Universidade de Aveiro Departamento de Biologia
Ano 2018

Maria Inês Ribeiro Ferreira

Nanoplastics toxicity: microalgae and rotifers studies

Toxicidade de nanoplásticos: estudos com microalgas e rotíferos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Professor Doutor Marcelino Miguel Guedes de Jesus Oliveira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Professora Doutora Isabel Maria Cunha Antunes Lopes, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave cafeína, microalgas, nanoplástico, polimetilmetacrilato, rotíferos, toxicidade

resumo

Atualmente é cada vez maior a quantidade de plástico produzido mundialmente. Este é um fator preocupante, uma vez que o plástico representa uma ameaça para o ambiente marinho quando não é devidamente descartado ou reciclado. A existência de nanoplásticos (partículas de plástico inferiores a 100 nm) no meio aquático constitui um perigo, não só pelas substâncias que podem ser adsorvidas, mas também pelos diversos efeitos negativos associados ao facto de se apresentarem na forma de nanopartícula. Os organismos aquáticos podem estar expostos a vários tipos de plásticos, como por exemplo, o polimetilmetacrilato. Deste modo, o primeiro objetivo deste trabalho foi realizar uma revisão da literatura e analisar os efeitos dos nanoplásticos em animais marinhos. A revisão mostrou que os nanoplásticos podem afetar os ecossistemas marinhos desde produtores a consumidores, no entanto, a informação disponível é ainda reduzida tornando-se necessário continuar a estudar este tema em diferentes organismos e com diferentes tipos de plásticos. Assim, o segundo objetivo foi avaliar os efeitos de nanopartículas de polimetilmetacrilato (~50 nm), polímero pouco estudado, nas microalgas *Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* e *Thalassiosira weissflogii* e no rotífero marinho *Brachionus plicatilis*. Os resultados demonstraram que nanoplástico tem a capacidade de afetar tanto o crescimento das algas marinhas, sendo que a mais sensível foi a *T. weissflogii* com uma concentração de efeito (EC_{50}) de 83.75 mg/L, como a sobrevivência dos rotíferos, sendo que o tipo L da espécie *B. plicatilis* foi o mais sensível com uma concentração letal (LC_{50}) de 13.27 mg/L. A sobrevivência deste organismo começa a ser afetada a partir de concentrações superiores a 9.38 mg/L. Por último, este trabalho teve como objetivo estudar o efeito, nas microalgas *T. chuii* e *N. gaditana*, da exposição simultânea a polimetilmetacrilato e um contaminante ambiental. Para este efeito foi selecionada a cafeína, considerada como um marcador de contaminação antropogénica. A cafeína afetou o crescimento das algas, tendo sido registada uma EC_{20} de 565.4 mg/L para a *T. chuii* e uma EC_{20} de 567.6 mg/L para a *N. gaditana*. O crescimento de ambas as microalgas foi significativamente afetado quando expostas à mistura de nanoplásticos com a cafeína.

keywords caffeine, microalgae, nanoplastics, polymethylmethacrylate, rotifers, toxicity

abstract

Nowadays the production of plastic is increasing all around the world. This is a worrying situation since plastic constitutes a threat to the marine environment when it is not properly discarded or recycled. The existence of nanoplastics (particles with less than 100 nm) in the marine environment may became dangerous, not only because of the substances that can be adsorbed, but also because of their expression as nanoparticles. Marine organisms can be exposed to several types of nanoplastics such as polymethylmethacrylate. Thus, the first object of this work was to do a literature review on the effects of nanoplastics on marine organisms. The review showed that nanoplastics affect all marine ecosystems from producers to consumers, however there is still a lot of information that is needed regarding different organisms or different types of plastics. Therefore, the second goal was to evaluate the effects of polymethylmethacrylate nanoplastics (~50 nm), a less studied polymer, on marine microalgae, *Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*, as well as on the marine rotifer *Brachionus plicatilis*. Nanoplastics significantly affected both growth rate of marine microalgae with *T. weissflogii* being the most sensitive one with an EC_{50} of 83.75 mg/L, and rotifers survival, where *B. plicatilis* type L was the most affected one with significantly results from 9.38 mg/L and a LC_{50} of 13.27 mg/L. The last goal of this work was to evaluate the effect, on marine algae *T. chuii* and *N. gaditana,* of a combined exposure between polymethylmethacrylate and an environmental contaminant. For this purpose, caffeine was selected as an anthropogenic contamination marker. Caffeine significantly affected the growth rate of both algae with an EC_{20} of 565.4 mg/L for *T. chuii* and an EC_{20} of 567.6 mg/L for *N. gaditana*. Growth rate of both marine microalgae was significantly affected when they were exposed to a mixture of nanoplastics and caffeine.

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CHAPTER I

General introduction

1. General introduction

Marine environment is exposed to various threats and marine litter, nowadays, is one of them. It includes metals, glass, ceramics, textiles, paper, timber and plastic which is the most harmful fraction of marine litter (Schneider et al., 2018). There are different plastic polymers, such as polymethylmethacrylate (PMMA) which is a type of plastic mainly used in medicine, automobile manufacturing, computer engineering or network configuration (Taguenang et al., 2008), sizes from macroplastics (> 5 mm) to nanoplastics (< 100 nm) and shapes of plastic. Microplastics (< 5 mm) have been proving to be transferred across trophic levels (e.g. from fish to a marine mammal) which may lead to a microplastic ingestion for any species whose feeding ecology involves the consumption of a whole prey (Nelms et al., 2018). Even in smaller plastics, nanoplastics, it has already been shown that they can be transferred through a freshwater food chain (Karin Mattsson et al., 2017), however there are no studies regarding marine food chains. Despite the number of nanoplastic studies is increasing, there is still a lack of knowledge in what concerns the effects of mixtures between nanoplastics and other marine contaminants (e.g. caffeine). Caffeine is an anthropogenic marker since it is one of the most widely consumed drugs in the world.

Plastics can affect all types of marine organisms from bacteria or algae to fish and marine mammals. Microalgae are eukaryotic photosynthetic microorganisms that can be used to produce high value compounds (Mendes et al., 2003). Their rapid growth rate and their high lipid content carbohydrates, and proteins make microalgae one of the most promising biomass resources (Pleissner et al., 2013; Song et al., 2013). Rotifers constitute a phylum with about 2000 described species (Gómez et al., 2002). *Brachionus plicatilis* occurs in brackish habitats and it is considered one of the most common marine rotifers around the globe (Fontaneto et al., 2007). This specie has commercial value since it is commonly used for aquaculture purposes as live food for marine fish (Fontaneto et al., 2006) due to their small size, slow swimming behavior, and the way they provide nutrients that are essential for larval fish growth (Best et al., 2010). As well as in ecotoxicology assessments to evaluate toxicity on marine organisms since it can be cultured in laboratorial conditions and has a short reproduce time (Rico-Martínez et al., 2013).

Plastics have only been produced for around 100 years so even though marine organisms are able to adapt to some environmental conditions (e.g. temperature, pH, CO_2, salinity or carbonates) or changes that occur over geological time the development of adaptive responses of marine organisms to plastics have not yet occurred (Deudero & Alomar, 2015). Thus, the main objectives of this study were to do a literature review about the effects that nanoplastics (<100 nm) can cause to all marine species ever studied (chapter II) as well as determine effect and lethal concentrations for marine microalgae (*Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*) and rotifers (*Brachionus plicatilis*) when exposed to polymethylmethacrylate (PMMA) nanoplastics, furthermore analyze the difference between exposing marine algae to nanoplastics and a mixture of PMMA and caffeine (chapter III).

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CHAPTER II

Nanoplastics and marine organisms: what has been studied?

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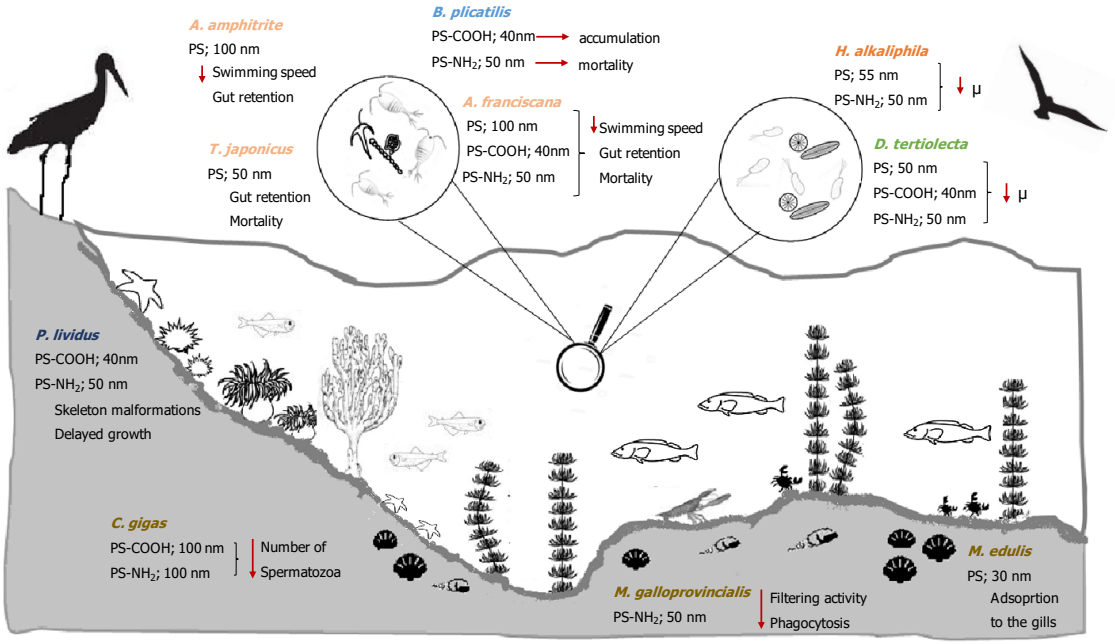

Abstract

Nowadays, there is an increased awareness on threats that marine litter may pose to the marine environment. This review describes the major concerns related to plastic pollution, namely in terms of toxicity of different types and sizes of nanoplastics (particles smaller than 100 nm) on marine organisms, either producers or consumers. The available data show that nanoplastics may negatively affect organisms from different phyla with reported effects ranging from alterations in reproduction to lethality. Nevertheless, no information regarding marine vertebrates (e.g., fish) was found. Data show a high potential for bioaccumulation/biomagnification along marine food chains, since they can easily be retained inside organisms. The lack of standardized methodology for nanoplastics detection and the poor or inexistent legislation makes nanoplastics an environmental challenge.

Keywords: ecological risks; effects; marine organisms; nanoplastic

1. Plastics

Marine litter, any persistent, manufactured or processed solid material that ends up in the sea is increasing around the world and becoming a threat to the marine ecosystem. Among the different materials that may be found within marine litter are plastics, which are nowadays recognized as emerging contaminants of concern. Plastics are defined as synthetic organic polymers that can be easily molded into different shapes and products (Worm et al., 2017), with high durability, light weight and cheap. These properties make plastics a support for a large variety of applications: from simple plastic bottles, containers for food products and consumer goods, up to the sectors of transport, construction, telecommunications and health care (Gourmelon et al., 2015). Their wide use increased their release into the environment, either deliberately (e.g., throw domestic and industrial effluents) or unintentionally (e.g., run-off) (Todd et al., 2010; Sá et al., 2018). Since the 1990s the annual plastic production increased from 1.7 to 335 million tones in 2016 (PlasticsEurope, 2017). Furthermore, it has been estimated that 4.8 to 12.7 million tons of plastic debris enter the ocean each year (Jambeck et al., 2015). The most produced plastic polymers are polypropylene (PP), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyvinyl chloride (PVC), polyurethane (PUR) polyethylene terephthalate (PET) and polystyrene (PS), being employed in the several manufacture industries, from electronics to health care, as illustrated in Figure 1. For instance, in a field study performed in the southern Adriatic sea, of a total of 120 samples (water and sediment), 80.6 % contained plastic debris, and 38.7% of the samples were composed of polystyrene plastics (Šilc et al., 2018).

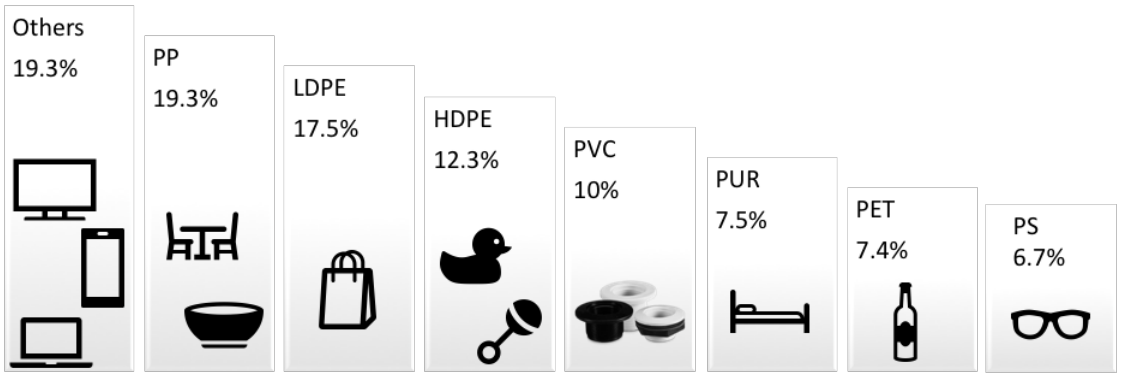

Figure II.1 – Representation of the percentages of the plastic polymers most produced in 2016 and example of products in which they are commonly employed. PP – polypropylene; LDPE – low-density polyethylene; HDPE – high-density polyethylene; PVC – polyvinyl chloride; PUR – polyurethane; PET – polyethylene terephthalate and PS – polystyrene. Adapted from PlasticsEurope (2017).

One of the concerns associated with plastic pollution is the occurrence of particles smaller than 5 mm, particularly in the low micro and nanosizes. Although there is no established definition of nanoplastic, it has been assumed that they fall within the range of other types of nanoparticles i.e. a size range from 1 to 100 nm (Koelmans et al., 2015; Gigault et al., 2018). Microplastics and/or nanoplastics may be divided in primary or secondary. Primary micro(nano)plastics are those that enter the ecosystem in their originally small size associated with a specific application and consumer products, such as, synthetic fibers, cosmetics, medicine and raw materials (Bessa et al., 2018; Tamminga et al., 2018; Wang et al., 2018). Their release into the environment is frequently associated with inadequacy of the disposal infrastructures at wastewater treatment plants (WWTP). For example, in a study addressing this issue, a WWTP located in the Baltic Sea was able to reduce the burden of plastics in wastewaters from hundreds to less than 10 particles per liter of wastewater. However, these values of particles per liter of wastewater were still 25 times higher than those reported for sea water samples (Talvitie et al., 2015). Alongside the disposal of primary micro(nano)plastics, their concentration might increase as a result of the degradation of macroplastics, the so called secondary micro(nano)plastics (Andrady, 2011; Cole et al., 2011). This process of breakdown happens because once in the environment,

polymers are susceptible to biological activity (such as the action of bacteria) and/or subjected to several abiotic processes (wind, rain, UV radiation, mechanical forces, photo-oxidation) (Andrady, 2003). Their action, solely or jointly, may promote a decrease in the size of the particles, first to micro and later to nanoplastics (Lambert & Wagner, 2016). The process of fragmentation/degradation has already been demonstrated to occur rapidly under laboratorial conditions. During the thermal cutting of polystyrene foam Zhang et al. (2012) found that most of the particles emitted were of sizes between 22 and 220 nm. Using disposable coffee cup lids, Lambert & Wagner (2016) showed that 56 days were enough to reach a concentration of 1.26×10^8 particles/mL of PS particles with an average size of 224 nm. The time required to reach particles of nano size depends on the size of the initial plastic (Koelmans et al., 2015). The degradation process will drastically reduce the average molecular weight of the polymer, further increasing their susceptibility to breakdown but at the same time, making them more available to be incorporated into the marine biomass (Andrady, 2011). Thus, if not properly disposed, reused or recycled, plastics may become a serious threat to the aquatic environment. The presence of plastic particles in freshwater, estuarine and marine environments has been reported in several studies, as showed in Figure 2, with reports of up to thousands of particles/m^2 (Carvalho & Neto, 2016). Nevertheless, the estuarine/marine environment is of most concern as it constitutes the final recipient of these particles that reach this environment through rivers, water runoff, wastewater discharges and transportation through wind. Recreational activities at the beach and ship-generated litter dumped by commercial boats, cruises or private vessels or fishing gear may also contribute to the discharge of microplastics to the marine/estuary compartment (Pruter, 1987; Sheavly & Register, 2007).

Figure II.2 – World map summarizing field studies that report the presence of plastics in freshwater, estuarine or marine environments. Different shaped symbols (squares and circles) represent plastics concentration expressed in particles/m^2 and particles/m^3, respectively. References are listed as follows: [1.](Goldstein et al., 2013); [2.3.](Gray et al., 2018); [4.](Eriksen et al., 2013); [5.](Carvalho & Neto, 2016); [6.](Rayon-viña et al., 2018); [7.](Sadri & Thompson, 2014); [8.9.10.11.](Tamminga et al., 2018); [12.](Collignon et al., 2012); [13.](Imhof et al., 2013); [14.](Xiong et al., 2018); [15.](Lee et al., 2013); [16.17.](Zhao et al., 2014).

There are three major problems related to plastics: a) toxicity towards biota caused directly by the plastics themselves; b) toxicity caused by additives added to plastics during the production process and c) their role as vectors for environmental contaminants and invasive/pathogenic organisms.

There is a huge concern about the additives that are added during plastics production. The most commonly used additives are phthalates, [e.g., bisphenol A (BPA), polybrominated diphenyl ethers (PBDE) and tetrabromobisphenol A (TBBPA)], mainly used as plasticizers, stabilizers and brominated flame retardants (Hermabessiere et al., 2017). These additives can increase the time of degradation of plastic enduring their permanence in the environment and may leach into the marine environment and become available to biota (Avio et al., 2017). They have been shown toxic to biota. For example, BPA has been reported to affect growth rate and sexual maturation, hormone levels in blood, reproductive organ function, immune function, enzyme activity and brain structure (vom Saal & Hughes, 2005).

The presence of micro and nanoplastics in the marine environment can affect biota and the environment through other pathways. Smaller plastics have a high surface area and adsorb hydrophobic substances from the marine environment, namely persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), polybrominated diphenyl ethers (PBDEs) and perfluorooctanoic acid (PFOA), as well as metals (Moore et al., 2007; Ashton et al., 2010; Frias et al., 2010; Andrady, 2011; Holmes et al., 2012; Velzeboer et al., 2014; Li et al., 2018). Rochman et al. (2013) found that LDPE, HDPE and PP plastic debris from San Diego Bay had a great affinity for chemical pollutants such as PCBs and PAHs. This ability to adsorb contaminants and release additives highlights the possibility of transferring these contaminants to biota.

The ubiquity of plastic particles and the recognition that macroplastics can be degraded to micro and nanoplastics and thus become more bioavailable to biota raises concerns on the molecular and physiological effects that these particles may lead to. Effects at behavioral and reproductive levels, in addition to the well reported effects of physical damage and false satiation attributed to macroplastics are some of the examples (Lazar & Gračan, 2011) and can be transversal from marine invertebrates to mammals. The known effects include alteration of hormone levels and enzyme activity, oxidative stress, growth inhibition, loss of energy and weight, retention on digestive tract as well as in immune and reproductive system and even mortality (Jin et al., 2018; Li et al., 2018; Naji et al., 2018; Xiong et al., 2018)..

2. Nanoplastics

One of the main problems associated with the presence of plastics in the environment is the fact that they may breakdown into smaller fragments, increasing their availability to be ingested by marine biota (Santos et al., 2009). In addition to being originated from plastic fragmentation, nanoplastics can also be produced to be included in products for coatings, biomedical purposes, drug delivery, medical diagnostics, electronics, magnetics and optoelectronics (Koelmans et al., 2015). Alongside the decrease in size and consequent

increase in surface area that promotes the adsorbance of other environmental contaminants, as already mentioned above, the particles may become more reactive. The nanoparticle formation changes the chemical and physical characteristics of the particle and, consequently, its availability and biological impact on aquatic organisms (Mattsson et al., 2015). Therefore, it is expected that at the nanoscale the characteristics of particles (e.g. strength, conductivity and reactivity) will differ substantially from macro and micro-sized ones (Klaine et al., 2012). The biological reactivity is frequently also increased with the decreased size. The nano size increases the ability of the particles to pass throw cellular boundaries and accumulate on organisms and the reactivity of the particles (Mattsson et al., 2015; Worm et al., 2017) with more atoms and molecules displayed on the surface which can lead to more reactive groups on it (Nel, 2006). Although an increasing number of studies are focusing on the effects of microplastics, the knowledge of the effects of nanoplastics are still scarce, especially regarding marine biota. Considering the hypothesis that reactivity increases at the nanoscale and that the marine ecosystems is the final recipient, it is urgent to gather the available information to identify knowledge gaps and set priorities and lines of investigation that should be addressed. Therefore, the objective of this review was to summarize published data on the effects of nanoplastics on marine biota, focusing in types of plastic that are being used and organisms are being studied, from producers to consumers.

3. Effects of nanoplastic particles

A literature review (in Scopus database) revealed 1699 articles focusing on microplastics; however, when the search was narrowed to the keyword “nanoplastics” the number decreased to 80. There were 26 documents when the keywords “nanoplastic” and “marine” were combined and only 20 when “nanoplastic” and “marine” are combined with the keyword “effects”. It is evident that more information is needed and the knowledge around nanoplastics is increasing in the last 2 years. From those 20 results, 14 are from 2017 and 2018. Gathering the information on the effects of nanoplastics will allow to have a broader perspective of what has already been achieved and to where should the science efforts on this matter be pointed out to fill knowledge gaps. Thus, a compilation of reported effects on marine organisms was included in Table II.1.

This review will focus mainly on the toxic effects that nanoplastics are known to cause on marine biota. A brief analysis of Table 1 immediately shows that all of the studies used PS as a model particle. This fact may be explained by the easy synthesis of nanoplastics of this polymer when compared to others. Still, the toxic effects exerted by PS may not correspond to the toxic effects caused by other polymers, emphasizing the urgent need to further generate information on this topic.

3.1. Effects on bacteria

Bacteria constitute a large domain of prokaryotic microorganisms. *Halomonas alkaliphile* is a specie from the Proteobacteria phylum. Sun et al. (2018) exposed, for two hours, this halophilic bacterium (bacteria that thrive in high salt concentrations) to 50 nm cationic amino (-NH_2) PS particles and 55 nm PS beads at 20, 40, 80, 160 and 320 μg/mL. For PS-NH_2, cell growth was significantly affected from 80 μg/mL onwards, with a maximum of inhibition (34%) found at 360 μg/mL. Similarly, PS beads decreased the cellular growth up to 32.7% at 360 μg/mL. A significant increase in the intracellular levels of reactive oxygen species (ROS) was detected after 0.5 and 2 h exposure to both types of plastics.

3.2. Effects on algae

Algae are photosynthetic, unicellular or pluricellular organisms, that contain chlorophyll, with no tissue differentiation or vascular transport organs. These organisms are vital to the wellbeing of marine ecosystems as they are the base of food webs, source of oxygen production and other nutrients (Mao et al., 2018). The effects of nanoplastics have already been assessed in these organisms. PS-NH_2 particles (50 nm) caused a significant inhibition on the growth rates of the unicellular green microalgae *Dunaliella tertiolecta*, with an estimated $EC_{50,72h}$ of 12.97 ± 0.57 μg/mL, whereas no effect was found after 72 h exposure to anionic carboxylated (-COOH) PS particles (40 nm) (Bergami et al., 2017). The observed effect may be associated with a pernicious effect on photosynthesis and ROS formation. In the same line of evidence and for the same species, Sjollema et al. (2016) observed a clear reduction on the average cell density (about 45%), that was translated in a 57% effect on cellular growth, after exposure to 250 μg/mL of 50 nm PS beads. These results suggest that

nanoplastics may impair algae growth rates. However, it is crucial to study other species.

3.3. Effects on rotifers

The Rotifera phylum include around 2200 described species, some of them from marine ecosystems. Manfra et al. (2017) exposed the marine rotifer *Brachionus plicatilis* to a concentration range of 0.5, 1, 5, 10, 25 and 50 µg/mL of PS-COOH (40 nm) and PS-NH_2 (50 nm) nanoplastics. For PS-COOH particles, although no mortality was found, gut retention was observed after 48 h of exposure. For PS-NH_2 particles, LC_{50s} of 13.04 ± 0.60 and 6.62 ± 0.87 µg/mL, were estimated after 24 and 48 h exposures, respectively.

3.4. Effects on mollusks

Mollusks are the largest marine phylum and contains the class Bivalvia where clams, oysters, cockles, mussels and scallops, organisms widely used in ecotoxicity studies are included (Brandts et al., 2018). *Crassostrea gigas* exposed to 0.1, 1, 10, 100 µg/mL of 100 nm PS-NH_2 and PS-COOH did not affect the percentage of viable cells in spermatozoa. However, 100 µg/mL of PS-COOH particles promoted the aggregation of spermatozoa, resulting in a decrease of 32% and 24% of single spermatozoa after 3 and 5 h of exposure, respectively. Spermatozoa exposed to 100 µg/mL of PS-COOH and PS-NH_2 showed an increase of 4–5 % in relative size after 1, 3 and 5 h exposure. Moreover, ROS levels were not significantly affected by PS-NH_2 but PS-COOH increased ROS production in 17.4 %, 59.4 % and 121 % after 1 h exposure to exposure 1, 10 and 100 µg/mL, respectively (González-Fernández et al., 2018). In the common, edible mussel, *Mytilus edulis*, exposure to 100, 200 and 300 µg/mL of 30 nm PS particles induced the production of pseudofeses, which increased with concentration increase (Wegner et al., 2012). This result suggests that PS particles are recognized as non or low nutritional food. A reduction in the filtration rate, dependent on the PS concentration was found. In *M. galloprovincialis*, reproduction fitness was affected by nanoplastics. Fertilized eggs of *M. galloprovincialis* exposed to PS-NH_2 (50 nm) particles presented a decrease in lysosomal membrane stability (50% at 50 µg/mL) as well as cytochrome c reduction (Canesi et al., 2015). Thus, this nanoplastics may impair cell metabolism/nutrition, signaling and repairing (cellular functions in

which the lysosome plays an important role), as well as inhibiting mitochondria activity. Canesi et al. (2015) reported also a decreased by 50% in phagocytosis at a concentration of 50 μg/mL of PS-NH_2. More recently, Balbi et al., (2017) reported that 48 h of exposure to 0.001 to 1 μg/mL of PS-NH_2 (50 nm) caused malformations of the D-larvae (early stage in the development of a veliger) of *M. galloprovincialis* and a delay in development at higher concentrations (2.5 to 10 μg/mL). An EC_{50} of 0.142 μg/mL was determined for larval development. A decrease in shell length of 20 to 30% was also observed in 48 hpf larvae at different concentrations (0.15, 1, 2.5 and 5 μg/mL).

3.5. Effects on arthropods

Phylum Arthropoda includes crustaceans and englobes crabs, lobsters, crayfish, shrimp and krill. In order to evaluate the lethal and sub-lethal effects of nanoplastics Gambardella et al. (2017) exposed two marine crustaceans (II stage nauplii of the barnacle *Amphibalanus amphitrite* and first instar larvae of the brine shrimp *Artemia franciscana*) to 0.001, 0.01, 0.1, 1 and 10 μg/mL of 100 nm PS particles. No significant effects on survival were found but PS nanoparticles affected swimming speed. In *A. amphitrite* there was a significant inhibition at 48 h in higher concentrations (1 and 10 μg/mL) whereas in *A. franciscana* swimming speed was inhibited at 24 h but significantly increased at longer exposure and higher concentrations. Both species ingested the nanoparticles and accumulated them in the gut after 24 and 48 h exposure. The brine shrimp species was also studied in the same larval stage by Bergami et al. (2017) although exposed to PS-COOH (40 nm) and PS-NH_2 (50 nm) particles at 0.5, 1, 1.5, 2.5, 5 and 10 μg/mL, to understand effects of nanoplastics at the molecular level. There were no significantly differences on organisms exposed to PS-COOH. However, in organisms exposed to 1 μg/mL PS-NH_2, the expression of two genes (*clap* and *cstb*) connected to growth which includes molting, organogenesis and tissue remodeling in early larvae was increased after 48 h of exposure and related to an increase in the number of molts. After 14 days exposure to PS-NH_2 nanoparticles, high mortality rates were registered, with an LC_{50} computed around 0.83 μg/mL. Bergami et al. (2016) also studied the marine shrimp *A. franciscana* up to Instar III Nauplius. In this study, organisms were exposed to 5, 10, 25, 50, and 100 μg/mL of PS-

COOH (40 nm) and PS-NH_2 (50 nm) with data showing that both nanoplastics may accumulate in biota, being retained inside the gut lumen. However, cationic particles were more harmful affecting brine shrimp larvae swimming (at 48 h), an effect that can limit their feeding ability. Furthermore, an increase of almost 50% in molts cycle was observed after 48 h exposure to PS-NH_2. Lee et al., (2013) exposed the marine copepod *Tigriopus japonicus* to 0.125, 1.25, 12.5 and 25 µg/mL of 50 nm PS particles and verified that particles could also accumulate in the gut lumen in this species. Survival started to be affected at concentrations of 1.25 µg/mL.

3.6. Effects on echinoderms

The phylum Echinodermata englobes marine invertebrates such as sea stars, sea cucumbers and sea urchins. The available studies with these organisms reveal that they may accumulate nanoplastics. Della Torre et al. (2014) reported that PS-COOH (50 µg/mL) nanoplastics accumulated inside the digestive tract of sea urchin (*Paracentrotus lividus*) embryos, with no relevant malformations in the embryos. However, PS-NH_2 (10 µg/mL) nanoplastics induced a higher toxicity, though not accumulating as PS-COOH particles. Several larvae presented malformations within a period of 6 to 48 hours post fertilization (hpf). The reported malformations included thickening and abnormal proliferation of the ectodermal membrane (6 hpf), undeveloped embryos (24 hpf), incomplete or absent skeletal rods, fractured ectoderm and reduced length of the arms (48 hpf). The EC_{50} computed for PS-NH_2 beads were of 3.82 µg/mL at 24 hpf and 2.61 µg/mL at 48 hpf. More recent studies with the same species revealed that, after exposure to 3 µg/mL of PS-NH_2 (50 nm) skeleton elongation was delayed, and 4 µg/mL induced malformations on skeletal rods and arms (Pinsino et al., 2017).

4. Final Considerations

The available studies with particles smaller than 100 nm were performed with PS. Thus, it becomes imperative to assess the effects of other types of plastics in a wide range of organisms. Particles that may cause severe damage in some organisms (e.g., PS-NH_2 to bacteria, algae or echinoderms larval

stages), may present a lower threat to others (e.g., rotifers), making it difficult to accurately conclude on their toxicity. Although studies have been performed to assess the amount of plastics in the marine environment, there is no information regarding the number of nanoplastics. Thus, it is hard to predict the ecological risk of nanoplastics in the marine environment. The available data shows that these particles, alone, may be harmful to the marine ecosystem from producers to consumers. However, the available studies are scarce, particularly in what concerns to the effects on marine vertebrates like fish that in addition to their ecological importance, also present high commercial value. The lack regulatory frameworks regarding the emission of plastics into the environment and legislation concerning nanoplastics in food may justify the limited available studies. Furthermore, detection methodology limitations do not allow the establishment of cause/effect associations nor potential links to human and environmental health (EFSA, 2016). The analysis of the available studies shows that there is a lack of knowledge on generational and long-term effects of nanoplastics as well as their potential to be transferred along a marine food chain. In microplastics food web transfer was already observed in several different marine species such as algae, zooplankton, mussels and crabs (Cole et al., 2013; Farrell & Nelson, 2013). The smaller microplastics have higher potential for accumulation in the tissues of organisms (Browne et al., 2008). Since nanoplastics are smaller particles, there is also a high probability for them to be incorporated in the diet of the organisms and, consequently, be transferred to other trophic levels. It is also imperative to study the interaction between nanoplastics and other contaminants because they may affect organisms differently.

Table II.1 – Effects of nanoplastic particles on marine organisms according to the type, size and concentration of the nanoplastic. Only studies about marine organisms and particles with less than 100 nm were included. Abbreviations stand for: PS - polystyrene; PS-COOH - anionic carboxylated polystyrene; PS-NH_2 – cationic amino polystyrene; nsw – natural sea water; asw – artificial sea water; LC/EC_x – lethal or sublethal concentration causing x % of effect; hpf – hours post fertilization; μ = growth rate.

Phylum/ Order	**Organism**	**Type of plastic**	**Size** (nm)	**Concentration** (µg/mL)	**Effects**	**Reference**
Proteobacteria Oceanospirillales	*Halomonas alkaliphila*	PS PS-NH_2	55 50	20, 40, 80, 160, 320	Intracellular ROS levels significantly increased. μ inhibited by 32.7% at 320 µg/mL for PS and 34% at 320 µg/mL for PS-NH_2	(Sun et al., 2018)
Chlorophyta Chlamydomonadales	*Dunaliella tertiolecta*	PS	50	25, 250	μ inhibited by 57%, at 250 µg/mL cell density reduced by 45% at 250 µg/mL	(Sjollema et al., 2016)
		PS-COOH PS-NH_2	40 50	0.5, 1, 5, 10, 25, 50 in nsw	EC_{50} for μ of 12.97 ± 0.57 µg/mL	(Bergami et al., 2017)
Echinodermata Camarodonta	*Paracentrotus lividus* (embryos)	PS-NH_2	50	3, 4 in nsw	Delay in development Deficient skeleton rods and arms	(Pinsino et al., 2017)
		PS-COOH PS-NH_2	40 50	50 10 in nsw	Larval malformations $EC_{50\ 24\ hpf}$ of 3.82 µg/mL; $EC_{50\ 48\ hpf}$ of 2.61 µg/mL, for PS-NH_2	(Della Torre et al., 2014)
Rotifera Ploimida	*Brachionus plicatilis*	PS-COOH PS-NH_2	40 50	0.5, 1, 5, 10, 20, 50 in nsw	PS-COOH accumulation in organisms; $LC_{50\ 24h}$ of 13.04 ± 0.60 µg/mL and $LC_{50\ 48h}$ of 6.62 ± 0.87 µg/mL for PS-NH_2	(Manfra et al., 2017)
Mollusca Ostreoida	*Crassostrea gigas*	PS-COOH PS-NH_2	100	0.1, 1, 10, 100	PS-COOH aggregates attached to the cells; decrease in the number of spermatozoa and ROS levels significantly increased in PS-COOH.	(González-Fernández et al., 2018)

	Mytilus edulis	PS	30	0, 100, 200, 300 in asw	Reduce filtering activity. Particles adsorbed to the gills.	(Wegner et al., 2012)
	Mytilus galloprovincia-lis	PS-NH_2	50	1, 5, 50 in asw	Cytochrome c reduced. Decrease in phagocytosis Strong lysosomal destabilization.	(Canesi et al., 2015)
	Mytilus galloprovincia-lis (48hpf larvae)	PS-NH_2	50	0.001, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20 in asw	$EC_{50,\ growth}$ of 0.142 µg/mL. Malformed and immature embryos. Decrease in shell length by 20 to 30%.	(Balbi et al., 2017)
Arthro-poda Sessilia	*Amphibalanus amphitrite* (II stage)	PS	100	0.001, 0.01, 0.1, 1, 10 in nsw	Decreased swimming speed Particles aggregation in the gut	(Gambardella et al., 2017)
Arthro-poda Anostra-ca	*Artemia franciscana* (1st instar larvae)	PS	100	0.001, 0.01, 0.1, 1, 10 in nsw	Decreased swimming speed Particles aggregation in the gut	(Gambardella et al., 2017)
	Artemia franciscana (1st instar larvae)	PS-COOH PS-NH_2	40 50	0.5, 1, 1.5, 2.5, 5, 10 in nsw	$LC_{50,14days}$ of 0.83 µg/mL. Induction of *clap* and *cstb* genes	(Bergami et al., 2017)
	Artemia franciscana (up to instar III Nauplius)	PS-COOH PS-NH_2	40 50	5, 25, 50, 100 in nsw	Difficulties in swimming: increase the number of molts; aggregation in the gut lumen.	(Bergami et al., 2016)
Arthro-poda Harpacti-coida	*Tigriopus japonicus*	PS	50	0.125, 1.25, 12.5, 25 in nsw	Gut retention; Survival affected at concentrations higher than 1.25 µg/mL.	(K. Lee et al., 2013)

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CHAPTER III

Nanoplastic effects on microalgae and rotifers

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2018

Abstract

The biota of marine ecosystems is currently exposed to plastics of different types, sizes and shapes and other environmental contaminants that may compromise their health status and consequently, ecosystems. Polymethylmethacrylate (PMMA) is a type of plastic for which little information is available in terms of potential effects to aquatic organisms, despite its use in different human activities. Caffeine is included in the high production volume chemicals, one of the most widely consumed drugs in the world, thus proposed as an anthropogenic marker. In this perspective, the study of the effects of PMMA alone and combined with caffeine may provide important information on the interaction of these particles with environmental contaminants. Accordingly, two specific objectives were defined for this study: i) to assess the effects of increasing concentrations of PMMA on four marine algae species (*Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*) and on three types of one marine rotifer species (*Brachionus plicatilis* type SS, S and L); ii) to verify if there is an interaction between PMMA and environmental contaminants, namely caffeine. The first objective was achieved by performing a battery of standard monospecific bioassays. To tackle the second objective, the growth rates of *T. chuii* and *N. gaditana* were evaluated when exposed to caffeine alone and when combined caffeine with PMMA. PMMA was able to influence the growth rate of all microalgae species with *T. weissflogii* being the most sensitive one (EC_{50} = 83.75 mg/L) while *T. chuii* was the less affected one (EC_{50} = 132.52 mg/L). PMMA also affected the survival of the rotifers with a LC_{50} of 13.27 mg/L for the most sensitive type of rotifer. Concerning caffeine an EC_{20} of 565.4 mg/L and 567.6 mg/L was estimated for *T.chuii* and *N. gaditana,* respectively. In the combined exposure, results showed than the mixture was able to significantly affect the growth rate of marine algae.

1. Introduction

Aquatic organisms are exposed to a variety of natural and anthropogenic stresses. Plastic debris are an example of anthropogenic-derived stressors due to their widespread use and durability (Abidli et al., 2018). Considering that there are several types of plastics polymers, their effects to marine biota can be very distinct, affecting organisms in different ways. Since there are no evidences that the amount of plastic ending up on the oceans is decreasing (Law et al., 2010; Goldstein et al., 2012), it becomes imperative to analyze the impacts of these debris. Polymethylmethacrylate (PMMA) is a plastic polymer that may be able to affect marine organisms and/or interact with other contaminants affecting organisms differently as other polymers do (Caron et al., 2018; Compa et al., 2018). Those contaminants can either be related to plastic production processes, or related to other pollution sources (Gauquie et al., 2015). Caffeine is a xanthine alkaloid compound and a central nervous system stimulant, consumed daily in coffee, tea, soft drinks, and chocolate. Thus, it one of the most widely consumed psychoactive substances in the world (Knee et al., 2010; Paíga & Delerue-Matos, 2017) which makes it being discharge through wastewaters and, later, discard into coastal waters (Comeau et al., 2008). Caffeine is one of the most commonly found organic chemicals in surface waters (Pollack et al., 2009) and as Dafouz et al., (2018) stated, caffeine levels present a chronic risk quotient higher than one for almost one third of seawater samples.

Microalgae are primary producers, so they are responsible for producing energy, oxygen and food. Microalgae have been used in toxicity tests to assess the toxic effects of compounds like metals (Hamed et al., 2017; Cameron et al., 2018) or drugs (Teixeira & Granek, 2017; Bácsi et al., 2018). However, no studies have been found concerning the toxic effects of nanoplastics to marine microalgae. Rotifers are aquatic invertebrates that have crucial roles such as energy transfer between producers and consumers in aquatic food chains (Han et al., 2018). Since they are small, they are also suitable for the earliest stages of fish and shrimp larvae (Dhont et al., 2013). Despite its importance in the aquatic environment, few studies are found in terms of the effects of nanoplastics to marine rotifers.

Therefore, the aim of this study was to evaluate the effects of PMMA nanoplastics, on the growth rates of four marine microalgae (*Tetraselmi chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*) and one marine rotifer (*Brachionus plicatilis*) through standard bioassays. Two types of assessment were performed: a) species (microalgae and rotifers) were exposed to PMMA nanoplastics solely; and b) species (microalgae) were exposed PMMA nanoplastics combined with caffeine.

2. Materials and methods

2.1. Nanoplastics

Polymethylmethacrylate (PMMA) characterization was performed in ultrapure water as well as seawater through dynamic light scattering (DLS) in order to analyze the nanoplastics behavior. PMMA in ultrapure water had an average size of 49.49 nm (Figure III.1-a) however, when the same nanoplastics are in saltwater its size increases as shown in figure III.1-b. Particles size increased immediately after being placed in saltwater to approximately 58.6 nm. After 1 hour the average size was 97.3 nm and they reached a size of 120.3 nm after 24 h. Their suspension stability was also evaluated through zeta potential assessment, with particles presenting a value of -22.3 mV in seawater. All executed tests used the same stock solution of PMMA (0.4395 g/mL).

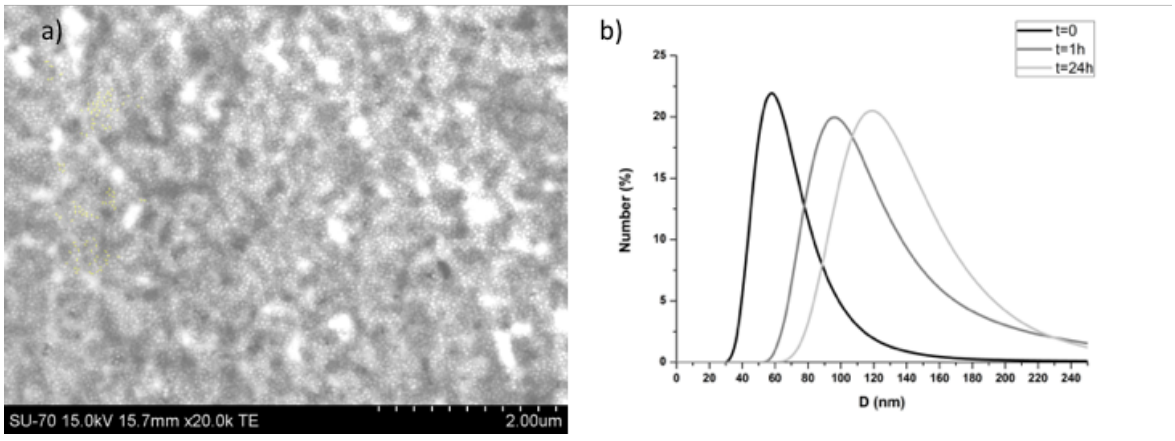

Figure III.1 – Characterization of polymethylmethacrylate (PMMA). a) PMMA nanoparticles seen at an electron microscope. b) Size characterization (after 0, 1 and 24 h) of PMMA nanoparticles in saltwater through dynamic light scattering (DLS).

2.2. Selection of test organisms

Microalgae (e.g. *Tetraselmis chuii* and *Nannochloropsis gaditana*) are used in aquaculture as a primary food source for larval and juvenile bivalves, as well as larvae of crustaceans and fish species (Brown et al., 1997). *T. chuii* is a prasinophyceae algae with cylindrical shape that has between 8 and 16 μm. It has a large distribution among various applications in laboratorial cultures (Cordero et al., 2005). *N. gaditana* belongs to the phylum Heterokonta and is a marine microalga that has gained increasing attention due to its promising role in biofuel production systems, because of its fatty acid profile and high lipid content (Alboresi et al., 2017; Jackson et al., 2018; Moraes et al., 2018). *Isochrysis galbana* is another example of a microalgae that is potentially promising to the food industry due to its significantly high lipid content (Bonfanti et al., 2018). This specie can grow at high temperatures, such as 30 ºC so it is used as food in tropical aquaculture (Silitonga et al., 2017). Marine diatoms are also an important part of microalgae. Approximately 20 % of total global primary production are due to marine diatoms (Gao et al., 2018). *Thalassiosira weissflogii* is a primary producer diatom widely used in toxicity tests as a sensitive test organism (Araújo & Souza-Santos, 2013).

The marine rotifer *Brachionus plicatilis* is an important organism for ecophysiology, ecotoxicology and environmental genomics (Dahms et al., 2011; Hagiwara & Yoshinaga, 2017) and it has already been used in many toxicity tests due to its small size, short generation cycle, high fecundity, and easy laboratory maintenance (Zheng et al., 2017; Han et al., 2018; Ponce et al., 2018).

2.3. Maintenance of test organisms

Four marine microalgae, *Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii* were used in this study. All species were maintained in F/2 medium (Guillard, 1975) made with natural seawater (NSW), previously filtered through a 200 nm filter and then autoclaved for 20 minutes at 121 ºC, 1 BAR (Uniclave 88, AJC). After sterilization, the medium was supplied with vitamins (B1, B12 and H). In the case of the diatom, culture medium was supplied with silica (22.5 g/L). Algae were kept under laboratorial conditions both of light (24 h light) and temperature (23 ± 1 ºC).

The marine rotifer *Brachionus plicatilis* was selected as a primary consumer. This species can be divided in three types according to their size: SS from 100 to 160 μm, S from 140 to 220 μm and L from 190 to 320 μm (Rahman et al., 2018). They were maintained in NSW, previously filtered through a 200 nm filter to remove organisms and suspended particles, and with salinity adjusted to 20, an optimal value for the growth of rotifers. Organisms were fed three times a week with *Tetraselmis chuii* (~2 * 10^5 cell/mL) (Kaneko et al., 2016) and maintained under laboratorial control conditions of temperature (23 ± 1 ºC) and light (24 h light).

2.4. Ecotoxicity assays

2.4.1. Methodology used to count microalgae

Cell counting is a precise method but rather time-consuming. Thus, for each algae species, a calibration curve was performed relating the absorbance of the samples with the number of cells present in that same sample, in the absence of contaminants. For each alga, a calibration curve was performed throughout an 8 days growth test. An algae concentration of 10^4 cell/mL was used to start the test and then, every day, algae were counted with a Neubauer chamber and absorbance (ABS) read in a spectrophotometer (Thermo Scientific Multiskan Spectrum). The cell density (number of cells/mL) was calculated through the absorbance measurements, obtained at specific wavelengths according to each algae species: 540 nm in *T. chuii* (Enache, 2013); 640 nm and 682 nm for *N. gaditana* (Gentile & Blanch, 2001; Santos-Ballardo et al., 2015); 660 nm and 680 nm for *I. galbana* (Sánchez et al., 2000; Lin et al., 2007)*;* and 440, 462 and 490 nm for *T. weissflogii* (Taguchi & Fujiki, 2001).

2.4.2. Growth inhibition assays of marine algae exposed to PMMA

The 96-h growth rate inhibition assays started after the establishment of the curves for the growth rates of each algae species (Fig. III.2). After performing several range-finding tests, to determine concentrations ranges inducing effects on algae growth, seven definitive concentrations of PMMA were chosen to allow a more accurate determination of $LC_{50,96h}$: 150.0, 168.8, 189.8,

213.6, 240.3, 270.3 and 304.1 mg/L for *T. chuii*, *N. gaditana* and *I. galbana* and 75.0, 94.1, 118.1, 148.3, 186.1, 233.5, 293.0 mg/L for the diatom *T. weissflogii*. The assays were performed according to the OECD guideline 201 (OECD, 2011), adapted to 24-well microplates. Three replicates were established per concentration plus a control (F/2 medium solely). Each replicate contained 900 µL of the nanoplastics solution (prepared in F/2 medium) and 100 µL of algal inoculum (at an initial cell concentration of 10^5 cells/mL). The tests lasted for 96 h and during the incubation period, test plates were kept at 20 ± 0.1 ºC with continuous light. At the end of the assays, cell density (number of cells/mL) for all tests was calculated through the Absorbance (ABS) measurements and average growth rate (µ), for each species (equation 2, 3, 4 and 5), was determined through equation 1:

1. $\mu ab = \frac{(lnDb - lnDa)}{tb - ta} \times 100$, where D_b is the cell density at the end of the assay, D_a is the cell density at the beginning of the assay and $t_b - t_a$ is the exposure time interval (96 h).

2.4.3. Growth inhibition assays of marine algae exposed to PMMA and caffeine

For the combined exposure assays, only two of the four species of marine microalgae were selected: *T. chuii* and *N. gaditana*. Firstly, the growth rates of the two algae species were evaluated under exposure to the following concentrations of caffeine (based on Aguirre-Martínez et al., (2015)): 350, 400, 450, 500, 550, 600 and 650 mg/L. To assess the toxicity of the mixture of caffeine and PMMA the selected concentrations were: 100 (P1) and 115 (P2) mg/L of PMMA and 250 (C1) and 350 (C2) mg/L of caffeine.

The test procedure followed the OECD guideline 201 (OECD, 2011) and it was executed as described in the previous section. Briefly, three replicates were established, each one had 900 µL of caffeine or PMMA combined with caffeine and 100 µL of algae inoculum. The same method as before was used to calculate cell density and determinate the average growth rate (equation 1).

2.4.4. Effect of PMMA on the survival of rotifers

The 48-h survival assays with rotifers were based on Rotoxkit M protocol (MicroBioTests Inc., Ghent, Belgium). Tests were performed in 24-well plates. Five PMMA concentrations (based on range finding tests) were tested 4.7, 9.4, 18.9, 37.5, 75.0 mg/L plus a negative control. Concentrations were obtained by diluting a stock solution of NP (439500 mg/L) with filtered NSW at a salinity of 20. Each well was filled with 1 mL of the nanoplastics desired concentration. Four replicates were assembled per treatment, with five organisms per replicate. Organisms were kept at 20 ± 1 ºC, in the dark and were not feed during this test. Percentage of survival was determined after 48 h of exposure.

2.5. Data analysis

Each data set of algae was checked for normality (Shapiro-Wilk) and an Equal Variance Test (Brown-Forsythe) If the algae passed both tests, then a one-way variance analysis (one-way ANOVA) was performed followed by the Dunnett test to assess possible differences between treatments and the respective control (with no nanoplastics added). If the algae did not pass the normality and equal variance tests a non-parametric ANOVA was calculated. For the combined exposure a Tukey test was used instead of a Dunnett test, in order to test differences between mixture and control, mixture and caffeine and mixture and PMMA. $p < 0.05$ was taken as the significant cutoff. Statistical analysis was performed using the software SigmaStat 4.0.

Effective concentration of nanoplastics and caffeine causing 50 % and 20 % of inhibition on algae growth (EC_{50} and EC_{20}, respectively) were calculated using the software Statistica. The concentrations causing 50 %, 20 % an 10 % of mortality on rotifers (LC_{50}, LC_{20} and LC_{10}, respectively) were computed using the software Probit.

3. Results

3.1. Calibration curves for marine algae

Calibration curves are showed in Fig. III.2 and the concentration of each algae in number of cells per milliliter can be determined through equations 2, 3, 4 and 5:

2. Conc (cells/mL) = ABS / 0.0000004 – 0.0088 (R^2 = 0.92), *Tetraselmis chuii*

3. Conc (cells/mL) = ABS / 0.0000004 – 0.0117 (R^2 = 0.97), *Nannochloropsis gaditana*

4. Conc (cells/mL) = ABS / 0.0000003 – 0.006 (R^2 = 0.96), *Isochrysis galbana*

5. Conc (cells/mL) = ABS / 0.0000002 – 0.0006 (R^2 = 0.99), *Thalassiosira weissflogii*

where ABS correspond to the absorbance read at 540 nm for *T. chuii*, 682 nm for *N. gaditana*, 680 nm for *I. galbana* and 490 nm for *T. weissflogii*.

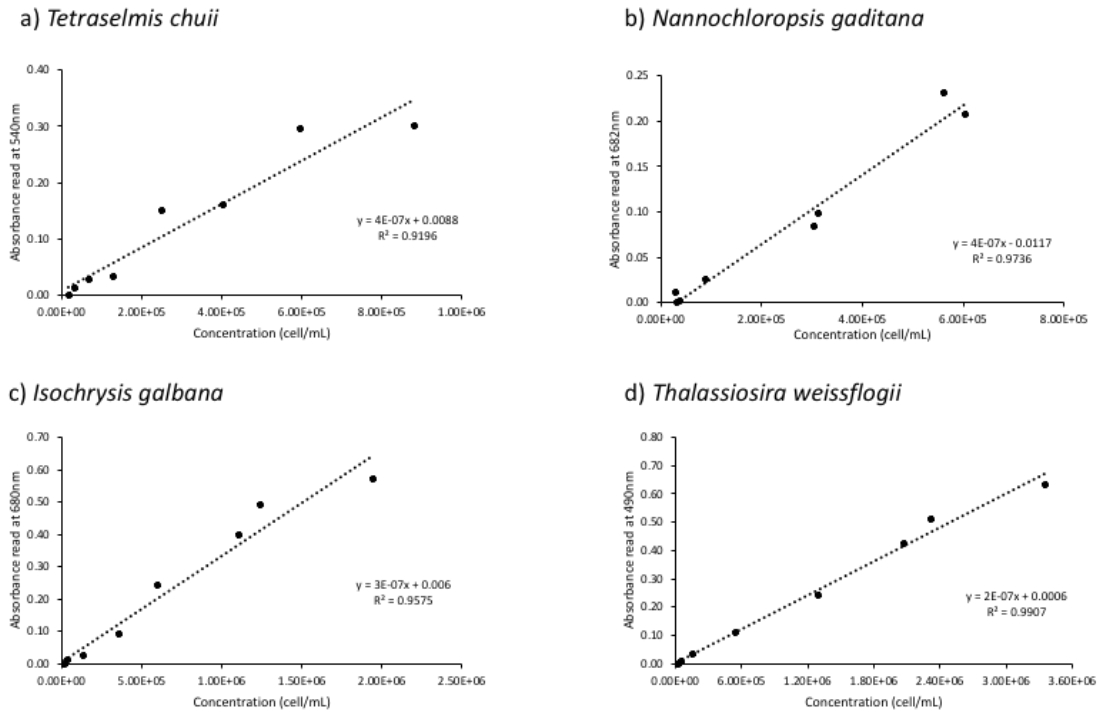

Figure III.2 – Calibrations curves for each of the studied algae species.

3.2. Growth inhibition assays of marine algae exposed to PMMA

After 96 h of exposure to PMMA nanoplastics, growth rate was significantly affected in every algae specie (Fig. III.3). Growth rate in *T. chuii* significantly decreased when compared to the control at all tested concentrations and growth rate was even 0 % at 189.8, 213.6, 240.3 and 304.1 mg/L. The same applies to *N. gaditana* which presented a growth rate of 0% at 213.6, 270.3 and 304.1 mg/L. The diatom *T. weissflogii* was also significantly affected at all concentrations of nanoplastics with the lower growth rate at 233.5 mg/L. For *I. galbana* growth rate was only significantly decreased at 240.3 and 304.1 mg/L of PMMA. Effective concentrations for algae exposed to

polymethylmethacrylate nanoplastics and caffeine are shown in Table III.1. The green microalgae *T. chuii* was found to be the most tolerant marine algae to PMMA with an EC_{50} of 132.52 mg/L, while the diatom *T. weissflogii* was found to be the most sensitive one with an EC_{50} of 83.40 mg/L.

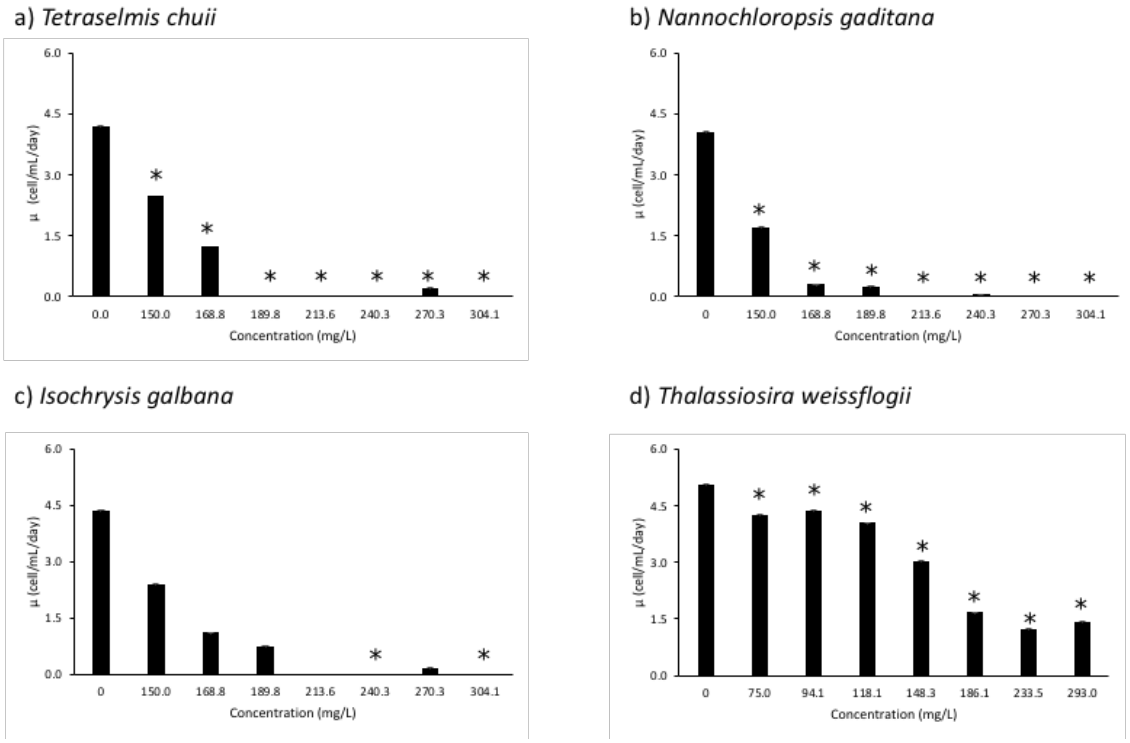

Figure III.3 – Bar plots representing the growth rate (μ) of algae exposed for 96 h to increase concentrations of polymethylmethacrylate (PMMA) nanoplastics. Vertical bars correspond to the error (n=3). *p < 0.05 (Dunnett's test).

Table III.1 – Effective concentrations causing X % of effect (EC_x) on the growth rate of marine algae after 96 h of exposure to polymethylmethacrylate nanoplastics (PMMA). Inside brackets represent the 95 % confidence limits. n.d. – not determined.

	Species	EC_x (mg/L)	
		EC_{50}	EC_{20}
PMMA	*Tetraselmis chuii*	132.5 (124.5-140.5)	117.4 (104.5-130.2)
	Nannochloropsis gaditana	116.5 (102.9-131.0)	n.d.
	Isochrysis galbana	123.8 (116.6-131.1)	106.3 (95.9-116.2)
	Thalassiosira weissflogii	83.4 (72.5-94.4)	48.9 (36.3-61.4)

3.3. Growth inhibition assays of marine algae exposed to PMMA and caffeine

Firstly, regarding the exposure to caffeine, after 96 h the growth rate of both marine microalgae was significantly affected (Fig. III.4). The growth rate of *T. chuii* was significantly decreased at 350, 500, 550, 600 and 650 mg/L when compared to control ($p < 0.001$ for every concentration). For *N. gaditana* growth rate was significantly decreased only at 550, 600 and 650 mg/L when compared to control ($p = 0.025$ for 550 mg/L; $p < 0.001$ for 600 and 650 mg/L). Effective concentrations for algae exposed to caffeine are shown in Table III.2. The results were very similar in the two algae, with *T. chuii* displaying and EC_{20} of 565.4 mg/L and *N. gaditana* an EC_{20} of 567.6 mg/L.

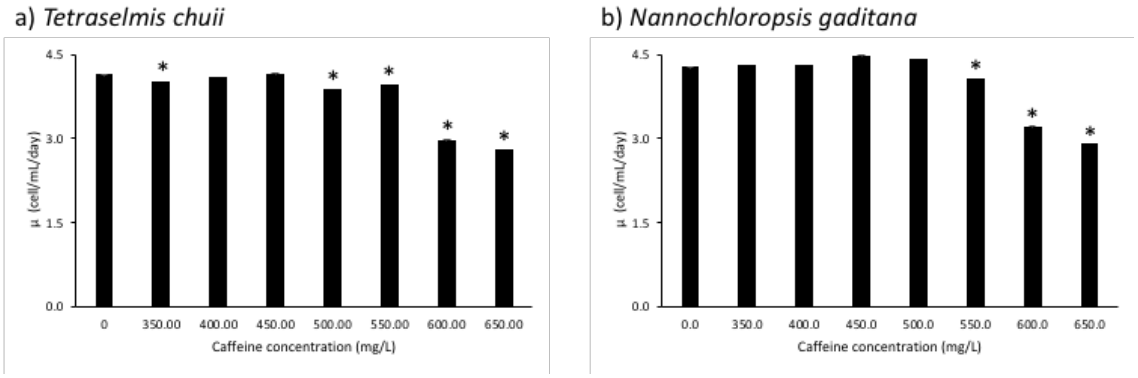

Figure III.4 – Bar plots representing the growth rate (μ) of algae exposed for 96 h to increase concentrations of caffeine. Vertical bars correspond to the error (n=3). *p < 0.05 (Dunnett's test).

Table III.2 – Effective concentrations causing X % of effect (EC_x) on the growth rate of marine algae after 96 h of exposure to caffeine. Inside brackets represent the 95 % confidence limits. n.d. – not determined; n.c. – not calculated.

	Species	**EC_x (mg/L)**	
		EC_{50}	EC_{20}
Caffeine	*Tetraselmis chuii*	n.c.	565.4 (554.3-578.7)
	Nannochloropsis gaditana	n.c.	567.6 (n.d.-582.2)

The results from the growth inhibition assay with the combined exposure of caffeine and PMMA are shown in Fig. III.5. For *T. chuii* growth rate was not significantly affected by exposure to the least amount of caffeine (C1), while for *N. gaditana*, growth rate was not significantly affected by exposure to caffeine (C1 and C2) when compared to control conditions; however, the growth rates of the two microalgae species, were significantly reduced at both PMMA concentrations (P1 and P2), as well as when exposed to the mixtures when compared to the control. Moreover, show that for *T. chuii* there were no significant differences in growth rate between P1 and P1 + C2, as well as between P2 and P2 + C2. However, there were significant differences between P1 and P1 + C1 and between P2 and P2 + C1. Regarding *N. gaditana* there were no differences only between the mixtures P2 + C1 and P2 + C2.

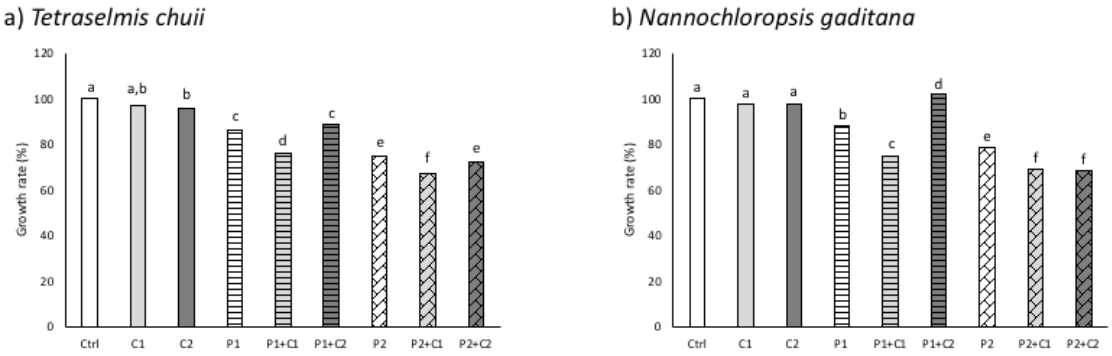

Figure III.6 – Bar plots representing the growth rate (%), with growth rate of control being considered 100 %, of algae exposed for 96 h to caffeine (C1 = 250 mg/L; C2 = 350 mg/L), polymethylmethacrylate (PMMA) (P1 = 100 mg/L; P2 = 115 mg/L) and a mixture of PMMA and caffeine (P1+C1 = 100 mg/L of PMMA with 250 mg/L of caffeine; P1+C2 = 100 mg/L of PMMA with 350 mg/L of caffeine; P2+C1 = 115 mg/L of PMMA with 250 mg/L of caffeine; P2+C2 = 115 mg/L of PMMA with 350 mg/L of caffeine). Different letters represent significative differences between the means ($p < 0.05$) recorded in the several concentrations (Tukey's test).

3.4. Survival assay with PMMA

The survival of rotifers was affected when they were exposed to PMMA nanoplastics for 48 h (Fig. III.6). *Brachionus plicatilis* type S and SS organisms were less affected, with significant effects on survival detected at the highest concentration, 75 mg/L ($p < 0.001$ and $p = 0.008$, respectively). On the other hand, type L rotifers shown significant decreased survival at concentrations equal or above 9.38 mg/L and 0 % at 75 mg/L ($p = 0.039$ for 9.38 mg/L; $p = 0.003$ for 18.75 mg/L; $p = 0.007$ for 37.5 mg/L and $p < 0.001$ for 75 mg/L). The survival of rotifers allowed the estimation of lethal concentrations for rotifers exposed for 48 h to PMMA nanoplastics (Table III.3). Type S rotifers was discovered to be the more tolerant species (LC_{50} = 37.59 mg/L), while L was discovered to be the most sensitive one with an LC_{50} of 13.27 mg/L).

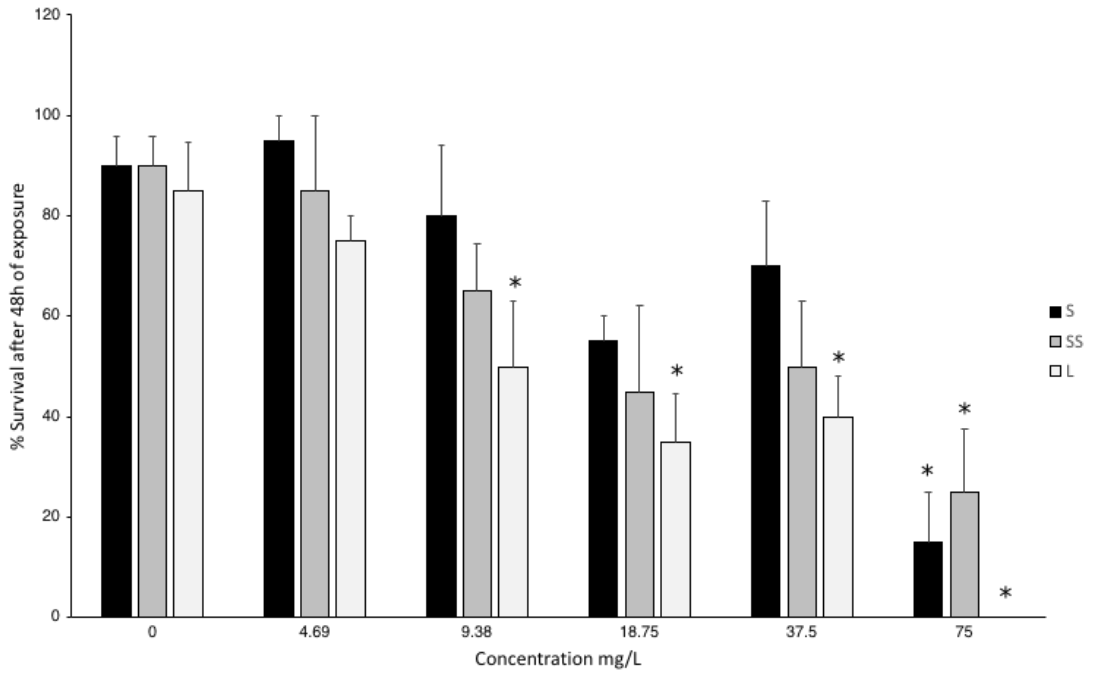

Figure III.6 – Bar plots indicating the percentage of survival after 48 h of three size categories of *Brachionus plicatilis* exposed to polymethylmethacrylate (PMMA). Vertical bars correspond to the error (n=4). *p < 0.05 (Dunnett's test).

Table III.3 – Lethal concentrations of polymethylmethacrylate (PMMA) nanoplastics causing X % of effect (LC_x) for each specie studied. Mortality was measure after 48 h. Inside brackets are represented the 95 % confidence limits. n.d. – not determined.

	Species	**LC_x (mg/L)**		
		LC_{50}	LC_{20}	LC_{10}
PMMA	*Brachionus plicatilis* Type S	37.6 (27.2-61.6)	13.8 (8.6-19.0)	8.18 (4.0-12.1)
	Brachionus plicatilis Type SS	29.3 (10.3-n.d.)	6.7 (n.d.-16.1)	3.1 (n.d.-9.3)
	Brachionus plicatilis Type L	13.3 (8.1-19.7)	3.8 (1.1-6.6)	2.0 (0.4-4.1)

4. Discussion

Nanoplastics have been found to affect marine organisms from bacteria to mollusks (Balbi et al., 2017; Sun et al., 2018). The PMMA nanoplastics appear to affect growth and mortality of marine microalgae and rotifers, respectively. It is difficult to find a report on PMMA effects on marine organisms,

so this may be the first report of PMMA effects on marine algae and rotifers. The exposure of microalgae to PMMA showed that this type of plastic can affect the growth at concentrations higher than 75 mg/L (*T. weissflogii*), 150 mg/L (*T. chuii* and *N. gaditana*) and 240.3 mg/L (*I. galbana*). The estimated EC confirmed that the diatom *T. weissflogii* was the most sensitive with an EC_{50} of 83.4 mg/L, followed by *N. gaditana* with an EC_{50} of 116.5 mg/L, *I. galbana* with an EC_{50} of 123.8 mg/L and the more resistant was *T. chuii* with an EC_{50} of 132.5 mg/L. Additionally, the diatom was the marine algae species where significant effects of PMMA started at lower concentrations (EC_{20} = 48.90 mg/L). Comparing the obtained data with those obtained in other studies, PMMA (~50 nm) appears to have more impact on microalgae growth rate more than polystyrene (PS) (50 nm) when comparing with the data obtained by Sjollema et al., (2016) in *Dunaliella tertiolecta* where a 57% decrease was observed at 250 mg/L. However, PMMA was less harmful for marine algae than cationic amino (-NH_2) PS particles (50 nm) where an EC_{50} of 12.97 mg/L was registered for *D. tertiolecta* after only 72h of exposure (Bergami et al., 2017). Thus, PMMA appears to be more dangerous than PS and less harmful than PS-HN_2 for marine microalgae. However, this comparison is not straightforward as species specific sensitivity must be taken into account. Studies of *T. chuii* exposed to microplastics (1-5 μm) show that the growth of this marine algae is not affected up to 41.5 mg/L (Davarpanah & Guilhermino, 2015; Prata et al., 2018) which seems to happen for *T. chuii* exposed to PMMA as well, considering that an EC_{20} of 117.4 mg/L was observed.

Effects of caffeine on marine organisms, such as mollusks and arthropods have already been studied (Aguirre-Martínez et al., 2013; Capolupo et al., 2016), but there are no studies concerning effects on *T. chuii* and *N. gaditana*. Growth rate of *T. chuii* was decreased at 350, 500, 550, 600 and 650 mg/L, as for *N. gaditana* growth rate was decreased at 550, 600 and 650 mg/L. Both algae appear to be more resistant to caffeine than *I. galbana* which had significant growth inhibition upon 96 h of exposure to 100 and 500 mg/L (Aguirre-Martínez et al., 2015). An EC_{20} of 565.4 mg/L for *T. chuii* and an EC_{20} of 567.6 mg/L for *N. gaditana* was found for caffeine, demonstrating that algae were more sensitive to PMMA than to caffeine.

There are no studies published about the effects on marine organisms of combined exposure of mixtures of PMMA with any other environmental contaminant. Thus, this report is the first regarding the effects of an exposure of PMMA and caffeine on marine organisms. Since both PMMA and caffeine independently affected the growth rate of *T. chuii* and *N. gaditana,* it was expected that when the microalgae were exposed to a mixture of those contaminants the growth inhibition was even higher. This hypothesis was verified for both *T. chuii* and *N. gaditana* in the mixture with the less amount of PMMA and caffeine (P1 + C1) and in the highest amount of PMMA and less caffeine (P2 + C1), as well as in the highest amount of PMMA and caffeine (P2 + C2) for *N. gaditana*. Regarding the effects of P1 + C2 and P2 + C2 for *T. chuii*, results showed that there are no significative differences between the mixture and the exposure to PMMA solely. On the other hand, for *N. gaditana* the mixture P1 + C2 appears to have an antagonistic effect when compared to PMMA exposure solely, this may be due to the mixture causing an intermediate level of stress which promotes a peak on the growth rate of this marine algae.

In the 48-h acute toxicity test, survival of *Brachionus plicatilis* type SS, S and L was affected by PMMA (~50 nm). Type L rotifers, the bigger sized ones, were significantly affect from 9.38 mg/L, while type S and SS were only affected at 75 mg/L. Since it had already been proved that nanoplastics are ingested and retained by rotifers (Manfra et al., 2017), the observed differences in survival of this marine rotifer can be due to the different sizes of the organism, being easier for the largest type of rotifers to incorporate PMMMA particles and, consequently, be more affected by them. Therefore, *B. plicatilis* type L was the most sensitive one with a LC_{50} of 13.3 mg/L and also the one that was affected at lower concentrations (LC_{10} = 1.97 mg/L), then type SS with a LC_{50} of 29.3 mg/L and, lastly, type S with a LC_{50} of 37.6 mg/L. These results show that PMMA is less harmful than PS-NH_2 (50 nm), since a LC_{50} of 6.62 mg/L was observed for the same rotifer specie by Manfra et al. (2017). On the other hand, *B. plicatilis* exposed to PS nanoplastics (100 nm) did not show any significant effect on mortality in concentrations up to 10 mg/L (Gambardella et al., 2018), suggesting a size related toxicity.

In summary, marine microalgae revealed to be more tolerant to PMMA than all types of *B. plicatilis*. In fact, the most sensitive microalgae (*T.*

weissflogii) was twice more tolerant than the most tolerant type of marine rotifer (*B. plicatilis* type S).

Nanoplastics affect, in different ways, organisms from several habitats and trophic levels. These data contribute to scientific knowledge about nanoplastics effects, however it is important to expose these species to different sizes and polymers of nanoplastics in order to evaluate the bioavailability, as well as combined exposures with different contaminants. Also, there is a need to investigate the effect of nanoplastics through the food chain and the mechanisms of bioaccumulation in order to see if these particles could be dangerous even for humans.

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CHAPTER IV

General discussion

1. General discussion and future perspectives

Plastic are becoming a serious threat to marine environment regardless their type, size and shape. All marine ecosystems are affected by them, species from zooplankton to whales are exposed to plastic. The literature review showed that currently, there is considerable amount of information concerning the effects of macro- and microplastics. However, and even though studies show that these macro- and microplastics can degrade into nanoplastics, few studies have focused on the effects of these particles to marine organisms. Regarding marine organisms the most studied organisms are arthropods and mollusks but, for algae and rotifers, a single species has been studied. Polystyrene is the most commonly studied plastic in the literature, however different kinds of plastic may cause different effects on the same organism, justifying further studies with other types of polymers like PMMA.

The effects of PMMA nanoplastics on marine microalgae and rotifers are completely unknown. This lack of knowledge justified the study of the effects of PMMA on producers (*Tetraselmi chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*) and primary consumers (*Brachionus plicatilis*) in chapter III. The results from ecotoxicological assays showed that PMMA significantly affects algae growth with an EC_{50} from 83.4 (*T. weissflogii*) to 132.5 mg/L (*T. chuii*) with *T. weissflogii* being the most sensitive one. In order to evaluate the effects of a combined exposure to nanoplastics and an environment contaminant, *T. chuii*, and *N. gaditana* were exposed to caffeine. The results showed an EC_{20} of 565.4 mg/L for *T. chuii* and an EC_{20} of 567.6 mg/L for *N. gaditana*. The results from the combined exposure of PMMA and caffeine showed that the mixture was able to affect both marine microalgae. Rotifers appeared to be more sensitive to PMMA than algae. The type L of *Brachionus plicatilis* was the most affected one with a LC_{50} of 13.3 mg/L and its survival was significantly affected even at 2.0 mg/L.

Overall this work shows the need to assess the effects of nanoplastics on different type of organism, present in different habitats. There is however the need for a proper characterization of the environmental levels, behavior and incorporation on biota in order to being possible to perform assays that provide environmentally relevant information. Furthermore, it is necessary to see the effects of nanoplastics in multigenerational studies as well as evaluate

epigenetics effects and not only lethality and reproductive ones. Considering the diversity of plastic polymers and that they can cause different effects on organisms it is important to test of more polymers and size ranges especially of the polymers more used by man.