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Influence of particle size on the ecotoxicity of low-density polyethylene microplastics, with and without adsorbed benzo-a-pyrene, in the clam *Scrobicularia plana*

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Declaração de autoria de trabalho

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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Ana Rita Rodrigues

ABSTRACT

Plastic was introduced massively into the world on the beginning of the 1950's and has accumulated in terrestrial and marine environments since then. Its world-wide production almost reached 350 million tons in 2017. MPs became bioavailable to predatory filter feeding organisms and others at higher trophic levels. This study investigated the ecotoxicological effects in the gills and digestive gland of the clam *Scrobicularia plana*, exposed to different sizes of low density polyethylene (LDPE) microplastics with and without adsorbed benzo-a-pyrene (BaP), to evaluate the potential effect of different sizes on microplastics accumulation and of microplastics as a source of chemical contamination once ingested. *S. plana* was exposed to 1 mgL^{-1} of two different sizes of microplastics, 4-6µm and 20-25 µm, with and without BaP (at environmentally relevant concentrations) for 14 days. To determine the ecotoxicological effects a set of biomarkers were analysed: oxidative stress including the quantification of antioxidant enzymes activities (superoxide dismutase - SOD and catalase - CAT) and biotransformation enzyme activity (glutathione-S-transferases - GST), oxidative damage (lipid peroxidation - LPO), and neurotoxicity (acetylcholinesterase - AChE). The condition index was evaluated to assess the overall health status of the organism. To have a better impact perspective of these contaminants in this bivalve species an integrated biomarker response index (IBR) and a Health Index was applied. All sizes of microplastics were significantly accumulated in MPs exposed clams when compared to control and pre-exposure. In relation to antioxidant enzyme activities, SOD was noted a higher response in the gills for the bigger size of LDPE + BaP MPs. CAT activity is lower in the gills showing a decrease for smaller and bigger + BaP MPs. GST had a significant increase for the bigger size with BaP only on the 7^{th} day. No significant differences either with size or time of exposure for the digestive gland. Oxidative damage was higher in the digestive gland for smaller virgin LDPE MPs with and without BaP. Neurotoxicity showed no inhibition responses during exposure time. Biomarkers alterations are apparently more related to the smaller size in gills and related to the virgin LDPE MPs in the digestive gland according to IBR results. As a final step a health index was performed demonstrating that the digestive gland was more affected by these microplastics.

Key words: Microplastic, benzo-a pyrene, *Scrobicularia plana*

RESUMO

Os plásticos foram introduzidos massivamente no mundo há 70 anos, seguindo de um aumento de produção nas últimas décadas devido ao seu consumo excessivo, acumulando-se em zonas terrestres e marinhas. A sua produção mundial atingiu 350 milhões de toneladas em 2017, 64,4 milhões dos quais são produzidas na Europa, o segundo maior produtor de plástico (18,5 %). São imensos os impactos dos microplásticos no meio marinho e o zooplâncton é a primeira espécie-alvo tornando os microplásticos bio disponíveis para organismos filtradores e consequentemente organismos de níveis tróficos mais elevados.

Este estudo teve como objetivo investigar os efeitos ecotoxicológicos nas brânquias e glândula digestiva da amêijoa *Scrobicularia plana*, quando expostas a diferentes tamanhos de microplásticos de baixa densidade (polietileno) com ou sem benzo-a-pireno adsorvido, com o intuito de avaliar o efeito potencial dos diferentes tamanhos de microplásticos acumulados na amêijoa e se esses microplásticos poderiam ser uma fonte de contaminação química, uma vez ingeridos. A *S. plana* foi exposta 1 mgL^{-1} de microplásticos de dois tamanhos diferentes, 4-6 µm e 20-25 µm, com e sem BaP, durante 14 dias. Para estudar esses efeitos, um conjunto de biomarcadores foram analisados, incluindo a quantificação da atividade das enzimas antioxidantes (superóxido dismutase, catalase e glutationa peroxidase), de biotransformação (glutationa-S-transferases), dano oxidativo (peroxidação lipídica), neurotoxicidade (acetilcolinesterase) e quantificação de microplásticos nas amêijoas expostas. O índice integrado de resposta a biomarcadores (IBR) e o índice de saúde (HIS) foram utilizados para avaliar o nível de stress durante o tempo de exposição.

Todos os diferentes tamanhos de microplásticos foram acumulados pelas amêijoas durante o tempo de exposição, com maior significância para micropartículas menores. Em relação à atividade das enzimas antioxidantes, a atividade da SOD demonstrou maior atividade nas brânquias para os tamanhos maiores quando o BaP estava presente. A GST demonstrou um aumento significativo para o tamanho maior com BaP apenas no 7º dia de exposição. Na glândula digestiva não demonstrou diferenças significativas. A atividade da CAT é menor nas brânquias demonstrando uma diminuição para ambos os

tamanhos de MPs+BaP. O dano oxidativo aumentou em ambos os tecidos. No entanto, níveis mais elevados foram observados na glândula digestiva para microplásticos de menor tamanho. A neurotoxicidade não mostrou respostas de inibição durante o tempo de exposição. Aparentemente, as alterações dos biomarcadores estão mais relacionadas ao menor tamanho das brânquias e aos MPs virgens na glândula digestiva, de acordo com os resultados do IBR. HIS demonstrou um elevado e maior impacto na saúde da glândula digestiva durante todo o tempo de exposição.

Palavras chave: microplástico, benzo-a pireno, *Scrobicularia plana*

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1. **INTRODUCTION**

3. RESULTS

LIST OF ABREVIATIONS

AChE	Acetylcholinesterase
ATC	Acetycholine solution
BaP	Benzo-a-pyrene
BHT	Butylated hydroxytoluene solution
CAT	Catalase
CDNB	1-chloro 2,4 dinitrobenzene
CI	Condition index
Ctrl	Control
DAM	Daily assay mixture
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DNTB	5,5' -dithio-bis (2- nitrobenzoic acid)
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
GSH	Reduced glutathione
GST	Glutathione-S-transferases
HCL	Hydrochloric acid
HDPE	High density polyethylene
HIS	Health Index
H_2O_2	Hydrogen peroxide
IARC	International Agency for Research on Cancer
IBR	Integrated Biomarker Response Index
KCL	Potassium chloride

LDPE	Low density polyethylene
LPO	Lipid peroxidation
MDA	Malondialdehyde
MPs	Microplastics
NaCl	Sodium chloride
NOAA	National Oceanic and Atmospheric Administration
PCA	Principal Component Analysis
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyl
PET	Polyethylene terephthalate
POPs	Persistent organic pollutant
PP	Polypropylene
pp'DDE	2,2-bis-(p-chlorphenyl)-1,1-dichlorethylene
PS	Polystyrene
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
UV	Ultra Violet radiation
4-HNE	4-hydroxyalkenals

1 Introduction

1.1. Pollution by plastics

Plastics are synthetic organic polymers of a great variety of materials designed to meet very different needs of thousands of end products. Plastics can be divided in two categories, thermoplastics and thermosets, depending on their origin. Thermoplastics can be melted when heated and hardened when cooled, being reversible materials, such as, polyethylene, polypropylene, polyvinyl-chloride and polystyrene. Thermosets undergo a chemical change when heated, creating a three-dimensional network, and after being heated and cooled these plastics cannot be re-melted (polyurethane, epoxy resins and unsaturated polyester) (Plastics – the Facts, 2018). The top 5 type of plastic material used since 2007 and comprising roughly 75% of Europe production, are low density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS) (Plastic – the Facts, 2010; Ribeiro et al., 2017).

Before plastic was massively introduced in the world at the beginning of the 1950's, industries conventionally used for packaging, materials such as paper and glass (Andrady, 2003). In the meantime, due to the cheapness, high durability, resistance and versatility, plastic has become an important packaging material to produce, that lead to its commercial success and incorporation in everyday life. It has rapidly increase in expendable, single use applications in many industry sectors, originating what we call "the throw away generation". Plastic quality has improved, but yet, plastics are a long-lasting source of litter that become a real world-wide environmental problem. Fast food packaging, water bottles, plastic bags, straws are some of the examples, that has minutes of use and years of litter accumulation, being accumulated in terrestrial environments, on shorelines even on the most remote islands, in the open ocean and in the deep sea (Barnes *et al*., 2009). Its world-wide production almost reached 350 million tons in 2017. Europe produced 60 million tons in 2016 and 64.4 million tons the year after, putting Europe in second place as plastic producer (18,5 %) (Plastics – the Facts, 2018).

The proportion of plastic contributing to municipal waste is about 10% of the total waste generated worldwide (Barnes et al., 2009). When population acknowledged that this waste would create a major environmental problem, several ways to minimize were

stablished, such as, recycling. However, the recycling percentage varies from country to country. Most of the European countries had packaging recycling rates no higher than 35% of the total waste (Plastics – the Facts, 2018).

Debris were constantly found floating at the sea surface, but mainly natural marine debris, such as, driftwood, shells, pumice and others. Only 40-50 years ago within the evolution of plastic, marine debris have started to accumulate more and more, being plastic the major component (Coe and Rogers, 1997; Barnes and Milner, 2005). Plastic can travel long distances following the oceanic circulation. Fouling by biofilms, epibiota or water logging may increase their weight and cause flocculation, sinking to greater depth and settling in benthic sediments (Barnes *et al.,* 2009).

The history of plastic industry has only ~70 years, which is not enough to know the exact degradation time in environmental conditions, however, it is estimated that plastic degradation in the environment will be a few hundred years but is likely to be far longer in deep sea and non-surface polar environments (Barnes *et al*., 2009; Sivan, 2011). The relatively cold hyaline temperatures of the oceans slow down degradation further by preventing photo-oxidation (Cole *et al.,* 2011).

1.2. Plastic Sources to the marine environment

1.2.1. Land

There are many ways that plastic from land can end up in the ocean, as a result of inappropriate waste management and improper human behaviour. Well operated landfills are usually closed systems, which are everyday covered by soil or synthetic materials to hold wind-blow debris (Barnes *et al*., 2009). The portion of plastic litter that does not reach the landfills will roam the earth's surface, travelling by wind until it reaches rivers, and consequently the ocean. Improper human behaviour will accelerate the process, by dumping and abandoning litter outside licensed collection points close to the sea, such as beaches (Barnes *et al*., 2009). In highly populated areas, land-based sources dominate the input of plastic waste into the marine environment, making a total of 5.6 million tons of marine debris every year, which is 88% of the total marine debris input (Barnes *et al*., 2009).

1.2.2. Sea

The ways in which the plastic reaches the marine environment are numerous, and difficult to control. Before the massive plastic production, materials used for fishing industries were made by natural fibres, such as, manila, cotton and hemp (Henderson, 2001). Those materials had less durability and lose 50% of their strength when in contact with seawater. However, in 1950's natural fibres were replaced by synthetic plastic material to produce mainly ropes and nets. Being plastic a high resistance material, fisherman did not have to waste time to repair fishing gear, see table 1.1. The fish industry, either commercial or recreational activities, greatly contributed to the amount of plastic debris found in the ocean, followed by those discarded by coastal tourism, marine vessels and marine industries (e.g. aquaculture, oil-rigs) that can directly enter the marine environment, posing risk to biota given its long-term degradation (Derraik, 2002).

Littering and dumping at sea from boats, recreational or commercial, has been prohibited for the first time in 1990 under the international shipping regulation MARPOL Annex V, expecting a reduced in ship-derived plastic debris (Barnes *et al.,* 2009; Ryan *et al*., 2009; Thompson *et al*., 2009). The London Dumping convention, in force since 1975 also had as main goal to promote the effective control of all sources of marine pollution and to take all practice steps to prevent pollution of the sea by dumping wastes and other matter. 87 states adhered to this convention (Leslie *et al.,* 2011).

Table 1.1. Summary of abandoned/discarded and lost polymer-containing fishing gear from around the world (taken from articles summarized by UNEP, 2009) (Hammer et al., 2012).

Region	Fishery/gear type	Indicator of gear loss
North Sea and NE Atlantic	Bottom-set gillnets	0.02–0.09% nets lost per boat per year
English Channel and North Sea (France)	Gillnets	0.2% (sole and plaice) to 2.11% (sea bass) nets lost per boat per year
Mediterranean	Gillnets	0.05% (inshore hake) to 3.2% (sea bream) nets lost per boat per year
Gulf of Aden	Traps	20% lost per boat per year
United Arab Emirates Sea Area	Traps	260,000 lost per year in 2002
Indian Ocean	Maldives tuna longline	3% loss of hooks/set
Australia (Queensland)	Blue swimmer crab trap fishery	35 traps lost per boat per year
NE Pacific	Bristol Bay king crab trap fishery	7,000–31,000 traps lost in the fishery per year
NW Atlantic	Newfoundland cod gillnet Fishery	5,000 nets per year
	Canadian Atlantic gillnet Fisheries	2% nets lost per boat per year
	Gulf of St Lawrence snow crab	792 traps per year
	Net England lobster fishery	20–30% traps lost per boat per year
	Chesapeake Bay	30% traps lost per boat per year
Caribbean	Guadeoupe trap fishery	20,000 traps lost per year

1.3. Macroplastics, Microplastics, and Nanoplastics

Plastic debris can be categorized into three strands: macroplastics, microplastics and nanoplastics. Macroplastics (> 5 mm) are visibly identified, mainly come from sectors of packaging, and cause the injury and death of marine birds, mammals and fish by entanglement and ingestion, and also promote the transportation of non-native marine species (Derraik, 2002; Gregory, 2009; Lozano and Mouat, 2009; Ribeiro et al., 2017).

The microplastics (< 5mm) are small plastic particles, used as scrubbers in cosmetics (exfoliating hand cleansers and facial srubs), air-blasting and also small plastic fragments derived from the breakdown of macroplastics (Derraik, 2002; Thompson et al., 2004; Avio et al., 2015). The term 'microplastic' was first used in 2004 by Thompson and colleagues (2004), in the paper ‘Lost at sea: where is all the plastic?’, to describe small fragments of plastic. In 2009 the first International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris was held in America.

Microplastics are also classified as primary and secondary fragments. Primary microplastics are plastics already manufacture with < 5 mm in products, such as, cosmetics (Gregory, 1996; Derraik, 2002; Browne *et al*., 2007). Secondary microplastics originate from bigger fragments who suffered from degradation/breakdown turning into smaller fragments, both at sea and on land (Thompson *et al*., 2004).

Nanoplastics is a lower limit following microplastics, are plastics below 100 nm, in at least one of its dimensions. Studying and monitoring these particle size represents a big issue due to the challenges posed in reliable detection (Bergmann *et al.,* 2015).

1.4 Physico-chemical characteristics

Plastics come in a great variety of shapes, size, colour and surface charge chemical composition that is influenced by the surrounding environment. The breakdown of plastic can be triggered by thermal, photolytic, chemical, and physical fragmentation, resulting in macroplastic turning into microplastic sizes, unmineralized and never fully degraded (Thompson *et al.,* 2004). Biofilms when formed creates a barrier for plastic that, consequently, inhibits degradation by UV exposure keeping them longer in the environment (Barnes *et al.,* 2009).

1.5 Marine organisms and environmental impacts

Primary producers are at the base of the food chain, with phytoplankton being an important food source to several marine organisms. Phytoplankton, such as algae, is capable to interact with MPs in a way that has consequences for both. Algae can form heteroaggregates with MPs influencing the vertical distribution and sedimentation rates of the MPs in the marine environment (Long *et al*., 2017). While these interaction increases sinking and sedimentation of MPs, chlorophyll concentration, photosynthesis, cell population growth and morphology of phytoplankton could be drastically altered by MPs aggregation (Yokota *et al*., 2017). Since the density of many plastic polymers is lower than seawater, they float in the marine environment. However, the relative density of floating microplastic may increase during its residence time in marine waters (Wang *et al*. 2016). Also, MPs can eventually sink due to fouling by microorganisms increasing

the density of positively buoyant MPs until the density reaches or exceeds that of seawater (Fazey & Ryan 2016), making benthic environment a MPs reservoir.

MPs are abundant in marine shallow sediments (Claessens *et al*., 2011, Alomar et al. 2016) and in deep-sea sediments (Woodall *et al.,* 2014, Fischer *et al.,* 2015). In sediments off the coast of Belgium, concentrations of MPs were 15 to 50 times higher than reported maximum concentrations of other similar study areas (Claessens *et al.,* 2011). This indicate that the distribution of MPs in marine sediments is related to the proximity of urban centers to the marine environment, as well as the intensity of industrial, maritime and recreational activities (Egbeocha *et al.,* 2018).

Ingestion is the most likely way by which marine fauna interact with microplastics, especially when the feeding mechanisms of the organisms are non-discriminatory and do not allow them to differentiate between food and plastic fragments (Lusher *et al*., 2016; Allen *et al*., 2017). Zooplankton is the first target species as they feed on phytoplankton, making MPs bioavailable to predatory and filter feeding organisms at higher trophic levels (Ayukai, 1987). Consequently, a large number of benthonic and filter feeding organisms, such as, copepods, bivalves, fish and whales could actively target or passively ingest MPs (Lusher, 2015). Smaller sizes are more likely to be ingested by a wide range of marine biota in pelagic and benthic ecosystems (Egbeocha *et al.,* 2018).

Filter feeding organisms, such as, bivalve mollusks have been reported to ingest MPs, either in their environment (Van Cauwenberghe *et al*., 2015) or in laboratory conditions (von Moos *et al*., 2012; Xu *et al*., 2017). Lack of food and abundance of MPs in the habitat can be responsible to a higher MPs ingestion, however, mussels in comparison to invertebrates, ingested much less MPs due to selection (Egbeocha *et al.,* 2018). Filter feeding organisms have a selection mechanism like preferential clearance on the ctenidia, pre-ingestive selection on the labial palps, post-ingestive selection in the stomach and differential absorption in the gut (Brillant & MacDonald 2000). Some bivalves can accumulate it for prolonged periods of time with the risk of adverse consequences (Egbeocha *et al.,* 2018). Many marine organisms egest within hours the MPs consumed, but also has been observed MPs in the gills, digestive glands, stomach and circulatory system of bivalves, remaining in the haemolymph up to 48 days (Browne *et al*., 2008; von Moos *et al*., 2012).

Prevalence of MPs in marine organisms has several consequences, summarized in table 1.2, which are important focal points of environmental research. So far effects, such as, digestive track obstruction and blockage, internal abrasion and ulceration have been reported, but still, MPs effects on organism *in situ* are largely unknown (Egbeocha *et al.,* 2018). These effects mentioned before, can cause false sense of fullness, starvation and physical deterioration which consequently leads to weakness, reduce reproductive fitness, ability of chasing prey, avoid predators, reducing growth rates and absorption of toxins, followed by the dead of the organisms (Galgani *et al*., 2010, Cole *et al*., 2013, Wright *et al*., 2013b). Regarding the ingestion of MPs by bivalve mollusks, effects can lead to a decrease in lysosomal membrane stability and increase in the formation of tight baal-like collections of immune cells referred to as granulocytomas (von Moos et al., 2012; Egbeocha *et al.,* 2018 that are capable of overcoming host encapsulation and inducing atrophy and autolysis of the digestive gland, and consequently represent a terminal condition (Lowe & Moore 1979). Fecundity and the energy allocated for maintenance and structural growth of the organisms can be affected by MPs ingestion, thereby impairing gametogenesis and the gamete quality of bivalve's mollusks (Sussarellu *et al*., 2016), affecting negatively the population and survival (Egbeocha *et al.,* 2018).

Table 1.2. Effects of MPs ingestion on marine organisms. The class and order of the species are given; polymer indicates the type of microplastic that was ingested by the respective species. PE: polyethylene, PS: polystyrene, HDPE: high-density polyethylene, PVC: polyvinyl chloride (adapted from Egbeocha *et al*., 2018).

Class	Order	Species	Effects	polymer	Reference
Bivalvia	Mytilida	Mytilus galloprovincialis	Reduced granulocyte:hyalinocyte ratio Reduced lysosomal membrane stability Increased nuclear alteration	PE, PS	Avio et al. (2015)
		Mytilus galloprovincialis	Increase in endpoint granulocytoma formation stability Decrease in lysosomal membrane	HDPE	von Moos et al. (2012)
		Mytilus edulis and Mytilus gallopr	Significant increase in the death of hemocytes Significant production of reactive oxygen species (ROS) Reduced granulocyte concentration Significantly increased capacity for phagocytosis Induction of glycolysis and digestive activity	PS	Paul-Pont et al. (2016)
		Mytilus edulis	Irregular phagocytic activities and oxidative status	PS	Browne et al. (2008)
	Ostreida	Crassostrea gigas	Increased food consumption due to digestive interference cau Increased absorption efficiency Disturbance in homeostasis Endocrine disruption Strong negative effects on reproductive health indices	PS	Sussarellu et al. (2016)
	Imparidentia	Atactodea striata	Reduction in microplastic clearance rate	PS	Xu et al. (2017)
Gastropoda	Littorinimorp	Crepidula onyx	Reduced growth rate	PS	Lo & Chan (2018)
Copepoda	Calanoida	Centropages typicus	Drastically reduced algae feeding	PS	Cole et al. (2013)
		Calanus helgolandicus	Decreased reproductive output Sustained reduction of ingested carbon biomass	PS	Cole et al. (2015)
		Calanus helgolandicus, Centropag	Reduction in the density and sinking rate of fecal pellets	PS	Cole et al. (2016)
	Cyclopoida	Paracyclopina nana	Developmental delays Increase in intracellular ROS Reduced fecundity Increase in phosphorylation	PS	Jeong et al. (2017)
Malacostra	Harpacticoid	Tigriopus japonicus	Significant decrease in fecundity Significantly longer nauplius (first larval) stage Mortality in nauplii and copepodites	PS	Lee
	Mysida	Neomysis japonica	Severe short-term toxicity affecting survival	PS	Wang et al. (2017)
	Decapoda	Carcinus maenas	Significant reduction in O2 consumption Slight but significant drop in the concentration of Na+ ions wit Slight but significantly higher concentration of hemocyanin wi	PS	Watts et al. (2016)
Echinoidea	Camarodont	Tripneustes gratilla	Slight reduction in T. gratilla size	PE	Kaposi et al. (2014)
Pisces	Perciformes	Dicentrarchus labrax	Intestinal alteration	PVC	Peda et al. (2016)
		Dicentrarchus labrax larvae	Significant increase in mortality	PE	Mazurais et al. (2015)
		Pomatoschistus microps	Reduced predatory performance and efficiency Decreased fitness	PE	de Sa et al. (2015)
	Beloniforme	Oryzias latipes	Significant changes in gene expression mediated by estrogen Abnormal proliferation of germ cells in male testicular tissue Single cell necrosis Severe glycogen depletion	PE	Rochman et al. (2013,
Polychaeta	Capitellidae	Arenicola marina	Decreased weight Decreased feeding activity	PS	Besseling et al. (2013)
		Arenicola marina	Significant reduction in feeding Significant increase in phagocytic activity of immune cells Significant reduction in energy reserve	PVC	Wright et al. (2013)

1.6 Contaminants associated to microplastics

Microplastics are widely reported to impact the biotic components of marine ecosystems. However, their interaction with abiotic components like marine chemical contaminants can have significant consequences for marine biota. In the marine environment, adsorption of contaminants by polymers was primarily studied with mesoplastic and microplastic debris. Hydrophobic organic contaminants have a greater affinity for plastics like polyethylene, polypropylene, and PVC, than for natural sediments (Teuten *et al*., 2009).

Marine waters act as sinks for persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (Egbeocha *et al*., 2018). Although the hydrophobicity of these contaminants makes them sparingly soluble in water, it also makes these chemical pollutants preferentially adsorb onto microplastics. Consequently, microplastics are regarded as harmful pollutants (Teuten *et al*., 2007).

Contaminants after being adsorbed onto MPs, can desorb from these particles afterwards. Desorption rate will increase with the increase in the surface area to volume ratio of the MPs (Teuten *et al*., 2009). Instead, they are ingesting MPs with adsorbed contaminants that might be responsible for the accumulation of contaminants. Different shapes and colors can, indeed, determinate not only what organisms intentionally or unknowingly consume them, but also can be leached into the digestive fluids and can be transferred to cells and tissues (Boerger *et al*., 2010). Toxicants may accumulate (figure 1.1) in the tissues to produce high tissue toxicant concentrations and then also increase through transfer within a food web (biomagnification). Higher trophic level organisms are exposed to enriched concentrations of contaminants via their prey. However, researchers have shown that some contaminants, like PAHs, do biomagnify with increasing trophic level (Takeuchi *et al.,* 2009).

Results from a 2008 feeding experiment proved that PCBs were transferred from contaminated plastics to streaked shearwater chicks. Chicks fed fish laced with polyethylene pellets that were contaminated by PCBs contained PCB residues that were threefold higher than that of the control group (Teuten *et al*., 2009).

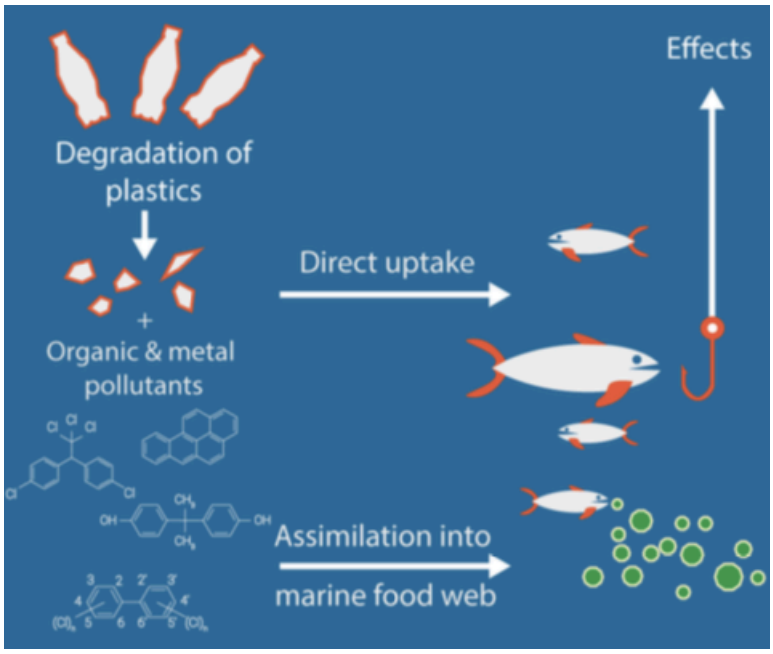

Figure 1.1. Marine contaminants in food web chain (Booth et al., 2017).

1.6.1 Benzo-a-pyrene

Benzo-a-pyrene (BaP) is the most commonly studied polycyclic aromatic hydrocarbon (PAHs) listed as persistent toxic substances by United Nations Environment Program and by the European Union. Persistent toxic substances mainly come from the incomplete pyrolysis of combustible organic material and anthropogenic sources, e.g. oil spills, litter incineration and from fossil fuel combustion, but can also be derived naturally due to several processes, such as, forest fires. PAHs enter the atmosphere first as gas or soot becoming available to the marine environment though rain and surface run off (Antunes *et al.,* 2013; Liu *et al.,* 2015; Châtel *et al.,* 2017).

BaP is ubiquitously distributed in coastal and marine environment, and due to its lipophilic nature, it resists degradation and has the potential to accumulate in organisms and biomagnify through the food web (Song *et al.,* 2016). Known by its carcinogenic properties is one of the most toxic PAHs for the humans, classified by the International Agency for Research on Cancer (IARC, 2011; Liu *et al.,* 2015; Châtel *et al.,* 2017).

1.7 Biomarkers

Biomarkers are measurable indicators of some biological disturbance or condition of the normal function of an organism responding to a toxicant (Nikinmaa, 2014). Ranging from molecular through cellular and physiological responses to behavioral changes, which can be related to exposure to or toxic effects of environmental chemicals (Peakall and Walker, 1994). To clarify any effects of exposure of those contaminants within microplastics to marine organisms, a set of biomarkers have been used including, oxidative stress. A good biomarker is: fast, cheap and easy to measure; measurements are specific to the toxicant; and a concentration or dose response relationship is shown. There are biomarkers of effect and exposure, which measures the disturbance resulting from exposure, and the biomarkers of exposure that indicates if the organism has been exposed to a toxicant (Nikinmaa, 2014).

1.7.1 Oxidative stress

Organisms consume molecular oxygen that has to be tetravalent reduced to water at the same time that is coupled to the oxidation of food and energy production (figure 1.2). This reduction leads to the production of reactive oxygen species (ROS) that include: superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($RO2^{\bullet}$), the alkoxyl radical ($RO^{\bullet}$) and the hydroperoxyl radical ($HO^{\bullet}$). Non-radical species of ROS are also part of oxidative damage including: hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL) single oxygen (O) and peroxynitrite ($ONOO^-$). Those different types of oxygen are produced as unwanted products by haem proteins and auto oxidation. Enzymes such as superoxide dismutase (SOD) and catalase (CAT) act as a defense system for ROS formation and are used as biomarkers of susceptibility of environmental contaminants (Livingston, 2001; van der Oost, Porte-Visa and van den Brink, 2005). These enzymes are produced into the cytoplasm and membrane-bound organelles. SOD protects the cell from dangerous chemical reactions, catalysing the portioning of the superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide and water thus reducing the potential for cellular oxidative damage to occur (Jo *et al*., 2008; Nikinmaa, 2014). Catalase (CAT) prevents cellular damage from ROS by reducing H_2O_2 to H_2O (Oliveira *et al.,* 2009; Solé, Kopecka-Pilarczyk and Blasco, 2009). Glutathione-S-transferases (GSTs) are mainly involved in detoxification but also have an antioxidant role with some

isoforms inhibiting lipid peroxidation (Solé, Kopecka- Pilarczyk and Blasco, 2009; Nikinmaa, 2014).

Biomarkers of oxidative stress rely on the fact that a change in the activity of the ROS defense system, such as, antioxidant enzymes, can occur as a result of environmental contamination. When the production of ROS exceeds antioxidant defenses cells will experience oxidative stress (Viarengo *et al.,* 2007).

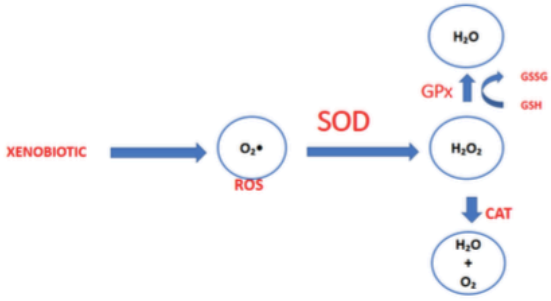

Figure 1.2 Schematic diagram of antioxidant ROS defense mechanism system.

1.7.2 Oxidative Damage

Oxidative damage happens when oxidative stress reaches a certain level that may cause oxidative damage to the DNA, proteins, carbohydrates and lipid membranes. Lipid peroxidation (LPO) is the process in which free radicals react in cell membranes and from lipid hydroperoxides resulting in cell damage. Hydroperoxides decompose unsaturated fatty acid double bonds and dismantle membrane lipids (Oliveira *et al*., 2009).

1.7.3 Neurotoxicity

Acetylcholinesterase, AChE, is known to be involved in catalysing the breakdown of acetylcholine and other choline esters and an essential enzyme in the transmission of the nerve impulse frequently used in marine pollution monitoring (Viarengo *et al.,* 2007; Tsangarisetal. 2010). It is also found in the neuromuscular junctions and chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transition.

AChE has been known to respond to low levels of contaminants in the environment, being inhibited by carbamate pesticides, some metals in invertebrates (van der Oost, Porte-Visa and van den Brink, 2005; Solé, Kopecka-Pilarczyk and Blasco, 2009).

1.8 Bioindicators

Bioindicator are species which indicate the harmful effects of contamination of an environment. These effects may be either measurable responses in the organism or changes in the number of species, or proportion of species within communities. The alterations in these organisms can be physiological, genetical, biochemical, morphological and ecological, that may be used as an estimation of ecosystem health if they occur in a wide range of environments (Moreira *et al*., 2011). Sessile organisms are particularly relevant at this matter due to their high tolerance to chemical exposure, sedentary lifestyle and low metabolism, reflecting the *in-situ* effects of contamination (Dixon *et al*., 2002; Moreira *et al*., 2011).

1.9 Scrobicularia plana

Scrobicularia plana is a long-living bivalve mollusc belonging to the family *Semelidae* (table 1.3), commonly named peppery furrow shell. This species normally inhabits in the intertidal areas and estuaries with muddy to sandy substrates with abundant organic detritus. This bivalve is a suspension-feeder, with long siphons, burying itself up to 20 cm deep in mud and sandy substrate. When buried, they make a star-shape at the sediment surface, what makes it easy to find. According to Montserrat et al. (2009), *S. plana* has a wide geographical distribution range from the Norwegian Sea to Senegal including the Mediterranean (Tebble, 1976; Casagranda and Boudouresque, 2005).

This species is a suspension-feeder which primarily feeds on particles of surface deposits, but also obtains part of the food by filtering suspended matter from the water column (Hughes, 1969; Moreira *et al*., 2011). Concentric growth rings are visible on the shell exterior, which is a pale grey and yellow color as can be seen in figure 1.3. Adults may grow up to 65 mm and are known to live up to 18 years (Green, 1957; Santos *et al.,* 2011).

Table 1.3. Classification of *S. plana* within the Animal Kingdom (Marinespecies.org., 2019)

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

Figure 1.3. *Scrobicularia plana* shell. Photo: Author

1.10 Objectives

The objectives of this thesis were to determine the ecotoxicological effects in *S. plana*, when exposed to contaminated and uncontaminated low density microplastics. *S. plana* was exposed to two different sizes of microplastics, 4-6µm and 20-25 µm, with and without BaP adsorbed for 14 days. To determine the ecotoxicological effects a set of biomarkers were analysed namely oxidative stress including the quantification of

antioxidant enzymes activities (superoxide dismutase - SOD and catalase - CAT) and biotransformation enzyme activity (glutathione-S-transferases - GST), oxidative damage (lipid peroxidation - LPO), and neurotoxicity (acetylcholinesterase - AChE) in the gills and digestive gland. The condition index was evaluated to assess the overall health status of the organism. To have a better impact perspective for the effect of these contaminants in this bivalve species an integrated biomarker response index (IBR) and a Health Index was applied.

2 Material and methods

2.1 Microplastics and contaminants

The LDPE microplastics (MPP-635G), used for contamination were obtained from MicroPowders Inc. (USA). Chemicals were obtained from Sigma Aldrich. Sorption of contaminants (BaP) to microplastic particles was conducted by the Man-Technology-Environment Research Centre, Department of Natural Science, Örebro University, Sweden.

2.2 Experimental design

Clams, *S. plana,* were collected in Cabanas de Tavira, Ribeira do Almargem in Ria Formosa, (N 37°7 59.75″ W 7 36′34.95″), (figure 2.1), at low tide in two different days and transported alive to the laboratory in thermal boxes filled with seawater from the same place. Clams were collected in April, when there was no sexual activity. A total of 420 clams were collected.

Figure 2.1. Location were *S. plana* samples was collected (Google maps).

Before sampling the clams, the top 30 cm of the sediments were collected from the same site and passed through a sieve (4mm) to eliminate possible macro-organisms and sediment debris. Afterwards, the sediment was dried at 60°C for 48h, to reduce organic matter content and volatile compounds. Then the sediments were rehydrated using the original % of water present when collected, calculated by the difference in wet

weight of a known volume of sediment after reaching constant dry weight at 60°C. The 25 L aquarium were filled with 4 L of sediments and 16 L of seawater and constant aeration was supplied using glass Pasteur pipettes at the bottom of plastic aeration tubes, to minimize plastic contact with the experimental medium. See figure 2.2.

In the laboratory the air temperature was set at constant 19°C to maintain constant seawater temperature. Clams were acclimatized for 3-4 days in the aquaria, with a photoperiod of 12 hours light to 12 hours dark. 84 clams were introduced in each aquarium.

Figure 2.2. Aquariums used for exposure with 4 L of sediment and 16 L of seawater and constant aeration. Photo: Author.

The exposure experiment consisted in 5 aquaria with 5 treatments. One aquarium represents the control and the remaining represent the different exposure treatments as figure 2.3 demonstrates. The clams, *S. plana,* were exposed to LDPE microplastics (4-5 and 20- 25 µm), at a concentration of 1 mgL^{-1}, with and without BaP adsorbed. The exposure lasted 14 days, and samples were collected at different times of exposure namely in the beginning and after 7 and 14 days of exposure.

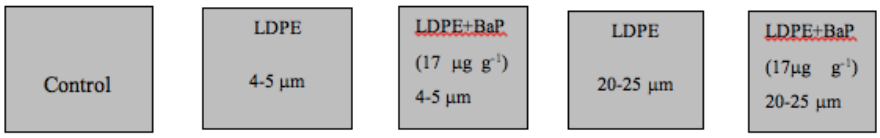

Figure 2.3. Experimental set up. Control aquarium (no addiction of MPs during all exposure). Two aquarium were contaminated with 4-5 µm with and without BaP adsorbed the two with MPs of 20-25 µm with and without BaP adsorbed.

During the experiment the water was changed every 48 hours as well as the application of microplastics. No food was given during the exposure to minimize the interaction between organisms and microplastics. Abiotic parameters were checked by measuring temperature (19.3 ± 0.2°C), salinity (35 ± 1), percentage of oxygen saturation (95.8 ± 1.8%), and pH (8.05 ±0.04), using a multiparametric probe (ODEON V3.3.0).

At each sampling time, clams, *S. plana,* were randomly collected from each aquarium before water changing and plastic addition. On day 0 before the addition of microplastics (MPs), 6 clams per treatment were collected, with no distinction being made between aquarium. After 7 days of exposure, 6 clams were collected from each aquarium for the analysis of enzymes activity, LPO, AChE and CI) were taken from each aquaria with a different treatment. At the 14th day of exposure, 6 clams per analysis (enzymes, LPO, AChE, CI, BaP and MPs accumulation) were taken from each aquarium. At each sampling, gills and digestive gland were dissected immediately, placed in micro-centrifuge tubes, flash frozen in liquid nitrogen and stored at -80°C for further analyses. For microplastic quantification, all the soft tissue was stored in aluminium foil at -20°C.

2.3 Condition Index

Condition index (CI) was assessed in 6 individuals from each treatment and time of exposure. At day 0 (pre-exposure) of the experiment 6 individuals were randomly sampled from each of the 5 aquariums. 6 individuals were sampled from each aquarium, per each day of exposure analysed (7th and 14th day), in order to determine the physiological status of both control and exposed clams during the duration of the experiment. Tissues were dried at 75°C until a constant dry weight was achieved.

Condition Index was assessed by calculating the percentage (%) of the ratio between dry weight of the soft tissues (g) and the dry weight (g) of the shell (Walne, 1976).

2.4 MPs Quantification

MPs quantification in the whole soft tissues of *S. plana*, was assessed on 6 individuals from day 0 (pre-exposure), and after the 14th day of exposure (6 replicates per treatment) and previously stored in aluminium foil at -20ºC.

Samples were dried for 48 hours at 75ºC to obtain the dry weight (g). In the flow chamber, samples were placed, individually, in glass flasks along with 3 mL of nitric acid to digest the organic matter for 24 hours, as it shows in figure 2.4. An extra of 5 mL of nitric acid was added, and flasks placed in a heating plate, at 60ºC, in order to evaporate the nitric acid as much as possible without losing sample material. Nitric acid was not added all at once to avoid flask overflow and consequently loss of sample. During the entire process each flask was covered with aluminium foil to avoid contamination.

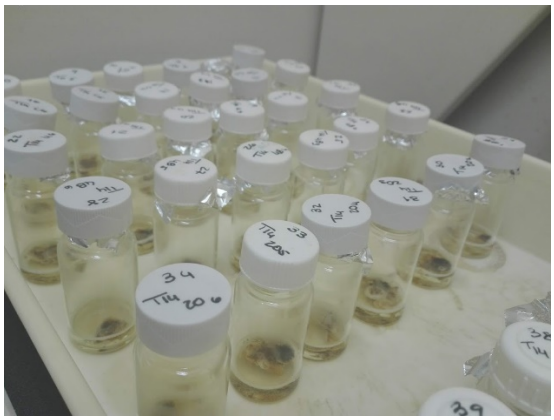

Figure 2.4. Flasks used for the digestion of the organic matter using nitric acid. Under the caps were used aluminum foil to avoid plastic contamination. Photo: Author.

After digestion, 20 mL of pre-filtered distillate water was also added in each flask, to avoid any contamination, and to dilute the nitric acid preventing filter damage. To eliminate unwanted particles, a density separation was performed using a NaCl solution (140 molL^{-1}). Each sample was, kept in this solution for 1 hour, in a beaker, along with 8 drops of Nile red dye. Nile red is a lipophilic stain that was used to bound to the MPs.

Each sample, after being separated by density, were filtrated using filtration equipment, as represented in figure 2.5A, and cellulose 0.2 μm filters to prevent MPs loss (figure 2.5B). Filtration was performed in a laminar flow chamber, only glass material was used during the entire procedure.

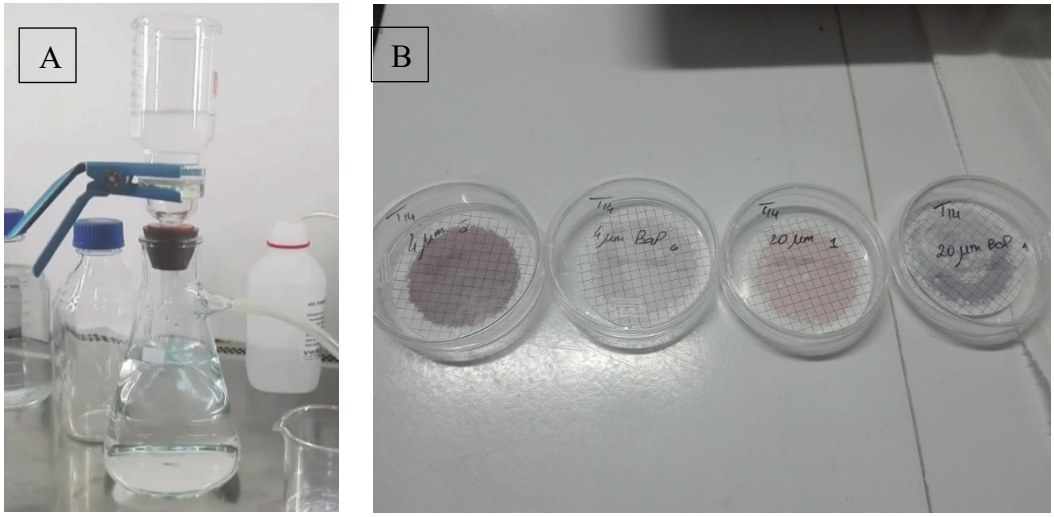

Figure 2.5. MPs filtration equipment in the laminar flow chamber (A); Cellulose 0.2 µm filters after filtration of the organic matter previously digested (B). Photo: Author.

MPs quantification was assessed in a fluorescence microscope, Leica DMLB, (figure 2.6A). MPs dyed with Nile red when expose to ultraviolet light emits fluorescent light, making the counting possible and easier, see figure 2.6B. Each filter had 100 grids, but due to the number of MPs in each filter, only 30 grids were counted and afterwards an estimation was made for the 100 grids.

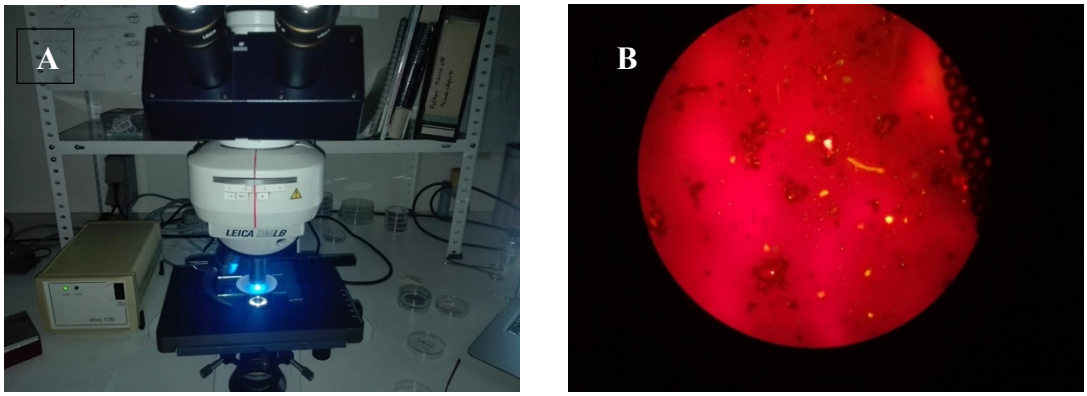

Figure 2.6. Fluorescent microscope used for the MPs filters quantification (A). Filter view from the fluorescent microscope. Yellowish dots represent the MPs (B). Photo: Author.

2.5 Enzyme Activity

2.5.1 Homogenisation of Tissues

For the determination of enzyme activities, 6 individuals from each treatment and time of exposure were collected. At day 0 (pre-exposure), 6 individuals were randomly sampled from each of the 5 aquariums. 6 individuals were sampled from each aquarium, per each day of exposure analysed (7^{th} and 14^{th}). For the determination of enzyme activities preserved tissues at -80 °C were defrosted, weighted and homogenized, on ice, in 5 mL of Tris sucrose buffer (Sucrose 0.5 M, Tris 20 mM, KCL 0.5 M, DTT 1 M, EDTA 1 mM, at pH 7.6). The homogenate was centrifuged at 500 *g*, for 15 minutes at 4 °C. The supernatant was transferred to another centrifuge tube and centrifuged a second time at 12000 *g*, for 45 minutes at 4 °C. Supernatant was recovered and divided in 4 aliquots, stored in Eppendorf tubes and frozen at -80 °C for further determination of SOD, CAT and GST activities and total protein concentration.

2.5.1.1 Superoxide Dismutase (SOD) activity

SOD activity was determined using the colorimetric method described by McCord and Fridovich, (1969). The homogenate of each tissue previously prepared was defrosted on ice and vortexed. 2.650 µL of phosphate buffer (50 mM, with EDTA 0.1 mM, at pH 7.8), 100 µL hypoxanthine (1.5 mM), 100 µL of cytochrome *c* oxidase (0.15 mM), 50 µL of the sample from each tissue and 100 µL of xanthine oxidase (56 mU/ml) were added. The percentage of inhibition of the absorbance of cytochrome *c* as a result of the superoxide anion generated by the xanthine/hypoxanthine reaction was measured at 550 nm for 1 minute using a spectrophotometer and used to determine the amount of SOD activity present. Samples were run in triplicate. SOD activity is expressed in Units (U) mg^{-1} protein, where 1 U of activity corresponds to the amount of sample required to cause 50% inhibition. The following formula was used to determine SOD activity:

$$\% \, I = | \, 1 - (\text{average } \Delta OD_{Sample}) / (\text{average } \Delta OD_{\text{Xanthine Oxidase}}) \, |$$

where:

I = Inhibition

ΔOD = Variation in Absorbance

2.5.1.2 Catalase (CAT) activity

CAT activity was determined by measuring the changes in absorbance at 240 nm that corresponds to the consumption of hydrogen peroxide (H_2O_2), according to Greenwald (1987). The homogenate of the gills and digestive gland collected at each time of exposure and prepared as described in 3.4.1were defrosted on ice and vortexed. 1900 µL of phosphate buffer (pH 7.5), 1000 µL H_2O_2 and 100 µL of sample, respectively were added on a quartz cuvette. The absorbance was read in a spectrophotometer at 240 nm for 1 minute, with two replicates per sample. CAT activity (µmol min^{-1} $mg^{-1}$of total protein concentration) was calculated according to the following formula:

$$\text{CAT activity} = \frac{\left(\frac{\Delta 0D}{40}\right) * \left(\frac{\text{Vol total}}{\text{Vol sample}}\right)}{\text{Prot (mg/ml)}}$$

where:

V total = volume of cuvette, 3 mL

Vol sample = 100 mL

40 M^{-1} cm^{-1} = extinction coefficient of H2O2

ΔOD = Variation in absorbance

2.5.1.3 Glutathione-S-Transferases (GST) activity

GST activity was determined on the cytosolic fraction of the clam gills and digestive gland following method described by Habig and Jakoby (1974), adapted to the microplate reader by McFarland *et. al.* (1999). Glutathione-S-transferases catalyze nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom (Hayes *et al.,* 2005). All GST families contain members that catalyze the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB)

and exhibit glutathione peroxidase activity toward hydroperoxide (CuOOH). Dinitrophenyl thioether is produced as a result of the reaction, whose absorbance was detected at 340 nm in a microplate reader Tecan (Infinite 200 Pro). Samples were run in triplicate, and absorbance was read every 30 seconds over a 3-minute period, at room temperature. The following formula was used to calculate GST activity (in nmol CDNB min^{-1} mg $protein^{-1}$):

$$GST\ activity = \frac{(Abss - Absb) * Voltotal * DF}{(9.6 * 0.6135 * Vols * [protmg/mL]) * 1000}$$

where:

Abss = Absorbance of the sample (OD/min)/t

Absb = Absorbance of the blank (OD/min)

Voltotal = Total volume per well (0.225 ml)

DF = Dilution Factor

CDNB Extinction coefficient = 9.6 (mM^{-1} cm^{-1}) Light path = 0.6135 (cm)

Vols = sample volume (0.025 ml)

2.6 Acetylcholinesterase (AChE) Activity

AChE activity was assessed in the gills tissue of 6 individuals at day 0 (pre-exposure), from each treatment, and at the 14^{th} day of exposure (6 replicates per treatment), according to Ellman's colorimetric modified protocol (Ellman's *et al.* 1961), where thiocholine is produced as AChE hydrolyses to acetylcholine. Thiocholine reacts non- enzymatically with DNTB releasing 5-mercapto-2-nitrobenzoato compound whose color is yellow. The increase of the absorbance of this yellow compound was measured at 405 nm, with an extinction co-efficient of ε = 13.6 mM^{-1} cm^{-1} in order to estimate the

amount of thiocholine produced which is proportional to the AChE activity (Colovic *et al.,* 2013).

The gills were defrosted on ice, weighted and homogenized in 5 mL of Tris HCl buffer (100 mM, pH 8.0) and 50 µL of Triton- X 100 (0.1%). The homogenate was centrifuged at 12000 *g,* for 30 minutes at 4ºC. After centrifugation, the supernatant was divided into aliquots and placed in two Eppendorf's using a pipette and stored at -80ºC. The first aliquot was used for the determination of AChE activity and the second for the determination of total protein concentration.

To determinate AChE activity, 50 µl of each gill was added, in triplicate, to a 96 well microplate. 200 µl of 5,5'-dithio-bis (2- nitrobenzoic acid) (DNTB, 0.75 mM) solution was added to each well and incubated for 5 minutes at room temperature. Then, 50 µl of acetylcholine solution (ATC, 3 mM) was added to each well to trigger the reaction, described below. The microplate was incubated for 10 minutes at room temperature. The absorbance was read at 405 nm using microplate reader Tecan (Infinite 200 Pro), in 30 second intervals for 5 minutes.

AChE activity, measured in nmol ACTC min^{-1} mg $protein^{-1}$, was quantified using the following formula:

$$AChE\ Activity = \frac{\Delta A405 * Volt}{\varepsilon * Lightpath * Volts * [proteins]} * 1{,}000$$

where:

ΔA405 = The variation of absorbance at 405 nm measured per minute.

Volt = 0.300 ml, the total volume of mixture per well.

Ɛ = DNTB Extinction Co-efficient = 13.6 mM cm^{-1}

Light path (The length of light passing through the microplate wells) = 0.8385 cm

Vols = Volume of the sample = 0.05 ml

[Proteins] = Concentration of proteins in the supernatant fraction quantified using the Bradford assay

2.7 Lipid Peroxidation (LPO)

LPO was assessed in the gills and digestive gland of 6 individuals collected from each treatment at day 0 (pre-exposure), and at the 7th and 14th day of exposure (6 replicates per treatment), according to the colorimetric method described by Erdelmeier *et al.* (1998). Both tissues were defrosted on ice, weighted and homogenized on ice, in 5 mL of Tris HCl buffer (0-02 M, pH 8,6) and 50 µL of butylated hydroxytoluene solution (BHT). The homogenate was centrifuged at 30000 *g*, for 45 minutes at 4ºC. The supernatant was divided into two aliquots that were stored at -80ºC. The first aliquot was used for the determination of LPO levels and the second for the quantification of total protein concentration.

To determinate LPO levels, 200 µL of gills and digestive gland samples from control, exposed to LDPE 4-5 and 20-25 µm size range virgin MPs and also adsorbed to BaP, were mixed with both, 650 µL of 1-methyl-2-phenylindone diluted in methanol, and 150 µL of methanesulfonic acid (15.4 M), and put into a water bath at 45ºC for 60 minutes. After the mixture was centrifuged at 15,000g for 10 minutes at 4ºC. 150 µL of the supernatant was added in quadruplicate to a 96 well microplate. The following reaction was used to determine lipid peroxidation through the quantification of Malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides: Two moles of N-methyl-2-phenylindole (chromogenic reagent) + one mole of MDA incubated at 45 ºC for 60 minutes. Malondialdehyde bis (dimethyl acetal) was used as a standard.

The absorbance was read using Tecan (Infinite 200 Pro) at 386 nm in a microplate reader, over 30 seconds at room temperature.

MDA and 4-HNE was quantified, according to the following formula:

$$MDA(nmol.mg^{-1}\,protein) = \frac{\left(\frac{Abs-b}{a}\right) * \left(\frac{\mu mol}{l}\right) * volume\ Tris(ml)}{\frac{Weight\ tissue\ (g)}{Total\ protein\ (\frac{mg}{g})}}$$

where:
Abs = Absorbance of the sample
a & b are obtained from the standard curve equation

2.8 Total Protein Concentration

To determine the total protein concentration in the cytosolic fraction of the gills and digestive gland. the Bradford method was used (Bradford, 1976) to normalize the enzyme activities and LPO levels. The principle of the method is based on the absorbance shift of Coomassie Brilliant Blue G-250 dye. Standard protein dilutions were prepared, from 0.005 - 1.0 mg ml^{-1}, using bovine serum albumin (BSA), to obtain the standard curve equation. Milli-Q water was used as a blank (0 mg ml^{-1} protein). Samples assessed from the tissue homogenisation (3.4.1.), were defrosted on ice, diluted 1/5 with Milli-Q water and vortexed. 5 µL of each sample, blank, or standard was added, in quintuplet, to a 96 well microplate reader. 200 µL of diluted Bradford solution (1:5) was added to each well. Microplates were incubated in a microplate reader using Tecan (Infinite 200 Pro) at 595 nm, for 20 minutes at room temperature. The increase in absorbance is proportional to the amount of bound dye and therefore to the amount of protein present in the sample. To determine total protein concentrations the following formula was used, and proteins are expressed as mg g^{-1} of wet tissue weight:

$$\text{Concentration (mg ml}^{-1}) = ((\text{Average Abs} * b)/(a))$$

And:

$$\text{Protein (mg g}^{-1}) = \text{Concentration} * (\text{vol Tris(ml)}/ \text{W tissue (g)})$$

where:

Concentration: takes into account the dilution factor used *a & b* are obtained from the standard curve equation

vol Tris = Buffer volume used to dilute the sample before homogenization = 5 ml

W tissue = Wet weight of gill tissue sample before homogenization

2.9 Integrated Biomarker Response Index (IBR)

Integrated biomarker response index relies on assessing biological effects measured through a set of biomarkers and CI, from gills and digestive glad tissue. This method was first defined by Beliaeff and Burgeot (2002), described by Serafim *et al*. (2012) and later modified by Sanchez *et al*. (2013), which is now called IBR version 2. A sum of the deviations between the different treatments with different LDPE MPs sizes with and without BaP, from each individual biomarkers and CI (Xi) was compared to the data of each biomarker and CI of the control group (X0) and log transformed (Yi) to reduce variance (Yi = log (Xi / X0)). Mean (μ) and standard deviation (σ) were calculated for Yi and data standardized using the following formula:

$$Zi = (Yi - \mu)/\sigma$$

Afterwards, the next area (A) was calculated as the difference between the mean of standardized biomarker response and CI (Zi) of each exposed group and the mean of the unexposed biomarker data and CI (Z0

$$A = Zi - Z0$$

Lastly, to obtain the IBRv2 index, the absolute value of A is summed for each parameter in each experimental condition:

$$IBR = \Sigma\,|A|$$

2.10 Health Index (HIS)

The Health Index (HIS) was developed and calibrated using biomarkers from active biomonitoring and subsequently applied on datasets obtained from either mesocosms experiments or field studies. The aim of the present method is to develop and test an objective decision-support expert system capable of integrating biomarker results into a five-level health-status index. The expert system is based on a set of rules derived

from available data on responses to natural and contaminant-induced stress of marine animals. Integration of parameters includes: level of biological organization; biological significance; mutual inter-relationship; and qualitative trends in a stress gradient. Details of the expert system and algorithm description are in Dagnino et al. (2007). The health index was applied in the gills and digestive gland considering biomarkers over a stress gradient yield characteristic trend such as: bell-shaped (SOD, CAT, GPx), increasing (LPO ad DNA damage) or decreasing (AChE, CI). The results were integrated into a five-level health status index such as A (high), B (good), C (moderate), D (poor) and E(bad), following the classification established by the European Union Water Framework Directive.

2.11 Statistical Analyses

Data are expressed as mean ± standard deviation (SD). Statistical differences between treatments (Ctrl, 4 μm, 4 μm+BaP, 20 μm, 20μm+BaP), time of exposure (T0, T7 and T14) and different tissues (gills and digestive gland) were assessed by using parametric tests, in this case, two-way ANOVA by Graphpad Prism 8. Significant ANOVA results were analyzed using Tukey's HSD test. A PCA test was performed using PAST 3. Graphics were performed using Graphpad Prism 8. Any difference with a p value ≤ 0.05 was considered significant.

3 Results

3.1 Condition Index (CI)

The CI of the organism's showed no significant differences between treatments for the same exposure day ($p > 0.05$) (Table 3.1) or between sampling times of the same treatment ($p > 0.05$). Results indicate that *S. plana* remained in good health during the whole experiment.

Table 3.1. Condition Index (mean± SD) from each treatment (Ctrl, 4-5 µm, 4-5 µm + BaP, 20-25 µm and 20-25 µm + BaP) at the different times of exposure.

	Day 0	Day 7	Day 14
	Mean ± SD	Mean ± SD	Mean ± SD
Ctrl	12.1±0.4	10.8±0.4	8±0.5
4-5µm	12.1±0.5	8.8±0.4	12±0.4
4-5µm + BaP	12.1±0.6	8.7±0.7	6.6±0.4
20-25µm	12.1±0.7	12±0.4	9.5±0.5
20-25µm + BaP	12.1±0.8	9±0.4	8.7±0.2

3.2 MPs accumulation

MPs accumulated in clams exposed to different sizes of MPs with and without BaP (figure 3.1). MPs accumulation in MPs exposed clams showed a significant increase through the exposure time and when compared to control after 14 days ($p>0.05$).

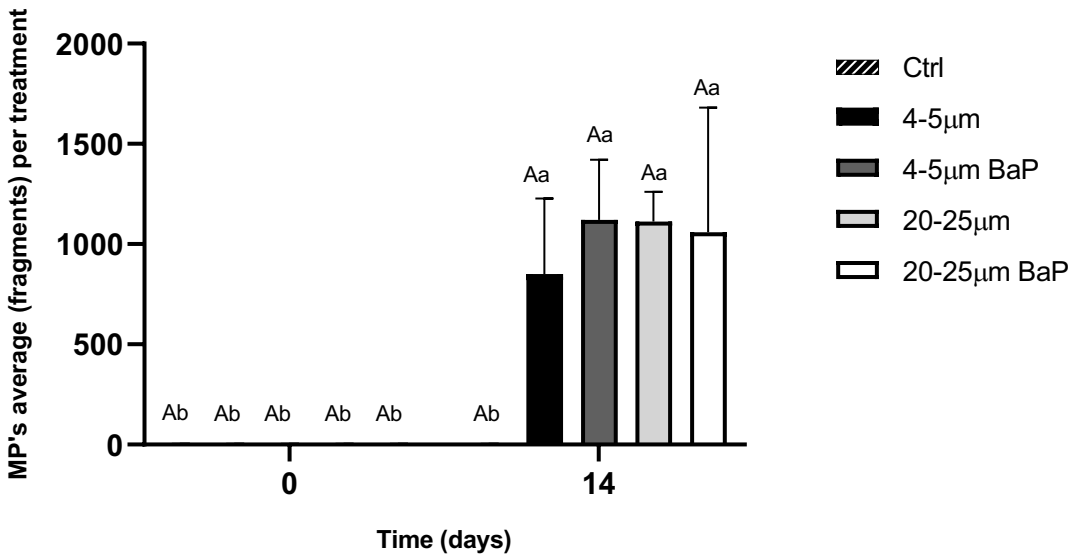

Figure 3.1. MPs quantification (mean ± SD) in the soft tissue of *Scrobicularia plana* for different treatments per time (0, 14). Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.3 Benzo-a pyrene Bioaccumulation

BaP accumulation in the clams whole soft tissues from the different treatments are in Table 3.2. Data shows that BaP accumulation increase with the size of MPs + BaP being higher in clams exposed to 20-25 μm+BaP.

Table 3.2. Benzo-a pyrene concentration after 14 days of exposure.

Treatment	Benzo-a pyrene (μg/kg WW)
Control (0)	1.00
Control (14)	0.50
4-5 μm	0.61
4-5 μm BaP	2.67
20-25 μm	0.67
20-25 μm BaP	4.94

3.4 Enzyme Activity

3.4.1 Antioxidant (SOD and CAT)

Antioxidant enzymes, SOD and CAT activities in clam gills and digestive gland for each MPs treatment with and without BaP (control, 4-5 μm, 4-5 μm + BaP, 20-25 μm and 20-25 μm + BaP) at different times are displayed in figure 3.2 and 3.3, respectively. SOD activity in the gills in clams exposed to 20-25 μm MPs+BaP significantly increases by day 7 when compared to day 0, followed by a significant decrease by day 14 when compared to both day 0 and day 7 (p<0.05). On day 7 the clams exposed to 20-25 μm MPs+BaP also have higher SOD values when compared to the other treatments (p<0.05). In clams exposed to 20-25 μm MPs treatment there is a significant decrease in SOD activity on day 7 when compared to the other treatments, except 4-5 μm MPs, on the same day (p<0.05), with values remaining significantly lower than control on day 14 (p<0.05). On day 14, all MPs treatments have lower SOD values than control (p<0.05, figure 3.2.A).

In relation to the digestive gland (figure 3.2B), SOD activity is lower than the gills. SOD activity in the digestive gland significantly decreases in clams exposed to the 4-5 μm MPs+BaP and 20-25 μm MPs+BaP treatment at the 14th day of exposure when compared to the other treatments (p < 0.05).

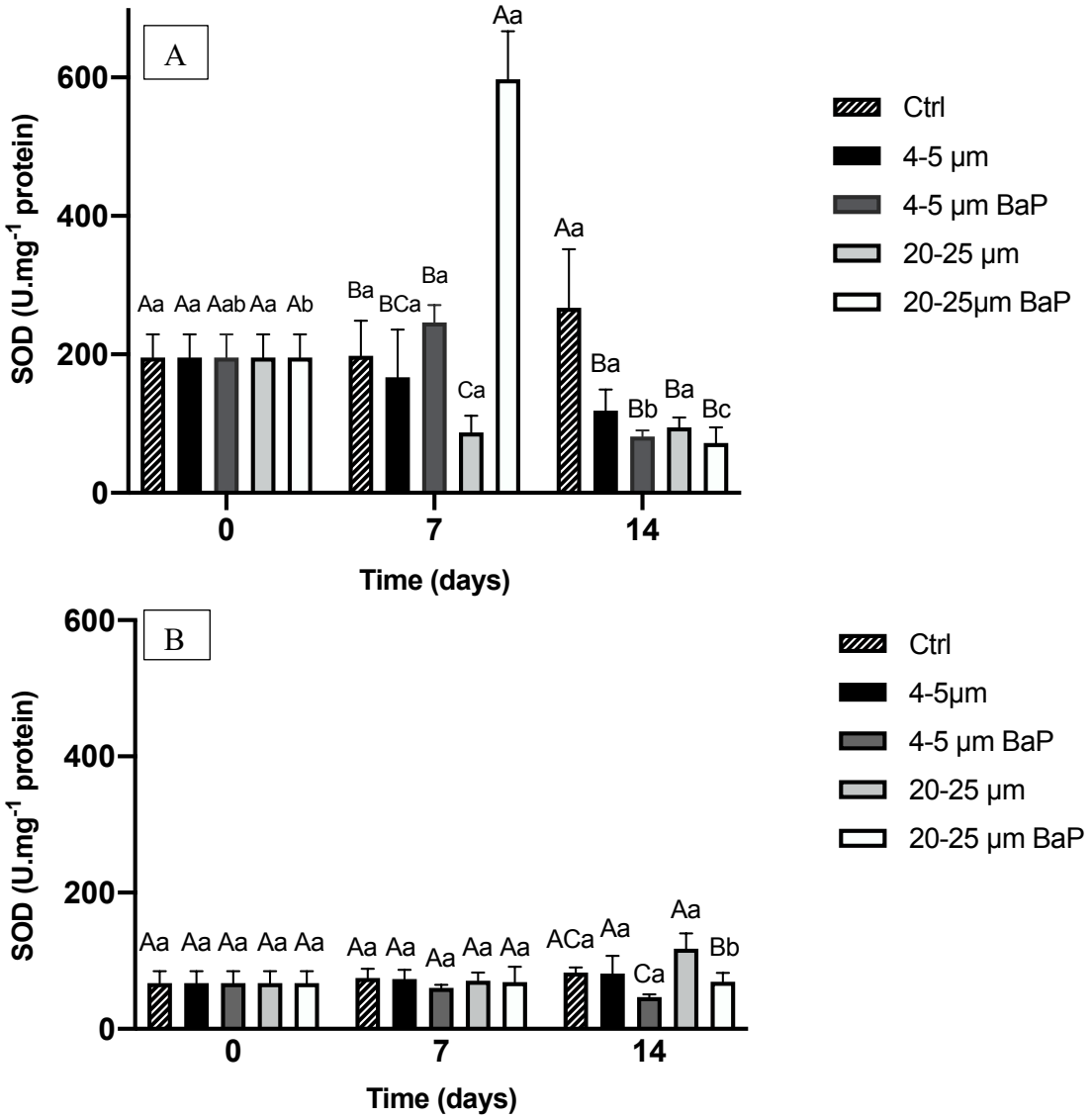

Figure 3.2. SOD activity (mean ± SD) in the gills (A) and digestive gland (B) of *Scrobicularia plana* for unexposed and exposed to 4-5 and 20-25 µm of MPs with and without BaP adsorbed in the beginning and after 14 days of exposure. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

CAT activity is much higher in the digestive gland than in the gills (Figure 3.3A). CAT activity in the gills, only shows a significant decrease for 4-5 µm MPs+BaP and 20-25 µm MPs+BaP treatment at the 7th day (p<0.05) when compared to control (Figure 3.3A). In the digestive gland only the 4-5 µm MPs+BaP on day 14 is significantly lower than 20-25 µm MPs (p<0.05) (figure 3.3.B).

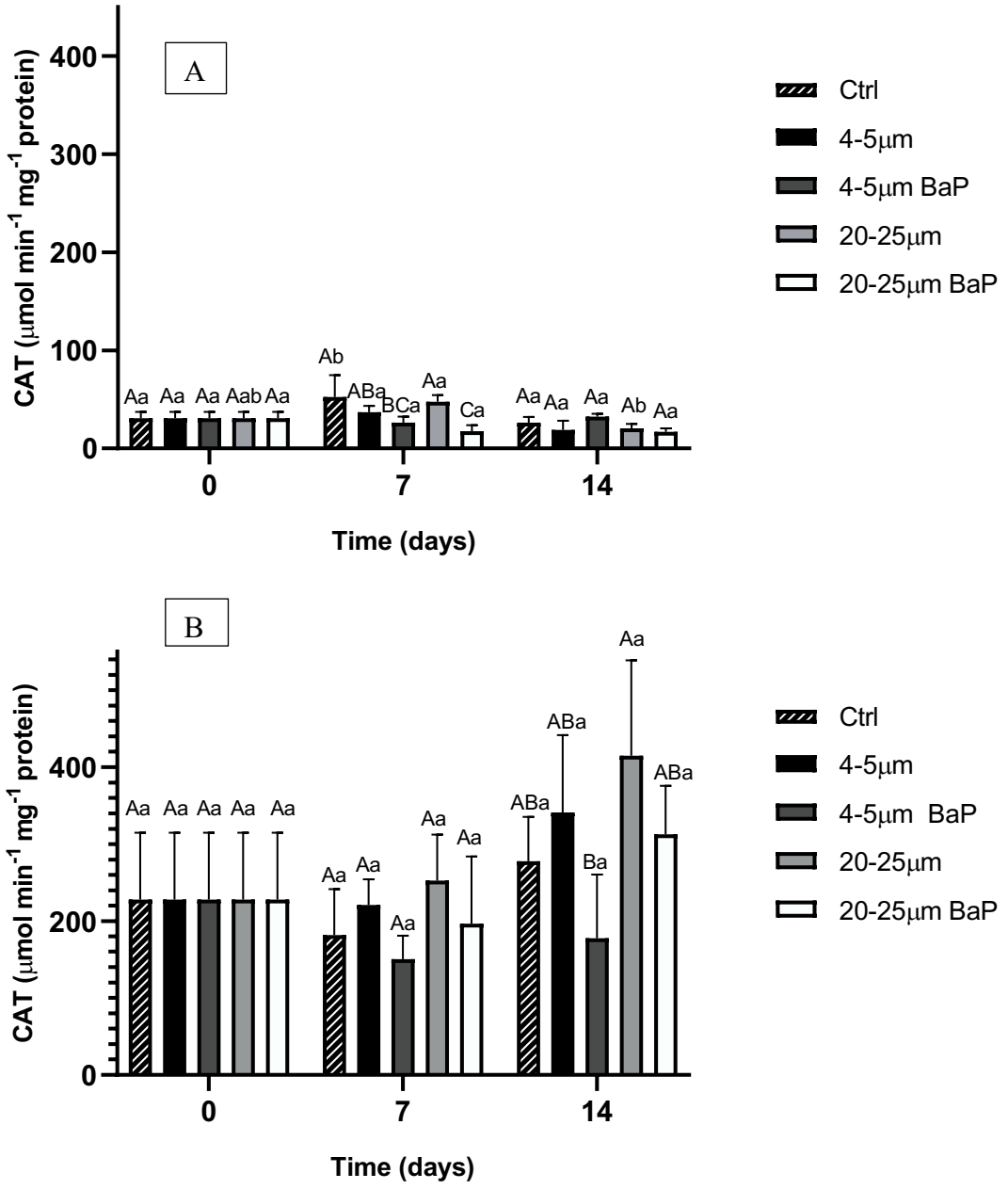

Figure 3.3. CAT activity (mean ± SD) in the gills (A) and digestive gland (B) of *Scrobicularia plana* for unexposed and exposed to 4-5 and 20-25 μm of MPs with and without BaP adsorbed in the beginning and after 14 days of exposure. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.4.2 GST activity

On day 7, a significant increase in GST activity was noticed in the gills for the clams exposed to 20-25 μm MPs+BaP, when compared to the other treatments and also in relation to both day 0 and day 14 ($p<0.05$) (figure 3.4.A). GST activity for the digestive

gland remain relatively unchanged through time with no significant differences between treatments ($p > 0.05$) (figure 3.4.B).

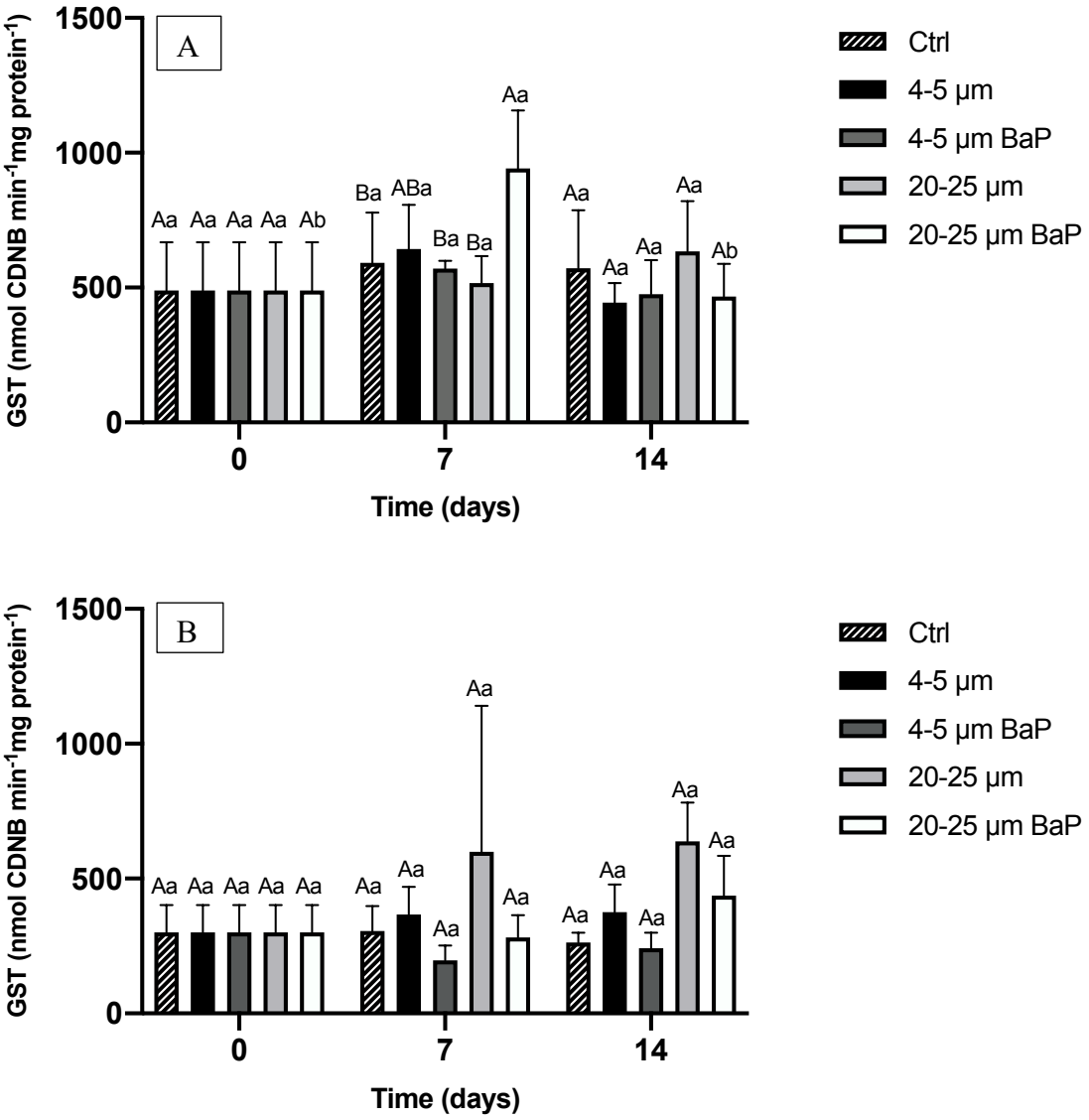

Figure 3.4. GST activity (mean ± SD) in the gills (A) and digestive gland (B) of *Scrobicularia plana* for unexposed and exposed to 4-5 and 20-25 µm of MPs with and without BaP adsorbed in the beginning and after 14 days of exposure. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.5 Acetylcholinesterase (AChE) activity

Significantly higher AChE activity was found in the gills of *S. plana* ($p < 0.05$), on day 14th when exposed to 4-5 µm MPs and to 20-25 µm MPs+BaP, when compared to

day 0 (figure 3.5). After 14th day of exposure there is a significantly higher AChE activity in clam gills exposed to 4-5 μm MPs when compared to controls and to 20-25 μm treatments ($p< 0.05$).

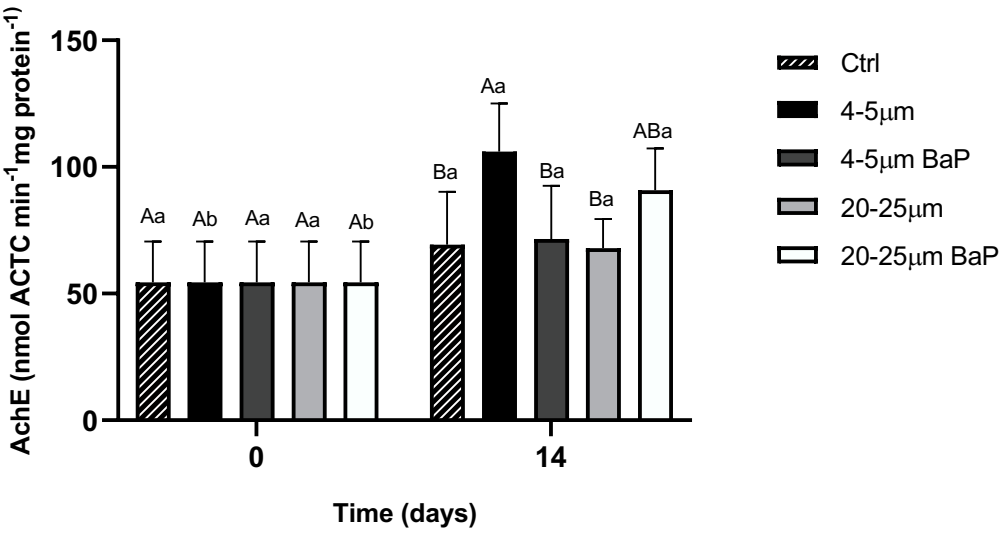

Figure 3.5 AChE activity (mean ± SD) in the gill of *Scrobicularia plana* for unexposed and exposed to 4-5 and 20-25 μm of MPs with and without BaP adsorbed in the beginning and after 14 days of exposure. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.6 Lipid peroxidation (LPO)

Only a significant increase in LPO levels occur in the gills of *S. plana* exposed to 4-5 μm MPs+BaP at day 14th ($p< 0.05$), when compared to the other treatments, except 4-5 μm MPs, and day 7. In the digestive gland of individuals contaminated with 4 -5 μm MPs, LPO levels were significantly higher at day 14 ($p< 0.05$), when compared to the other treatments except 4-5 μm MPs+BaP. Similarly, higher values were observed significantly different from control (figure 3.6.).

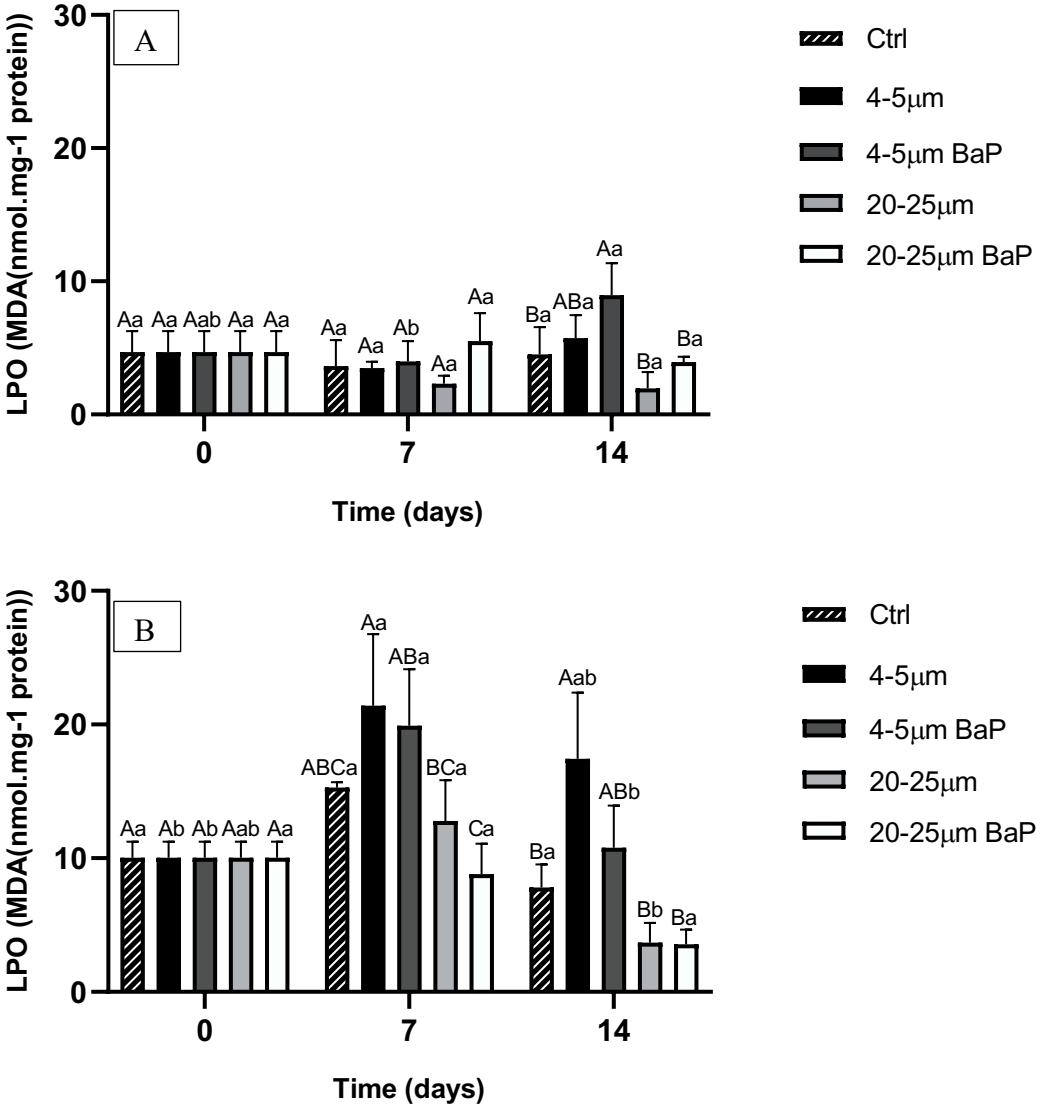

Figure 3.6. LPO activity (mean ± SD) in the gills (A) and digestive gland (B) of *Scrobicularia plana* for unexposed and exposed to 4-5 and 20-25 μm of MPs with and without BaP adsorbed in the beginning and after 14 days of exposure. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.7 PCA

PCA was applied to all the data obtained for gills and digestive gland to explain the effects of the different biomarkers response. To have a more confident variance the percentage of 3 factors were used that represent % of total variance. Figure 3.7 represents the relation between PC1 (39.4%) and PC2 (21.2%) and figure 3.8 between PC1 (39.4%) and PC3 (15.6%).

Regarding PC1, MPs, LPO levels, AChE, CAT activity and CI are in the positive axis and are positively related to DG treatments and a negative relationship with gills treatments while SOD and GST activities are in the negative side of PC1 and have a negative relationship with DG treatments and a positive relationship with gills treatments. All the data for the digestive gland remains on the positive side of PC1 where there is a clear separation of the different sizes (4-5 μm and 20-25 μm). Gills data are on the negative side with no clear size grouping.

MPs, AChE activity, LPO and GST activity are in the positive side of PC2 and are positively related to the majority of the treatments. However, SOD activity, CAT activity, and CI were in the negative side of PC2 and negatively related mainly to the control treatments.

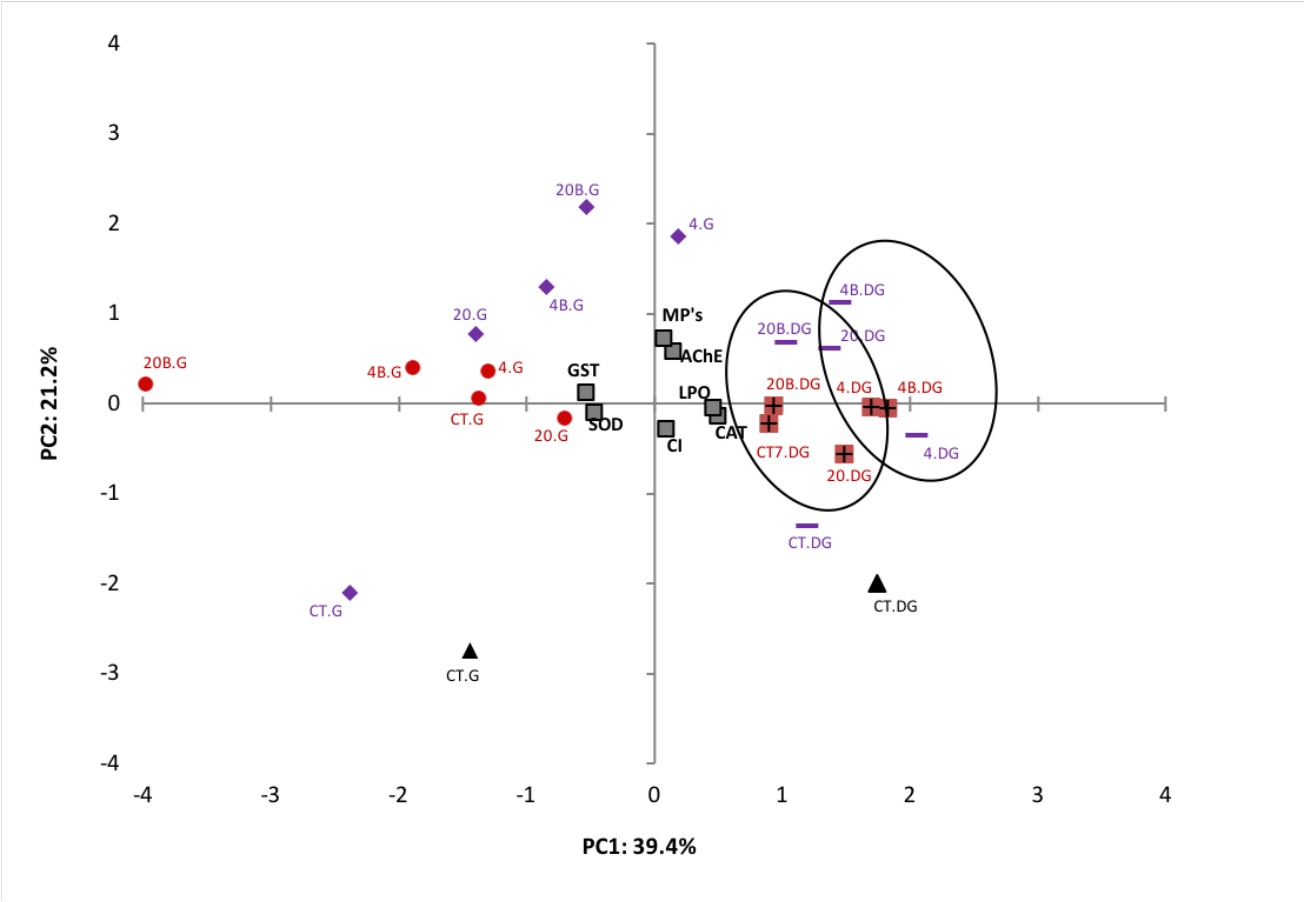

Figure 3.7. PCA of a battery of biomarkers (SOD, CAT, LPO, AChE and GST) in the gills (G) and digestive gland (GD) of a clam *S. plana*, for different sizes of LDPE MPs with (4-5 µm BaP *(4B)* and 20-25 µm BaP *(20B)*) and without (4-5 µm *(4)* and 20-25 µm *(20)*) BaP adsorbed at different times of exposure (T0 (black), T7 (red) and T14 (purple)) for $p<0,05$.

In relation to PC3 (figure 3.8), only MPs and CAT activity have a negative correlation, when compared to the other biomarkers. There is no clear separation between time, size of LDPE MPs or between contaminated/uncontaminated clams.

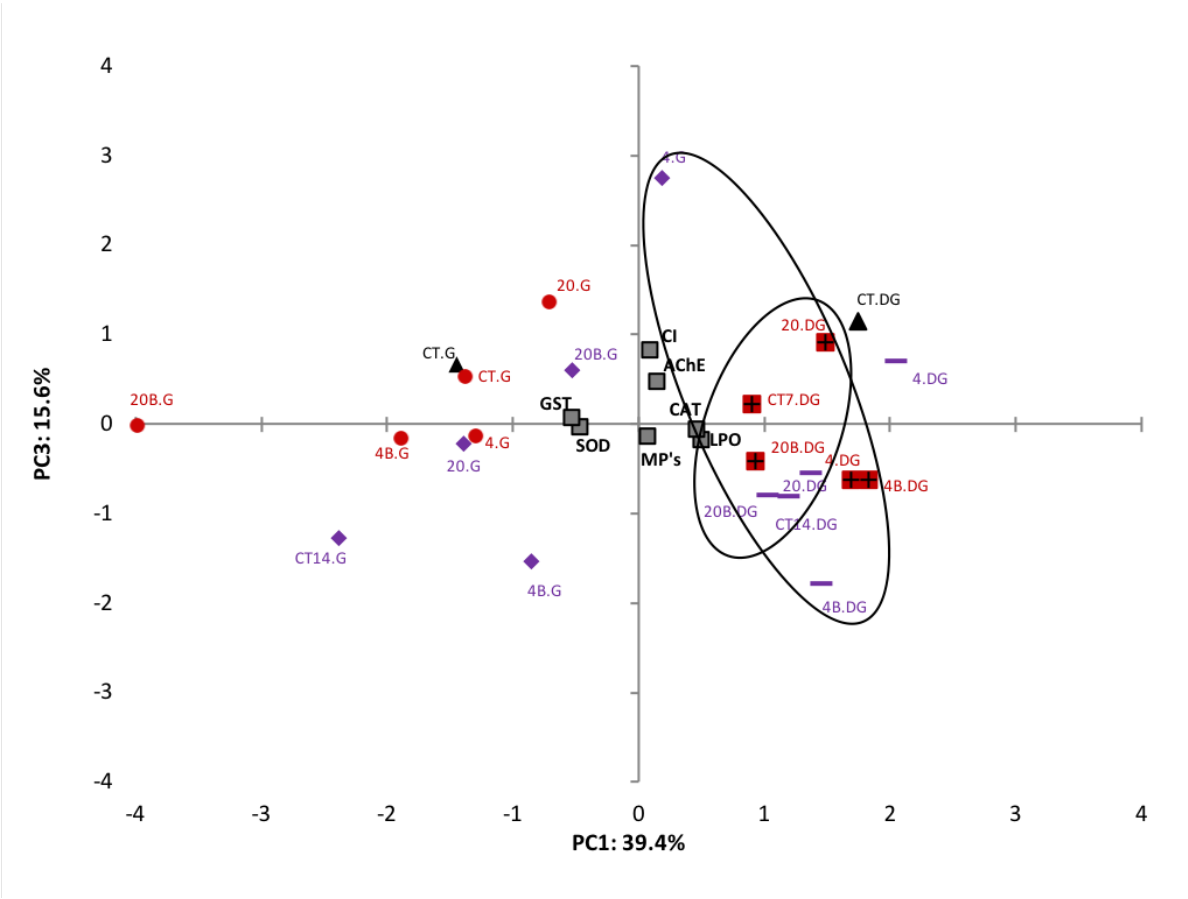

Figure 3.8. Principal component analysis (PCA) of a battery of biomarkers (SOD, CAT, LPO, AChE and GST) in the gills (G) and digestive gland (GD) of a clam *S. plana,* for different sizes of LDPE MPs with (4 µm BaP *(4B)* and 20 µm BaP *(20B)*) and without (4-5 µm *(4)* and 20-25 µm *(20)*) BaP adsorbed at different times of exposure (T0 (black), T7 (red) and T14 (purple)) for $p<0,05$.

3.8 IBR

IBR was calculated using all biomarkers and CI data for the gills (figure 3.9A) and digestive gland (figure 3.9B) of clams exposed to LDPE MPs with and without adsorbed BaP. IBR index was higher in the gills of clams exposed to 4-5 µm LDPE MPs whenever they were contaminated with BaP or not, when compared with those of the bigger size. In the digestive gland however, there was no size effect and IBR was lower in BaP contaminated LDPE MPs when compared with uncontaminated MPs (figure 3.9B).

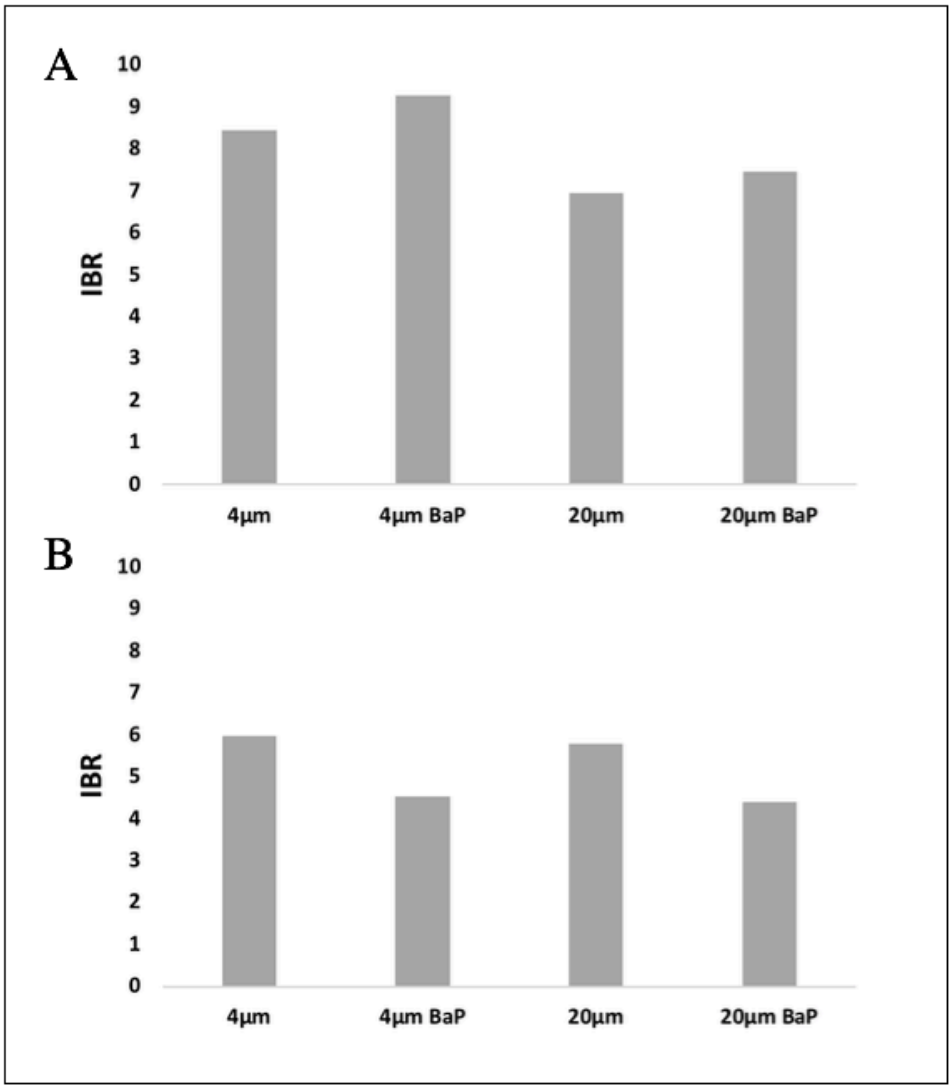

Figure 3.9. IBR in the gills (A) and digestive gland (B) of *Scrobicularia plana*, for the different treatments.

3.9 Health Index

Health index was applied in the gills and digestive gland using the same variables as in the case of IBR. HIS results (table 3.3) indicate that, in the gills of clams exposed to 4-5 µm MPs with BaP adsorbed were considered in a pathological state while for the other treatments gills were considered healthy. In the digestive gland, the HIS index was different, clams exposed to LPDE of 4-5 µm were considered in a pathological state, while those 4-5 µm MPs+BaP and 20-25 µm as medium stress, and in clams exposed to 20-25 µm MPs + BaP the digestive gland showed low stress.

Table 3.3. HIS, in the gills and digestive gland of *Scrobicularia plana* for all treatments. A (Healthy); B (Low Stress); C (Medium Stress) and E (Pathological).

Treatment/ Tissue	Gills	Digestive gland
4 µm	A+	E
4 µm + BaP	E	C
20 µm	A+	C+
20 µm + BaP	A	B

4 Discussion

Microplastics are a major concern to marine environment due to their bioavailability to organisms. Ingestion is the most likely way by which marine fauna interact with microplastics, especially when the feeding mechanisms of the organisms are non-discriminatory and do not allow them to differentiate between food and plastic fragments (Lusher *et al*., 2016; Allen *et al*., 2017). As the inhalant currents move water through the gills, food particles are sorted and captured by the cilia and incorporated into mucous strings. Rejected particles are moved away and expelled with the exhalent current. Accepted particles are moved through the labial palps to an oral groove and finally into the mouth and digestive tract. Small nutrient particles are moved into the digestive gland where the food particles are digested (Langdon and Newell, 1996; Gosling, 2015). Results did not demonstrate significant differences between bioaccumulation of different sized microplastics. On the other hand, BaP accumulation in the clams whole soft tissues showed that BaP accumulation of (4 - 5 μm) MPs + BaP was lower than in bigger microparticles (20 - 25 μm) (Figure 3.1), presuming that bigger particles are better at delivering BaP to the organisms, can be explain by the fact that smaller MPs are excreted immediately in bulk, and larger MPs are excreted more slowly in bulk, having more time of retention for contaminants desorption according to Kinjo et al., (2019).

These xenobiotics will trigger an oxidative stress response which is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense capacity. Organisms consume molecular oxygen that has to be tetravalent reduced to water at the same time that is coupled to the oxidation of food and energy production. This reduction leads to the production of ROS which can be armful for the clams. The antioxidant enzymes (SOD, CAT, GST and LPO) used has a biomarkers have an important role in protecting the cells acting as a defense mechanism by catalyzing the superoxide anion radical into hydrogen peroxide and water, thus reducing the potential for cellular oxidative damage to occur (Jo *et al*., 2008; Nikinmaa, 2014).

Gills tissue, being the major organ responsible for filtration, are the first site of MPs uptake in mussels (Browne *et al.,* 2008; Ribeiro *et al*., 2017), with *S. plana* also ingesting particles through inhalation siphon, being consequently transported to mouth and digestive gland (Hughes, 1969). Results indicate a time and tissue dependent

oxidative stress response that varies depending on the treatment used and the biomarker assessed. SOD activity only showed a bell shape behavior in gill tissues exposed to 20-25 µm MPs + BaP after 7 days significantly decreasing after 14 days of exposure (figure 3.2.A). An increase in SOD activity means that the first line of defense is protecting the cell against oxidative stress (Livingston, 2001**;** van der Oost, Porte-Visa and van den Brink, 2005), acting against exposure to MPs with BaP adsorbed. O'Donovan (2018), also shown a similar behaviour in clams exposed to MPs adsorbed to BaP but with smaller size (10-13 µm), hypothesizing that the observed increase could result from the toxicity of the adsorbed contaminant. Similar behavior was observed by Ribeiro (2017) showing an increase in SOD levels in the same clams species exposed to the same size of polystyrene MPs. Taking in consideration that BaP bioaccumulation was higher in clams exposed to 20–25 µm (table 3.2) and SOD activity was also higher in the gills of clams exposed to the bigger particles, size related to the retention time of MPs accumulation influence BaP accumulation in organisms.

Catalase is the second line of defense, preventing cellular damage from reactive oxygen species (ROS) by reducing H_2O_2 to H_2O+O_2 (Oliveira *et al.,* 2009; Solé, Kopecka-Pilarczyk and Blasco, 2009). ROS are chemically reactive chemical species containing oxygen that play an important role in apoptosis induction under both physiologic and pathologic conditions (Simon *et al*., 2000; Pittura *et al.,* 2018). In the gills CAT activity remained unchanged with exposure time (figure 3.3A). Meanwhile, in the digestive gland (figure 3.3B) CAT activity was much higher than in the gills, showing only an increase at the 14th day, but not significantly higher than control. These responses indicate that CAT activity is likely not the antioxidant defense mechanism used by *S. plana* or the exposure time was not enough to induce significant responses. Avio *et al.*, (2015) noted an inhibition of CAT activity in the digestive tissue of the marine mussel *M. galloprovincialis* exposed to 1000-100µm and <100 µm MPs (PE and PS). Ribeiro (2017), shows a late increase response in CAT activity in *S. plana* gills after three days of exposure to PS MPs (20 µm) and inhibited in the digestive gland after 7 days of exposure. Therefore, CAT activity seems to be particle size dependent, but this was not significant in the present study.

GST is usually associated with phase II biotransformation involved in detoxification but also has an antioxidant role in the metabolism of lipophilic organic compounds by catalyzing the conjugation of the reduced form of glutathione (GSH) to

xenobiotic substrates, playing a significant role in the detoxification of the toxic endogenous substances by converting reactive lipophilic molecules into non-reactive ones which can be more easily excreted by the organism (Hoarau, 2002; Lesser, 2006; Solé, Kopecka- Pilarczyk and Blasco, 2009; Nikinmaa, 2014). *S. plana* may use this detoxification mechanism to deal with the exposure of BaP to MPs in the gills, due to the significant increase of GST activity for 20-25 µm+BaP after 7 days ($p < 0.05$) (figure??). GST activity in the digestive gland remained relatively stable through time. Ribeiro *et al*., (2017) also showed an GST activity increase in the gills of *S. plana* when exposed to 20 µm PS MPs. GST activity seems to trigger a short response to the ROS formation in both virgin LDPE and contaminated MPs but only for bigger size particles.

Oxidative damage will happen when oxidative stress reaches a certain level that may cause damage to DNA, proteins carbohydrates and lipid membranes. Lipid peroxidation is the process in which free radicals react in cells membranes to form lipid hydroperoxides resulting in cell damage (Oliveira *et al*., 2009). Results indicate a treatment and tissue dependent LPO levels, indicating a significant increase in the gills for clams exposed to LDPE+BaP MPs (4-5 µm) after 14 days and in the digestive gland exposed to both virgin and contaminated smaller LDPE MPs (4-5 µm) after 7 days, decreasing after 14 days of exposure. In this way, it may be hypothesized that an increase in lipid peroxidation may result from inefficient oxidative stress reduction mechanism in processing the excess of ROS, when exposed to smaller particles.

Acetylcholinesterase is responsible for the breaking down of acetylcholine and other choline esters responsible for neurotransmission, being found in neuromuscular junctions and chemical synapses of the cholinergic type (Viarengo *et al.,* 2007; Tsangarisetal. 2010). AChE activity increased over time in all treatments, but only significant for 4-5 µm and 20-25 µm MPs +BaP, showing no inhibition for AChE activity (figure 3.4). Organisms when exposed to MPs, an inhibition of AChE activity would be expected as it was described for juveniles of the common goby *Pomatoschistus microps* exposed to PE microspheres (1-5 µm) for 96h, with and without absorbed contaminants (pyrene) (Oliveira *et al*., 2013). Ribeiro et al. (2017), also had an inhibition response of AChE activity to PS MPs exposure for *S. plana*. However, O'Donovan et al. (2018) also showed an increase of AChE activity in the same clam species after the same time of exposure to LDPE MPs (11-13 µm) contaminated with the same concentration of BaP.

IBR calculated using all biomarkers and CI data for the gills and digestive gland. Results that in clam gills, the smaller size-range of MPs has a higher IBR than bigger MPs. Relatively to the digestive gland, MPs without BaP adsorbed have a higher IBR level (figure 3.9.) which might result from the responses of antioxidant enzymes (SOD and CAT) (Serafim *et al*., 2012). Health index was created to develop and test an objective decision-support expert system capable of integrating biomarker results into a five-level health-status index, based on a set of rules derived from available data on responses to natural and contaminant-induced stress (Dagnino *et al*., 2007). Results demonstrate a lower health status for the digestive gland, where the up taken particles stay longer for digestion, this might be the reason for the higher levels of HIS.

5. Conclusion

Since the introduction of plastic in our everyday life, debris was accumulated in several places including the ocean. Impact of its accumulation are numerous and diverse and affect the marine biota. The efforts to minimize, through recycling, for example, have been more and more explored. Several plastic polymers adsorb contaminants from the surrounding environment, acting as a potential vector for these chemicals that once ingested by marine organisms can affect them. Concentration of contaminants adsorbed in MPs has a great importance in order to find specific and efficient ways to reduce them, otherwise it will become bioaccumulated and or biomagnified.

The main conclusions of this theses, under the experimental conditions, are:

- ∴ All sizes of microplastics were significantly accumulated in MPs exposed clams when compared to control and pre-exposure.
- ∴ Bigger particles are more successful at delivering BaP to the clams.
- ∴ Antioxidant enzyme SOD has a higher response in the gills for the bigger size of LDPE + BaP microplastics.
- ∴ A significant increase in GST activity was noticed in the gills for the clams exposed to 20-25 μm MPs+BaP, when compared to the other treatments and also in relation to both day 0 and day 14.
- ∴ AChE had no inhibition response for any of the MPs treatments.
- ∴ LPO levels were higher in the digestive gland for smaller virgin size LDPE MPs with and without BaP.
- ∴ Antagonistic effects of LDPE and BaP occur in some biomarkers mainly in the bigger size of MPs.
- ∴ Integrated Biomarker Response had more impact in the gills and in LDPE MPs smaller size.
- ∴ Health Index (HIS) demonstrate that the digestive gland was the more affected.

6. Further studies

During the experimental design instead of using 5 aquariums (control; 4-5 μm MPs; 4-5 μm MPs + BaP; 20-25 μm MPs and 20-25 μm MPs+ BaP), an extra aquarium should be used only with the contaminant BaP. These might be important to determinate if some data is the result of MPs combined with contaminant, only MPs or also only the contaminant itself. Also increase the exposure and sampling days in order to see how data behaves in a longer timescale. If enzymes responsible for oxidative stress for instance can adapt after some time of exposure or if they are permanently affected over time.

In this study, MPs quantification was assessed using the total soft tissue of the organism. In this way MPs it was not possible to know the distribution of the MPs in the tissues. It would be interesting to know in which tissue, gills or digestive gland, had more microplastics accumulation to relate to the biomarker analysis. For further studies regarding MPs quantification, it would be necessary to do blanks for each treatment to have a comparison factor to minimize error while quantification method.

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