metabolic microbial pathway need to be considered further to predict a more realistic scenario for environment risk assessment of MP.

References

Ajith, N., Sundaramanickam, A., Parthasarathy, S., Sathish, M., Janakiraman., S., 2020. Global distribution of microplastics and its impact on marine environment—a review. Environmental Science and Pollution Research volume 27, pages 25970–25986. https://doi.org/10.1007/s11356-020-09015-5

Alessio, G., Pierluigi, S., Giulio, P., et al., 2018. Comparative effects of ingested PVC micro particles with and without adsorbed benzo(a)pyrene vs. spiked sediments on the cellular and sub cellular processes of the benthic organism *Hediste diversicolor*. Front. Mar. Sci. 5, 99–111. https://doi.org/10.3389/fmars.2018.00099

Anderson, A.G., Grose, J., Pahl, S., Thompson, R.C., Wyles, K.J., 2016. Microplastics in personal care products: exploring perceptions of environmentalists, beauticians and students. Mar. Pollut. Bull. 113, 454-460. https://doi.org/10.1016/j.marpolbul.2016.10.048

Artham, T., Sudhakar, M., Venkatesan, R., Nair, C.M., Murty, K.V.G.K., & Doble, M. 2009. Biofouling and stability of synthetic polymers in sea water. Int Biodeter Biodegr. 63: 884-90. https://doi.org/10.1016/j.ibiod.2009.03.003

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to mussels. Environ. Pollut. 198, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021

Bakir, A., Rowland, S.J., Thompson, R.C., 2012. Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar. Pollut. Bull. 64, 2782–2789. https://doi.org/10.1016/j.marpolbul.2012.09.010

Basili, M., Quero, G. M., Giovannelli, D., Manini, E., Vignaroli, C., Avio, C. G., De Marco, R., Luna, G. M., 2020. Major Role of Surrounding Environment in Shaping Biofilm Community Composition on Marine Plastic Debris. Frontiers in Marine Science, 7. https://www.frontiersin.org/article/10.3389/fmars.2020.00262

Batel, A., Linti, F., Scherer, M., Erdinger, L., Braunbeck, T., 2016. The transfer of benzo [a]pyrene from microplastics to Artemia nauplii and further to zebrafish via a trophic food web experiment - cyp1a induction and visual tracking of persistent organic pollutants. Environ. Toxicol. Chem. 35 (7), 1656-1666. https://doi.org/10.1002/etc.3361

Batel, A., Borchert, F., Reinwald, H., Erdinger, L., Braunbeck, T., 2018. Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (Danio rerio) gills and zebrafish embryos. Environ. Pollut. 235, 918–930. https://doi.org/10.1016/j.envpol.2018.01.028

Beckmann, S., Luk, A. W. S., Gutierrez-Zamora, M.-L., Chong, N. H. H., Thomas, T., Lee, M., et al., 2019. Long-term succession in a coal seam microbiome during in situ biostimulation of coalbed-methane generation. ISME J. 13, 632–650. http://doi.org/10.1038/s41396-018-0296-5

Beiras, R., Bellas, J., Cachot, J., et al., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard. Mater. 360, 452–460. https://doi.org/10.1016/j.jhazmat.2018.07.101

Boyle, D., Catarino, A. I., Clark, N. J., Henry, T. B., 2020. Polyvinyl chloride (PVC) plastic fragments release Pb additives that are bioavailable in zebrafish. Environmental Pollution 263, 114422. https://doi.org/10.1016/j.envpol.2020.114422

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T. et al., 2011. Accumulation of microplastic on shorelines worldwide: sources and sinks. Environ Sci Technol 45:9175–9179. https://doi.org/10.1021/es201811s

Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr. Biol. 23, 2388-2392. https://doi.org/10.1016/j.cub.2013.10.012

Brzeszcz, J., and Kaszycki, P., 2018. Aerobic bacteria degrading both n-alkanes and aromatic hydrocarbons: an undervalued strategy for metabolic diversity and flexibility. Biodegradation 29, 359–407. http://doi.org/10.1007/s10532-018-9837-x

Carpenter, E. J., Smith, K. L. Jr, 1972. Plastics on the Sargasso sea surface. Science 175:1240–1241. DOI: 10.1126/science.175.4027.1240

Catarino, A.I., Macchia, V., Sanderson, W.G., Thompson, R.C., Henry, T.B., 2018. Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres during a meal. Environ. Pollut. 237, 675-684. https://doi.org/10.1016/j.envpol.2018.02.069

Chen, Q., Yin, D., Jia, Y., Schiwy, S., Legradi, J., Yang, S., Hollert, H., 2017. Enhanced uptake of BPA in the presence of nanoplastics can lead to neurotoxic effects in adult zebrafish. The Science of the total environment vol. 609 (2017): 1312-1321. http://doi.org/10.1016/j.scitotenv.2017.07.144

Chua, E. M., Shimeta, J., Nugegoda, D., Morrison, P. D., Clarke, B. O., 2014. Assimilation of Polybrominated Diphenyl Ethers from Microplastics by the Marine Amphipod, *Allorchestes Compressa*. Environ. Sci. Technol. 48, 8127– 8134. https://doi.org/10.1021/es405717z

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

Cormier, B., Batel, A., Cachot, J., Bégout, M., 2019. Multi-Laboratory Hazard Assessment of Contaminated Microplastic Particles by Means of Enhanced Fish Embryo Test With the Zebrafish (*Danio rerio*). 7. pp. 1–14. https://doi.org/10.1002/wer.1203

Costa, P.M., et al., 2010. Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]Pyrene. Environ. Pollut. 158 (10), 3338-3346. https://doi.org/10.1016/j.envpol.2010.07.030

De Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple species: proposed methodologies. Environ. Toxicol. Chem. 24, 2665-2672. https://doi.org/10.1897/04-639R.1

Di Natale, M., Bennici, C., Biondo, G., Masullo, T., Monastero, C., Tagliavia, M., Torri, M., Costa, S., Ragusa, M. A., Cuttitta, A., Nicosia, A., 2019. Aberrant gene expression profiles in Mediterranean sea urchin reproductive tissues after metal exposures. Chemosphere 216:48-58. https://doi.org/10.1016/j.chemosphere.2018.10.137

Dussud, C., Meistertzheim, A. L., Conan, P., Pujo-Pay, M., George, M., Fabre, P., et al., 2018. Evidence of niche partitioning among bacteria living on plastics, organic particles and surrounding seawaters. Environ. Pollut. 236, 807–816. http://doi.org/10.1016/j.envpol.2017.12.027

Eerkes-Medrano, D., Thompson, R. C., Aldridge, D. C., 2015. Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritization of research needs. Water Res. May 15;75:63-82. https://doi.org/10.1016/j.watres.2015.02.012

Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9, e111913. https://doi.org/10.1371/journal.pone.0111913

Eriksen, T.H., Schober, E., 2017. Waste and the Superfluous: an Introduction. Soc. Anthropol. 25 (3), 285–287. https://doi.org/10.1371/journal.pone.0111913

Erni-Cassola, G., Wright, R. J., Gibson, M. I., and Christie-Oleza, J. A., 2020. Early colonization of weathered polyethylene by distinct bacteria in marine coastal seawater. Microb. Ecol. 79, 517–526. http://doi.org/10.1007/s00248-019-01424-5

Flemming, H.-C., 1995. Sorption sites in biofilms. Water Sci. Technol., 32, 27–33. https://doi.org/10.1016/0273-1223(96)00004-2

Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., Nadal, A., Romano, D., 2018. Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preven- tive measures. Environ. Sci. Eur. 30, 13. https://doi.org/10.1186/s12302-018-0139-z

Galloway, T.S., Lewis, C.N., 2016. Marine microplastics spell big problems for future generations. P. Natl. Acad. Sci. USA 113, 2331-2333. https://doi.org/10.1073/pnas.1600715113

Gaylor, M.O., Harvey, E., Hale, R.C., 2012. House crickets can accumulate polybrominated diphenyl ethers (PBDEs) directly from polyurethane foam common in consumer products. Chemosphere 86, 500–505. https://doi.org/10.1016/j.chemosphere.2011.10.014

Goldstein, M. C., Rosenberg, M., Cheng, L., 2012. Increased oceanic microplastic debris enhances oviposition in an endemic pelagic insect. Biology Letters 8: 817– 820. doi: 10.1098/rsbl.2012.0298

Henry, T.B., McPherson, J.T., Rogers, E.D., Heah, T.P., Hawkins, S.A., Layton, A.C., Sayler, G.S., 2009. Changes in the relative expression pattern of multiple vitellogenin genes in adult male and larval zebrafish exposed to exogenous estrogens. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 154, 119–126. https://doi.org/10.1016/j.cbpa.2009.05.009

Henry, T.B., Wileman, S.J., Boran, H., Sutton, P., 2013. Association of Hg^{2+} with aqueous $(C_{60})n$ aggregates facilitates increased bioavailability of Hg2þ in zebrafish (*Danio rerio*). Environ. Sci. Technol. 47, 9997-10004. https://doi.org/10.1021/es4015597

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., Duflos, G., 2017. Occurrence and effects of plastic additives on marine environments and organisms: A review, Chemosphere. https://doi.org/10.1016/j.chemosphere.2017.05.096

Herzke, D., Anker-Nilssen, T., Nøst, T.H., Götsch, A., Christensen-Dalsgaard, S., Langset, M., Fangel, K., Koelmans, A.A., 2016. Negligible impact of ingested microplastics on tissue concentrations of persistent organic pollutants in northern fulmars off coastal Nor- way. Environ. Sci. Technol. 50:1924–1933. http://dx.doi.org/10.1021/acs.est.5b04663

Holmes, L.A., Turner, A., Thompson, R.C., 2012. Adsorption of trace metals to plastic resin pellets in the marine environment. Environ. Pollut. 160:42–48. http://dx.doi.org/10. 1016/j.envpol.2011.08.052.

Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E., Svendsen, C., 2017. Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci. Total Environ. 586, 127–141. https://doi.org/10.1016/j.marpolbul.2016.09.004

Imhof, H.K., Ivleva, N.P., Schmid, J., Niessner, R., Laforsch, C., 2013. Contamination of beach sediments of a subalpine lake with microplastic particles. Curr. Biol. 23, R867-R868. https://doi.org/10.1016/j.cub.2013.09.001

Jahnke, A., Arp, H.P.H., Escher, B.I., Gewert, B., Gorokhova, E., Kühnel, D., Ogonowski, M., Potthoff, A., Rummel, C., Schmitt-Jansen, M., Toorman, E., MacLeod, M., 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ. Sci. Technol. Lett. 4, 85e90. https://doi.org/10.1021/acs.estlett.7b00008

Joye, S. B., Teske, A. P., and Kostka, J. E., 2014. Microbial dynamics following the Macondo oil well blowout across the Gulf of Mexico environments. BioScience 64, 766–777. http://doi.org/10.1093/biosci/biu121

Kais, B., Schneider, K.E., Keiter, S., Henn, K., Ackermann, C., Braunbeck, T., 2013. DMSO modifies the permeability of the zebrafish (*Danio rerio*) chorion- implications for the fish embryo test (FET). Aquat. Toxicol. 140-141, 229-238. https://doi.org/10.1016/j.aquatox.2013.05.022

Kesy, K., Oberbeckmann, S., Kreikemeyer, B., et al., 2019. Spatial environmental heteroge- neity determines young biofilm assemblages on microplastics in Baltic Sea mesocosms. Front. Microbiol. 10, 1–18. DOI: 10.1016/B978-0-12-803581-8.11722-9

Khan, F.R., Syberg, K., Shashoua, Y., Bury, N.R., 2015. Influence of polyethylene microplastic beads on the uptake and localization of silver in zebrafish (*Danio rerio*). Environ. Pollut. 206, 73-79. https://doi.org/10.1016/j.envpol.2015.06.009

Kimes, N. E., Callaghan, A. V., Aktas, D. F., Smith, W. L., Sunner, J., Golding, B. T., et al., 2013. Metagenomic analysis and metabolite profiling of deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. Front. Microbiol. 4:50. http://doi.org/10.3389/fmicb.2013.00050

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

Le Bihanic, F., Clérandeau, C., Cormier, B., Crebassa, J.C., Keiter, S.H., Beiras, R., Morin, B., Bégout, M.-L., Cousin, X., Cachot, J., 2020. Organic contaminants sorbed to microplastics affect marine medaka fish early life stages development. Mar. Pollut. Bull. 154, 111059. https://doi.org/10.1016/j.marpolbul.2020.111059

Lee, H., Shim, W. J., Kwon, J. H., 2014. Sorption capacity of plastic debris for hydrophobic organic chemicals. Sci. Total Environ., 470, 1545–1552. https://doi.org/10.1016/j.scitotenv.2013.08.023

Liu, X., Zheng, M., Wang, L., Ke, R., Lou, Y., Zhang, X., Dong, X., Zhang, Y., 2018. Sorption behaviors of tris-(2,3-dibromopropyl) isocyanurate and hexabromocyclododecanes on polypropylene microplastics. Mar. Pollut. Bull. 135, 581–586. https://doi.org/10.1016/j.marpolbul.2018.07.061

Li, X., Mei, Q., Chen, L., Zhang, H., Dong, B., Dai, X., He, C., Zhou, J., 2019. Enhancement in adsorption potential of microplastics in sewage sludge for metal pollutants after the wastewater treatment process. Water Res. 157, 228–237. https://doi.org/10.1016/j.watres.2019.03.069

Liao, Y.L., Yang, J.Y., 2019. Microplastic serves as a potential vector for Cr in an in-vitro human digestive model. Sci. Total Environ. 703, 134805. https://doi.org/10.1016/j.scitotenv.2019.134805

Liu, X., Xu, J., Zhao, Y., Shi, H., Huang, C.H., 2019. Hydrophobic sorption behaviors of 17Β- estradiol on environmental microplastics. Chemosphere 226, 726–735. https://doi.org/10.1016/j.chemosphere.2019.03.162

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25, 402–408. https://doi.org/10.1006/meth.2001.1262

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

Lu, K., Qiao, R., An, H., Zhang, Y., 2018. Influence of microplastics on the accumulation and chronic toxic effects of cadmium in zebrafish (Danio rerio). Chemosphere 202, 514–520. https://doi.org/10.1016/j.chemosphere.2018.03.145

Oberbeckmann, S., Osborn, A. M., and Duhaime, M. B., 2016. Microbes on a bottle: substrate, season and geography influence community composition of microbes colonizing marine plastic debris. PLoS One 11:e0159289. http://doi.org/10.1371/journal.pone.0159289

Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby Pomatoschistus microps (Teleostei, Gobiidae). Ecol. Indicat. 34, 641e647. https://doi.org/10.1016/j.ecolind.2013.06.019

Paul-Pont, I., et al., 2018. Constraints and priorities for conducting experimental exposures of marine organisms to microplastics. Front. Mar. Sci. 5. https://doi.org/10.3389/fmars.2018.00252

Peeken, I., Primpke, S., Beyer, B., Gütermann, J., Katlein, C., Krumpen, T., Bergmann, M., Hehemann, L., Gerdts, G., 2018. Arctic Sea Ice Is an Important Temporal Sink and Means of Transport for Microplastic. Nat. Commun., 9, 1505. doi:10.1038/s41467-018-03825-5

Pittura, L., Avio, C.G., Giuliani, M.E., d'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the mediterranean mussels, *Mytilus galloprovincialis*. Front. Mar. Sci. 5, 103. https://doi.org/10.3389/fmars.2018.00103

Prince, R. C., Gramain, A., and Mcgenity, T. J., 2010. “Prokaryotic hydrocarbon degraders,” in Handbook of Hydrocarbon and Lipid Microbiology, ed. K. N. Timmis (Berlin: Springer Berlin Heidelberg), 1669–1692. http://doi.org/10.1007/978-3-540-77587-4_118

Qiao, R.X., Lu, K., Deng, Y.F., et al., 2019. Combined effects of polystyrene microplastics and natural organic matter on the accumulation and toxicity of copper in zebrafish. Sci. Total Environ. 682, 128–137. https://doi.org/10.1016/j.scitotenv.2019.05.163

Ragusa, M.A., Costa, S., Cuttitta, A., Gianguzza, F., Nicosia, A., 2017b. Coexposure to sulfamethoxazole and cadmium impairs development and attenuates transcriptional response in sea urchin embryo. Chemosphere, 180, 275–284, https://doi.org/10.1016/j.chemosphere.2017.04.030

Reisser, J., Shaw, J., Hallegraeff, G., Proietti, M., Barnes, D. K. A., Thums, M., et al., 2014. Millimeter-Sized Marine Plastics: A New Pelagic Habitat for Microorganisms and Invertebrates. PLoS ONE 9(6): e100289. https://doi.org/10.1371/journal.pone.0100289

Risso-De Faverney, C., De Sousa, G., Lafaurie, M., Rahmani, R., 1999. Expression and induction of CYP1A1 in black seabream (*Spondyliosoma Cantharus*) hepatocyte cultures: effects of heavy metals. Polycycl. Aromat. Comp. 13 (3), 285-299. https://doi.org/10.1080/10406639908020551

Rochman, C.M., Hentschel, B.T., Teh, S.J., 2014. Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. PLoS One 9, e85433. https://doi.org/10.1371/journal.pone.0085433

Rummel, C.D., Jahnke, A., Gorokhova, E., Kühnel, D., Schmitt-Jansen, M., 2017. Im- pacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. Environ. Sci. Technol. Lett. 4 (7), 258-267. https://doi.org/10.1021/acs.estlett.7b00164

Sandvik, Morten, et al., 1997. Interaction of benzo[a]pyrene, 2,3,30 ,4,40 ,5- hexachlorobiphenyl (PCB-156) and cadmium on biomarker responses in flounder (*Platichthys flesus L*.). Biomarkers 2 (3), 153-160. https://doi.org/10.1080/135475097231689

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. Environ. Sci. Technol. 38, 228A–231A. https://doi.org/10.1021/es040548w

Senathirajah, K., Attwood, S., Bhagwat, G., Carbery, M., Wilson, S., Palanisami, T.,2021. Estimation of the mass of microplastics ingested – A pivotal first step towards human health risk assessment. Journal of Hazardous Materials, Volume 404, Part B. https://doi.org/10.1016/j.jhazmat.2020.124004

Sleight, V.A., Bakir, A., Thompson, R.C., Henry, T.B., 2017. Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar. Pollut. Bull. 116, 291e297. https://doi.org/10.1016/j.marpolbul.2016.12.055

Sorrentino, C., Roy, N.K., Courtenay, S.C., Wirgin, I., 2005a. Co-exposure to metals modulates CYP1A MRNA inducibility in atlantic tomcod Microgadus tomcod from two populations. Aquat. Toxicol. 75 (3), 238-252. https://doi.org/10.1016/j.aquatox.2005.08.006

Souza, I. C., Duarte, I. D., Pimentel, N. Q., Rocha, L. D., Morozesk, M., Bonomo, M. M., Azevedo, V. C., Pereira, C. D. S., Monferrán, M. V., Milanez, C. R. D., Matsumoto, S. T., Wunderlin, D. A., Fernandes, M. N., 2013. Matching metal pollution with bioavailability, bioaccumulation and biomarkers response in fish (*Centropomus parallelus*) resident in neotropical estuaries. Environ Pollut 180:136–144. https://doi.org/10.1016/j.envpol.2013.05.017

Thompson, H. F., Summers, S., Yuecel, R., and Gutierrez, T., 2020. Hydrocarbon-degrading bacteria found tightly associated with the 50-70 µm Cell-size population of eukaryotic phytoplankton in surface waters of a Northeast Atlantic Region. Microorganisms 8:1955. http://doi.org/10.3390/microorganisms8121955

Tourinho, P.S., Kočí, V., Loureiro, S., van Gestel, C.A.M., 2019. Partitioning of chemical con- taminants to microplastics: sorption mechanisms, environmental distribution and effects on toxicity and bioaccumulation. Environ. Pollut. 252, 1246–1256. https://doi.org/10.1016/j.envpol.2019.06.030

Trevisan, R., Voy, C., Chen, S. X., and Di Giulio, R. T., 2019. Nanoplastics decrease the toxicity of a complex PAH mixture but impair mitochondrial energy production in developing zebrafish. Environ. Sci. Technol. 53, 8405–8415. https://doi.org/10.1021/acs.est.9b02003

Turrini, P., Tescari, M., Visaggio, D., Pirolo, M., Lugli, G.A., Ventura, M., Frangipani, E., Visca, P., 2020. The microbial community of a biofilm lining the wall of a pristine cave in Western New Guinea
Microbiol. Res., 241, Article 126584. https://doi.org/10.1016/j.micres.2020.126584

van den Hurk, P., Faisal, M., Roberts Jr., M.H., 2000. Interactive effects of cadmium and benzo[a]pyrene on metallothionein induction in mummichog (*Fundulus heteroclitus*). Mar. Environ. Res. 50, 83-87. https://doi.org/10.1016/S0141-1136(00)00098-2

Vaksmaa, A., Knittel, K., Abdala, A.A., Goudriaan, M., Ellrott, A., Witte, H.J., Vollmer, I., Meirer, F., Lott, C., Weber, M., Engelmann J.C., Niemann, H., 2021. Microbial Communities on Plastic Polymers in the Mediterranean Sea. Frontiers in Microbiology, 12 https://www.frontiersin.org/article/10.3389/fmicb.2021.673553

Wagner, M., Scherer, C., Alvarez-Munoz, D., Brennholt, N., Bourrain, X., Buchinger, S., Fries, E., Grosbois, C., Klasmeier, J., Marti, T., Rodriguez-Mozaz, S., Urbatzka, R., Vethaak, A., Winther-Nielsen, M., Reifferscheid, G., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Environ. Sci. Eur, 26, 12. https://doi.org/10.1186/s12302-014-0012-7

Wang, F., Shih, K.M., Li, X.Y., 2015. The partition behavior of per- fluorooctanesulfonate (PFOS) and perfluorooctanesulfonamide (FOSA) on microplastics. Chemosphere 119, 841e847. https://doi.org/10.1016/j.chemosphere.2014.08.047

Wayman, C., and Niemann, H., 2021. The fate of plastic in the ocean environment – a minireview. Environ. Sci. Process. Impact. 23, 198–212. http://doi.org/10.1039/d0em00446d

Wen, G., Kotzsch, S., Vital, M., Egli, T., Ma, J., 2015. BioMig - A Method to Evaluate the Potential Release of Compounds from and the Formation of Biofilms on Polymeric Materials in Contact with Drinking Water. Environ. Sci. Technol. 2015, 49, 11659–69. https://doi.org/10.1021/acs.est.5b02539

Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol., 23, R1031–R1033. https://doi.org/10.1016/j.cub.2013.10.068

Writer, J. H.; Ryan, J. N.; Barber, L. B. Role of Biofilms in Sorptive Removal of Steroidal Hormones and 4-Nonylphenol Compounds from Streams. Environ. Sci. Technol. 2011, 45, 7275− 7283. https://doi.org/10.1021/es2008038

Xu, B., Liu, F., Brookes, P.C., Xu, J., 2018. The sorption kinetics and isotherms of sulfamethoxazole with polyethylene microplastics. Mar. Pollut. Bull. 131, 191–196. https://doi.org/10.1016/j.marpolbul.2018.04.027

Yakimov, M. M., Timmis, K. N., and Golyshin, P. N., 2007. Obligate oil-degrading marine bacteria. Curr. Opin. Biotechnol. 18, 257–266. https://doi.org/10.1016/j.copbio.2007.04.006

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “plastisphere”: microbial communities on plastic marine debris. Environ. Sci. Technol. 47 (13), 7137-7146. https://doi.org/10.1021/es401288x

Zhang, X., Zheng, M., Wang, L., Lou, Y., Shi, L., and Jiang, S., 2018. Sorption of three synthetic musks by microplastics. Mar. Pollut. Bull. 126, 606–609. https://doi.org/10.1016/j.marpolbul.2017.09.025

Zhang, R., Wang, M., Chen, X., Yang, C., Wu, L., 2020. Combined toxicity of microplastics and cadmium on the zebrafish embryos (*Danio rerio*). Sci. Total Environ. 743, 140638. https://doi.org/10.1016/j.scitotenv.2020.140638

Ziccardi, L. M., Edgington, A,, Hentz, K., Kulacki, K. J., Kane, Driscoll, S., 2016. Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. Environ Toxicol Chem., Jul; 35(7):1667-76. PMID: 27093569. https://doi.org/10.1002/etc.3461

Chapter 3: How artificially aging influences bioavailability of metals and BDE-47 by microplastics on *P. lividus* embryos: a dual approach

3.1 Abstract

A potential environmental risk of MicroPlastics (MPs) is associated with their ability to interact with diverse environmental chemicals. Once reached marine environment, MPs are affected by several biotic and abiotic factors, which may alter their surface structure as well their sorption behavior. Aging process plays a key role in influencing the interactions with environmental chemicals and adds complexity to the vector role of MPs. The present study aimed to improve understanding of the sorption of different type of chemicals by microplastics and to clarify the importance of MP-mediated contaminant transfer exposing organisms to significant ecotoxicological risk, especially when more realistic conditions (i.e. mixture of chemicals and aging conditions), are simultaneously considered. Two different polymers - polystyrene microparticles (PS) and polyamide fibers (PA) – and two different kinds of environmental pollutants - 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and metal ions (Cd and Cu) alone or in mixture – have been used as sorbent and sorbate. A dual approach integrating chemical and molecular investigations was followed to investigate the influence of artificially aging (via seawater soaking and UV radiation) on the sorption of these model contaminants and to provide estimations of the role of MPs on the bioavailable fractions of these compounds. A high decrease of molecular weight for both polymers emerged after photo-oxidation process. The effects of aging on sorption process of contaminants by MPs were found to depend by contaminant type and, in a lesser way, by polymer type. No change in the association with Cd for polymers have been detected. On the other hand, a strong release of Cu but not BDE-47 was observed for both polymers tested after photo-exposure. In addition, a competition mechanism affecting the sorption of Cu by PS microparticles but not PA fibers in the blend Cu/BDE-47 have been also observed. The evaluation of the effects of these contaminants in combined exposures with microplastics did not highlighted specific morphological effects on *P. lividus* embryos; however, significant changes in gene expression of members belonging to stress response pathway and skeletogenic and endodermal Gene Regulatory Networks (GRNs) were observed. Changes in the interactions between sorbent and sorbate occurred after aging process led to establishment of molecular mechanisms perturbing the canonical developmental program as well as the oxidative status. This study provides insights to shed light on mechanisms and the potential hazards of MPs in marine environments after photodegradation

3.2 Introduction

The distribution of microplastics (MPs) - plastic particles with size ranging from 1 µm to 5 mm - in the marine environment has received an increasing attention over recent years. The widespread distribution in several areas of worldwide (including oceans, rivers, lakes, sediments and even in Artic sea ice) (De la Torre et al., 2020; Zhao et al., 2020) together with their small size, makes microplastics readily bioavailable to marine biota. As consequence, MPs can be considered an issue to ecosystems and to human health (Hodson et al., 2017; Vethaak et al., 2016). Microplastics can affect species of marine organisms with different feeding strategies, from suspension and filter-feeders to deposit feeders (Cole et al., 2013; Wright et al., 2013). However, their potential ecological risk is not only related to MPs ingestion process but also to chemical pollutants adsorbed on them (Nobre et al., 2015). Microplastics are widely known to interact with several kind of organic pollutants present in environments (such as polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), pesticides and pharmaceuticals as well as metal ions (Brennecke et al., 2016; Camacho et al., 2019). Sorption of chemicals onto MPs has led to considered them as vectors of bioavailable hazardous chemicals into aquatic biota (Teuten et al., 2009; Hirai et al., 2011) as emerged from studies reporting bioaccumulation of chemicals in tissues of marine organisms in addition to xenobiotic toxicity (Avio et al., 2015; Batel et al., 2018). However, studies suggested a negligible vector role for MPs, since chemical bioaccumulation from a MP-mediated chemical exposure pathway is very low compared to other exposure pathways, such as uptake via food, suspended organic material or aqueous exposure (Gouin et al., 2011; Koelmans et al., 2016, 2013). Although the contribute of plastic to chemical distribution and accumulation in aquatic environment seems to be of minor importance (Koelmans et al., 2016), they become relevant in the areas with increasing load of MPs or in MPs hot spots; therefore, the question on whether MPs will significantly alter the impacts of pollutants on organisms remains unclear.

The knowledge of data regarding the interactions and the sorption mechanisms occurring between the sorbent (microplastic polymer) and sorbate (chemical contaminant) is crucial to improve our understanding on MP-mediated contaminant transfer as well as the impact of MPs on the marine environment. Partitioning of environmental chemicals into MPs is highly dependent on the physical and chemical properties of polymer matrix, such as polymer type, crystallinity, functional groups as well as surface area, size and molar masses distribution (Torres et al., 2021). For example, small plastic particles having a high surface area, tend to have an higher sorption coefficient (K_d) than larger ones as emerged in studies on the sorption behavior of organic compounds on polystyrene (PS) particles that differed from size (Wang et al., 2019; Li et al., 2019). Plastic polymers with higher crystallinity, such as PS, make contaminant access into the plastic more difficult than amorphous ones, such as polypropylene (PP) and polyethylene (PE), thus favoring the interaction of chemicals onto their surface (adsorption process) rather than into the matrix (absorption process) (Zhao et al., 2020; Rodrigues et al., 2019). Also, peculiar properties of sorbate, including hydrophobicity and functional groups, influence chemical sorption on plastic polymer as well as desorption processes, governing the influx of chemical contaminants in aquatic biota (Mei et al., 2020).

To date, most studies on sorption of chemical pollutants to MPs were focused mainly on virgin plastic particles (Zhang et al., 2019; Wang and Wang, 2018; Xu et al., 2018). When MPs are in virgin state, their hydrophobic surface interacts with several hydrophobic organic pollutants (POPs). However, MPs in natural environments undergo weathering processes and biofoulant attack that over time may lead to a progressive degradation of plastic polymers and affect their sorption capacity as well as their behavior in aquatic environments (Velez et al., 2018). The dominant cause of degradation of plastics outdoors is solar UV radiation, which causes a sequence of chemical reactions involving free radicals generation by breaking of C - H bonds inside the chains of the polymers and oxygen-containing functional groups increase (i.e. hydroxyl and carbonyl groups) (Andrady 2011; Celina, 2013). Consequently, the interaction between free radicals with oxygen, lead to creation of new functional groups on plastic surface, decreasing their hydrophobicity and reducing the potential retention of hydrophobic chemicals (Endo et al., 2005; Alimi et al., 2018). However, during advanced stages of degradation and under oxidizing conditions, plastic polymers experience molecular modifications due to cross-linking reactions and/or chain scissions. The occurrence of these processes whose mechanisms depends on the polymer nature, can lead to macromolecular fragmentation in particles having lower molecular weight, with consequent higher surface area prone for chemicals sorption (Frias et al., 2010; Rodrigues et al., 2019). Studies focused on the effect of UV aging on sorption of POPs highlighted how not all polymers have the same resistance to weathering depending on their intrinsic characteristics but also on chemicals characteristics. For example, aging of PP had no effect on benzene, toluene, ethyl benzene and xylene sorption (BTEX) compared to aged PS, for which a decreased sorption has been observed (Fisner et al., 2017). Some other studies reported higher phenanthrene and difenoconazole sorption on weathered PP pellets (Karapanagioti and Klontza, 2008; Geodecke et al., 2017).

Currently, the uptake and distribution of chemicals onto virgin and aged plastic particles is increasingly being investigated, although few studies have focused on their transfer and toxicity in aquatic organisms (Bejgarn et al., 2015; Rochman et al., 2014). From an ecotoxicological point of view, the portion of compounds that can be desorb from plastics represents the bioaccessible fraction able to determine a potential ecotoxicity (Ortega-Calvo et al., 2015). Since this accessible fraction may undergo alterations due to aging of plastic particles, more data is required on the bioaccessible fraction of co-contaminants to organisms, especially when complex mixtures formed by toxicants and MPs are considered. Among hydrophobic organic chemicals found sorbed to MPs, polybrominated diphenyl ethers (PBDEs) commonly used as flame retardant in several products, including electronic appliances, textiles, plastics and many other consumer goods (Hites, 2004), are the most detected in the environment and commercially produced POPs (Guan et al., 2007; Hassanin et al., 2004). Likewise, other organic pollutants, having a relative high log K_{0w}, PBDEs show a greater affinity for

hydrophobic surface of plastics compared to seawater, establishing with them hydrophobic interactions. PBDEs could have several negative impacts on aquatic invertebrates, including neurotoxicity, genotoxicity, developmental defects and endocrine disrupting properties (Breitholtz and Wollenberger, 2003; Díaz-Jaramillo et al., 2016). Studies focused on evaluating the concentration of PBDEs sorbed to microplastics from seawater or other matrices showed that these were mostly composed of lower-brominated PBDEs (Guan et al., 2007; Hassanin et al., 2004; Yeo et al., 2020), especially BDE-47, the main congener detected in biological samples and in marine environments (Ge et al., 2018; Shao et al., 2018). Although it has been shown that MPs and PBDEs can change the dynamics of bioaccumulation of different PBDEs congeners (Chua et al., 2014), to date, no studies reported the effects of co-exposure to these pollutants, individually and in combination, with respect to transcriptional profile response of specific genes. Likewise, organic compounds, also MPs pollution and their interactions with heavy metal (HMs) ions have gained global concern (Guo and Wang, 2020). Several studies reported interactions between the different types of polymers (i.e. PE, PP, PS, PA, PVC) and metals (such as Cr, Co, Ni, Cu, Zn, Cd, Pb, Ag and Hg). It seems that the lack of surface modifications on the original/virgin MPs would make their sorption negligible (Tang et al., 2020) in comparison to beached/eroded/weathering MPs (Turner and Holmes, 2015). Several laboratory studies showed a sorption capacity of Cd, Pb, Cu, and Zn ions onto the aged MPs several times higher than virgin ones (Guo and Wang, 2020; Brennecke et al., 2016; Bandow et al., 2017). Thus, the influence of aging on metals sorption process should be considered. To date, what properties (size, shape, or type) of MPs enhances or mitigates the impact of HMs to test organisms appears unclear. Studies distinguishing between the effects of the aged/real synthetic polymer itself and related sorbed chemicals are still scarce (Huang et al., 2021, Cao et al., 2021).
The findings on toxic effects arising from MPs focus on organisms able to ingest MPs with co-contaminants (Koelmans et al., 2016, Oliveira et al., 2012), there is missing information about organisms unlikely to ingest them but have them in surroundings. The use of planktonic early life stages to evaluate the chemical toxicity associated to pollutants sorbed onto MPs is important to help in the understanding of the impacts on critical biological processes such as larval development, which could change the structure of populations and communities. In light of this, the echinoderm Early Life Stages (ELSs) are generally recognized as the most vulnerable phases for sea urchin life cycle and extremely sensitive to chemical pollutants, including organic toxicants and HMs (Pagano et al., 2017; Masullo et al., 2020; Anselmo et al., 2011). Thus, their regular development process is frequently used to evaluate the effects of many contaminants and for an assessment of pollution in marine environment.

The aim of this study was to investigate the influence of artificially aging of two different type of virgin plastic polymers on sorption of two model contaminants. Polyamide 6.6 - PA66 - microfibers and polystyrene microspheres (PS), polybrominated diphenyl ethers – PBDEs - congener 2,2′,4,4′-tetrabromodiphenyl ether - BDE-47- and trace metals such as Cadmium - Cd- and Copper - Cu have been used. Consequently, their bioavailability in *Paracentrotus lividus* embryos was analyzed. The sorption capacities and behavior of these chemicals on virgin and aged MPs have been evaluated through a chemical approach. A molecular approach has been used to unveil possible variations in the transcriptional profile of members of the *P. lividus* Gene Regulatory Networks (GRN) involved in skeletogenesis (*sm50, p16, msp130*) and endo-mesodermal specification (*foxa, hox11/13b*). In addition, stress response genes (*hsp70, hsp60*) and metal scavenging (*mt4*) at 24 h post- fertilization (hpf) corresponding to the blastula/gastrula stage were investigated. After exposure, the morphology of embryos was also analyzed to evaluate developmental defects and skeletal malformations induced by co-contaminants available fraction. In addition, since the simultaneous exposure of organisms to different contaminants may result in variable levels of toxicity due to neutralizing, additive or synergistic effects (Ragusa et al., 2017b; Wah Chu and Chow, 2002), mixture of the selected co-contaminants were also explored.

3.3 Materials and methods

3.3.1 Overview of study

Two series of experiments were carried out to investigate how the presence of two types of MPs, PS microparticles (Experiment 1) and PA fibers (Experiment 2), both virgin and artificially aged, modify acute effects of three different environmental pollutants Cd, Cu and BDE-47 on the larvae of the sea urchin *P. lividus*. The acute effects were measured by differential gene expression profile of sea urchin embryos, whilst differences in sorption of trace metals as well as organic chemicals to MPs aged or non-aged were measured in aqueous phase using ICP-MS and GC-MS, respectively.

3.3.2 Microplastics and chemicals

This study was carried out using virgin plastic polymers to simplify the matrix of contaminant adsorption. Polystyrene (PS) microparticles were selected in this study both for their high occurrence in marine ecosystems and for the high sorbing capacity of hydrophobic chemicals as well as metal ions (Ziccardi et al., 2016). Whilst Polyamide (PA) or Nylon 66 fibers were chosen not only for their widely distribution in natural environments but also for their high susceptibility to degradation and weathering in the natural environment (Thomas and Hridayanathana, 2006). Virgin (additive free) PS microplastics (hereafter PS-MP) with particle size ranged from 1 to 1.49 µm (cat. N° PP10-10-100) were purchased from Spherotech Inc. (IL-USA). PA 66 fibers, having size from 300 µm until to ~566 µm, were kindly gifted from Dr Mariacristina Cocca of Institute for Polymers, Composites and Biomaterials, National Research Council of Italy (Naples). A list with some characteristics of plastic materials used in this study is showed in Table 3.1 (MPs materials).

Table 3.1: Details of microplastic materials used in the experiments

MP material	Supplier	Polymer Type	Form	Size (µm)	Density (g/cm³)
PS	Spherotech Inc.	Polystyrene microspheres	Aqueous (10% w/v)	1 – 1.49	1.05
PA 66	IPCB-CNR	Polyamide fibers	Solid	566	1.31

Analytical grade cadmium and copper chloride (99,9 % purity) purchased from Sigma-Aldrich (Germany) were used as cadmium and copper sources. Stock solutions of Cadmium and Copper chloride with a Cd and Cu concentrations of 1000 µg L^{-1} were prepared in Artificial Sea Water (ASW). A stock solution of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47,purity ≥97.0%, Sigma-Aldrich, Germany) was prepared at concentration of 1000 µg L^{-1}. Considering very low PBDE solubility in seawater, dimethyl sulfoxide (DMSO, ≥99,5 %, liquid, Sigma-Aldrich, St. Louis, MO, USA) was used as a co-solvent; however, the final concentration of DMSO (0.01%, %v/v) never exceeded the NOEC previously found for this chemical for each testing organism.

3.3.3 Aging experiments

The PS particles and PA fibers were artificially irradiated through a photo-oxidative aging process. Before UV light exposure, for each of the polymers types investigated (PS and PA), six different treatment groups (twelve groups in total) were prepared: 1) control group (PS or PA alone); 2) MPs + Cd group; 3) MPs + Cu group; 4) MPs + BDE-47 group; 5) MPs + BDE-47 + Cd group; 6) MPs + BDE-47 + Cu group. Another set including six treatment groups for each polymer (PS and PA) without UV exposure were also set up for comparison. The exposure solutions were prepared by adding a volume of 100 µL of 10% w/v aqueous suspension (PS microparticles) and an amount of 10 mg (PA fibers) into each glass vials with 20 mL of artificial seawater (ASW) (Instant Ocean Aquarium System), salinity 35 ± 1‰. The MPs concentration chosen (0.5 mg mL^{-1}) was similar to that used in previous MP sorption studies (Bakir et al., 2012; Boyle et al., 2020; Sleight et al., 2017). MPs were spiked with selected contaminants (Table 3.2); briefly, the exposure solution for MPs + Cd group was prepared by adding a volume of 100 µl Cd stock solution (1000 µg L^{-1}) to 20 mL ASW containing MPs, in order to reach a final concentration of 5 µg L^{-1}. The same volume was also taken from Cu and BDE-47 stock solution to prepare the other exposure solutions. For the mixture solution, consisting of combinatorial exposure to Cd and BDE-47 or Cu and BDE-47, we used the same contaminants concentrations as for single exposures (Table 3.2). After that, treatments groups (aging conditions) have been subjected to accelerated photo-oxidative degradation; parallel, the other treatments groups (non-aging conditions) were covered with aluminum foil.

Positive controls (contaminants- Cd, Cu, BDE-47, Cd/BDE-47 mix and Cu/BDE-47 mix without MPs) were also included in each aging and non-aging conditions tested.

Table 3.2 Concentrations of selected contaminants

Treatment	**[µg L^{-1}]**
Cd	5
Cu	5
Zn	5
Mix*	∑*(5+5+5)

*Mix consist of all the contaminants at the same concentrations used for single exposure.

3.3.4 Photo-oxidative aging of MPs

Photo-oxidative aging was carried out on a QUV PANEL (figure 3.1 a) apparatus at 60 °C with continued exposure to UV radiation for up to 21 days with an irradiance of 0.7 $W/m^2/nm$. This exposure roughly corresponded to a 6-months exposure at the latitude of Arizona area considered a benchmark location due to high annual UV and high year-round temperatures (Fedor, G. R., and Brennan, P. J., 1996). The irradiance of the UV lamps (UVA 340 lamps) has a broad band with a maximum at 340 nm. Since glass vials containing microplastics in seawater were used to simulate the exposure, only the part of the spectrum from 350nm to 420 nm was responsible for aging phenomena. For each plastic material (PS and PA 66), two set of samples (exposure experiments and chemical analyses) were prepared in experimental replicate (n=3 treatment^{-1}), for two different times of exposure: 0 (T_0) and 21 days (T_{21}). At least two separate samples were analyzed at each exposure time. A reference sample containing only artificial seawater was included in addition to control DMSO and control samples for each plastic type selected. After 21 days of exposure, one set of glass vials containing aged MPs (figure 3.1 b) was collected and then used for the exposure experiments, while the second set of aged MPs was collected for physico-chemical analysis.

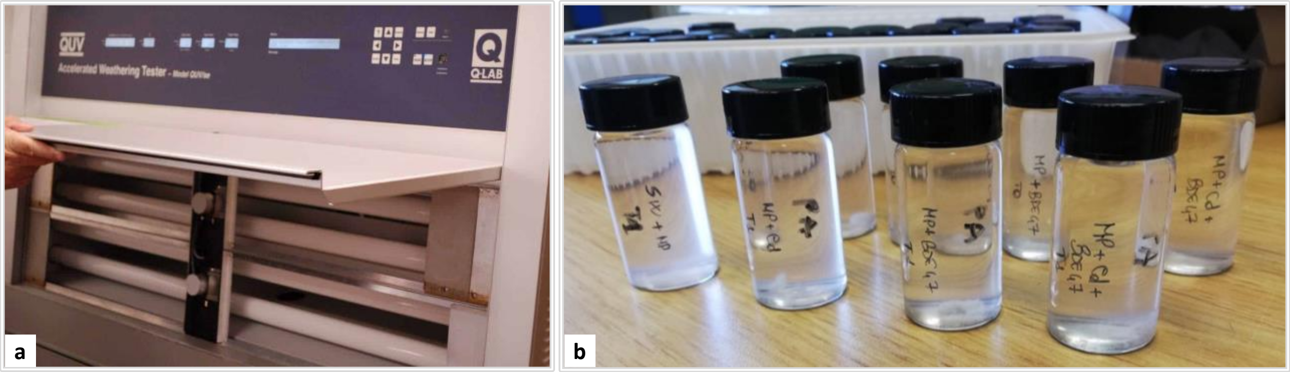


Figure 3.1: QUV PANEL system used for photo-oxidation of MPs (a) Glass test vials with aged MPs used for exposure experiment.

3.3.5 Sea urchin sampling and embryo exposure to co-contaminants and MPs

Adult *P. lividus* were collected in the South- Western coast of Sicily, nearby Capo Granitola, Trapani, Italy (Latitude 37° 34′ 25.3″ N; Longitude 12° 38′ 22.3″ E) and transported to the laboratory within 1 h after collection. The animals were acclimated for 15 days in an aquarium with ASW, with 12 h:12 h light: dark photoperiod, continuous aeration and temperature 17.0 ± 1.0 °C and pH 7.80 ± 0.10 monitored daily. During the acclimation, individuals were fed ad libitum every day with dehydrated macroalgae (Sera Marin Gourmet Nori), and feeding was interrupted 2 days before experimental sampling. The health status of the sea urchins was monitored through continuous observation and no mortality occurred during the acclimation period. After acclimation, gamete release was induced by vigorously shaking ripe organisms, followed by an injection of 0.5 M KCl for stubborn adults. Sperm from each male was pooled and maintained dried in an Eppendorf in cold conditions (+ 4°C) until fertilization, while eggs were filtered through 100 µm mesh to remove debris and shed into a beakers previously filled with ASW. Spawned oocytes were left to settle , washed three times with ASW and then diluted to a final concentration of 95000 oocytes mL^{-1}. Once sperm mobility had been checked, sperm was then diluted into artificial seawater (1:500) and the fertilization was executed by gently mixing the sperm and egg suspension with a sperms/eggs ratio corresponding to 100:1. Finally, fertilized eggs before the first cleavage were then added to each treatment (aging and non-aging conditions) glass vials, at a density of 1000 embryos mL^{-1}, and left to develop in a static incubator at 18°C on a 12:12 light dark cycle until the pluteus stage, approximately 48 hours post fertilization (hpf). Three replicates per treatment were tested.

3.3.6 Morphological analysis

To qualitatively assess the toxicity of chemical combined exposure (MPs, HMs, BDE-47 alone or mixture of them), the development was followed using the Olympus BX50 optical microscope and live images of a sub-sample of embryos from each treatment at 24 and 48 hpf were taken. Embryos showing morphological deviations from untreated ones (i.e. embryos that do not reached the pluteus stage of development or not showed fully developed skeletal rods and/or archenteron) were considered abnormal. The percentages of embryos indicating normal and abnormal phenotype were then determined by counting about 100 embryos/replicate in each treatment using the Olympus BX50 optical microscope.

3.3.7 Cd and Cu determination by ICP-MS analysis

Metal concentrations were determined with an ICP/MS Nexion 300X (Perkin Elmer Inc. Waltham, Massachusetts, U.S.A.) using the kinetic energy discrimination mode (KED) for interference suppression. Prior analysis, samples were diluted, acidified with nitric acid, and added with the internal standards required for the determination. Each determination was performed three times. The accuracy of the analytical determination was confirmed by measuring a standard reference material, ERM-CA403 seawater, without observing an appreciable difference. The sensitivity for cadmium and copper determination was 100 ppt.

3.3.8 BDE-47 determination by GC-MS

BDE-47 concentrations were determined using a GC/MS system (GC-2020, Shimadzu Corporation), coupled with a triple quadrupole mass spectrometer (Mass Detector TQ8040, Shimadzu Corporation) as detector. Each sample has been introduced into gas chromatograph through an autosampler for liquid sample AOC-20i (Shimadzu Corporation). Chromatography separation was carried out injected 1 µL of each sample into a DB-5ms column with an internal thickness of 0,25 µm, a diameter of 250 µm and a length of 30 m. The temperature of injector was 250 °C in spitless mode. Calibration curve for BDE-47 analysis (figure 3.2, table 3.3) was obtained through internal standard method, within the concentration range of 20-250 µg L^{-1} for BDE-47 in hexane solution. For each sample, 15 mL were extracted with two 15 mL aliquots of hexane, brought to dry and suspended again with 500 µL of deuterated internal standard diluted solution (BDE-47 Mass Labeled-2,2',4,4'-tetrabromo [13C12] diphenyl ether). The recovery percentage of extraction procedure was found to be 40%.

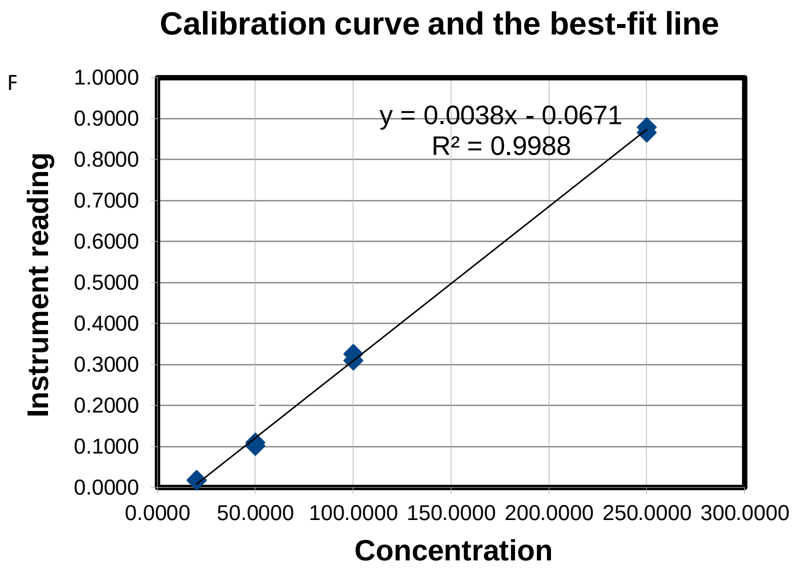


Figure 3.2: Calibration curve and the best-fit line for BDE-47 GC-MS analysis.

Table 3.3 Slope and intercept values of calibration curve used for BDE-47 GC-MS analysis

	Value	Error (σ)	% Error
Slope	0.0038	0.0001	1.39%
Intercept	-0.0671	0.0072	-10.70%

3.3.9 Analytical methods to characterize weathered plastic polymers

The microscopic structure of microplastics used in this study was characterized before and after irradiation by Scanning Electron Microscope (SEM). MPs were placed on a sticky sample holder and coated with a thin layer of gold (<10nm) to impart them conductivity. The coated samples were analyzed with a Thermo Phenom ProX Generation 5 microscope using a Secondary Electron Detector (SED) as detector and a 10 kV acceleration voltage. The molecular weight measurements of polymers after photodegradation exposure, were determined using Size-Exclusion Chromatography (SEC) applied to separate particles in a sample by size (Flavel et al., 2013). SEC analyses were performed on a Waters 600 A apparatus, equipped with five Ultrastyragel columns (7.8 × 300 mm, in the order 105, 103, 500, 104, and 100 Å pore size) connected in series, and a Waters R401 differential refractometer. Then 90 µL of a THF polymer solution was injected and eluted with a THF flow rate of 1 mL min^{-1}. The molar masses of PS samples were determined by using Polymer Lab Caliber software. The calibration curve was obtained with a set of primary polystyrene standards. An Ubbelhode viscometer was used to determine the medium molar mass of PA 66. Intrinsic viscosity [η] measurements were performed at 25 °C and PA 66 (0.5 g/dL) was dissolved in formic acid (90%). Molecular weights were calculated using Mark-Houwink-Kuhn-Sakurada (MHKS) equation $[\eta]=KM_v^{\alpha}$, where η is the intrinsic viscosity , K and α are constants for a particular polymer solvent system, and M_v is Molecular weight. In addition, a Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) analysis was performed to give valuable information about degradation products, end groups and monomeric repeat unit.

3.3.10 RNA extraction and first cDNA synthesis

Total RNA was extracted from control and exposed *P. lividus* embryos (~1000 embryos mL^{-1}) at 24 hpf using TRIzol Reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's instructions. RNA concentrations and purity were spectrophotometrically verified using Qubit® 2.0 Fluorometer. RNA integrity was evaluated using a 1.5% agarose denaturing gel and RNA was stored at −80 °C for future use. The subsequently treatment of samples with DNase (Deoxyribonuclease I, Amplification Grade, Sigma-Aldrich) removed any residual genomic DNA contamination, while DNase I was inactivated by adding 25 mM EDTA. The synthesis of cDNA was carried out using *SuperScript VILO cDNA Synthesis Kit* (Invitrogen Corporation, Carlsbad, USA) on 500 ng DNase I treated RNA, according to the manufacturer's instructions. A 1:10 cDNA dilution was then tested by Polymerase Chain Reaction using *18S* rRNA and after that diluted 1:10 in nuclease free water before to use it in Quantitative PCR experiments.

3.3.11 Gene expression by real-time quantitative polymerase chain reaction (RT-qPCR)

The qPCRs were performed using a PCR machine ABIPRISM 7500 System (Applied Biosystems, Forster City, USA) with *Power SYBR Green* as detection chemistry (Applied Biosystems, Forster City, USA). The 18S ribosomal RNA was selected as control gene based on its expression stability in all tested conditions (Stamateris et al., 2010; Ragusa et al., 2013; Vergara-Amado et al., 2017; Di Natale et al., 2019). Primer sequences used in this study were *Forkhead box transcription factor a (foxa), Homeodomain containing transcription factor (hox11/13b), Metallothionein 4 (mt4), heat shock protein 70 and 60 (hsp70 and hsp60), Spicule matrix protein (sm50), Biomineralization protein (p16)* and *Mesenchyme-specific cell surface glycoprotein (msp130)* (Table 3.4). A normalization factor based on geometric averaging of the expression level of these reference genes was also calculated and used to quantify the expression levels of the target genes as reported elsewhere (Nicosia et al., 2018). PCR efficiency of the target and reference genes, serial dilutions of pooled cDNAs from both control and treated samples were carried out (data not shown). Each reaction was repeated in triplicate with appropriate no-template controls included in each run and with dissociation analysis performed within the qPCR run to verify the specificity of the primer pair used. Quantitative real-time PCRs were conducted according to the manufacturer's recommended procedures and data analysis was performed according to the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The amplification conditions included: initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 30s and 60 °C for 50s, followed by a melting curve from 60 to 95 °C. Amplicons were detected by agarose gel analysis after each PCR to confirm the amplification of the specific gene. Differently from chapter 2, as the fold-change values (both for downregulation – values under "0" and up-regulation – values above "0") obtained were more high and difficult to clearly represent by barplot, data fold-changes have been represented in log (e)-transformed values. Therefore, relative expression for control treatments has been calculated as 0.

Table 3.4: Genes and oligonucleotide primers used in this study

Gene name	Gene symbol	Primer sequence (5' – 3')
Forkhead box transcription factor a	*foxa*	CAGGTATGGGAAGCATGGGA GCGTATCTCATCGACATGGC
Homeodomain containing transcription factor	*hox11/13b*	TTGCGACGTTCACAACAACT GTGAGATCGAGAGCCTGTGA
Spicule matrix protein 50	*sm50*	GATGGCACACCAGCTTATCC CTGACGCTTCATGACTGGAG
Biomineralization protein p16	*p16*	AGCAGGAGCAGTCGGAGATAC CATCATCACTTCCCATATCGC
Mesenchyme-specific cell surface glycoprotein	*msp130*	ATACATGGCAACCCAAGAAG CGATTCCAACGAAGATGAGT
18S ribosomal RNA	*18s*	GAATGTCTGCCCTATCAACTTTCG TTGGATGTGGTAGCCGTTTCTC
Metallothionein 4	*mt4*	GCTCAAAATCTTCAACATGGCTAATGA AGCACTTTCCAGTTTCACAACAAGC
Heat shock protein 70	*hsp70*	GGGTACGACCTATTCCTGTGTTG CTTAGCAGCATCTCCAATCAGTC
Heat shock protein 60	*hsp60*	GAATATCCAGTGTACTCCGAC GCATCAGCTAAGAGGTCAACAC

3.3.12 Statistical analysis

Gene expression results were presented as mean value ± SD. Graphs and statistical analyses were performed using R software v. 3.5.0 (R Core Team, 2018). All data were tested for normality and homogeneity of variances using Shapiro-Wilk test and Leven's test, respectively. Significant differences between values of different treated groups and the reference control groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test. The *p* values lower than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Characterization of the aged microplastics

The SEM micrographs of virgin and aged PS microparticles are shown in Figure 3.3. The surface of virgin PS microparticles was relatively smooth and appeared spherical in shape, while the surface of aged PS has undergone some changes. Particularly, for aged PS, a decrease in mean size of particles was observed (from 900 to 800 nm) in addition to a loss of sphericity and to appearance of halo. Conversely, the electron micrographs did not evidence signs of tear and crack lines on the micro-spheres surface.

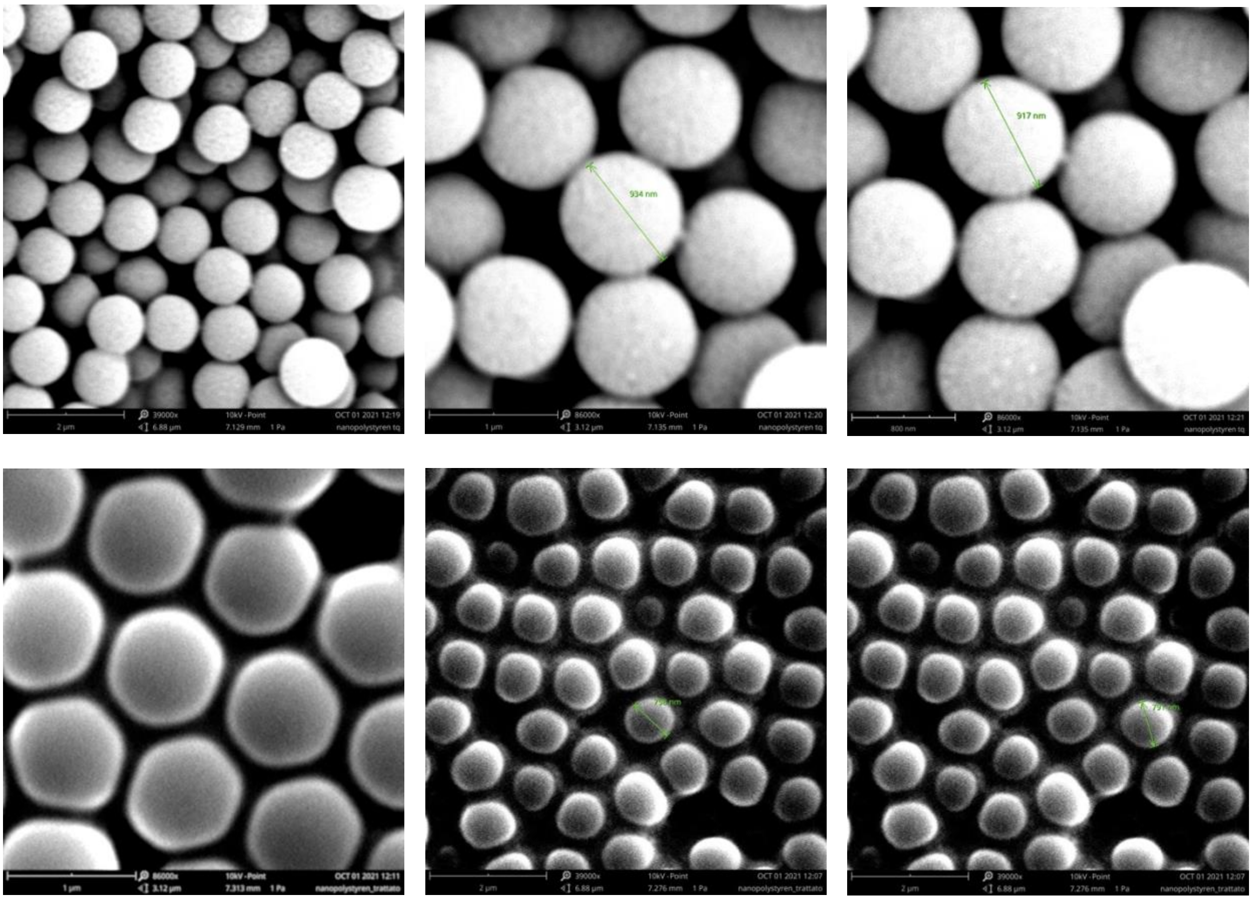


Figure 3.3: SEM images of virgin (upper) and aged (lower) PS microparticles exposed to photo-degradation for 21 days.

The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of PS microparticles decreased after being exposed to 21 days of light irradiation (Table 3.5). At the end of photodegradation, the M_n of the weathered PS microparticles decreased by 43.2 %. The potential influence of contaminants into oligomers generation and MMs decrease was also evaluated. Co-presence of contaminants, such as Cd, Cu or BDE-47 or a mixture of them, in addition to MPs, highlighted a change in molar mass distribution for PS only for Cu. A higher decrease of MMs (almost 50%) emerged, in fact, after 21 days of photo-degradation, when Cu was present in the solution with PS, both alone and in mixture with BDE-47 (Table 3.5). On the other hand, no change in MMs or a particular influence on degradation process was observed by BDE-47 and Cd contaminants on PS microparticles, as well as on PA fibers.

Table 3.5: Molecular weights and molecular weights distribution of PS microparticles alone or in presence of the selected contaminants, before and after photo- exposure (0, 21 days).

Plastic type	Aging time (days)	M_n (g/mol)	M_w (g/mol)	Weight after aging time (%)	PDI (M_n/M_w)
PS	0	37946	212246		5.59
	21	21551	119596	43.2	5.54
PS+BDE	0	37612	212013		5.49
	21	21231	116586	43.5	5.64
PS+ Cu	0	37521	212464		5.66
	21	19150	114210	48.9	5.96
PS+BDE+ Cu	0	37261	213970		5.74
	21	18712	113775	49.7	6.08

* M_n = number-average molecular weight; M_w = weight-average molecular weight; M_n/M_w = Index polydispersity.

The viscometry measurements used to obtain the connected molecular weights (Mv) of PA fibers on basis on quantitative relationships between their viscosity and molecular weight showed a values decrease of the 36% after 21 days of light irradiation (Figure 3.4). Intrinsic viscosity [η] at different times (0, 7 and 21 days) of photo-exposure. was $[\eta]=35{,}3 \times 10^{-3} \bullet M_v^{0{,}786}$.

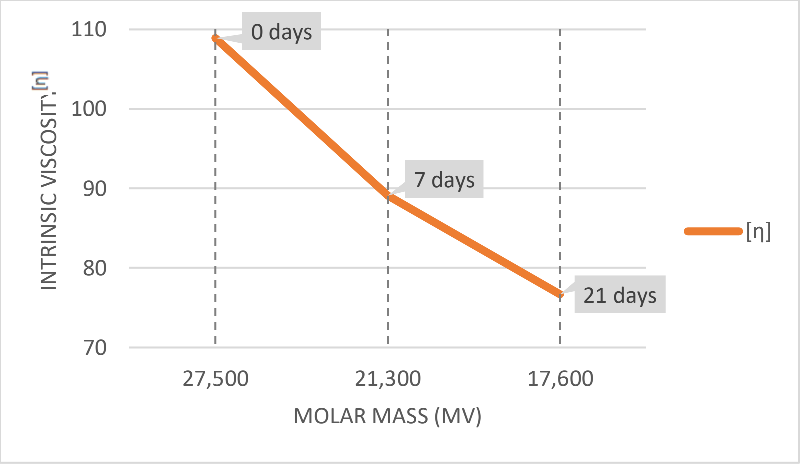


Figure 3.4: Intrinsic viscosity and Molar Mass of PA fibers obtained by viscometry analysis at different times of photo-exposure. Measuring temperature, 25 °C; PA concentration, [0.5 gr/dL]; solvent for viscometry analysis, 90% formic acid.

New peaks, not observed in virgin sample, appeared by MALDI-MS analysis at 928.08, 944.06, 960.08, 1072.22, 1142.32 m/z for photo-oxidized PA fibers (figure 3.5 a-b). These new peaks were attributed to four new structures (Table S1 showed, Appendix B), some of which have amide or carboxylic as end groups. However, the MALDI spectrum of aged PS (Figure 3.6) collected after photo-exposure, generated photo-degradation products having similar structure to virgin PS (Table S2 showed, Appendix B).

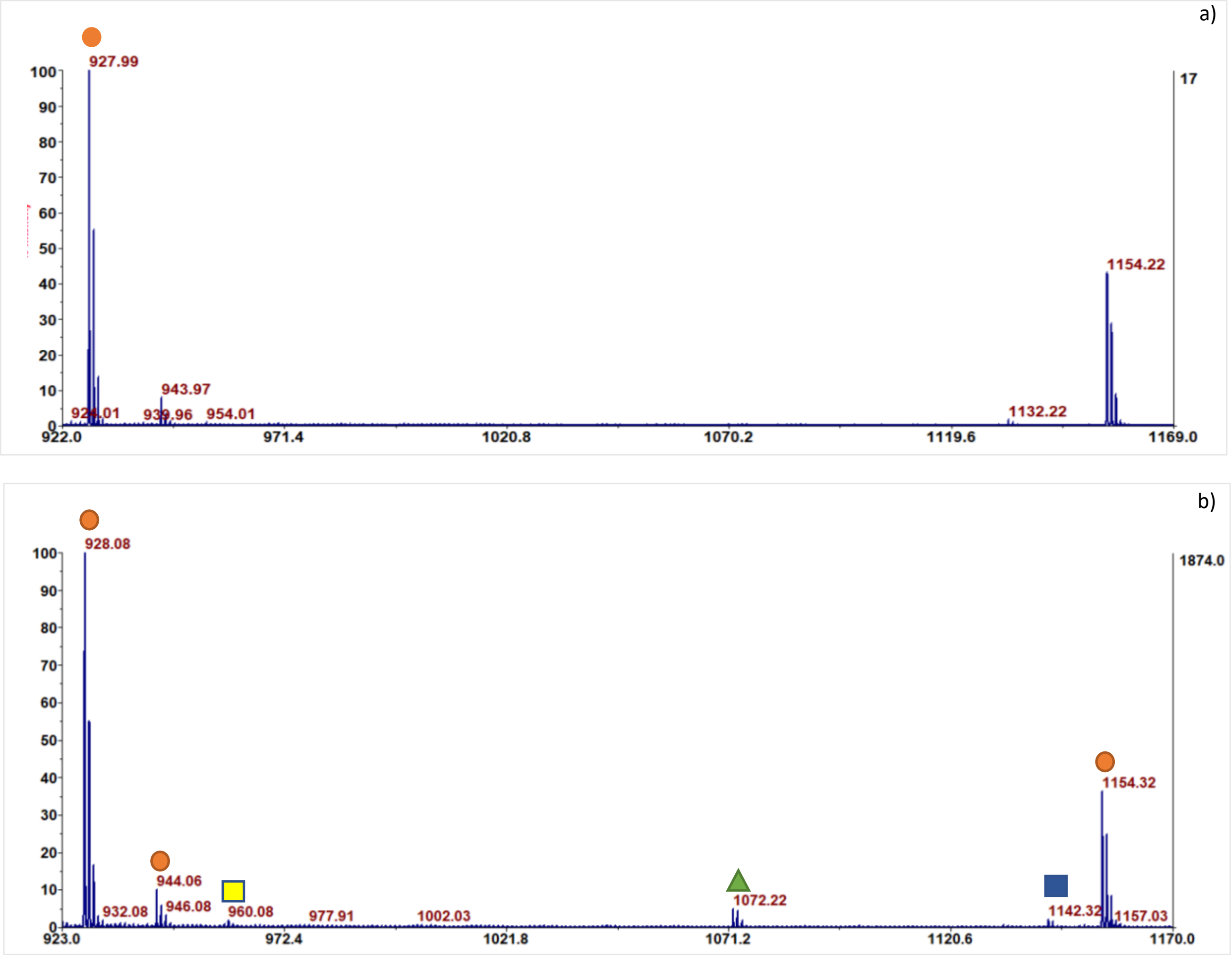


Figure 3.5: MALDI spectra of a) virgin and b) aged PA 6,6 fibers, recorded in positive reflectron mode.

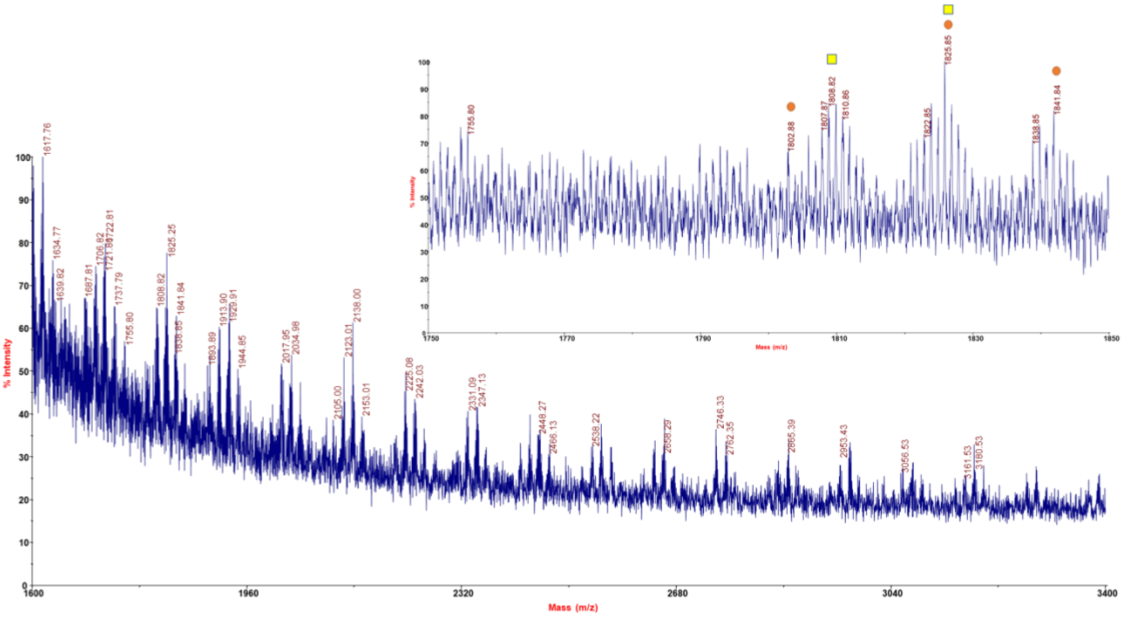


Figure 3.6: MALDI spectrum of PS microparticles after 21 days of photo-exposure. In the enlarged part is reported the mass range 1750-1850 m/z.

3.4.2 Effects of aging on sorption process of contaminants on microplastics

The results related to Cu concentration in the solutions containing non-aged or aged PS and PA showed that the concentrations of Cu in the combined Cu-PS solutions decreased in a significative way for both virgin and aged PS microparticles in comparison to single Cu solutions, although for aged PS that decrease appeared to be lower (Figure 3.7 a). In addition, co-presence of Cu and BDE-47 in the mixture with virgin and aged PS did not change the result obtained for single Cu-PS solutions, although a higher release of Cu in solution emerged in photo-exposed PS microparticles when compared to single Cu-aged PS ones. A significant decrease in Cu concentration was also observed in the solutions with virgin PA fibers (Figure 3.8 a), although the decrease detected was greater for PS microparticles if compared to PA fibers; however, after 21 days of photo-oxidative aging, the concentration of Cu significantly increased reaching levels comparable to single Cu solutions. On the contrary of PS microparticles, the addition of BDE-47 to the mixture with Cu to PA fibers seemed slightly to influence its sorption process as revealed from the greater release of Cu in the aqueous phase; however, after photo-exposure, the Cu level reached values similar to those of single Cu-aged PA solutions (Figure 3.8a). No evidence of sorption of Cadmium both for PS microparticles and PA fibers emerged before and after photo-exposure, if not a slightly decrease in Cadmium concentration for single Cd- aged PA solutions (Figure 3.7 and 3.8 b).

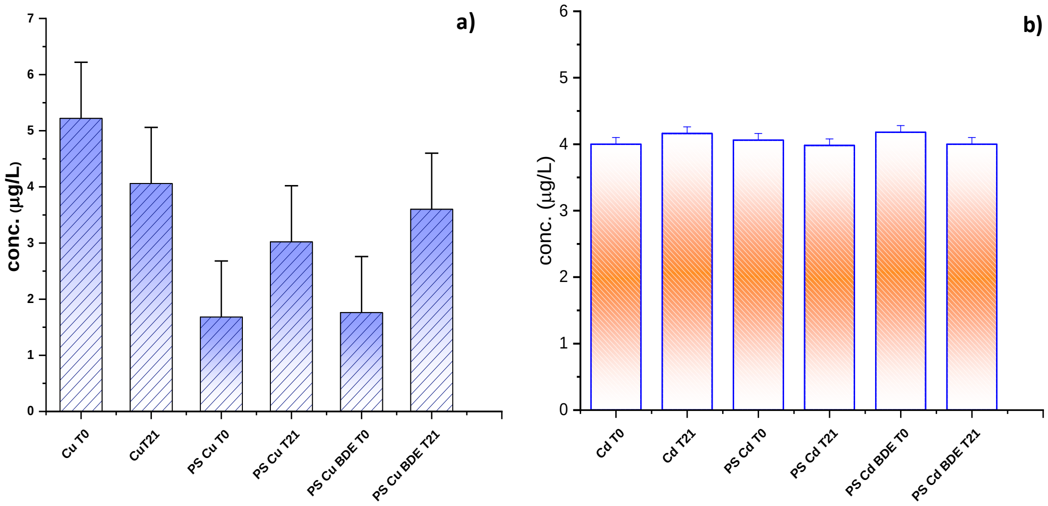


Figure 3.7: Determination of a) Cu and b) Cd concentrations alone or in mix with BDE-47 in solutions containing non-aged (T0) and aged (T21) PS microparticles; Cu, Cd and BDE-47 concentrations were 5 $\mu g\ L^{-1}$, PS concentration was 0.5 $mg\ L^{-1}$.

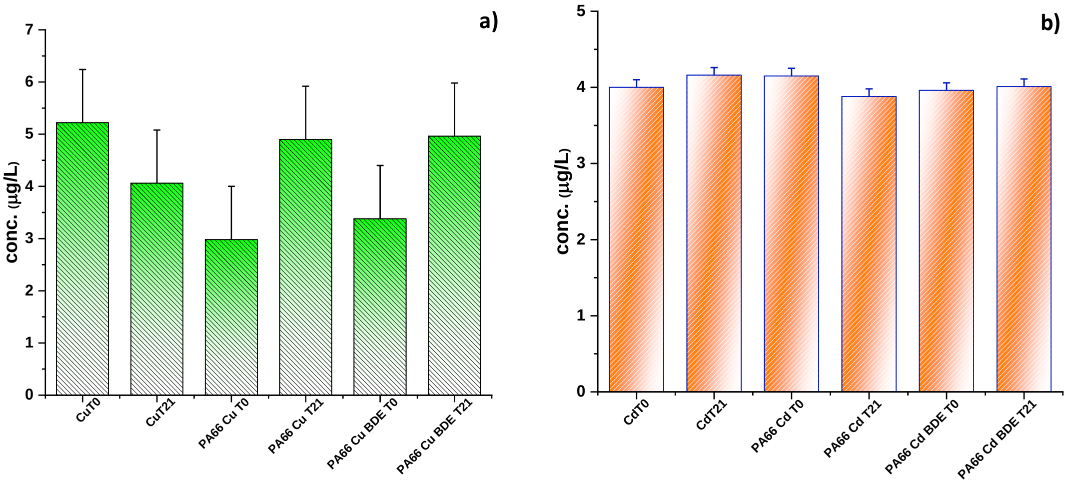


Figure 3.8: Determination of a) Cu and b) Cd concentrations alone or in mix with BDE-47 in solutions containing non-aged (T0) and aged (T21) PA fibers; Cu, Cd and BDE-47 concentrations were 5 µg L^{-1}, PA concentration was 0.5 mg L^{-1}.

Finally, a decrease of BDE-47 concentration emerged from GC-MS analysis in all treatments (non-aged and aged) with either PS microparticles and PA fibers, as the BDE levels found were below the instrument detection limit (Figure 3.9).

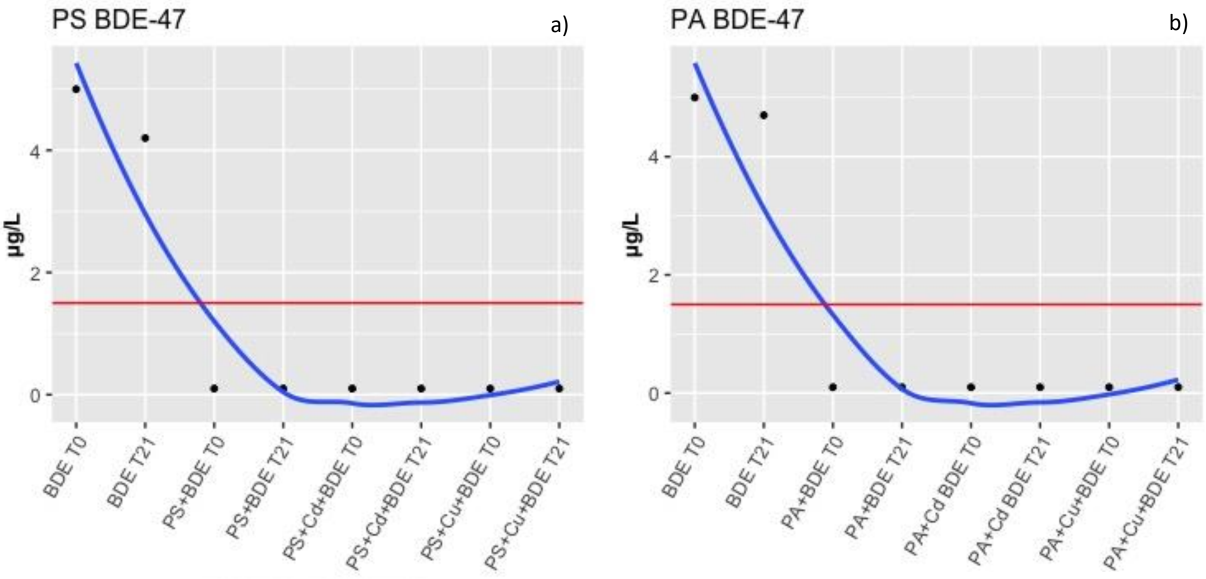


Figure 3.9: Determination of BDE-47 concentrations alone or in mix with Cu or Cd in solutions containing non-aged (T0) and aged (T21) PS a) or PA b). Cu, Cd and BDE-47 concentrations were 5 µg L^{-1}, PA and PS concentrations were 0.5 mg L^{-1}. Red line indicates the instrument detection limit (1,5 µg L^{-1}).

3.4.3 Morphological changes induced by co-contaminants and MPs treatments

Control group embryos (organisms without MPs and contaminants) observed at 24 hpf shown a morphological structure corresponding to the gastrula stage (Figure 3.10). Delay in endoderm invagination in Cd exposed embryos and abnormal embryos were evident after Cu exposure; while BDE 47 exposure does not provide evident aberrant phenotypes. Conversely, joint exposures to BDE 47, Cd and/or Cu more severely affected the developmental process, and additionally combined exposure to BDE47 and Cu appear to accelerate the development since at 24 hpf some embryos showing a prima-like phenotype appeared. PA and PS exposures do not alter embryo morphology resulting in embryos with the proper invagination of a canonical gastrula (Table 3.6).

Table 3.6: Description of morphological changes observed for each exposure group

Exposure group	Development	Description
SW	Normal	
PS	Normal	
PA	Normal	
Cd	Abnormal	Endodermal invagination delay, no gastrulation
Cu	Abnormal	Endodermal invagination delay, no gastrulation
BDE-47	Normal	Gastrula
Cd+BDE-47	Abnormal	Endodermal invagination delay, no gastrulation
Cu+BDE-47	Abnormal	Prisma, accelerated development
PS+Cd	Abnormal	Endodermal invagination delay, no gastrulation
PA+Cd	Normal	Gastrula
PS+Cu	Normal	Gastrula
PA+Cu	Normal	Gastrula
PS+BDE-47	Normal	Gastrula
PA+BDE-47	Normal	Gastrula
PS+Cd+BDE-47	Abnormal	Incorrect developed gastrula, malformations
PA+Cd+BDE-47	Normal	Gastrula
PS+Cu+BDE-47	Abnormal	Incorrect developed gastrula, malformations
PA+Cu+BDE-47	Normal	Gastrula

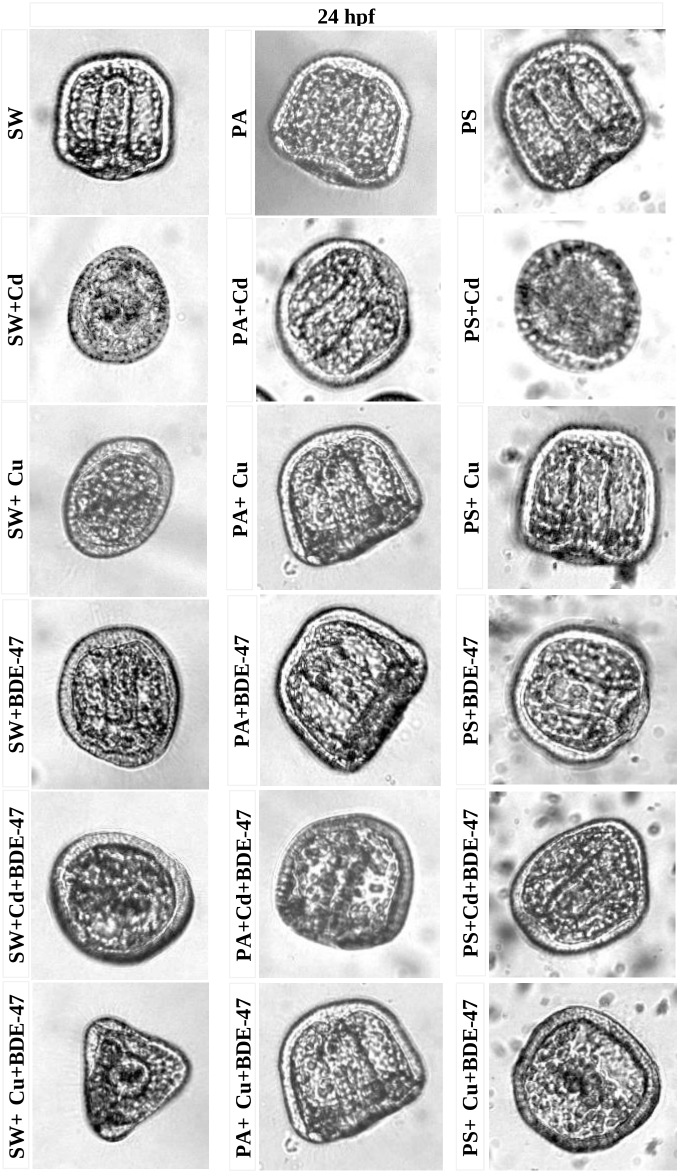


Figure 3.10: Phenotypic comparison of *P. lividus* embryos at 24 hours post fertilization, corresponding to gastrula stage. Embryos showed, correspond to different exposure conditions, including controls (without MPs), non-aged PA fibers and PS microparticles in combination with the individual selected contaminants or whit a mixture of them.

3.4.4 Pathway-focused mRNA expression analysis

Expression profiles of genes involved in stress response and metal scavenging

The embryos exposed to cadmium or copper, alone or in mixture with BDE-47 in association with non-aged or aged PS microparticles, showed a great variation in *HSPs* gene expression (Figure 3.11 a-c and 3.12 d, e) with respect to control groups. In details, among the stress genes, *hsp60* and *hsp70* were significant up-regulated ($p<0.05$) in embryos exposed to individual cadmium and copper solutions compared to negative control (Figure 3.11 a and b); while *mt4* resulted downregulated. In particular, no changes in expression after Cd with non-aged or aged PS were observed (Figure 3.11 a) as well as with non-aged or aged PA fibers exposures (see Figure S1 a, Appendix A, Supplementary material). However, a significant reduction of up-regulation of *hsp60 and hsp70* mRNA levels ($p<0.001$) occurred in embryos exposed to Cu and non-aged PS microparticles, although in presence of aged PS/Cu that reduction was less significant ($p<0.05$) (Figure 3.11 b). Embryos exposed to PS microparticles showed no significant difference between aged and non-aged PS, except for a significant transcriptional upregulation of *hsp70* mRNA level ($p<0.05$) in embryos exposed to aged PS emerged (Figure 3.11 a-c and 3.12 d, e). This *hsp70* overexpression did not occur in aged PA fibers treatment, since all three genes appeared downregulated (Figure S1 a-c, Appendix B).

The transcriptional profile of *mt4, hsp60* and *hsp70* was also analyzed in embryos exposed to organic contaminant BDE-47 (Figure 3.11 c). Unlike exposure to metals, embryos treatment with BDE-47, showed a significant upregulation of *mt4, hsp60* ($p<0.05$) and *hsp70* ($p<0.01$) genes compared to untreated control. The combinatorial exposure to PS microparticles and BDE-47 resulted in 2 different transcriptional profiles related to non-aged or aged microparticles. Indeed, although the upregulation of *mt4*, *hsp60* and *hsp70* mRNA levels in embryos exposed to aged PS microparticles with BDE-47 was maintained without significant changes in expression, the exposure in presence of non-aged PS microparticles led to a switch from an upregulation to a significant downregulation ($p<0.05$). However, in the combinatorial exposure to non-aged PA fibers/BDE-47, the upregulation observed in the individual treatment with BDE-47 was maintained (see Figure S1 b, Appendix B).

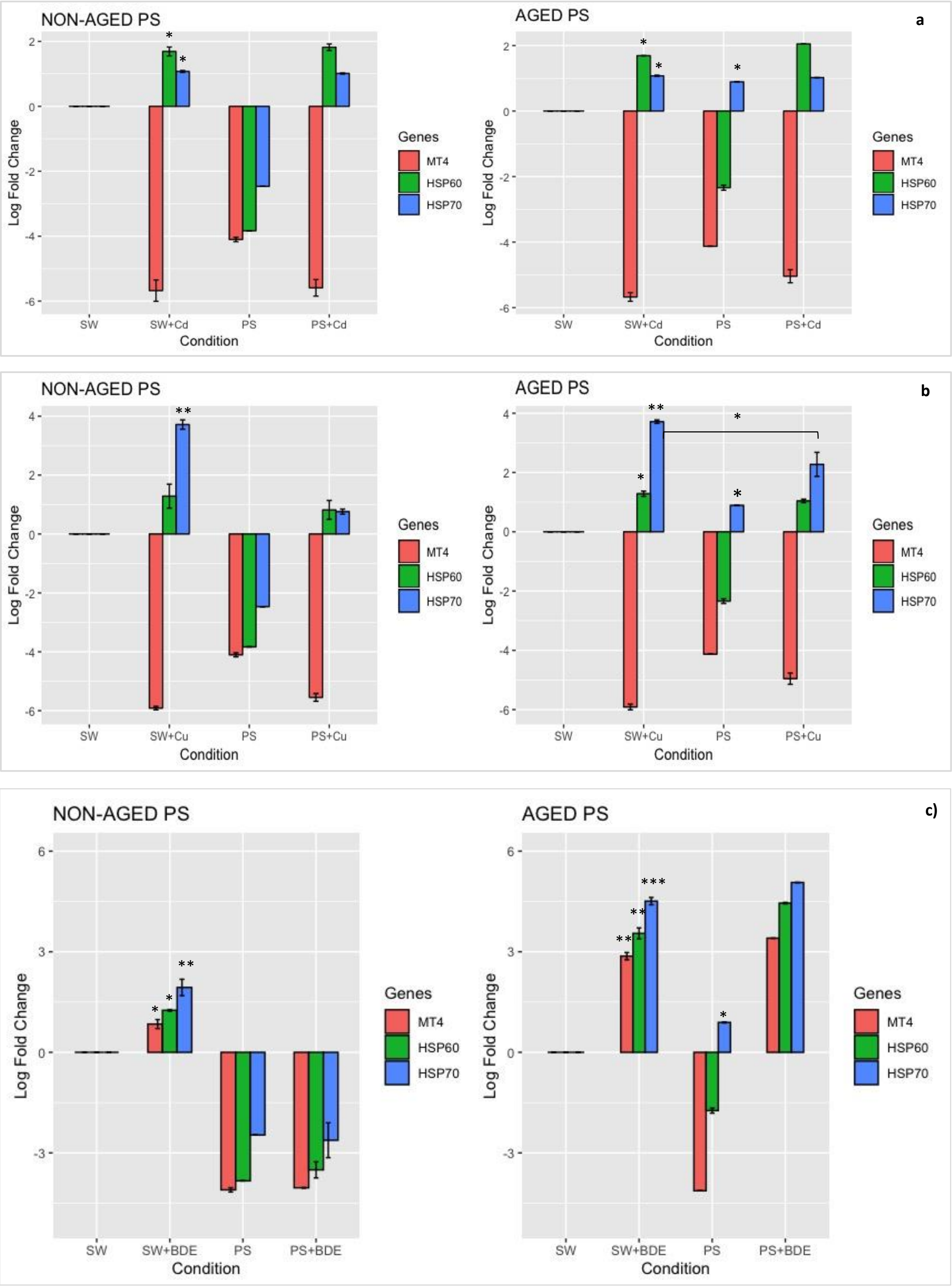


Figure 3.11: RT- qPCR results showing the mRNA levels of *metallothionein 4*, *mt4,* and *heat shock proteins, hsp60* and *hsp70,* with respect to reference gene 18S, in embryos at 24 hpf (gastrula stage) exposed to a) Cd or b) Cu or c) BDE-47 without or in association with non-aged or aged PS. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at *p* <0.05 (*); *p* < 0.01 (**); *p* < 0.001 (***); one-way ANOVAs with Tukey's test.

The transcript levels of *mt4* and *HSPs* genes were also evaluated in embryos exposed to mixture of each metals with BDE-47. The qPCR analyses revealed that co-exposure differentially affected the expression of examined transcripts in the 2 mixtures selected (Figures 3.12 d and e) in comparison to single metal exposure (Figure 3.11 a and b). Specifically, in embryos exposed to Cd/BDE-47 and Cu/BDE-47 mixtures, all the 3 transcripts appeared upregulated or downregulated, respectively, than untreated control. However, a significant reduction of the *hsp60* (*p*<0.001) and *hsp70* (*p*<0.01) mRNA levels was observed when non-aged PS microparticles were added into Cd/BDE-47 mixture (Figure 3.12 d). That reduction, in presence of aged PS microparticles, changed in a significant down-regulation (Figure 3.12 d). Contrarily, for Cu/BDE-47 mixture, although addition of non-aged PS microparticles did not change gene expression profile, with aged PS microparticles a significant upregulation of *mt4* (*p*<0.001) and *hsp70* (*p*<0.001) occurred, compared to exposure without PS (Figure 3.12 e). No change in gene expression profiles after exposure to Cd/BDE-47 mixture with both unaged and aged PA fibers was observed (Figure S1 c, Appendix B).

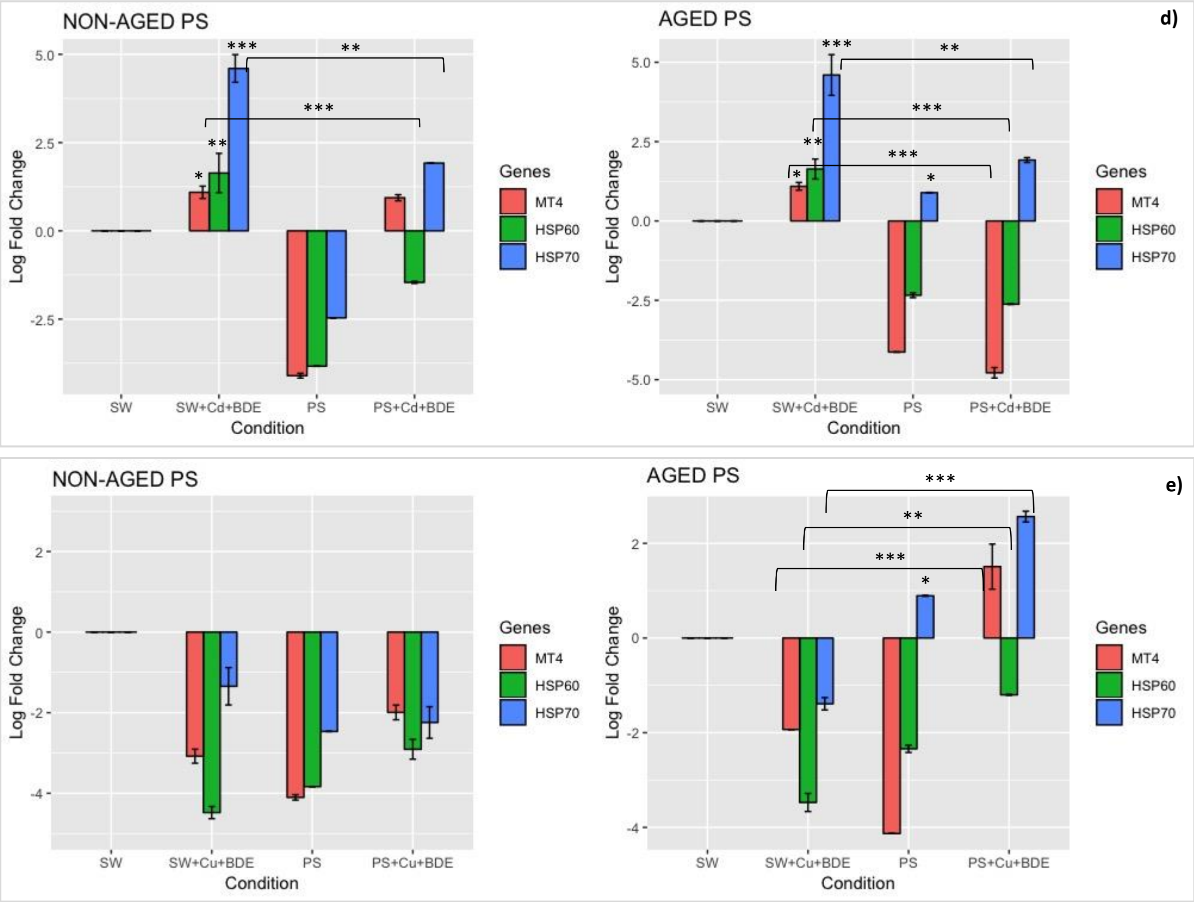


Figure 3.12: RT- qPCR results showing the mRNA levels of *metallothionein 4*, *mt4,* and *heat shock proteins, hsp60* and *hsp70,* with respect to reference gene 18S, in embryos at 24 hpf (gastrula stage) exposed to the mixture of each metals d) Cd or e) Cu with BDE-47 without or in association with non-aged or aged PS. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at *p* <0.05 (*); *p* < 0.01 (**); *p* < 0.001 (***); one-way ANOVAs with Tukey's test.

Expression profiles of developmental regulators of skeletogenic differentiation

The mRNA levels of members of the *P. lividus* GRN involved in the skeletogenic differentiation (*sm50*, *p16* and *msp130*) were analyzed in embryos exposed to individual co-contaminant selected (Figure 3.13 a-c) or to mixture of each metal with BDE-47 (Figure 3.14 d, e) in association with non-aged or aged PS microparticles.

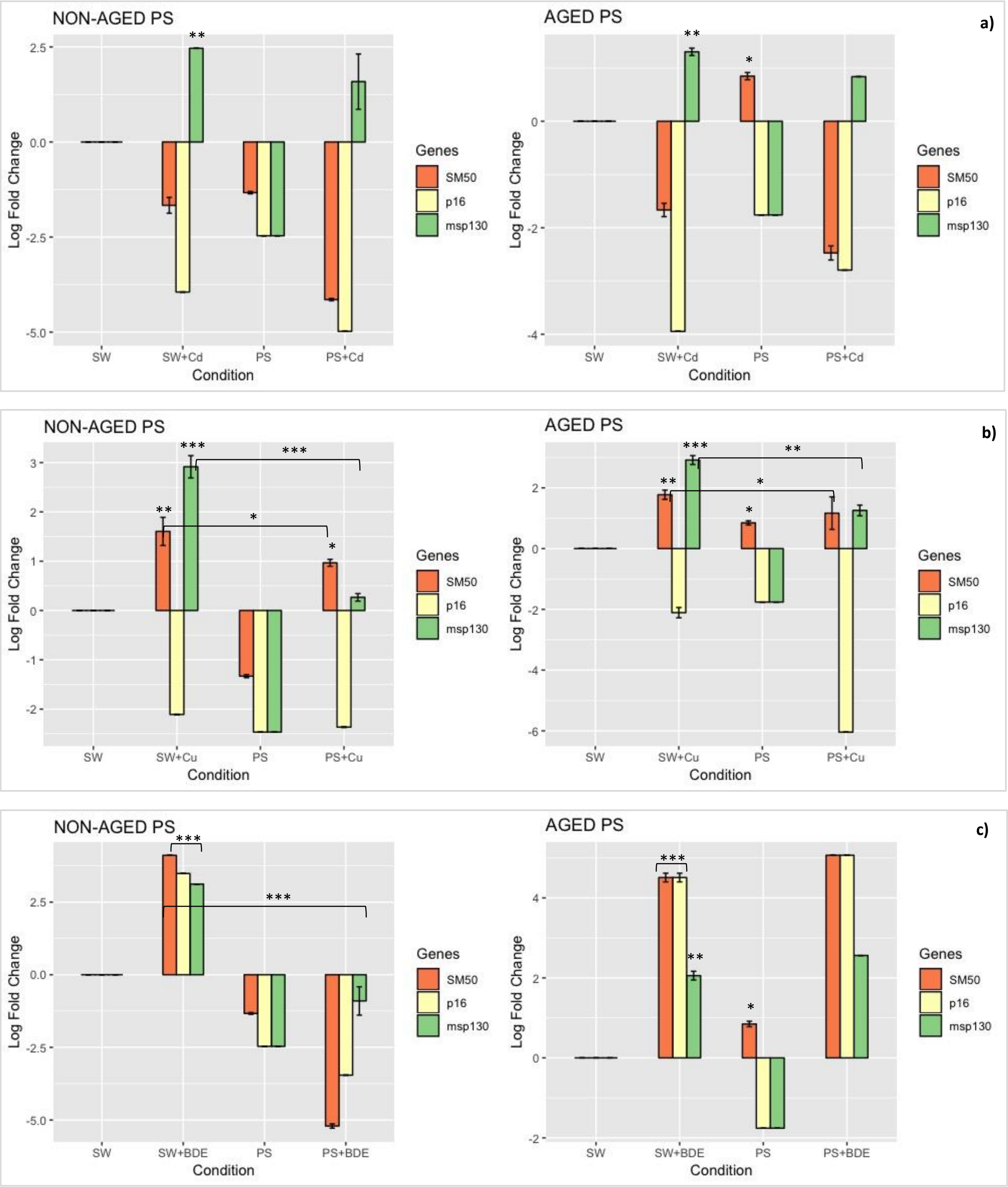


Figure 3.13: RT- qPCR results showing the mRNA levels of *spicule matrix protein 50*, *sm50, biomineralization protein, p16*, and *mesenchyme-specific cell surface glycoprotein, msp130,* in embryos exposed to a) Cd or b) Cu or c) BDE-47 without or in association with non-aged or aged PS, with respect to 18S at 24 hpf (gastrula stage). Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at p <0.05 (*); p < 0.01 (**); p < 0.001 (***); one-way ANOVAs with Tukey's test.

No significant differences emerged from the comparison between aged or non-aged conditions, except for a significant upregulation of *sm50* transcript (p<0.05) observed only in embryos exposed to aged PS. This *sm50* over-expression did not occur in aged PA fibers treatment, since all three genes appeared downregulated (Figure S2 a-c, Appendix B). Among the skeletogenic genes, the expression of *spicule matrix protein 50*, *sm50*, in embryos exposed to Cd or Cu alone or with aged or non-aged PS, appeared down-regulated or up-regulated, respectively.

The embryos exposed to BDE-47 with aged or non-aged PS microparticles, showed a different transcriptional profile of all 3 genes investigated as a down-regulation was observed for non-aged PS microparticles if compared with aged PS microparticles (Figure 3.13 c). However, in the combinatorial exposure to BDE-47 and non-aged PA fibers, no down-regulation emerged; rather, a general up-regulation of all 3 genes was observed (Figure S2 b, Appendix B). The *biomineralization protein p16*, *p16*, was down-regulated in almost all the exposures tested, except for embryos exposed to BDE-47 alone or in association with aged PS. Its expression was significant reduced (*p*<0.001) in embryos exposed to non-aged PS/BDE-47 compared to control without PS. However, no significant changes in *p16* mRNA levels emerged after exposure to aged PS/BDE-47 (Figure 3.13 c). A general up-regulation *of msp130* mRNA levels was found, although in presence of aged or non-aged PS its expression was reduced. In particular, a significant reduction of *p16* mRNA transcript was found in non-aged or aged PS/Cu co-exposures (*p*<0.001 and *p*<0.01, respectively) as well as in non-aged PS/BDE-47co-exposure compared to exposures without PS (Figure 3.13 c).

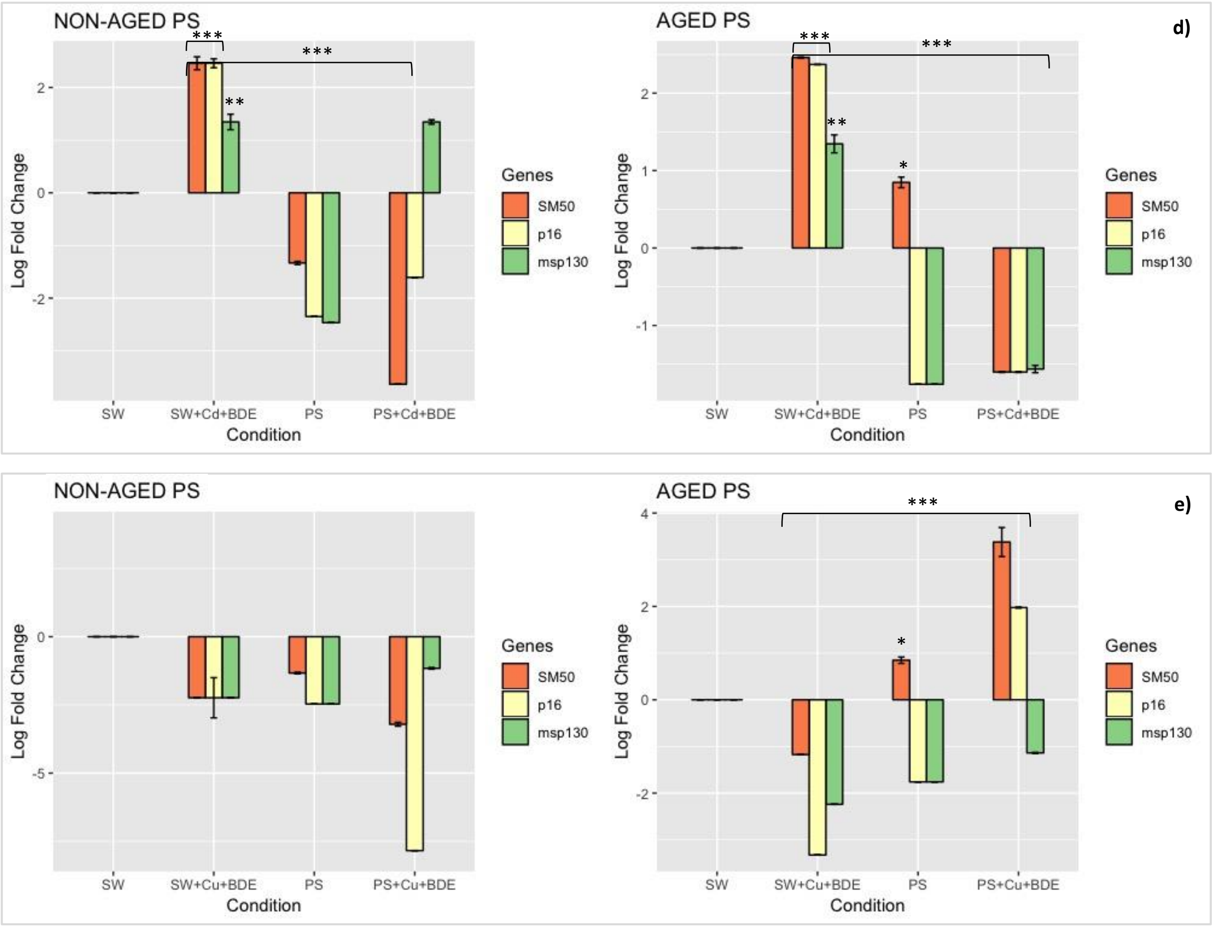


Figure 3.14: RT- qPCR results showing the mRNA levels of s*picule matrix protein 50*, *sm50, biomineralization protein, p16*, and *mesenchyme-specific cell surface glycoprotein, msp130,* with respect to reference gene 18S, in embryos at 24 hpf (gastrula stage) exposed to the mixture of each metals d) Cd or e) Cu with BDE-47 without or in association with non-aged or aged PS . Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at *p* <0.05 (*); *p* < 0.01 (**); *p* < 0.001 (***); one-way ANOVAs with Tukey's test.

The qPCR analyses of embryos exposed to mixture of each metals with BDE-47 showed that co-exposure differentially affected the expression of examined transcripts in the 2 mixture selected (Figures 3.14 d and e). Significant differences emerged from the comparison between aged or non-aged conditions as observed in embryos exposed to Cd and BDE-47 mixtures, where the presence of PS microparticles significantly reduced the transcript levels of *sm50* and *p16* genes (p<0.001) in non-aged treatments and of all transcripts in aged ones (p<0.001) (Figures 3.14 d). However, in embryos exposed to Cd/BDE-47 mixture with aged PA fibers, the upregulation of *sm50*, *p16* and *msp130* was maintained (Figure S2 c, Appendix B). In the mixture of Cu and BDE-47, although the presence of non-aged PS microparticles determined no significant changes in mRNA transcripts compared to control without PS, a significant upregulation of *sm50* and *p16* genes (p<0.001) occurred in embryos exposed to the same mixture with aged PS microparticles (Figures 3.14 e).

Expression profiles of developmental regulators of endodermal specification

The transcriptional profile of members of the *P. lividus* GRN involved in the endodermal specification, including *foxA and hox11/13b,* was analyzed in embryos at 24 hpf exposed to non-aged or aged PS microparticles in association with cadmium, copper as well as BDE-47, tested individually or in mix (Figure 3.15 a-e).

The expression of both genes was differentially affected in embryos exposed to metals individually tested. In particular, an overall significant transcriptional upregulation of *foxa* and *hox11.13b* mRNA levels (p<0.05) emerged in embryos exposed to individual Cd treatment compared to untreated controls. In presence of aged PS a significant downregulation of *hox11.13b* (p<0.001) was observed compared to control exposure without PS (Figure 3.15 a). That downregulation involved also *foxa* transcript level in the co-exposure to aged PA fibers and Cd (Figure S3 a, Appendix B).

Embryos exposed to Cu showed a different transcriptional profile for both genes, since they were downregulated (Figure 3.15 b). A different transcriptional profile between aging and non-aging conditions was observed after BDE-47 treatment. Specifically, embryos co-exposed to non-aged PS/BDE-47 *foxa* and *hox11.13b* mRNA were significantly down-regulated (p<0.001) than exposure without PS. However, no significant changes for both genes occurred in aged PS/BDE-47 co-exposure compared to exposure without PS in aging conditions (Figure 3.15 c).

Similarly to what observed in the other transcriptional pathways investigated, once again RT-qPCR analysis revealed a positive fold change in mRNA levels (significant induction of *foxa* transcript – p<0.05) in embryos exposed to aged PS microparticles if compared with non-aged ones (Figure 3.15 a-c, d, e). No changes in gene expression occurred in PA fibers exposures at the same conditions (Figure S3 a-c, Appendix B).

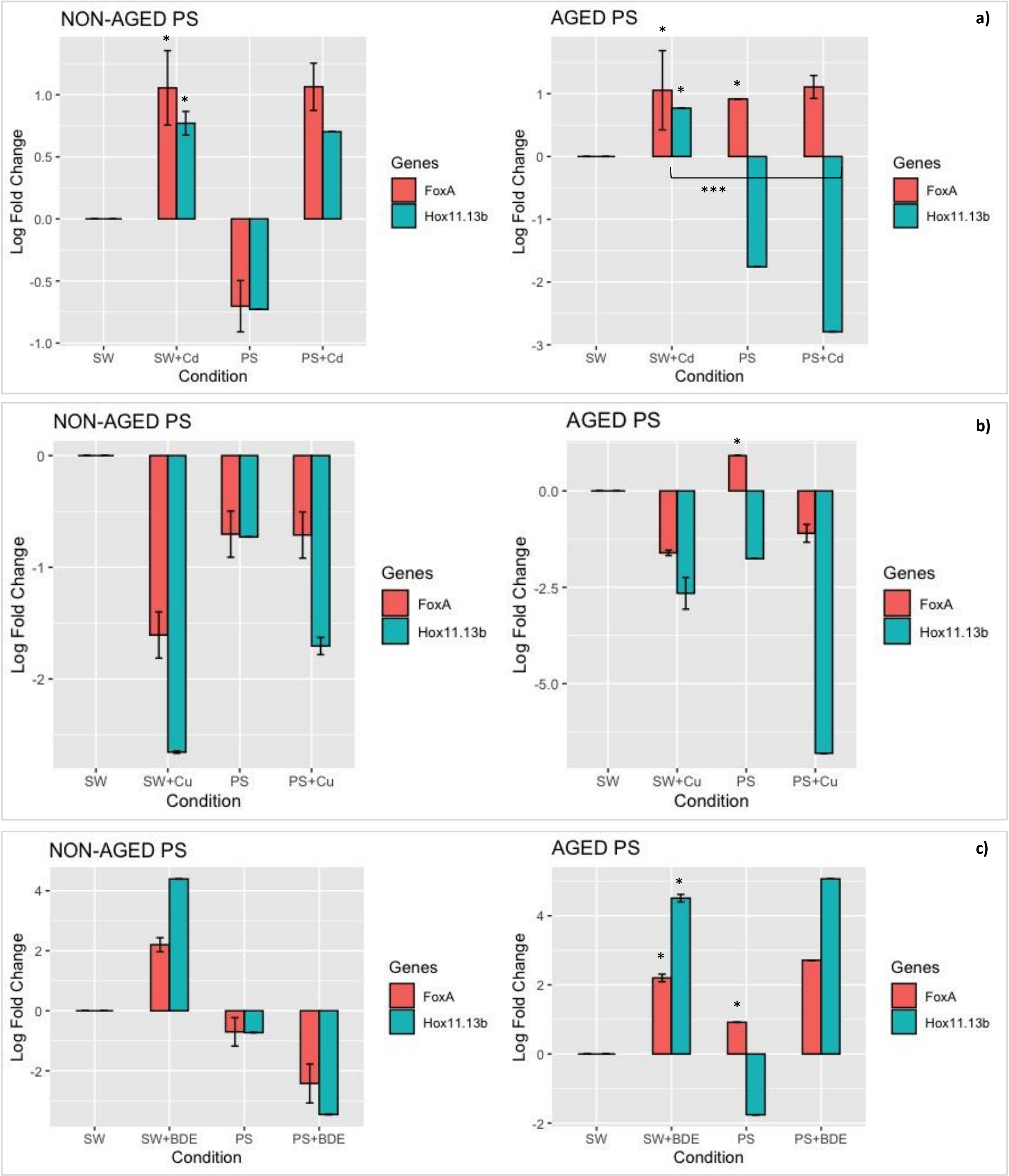


Figure 3.15: RT- qPCR results showing the mRNA levels, *Forkhead box transcription factor a*, *foxA* and *Homeodomain containing transcription factor, hox11/13b*, with respect to reference gene 18S, in embryos at 24 hpf (gastrula stage) exposed to a) Cd or b) Cu or c) BDE-47 without or in association with non-aged or aged PS, with respect to 18S at 24 hpf (gastrula stage). Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at *p* <0.05 (*); *p* < 0.01 (**); *p* < 0.001 (***); one-way ANOVAs with Tukey's test.

The transcript levels of these genes in embryos exposed to mixture of each metal with BDE-47 (Figure 3.16 d-e) revealed that *foxa* and *hox11.13b* mRNAs were significantly up-regulated (*p*<0.001) in embryos exposed to

Cd/BDE-47 blend compared to untreated exposures. However, when PS microparticles were added into the mixture, a change in gene expression profile occurred. Specifically, a significant down-regulation ($p<0.001$) of both genes emerged in presence of non-aged PS microparticles; while, a significant up- and down-regulation of *foxa* ($p<0.01$) and *hox11.13b* ($p<0.001$) genes, respectively, occurred when aged PS microparticles were added to the mixture if compared to exposure without PS (Figure 3.16 d). Nonetheless, in embryos exposed to Cd/BDE-47 mixture in presence of aged PA fibers an over-expression of both genes was found (Figure S3 c, Appendix B).

Finally, the mRNA levels detected in embryos co-exposed to Cu and BDE-47 (Figure 3.16 e) showed a transcriptional profile similar to single Cu treatment, given the significant down-regulation for both genes ($p<0.001$) observed. Although the addition of non-aged PS microparticles did not significantly change gene expression profile in the mixture (Figure 3.15 b), a significant up-regulation of *foxa* and *hox11.13b* mRNA levels ($p<0.001$) was detected in the exposure mixture with aged PS microparticles compared to exposure without PS at the same conditions (Figure 3.16 e).

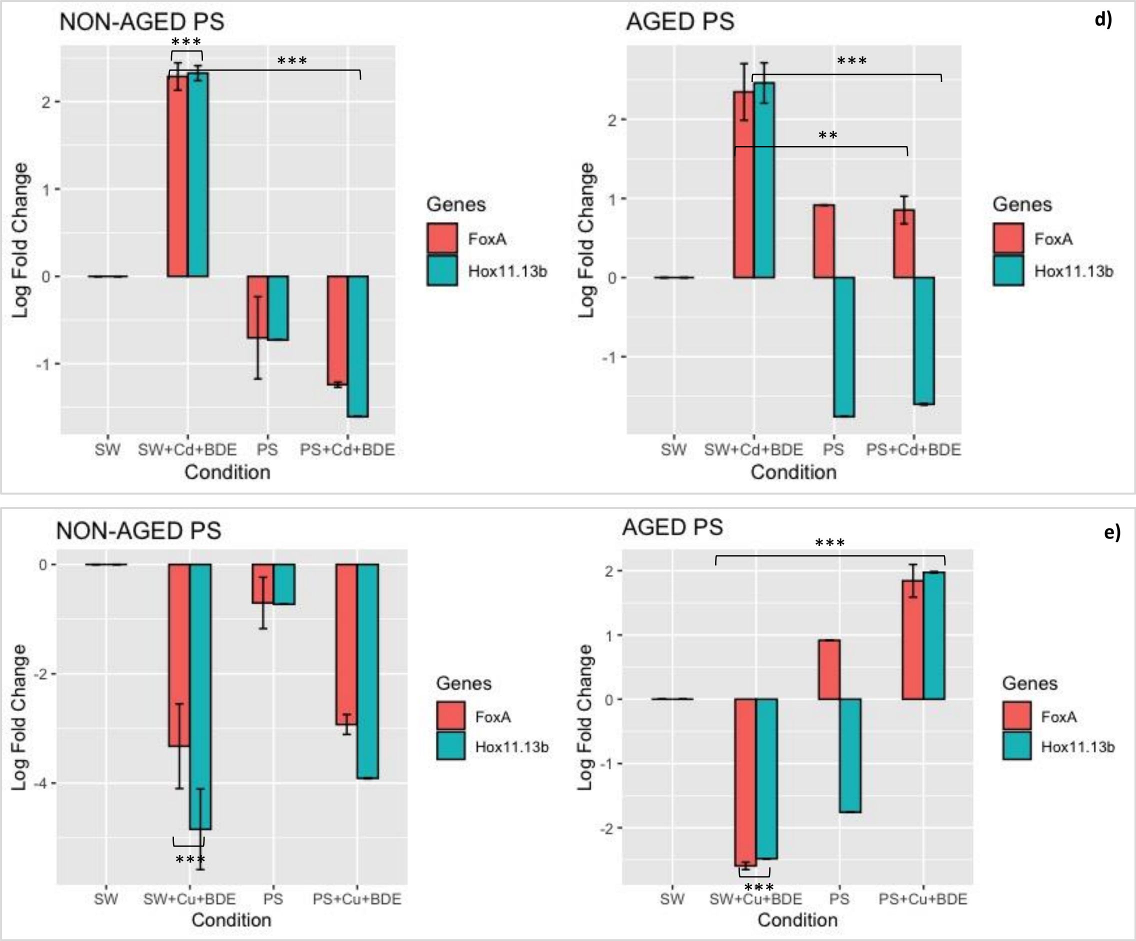


Figure 3.16: RT- qPCR results showing the mRNA levels, *Forkhead box transcription factor a*, *foxa* and *Homeodomain containing transcription factor, hox11/13b*, with respect to reference gene 18S, in embryos at 24 hpf (gastrula stage) exposed to the mixture of each metals d) Cd or e) Cu with BDE-47 without or in association with non-aged or aged PS. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***); one-way ANOVAs with Tukey's test.

3.5 Discussion

The results of this study provide evidence that the effect-based molecular approach provide estimate of bioavailable fractions of compounds associated with non-aged and artificially aged MPs. Changes in gene expression observed after individual chemical treatments in presence of MPs revealed that aging process can influence acute effects of cadmium, copper and BDE-47 on *P. lividus* embryos. Herein, for the first time, BDE-47 effects on early life stages of *P. lividus* were analyzed by following the expression of genes involved in several physiological processes as well as changes in bioavailability of this organic pollutant when non-aged or aged MPs were added into the solutions. Despite the fact that MPs can sorb environmental pollutants with different molecular structures and hydrophilicity/hydrophobicity properties, changes in the interactions between sorbent and sorbate occurred after aging process and led to establishment of molecular mechanisms perturbing the canonical developmental program as well oxidative status.

3.5.1 Characterization of the aged MPs and sorption behavior of contaminants

The SEM images showed a reduction of size with loss of sphericity and some sign of deformation on the surface of aged PS particles in comparison to non-aged microparticles. Although the first visual effects of polymer degradation are changes in color and crazing of the surface (Gewert et al., 2015), it has been observed that the changes on the surface topography, such as the presence of cracks and fissures, are to attribute to associated biota (Reisser, J. et al., 2014; Kowalczyk, A. et al., 2016). Naturally weathered samples have generally smoother surfaces without any significant cracks or holes despite the fact that they exposed to physical/mechanical treatment (i.e. waves, temperature and UV irradiation) (Syranidou E. et al., 2017). As indicated by Meides et al., 2021, in weathering simulation, cracks become visible on the PS particle surfaces for solar exposure simulation >800 h, a time more long in comparison to that used in this study (~504 h). Thus, the smooth surfaces without significant clearly visible cracks lines or holes observed on aged PS microparticles, represents a first degradation stage associated with an exposure period between 0 and 500 h, and characterized by a photooxidation in a near-surface layer and a decrease of molecular weight induced by irradiation. Actually, obtaining a suitable contrast level in SEM micrographs of these samples has been quite challenging, probably due to their small sizes (1 – 1.49) and to the presence of salts in the solution of PS microparticles that created a background signal. A wide decrease in molar mass for both PS microparticles and PA fibers was observed upon photodegradation as emerged by SEC and viscosimetric analyses. Size exclusion chromatography allows separating macromolecules by their hydrodynamic volume, and represents a fundamental technique for soluble polymer characterization that surprisingly have not be used commonly for the characterization of MMs of microplastics. A decrease by 43.2 % in number-average molecular weight (M_n) for PS microparticles exposed to 21 days of light irradiation was in fact detected, as well as for PA fibers (by

36%). Also the weight-average molecular weight (M_w) followed the same trend. Since the molar masses of a polymer are proportional to the number of monomers units forming the polymer chain, a decrease in the molar masses is inevitably related to a decrease of the chain length (Yousif and Haddad, 2013; Ter Halle et al., 2017). In addition, the polydispersity index (PDI, M_w/M_n) of the weathered microparticles exhibited almost constant values after aging process suggesting the occurrence of a no-selective bond scission along the macromolecular chain. Interestingly, when Cu was present in the solution with PS, both alone and in mixture with BDE-47, a higher decrease of molar mass (almost 50%) have been observed for photo-exposed PS in comparison to non-aged microparticles. This result could be probably related to catalytic mechanisms influencing photo degradation processes occurring MPs that consequently could altered the stability of polymer. The length of the polymer backbone as well as its morphology have important consequences on microplastic behavior in the marine environment. In fact, when a microplastics breaks down into smaller microscopic fragments, these fragments are likely to possess properties that may differ from their parental fragments, since upon aging, the smaller fragments are more oxidized and have a lower molar mass. This certainly affects the mode of interaction of these microparticles with organisms and should be considered in exposure studies as well as in the bioavailability of chemicals.

The appearance of new peaks in MALDI spectra, observed in photo-exposed PA fibers but not in virgin samples, highlighted a probably occurrence of a degradative process. When radiation was absorbed by polymer, a photo-oxidation process occurs and free radicals generate due to breaking of C−H bonds inside the chains of the polymers (White and Turnbull, 1994; Wypych, 2003). However, although in aged PA fibers four new peaks, attributable to new structures with amide or carboxylic group in their functional groups appeared, for PS microparticles no change in their functional groups emerged after photo-exposure. The observation of new functional groups as carboxyl, aldeidic, amidic and hydroxyl groups demonstrated that MPs aging was a result of oxidation. Weathering process modified the polarity of PA fibers as well as their sorption features due to a shift from hydrophobic to even more hydrophilic character of the system, although PA due its hydrophilic groups such as amides is itself a more hydrophilic polymer compared to PS where no hydrophilic functional group is present.

The effects of aging on sorption process of contaminants on microplastics evaluated by ICP-MS and GC-MS, were dependent on the type of contaminant and, in a lesser way, on the type of polymer tested. As revealed from data obtained by ICP-MS analysis (Figure 3.10 and 3.11), Cu content in the solutions containing virgin PS microparticles, as well as PA fibers, drastically decreased, if compared to negative control (without any contaminant and microplastics). Our outcome confirmed that particle size was an important factor determining the adsorption of metals onto MPs. As shown in figure 2.10 and 2.11, the Cu adsorption capacity for the two virgin microplastic materials is PS > PA owing to greater surface area of smaller microplastic particle size as suggested by Wang et al., 2019. Thus, the increase in metal adsorption by virgin polymers is reasonable due to electrostatic interactions, van der Waals forces, and π–π interactions (Fu et al., 2020; Lin et al., 2021; Torres et al., 2021). PS-MPs have a negative charge on their surface (Lin et al., 2021)., thus the electrostatic attraction between these negatively charged MPs and positively charged metal ions promotes the adsorption behavior; however, they have different adsorption capacities for different metal ions also ascribed to their specific physical and chemical properties (Li et al., 2019). Although from the other experimental results, it can be seen that the adsorption of heavy metals by PS increases with increasing aging degree (Guo et al., 2018), in this study contrasting results were obtained in relation to aging effect of both microplastic types. Specifically, higher Cu concentration in the solutions was measured. The decreased Cu adsorption on aged microplastics can be attributed to abiotic factors such as salinity and aged surface features that may decrease the adsorption capacity of microplastics for heavy metals. As emerged from other studies, the weathering did not always improve the adsorption process and in some cases, changes in the surface of microplastic polymers may reduce the rate of the adsorption process (Huang et al., 2021) as well as the electrostatic interactions between metals and microplastics. However, it should be noted that, since generally the degree of desorption depends on the interaction forces between the adsorbent and the adsorbate (Wang et al., 2019), it is reasonable to suppose that the high desorption rate observed for Cu in both tested polymers can be is due to a variation in the binding forces between metals and MPs. In particular, weaker binding interactions provided by physical adsorption can dominate on chemical sorption process. As consequence, become plausible to consider that for both aged plastic polymers the break of the chain length observed as reduction of molecular weight, may contribute to a release of metals in solutions as result of a weak physical interactions dominating the adsorption mechanism. Concerning Cd sorption, ICP-MS analysis did not highlighted any decrease in concentration for both polymer types tested or differences between non-aged/aged conditions. Although some studies provided evidences that MPs can accumulate Cd thus posing risks to the organisms exposed to them (Zhang et al., 2020; Lu et al., 2018), it should be noted that in these studies the adsorption capacity of MPs for Cd are related to freshwater conditions. However, the adsorption rate may vary depending adsorption conditions as well as environmental conditions (Wang et al., 2019); herein, the adsorption characteristics of metal by MPs were investigated using artificial seawater as medium for batch experiments and the salinity of the surrounding aquatic medium can alter the adsorption behaviors of pollutants onto plastics. In particular, increased salinity levels generally lead to lower sorption capacities of for heavy metals as for example Cd, Co and Ni on PE (Holmes et al., 2014). As emerged in this study, results for Cd adsorption are consistent with this effect of salinity, since no significant difference was observed in Cd concentration in the solutions containing virgin PS microparticles and PA fibers. Thus, it is reasonable to suppose that in presence of NaCl, the competing effect of the background electrolyte ions causes the competition between Na and Cd for the adsorption sites. In addition, co-presence of Cl^- and Cd forming several complexes including $CdCl^+$ or $CdCl^-_3$, as well as enhanced aggregation of MPs as result of an higher ionic strength, could lead to a further Cd adsorption onto MPs decrease (Wang et al., 2019; Hall et al., 1995). On the other hand, the higher sorption of BDE-47 onto PS and PA was not influenced by salinity, as showed by previous studies according to which organics with hydrogen bonds in molecules are not sensitive to the concentration of NaCl (Bakir et al., 2014; Zhang et al., 2018). This observation suggests that the sorption process may not be mediated by ion-exchange sorption but rather by intramolecular hydrogen bonds. Wu et al., 2020 suggested that the aged PS have a low sorption capacity for BDE-47, if compared with virgin PS, and that environmental aging processes and photooxidation can lead to the formation of polar functional groups, thereby decreasing the sorption of hydrophobic organic pollutants. Conversely to what reported in Wu et al., 2020, in the present study the sorption of BDE-47 on both PS microparticles and PA fibers was not affected by aging, as the BDE levels found were below the detection limit. Our results appeared in line with MALDI spectra from which no appearance of oxygen-containing functional groups have been detected. Indeed, the lack of polar functional groups in addition to the reduction in molar mass emerged by SEM analysis, may be led to the higher sorption capacity showed by BDE-47 onto polymers tested. Since smaller microscopic fragments generated after photo-exposure have a total surface greater than the larger parental particles, smaller particles present a greater potential for interactions with organic contaminant. In addition, also salinity may have a key role in reducing the appearance of oxygen-containing functional groups as well as influencing the sorption of hydrophobic organic pollutants (Wu et al., 2020).

Combined exposure to Cu and BDE-47 caused significantly higher release of Cu in solution containing photo-exposed PS microparticles if compared to the treatment with single Cu-aged PS. This may be due to the reduction in molar mass observed for PS microparticles after aging that has led to a release of metal in solution. In addition, the presence of an organic chemical such as BDE-47, by competing with Cu ions for the adsorbing sites, contributed to reduce the availability of surface sorption sites for Cu. However, the presence of BDE-47 in the mixture with aged PA fibers did not influence Cu release if compared to single Cu-aged PA, probably due to different hydrophobicity of PA than PS and for which no real competition between two contaminants took place. Therefore, the effects of aging on sorption process of contaminants on MPs were dependent on the type of contaminant and, in a lesser way, on the type of polymer. MPs tested have different adsorption capacities for different metal ions. Both non-aged MPs can sorb Cu metal ions, although PS microparticles largely; while no association with Cd have been detected. The aging treatment changed the MPs surface to varying degree. A 21-days photooxidation led to a high decrease of molecular weight for both polymers, PS microparticles and PA fibers; however, while aged PA fibers have the appearance of oxygen-containing functional groups on the surface, aged PS microparticles did not show this behavior. The decrease of sorption capacity observed for Cu mostly likely could be related to reduction of molecular weight after UV exposure that lead to the promotion of weak physical interactions that prevail the adsorption mechanism. Aging treatment may not change the low adsorption capacity detected for Cd since salinity may play a pivotal role on the interactions between Cd and both polymers. Photo-exposure not changed the high sorption of

BDE-47 on either PS microparticles or PA fibers; in this case, the absence of polar functional groups maintained the hydrophobicity of polymer whilst the reduction in molar mass, generating smaller particles with a higher total surface area, enhanced the interactions between sorbate and sorbent and consequently BDE-47 sorption. Finally, after aging, a competition mechanism may also affect the sorption of Cu on PS microparticles but not PA fibers, in the combined exposure with BDE-47.

3.5.2 Pathway-focused gene expression profiling in response to co-contaminants and MPs treatments

Experiments focused towards determining the effects on development *of P. lividus* embryos which came in contact with small PS microparticles (1-1.49 µm) or large PA fibers (>500 µm) after fertilization did not show strong developmental defects. Although this result is according with some studies using similar MPs concentration (Thomas et al., 2020; Trifuoggi et al., 2019) other studies have noted a significant increase in developmental deformities and anomalies at MPs concentrations lower than that used in this study (Messinetti et al., 2018) especially for sea urchins belonging to different species. Thus, a different interspecies sensitivity to microplastics as well as a different life stage sensitivity may lead to different responses that in turn could have different impacts at the community level (Thomas et al., 2020). Based on the morphological observations, exposure to both microplastics reduced embryo aberrant phenotypes. As consequence, the RT-qPCR results related to embryos exposed to PS/PA microplastics and contaminants were not expected to be affected. Nevertheless, it was important to profile the gene expression pattern of key factors involving in stress response regulation as well as in molecular mechanisms orchestrating the regulatory network of embryo development. The analysis of mRNA levels of target genes allows the detection of early warning signs of possible damage, since changes at the molecular level occur prior to manifestations at higher levels of biological organization (Teles et al., 2016). In fact, the gene expression is an indication that the organisms responds to a stress, which may not be visible at a phenotypic level, because the expression is part of the adjustment mechanism of the organism. However, long term exposure means that the organism is using more energy to keep functioning while responding to the stress, which may result in effects at later stagesThe presence of MPs can affect the availability of contaminants for embryos and consequently, influence their toxic effects. Thus, in this study acute effects at transcriptional level of sea urchin embryos exposed to different treatments groups (in aging or non-aging conditions) were investigated.

The heat shock protein (*hsps*) plays a protective role as chaperones in protein folding in order to prevent their aggregation also in response to different stress, including metals (Roccheri et al., 2004; Gupta et al., 2010). The protective function of *hsp70* and *hsp60* against oxidative stress associated to metal exposure (Liu et al., 2014; Nicosia et al., 2014, 2015; Guo et al., 2018) as well as induced by micro- and nanoplastics is well documented (Varò et al., 2019; Imhof et al., 2017; Pinsino et al., 2017; Brandts et al., 2018). In the present study, the increase in *hsp70* and *hsp60* levels in embryos treated with individual cadmium or copper solutions without MPs, appeared according with previous studies reporting the increase in *HSPs* levels in larvae or tissues of *P. lividus* after metal exposure (Morroni et al., 2019; Migliaccio et al., 2015; Di Natale et al., 2019). However, in this study, PS microparticles exposure did not lead to an upregulation of heat shock proteins as shown in other findings on a range of organisms exposed to functionalized MPs (Varò et al., 2019; Brandts et al., 2018). Rather, the gene expression profile of both *hsp70* and *hsp60* appeared indiscriminately downregulated both in aged or non-aged PS microparticles individual exposure, except for a slightly but significant transcriptional upregulation of *hsp70* in exposures with aged MPs. Although upregulation of *HSPs* expression can be considered as a rule in organisms exposed to pollutants, their downregulation following exposure has also been reported (Gupta et al., 2010; Gomes et al., 2013; Izagirre et al. 2014). At least two no-excluding effects may have contribute to transcription level reduction of stress response genes after PS exposure. First, the downregulation of this gene transcript does not necessarily reflect low *hsp* protein level, since high levels of *hsp70* have shown to lead to inactivation of its own transcription factor, through a negative feedback mechanism (Pinsino et al., 2017). Second, the surface of MPs used in this study did not have specific surface modifications such as carboxylated groups (-COOH) or unsaturated amine (NH2) for which have been shown to induce a cellular response against oxidative stress (Della Torre et al., 2014). Moreover, it should be noted that virgin microplastics are generally supposed to be free from any additives and/or residual monomers. Some studies revealed a certain toxicity likely associated either by the migration of toxic residual or unreacted quantities of styrene/ ethylene monomers (used in production process) from the virgin PS microspheres (Martin-Gòmez et al., 2017). Aging process may facilitate or enhance the leaching of secondary chemicals (Bejgarn et al., 2015). Thus, in this study, an influence of aging process on changes in expression profile detected after exposure to aged PA/PS could be hypothesized. The reduction in molecular weight observed after PS/PA photoexposure may led to a probably leaching process of secondary toxic products that in turn may have induced *hsp70*-mediated response observed. However, it is reasonable to suppose that such induction was polymer type-dependent, since no induction of stress genes after exposure to aged PA fibers emerged. Therefore these results confirm that surface chemistry of MPs strongly influence the toxic potential of them and their interactions. Given the capacity for leached compounds to cause toxicity at several levels even to very low concentrations (Rochman et al., 2014), it is crucial that chemical profiling of microplastics used in toxicity testing becomes more commonplace (Cole et al., 2019). Finally, although the evaluation of MPs ingestion was beyond the scope of this study, it is not possible to exclude the occurrence of an ingestion process, since the size of the microparticles selected for this study was sufficiently small to be ingested and uptake by sea urchins. As showed by Della Torre et al. 2014, MPs could adhere to the external surface of blastulae, be internalized during invagination of primary mesenchyme cells and then, be accumulated in the digestive tract during the gastrulation stage.

The reduction of transcriptional levels of *HSPs* detected in embryos co-exposed to non-aged PS and Cu may indicate an increase in Cu sorption capacity and consequently a lesser bioavailability of copper for embryos, if compared to control without PS microparticles. However, aging process influenced sorption capacity of Cu by MPs and then its bioavailability. The increase of *hsp70* transcript level observed in embryos exposed to aged PS/Cu combination permit to suppose a decrease in sorption capacity of Cu by MPs. As consequence, the higher release of Cu in solution may lead to a higher bioavailability of it for embryos. On the other hand, aging process did not appear to affect Cd bioavailability, since no changes in gene expression were observed after PS/PA non-aged and aged treatments. Thus, changes in gene expression observed would seem to correlate with data obtained from chemical analysis and may represents a very sensitive tool for a cellular damage analysis in response to MPs exposure. This interpretation of results is also supported by general decrease in stress response genes detected in embryos exposed to combined treatment with non-aged PS/BDE-47. On basis of these considerations, it would have expected a decrease of transcript levels in aged PS microparticles exposure, for which no decrease in sorption of BDE-47 by GC-MS analysis was emerged. This result herein obtained could be explained by unknown toxic chemicals probably leached from MPs after UV exposure. Thus, appear reasonable to suppose that the induction of genes involved in stress response observed in embryos co-exposed to aged PS/BDE-47 is resulting from a cumulative or synergetic effects by inert particle and various leaching chemicals. In addition, since the molecules of PBDE are aromatic conjugate structure, they have the property of absorbing UV spectrum energy. Therefore, the molecules could eliminate bromine atom and degradate (Liu et al., 2019; Zhao et al., 2015). Consequently, albeit BDE-47 bioavailability was low (< 1.5 µg L^{-1} indicated as instrument detection limit for GC-MS), an additive effect of degradation BDE-47 by-products UV-induced cannot be excluded. Effectively, embryos exposed to BDE-47 without MPs in the aging conditions showed a more significant induction of genes belonging to the different pathways investigated (i.e. *hsp70*, $p<0.001$) than to same exposure in non-aging conditions (i.e. *hsp70*, $p<0.01$). On the other hand, the opposite expression profile observed after exposure to non-aged PA fibers with BDE may be attributed to higher hydrophilicity of polymer than PS. In fact, more hydrophilic MPs, such as polyamide, seem to have lower sorption affinity for non-polar organic compounds (Hüffer & Hofmann, 2016). Thus, a lower sorption of BDE-47 by PA in comparison to PS could be hypothesized. As consequence, even at very low concentration, the bioavailable fraction of BDE-47 could be responsible of the cellular response against oxidative stress observed. Metallothioneins (MTs) is an ubiquitous system composed either constitutive that inducible proteins which is known to counteract the metal-induced cytotoxicity in different systems (Amiard et al., 2006; Arini et al., 2015; Chaâbene et al., 2018), including the sea urchin embryo exposed to metals (Ragusa et al., 2013, 2017a; Migliaccio et al., 2015). It has been reported that during sea urchin development, embryos express *MT7* and MT8 transcripts at high levels. Conversely, *mt4, MT5* and *MT6* are expressed at low levels and were found induced by metal exposures (Ragusa et al., 2013, 2017a). Thus, the effects of single or combined metal or MPs exposures were herein analyzed on the expression of sea urchin inducible metallothionein 4 (*mt4*). Surprisingly, metal exposures (both Cd and Cu treatments) did not activate the expression of the inducible metallothionein, indeed the *mt4* mRNA expression level was negatively affected, while his level appeared increased after BDE-47 exposure. The lack of *mt4* upregulation has been also described elsewhere. The results of this study appeared in fact consistent with other studies where model organisms exposed to moderate anthropogenic activity and metal contaminations, did not show a marked increase of MT protein amount (Strogyloudi et al., 2014). Moreover, it is known that metals induce MTs expression in a concentration dependent manner; for example, Ragusa et al., 2013, 2017a reported for cadmium exposure a concentration threshold for the *PlMT4* activation equal to 1–10 µM, a much higher concentration range than that used in this study (5 µg L^{-1}). Therefore, it is reasonable to suppose that sea urchin exposures at non-lethal conditions may result in tolerance to metal toxicity, although for organic chemicals that tolerance di not subsist. Further researches are required to evaluate the *MTs* mRNA expression levels (including constitutive *MTs*) at much higher metal concentrations as well as over other development stages and exposure times after BDE-47 treatment.

The RT-qPCR analysis revealed that mixture exposures differently affected *mt4* and *HSPs* transcriptional expression compared to control groups. It has been shown that several pollutants, usually present at the same time in the aquatic environment, may produce either neutralizing, additive or synergistic effects, thus resulting in variable degrees of toxicity (Di Natale et al., 2019; Ragusa et al., 2017b). According to this, albeit *mt4* mRNA was downregulated in individual exposure to Cd, in embryos exposed to Cd/BDE-47 mixture its transcript was found increased. In addition, *hsps* genes shown a more marked upregulation if compared to control treatment with each individual contaminant. Therefore, it is reasonable to suppose that neutralizing or synergistic mechanisms have herein acted to upregulate mRNA expression of *mt4* and *HSPs*, respectively. Similarly, the *HSPs* and *mt4* upregulation observed in individual contaminant exposures were abrogated when Cu/BDE-47 mixture was tested. Thus, the decrease in the mRNA levels of stress and metal scavenging genes may likely be associated to neutralizing activity exerted by Cu/BDE-47 blend. The addition of both non-aged/aged PS microparticles into the Cd/BDE-47 mixture seem to reduce the induction of genes involved in stress response respect to control groups. Given higher Cd bioavailability emerged from chemical analysis, it would have expected an expression profile similar to that obtained after combined treatment with non-aged PS and Cd. Therefore, it could be hypothesized that the presence of BDE-47, although present in solution at very low concentration, may abrogate the actions of specific members of the pathways that have acted to downregulate *mt4* or to upregulated *hsp60* mRNA in non-aged PS/Cd co-exposure. Curiously, in presence of aged PS microparticles, the transcriptional upregulation of *mt4* was abrogated; this result could be support the hypothesis that a possible leaching process occurred after polymer aging, influencing the transcriptional upregulation of *mt4*. According to the low bioavailable fraction of Cu and BDE-47 detected by chemical analyses, embryos exposed to Cu/BDE-47 mixture showed a general downregulation pattern in presence of non-aged PS microparticles. However, the expression profile emerged from embryos co-exposed to Cu/BDE-47 with aged PS microparticles lead to suppose a probably influence of aging process on stress response regulatory mechanisms. The significant upregulation of *mt4* and *hsp70* transcript levels may be associated to the increase in Cu bioavailability. This, in addition to chemical products leached out from aged microparticles may have acted in a neutralize, synergistic or additive manner to determine the induction of the cellular response against oxidative stress observed.

Generally, for a normal development, an accurate control of gene expression is required. For example, in the gene regulatory network related to skeletogenic differentiation of *P. lividus*, the maternal factor *Ets1* activates the expression of several genes that in turn, cooperating, lead to activate the expression of terminal differentiation genes such as spicule matric gene *sm50, msp130* and *p16* (Ben-Tabou and Davidson, 2007; Gildor et al., 2015). As reported by different investigations, environmental stressors including metals (Masullo et al., 2019; Morroni et al., 2019), X-rays (Matranga et al., 2010), bioactive substances (Varrella et al 2016) and UVB (Bonaventura et al., 2005) affect the expression of the skeletogenic genes. Therefore, changes in the expression of these genes may be due to the need to carry on the development under stressful conditions. The expression level of skeletogenesis genes investigated appears in line with previous data showing an upregulation of *sm50* after Cu treatment and a general downregulation of *sm50* and *p16* genes as well an upregulation of *msp130* transcript after Cd treatment, at stage development considered (Migliaccio et al., 2015; Masullo et al., 2021; Morroni et al., 2019). Similarly to expression profiles showed by stress response genes, no evident changes in expression of members of skeletogenic differentiation emerged after exposure to PS microparticles (both non-aged and aged), except for the *sm50* upregulation observed in the solution containing aged PS microparticles. That induction may be associated to degradation process took place after UV exposure which likely led to release of unknown toxic chemicals by MPs. However, as already showed for stress response pattern, the induction appeared polymer-dependent, since no induction after exposure to aged PA fibers emerged.

Although no particular changes in gene expression were found in embryos co-exposed to Cd with aged and aged PS microparticles, co-exposure to aged PA fibers and Cd led to a different gene expression profile respect to unaged fibers. The different data herein obtained could be explained by the appearance new functional groups detected in PA photo-exposed which are able to make interaction with cadmium, reducing its bioavailability. The addition of PS microparticles in the combinatorial treatment with Cu reduced the induction of genes involved in skeletogenic differentiation respect to control groups. However, the upregulation of *msp130* transcript found in embryos after exposure to aged PS and Cu appeared to match with the greater release of Cu in solution detected by chemical analyses. Moreover, a synergistic mechanism associated to secondary chemicals derived from polymer aging process may explain the faintly change in gene expression observed for *p16* mRNA after exposure to aged PS microparticles.

The expression profile associated to exposure of embryos at BDE-47 highlighted a significant upregulation of all genes involved in skeletogenesis process. The addition of non-aged PS microparticles reduced the induction of *sm50, p16* and *msp130* mRNAs, according to a lesser bioavailability of BDE-47 detected by chemical analyses. However, once again, aging process negatively affected gene expression leading to significant upregulation of all three genes at levels comparable to untreated controls. Although no change in BDE-47 sorption capacity emerged, cumulative effect attributable to leached chemicals from photoexposed PS microparticles could be hypothesized. In addition, low concentration of BDE-47 (below the detection limit) as well as BDE-47 by-products UV-generated may have contribute to aberrant expression of genes involved in skeletogenesis process.

In the mixture of BDE-47 with Cd or Cu, gene expression profile appeared faintly related to that activated by single contaminant. In particular, since in the mixture with cadmium and PS microparticles (both non-aged and aged) Cd availability appeared no changed, a dose-dependent process may be hypothesized in relation to BDE-47. Indeed, the decreased expression of skeletogenesis genes, permit to suppose that co-occurrence of Cd and BDE-47 in absence of MPs may synergize the actions of specific members that have acted to upregulate *sm50* and *p16* mRNA expression. As consequence, when non-aged PS microparticles were added to the blend, the lesser BDE-47 bioavailability detected in comparison to control treatments may be responsible for the loss of synergistic effect hypothesized. On the other hand, the general downregulation found in embryos exposed to Cd/BDE-47 mixture in association with aged PS microparticles highlighted the influence of aging process on gene expression. A probably leaching process of chemicals from aged PS may have contribute to generate expression profiles poorly related to those detected by non-aged PS. Conversely, the higher hydrophilicity of PA respect to PS, in addition to the reduction in molecular weight detected after photo-exposure, may lead to a higher bioavailability of BDE-47 and consequently to determine induction of all 3 genes observed in Cd/BDE-47 mixture in association with aged PA.

Finally, in the mixture of BDE-47 with copper the lesser BDE-47 bioavailability detected in presence of either non-aged that aged PS microparticles, is also coupled to a change in Cu bioavailability. The co-presence of Cu and BDE-47 may abrogate the actions of specific members able to determine the upregulation of skeletogenesis genes observed in individual treatments. Albeit the addition of non-aged PS to the Cu/BDE-47 blend did not change the expression profile of skeletogenesis genes (probably due to comparable bioavailability of both chemicals tested) a significant change emerged in presence of aged PS microparticles. The lesser BDE-47 bioavailability, in addition to the greater Cu availability detected after PS photoexposure, may be responsible for the loss of neutralizing effect supposed. Moreover, the leaching process of chemicals from aged PS may have contribute to generate expression profiles poorly related to those detected by non-aged PS.

Among the genes involved in endodermal differentiation process (*foxa and hox11.13b*), all were found affected by cadmium, copper and BDE-47 individual treatment. The data herein obtained appeared in line with previous studies on the expression pattern of these specific genes observed in embryos exposed to metals (Masullo et al., 2021; Morroni et al., 2019). However, there are no previous data about the expression of these genes in response to BDE-47 or in response to the combination of each selected chemical with microplastics. As expected, the gene expression profile emerged from embryos co-exposed to Cd and non-aged PS microparticles (or PA fibers), provided evidence that MPs, albeit faintly, perturb the normal pattern of endodermal specification. However, aging process have led to a change in gene expression pattern either in PS microparticles individual treatment that in the combinatorial exposure with Cd. Despite no significant change in Cd bioavailability occurred after photoexposure, these findings confirm once again the leaching process hypothesis that may lead to synergistic mechanisms able to provide gene expression profiles not overlapping to those occurring in response to virgin/non-aged MPs. On the other hand, the different structure of the polyamide in addition to its different ability to make interactions after photo-exposure could explain the general downregulation found after exposure to aged PA fibers and Cd.

No copper related-differences were measured in *foxa and hox11.13b* transcript levels, which resulted in an overall downregulation. These finding appears in agreement with Morroni et al 2019, where at gastrula stage genes belonging to development/differentiation started to react to copper decreasing their expression levels (Morroni et al., 2019). Given the lower Cu bioavailability measured in the aqueous phase containing non-aged PS microparticles, no change in gene expression were found, except for the greater downregulation of *hox11.13b* mRNA observed in aged PS/Cu co-exposure. Therefore, it could be hypothesized that in sea urchin embryos, co-presence of copper and photodegraded products PS-derived, may synergize the downregulation mechanisms acting on regulators of endodermal specification.

Similarly to what occurred with other stress response and skeletogenesis pathways, differences in gene expression were once again occurred after co-exposure to BDE-47 and MPs.

Considering that no changes in BDE-47 bioavailability have been detected among treatments carried out, the upregulation of *foxa* and *hox11.13b* mRNAs found in BDE-47/aged PS exposure may be explained by changes in interactions polymer aging-induced.

Moreover, different effects after co-exposure of each metal with BDE-47 have been generated. Interestingly, both positive and negative fold changes in mRNA levels observed in response to single contaminant appeared mitigate. In particular, co-exposure to Cu and BDE-47 blend neutralized the transcriptional induction of *foxa* and *hox11.13b* mRNA highlighted in individual exposure to BDE-47. On the other hand, a synergistic effect leading to a higher induction of both genes could be hypothesized in Cd and BDE-47 co-exposure.

Since combinatorial exposure may result in unpredictable toxicity effects (Wah Chu and Chow, 2002), these findings confirm once again that the co-occurrence of different stressors, triggering multiple pathways, may provide gene expression profiles that are not overlapping to those occurring in response to a single contaminant.

Curiously, addition of MPs to the mixtures led to a change of transcriptional effects occurred in untreated control groups. Given the lower BDE-47 bioavailability and the unchanged bioavailable fraction of Cd measured in the solutions containing both non-aged and aged MPs, an induction of both genes would been expected. Thus, the dual downregulation found after co-exposure to Cd/BDE-47/non-aged PS microparticles could be explained by the occurrence of regulatory mechanisms acting in BDE-47 dose-dependent manner.

Indeed, although at low concentration, BDE-47 may cooperate with Cd into generation of a new gene expression profile faintly related to that deriving from individual contaminant exposure. On the other hand, in Cu/BDE-47 mixture, the presence of non-aged PS microparticles that lead to a lesser BDE-47 and Cu bioavailability, did not affect negative fold changes in *foxa* and *hox11.13b* mRNA levels.

Curiously, addition of aged MPs to Cd/BDE-47 mixture led to further new transcriptional profile that appeared different for PS and PA. In particular, expression pattern related to PS appeared similar to that of Cd/PS individual treatment; on the contrary, gene expression profile for PA was found similar to that of BDE-47/PA individual treatment. Thus, it is likely to suppose the occurrence of aging processes differentially acting in PS and PA as well as in the mixture of each metal with BDE-47. Given the lesser and greater BDE-47 and Cu bioavailability, respectively emerged in embryos co-exposed to Cu/BDE-47 and aged PS microparticles, an overall downregulation was expected. Therefore, the different data herein obtained could be explained by the co-presence of copper and photodegraded products. These, in association with low BDE-47 levels (below instrument detection limit) may have led to abrogate the actions of members of the pathways that have acted to downregulate *Fox A* and *hox11.13b* mRNA expression in untreated control groups as well as in exposure with aged PS and Cu.

Overall, this study provided suitable tool for assessing the microplastic effects upon early life stages of marine organisms evaluating the release of hazardous compounds from pristine and aged polymers. Although the effect-based molecular approaches provide estimates of bioavailable fractions of the compounds, the interactions between compounds may hinder by the specificity of the selected biological response. As emerged from different exposures herein carried out, co-occurrence of different stressors may interact with different receptors able to trigger response via the same pathways. Consequently, gene expression profiles quite different from those provided in response to a single contaminant may be generate. Thus, molecular approach represents a valuable tool to determine bioavailability of chemicals sorbed or desorbed by MPs on gene expression level, but places limits when mixture of pollutants are considered. Since chemical sorption/desorption and following uptake in organisms depend on physicochemical properties of sorbate and sorbent, a multidisciplinary approach based on evaluation of these variables should be considered in vector studies with vivo models.

3.6 Conclusions

This study offers a first molecular insight into how presence of non-aged or aged MPs modifies the effects of cadmium, copper and BDE-47 or mixture of them, on *P. lividus* embryos. The knowledge of toxic effects caused by the exposure to microplastics and the comprehension of factors influencing their vector role in sea urchin, permit to highlight potential ecological impacts associated to increased levels of microplastics observed in Mediterranean Sea as well as co-contaminants adsorbed on them.

Despite no specific morphological effects after MPs and contaminants combinatorial exposure emerged, the profiling of transcriptional response of embryos frequently showed significant changes in gene expression. This lead to consider that changes in transcriptional expression levels are more sensitive than the observation of morphological effects confirming that molecular evidences may be used as early indicators of stress conditions or pre-pathological developmental alterations also after combinatorial MPs/contaminants exposures (Ruocco et al., 2017; Aardema and MacGregor, 2002). The most studies focused on real toxic assessment MPs-associated generally lack evidences at molecular level from through which a more detailed insight of the risks arising from MPs as well as contaminants associated with them risks can be provided.

The results herein presented essentially attempt to improve understanding of the sorption of different type of chemicals by microplastics and to clarify the importance of MP-mediated contaminant transfer exposing organisms to significant ecotoxicological risk. Molecular endpoints revealed that aging process can influence acute effects of cadmium, copper and BDE-47 on *P. lividus* embryos. The presence of suspended MPs can reduce the total fraction available and their toxicity but in contaminant-dependent manner. Despite the higher sorption capacity observed for Cu onto pristine/non-aged MPs, aging process and low molecular weight fragments generated influenced the interaction with it. However, although Cu bioavailable fraction was lesser than untreated control group, a perturbation in the canonical developmental program as well the oxidative status occurred.

To the best of my knowledge, this work to date represents the first study exposing *P. lividus* embryos to hydrophobic organic pollutant BDE-47 alone or in combination with MPs. Data from BDE-47/MPs exposures showed changes in gene expression profile also at very low concentrations if compared to control, emphasizing the relevance of studying effects of these emerging contaminants at low concentrations. However, additional research on BDE-47 sorption capacity by MPs for example at increasing doses of it, as well over other embryo sea urchin development stages are required. Although the effect-based molecular approaches provide estimations of bioavailable fractions of the compounds, co-presence of different pollutants may pose limitations to the specificity of the biological response investigated. In addition, considering generation of photodegradation products (i.e., chemical additives, polymer fragments, chemical by-products), the use of model/pristine MPs in ecotoxicological investigations may led to an underestimation of the ecological risk of MPs in marine environment. Thus, in order to determine an ecological impact of MPs under environmentally relevant exposure conditions, an evaluation of secondary chemicals leached from pristine MPs should be considered before toxicity testing. Overall, these data support the utility of molecular tools to analyze the effect of microplastics associated with contaminants, maintaining the general concern about their ability to pose a potential ecological risk for this model organism. Given that interactions between different contaminants and plastic polymers provide an important insight into the development of a risk assessment framework for MPs, this study provides insights to shed light on mechanisms and the potential hazards of MPs in marine environments after photodegradation.

References

Aardema, M.J., MacGregor, J.T., 2002. Toxicology and genetic toxicology in the new era of “toxicogenomics”: impact of “-omics” technologies. Mutat. Res. 499, 13e25. https://doi.org/10.1016/S0027-5107(01)00292-5

Alimi, O.S., Farner Budarz, J., Hernandez, L.M., Tufenkji, N., 2018. Microplastics and nanoplastics in aquatic environments: aggregation, deposition, and enhanced contaminant transport. Environ. Sci. Technol. 52, 1704–1724. https://doi.org/10.1021/acs.est.7b05559

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160e202. https://doi.org/10.1016/j. aquatox.2005.08.015

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Anselmo, H.M.R., Koerting, L., Devito, S., van den Berg, J.H.J., Dubbeldam, M., Kwadijk, C., Murk, A.J., 2011. Early life developmental effects of marine persistent organic pollutants on the sea urchin *Psammechinus miliaris*. Ecotoxicol. Environ. Saf. 74, 2182–2192. https://doi.org/10.1016/j.ecoenv.2011.07.037

Arini, A., Gourves, P.Y., Gonzalez, P., Baudrimont, M., 2015. Metal detoxification and gene expression regulation after a Cd and Zn contamination: an experimental study on *Danio rerio*. Chemosphere 128, 125e133. https://doi.org/10.1016/j.chemosphere.2015.01.022

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d’Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to mussels. Environ. Pollut. 198, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021

Bakir, A., Rowland, S.J., Thompson, R.C., 2012. Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar. Pollut. Bull. 64, 2782–2789. https://doi.org/10.1016/j.marpolbul.2012.09.010

Bakir, A., Rowland, S.J., Thompson, R.C., 2014. Transport of persistent organic pollutants bymicroplastics in estuarine conditions. Estuar. Coast. Shelf Sci., 140, 14–21. https://doi.org/10.1016/j.ecss.2014.01.004

Bandow, N., Will, V., Wachtendorf, V., and Simon., F.G., 2017. Contaminant release from aged microplastic. Environ. Chem. 14, 394–405. https://doi.org/10.1071/EN17064

Batel, A., Borchert, F., Reinwald, H., Erdinger, L., Braunbeck, T., 2018. Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (*Danio rerio*) gills and zebrafish embryos. Environ. Pollut. 235, 918–930. https://doi.org/10.1016/j.envpol.2018.01.028

Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. Chemosphere 132, 114–119. http://dx.doi.org/10.1016/j.chemosphere.2015.03.010

Ben-Tabou De-Leon, S., Davidson, E.H., 2007. Gene regulation: Gene control network in development. Annu. Rev. Biophys. Biomol. Struct. 36, 191–212. https://doi.org/10.1146/annurev.biophys.35.040405.102002

Bonaventura, R., Poma, V., Costa, C., Matranga, V., 2005. UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochem. Biophys. Res. Commun. 328, 150–157. https://doi.org/10.1016/j.bbrc.2004.12.161

Boyle, D., Catarino, A. I., Clark, N. J., Henry, T. B., 2020. Polyvinyl chloride (PVC) plastic fragments release Pb additives that are bioavailable in zebrafish. Environmental Pollution 263, 114422. https://doi.org/10.1016/j.envpol.2020.114422

Brandts, I., Teles, M., Gonçalves, A.P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M.A., Soares, A.M.V.M., Tort, L., Oliveira M., 2018. Effects of nanoplastics on *Mytilus galloprovincialis* after individual and combined exposure with carbamazepine. Sci. Total Environ. 643, 775–784. https://doi.org/10.1016/j.scitotenv.2018.06.257

Breitholtz, M., Wollenberger, L., 2003. Effects of three PBDEs on development, reproduction and population growth rate of the harpacticoid copepod *Nitocra spinipes*. Aquat Toxicol. Jun 19; 64(1):85-96. https://doi.org/10.1016/S0166-445X(03)00025-0

Brennecke, D., Duarte, B., Paiva, F., Caçdor, I., Canning-Clode, J., 2016. Microplastics as vector for heavy metal contamination from the marine environment. Estuar. Coast. Shelf Sci. 178, 189–195. https://doi.org/10.1016/j.ecss.2015.12.003

Camacho, M., Herrera, A., Gómez, M., Acosta-Dacal, A., Martínez, I., Henríquez-Hernández, L.A., Luzardo, O.P., 2019. Organic pollutants in marine plastic debris from Canary Islands beaches. Sci. Total Environ. 662, 22–31. https://doi.org/10.1016/j. scitotenv.2018.12.422

Cao, Y., Zhao, M., Ma, X., Song, Y., Zuo, S., Li, H., Deng, W., 2021. A critical review on the interactions of microplastics with heavy metals: Mechanism and their combined effect on organisms and humans. Sci Total Environ. Sep 20; 788:147620. https://doi.org/10.1016/j.scitotenv.2021.147620

Celina, M. C., 2013. Review of polymer oxidation and its relationship with materials performance and lifetime prediction. Polym. Degrad. Stabil. 98, 2419–2429. https://doi.org/10.1016/j.polymdegradstab.2013.06.024

Chaâbene, Z., Rorat, A., Rekik Hakim, I., Bernard, F., Douglas, G.C., Elleuch, A., Vandenbulcke, F., Mejdoub, H., 2018. Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*). Chemosphere 197, 123e134. https://doi.org/10.1016/j.chemosphere.2017.12.146

Chua, E. M., Shimeta, J., Nugegoda, D., Morrison, P. D., Clarke, B. O., 2014. Assimilation of Polybrominated Diphenyl Ethers from Microplastics by the Marine Amphipod, *Allorchestes Compressa*. Environ. Sci. Technol. 48, 8127– 8134. https://doi.org/10.1021/es405717z

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T. S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646−6655. https://doi.org/10.1021/es400663f

Cole, M., Coppock,R., Lindeque, P., Altin, D., Reed, S., Pond, D. W., Sørensen, L., Galloway, T. S., and Booth, A. M., 2019. Effects of Nylon Microplastic on Feeding, Lipid Accumulation, and Moulting in a Coldwater Copepod. Environ. Sci. Technol. 2019, 53, 12, 7075–7082. https://doi.org/10.1021/acs.est.9b01853

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K.A., Corsi, I., 2014. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *Paracentrotus lividus*. Environ. Sci. Technol. 48, 12302–12311.

De-la-Torre, G. E., 2020. Microplastics: an emerging threat to food security and human health. J Food Sci Technol. 57, 1601-1608. https://doi.org/10.1007/s13197-019-04138-1

Di Natale, M., Bennici, C., Biondo, G., Masullo, T., Monastero, C., Tagliavia, M., Torri, M., Costa, S., Ragusa, M. A., Cuttitta, A., Nicosia, A., 2019. Aberrant gene expression profiles in Mediterranean sea urchin reproductive tissues after metal exposures. Chemosphere 216:48-58. https://doi.org/10.1016/j.chemosphere.2018.10.137

Díaz-Jaramillo, M., Miglioranza, K. S. B., Gonzalez, M., Barón, E., Monserrat, J. M., Eljarrat, E., Barceló, D., 2016. Uptake, metabolism and sub-lethal effects of BDE-47 in two estuarine invertebrates with different trophic positions. Environ Pollut. 213:608-617. https://doi.org/10.1016/j.envpol.2016.03.009

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R., Date, T., 2005. Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. Mar.Pollut. Bull. 50, 1103–1114. https://doi.org/10.1016/j.marpolbul.2005.04.030

Fedor, G. R., and Brennan, P. J., 1996. Comparison between natural weathering and fluorescent UV exposures: UVA-340 lamp test results. United States: N. p., 1996. Web. https://www.q-lab.com/documents/public/bc750e56-b4d6-41fc-a5a1-2a163b4ba5a3.pdf

Fisner, M., Taniguchi, S., Moreira, F., Bicego, M. C., Turra, A., 2017. Colour spectrum and resin-type determine the concentration and composition of Polycyclic Aromatic Hydrocarbons (PAHs) in plastic pellets. Mar. Pollut. Bull. 122, 323–330. https://doi.org/10.1016/j.marpolbul.2017.06.072

Flavel, B. S., Kappes, M. M., Krupke, R., and Hennrich, F., 2013. Separation of Single-Walled Carbon Nanotubes by 1-Dodecanol-Mediated Size-Exclusion Chromatography. *ACS Nano 7* (4), 3557-3564. https://doi.org/10.1021/nn4004956

Frias, J.P.G.L., Sobral, P., Ferreira, A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. Mar. Pollut. Bull. 60, 1988–1992. https://doi.org/10.1016/j.marpolbul.2010.07.030

Fu, Q., Tan, X., Ye, S., Ma, L., Tang, Y., 2020. Mechanism analysis of heavy metal lead captured by natural-aged microplastics. Chemosphere 270, 128624. https://doi.org/10.1016/j.chemosphere.2020.128624

Ge, W., Mou, Y., Chai, C., Zhang, Y., Wang, J., Ju, T., Jiang, T., Xia, B., 2018. Polybrominated diphenyl ethers in the dissolved and suspended phases of seawater from Sanggou Bay, east China. Chemosphere 203:253-262. https://doi.org/10.1016/j.chemosphere.2018.03.184

Goedecke, C., Mülow-Stollin, U., Hering, S., Richter, J., Piechotta, C., Paul, A., Braun, U., 2017. A first pilot study on the sorption of environmental pollutants on various microplastic materials. J. Environ. Anal. Chem. 4, 191. doi:10.41722380-2391.1000191

Gewert,B., Plassmanna, M. M., and MacLeod, M., 2015. Pathways for degradation of plastic polymers floating in the marine environment. Environ. Sci.: Processes Impacts, 17, 1513-1521. https://doi.org/10.1039/C5EM00207A

Gildor, T., De-Leon, S.B.-T., 2015. Comparative Study of Regulatory Circuits in Two Sea Urchin Species Reveals Tight Control of Timing and High Conservation of Expression Dynamics. PLoS Genet. 11, e1005435, https://doi.org/10.1371/journal.pgen.1005435

Gomes, T., Pereira, C. G., Cardoso, C., Bebianno, M. J., 2013. Differential protein expression in mussels *Mytilus galloprovincialis* exposed to nano and ionic Ag, Aquatic Toxicology, Volumes 136–137, Pages 79-90, https://doi.org/10.1016/j.aquatox.2013.03.021

Gouin, T., Roche, N., Lohmann, R., Hodges, G., 2011. A thermodynamic approach for assessing the environmental exposure of chemicals absorbed to microplastic. Environ. Sci. Technol. 45, 1466–1472. https://doi.org/10.1021/es1032025

Guo, X., Wang, J., 2020. Projecting the sorption capacity of heavy metal ions onto microplastics in global aquatic environments using artificial neural networks. J. Hazard. Mater. 402, 123709. https://doi.org/10.1016/j.jhazmat.2020.123709

Guo, X.T., Pang, J.W., Chen, S.Y., Jia, H.Z., 2018. Sorption properties of tylosin on four different microplastics. Chemosphere 209, 240–245. https://doi.org/10.1016/j.chemosphere.2018.06.100

Gupta, S. C., Sharma, A., Mishra, M., Mishra, R. K., Chowdhuri, D. K., 2010. Heat shock proteins in toxicology: how close and how far? Life Sci., Mar 13;86(11-12):377-84. https://doi.org/10.1016/j.lfs.2009.12.015

Hassanin, A., Breivik, K., Meijer, S. N., Steinnes, E., Thomas, G.O., Jones, K.C., 2004. PBDEs in European background soils: levels and factors controlling their distribution. Environ Sci Technol., Feb 1;38(3):738-45. https://doi.org/10.1021/es035008y

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., 2011. Organic micropollutants in marine plastic debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull. 62, 1683–1692. https://doi.org/10.1016/j.marpolbul.2011.06.004

Hites, R. A., 2004. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. Environmental Science & Technology 38, 945–956. https://doi.org/10.1021/es035082g

Hodson, M.E., Duffus-Hodson, C.A., Clark, A., Prendergast-Miller, M.T., Thorpe, K.L., 2017. Plastic bag derived-microplastics as a vector for metal exposure in terrestrial invertebrates. Environ. Sci. Technol., 51 (8), pp. 4714-4721. https://doi.org/10.1021/acs.est.7b00635

Holmes, L.A., Turner, A., Thompson, R.C., 2014. Interactions between trace metals and plastic production pellets under estuarine conditions. Mar. Chem. 167, 25e32. https://doi.org/10.1016/j.marchem.2014.06.001

Huang, D., Tao, J., Cheng, M., Deng, R., Chen, S., Yin, L., Li, R., 2021. Microplastics and nanoplastics in the environment: Macroscopic transport and effects on creatures. J Hazard Mater., Apr 5; 407:124399. https://doi.org/10.1016/j.jhazmat.2020.124399

Imhof, H.K., Rusek, J., Michaela Thiel, M., Wolinska, J., Laforsch, C., 2017. Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? PLoS One. https://doi.org/10.1371/journal.pone.0187590

Izagirre, U., Errasti, A., Bilbao, E., Múgica, M., Marigómez, I., 2014. Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*. Aquat Toxicol. ; 149:145-56. https://doi.org/10.1016/j.aquatox.2014.01.013

Hall Jr., L.W., Ziegenfuss, M.C., Anderson, R.D., Lewis, B.L., 1995. The effect of salinity on the acute toxicity of total and free cadmium to a Chesapeake Bay copepod and fish. Mar. Pollut. Bull. 30, 376e384. https://doi.org/10.1016/0025-326X(94)00186-D

Hüffer, T., Hofmann, T., 2016. Sorption of non-polar organic compounds by micro- sized plastic particles in aqueous solution. Environ. Pollut. 214, 194e201. https://doi.org/10.1016/j.envpol.2016.04.018

Karapanagioti, H.K., Klontza, I., 2008. Testing phenanthrene distribution properties of virgin plastic pellets and plastic eroded pellets found on Lesvos island beaches (Greece). Mar. Environ. Res., 65, 283–290. https://doi.org/10.1016/j.marenvres.2007.11.005

Koelmans, A.A., Besseling, E., Wegner, A., Foekema, E.M., 2013. Plastic as a carrier of POPs to aquatic organisms: A model analysis. Environ. Sci. Technol., 47, 7812–7820. https://doi.org/10.1021/es401169n

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

Kowalczyk, A., Chyc, M., Ryszka, P. & Latowski, D., 2016. Achromobacter xylosoxidans as a new microorganism strain colonizing high-density polyethylene as a key step to its biodegradation. Environ. Sci. Pollut. Res. 1–8. DOI 10.1007/s11356-016-6563-y

Li, X., Mei, Q., Chen, L., Zhang, H., Dong, B., Dai, X., He, C., Zhou, J., 2019. Enhancement in adsorption potential of microplastics in sewage sludge for metal pollutants after the wastewater treatment process. Water Res. 157, 228–237. https://doi.org/10.1016/j.watres.2019.03.069

Lin, Z.K.L, Hu, Y.W., Yuan, Y.J., Hu, B.W., Wang, B.L., 2021. Comparative analysis of kinetics and mechanisms for Pb (II) sorption onto three kinds of microplastics. Ecotoxicol. Environ. Saf. 208, 111451. https://doi.org/10.1016/j.ecoenv.2020.111451

Liu, H., He, J., Chi, C., Shao, J., 2014. Differential HSP70 expression in *Mytilus coruscus* under various stressors. Gene 543, 166e173. https://doi.org/10.1016/j.gene. 2014.04.008

Liu, P-Y., Zhang, Y.-N., Gao, Z., Li, Z-S., 2019. Study on photodegradation pathway and mechanism of 2,2′,4,4′-Tetrabromodiphenyl ether, Emerging Contaminants, Volume 5, Pages 45-52. https://doi.org/10.1016/j.emcon.2019.02.005

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lu, K., Qiao, R., An, H., Zhang, Y., 2018. Influence of microplastics on the accumulation and chronic toxic effects of cadmium in zebrafish (*Danio rerio*). Chemosphere 202, 514–520. https://doi.org/10.1016/j.chemosphere.2018.03.145

Martínez-Gòmez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Mar. Environ. Res. 130, 69e76. https://doi.org/10.1016/j.marenvres.2017.06.016

Masullo, T., Biondo, G., Di Natale, M., Tagliavia, M., Bennici, C.D., Musco, M., Ragusa, M.A., Costa, S., Cuttitta, A., Nicosia, A., 2021. Gene Expression Changes after Parental Exposure to Metals in the Sea Urchin Affect Timing of Genetic Programme of Embryo Development. Biology 2021, 10, 103. https://doi.org/10.3390/biology10020103

Matranga V., Zito F., Costa C., Bonaventura R., Giarrusso S., Celi F., 2010. Embryonic development and skeletogenic gene expression affected by X-rays in the Mediterranean sea urchin *Paracentrotus lividus*. *Ecotoxicology,* 19:530–537. DOI 10.1007/s10646-009-0444-9

Mei, W., Chen, G., Bao, J., Song, M., Li, Y., Luo, C., 2020. Interactions between microplastics and organic compounds in aquatic environments: a mini review. Sci. Total Environ. 736, 139472. https://doi.org/10.1016/j.scitotenv.2020.139472

Meides, N., Menzel, T., Poetzschner, B., Löder, M. G. J., Mansfeld, U., Strohriegl, P., Altstaedt, V., Senker, J., 2021. Reconstructing the Environmental Degradation of Polystyrene by Accelerated Weathering. Environ Sci Technol., Jun 15; 55(12):7930-7938. https://doi.org/10.1021/acs.est.0c07718

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., Pennati, R., 2018. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environ. Pollut. 237, 1080–1087. https://doi.org/10.1016/j.envpol.2017.11.030.

Migliaccio, O., Castellano, I., Cirino, P., Romano, G., Palumbo, A., 2015. Maternal exposure to cadmium and manganese impairs reproduction and progeny fitness in the sea urchin *Paracentrotus lividus*. PLoS ONE 2015, 10, e0131815. https://doi.org/10.1371/journal.pone.0131815

Morroni, L., Sartori, D., Costantini, M., Genovesi, L., Magliocco, T., Ruocco, N., Buttino, I., 2019. First molecular evidence of the toxicogenetic effects of copper on sea urchin *Paracentrotus lividus* embryo development. Water Res., 160, 415–423. https://doi.org/10.1016/j.watres.2019.05.062

Nicosia, A., Maggio, T., Mazzola, S., Gianguzza, F., Cuttitta, A., Costa, S., 2014. Characterization of small HSPs from *Anemonia viridis* reveals insights into molecular evolution of alpha crystallin genes among cnidarians. PloS One 9, e105908. https://doi.org/10.1371/journal.pone.0105908.

Nicosia, A., Salamone, M., Mazzola, S., Cuttitta, A., 2015. Transcriptional and biochemical effects of cadmium and manganese on the defense system of *Octopus vulgaris* paralarvae. Biomed. Res. Int., 2015. https://doi.org/10.1155/2015/437328

Nicosia, A., Bennici, C., Biondo, G., Costa, S., Di Natale, M., Masullo, T., Monastero, C., Ragusa, M.A., Tagliavia, M., Cuttitta, A., 2018. Characterization of Translationally Controlled Tumour Protein from the Sea Anemone *Anemonia viridis* and Transcriptome Wide Identification of Cnidarian Homologues. Genes, 9, 30. https://doi.org/10.3390/genes9010030

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereora, C.D.S., Turra, A., 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). Mar. Pollut. Bull. 92, 99–104. https://doi.org/10.1016/j.marpolbul.2014.12.050

Ortega-Calvo, J.J., Harmsen, J., Parsons, J.R., Semple, K.T., Aitken, M.D., Ajao, C., Eadsforth, C., Galay-Burgos, M., Naidu, R., Oliver, R., Peijnemburg, W.J.G., Römbke, J., Streck, G., Versonnen, B., 2015. From bioavailability science to regulation of organic chemicals. Environ. Sci. Technol., 49, pp. 10255-10264. https://doi.org/10.1021/acs.est.5b02412

Pagano, G., Guida, M., Trifuoggi, M., Thomas, P.J., Palumbo, A., Romano, G., Oral, R., 2017. Sea urchin bioassays in toxicity testing: I. Inorganics, organics, complex mixtures and natural products. Expert Opin. Environ. Biol. 6, 1. https://doi.org/10.4172/2325-9655.1000142

Pinsino, A., Bergami, E., Della Torre, C., Vannuccini, M. L., Addis, P., Secci, M., Dawson, K. A., Matranga, V., Corsi, I., 2017. Amino-modified polystyrene nanoparticles affect signalling pathways of the sea urchin (*Paracentrotus lividus*) embryos. Nanotoxicology, Mar; 11(2):201-209. https://doi.org/10.1080/17435390.2017.1279360

Ragusa, M.A., Costa, S., Gianguzza, M., Roccheri, M.C., Gianguzza, F., 2013. Effects of cadmium exposure on sea urchin development assessed by SSH and RT-qPCR: Metallothionein genes and their differential induction. Mol. Biol. Rep., 40, 2157–2167, doi:10.1007/s11033-012-2275-7

Ragusa, M., Nicosia, A., Costa, S., Cuttitta, A., Gianguzza, F., 2017a. Metallothionein Gene Family in the Sea Urchin *Paracentrotus lividus*: Gene Structure, Differential Expression and Phylogenetic Analysis. Int. J. Mol. Sci., 18, 812, doi:10.3390/ijms18040812.

Ragusa, M.A., Costa, S., Cuttitta, A., Gianguzza, F., Nicosia, A., 2017b. Coexposure to sulfamethoxazole and cadmium impairs development and attenuates transcriptional response in sea urchin embryo. Chemosphere, 180, 275–284, https://doi.org/10.1016/j.chemosphere.2017.04.030

Reisser, J., Shaw, J., Hallegraeff, G., Proietti, M., Barnes, D. K. A., Thums, M., et al., 2014. Millimeter-Sized Marine Plastics: A New Pelagic Habitat for Microorganisms and Invertebrates. PLoS ONE 9(6): e100289. https://doi.org/10.1371/journal.pone.0100289

Roccheri, M.C., Agnello, M., Bonaventura, R., Matranga, V., 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem. Biophys. Res. Commun., 321, 80–87. https://doi.org/10.1016/j.bbrc.2004.06.108

Rochman, C.M., Hentschel, B.T., Teh, S.J., 2014. Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. PLoS One 9, e85433. https://doi.org/10.1371/journal.pone.0085433

Rodrigues, J.P., Duarte, A.C., Echeandía, J.S., 2019. Significance of interactions between microplastics and POPs in the marine environment: A critical overview. Trends Analyt. Chem., 111, 252–260. https://doi.org/10.1016/j.trac.2018.11.038

Ruocco, N., Feedele, A.M., Costantini, S., Romano, G., Ianora, A., Costantini, M., 2017. New inter-correlated genes targeted by diatom-derived polyunsaturated aldehydes in the sea urchin *Paracentrotus lividus*. Ecotoxicol. Environ. Saf. 142, 355e362. https://doi.org/10.1016/j.ecoenv.2017.04.022

Shao, Y., Han, S., Ma, L., Luo, M., Yang, G., Liu, W., Xu, D., 2018. Polybrominated diphenyl ethers in surface waters around Beijing: Occurrence, distribution and sources, Applied Geochemistry, Volume 98, Pages 58-64, ISSN 0883-2927, https://doi.org/10.1016/j.apgeochem.2018.09.011

Sleight, V.A., Bakir, A., Thompson, R.C., Henry, T.B., 2017. Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar. Pollut. Bull. 116, 291e297. https://doi.org/10.1016/j.marpolbul.2016.12.055

Stamateris, R.E., Rafiq, K., Ettensohn, C.A., 2010. The expression and distribution of Wnt and Wnt receptor mRNAs during early sea urchin development. Gene Expr. Patterns, 10, 60–64. https://doi.org/10.1016/j.gep.2009.10.004

Strogyloudi, E., Pancucci-Papadopoulou, M.-A., Papadopoulos, G.L., 2014. Metal and metallothionein concentrations in *Paracentrotus lividus* from Amvrakikos gulf (Ionian sea-Greece). Environ. Monit. Assess. 186, 5489e5499. https://doi.org/10. 1007/s10661-014-3798-2

Syranidou, E., Karkanorachaki, K., Amorotti, F. et al., 2017. Biodegradation of weathered polystyrene films in seawater microcosms. Sci Rep 7, 17991. https://doi.org/10.1038/s41598-017-18366-y

Tang, S., Lin, L., Wang, X., Feng, A., and Yu, A., 2020. Pb(II) uptake onto nylon microplastics: Interaction mechanism and adsorption performance. J. Hazard. Mater. 386:121960. https://doi.org/10.1016/j.jhazmat.2019.121960

Teles, M., Fierro-Castro, C., Na-Phatthalung, P., Tvarijonaviciute, A., Trinidade, T., Soares, A.M.V.M., Tort, L., Oliveira, M., 2016. Assessment of gold nanoparticle effects in a marine teleost (*Sparus aurata*) using molecular and biochemical biomarkers. Aquat. Toxicol. 177, 125–135. https://doi.org/10.1016/j.aquatox.2016.05.015

ter Halle, A., Ladirat, L., Martignac, M., Mingotaud, A.F., Boyron, O., Perez, E., 2017. To what extent are microplastics from the open ocean weathered? Environ. Pollut. 227, 167e174. http://dx.doi.org/10.1016/j.envpol.2017.04.051

Teuten, E.L., Saquing, J.M., Knappe, D.R., Barlaz, M.A., Jonsson, S., Bjorn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., et al., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philos. Trans. R. Soc., 364, 2027–2045. https://doi.org/10.1098/rstb.2008.0284

Thomas, S.N., Hridayanathana, C., 2006. The effect of natural sunlight on the strength of polyamide 6 multifilament and monofilament fishing net materials. Fish. Res. 81, 326–330. https://doi.org/10.1016/j.fishres.2006.06.012

Thomas, P. J., Oral, R., Pagano, G., Tez, S., Toscanesi, M., Ranieri, P., Trifuoggi, M., Lyons, D. M., 2020. Mild toxicity of polystyrene and polymethylmethacrylate microplastics in *Paracentrotus lividus* early life stages. Marine Environmental Research, Volume 161, 105132, ISSN 0141-1136, https://doi.org/10.1016/j.marenvres.2020.105132

Torres, F. G., Dioses-Salinas, D. C., Pizarro-Ortega, C. I., De-la-Torre, G. E., 2021. Sorption of chemical contaminants on degradable and non-degradable microplastics: Recent progress and research trends. Science of The Total Environment, Volume 757, 143875, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2020.143875

Trifuoggi, M., Pagano, G., Oral, R., Pavičić-Hamer, D., Burić, P., Kovačić, I., Siciliano, A., Toscanesi, M., Thomas, P.J., Paduano, L., Guida, M., Lyons, D.M., 2019. Microplastic-induced damage in early embryonal development of sea urchin *Sphaerechinus granularis*. Environ. Res. 179, 108815. https://doi.org/10.1016/j.envres.2019.108815.

Turner, A., Holmes, L.A., 2015. Adsorption of trace metals by microplastic pellets in fresh water. Environ. Chem. 12, 600–610. https://doi.org/10.1071/EN14143

Varó, I., Perini, A., Torreblanca, A., Garcia, Y., Bergami, E., Vannuccini, M. L., Corsi, I., 2019. Time-dependent effects of polystyrene nanoparticles in brine shrimp *Artemia franciscana* at physiological, biochemical and molecular levels. Science of The Total Environment, Volume 675, Pages 570-580, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2019.04.157

Varrella, S., Romano, G., Costantini, S., Ruocco, N., Ianora, A., Bentley, M.G., Costantini, M., 2016. Toxic Diatom Aldehydes Affect Defence Gene Networks in Sea Urchins. PLoS ONE, 11, e0149734, https://doi.org/10.1371/journal.pone.0149734

Velez, J. F. M., Shashoua, Y., Syberg, K., Khan, F. R., 2018. Considerations on the use of equilibrium models for the characterisation of HOC-microplastic interactions in vector studies. Chemosphere, Volume 210, Pages 359-365, ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2018.07.020

Vergara-Amado, J., Silva, A.X., Manzi, C., Nespolo, R.F., Cárdenas, L., 2017. Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*. J. Therm. Biol., 68, 104–109, https://doi.org/10.1016/j.jtherbio.2017.03.009

Vethaak, A. D., and Leslie, H. A., 2016. Plastic Debris Is a Human Health Issue. Environ. Sci. Technol., 50, 13, 6825–6826. https://doi.org/10.1021/acs.est.6b02569

Oliveira, M., Ribeiro, A., Guilhermino, L., 2012. Effects of short-term exposure to microplastics and pyrene on *Pomatoschistus microps* (Teleostei, Gobiidae). Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 163, S20. https://doi.org/10.1016/j.cbpa.2012.05.063

Wah Chu, K., Chow, K.L., 2002. Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquat. Toxicol. 2002, 61, 53–64, https://doi.org/10.1016/S0166-445X(02)00017-6

Wang, W., Wang, J., 2018. Comparative evaluation of sorption kinetics and isotherms of pyrene onto microplastics. Chemosphere 193, 567e573. https://doi.org/10.1016/j.chemosphere.2017.11.078

Wang, F., Yang, W., Cheng, P., Zhang, S., Zhang, S., Jiao, W., Sun, Y., 2019. Adsorption characteristics of cadmium onto microplastics from aqueous solutions. Chemosphere, Volume 235, Pages 1073-1080, ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2019.06.196

White, J.R., Turnbull, A., 1994. Weathering of polymers: Mechanisms of degradation and stabilization, testing strategies and modelling. J. Mater. Sci. 1994, 29, 584–613. https://doi.org/10.1007/BF00445969

Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol., 23, R1031–R1033. https://doi.org/10.1016/j.cub.2013.10.068

Wu, J., Xu, P., Chen, Q., Ma, D., Ge, W., Jiang, T., Chai, C., 2020. Effects of polymer aging on sorption of 2,2',4,4'-tetrabromodiphenyl ether by polystyrene microplastics. Chemosphere, Volume 253, 126706, ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2020.126706

Wypych, G., 2003. Handbook of Material Weathering, 3rd ed.; Society of Plastics Engineer's Inc.: Toronto, ON, Canada; p. 59.

Xu, P., Ge, W., Chai, C., Zhang, Y., Jiang, T., Xia, B., 2019. Sorption of polybrominated diphenyl ethers by microplastics. Mar. Pollut. Bull. 145, 260–269. https://doi.org/10.1016/j.marpolbul.2019.05.050

Huang, Y., Ding, J., Zhang, G., Liu, S., Zou, H., Wang, Z., Zhu, W., Geng, J., 2021. Interactive effects of microplastics and selected pharmaceuticals on red tilapia: role of microplastic aging. Sci. Total Environ., 752, Article 142256. https://doi.org/10.1016/j.scitotenv.2020.142256

Yeo, B. G., Takada, H., Yamashita, R., Okazaki, Y., Uchida, K., Tokai, T., Tanaka, K., Trenholm, N., 2020. PCBs and PBDEs in microplastic particles and zooplankton in open water in the Pacific Ocean and around the coast of Japan. Mar Pollut Bull., Feb;151:110806. https://doi.org/10.1016/j.marpolbul.2019.110806

Yousif, E., Haddad, R., 2013. Photodegradation and photostabilization of polymers, especially polystyrene: review. SpringerPlus 2, 398. https://doi.org/10.1186/2193-1801-2-398

Zhang, X., Zheng, M., Wang, L., Lou, Y., Shi, L., and Jiang, S., 2018. Sorption of three synthetic musks by microplastics. Mar. Pollut. Bull. 126, 606–609. https://doi.org/10.1016/j.marpolbul.2017.09.025

Zhang, S., Han, B., Sun, Y., Wang, F., 2019. Microplastics influence the adsorption and desorption characteristics of Cd in an agricultural soil. J. Hazard. Mater. 388, 121775. https://doi.org/10.1016/j.jhazmat.2019.121775

Zhang, R., Wang, M., Chen, X., Yang, C., Wu, L., 2020. Combined toxicity of microplastics and cadmium on the zebrafish embryos (*Danio rerio*). Sci. Total Environ. 743, 140638. https://doi.org/10.1016/j.scitotenv.2020.140638

Zhao, Q., Zhao, H., Quan, X., He, X. & Chen, S., 2015. Photochemical formation of hydroxylated polybrominated diphenyl ethers (OH-PBDEs) from polybrominated diphenyl ethers (PBDEs) in aqueous solution under simulated solar light irradiation. Environ. Sci. Technol.49, 9092–9099. https://doi.org/10.1021/acs.est.5b01240

Zhao, L., Rong, L., Xu, J., Lian, J., Wang, L., Sun, H., 2020. Sorption of five organic compounds by polar and nonpolar microplastics. Chemosphere 257, 127206. https://doi.org/10.1016/j.chemosphere.2020.127206

Ziccardi, L. M., Edgington, A,, Hentz, K., Kulacki, K. J., Kane, Driscoll, S., 2016. Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. Environ Toxicol Chem., Jul; 35(7):1667-76. PMID: 27093569. https://doi.org/10.1002/etc.3461

Chapter 4: Conclusions

The environmental concern about microplastics is growing due to their potential of adsorbing chemical pollutants, thus providing an unknown source of exposure for aquatic organisms. The identification of mechanisms influencing the interactions between MPs and co-contaminants is essential to assess the MPs toxicity once entered in the aquatic environment. The evidences suggest that the weathering factors such as UV-mediated aging and MPs-associated biofilm, changing MPs physico-chemistry properties, influence chemicals sorption ability and their bioavailability. A big challenge in the assessment of MPs potential impact on toxicological level concerns the sorption behavior and multiple interactions involving two or more contaminants simultaneously. In realist aquatic scenarios MPs are seldom associated to a single chemical, rather, a complex mixture of pollutants usually is present on them. Chapter 2 and 3 of the present thesis explored sorption capacities of different MPs with representative groups of environmental contaminants such as PAHs, PBDEs and metal ions, considering the adsorption phenomena of both the single contaminant and the mixture of different contaminants. The sorption processes were evaluated using sorbed contaminants bioavailability measurements in ELS of model invertebrate and vertebrate organisms.

Chapter2 described the sorption of Benzo[a]pyrene and Cadmium on HDPE-MPs-associated biofilm. Biofilm presence on MPs differentially influenced co-contaminants bioavailability to ELS zebrafish. Biomarker gene expression data (*cyp1a* and *mt2)* showed that the single short-time exposures lead to a greater reduction in organic compound bioavailability than inorganic counterpart in zebrafish larvae. Co-presence of both contaminants identified patterns of bioavailability slightly relating to those shown for each single contaminant. The presence of biofilm can create additional active sites promoting B[a]P sorption and consequently, higher Cd bioavailability to zebrafish larvae. This result contrasts the idea that biofilm sorptive properties increasing hydrophilicity of the plastic surface, enhances the accumulation of metals at the expense of organic molecules (i.e. B[a]P). Metabolic predictions inferred by shotgun metagenomic analysis would seem to support pattern of B[a]P bioavailability observed. The main metabolic modules complete above 50% included different biosynthesis pathways as well as aromatic degradation pathway. This lead to hypothesize that the microbial (Archaea, Bacteria, and Fungi) diversity of biofilm could use organic compounds as alternative carbon and energy sources to support the different biosynthesis pathways. Therefore, biofilm microbial composition on HDPE-MPs on one side can provide additional sorptive phase for hydrophobic molecules and influencing their bioavailability to zebrafish larvae on the other.

Additional research on colonization processes is needed to fully understand the basic processes that are involved in the formation of a biofilm, with a particular focus on biofilm−MP interactions. It should be evaluated the key factors that influence the physicochemical behavior of MP (e.g., particle properties and surface characteristics and/or absorbing molecules) and how these factors act on different MP materials, on different pollutants and on different mixture of them.

Chapter 3 identified sorption of 2,2’,4,4’-tetrabromodiphenyl ether , Cadmium and Copper by artificially aged MPs (PS and PA 6,6). Aging process led to high decrease of molecular weight of both polymers and generation of new oxygen-containing functional groups only for PA. The effects of aging on sorption process by MPs were found to depend by contaminant type and, in a lesser way, by polymer type. Among metal ions tested, sorption of copper was strongly affected by MPs photo-exposure, whilst no change in the association with Cd fand BDE-47 for both polymers have been detected. However, in the blend Cu/BDE-47, a hypothesis of a competition mechanism affecting the sorption of Cu by PS microparticles but not PA fibers was postulated. This work is the first to comprehensively investigate the sorption of BDE-47 on two different polymer types under aging conditions using bioavailability of sorbed chemicals (alone and in mixture) to evaluate sorption processes at the organism level and acute effects associated with them. Albeit from a morphological perspective, MPs presence tend to reduce morphological alterations on *P. lividus* embryos compared to their absence in medium, significant changes at transcriptional level emerged.

Based on variations in gene expression of members belonging to stress response pathway and skeletogenic and endodermal Gene Regulatory Networks (GRNs), changes in the interactions between sorbent and sorbate occurring after photo-exposure, led to establishment of molecular mechanisms perturbing the canonical developmental program as well the oxidative status. Therefore, molecular endpoints revealed that aging process can influence acute effects of cadmium, copper and BDE-47 on *P. lividus* embryos.

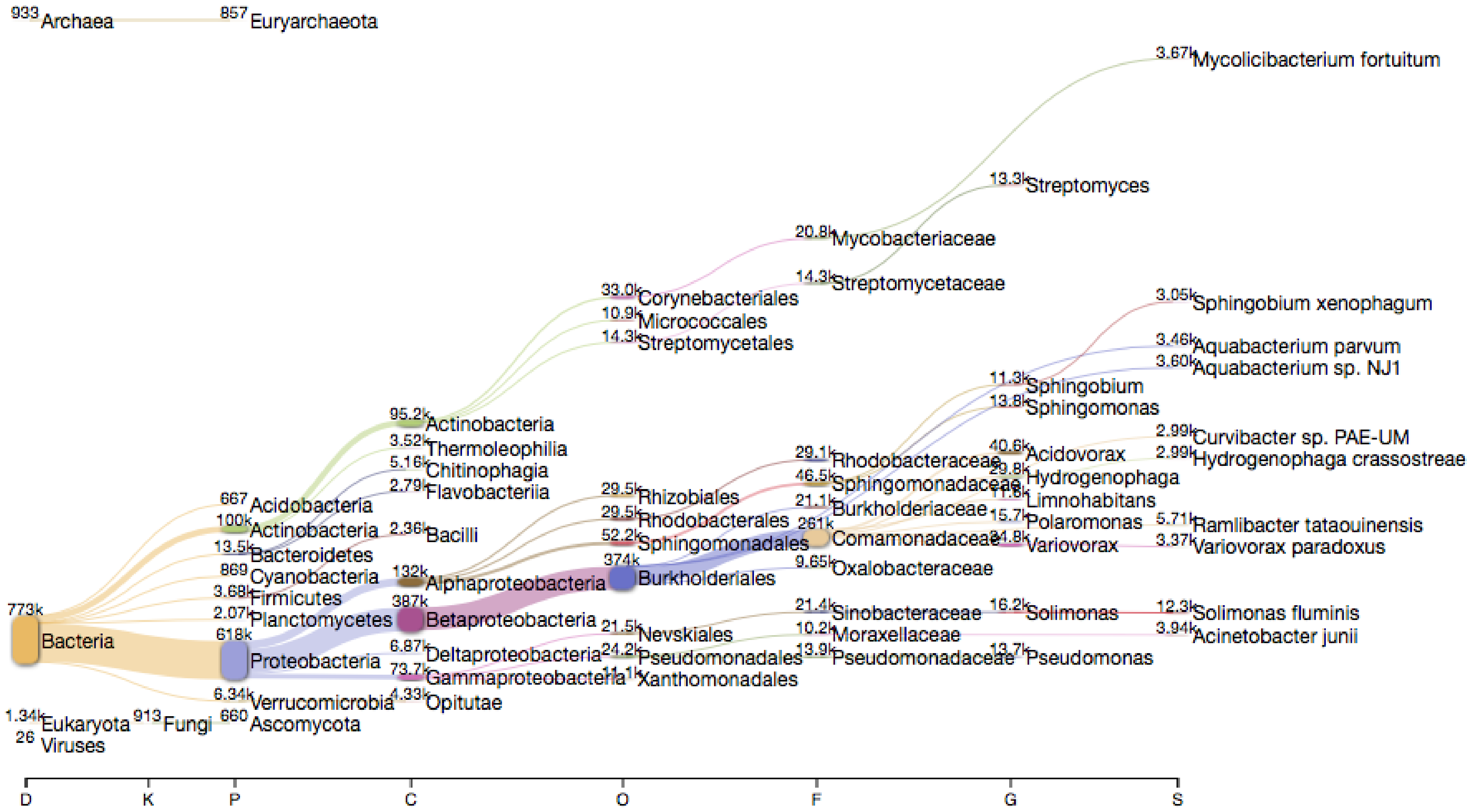
The effect-based molecular approaches provide estimations of bioavailable fractions of the compounds. However, co-presence of different pollutants may pose limitations to the specificity of the biological response investigated since co-occurrence of different stressors may provide gene expression profiles quite different from those provided in response to a single contaminant. Further specific evaluations on BDE-47 sorption capacity by MPs would be need to fully evaluate the effects of these emerging contaminants also at very low concentrations, as emerged from this study. Moreover, additional investigations on other embryo sea urchin development stages would be interesting.

Overall, the results of the present thesis maintain the general concern about the ability of MPs to pose a potential ecological risk for aquatic organisms. Additionally, gene expression analysis is confirmed a relevant environmental tool as well as an early warning indicator to analyze bioavailability of chemicals MPs-associated and to evaluate MPs toxicity in the aqueous phase.

The challenge for MPs research is to account for the interactions between diverse plastic materials undergoing weathering and to characterize colonization by microorganisms in various environmental settings. The conduction of integrative approaches can provide insights on sorption capacities of certain contaminants, on their toxicity and bioavailability, as well as to determine a proper risk assessment associated to MPs and to the response of biological systems to MPs pollution.

Appendix A

Species abundance data and phylogenetic relationships (Genome sample 1)



(Genome sample 2)

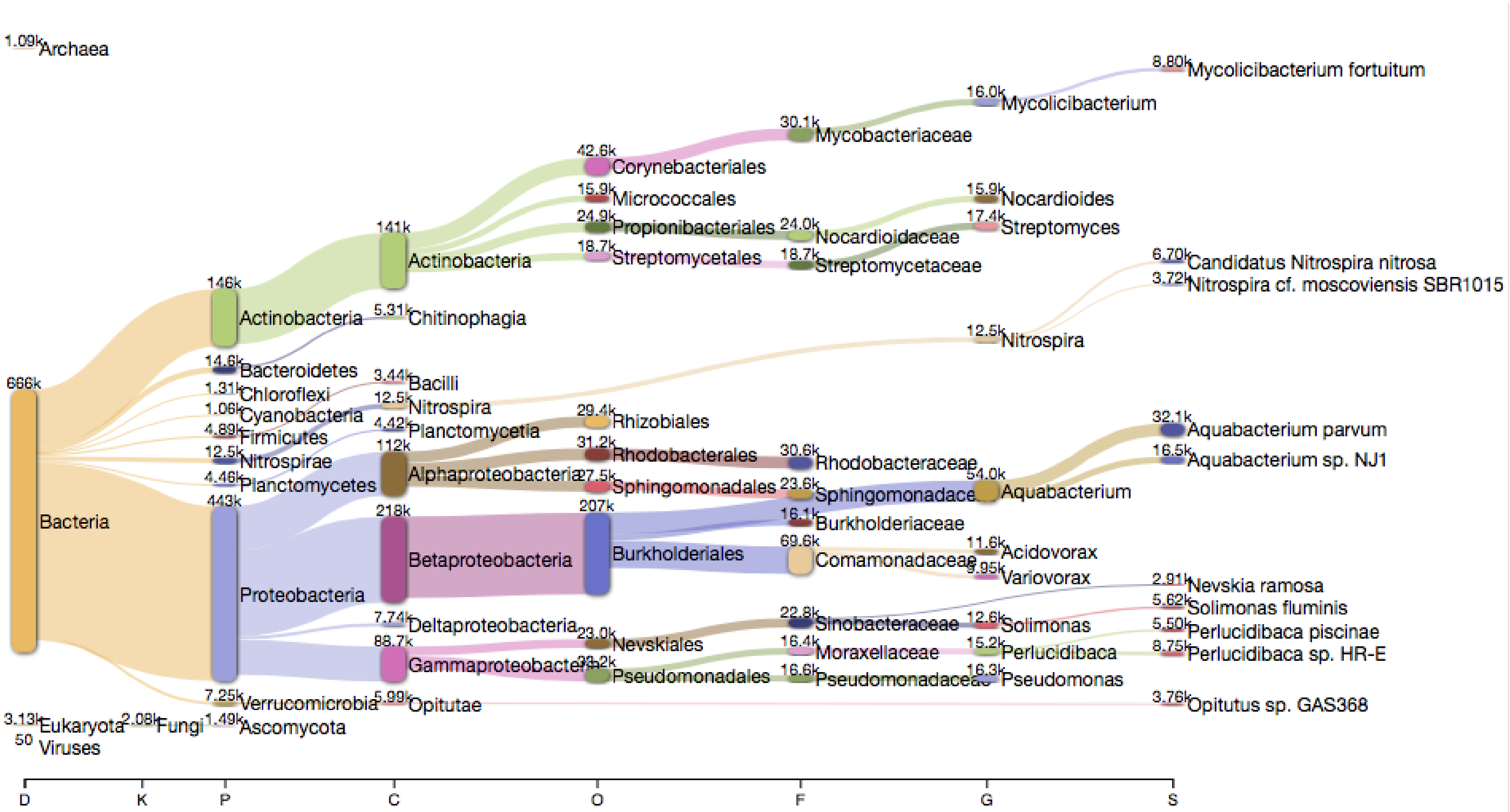


Figure S1: Sankey visualization of the relative abundance species from two samples of biofilm lining the HDPE-MPs (U1, U2). D, Domain; P, Phylum; F, family; G, genus; D, domain.

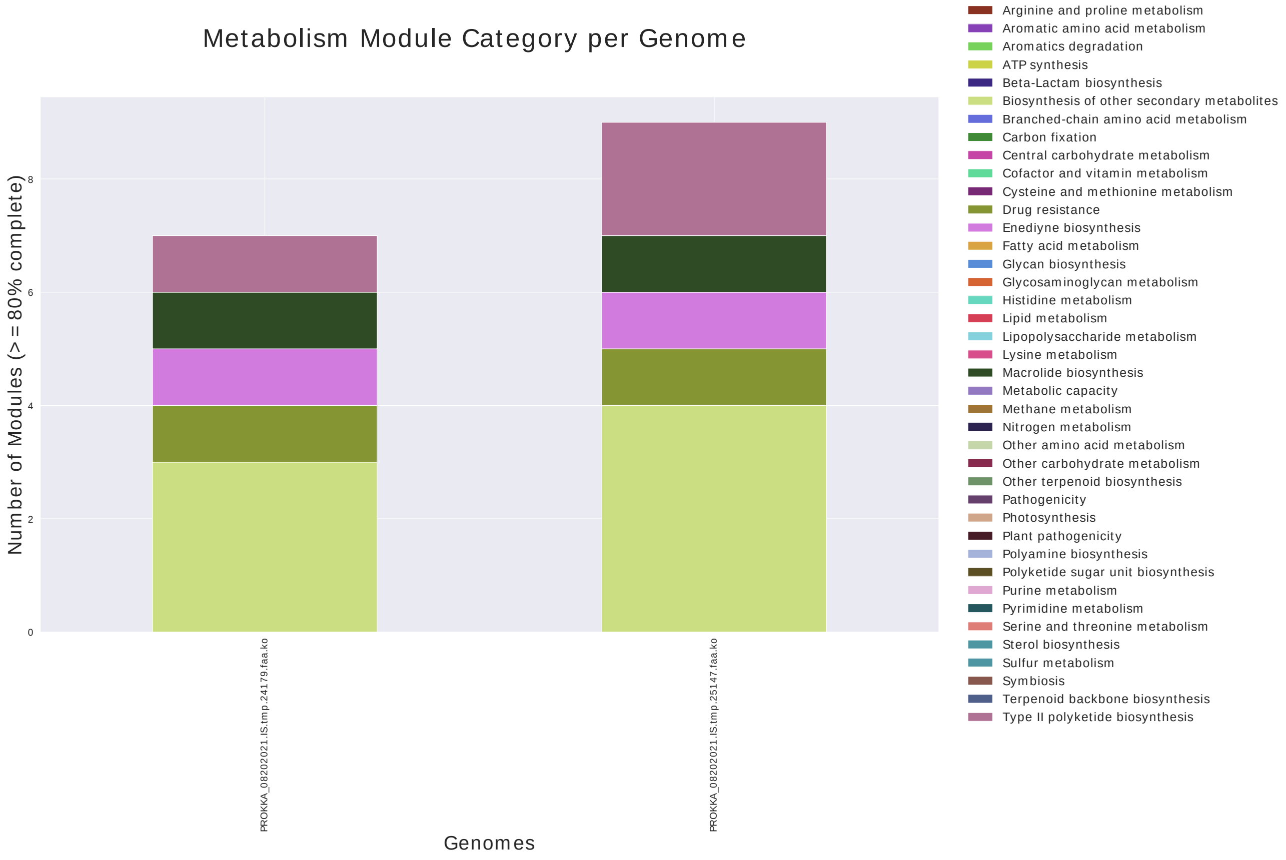


Figure S2: Barplots of module with completeness above 80% grouped by the category (pathway). The different colors of the modules indicate which pathway they belong to (right box).

Completeness of metabolic pathways

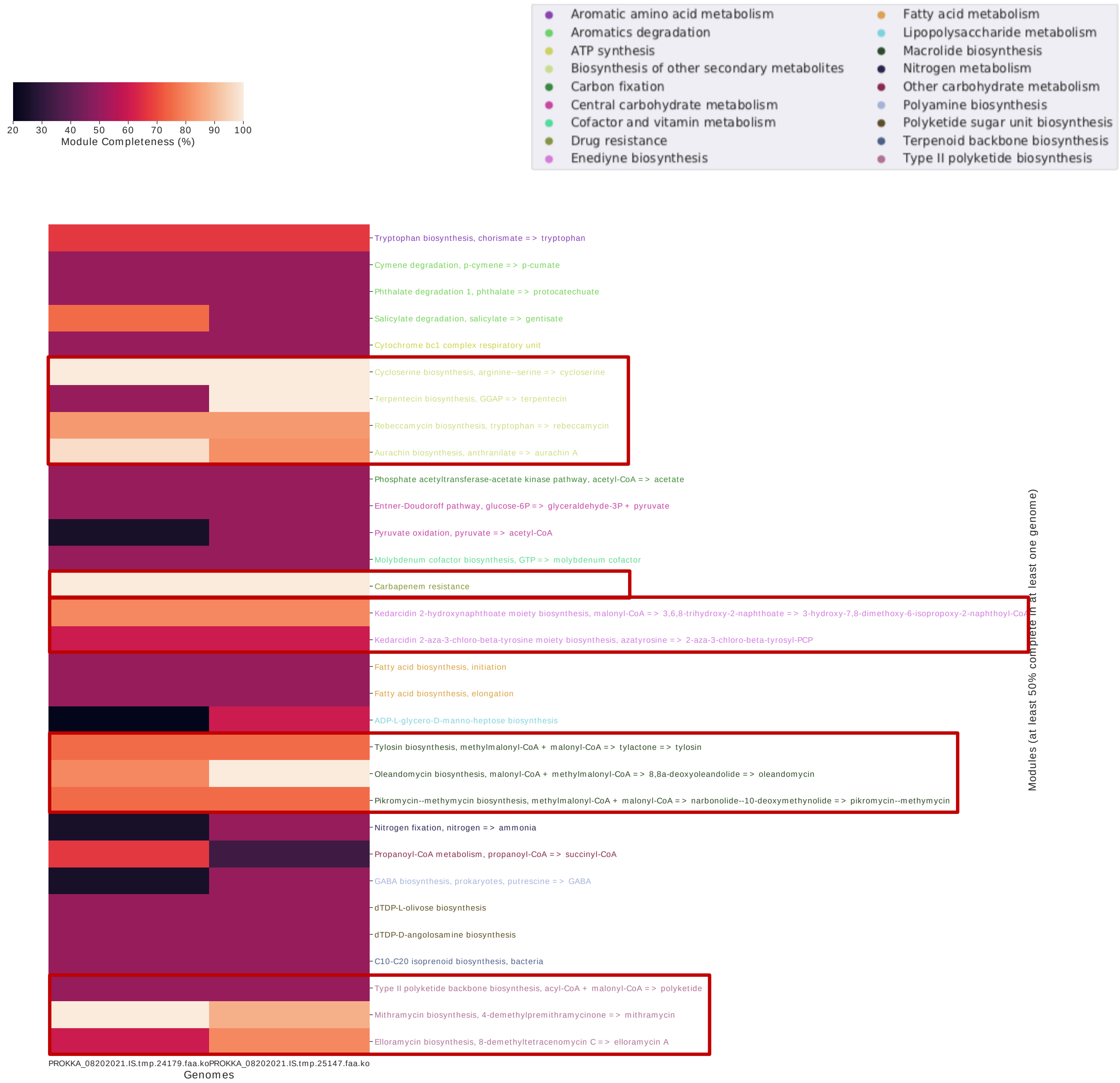


Figure S3: Module completeness heatmap for examined genomes. Categories that were not found in both examined genomes have been removed. The different colors of the modules indicate which pathway they belong to (upper right box).

Appendix B

Table S1: Polyamide 6,6 structures and corresponding masses

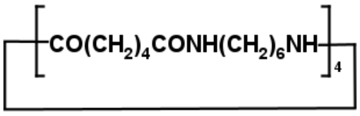
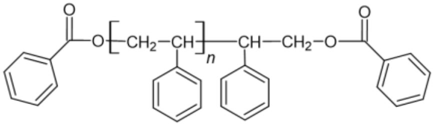
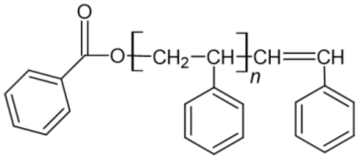
Symbols	Original structures	MNa$^+$	MK$^+$	MH$^+$
🟠	 $[CO(CH_2)_4CONH(CH_2)_6NH]_4$	927.99	943.97	1132.22
	Photo-oxidized structures			
Isobaric species 🟠	$CH_3CO-[NH(CH_2)_6NHCO(CH_2)_4CO]_3-NH(CH_2)_6NHCOCH_2CH{=}CH_2$	928.08	944.06	
	$CH_3(CH_2)_3CONH(CH_2)_6NH-[CO(CH_2)_4CONH(CH_2)_6NH]_3-COCH_3$	944.06		
	$NH_2CO(CH_2)_4CO-[NH(CH_2)_6NHCO(CH_2)_4CO]_3-NH(CH_2)_5CHO$			
	$CHO(CH_2)_3CONH(CH_2)_6NH-[CO(CH_2)_4CONH(CH_2)_6NH]_3-CHO$			
🟨	$HOOC(CH_2)_3CONH(CH_2)_6NH-[CO(CH_2)_4CONH(CH_2)_6NH]_3-CHO$	960.08		
	$HOOC(CH_2)_4CO-[NH(CH_2)_6NHCO(CH_2)_4CO]_3-NHCO(CH_2)_5NH_2$			
	$NH_2CO(CH_2)_4CO-[NH(CH_2)_6NHCO(CH_2)_4CO]_3-NH(CH_2)_5COOH$			
▲	$NH_2CO(CH_2)_4CO-[NH(CH_2)_6NHCO(CH_2)_4CO]_4-NH_2$	1072.22		
	$NH_2(CH_2)_6NH-[CO(CH_2)_4CONH(CH_2)_6NH]_4-CHO$			
🟦	$NH_2(CH_2)_6NH-[CO(CH_2)_4CONH(CH_2)_6NH]_4-CO(CH_2)_3CHO$	1142.32		

Table S2: Polystyrene structures and corresponding masses.

Symbols	Structures	MNa^+	MK^+	MH^+
🟠		**1825**	**1841**	**1802**
🟨		**1809**	**1825**	**1786**

Expression profiles of *mt4*, hsp60 and hsp70 in embryos exposed to PA fibers

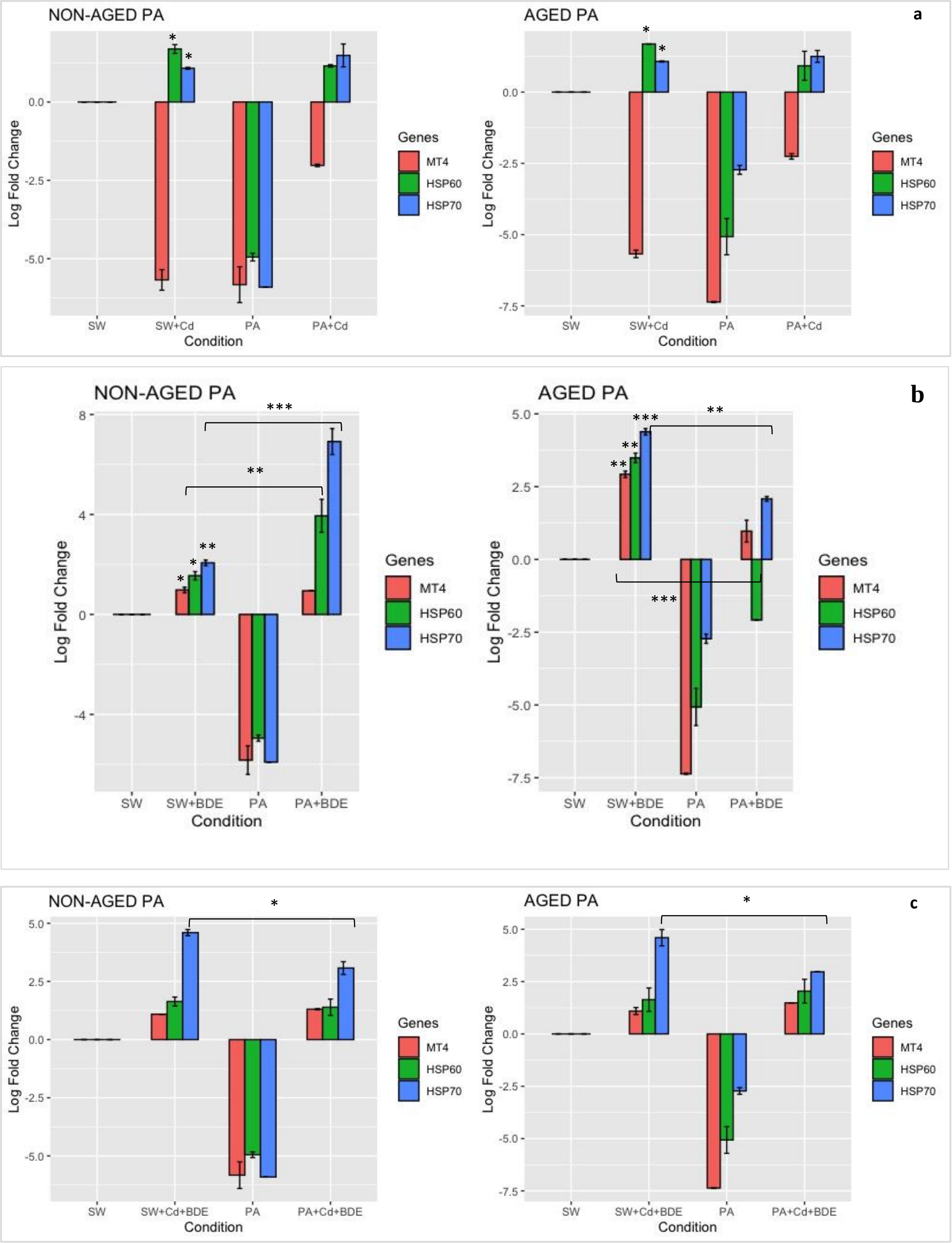


Figure S1: RT- qPCR results showing the mRNA levels of *metallothionein 4*, *Mt4,* and *heat shock proteins, hsp60* and *hsp70,* with respect to reference gene *18S*, in embryos at 24 hpf (gastrula stage) exposed to a) Cd or b) BDE-47 or c) mixture of them, without or in association non-aged or aged PA, with respect to *18S* at 24 hpf (gastrula stage). Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at p <0.05 (*); p < 0.01 (**); p < 0.001 (***); one-way ANOVAs with Tukey's test.

Expression profiles of *sm50, p16* and *msp130* in embryos exposed to PA fibers

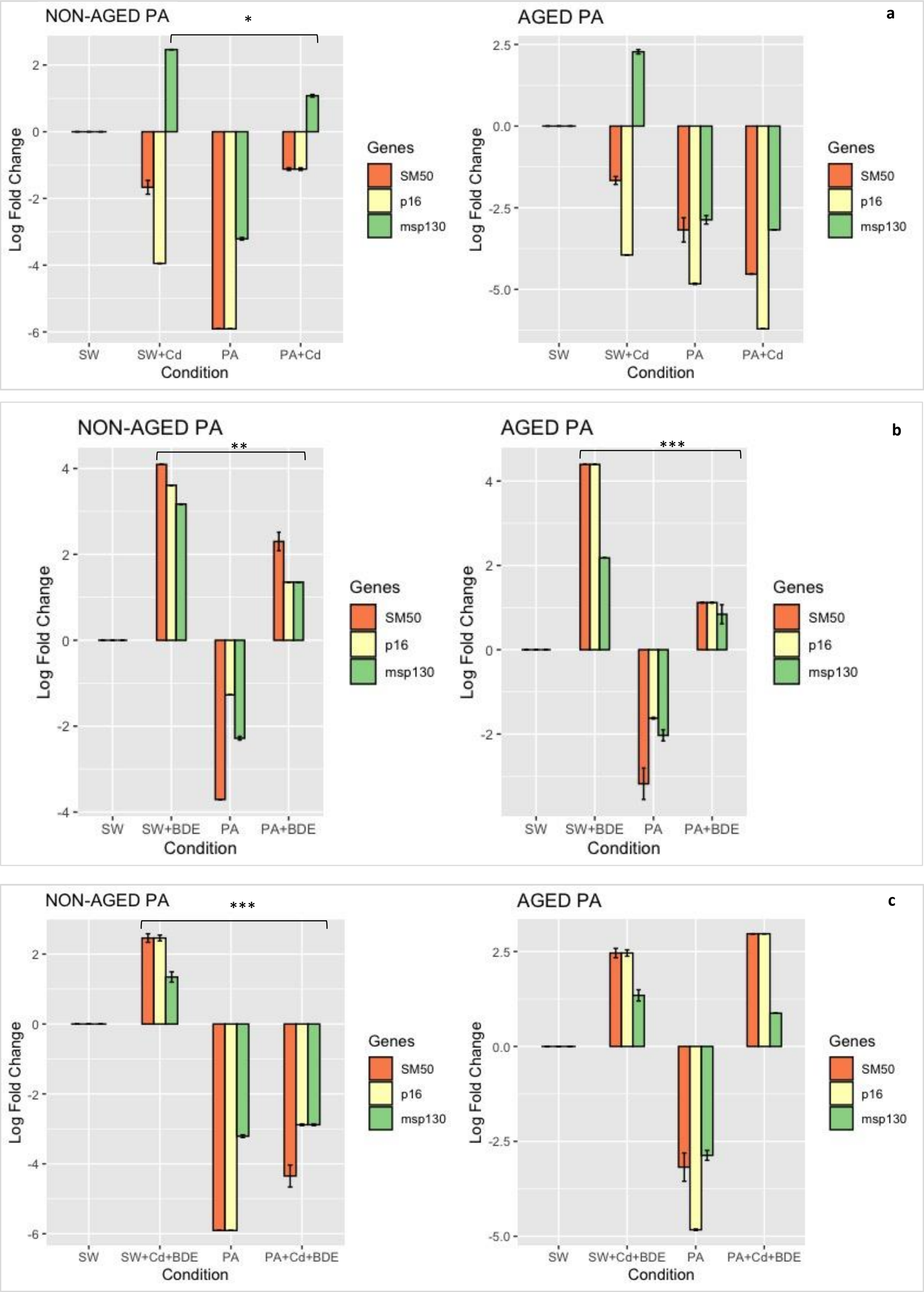


Figure S2: RT- qPCR results showing the mRNA levels of s*picule matrix protein 50*, *sm50, biomineralization protein, p16*, and *mesenchyme-specific cell surface glycoprotein, msp130,* in embryos exposed to a) Cd or b) BDE-47 or c) mixture of them, without or in association non-aged or aged PA, with respect to *18S* at 24 hpf (gastrula stage). Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at *p* <0.05 (*); *p* < 0.01 (**); *p* < 0.001 (***); one-way ANOVAs with Tukey's test.

Expression profiles of *foxa* and *hox11.13b* in embryos exposed to PA fibers

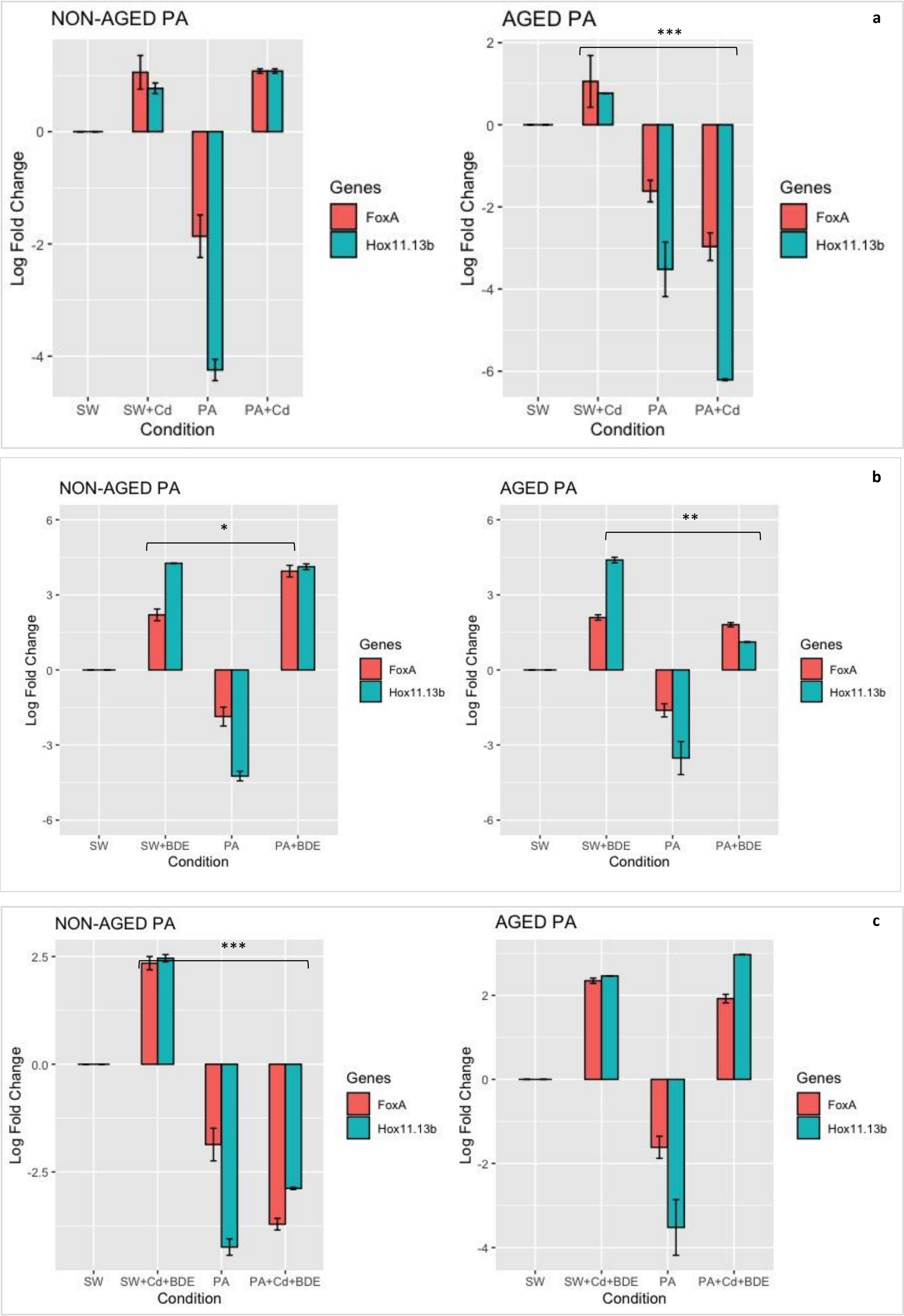


Figure S3: RT- qPCR results showing the mRNA levels, *Forkhead box transcription factor a*, *foxa* and *Homeodomain containing transcription factor, hox11/13b*, with respect to reference gene *18S*, in embryos at 24 hpf (gastrula stage) exposed to a) Cd or b) BDE-47 or c) mixture of them, without or in association with non-aged or aged PA, with respect to *18S* at 24 hpf (gastrula stage). Data are reported as a fold difference in the expression levels of the analyzed genes, compared to controls (mean ± SD), in a logarithmic scale. Values were considered statistically significant at p <0.05 (*); p < 0.01 (**); p < 0.001 (***); one-way ANOVAs with Tukey's test.