

Francisca Inês Gomes Novais Ribeiro

**Polystyrene Microplastics Accumulation
and Biomarkers Response in *Scrobicularia plana***



UAlg **FCT**

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

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**Polystyrene Microplastics Accumulation
and Biomarkers Response in *Scrobicularia plana***

Mestrado em Biologia Marinha

Trabalho efetuado sob a orientação de:
Professora Doutora Maria João Bebianno



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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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ABSTRACT

Nowadays there is an increasing resilience of plastics as an everyday item. With the rapid increase in their production and spread, plastic debris are accumulating in the marine environment where they are fragmented into smaller pieces. One of the most produced polymer, and accordingly, more common in the marine environment is the polystyrene (PS). Ranges of organisms, especially invertebrates, are vulnerable to the exposure of microparticles. However, the impacts of microplastics (< 5mm) in the marine systems are poorly understood. The aim of this study was to assess the ecotoxicity of PS microplastics in different tissues of the peppery furrow shell *Scrobicularia plana* and select the most appropriate biomarkers to evaluate microplastics effects.

Clams were exposed to 1 mg L⁻¹ of PS microplastics (20 µm) for 14 days, followed by a 7 days depuration. Microplastics accumulation in gills and digestive gland was analysed through Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) and their effects by a battery of biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxidases and glutathione-S-transferases), genotoxicity (comet assay to evaluate DNA damage), neurotoxicity (acetylcholinesterase activity) and oxidative damage (lipid peroxidation).

Results indicate that microplastics were accumulated in both organs, but more significant in the gills and were not completely eliminated after 7 days of depuration. Microplastics accumulation induced an oxidative stress response in both tissues. The overall results on oxidative stress biomarkers indicated that short-term exposure to PS microplastics induce major perturbations, as revealed by the effects on the total antioxidant capacity, DNA damage, neurotoxicity and thus oxidative damage.

The results highlighted the potential source of PS toxicity for human health and the marine environment and that *S.plana* is a significant target of PS microplastics ecotoxicity and can be a suitable biomonitor for assess their environmental risk.

Key words: ecotoxicology, accumulation, neurotoxicity, oxidative stress, genotoxicity, *Scrobicularia plana*

RESUMO

Hoje em dia há uma resiliência crescente dos plásticos como um item do dia-a-dia para fins comerciais, industriais e terapêuticos. No entanto, a sua produção, o rápido crescimento e distribuição tem dado origem a sérias implicações ambientais. O consumo de plásticos em muitos países europeus indica que as resinas plásticas mais utilizadas desde 2007 são polietileno de baixa densidade (PEBD) e polietileno de alta densidade (HDPE), polipropileno (PP), cloreto de polivinilo (PVC), polietileno tereftalato (PET) e poliestireno (PS). O poliestireno (PS) é um dos plásticos mais utilizados em todo o mundo e tem sido detetado nos oceanos sob a forma de micro e nano partículas. Tem-se verificado que o PS tem um impacto ambiental considerável, nomeadamente em espécies marinhas. Recentemente, foram identificadas no ambiente marinho partículas microscópicas omnipresentes - os microplásticos - definidos como partículas com menos de 5 mm de diâmetro, de acordo com a National Oceanic and Atmospheric Administration dos Estados Unidos da América. São considerados um poluente marinho emergente e, até à data, têm sido detetados em muitos habitats e numa variedade de espécies marinhas e de água doce. Assim, é importante entender a sua distribuição no ambiente marinho e as implicações sobre os habitats, biodiversidade e bem-estar das espécies marinhas.

Os efeitos biológicos dos microplásticos nos organismos dependem do seu tamanho sendo que, quanto menor, maior será a acumulação e o efeito a nível celular. Apesar da preocupação relacionada com a ingestão, os efeitos dos microplásticos em populações marinhas e as suas implicações para a cadeia alimentar ainda não são bem conhecidos. Os invertebrados marinhos são particularmente suscetíveis aos microplásticos, por causa do tamanho e modo de alimentação. Uma vez que o modo de ação e o risco biológico dos microplásticos ainda não são muito claros, esta dissertação avaliou a acumulação e os efeitos dos microplásticos de poliestireno (20 μm) na lambujinha *Scrobicularia plana*, de forma a avaliar o potencial risco ecotoxicológico para os diferentes níveis de organização biológica e seleccionar o biomarcador mais apropriado para determinar os efeitos dos microplásticos.

Relativamente à parte experimental, após a recolha, os animais tiveram um período de aclimação de 7 dias. Seguidamente foram expostos a uma concentração de PS microplásticos (1 mg L^{-1}) durante 14 dias, juntamente com um grupo de controlo, ao qual se seguiu um período de depuração de 7 dias. Os animais foram recolhidos em diferentes dias de exposição, nomeadamente nos dias 0, 3, 7, 14 e 21.

Inicialmente, as características dos microplásticos e o seu comportamento na água do mar foram analisados em termos de forma, tamanho, carga superficial (potencial zeta), agregação, turbidez e taxa de sedimentação. Seguidamente, avaliou-se a acumulação dos microplásticos nas brânquias e na glândula digestiva através da observação ao microscópio ótico das partículas presentes na hemolinfa, e pela técnica de espectroscopia por refletância difusa no infravermelho com transformação de Fourier (DRIFT).

Para o estudo da toxicidade dos microplásticos de PS uma bateria de biomarcadores foi analisada nas brânquias e na glândula digestiva incluindo: stress oxidativo (superóxido dismutase - SOD, catalase - CAT, glutathione peroxidases - GPx e glutathione-S-transferases - GST), genotoxicidade (danos no ADN), neurotoxicidade (actividade da enzima acetilcolinesterase), e dano oxidativo (peroxidação lipídica).

Os microplásticos usados neste estudo foram micropartículas esféricas com um tamanho de $20 \pm 0.02 \mu\text{m}$ e densidade de 1.05 g cm^{-3} . Em água do mar, os microplásticos de PS tendem a formar pequenos agregados com uma carga superficial negativa (potencial zeta = $-12.4 \pm 2.36 \text{ mV}$). A taxa de sedimentação rápida e lenta dos microplásticos na água do mar foi de 1.04×10^{-1} e $1.16 \times 10^{-3} \text{ h}^{-1}$, respetivamente, confirmando a sua tendência para sedimentar nos tanques de exposição, após as primeiras duas horas.

No que diz respeito à acumulação dos microplásticos de PS nos tecidos, as brânquias apresentaram um padrão de acumulação crescente ao longo do tempo de exposição, com uma possível recuperação no final do período de depuração, através da eliminação dos microplásticos de PS quase na sua totalidade. Em relação à glândula digestiva, a acumulação de microplásticos de PS é evidente, no entanto, não apresentou o mesmo padrão de aumento observado nas brânquias. Os resultados indicaram que a acumulação foi mais eficiente nas brânquias do que na glândula digestiva.

A toxicidade dos microplásticos de PS nas lambuginhas é dependente do tecido e do tempo de exposição e envolve mudanças na atividade das enzimas antioxidantes, stress oxidativo, neurotoxicidade e danos no ADN.

As brânquias são o órgão que responde mais ativamente ao stress oxidativo induzido pelos microplásticos de PS, com efeitos associados ao aumento da atividade das enzimas antioxidantes (SOD, CAT, GPx) e de biotransformação (GST). Na glândula digestiva verificou-se um aumento da atividade da SOD, CAT e GPx. Comparando as atividades das enzimas antioxidantes e de biotransformação dos dois órgãos (brânquias e glândula digestiva), a atividade da CAT foi a única que aumentou na glândula digestiva

em relação às brânquias. Após o período de depuração verificou-se um aumento da atividade da SOD e GPx nas brânquias. Na glândula digestiva ocorreu um aumento da atividade da CAT e uma diminuição da atividade da GST.

Verificou-se ainda um efeito genotóxico e neurotóxico causado pelos microplásticos de PS. O efeito genotóxico traduziu-se pelo aumento da percentagem de ADN presente na cauda do cometa (DNA Tail) e no comprimento da cauda do cometa e pela proporção de ADN presente na cauda (Olive Tail Moment). Também no período de depuração se verificou um aumento para estes dois parâmetros. O efeito neurotóxico dos microplásticos de PS é suportado pela diminuição da atividade da acetilcolinesterase após o primeiro dia de exposição.

De uma forma geral, o dano oxidativo foi maior na glândula digestiva do que nas brânquias. Nas brânquias o dano foi menor após o início da exposição aos microplásticos. Na glândula digestiva verificou-se um aumento progressivo até ao 7º dia. Após o período de depuração, apenas se verificaram diferenças significativas na glândula digestiva, com uma diminuição do nível de LPO em relação ao ultimo dia de exposição (dia 14).

Analisando os resultados no seu conjunto, as brânquias aparentam ser um órgão essencial na proteção de *S. plana* contra o efeito dos microplásticos de PS, uma vez que a resposta das enzimas antioxidantes e de biotransformação foi mais notória neste órgão do que na glândula digestiva, traduzindo-se numa maior toxicidade. Estes resultados indicam que, possivelmente, *S. plana* lida com a produção de espécies reativas de oxigénio (ROS) através da indução das defesas antioxidantes, o que, por conseguinte, limita o ataque de ROS nas membranas celulares, impedindo que haja peroxidação lipídica nas brânquias.

O período de depuração não parece ser suficiente para a eliminação dos microplásticos de PS. Durante o período de depuração, as brânquias de *S. plana* aparentam possuir baixa capacidade de eliminação de PS, sendo o principal órgão de contacto com os microplásticos, enquanto que a glândula digestiva parece eliminar mais facilmente as micropartículas.

Palavras-chave: ecotoxicologia, acumulação, neurotoxicidade, stress oxidativo, gentoxicidade, *Scrobicularia plana*

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CAT	Catalase
CDNB	1-Chloro-2,4-dinitrobenzene
ChE	Cholinesterase
CI	Condition Index
CTR	Control treatment
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
DDT	Dichlorodiphenyltrichloroethane
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DRIFT	Diffuse Reflectance Infrared Fourier Transform Spectroscopy
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELS	Electrophoretic Light Scattering
GPx	Glutathione peroxidases
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-Transferases
H ₂ O ₂	Hydrogen peroxide
HDPE	High density polyethylene
KBr	Potassium bromide
LMA	Low melting point agarose
LPDE	Low density polyethylene
LPO	Lipid peroxidation
MCT	Mercury cadmium telluride
MDA	Malondialdehyde
MICR	Microplastics treatment
MQ	Mili-Q

NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NMA	Normal melting point agarose
NPs	Nanoparticles
OM	Optical microscope
OTM	Olive Tail Moment
PAH	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PBTs	Polychlorinated biphenyls
PCA	Principal Component Analysis
PET	Polyethylene terephthalate
POPs	Persistent Organic Pollutants
PP	Polypropylene
PS	Polystyrene
PSW	Plastic Solid Waste
PVC	Polyvinyl chloride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
SR	Sedimentation rate
STD	Standard deviation
ζ-potential	Zeta potential

CHAPTER 1. INTRODUCTION

1.1. Plastics production

The term “plastic” defines a sub-category of a larger class of materials called polymers. Polymers are very large molecules that have long chain molecular architecture and, therefore, high average molecular weight. They may consist of homopolymers, which are repeating identical units or different sub-units in various possible sequences - copolymers. These polymers, which can be shaped by heat, are generally referred to as “plastic” materials. These include both virgin plastic resin pellets (easily transported prior to manufacture of plastic objects) as well as resins mixed with numerous additives to enhance the performance of the material (Kershaw, 2015).

Nowadays there is an increasing resilience on plastics as an everyday item, however, their rapid growth production and distribution has serious environmental implications (Lusher, 2015). Plastics are used in everyday life and in several items: in cars, electronic equipment, furniture, footwear, construction, food packages, among others (Pinto, 2012). The largest plastics producers are the sectors of packaging (39%) and construction (20.6%), followed by transportation, agriculture, household and electronics (Pinto, 2012).

Its high durability and resistance to degradation, coupled with high consumption and low recycled volumes, contributed to the continuous increase of plastics in the environment in recent decades (Keane, 2007). Thus, there is an accumulation of plastic material and a growing need for the production of new ones (Pinto, 2012). In modern society, plastics have reached a critical status for medical, commercial and industrial applications. The annual production of plastics increased considerably from 1.5 million tons in 1950 (decade where the commercial development of polyolefins, polypropylene and polyethylene - started), to approximately 311 million tons in 2014 (PlasticsEurope, 2015; Wright *et al.*, 2013b), representing an increase of 9% per year, approximately. Figure 1.1 shows the evolution of world plastic production during that period.

The world's greatest producer of plastics in 2014 was China, with a production of 67.6 million tons, followed by the European Union and North America, with 52 and 49.4 million tons, respectively (PlasticsEurope, 2015). Of the total production, about 60% was transformed into plastic solid waste (PSW) (Al-Salem *et al.*, 2010).

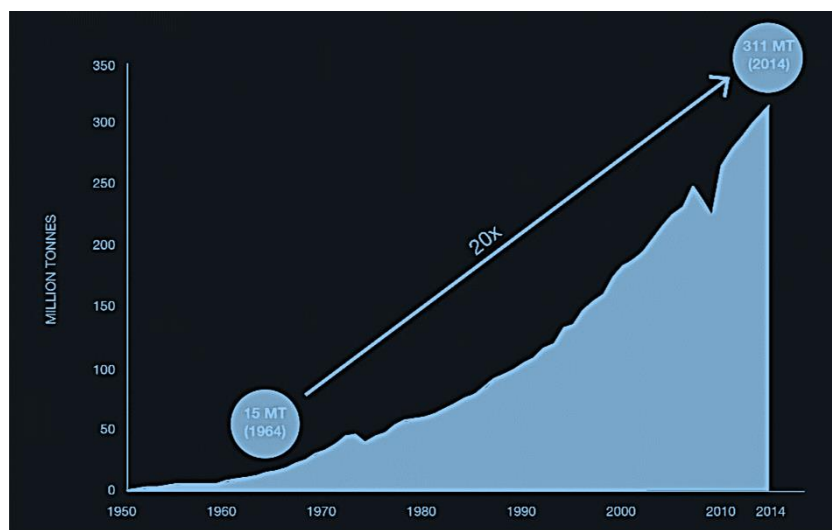


Figure 1.1. Worldwide plastic production between 1950 and 2014 (source: World Economic Forum (2016), adapted from PlasticsEurope (2015))

The consumption of plastics in many European countries indicates that the plastic resins most used since 2007 are low density polyethylene (LDPE) and high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS). Table 1.1 features the different plastic resins and their applications.

Table 1.1. Plastic resins and their applications (adapted from: Pinto (2012))

Types of resins	Characteristics	Applications
Low density polyethylene (LDPE)	Low electrical and thermal conductivity; resistant to chemical action. Many features and mechanical properties are maintained below 60 ° C.	Electronic material, agriculture and construction.
High density polyethylene (HDPE)	High density. Opaque material. Easy to be processed, tougher and with better mechanical properties than LDPE. Resistance to chemicals, but not to strong oxidizing agents.	Packages, electronic material.
Polypropylene (PP)	<u>Homopolymer</u> : electrical and mechanical strength; resistant to high temperatures. <u>Copolymer</u> : transparent, more flexible and resilient than the homopolymer.	Car industry, packages, toys and electronic material.
Polyvinyl chloride (PVC)	Resistant to high temperatures; flexible with much elasticity; resistance to many chemicals; good mechanical strength. High resilience to low temperatures. Easy to be sterilized.	Construction, packages, industrial processes, toys, footwear.

Polystyrene (PS)	<p><u>Crystal</u>: electrical insulating of high molecular weight and low water absorption. Bright, transparent and sensitive to light. Good thermal stability.</p> <p><u>Expanded</u>: high mechanical strength; loses properties at temperatures ≥ 88 °C. Resistant to acids, bases and salts. Flammable. Low adsorption of water.</p> <p><u>High and medium impact</u>: sensitive to UV radiation. Translucent or opaque. Rigid and impact-resistant; not resistant to high temperatures. Thermally stable.</p>	Packages and electronic material.
Polyethylene terephthalate (PET)	Good mechanical strength and lower impact resistance. Impermeable to gases. Water adsorption capacity.	Packages and electronic material.

This production volume (Figure 1.1), coupled with their high durability and low weight, leads to the widespread and accumulation of discarded plastic in landfills and, as litter, in terrestrial and aquatic habitats worldwide (Derraik, 2002; Moore, 2008; Thompson *et al.*, 2004).

1.1. Origin and the presence of plastics in the marine environment

The presence of plastics in the oceans was first reported in 1970, however it drew minimal interest of the scientific community (Andrady, 2011; Fowler, 1987). In the following decades, with the ecological effects of plastics, the subject began to gain scientific notoriety (Fowler, 1987).

The literature indicates the predominance of plastics amongst the marine litter (Gregory & Ryan, 1997). It is not possible to obtain reliable estimates of the amount of plastic debris reaching the marine environment, but the amounts are, however, quite substantial (Derraik, 2002). The production trend or the volume of particular polymer types does not correspond to the pattern of plastic litter observed. In fact, the variety of resin types produced reflects the composition of plastic debris recovered from the marine environment, but there are many social, economic, technical and environmental factors that influence the distribution and composition of plastic litter (Kershaw, 2015).

The major sources of plastic debris in the sea are fishing fleet (Cawthorn, 1989), recreational fishing and boats (UNESCO, 1994). Plastic materials also end up in the marine environment due to marine recreational activities (Pruter, 1987; Wilber, 1987).

Plastic also reaches the sea, as litter, carried by rivers and municipal drainage systems (Williams & Simmons, 1997). The major inputs of plastic litter from land sources occur in densely populated or industrialized areas (Derraik, 2002). It is estimated that with the migration of population to coastal areas, the influx of plastic waste in the ocean increased (Andrady, 2011) and that about 10% of the produced plastics enters the sea (Thompson *et al.*, 2004).

In the sea, these versatile and non-biodegradable polymers are found in the form of larger items (macroplastics), including hulls of boats and fishing nets many meters long, and tiny fragments, micrometres in length (microparticles), and potentially, also at the nano-scale level (Browne *et al.*, 2008; Canesi *et al.*, 2015).

Particles of macroplastic (>1 mm) can be transported thousands of kilometres and contaminate relatively distant locations (Browne *et al.*, 2010). Plastic debris accumulate along strandlines (Thornton & Jackson, 1998), in the open ocean (Shaw & Day, 1994), and on the seafloor (Galgani *et al.*, 2000). Many data suggest that physical factors determine the spatial distribution of plastic, such as wind (Williams & Tudor, 2001), wave-action (Thornton & Jackson, 1998), and density of plastic (Thiel *et al.*, 2003). This last factor will determine whether they float or sink and the role in influencing spatial patterns of accumulation. The polystyrene is the third densest resin among the more common plastics found in the marine environment, with a specific gravity of about $1.04 - 1.09 \text{ g cm}^{-3}$ (Andrady, 2011).

Most plastics are resistant to biodegradation, but will break down gradually through mechanical action, since the mechanical integrity of plastic depends on its molecular weight and, therefore, any significant degree of degradation inevitably weakens the material (Thompson *et al.*, 2004). When exposed to UV-B radiation, to the oxidative properties of the atmosphere and to the hydrolytic properties of seawater, these plastics brittle and break into smaller pieces, until they become microplastics.

Microplastics are defined as particles that are less than 5 mm in diameter, according to the National Oceanic and Atmospheric Administration of the United States of America (NOAA, 2015). Its presence in the ocean comes from a variety of sources and are distributed according to the currents (Lusher, 2015). Their origin can be natural (such as silk or cotton) or synthetic, such as polystyrene (PS) or polyethylene (PE) (Pinto, 2012).

The distinction between primary and secondary microplastics is based on whether the particles were originally manufactured to be that size (primary) or whether they have

resulted from the breakdown of larger items (secondary) (Kershaw, 2015). The primary source of microplastics may include polyethylene (PE), polypropylene (PP) and polystyrene (PS) from cleaning products or cosmetics (Fendall & Sewell, 2009), or from industries or industrial effluents (Lusher, 2015).

The secondary source is the degradation of plastics under marine conditions, that dramatically reduces the molecular weight of the polymers (Andrady, 2011). Ideally, these particles may also undergo further degradation by microbial action, releasing carbon (Andrady, 2011). The digestion is complete when the carbon present in plastics is converted into CO₂, water and biomass (Andrady, 1994). To date, there is no information on the complete mineralization of plastic in the marine environment because of the temperature and oxygen concentration (Andrady, 2011).

An exhaustive quantitative description of the relative abundance of microplastic compared to macroplastic debris remains to be accomplished. Although microplastics greatly exceed large plastic items in marine systems, they are still only a small proportion of the total mass of plastics in the ocean. This means that even if we were able to stop the discharge of macroplastics into the sea, the on-going degradation of the larger items already in the sea and on the beaches would result in a sustained increase in microplastics for many years to come (Browne *et al.*, 2010).

Microplastics have become a growing issue in such a way that the Marine Strategy Framework Directive (MSFD N° 2008/56/EC) highlights microplastics and its associated chemicals as one of the major policy descriptors to determine the impact on the marine environment (Zarfl *et al.*, 2011).

In general, microplastics have been documented in most habitats in the open ocean, seas and beaches, in surface waters, in the water column and in the deep ocean (Lusher, 2015), and recently, in freshwater systems (Eerkes-Medrano *et al.*, 2015). In Portugal, 62% of microplastics were identified in the North Atlantic by trawling, with a density of 580 000 particles per km². 61% of the water samples collected in the Portuguese coast contained microplastics, and the concentrations were higher in the Costa Vicentina and Lisbon (0.036 and 0.033 particles m⁻³, respectively) than in the Algarve and Aveiro (0.014 and 0.002 particles per m³, respectively) (Lusher, 2015).

Manufactured nanoparticles are the latest trend of nanotechnology. They are used in a variety of applications including cosmetics, electronics, molecular biology, medicine, between others (Ward & Kach, 2009). Plastic nanoparticles - nanoplastics – are commonly defined as particles of plastic which are less than 100 nm (Koelmans, 2015).

These particles derived from post-consumer waste as well as from meso and microplastics after suffering degradation. However, the question remains about the origin of nanoparticles from the plastics breakdown (Andrady, 2011).

1.2. The impact of plastics in marine organisms

The occurrence of plastic in the ocean and the potential impact to marine organisms are of the growing concern (Canesi *et al.*, 2015). The fact of microplastics have such a small size, actively contributes to its bioavailability and accumulation in organisms of lower trophic classes. Many of them, as they have little selectivity, catch everything that has an appropriate size and may easily be ingested by marine invertebrates, which are the basis of most food chains (Thompson *et al.*, 2004). Therefore, ingestion is the main interaction between marine organisms and microplastics (Lusher, 2015), probably due to confusion with the prey (Andrady, 2011; Moore, 2008). As the particles interact with plankton and sediments, both organisms that feed on suspended particles, and the ones that feed on the bottom, are at risk of, accidentally or selectively, ingest plastic (Lusher, 2015). Plastics were ingested by marine mammals (Laist, 1997), cetaceans (Clapham *et al.*, 1999), birds (Mallory, 2008), sea turtles (Mascarenhas *et al.*, 2004), zooplankton (Cole *et al.*, 2013), larvae and adult fish (Browne *et al.*, 2013; Lusher, 2015; Rochman *et al.*, 2014).

However, the selectivity is related to the size of the affected organisms and the particles they find (Lusher, 2015). Particles with less than 20 μm are likely to be ingested and egested (Lee *et al.*, 2013) by small organisms (Thompson *et al.*, 2004; Wright *et al.*, 2013b). Microplastics with size between 1 and 5 mm can compromise the nutrition and digestion (Codina-García *et al.*, 2013). The ingestion of plastics with a greater size, can cause serious external and internal injuries, ulcers, digestive tract blockage, false sense of fullness, loss of feeding capacity, impairment, inability to avoid predators or death (Gall & Thompson, 2015).

Microplastic ingestion was also documented for a wide range of marine vertebrates and invertebrates. Interactions were recorded from field of wild populations (Table 1.2) and during controlled laboratory studies (Table 1.3), both indicating microplastic ingestion. From ecotoxicological studies involving microplastics and their interactions and effects on aquatic organisms in seawater species, fish are the main group

studied, followed by Malacostraca, Bivalvia, Polychaeta, Mammalia and Echinoidea (Lusher, 2015).

Table 1.2. Evidence of microplastics ingestion in marine organisms

Species	Microplastics (%)	Mean (\pm SD) number of particles/ individual	Type and size (μ m)	Reference
Phylum Arthropoda				
<i>Gammarus pulex</i>	–	–	Acrylic 29.5 \pm 26	Imhof <i>et al.</i> (2013)
<i>Notodromas monacha</i>	–	–	Acrylic 29.5 \pm 26	Imhof <i>et al.</i> (2013)
Phylum Annelida				
<i>Lumbriculus variegatus</i>	–	–	Acrylic 29.5 \pm 26	Imhof <i>et al.</i> (2013)
Phylum Mollusca				
<i>Mytilus edulis</i>	–	3.7 per 10 g tissue	Fibres 300-1000	De Witte <i>et al.</i> (2014)
<i>Mytilus edulis</i>	–	0.36 (\pm 0.07) g ⁻¹	5 - 25	Van Cauwenberghe and Janssen (2014)
<i>Cassostrea gigas</i>	–	0.47 (\pm 0.16) g ⁻¹	5 - 25	Van Cauwenberghe and Janssen (2014)
Phylum Crustacea				
<i>Lepas spp.</i>	33.5	1-30	1.41	Goldstein and Goodwin (2013)
<i>Nephrops norvegicus</i>	83	–	–	Murray and Cowie (2011)
<i>Crangon crangon</i>	–	11.5 fibres per 10 g shrimp	300-1000	Devriese <i>et al.</i> (2015)
Phylum Chaetognatha				
<i>Parasagitta elegans</i>	100	–	0.1-3 PS	Carpenter and Smith (1972)
PS (Polystyrene)				

Whilst it is apparent that microplastics have become widespread and ubiquitous, the information concerning the accumulation, mode of action and biological impacts of this emerging pollutant in marine organisms is still scarce (Wright *et al.* (2013b). Table 1.3 summarizes the information about the ecotoxicological effects of microplastics in several aquatic organisms.

Table 1.3. Effects of microplastics to aquatic organisms

Species	Microplastics		Exposure		Effects	Reference
	Type	Size (µm)	Concentration (particles ml ⁻¹)	Duration		
Phylum Annelida Class Polychaete	Acrylic, alkyd resin, PE, PP, polyvinyl alcohol	20-2000	1.5 g L ⁻¹	–	Ingestion	Thompson <i>et al.</i> (2004)
<i>Arenicola marina</i>	UPVC	130	0-5% by weight	48 hours 24 days	Intake, decrease feed rate, increase phagocytic activity, reduced lipid and energy reserves	Wright <i>et al.</i> (2013a)
	UPVC	230	1500 g of sediment mixture adsorbed with 5% of microparticles	–	Ingestion, oxidative stress	Browne <i>et al.</i> (2013)
	PS	400-1300	0,1,10,100 g L ⁻¹	28 d	Ingestion, decrease feeding rate, weight loss	Besseling <i>et al.</i> (2012)
	PE, PS	(<100)	110 MPs g ⁻¹ of sediment	14 d	Increase EC Increase protein content	Van Cauwenberghe <i>et al.</i> (2015)
	Ficoll – inert polymer	3-10	5 µL ⁻¹	20 min	Ingestion	Bolton and Havenhand (1998)
	PLA, PE, PVC	1.4-707	n.s	31 d	Increase metabolic rate	Green <i>et al.</i> (2016)
Class Clitellata						
<i>Galeolaria caespitosa</i>	PS	3 e 10 fluorescently labeled	635, 2240, 3000	1, 24 h	Ingestion, size selection, egestion	Cole <i>et al.</i> (2013)
<i>Marenzelleria spp.</i>	PS	10	2000	3 h	Ingestion	Setälä <i>et al.</i> (2014)
Phylum Mollusca						
Class Bivalvia						
<i>Mytilus galloprovincialis</i>	PE,PS, PE-PYR e PS_PYR	<100	20 g L ⁻¹	6 d	Adsorption of pyrene not differ between PS and PE; bioaccumulation in digestive tissues and gills	Avio <i>et al.</i> (2015)
<i>Mytilus edulis</i>	PS fluorescently labeled	2	0.51 µg L ⁻¹	12 h	Uptake accumulation in gut; and hemolymph after 3 d	Browne <i>et al.</i> (2008)

		4-16		3 d		
		3 and 9.6				
	PS	10	50	14 d	Greater accumulation of smaller particles; no significant effects on metabolism	Van Cauwenbergh <i>et al.</i> (2015)
		30	50			
		90	10			
			110 ml ⁻¹ (Total concentration)			
	HDPE	0-80	2.5 g L ⁻¹	3, 6, 12, 24, 48 and 96 h	Uptake; retention in gut and transfer into the lymphatic system; immune response	von Moos <i>et al.</i> (2012) Köhler (2010)
	PS Microspheres fluorescently labeled	0.5	50 µg per 400 ml seawater	1 h	Uptake; trophic transfer to <i>Carcinus maenas</i>	Farrell and Nelson (2013)
	PS	10	2 × 10 ⁴	30 min	Intake	Ward and Targett (1989)
	PS	10, 30	3.10 × 10 ⁵	–	Intake	Claessens <i>et al.</i> (2013)
	PS	30 nm	0.1-0.3 g L ⁻¹	8 h	Excretion in pseudofaeces; decrease of feeding rate	Besseling <i>et al.</i> (2012)
<i>Mytilus trossulus</i>	PS	10	1000	–	Intake	Ward <i>et al.</i> (2003)
<i>Crassostrea virginica</i>	PS	10	1000	45 min	Intake and egestion	Ward and Kach (2009)
<i>Crassostrea gigas</i>	PS	2, 6 µm	0.023 mg L ⁻¹	2 month	Decreases in oocyte number, diameter, and sperm velocity; decrease of larval development; endocrine disruption	Sussarellu <i>et al.</i> (2016)
<i>Placopecten magellanicus</i>	PS	15, 10, 16, 18, 20	1.05	1 h	Intake, retention and egestion	Brillant and MacDonald (2002)
Phylum Echinodermata						
Class Holothuridea						
<i>Apostichopus californicus</i>	PS	10, 20 µm	2.4 µl ⁻¹	-	Intake, retention	Hart (1991)

<i>Thyonella gemmata</i>			10 g PVC, 60 g resin			
<i>Holothuria grisea</i> , <i>Holothuria floridana</i> , <i>Cucumaria frondosa</i>	PVC, nylon, resin pellets	0.25-15 mm	2 g nylon line added to 600 ml of silica sand	20 - 25 h	Selective intake	Graham and Thompson (2009)
Class Echinoidea						
<i>Tripneustes gratilla</i>	PE fluorescently labeled	32-35	1,10,100,300	5 d	Intake and egestion	Kaposi <i>et al.</i> (2014)
<i>Dendraster excentricus</i>	PS	10, 20	2.4 μL^{-1}	–	Intake and egestion	Hart (1991)
<i>Strongylocentrotus sp.</i>	PS	10, 20	2.4 μL^{-1}	–	Intake and egestion	Hart (1991)
<i>Lytechinus variegatus</i>	PE	n.s.	250 ml of MPs L^{-1}	24 h	Anomalous larvae development	Nobre <i>et al.</i> (2015)
<i>Paracentrotus lividus</i>	PS	0.04-0.05	2.61-50 $\mu\text{g ml}^{-1}$	48 h	Increase of MPs accumulation Increase Abcb1 gene Increase cas8 gene	Della Torre <i>et al.</i> (2014)
Class Ophiuroidea						
<i>Ophiopholis aculeata</i>	PS	10, 20	2.4 μL^{-1}	–	Intake and egestion	Hart (1991)
Class Asteroidea						
<i>Dermasterias imbricata</i>	PS	10, 20	2.4 μL^{-1}	–	Intake and egestion	Hart (1991)
Phylum Arthropoda						
Subphylum Crustacea						
Class Maxillopoda						
<i>Semibalanus balanoides</i>	Synthetic polymers	20-2000	1 g L^{-1}	–	Intake	Thompson <i>et al.</i> (2004)
Subclass Copepoda						
<i>Tigriopus japonicus</i>	PS	0.05 0.5	9.1 x 10^{11} 9.1 x 10^8	24 h	Intake, egestion, mortality, reduced fertility rate	Lee <i>et al.</i> (2013)
<i>Acartia tonsa</i>	Plastic particles	6 10-70	5.25 x 10^5 3000-4000	15 min	Intake, particle size selection	Wilson (1973)
<i>Acartia spp.</i>	PS	10	2000	3 h	Intake	Setälä <i>et al.</i> (2014)
<i>Eurytemora affinis</i>	PS	10	1000, 2000, 10000	3 h	Intake and egestion	Setälä <i>et al.</i> (2014)

<i>Limnocalanus macrurus</i>	PS	10	1000, 2000, 10000	3 h	Intake	Setälä <i>et al.</i> (2014)
<i>Temora longicornis</i>	PS fluorescently labeled	20	100	12 h	Ingestion 10.7± 2.5 particles per organism	Cole <i>et al.</i> (2014)
<i>Calanus helgolandicus</i>	PS	20	75	24 h	Intake and egestion	Cole <i>et al.</i> (2015)
Class Branchipoda						
<i>Daphnia magna</i>	Acrylic	29.5 ± 26 µm	–	–	Intake	Imhof <i>et al.</i> (2013)
Class Malacostraca						
<i>Orchestia gammarellus</i>	Acrylic, alkyd resin, PE, PP, polyvinyl alcohol	20-2000	1 g per organism (n=150)	–	Intake	Thompson <i>et al.</i> (2004)
<i>Talitrus saltator</i>	PE	10-45	10% per dry weight food	24 h	Intake, egestion after 2 h	Ugolini <i>et al.</i> (2013)
<i>Allorchestes compressa</i>	LDPE	11-700	0.1 g/L	72 h	Intake, egestion after 36 h	Chua <i>et al.</i> (2014)
<i>Neomysis integer</i>	PS	10	2000	3 h	Intake	Setälä <i>et al.</i> (2014)
<i>Mysis relicta</i>	PS	10	2000	3 h	Intake and egestion	Setälä <i>et al.</i> (2014)
<i>Carcinus maenas</i>	PS fluorescently labeled	8 – 10	4 x 10 ⁴	24 h, 21 d	Uptake by gills and mouth, retention and excretion	Watts <i>et al.</i> (2014)
	PP fibres	500	0, 0.6, 1.2, 2.0 mg added to 2 g of food	4 weeks	Decrease intake of food over time; growth rate reduction; microfibers accumulation in digestive tract - changing of microfibers properties by passing the digestive tract	Watts <i>et al.</i> (2015)
<i>Nephrops norvegicus</i>	PP microfibers	5 mm	10 fibres per 1 cm ³ of organism	–	Intake	Murray and Cowie (2011)
Class Branchiopoda						
<i>Bosmina coregoni</i>	PS	10	2000, 10000	3 h	Intake	Setälä <i>et al.</i> (2014)
Phylum Chordata						
<i>Pomatoschistus microps</i>		1-5	18.4, 184 µg L ⁻¹	96 h	Intake, modulation of bioavailability and biotransformation of pyrene, decrease of energy, inhibition of AChE activity	Oliveira <i>et al.</i> (2013)

	PE	1-5	18.4, 184 $\mu\text{g L}^{-1}$	96 h	Intake, modulation of bioavailability and biotransformation of pyrene, decrease of energy, inhibition of AChE activity	Oliveira <i>et al.</i> (2013)
		420-500	30 particles per 300 ml water	96 h	Intake (confusion with food– <i>Artemia nauplii</i>)	de Sá <i>et al.</i> (2015)
	PE	1-5	0.184 mg L^{-1}	96 h	Inhibition of AChE activity	Luís <i>et al.</i> (2015)
<i>Gadus morhua</i>	Plastic particles	2, 5 mm	–	–	Intake, egestion, particles of 5 mm for a long period. Emptying of plastics improved by giving additional meals.	Dos Santos and Jobling (1992)
<i>Sfelliifer brasiliensis</i>	Nylon	–	–	–	Intake (higher in adults than larvae) ; presence of biofilms in microplastics	Dantas <i>et al.</i> (2012)
<i>Cathorops spixii</i> , <i>Cathorops agasizii</i> , <i>Sciades herzbergii</i>	Nylon	–	–	–	Intake by 17 to 33% of total organisms. Selective intake according to size	Possatto <i>et al.</i> (2011)
<i>Eugerres brasiliensis</i> , <i>Eucinostomus melanopterus</i> , <i>diapterus rhombeus</i>	Nylon	1-5 mm	–	–	Intake by 4.9 to 33.4 % of total organisms. Selective intake according to size	Ramos <i>et al.</i> (2012)
<i>Gobio gobio</i>	Fibers and plastic pellets	–	–	–	Intake by 12 of total organisms. 7 in 11 fishes had microplastics in the system.	Dris <i>et al.</i> (2015)
<i>Oryzias latipes</i>	PE	3 mm	10% particles per weight	2 months chronic exposure	Altered gene expression, decreased choriogenin regulation in males and decreased vitellogenin and choriogenin in females	Rochman <i>et al.</i> (2014)
	LDPE	3 mm	10% particles per weight	2 months chronic exposure	Toxicity in the liver, pathologies, hepatic stress	Rochman <i>et al.</i> (2013)
<i>Dicentrarchus labrax</i>	PE	10-45	10-100 MPs mg^{-1} of diet	36 d	Increase mortality Increase CYP P450	Mazurais <i>et al.</i> (2015)

PS (Polystyrene) **PE** (Polyethylene) **PP** (Polypropylene) **LDPE** (Low Density Polyethylene) **HDPE** (High-density Polyethylene) **UPVC** (Polyvinyl chloride) **PE-PYR** (Pyrene Treated Polyethylene) **PS_PYR** (Pyrene Treated Polystyrene)

1.3. Microplastics as contaminants to marine organisms

In addition to the direct physical impact caused by the intake of microplastics, they can be a vehicle for accumulation and transfer of other pollutants (Browne *et al.*, 2013). These chemicals are divided into two groups: the first comprises the additives, monomers and by-products of the molecules component of the plastics, and the second,

hydrophobic chemicals that are adsorbed from surrounding seawater, due to affinity of those chemicals for the hydrophobic surface of the plastics (Teuten *et al.*, 2009). This includes persistent organic pollutants (POPs) and bioaccumulative and toxic substances (Browne *et al.*, 2013; Engler, 2012), including polychlorinated biphenyls (PBTs), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT), polycyclic aromatic hydrocarbons (PAHs) and the other petroleum hydrocarbons (Chua *et al.*, 2014; Mato *et al.*, 2001; Rios *et al.*, 2007; Teuten *et al.*, 2009). Marine microplastics can also be covered with biofilm communities that act like a reservoir for POPs. In this case, there is a great probability of transfer of these chemicals along with the microplastics to marine organisms (Eerkes-Medrano *et al.*, 2015), especially to lower trophic levels (Arnot & Gobas, 2004).

With the increasing microplastic contamination in marine ecosystems, large concentrations of microplastics and additives can harm ecophysiological functions performed by organisms (Browne *et al.*, 2013). Ingestion of contaminated microplastics and bioaccumulation of sorbed chemicals have been documented in several organisms. In fish (Rochman *et al.*, 2013) where, when exposed to a mixture of polyethylene with chemical pollutants sorbed from the marine environment, they bioaccumulate and suffer liver toxicity and pathology. Lugworms were also affected (Browne *et al.*, 2013), by the uptake of Triclosan from PVC, which reduced the ability of worms to engineer sediments and cause mortality. The marine amphipod *Allorchestes compressa* assimilated PBDEs, derived from microplastics, into the tissues (Chua *et al.*, 2014). Avio *et al.* (2015) observed PAH accumulation in digestive tissues, hemolymph and gills of *M. galloprovincialis*.

If marine organisms ingest microparticles, it is possible that microplastics-associated POPs and other additive chemicals are delivered to different tissue types and locations (Zettler *et al.*, 2013) although, until to date, this was not totally clarified (Browne *et al.*, 2013; Chua *et al.*, 2014).

1.4. Biomarkers

In bivalves, the microplastics uptake relates directly to their feeding strategy, since they are filter feeders. The microparticles are inert and, after ingestion, pass through the cell membrane and are incorporated into tissues, particularly in the gut cavity (Wright *et al.*, 2013b). After passing to the circulatory system, microplastics are transferred to other

organs, via hemolymph, where they can be retained for several weeks, accumulate in several organs and cause adverse effects (Browne *et al.*, 2008). Exposure experiments demonstrated significant biological effects including weight loss, reduced feeding activity, increased phagocytic activity, transference to the lysosomal system and inhibition of AChE activity (Lusher, 2015). Microplastics accumulation can also be transferred to higher trophic levels (Wright *et al.*, 2013b).

To clarify any effects of exposure to microplastics, a set of biomarkers are employed: the analysis of DNA damage, the activities of antioxidant enzymes that enable to maintain the cellular integrity (SOD, CAT, GPx), and glutathione S-transferases (GST) participating in biotransformation and protection against oxidative stress, the activity of the enzyme acetylcholinesterase (AChE) involved in neuro and neuromuscular transmission, and the levels of lipid peroxidation (LPO) as indicative of oxidative damage in lipids.

1.5. Oxidative stress

Most of the oxygen consumed by the animals is reduced to water along with oxidation of food intake and energy production. It is estimated that about 1 to 3% of oxygen consumed by animals is converted into ROS. These compounds are continuously produced due to the presence of xenobiotics/contaminants, mainly as "unwanted bi-products" of the biotransformation from endogenous processes, which include self-oxidation of heme proteins and electrons nuclear transport (de Zwart *et al.*, 1999; Livingstone, 2001).

Even without pollution and stress factors, the cellular metabolism of aerobic organisms involves the production of reactive oxygen species (ROS), that include the anion superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), the peroxy radical, the hidroperoxil radical, hypochlorous acid and peroxynitrite (considered by some as a reactive nitrogen species – RNS) (Halliwell & Gutteridge, 1999; Kappus, 1987; Valavanidis *et al.*, 2006). These ROS are essential for the physiological control of cell functions in biological systems (Halliwell & Gutteridge, 1999).

Organisms have developed non-enzymatic and enzymatic antioxidant defence mechanisms to prevent and eliminate the effects of ROS, and repair of oxidized component systems. Aquatic organisms in the presence of a stressor agent can induce the

production of ROS, which can cause oxidative damage, indicative of toxicity (Livingstone, 2001).

The impact of plastic ingestion (stressor agent) in marine organisms include oxidative stress, and thus, oxidative damage (Table 1.3) (Browne *et al.*, 2013; Galloway & Lewis, 2016; Luís *et al.*, 2015; Oliveira *et al.*, 2012; Oliveira *et al.*, 2013).

When there is an imbalance between the production of ROS and the detoxification of biological systems, oxidative stress occurs (Valavanidis *et al.*, 2006).

Due to the high reactivity of ROS, most components of cellular structures and function can be targets of oxidative damage (Kappus, 1987). ROS production associated with exposure to contaminants can inhibit the activity of antioxidant defences, leading to oxidation of essential cellular components such as proteins, DNA, carbohydrates and lipids, in the tissues of exposed organisms (Halliwell & Gutteridge, 1999; Shi *et al.*, 2005) (Figure 1.2).

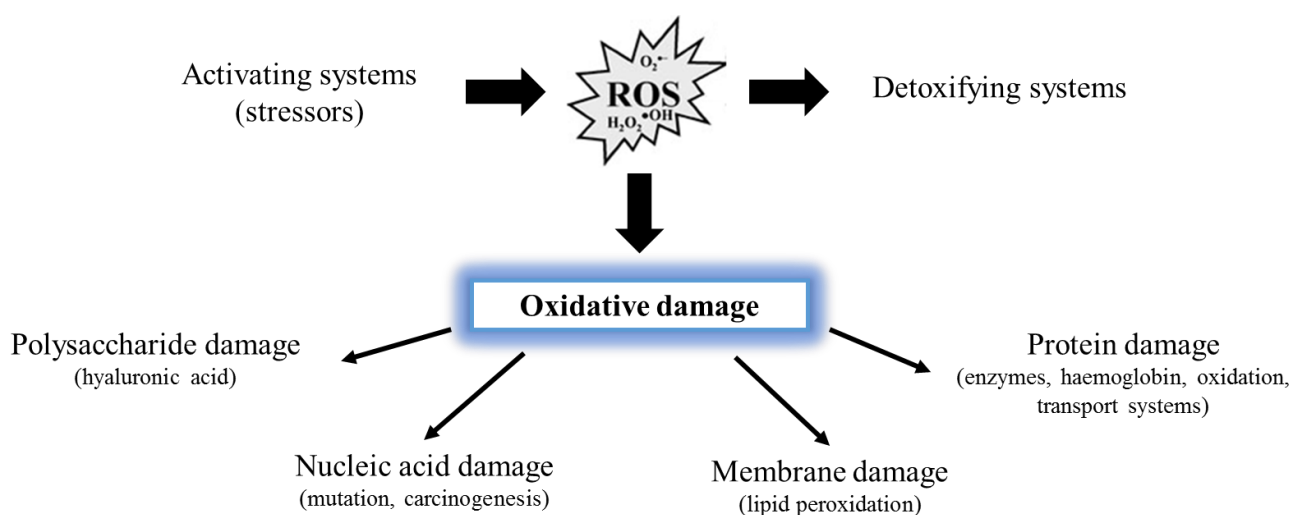


Figure 1.2. Generalized scheme for oxidative damage to macromolecules of living organisms (adapted from: Lackner (1998))

1.5.1. Antioxidant enzymes

The production of ROS needs to be balanced through enzymatic and non-enzymatic antioxidant defences. In aerobic organisms, intracellular antioxidant enzymes are responsible for the neutralization of ROS (Fenech & Ferguson, 2001).

The use of antioxidant enzymes as biomarkers have been widely applied in toxicity studies with aquatic organisms (e.g. Bebianno *et al.*, 2014, Silva *et al.*, 2012) to evaluate the effects caused by a contaminant. In figure 1.3 there is an explanatory diagram

of the mechanism of action of each enzyme, and how they act in a coordinated fashion to be effective in the ROS removal.

Superoxide dismutase (SOD) catalyses the dismutation of the radical superoxide (a major reactive oxygen species) to hydrogen peroxide. This enzyme occurs in the cytoplasm and mitochondria of cells (Halliwell & Gutteridge, 1999).

Catalase (CAT) is located in the peroxisomes, and decomposes the hydrogen peroxide into water and oxygen, being also involved in the metabolism of fatty acids. (Halliwell & Gutteridge, 1999).

The activity of glutathione peroxidases (GPx) catalyses the levels of hydrogen peroxide (H_2O_2) and lipid hydroperoxides (Júnior *et al.*, 2001). Glutathione reductase (GR) does not directly act in the removal of ROS, being responsible for the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), continuing the action of glutathione peroxidases and glutathione S-transferases (Halliwell & Gutteridge, 1999).

The superfamily of glutathione S-transferases (GST) comprises eukaryotic and prokaryotic phase II metabolic isozymes. Most of them catalyses the conjugation of GSH (reduced glutathione) with xenobiotic substrates for the purpose of detoxification (Ioannides, 2002). The GST catalyses the reduction of lipid peroxides and so it is, therefore, important in preventing the oxidative damage (Zanette *et al.*, 2011; Zhao *et al.*, 1999), also having the function of antioxidant enzyme.

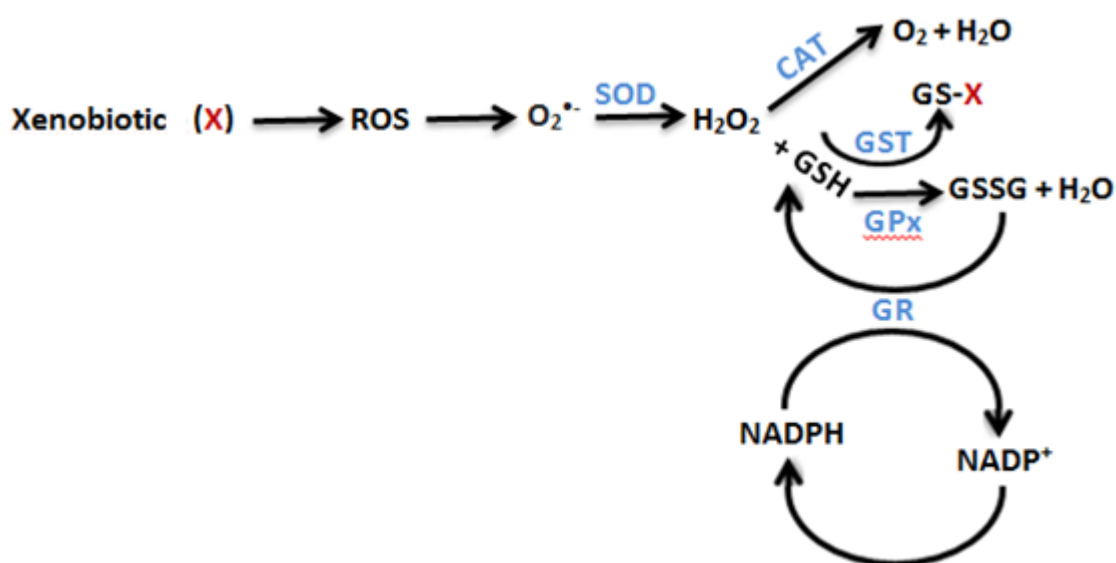


Figure 1.3. Representative scheme of the action of antioxidant enzymes (SOD, CAT, GPx and GR) and GST

1.6. DNA damage

It is known that superoxide radicals directly or indirectly damage DNA, resulting in strand scission and chromosome breakage (Brawn & Fridovich, 1981).

DNA alterations in aquatic organisms have proved to be a very suitable method to evaluate the genotoxic contamination of the environment, allowing the detection of effects after exposure to low concentrations of contaminants, in a variety of aquatic species (Frenzilli *et al.*, 2009; Nacci *et al.*, 1996), such as vertebrates *Salmo trutta fario* (Belpaeme *et al.*, 1996) and *Cyprinus carpio* (Pandrangi *et al.*, 1995)). The comet assay is more sensitive than other methods commonly used in genetic ecotoxicology (Frenzilli *et al.*, 2009) and has been successfully used in invertebrates namely: *Nereis diversicolor* (Catalano *et al.*, 2012; Maranho *et al.*, 2014), including bivalve molluscs (Jha, 2008): *Mytilus galloprovincialis* (Gomes *et al.*, 2013), *Scrobicularia plana* (Petridis *et al.*, 2009), *Perna viridis* (Siu *et al.*, 2004), among others.

Given that, until today, studies with nanoparticles and quantum dots evidence DNA damage using the Comet assay (Gomes *et al.*, 2013; Rocha *et al.*, 2014), the possible genotoxicity of microplastics is an important topic of research, alongside the development of a robust assay that can be used for general screening of anthropogenic impacts.

1.7. Neurotoxicity

Animals are extremely sensitive to environmental contamination, that may affect their neurological and behavioural activities (Costa, 1996; Døving, 1991; Silbergeld, 1993). The main role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh) into choline and acetic acid (Lackner, 1998; Lionetto *et al.*, 2003).

Its inhibition is directly linked with the mechanisms of toxic action of pollutants (Hernández *et al.*, 1998). Even at low concentrations, these compounds can inhibit AChE, which leads to accumulation of acetylcholine at central cholinergic synapses and at vertebrate neuromuscular junctions (Høy *et al.*, 1991; Sancho *et al.*, 1997). As a result, these disturbances can affect locomotion and equilibrium in exposed organisms (Bretaud *et al.*, 2000).

In aquatic organisms there is considerable diversity in the biochemical properties and distribution of AChE as well as in their sensitivity to anticholinesterase agents (Habig & Di Giulio, 1991). Therefore, measurements of acetylcholinesterase activity has been routinely used as a biomarker of exposure to certain groups of contaminants that have the potential to inhibit AChE such as: organophosphate and carbamate insecticides (Grue *et al.*, 1997; Williams & Sova, 1966), pesticides (Davies *et al.*, 1994), herbicides (dos Santos Miron *et al.*, 2005), metals (Garcia *et al.*, 2000; Gill *et al.*, 1990), pharmaceuticals (Luís *et al.*, 2015), between others.

More recently, the acetylcholinesterase activity has also been used to infer the effects of microplastics on cholinergic, neurological and neuromuscular transmission (Avio *et al.*, 2015; dos Santos Norberto, 2014; Ferreira *et al.*, 2016; Oliveira *et al.*, 2012; Oliveira *et al.*, 2013).

1.8. Lipid peroxidation

The most typical reaction induced by reactive oxygen species implies the peroxidation of unsaturated fatty acids (Kappus, 1987). The reaction sequence starts with a radical (e.g., OH^\bullet) which removes one proton from the hydrocarbon tail of the fatty acid and leaves the radical of the acid. This radical experiences isomerization and oxidation with molecular oxygen, producing a peroxy radical of the fatty acid. In turn, peroxy radicals remove protons from other molecules and become hydroperoxides. Since these protons may be from another fatty acid, a new cycle is started. Therefore, lipid peroxidation proceeds via a chain reaction until the chain is interrupted, by either the dimerization of two radicals, or until the proton is removed from a substance which forms relatively stable radicals (radical scavengers). Through this chain reaction, only one initiating radical may lead to the peroxidation of hundreds of fatty acids (Lackner, 1998). The resulting hydroperoxides are unstable and decompose by chain cleavage to a very complex mixture of aldehydes, ketones, alkanes, carboxylic acids and polymerisation products (Esterbauer *et al.*, 1982).

The only mechanism that produces malondialdehyde in biological systems is lipid peroxidation. Malondialdehyde is not the major product of lipid peroxidation however, it is a typical degradation product. This fact coupled to the high sensitivity of the

thiobarbituric acid test (described in materials and methods section), have greatly inspired reactive oxygen species research (Lackner, 1998).

Therefore, LPO was found to be suitable as a biomarker of effect (Ahmad *et al.*, 2008; Lackner, 1998; Livingstone, 2001).

1.9. *Scrobicularia plana* characterization

Invertebrates are a very large and diverse group of organisms. They are very useful in monitoring studies with special emphasis on sessile individuals (Dixon *et al.*, 2002). Within this group there is the Bivalvia Class, Phylum Mollusca (Table 1.4), which is composed of approximately 15 000 species, most of them marine (Campbell *et al.*, 1994), including the *Scrobicularia plana* (da Costa, 1778).

Table 1.4. Scientific classification of *Scrobicularia plana* (Source: WoRMS <http://www.marinespecies.org>)

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia
Subclass	Heterodonta
Infraclass	Euheterodonta
Superorder	Imparidentia
Order	Cardiida
Superfamily	Tellinoidea
Family	Semelidae
Genus	<i>Scrobicularia</i>
Species	<i>Scrobicularia plana</i>

S. plana has an oval, flat shell with an exterior pale yellow grey colour and white in the interior (Figure 1.4). The size vary between 4 to 6 cm length with an outer surface with concentric lines (Campbell *et al.*, 1994). Figure 1.5 shows the internal appearance of *S.plana*.

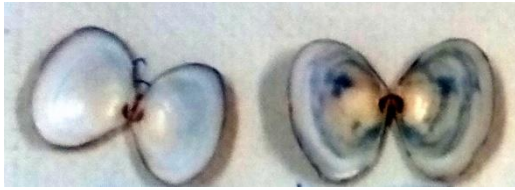


Figure 04. Specimens of *S. plana*. **A** – Interior of the shell; **B**- outer surface of the shell



Figure 1.5. Internal appearance of *S. plana*

S. plana is gonochoristic, and sexual maturity occurs 2 to 3 years after settlement, corresponding to a shell length greater than 20 mm (Hughes, 1970; Rodríguez-Rúa *et al.*, 2003). Regarding the reproduction cycle, *S. plana* development of gonads occurs from the beginning of February until the end of October, the spawning season is from March to September, and the maximum spawning peak usually occurs in the second half of May and July. October to January represents the inactive reproductive period, during which about more than half of the total population is not sexually determined. This reproductive cycle is influenced by environmental factors, such as water temperature and chlorophyll availability (Rodríguez-Rúa *et al.*, 2003).

S. plana inhabits the intertidal zone of estuarine muds (Green, 1957), where there is abundance of organic detritus and most pollutants are present (Wootton & Pipe, 2003). This bivalve species is the most representative species of the Atlantic and the Mediterranean coasts. It has a wide geographical distribution: from Norway (in the north) to Senegal (in the south) in the Atlantic ocean, and in all of the Mediterranean, except the Black Sea (Campbell *et al.*, 1994; Parenzan, 1974) (Figure 1.6). Its economic and commercial interest has increased in recent years (FAO, 2014; González De Canales *et al.*, 2009)

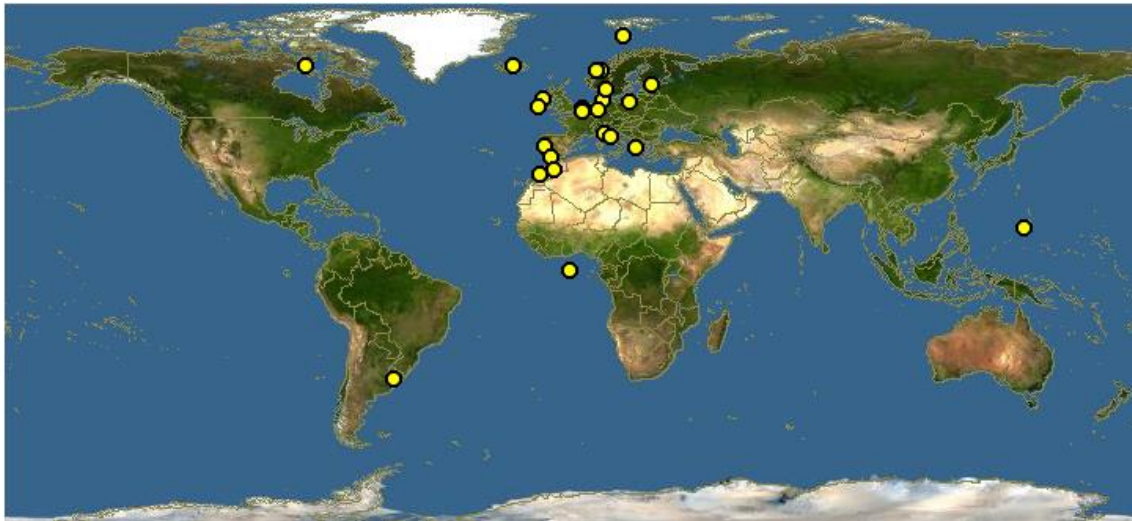


Figure 1.6. World distribution of *Scrobicularia plana* (Source: Discover life <http://www.discoverlife.org/>)

S. plana is a burrowing deposit-feeding bivalve with a filtration efficiency approaching 100% for particles of 4-40 μm , and much of the filtered material is ingested (Hughes, 1969).

1.9.1. *S. plana* as a bioindicator of environmental contamination

Sentinel species have been used to define the status and the evolution of the quality of the marine environment (Viarengo & Canesi, 1991). Several features make bivalves particularly important as sentinel species: they are sessile, filter-feeders and accumulate particles from the water, allowing to measure stress in their tissues (Canesi *et al.*, 2012). They are resistant to a variety of contaminants and environmental factors (such as temperature or salinity) having the ability to survive in highly stressful environments. They are easily collected and easily maintained in the laboratory. Since they aggregate in large populations, it is possible to repeat sampling over a given period and evaluate the environmental contamination in a given area. As these bivalves have a worldwide distribution (both fresh and salty water), and there is enough information regarding its biology and response to environmental conditions (Canesi *et al.*, 2012; Rocha *et al.*, 2015), the results obtained in experimental studies can be compared.

This bivalve, in direct contact with sediments, through physical contact and ingestion of sediment particles, is a suitable indicator of sediment-associated contaminants (Mouneyrac *et al.*, 2008). Moreover, *S. plana* forms an important part of

the diet of wading birds, crabs and benthic fish (Hughes, 1970) and if contaminants are available, they can be transferred through the food chain.

Concluding, its wide geographic distribution, high tolerance of exposure to contaminants, the type of sedentary life, its low metabolism, the commercial importance, and increasing knowledge about the species, defined it as an excellent candidate to be used in monitoring studies of aquatic ecosystems (Solé *et al.*, 2009).

1.10. Objectives

Since the microplastics mode of action and biological risk are not yet clear, the aim of this study is to investigate the accumulation and mode of action of the polystyrene microparticles in the different tissues of the peppery furrow shell *S. plana* and assess the potential ecotoxicological risk of this emerging contaminant. Polystyrene (PS) is one of the most largely used plastics worldwide, it is found in the oceans as micro and nano debris and has a considerable impact in marine species, such as bivalves.

The effects of microplastics accumulation in gills and digestive gland of *S. plana* will be evaluated using a battery of biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxidases), glutathione-S-transferases, genotoxicity (comet assay to evaluate DNA damage), neurotoxicity (acetylcholinesterase activity) and oxidative damage (lipid peroxidation), considered the most appropriate to assess microplastic effects.

The aim of this work was to answer the following specific questions:

1. Do microplastics accumulate in *S. plana* tissues?
2. Do microplastics have the potential to induce cellular oxidative stress and/or neurotoxicity in *S. plana*?
3. Are plastic particles responsible for DNA damage in the cells of *S. plana*?
4. Can this species be a good sentinel to assess the effects of microplastics in marine organisms?

CHAPTER 2. MATERIALS AND METHODS

2.1. Microplastics characterization

Monodisperse PS microplastics were obtained from Sigma-Aldrich (Germany) with the particle size 20 μm and density 1.05 g cm^{-3} . A stock solution (100 mg L^{-1}) was prepared in ultrapure water ($18 \text{ M}\Omega/\text{cm}$), and before every water renewal, sonicated for 30 minutes (Ultrasonic bath VWR International, 230 V, 200 W, 45 kHz frequency), and kept in constant shaking before the addition to the tanks. Another solution of 100 mg L^{-1} of polystyrene microplastics was prepared in natural seawater ($S = 35$), and sonicated for 15 minutes immediately prior to analysis

The microplastic size was determined by optical microscopy (OM) and dynamic light scattering (DLS), and the surface charge (zeta potential) by electrophoretic light scattering (ELS). Zeta potential values of the microparticles were determined by electrophoresis mobility measurements at 25°C using a DLS particle sizer (ZetaSizer Nano ZS90, Malvern Inc.) in a disposable polycarbonate capillary cell (DTS1061). Malvern particle-size analyses was carried out, in a Malvern Mastersizer instrument using ultrapure water ($18 \text{ M}\Omega/\text{cm}$) with 1 g of an antifloculating/dispersive agent (sodium hexametaphosphate) per litre, added to deflocculate clay minerals.

2.2. Sedimentation rate

The sedimentation rate (SR) of 1 mg L^{-1} of microplastics was measured by the change of turbidity with time (0 -24 h), as described in Sousa and Teixeira (2013). The SR relates to the normalized microparticle turbidity C/C_0 , where C is the turbidity at time t and C_0 the initial turbidity at time 0. The SR is faster during the first two hours, which represents a decrease of 5%, and slows down after this period of time. Then, the SR is given by the expression $\delta(C/C_0)/\delta t$, estimated from the decrease in turbidity (C/C_0), which occurred within the first two hours for the fast sedimentation (fast SR) conditions and within 3-24 h for slow sedimentation (slow SR) conditions (Keller *et al.*, 2010).

2.3. Laboratory assay

S. plana (38 ± 5 mm shell length) were collected in Cabanas de Tavira, Ribeira do Almargem (N 37°7'59.75 " W 7 36'34.95 ") and transferred to the laboratory, where they were acclimated during 7 days at constant aeration and maintained at 19 °C, with a photoperiod of 12h light and 12h darkness. Clams were inserted in 25 liters glass aquaria (3 clams/L) filled with 20 L of natural seawater ($S = 35$). Clams were divided into two groups: control and exposed to 1 mg L^{-1} of PS microplastics for 14 days in a triplicate design, followed by 7 days of depuration (Figure 2.1). The water was changed every 24 hours with subsequent addition of PS microplastics from the polystyrene stock microplastics solution (prepared in ultrapure water ($18 \text{ M}\Omega/\text{cm}$) and sonicated for 30 minutes (Ultrasonic bath VWR International, 230 V, 200 W, 45 kHz frequency), before renewal. During the experiment abiotic parameters were checked in all tanks by measuring temperature ($18.0 \text{ }^{\circ}\text{C}$), salinity (35.2), percentage of oxygen saturation (93.0 %) and pH (7.8), with the multiparametric probe TRIPOD (from PONSEL). Clams were only fed with natural seawater to avoid any interaction of microplastics and food. Unexposed and exposed clams were collected after 0, 3, 7 and 14 days of exposure, and after the 7 days of depuration (Figure 2.1). Gills, digestive gland, and remaining tissues (mantle, foot, and adductor muscles) were dissected and stored at $-80 \text{ }^{\circ}\text{C}$, until future analysis and the hemolymph was collected, at each sampling time, from the posterior adductor muscle of the *S. plana* with a sterile hypodermic syringe 1 ml (12 mm x 12:33). No significant mortality was observed between treatments, at the end of the accumulation and depuration period ($p > 0.05$).

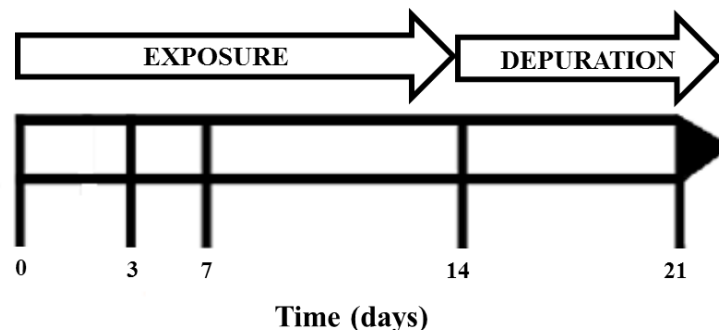


Figure 2.1. Representative scheme of the experiment. Exposure of 100 mg L^{-1} of PS microplastics for 14 days and 7 days depuration

2.4. Condition index

To assess the physiological status of control and exposed clams to PS microplastics, soft tissues and shells were weighted, and the condition index (CI) was determined as the percentage (%) of the ratio between drained weight of the soft tissues (g) and total weight (g), according to Gomes *et al.* (2013).

2.5. Tissue preparation for microplastics accumulation

To confirm if the microplastics entered the organism by the inhalant siphon, and then transported to other organs, frozen aliquots of hemolymph of clams exposed to PS microplastics for 14 days, were observed under an optical microscope.

Furthermore, gills and digestive gland collected at different times of exposure were lyophilized at -40 °C, during approximately 48 hours, with a Modulyo freeze dryer prior to DRIFT analysis.

2.5.1. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT)

For the DRIFT spectroscopic analysis, the lyophilized samples were diluted (~1:2) in KBr (from Aldrich, FTIR grade) and grinded in an agate mortar. After, this mixture was placed in a 11 mm diameter sample cup and manually pressed to obtain a very smoothed surface.

The infrared analysis was made using a Fourier transform spectrophotometer Mattson RS1, with a wide band MCT (mercury cadmium telluride) detector, in the range 400-4000 cm^{-1} with 4 cm^{-1} resolution. Since the samples were solid, the analysis was performed in Diffuse Reflectance mode (DRIFT). The DRIFT spectra result from the ratio of 500 single-beam scans of the sample to the same number of background scans for pure KBr.

The diffusely reflected radiation, used in this infrared analysis mode, is all the light reflected from the sample with an angle of reflection different from the angle of incidence. In fact, the reflected radiation with the same angle as the incidence radiation (called specular reflection) can be a problem and must be avoided, so the accessory used,

a Graseby/Specac Selector, is the adequate to collect all the radiation reflected diffusely except the specular.

The spectra, in reflectance units (R_∞) were transformed into Kubelka-Munk units ($f(R_\infty)$) using the FIRST software according to the two parameters Kubelka-Munk equation (Mitchell, 1993; Stuart, 2005):

$$f(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty}$$

The utilization of the Kubelka-Munk equation (also designated as remission function) implies that no light is transmitted, which means that all the light must be reflected or absorbed. Therefore, the value of reflectance measured, R_∞ , corresponds to the absolute reflectance of an "infinitely thick" sample. The infinitely thick condition is generally fulfilled for samples that are 3-5 mm thick, experimentally corresponding to the sample cup deep.

No other mathematical treatment of the spectra was made, such as, baseline correction or smooth.

2.6. Tissue preparation for biomarker analysis

Previous to analysis of the enzymatic activities the tissues (gills and digestive glands) of control and microplastic exposed clams were weighed and rapidly buffered in Tris-HCl buffer (50 mM Tris-HCl, 250 mM Sucrose, 5mM $MgCl_2$, 1mM DTT, pH=7.6) (the tissue-to-buffer ratio was 1:3 wet weight tissue/volume of buffer), homogenized in an ice bath and centrifuged at 10000 g, for 10 minutes, at 4 °C, using a biofuge stratus 230 V centrifuge (Thermo scientific, Germany). Both the cytosolic and mitochondrial fractions were stored at -80 °C for future analysis.

2.7. Total protein concentrations

For normalizing biomarker results, total protein concentrations were determined in the cytosolic fraction following the Bradford method (Bradford, 1976). Bovin Serum Albumin (Sigma-Aldrich) was used as a standard solution (1 mg ml⁻¹). A blank and six standards were prepared (0.005, 0.01, 0.025, 0.05, 0.1 and 0.5 BSA mg ml⁻¹). The method

involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The reagent is reduced in proportion to the amount of protein present, resulting in a change of colour, from red to blue, whose absorbance is measured at 595 nm using a microplate reader. Protein concentrations are expressed as mg per g of wet weight tissue.

2.7.1. Superoxide dismutase (SOD)

SOD activity was determined in the cytosolic fraction by the percentage of inhibition of the absorbance at 550 nm (McCord & Fridovich, 1969). Results are expressed in units (U), where 1 U of activity corresponds to the amount of sample needed to cause 50% of inhibition of the reduction of cytochrome *c* by the xanthine oxidase/hypoxanthine system. Results are expressed as U mg⁻¹ protein.

2.7.2. Catalase (CAT)

CAT activity was determined by measuring the consumption of hydrogen peroxide (H₂O₂) at 240 nm, with a molar extinction coefficient of 40 M⁻¹cm⁻¹ (Greenwald, 1987). The reaction starts by the addition of sample to the cuvette containing phosphate buffer (80 mM) and hydrogen peroxide (150 mM), and the activity is measured for one minute. Results are expressed as μmol min⁻¹ mg⁻¹ of total protein.

2.7.3. Glutathione peroxidase (GPx)

GPx activity was measured indirectly by the joint reaction with glutathione reductase (GR), with the substrate hydrogen peroxide (H₂O₂). The activity was observed by the decrease of the absorbance of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm ($\epsilon_{340}(\text{NADPH}) = 0.005598 \text{ uM}^{-1} \text{ cm}^{-1}$), that was consumed during the regeneration of reduced glutathione (GSH) due to the reduction of oxidized glutathione (GSSH) (Lawrence & Burk, 1978). The reaction initiates by adding 50 μl of substrate (1.25 mM H₂O₂ or 1 mM Cumene hydroperoxide) to the sample, and the absorbance is measured for 5 minutes, in intervals of 30 seconds, at 28 °C. The activity is expressed as as nmol min⁻¹ mg⁻¹ of total protein concentration.

2.7.4. Glutathione-S-transferase (GST)

GST activity was measured by using an adapted method described by Habig *et al.* (1974). It was determined by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) and the increase of absorbance measured at 340nm ($\epsilon_{340}(\text{CDNB}) = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The activity of GST was measured every 30 seconds over 3 minutes, and calculated through the slope of the linear variation of the absorbance. The activity is expressed in $\mu\text{mol CDNB min}^{-1} \text{ mg protein}^{-1}$.

2.8. Oxidative damage

Before the analysis of oxidative damage, gills and digestive gland were weighed and rapidly buffered in 0.02M Tris-HCl (0.1 M HCl, 0.2 M Tris, pH=8.6) (the tissue-to-buffer ratio was 1:3 wet weight tissue/volume of buffer). Then, 10 μl of BHT (Butylated hydroxytoluene) were added, per each ml of Tris-HCl [0.02M]. The samples were homogenized in an ice bath and centrifuged at 30000 g, for 45 minutes, at 4 °C, using a biofuge stratus 230 V centrifuge (Thermo scientific, Germany). Both the cytosolic and mitochondrial fractions were stored at -80 °C for future analysis.

Lipid peroxidation (LPO) was quantified based on the method described by Erdelmeier *et al.* (1998) - determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon the decomposition by polyunsaturated fatty acid peroxides. The tissue supernatant (200 μL) was incubated at 45 °C, for 60 minutes, with 650 μL of 1-methyl-2-phenylindone diluted in methanol and 150 μL of methanesulfonic acid. The absorbance was measured at 586 nm and LPO levels are expressed as nmol malondialdehyde (MDA) + 4-HNE g^{-1} per g of wet weight tissue.

2.9. Acetylcholinesterase (AChE) activity

Gills were homogenized on ice in five volumes of a Tris-HCl buffer (100 mM, pH 8.0) containing 10% Triton X-100 and centrifuged at 12000g, for 30 minutes, at 4° C. Anti-cholinesterase activity was measured by the modified Ellman's colorimetric method (Ellman *et al.*, 1961), assessed by the addition of Ellman's reagent – DTNB - using

AChEI (acetylcholine) as substrate, for the estimation of respective thiocoline (ChE). The absorbance is measured, at 405 nm (coefficient of extinction of $\varepsilon = 13.6 \text{ mM}^{-1}.\text{cm}^{-1}$), to estimate the amount of ChE liberated by the reaction, which is proportional to the AChE activity (Colovic *et al.*, 2013).

2.10. Genotoxicity

2.10.1. Cell viability

The hemolymph was centrifuged at 3000 rpm for 3 min (4 °C) and cell viability assessed by staining a 100 μl of a subsample, from each experimental condition, with 100 μl of trypan blue (0.4% in physiological solution; v/v) and the percentage of live cells measured by randomly counting 200 cells, with a Neubauer chamber.

2.10.2. Comet assay

DNA strand breaks (single and double) represent one of the major oxidative damage to DNA via oxidative stress that is generally assessed by the comet assay.

Genotoxicity was estimated using the comet assay in a slightly modified version of Singh *et al.* (1988) and described in Almeida *et al.* (2011). The comet assay was first described by Ostling and Johanson (1984) as a method for detection of DNA damage in single cells. It is the most accurate method for quantifying DNA oxidation (Collins, 2009). This method is fast, inexpensive and applicable to any type of cell. It is not necessary that cell division occur so that you can see damage, which offers a great advantage when applied to poikilotherms individuals, in which the rate of cell division is extremely slow (Wilson *et al.*, 1998).

Microscopic slides were coated with 0.65% normal melting point agarose (NMA), in Tris-acetate EDTA. After collection, hemolymph cells were centrifuged at 3000 rpm for 3 min (4 °C), and the pellets with isolated cells suspended in 0.65% low melting point agarose (LMA) in Kenny's salt solution, and casted on the microscope slides. Afterwards, the slides with the embedded cells were immersed in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide, 1% Sarcosil, pH 10, 4

°C), for the diffusion of cellular components and DNA immobilization in agarose. Following the lysis step, slides were placed in an electrophoresis chamber containing electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C), gently submerged and left in this solution for 15 minutes, to permit DNA unwinding. The electrophoresis was carried out and, once concluded, the slides were removed and immersed in a neutralizing solution (0.4 mM Tris, pH 7.5) and rinsed with ultrapure water. Then, the slides were allowed to dry for 48 hours, from which the analysis in fluorescence microscope was made possible.

Afterwards, the slides were stained with 4,6-diamidino-2- phenylindole (DAPI, 1 mg mL⁻¹) and the presence of comets analysed using an optical fluorescence microscope (Axiovert S100) coupled to a camera (Sony). Fifty randomly chosen cells for each slide (25 in each gel from each organism) were scored with the Komet 5.5 image analysis system (Kinetic Imaging Ltd) at a total magnification of x400. Different parameters of the comet, including the olive tail moment (product of comet tail length and proportion DNA in comet tail – Figure 2.2), comet tail length (in micrometres, measured from the edge of the comet head) and amount of DNA in the comet tail (proportion based on tail intensity) were measured, and results are expressed as mean \pm STD. During the entire procedure, great care was taken to avoid exposing the cells and slides to light and heat.

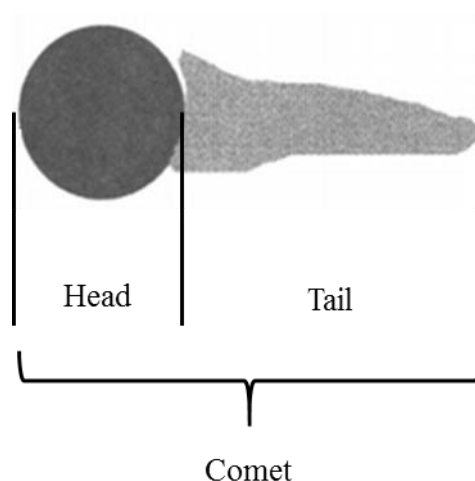


Figure 2.2. Diagram of typical comet showing distribution of DNA in head and tail

2.11. Statistical analysis

Statistical analyses were carried out using the Statistica 7.0 software (Statsoft Inc., 2005, USA). The results were compared using parametric tests (two-way ANOVA, followed by the Tukey's test) and non-parametric tests (Kruskal-Wallis), depending on the distribution of the data, at a 5% significant level. The homogeneity of variances was verified with the Levene's test. Principal component analysis (PCA) for gills and digestive gland was used to evaluate the influence of PS microplastics in the determined biomarkers, in exposed clams, along the period of exposure and assess the overall results. Statistical significance was defined at $p < 0.05$.

CHAPTER 3. RESULTS

3.1. PS microplastics characterization

The results from the PS microplastics characterization are summarized in Table 3.1.

Table 3.1. Characterization of PS microplastics using different techniques

Particle characterization	Method	PS microplastics
Particle size (μm) ^a	OM	20 ± 0.02
Density (g cm^{-3}) ^a	–	1.05
Mean particle diameter (μm) ^c	DLS	18.4 ± 1.33
Zeta (ζ) potential (mV) ^{b c}	ELS	Sea water: -12.4 ± 2.36 Mili-Q water: -52.6 ± 2.34

a. Original solution of PS microplastics from Sigma Aldrich (4.3×10^6 particles mL^{-1})

b. 100 mg L^{-1} of microplastics dispersed in natural seawater

c. 100 mg L^{-1} of microplastics dispersed in ultrapure water

OM. Optical microscope

DLS. Dynamic light scattering

ELS. Electrophoretic light scattering

Figure 3.1 shows PS microplastics observed under OM and Figure 3.2 the solution of PS microplastics in natural sea water (100 mg L^{-1}). The ocular ruler, present in one of the eye pieces of the microscope, was calibrated with a 1 mm stage micrometer (stage ruler), and the size was observed with the magnification x100. Table 3.1 indicates that

the particles have the size specified by the manufacturer (20 μm) and Figure 3.2 confirm that the particles have that size and the tendency to aggregate/agglomerate in natural seawater. DLS measurements show that d_h (hydrodynamic diameter) of PS microplastics is 18.4 ± 1.33 , which is similar to the size specified by the manufacturer.

ζ - potential measurements show that PS microplastics have highest negative surface charge in seawater (-12.4 ± 2.36 mV) than in Milli-Q water (-52.6 ± 2.34 mV) ($p < 0.05$), showing the tendency of these PS microplastics to aggregate due to low repulsion forces.

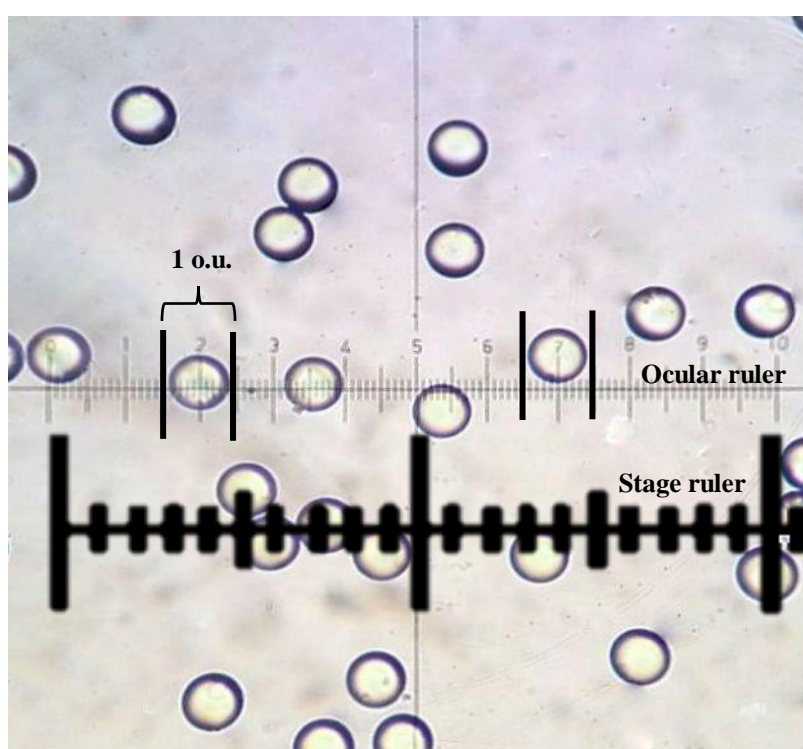


Figure 3.1. PS microplastics in aqueous solution observed under the OM (magnification: 100 x). One ocular unit (o.u.) corresponds to 20 μm .

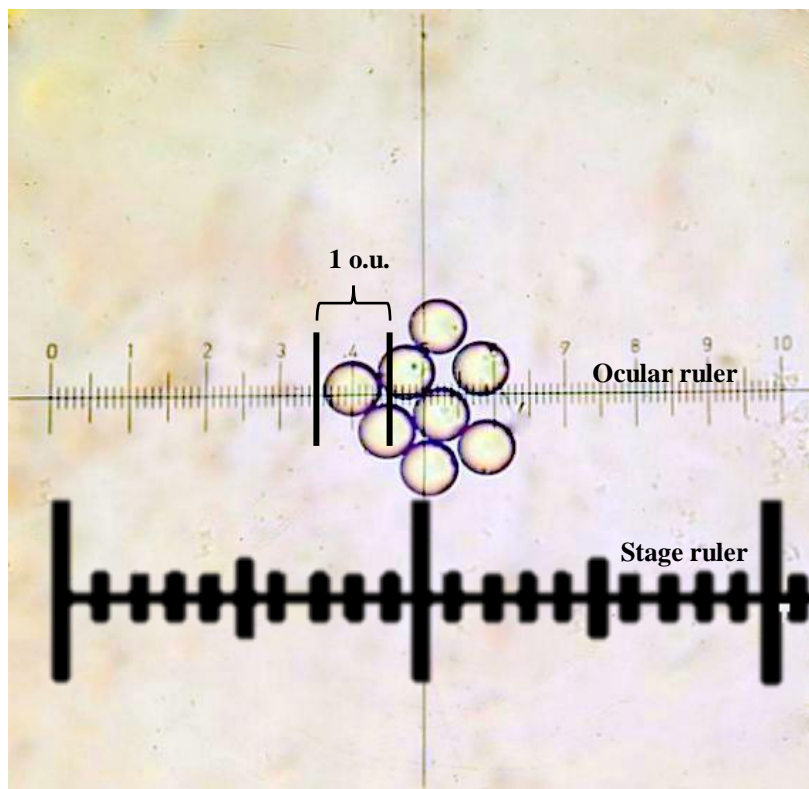


Figure 3.2. PS microplastics in natural sea water (100 mg L^{-1}) observed under the OM (magnification: $100\times$). One ocular unit (o.u.) corresponds to $20 \mu\text{m}$.

3.2.Sedimentation rate

Turbidity (C/C_0) of PS microplastics suspension was measured to assess the sedimentation rate (SR) (Figure 3.3). No significant differences in turbidity decrease were observed over time (24 h) between Mili-Q water (97.94%) and seawater (94.65 %) ($p > 0.05$). However, a sharp decrease in turbidity during the first 2 hours is observed in MQ water compared to seawater ($p < 0.05$).

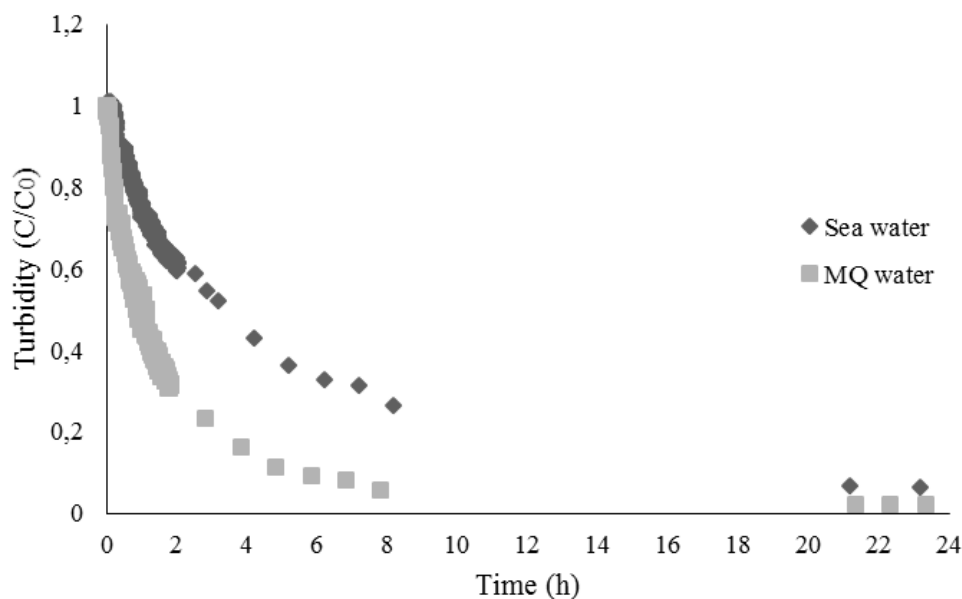


Figure 3.3. Turbidity of PS microparticles for 24 hours in MQ water and in sea water. C/C_0 is the normalised microparticle turbidity where C is the turbidity at time t and C_0 the initial turbidity at time 0.

During the first 2 hours, an increase in SR is observed in MQ water ($1.68 \times 10^{-1} \text{ h}^{-1}$) in relation to seawater ($1.04 \times 10^{-1} \text{ h}^{-1}$), while for the last 22 hours there is a decrease in SR for MQ water ($3.05 \times 10^{-4} \text{ h}^{-1}$) compared to seawater ($1.16 \times 10^{-3} \text{ h}^{-1}$) showing that, after the initial time, PS microplastics tend to sediment faster in natural seawater ($p < 0.05$) (Figure 3.4).

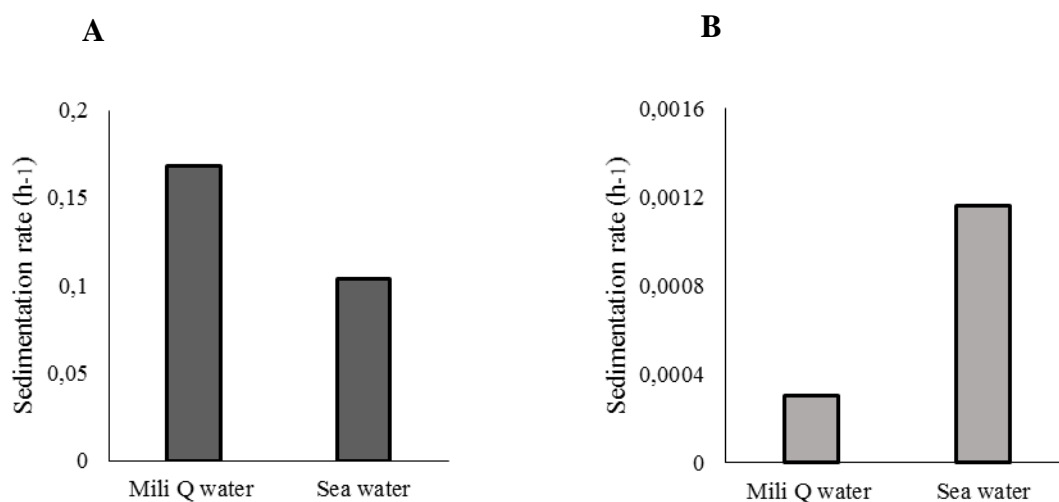


Figure 3.4. Fast SR (A) (over the first 2 hours) and slow SR (B) (over the remaining 22 hours) of PS microplastics suspended in MQ water and sea water.

3.3. Condition index

No significant changes were observed in the condition index, between unexposed and exposed organisms, during the accumulation (control: 33.05 ± 4.76 %; exposure: 31.53 ± 5.30 %; $p > 0.05$) and depuration (control: 31.31 ± 4.58 % microplastics: 31.83 ± 4.72 %; $p > 0.05$), indicating that the organisms were in good health during the experiment.

3.4. Microplastics accumulation

The PS microplastics present in the hemolymph observed under the OM are in figure 3.5. The presence of a small aggregate of PS microplastics is observed in image A, and 2 polystyrene particles in image B (highlighted by two circles) indicating that like for seawater there is a tendency for the PS microplastics to form small aggregates/agglomerates in the hemolymph.

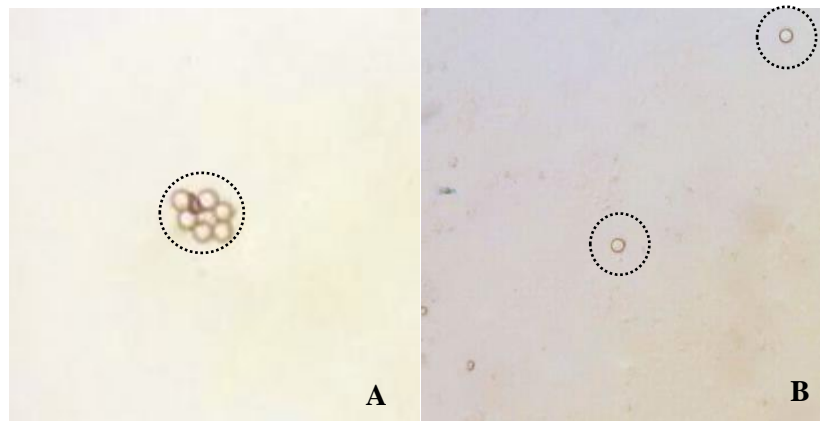


Figure 3.5. PS microplastics in the hemolymph (A and B), observed under the OM (magnification 40x). The presence of PS is highlighted by the dark shaded circles.

3.4.1. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT)

For both organs, samples of negative controls (without microplastics - T0) from the initial time of the experiment (without PS microplastics), positive controls (with 1 mg L⁻¹ of PS microplastics added) and of organisms exposed to microplastics during 3 (T3), 7 (T7) and 14 days (T14) were analysed. Unexposed organisms after 7 and 14 days of exposure and 7 days of depuration were also tested (T21).

In the case of living organisms, infrared spectrum of each "individual" may show some differences, either in band relative intensities or in band profile. The spectra were normalized at the maximum absorption to eliminate the path length variation (in the case of DRIFT the diffusion deep of the radiation inside the sample) and to reduce the global intensity differences between each measurement since the amount of sample (local concentration) analysed cannot be completely controlled (Davis & Mauer, 2010).

In this assay, the microplastic is the polystyrene (PS). A preliminary analysis of the PS used in the experimental work (obtained from a manufacturer) was made together with the positive and the negative controls of the digestive gland of *S.plana* (Figure 3.6).

The PS bands at 755, 700 and 541 cm⁻¹ are the more intense in the spectrum and can be related to deformation modes of the polymer benzene ring. Comparing the DRIFT spectra of the negative control of the digestive gland (Dg-T0) (Figure 3.6 A) with the positive control with more microplastic load (Dg-Ctr-p2), and with the one of polystyrene, it become clear that the spectra of tissue with microplastic present modifications in several spectral regions. Nevertheless, since the region 900-450 cm⁻¹ is the one that presents the most strong absorption bands in the polymer infrared spectrum, it is in this region that the presence of PS micoplastic can be more easily detected.

In figure 3.6 B there is an expansion of the 900-450 cm⁻¹ region where the presence of microplastics in the positive controls is very clear (Dg-Ctr-p1 and Dg-Ctr-p2). The spectra have significant changes in the region 625 a 475 cm⁻¹, with an increase in the relative intensity at ~540 cm⁻¹, wherein the polymer has a strong band. Moreover, the strongest PS bands at 700 and 756 cm⁻¹ are also clear in the positive control samples, indicating that PS microplastics are present. The greatest increase in the case of Dg-Ctr-p2 sample shows that the polymer is in high amounts in this sample.

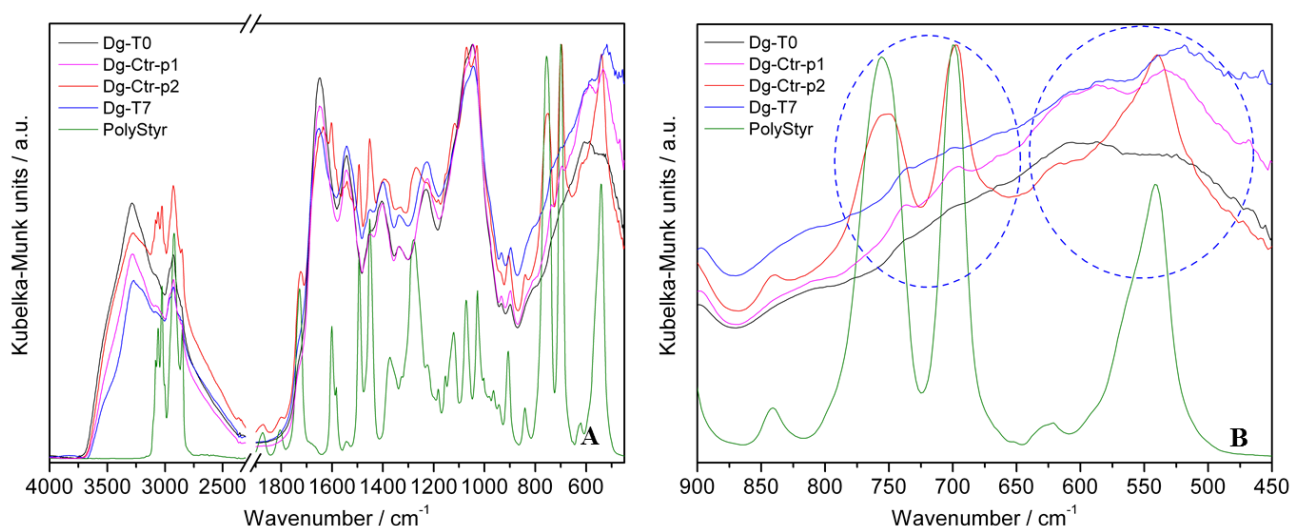


Figure 3.6. A. DRIFT spectrum of digestive gland from negative control (Dg-T0), positive controls (Dg – Ctr- p1 and p2), a digestive gland from a clam exposed to microplastics (Dg-T7) and the PS used in the assay (PolyStyr). B. Expansion of the region 900-450 cm^{-1} of DRIFT spectrum from figure A. The presence of PS is highlighted by the blue circle.

The PS effect on the DRIFT spectrum of the *S. plana* gills are presented in Figure 3.7, containing both a positive and negative controls of gills. In the regions 3800-2500 cm^{-1} and 1000-450 cm^{-1} there is a clear evidence of the presence of PS on the positive control. In both regions there are bands that evidently show the presence of microplastics in the positive control, when compared with the negative controls (Gi-g3, 4, 5 and 6-T0), marked by the surrounded blue regions.

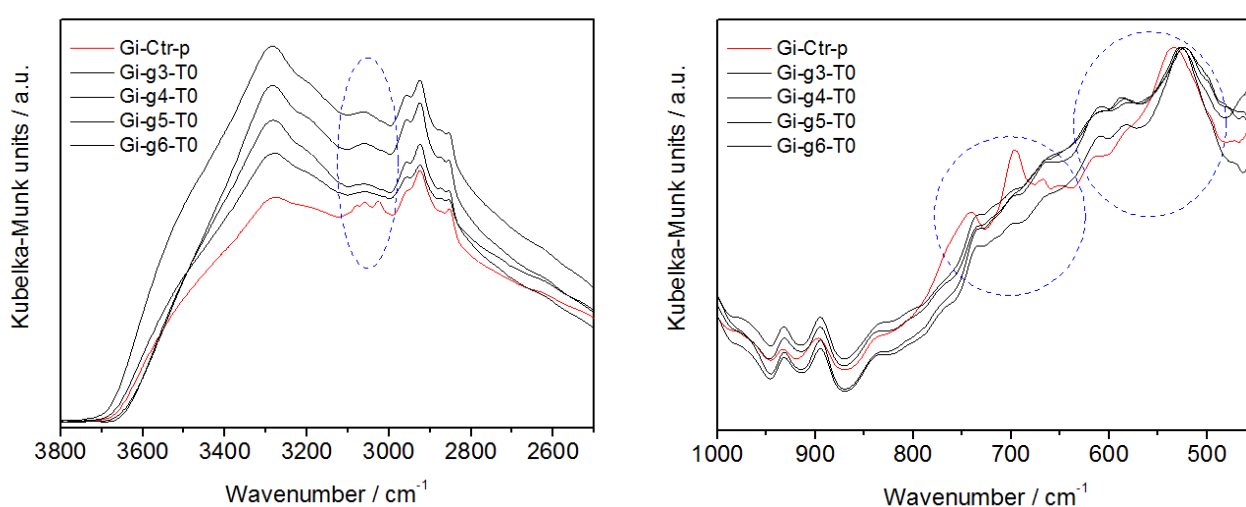


Figure 3.7. Expansion of the regions 3800-2500 cm^{-1} and 1000-450 cm^{-1} of DRIFT spectra from a positive control (Gi-Ctr-p) and negative controls (Gi-g3, 4, 5 and 6-T0), in gills of *S. plana*. The presence of PS is highlighted by the blue circle.

Figures 3.8 and 3.9 show DRIFT analysis of gills, obtained from several replicates of each sampling day. According to the DRIFT analysis, the gills exhibited an increasing pattern of PS microplastic accumulation along the exposure days. As it can be seen in Figure 3.8 A, which compares the samples spectra from the initial time and the 3rd day, there are bands that show the presence of PS microplastics marked by the surrounded blue regions. In figure 3.8 B, the same bands represent the presence of microplastics with a higher intensity, indicating that on day 7, more particles of PS microplastics were accumulated. In Figure 3.8 C, even a higher intensity of the samples from day 14 in relation to day 0 is noticed, also in the surrounded blue regions. In the last figure 3.8 D there is a comparison between the day 14 and the 21st, where it can be seen that the intensity of the bands between the two days is similar, indicating a possible recover of the organisms at the end of the depuration.

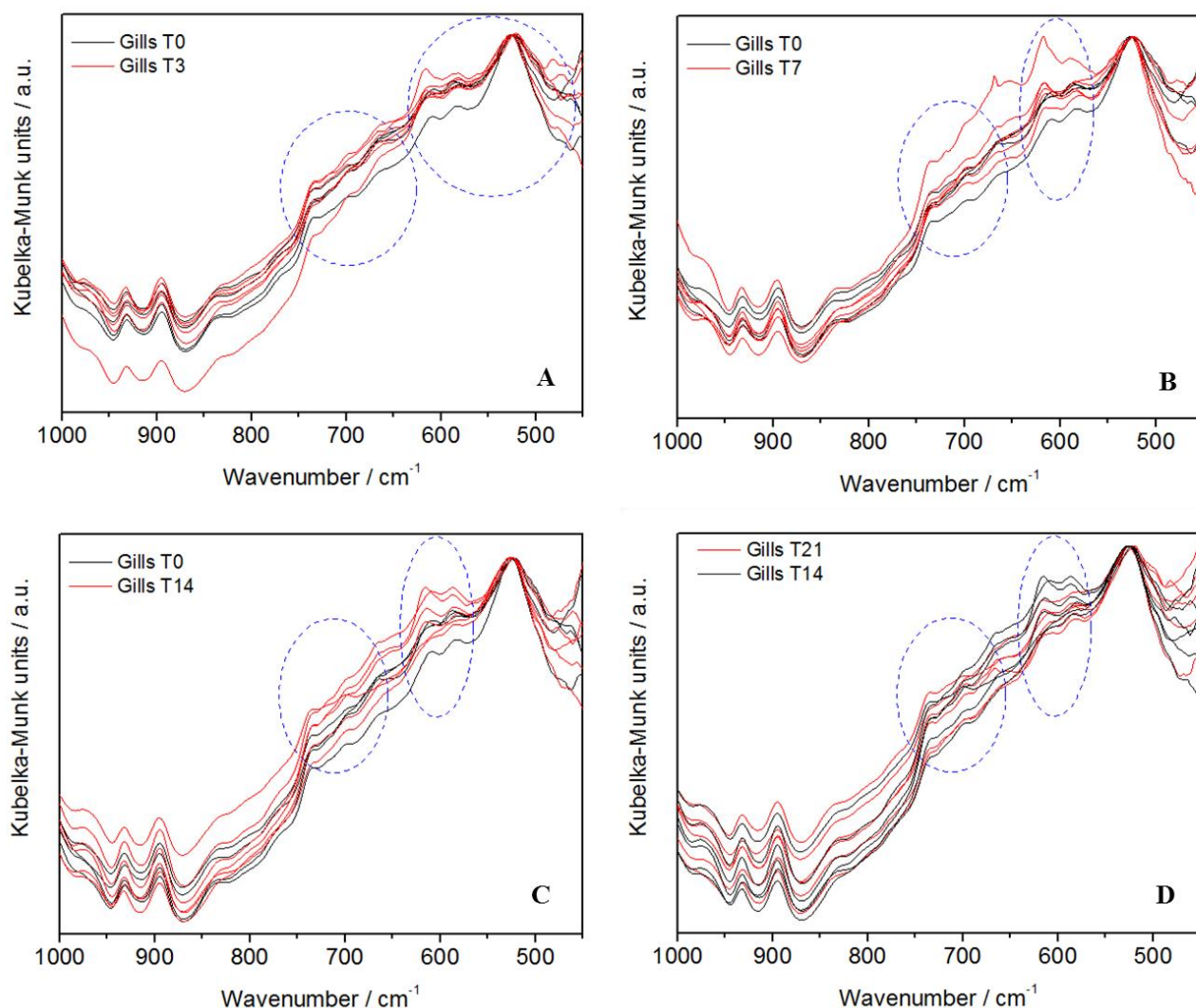


Figure 3.8. Expansion of regions 1000-450 cm⁻¹ of DRIFT spectra from in gills of *S. plana* from day 0 and day 3 of the experiment (A), day 0 and day 7 (B) day 0 and 14 (C) and day 14 and 21 (D). The presence of PS is highlighted by the blue circle.

Comparing the spectra between day 0 and day 21 (Figure 3.9) it seems that the pattern of the bands is similar between the two. However, the recover is not so evident when the comparison is made between the 21st day with the last day of exposure (day 14), since some bands were detected with a higher intensity in relation do day 0, indicating that, possibly, the gills still were unable to eliminate the microparticles accumulated at the end of the depuration period.

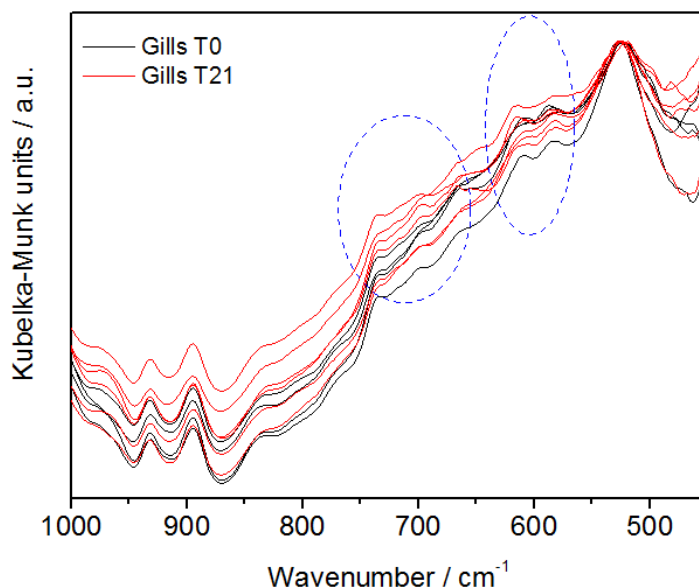


Figure 3.9. Expansion of regions 1000-450 cm⁻¹ of DRIFT spectra from gills of *S. plana* from day 0 and day 21 of the experiment . The presence of PS is highlighted by the blue circle.

Figure 3.10 shows DRIFT analysis of the digestive gland, obtained from several replicates of each sampling day. The accumulation of PS microplastics is evident. However, it does not appear the accumulation pattern was imilar to the gills (Figure 3.10). In figure 3.10 A there are no big differences in the intensity of the bands between day 0 and day 3. In figure 3.10 B, some samples from the T7 show a bigger intensity. The same happens in figure 3.10 C with just one replicate from the 14th day. Finally, in the last figure (3.10.D), a slight decrease in the band intensity seems to occur in relation to day 21, however not so clear as in the case of the gills.

The overall results indicate that the accumulation of PS microplastics was more efficient in the gills than in digestive gland of *S.plana*.

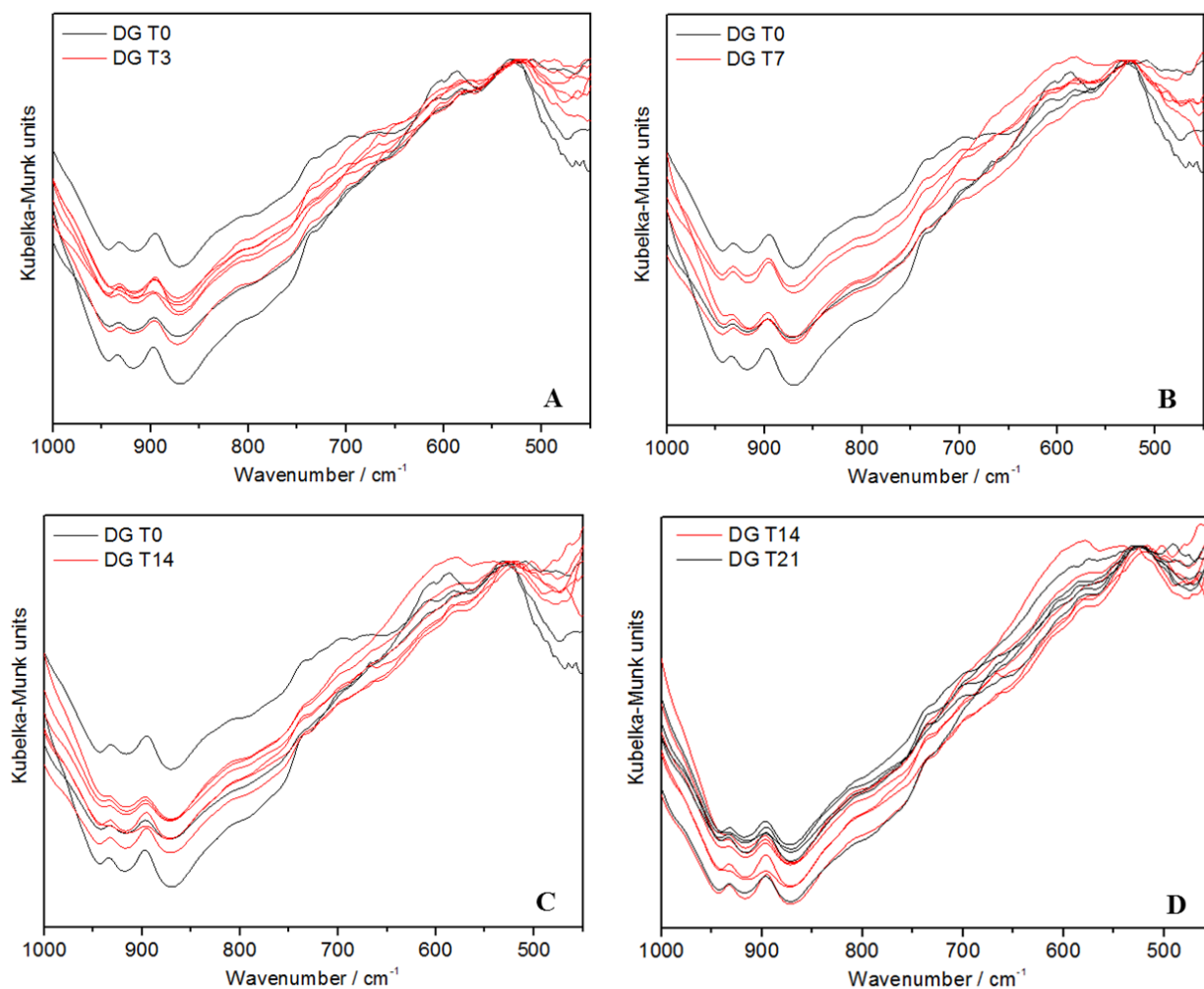


Figure 3.10. Expansion of regions 1000-450 cm^{-1} of DRIFT spectra of digestive gland of *S. plana* from day 0 and day 3 of the experiment (A), day 0 and day 7 (B), day 0 and 14 (C) and days 14 and 21 (D).

3.5. Enzymatic activity

The antioxidant enzymes activities (SOD, CAT, GPx) and GST in the gills and digestive gland of *S. plana* are presented in Figure 3.11 and show a tissue specific response

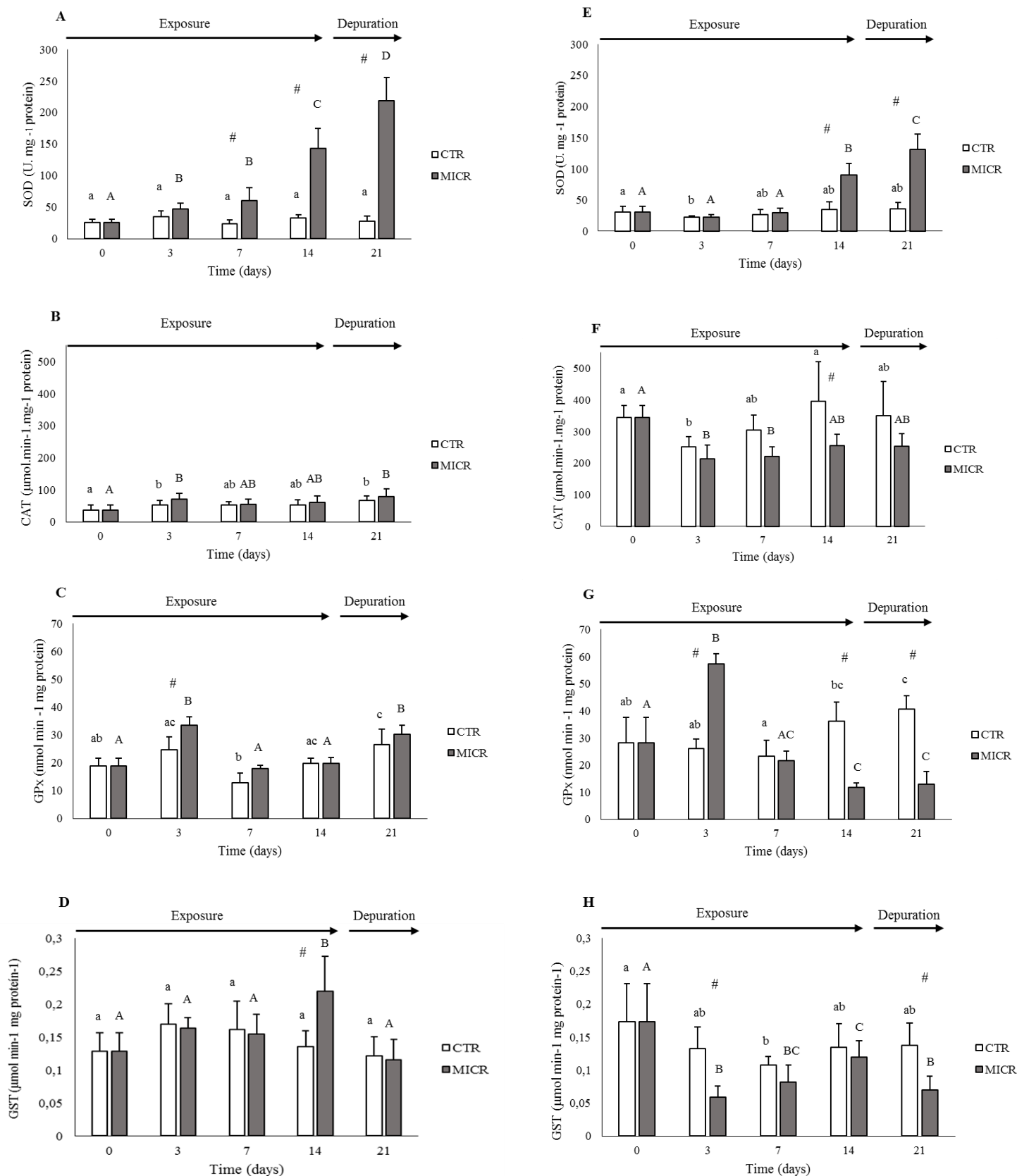


Figure 3.11. SOD, CAT, GPx and GST activities (mean \pm SD) in the gills (A, B, C and D) and digestive gland (E, F, G and H) of *S. plana* for control (CTR) and PS microplastics (MICR 1 mg L⁻¹) during exposure and depuration. Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

When the enzymatic activities of antioxidant and biotransformation enzymes are compared between the two organs (gills and digestive gland), only CAT had a higher activity in the digestive gland compared to the gills ($p < 0.05$).

In the gills, SOD activity in the control clams remained unchanged during all the experimental period. The exposure to PS microplastics induced an increase in SOD activity with the time of exposure, significant after 7 and 14 days of exposure ($p < 0.05$). In the depuration period SOD activity continue to increase in relation to the last day of the accumulation period ($p < 0.05$).

CAT activity did not evidence any significant difference for each treatment and among treatments however, the activity increased after 3 days of exposure in the control group, compared to the initial time ($p < 0.05$). There was a slight increase after the elimination period for both treatments, however not significant ($p > 0.05$).

For the control group, GPx showed some fluctuations between the 3rd and the 7th day, with a decrease in GPx activity, and with an increase between the 7th and the 14th day ($p < 0.05$). In exposed clams, GPx activity only significantly increase in clams exposed to PS microplastics after the 3rd day of exposure, compared to controls ($p < 0.05$). Similar to SOD, GPx activity increased at the end of the depuration period, but was not significantly different between treatments ($p < 0.05$).

Regarding GST there was an increase in GST activity for the control group after day 3 in relation to day 0 ($p < 0.05$). Similarly, an activity increment between days 0 and 3 was noticed for MICR, and on the 14th day of exposure ($p < 0.05$). On the 21st day there was a significant decrease in GST activity in clams exposed to PS microplastics ($p < 0.05$).

Concerning the digestive gland, SOD activity for control groups, like for the gills was similar for all exposure period, with the exception of day 3, where there was a significant decreased in SOD activity in respect to day 0 ($p < 0.05$). For the exposure group, like for the gills, SOD activity increased with time, but was only significantly different after the 14 days of exposure ($p < 0.05$). At the end of the depuration period, there was a significant increase in SOD activity compared to the 14th day, and to controls ($p < 0.05$).

CAT activity in digestive gland varied for the control group: decreased on day 3, and remained unchanged on day 7 and increased again on day 14 ($p < 0.05$). In the contaminated group differences were detected on day 14, compared to controls ($p < 0.05$). For the elimination period, no differences were noticed ($p > 0.05$).

Moreover, like for the gills, GPx activity in controls showed some fluctuations and increased on the 3rd day, decreased on the 7th and increased again on the 14th day of exposure ($p < 0.05$). Regarding the exposed organisms, GPx activity significantly increased on day 3 and significantly decreased after that time ($p < 0.05$). In the depuration period the activity remained unchanged compared to day 14 ($p < 0.05$).

GST activity in the control group decreased after the day 0 showing some fluctuations ($p < 0.05$). Regarding the organisms exposed to PS microplastics, GST activity only significantly decreased on day 3 ($p < 0.05$). This decrease was similar at the end of the depuration period ($p < 0.05$) and was significantly different from day 14 ($p < 0.05$).

3.6.Comet assay

Genotoxic effects for both treatments (CTR and MICR), analysed by comet assay, and expressed as % of tail DNA, Olive Tail Moment and Tail Length are in Figure 3.12 (A, B and C, respectively).

Regarding the percentage of DNA in the comet tail, no differences were found among the control group ($p > 0.05$). In the MICR treatment, there was a tendency to increase, but only significant after the 7th day ($p < 0.05$). Concerning the depuration period, significant differences were observed between treatments and in relation to the end of the exposure period (14th day) ($p < 0.05$).

The olive tail moment varied a little in the control group, with a decrease on the 3rd day, in relation to the initial time ($p < 0.05$). In clams exposed to MICR, significant differences were found on the 7th day of experiment, with a higher OTM in the MICR group ($p < 0.05$). In relation to the end of the elimination period, the olive tail moment was significantly higher in clams that were previously exposed to MICR and in relation to controls ($p < 0.05$).

The tail length showed fluctuations in the CTR and a decrease after the day 0 for the following days of exposure ($p < 0.05$). The MICR showed the same pattern as the CTR ($p < 0.05$). Significant differences between treatments were only found for the end of the depuration period, with the tail length being higher in clams previously exposed to MICR ($p < 0.05$).

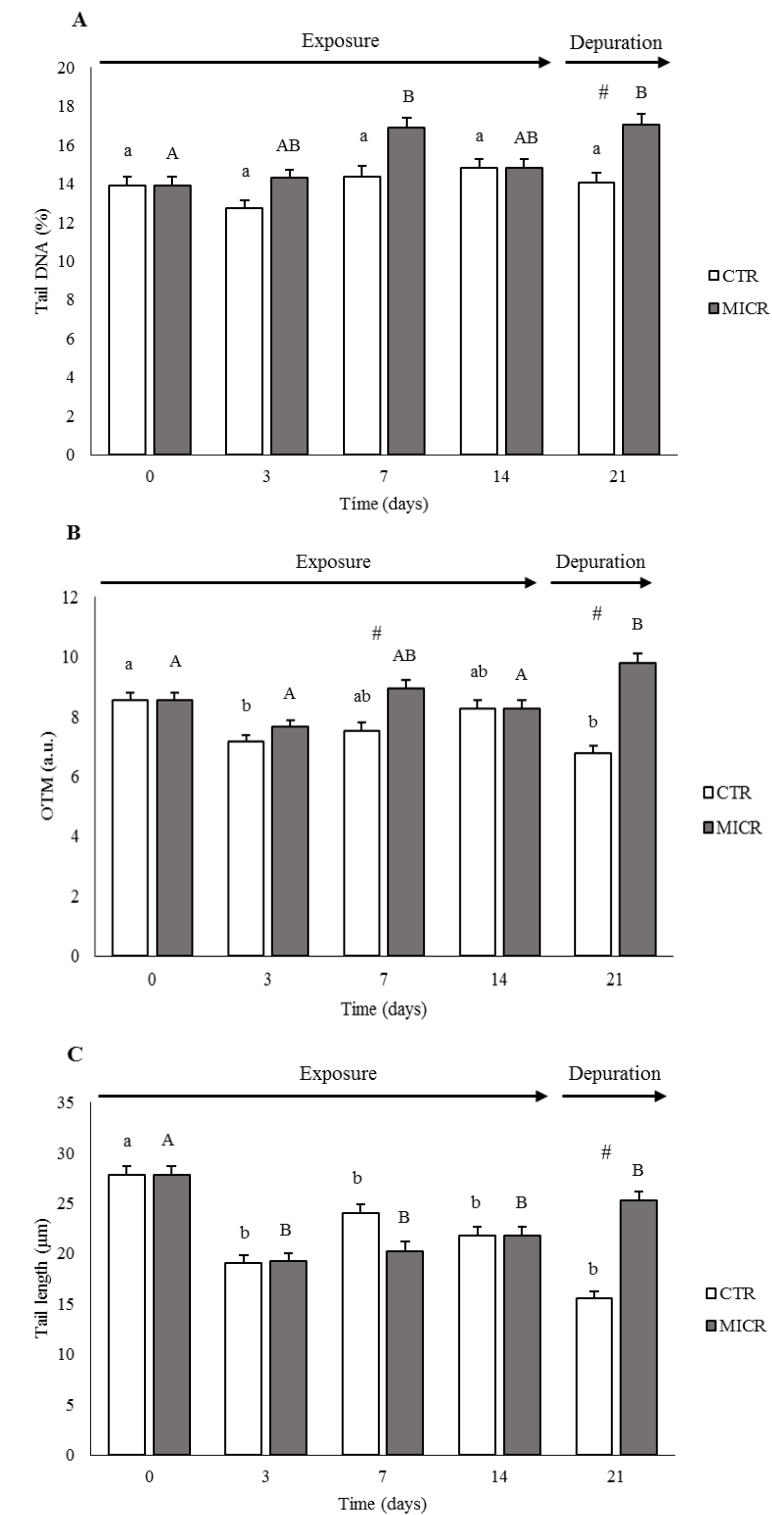


Figure 3.12. DNA damage (average \pm SEM) in the haemocytes of *S. plana* expressed as tail DNA % (A) and OTM (a.u.) (B) and Tail length (μm) (C) for control (CTR) and PS microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

3.7.AChE activity

The activity of AChE in the gills is present in Figure 3.13.

Regarding the control, AChE activity remained unchanged, with the exception of 7th day, where it significantly decreased ($p < 0.05$). In the MICR group, for all the experimental days, the AChE activity significantly decreased after the initial time (day 0) ($p < 0.05$) and was significantly lower on the 3rd day of experiment ($p < 0.05$). The same decrease in AChE activity was observed between controls and MICR exposed clams in the depuration period, but similar when compared to the last day of exposure ($p > 0.05$).

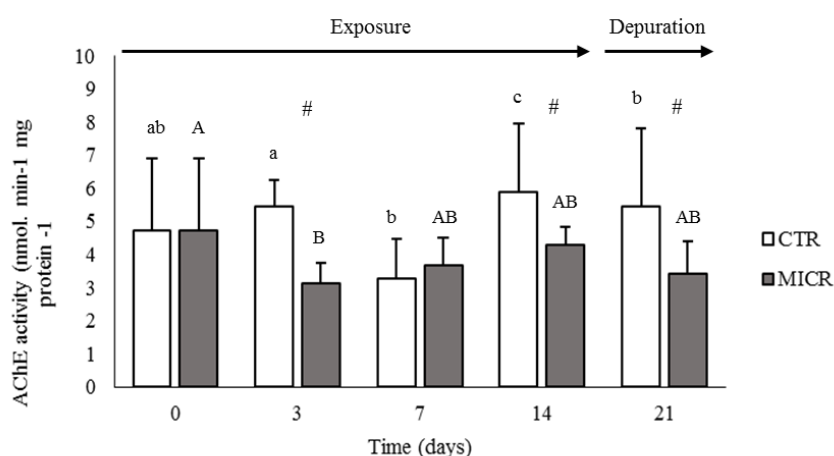


Figure 3.13. AChE activity in the gills of *S. plana* (average \pm SD) for control (CTR), and microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

3.8.Oxidative damage

LPO levels for gills (A) and digestive gland (B) are present in Figure 3.14. The oxidative damage was higher in digestive gland, comparing to the gills, for both treatments, at different days ($p < 0.05$).

Concerning the gills, the LPO levels remained unchanged, for controls, in exposed organisms. In exposed clams, although they remain unchanged, levels decreased compared to the initial time of the experiment (day 0) and to the other sampling days ($p < 0.05$).

Regarding digestive gland, the control group had similar LPO levels during the time of exposure. In clams exposed to PS microplastics, significant differences occurred on day 7, in relation to the control group ($p < 0.05$).

Regarding the elimination period, significant differences were found between treatments for both gills and digestive gland ($p < 0.05$), but only in digestive gland. LPO levels significantly decreased when comparing to the control and to the 14th day, for the organisms previously exposed to MICR ($p < 0.05$).

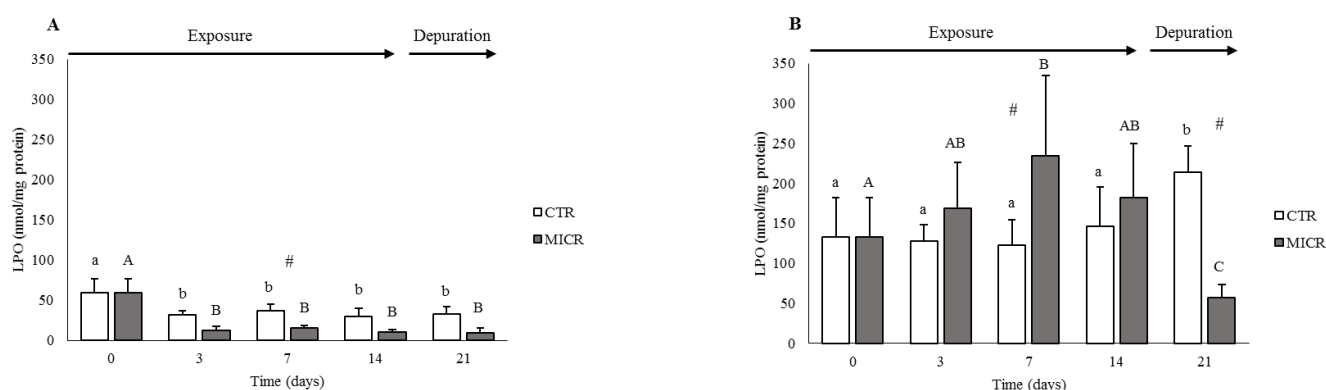


Figure 3.14. LPO (mean \pm SD) in gills (A) and digestive gland (B) of *S. plana* for control (CTR) and microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

3.9. Principal component analysis (PCA)

PCA was applied to all the data obtained for the gills and digestive gland to help to explain the effects of PS microplastics on biomarkers response (Figure 3.15).

Regarding the gills, the two principal components represent 69.3% of total variance, with PC1 representing 42.4 % and PC2 26.9 % (Figure 3.15 A). Regarding the time, the overall PCA indicates a separation between the initial time of the experiment (T0) and the remaining days, and a clear separation between exposed clams and the control, in both axis.

On the other hand there is a clear separation of the sampling days suggesting a specific response of *S. plana* gills due to the time of exposure, with days 7 and 14 being the most influential. CAT, GPx, SOD and the genotoxic parameters are present in the positive part of PC1, and AChE and LPO in the negative one. In PC2 axis SOD, LPO and the parameters of genotoxicity are present in the negative part.

In respect to the digestive gland, the two principal components represent 69.2% of total variance, with PC1 representing 41.8 % and PC2 27.4 % (Figure 3.15 B). In this case, the separation of the time of exposure is notorious, although the separation of the initial time is not as clear as in the gills. With the exception of the initial time (T0), all the clams exposed to PS are in the negative part of the PC1. Besides that, SOD, CAT, GST and the genotoxic parameters are also in the negative part of PC1 and GPx and LPO in the positive one.

The results from the two PCAs show that the enzymatic and genotoxic response is opposite between the two organs, which demonstrates that they exhibit different responses in dealing with the PS microplastics exposure.

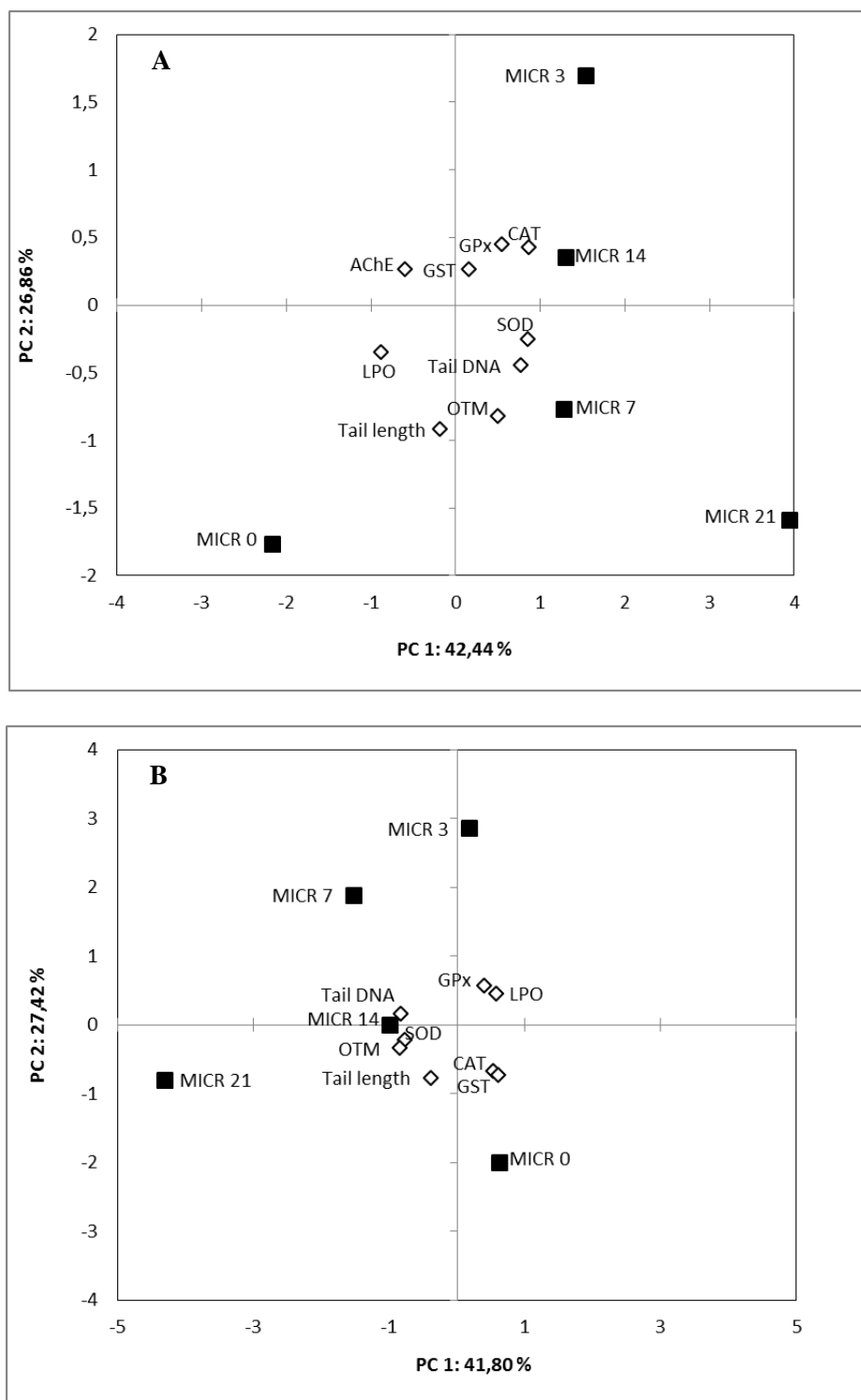


Figure 3.15. Principal component analysis (PCA) of a battery of biomarkers in the gills (A) and digestive gland (B) of *S. plana* from exposed clams

CHAPTER 4. DISCUSSION

Several studies are reported in the literature about the effects of microplastics in living organisms, including diverse aquatic species, but in bivalves is scarce (Avio *et al.*, 2015; Sussarellu *et al.*, 2016; Van Cauwenberghe *et al.*, 2015). As far as we know this is the first study where microplastics toxicity was assessed in *S. plana*, a marine deposit-feeder bivalve, used as a sensitive bioindicator to assess accumulation and toxicity of several contaminants (Buffet *et al.*, 2014; Coelho *et al.*, 2014; Riba *et al.*, 2005).

Polystyrene is one of the most used plastics worldwide (Canesi *et al.*, 2015) commonly detected in the marine environment (Andrady, 2011). It is ubiquitous within sea-surface samples collected around the globe (Hidalgo-Ruz & Thiel, 2013). Once in the marine environment, this polymer experiences changes in their properties that end up in the degradation process of the plastic (Andrady, 1994). Besides, the behaviour and fate of PS microplastics in the aquatic environment is highly dependent on their specific properties, such as size, shape, surface charge and environmental conditions, their toxicity is due to the partial degradation of microplastics due to UV-B radiation and the properties of the atmosphere and of the seawater (Andrady, 2011). Furthermore, the size of the microplastic chosen for this experiment (20 μm) is commonly used in cosmetics and personal care products (Fendall & Sewell, 2009).

In this study, PS microplastics characterization was performed by a combination of multiple techniques (OM, DLS, ELS and DRIFT) using both natural seawater from the Ria Formosa Lagoon (Portugal) and ultrapure water (Milli-Q) (Table 3.1 and Figures 3.1 - 3.10). The combination of multiple analytical techniques for PS microplastics characterization provides a more comprehensive analysis for the hazard and risk characterization of these particles. The PS microparticles used in this assay are of the same size and of a spherical shape and tend to form small aggregates in seawater. Therefore it is hypothesised that the toxic effects of PS microplastics are caused by the damage that these particles generate in tissues, although it could be much higher if microparticles were heterogeneous and not of the same size and shape (Frias, 2015). Most of the microplastics found in the marine environment are secondary microplastics, resulting from the degradation of larger pieces, and thus, with different sizes and sharp edges, that may contribute to internal cuts in the digestive tract, for example (Browne *et al.*, 2008; von Moos *et al.*, 2012). One of the main questions related to laboratory

experiments is precisely whether these procedures simulate field conditions and mimic the natural environment (Phuong *et al.*, 2016). So, in the future, experiments with microplastics with different sizes and shapes need to be performed in order to achieve a more realistic scenario.

S. plana can uptake PS microplastics from seawater across the gills. Results showed that PS microplastics were accumulated in gills and in digestive gland (Figures 3.8 - 3.10) and that accumulation in the gills increase with the time of exposure, with a consequent recovery after the depuration period. PS microplastics are trapped in the gills, the first organ in contact with particles and accumulate there. The microparticles are also ingested through the inhalant siphon of the clams, subsequently transported to the mouth and in the haemolymph to the digestive tract and to the digestive gland for intracellular digestion (Hughes, 1969). The results from this experiment show that the PS microparticles accumulate in the digestive gland (Figure 3.10), although without exhibiting an increasing pattern of accumulation as it was observed in the gills (Figure 3.8). Once in the digestive gland, since PS microplastics cannot undergo total digestion most of them are eliminated (Andrady, 2011). PS microplastics accumulation in the gut of *Mytilus edulis* was also demonstrated by Browne *et al.* (2008). A previous study testing the toxicity of gold nanoparticles (Au NPs) in *S. plana* refers that all accumulated nanoparticles were almost exclusively in the digestive gland, although they have also been accumulated in gills. All these results highlight the accumulation of both nano and microparticles essentially in the digestive tract of this species. However, the present results exhibit a completely contradictory response that can be due to the fact that an incomplete quantification of the accumulation by weight of tissues was made. This emphasizes the need that further data about the impact of PS microplastics in the tissue accumulation of bivalves, especially in *S. plana*, are needed. A future study to investigate whether the microparticles are eliminated in the pseudo-faeces of *S. plana* would also be useful to increase the knowledge about the accumulation and metabolism of PS microplastics.

In the present investigation, a battery of biomarkers was applied to investigate the possible effects and toxicity of PS microplastics in the gills and digestive gland of the marine bivalve *S. plana*. Even at a low concentration of PS microplastics (1 mg L⁻¹), the results indicate an oxidative stress response in gills and digestive gland cells, that are tissue and exposure time dependent, and that PS induce major perturbations, as revealed

by the effects on the total antioxidant capacity, DNA damage, neurotoxicity and thus oxidative damage (Figures 3.11 - 3.14).

Micro-PS exposure significantly modulates the cell oxidative system, associated to micro-specific properties and ROS generated from them. PS microplastics induce ROS production, antioxidant enzymes alterations and oxidative stress in this clam's species, wherein the digestive gland showed more pronounced enzymatic activities changes compared to the gills (Figure 3.11). In fact, SOD activity increased with the time of exposure, in both organs (Figure 3.11 A and B), suggesting that SOD is the first defence line to protect against oxidative stress induced by this stressor agent. The present data confirm that microplastics accumulation cause oxidative stress, since particles can cause injuries in the tissues and induce stress. SOD actively responds to the presence of PS microplastics, which reflects the need for a greater capacity to rapidly convert $O_2^{\bullet-}$ into the less damaging H_2O_2 , and thus, contribute to prevent cellular oxidative damage (Jo *et al.*, 2008). The same response was also detected in marine mussels *Mytilus spp* exposed to a concentration of $32 \mu g L^{-1}$ of PS microplastics, with 2 and 6 μm , after 14 days (Paul-Pont *et al.*, 2016).

CAT is involved in the removal of hydrogen peroxide - the main precursor of hydroxyl radical in aquatic organisms - and acts as a defence mechanism toward the exogenous source of H_2O_2 (Regoli & Giuliani, 2014). However, CAT is not the antioxidant defence mechanism used by *S. plana* to respond to PS microplastics toxicity. The differences in CAT activity were mainly observed in the digestive gland (Figure 3.11 D), where, although not significant, there was a decrease in activity after the first day of the experiment. This result is in agreement with those obtained by Avio *et al.* (2015), where an inhibition of CAT activity was measured in first defence line against microplastics in the digestive tissue of the marine mussel *Mytilus galloprovincialis*. CAT and GPx are both involved in the removal of H_2O_2 , while glutathione peroxidases are mainly responsible for eliminating metabolically produced H_2O_2 . Comparing the two enzymes, CAT was inhibited and only GPx had a positive response. The variation of these enzymes suggest different mechanisms and cellular pathways for H_2O_2 formation in tissues exposed to PS microplastics (Avio *et al.*, 2015). Regarding GPx, the two tissues showed a similar pattern during exposure (Figure 3.11 E and F), with an increase in activity after the beginning of the experience, suggesting a defence mechanism, and a posterior decrease that could indicate a higher availability of free ROS levels originated by microplastics accumulation, and thus, clams lose the capability to deal with the effect

of this stressor. Glutathione peroxidases are known as particularly sensitive in revealing the early onset of a prooxidant challenge, even at low levels of environmental disturbance, since they catalyse the levels of H_2O_2 originated by SOD (Regoli & Giuliani, 2014). The initial increase of GPx activity can be due to the initial interaction with PS microplastics that induce the production of H_2O_2 , produced by SOD activity. After this, GPx is inhibited, which further strengthens the PS microplastics toxicity. In *S. plana* environmentally exposed to mercury, a decrease in GPx activity in the whole soft tissue occurred at the contaminated sites, which contributed to the higher toxicity of mercury (Ahmad *et al.* 2011). Such ROS perturbations were also observed in mussels (*Mytilus galloprovincialis*) exposed to polystyrene (PS) and polyethylene (PE) alone or in combination with pyrene. The pro-oxidant challenge induced by microplastics on mussels was supported by the lack of significant variations for malondialdehyde, lipofuscin and neutral lipids in digestive tissues. The ROS production led to an inhibition of catalase and Se-dependent glutathione peroxidases (Avio *et al.*, 2015). Browne *et al.* (2013) showed that UPVC microparticles induced an oxyradical production in *Arenicola marina*, and that lugworms that ingested sediment with PVC reduce the capacity more than 30% to deal with oxidative stress. PS microbeads also led to an increase in ROS production in haemocytes and the enhancement of anti-oxidant and glutathione-related enzymes in mussel tissues (Paul-Pont *et al.* (2016). GST is usually associated with phase II biotransformation metabolic operations, involved in the metabolism of lipophilic organic compounds by catalysing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates, and has also a protective role against oxidative stress (Lesser, 2006). *S. plana* is using detoxification to deal with the exposure to PS microplastics, by catalyzing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates (Lesser, 2006), playing a significant role in the detoxification of the reactive products from lipid peroxidation. GST is a biomarker of defence that actively responds in gills indicating a presumptive protective role to stress. Gills showed an increase in GST activity (Figure 3.11 G), while the digestive gland had the opposite response (Figure 3.11 H). Because gills are the first organ in contact with PS microplastics, GST tries to help to eliminate the metabolically digested PS. The induction of GST-CDNB in the exposure to organic pollutants, such as pp'DDE (2,2-bis-(p-chlorophenyl)-1,1-dichlorethylene) in gills extracts of *Mytilus galloprovincialis* (Hoarau *et al.*, 2002; Khessiba *et al.*, 2001), can corroborate our results, and support the idea that GST could be used as a biomarker of exposure in the gills. In the digestive gland, there was a decrease in GST activity that is

supported by the findings of Avio *et al.* (2015), where PS-exposed mussels experienced a decrease in GST activity in the digestive gland, after 14 days of exposure.

Looking to the overall results, gills are an essential organ to protect *S. plana* from PS microplastics stress, since the response of antioxidant and biotransformation enzymes was more notorious than in digestive gland.

In the depuration period, it is relevant to highlight the increasing activity of SOD, also in both organs (Figure 3.11 A and B), and for GPx in gills (Figure 3.11 E). On the other hand, there was a decrease of GST activity in both organs (Figure 3.11 G and H). The increasing enzymatic activity at the end of the depuration period can be due to an impairment of the filtration activity or to the presence of remaining microparticles in the gills and digestive gland, that may induce an enzymatic response (Paul-Pont *et al.*, 2016). This response suggests the inability of these tissues of *S. plana* to eliminate microplastics, or the capacity to recover after this elimination period. Because there are not many information about the effects during the depuration period, probably 7 days of depuration were not enough for *S. plana* to completely recover from the exposure of PS microplastics.

We can hypothesize that ROS are produced as a result of microplastics uptake and possibly injury of gills and/or internalization by the digestive gland cells, since it is known that this material is capable of crossing cell membranes, leading to cell damage (Browne *et al.*, 2008; Rosenkranz *et al.*, 2009; Van Cauwenberghe *et al.*, 2015).

Lysosomal membranes are highly susceptible to oxidative effects of ROS which can be generated throughout a complex cascade of direct reactions and indirect mechanisms (Regoli & Giuliani, 2014). The cell is the site where contaminant accumulation, metabolism and toxicity occur, and so, lysosomal alterations such as lysosomal membrane destabilisation is an example of lipid peroxidation. Lysosomes in molluscan digestive cells accumulate contaminants that in turn can provoke significant alterations in these organelles (Cajaraville *et al.*, 2000). Concerning the oxidative damage to lipids (Figure 3.14 A), gills experienced a decrease in LPO levels after the beginning of the experiment (day 0). Moreover, the establishment of protection through the antioxidant enzymes activities seems related with the decreasing trend of LPO levels in the gills. These results possibly indicate that *S. plana* deals with the production of ROS by inducing antioxidant defences, which consequently limits the attack of ROS on membrane lipids, preventing LPO. In digestive gland, there is an increasing tendency of the LPO levels (Figure 3.14 B), and the main difference was on day 7, where the damage

was maximum. If we compare this result with the enzymatic activities, they were always lower on day 7, with a posterior recovery after one week of exposure, with the exception of GPx that was lower. The significant rise of ROS in digestive gland haemocytes of *Mytilus spp.* upon 7 days of micro-PS exposure (mix of 2 and 6 μm) seemed to be well controlled, as no antioxidant markers were activated and no sign of lipid peroxidation was observed (Paul-Pont *et al.*, 2016). On the other hand, Ahmad *et al.* (2011) showed an increase in LPO levels in both gills and digestive gland of *S. plana*, in response to environmental mercury. In the present work, no lipid peroxidation was observed in the gills due to the efficient response of the antioxidant defence system, while the increased LPO levels in the digestive gland is related with the decrease of antioxidant enzymes activities in this organ. Because the enzymatic defences in the digestive gland are not actively responding to the PS microplastics exposure, it results in an inflammation response and a lysosomal membrane destabilization, as a cellular host response to PS microplastics. The same response occurred in mussels (*Mytilus edulis* L.) after the exposure to high-density polyethylene (HDPE) particles (von Moos *et al.*, 2012). After the depuration period, the LPO levels although in the MICR exposure group decrease, this decrease was different among treatments, for both organs. Moreover, the damage decreased after the 7 days of elimination in digestive gland. The lack of lipid peroxidation in the gills and the low damage in the digestive gland of exposed clams may be an expected response due to the assumption of the oxidative stress-generating potential of these microplastics.

The Comet assay is a sensitive, rapid and economic technique for the detection of strand breaks and can be regarded as a good biomarker of genotoxicity in aquatic species (Jha, 2008). Moreover, Petridis *et al.* (2009) demonstrated that the blood cells of *S. plana* are suitable for screening genotoxic effects, using the Comet assay. In this experiment, genotoxic effects induce DNA strand breaks in PS exposed haemocytes of *S. plana* via different modes of action. In mussels treated with PE microplastics a significant enhancement of DNA strand breaks were detected in haemocytes (Avio *et al.*, 2015). DNA strand breaks represented the first form of damage caused by the enhanced production of reactive oxygen species in response to microplastics in the haemocytes of the marine mussel *Mytilus galloprovincialis*. The % Tail DNA and the OTM in *S. plana* in a general way increased with the time of exposure (Figure 3.12 A and B). Tail length (Figure 3.12 C) varied in every exposure days in relation to the initial state, in microplastics-exposed organisms. However, this alteration was also verified in the control

group, and because there were no differences among treatments, this parameter is not a good indicator of genotoxicity in this species. Therefore, taking into consideration only the Tail DNA and the OTM we can predict that there is a genotoxic effect in the haemocytes of *S. plana*.

Regarding the elimination period, there was an increase of both Tail DNA (%) and OTM, in exposed organisms. It is, therefore possible that the formation of ROS continues, and thus, results in DNA damage. This result is supported by the higher LPO level on day 7, where the Tail DNA (%) and OTM parameters were higher. Possibly, the incapacity of dealing with ROS production on this specific day, followed by a low enzymatic efficiency, led to genotoxic effects. The mechanism of genotoxicity of PS microplastics remains unknown, so it is suggested that it can be related to the direct interaction with DNA or nuclear proteins, as it occurs with nanoparticles, formation of strand-breaks through indirect mechanism by ROS production and oxidative stress (Rocha *et al.*, 2014), causing damage by covalently binding to DNA (Hossain & Huq, 2002) or by inhibiting DNA synthesis (Hidalgo & Dominguez, 1998) and thus preventing cell division and DNA replication (Singh *et al.*, 1988). The present results indicated that genotoxic effects of PS microplastics in *S. plana* were mainly related to ROS generation and oxidative stress, although the interaction of the PS microparticle with DNA should be due to PS microplastics accumulation.

PS microplastics caused significant reduction in AChE activity in gills (Figure 3.13 A), after the first day of experiment, indicating adverse effects in cholinergic neurotransmission, and thus, potentially in nervous and neuromuscular function. The ability of microplastics to inhibit AChE activity was also described in juveniles of the common goby *Pomatoschistus microps* exposed to PE microspheres (1-5 μm) for 96 hours, dosed alone or in combination with pyrene (Oliveira *et al.*, 2013) and alone or in combination with chromium (Luís *et al.*, 2015). There were no differences in the elimination period, indicating that the effects caused by the PS microplastic accumulation are not reversible. Our results support the hypothesis that anticholinesterase effects of microplastics should be taken in consideration due to the abundance of these particles in the marine environment and the pivotal role of AChE in neurotransmission of fundamental physiological (e.g. growth, reproduction) and behavioural processes that directly or indirectly may influence individual and population fitness (Avio *et al.*, 2015). The mechanisms of action still remain to be elucidated in this species.

Despite existing data about the increasing occurrence of microplastic in the marine environment (Andrady, 2011; Cole *et al.*, 2011; Wright *et al.*, 2013b), less is known about their biological effects. The present data are the first that evaluate the elimination of PS microplastics in marine bivalves. In light of this, further studies are needed to investigate the capacity to recover from the potential effects of PS and other microplastics in marine organisms. The evaluation of the biological effects of microplastics also requires a molecular-level of understanding of how they interact with cells in a physiological environment, but until to date the functional implications at cellular level still remains to be elucidated.

The microplastics impact in the marine environment should be carefully assessed, because in addition to the effects observed in marine organisms, the uptake of microplastics (in this case by clams) or nanoplastics may represent another route by which plastics enter the food web at large. The trophic transfer of microplastics is widely documented (Setälä *et al.*, 2014) and the potential for biomagnification of plastic particulates up the food chain is of particular concern for organisms at higher trophic levels and, ultimately, for human health (Cole & Galloway, 2015). Right now the priority should be to reduce the plastics inputs, since plastic waste is one of the most threatening emergent contaminant in the marine environment (Galloway & Lewis, 2016). Before it is too late, decision makers, scientists and the population should be warned to reduce the level of plastic consumption and waste, to avoid permanent alterations in the marine ecosystem.

5.1. Conclusions

An overview of the final conclusions is summarized as follows:

- *S. plana* is an important target for the ecotoxicity of PS microplastics and represents a suitable model for characterizing their impact in the marine environment.
- The environmental risk assessment (ERA) of PS microplastics in the marine environment depends on their behaviour and fate in seawater, which strongly influences their bioavailability, uptake, accumulation and toxicity in clams.

- PS microplastics are uptaken by clams mainly by the gills where they tend to accumulate. The presence of PS microplastics was evident in the digestive gland however, they are eliminated.
- Main mechanisms of toxicity of PS microplastics in clams involve ROS production, changes in antioxidant enzymes activities, DNA damage and neurotoxicity.
- Tissue-specific sensibility is involved in clam's responses to oxidative stress.
- In gills, PS microplastics toxicity is related to changes in SOD, CAT, GPx, GST activities and exposure time. In the digestive gland is related to SOD, CAT and GPx activities and exposure time.
- Gills had a more effective response to oxidative stress than digestive gland.
- The genotoxicity and neurotoxicity of PS microplastics is time dependent, having an increase in both genotoxicity and neurotoxicity along the experimental period.
- Detoxification processes of PS microplastics in clam's tissues were inefficient, indicating their potential trophic transfer and risks to human health.
- The multibiomarker approach represents a sensitive tool to assess the ecotoxicity of PS microplastics in *S. plana*, wherein biomarkers of the genotoxicity (DNA damage), oxidative stress (SOD, CAT, GPx and GST) and oxidative damage (LPO) should be incorporated as complementary tools in biomonitoring programmes to assess the risk associated with the presence of PS microplastics in the marine environment.

5.2. Future perspectives

Taking in consideration the results of this thesis, some key points are suggested for future research to better understand the ecotoxicity and environmental risk of PS microplastics in the marine environment:

- Identification and quantification of microparticles accumulated in the tissues, through histology methods.
- Analysis of pseudofaeces to quantify the PS microplastics elimination in *S. plana* and the efficiency of the detoxifying system.

- Molecular characterization and functional analysis of antioxidant enzymes isoforms from clams exposed to PS microplastics to understand the tissue-specific susceptibility to oxidative stress related to micro-specific properties.
- Infer the toxicokinetics and tissue distribution of PS microplastics in clams.
- Access the immunotoxicity and cytogenotoxicity of PS microplastics in clams
- Applications of proteomics, transcriptomics and metabolomics technologies to describe gene and protein expression changes and metabolic profiles in clams exposed to PS microplastics.
- Simulate field conditions and mimic the natural environment of *S. plana* and perform a multi experimental work with microplastics of different sizes and shapes and different materials in order to achieve a more realistic scenario.
- Perform an experimental work with a longer depuration period.
- Assessment of ecotoxicity of PS microplastics in other relevant environmentally conditions, such as multispecies exposures and mesocosms.

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