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Maria Inês Santos Simões

Toxic effects of paraffin microparticles in *Mytilus* spp.

Efeitos tóxicos de micropartículas de parafina em *Mytilus* spp.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor Bruno André Fernandes de Jesus da Silva Nunes, Equiparado a Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e sob coorientação do Doutor Bruno Branco Castro, Professor Auxiliar do Departamento de Biologia da Universidade do Minho.

Aos meus pais, pelo incansável apoio, motivação e força. Não só nesta fase, mas sempre.

o júri

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palavras-chave

Microplásticos, cosméticos, partículas de parafina, *Mytilus* spp., ecossistema marinho, efeitos bioquímicos.

resumo

Os plásticos são considerados um dos principais poluentes ambientais, principalmente em ecossistemas aquáticos. Grande parte dos plásticos são libertados no ambiente sem qualquer tratamento, o que contribui para a sua dispersão ambiental, particularmente na forma de pequenas partículas ou fragmentos (microplásticos). O ambiente aquático, e em particular os oceanos, é a localização final para o depósito de grandes quantidades de plástico, com partículas de vários tamanhos e com vários constituintes químicos. A exposição dos organismos aos microplásticos pode resultar em contaminação física (bloqueio ou danos no sistema digestivo) e química (libertação de compostos tóxicos), o que pode levar a consequências nocivas. A presença de microplásticos em meio marinho pode ocorrer também por introdução direta no meio, na forma de microesferas utilizadas em cosméticos. Os efeitos ecotoxicológicos dos plásticos levaram alguns produtores de cosméticos a oferecer alternativas viáveis às microesferas de plástico, tais como partículas de parafina. No entanto, a viabilidade desta alternativa carece de um profundo conhecimento das propriedades ecotoxicológicas das partículas de parafina em comparação com as esferas de microplásticos. Assim sendo, o principal objetivo deste estudo foi avaliar os potenciais efeitos tóxicos de micropartículas de parafina em processos fisiológicos chave no molusco marinho *Mytilus* spp.. Para atingir este objetivo, este trabalho avaliou os efeitos agudos de três densidades (5, 20 e 80 mg/l) e quatro gamas de tamanho de partículas de parafina (100-300 µm, 300-500 µm, 400-850 µm and 800-1200 µm) em mexilhões expostos durante 96 h na presença e na ausência de alimento. Os parâmetros toxicológicos avaliados foram as atividades de quatro enzimas envolvidas no metabolismo (glutationa-S-transferases, GSTs) e defesas antioxidantes (catalase, CAT; glutationa redutase, GRed; e glutationa peroxidase, GPx). No geral, partículas mais pequenas (100-300 µm) quase não causaram efeito nas atividades das quatro enzimas testadas. Pelo contrário, partículas maiores (800-1200 µm) causaram efeitos em todas as atividades enzimáticas. A atividade de GRed foi a menos afetada pela exposição, enquanto que a atividade das GSTs foi o parâmetro mais afetado. O efeito da densidade das partículas foi maioritariamente observado para o tratamento sem alimento, e esta influência foi mais significativa para a densidade de partículas mais elevada (80 mg/l). No geral, os dados obtidos neste trabalho sugerem que, em densidades próximas às que são encontradas atualmente no ambiente, não é possível antecipar efeitos tóxicos ao nível do equilíbrio redox e de metabolismo de biotransformação em mexilhões.

keywords

Microplastics, cosmetics, paraffin particles, *Mytilus* spp., marine ecosystem, biochemical effects.

abstract

Plastics are considered one of the main environmental pollutants, especially in aquatic ecosystems. Most plastics are dumped into the environment without any treatment, which contributes to their dispersion, particularly in the form of small particles or fragments (microplastics). The aquatic environment, and in particular the oceans, are the final location for the deposit of large quantities of plastics, of various particles sizes and chemical constituents. Exposure of organisms to microplastics can result in physical (blockage or damage of the digestive tract) and chemical contamination (release of toxic compounds), which can lead to deleterious consequences. The presence of microplastics in the marine environment can also occur by direct introduction in the medium, in the form of microspheres used in cosmetics. Ecotoxicological effects of microplastics have led some cosmetic producers to offer viable alternatives to plastic microspheres, such as paraffin beads. However, the viability of such alternatives requires knowledge of the toxic properties of paraffin particles when in comparison with those posed by microplastic spheres. So, the main objective of this study was to evaluate the potential toxic effects of paraffin microparticles on key physiological processes of the marine mollusc *Mytilus* spp.. To attain this objective, this work assessed the acute effects of three densities (5, 20 and 80 mg/l) of four size ranges of paraffin particles (100-300 µm, 300-500 µm, 400-850 µm and 800-1200 µm) on mussels exposed for 96 h with and without food. The here-quantified toxicological parameters were the activities of four enzymes involved in metabolism (Glutathione-S-transferases, GSTs), and antioxidant defense (catalase, CAT; glutathione reductase, GRed; and glutathione peroxidase, GPx). In general, smaller microparticles (100-300 µm) caused nearly no effects on the activity of the four enzymes tested. On the contrary, larger particles (800-1200 µm), caused effects on every enzymatic activity. GRed activity was the least affected by the exposure, while GSTs was the most affected parameter. The effect of particle density was mainly observed for the treatment without food supply, and this influence was more significant for the highest particle density (80 mg/l). Overall, data obtained in this work suggest that, in the densities close to those found today in the environment, it is not possible to anticipate toxic effects on redox equilibrium and biotransformation metabolism in mussels.

General Index

List of abbreviations and acronyms

ANOVA – analysis of variance

CAT – catalase

DDT – dichlorodiphenyltrichloroethane

g – grams

g^{-1} – per gram

g ml^{-1} – gram per millilitre

GPx – glutathione peroxidase

GRed – glutathione reductase

GSH – glutathione (reduced)

GSSG – glutathione (oxidized)

GST – glutathione S-transferase

h – hours

HBCDD – hexabromocyclododecane

HCB – hexachlorobenzene

HCBD – hexachlorobutadiene

HO_2• - hydroperoxyl radical

H_2O – water

H_2O_2 – hydrogen peroxide

Kg^{-1} – per kilogram

Km^{-2} - per square kilometre

l – liter

l^{-1} - per liter

m^{-2} – per square metre

m^{-3} – per cubic metre

MAHS – monocyclic aromatic hydrocarbons

mg/l – milligrams per liter

mm – millimetre

$mM^{-1}cm^{-1}$ – millimolar per centimetre

NADPH – nicotinamide adenine dinucleotide phosphate

nm - nanometre

•NO – nitric oxide radical

•NO_2 – nitrogen dioxide radical

O_2 – chemical formula for oxygen

$O_2^{-\bullet}$ - superoxide anion radical

•OH – hydroxyl radical

$ONOO^-$ - peroxynitrite

PAH – polycyclic aromatic hydrocarbons

PBDE – polybrominated diphenyl ethers

PCB – polychlorinated biphenyls

PCDD – polychlorinated dibenzo-p-dioxin

PCDF – polychlorinated debenzofurans

PE – polyethylene

PMMA – polymethyl methacrylate

POPs – persistent organic pollutants

PP – polypropylene

PS – polystyrene

PVC – polyvinyl chloride

RNS – reactive nitrogen species

RO• - alkoxyl radical

ROO• - peroxyl radical

ROS – reactive oxygen species

SCCPs – short chain chlorinated paraffins

SOD – superoxide dismutase

µm – micrometre

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1. General Introduction

1.1. Plastics as environmental contaminants

The release of plastic waste into the environment is a problem of growing concern (European Comission 2011). Plastic polymers are particularly important given their wide use in different areas of human activity (e.g., commercial, industrial, and medicinal), giving rise to a huge diversity of objects (Wang et al., 2016). The specific characteristics that turn plastics into highly successful raw materials to obtain a wide array of common objects include low density, good mechanical properties and low production cost, which allow the use of plastics in diverse industries and also in everyday life (PlasticsEurope and EPRO, 2016). Over the past 60 years, plastic production has increased dramatically worldwide, from 0.5 million tonnes per year, in 1960, to 300 million tonnes in 2013 (Avio et al., 2017) (Figure 1). Due to this increase in production, the high durability and resistance of plastics to chemical and physical degradation, along with poor waste management and inefficiency of wastewater treatment systems – but also as a consequence of human behaviour – this material has accumulated in the environment, particularly in aquatic systems (Bakir et al., 2012; Bergmann et al., 2015) which are the final sink for these compounds.

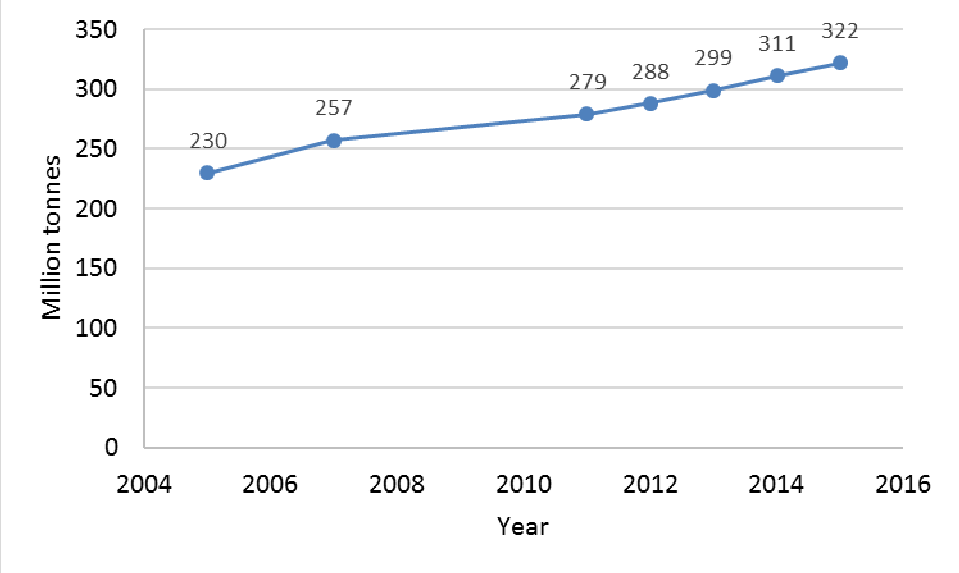

Figure 1 - Plastic production worldwide, in million tonnes, between 2005 and 2015. Adapted from PlasticsEurope and EPRO (2016). *Plastics – the facts*.

Plastic is a generic name for a vast group of synthetic polymers derived mostly from natural gas or crude oil (or their processing and refinement), and they can be of various chemical types (Khoo et al., 2010; Bejgarn et al., 2015). Consequently, various types of plastic can be found in the marine environment, namely polyethylene (PE; usually the most abundant) followed by polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS) (Andrady, 2011). In March 2011, the Global Declaration for Solutions on Marine Litter was launched by 47 associations involved in plastics management from regions all across the globe. Since 2011, 65 associations in 34 countries have signed the Global Declaration of the Global Plastics Associations for Solutions on Marine Litter, and 260 projects are underway that encourage and increase recycling and recovery, foster local, regional and global partnerships, promote better product stewardship and increase ocean pollution cleanup and marine litter prevention programs (PlasticsEurope and EPRO (2016).

Despite their frequent and widespread presence in the environment, plastics are not currently included in monitoring programs, since their ecotoxicological effects are not yet fully understood, which makes them emerging contaminants (Aristi et al., 2016). Although large fragments of plastic cause obvious problems to some life forms (such as choking, trapped in nets, intestinal blockage and starvation of sea turtles, birds or mammals) (Gregory, 2009; Andrady, 2011), micro-sized plastic fragments (microplastics) constitute a distinct hazard given their higher potential to be transferred along the food web (Auta et al., 2017).

1.2. Microplastics

Although some plastics are likely to be recycled, most of them are dumped into the environment, where their full degradation takes centuries to occur (Cole et al., 2011). The degradation of plastics in the aquatic environment is slow and may result in the formation and release of smaller fragments called microplastics (< 5 mm) (Andrady, 2011) (Table 1). Microplastics can be formed due to photooxidation processes (which make them more brittle) (Bejgarn et al., 2015) and mechanical erosion, namely on coastal areas, such as sandy beaches (Cole et al., 2011).

Table 1 - Distribution of microplastics (in percentage) and their concentrations in the marine environment. Adapted from (Auta et al., 2017).

Marine environment	Distribution (%)	Concentration	Reference
Northwestern Atlantic	60	2500 particles km^{-2}	(Law et al., 2010)
Western Atlantic	-	0.808 to 1.24 g ml^{-1}	(Morét-Ferguson et al., 2010)
Laurentian Great Lake	20	43,000 particles km^{-2} to 466,000 particles km^{-2}	(Eriksen et al., 2013)
Portuguese Coast	53	32–362 items m^{-2}	(Antunes et al., 2013)
Jade Bay, Southern North Sea	70	1770 particles L^{-1}	(Dubaish & Liebezeit, 2013)
North East Atlantic Ocean	89	2.46 particles m^{-3}	(Lusher et al., 2014)
Yangtze Estuary and East China Sea	90	0–144 particles m^{-3}	(Zhao et al., 2014)
Artic Polar Waters	95	0-1.31 particles m^{-3}	(Lusher et al., 2015)
Mediterranean Sea	74	0.90 ± 0.10 microplastics g^{-1}	(Alomar et al., 2016)
Beaches of Guanabara Bay, Southeast Brazil	56	12–1300 particles m^{-2}	(Carvalho & Baptista Neto, 2016)
Swedish Coast	-	150–2400 particles m^{-3} to 68,000–102,000 particles m^{-3}	(Lönnstedt & Eklöv, 2016)
Chinese Bohai Sea	-	63–201 items kg^{-1}	(Yu et al., 2016)

The presence of microplastics in the marine environment can result from the breakdown of larger plastic material (secondary source), but also due to their direct introduction in the aquatic medium (primary source) (Napper et al., 2015). Primary sources include the use of micronized plastics in cosmetics, spillage of pre-production plastic pellets and powders used for industrial applications, as well as the result of

shredding plastic items (e.g., on board of ships) (Fendall & Sewell, 2009; Andrady, 2011). The relative contribution of primary and secondary sources is currently unknown. Among the primary sources, the use of plastics in exfoliating products is particularly worrying, given the vast array of products and the potential high number of users worldwide (Fendall & Sewell, 2009; Chang, 2013; Rochman et al., 2015). The type of plastics used in these products is mostly polyethylene, because it is smoother than several natural products, such as fruit cores (Chang, 2015).

Cosmetic microplastics are eventually transported to wastewater treatment plants, where some will be captured in oxidation tanks, while others, due to their small size, will bypass filtration systems until they reach marine ecosystems (Leslie et al., 2014; Napper et al., 2015; Rochman et al., 2015). Depending on the size scale of the particles, the organism and the medium, these small-sized plastic particles are of great concern due to their potential for bioaccumulation, which increases with decreasing particle size; the smaller the particles, the easier they are ingested by aquatic organisms, favoring the transfer of contaminants present in the plastic along the food chain (Cole et al., 2011; Setälä et al., 2014). Microplastics can be ingested by various organisms, including plankton (Setälä et al., 2014; Vroom et al., 2017), fish (Neves et al., 2015; Alomar et al., 2017; Murphy et al., 2017; Wang et al., 2017), birds (Herzke et al., 2016) and even mammals (Lusher et al., 2017).

1.3. Biological effects of microplastics

Exposure of organisms to microplastics can result in two different types of effects: physical/mechanical and chemical. In the first case, these may result from difficulties in mobility, formation of fat deposits (Derraik, 2002), blockage in the segregation of gastric enzymes, or deleterious effects on the respiratory and digestive systems (Lambert et al., 2014). Digestive blockage leads to a decrease in food consumption and, consequently, causes malnutrition (Derraik, 2002; Lambert et al., 2014). This is the most evident biological effect supported by multiple reports of marine animals with plastics in the gastrointestinal tract (Derraik, 2002; Lambert et al., 2014).

On the other side, the ingestion or contact with plastics can lead to toxic effects. The chemical impacts of microplastics usually result from the release of multiple

compounds that are part of the matrix composition of these particles, such as the polymer or monomer of the plastic itself, and the additives that confer color, stability, flexibility, flame resistance, resistance to abrasion and to colonization by microorganisms, as well as pleasant characteristics to the touch. (Lithner et al., 2011; Fries et al., 2013; Koelmans et al, 2014). Chemical compounds released by the microplastics are called plastic leachates, and the toxicity of such chemicals from different types of plastic has already been documented, including in crustaceans (Lithner et al., 2012; Bejgarn et al., 2015), worms (Koelmans et al., 2014; Browne et al., 2013) and fish (Koelmans et al., 2014). Adverse chemical effects of plastics have been described, namely increased/decreased heart rate, neurotoxicity, pericardial edema, decreased levels of steroid hormones with consequent inhibition of growth, and delayed sexual maturation in fish (Azzarello & Vleet, 1987; Derraik, 2002; Lambert et al., 2014).

Additionally, plastic particles play a role as a dispersion vehicle of adsorbed contaminants (Teuten et al., 2009; Bakir et al., 2016; Gandara e Silva et al., 2016), which can be toxic (Engler, 2012). Microplastics have a high adsorption capacity, especially of hydrophobic contaminants, including several persistent organic pollutants (POPs) (Andrady, 2011; Van et al., 2012), due to their hydrophobic nature and high available surface area (Engler, 2012). This is mainly relevant in polyethylene-based microplastics because of their low degradation capacity in the aquatic environment (Rios et al., 2010). POPs are a group of pollutants that are semi-volatile, bioaccumulative, persistent, lipophilic and toxic (Bais et al., 2008), and there is a wide diversity of these contaminants such as pesticides (DDTs, lindane), industrial chemicals (hexachlorobenzene – HCB, hexabromocyclododecane – HBCDD, polychlorinated biphenyls – PCB, polybrominated diphenyl ethers - PBDE) and POPs from unintentional production (Hexachlorobenzene – HCB, Hexachlorobutadiene – HCBD, polychlorinated dibenzo-p-dioxins – PCDD, polychlorinated dibenzofurans – PCDF) (Stockholm Convention 2008).

These chemicals have high water-polymer distribution coefficient, favoring their adsorption by microplastics (Andrady, 2011). These compounds can then be transferred to the organisms that ingest or breathe microplastics particles, as Bakir et al. (2016) demonstrated for DDT, DEHP (phthalate) and phenanthrene (polycyclic aromatic

hydrocarbon-PAH). Given their lipophilicity, these compounds may potentially be bioaccumulated and cause various biological effects such as endocrine disruption, neurotoxicity, behavioral changes, genotoxicity, immune dysfunction, reproductive effects and biochemical responses (Vasseur & Cossu-Leguille, 2006). All of these chemicals are of particular concern for human health and for the environment (Engler, 2012).

1.4. Alternatives to microplastics

One source of microplastic that has received a large amount of attention is microbeads. These are plastic fragments measuring 5 µm to 1 mm, which are used in several cosmetics products including face and body washes (Rochman et al., 2015), shampoos, facial masks, makeup, etc. (Leslie et al., 2014; Napper et al., 2015). These microbeads are synthetic, non-degradable, water insoluble and with several additives that give them the desired properties (Leslie et al., 2014). The majority of them are PE, but microbeads can also be made of PP, PE terephtalate (PET), polymethyl methacrylate (PMMA) and nylon (Napper et al., 2015). Some studies estimated that some cosmetic products contain approximately as much plastic as in the plastic container packaging (UNEP, 2015). Another study estimated that microbeads represent approximately 25% of the total mass of plastics that have accumulated in the North Atlantic Subtropical Gyre in a year (Gouin et al., 2011).

Microbeads have replaced natural materials such as pumice and walnut husks as exfoliating agents (Cheung & Fok, 2016); however, this situation has been somewhat reverted, since public attention over toxic effects of microplastics is growing (Cheung & Fok, 2016). Countries such as the United States of America, Canada (Rochman et al., 2015) and Australia have forbidden the use of microbeads in cosmetics (Schroeck, 2015). Several marketing campaigns have already been implemented by various brands in order to increase the public awareness for the harmful effects of microbeads (Rochman et al., 2015), and they now offer natural alternative products (Chang, 2015). This situation has led to the re-introduction of nutshells as an abrasive material (Chang, 2013). Other brands have also agreed to replace microbeads by other particles. However, the advantages of such alternatives are not yet fully clear.

Among the proposed alternatives, one can find a multiplicity of materials, such as seeds of various fruits (strawberries, blueberries, grapes, kiwi and raspberry), orange peels, cocoa, coconut, almonds or nuts, coconut fiber, corn on the cob, salt, rice, sugar and macadamia (Bergmann et al., 2015). Another alternative to microbeads is the use of other hydrocarbons (preferably from a natural origin), such as paraffin or paraffin wax, due to its excellent skin tolerance, high protecting and cleansing performance and broad viscosity options (Petry et al., 2017). Paraffin is considered by some a bioplastic, when its origin is natural; this is more appealing to companies and consumers, since paraffin can be synthesised from renewable materials (Leslie et al., 2014; Verschoor et al., 2014).

Paraffin (n-alkane) is a petroleum-derived compound formed by linear or branched chains, with exclusively saturated bonds (formula C_nH_{2n+2}). This material can be found in its gaseous (< 5 carbons), liquid (5 to 15 carbons) or solid form (> 15 carbons), and in this last case it is called *paraffin wax* (Freund & Mózes, 1982), which is the form by which it can be incorporated into cosmetics. Being an alkane, paraffin has potential advantages over plastics, because it can be used as a source of carbon and energy by several prokaryotic and eukaryotic microorganisms (Hankin & Kolattukudy, 1968; Wentzel et al., 2007) and larger animals (Yin et al., 1995; Stetten, 2017), thus contributing to its biodegradability. However, the number of studies about the ecotoxicity of paraffins is still limited, although one of the arguments for choosing this material is related to the fact that it is theoretically inert and less toxic, which is suggested by its etymology (from German and from Latin *parum* = 'little' + *affinis* = 'related', supporting its low reactivity). This assumption was suggested by Shubik et al. (1962), who studied the safety of petroleum waxes as incidental additives to human food, and also by Smith et al. (1995), who studied the effects of mineral oils in rats and beagle dogs. Despite this, other studies suggest that these particles have toxic effects; Miller et al. (1996) and Griffis et al. (2010) suggested that waxes, via diet, cause inflammatory effects in the liver and mesenteric lymph nodes in rats. Tagwireyi et al. (2006) documented that paraffin (kerosene) ingestion is the most common form of childhood poisoning in Zimbabwe.

Given the available evidence in the literature, it is not licit to rule out the toxicity of paraffin, especially because paraffin waxes can contain, not only a high portion of

aliphatic hydrocarbons, but also small amounts of aromatic hydrocarbons. Aromatic hydrocarbons have one or several aromatic rings usually substituted with different alkyl groups, and they are mainly divided as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs) (Varjani, 2017). It is generally agreed that aromatic hydrocarbons, specially benzene, are more dangerous than aliphatic hydrocarbons (Von Oettingen, 1942).

Plastic has malleable qualities, poor water solubility and limited reactivity, which means that it can also be viewed as plastic (Leslie et al., 2014; Verschoor et al., 2014). Paraffin beads can thus be seen as a theoretically less toxic microplastic and an alternative to other synthetic polymers. However, they can also play a role as a dispersion vehicle of adsorbed contaminants in the marine environment. This is yet to be clarified, as well as the potential toxicity of paraffin if ingested by marine organisms. A recent study has already reported the presence of paraffin particles in the Mediterranean Sea, although in small amounts (0.8% of a total of 4050 particles found) (Suaria et al., 2016).

1.5. Potential eco-receptors and choice of test organism

One of the primary risks associated with microplastics is their bioavailability for marine organisms (Li et al., 2016). The small size of microplastics makes them available to several marine invertebrates (Van Cauwenberghe et al., 2015). The uptake of microplastics by these organisms will depend on their size, shape and density, parameters that will determine their position on the water column (Browne et al., 2007). Smaller or less dense microparticles sediment at slower rate and, therefore, end up suspended in the water column, while larger or denser ones tend to sink, making them available to filter- or deposit-feeding organisms, respectively (Van Cauwenberghe et al., 2015). Ingestion of microplastics has already been confirmed for several organisms, with different feeding strategies, such as amphipods, lugworms, barnacles (Thompson et al., 2004), echinoderms (Graham & Thompson, 2009), decapods (Murray & Cowie, 2011) and bivalves (Santana et al., 2016). Among them, bivalves stand out because of their extensive filter-feeding activity, which exposes them directly to microplastics present in the environment (Li et al., 2016). Bivalves are also a good model to evaluate physiological and biochemical changes when exposed to inorganic and organic contaminants (Apeti et al.,

2010; Lavradas et al., 2016; Freitas et al., 2017). Their use in biomonitoring studies is quite common, since many of the species are also consumed by humans (Caetano, 2014).

Microplastics can be ingested directly or indirectly by the organisms. Indirect consumption of plastics results from trophic transfer of microplastics (Farrell & Nelson, 2013). A special concern has been raised in aquaculture of bivalves; because this is mainly conducted in open systems, cultured organisms are constantly exposed to pollutants present in the seawater, including microplastics (Van Cauwenberghe & Janssen, 2014). These contaminants can therefore be subsequently transferred to humans (Table 2).

Table 2 – Microplastics found inside different species of cultured and wild bivalves around the world. Adapted from "Plastic Ingestion by Bivalves." *Blastic*, www.blastic.eu/knowledge-bank/impacts/plastic-ingestion/bivalves/.

Species	Amount of microplastics	Area
Mytilus edulis	178/individual (cultured) 116/individual (wild)	Nova Scotia, Canada
mainly *Mytilus edulis*	0.35/g of wet tissue (cultured), 2.6 – 5.1/g of wet tissue (wild)	Belgian-Dutch coastline
Mytilus edulis	0.2/g of wet tissue (wild)	French-Belgian-Dutch coastline
Mytilus edulis	0.36/g of wet tissue before depuration, 0.24/g of wet tissue after depuration (cultured)	Germany
Crassostrea gigas	0.6/individual (cultured)	east coast of USA
Crassostrea gigas	0.47/g of wet tissue before depuration, 0.35/g of wet tissue after depuration (cultured)	Brittany, France
Venerupis philippinarum	1.7/g wet tissue or 12/individual (cultured), 0.9/g wet tissue or 9/individual (wild)	British Columbia, Canada

Mussels are ecologically representative benthic filter feeders, and have been extensively used in biomonitoring of marine environments (Li et al., 2016). Many factors contribute for their use as sentinels, namely their wide distribution, filtering capability for breathing and ingestion (Pereira, 2009), resistance to a wide variety of contaminants, long

life cycle which allows monitoring bioaccumulation of a variety of contaminants (Teixeira et al., 2017), sedentary behaviour (Faggio et al., 2016), easy capture and maintenance in laboratory conditions, and large populations, which allows repeated samplings in the same area (Gomes, 2012). Mussels also present low metabolic rates of detoxification, making them particularly susceptible to xenobiotic compounds (Amiard-Triquet et al, 1986; Pereira, 2009). Experiments focusing on microplastics ingestion demonstrated that mussels ingest these microparticles and that they have the potential to translocate them from the digestive tract to the circulatory system (Browne et al., 2008; Avio et al., 2015). By virtue of all these features, the blue mussel (*Mytilus edulis* complex) was chosen as the model organism.

Individuals of *Mytilus* spp. can filter contaminants and particles directly from the water through the gills, or indirectly through the digestive system. The particles trapped on gills and then transported to lip palps and to the mouth, thus entering the digestive gland for absorption into the gut epithelium via phagocytosis, or they can be egested via faeces (Gomes, 2012; Anderson et al., 2016). Digestive cells are highly adapted for absorbing microparticles (>100 nm); if microplastics reach the marine environment, they are likely to be filtered by these organisms and accumulate in their tissues (Moore, 2006; Gomes, 2012). *Mytilus* spp. are a sessile suspension feeders, and the gills and digestive gland are well known target organs for xenobiotic effects, in which cellular alterations and physiological responses can detected using a range of standardized procedures, as enzymatic biomarkers (von Moos et al., 2012).

1.6. Potential cellular targets and biomarkers

Organisms can eliminate chemicals by immediate excretion, that does not require metabolism, or chemicals can be structurally altered, by entering the process of biotransformation, in order to acquire characteristics that grant them higher water solubility. If excretion does not occur, compounds may accumulate in the tissues of the organisms, which can induce a metabolic response of biotransformation, and its increase can be quantified. The induction of the enzymatic system responsible for the metabolism of hydrocarbons converts accumulated products into others that can be excreted to reduce their content in the tissues and their toxicity. In order to understand which are the

molecular, cellular and physiological/ targets of microplastics/paraffins, it is imperative to understand if they are metabolized by the organisms. Since some plastics are polymers chemically synthesized via polymerization from petroleum products (Chidambarampadmavathy et al., 2017) and paraffin being a hydrocarbon itself, there might be some affinities in their metabolism. Our focus is on paraffin, which is composed of long hydrogenated chains of carbon, and is thus very likely to be metabolized by living beings (Lopes, 2009).

The biotransformation of compounds, such as hydrocarbons, occurs in all organs and tissues, although the liver (in vertebrates) and the digestive gland (in bivalves) are the main detoxifying organs (Michel et al, 1993). Overall, hydrocarbon metabolism includes three phases: phases I and II involve the conversion of the lipophilic, non-polar xenobiotic into a more water-soluble metabolite, which can then be eliminated more easily from the cell in phase III. Phase I includes oxidation, reduction, hydrolysis or acetylation in order to expose or bind a functional group converting the resulting compounds in more polar or hydrophilic species, and therefore, more likely to enter the metabolic pathways (Barreira, 2007; Lopes, 2009). The first step in aliphatic (n-alkanes) and aromatic hydrocarbon metabolism is oxidation of the organic chemical with molecular oxygen catalysed by monooxygenases, and this transformation relies on the action of enzymatic systems such as the cytochrome P450-dependent monooxygenase (Barreira, 2007; Lopes, 2009). In aliphatic hydrocarbons, this oxidation gives rise to a primary alcohol followed by an aldehyde and a monocarboxylic acid, and in aromatic hydrocarbons, it originates diols followed by ring cleavage and formation of common intermediates (catechol, protocatechol and gensinate) (Simões, 2009). Products from this phase are more hydrophilic than the parent one and move from the endoplasmic reticulum to the cytosol, where the enzymes responsible for phase II metabolism are located (Barreira, 2007; Lopes, 2009). However, the product of detoxifying enzymes can be more toxic than the parent compound (Michel et al., 1993; Barreira, 2007). A second step of biotransformation may thus be necessary; phase II metabolism is conducted by conjugative enzymes, such as glutathione S-transferase (GST), UDP-glucoronyl transferase, UDP-glucosyl transferase, sulphotransferase and amino acid conjugases,

which will attach an endogenous and polar cell constituent (glutathione, sulphate, glucoronide, amino acid) to the compound or metabolites resulting from phase I. Conjugation reactions give rise to products highly soluble in water, generally non-toxic, ionizable and easily excretable (Sheehan et al., 2001; Van der Oost et al., 2003). Phase III metabolism is conducted by enzymes (peptidases, hydrolases and β-lyase), which catalyse the product of the conjugated metabolites to form easily excretable products (Van der Oost et al., 2003).

One of the likely consequences of hydrocarbon metabolism is the production of free radicals derived from oxygen (ROS) and nitrogen (RNS). ROS are chemical entities containing typically one or more unpaired electrons, such as the superoxide anion radical ($O_2^{-}\bullet$), hydroperoxyl radical ($HO_2\bullet$), hydroxyl radical (•OH), peroxyl (ROO•) and alcoxyl (RO•), which are continuously formed in small amounts by normal metabolism processes (Barreira, 2007). More recently, it was discovered that an important nitrogen radical is also formed in organisms, the nitric oxide radical (•NO). Nitric oxide can react with O_2 to form the more reactive radical nitrogen dioxide ($\bullet NO_2$) and with $O_2^{-}\bullet$ to give rise to peroxynitrite ($ONOO^{-}$), a non-radical but a highly oxidizing species. These nitrogen derivatives are designated *reactive nitrogen species* (RNS) (Radi et al., 2001). Both ROS and RNS are highly beneficial to the organisms by playing an important role in cell signalling, apoptosis, gene expression and ion transportation (Beyer et al., 2017). However, when they are produced in excess they can damage biomolecules, lipids (lipid peroxidation), proteins (protein oxidation), DNA (DNA oxidation) and carbohydrates, promoting an oxidative stress scenario (Gravato et al., 2006; Oliva et al., 2012; Klein et al., 2017), which is described by an adverse condition that results from an imbalance between concentrations of ROS and RNS and the capacity of the organism's antioxidant defence system to neutralize these oxidative molecules (Beyer et al., 2017).

When organisms are exposed to oxidative stress conditions, antioxidant defences are triggered in order to detoxify and control the amount of ROS produced (Correia et al., 2016). These antioxidant defences can be enzymatic and non-enzymatic. Non-enzymatic antioxidant defences include vitamins C and E, beta carotene and glutathione (GSH). The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione

reductase (GRed) and glutathione peroxidase (GPx). Since superoxide is the primary ROS produced, its dismutation by SOD into hydrogen peroxide (H_2O_2) is of primary importance (Zelko et al., 2002; Birben et al., 2012). H_2O_2 is nevertheless a harmful by-product, which requires elimination or degradation (Correia et al., 2016). To prevent further cellular and tissue damage, H_2O_2 is reduced to water by CAT and GPx. GPx uses GSH to reduce H_2O_2 to the corresponding alcohol (Arthur, 2000; Birben et al., 2012). The requirement of NADPH is very common to these enzymes as a reducing equivalent. NADPH maintains CAT in the active form and is used as a cofactor by GRed; the latter converts GSSG to GSH, so it can be used again by GPx (Kirkman et al., 1999; Birben et al., 2012). Some GSTs isoforms also work as an antioxidant, by inactivating secondary metabolites such as unsaturated aldehydes, epoxides and hydroperoxides (Birben et al., 2012).

Both biotransformation and antioxidant defences occur preferably in gill and digestive gland of mussels, namely in *Mytillus* sp. (Beyer et al., 2017), and there are several studies that detected these biochemical defences against hydrocarbons in these organism (Lima et al., 2007; Serafim et al., 2008; Nogueira et al., 2015; González-Fernández et al., 2016 a, b; Benali et al., 2017; González-Fernández et al., 2017; Perić et al., 2017).

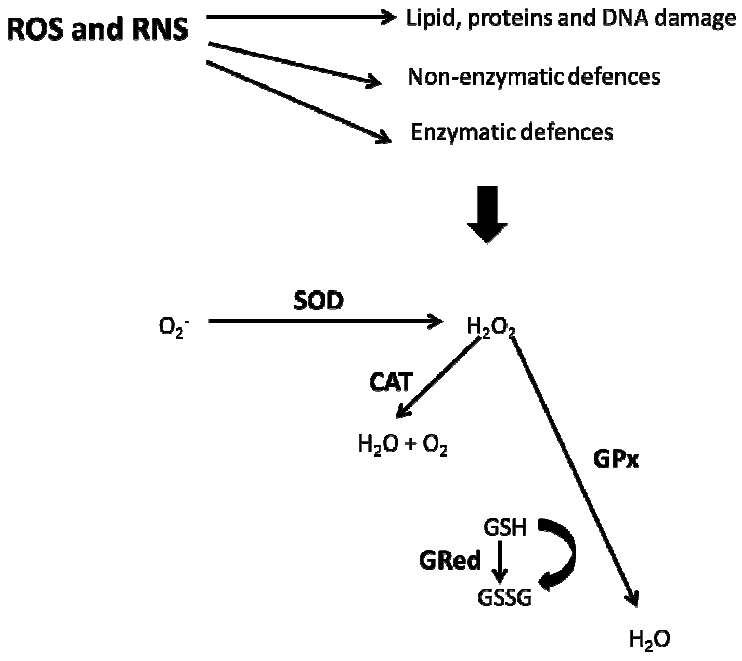

Figure 1 - Scheme of free radical defence mechanisms. Adapted from (Barreira, 2007).

By measuring biological endpoints in response to toxicants, such as biotransformation and oxidative stress defence mechanisms, one is using them as biomarkers to indicate a change in the level of stress or physiological condition. Biomarkers are selected biological endpoints, measured in species of interest and may be biochemical, physiological, histological, behaviour indicators or adverse health effects evaluated after exposure to an environmental contaminant (Forbes et al., 2006; Hook et al., 2014). They normally correspond to primary responses of a biological system after exposure to a chemical or other stressor that anticipate responses at higher hierarchical levels (Forbes et al., 2006). Such biological responses work as biomarkers by contributing to understand mechanisms of chemical impacts.

1.7. Objectives

This works intends to evaluate the ecotoxicological short-term effects of paraffin particles (putative substitutes of more noxious microplastics) used in cosmetics in *Mytilus* spp.. This work intended to understand the potential direct effects of relevant to worst-case-scenario particle densities, at the enzymatic level. A particular emphasis was put on biotransformation (GSTs) and oxidative stress (CAT, GPx, GRed), which may serve as an early sign of metabolic changes. To attain this purpose, this work aimed at evaluating the sub-lethal effects of four size ranges of paraffin particles on *Mytilus* spp. via diet, during a 96 h of exposure under feeding regimes. The specific goals included evaluating biochemical responses to exposure to paraffin microspheres and assessing the effect of particle density and presence of food (as a potential confounding factor).

2. Material and Methods

2.1. Collection and acclimation of test organisms

Organisms of the *Mytilus* spp. were manually collected during the low tide period, near the mouth of Ria de Aveiro, Portugal. This area corresponds to a brackish coastal lagoon located in the northwest of the Portuguese shore (Lopes & Silva, 2006). Ria de Aveiro presents a complex morphology, being constituted by several branched channels, and is connected to the Atlantic Ocean by only one exit (Coelho, 2009). The sampling site is sheltered from wave action but it is subjected to tidal cycles; salinity at the time of sampling was 30. The *Mytilus* spp. populations in Ria de Aveiro are comprised of two species: *Mytilus edulis* and *Mytilus galloprovincialis* (Moreira, 2008). Besides being very similar morphologically, there is strong evidence of mixed populations and the presence of interspecific hybrids (Daguin et al, 2001; Lourenço et al., 2015). For this reason, we will assume our specimens to be *Mytilus* spp.

After collection, 300 organisms were immediately transported to the laboratory. There, animals were subjected to a depuration, quarantine and acclimation period, for approximately 15 days. During this period, animals were kept in 15 l aquaria with artificial seawater (salinity 30; made from synthetic Tropic Marin® SEA SALT from Tropic Marine Center), continuous aeration, at a temperature of 20±1 °C, and a photoperiod of 16 h L:8 h D. Mussels were fed every two days with a *Chlorella vulgaris* suspension ($1x10^5$ cells/ml) (Caetano, 2014). During the acclimation period, the water was renewed every week and dead organisms were immediately discarded. Organisms were considered dead when their shells gaped and failed to shut after external stimulus. The batch was considered able for experiments when mortality did not exceed 10 %.

2.2. Paraffin microparticles

Four size ranges of paraffin particles were used to evaluate toxic effects on the selected test organism: 100-300 µm (size 1), 300-500 µm (size 2), 400-850 µm (size 3) and 800-1200 µm (size 4). The particles were obtained from a company that operates on the wholesale market of excipients for the dermocosmetic industry. For each size range, we selected three densities of exposure, based on a worst-case scenario. In a study

measuring the density of microplastics found in the North Pacific Subtropical Gyre, a maximum of 0.25 mg/l of microparticles was recorded (Goldstein et al., 2012); we used this value as a benchmark for the density of microplastics in coastal waters, given the lack of more estimates. The tested densities of paraffin microparticles were significantly above this value: 5 mg/l, 20 mg/l and 80 mg/l.

2.3. Experimental conditions

2.3.1. Pre-test

After acclimation, 15 mussels were randomly distributed by three groups: a group was exposed to paraffin particles for 2 h, another group for 6 h, and a third group exposed for 24 h. Each group was divided into five replicates, of one individual per replicate, plus two more replicates without any mussel (only paraffin particles). The purpose of this test was to record the amount of paraffin filtered along time, and observe the final fate of ingested paraffin particles by mussels, especially gills (which work as sieves for particulate matter suspended in water) and digestive glands. To attain this objective, the paraffin particles were previously dyed with Sudan Black B, which is a lipophilic dye (that does not leach into the aqueous solution) and allows the visualization of paraffin particles in tissues of dissected animals. Glass jars (with 200 ml of artificial seawater) were used for the exposures, under permanent aeration. For the sake of this experiment, paraffin particles of size 2 at a density of 80 mg/l were used. Along this test, the mussels were not fed. At the end of the assay, microparticles that remained in the medium were filtered, air-dried, and weighed. The aim of this step was to quantify the amount of particles filtered by the mussels, by comparing the remaining mass of particles in the experimental units with mussels, with that of the controls (without mussels). Two mussels of each group were sacrificed and dissected to confirm the presence of paraffin microparticles in the tissues; the gills and digestive glands were removed and observed with a stereoscope. Photos were taken with Stemi 508 Stereo Microscope under a 6.3 x magnification.

2.3.2. Test

In this test, mussels were exposed by being randomly distributed along four particle densities: 0 (control), 5, 20 and 80 mg/l of paraffin. Each of these experimental conditions was divided into seven replicates, with one individual mussel per replicate. In order to understand if the presence of food influences the filtration of the particles, the treatment was duplicated: half of the experimental animals were fed with the already-mentioned algal suspension, in a final concentration of $1x10^5$ cells/ml, and the other half was exposed without food. So, a total of 8 treatments and 56 experimental units were used: 2 feeding regimes (food vs. no food) × 4 particle densities (0, 5, 20, 80 mg/l) × 7 replicate vessels (1 individual per vessel). This experiment was separately carried out for each one of the four size ranges of paraffin particles (size 1-4), for logistic reasons.

The experiment lasted 96 h. Each replicate was exposed in 200 ml glass jars with permanent aeration to promote the dispersion of the paraffin particles, and to ensure that the dissolved oxygen did not become limiting for the organisms. During the experiment, artificial seawater and particles were renewed every 24 h to maintain the particle density in the medium and discard excretion products from mussels. Chemical parameters were monitored, such as temperature, conductivity, pH and dissolved oxygen concentration.

After exposure, all mussels were sacrificed and dissected; the gills and the digestive gland were removed for biochemical analysis. Sacrifice was performed on ice-cold water (4°C), and samples were frozen at -80°C until the performance of enzymatic assays.

2.4. Enzymatic biomarkers

Tissue samples were homogenized on ice (4°C) in a volume of 1 ml of 50 mM phosphate buffer pH = 7.0 with Triton X-100 0.1% (homogenization buffer), using a Branson Sonifier 250 (constant cycle ultrasounds for about 30 seconds). After this step, homogenates were centrifuged at 15 000 g for 10 min at 4°C. Supernatants were recovered and stored (at -80°C) for the determination of all oxidative stress biomarkers.

Measurement of glutathione S-transferases (GSTs) activity was based on the principle that these enzymes catalyse the conjugation of the glutathione with multiple electrophilic compounds. They catalyse the conjugation reaction between 2,4-

dinitrochlorobenzene (CDNB) and reduced glutathione (GSH), forming a thioether that can be monitored following the increase in absorbance at 340 nm (ε = 9.6 mM^{-1} cm^{-1}). Results were expressed in terms of total soluble protein present in the samples (nmol min^{-1} mg^{-1} protein) (Habig et al., 1974). GSTs activities were quantified in digestive glands of the mussels.

Catalase (CAT) has the double function of decomposing hydrogen peroxide (H_2O_2) and oxidising hydrogen donors (methanol, formic acid and phenols) with peroxide consumption. The activity of this enzyme was determined by monitoring the decomposition of H_2O_2 at 240 nm (ε = 0.0394 mM^{-1} cm^{-1}). CAT activity was expressed in terms of total soluble protein present in the samples (nmol min^{-1} mg^{-1} protein) (Aebi, 1984). CAT activity was quantified in digestive glands of the mussels.

Glutathione peroxidase (GPx) catalyses the reduction of various peroxides using glutathione (GSH) as the reducing agent; as such, it converts GSH into its oxidised form (GSSG). GPx activity was indirectly measured by monitoring the oxidation of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) when GSSG is reduced back to GSH by the activity of glutathione reductase (GRed). This reaction is initiated by adding a specific peroxide: H_2O_2 allows determining the activity of selenium dependent GPx, while cumene hydroperoxide is used for determining total GPx activity. The results were, once again, expressed in terms of total soluble protein present in the samples (nmol min^{-1} mg^{-1} protein) (Flohé & Günzler, 1984). GPx activity was quantified in gills and digestive glands of the mussels.

Glutathione reductase (GRed) reduces oxidised glutathione (GSSG) to GSH, recycling the latter to act as a ROS scavenger or as a substrate for conjugation reactions. This activity was measured by monitoring the oxidation of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). Enzymatic activity was also expressed in terms of total soluble protein present in the samples (nmol min^{-1} mg^{-1} protein) (Carlberg & Mannervik, 1985). GRed activity was quantified in digestive glands of the mussels.

Total soluble protein concentration of samples was determined for every sample using the method by Bradford (1976). This method involves binding a dye (Bradford reagent) to the proteins, giving rise to a blue coloured and stable complex capable of being measured at 595 nm (Bradford, 1976). Protein standards were prepared using Y-

globulin (1 mg/mL), and the total protein content of samples was estimated from the resulting calibration curve.

All enzymatic activities were determined by spectrophotometry, using a Thermo Scientific Multiskan Spectrum 96-well microplate reader.

2.5. Statistical analysis

Experiments conducted with each particle size were analysed separately. For each particle size, biochemical parameters were statistically analysed with Two-Way Analysis of Variance (ANOVA) to test for differences across feeding regimes (food vs. no food) and particle densities (0, 5, 20, 80 mg/l), as well as their interaction. When a significant interaction between the two factors was recorded, data were divided into two datasets, one for each feeding regime, and each subset was analysed with a One-Way ANOVA to test for differences across particle densities. In the presence of a significant effect of particle density, we used Dunnett's test to discriminate significant differences relatively to the control group (Dunnett, 1955). All statistical analyses were performed using SPSS Statistics 24. The adopted level of significance was 0.05.

3. Results

3.1. Pre-test

The amount of particles filtered by mussels could not be recorded during the experiment, due to the material released by mussels during the experimental periods, namely faeces and byssal threads. These materials were also retained by the filter, thus interfering with the measurement of the mass of suspended particles. Therefore, it was impossible to quantify only the paraffin particles. However, it was possible to distinctly observe microparticles on the gills and inside digestive glands after 2 and 6 h of exposure, and only on digestive gland at 24 h (Figure 3).

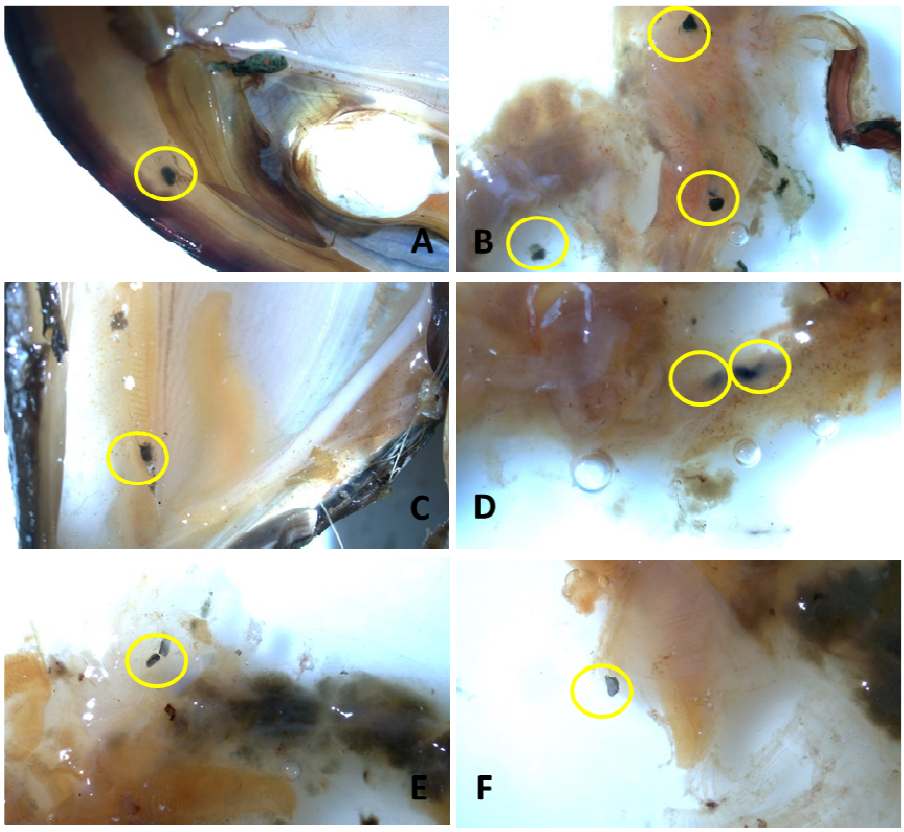

Figure 2 - Close-up (6.3 × magnification) of dissected *Mytilus* spp. exposed to suspended paraffin particles (at a density of 80 mg/l), showing the presence of microparticles in the mantle (A), digestive gland (B, D, E, F) and gills (C). Each pair of photos represents animals collected at different timings, namely after 2 h (A-B), 6 h (C-D) and 24 h (E-F) of exposure to the microparticle suspension. Paraffin microparticles, measuring 300–500 µm in diameter, were previously dyed with Sudan Black B to facilitate visualisation

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3.2. Toxicity assessment: enzymatic biomarkers

During the period of exposure to the paraffin particles, almost no mortality was observed. The exception was recorded for organisms exposed to the finer particles (100-300 µm), for which five deaths occurred at the highest concentration (two in the presence of food and three without food). Only one occasional death was observed in the remaining experimental units for the whole experiment.

GSTs activity was not affected (two-way ANOVA: $F_{3,43}$=0.836; p=0.482) by the finer paraffin particles (size 1; Figure 4A), but significant differences were detected between the two feeding regimes (two-way ANOVA: $F_{1,41}$=5.947; p=0.019). Indeed, GSTs activity was overall higher in the presence of food (Figure 4A). A significant interaction between feeding regime and particle density was observed for size 2 (two-way ANOVA: $F_{1,47}$=3.385; p=0.026). This interaction resulted from the fact that paraffin particles affected GSTs activity only in the absence of food (one-way ANOVA: $F_{3,24}$=3.413; p=0.034), with a significant decrease between the control and the highest particle density being observed (Dunnett test, p=0.011). In fact, for this size range and in the absence of food, GSTs activity tended to linearly decrease with the increase of paraffin particles density (Figure 4B). This confounding effect resulting from the presence/absence of food (i.e., a significant feeding regime × particle density interaction) was also observed for larger particles, i.e. size 3 (two-way ANOVA: $F_{3,48}$=13.100; p<<0.001) and size 4 (two-way ANOVA: $F_{3,45}$=3.742; p=0.017). This effect was reflected by a significant increase observed when comparing the control and the second highest particle density (Dunnett test, p<<0.001) for size 3 (Figure 4C), but only in the absence of food (one-way ANOVA: $F_{3,24}$=14.678; p<<0.001). A similar scenario was also observed for size 4 (Figure 4D), with a significant increase of GSTs activity at the highest particle density (Dunnett test, p=0.025) in the absence of food (one-way ANOVA: $F_{3,24}$=3.250; p=0.039).

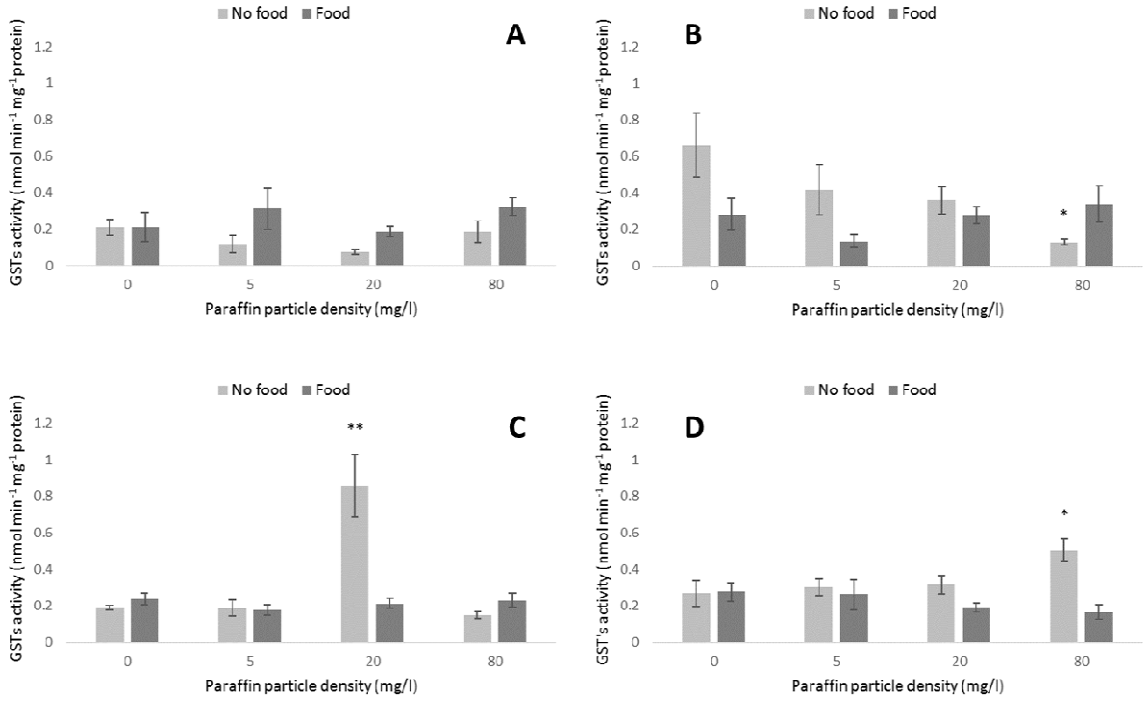

Figure 3 – Effects of suspended paraffin particles on GSTs activity of *Mytilus* spp. in the digestive gland, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

CAT activity was not affected by the smaller paraffin particles, size 1 (two-way ANOVA: $F_{3,40}$=1.411; p=0.254) (Figure 5A). A significant interaction between feeding regime and particle density was observed for size 2 particles (two-way ANOVA: $F_{3,48}$=3.583; p=0.020), and this interaction resulted from the fact that particles affected CAT activity only in the absence of food (one-way ANOVA: $F_{3,24}$=4.577; p=0.011). However, no differences between animals from the control treatment and those exposed to distinct particle densities were recorded (Dunnett test, p=0.207; p=0.971; p=0.168) (Figure 5B). For size 3, a significant interaction between feeding regime and particle density was observed (two-way ANOVA: $F_{3,48}$=12.695; p<<0.001). In the absence of food (one-way ANOVA: $F_{3,24}$=26.950; p<<0.001), a significant increase between the control and the second highest particle density was observed (Dunnett test, p<<0.001); in the presence of food (one-way ANOVA: $F_{3,24}$=9.672; p<<0.001) we recorded an increase in CAT activity on the two lowest particle densities (Dunnett test, p<0.05) (Figure 5C). This

significant interaction between feeding regimes and particle density was also observed for the larger paraffin particles (size 4; two-way ANOVA: $F_{3,46}$=6.858; p=0.001). CAT activity was affected by this size range only in the absence of food (one-way ANOVA: $F_{3,24}$=7.684; p=0.001), with a significant increase between the control and the highest particle density (Dunnett test, p=0.001) (Figure 5D).

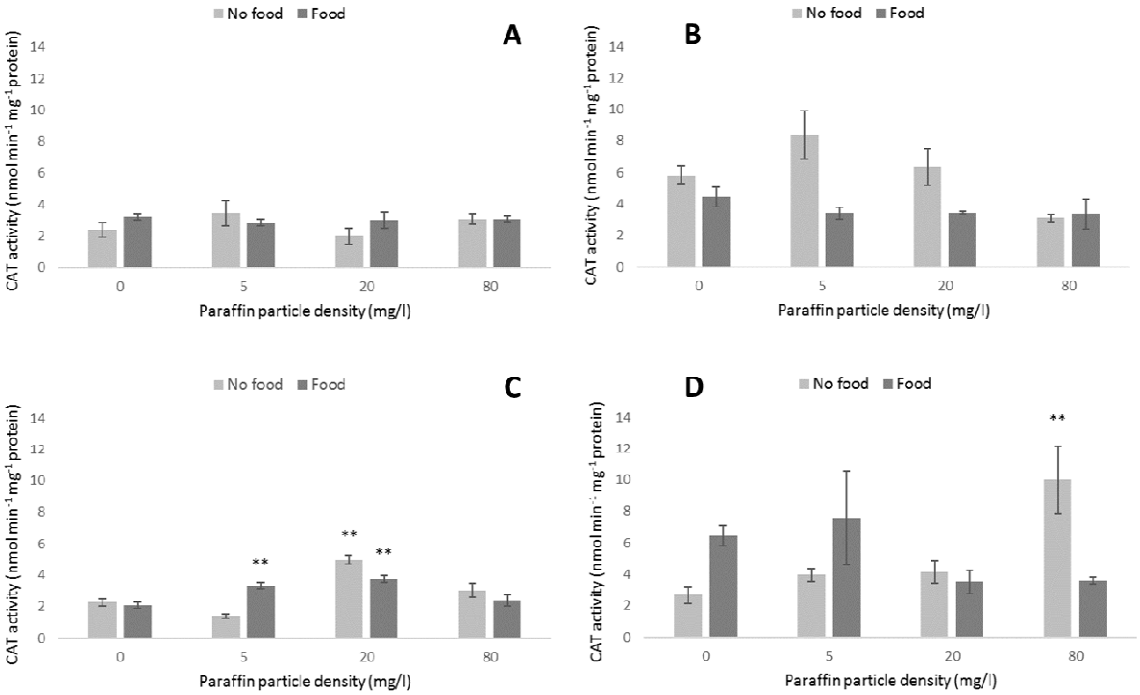

Figure 4 - Effects of suspended paraffin particles on CAT activity of *Mytilus* spp. in the digestive gland, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

GRed activity was not affected by any of the size ranges of paraffin particles (two-way ANOVA): size 1 - $F_{3,41}$=1.774, p=0.167 (Figure 6A); size 2 - $F_{3,47}$=0.980, p=0.410 (Figure 6B); size 3 - $F_{3,48}$=1.339, p=0.273 (Figure 6C); size 4 - $F_{3,45}$=1.609, p=0.201 (Figure 6D). However, significant differences were detected between the two feeding regimes for the larger paraffin particles (size 4; two-way ANOVA: $F_{1,45}$=4.637; p=0.037). Indeed, GRed activity was overall higher in the absence of food for organisms exposed to size 4 particles.

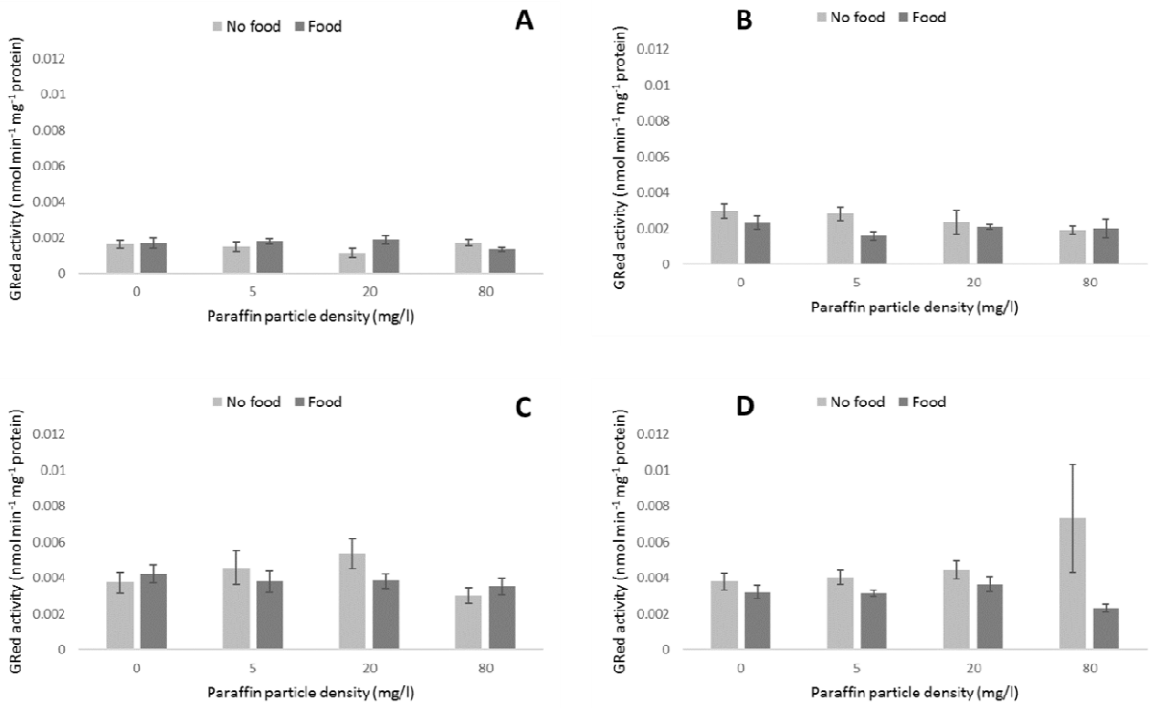

Figure 5 - Effects of suspended paraffin particles on GRed activity of *Mytilus* spp. in the digestive gland, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

Total GPx activity in the gills of *Mytilus* spp. was not affected by any of the sizes of paraffin particles (two-way ANOVA): size 1 - $F_{3,40}$=0.560, p=0.644 (Figure 7A); size 2 - $F_{3,46}$=2.172, p=0.104 (Figure 7B); size 3 - $F_{3,47}$=0.964 p=0.417 (Figure 7C); size 4 - $F_{3,43}$=0.758, p=0.524, (Figure 7D). However, significant differences were detected between the animals subjected to the two feeding regimes, for the larger paraffin particles (size 4; two-way ANOVA: $F_{1,43}$=15.366; p<<0.001). Similarly to GRed, total GPx activity was overall higher in the absence of food and for size 4.

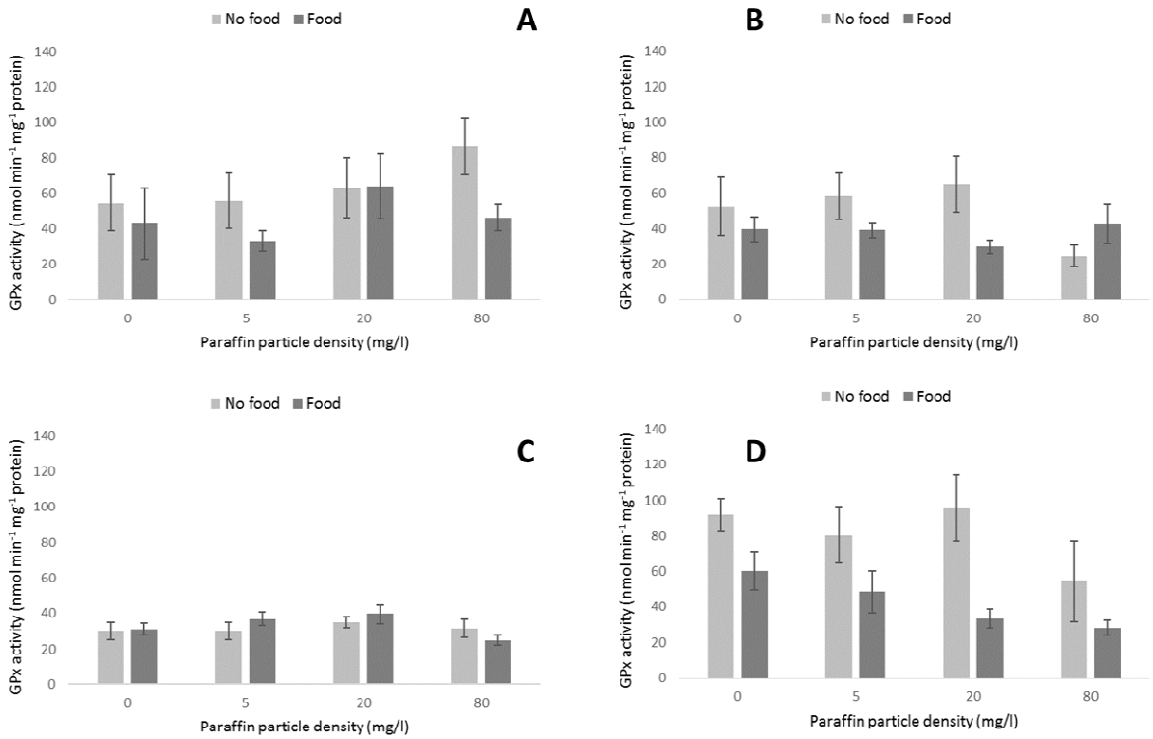

Figure 6 - Effects of suspended paraffin particles on total GPx activity of *Mytilus* spp. in the gills, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

Total GPx activity in the digestive gland of *Mytilus* spp. was not affected by paraffin particles size 1 (two-way ANOVA: $F_{3,41}$=1.002; p=0.402). However, significant differences were detected between the two feeding regimes (two-way ANOVA: $F_{1,48}$=15.444; p<<0.001). Indeed, total GPx activity was generally higher in the absence of food (Figure 8A). A significant interaction between feeding regime and particle density was observed for size 2 particles (two-way ANOVA: $F_{3,48}$=3.664; p=0.019), and this interaction resulted from the fact that particles affected GPx only in the absence of food (one-way ANOVA: $F_{3,24}$=3.043; p=0.048). However, no differences were recorded between the control and treatments with paraffin particles (Dunnett test, p=0.197; p=0.780; p=0.555) (Figure 8B). For size 3, a significant effect of the particle density was observed (two-way ANOVA: $F_{3,48}$=4.033; p=0.012). This was reflected by an increase in total GPx activity in the second

highest density (Dunnett test, p=0.006; Figure 8C). A significant effect of particle density was also observed for size 4 (two-way ANOVA: $F_{3,46}$=3.843; p=0.015), but in this case, it was possible to observe a decrease between the control and highest density was observed (Dunnett test, p=0.018; Figure 8D).

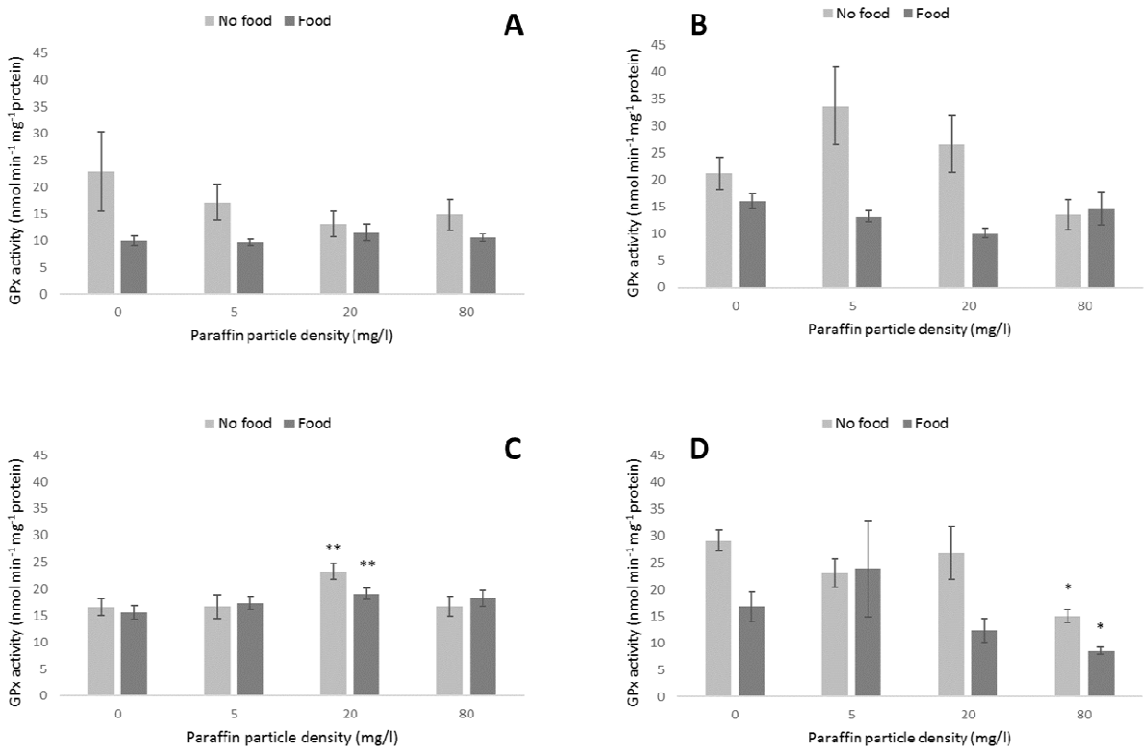

Figure 7 - Effects of suspended paraffin particles on total GPx activity of *Mytilus* spp. in the digestive gland, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

Selenium dependent GPx activity in the gills was not affected by paraffin particles of size 1 (two-way ANOVA: $F_{3,41}$=0.599; p=0.19; Figure 9A), size 2 (two-way ANOVA: $F_{3,46}$=2.365; p=0.083; Figure 9B) or by size 3 (two-way ANOVA: $F_{3,47}$=0.123; p=0.946; Figure 9C). However, for the largest particles (size 4), a significant decrease in enzymatic activity was observed (two-way ANOVA: $F_{3,43}$=2.941; p=0.044) at the highest particle density (Dunnett test, p=0.014; Figure 9D).

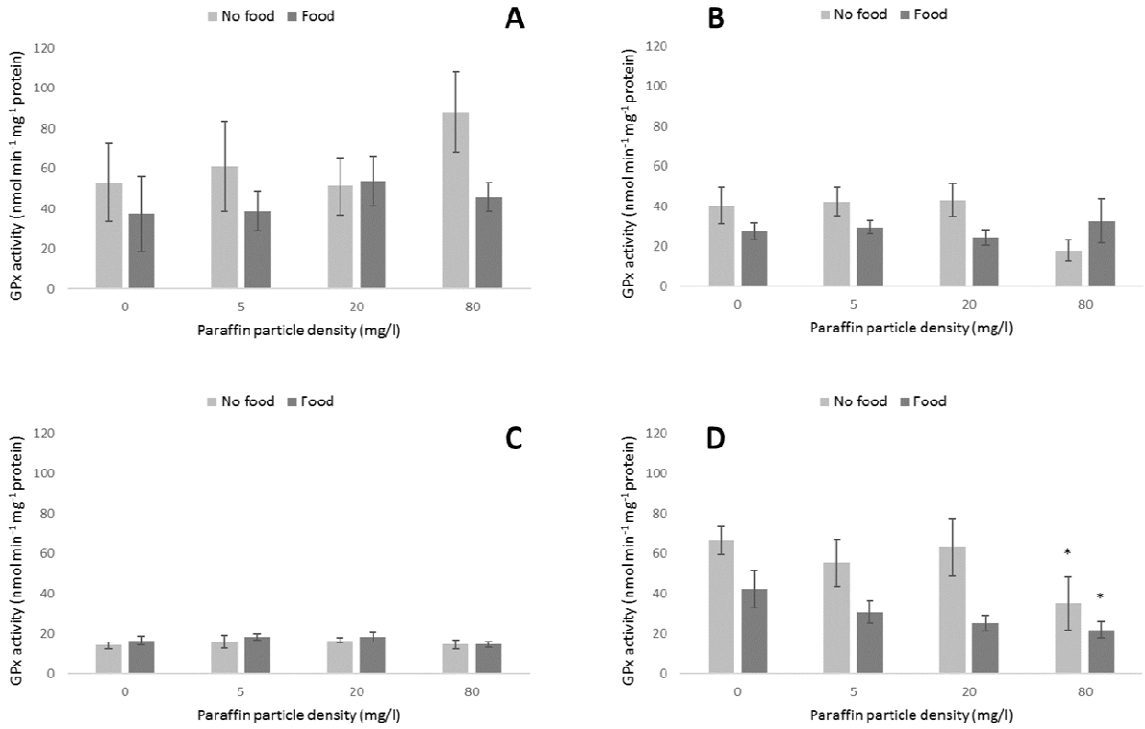

Figure 8 - Effects of suspended paraffin particles on selenium dependent GPx activity of *Mytilus* spp. in the gills, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

Selenium-dependent GPx activity in the digestive gland was not affected by paraffin particles size 1 (two-way ANOVA: $F_{3,41}$=0.538; p=0.659) or by size 2 (two-way ANOVA: $F_{3,48}$=1.898; p=0.143); however, significant differences were detected between the two feeding regimes for both paraffin particle sizes: size 1 two-way ANOVA - $F_{1,41}$=7.216; p=0.100 (Figure 10A); size 2 two-way ANOVA - $F_{1,48}$=10.887, p=0.002 (Figure 10B). Indeed, selenium-dependent GPx activity was overall higher in the absence of food for both paraffin particle sizes. With respect to size 3, we observed a significant interaction between feeding regime and particle density (two-way ANOVA: $F_{3,48}$=4.263; p=0.010). This interaction resulted from the fact that paraffin particles affected Se GPx activity only in the presence of food (one-way ANOVA: $F_{3,24}$=7.091; p=0.001), with a significant increase between the control and the highest particle density being observed (Dunnett test,

p=0.002; Figure 10C). The opposite trend was recorded for size 4 particles, with a significant decrease being observed (two-way ANOVA: $F_{3,46}$=3.591; p=0.021) between the control and the highest density (Dunnett test, p=0.010; Figure 10D).

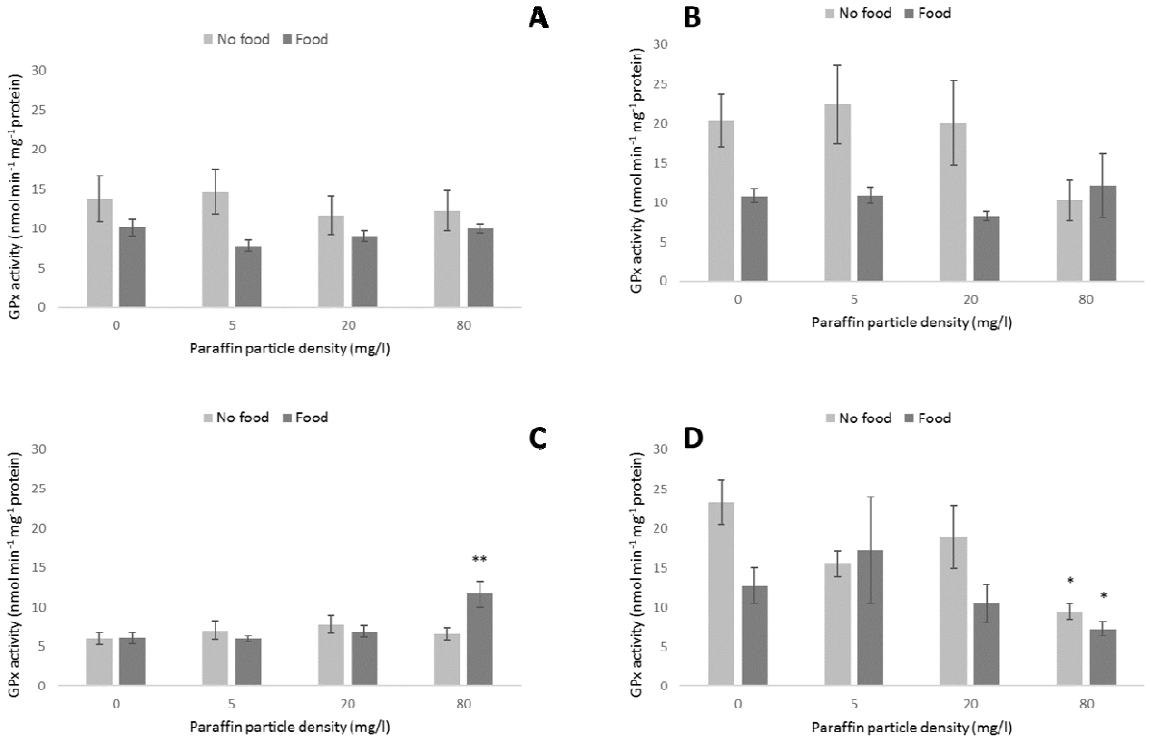

Figure 9 - Effects of suspended paraffin particles on selenium dependent GPx activity of *Mytilus* spp. in the digestive gland, across four microparticle densities and in the presence or absence of food (microalgal suspension). Experiments were conducted separately for four particle size classes: (A) 100-300 µm; (B) 300-500 µm; (C) 400-850 µm; (D) 800-1200 µm. Results are expressed as mean ± standard deviation (n=7); asterisks represent statistically significant differences relatively to the control (0 mg/l).

Table 3 – Summary the effects of suspended paraffin particles of four particle size classes (100-300 µm; 300-500 µm; 400-850 µm; 800-1200 µm) on GSTs, CAT, GRed and GPx activity of *Mytilus* spp.. The factors that were significant are highlighted in bold (feeding regimes and particle densities, or their interaction); the particle densities that significantly differed from the control are also discriminated (5, 20, or 80 mg/l).

	Particle size			
Biomarkers	100-300 µm (size 1)	300-500 µm (size 2)	400-850 µm (size 3)	800-1200 µm (size 4)
GSTs	**feeding regime** overall higher activity in the presence of food	**interaction** linear decrease in activity ONLY in the absence of food (at 80 mg/l)	**interaction** irregular increase in activity ONLY in the absence of food (at 20 mg/l)	**interaction** linear increase in activity ONLY in the absence of food (at 80 mg/l)
CAT	**no effect**	**no effect**	**interaction** irregular increase in activity in the absence (at 20 mg/l) AND in the presence of food (at 5 and 20 mg/l)	**interaction** linear increase in activity ONLY in the absence of food (at 80 mg/l)
GRed	**no effect**	**no effect**	**no effect**	**feeding regime** overall higher activity in the absence of food
Total GPx - (gills)	**no effect**	**no effect**	**no effect**	**feeding regime** overall higher activity in the absence of food
Total GPx - (digestive gland)	**feeding regime** overall higher activity in the absence of food	**no effect**	**particle density** irregular increase in activity (at 20 mg/l)	**particle density** decrease in activity (at 80 mg/l)
Se-dependent GPx - (gills)	**no effect**	**no effect**	**no effect**	**particle density** decrease in activity (at 80 mg/l)
Se-dependent GPx - (digestive gland)	**feeding regime** overall higher activity in the absence of food	**feeding regime** overall higher activity in the absence of food	**interaction** linear increase in activity ONLY in the presence of food (at 80 mg/l)	**particle density** decrease in activity (at 80 mg/l)

Table 3 summarizes all the results described above, for a more integrated interpretation of the data.

Considering the obtained results and the analysed effects and interactions, it is reasonable to observe that the lower-sized paraffin particles (size 1 and 2) were much

less prone to cause effects on the four analysed biomarkers (except for GSTs in size 2). On the opposite side, the larger the paraffin particles (sizes 3 and 4), the greater the extent of effects on selected parameters, with the involvement of the activity of almost all enzymes. The feeding regime significantly altered the enzymatic activities of all biomarkers, or confounded (i.e., interacted with) the effect of particle size (except for Se-dependent GPx measured in the gills).

GRed activity was the less responsive biomarker either to feeding regime or paraffin particles. On the opposite end, GSTs activity was the most responsive enzymatic biomarker, with significant differences being observed for all paraffin particle size classes, and in most cases being confounded by feeding regime. Two important trends in the data were the observations that: (i) often, an effect due to the paraffin particles was only observed in the absence of food; (ii) the most frequent particle density causing a significant effect in the measured biomarkers (lowest observed effect level – LOEL) was 80 mg/l.

4. General Discussion

4.1. Exposure and absorption to paraffin microparticles

Results from the pre-test showed that it was possible to distinctly observe microparticles in gills and inside digestive glands after 2, 6 and 24 h of exposure (see Figure 3). This is in alignment with the known physiology of the tested organism, since individuals of *Mytilus* spp. can filter contaminants and particulate matter directly from the water through gills, or indirectly through digestive system (Gomes, 2012). Multiple studies concerning the feeding of *Mytilus* spp., including endoscopic examination, have shown that microparticles are filtered by gills, where filamentous cilia are able to capture particles from the external media, and particles can be either directly absorbed by transmembrane crossing (in case of lipophilic compounds) (Shumway & Parsons, 2016) or, rapidly transported to the labial palps and to the mouth, thus entering the digestive gland for absorption (Ward et al., 1991, 1993, 1998; Browne et al., 2008). Taking into account the chemical nature of paraffin (hydrocarbons), both gill and digestive absorption pathways are clearly possible. As such, our results will be discussed assuming direct contact of paraffin microparticles with external (gills) and internal organs (digestive gland) and subsequent absorption of hydrocarbons released from the particles.

4.2. Mortality during exposure to paraffin microparticles

During exposure to the particles, almost no mortality was observed, confirming the overall good condition of the laboratory organisms. This finding may also indicate that the tested particles had low toxicity, in general. A notable exception to this trend was a group of five dead organisms, after being exposed to the finer particles (size 1) at the highest density. This was an unexpected result, given the theoretically low toxicity of paraffin. However, increases in the mortality rate along with the increase of particle densities, namely microplastics, is a common outcome, not only with mussels (Rist et al., 2016) but also with crustaceans (Jemec et al.2016; Bergami et al., 2017), polychaetes (Thit et al., 2015) and fish larvae (Mazurais et al., 2015). The here obtained data showed that this pattern only occurred for the smallest size of paraffin particles; for the same mass (density) of particles, the smaller particles are always more numerous than larger

particles. Thus, the observed effect may have to do with a higher number of particles present in suspension, because too many particles can lead to valve closure in order to prevent tissue damage, namely in gills, which would result in inhibition of gas exchange (Jørgensen, 1990; Ortmann & Grieshaber, 2003). This situation can ultimately lead to depletion of oxygen reserves, compromising essential life functions, such as ATP synthesis (Ortmann and Grieshaber, 2003; Rist et al., 2016).

4.3. Biochemical responses to paraffin microparticles

The marine environment is characterized by many kinds of environmental pollutants originating from direct discharge, atmospheric deposition or run off from land. All marine organisms have some degree of adaptation to metabolize the toxicants that may enter their internal environment (Zhang et al., 2014). In the case of hydrocarbons, such as the case of the paraffin released by cosmetic microbeads, most organisms are able to metabolize and excrete/eliminate these recalcitrant and lipophilic contaminants. Such metabolism involves many stages and many cellular players (see Introduction), and may cause oxidative stress or depletion of antioxidants (e.g.: glutathione).

GSTs was the most responsive biomarker to the exposure to paraffin microparticles, increasing in some situations, while decreasing in others. This increase may indicate the activation of a specific pathway of the phase II biotransformation mechanism, where GSTs conjugated glutathione with the functional groups that are either present on the xenobiotic or were introduced during phase I, in order to turn it into a product highly soluble in water, non/less toxic, ionizable and easily excretable (see introduction). This biotransformation mechanism was already described, and for aliphatic hydrocarbons (Hou, 2000; Shabaruddin et al., 2012). There is limited experimental evidence on possible biochemical toxicity mechanisms of aliphatic hydrocarbons. The liver is a target organ in repeated dose studies with rats and mice; Kuroda et al. (2013) studied the effects of ozocerite (mainly consisting in aliphatic hydrocarbons) in rats, and observed that GSTs in the liver increased in all treated groups. A study conducted by Johnson (1965) tested the influence of specific aliphatic compounds on rat liver glutathione levels, and put the hypothesis that several of them led to a depression of liver GSH levels, which was associated to the fact that these compounds were all substrates for GSTs. Studies

conducted with other organisms are mainly with aromatic hydrocarbons and short chain chlorinated paraffins (SCCPs); Burýšková et al. (2006) observed a significant induced detoxification by GSTs in *Xenopus laevis* embryos exposed to a commercial mixture of SCCP.

An increase on CAT activity (in paraffin particle sizes 3 and 4) and on GPx (size 3) activities in digestive gland was observed, so an antioxidant defence appears to have been activated. A decrease on GPx activity was also observed (size 4), which can be related to the activation of other defence pathways responsible for the reduction of hydrogen peroxide like CAT (Birben et al., 2012). The metabolism of hydrocarbon compounds may lead to the production of free radicals derived from oxygen (ROS) and nitrogen (RNS), and exposure to these oxygen/nitrogen reactive species often leads to an oxidative stress scenario (Zhang et al., 2014). When organisms are exposed to chemicals whose metabolism may result in pro-oxidative conditions, antioxidant defences are triggered in order to detoxify and scavenge the excess of ROS/RNS that is likely to be produced (Correia et al., 2016). These antioxidant defences can be enzymatic and non-enzymatic, and the majority of enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRed) and glutathione peroxidase (GPx) (Birben et al., 2012). On the other hand, our results may be justified considering that aliphatic compounds, namely aliphatic alcohols, serve as specific substrates for CAT (Metelitsa & Popova, 1979; Hnaien et al., 2010), and a study has already shown that cyclohexane induces GPx activity (Prasad et al., 2015). GPx increased activity was mainly detected in digestive gland, which may indicate that the organisms absorbed the paraffin particles mainly through the digestive system. Many studies have focused on the utility of antioxidants as biomarkers for bivalves (Lima et al., 2007; Lüchmann et al., 2011; Zhang et al., 2014; Sandrini-Neto et al., 2016), however oxidative stress responses to aliphatic hydrocarbons have been mainly studied in microorganisms or rat liver.

GRed activity was the less responsive biomarker, since it was not modulated by either the feeding regime or paraffin particles (size and density). Opposite results were found by Lima et al. (2007), who observed that GRed activity stood out a as suitable biomarker of detection of aliphatic hydrocarbons in the digestive gland of *Mytilus*

galloprovincialis. This situation could be clarified with GSH, GSSG and NADPH quantification. GPx promotes the oxidation of GSH to GSSG to eliminate organic and inorganic peroxides from the organism. As GSSG accumulates, and to maintain the cellular redox balance, it must be reduced to GSH by GRed and, if the production of GSSG surpasses the regeneration of GSH, GSSG accumulates and is translocated outside the cell by specific transporters to avoid NADPH exhaustion (Lima et al., 2007). However, without these data, results suggest that, despite the unchanged activity of GRed, antioxidant systems of *Mytilus spp.* could have remained active enough to prevent oxidative deleterious effects.

4.4. Differences across paraffin particle sizes

Suspension feeding bivalves tend to increase their filter efficiency in the presence of very small food particles (Møhlenberg, 1988; Strohmeieret al., 2012); however, it has been reported that *Mytilus* spp. can ingest zooplankton organisms up to 3 mm (Lehane & Davenport, 2002). So, all four size ranges of paraffin particle used in this study are likely to be ingested by the organisms; this is especially true for the finer particles, since phytoplankton (20-200 µm) is the primary food source of mussels (Lehane & Davenport, 2002). However, the results obtained in our experiment show that the lower-sized paraffin particles (size 1 and 2) were less prone to cause effects on the four enzymes that were analysed. In theory, the small particles have a large relative surface, therefore they would be more susceptible to erosion/abrasion and digestion, which would lead to a loss of hydrocarbons, thus making these results somewhat contradictory. The lack of effects caused by the lower-sized particles may be explained by the protective role of mucus. The process by which bivalves capture particles from the external media, and transports them to the mouth and down to the digestive gland may or may not involve mucus to prevent the absorption of the particles, and this segregation may be induced by higher concentrations of suspended particles in the medium (Beninger et al., 1999; Riisgård et al., 2011). This may be the case in the smaller particles. The particle quality (organic or inorganic) is detected by the labial palps and this contributes to sorting non-food particles that, by the mucus, are ultimately washed away from gills (Jørgensen, 1996; Riisgård et al., 2011). Particles that end up stuck in the mucus are transported with rejection tracts to

be transformed in pseudofaeces and ultimately ejected (Foster-Smith, 1978; Riisgård et al., 2011). Additionally, Clausen & Riisgård (1996) observed that very high algal concentrations in the medium reduce the filtration rate by mussels, and Hornbach et al. (1984) observed that filtration rates decreased as particle concentration increased over a range of 2-64 mg/l. This shows that this is an apparently saturable mechanism, and which is subject not only to the size, but also to the effects of the density of particles in the medium. This may have affected the absorption of the finer paraffin particles and consequently their effects.

4.5. Influence of feeding regime in biomarkers

Effects due to the paraffin particles were mainly observed in the absence of food (except for Se-dependent GPx measured in the digestive gland and for CAT in size 3) but not in the presence of food. This shows a potentially confounding factor of food or feeding regime in the assessment of biomarkers. The experiments conducted by Ward et al. (2003) examined the roles of diet quality and concentration on particle processing by the ctenidia and filtration of four species of bivalve, and observed a significant increase with increasing diet quality, and a significant decrease with increasing particle concentration. Wettability (hydrophobic/hydrophilic) and electrostatic charge, have been suggested to play possible roles in food selection by bivalves (Rosa et al., 2013). Wettability has been demonstrated that can be related to particle selection by *Daphnia magna*, with hydrophilic particles being retained more efficiently than hydrophobic ones (Gerritsen & Porter, 1982) and charged particles are more easily filtered than neutral ones by brittle star *Ophiopholis aculeate* (Labarbera, 1978) and larval clam *Mercenaria mercenaria* (Solow & Gallager, 1990). Other studies demonstrated that bivalves can select living particles from non-living detritus on the gills, as showed by Ward et al. (1997). Together, these results suggest that *Mytilus* spp. may be able to sort algae cells from the paraffin particles, which can explain the lack of effects in the presence of food; it is this licit to hypothesize that paraffin particles might not be filtered, or at least they were in less quantity. Another study by Levinton et al. (2002) concluded that bivalves distinguish among particles of different chemical composition, and respond by changing their clearance rates and their selectivity, because in the absence of food bivalves tend to

increase their rate of filtration, which expose them more to the particles. This fact seems more likely to explain our results since there were also effects in the presence of food. Additionally, algae are rich in antioxidant molecules, from vitamins to fatty acids, which aid in protecting cells against oxidation under conditions of various stresses (Wang et al., 2008; Wu et al., 2015). *Chlorella vulgaris*, the green microalgae used in this experiment, possesses indeed a vast array of antioxidant defences, as shown by Wang et al. (2008). This can also explain the lack of effects in all enzymes activities in presence of food, since antioxidant defences present in the microalgae could have been activated, or the organisms may have incorporated these defences.

4.6. Microplastics vs. paraffin microparticles

Microplastics can be ingested by various organisms, and there are some studies that focus on biochemical responses after exposure to microplastics, namely in clam *Scrobicularia plana* exposed to 1 mg/l of 20 µm polystyrene microplastics (Ribeiro et al., 2017), zebrafish *Danio rerio* exposed to 20, 200 and 2000 µg/l of 5 and 20 µm polystyrene microplastics (Lu et al., 2016) and marine mussels *Mytilus* spp. exposed to 32 µg/l of 2 and 6 µm polystyrene microplastics (Paul-Pont et al., 2016). These studies exposed the organisms to lower densities than the ones used in our study, and both observed biochemical responses including DNA damage, neurotoxicity and enhancement of anti-oxidant and glutathione-related enzymes. However, particle size influences the distribution of microplastics in biological systems; for instance, 10 µm plastics can be transported to the circulatory systems in mussels (Browne et al., 2008), and 8-10 µm plastics may accumulate in gills and gut of crustaceans (Watts et al., 2014). In our study, the most frequent particle density causing a significant effect in the measured biomarkers was the highest one – 80 mg/l so, only by comparing these studies, it appears that microplastics promote toxic effects at lower densities. However, more studies have to be made in order to evaluate the toxic effects of paraffin particles, including not only biochemical responses but other potential targets, like DNA damage, neurotoxicity – these are effects that have already been reported for microplastics (Oliveira et al., 2013; Lu et al., 2016; Deng et al., 2017; Ribeiro et al., 2017) and lipid metabolism, not only for the previous reason but also because paraffin can be used as a source of carbon and

energy by several prokaryotic and eukaryotic microorganisms (Hankin & Kolattukudy, 1968; Wentzel et al., 2007) and larger animals (Yin et al., 1995; Stetten, 2017).

The amount of paraffin particles currently found in the environment is very small when compared to microplastics. A study reported the amount of micro- and meso-plastics floating in Mediterranean waters, of which only 0.8% of a total of 4050 particles were paraffins (Suaria et al., 2016). So, organisms are currently much more exposed to microplastics than to paraffin particles. In our study, the tested densities of paraffin microparticles were based on a worst-case scenario study that measured a maximum of 0.25 mg/l of microplastics in the North Pacific Subtropical Gyre (Goldstein et al., 2012). Since the most frequent particle density causing a significant effect in the measured biomarkers was 80 mg/l (highest density), it is unlikely that oxidative insult or damage may occur with the densities of paraffin particles close to those found today in the environment. However, in order to keep it that way, dumping of plastic-based products in the environment must be avoided, even though the toxicity scenarios are unlikely to occur.

5. Conclusion

The ecotoxicological acute effects of paraffin particles were analysed in *Mytilus* spp.. These microparticles promoted effects on phase II biotransformation metabolism, GSTs activity was the most responsive enzymatic biomarker with significant differences being observed for all paraffin particle size classes, and on enzymatic antioxidant defences, supported by alterations in CAT and GPx activities. On the contrary, GRed was the less responsive biomarker either to feeding regime or to paraffin particles. The effects observed were not always consistent or very pronounced, indicating that the biochemical and cellular insult was not very intense. However, research should be pursued for a proper assessment of the environmental risk posed by these particles. As for particle sizes, the larger the paraffin particles, the greater the extent of effects on selected parameters, with the involvement of the activity of almost all enzymes for particles size 3 and 4. Effects due to the paraffin particles were mainly observed in the absence of food, and the most frequent particle density causing a significant effect in the measured biomarkers (lowest observed effect level – LOEL) was 80 mg/l. Since this study was conducted based on a worst-case scenario, it is unlikely that these results occur with the densities of paraffin particles close to those found today in the environment. As for the question if paraffin is a suitable replacement or alternative to microplastics in cosmetics, it seems that at least it does not promote toxic effects at the densities that other microplastics do.

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