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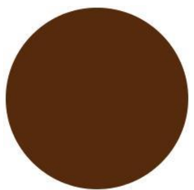

Effects of microplastics, nanoparticles and other environmental contaminants on marine organisms

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D. ICBAS 2018

U.PORTO

PROGRAMA DOCTORALCIENCIAS DO MAR E DO AMBIENTE

ÁREA DE ESPECIALIZAÇÃO EM QUALIDADE AMBIENTAL

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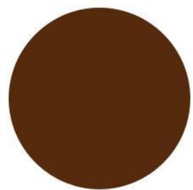

Elham Davarpanah

Effects of microplastics, nanomaterials and other environmental contaminants on marine organisms

Tese de Candidatura ao grau de Doutor em Ciências do Mar e do Ambiente

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Abstract

Currently there is a high concern about products containing microplastics and nanomaterials. This concern comes from the widespread use and production of these substances, inappropriate disposal and environmental contamination, and the adverse effects that they may cause on living organisms and humans. Moreover, the ability of microplastics to adsorb other pollutants in the marine environment is a growing concern due to the potential toxicological impacts of chemicals associated to microplastics on aquatic organisms. The knowledge about the environmental fate and behaviour of microplastics and nanomaterials, toxicological interactions between microplastics and nanoparticles, and between them and other environmental contaminants is still limited. This knowledge is most important to improve human and environmental risk assessment of these substances.

The main goal of the present thesis was to investigate if microplastics are able to modify the toxicity of other environmental contaminants, namely copper and gold nanoparticles, to the marine microalgae *Tetraselmis chuii*. This species was selected as test organism mainly because is an abundant species in several marine ecosystems where it is an important component of the phytoplankton community and has been widely used as representative of primary producers in ecotoxicological studies. To reach the main goal four null hypotheses were tested: (H_{01}) exposure to microplastics (1-5 μm spheres) at concentrations up to the low ppm range does not affect the average specific growth rate (hereafter indicated as population growth) of *T. chuii*; (H_{02}) microplastics do not interact with the effects of copper on *T. chuii* population growth; (H_{03}) exposure to concentrations of gold nanoparticles in the low ppm range does not affect the population growth rate of *T. chuii*; (H_{04}) microplastics do not change the toxicity of gold nanoparticles to *T. chuii*.

The Thesis is organized in seven Chapters. Chapter I corresponds to the general introduction of the Thesis. Chapter II is a review of the literature regarding microplastic environmental contamination, challenges and effects. Chapter III is a review on the environmental contamination by metalic nanoparticles, challenges and effects. Chapter IV corresponds to an experimental study where the null hypotheses H_{01} and H_{02} were tested. Chapter V corresponds to an experimental study where the null hypotheses H_{03} and H_{04} were tested. Chapter VI corresponds to the general discussion and conclusions, and Chapter VII is the list of references.

Overall, Chapters II and III indicate that despite the considerable amount of work done in the least years regarding the environmental contamination by microplastics and metallic nanoparticles, and their biological effects, there are several practical difficulties in accessing

the environmental contamination and effects of these particles, and the knowledge on their biological and ecological effects is still limited, especially regarding mixture toxicity.

In Chapter IV, a first 96 h toxicity bioassay testing several concentrations of microplastics alone (0.046 to 1.472 mg/l) was carried out. No significant ($p > 0.05$) effects of microplastics on *T. chuii* population growth were found, indicating that the microplastics tested were not toxic to *T. chuii* up to 1.472 mg/l and leading to the acceptance of H_{01}. Then, a second bioassay testing the effects of copper alone (0.02 to 0.64 mg/l) on *T. chuii* population growth was conduct. Copper alone significantly ($p \leq 0.05$) decreased the population growth of *T. chuii*. The 10 % (EC_{10}), 20 % (EC_{20}) and 50 % (EC_{50}) effect concentrations and corresponding 95 % confidence intervals (95 % CI) were: 0.009 (95 % CI: 0.004 - 0.016 mg/l), 0.023 mg/l (95 % CI: 0.013 – 0.035 mg/l) and 0.139 mg/l (95 % CI: 0.106 – 0.187 mg/l), respectively. Therefore, in the range of concentrations tested, copper was toxic to *T. chuii*. Finally, a mixture bioassay to test the second null hypothesis was carried out. The treatments were: control; 0.184 mg/l of microplastics alone; 0.64 mg/l of copper alone and 6 mixtures, each one containing 0.184 mg/l of microplastics and one of the following copper concentrations: 0.02, 0.04, 0.08, 0.16, 0.32 or 0.64 mg/l. Based on the copper concentration, the EC_{10}, EC_{20} and EC_{50} of the mixture were: 0.012 mg/l (95 % CI: 0.006 – 0.020 mg/l), 0.029 mg/l (95 % CI: 0.017 – 0.041 mg/l), and 0.145 mg/l (95 % CI: 0.113 – 0.189 mg/l), respectively. No significant ($p > 0.05$) differences between the toxicity curves of copper alone and in the presence of microplastics were found. These findings indicated that the microplastics tested did not change significanty the toxicity of copper to *T. chuii* and lead to the acceptance of H_{02}.

In Chapter V, the toxicity of gold nanoparticles to *T. chuii* and the potential influence of microplastics on the toxicity of gold nanoparticles to *T. chuii* were investigated (to test H_{03} and H_{04}, respectively). The treatments were: control; citrate-control; gold nanoparticles alone (0.1, 0.3 and 3 mg/l); microplastics alone (0.3, 0.9 and 4 mg/l) and 3 mixtures of the two substances (0.1 mg/l gold nanoparticles + 0.3 mg/l microplastics, 0.3 mg/l gold nanoparticles + 0.9 mg/l microplastics, 3 mg/l gold nanoparticles + 4 mg/l microplastics). The treatment containing citrate had no significant differences in relation to the control group, indicating that the tested concentration of this substance was not toxic to *T. chuii*. Gold nanoparticles alone and microplastics alone did not cause significant ($p > 0.05$) decrease of *T. chuii* population growth up to 3 mg/l and 4 mg/l, respectively. The mixture containing the highest concentrations of both substances significantly ($p \leq 0.05$) reduced the population growth of the microalgae by 27 %. Therefore, the mixture was more toxic to *T. chuii* than its components individually, leading to the rejection of H_{04}.

Overall, the findings of the present thesis indicated that copper, and the microplastics and gold nanoparticles tested have a relatively low toxicity to *T. chuii*. Therefore, in the most part of marine ecosystems, these substances are not expected to cause significant negative impacts on natural populations of *T. chuii* after short-term exposure. The results of Chapters IV and V also show that microplastics may influence or not the toxicity of other contaminants to a particular species, depending of the specific properties of the microplastics and of the other contaminants tested. Moreover, the results of Chapter V and studies published in the literature, indicate that microplastics are able to increase the toxicity of other contaminants (*e.g.* some types of nanoparticles and pharamaceuticals) to *T. chuii* and other aquatic organisms. Therefore, further studies on the combined effects of microplastics and other environmental contaminants on microalgae and other aquatic organisms are needed, especially under long-term exposures and different abiotic conditions.

Key words: gold nanoparticles; microplastics; copper; mixture toxicity; marine primary producers; microalgae; *Tetraselmis chuii*

Resumo

Atualmente existe grande preocupação relativamente a produtos contendo microplásticos e nanomateriais. Essa preocupação vem do amplo uso e produção dessas substâncias, do descarte inadequado e dos efeitos adversos que podem causar em organismos vivos e no Homem. Além disso, a capacidade dos microplásticos para adsorver outros poluentes no ambiente marinho é igualmente uma preocupação devido à elevada toxicidade de muitos desses agentes.

O conhecimento sobre o destino, comportamento e interações toxicológicas entre microplásticos e nanomateriais, e entre estes e outros contaminantes ambientais é limitado. Esse conhecimento é muito importante para melhorar avaliação de risco dessas substâncias.

O objetivo central da presente Tese foi investigar se os microplasticos são capazes de modificar a toxicidade de outros contaminantes ambientais, nomeadamente cobre e nanopartículas de ouro, para a microalga marinha *Tetraselmis chuii*. Esta espécie foi selecionada como organismo teste principalmente por ser uma espécie abundante em vários ecossistemas marinhos, sendo uma componente importante da sua comunidade fitoplanctónica, e porque tem sido muito utilizada como espécie representativa de produtores primários em estudos ecotoxicológicos. Para atingir este objetivo central foram testadas quatro hipóteses nulas: (H_{01}) a exposição a microplásticos (esferas de 1-5 μm de diâmetro) até concentrações na gama inferior das inferior das partes por milhão (ppm) não afeta a taxa específica média de crescimento (doravante indicada como crescimento populacional) de *T. chuii*; (H_{02}) os microplásticos não interagem com a toxicidade do cobre para *T. chuii*; (H_{03}) a exposição a concentrações de nanopartículas de ouro na gama inferior das ppm não afeta o crescimento populacional de *T. chuii*; (H_{04}) os microplásticso não modificam a toxicidade das AuNP para *T. chuii*.

A Tese está organizada em sete Capítulos. O Capítulo I corresponde à introdução geral da Tese. O Capítulo II é uma revisão da literatura relativamente à contaminação ambiental por microplásticos, desafios ao conhecimento e efeitos destas partículas. O Capítulo III é uma revisão da literatura sobre o problema da contaminação ambiental por nanopartículas metálicas, desafios e efeitos destas partículas. O Capítulo IV corresponde a um estudo experimental onde as hipóteses nulas H_{01} e H_{02} foram testadas. O Capítulo V corresponde a um estudo experimental onde as hipóteses nulas H_{03} e H_{04} foram testadas. O Capítulo VI corresponde à discussão geral e conclusões e o Capítulo VII é a lista de referências.

De forma resumida, os Capítulos II e III indicaram que, apesar do número considerável de trabalhos sobre os efeitos biológicos de microplásticos e nanopartículas metálicas

efetuados nos últimos anos, ainda há dificuldades práticas consideráveis na avaliação da contaminação ambiental por estas substâncias e dos seus efeitos e que o conhecimento sobre os seus efeitos biológicos e ecológicos ainda é limitado, especialmente no que se refere à toxicidade de misturas.

No Capítulo IV, foi efetuado um primeiro bioensaio de toxicidade com duração de 96 h onde foram testadas várias concentrações de microplásticos isoladamente (0.046 a 1.472 mg/l). Não foram encontratados efeitos significativos ($p > 0.05$) dos microplásticos testados no crescimento populacional de *T. chuii*. Estes resultados indicam que os microplásticos não foram tóxicos para a microalga até 1.472 mg/l, pelo que se aceitou a H_{01}. De seguida foi efetuado um bioensaio onde se investigaram os efeitos de várias concentrações de cobre isoladamente (0.02 a 0.64 mg/l). O cobre dimuniu significativamente o crescimento populacional de *T. chuii*, com concentrações efetivas para 10 % (EC_{10}), 20 % (CE_{20}) e 50 % (CE_{50}) da população da microalga (e correspondentes intervalos de confiança a 95 % - 95 % IC) iguais a 0.009 mg/l (95 % CI: 0.004 – 0.016 mg/l), 0.023 mg/l (95 % CI: 0.013 – 0.035 mg/l) e 0.139 mg/l (95 % CI: 0.106 – 0.187 mg/l), respetivamente. Portanto, na gama de concentrações testada, o cobre foi tóxico para *T. chuii*. Finalmente, foi efetuado um bioensaio de misturas para testar a H_{01}. Neste bioensaio, foram avaliados os efeitos de misturas de cobre (concentrações semelhantes às do segundo bioensaio) e dos microplásticos (0.184 mg/l) testados no primeiro ensaio, no crescimento populacional de *T. chuii*. Com base nas concentrações de cobre, as CE_{10}, CE_{20} e CE_{50} e respetivos 95 % CI, determinados após 96 horas de exposição foram 0.012 mg/l (0.006 – 0.020mg/l), 0.029 mg/l (0.017 – 0.041 mg/l) e 0.145 mg/l (0.113 – 0.189 mg/l), respectivamente. A comparação das curvas de toxicidade do cobre na ausência e na presença de microplásticos através de uma Análise de Covariância indicou diferenças não significativas ($p > 0.05$) entre elas, levando à aceitação da H_{02}.

No Capítulo V, foram testadas as hipóteses nulas H_{03} e H_{04}. Após estudos preliminares que incluíram a caracterização das nanoparticulas de ouro e o seu comportamento no meio de teste, foi efetuado um bioensaio em que culturas de *T. chuii* foram expostas durante 96 h aos seguintes tratamentos: controlo; controlo-citrato; 0.1 mg/l de nanoparticulas de ouro; 0.3 mg/l de nanoparticulas de ouro, 3 mg/l denanoparticulas de ouro; 0.3 mg/l de microplásticos; 0.9 mg/l de microplásticos; 4 mg/l de microplásticos; 0.1 mg/l de nanoparticulas de ouro + 0.3 mg/l de microplásticos (Mix 1); 0.3 mg/l de nanoparticulas de ouro + 0.9 mg/l de microplásticos (Mix 2); 3 mg/l de nanoparticulas de ouro + 4 mg/l de microplásticos (Mix 3). Foram encontradas diferenças significativas ($p < 0.05$) no crescimento populacional das culturas de *T. chuii* expostas a distintos tratamentos. Nas gamas de concentrações testadas, o citrato, os tratamentos contendo apenas

nanoparticulas de ouro ou apenas microplasticos, e as misturas Mix 1 e Mix 2 não induziram efeitos significativos ($p > 0.05$) no crescimento populacional da microalga. Assim, foi aceite a H_{04}. A mistura contendo a maior concentração de nanoparticulas de ouro e a maior concentração de microplásticos causou uma redução significativa ($p \leq 0.05$) do crescimento populacional (27 %) em relação à média das culturas do grupo controlo, indicando que a mistura foi mais tóxica para *T. chuii* do que os seus componentes quando testados isoladamente, o que levou à rejeição da H_{04}.

Em resumo, os resultados do presente estudo indicaram que o cobre, os microplásticos e as nanoparticulas de ouro, nas concentrações testadas, têm uma toxicidade relativamente baixa para *T. chuii*. Assim, não é expectável que na maior parte dos ecossistemas marinhos estes agentes possam causar efeitos adversos nas populações naturais de *T. chuii* após exposições relativamente curtas. Os resultados dos Capítulos IV e V também demonstraram que os microplásticos podem influenciar ou não a toxicidade de outros contaminantes ambientais para uma determinada espécie, dependendo principalmente das propriedades específicas dos microplásticos e dos outros contaminantes testados. Os resultados do Capítulo V e estudos publicados na literatura indicam que os microplásticos podem aumentar a toxicidade de outros contaminantes ambientais (*e.g.* alguns tipos de nanopartículas e fármacos) para *T. chuii* e outros organismos. Em virtude destas evidências, é necessário efetuar mais estudos sobre os efeitos combinados de microplásticos e outros contaminantes ambientais em microalgas e outros organismos, sobretudo considerando exposições a longo prazo.

Palavras-chave: nanopartículas de ouro; microplásticos; cobre; toxicidade de misturas; produtores primários marinhos; microalgas; *Tetraselmis chuii*

Abbreviations

AChE	Acetylcholinesterase
Ag	Silver
Al	Aluminium
ANCOVA	Analysis of Co-Variance
ANOVA	Analysis of Variance
AP	Amphiphilic
AuNP	Gold nanoparticles
BaP	Benzo(a)pyrene
BPA	Bisphenol A
CeO_2	Cerium (IV) oxide
Cu	Copper
CuO	Copper (II) oxide
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
F	Fluorescence
Guillard's medium	F/2
Fe	Iron
Fe_3O_4	Iron (III) oxide
Fw	Freshwater
HDPE	High-density polyethylene
IDH	Isocitrate dehydrogenase
EC_{50}	Median Effective Concentration
LDH	lactate dehydrogenase
LDPE	Low-density polyethylene
m^3	Cubic meter
nm	nanometer
Mn	Manganese
MP	1-5 nm plastic micro-spheres used as model of microplastics
NPs	Nanoparticles
OECD	Organization for Economic Co-operation and Development
O.D.	Optical Density
PAHs	Polycyclic aromatic hydrocarbons
PAMAM	Polyamidoamine
PBDEs	Polybrominated diphenyl ethers
PBT	Polybutylene terephthalate

PCBs	Polychlorinated biphenyls
PE	Polyethylene
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
PS	Polystyrene
PS-E	Expanded polystyrene
PVC	Polyvinyl chloride
PVP	Polyvinyl pyrrolidone
PUR	Polyurethane
SnO_2	Tin (VI) oxide
Sw	Seawater
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
u.p.	Ultra-pure
Zn	Zinc
ZnO	Zinc oxide
λ	Wavelength

This thesis is dedicated to:

Masoud

&

Toranj

the completion of this work would not have been possible without their love, patience, support, understanding and help.

Aknowledgments

I wish to express my deep gratitude to my PhD supervisor, professor. *Lúcia Maria das Candeias Guilhermino*, who allowed me to realize this Thesis. She always supported me during the thesis work and gave me valuable advice. She is an excellent scientist and a person that I appreciate a lot. I have been a lucky person for having such a supervisor who continuously cared about my research work, and who so promptly replayed to my questions and queries. I thank her for to have had the courage to re-read the thesis manuscript with a comprehensive corrections and helpful comments.

I am also very grateful for the help of the technicians I met during my thesis. Thank you also to my colleagues in the laboratory.

I would like to thank all my family, my parents, my sister *Elahe*, and my brother *Amin*, who were able to accompany me even in the difficult moments of the Thesis.

I could not have completed this degree without the support and love of my husband *Masoud*, and I cannot thank him enough for standing behind me and my studies. The word also goes for *Toranj*, my daughter, who has been the light of my life for the last four years and who has given me the extra strength and motivation to get things done.

The author had a PhD fellowship from the Foundation for the Science and Technology of Portugal (FCT) (SFRH/BD/ SFRH / BD / 94099 / 2013), QREN - POPH – “Tipologia 4.1 - Formação Avançada”, co-founded by the European Social Fund and national funds from the “Ministério da Educação e Ciência”.

Research work included in this PhD Thesis was developed in the scope of the following projects:

"SIGNAL – Effects of pollution on estuarine zooplankton-zooplanktivorous fish ecological interactions in relation to climate changes", funded by the Portuguese "Fundação para a Ciência e a Tecnologia, I.P." (PTDC/AAC-AMB/110331/2009) and by the COMPETE - Operational Competitiveness Program (FCOMP-01-0124-FEDER-013876) of the European Regional Development Fund.

"PLASTICGLOBAL – Assessment of plastic-mediated chemicals transfer in food webs of deep, coastal and estuarine ecosystems under global change scenarios", co-funded by FCT, Portugal, with national funds (FCT/MCTES, "orçamento de Estado", project reference PTDC/MAR-PRO/1851/2014) and the ERDF through the COMPETE 2020 (POCI-01-0145-FEDER-016885) and Lisboa 2020 (LISBOA-01-0145-FEDER-016885) programmes.

This Thesis was also funded by the Institute of Biomedical Sciences of Abel Salazar of the University of Porto (ICBAS-UP), by the Laboratory of Ecotoxicology of ICBAS-UP (ECOTOX), and by the Research Team of Ecotoxicology, Stress Ecology and Environmental Health (ECOTOX) of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR).

In the scope of this PhD Thesis, one article was published in an international scientific journal, one was submitted to an international scientific journal, and two are in preparation, as further indicated:

Davarpanah, E.; Guilhermino, L. (2015). Single and combined effects of microplastics and copper on the population growth of the marine microalgae *Tetraselmis chuii*. Estuarine, Coastal and Shelf Science, 167. 269-275. (corresponds to Chapter IV and is included in the Thesis with the permission of the publisher included in the Annex I)

Davarpanah, E.; Guilhermino, L. (2015). A mixture of microplastics and gold nanoparticles is more toxic to the marine microalgae *Tetraselmis chuii* than the substances individually. Submitted to Science of the Total Environment (part of the work of Chapter V).

Davarpanah, E.; Guilhermino, L. (in preparation). Tentative title: Factors contributing to the uncertainty in the assessment of aquatic ecotoxicological effects of metallic nanomaterials – what we known and what we need to know.

Davarpanah, E.; Guilhermino, L. (in preparation). Tentative title: what we know about challenges and effects of microplastics in the marine environment.

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Chapter I:

General Intoduction

1.1. Introduction

Pollution of coastal and marine ecosystems is increasing globally. Industrial and domestic discharges into the marine environment, high population density close to coasts and the hydrographic basins are major contributors to the transportation of a considerable number of chemical pollutants to estuaries and other coastal ecosystems (Vikas and Dwarakish, 2015; Lebreton *et al.*, 2017). Disposal and spreading of contaminants such as petroleum products, heavy metals, plastics, nanoparticles, induce adverse effects on the marine environment and on its biota even at low concentrations (Mearns *et al.*, 2014). Therefore, concerns about the potential harmful effects of these substances on the health of marine organisms and humans consuming contaminated species have increased (Fleming *et al.*, 2006; Wright and Kelly, 2017). Several global pollutants present in the marine environment have impact on human food, water, climate, carbon cycle and disease control (Pinto *et al.*, 2014; Singh *et al.*, 2017).

Among marine ecosystems, estuaries and other coastal areas as highly productive ecosystems, produce important services to the human society, support an important range of biota (*e.g.* as nursery area or migration routes) and crucial chemical, physical and biological processes (Roessig *et al.*, 2004; Martinho *et al.*, 2007; Dolbeth *et al.*, 2008; Weckström *et al.*, 2017). In general, the main attributes of estuarine ecosystems are: the salinity gradient which is in great part responsible for the distribution of organisms in these systems (Leitão *et al.*, 2007; Telesh and Khlebovich, 2010; Cloern *et al.*, 2017); the water circulation that influences the transport of organisms, nutrients and oxygen cycling; and the presence of a wide variety of chemicals released from several sources including classical (*e.g.* metals, persistent organic pollutants (POPs) and detergents) and emerging ones (*e.g.* pharmaceuticals, nanomaterials and microplastics, hereafter indicated as MP) (Basset *et al.*, 2013; Vikas and Dwarakish, 2015; Avio *et al.*, 2017). All these chemicals can cause toxic effects of the biota (Setälä *et al.*, 2014; Stewart *et al.*, 2014; Stockdale *et al.*, 2015; Ribeiro *et al.*, 2016; Wright and Kelly, 2017; Barboza *et al.*, 2018a).

In marine ecosystems, the phytoplankton is in the basis of food web, it influences the fate of several types of contaminants and can be used as bioindicator of environmental contaminants (Conti *et al.*, 2007; Parmar *et al.*, 2016). Among marine phytoplankton, microalgae are important food sources for several kinds of fish, shriMP, shellfish and other organisms, have been used as model organisms in ecotoxicological studies to investigate the effects of environmental contaminants on marine primary producers (Doi *et al.*, 2008; Vaz *et al.*, 2016). Their important environmental performance, their rapid growth rate, low-cost growth environments, high surface-to-volume ratios, and a high sensitivity to a large

number of environmental contaminations make them convenient test organisms (Klain *et al.*, 2008; Morales-Sánchez *et al.*, 2015). Moreover, even if very low levels of environmental contaminants do not cause significant effects on microalgae, they can have adverse effects on higher trophic levels through bioaccumulation in microalgae. Therefore, it is important to investigate the toxicity of environmental contaminants on marine microalgae in relation to environmental and human health, and more studies are needed, particularly in relation to emerging contaminants, such as microplastics and nanomaterials, and mixtures of environmental contaminants.

1.2. Plastics and marine litter

Plastics as a subcategory of polymers (*e.g.* synthetic or semi-synthetic organic solids) are suitable materials for a wide range of uses. The physicochemical properties of plastic materials allow producing several types of products (Hopewell *et al.*, 2009). Hence, plastics are widely used in a variety of fields, such as medicine, energy, aerospace, electronics, automobile industry and building construction, and their usage global trend is increasing (Andrady and Neal, 2009; Hidalgo-Ruz *et al.*, 2012; Duis and Coors, 2016). Around 60 % of this huge production in the world is used in packaging and construction and 20 % is used in household appliances, furniture, sport, health and safety equipment's (Andrady and Neal, 2009; PlasticsEurope, 2017). Because of several characteristics, including relative low-cost manufacture and low weight, they have been replacing other materials (*e.g.* glass, metals) in several applications (ThoMPon *et al.*, 2009; North and Halden, 2013). As a result, the plastic global production has been increasing over decades (Statista, 2015). According the estimates from the European Association of Plastics Manufacturers, the worldwide plastic production increased from 1.7 million metric tons in 1950 to 335 million metric tons in 2016 (PlasticsEurope, 2017). The most produced types of plastics are polyethylene, polypropylene, polyvinyl chloride, polystyrene, polyurethane and polyethylene terephthalate (PlasticsEurope, 2017). Among them, polyethylene (Leslie, 2014), followed by polypropylene (Andrady, 2011; Leslie, 2014) have the highest production rates.

During the plastic production process, use and reuse, release of plastics into the environment occurs both intentionally and unintentionally. The most part (~ 80 %) of the plastic debris present in the marine environment come from land-based activities (Andrady, 2011; Lambert *et al.*, 2014), including unacceptable plastic waste disposal (Gregory, 2009; Lambert *et al.*, 2014), wastewater discharges (ThoMPon *et al.*, 2004; Fendall and Sewell, 2009; Duis and Coors, 2016), air blasting technologies (Cole *et al.*, 2011; Sharma and Chatterjee, 2017), among several others. Main rivers are most important contributors to marine plastic pollution, by entering between 1.15 and 2.41 million tons of plastic per year

to the ocean (Lebreton *et al.,* 2017). Sea-based origins of plastics include fishing and recreational vessels, loss of nets and other devices during fishing, military activities, oil and gas platforms, and aquaculture farms (Andrady, 2011; Cole *et al.*, 2011; Lambert *et al.*, 2014).

As the result of high production, use, and high environmental persistence (Moore, 2008; Gouin *et al.*, 2011; Andrady, 2017), plastics have been accumulating in the marine environment over decades (Doyle *et al.*, 2011; Sá *et al.*, 2015; Li *et al.*, 2016; Villarrubia-Gómez *et al.,* 2017). In the marine ecosystem, plastic debris have been detected on shorelines, water column and sediments (Barnes *et al.*, 2009; Van Cauwenberghe *et al.*, 2014; Eriksen *et al.*, 2014; Villarrubia-Gómez *et al.,* 2017), accounting for about 60 % to 80 % of all marine debris (ThoMPon *et al.*, 2009). They were found even in remote areas, such as the Arctic and Antarctic (Cole *et al.*, 2011, Lambert *et al.*, 2014; Cincinelli *et al.*, 2017; Cózar *et al.,* 2017; Waller *et al.,* 2017).

Some of the properties of the most common plastic polymers with relevance for their fate in the marine environment are listed in the Table 1-1. Buoyancy as one of them is directly related to the density of the plastic and the density of seawater (Ryan, 2015; Kooi *et al.*, 2016). Most types of plastic will float in the sea because their density is lower than seawater density. The others, such as polyvinyl chloride, which is denser than seawater, will therefore be sinked. In addition, the bouyancy capability of plastics is also influenced by biofouling because the accumulation of microorganisms on the polymer surface will increase the plastic density (Andrady, 2011; Fazey and Ryan, 2016; Kaiser *et al.*, 2017; Kooi *et al.*, 2017). It should be noted that fragmentation of plastics does not affect their bouyancy since this process will not change the plastic density (Barnes *et al.*, 2009; Kooi *et al.*, 2016).

Table 1-1: Properties of most common plastic polymers. HDPE - High-density polyethylene. LDPE - Low-density polyethylene. PE - Polyethylene. PET - Polyethylene terephthalate. PP - Polypropylene. PS - Polystyrene. PS-E - Expanded polystyrene. PVC - Polyvinyl chloride. PUR - Polyurethane.

Plastic type	Density (g/cm^3)	Buoyancy (if clean)	Specific gravity	Common application	References
PE (LDPE, HDPE)	0.925	+	0.91- 0.95	Microbead pellets, plastic bags, storage containers, netting, packaging films, bubble wrap	Klyosov, 2007 Andrady, 2011
PP	0.91	+	0.90- 0.92	Bottle caps, rope, fishing line	Klyosov, 2007 Andrady, 2011
PVC	1.44	-	1.16- 1.30	Film, pipe, electrical cable	Klyosov,2007 Andrady, 2011
PS, PS-E	1.05	-	1.04- 1.09/ 0.01- 1.05	Containers, cool boxes, disposable cups, foam board, drinking straw, packaging peanuts	Andrady, 2011
PUR	1.20	-	0.045- 1.25	Medical devices and implants, furniture, thermal insulation, straps, athletic footwear, coatings	Arnold *et al.*, 2012; Tan *et al.,* 2018
PET	1.38	-	1.34- 1.39	Bottles, srtrapping	Andrady, 2011

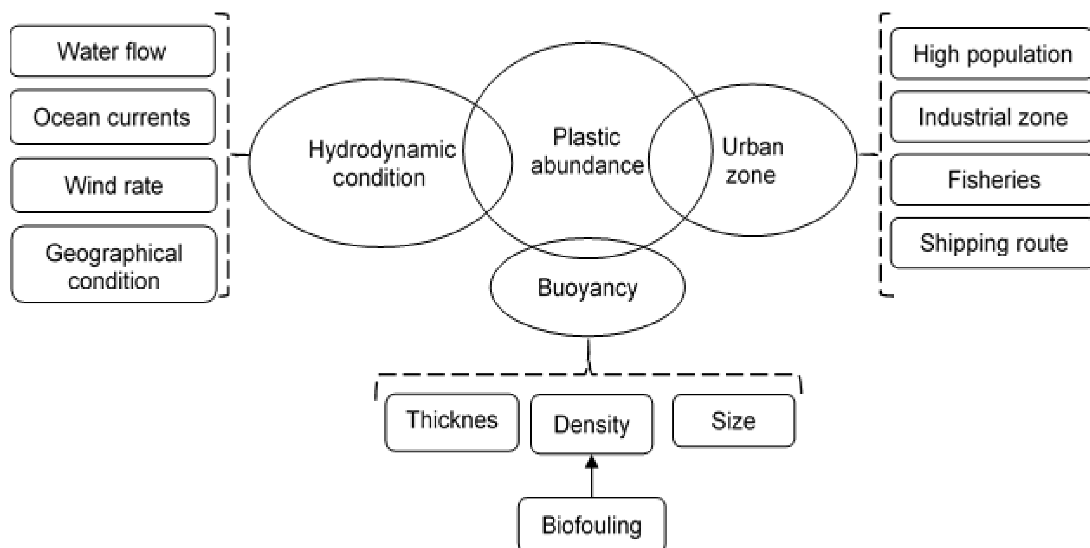
* Seawater density: 1.02 - 1.03 (g/cm)

In addition to the factors previously discussed, as shown in Figure 1-1, the size of plastics is another factor that influences the floating and mixing of plastic debris in the water column, sediments and beach sand (Ryan *et al.*, 2009; Cole *et al.*, 2011; Cózar *et al.*, 2014). The average size of plastic particles in the marine environment has been decreasing over time due to the successive breakdown of macroplastics, and the significant growth in the use of very small plastic particles (*e.g.* in cosmetics and cleaning products) (Derraik, 2002; Barnes *et al.*, 2009; Browne *et al.*, 2010; Juliano *et al.*, 2017). Thickness is also an important factor influencing their bouyancy in the seawater (Barnes *et al.*, 2009; Ryan *et al.*, 2009; ThoMPon, 2015).

The environmental conditions of the different regions and specific areas also influence the fate and behaviour of plastics in the marine environment. Some of them are hydrodynamic conditions (*e.g.* water flow in estuaries, ocean currents, tides), air circulation, deepness and distance to the coast among others (Figure 1-1) (Moore, 2008; Barnes *et al.*, 2009; Ryan, 2015; Kooi *et al.*, 2016; Avio *et al.*, 2017). For example, the abundance and

accumulation of plastic debris in areas of low water volume and / or circulation (*e.g.* bays, coastal lagoons) is greater than in the open sea and oceans (Collignon *et al.*, 2012). The distance to sources of plastics also influences the abundance of plastics (Avio *et al.*, 2017). The concentration of plastics is predominantly high in marine waters near the main shipping routes and marine ecosystems near industrial, urban, sewage treatment plants and aquaculture sites (Barnes *et al.*, 2009; Eriksen *et al.*, 2014; Moore, 2014; Avio *et al.*, 2017; Gallo *et al.*, 2018). For example, the Mediterranean Sea, due to its densely populated coastlines, reduced water flow and high shipping, has higher plastic density than several other seas (Barnes *et al.*, 2009; Pasquini *et al.*, 2016).

Figure 1-1: Main factors affecting the frequency of plastic in marine environment.



The study of Eriksen *et al.* (2014) indicated that a considerable amount of plastic debris is floating at seas and oceans, from subarctic to tropical waters in the both northern and southern hemispheres. However, they estimated that only about 0.1 % of the global plastic production is at the sea surface. In another study by Jambeck *et al.* (2015), the share of coastal countries in sea surface contamination was reported to be 1.7 to 4.6 percent of the world's annual production of plastic. In general, estimating the real abundance of marine plastic debris is a very complicated task for several reasons, including the high specific gravity of some types of plastics, the impossibility of measuring very small-sized plastics, the potential accumulation in remote and unknown areas, the limitation or lack of information on their presence in some areas (Ryan *et al.*, 2009; Andrady, 2011; Woodall *et al.*, 2014; Andrady, 2017).

In general, the plastics present in the environment are divided into microplastics, hereafter indicated as MP, (diameter lower than 5 mm) and macroplastics (diameter higher than 5 mm) (Lambert *et al.*, 2014). Moreover, recently several researchers considered the very small particles below 1 µm as nanoparticles (Andrady, 2011; Ter Halle et al., 2016).

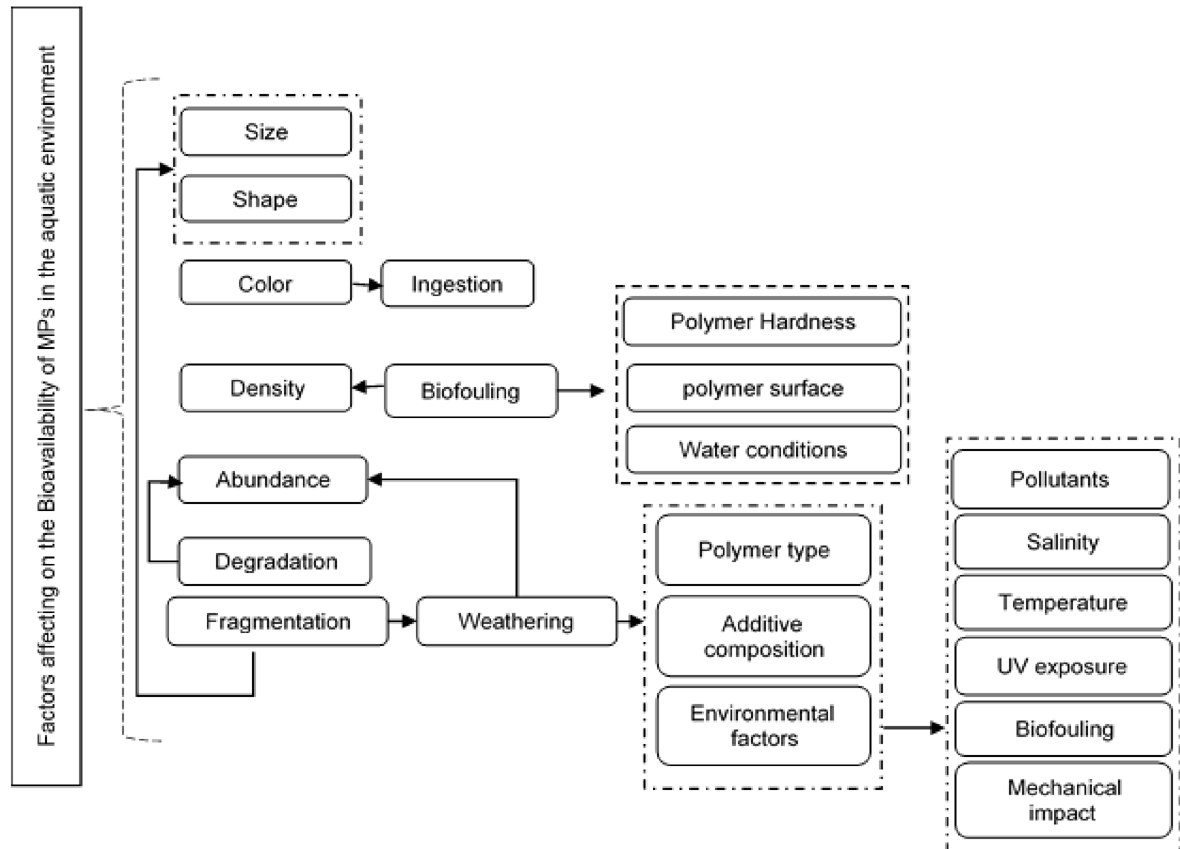
Toxicity of larger plastic particles in the marine environment has been also studied, but there is insufficient data on the toxicity and ecological treatment of small particles (Derraik, 2002). MP are found in the marine environments, including seas and oceans (Eriksen *et al.*, 2014; Anderson *et al.*, 2016), estuaries and coastal systems (Browne *et al.*, 2010; Zhao *et al.*, 2014,; Lima *et al.*, 2014; Zhang *et al.*, 2017; Yu *et al.*, 2018), even in the remote Arctic (Hubard *et al.*, 2014; Bergmann *et al.*, 2017) and Antarctic oceans (Cincinelli *et al.*, 2017; Waller *et al.,* 2017). Samples of MP have been collected for example in beaches (Browne *et al.*, 2011; Frias *et al.*, 2015), surface waters (Law and ThoMPon, 2014), marine sediments (Van Cauwenberghe *et al.*, 2015; Kedzierski *et al.*, 2016), and biota (Desforges *et al.*, 2015; Anderson *et al.*, 2017; Barbosa *et al.*, 2018b; Machado *et al.*, 2018) and in all the other compartments. Different densities and concentrations of MP have been reported (Cincinelli *et al.*, 2017; Zhang *et al.*, 2017; Di and Wang, 2018) but densities of 150-2400 particle/m^3 (Norén 2007) and 10^4 particles/m^3 have been often reported in surface waters as high concentration (Andrady 2017). The most part of the MP present in the marine environment are believed to result from the breakdown of larger plastic fragments by several processes (Barnes *et al.*, 2009; Andrady, 2011, 2017). In addition, natural biopolymers may also exist in the oceans (Andrady, 2011), but in comparison to synthetic plastics they are usually degradable and less hydrophobic (Rochman *et al.*, 2015).

The percentage of MP in the collected samples from different coastal locations varies greatly. For example, MP were found in 60 % of samples collected in the northwestern Atlantic (Law *et al.*, 2010), in 61 % of samples collected in Portuguese coastal waters (Frias *et al.*, 2014), in 74 % of samples collected around Corsica in the western Mediterranean (Collignon *et al.*, 2014), in 89 % of samples collected in the Celtic Sea (Lusher *et al.*, 2014) and in 97 % of samples collected in an estuary of the North Sea (Dubaish *et al.*, 2013). Moreover, the method of sampling may affect abundance of MP due to the sea conditions at recording time (Kukulka *et al.*, 2012; Collignon *et al.*, 2014; Lushar *et al.*, 2014).

The physical properties of the MP (*e.g.* size, shape, density, color, abundance, degredation, weatherability), the chemicals incorporated during their manufacture, the abiotic characteristics of the area and specific ecosystem, and the biota have significant impact on their behaviour and fate in the marine environment (Andrady, 2011), as shown in Figure 1-2. Properties of MP and abiotic properties of the system influence their toxicity to

the biota and their impacts on ecosystem functioning and services provided to the human society (Green *et al.*, 2017; Smith *et al.,* 2018).

Figure 1-2: The effect of several factors on microplastics bioavailability.



1.2.1. Effects of microplastics on organisms

The impact of MP on the marine biota is done in different ways: several fish and bird species have been found to ingest MP, frequently because they have confused these particles with real prey (Ryan, 2009; Lusher *et al.*, 2014; Sá *et al.*, 2015) but uptake through gills probably also occurs (Fossi *et al.*, 2014). Ingestion of MP by other species such as zooplanktonic ones (Cole *et al.*, 2013; Cole *et al.*, 2015), bivalves (Von Moos *et al.*, 2012; Van Cauwenberghe and Janssen, 2014), large vertebrates such as harbour seals (*Phoca vitulina*) (Rebolledo *et al.*, 2013), the Mediterranean basking shark (*Cetorhinus maximus*) and the fin whale (*Balaenoptera physalus*) (Fossi *et al.*, 2014), among other types of organisms, was also reported. In addition, MP may adsorb to organisms' surface and cause adverse effects (Prata *et al.*, 2018). For example, sorption of nano-sized plastic particles (NPs) to freshwater and saltwater microalgae (*Chlorella* and *Scenedesmus*) had adverse effects on photosynthesis and increased the production of reactive oxygen species (ROS) (Bhattacharya *et al.*, 2010).

The size and shape of MP are considered important factors affecting ingestion and egestion rates of these particles in exposed organisms (Watts *et al*., 2015; Gray and Weinstein, 2017). MP size is also an important parameter regarding their reactivity with the body surface of some organisms, such as microalgae, and the toxicity induced (Zhang *et al*., 2017; Prata *et al*., 2018). Also, the color, abundance and distribution of MP in the environment influence their absorption by some species (Wright *et al*., 2013; Anderson *et al*., 2016; Welden *et al*., 2018). By investigating the physical properties of MP which influence their ingestion and residence times in organisms, our knowledge about MP trophic transfer will increase (Au *et al.,* 2017).

1.2.2. Chemicals associated with microplastics

The MP present in the environment generally contain various types of chemicals that are incorporated during their synthesis or are attached during their permanence in the environment (Andrady, 2011; Cole et al., 2011; Browne et al., 2013; Wright et al., 2013; Ivar do Sul and Costa, 2014). Several physicochemical properties of MP such as size, shape, surface area, residence time and hydrophobicity control the amount of these chemicals in the MP (Wright et al., 2013; Chubarenko et al., 2016). Plastics and MP collected from beaches, sediments and water of different regions have been found to contain several types of persistent organic pollutants (POPs) (Table 1-2), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, among others (Mato *et al*., 2001; Endo *et al*., 2005; Teuten *et al*., 2007; Hirai *et al*., 2011; Fries and Zarfle, 2012; Besseling *et al*., 2013; Bakir *et al*., 2014; Beckingham and Ghosh, 2016). Moreover, several metals, including copper, chromium, cadmium, and others (Table 1-2) have been found to adsorb to MP (Ashton *et al*., 2010; Holmes *et al*., 2012; Turner and Holmes, 2015; Munier and Bendell, 2018).

Table 1-2: Chemicals associated with plastic particles. Al - Aluminium. Cu - Copper. Fe - Iron. Mn - Manganese. BaP - benzo(a)pyrene. DDEs - Dichlorodiphenyldichloroethylene. DDTs - Dichlorodiphenyltrichloroethane. HDPE - High-density polyethylene. HCHs - hexachlorocyclohexanes. LDPE - Low-density polyethylene. PBDEs - Polybrominated diphenyl ethers. PAHs - Polycyclic aromatic hydrocarbons. PCBs- Polychlorinated biphenyls. PE - Polyethylene. PET- Polyethylene terephthalate. PP - Polypropylene. PS - Polystyrene. PVC - Polyvinyl chloride. Zn - Zinc.

Type of plastic	**Associated contaminants**	**Results**	**Reference**
PE PP	DDE, PCBs, Nonylphenols	Sorption from seawater after 6 days	Mato *et al.*, 2001
PE silicone rubber	Hydrophobic chemicals	Uptake of chemicals by polymers decreased by increasing the water turbulence due to the thinning of diffusive boundary layer at the surface of the polymer	Booij *et al.*, 2003
PE	PCBs	Hydrophobicity of PCB increased after 128 days.	Endo *et al.*, 2005
Resin pellets, Aged plastic fragments	PAHs, PCBs, pesticides	Observed in more than 50 %, 40 %, and nearly 80 % of the samples, respectively.	Rios *et al.*, 2007
PE, PP, PVC	Phenanthrene	Sorption of Phenanthrene was highest to the LDPE and lowest for the PVC	Teuten *et al.*, 2007
Pellets	Phenanthrene	Highest distribution coefficients for PE	Karapanagioti and Klontza, 2008
PE	Al, Fe, Mn	Adsorption of metals from water to new polyethylene pellets	Ashton *et al.*, 2010
Pellets	DDTs, HCHs PAHs, PCBs	The possibility uses of plastic pellets for global contaminant monitoring in the sea.	Karapanagioti *et al.*, 2011
PE	DDT, PAHs, PBDEs, alkylphenols PCBs, PCB	Levels of PCBs and PAHs were higher on urban beaches compared to those found in the open ocean and remote beaches	Hirai *et al.*, 2011
new and beached PE	trace metal	Higher adsorption for littered pellets than new ones. Concentrations of metals on plastics and in nearby sediment have been found to be similar	Holmes *et al.*, 2012
Pellets	PCBs	Aassociated concentrations for plastic collected from beaches in Porto and Lisbon higher than those from rural sites In the Portuguese coast higher contaminated pellets collected in industrial, urbanized area and harbors	Frias *et al.*, 2013

Type of plastic	Associated contaminants	Results	Reference
Pellets	DDTs, HCHs, Hopanes, PAHs, PCBs	Higher levels of PCBs and PAHs in plastic pellets collected near urban coastal areas compared to rural sites	Mizukawa *et al.*, 2013
LDPE, HDPE, PET PVC	PAHs PCBs,	Highest sorption for LDPE and HDPE and lowest for PVC and PET	Rochman *et al.*, 2013
micrometer-size PE, nano-size PS	PCBs	Stronger sorption to nano-sized PS (due to aromaticity and the surface-to-volume ratio) Reduction availability of sorption sites on the surface of PS directly effect on the distribution coefficients. The presence of salt in water increased polymer-water distribution coefficients of PCBs for both	Velzeboer *et al.*, 2014
PS, PVC	Cu, Zn	The adsorption of Cu was significantly higher in PVC fragments than in PS, because of higher surface area and polarity. Concentrations of Cu and Zn increased significantly on PVC and PS during the experiment.	Brennecke *et al.*, 2016
LDPE	BaP	Virgin and contaminated LDPE was greatly influenced by accumulation of BaP MP can transfer adsorbed organic contaminants like BaP to tissues of marine organisms	Pittura *et al.*, 2018

Virgin and aged MP in the marine environment may accumulate metals (Ashton et al., 2010; Holmes et al., 2014; Brennecke et al., 2016; Munier and Bendell, 2018) due to the increasing reactivity of their surface due to the presence of biofilms and chemical precipitates (Gambino and Cappitelli, 2016). Moreover, some of these chemicals, such as POPs, accumulate on MP and their concentrations on the MP are significantly higher than in the water (Hirai *et al.*, 2011; Holmes *et al.*, 2012; Bakir *et al.*, 2014). Furthermore, photo-oxidative weathering increases the polarity of the polymer (Mato *et al.*, 2001) and accumulation of biofilms (Gambino and Cappitelli, 2016; Rummel *et al.*, 2017). In addition, the hydrogenous precipitates increase the charge, roughness, porosity and hydrophobicity of the surface of MP (Artham *et al.*, 2009; Anderson *et al.*, 2016; Kwon *et al.*, 2017; Anderson *et al.*, 2018).

Consequently, the ingestion, uptake and sorption of MP by marine organisms may considerably increase their exposure to other chemicals and transfer of these chemicals through the food web may occur (Wright *et al.*, 2013; Watts *et al.*, 2014; Lin, 2016; Critchel and Hoogenboom, 2018) increasing the risk for high predators and humans consuming

contaminated preys (Bakir *et al.*, 2012; Farrell and Nelson, 2013; Fossi and Depledge, 2014; Setälä *et al.*, 2014; Carbery *et al.*, 2018). Furthermore, MP may also interact with the biotransformation and toxicity of other environmental contaminants in marine species, such as PAHs (Oliveira *et al.*, 2013; Avio *et al.*, 2015; Koelmans *et al.*, 2016), metals (Fries *et al.* 2014; Khan *et al.* 2015; Luís *et al.*, 2015; Ferreira *et al.*, 2016; Barboza *et al.*, 2018a), pharmaceuticals (Fonte *et al.*, 2016; Prata *et al.*, 2018), among others.

The toxic effects of MP to marine organisms are influenced by several factors, such as their bioavailability, their concentration, their size, their bioaccumulation in the organism, the body mass of exposed organisms, among others (Brennecke *et al.*, 2017). In marine organisms, MP were found to cause mortality (Luís *et al.*, 2015; Pacheco *et al.*, 2018), false food satiation, decreased growth (Karami *et al.*, 2016;), behavioural changes (Barboza *et al.*, 2018a,b; Critchell and Hoogenboom, 2018), decrease of the predatory performance (Sá *et al.*, 2015), neurotoxicity (Oliveira *et al.*, 2018; Barboza *et al.*, 2018a), oxidative stress (Avio *et al.*, 2015; Barboza *et al.*, 2018a; Ribeiro *et al.*, 2017), among several others.

1.3. Objectives and outline of the Thesis

The main objective of the present Thesis was to investigate the effects of very small MP (1–5 μm diameter) on the population growth of the marine microalgae *Tetraselmis chuii*, individually and in mixture with copper or gold nanoparticles (AuNP). *T. chuii* was selected as test organism mainly because is an abundant species in several marine ecosystems where its populations are often an important component of the phytoplankton community. Moreover, this species has been widely used as representative of primary producers in ecotoxicological studies (Nunes *et al.*, 2005; Ferreira *et al.*, 2007; Debelius *et al.*, 2009; Vieira and Guilhermino, 2012). To reach the main goal four null hypotheses were tested: (H_{01}) exposure to microplastics (1-5 μm spheres) at concentrations up to the low ppm range does not affect the average specific growth rate (hereafter indicated as population growth) of *T. chuii*; (H_{02}) microplastics do not interact with the effects of copper on *T. chuii* population growth; (H_{03}) exposure to concentrations of gold nanoparticles in the low ppm range does not affect the population growth rate of *T. chuii*; (H_{04}) microplastics do not change the toxicity of gold nanoparticles to *T. chuii*.

The Thesis is organized in seven Chapters. Chapter I corresponds to the general introduction, objectives and outline of the Thesis. In Chapter II, a revision of the challenges posed by the global contamination by MP and the effects of these particles on microalgae are presented. Chapter III is a revision of the effects and the factors influencing the ecotoxicity of nanoparticles. Chapter IV is an experimental work where the effects of MP

alone and in mixture with copper on the population growth rate of *T. chuii* were investigated. Chapter V investigated the effects of AuNP, alone and in mixture with MP, on the population growth rate of *T. chuii*. Chapter VI corresponds to the general discussion and conclusions of the Thesis. Finally, Chapter VII corresponds to the list of the references cited in the other Chapters.

Chapter II:

Microplastic challenges and effects

Abstract

The number of MP in the marine environments has increased significantly due to increased production of various types of plastics. For this reason, MP are a new global pollutant and a serious threat to the marine environment from coastal to remote areas. Therefore, several studies have been carried out to understand the contamination, accumulation and impact of MP in the marine environment and their effects on the marine biota. The results of such studies showed ingestion and accumulation of MP in various types of marine species, including planktonic ones, bivalves and fish, with potential negative effects to human food safety, health and wellbeing.

Due to the high number of studies published, regular literature reviews are needed to compilate, improving the understanding and summarizing the main findings. Therefore, this chapter reviewed the published literature with the objective of understanding: (1) how aquatic organisms may be exposed to MP and associated contaminants and (2) the potential bioaccumulation of chemicals associated to MP and their toxicity to aquatic biota.

2.1. Introduction

The number of MP in the aquatic environment has been increasing due to the growth of global plastic production and use (Law and ThoMPon 2014; Wagner *et al.*, 2014; Eerkes-Medrano *et al.*, 2015). So, the abundance of MP is highly influenced by the human population (Browne *et al.*, 2011; Anderson *et al.*, 2016). This global pollution by MP has raised safety concerns about the marine environment. One major reason for this concern is the small size of MP that is in the same range of several sediment particles and some plankton organisms (Wright *et al.*, 2013; Van Cauwenberghe *et al.*, 2015) which leads to their ingestion by filter and deposit feeders, detritivores and planktivores species (Browne *et al.*, 2008; Graham and ThoMPon, 2009; Avio *et al.*, 2015; Steer *et al.*, 2017).

Recent studies have shown that at least 693 species of aquatic species are affected by plastic debris in terms of lethal and sublethal effects (Gall and ThoMPon, 2015; Kühn *et al.*, 2015). Through different processes, MP and associated chemicals (*e.g.* chemicals incorporated during MP manufacture and use, and/or adsorbed during their permanence in the environment) may negatively affect the organisms of aquatic environments through direct and indirect effects (Figure 2-1) (Farrell and Nelson, 2013; Wright *et al.*, 2013; Setälä *et al.*, 2014).

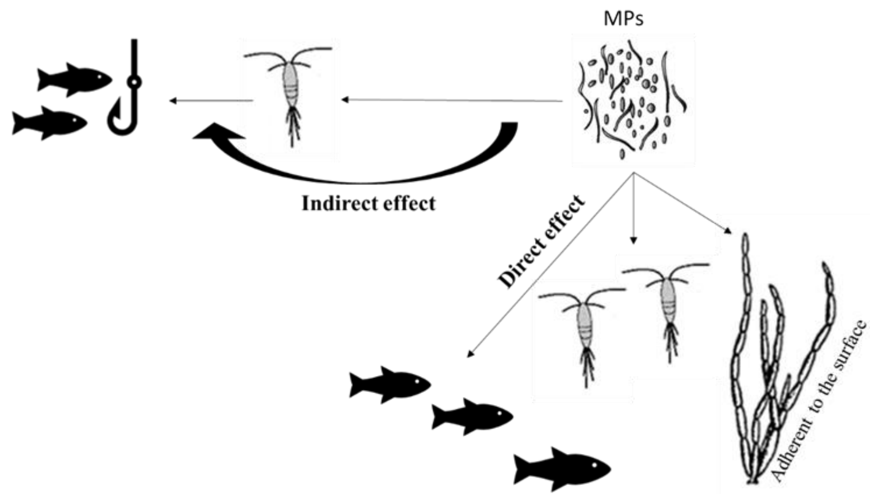


Figure 2-1: Direct and indirect exposure of microplastics of different trophic levels in marine systems. Direct exposure: ingestion of microplastics as food and through the body surface; Indirect effect: consumption of prey that ingested microplastics. MP-microplastics.

Results from both laboratory and field studies have demonstrated that numerous marine organisms of different sizes (*e.g.* plankton, fish, whales) ingest MP (Browne *et al.*, 2008; Graham and ThoMPon, 2009; Tedesco *et al.*, 2010; Von Moos *et al.*, 2012; Cole *et al.*, 2013; Fossi *et al.*, 2014; Avio *et al.*, 2015; Costa and Barletta, 2015; Sá *et al.*, 2015; Jovanović, 2017; Steer *et al.*, 2017; Canniff and Hoang, 2018). MP can be directly attached to the surface of cells (*e.g.* microalgae) or cause adverse effects on fish, mollusks, crabs, and other animals through gills and ingestion. For example, a study indicated that ingestion of polystyrene microspheres (8–10 µm) by the crab, *Carcinus maenas,* from pre-exposed food is higher than the absorption of the MP through the gills (Watt *et al.*, 2014). MP can also cross the membranes and enter into the cells. For example, Browne *et al.* (2008) showed the transfer of MP (3, 9.6 µm) from the gut to the circulatory system and their storage in the tissues of the blue mussel (*Mitilus edulis*). Recently, in studies where fish were exposed to MP, the presence of the particles in the brain was shown (Mattsson *et al.*, 2017; Rainieri *et al.*, 2018). Therefore, there are concerns about the effects of MP on animal, ecosystem and human health (Van Cauwenberghe *et al.*, 2014; Ferreira *et al.*, 2016; Wright and Kelly, 2017; Barboza *et al.*, 2018c; Martins and Guilhermino, 2018).

In addition, absorption of MP by animals is also a way to increase the bioavailability of other chemicals that MP contain (Teuten *et al.*, 2007, 2009; Rochman, 2015). The small size of MP due to the large ratio of surface area to volume, allows organic pollutants and other contaminants easily adsorb on their surface from aquatic environments (Gauquie *et al.*, 2015). Nevertheless, it is still unclear, how big is the contribution of MP to the entry of other contaminants into organisms (Hollman *et al.*, 2013; Koelmans *et al.*, 2016).

For reliable and reproducible results on toxicological effects of MP, a number of appropriate parameters should be considered, including the physical and chemical properties of MP (Wright *et al.,* 2013; Chubarenko *et al.*, 2016; Lambert *et al.*, 2017; Gallo *et al.,* 2018). For example, the toxicity of MP is affected by their size and shape because their intake and absorption are affected by such properties, as well was the adsorption of other contaminants to the MP surface (Chubarenko *et al.*, 2016; Lambert *et al.*, 2017). Additionally, high MP concentration can cause agglomeration of the particles. Therefore, not only the physical properties of the MP should be investigated, but also their chemical properties to prevent unpredictable outcomes (Wright *et al.*, 2013; Lambert *et al.*, 2017; Gallo *et al.,* 2018; Smith *et al.*, 2018). A fundamental knowledge is needed to address these challenges related to the proper investigation of the toxic properties of MP.

2.2. Toxicity of microplastics

The study of MP behaviour and toxicity in the aquatic environment has been the subject of intense scientific debate over the past years (Lambert *et al.*, 2017). The toxicity behaviour of MP can significantly vary according to the type, size, shape and other properties of the MP, as well as the additives and environmental contaminants that MP contain. Nevertheless, there are few studies that addressed the interaction of these multiple parameters on the toxicity of MP.

As previously indicated, the shape of MP is an important factor. The shapes of MP may differ from spherical to a more complex shape but the most common shape of MP in the marine environment is a fibrous form (ThoMPon *et al.*, 2004; Claessens *et al.*, 2011). For example, the reduction in the growth of the crab, *Nephrops norvegicus,* exposed to MP is due to the incomplete digestion of polypropylene fibers (Murray and Cowie, 2011). In another study, it was shown that fibrous polypropylene MP were more toxic to exposed organisms than MP having spherical shapes (Wright *et al.*, 2013). Regarding to the increasing studies on MP, some important parameters such as size can be used to compare the frequency and distribution (Costa *et al.*, 2010; Claessens *et al.*, 2011; Lusher *et al.*, 2015; Gewert *et al.*, 2017).

The distribution and behaviour of MP in the aquatic environment are influenced by some factors, including the nature and location of the MP sources, as well as the complex interaction of physical (*e.g.* size and shape), chemical (*e.g.* polymer type) and biological processes (Auta *et al.*, 2017; Costa *et al.*, 2017). The spatial distribution of MP from beaches and costal sediments to deep sea is influenced by ocean currents (*e.g.* tidal force, intertidal waves), weather patterns (floods, tsunamis, hurricanes, and tornados) (Barnes *et al.*, 2009;

Kukulka *et al.*, 2012) and other processes (*e.g.* wastewater discharges). Therefore, these evaluations show that in toxicological research not only the abundance of MP, but also the distribution of size, shape and polymer groups should also be considered (Auta *et al.*, 2017).

MP biofouling (accumulation of several microorganism on the surface of MP) is another factor that may influence the toxicity and behaviour of MP in the marine environment (Kooi *et al.*, 2016; Kaiser *et al.*, 2017). The biofouling can change the density of MP (Kooi *et al.*, 2017). Experimental studies showed that biofouling of large plastics can increase their densities, and subsequently lead to their sinking (Kaiser *et al.*, 2017). For this reason, the sedimentation rate of MP in the water column depends on the type of polymer and living organisms that are on the MP (Andrady, 2017). For example, the marine phytoplankton species, *Chaetoceros neogracile,* that colonized polystyrene created larger and stronger aggregates that sinked faster than those resulting from MP colonization by *Rhodomonas salina* (Long *et al.* 2016). In another study, the effect of biofouling on low- and high-density polyethylene MP was compared after exposure to seawater for 12 weeks; then faster sedimentation of high-density polyethylene was shown, while both polymers have negative buoyancy in seawater (Fazey and Ryan, 2016). Also, biofouling may change in the water column, generally decreasing with the increase of depth due to limitations of organisms depending on light and changes in water density and temperature (Wang *et al.* 2016). As an example, the speed of biofouling on polyethylene and polypropylene debris in the pelagic area is quicker than benthic areas (Eich *et al.*, 2015). Furthermore, the surface charge of the particles has an impact on their aggregation, biofouling and sedimentation in the pelagic and benthic areas (Nolte *et al.*, 2017a; Andrady, 2017).

The increasing volume of scientific evidences indicates that MP are entering marine food webs through absorption and accumulation by various marine species, with potential adverse effects on such species, their predators and humans consuming them (Setälä *et al.*, 2014; Van Cauwenberghe and Janssen, 2014; Carbery *et al.*, 2018). The potential hazards of MP to human and environmental health opened a new field of study in marine toxicology research (Wright and Kelly, 2017). However, a key concern is a variety of sources of uncertainty. Due to this uncertainty, the contribution of MP toxicity to human health has been addressed in a small number of studies with *in vivo* or *in vitro* models (Fleming *et al.*, 2006; Van Cauwenberghe and Janssen, 2014; Wright and Kelly, 2017; Carbery *et al.*, 2018).

2.3. Sorption capacity of microplastics

As before indicated, MP can be a major source of organic and inorganic contaminants to living organisms. In general, the surface-to-volume ratio, as well as the hydrophobic level (Figure 2-2), make the MP easily contaminated with POPs which may increase the toxicity of MP (Teuten *et al.*, 2009; Cole *et al.*, 2011; Brennecke *et al.*, 2016; Koelmans *et al.*, 2016).

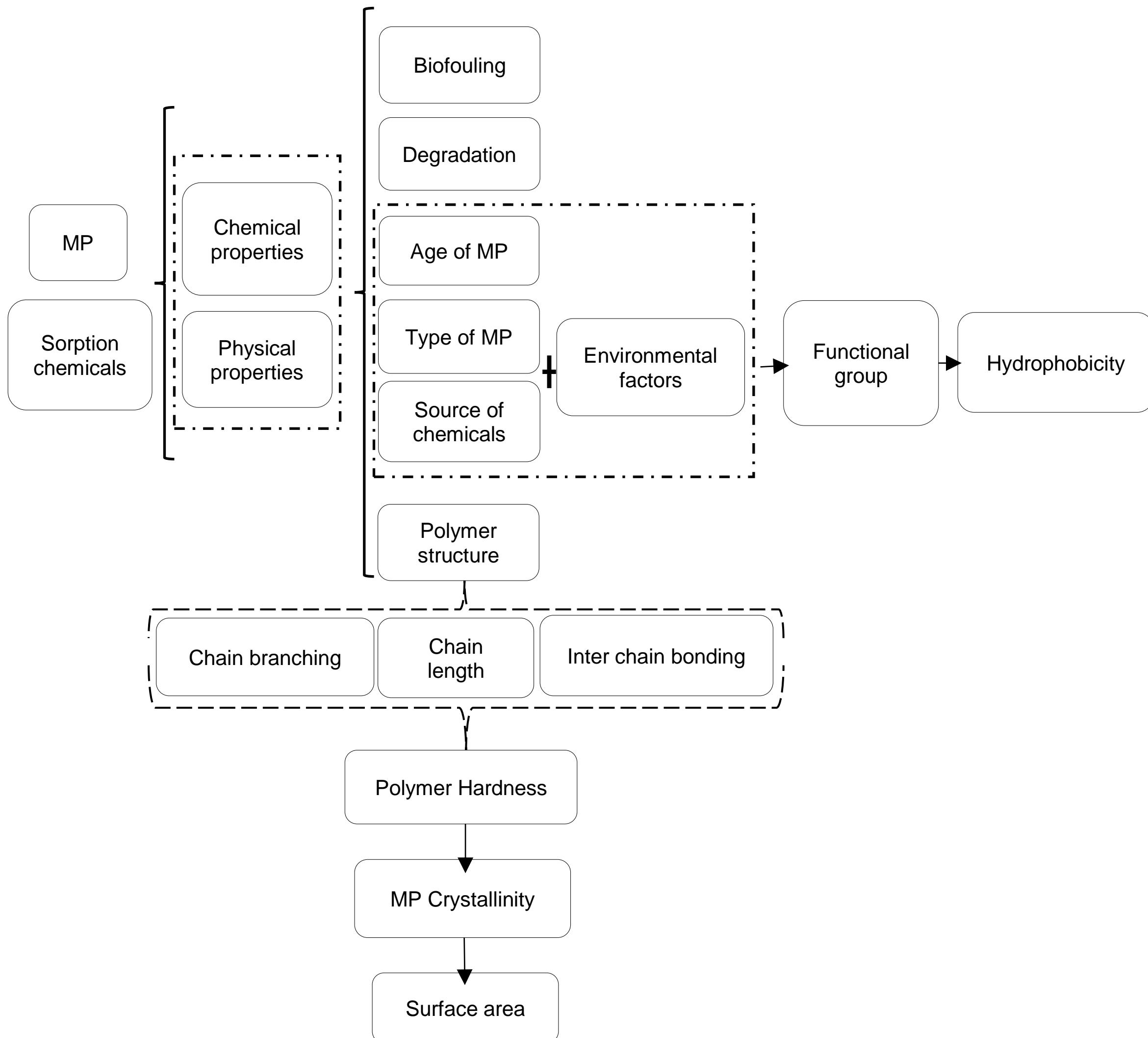


Figure 2-2: Factors affecting the sorption capacity of microplastics.

The continuous consumption of contaminated MP increases the probability of bioaccumulation of POPs as well as other contaminants in fish and mammals (Eriksen *et al.*, 2014; Desforges *et al.*, 2014). Furthermore, the organic matter present in the water and environmental conditions influence the MP reactivity with surrounding water molecules and contaminants and POPs transport (Koelmans *et al.*, 2016). In the same way, the origin of MP is an important factor in characterizing their structure and their sorption capacity.

One parameter that can increase the sorption of contaminants such as heavy metals and organic pollutants is the surface of MP, (Holmes *et al.*, 2012). Another parameter is the

crystallinity of MP, dictating the available surface area for contaminant sorption, that significantly influences the interaction of MP with pollutants and natural organic matter. The degree of crystallinity of MP is a function of production parameters including cooling rate, solvents and the type of polymers (Guo *et al.*, 2012). The degree of crystallinity of MP is defined based on chain length, chain branching, and inter-chain bonding, which are three structural properties of polymers (Figure 2-2) (Wright *et al.*, 2013; Chubarenko *et al.*, 2016). There is a direct correlation between the length of the polymer chain and its hardness as well as surface area for sorption of other pollutants (Wright *et al.*, 2013; Hüffer *et al.*, 2018). Also, the branching in the polymer chain would influence the surface reactivity for the adsorption of pollutants. For example, methyl groups in polypropylene, on the one side of the carbon chain, produce a higher degree of crystallinity than polyethylene (Guo *et al.*, 2012). Hence, MP such as polypropylene absorb more pollutants, such as PAHs, because of their higher adsorption sites on their surface. Inter-chain bonding in the MP will also affect the degree of PAHs sorption, greater sorption may occur with the wide-open alignment of polymer chains. Also, MP behave like hydrophobic materials when they contain functional groups same as particulate organic matter (Bakir *et al.*, 2014). Hence, MP are a significant contaminant when compared to other natural particulate matter (*i.e.* organic matter, sediment) (Koelmans *et al.*, 2016). Due to these complexities, one question indicated in many scientific works is whether the toxicity of MP in real scenarios can be extrapolated from laboratory studies.

Another factor that can significantly influence contaminant sorption is the degradation of MP through physical, thermal and oxidative changes (Barnes *et al.*, 2009). The hydrophobicity of the polymer can be reduced by the addition of functional oxygen groups (*e.g.* carbonyl, carboxyl), which leads to the reduction of MP surface available to adsorb hydrophobic compounds (Endo *et al.*, 2005). Aging of MP leads to cracking of the surface and increases the surface area availablefor the sorption of POPs and other environmental contaminants (Chubarenko *et al.*, 2016).

2.4. Transfer of microplastics and associated chemicals in the food webs

MP and associated chemicals can be transferred from one trophic level to the upper one (Browne *et al.*, 2008; Desforges *et al.*, 2015; Nelms *et al.*, 2018). However, it is not yet clear exactly how much MP contribute to the spread of other pollutants (Hollman *et al.*, 2013; Koelmans *et al.*, 2014). Today, concerns about the toxic effects of MP and trophic transfer of associated chemicals have. The trophic transfer of MP and associated chemical may be increased by higher body temperatures, lower pH and the presence of intestinal surfactants

(Bakir *et al*., 2014). For example, Powell *et al*., (2010) showed that the acidic pH of the stomach and the presence of gastrointestinal enzymes will help to remove adsorbed chemicals from the surface of MP.

The trophic transfer of MP was shown in laboratory feeding studies with ten zooplankton taxa exposed to polystyrene microspheres, and the trophic transfer of MP through mysid shriMP has been identified (Setälä *et al*., 2014). In field studies, ingestion of MP by 36 % of individual collected fish has been confirmed (Lusher *et al*., 2014). Similarly, MP were found in the gut contents of 62 % of Norway lobsters (*Nephrops norvegicus*) analysed (Murray and Cowie, 2011). In another study, MP ingested by blue mussels were found in the hepatopancreas, ovary and gills of the crab *Carcinus maenas* (Farell and Nelson, 2013).

Since MP are present in the wild, as well as in aquaculture areas, their presence (0.07 - 5.47 particles/g) in Manila clams (*Venerupis philippinarum*) that are commonly used by humans as food was also confirmed (Davidson and Dudas, 2016). In addition, MP fibers (200 - 1500 µm) were detected in blue mussels (*Mytilus edulis*) from a supermarket (De Witte *et al*., 2014). Higher concentrations of MP were observed in supermarket-purchased clams (*Crassostrea gigas*) in comparison to *Mytilus edulis* from a mussel farm (Van Cauwenberghe and Janssen, 2014). Ingestion of whole organisms can lead to the transfer of MP from the environment to humans (Cauwenberghe and Janssen, 2014; Karami *et al*., 2017a). In case of fish consumption, the process is different from shellfish, since generally the whole body is not consumed and therefore usually most of the MP ingested are removed before human consumption. More information on the bioaccumulation, trophic transfer of MP and their impacts on food webs is needed.

The trophic transfer of contaminants associated to MP is confirmed by a study that demonstrated transfer of polyethylene MP and adsorbed benzo[a]pyrene from *Artemia sp.* to zebrafish (Batel *et al*., 2016). Ingestion of MP potentially can affect processes at population and ecosystem levels as well as transmission of chemicals and biological resources (*e.g.* invasive species, pathogens) (Zettler *et al*., 2013; Osborn and Stojkovic, 2014) which are a threat to marine biodiversity. Further research on whether MP may act also as a method for the elimination of other environmental pollutants from organisms is needed.

2.5. Bioaccumulation and toxicological effects of microplastics and associated chemicals

The small size of MP makes them a potential threat for a wide range of marine organisms (*e.g.* phytoplankton, zooplankton, polychaetes, crustaceans, bivalves, fish) (Derraik 2002;

Barnes *et al.*, 2009; Fendall and Sewell, 2009; ThoMPon *et al.*, 2004; Cole *et al.*, 2011; Farrell and Nelson, 2013; Setäla *et al.*, 2014; Davarpanah and Guilhermino, 2015; Barboza *et al.,* 2018 a,b; Prata *et al.,* 2018). MP can induce physical effects on marine organisms (*e.g.* blockage or damage of the feeding system and digestive tract with associated inflammation and fibrosis) (Wright *et al.*, 2013; Ogunola and Palanisami, 2016). In addition, MP can cause chemical toxicity by transferring other hazardous chemicals to marine organisms (*e.g.* additives, monomers, absorbed chemicals) (Bakir *et al.*, 2016). Based on the method and type of study, the literature review can be grouped into three categories, namely: field studies, laboratory studies, and modelling studies.

2.5.1. Field studies

Several field studies showed consumption of MP by fish and shellfish (Murray and Cowie, 2011; Lusher *et al.*, 2013, Neves *et al.*, 2015; Steer *et al.*, 2017), and a relatively small number of them investigated the transfer of contaminants from MP to lower trophic levels (Table 2-1). Lavers *et al.*, (2014) observed the high concentrations of chromium and silver in the flesh-footed shearwater (*Puffinus carneipes*). They stated that the high concentration of these metals had a positive relationship with plastics ingested. There are several indications that there is a positive correlation between the concentration of associated chemicals with density of MP in the waters (Koelmans *et al.*, 2016). Regarding this, Rochman *et al.*, (2014b) indicated that concentration of PBDEs in tissue of Japanese rice fish (*Oryzias latipes)* had relation with the concentration of plastic in the water column.

Table 2-1: Example of studies on the effects of chemicals associated to microplastics in field studies. PCBs - Polychlorinated biphenyls. MP - microplastics. PBDEs - Polybrominated diphenyl ethers.

Associated chemicals	Species	Results	References
PCBs	*Puffinus gravis*	PCBs observed in the abdominal adipose tissues that were correlated with ingested MP.	Ryan *et al.*, 1988
Nonylphenol	*Seriola lalandi*	10 % of the sampling fish contained synthetic debris; nonylphenol was detected in one-third of samples.	Gassel *et al.*, 2013
Phthalates organochlorines	*Cetorhinus maximus, Balaenoptera physalus*	Ingestion of MP	Fossi *et al.*, 2014
Chromium; silver	*Puffinus carneipes*	High concentrations of chromium and silver had a positive relationship with plastic ingested.	Lavers *et al.*, 2014

PBDEs	*Oryzias latipes*	Concentration of PBDEs in fish tissue had relation with plastic accumulation in the water column.	Rochman *et al.*, 2014b

2.5.2. Laboratory studies

In some laboratory experiments, it has been shown that both chemicals associated with the production of plastics and other chemicals that may be adsorbed to MP can accumulate in some organisms. These studies confirm the accumulation of chemicals on plastics and their transfer to aquatic organisms (Koelmans *et al.*, 2016).

The physical and chemical effects of MP and associated chemicals on different groups of organisms were investigated. The results of some of these studies are summarized in Tables 2-2, 2-3 and 2-4. Direct toxic effects of MP can occur after ingestion and transfer of the particles into cells, tissues and body fluids. Also, MP connected to external surfaces of organisms may restrict their movements and feeding (Besseling *et al.*, 2013; Cole et al., 2013; Watts *et al.*, 2014; Cole et al., 2015; Rehse *et al.*, 2016). Regarding the ability of MP to adhere to the external surface of algae (Gutow *et al.*, 2015), photosynthesis can be reduced, and oxidative stress levels are affected (Bhattacharya *et al.*, 2010; Zhang *et al.*, 2017a). However, in another study on the effect of polystyrene on *Dunaliella tertiolecta*, photosynthesis was not affected and only the growth rate decreased (Sjollema *et al.*, 2016). The indirect toxic effects of MP on cyanobacteria may be due to hazardous chemicals transferred from the MP (Yokota *et al.*, 2017).

Table 2-2: Examples of studies on the effects of microplastics and chemicals associated to them on algae. HDPE - high-density polyethylene. MP - micropastics. nm - nanometer. PE - Polyethylene. PP - Polypropylene. PS - Polystyrene. PVC - Polyvinyl chloride.

Type and size of microplastics	**Associated contaminants**	**Species**	**Results**	**References**
PS 20 nm	-	*Scenedesmus*	The adsorption of MP on the external surface of algae decreased photosynthesis and caused oxidative stress	Bhattacharya *et al.*, 2010
PE microspheres 1-5 μm	Copper	*Tetraselmis chuii*	No significant differences between the toxicity of copper in the presence and absence of MP were found	Davarpanah and Guilhermino, 2015
PS 10 μm	-	*Fucus vesiculosus*	MP can easily adhere to the algae surface.	Gutow *et al.*, 2015
PS 0.05, 0.5 ,6 μm	-	*Dunaliella tertiolecta*	The growth rate decreased by all of three sizes of PS but did not affect on photosynthetic efficiency These effects increased with decreasing particle size.	Sjollema *et al.*, 2016
PP, HDPE 400-1000 μm	-	*Chlamydomas reinhardtii*	Significant interactions and rapid formation of hetero-aggregates were observed. The growth rate decreased.	Lagarde *et al.*,2016
PS microspheres 2 μm	-	*Tisochrysis lutea, Heterocapsa triquetra, Chaetoceros neogracile*	The micro-PS did not affect on microalgal growth and chlorophyll, but type of species affected on the distribution of micro-PS in algal cultures.	Long *et al.*, 2017
PVC 1 μm	-	*Skeletonema costatum*	photosynthesis (chlorophyll content and photosynthetic efficiency) reduced due to adsorption of MP to algae surfaces, and the formation of aggregates	Zhang *et al.*, 2017a
Polymer microspheres 1–5 μm	Procainamide Doxycycline	*Tetraselmis chuii*	MP-pharmaceutical mixtures were more toxic than pharmaceuticals alone	Prata *et al.*, 2018

Table 2-3: Examples of studies on the effects of microplastics and chemicals associated to them on invertebrates. AuNP - Gold nanoparticles. MP - microplastics. nm - nanometer. PBDEs - Polybrominated diphenyl ethers. PCBs - Polychlorinated biphenyls. PE - Polyethylene. PS - Polystyrene. PVC - Polyvinyl chloride. PUR - Polyurethane. μm - micrometer.

Type and size of microplastics	Associated contaminants	Species	Results	References
PE	PBDEs	*Allorchestes compressa*	The presence of MP reduced the absorption of PBDEs. PBDEs stored within the MP were less than free PBDEs.	Chua *et al.*, 2014
PS 0.4-30.6 μm	-	15 Different species of Zooplankton	Ingestion of PS observed in thirteen (87 %) taxa (exceptions siphonophores and chaetognaths). The taxa, the stage of life and the size of the bead influence on the absorption of PS. Feeding activity reduced and feeding organs blocked. MP connected to the external carapace and with >4000 particles/ml significantly decreased feeding	Cole *et al.*, 2013
	-	*Centropages typicus*	Concentration of MP had negative relationship with ingestion rate of algal by copepod.	
	-	*Calanus helgolandicus*	Feeding capacity and ingestion of food significantly reduced. Prolonged exposure to PS significantly increased mortality rates and decreased viability (egg hatching success and survival)	
PS 20 μm	-	*Calanus helgolandicus*	The consumption of algae was decreased.	Cole *et al.* 2015
	-	*Lytechinus variegatus*	Plastic pellets (virgins and beach-collected) affected embryonic development, but effects of virgin ones were more intense.	Nobre *et al.*, 2015
PS	PCBs	*Arenicola marina*	Significant effects of PS on the organisms' fitness and bioaccumulation observed. The feeding activity reduced. Concentrations of PCBs in tissue increased by mixture of PS-PCBs.	Besseling *et al.*, 2013
PVC	nonylphenol, phenanthrene, PBDE-47	*Arenicola marina*	Nonylphenol reduced the immunity, and PVC reduced the antioxidant capacity.	Browne *et al.*, 2013

Type and size of microplastics	Associated contaminants	Species	Results	References
HDPE > 0−80 µm	-	*Mytilus edulis*	Uptake of MP (0–80 µm) into digestive tubes with translocation into cells and cell organells (lysosomes).	von Moos *et al.*, 2012
PS 30 nm	-	*Mytilus edulis*	Reduced filtering/feeding activity	Wegner *et al.*, 2012
PE, PS	Pyren	*Mytilus gallaprovincialis*	Concentration of free pyrene in the gills and digestive glands of the mussels was greater than those measured on the contaminated MP. In the short-term exposure, physical impacts of MP have been more than chemical impacts. In the long-term exposure, pyrene-contaminated MP could be a potential risk for the condition of the mussels.	Avio *et al.*, 2015
PS 2 and 6 µm	Fluoranthene	*Mytilus edulis, Mytilus galloprovincialis*	MP alone changed oxidative and energetic processes and increased hemocyte mortality The presence of fluoranthane has effect on the antioxidant levels and caused cellular and tissue damage.	Paul-Pont *et al.*, 2016
32 types of plastic products	-	*Daphnia magna*	PUR had the most toxic effect.	Lithner *et al.*, 2009
PE	-	*Daphnia magna*	Ingestion of both primary and secondary MP decreased the algae consumption by *D. magna*.	Ogonowski *et al.*, 2016
Fluorescent red microspheres 1-5 µm	AuNP	*Daphnia magna*	Parental mortality and immobile juveniles are caused by the influence of AuNP and MP alone. Toxicity of mixtures were higher than toxicity of AuNP and MP alone.	Pacheco *et al.*,2018
PS 10 µm	-	mysid shriMP, copepods, cladocerans, rotifers, polychaete larvae and ciliates	Polychaete larvae of the genus *Marenzelleria* ingested the highest portion of MP. MP in fecal pellets were reabsorbed by copepods and mysids.	Setälä *et al.*, 2014

Table 2-4: Examples of studies on the effects of microplastics and chemicals associated to them on vertebrates. AuNP- Gold nanoparticles. AChE - Acetylcholinesterase. IDH - Isocitrate dehydrogenase. LDPE - Low-density polyethylene. MP - microplastics. mm - millimeter. PAHs - Polycyclic aromatic hydrocarbons. PBDEs- Polybrominated diphenyl ethers. PCBs - Polychlorinated biphenyls. PE - Polyethylene. PS - Polystyrene. μm - micrometer.

Type and size of microplastics	Associated contaminants	Species	Results	References
PE 0.1–4.5 mm	PBDEs PAHs PCBs	*Oryzias latipes*	Bioaccumulation of PBDEs and some PCBs in fish, along with MP materials, caused liver toxicity (*e.g.* glycogen depletion, fatty vacuolation, and single cell necrosis). Concentrations of hydrophobic organic compounds in the tissues were greater than those in control. PBDEs transfered from MP to organisms (PBDEs bioaccumulation increased in those fed with contaminated MP).	Rochman *et al.*, 2013
PE <1mm		*Oryzias latipes*	Gene expression in male and female fish exposed to the MP changed. The chemicals in the MP may induce endocrine-disrupting effects.	Rochman *et al.*, 2014b
PE 1–5 μm	Pyrene	*Pomatoschistus microps*	AChE activity decreased in fishes exposed to MP alone and mixture with pyren but IDH activity only decreased with mixture.	Oliveira *et al.*, 2013
PE 1–5 μm	AuNP	*Pomatoschistus microps*	Exposure to AuNP alone reduced the predatory performance. MP did not change the AuNP toxicity.	Ferreira *et al.*, 2016
L*DPE* < 60 μm	Phenanthrene	*Clarias gariepinus*	Virgin LDPE caused toxicity and modulated the adverse impacts of phenanthrene.	Karami *et al.*, 2016

The physicological signs of stress and exposure biomarkers have been observed in organisms after ingestion of chemicals associated with MP. Toxicological studies of MP-associated chemicals are limited to several experimental studies and may not be accurate enough as did not reflect environmental concentrations of these chemicals and realistic exposure scenarios (Koelmans *et al.*, 2014; Gall and ThoMPon, 2015).

In addition, although MP alone have adverse effects on organisms, the simultaneous exposure to MP and other environmental contaminants present in the water can have a greater impact (Browne *et al.*, 2013; Oliveira *et al.*, 2013; Rochman *et al.*, 2013; Pacheco *et al.*, 2018; Prata *et al.*, 2018), and more research is needed

2.5.3. Modeling studies

It is difficult to investigate the contribution role of MP and associated contaminants in both field and experimental studies (Holmes *et al.*, 2012; Bakir *et al.*, 2014). Accordingly, the modeling of the equilibrium division was performed to provide evidence of the indirect transmission of associated chemicals to organisms compared to other exposure routs (EPA, 2016). Both experimental and modeling studies predicted bioaccumulation up to a factor of two to three if plastic is the only source of absorption. However, according to more environmentally effective factors (*e.g.* all exposure routs), MP play a relatively small role in comparison to other routes (Koelmans, 2016).

Table 2-5: Examples of studies on the effects of microplastics and chemicals associated to them in modelling studies. BPA - Bisphenol A. DDT - Dichlorodiphenyltrichloroethane. MP - Microplastics. PBDEs - Polybrominated diphenyl ethers. PBT - Polybutylene terephthalate. PCBs - Polychlorinated biphenyls. PE - Polyethylene. PS - Polystyrene. PVC - Polyvinyl chloride. PUR - Polyurethane.

Type and size of microplastics	Associated contaminants	Species	Results	References
PE	PBT	*piscivorous fish*	Bioaccumulation of PBT reduced due to high absorption of the chemicals by MP	Gouin *et al.*, 2011
PS	PCBs	*Arenicola marina*	A bioaccumulation model used to evaluate the accumulation of PCBs from MP	Koelmans *et al.*, 2014
PS	PCBs	*Arenicola marina*	Small contribution of MP in the accumulation of PCBs observed in contrast to other exposure pathways	Besseling *et al.*, 2013
Marine MP	nonylphenol BPA	*Arenicola marina; Gadus morhua*	Bioaccumulation of nonylphenol and BPA was very low through MP ingestion.	Koelmans *et al.*, 2015
MP	PCBs PBDEs DDTs	*Fulmarus glacialis*	MP used as a passive sampler	Herzke *et al.* 2016

For example, contribution of polystyrene for the accumulation of PCBs in the lugworm (*Arenicola marina*) was small when compared to other exposure pathways (Besseling *et al.*, 2013). In another sudy, Koelmans *et al.*, (2015) showed that the bioaccumulation of nonylphenol and BPA was very low through ingestion of MP in the lugworm (*Arenicola marina)* and the Atlantic cod (*Gadus morhua).* Some modeling studies using invertebrates, fish, and seabirds as case studies explain that while the capacity of MP to adsorb chemicals is high, disposing of ingested chemicals may not be a significant factor because their

bioaccumulation from natural prey may be higher than from MP (Koelmans *et al.*, 2016; Ziccardi *et al.*, 2016).

2.6. Future research and monitoring of microplastics in the biota

Contamination of the aquatic biota by MP in natural habitats has grown and the reports on the worldwide concentrations of various types of MP show an increasing trend (Wright *et al.*, 2013; Paul-Pont *et al.*, 2016). The toxicity of MP significantly varies with several parameters such as size, shape, crystallinity and strong correlation between the different parameters, though one must consider in pushing toxicological test towards a robust experimental evaluation of MP (Wright *et al.*, 2013). However, there are still researches in this field to be addressed. Studies should not only study the physicochemical properties of MP but should also consider the behaviour of MP in relation to other contaminants (Brennecke *et al.*, 2016; Sleight *et al.*, 2017).

Despite the significant development in detection techniques for the minimization of the uncertainties associated with the extraction and quantification of MP, a consistency between the techniques and units is still lacking. Though, one main aspect in understanding the behaviour of MP is related to standardization and harmonization to establish a common monitoring protocol. The development of this protocol is a key factor for the reliability of MP data, which allows the expansion of both intra and inter laboratory results.

Chapter III:

Challenges and effects of metallic nanomaterials

Abstract

Since the use of nanometals has increased in many applications, understanding their potential toxicity is of great importance. Despite the advances in past years, the knowledge about environmental concentrations of these pollutants and their ecotoxicological effects is still limited. One major barrier to the advancement in nanotechnology is the potential uncertainty associated with the determination of the concentration of nanomaterials in complex matrices (*e.g.* natural water, sediment), and the toxicity test method used for these materials. The central goal of the present study was to summarize and discuss what is known and what needs to be done to overcome the uncertainties in aquatic ecotoxicity of metallic nanomaterials. Among the various sources of uncertainties, physicochemical properties of nanometals including particle size, shape as well as agglomeration, solubility and experimental approaches need to be investigated more carefully.

3.1. Introduction

Nowadays, nanotechnology or more specifically nanomaterials are used in various industrial products and applications. Figure 3-1 shows the use of nanomaterials in many industrial areas.

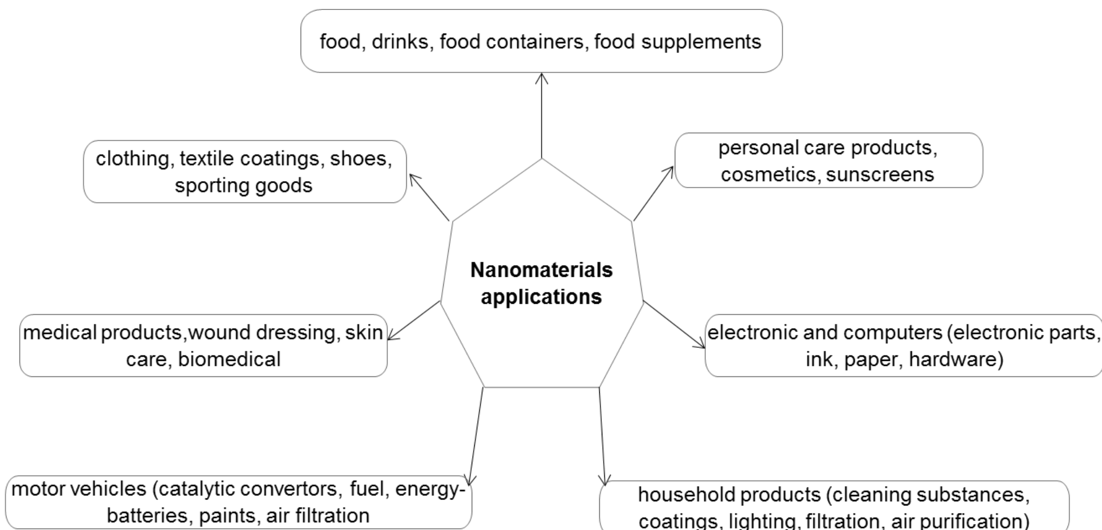


Figure 3-1: Examples of nanomaterials applications in industry.

Increasing the production and use of nanomaterials and their entry into aquatic environments during and after production and use, as well as its subsequent effects on the environment and human health, has become increasingly a major concern (Weinberg *et al.,* 2011). To investigate their effects, at first the environmental concentrations of metallic nanomaterials in aquatic ecosystems should be known. However, environmental

concentrations of nanomaterials are largely unknown (Griffitt *et al.*, 2008: Weinberg *et al.,* 2011; Proulx and Wilkinson, 2014; Conway *et al.,* 2015). The main reason for this is the lack of cost-effective technology to measure the concentrations of a wide range of nanometals in complex matrices such as natural waters and sediments. Moreover, the effects of metallic nanomaterials on aquatic organisms and ecosystems are still poorly understood and the most part of the existing knowledge resulted from relatively high exposure concentrations likely not ecologically relevant that refer to a small number of metallic nanomaterials. Nanomaterials which are released to the aquatic environment are both organic and inorganic (Figure 3-2). Therefore, it is important to consider the type of waste material in terms of their effects during the life cycle of nanomaterials in the aquatic environment.

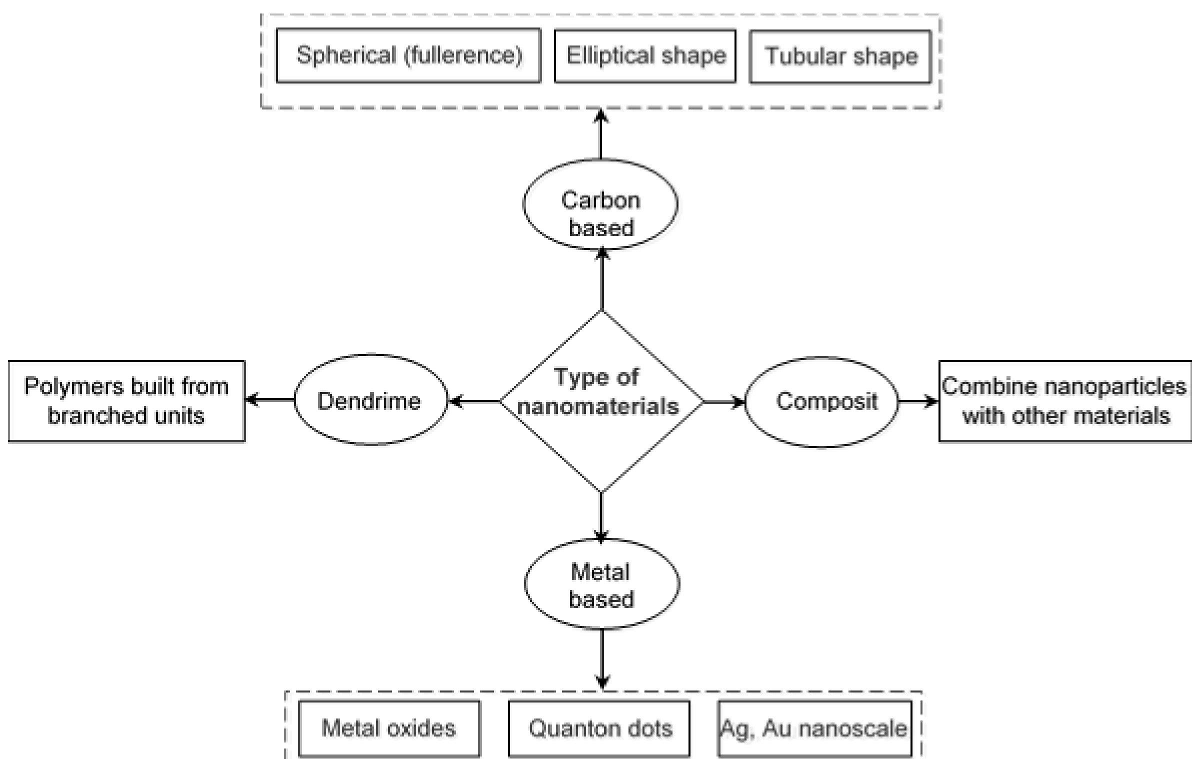


Figure 3-2: Wide categorization of engineered nanomaterials

Metallic nanomaterials can induce several types of toxic effects on freshwater and marine species at concentrations in the ppm or lower ranges. Furthermore, studies with aquatic food chains and aquatic ecosystem models showed that some metallic nanomaterials (*e.g.* AuNP) can be bioaccumulated by some species (Tedesco *et al.*, 2008; Tedesco *et al.*, 2010; Gilroy *et al.*, 2014 (Table 3-1) and increase the risk of exposure and toxic effects to their predators and human through consumption of contaminated aquatic species (Ferreira *et al.*, 2016).

Due to the widespread use of nanomaterials, limited knowledge, and the large variety of aquatic species and nanomaterials, further work regarding their presence and impacts on ecosystems is needed.

Table 3-1: Examples of studies on bioaccumulation and effects of some nanomaterials in aquatic species. Ag- Silver. AuNP - gold nanoparticles. CeO_2 - Cerium (IV) oxide. CuO - Copper (II) oxide, Fe_3O_4 - *Iron (III) oxide.* PS - Polystyrene. PSNPs - Polystyrene nanoparticles. TiO_2 - Titanium dioxide. SnO_2 - Tin (VI) oxide. ZnO - Zinc oxide.

Nanomaterials	Organisms	Effects	Reference
ZnO, CuO, TiO_2	*Pseudokirchneriella subcapitata*	The most toxic nanoparticle was ZnO followed by CuO and TiO_2.	Aruoja *et al.,* 2009
AuNPs	*Pseudokirchneriella subcapitata*	AuNPs influenced on the algae with weaken the cell wall. AuNP did not able to penetrate the cell.	Botha *et al.,* 2015
AuNPs, PS	*Pseudokirchneriella subcapitata*	PSNPs adsorbed to the algal cells and the cell wall changed.	Nolte *et al*., 2017a
Citrate-Au	*Chlorella autotrophyca;* *Rhodomonas salina*	No acute toxicity was recorded at ecological concentrations up to 0.3 mg/l.	Blasco *et al*., 2012
Au	*Ankistrodesmus falcatus;* *Daphnia magna*	Trophic transfer of AuNP was observed (from algae to daphnia). Most AuNPs stayed in the digestive tube and were removed by excretion; so, it was no influence on reproduction.	Gilroy *et al.,* 2014
Citrate-Au	*Mytilus edulis*	Accumulation and oxidative stress in the digestive gland and gills.	Tedesco *et al*., 2008
AuNP	*Mytilus edulis*	95 % of AuNP were accumulated in the digestive gland; increase of lipid peroxidation levels; decrease of thiol-containing proteins.	Tedesco *et al*., 2010
CuO	*Scrobicularia plana;* *Hediste diversicolor*	Oxidative stress (catalase and glutathione *S*-transferase activities) increased; and burrowing behaviour in both species affected.	Buffet *et al*., 2011
SnO_2; CeO_2; Fe_3O_4	*Paracentrotus lividus*	Stressful effects on immune cells were detected.	Falugi *et al*., 2012

Ag	*Danio. rerio*	Single particles (5-46 nm) were transferred into and out embryos through chorion pore canals. Abnormalities were observed in the embryos.	Hydutsky *et al.*, 2007
Ag	*Cyprinodon variegatus*	Thickness of epithelia gill tissue was increassed and gene expression in adults and juveniles was changed.	Griffitt *et al.*, 2012
Ag	*Pimephales promelas*	Early life stage and mortality in embryos affected.	Laban *et al.* 2010
Ag	*Perca fluviatilis*	The diffusion conductance of gills during low water oxygen capacity was reduced.	Bilberg *et al.*, 2010

The evaluation of the toxicity of metallic nanoparticles in aquatic ecosystems is challenging due to several factors that can lead to uncertainty in the results. For example, the type of the metallic nanomaterial (*e.g.* gold, silver, copper) its solubility in water, its size, shape, surface coating and other physicochemical properties can influence its toxicity to aquatic organisms (Oukarroum *et al.*, 2012a; Rose *et al.,* 2012; Yah, 2013; Shin *et al.,* 2015; Yung *et al.,* 2017a). For instance, surface properties determine the stability and mobility of metallic nanoparticles in aquatic systems and their interactions with the organisms (Navarro *et al.*, 2008; Bantz *et al.*, 2014). However, the same type of metallic nanoparticles with different inorganic or organic coating agents and various surfactants used to stabilize them may lead to different toxicity (Zhao *et al.*, 2015; Van Haute *et al.*, 2018). For this reason, nanomaterials should be characterized for the toxicological study purposes (Figure 3-3).

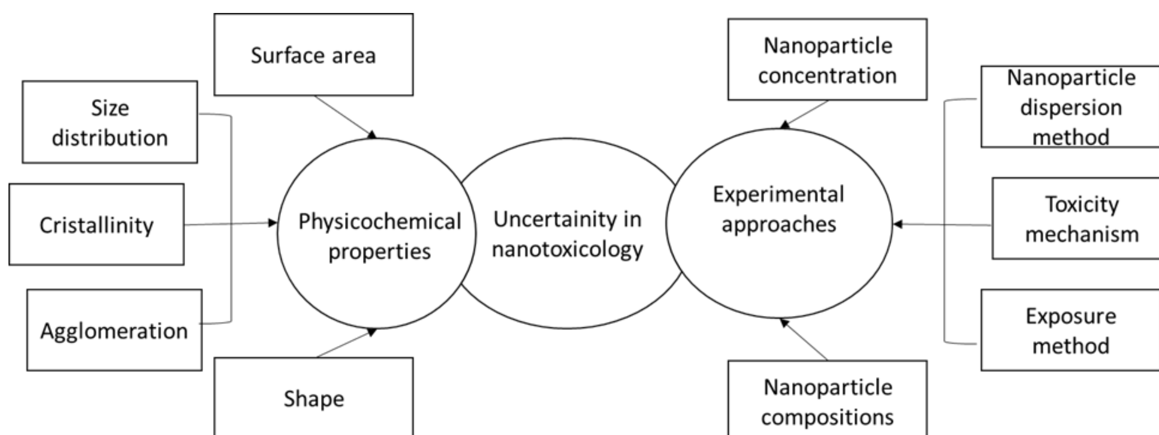


Figure 3-3: Important properties of nanoparticles that are affected on the uncertainty.

To characterize the physical properties of nanoparticles, aggregation/agglomeration, size, dissolution, chemical composition and some key abiotic factors in the aqueous media, such as pH, presence of natural organic matter, and ionic strength should beinvestigated. However, obtaining comprehensive characterization can not always be possible due to different characterization equipment. In addition to the properties of the particles, the exposure conditions also influence the toxicity of metallic nanomaterials. For example, temperature and the type of test medium have great influence because generally metallic nanoparticles aggregate in aqueous media, and several factors can affect the aggregation of the particles (*e.g.* temperature, pH, salinity, ionic strength, and concentration of organic compounds of test medium) (Keller *et al.*, 2010; Oukarroum *et al.*, 2012b; Baalousha, 2017; Yung *et al.,* 2017b; Khan *et al.,* 2018). Therefore, the characterization of the metallic nanoparticles and their behaviour under different environmental conditions represents useful knowledge for their environmental risk assessment and safety management.

The particle size distribution of nanomaterials is the most important criterion that affects the functionality of nanomaterials such as rheology, film gloss, surface area and packing density (Rodríguez-López *et al.*, 2010; Guisbiers *et al.*, 2012; Shin *et al.*, 2015). For example, smaller particles due to their larger specific surface area provide additional properties such as rapid transfer of mass, effective adhesion to substrates, and good suspension in the solution in comparison to bulk materials (Uglov *et al.*, 2016; zhang *et al.*, 2017c). In this sense, without the proper characterization of particle size and their size distribution, the validity of nanotoxicology analysis is questionable. Furthermore, particle size determines how easy nanomaterials can be transferred to different organisms. Therefore, determining the particle size distribution is an important factor in understanding

the scale of environmental pollution in aqueous medium. In view of the above discussion, toxicological studies must include careful control of the size-dependent effects.

It is generally believed that as the size of particles reduces from microscale to nanoscale, they become more toxic because of their increased surface area to their volume ratio as well as the higher number of atoms on their surface for chemical reactions (Brown *et al.,* 2001; Dick *et al.,* 2003; Duffin *et al.,* 2007; Warheit *et al.,* 2009). However, in aqueous environments, nanoparticles show a large tendency towards agglomeration due to high surface energy and neutral pH that is required for ecotoxicological experiments (Table 3-2) (Chithrani *et al.,* 2006; Bihari *et al.,* 2008; Lundqvist *et al.,* 2008; Dobrovolskaia *et al.,* 2009; Wiesner *et al.,* 2009; Jahan *et al.,* 2017). Agglomeration reduces the effective specific surface area that would decrease metal ion release and reactive oxygen species generation. In previous studies dealing with agglomeration, sonication devices or biological surfactants have been used (Keller *et al.,* 2010; Sperling & Parak 2010; Gao *et al.,* 2012; Oh *et al.,* 2013; Park *et al.,* 2013; Durand-Gasselin *et al.,* 2014; Behra 2015). However, effectiveness of such strategies is questionable because the particle size distribution after sonication is not known. Ignoring agglomeration in the context of toxicity evaluations would lead to wrong conclusions.

Table 3-2: Size of some nanoparticles as individual and aggregated form. AuNPs – Gold nanoparticles. CeO_2 - Cerium (IV) oxide. nm - Nanometer. TiO_2 - Titanium dioxide. SnO_2 - Tin (VI) oxide. ZnO - Zinc oxide.

Materials	diameter (nm)	Primary size (nm)	Hydrodynamic diameter(nm)	References
Citrate-coated Au	5	2-5	200	Bihari *et al.,* 2008
Citrate-coated Au	17	20	150	
TiO_2		23-31	187-211	Keller *et al.,* 2010
CeO_2		7-9	215-229	
ZnO		23-27	201-209	
AuNPs	30	22-34	76-100	Dobrovolskaia *et al.,* 2009
AuNPs	50	45-55	100-150	
AuNPs	13	9.04-14.76	83.7-96.16	Cooper, 2015
AuNPs	16	19.59-28.23	110.28-113.98	
AuNPs	36	32.43-45.35	80.61-90.85	

Different shapes of nanoparticles may affect their retention time inside organisms and thus theirtoxicity. The shape of nanoparticles can directly affect their transport into biota (Albanese *et al.*, 2012; Guo *et al.*, 2013; Kumar *et al.*, 2013) and their toxicity. For example disk-shaped nanosilvers are more toxic to zebrafish embryos compared to sphere and rode ones due to the presence of surface defects (George *et al.,* 2012). Hua *et al., (*2014) compared the toxicity of zinc oxide nanosticks and nanoshperes and cuboidal shape and concluded that nanosticks led to higher toxicity compared to other shapes. A recently published article (Favi *et al* .,2015) showed that nanostar gold nanoparticles were less toxic than spherical gold nanoparticles to fibroblast and endothelia cells. Therefore, considering how biological creatures react to different shapes of nanoparticles is important to understand the toxicity of nanomaterials.

Surface functionality is one of the factors influencing nanomaterials toxicity to marine organisms. This parameter significantly affects the surface properties of nanometals such as surface charge, surface crystallinity, and surface topology (Nel *et al.*, 2009; Xu *et al.*, 2010; George *et al.*, 2012; Kim *et al.*, 2013; Kim *et al.*, 2014). Among other things, crystal defects have been shown to enhance the functionality of nanometals Atoms at crystal defect sites are more reactive as compared to those in an equivalent defect-free crystalline structure (Holzinger *et al.,* 2014). By reducing the size of crystalline materials to nanoscale, their crystal defect sites increase with a similar composition and equal mass (Kishen, 2015). In the context of biological evaluation, reactivity of crystal defects by influence on reactive oxygen species or interaction with biomolecules leading to high toxicity. Therefore, some toxicological studies reduced surface crystal defects by using surfactants, coatings and soluble materials to mitigate the toxicity of nanoparticles (Yah, 2013; Kim *et al.*, 2013; Bozich *et al.,* 2014; Saei *et al.*, 2017). The surface functionality of nanometals could vary considerably and due to this reason, it is crucial to study the toxic effects of such materials on marine organisms with regard to their environment and their surface chemistry. Hence, the uptakes of nanometals with different surface reactivity could provide a deeper insight into how changes in the functionality of nanometals and their toxicity are related.

Solubility has been shown to be strongly involved in the cytotoxic response (Borm, 2005; Wong *et al.,* 2013). Therefore, understanding the solubility of nanoparticles in the aquatic environment, especially in the marine water, in terms of the tendency of nanoparticles to attach to the bigger agglomerates, which attempt to settle in the solution, or the tendency to dissolve are principal parameters. Solubility of nanomaterials should increase exponentially with surface area, and since there is an inverse correlation between surface area and particle size, moving from the microscale to the nanoscale would help dissolution rate (Kaptay, 2012). Dissolution of nanometals, such as Au and Ag (Cherevko *et al.,* 2014;

Bardaxoglou *et al*., 2017), first requires the formation of an oxide layer on their surface in solution. After dissolving the oxide layer, increasing the dissolution of nanometals requires the presence of an oxidant [*e.g.*, H_2O_2, O_2, Cl^-] in the solution (Wong *et al*., 2013). For instance, Cherevko *et al.,* (2014) showed that an optimum concentration of Cl^-, results in the minimization of the size ofAuNP. The effects of oxidants on the chemistry of nanometals in aquatic environments are complex, because the oxidation of nanometals could affect pH of the solution and subsequently the degree of their toxicity (Wang *et al.,* 2016a). This suggests that solubility is another important factor that influences the toxicity of nanoparticles.

3.2. Gold nanoparticles

Due to exceptional physical and chemical characteristics including optical response, chemical and physical constancy, surface function, relatively low toxicity to several biological systems, and biocompatibility (Huang and El-Sayed, 2010; Yeh *et al.,* 2012; Gong *et al.,* 2015), AuNP have been used in several industries, as shown in Figure 3-4, For example, in biomedicine, (*e.g.* applications inbioimaging, drug delivery and tissue engineering) (Wang *et al*., 2012; Versiani *et al*., 2016; Kong *et al*., 2017; Elahi *et al*., 2018).

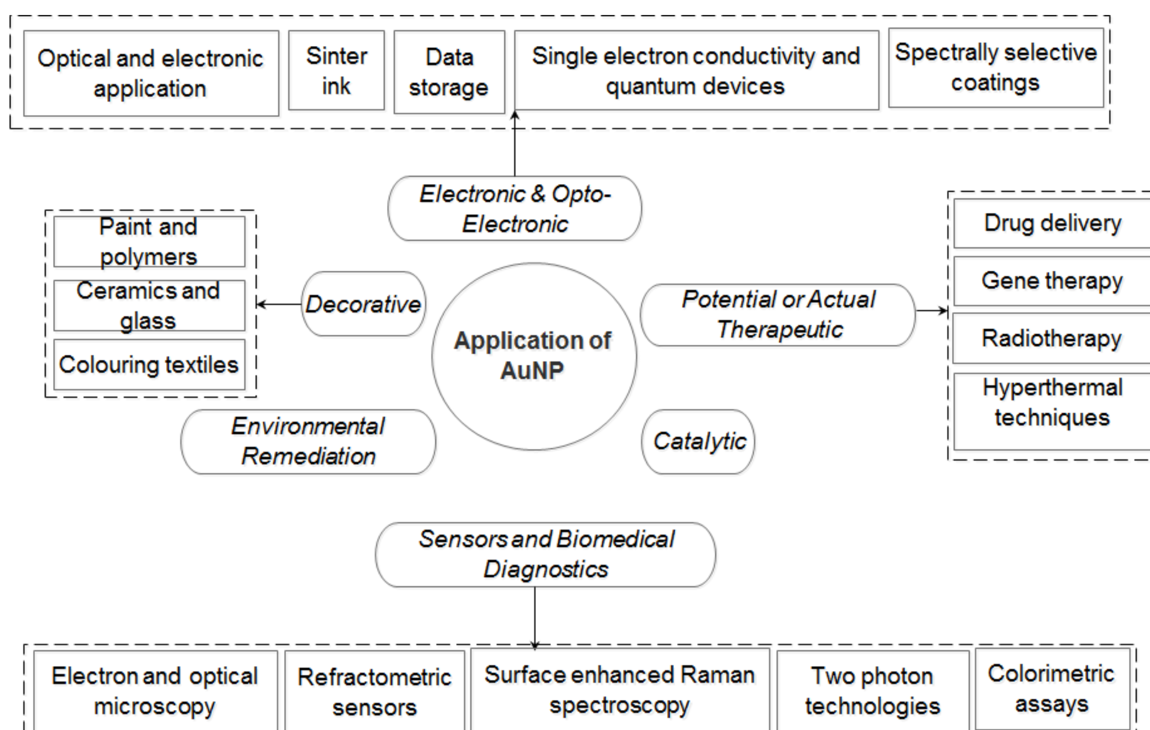


Figure 3-4: Applications of gold nanoparticles. AuNP- Gold nanoparticle.

Such multifunctional applications of AuNP lead to their investigation in several fields, including chemistry, physics, biology, engineering, among others to extract new findings of

AuNP for more potential applications (Dorsey *et al.,* 2013; Versiani *et al.*, 2016). For example, in the field of neuroscience, AuNP have been increasingly used over the last decade. The incorporation of AuNP in neuroscience research is thought to help finding new medication methods for treating some diseases, especially those not having yet an effective treatment (Paviolo and Stoddart, 2017).

The production and use of AuNP result in their release to the environment with potential negative impacts on environmental and human health that need to be further addressed. The toxicity of AuNP has been investigated in several models, both *in vitro* and *in vivo* (Li *et al.*, 2010; Van Hoecke *et al.*, 2011; Li and Chen, 2015; Chen *et al.*, 2017; Ferreira *et al.*, 2016; Nolte *et al.*, 2017a). However, the comparison of the published data to understand the toxicity of AuNP is not an easy task because the toxicity AuNP is influenced by several factors, including the physical and chemical properties of the particles tested, environmental conditions of the toxicity assays, the biological model used, among others.

The entrance of AuNP into cells and their biological activity depend on the size of the particles and of the biological system (Xie *et al.*, 2017; Zhang *et al.*, 2017c). The small size of AuNP facilitate their entry into cells and make them appropriate for biomedical and biological uses (Li and Monteiro-Riviere, 2016; Xie *et al.,* 2017). For example, AuNP with an average diameter of 18 nm can enter into cells without damaging them and cause no significant adverse effects (Connor *et al.*, 2005); AuNP with an average diameter of 1 nm can enter into the cell, cross nuclear membrances, and attach to deoxyribonucleic acid (DNA) without adverse effects (Tsoli *et al.,* 2005). Another important factor in *in vivo* studies is the circulation time. Regarding this, small AuNP can circulate faster than those with bigger sizes (De Jong *et al.*, 2008). The internalization of AuNP (10-100 nm) into the human Hella cells depends largely on their size (50 nm being considered the optimum size), also spheres shape were taken up more efficiency than nanorod forms (Chithrani *et al.*, 2006). In addition, in another study, Sathishkumar *et al.*, (2015) examined the toxicity of AuNP (20-50 nm) to A549 cells (human lung adenocarcinoma epithelial) and confirmed that size and coating affect the cytoxicity of AuNP. In this regard, the results of Khlebtsov and Dykmana, (2011) showed that AuNP with size 1-2 nm had the lowest cellular toxicity. They stated that AuNP in this size can bind to important biomolecules such as DNA and affect the function of cellular molecular processes.

3.2.1. Effects of gold nanoparticles on aquatic organisms

Due to the widespread use of AuNP, their entry into the aquatic environment threatens the health of the environment (Lopez-Sanchez *et al.,* 2011). Although there are several

researches on AuNP, their fate and behaviour are still unclear, particularly in natural aquatic ecosystems (Table 3-3).

The toxicity of AuNP to microalgae have been investigated, mainly because microalgae are considered good model organisms, have ecologically relevant roles, and in the wild interferences with their populations may affect the rest of the ecosystem (Renault *et al.*, 2008; Ji *et al.*, 2011; Baker *et al.*, 2014). In some studies, on the toxicity of AuNP to algae, coating, size and colloidal stability have examined (Renault *et al.*, 2008; Hartmann *et al.*, 2013; Nur, 2013; Van Hoecke *et al.*, 2013; Larguinho *et al.*, 2014; Behra *et al.*, 2015; Iswarya *et al.*, 2016).

Most studies showed that the toxic effects of AuNP on the population growth rate of microalgae is moderate in terms of the effective concentration in mg/l (Blasco *et al.*, 2012; Van Hoecke *et al.*, 2013). An interesting point in studies that reported AuNP toxicity on microalgae growth is that the TEM analysis does not show the presence of particles in the cells (Renault *et al.* 2008; Hartmann *et al.* 2013; Van Hoecke *et al.* 2013). It is reported that negative-charged AuNP has little absorption to algae (Garcia-Cambero *et al.* 2013; Hartmann *et al.* 2013; Van Hoecke *et al.* 2013; Botha *et al.*, 2015; Nolte *et al.*, 2017). Research by Renault *et al.*, (2008) showed that the growth rate of *Scenedesmus subspicatus* decreased (up to 20-50 %) by exposure to AuNP (10 nm) coated with amine after 24 hours. They explained that AuNP was attached to algae cell wall without entering into to the cell cytoplasm, but even so they may be transferred to upper trophic levels though microalgae ingestion. Another showed microalgae growth inhibition and photosynthetic activity reduction in microalgae exposed to AuNP and concluded the entrance of the particles into the cells is not needed to toxicity (Perreault *et al.*, 2012).

In addition, same materials may show different results on toxicity because of variability in their surface defects and preparation methods. Even if AuNP is not toxic without the coating, the coating surface may have a little toxic effect, when AuNP interacts with the cell wall of the bacterium.

Table 3-3: Examples of studies on influence of gold nanoparticles on unicellular organisms. AuNP - gold nanoparticles. LDH - lactate dehydrogenase. nm - nanometer. NPs - nanoparticles. PEG - poltethylene glycol. Ps - Polestyrene. PSNPs - Polystyrene nanoparticles. PVP - Polyvinyl pyrrolidone

Size (nm)	Species	Effect	References
Amine-AuNP (10)	*Scenedesmus subspicatus*	The initial number of cells decreased even at the lowest concentration (50 % algal mortality was observed after 24h) No uptake was observed into the intracellular environment.	Renault *et al*., 2008
Glycodendrimers coated AuNP	*C. reinhardtii* (wildtype and mutant)	The observed aggregation in wild-type strains was probably due to NPs interaction with cell walls. Although NPs penetrate the cytoplasm of both cells, inhibition of growth and photosynthetic activity has been reported only for wildtype strain, which indicates that the toxic effects associated with wall interaction and aggregation are greater than the presence of gold in the cytoplasm.	Perreault *et al*., 2012
Amphiphilic-AuNP; pegylated amphiphilic-AuNP (4-5)	*Pseudo subcapitata*	Absorption or direct interaction between particles and algal cells was not observed, but it was shown that AuNP has the potential to reduce algal growth rate. Moderate toxicity was observed. The pegylated AuNP were less toxic compared to the amphiphilic coated particles.	Van Hoecke *et al*., 2013
Citrate-AuNP (20-30)	*Chlorella autotrophyca* *Rhodomonas salina*	No acute toxicity was recorded at ecological relevant concentrations for assayed AuNP.	Blasco *et al*., 2012
(PVP)-capped citrate-capped AuNP	*Pseudomonas Flourescens*	Surface capping agents influenced on the AuNP toxicity. Toxic effect of PVP-capped AuNP was much more than citrate-capped AuNP.	Nur, 2013
Citrate-AuNP PEG-AuNP	*Dunaliella salina*	Approximately 76 % of the initial amount of AuNP (and 36 % for PEGylated AuNP) is absorbed by microalgae. No significant morphological alterations were observed No stress was detected in the microalgae population	Larguinho *et al*., 2014
citrate-coated AuNP (5)	*Chlamydomonas reinhardtii*	No significant toxicity was determined. There were no effects on the granularity or morphology on short- and long-term exposure due to negative surface charge of algae. Particles with diameter less than 3nm displayed higher chemical reactivity and toxicity.	Behra *et al*., 2015

AuNP	*Pseudokirchneriella subcapitata*	The absorption of AuNP has been influenced by the interaction between algae and the environment, which has also affected vital changes due to the weakening of the wall. Toxicity enhanced with releasing of free metal ions. AuNP did not able to penetrate the plasma.	Botha *et al.*, 2015
PVP-AuNP/30 PVP-AuNP/40	*Chlorella sp.*	The highest toxicity was related to AuNP (30nm) which increased membrane damage and LDH release. PVP-AuNP was less toxic than citrate-AuNP. PVP-AuNP (30) was more stable than the citrate-AuNP (30 and 40).	Iswarya *et al.*, 2016
AuNP PS	*Pseudokirchneriella subcapitata*	Increasing the absorption of PSNPs into algal cells caused a change in cell wall	Nolte *et al.*, 2017

The toxic effects of AuNP were also investigated in organisms of higher tropic levels, such as daphnids, polychaetes, crustaceans, bivalves and fish. The results of some of these studies are shown in Table 3.4.

For example, in a study where the daphnid *Daphnia magna* was exposed for 12 hours to AuNP (17 – 23 nm), the particles were found in the gut butnot in other organs and tissues. However, AuNP accumulation in the gut reduces food intake (Lovern *et al.*, 2008). In another study with *D. magna*, the estimated 50 % lethal concentration (LC_{50}) of AuNP (15 nm) after 48 hours was 65 - 75 mg/l (Li *et al.*, 2010, Table 3-4). In a recent 21-day chronic study with *D. magna*, 0.2 and 2 mg/l of ~ 5 nm AuNP caused parental mortality, decreased the number of mobile juveniles and caused the release of immobile juveniles and aborted eggs, findings that raise concern regarding the long-term exposure of animals and humans to AuNP (Pacheco *et al.*, 2018).

The toxicity of AuNP was also investigated in fish, such as *Danio rerio, Pomatoschistus microps* and *Oncorhynchus mykiss.* In *Danio rerio*, AuNP (5 – 10 nm) had no significant effect on the metabolic activity up to 17.4 mg/L, but the levels of reactive oxygen species (ROS) in the cells increased (Farkas *et al.*, 2010). Moreover, the different biological responses from *Danio rerio* were reported with AuNP (1.5 nm) (Truong *et al.*, 2013). Also, mitochondrial disorders in brain and muscle of *D. rerio* exposed to AuNP (12 - 50 nm) were found (Geffroy *et al.,* 2012). Ferreira *et al.*, (2016) showed that the predatory performance of *Pomatoschistus microps* decreased under exposure to 0.2 mg/l of AuNP (5 nm).

Table 3-4: Some example of Influence of gold nanoparticles on aquatic organisms. AuNPs - gold nanoparticles. nm - nanometer.

Nanomaterial (Size (nm))	Species	Effects	References
Citrate-AuNP (20)	*Daphnia magna*	AuNP distributed along the gut and eventually were eliminated from the digestive tract within 24h	Lovern *et al.*, 2008
Citrate-AuNP (20)	*Daphnia magna*	AuNP has changed the swimming pattern of the *Daphnia magna* by covering the outer curtains	Li *et al.*, 2010
AuNP (5)	*Daphnia magna*	The number of mobile juveniles decreased. Caused the release of immobile juveniles and aborted eggs.	Pacheco *et al.*, 2018
AuNP (13)	*Mytilus edulis*	No effects were observed	Tedesco *et al.*, 2008
Mercaptopropionicacid-AuNP (5)	*Mytilus edulis*	Effects of larger AuNP on the oxidative metabolism of mussels less than smaller ones.	Tedesco *et al.*, 2010
AuNP (21)	*Ankistrodesmus falcatus* *Daphnia magna*	Trophic transfer of AuNP were observed from *Ankistrodesmus falcatus* to *Daphnia magna*. Most of AuNPs in the digestive tube did not influence on reproduction and was removed with excretion.	Gilroy *et al.*, 2014
Citrate-AuNP (5-10)	*Oncorhynchus mykiss*	No effects on metabolic activity were observed in vitro up to 17.4 mg/l. Cellular ROS levels was increased.	Farkas *et al.*, 2010
AuNP (1.5)	*Danio rerio* (embryos)	Different biological responses were reported by functionalised AuNP with 2-mercaptoethanesulfonic acid, N, N, N-trimethylammoniumethanethiol, or 2-(2-(2-mercaptoethoxy) ethoxy) ethanol.	Truong *et al.,* 2013
AuNP (12–50)	*Danio rerio* (Adult)	Mitochondrial disorders were appeared in brain and muscle	Geffroy *et al.,* 2012
AuNP (5)	*Pomatoschistus microps*	The predatory performance decreased up to 0.2 mg/l of AuNP. Increasing of temperature directly influence on the concentration of Au in fish exposed to AuNP.	Ferreira *et al.,* 2016

Most of studies on AuNP have shown that mortality rate under acute exposure (very high concentration, short exposure time) is relatively low (Behra *et al.*, 2015). But in long term

exposure, chronic effects may be observed, as already indicated for *D. magna* (Pacheco *et al.*, 2018). However, there is not much information in this area due to lack of studies. Even though some works have been done about nanotoxicology and nanoecotoxicology during the last years, the issue of nanoecotoxicology remains an unclear area with several uncertainties associated with the resulting toxicological studies.

To understand the effect of AuNP on microalgae, in order to obtain more accurate results, a good experimental design is needed, and this is still an open chapter in the microalgae study. Due to the high reactivity of nanoparticles with many substances in aquatic environments (artificial or natural) (Ray *et al.*, 2009; Perreault *et al.,* 2012), It is not easy to test nanoparticles toxicity in microalgae, especially in the marine environment. The standardization of nanoparticles tests for any organism, including microalgae, is necessary (Hartmann *et al.*, 2012). However, there are some studies in the literature that investigated the effects of different AuNPs in some freshwater and marine microalgae species, as shown Table.3-5.

Table 3-5: Toxicity data of gold nanoparticles on different microalgae. Ag- Silver. AP- Amphiphilic. AuNP- Gold nanoparticles. EC50- Median Effective Concentration. Fw- freshwater. nm-Nanometer. PAMAM- Polyamidoamine PEG- Polyethylene glycol. Sw- seawater.

Nanoparticle (Size (nm))	**Species**	**Sw /Fw**	**Endpoint**	**Results**	**Reference**
Citrate-AuNP (10)	*Scenedesmus subspicatus*	Fw	EC_{50}, 24h	48.5 mg/l	Renault *et al.*, 2008
AP-AuNP (4-5)	*Pseudokirchneriella subcapitata*	Fw	EC_{50}, 72h	0.0037 mg/l	van Hoecke *et al.*, 2011
AP-PEG-AuNP (4-5)				0.02 mg/l	
Mannose-AuNP (2)	*Chlamydomonas reinhardtii* (wild type)	Fw	Growth inhibition, 48h	0.012 mg/l, 60 % Inhibition	Perreault *et al.*, 2012
	Chlamydomonas reinhardtii (wall-lacking mutant)			No inhibition	
PAMAM-AuNP (1.5-2.5)	*Chlamydomonas reinhardtii*	Fw	EC_{50}, 30 min	114 ± 34 mg/l	
			EC_{50}, 24h	83 ± 26 mg/l	
	Rhodomonas salina				

Nanoparticle (Size (nm))	Species	Sw /Fw	Endpoint	Results	Reference
Citrate-AuNP	*Chlorella autotrophyca*	Sw	EC_{50}, 72h	> 0.295mg/l	Moreno-Garrido *et al.*, 2012
	Cylindrotheca closterium				
	Phaeodactylum tricornutum				
	Rhodomonas salina				
	Pleurochrysis pseudoroscoffensis				
AuNP (12.5)	*Scenedesmus subspicatus*	Fw		No adverse effect	García-Cambero,2013
AuNP (25)	*Pseudokirchneriella subcapitata*	Fw	Growth inhibition, 72h	83 (27;250) mg/l	Hartmann *et al.,*2013
Citrate-Ag-AuNP	*Phaeodactylum*	Sw	EC_{50}, 72h	0.195± 0.045 mg/l	Pérez *et al.*, 2014
	Tricornutum *Nitzschia Palea*	Fw		0.648 ± 0.24 mg/l	
AuNP (14)	*Dunaliella salina*	Sw	Au uptake, 72h	17.91 mg/l	Larguinho *et al.*, 2014
AuNP-PEG (14)				8.51 mg/l	
Citrate capped AuNP (17)	*Ankistrodesmus falcatus*	Fw	96h		Gilroy *et al.*, 2014
AuNP	*Pseudokirchneriella subcapitata*	Fw	Growth inhibition, 72h	1.91 mg/l	Botha *et al.*, 2015
Carbonate-coated AuNP (2-5)	*Chlamydomonas reinhardtii* (Wild type)	Fw	EC_{50},1h	6.04 mg/l	Behra *et al.*, 2015
			EC_{50},2h	1.89 mg/l	
citrate-AuNP (2-5)	*Chlamydomonas reinhardtii* (Mutant)		EC_{50},1h	2.78 mg/l	
			EC_{50},2h	1,73 mg/l	

The difference in results is due to several factors, including the AuNP type (*e.g.* coating, size, synthesis method), bioassay design (*e.g.* type of media or exposure time), initial cellular density and test organism sensitivity, which potentially affect toxicity. Therefore, to obtain appropriate standardization, the minimum standard conditions must be maintained (*e.g.* 10^4 initial cellular density, 72-96 h exposure and widely accepted media for marine and

freshwater species). After achieving this minimum standard, by making changes to them, certain aspects of the research can be done.

3.3. Gaps of knowledge and further research

The knowledge on the physicochemical properties of metallic nanoparticles (e.g. size, shape, degree of agglomeration and coating) and the characteristics of the environment (e.g. pH, temperature, salinity and ionic strength) is essential to understand both the fate and behaviour of nanoparticles in the aquatic environment, their uptake and distribution within organisms, and their interactions with other pollutants (Fabrega *et al.*, 2011; Schirmer *et al.*, 2013; Velzeboer, 2014; Peijnenburg *et al.*, 2015; Zhang *et al.*, 2016; Peng *et al.*, 2017). Data on biological effects show that nanoparticles can be toxic to bacteria, algae, invertebrates and fish species, as well as mammals (Klain *et al.,* 2008). Furthermore, the sensitivity to nanomaterials can be different in diverse biota; and in some cases algae are sensitive to these materials (Stuart *et al.,* 2013b; Oukarroum *et al.,* 2015b).

Understanding the mechanism of toxicity (e.g. disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species, and release of toxic constituents) may be challenging (Oukarroum *et al.,* 2012a) since the toxicity of nanometals varies due to their differences in the methods of synthesis, concentration, solubility and the presence of additives. In addition, same materials may show different results on toxicity because of variability in their surface defects and preparation methods. These emphasize that without proper characterizations of physicochemical properties of nanometals, a general conclusion on toxicity of these materials might be difficult.

Due to the ecological and economic importance of marine microalgae and the limited number of studies available, more research on the toxicity of nanoparticles to these organisms is needed, especially under exposure to nanoparticles and other environmental contaminants.

Chapter IV:

Single and combined effects of microplastics and copper on the population growth of the marine microalgae *Tetraselmis chuii*

This chapter is published as: Davarpanah, E. & Guilhermino, L. (2015). Single and combined effects of microplastics and copper on the population growth of the marine microalgae *Tetraselmis chuii*. *Estuarine and Costal Shelf Science,* 167A*,* 269-275 (included in the present Thesis with permission of the publisher included in the Annex I).

Abstract

As the accumulation of microplastics continues to rise in the marine environment, more knowledge on their potential toxic effects on marine organisms is needed to assess their risks to environmental and human health. Thus, the goal of the present study was to investigate the effects of fluorescent red polyethylene plastic micro-spheres 1-5 µm diameter (used as microplastic model and hereafter indicated as MP), alone and in mixture with copper, on the population growth of the marine microalgae *Tetraselmis chuii*. Two null hypotheses were tested: (H_{01}) Exposure to MP concentrations in the ppb range does not affect the average specific growth rate of *T. chuii*; (H_{02}) MP do not interact with the toxicity of copper to *T. chuii*. In laboratory bioassays, *T. chuii* cultures were exposed for 96 h to MP concentrations ranging from 0.046 to 1.472 mg/l), concentrations of copper alone ranging from 0.02 to 0.64 mg/l, and the same concentrations of copper in the presence of 0.184 mg/l of MP in test media. No significant effects of MP on *T. chuii* population growth were found ($p > 0.05$), leading to the acceptance of H_{01}. Copper alone significantly decreased the population growth of *T. chuii* with EC_{10}, EC_{20} and EC_{50} of 0.009, 0.023 and 0.139 mg/l, respectively. The corresponding values in the presence of MP were 0.012, 0.029 and 0.145 mg/l, respectively. Moreover, no significant differences between the toxicity curves of copper in the presence and absence of MP were found ($p > 0.05$), leading to the acceptance of H_{02}. Despite these findings, because microplastics are known to adsorb and accumulate copper, aged pellets more than virgin ones, and the toxicity of smaller particles may be higher, further studies on the combined effects of copper and microplastics on microalgae should be performed, especially under long-term exposures to nano-sized aged microplastics.

Key words: copper; microplastics; combined effects; marine primary producers; *Tetraselmis chuii*

4.1. Introduction

Plastics are suitable materials for a wide range of uses, their manufacture has a relatively low cost, and they have been replacing other materials such as glass and metals in several applications (U.S. Department of interior, 1993; Rooney *et al.*, 2000; ThoMPon *et al.*, 2009). Therefore, their global production has been increasing over decades (Plastics Europe,

2013) to respond to the needs of several types of industry and demands of an increasing industrialized human society growing exponentially. As the result of their high production and use, and difficult degradation (Mato *et al.*, 2001; Moore, 2008; Artham *et al.*, 2009; Fendall and Sewell, 2009; Gouin *et al.*, 2011), plastics have been accumulating in the environment (Barnes *et al.*, 2009; Doyle *et al.*, 2011; Sá *et al.*, 2015), and are now considered pollutants of high concern, especially small sized particles under 5 mm, known as microplastics (Cole *et al.*, 2013; NOAA, 2015). Due to the limited knowledge on the environmental fate and effects of microplastics in different types of ecosystems still existing despite the studies done in the last years mainly in the marine environment (Andrady 2011, Cole *et al.* 2011; Wright *et al.*, 2013; Ivar do Sul and Costa 2014), more research is urgently needed to assess their risks to environmental and human health (Fossi *et al.*, 2014; Galgani *et al.* 2014).

The marine biota is able to uptake microplastics by different ways. For example, several fish and bird species have been found to ingest microplastics apparently because they confound these particles with real prey (Ryan, 2008; Lusher *et al.*, 2013; Sá *et al.*, 2015) but uptake through gills probably also occurs (Fossi *et al.*, 2014). Plankton species (Costa and Barletta, 2014; Lima *et al.*, 2014; Setälä *et al.*, 2014), filter feeders, including zooplankton species (Cole *et al.*, 2013), bivalves (Von Moos *et al.*, 2012), large vertebrates such as harbour seals (Phoca vitulina) (Rebolledo *et al.*, 2013), the Mediterranean basking shark (Cetorhinus maximus) and the fin whale (Balaenoptera physalus) (Fossi *et al.*, 2014), among other types of organisms, have been found to uptake microplastics. They may also adsorb to organisms and cause adverse effects. For example, sorption of nano-sized plastic particles to freshwater and saltwater microalgae (Chlorella and Scenedesmus) resulting in adverse effects on photosynthesis and increase in the production of reactive oxygen species (ROS) that are toxic were found (Bhattacharya *et al.*, 2010). Despite the interest of studying the effects of microplastics in marine microalgae, the studies done so far are very scarce and thus more knowledge needs to be produced due to the crucial role of microalgae populations in phytoplankton communities which are in general the basis of marine ecosystems.

Microplastics present in the marine environment may contain several types of chemicals introduced during their synthesis and/or bound during their permanence in the environment (Andrady, 2011; Cole *et al.*, 2011; Browne *et al.*, 2013; Wright *et al.*, 2013; Ivar do Sul and Costa, 2014). Plastics and microplastics collected in beaches, sediments and water of different regions have been found to contain several types of persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, among others (Mato *et al.*, 2001; Endo *et al.*, 2005; Teuten *et al.*, 2007;

Fries and Zarfle, 2012; Hirai *et al.*, 2011; Frias *et al.*, 2010, Besseling *et al.*, 2013), and metals, including copper, chromium, cadmium, and several others (Ashton *et al.*, 2010, Holmes *et al.*, 2012). Also, virgin and aged microplastics deployed in the field were found to accumulate metals (Holmes *et al.*, 2014). Moreover, the concentrations of some of these chemicals such as hydrophobic persistent organic pollutants (POPs) are considerably higher in the microplastics than in the water from where they were collected due to their large surface area to volume ratio (Mato *et al.*, 2001; Hirai *et al.*, 2011; Holmes *et al.*, 2012; Bakir *et al.*, 2014). Furthermore, photo-oxidative weathering increases the polarity of the polymer (Mato *et al.*, 2001) and accumulations of biofilms and hydrogenous precipitates increase the charge, roughness, porosity and hydrophobicity of the surface (Artham *et al.*, 2009).

Therefore, the ingestion, uptake and sorption of microplastics by marine organisms may considerable increase their exposure to these chemicals and thus the probability of toxic effects being induced. In addition, ingested and/or up taken microplastics and the chemicals that they carry may be transferred along the food chain and some may be biomagnified during the process increasing the risks to top predators and humans consuming contaminated preys (Fossi and Depledge, 2014). Furthermore, microplastics may interact with the biotransformation and toxicity of other environmental contaminants (Oliveira *et al.*, 2013). Such interactions that may have important implications for risk assessment have been poorly investigated and to the best of our knowledge they were not investigated in marine microalgae so far.

The goal of the present study was to investigate the effects of polyethylene plastic spheres (1–5 µm diameter), hereafter indicated as microplastic, alone and in combination with copper, on the population growth of the marine microalgae *Tetraselmis chuii*. Two null hypotheses were tested: (H_{01}) exposure to microplastic concentrations in ppb range does not affect the average specific growth rate of *T. chuii*; (H_{02}) microplastic do not interact with the toxicity of copper to *T. chuii*.

T. chuii was selected as test organism mainly because is an abundant species in several marine ecosystems where its populations are often an important component of the phytoplankton community and has been widely used as representative of primary producers in ecotoxicological studies (Nunes *et al.*, 2005; Ferreira *et al.*, 2007; Debelius *et al.*, 2009; Vieira and Guilhermino, 2012).

Microplastic were selected as model of microplastics mainly because polyethylene is one of the most produced plastic polymers and one of the most found in the marine environment and in marine biota (Andrady *et al.*, 2011 Harshvardhan and Jha, 2013; Wright *et al.*, 2013),

and were used as test substance in previous studies of our laboratory carried out with fish (Oliveira *et al.*, 2013).

Copper was selected as the second test substance because is an essential metal (Hill *et al.*, 1996; Franklin *et al.*, 2002; Debelius *et al.*, 2009), a common contaminant of marine ecosystems (Bhargava *et al.*, 2008; Levy *et al.*, 2008; Debelius *et al.*, 2009; Jianrong and Qiran, 2009; Flouty and Estephane, 2012). Naturally, concentrations of copper from 0.03 to 0.23 µg/L in surface seawaters, from 0.20 to 30 µg/L in freshwater, and from 0.001 to 0.1 µg/L in the open ocean were reported (Grosell, 2011). Copper concentrations in locations receiving anthropogenic inputs can vary from levels that approach regional natural background ones to 100 µg/L or more, with reported concentrations in the 200,000 µg/L range in mining areas (USEPA, 2007). Copper was found in microplastics collected and deployed in the wild (Ashton *et al.*, 2010; Holmes *et al.*, 2012), and its sorption to microplastics was previously investigated (Holmes *et al.*, 2012, 2014). Briefly, copper adsorbs to microplastics in relation to its concentrations in the seawater through a process that can be modelled as shown by Holmes *et al.*, (2014). In addition, the toxicity of copper to *T. chuii* and other microalgae species was already investigated with EC50 values ranging from 0.004 mg/l in Isochrysis sp. to 11.7 mg/l in *Chlorococcum sp.* (Table 4-1). Evidences suggest that both ionic and non-ionic copper species are able to interact with the surface of MP particles and that copper adsorption has a relatively low dependence of water pH and salinity (Holmes *et al.*, 2014).

Table 4-1: Concentrations of copper inducing 50 % of inhibition on the average specific growth rate (EC_{50}) of different microalgae species reported in the literature. Exp. - exposure time. Temp. - Temperature at which the bioassays were conducted. Photop. - photoperiod indicated as the number of hours under light conditions. 95 % CI – 95 % confidence intervals determined for the EC_{50}.

Microalgae	Exp. (h)	Test medium	Temp. (ºC)	Photop. (h)	Growth rate[a]	EC_{50} (mg/l) (95 % CI)	Ref.
Chaetoceros calcitrans	96	walne	28 ± 1	12	-	0.07	Ismail *et al.*, 2002
Chaetoceros sp.	72	NO3(124Mm $PO^{4}{}_{3}{}^{-}$(4mM)	20 ± 1	24	-	0.088	Debelius *et al.*, 2009
Chlorella sp.	72	Jaworski	27	12	1.7 ± 0.1	0.0073 0.0067 - 0.008	Franklin *et al.*, 2002
Chlorococcum littorale	72	Daigo IMK	22	12	-	10.2	Satoh *et al.*, 2005
Chlorococcum sp.	72	Daigo IMK	22	12	-	11.7	Satoh *et al.*, 2005

Microalgae	Exp. (h)	Test medium	Temp. (ºC)	Photop. (h)	Growth rate[a]	EC_{50} (mg/l) (95 % CI)	Ref.
Dunaliella minuta	96	LDM	15 ± 1	16	-	0.48	Visviki and Rachlin, 1991
Dunaliella tertiolecta	72	F/2	21	12	1.39 ± 0.02	0.530 0.450 - 0.600	Levy *et al.*, 2008
Isochrysis galbana	96	walne	28 ± 1	12	-	0.04 0.03 - 0.041	Ismail *et al.*, 2002
Isochrysis galbana	72	Daigo IMK	22	12	-	4.2	Satoh *et al.*, 2005
Isochrysis galbana	72	NO3(124mM) $PO^{4}{}_{3}^{-}$(4mM)	20 ± 1	24	-	0.058	Debelius *et al.*, 2009
Isochrysis galbana	96	Conway	25 - 28	24	1.6	0.91 0.53 - 2.92	Yap *et al.*, 2004
Isochrysis sp.	72	F/2	27 ± 2	12	1.85	0.004 0.0038 - 0.0042	Levy *et al.*, 2007
Phaeodactylum tricornutum	72	F/2	21	12	1.78 ± 0.08	0.008 0.0047 - 0.0083	Levy *et al.*, 2008
Prasinococcus sp.	72	Daigo IMK	22	12	-	5.4	Satoh *et al.*, 2005
Scenedesmus subspicatus	72	OECD	25 ± 2	12	-	0.34	Ma *et al.*, 2003
Selenastrum capricornutum	72	U.S. EPA	24	24	1.3 ± 0.2	0.0075 0.0068 - 0.0082	Franklin *et al.*, 2002
Synechococcus sp.	72	Daigo IMK	22	12	-	5.3	Satoh *et al.*, 2005
Tetraselmism chuii	72	NO3(124mM) $PO^{4}{}_{3}^{-}$(4mM)	20 ± 1	24	-	0.33	Debelius *et al.*, 2009
Tetraselmis sp.	96	walne	28 ± 1	12	-	0.37 0.34 - 0.41	Ismail *et al.*, 2002
Tetraselmis sp.	72	F/2	21	12	1.37 ± 0.26	0.047 0.046 - 0.049	Levy *et al.*, 2008
Tetraselmis tetrathele	96	walne	28 ± 1	12	-	0.13	Ismail *et al.*, 2002
Tetraselmis tetrathele	72	Daigo IMK	22	12	-	7.4	Satoh *et al.*, 2005

4.2. Material and Methods

4.2.1. Tested substances and other chemicals

Analytical grade (≥ 99.6 % purity) copper sulphate, purchased from Merck (Germany), was used as copper source. Fluorescent red polyethylene microspheres (1-5 µm diameter), were purchased from Cospheric (USA). All the other chemicals used for microalgae culture and bioassays were of analytical grade and purchased from Sigma-Aldrich (Germany) and Merck (Germany).

The parental culture and test medium were F/2 Guillard's medium (Guillard, 1975), hereafter indicated as F/2. It was prepared by dilution $NaNO_3$, NaH_2PO_4, trace metals and vitamins stock solutions in ultra-pure (u.p.) water. After that to prepare test medium, 1 ml of each $NaNO_3$, NaH_2PO_4, trace metals stock solution and 0.5 ml of vitamins stock solution were dissolved in 1 liter of seawater. The seawater was artificially prepared in the laboratory then filtered through natural filter (0.45 µm membrane filter cartiladge) and standardizing salinity to 30. Immediately after preparation, all the parental cultures and test media were sterilised by autoclave (121ºC for 35 min).

4.2.2. *T. chuii* cultures and general conditions of the bioassays

T. chuii has been cultured in our laboratory for several years. The parental cultures for this study were maintained in a chamber with control of temperature (20 ± 1°C) and photoperiod (photon flux density of 90 µE /m2/s); 24h light). All the material used for culture maintenance and testing was previously sterilised through autoclave (121°C for 35 min). Parental and tested cultures were prepared and maintained under aseptic conditions to minimize the risk of contamination, with air supply filtered (Eheim filters). All the cultures were shaken twice a day to avoid cell precipitation, culture medium was partially renewed every 3 day during the exponential growth phase, and each culture was maintained for a maximum of 21 days.

All the bioassays were carried out in temperature (20 ± 1ºC) and photoperiod (24h light; photon flux density of 90 µE /m2/s) controlled chambers (Bronson climate chambers, The Netherlands), using 500 ml glass backers filled with 400 ml of F/2 medium previously sterilized by autoclave (121°C for 35 min). An appropriate volume of microalgae parental culture in exponential growth phase was inoculated to each test medium to obtain the concentration of $1{\times}10^4$ cells/ml in the 400 ml of F/2 medium which was previously sterilised as previously indicated (section 4.2.1.). Continuous filtered air supply (Eheim filters) was provided to all the cultures. In all the bioassays, three replicate cultures were used per treatment, and they were shaked twice a day to decrease algae cell precipitation. At the

beginning of each bioassay (0 h) and after 24 h, 48 h, 72 h and 96 h, samples were collected to measure temperature and pH (pH meter 3310 Jenway, UK), and to count algae cells. An Improved Neubauer haemacytometer (PRECICOLOR HBG, Germany) was used to perform all the cell counts. The criterion indicative of toxic effects was the inhibition of *T. chuii* average specific growth rate determined as indicated in section 4.2.6. Three bioassays were performed under the general conditions previously described: a first one testing the effects of MP alone; a second one testing the effects of copper alone; a final one testing the effects of copper in the presence of MP. The specific procedures of these bioassays are described in the following sections.

4.2.3. Exposure conditions of the bioassay testing microplastics alone

A stock solution of MP in u.p. water with a concentration of 117.760 mg/l was prepared. This solution was serial diluted in u.p. water to obtain stock solutions with concentrations of 3.68; 7.36; 14.72; 29.44 and 58.88 mg of MP/ml. The appropriate volume of each solution was added to 400 ml of F/2 test medium previously sterilised as indicated in section 4.2.1. The MP final concentrations in test medium of different treatments were: 0.046, 0.092, 0.184, 0.368, 0.736 and 1.472 mg/l. The control cultures were exposed to F/2 only. After addition of MP to test medium, the inoculation of all treatments with microalgae was performed as described in section 4.2.2. Three replicate cultures were used per treatment and all the other conditions and procedures were similar to those described in sections 4.2.1 and 4.2.2.

4.2.4. Exposure conditions of the bioassay testing copper alone

A second bioassay was performed to test the effects of copper (as individual test substance) on *T. chuii* culture growth. For this, a stock solution of copper sulphate with a Cu concentration of 256 mg/l was prepared in u.p. water. This solution was serial diluted in u.p. water to obtain stock solutions with concentrations: 8; 16; 32; 64 and 128 mg of Cu/ml. The appropriate volume of each solution was added to 400 ml of F/2 test medium previously sterilised as indicated in section 4.2.1. The final concentrations of Cu in test media were: 0.02; 0.04; 0.08; 0.16; 0.32 and 0.64 mg/l. The control cultures were exposed to F/2 medium only. Then, the inoculation of all treatments with microalgae was performed as described in section 4.2.2. Three replicate cultures were used per treatment and all the other conditions and procedures were similar to those described in sections 4.2.1 and 4.2.2.

4.2.5. Exposure conditions of the bioassay testing copper in the presence of microplastics

Finally, a bioassay was carried out to investigate the effects induced by copper on *T. chuii* culture growth in the presence of MP. For this, stock solutions of copper were prepared similar to those described in section 4.2.4 to obtain final concentrations of 0.02; 0.04; 0.08; 0.16; 0.32 and 0.64 mg Cu/l. A stock suspension of MP in u.p. water with a concentration of 14.72 mg/l was prepared. The MP final concentration in test medium of different treatments was 0.184 mg/l. The following treatments were included in the bioassay: control (F/2 medium alone); copper alone (0.64 mg/l); MP alone (0.184 mg/l); and the following combined (Cu + MP) treatments (mg/l): 0.02 Cu + 0.184 MP; 0.04 Cu + 0.184 MP; 0.08 Cu + 0.184 MP; 0.16 Cu + 0.184 MP; 0.32 Cu + 0.184 MP and 0.64 Cu + 0.184 MP. All the other conditions and procedures were similar to those described in previous sections.

4.2.6. Statistical analyses of data

The average specific growth rate and the percentage of growth inhibition were determined from cell counts according the OECD Guideline 201 (OECD, 2006). Briefly, the average specific growth rate (X) for a specific period was determined as the logarithmic increase of biomass (using cell counts as surrogate parameter) in each test beaker for 5 days, as:

$$X_{(i-j)} = (\ln A_j - \ln A_i) / (t_j - t_i)$$

where: $X_{(i-j)}$ is the average specific growth rate from time i to j; A_j is the biomass at the time j; A_i is the biomass at the time i.

The percentage of growth rate inhibition (Ir) in each replicate was calculated relatively to the mean of the control replicates as:

$$Ir\ (\%) = ((X_c - X_t) / X_c) \times 100;$$

where: X_c is the mean of average growth rate in the control treatments; and X_t is the average growth rate in each replicate containing the tested substance(s).

For each bioassay, the mean of average specific growth rate obtained in different treatments were compared by a one-way Analysis of Variance (ANOVA) after checking data normality (Kolmogorov–Smirnov normality test) and homogeneity of variances (Bartlett's test) (Zar, 1999). There was no need of making any data transformation because the ANOVA assumptions were fullfield. When significant differences among treatments were found, the Tukey's multicomparison test was used to discriminate significant different treatments. A two-way ANOVA (2-ANOVA) with interaction was used to compare the effects

of copper in the absence and presence of MP (main factors: copper concentrations and MP presence). The 10 %, 20 % and 50 % effect concentrations (EC_{10}, EC_{20} and EC_{50}, respectively) were calculated from the toxicity curves obtained by plotting the log transformed copper concentrations against the corresponding probit transformed percentages (%) of average specific growth rate inhibition. The toxicity curves of the bioassays testing copper alone and in combination with MP were compared by the Analysis of Co-variance (ANCOVA), MP presence as fixed variable, and copper concentrations as co-variate. All the statistical analysis was performed using the SPSS© software package 22. The significance level was 0.05.

4.3. Results and Discussion

In all the bioassays, the variation of temperature and pH in each beaker were lower than 1 ºC and 1 pH unit, respectively. The coefficient of variation of average specific growth rates recorded in control treatments during the test period was lower than 10 %. The average specific growth rate calculated from the control treatments of the three bioassays and standard deviation was 0.711 ± 0.018 logarithm cells increase over 4 days. This average specific growth rate compares with the corresponding values reported for *T. chuii* in the literature. For example, average specific growth rates of 0.76 were previously reported for this microalga in F/2 medium at 20ºC (Vieira and Guilhermino, 2012).

4.3.1. Effects of microplastics alone

The effects of MP, when tested as single substance, on the average specific growth rate of *T. chuii* are shown in Figure 4-1. Despite a small concentration-related decrease of the average specific growth rate at increasing MP concentrations, reaching ≈ 24 % of growth inhibition, no significant differences among treatments were found (ANOVA: $F_{(6, 14)} = 1.707$, $p = 0.192$). Therefore, exposure of *T. chuii* for 96 h to MP concentrations up to 1.472 mg/l had no significant effects on its average specific growth rate, leading to the acceptance of the first null hypothesis (H_{01}). At least three hypotheses that are not mutually exclusive may be raised to explain the lack of significant effects of the MP tested on *T. chuii* population growth: the potential precipitation of MP particles, the size of the plastic particles tested, and the concentrations tested. Regarding the first hypothesis, if precipitation of MP occurred, then their concentration in test media would have been reduced and thus their adverse effects are expected to be reduced. In a previous study, where the effects of the same particles on marine fish were investigated, a decay from 27 % to 37 % of the MP concentrations in test media (artificial salt water) over 96 h was reported suggesting that at least some precipitation of the MP occurred (Luís *et al.*,2015). Thus, despite the differences

of test media used in the two studies, it is possible that some MP precipitation occurred during the *T. chuii* bioassay, decreasing the tested concentrations of MP and thus their toxic effects. MP size is important in relation to the effects caused by these pollutants on the marine biota, both in relation to their potential ingestion by confusion with prey and by their biological uptake by other processes (Wright *et al.*, 2013). The original diameter of the MP tested was 1 - 5 µm and larger particles may have been formed due to aggregation. *T. chuii* cells have about 12 – 16 µm long and 7 – 10 µm broad (Hori *et al.*, 1986). Thus, the uptake of MP by *T. chuii* is unlikely. However, MP can adsorb to organisms (Wright *et al.*, 2013) and cause toxic effects by this way, for example through mobility reduction. Nanoplastic sorption to microalgae (*Chlorella* and *Scenedesmus*) negatively interfering with photosynthesis and increasing the production of reactive oxygen species was reported (Bhattacharya *et al.*, 2010). Despite the no significant effects found in *T. chuii*, a slight decrease of the average specific growth rate with the MP concentration increase, reaching ≈ 24 % at 1. 472 mg/l was found (Figure 4-1). These results suggest that significant effects may be induced at higher MP concentrations. Therefore, it is important to further investigate the effects of MP and nanoplastics on microalgae, especially on functional parameters (*e.g.* chlorophyll production) and on the average specific growth rate under longer exposures.

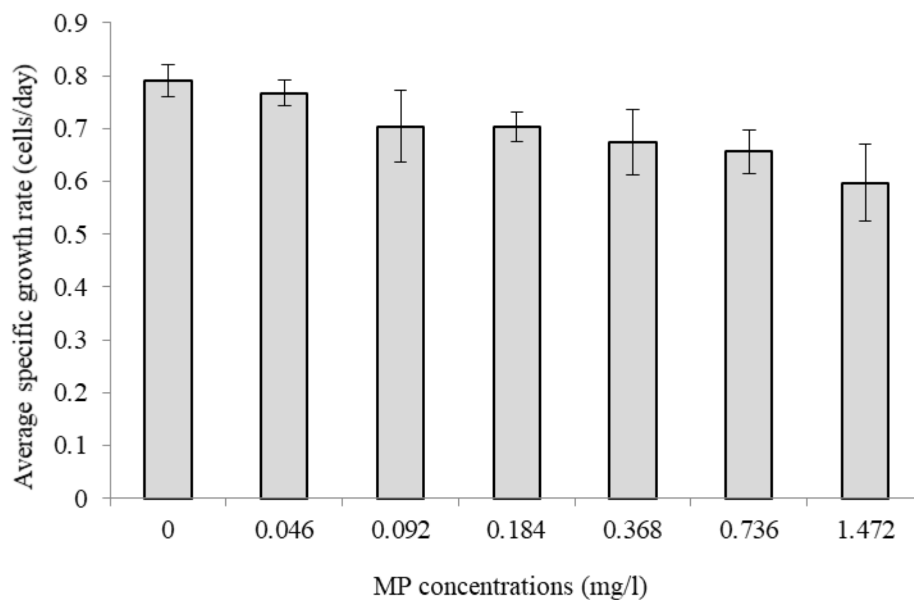


Figure 4-1: *Tetraselmis chuii* average specific growth rate (expressed as the logarithm of the increase of the cell number per day) after 96 h of exposure to different concentrations of microplastics alone.Values are the mean of three replicates per treatment with correspondent S.E.M. bars.

4.3.2. Effects of copper alone

In the bioassay testing the effects of copper alone (Figure 4-2), significant differences among treatments were found (ANOVA: $F_{6, 14} = 19.665$, $p = 0.000$). Copper concentrations

equal or higher than 0.08 mg/l significantly decreased the average specific growth rate of *T. chuii* (Figure 4-2) indicating that in the range of concentrations tested copper was able to negatively affect this species. The lowest concentrations tested (0.020 and 0.040 mg/l) are ecologically relevant because copper concentrations of 0.03 to 0.23 µg/l were found in the water of contaminated aquatic systems, such as surface seawaters (U.S. Environmental Protection Agency, 2007). The estimated EC_{10}, EC_{20} and EC_{50} of copper on *T. chuii* average growth rate are shown in Table 4-2.

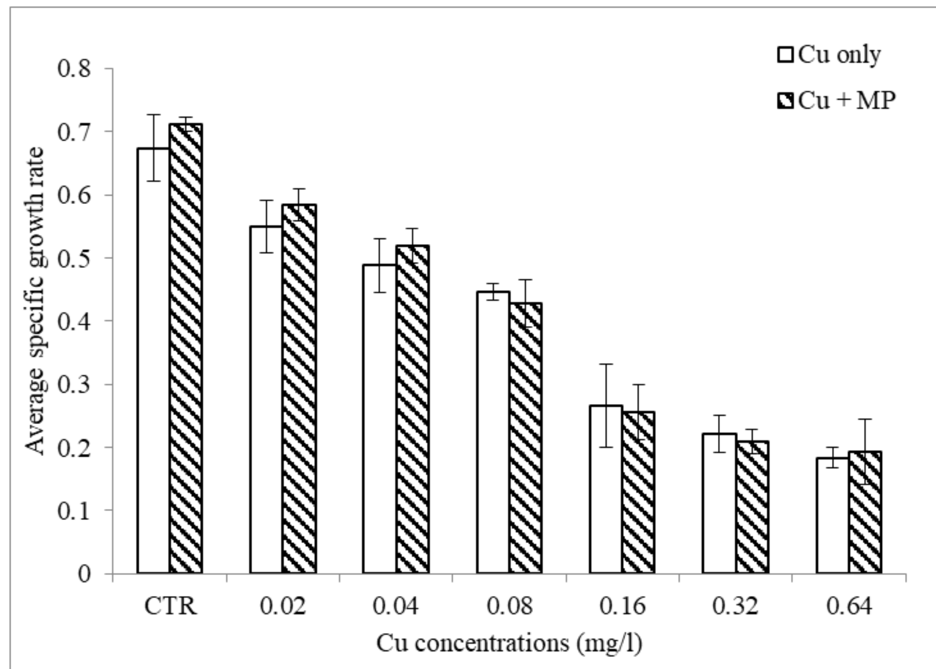


Figure 4-2: *Tetraselmis chuii* average specific growth rate (expressed as the logarithm of the increase of the cell number per day) after 96 h of exposure to different concentrations of copper alone (white columns) and in the presence of 0.184 mg/l of microplastics (white/black pattern columns). Values are the mean of three culture replicates per treatment with correspondent S.E.M. bars. Different letters indicate statistically significant differences (one-way ANOVA and the Tukey's multicomparison test at $p \leq 0.05$) among treatments of each bioassay, with small letters for the bioassay where copper alone was tested, and caps letters for the mixture (different copper concentrations in the presence of 0.184 mg/l of the microplastics tested). CTR – control treatment where the cultures were exposed to F/2 medium only).

Table 4-2: Estimated concentrations of copper (alone and in the presence of MP) inducing an inhibition of 10 % (EC_{10}), 20 % (EC_{20}) and 50 % (EC_{50}) on *Tetraselmis chuii* average specific growth rate after 96 h of exposure. The 95 % confidence intervals are indicated within brackets.

Tested substance(s)	EC_{10} (mg/l)	EC_{20} (mg/l)	EC_{50} (mg/l)
Copper alone	0.009 (0.004 – 0.016)	0.023 (0.013 – 0.035)	0.139 (0.106 – 0.187)
Copper + MP	0.012 (0.006 – 0.020)	0.029 (0.017 – 0.041)	0.145 (0.113 – 0.189)

The 96 h EC_{50} of copper determined in the present study (0.139 mg/l) is comparable to the 72 h EC_{50} previously determined for *T. chuii* (0.33 mg/l) at 20ºC by other authors (Debelius *et al.*, 2009), and is in the range of the corresponding values calculated for other microalgae species after exposure for 72 h or 96h at distinct temperatures (Table 4-1). The 72 h and 96 h EC_{50}s determined at 20 ± 1ºC for *T. chuii* equal to 0.33 mg/l (Debelius *et al.*, 2009, Table 4-1) and 0.139 mg/l (Table 4-2), respectively, are higher than the 72 h EC_{50}s determined for *Chaetoceros sp.* (0.088 mg/l, Debelius *et al.,* 2009), *Isochrysis galbana* (0.058 mg/l, Debelius *et al.* 2009), *Phaeodactylum tricornutum* (0.008 mg/l, Levy *et al.*, 2008) and *Tetraselmis sp.* (0.047 mg/l, Levy *et al.*, 2008) at 20ºC or 21ºC, and lower than the corresponding values determined for *Dunaliella tertiolecta* (0.530 mg/l). Although the different experimental conditions used in distinct studies make difficult a direct comparison of sensitivity to copper among species, these EC_{50}s suggest that *T. chuii* is more sensitive to copper than *D. tertiolecta* and less sensitive to this metal than *Chaetoceros sp., I. galbana, P. tricornutum and Tetraselmis sp.* Overall, the results of the present study (Table 4-2) and those summarized in Table 4-1 indicate that copper is able to significantly decrease the population growth of microalgae at concentrations in the ppb or low ppm range. Copper is an essential metal for several organisms. Thus, in real marine scenarios, the existing communities of bacteria and other species relying on copper (Ma *et al.*, 2003) may influence its water concentrations and availability and thus the exposure and resulting effects on *T. chuii*. Microalgae populations are in general important components of the community of primary producers of aquatic ecosystems. Thus, the inhibition of their growth in the wild by copper may reduce the diversity of the phytoplankton community, the primary production and the availability of food to phytoplanktivorous species, potentially leading to adverse effects in the whole ecosystem and the services it provides.

4.3.3. Influence of microplastics in the copper toxicity

The combined effects resulting from the simultaneous exposure of *T. chuii* to copper and MP are shown in Figure 4-3. Significant differences among treatments were found ($F_{6, 14}$ = 37.095, p = 0.000). In the presence of MP, copper significantly reduced the microalgae population growth at concentrations equal or higher than 0.04 mg/l. The comparison of the two bioassays-results through a two-way ANOVA with interaction indicated significant differences among treatments with different copper concentrations ($F_{6, 29}$ = 51.115, p = 0.000), no significant differences between treatments with and without MP ($F_{1, 29}$ = 0.072, p = 0.790), and no significant interaction between the two factors ($F_{5, 29}$ = 0.187, p = 0.965). Thus, in the concentration tested (0.184 mg/l) MP did not influence significantly the effects of copper on the average specific growth rate of *T. chuii*.

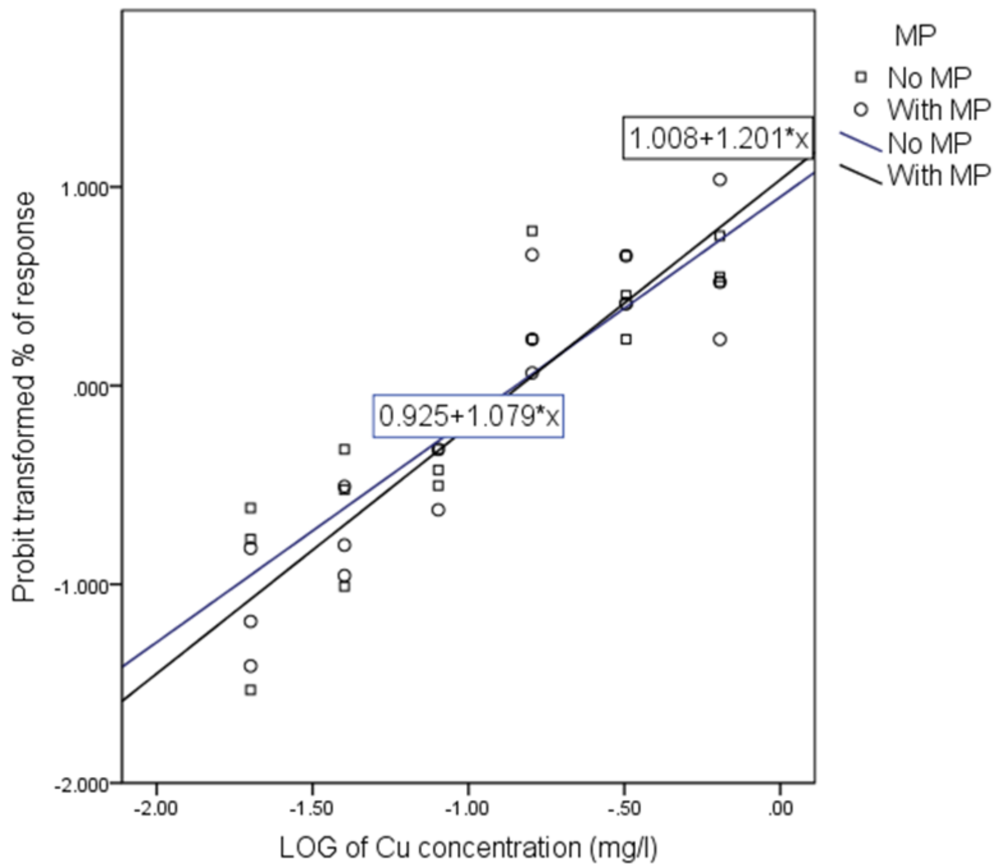


Figure 4-3: Curves of toxicity (log of the concentration versus probit transformed percentages of specific average growth rate inhibition) after 96h of exposure of *Tetraselmis chuii* exposure to copper alone and in the presence of microplastics (0.184 mg/l) in test media.

The toxicity curves of copper alone and combined with MP are shown in Figure 4-3. No significant differences between them were found (ANCOVA, log copper concentrations: $F_{1, 33} = 148.130$, $p = 0.000$, contributing with 81.8 % of the variance; presence of MP: $F_{1, 33} = 0.083$, $p = 0.775$, contributing with 0.3 % of the variance). Thus, there were no significant differences in the EC_{10}, EC_{20} and EC_{50} of copper in the presence and absence of MP. Therefore, in the concentration tested, MP did not influence the toxicity of copper to *T. chuii*, leading to the acceptance of H_{02}.

Previous studies conducted in field and laboratory conditions showed that several types of microplastics, including polyethylene pellets, adsorb and accumulate copper from seawater (Ashton *et al.*, 2010; Holmes *et al.*, 2012, 2014). Evidences suggest that copper ionic and non-ionic forms interact with the microplastics surface (Holmes *et al.*, 2012, 2014). The binding of copper to microplastics in test medium may decrease the bioavailability of the metal to organisms, especially if the resulting copper- microplastics complex is less or not up taken, resulting in a decreased toxicity of the metal in the presence of MP. However, the incorporation and accumulation of copper by microplastics may also lead to an

increased exposure and toxicity if the resulting particles are up taken by the organisms. In the present study, no significant differences on the effects of copper on *T. chuii* in the absence and presence of microplastic were found, suggesting that if binding of copper to the microplastic tested occurred in test medium, the magnitude of the process was not high enough to influence the toxicity. Although, it will be of interest to further investigate the potential effects of microplastics and copper interactions in microalgae in long-term studies, especially with environmental aged plastic particles because they tend to interact more with metals than virgin ones (Holmes *et al.*, 2014).

4.4. Conclusions

Microplastic (polyethylene spheres 1–5 µm diameter) had no significant effects on the average specific growth rate of *T. chuii* after 96 h of exposure to concentrations up to 1.472 mg/l, leading to the acceptance of the first null hypothesis of the present study. When tested alone, copper significantly ($p < 0.05$) decreased the average specific growth rate of *T. chuii*, with 96 h EC_{10}, EC_{20} and EC_{50} of 0.09, 0.023 and 0.139 mg/l, respectively. The corresponding values determined in the presence of MP (0.184 mg/l) were 0.012, 0.029 and 0.145 mg/l, and no significant differences among the toxicity curves were found ($p > 0.005$). These results indicate that microplastics did not influence the copper induced toxicity on *T. chuii* in the range of concentrations tested, thus leading to the acceptance of the second null hypothesis. Despite these findings and because microplastics are able to adsorb and accumulate copper (Holmes *et al.*, 2012, 2014), and the biological effects of very small particles may be higher than those of micro-sized ones, the combined effects of copper and microplastics on microalgae should be further investigated, especially under long-term exposure to nano-sized and aged particles, using functional parameters (*e.g.* chlorophyll production) additionally to population growth.

Chapter V:

Single and combined effects of microplastics and gold nanoparticles on the population growth of the marine microalgae *Tetraselmis chuii*

Abstract

The widespread use of microplastics and nanomaterials resulting in environmental contamination is of high concern. Microplastics have been found to modulate the toxicity of other environmental contaminants. Thus, the potential influence of microplastics on the toxicity of gold nanoparticles to the marine microalgae *Tetraselmis chuii* was investigated. In a laboratory bioassay, *T. chuii* cultures were exposed for 96 h to ~ 5 nm diameter gold nanoparticles (AuNP) and to virgin 1-5 µm diameter microplastics (MP). The treatments were: control; citrate-control; AuNP alone (0.1, 0.3 and 3 mg/l); MP alone (0.3, 0.9 and 4 mg/l) and 3 mixtures of the two substances (0.1 mg/l AuNP + 0.3 mg/l MP; 0.3 mg/l AuNP + 0.9 mg/l MP; 3 mg/l AuNP + 4 mg/l MP). The effect criterion was the inhibition of the average specific growth rate. AuNP alone and MP alone did not cause significant ($p > 0.05$) decrease of *T. chuii* average specific growth rate up to 3 mg/l and 4 mg/l, respectively. The mixture containing the highest concentrations of both substances significantly ($p \leq 0.05$) reduced the average specific growth rate of the microalgae by 27 %. Therefore, the mixture was more toxic to *T. chuii* than its components individually. Overall, the results of the present study indicated that the MP and AuNP tested have a relatively low toxicity to *T. chuii* and the presence of MP in the water increased the toxicity of AuNP to *T. chuii*. These findings stress the need of more research on the toxicity of mixtures containing microplastics and nanomaterials.

Keywords: microplastics, gold nanoparticles, microalgae, *Tetraselmis chuii*, nanotoxicology

5.1. Introduction

The contamination of the marine environment by microplastics is of high concern because they have found globally (Lusher, 2015; Beer *et al.*, 2018). Although the amounts of microplastics found in marine waters varies considerably, most of the samples collected were found to contain them. For example, the following percentages of water samples containing microplastics were reported: 60 % in the Northwest Atlantic Ocean (Law *et al.*, 2010), 61 % in the Portuguese coastal waters (Frias *et al.*, 2014), 74 % around Corsica in the Western Mediterranean (Collignon *et al.*, 2014), 89 % in the Celtic Sea (Lusher *et al.*, 2014) and 97 % in an estuary of the North Sea (Dubaish *et al.*, 2013). The concentrations of microplastics in marine waters reported in the literature are also variable but concentrations about 100,000 particles/m^3 were already reported as indicated in Andrady (2017). The removal of microplastics from the environment is complex due to the lake of cost-effective technology (Browne *et al.* 2011; Claessens *et al.* 2011; Paul-Pont *et al.* 2016).

The marine biota is able to uptake microplastics by different ways (Ryan, 2008; Lusher *et al.*, 2013; Fossi *et al.*, 2014; Sá *et al.*, 2015; Lin, 2016). The microplastics present in the marine environment generally contain other chemicals and toxicological interactions among these chemicals may occur inside organisms that ingested microplastics (Engler, 2012; Koelmans *et al.*, 2017; Peng *et al.*, 2017a). After ingestion or up take of microplastics by organisms, both the particles and the chemicals that they contain may be transferred along the food chain and some may be biomagnified during the process increasing the risks to top predators and humans consuming contaminated preys (Bakir *et al.*, 2012; Farrell and Nelson, 2013; Setälä *et al.*, 2014; Fossi and Depledge, 2014; Prata *et al.,* 2018).

The effects of some types of microplastics, including particles in the micro and nano size ranges, have been studied in microalgae. Some of the adverse effects that were reported in microalgae exposed to microplastics include reduced population growth rate and photosynthetic activity, increased production of reactive oxygen species, excessive expression of genes involved in the biosynthesis of glucose, among several other adverse effects (Bhattacharya *et al.*, 2010; Besseling *et al.*, 2014; Lagarde *et al.*, 2016; Sjollema *et al.,* 2016; Nolte *et al.,* 2017; Zhang *et al.*, 2017). However, no significant effects on population growth rate were also reported (Davarpanah and Guilhermino, 2015; Sjollema *et al.,* 2016; Prata *et al.,* 2018). The type of the polymer, the size of the particles and other properties of microplastics are believed to contribute to such differences (Claessens *et al.*, 2011; Wright *et al.*, 2013; Setälä *et al.*, 2014; Van Cauwenberghe *et al.*, 2015). Thus, more research is needed, especially because of the determinant role of microalgae as primary producers in marine ecosystems (Doi *et al.*, 2008; Vaz *et al.*, 2016).

Another group of contaminants of emerging concern are nanomaterials, such as nanometals due to their high and wide use in diverse technological applications (Salata, 2004; Zhuang and Gentry, 2011; Khlebtsov and Dykman, 2012; Ibrahim *et al.*, 2016). Gold, silver and platinum nanomaterials are some examples of nanometals which have been increasingly used in biomedical and several other applications (Li *et al.*, 2010). Nevertheless, despite a continues progress in the development and enhancement of properties of nanometals, the knowledge on their environmental fate, behaviour and effects is still very limited and even more in the marine environment (Klain *et al.*, 2008; Baker *et al.*, 2014; Dale *et al.*, 2015).

Gold nanoparticles (AuNP) have unique characteristics that make them very suitable to be used in several applications, including in biomedicine (Chen *et al.*, 2010; Das *et al.*, 2011; Chen *et al.*, 2017) but the knowledge of their toxicity and other effects in the marine biota is still scarce, including in marine microalgae. The studies published on the toxic effects of AuNP on microalgae are limited and more knowledge is needed (Renault *et al.*, 2008;

Hartmann *et al.*, 2013; Van Hoecke *et al.*, 2013, Botha *et al.*, 2015; Behra *et al.*, 2015) specially because the high diversity of this group of organisms and the potential different sensitivities of distinct species.

The main goal of the present study was to investigate the effects of AuNP alone and in mixture with microplastics, on the marine microalgae *Tetraselmis chuii.* This species was selected as test organism mainly because is an abundant species in several marine ecosystems and has been widely used as representative of primary producers in ecotoxicological studies (Nunes *et al.*, 2005; Ferreira *et al.*, 2007; Debelius *et al.*, 2009; Vieira and Guilhermino, 2012).

5.2. Material and Methods

5.2.1. Tested substances and other chemicals

A suspension of spherical AuNP (5 ± 2 nm diameter) in citrate buffer, purchased from Sigma-Aldrich (Germany), lot number MKBP4643V, was used as source of these particles. According the manufacturer, the particles had the maximum absorbance in the range of 510-525 nm. This type of AuNP was selected because it was previously tested in *P. microps* (Ferreira *et al.*, 2016) and *D. magna* (Pacheco *et al.*, 2018) providing some basic knowledge for the present study.

Red fluorescent polymer microspheres, hereafter indicated as MP, of unknown composition, lot number 4–1006-1053, were purchased from Cospheric (USA). According to the manufacturer, 1 mg of the product contains about 1.836E+8 spheres, the particles had 1.3 g density, excitation and emission wavelengths of 575 and 607 nm, respectively, and 1 - 5 µm of diameter. This type of microplastics was previously tested in bioassays with *T. chuii* (Prata *et al.*, 2018), providing some basic knowledge.

All the other chemicals used for microalgae culture and bioassays were of analytical grade and purchased from Sigma-Aldrich (Germany) and Merck (Germany).

5.2.2. *T. chuii* parental cultures

T. chuii has been cultured in our laboratory for several years. The parental cultures for this study were maintained in a chamber (Bronson PGC 1400, The Netherlands) with control of temperature (20 ± 1°C) and photoperiod (photon flux density of 90 µ E /m^2/s); 16h light, 8h dark). The culture medium was F/2 Guillard's medium (Guillard, 1975), prepared as indicated in Davarpanah and Guilhermino (2015), filtered through glass filters (Munktell, 47 mm diameter, 0.45 µm), and with salinity standardized to 30. It was also used as test medium during the bioassay and will be hereafter indicated as test medium. All the test

medium and material used for culture maintenance were sterilized by autoclave (120˚C for 35 min) immediately after preparation, and all the procedures were carried out under anoxic conditions to minimize the risk of culture contamination. All the cultures were shaken twice a day to reduce cell sedimentation and aggregation, test medium was partially renewed at every three days during the exponential growth phase, and each culture was maintained for a maximum of 21 days.

5.2.3. Experimental design and exposure conditions of the bioassay

The bioassay was carried out in a chamber (Bronson PGC 1400, The Netherlands) with control of temperature and photoperiod under the abiotic conditions and using the test medium indicated in the previous section. Generally, it followed the OECD guideline 201 (OECD, 2011), with adaptations as further indicated. The exposure period was 96 h (4 days), glass test beakers filled with 400 ml of medium (containing the tested substances or not), 3 replicate cultures per treatment, and static conditions were used. Each test culture was started by inoculating the appropriate volume of a microalgae parental culture in exponential growth phase into test medium to obtain the concentration of 1×10^4 cells/ml in test medium. The treatments were: control (test medium only); citrate (test medium containing 28.86 ml / 400ml of citrate corresponding to the highest concentration of citrate in treatments containing AuNP); 3 treatments containing the nominal concentrations of 0.2 mg/l, 1 mg/l and 5 mg/l of AuNP alone; 3 treatments containing the nominal concentrations of 0.2 mg/l, 1 mg/l and 5 mg/l of MP alone; 3 binary mixtures containing the nominal concentrations of 0.2 mg/l, 1 mg/l and 5 mg/l of each of the test substances (AuNP and MP). Such concentrations were selected based on Davarpanah and Guilhermino (2015) and additional preliminary bioassays. The treatment containing citrate alone was prepared by diluting a stock solution (28.7 mg/l of citrate in u.p. water) into test medium. Treatments containing AuNP alone were prepared by diluting the commercial AuNP solution (69.3 mg/l in u.p. water) into test medium. Treatments containing MP alone were prepared by diluting a stock colloidal solution (MP concentration of 200 mg/l in u.p. water) into test medium. Mixtures were prepared by diluting the same AuNP and MP commercial or stock solutions, respectively, into test medium. The effect criterion was the inhibition of the average specific growth rate calculated as indicated in OECD (2011), using the number of cells as surrogate parameter. At the beginning of each bioassay (0 h) and after 24 h, 48 h, 72 h and 96 h, samples were collected to measure pH (pH meter 3310 Jenway, UK), to count algae cells using an Improved Neubauer haemocytometer (PRECICOLOR HBG, Germany), and to determine the actual concentrations of MP and AuNP in medium.

5.2.4. Behaviour and concentrations of gold nanoparticles in test medium

A preliminary study to characterize the AuNP and investigate their behaviour in the test medium used over 96 h was carried out before the bioassay. The diameter, the percentage of spheres, and the variation of actual concentrations over 96 h were determined with basis on their UV-Vis spectrophotometric properties using methodologies considered adequate for this purpose (Haiss, *et al.*, 2007; Amendola and Meneghetti, 2009; Ferreira *et al.*, 2016). Briefly, 6 series of solutions with ~ 5 nm AuNP nominal concentrations ranging from 10 to 0.16 mg/l were prepared by serial dilution (1:2 v/v): 3 in u.p. water and 3 in test medium. To investigate if *T. chuii* cells interfere with absorbance of AuNP used to determine the actual concentrations of the particles, an additional series of solutions with the same nominal concentrations was prepared in test medium containing a microalgae concentration of 1×10^4 cells/ml. The UV-Vis absorbance spectra (200 – 900 nm) of the solutions were determined (SpectraMax (M2e) Spectrophotometer, USA) immediately after their preparation (0 h) and after 24 h, 48 h, 72 h and 96 h of maintenance in the abiotic conditions of the bioassay. The plasmon resonance peak (PRP) of AuNP solutions with a concentration of 10 mg/l was detected at 522 nm in u.p. water solutions, at 556 nm in both test medium and test medium with microalgae solutions (Figure 5-1). The presence of *T. chuii* cells induced almost no changes in the absorbance spectrum of solutions in test medium (Figure 5-1). In test medium solutions with concentrations below 0.625 mg/l the PRP was not evident and a high variability in the absorbance readings was found. Thus, for all the aqueous media, only the solutions with concentrations between 10 and 0.625 mg/l were further used.

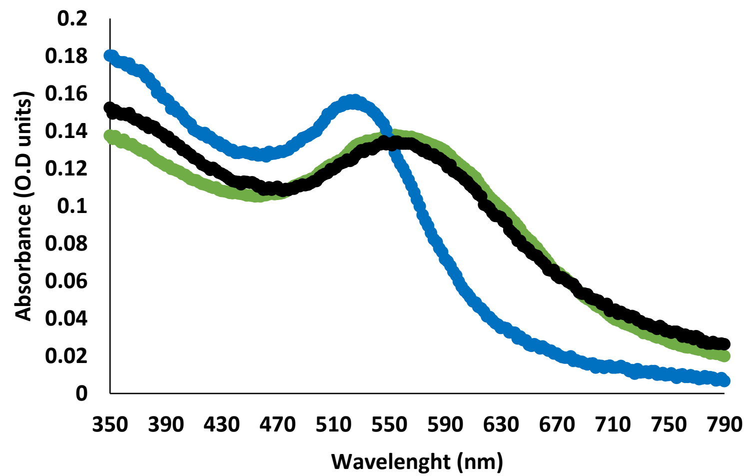


Figure 5-1: Representative spectra of 10 mg/l gold nanoparticles solutions in u.p. water (blue), test medium (black), and test medium with *Tetraselmis chuii* (green) immediately after the preparation of the solutions (0 h).

The u.p. water and test medium solutions with ~ 5 nm AuNP nominal concentrations of 2.5, 5 and 10 mg/l were maintained for 96 h in the abiotic conditions of the bioassay to determine the diameter, the percentage of spheres and the ~ 5 nm AuNP actual concentrations over time.

The diameter of AuNP particles were determined at 0 h and 96 h in u.p water (522 nm) and test medium (556 nm), according to (Haiss, *et al.*, 2007) as indicated in the equation 1.

$$d = \exp\left(B1\frac{Aspr}{A450} - B2\right) \qquad \text{(Equation 1)}$$

Where *d* is the estimated diameter of the particles; *Aspr* is the absorbance at the plasma resonance peak; *A*450 is the the absorbance at 450 nm; and *B1* and *B2* are empirical constants (approximated 3.00 and 2.20, respectively).

The percentage of spherical particles was determined at 0h and 96h from the absorbance at the PRP (522 nm in u.p water and 556 nm in test medium), using the method of Amendola and Meneghetti (2009) based on the Mie model.

To determine the actual concentrations of ~5 nm AuNP in the solutions, for each medium (u.p. water and test medium) separately, the nominal concentrations were plotted against the absorbance (522 nm for u.p. solutions and 556 nm for test medium solutions), the correlation between the two variables was determined using the Pearson's correlation coefficient (hereafter indicated as *r*), and a linear regression model was fitted to the data of each series of solutions. Positive and significant correlations (Pearson's correlation

coefficient, *r*) between the absorbance at the PRP and the ~ 5 nm AuNP nominal concentrations were found for solutions prepared in u.p. water (N = 18, r = 0.997, p < 0.001), test medium (N = 18, r = 0.996, p < 0.001). The linear regression model fitted to u.p. water solutions was: actual concentration of ~ 5 nm AuNPconcentrations (mg/l) = - 0.250 + 64.855 x absorbance (optical density units – O.D units), coefficient of determination (R) = 99.5 % (model 1). The model fitted to test medium solutions was: actual concentration of ~ 5 nm AuNP (mg/l) = - 0.093 + 75.407 x absorbance (O.D. units), R = 99.3 % (model 2) as shown in the Figure 5-2.

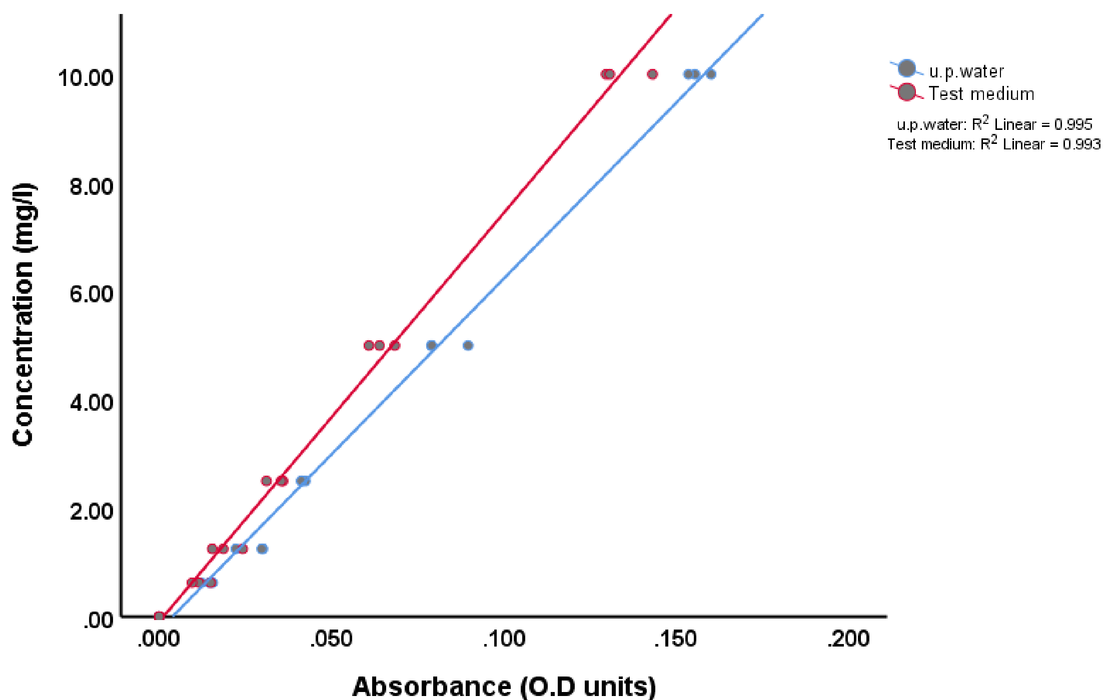


Figure 5-2: Calibration curve of the ~5nm gold nanoparticles (AuNP) in u.p water (blue line) and test medium (red line) with the linear models fitted to the data. Using the absorbance as independent variable and the nominal concentration of the AuNP suspensions as dependent variable, according its further use to determine the actual AuNP concentrations in test media of the bioassays. R^2 – Coefficient of determination. O.D – optical density.

The model fitted to data of solutions in test medium was used to determine the actual concentrations of ~5 nm AuNP from the absorbance readings of test medium samples collected during the bioassay (0 h, 24 h, 48 h, 72 h and 96 h).

The limit of detection (LOD) and the limit of quantification (LOQ) of ~ 5 nm AuNP actual concentrations in test medium, calculated using the model 2, were 0.6 mg/l and 1.8 mg/l, respectively. Therefore, during the bioassay was only possible to determine the actual concentrations in treatments containing 5 mg/l of AuNP.

The deviation of actual AuNP concentration from nominal one at the beginning of the bioassay (0 h) was determined per beaker according to Prata *et al.* (2018): deviation (%) =

module of 100 – (actual concentration x 100 / nominal concentration). The decrease of the 5 nm AuNP actual concentrations in test medium during the bioassay, hereafter indicated as decay was determined as indicated in Fonte *et al.* (2016) for other particles: decay (%) = 100 – (actual concentration after 24 h, 48 h, 72h, 96h x 100 / actual concentration at 0 h). Because the decay of the test substance was higher than 20 %, the exposure concentrations were calculated as the geometric mean of the actual concentrations determined at 0 h, 24 h, 48 h, 72 h and 96 h, as recommended in the OECD guideline (OECD, 2011). For each culture with ~ 5 nm AuNP nominal concentration of 5 mg/l where the actual concentration at 96 h was not zero, the exposure concentration during the bioassay was calculated as the geometric mean of the actual concentrations determined at 0 h, 24 h, 48 h, 72 h and 96 h (OECD, 2011). For each culture with nominal concentration of 5 mg/l where the actual concentration of ~ 5 nm AuNP at 96 h was zero, the exposure concentration was determined as: (geometric mean of actual concentrations from 0 h to 72 h x 3 + midpoint of the actual concentrations at 72 h and 96 h x 1) / 4. For each culture with ~ 5 nm AuNP nominal concentration of 1 mg/l or 0.2 mg/l, the exposure concentration was estimated based on the nominal concentrations (NC) and the proportions of decay per day calculated for cultures with 5 mg/l (D). First, the concentrations at 24 h, 48 h, 72 h and 96 h were calculated for each culture as: NCt2 = NCt1 - (NCt1 x Dt2), where t2 is the time (h) of the estimated concentration, and t1 is 24 h before. Then, the geometric mean of the estimated concentrations at 24 h, 48 h, 72 h and 96 h for each culture was calculated. When the values at 96 h were zero, the estimated exposure concentrations were calculated as indicated for the treatments with a nominal concentration of 5 mg/l and actual concentrations of zero at 96 h. The means of the estimated exposure concentrations were used to express the biological results.

5.2.5. Concentrations of microplastics in test medium

The determination of MP concentrations in test medium during the bioassay was performed by spectrofluorometry, following the general procedure indicated in Luís *et al.* (2015), with minor adaptations regarding the concentrations and emission/excitation wavelength of the particles and test medium used (Prata *et al.*, 2018). Three independent suspensions of MP with a concentration of 12.5 mg/l were prepared in u.p. water and test medium separately. Each solution was serial diluted (1:2 v/v) to obtain additional solutions in a final range of concentrations between 12.5 mg/l and 0.098 mg/l. The fluorescence was read in a spectrofluorometer (Jasco FP-6200) using an excitation wavelength of 575 nm and an emission wavelength of 607 nm. After discounting the values of test medium without MP, the correlation between the fluorescence values and the concentrations of MP (for the solutions with concentrations between 12.5 and 0.098 mg/l) was determined in u.p water

solutions: N = 27, r = 0.990, p < 0.001 and and the following linear regression model was fitted to the data: MP concentrations (mg/l) = - 0.25 + 0.14 x fluorescence; R = 98.1 % . In test medium solution (N = 27, r = 0.992, p < 0.001) and the following linear regression model was fitted to the data: MP concentrations (mg/l) = - 0.018 + 0.019 x fluorescence (F units), R = 98.3 % (Figure 5-3). The model fitted to data of solutions in test medium was used to determine the actual concentrations of MP from the readings fluorescence of test medium samples collected during the bioassay (0 h, 24 h, 48 h, 72 h and 96 h). The decrease of MP concentrations during the bioassay was determined as previously indicated for AuNP.

The limit of detection (LOD) was 0.015 mg/l and the limit of quantification (LOQ) was 0.043 mg/l. The decrease of the actual concentrations of MP in test media during the bioassay, hereafter indicated as MP decay, was calculated as previously indicated for AuNP. Because the MP decay over 96 h was higher than 20 %, the estimated exposure concentrations of MP during the bioassay were determined as the geometric mean of the actual concentrations determined at 0 h, 24 h, 48 h, 72 h and 96 h (OECD, 2011).

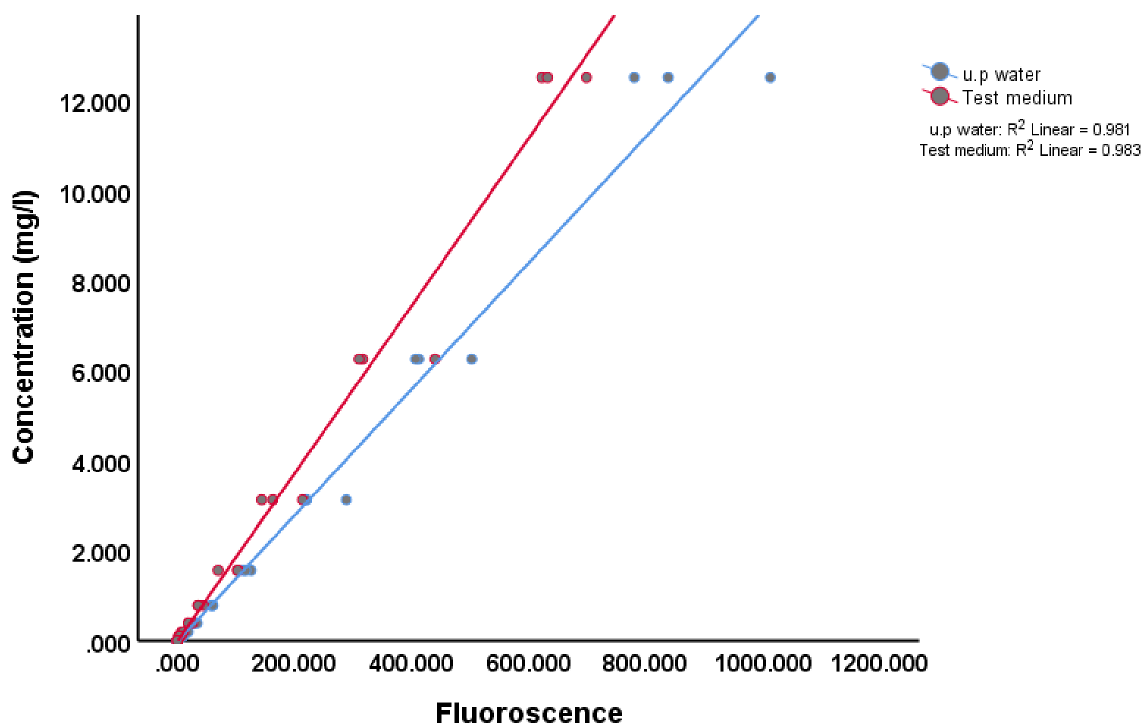


Figure 5-3: Calibration curve of the red fluorescent polymer microspheres (MPs) in test medium (red line) and in u.p water (blue line) with the linear models fitted to the data. Using the fluorescence values as independent variable and the nominal concentrations of the MP as dependent variable, according its further use to determine the actual MPs in test medium of the bioassays. R^2 – Coefficient of determination.

Therefore, the total mean (± SD, N = 6) of the exposure concentrations of treatments with the same nominal concentration of MP (0.2, 1 or 5 mg/l) were: 0.34 ± 0.02 mg/l; 0.88 ±

0.09 mg/l; and 3.9 ± 0.2 mg/l. The biological results were expressed in relation to 0.3, 0.9 or 4 mg/l of MP.

5.2.6. Statistical analyses of data

The average specific growth rate was calculated over 3 days and 4 days as indicated in OECD (2011), using the number of cells as surrogate parameter, and expressed as the logarithm of the cell number increase per day (day^{-1}). The percentage of average specific growth rate inhibition was also calculated according to OECD (2011).

The curves of AuNP solutions in u.p. water, test medium and test medium with microalgae were compared with the Analysis of Covariance (ANCOVA). Abiotic and biotic parameters are expressed as the mean ± standard deviation (SD) or as the mean ± standard error of the mean (SEM). Each data set was checked for normal distribution (Kolmogorov–Smirnov normality test) and homogeneity of variances (Levene's test) and transformed if necessary; percentage data were arcsine transformed (Zar, 1999). Abiotic variables were analysed by one-way Analysis of Variance (1-ANOVA) or two-way Analysis of Variance (2-ANOVA). When significant differences were found, the Tukey's multi-comparison test was used to discriminate statistically significant differences. Biological data were analysed by the Kruskal-Wallis test and a nonparametric Tukey-type test (Zar, 1999) because the requirements of ANOVA could not be achieved and used to determine the no observed effect concentration (NOEC) and the lowest observed concentration (LOEC) that may be of interest for environmental risk assessment.

All the statistical analyses were performed using the SPSS© software package 25. The significance level was 0.05.

5.3. Results and Discussion

5.3.1. Preliminary study to characterize the AuNP and their behaviour over time

The absorption spectra of representative AuNP solutions in both u.p. water and test medium with a concentration of 10 mg/l, determined immediately after the preparation of the solutions previously to the bioassay, are shown in Figure 5-1. As previously indicated, in u.p. water, the PRP was recorded at 522 nm, which is in agreement with the values reported in the literature (Haiss *et al.*, 2007; Amendola and Meneghetti, 2009; Ferreira *et al.*, 2016; Pacheco *et al.*, 2018) and the manufacturer indications (5.2.1). In test medium, the PRP was recorded at 556 nm. the shape of the spectra, and the significant differences in relation to the calibration curve in u.p. water, suggest some changes of the particles

shortly after dilution of the commercial solution into test medium. Because the wavelength of AuNP solutions increases when the particles become larger (Haiss *et al.*, 2007; Pamies *et al.*, 2014), these findings suggest some aggregation of AuNP causing a shift of the PRP to 556 nm shortly after dilution of the commercial solution into test medium. Such aggregation of some of the particles is likely due to the salt (salinity 30) and other compounds that the test medium contains in order to support the growth of *T. chuii*.

Significant differences of the PRP between AuNP solutions in u.p. water and test medium recorded (ANCOVA, aqueous medium: $F_{1, 27} = 36.120$, $p = 0.035$, $p < 0.001$; 5 nm AuNP concentrations: $F_{1, 27} = 2382.151$, $p < 0.001$) immediately after their preparation may be due to a higher ionic strength in test medium solutions than in u.p. water ones. The higher ionic strength of test medium solutions relatively to u.p. water ones is due to the salt and other materials such as metals in the medium which allow particles to have more contact leading to more aggregation (French, *et al.*, 2009; Chekli, *et al.*, 2013). Thus, the model 1 and the model 2 were used to determine the concentrations of the particles in u.p. water and test medium solutions (Table 5-1), respectively, over time in the preliminary assay made before the bioassay (hereafter indicated as preliminary assay).

The model fitted to solutions prepared in test medium with microalgae was: actual concentration of ~ 5 nm AuNP (mg/l) = 0.042 + 71.370 x absorbance (OD), R = 99.8 %. No significant differences between test medium and test medium with microalgae solutions were found (ANCOVA, aqueous medium: $F_{1, 17} = 0.105$, $p = 0.0.750$; ~ 5 nm AuNP concentrations: $F_{1, 17} = 2178.918$, $p < 0.001$). Thus, the model 2 was used to determine the actual concentrations of ~ 5 nm AuNP in all the solutions prepared in test medium (with and without microalgae).

The diameter of the AuNP in u.p. water and test medium determined immediately after preparation of the solutions are shown in Table 5-1. The mean and standard deviation (SD) of all the suspensions in u.p. water and in test medium were 3.9 ± 0.3 nm and 4. ± 0.7 nm, respectively. These calculated diameters are in the range of manufacturer diameter (5 ± 2 nm, mean ± SD). Overall these findings indicate that despite some aggregation, the size and shape of the most part of the particles was not significantly changed immediately after the dilution of the commercial solution into test medium, in good agreement with previous findings of the same AuNP in other saline medium (Ferreira *et al.*, 2016). Comparable findings were also found by Pamies *et al.* (2014) with different AuNP and aqueous medium.

Table 5-1: Absorbance of AuNP solutions in u.p. water (at 522 nm) and in test medium (at 556 nm) measured at 0 h used to calculate the diameter of AuNP. By the equation1 of section 5.2.4 (Haiss et al., (2007). For each concentration and medium, the values are the mean of 3 solutions prepared independently with the corresponding standard deviation (within brackets). Conc.- concentration. Nom.- nominal concentration. Abs. peak - optical density at the absorbance peak (522 nm in u.p.

water; 556 nm in test medium). Act. - actual concentration. Abs.450 - Absorbance at 450 nm. O.D. - optical density units. Sph – Spheres.

Nom. AuNP Conc. (mg/l)	u.p. water					Test medium				
	Abs. peak (O.D.) Mean (±SD)	**Abs. 450 (O.D.) Mean (±SD)**	**Act. Con.**	**Diameter (nm)**	**Sph. (%)**	**Abs. peak (O.D.) Mean (± SD)**	**Abs. 450 (O.D.) Mean (± SD)**	**Act. Con.**	**Diameter (nm)**	**Sph (%)**
0.625	0.014 (±0.002)	0.012 (±0.002)	0.663 (±0.111)	3.784 (±0.320)	96	0.010 (±0.001)	0.009 (±0.001)	0.809 (±0.206)	3.306 (±1.990)	83
1.25	0.024 (±0.001)	0.021 (±0.003)	1.285 (±0.094)	3.331 (±0.845)	98	0.019 (±0.004)	0.017 (±0.004)	1.370 (±0.336)	3.427 (±0.578)	92
2.5	0.042 (±0.001)	0.035 (±0.001)	2.450 (±0.043)	4.031 (±0.366)	100	0.035 (±0.001)	0.012 (±0.001)	2.481 (±0.199)	4.081 (±0.174)	99
5	0.083 (±0.006)	0.069 (±0.006)	5.103 (±0.397)	4.057 (±0.252)	100	0.041 (±0.006)	0.018 (±0.002)	4.719 (±0.704)	4.4 (±1.33)	100
10	0.156 (±0.003)	0.128 (±0.004)	9.889 0.128 (±0.220) (±0.004)	4.294 (±0.187)	100	0.134 (±0.007)	0.042 (±0.007)	10.047 (±0.565)	4.100 (±0.489)	100
Overall Mean (±SD)				3.900 (±0.261)	98				4.057 (±0.736)	95

Ancova	Diameter			% Spheres		
	Factor	*F*	*p*	Factor	*F*	*p*
	Media	$F_{(1,24)} = 0.279$	0.602	Media	$F_{(1,24)} = 20.296$	0.000
	AuNP conc.	$F_{(4,24)} = 2.250$	0.092	AuNP conc.	$F_{(4,24)} = 13.999$	0.000

The spectra of AuNP solutions in test medium containing microalgae cells changed over time as illustrated in Figure. 5-4. The PRP shifted to longer wavelengths over time: from 556 nm at 0h to 576 nm at 24 h, 588 nm at 48 h and 592 nm at 72h. The absorbance at the PRP decreased and the peak became more round over time, suggesting a reduction of the ~ 5 nm AuNP concentration along time. At 72 h, a second peak ~ 640 nm appeared, suggesting the presence of larger aggregates. After 96 h, the peak at 556 was not evident and the second peak was shift to ~ 680 nm. Because AuNP in saline solutions are known to aggregate and sedimentation of large aggregates occurs and increases over time moving the PRP to higher wavelengths (Gilroy et al., 2014; Pamies *et al.*, 2014; García-Negrete *et al.*, 2013), the changes in the spectra suggest that larger aggregates were formed over time and that sedimentation of some aggregates occurred. Moreover, the interaction of AuNP as an aggregate or a single particle with cell wall can change the refraction index and shift the wavelengths to 575 nm and 592 nm (Gilroy *et al.*, 2014). The simulation of these results also confirmed binding of AuNP (aggregate form) to the *Ankistrodesmus.* It was in agreement with the binding of AuNP to the surface of another chlorophyte algae *Scenedesmus* (Renault *et al.* 2008).

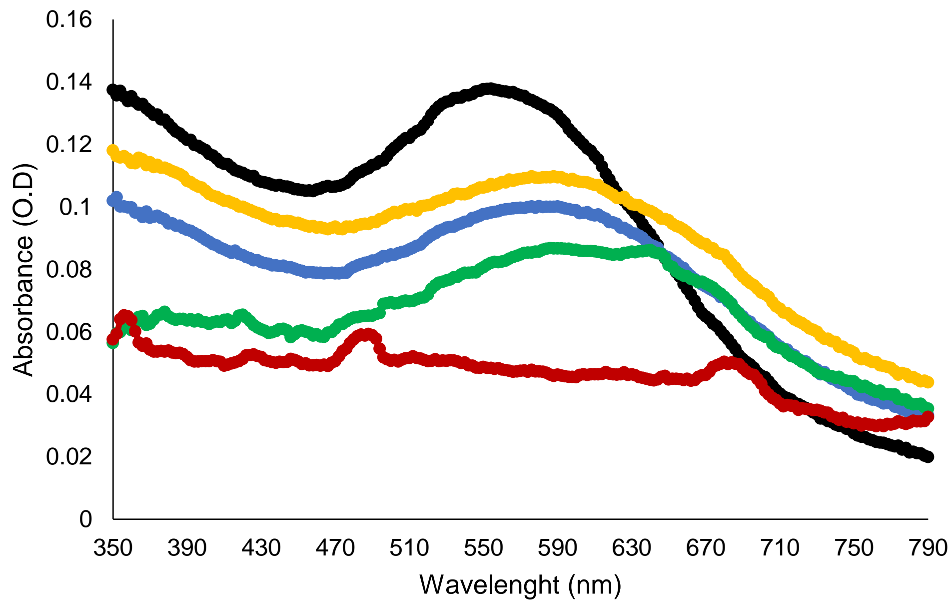


Figure 5-4: Representative UV-Vis spectra of a solution with a concentration of 10 mg/l of AuNP in test medium with microalgae cells at 0 h (black line), 24 h (orange line), 48 h (blue line), 72 h (green line) and 96h (red line). O.D- Optical density.

In good agreement with the changes of spectra (Figure 5-4), the mean of ~ 5 nm AuNP actual concentrations decreased over time (Table 5-2). At 0 h, the means (± SD) of ~ 5 nm AuNP actual concentrations (determined using the model 1) in solutions with nominal concentrations of 2.5, 5 and 10 mg/l were 2.50 ± 0.04 nm, 5.1 ± 0.4 nm and 9.9 ± 0.2 nm, respectively, with deviations from the nominal ones in individual replicates ranging from 1 to 11 %. These findings indicate that the dilution of the commercial solution in u.p. water did not change immediately the properties of the particles. At 96 h, the mean AuNP decay ranged from 70 to 74 % and no significant differences among solutions with different concentrations of the particles occurred ($F_{2, 6}$ = 0.504, p = 0.628) indicating that the concentration did not influence significantly the ~ 5 nm AuNP decay in the range of concentrations tested. The total mean (± SD) of ~ 5 nm AuNP concentrations decay was 71 ± 6 %.

Regarding the diameter of the particles, no significant differences among concentrations, significant differences along time, and no significant interaction between the two factors were found (2-ANOVA, concentrations: $F_{2, 30}$ = 0.132, p = 0.877; time: interaction: $F_{8, 30}$ = 0.489, p = 0.854). At 96 h the diameter was significantly lower (3 ± 1 nm, mean ± SD) from the diameter at 0 h. The diameter of the particles decreased significantly ($F_{4, 32}$ = 5.308, p = 0.002) along time (Table 5-2), with the differences becoming significantly different from 0 h after 48 h (1.3 nm ± 1.4 SD), 72 h (1.5 nm ± 1.5 SD) and 96 h (1.7 nm ± 0.9 SD). This finding seems to be in contradiction with the hypothesis that the decay of the ~ 5 nm AuNP

actual concentrations was due to the formation of large particles through aggregation. However, as aggregation occurs, the particles that are not aggregated and remain in the water column may be those with lower size, thus explaining why the mean diameter apparently decreased. Also, at 72 and 96 h, the absorbance values at the PRP and at 450 nm are relatively low and this may introduce error in the diameter calculation.

In relation to the percentage of spherical particles, at 0 h, the percentage of spherical particles was 100 % in all the concentrations and replicates, thus in agreement with the manufacturer (> 95 % spheres). Significant differences among concentrations, distinct periods of time and a significant interaction were found (2-ANOVA, concentrations: $F_{2, 30}$ = 27.891, $p < 0.001$; time: $F_{4, 30}$ = 1501.164, $p < 0.001$; interaction: $F_{8, 30}$ = 5.344, $p < 0.001$). The total mean (± SD) of the percentages of spheres were 58 ± 35 % at 2.5 mg/l, 62 ± 34 % at 5 mg/l and 67 ± 32 % at 10 mg/l. The total mean (± SD) of the percentages of spheres at different times were 100 % at 0 h, 89 ± 3 % at 24 h, 72 ± 10 % at 48 h, 37 ± 5 % at 72 h and 13 ± 6 % at 96 h. The decrease of the percentage of spherical particles over time indicates that the particles became more irregular along time and suggests aggregation of some of the particles (Pamies *et al.*, 2014; Yang *et al.* 2017). Overall, these findings indicate that the concentrations of ~ 5 nm AuNP in test medium decreased over time and suggest that the decay was due to the formation of particles aggregates that increased their size over time, likely with sinking of large aggregates on the beakers bottom. These findings are in agreement with previous studies investigating the behaviour of AuNP in other saline media (Pamies *et al.*, 2014; García-Negrete *et al.*, 2013; Ferreira *et al.*, 2016).

Table 5-2: Mean and standard deviation (SD) of the diameter of AuNP particles, percentage (%) of spheres, actual concentrations (Actual conc) of ~ 5 nm AuNP and their decay along time (0 h, 24 h, 72 h, 96 h) in solutions of the calibration curves prepared in ultra-pure water and test medium (medium) with nominal concentrations (NC) of 2.5, 5 and 10 mg/L. N = 3. * less than 3 values. Different letters indicate statistically significant difference among periods of time.

NC (mg/l)	0 h	24 h	48 h	72 h	96 h	Decay 0-96 h (%)
Solution in ultra pure water						
Absorbance at 522 nm						
2.5	0.042 ± 0.001	0.043 ± 0.002	0.046 ± 0.004	0.043 ± 0.002	0.045 ± 0.009	
5	0.083 ± 0.006	0.084 ± 0.001	0.091 ± 0.007	0.081 ± 0.003	0.086 ± 0.009	
10	0.156 ± 0.003	0.166 ± 0.007	0.17 ± 0.02	0.162 ± 0.002	0.171 ± 0.006	
Absorbance at 450 nm						
2.5	0.035 ± 0.001	0.038 ± 0.004	0.040 ± 0.005	0.041 ± 0.003	0.05 ± 0.01	
5	0.069 ± 0.006	0.076 ± 0.008	0.080 ± 0.007	0.075 ± 0.007	0.09 ± 0.02	
10	0.128 ± 0.004	0.15 ± 0.01	0.16 ± 0.02	0.15 ± 0.02	0.16 ± 0.02	
Actual concentrations of ~ 5 nm AuNP (mg/l)						
2.5	2.50 ± 0.04	2.6 ± 0.1	2.8 ± 0.3	2.6 ± 0.1	2.7 ± 0.6	0
5	5.1 ± 0.4	5.25 ± 0.08	5.7 ± 0.4	5.1 ± 0.2	5.4 ± 0.6	3 ± 5
10	9.9 ± 0.2	10.5 ± 0.4	11 ± 1	10.3 ± 0.1	10.8 ± 0.4	3 ± 4
Diameter (nm)						
2.5	4.0 ± 0.4	3.4 ± 0.6	4 ± 2	2.6 ± 0.5	2.3 ± 1.3	
5	4.1 ± 0.3	3.2 ± 0.9	3.3 ± 0.2	3.0 ± 0.8	3 ± 1	
10	4.3 ± 0.2	3.1 ± 0.4	3.1 ± 0.3	3.2 ± 0.9	3 ± 1	
Total	4.1 ± 0.3 a	3.2 ± 0.6 a, b	3.5 ± 1.2 a, b	3.0 ± 0.7 a, b	3 ± 1 b	
Percentage of spheric al particles						
2.5	100 ± 0	87 ± 2	63 ± 7	31 ± 5	8 ± 7	
5	100 ± 0	89 ± 3	70 ± 4	39 ± 3	11 ± 2	
10	100 ± 0	91 ± 2	83 ± 3	39 ± 2	20 ± 2	
Total	100 ± 0 a	89 ± 3 b	72 ± 10 c	37 ± 5 d	13 ± 6 e	
Solution in test medium						
Absorbance at 556 nm						
2.5	0.035 ± 0.001	0.013 ± 0.002	0.009 ± 0.002	0.010 ± 0.003	0.012 ± 0.002	
5	0.064 ± 0.004	0.024 ± 0.004	0.020 ± 0.006	0.014 ± 0.003	0.018 ± 0.007	
10	0.134 ± 0.007	0.043 ± 0.03	0.040 ± 0.009	0.035 ± 0.002	0.042 ± 0.003	

			Absorbance at 450 nm			
2.5	0.012 ± 0.002	0.011 ± 0.007	0.011 ± 0.007	0.010 ± 0.005	0.009 ± 0.007	
5	0.018 ± 0.007	0.026 ± 0.011	0.026 ± 0.011	0.020 ± 0.009	0.014 ± 0.007	
10	0.042 ± 0.003	0.056 ± 0.016	0.056 ± 0.016	0.037 ± 0.019	0.028 ± 0.015	
			Actual concentrations of ~ 5 nm AuNP (mg/l)			
2.5	2.5 ± 0.1	0.8 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	70 ± 7
5	4.7 ± 0.3	1.7 ± 0.3	1.4 ± 0.4	0.9 ± 0.2	1.2 ± 0.5	74 ± 10
10	10.1 ± 0.6	3.1 ± 0.3	2.9 ± 0.7	2.5 ± 0.1	3.1 ± 0.2	70 ± 1
			Diameter (nm)			
2.5	4.1 ± 0.2	1.3 ± 0.6	0.65 ± 0.09	0.8 ± 0.3	0.2 ± 1.7	
5	4.4 ± 0.4	3 ± 4	2 ± 2	2 ± 2	1.34 ± 0.08	
10	4.1 ± 0.4	1.2 ± 0.5	1.1 ± 0.7	1.3 ± 0.9	1.74*	
Total	4.2 ± 0.3	2 ± 2	1 ± 1	1.5 ± 1.5	1.7 ± 0.9	
			Percentage of spherical particles			
2.5	100 ± 1	75 ± 1	57 ± 6	30 ± 6	3 ± 1	
5	99 ± 1	83 ± 2	63 ± 2	35 ± 3	7 ± 2	
10	100 ± 0	85 ± 3	81 ± 4	32 ± 2	18 ± 3	
Total	99 ± 1 a	81 ± 5 b	67 ± 11 c	32 ± 4 d	9 ± 6 e	

5.3.2. General conditions of the bioassay

In test medium of individual beakers, the variation of temperature was lower than 1ºC and the maximum of pH variation was 1 pH units. After 3 days (72 h), the mean coefficient of variation for section-by-section specific growth rates was 25 %, and the coefficient of variation of the average specific growth rates recorded in control cultures during the test period was 4 %. The average specific growth rates calculated for the control cultures over a period of 3 days were 0.97 day^{-1}, 0.99 day^{-1} and 0.91 day^{-1}, and the factors of biomass increase in individual cultures of the control group ranged from 15 to 20 folds. Because in one of the control replicates, the average specific growth rate was lower than 0.92 day^{-1} and the factor of biomass increase was lower than 16, the bioassay was continued as recommended in the OECD guideline (OECD, 2011). After 4 days (96 h), the mean coefficient of variation for section-by-section specific growth rates was 23 %, and the coefficient of variation of the average specific growth rates recorded in control cultures during the test period was 3 %. The average specific growth rates calculated for the control cultures over a period of 4 days were 1.02 day^{-1}, 1.03 day^{-1} and 0.97 day^{-1}. At this time, the factors of biomass increase in control cultures ranged from 49 to 61. Thus, according to the OECD guideline (OECD, 2011), the bioassay can be considered valid.

5.3.3. Behaviour and concentrations of AuNP during the bioassay

The actual concentrations of ~ 5 nm AuNP in treatments with a nominal concentration of 5 mg/l determined during the bioassay are indicated in Table 5-3. At 0 h, the deviation of actual concentrations relatively to nominal ones in individual replicates ranged from 3 to 21 %. Significant differences in the concentrations of ~ 5 nm AuNP among periods of time, no significant differences between treatments with AuNP alone and in mixture with MP, and no significant interaction were found (2-ANOVA, time: $F_{4, 20} = 44.196$, $p < 0.001$; $F_{1, 20} = 0.224$, $p = 0.641$; interaction: $F_{4, 20} = 1.014$, $p = 0.424$). Therefore, the particles changed over time, in good agreement with the preliminary study. Moreover, the presence of MP did not influence the changes of ~ 5 nm AuNP concentrations over time. The percentages of ~ 5 nm AuNP decay after 24 h, 48 h, 72 and 96 h were 17 %, 20 %, 34 % and 99 %, with an overall (0 – 96 h) mean decay of 99 % (Table 5-1). The estimated exposure concentration during the bioassay for treatments with a nominal concentration of 5 mg/l of ~ 5 nm AuNP was 3 ± 0.7 mg/l. The estimated exposure concentrations during the bioassay for treatments with nominal concentrations of 1 mg/l and 0.2 mg/l were 0.3 and 0.1 mg/l respectively. Such concentrations of AuNP are higher than those found or expected to be found in environmental waters (up to the low ppb range) (Boxall *et al*, 2007; Bäuerlin *et al*., 2017; Markus *et al*., 2018).

Table 5-3: Mean (N = 3) and standard deviation (SD) of ~ 5 nm gold nanoparticles (AuNP) actual concentrations determined in test medium of treatments containing the nominal concentration of 5 mg/l AuMP alone (N = 3) and in mixture with microplastics (N = 3), and AuNP decay over 96 h (decay). Different letters after the mean indicate statistically significant differences (2-ANOVA + Tukey's test, p ≤ 0.05). Mix-H – mixture containing 5 mg/l of AuNP and 5 mg/l of microplastics (nominal concentrations).

Treatment	**~ 5 nm AuNP actual concentrations (mg/l)**				
	0 h	24 h	48 h	72 h	96 h
5 mg/l (alone)	5.4 ± 0.2	4.1 ± 0.3	3.6 ± 0.8	2.9 ± 0.3	0 ± 0
Mix-H	5.7 ± 0.5	5.1 ± 0.5	3.8 ± 1.7	2.1 ± 1.4	0.08 ± 0.1
Average	5.5 ± 0.4 a	4.6 ± 0.7 a,b	3.7 ± 1.2 b,c	2.5 ± 1.0 c	0.04 ± 0.1 d
	~ 5 nm AuNP				
	(0 - 24 h)	(24 - 48 h)	(48 – 72 h)	(72 – 96 h)	Total decay
5 mg/l (average)	17 %	20 %	34 %	99 %	99 ± 2

In addition, the decay of AuNP in presence of algae was more than its absence. This means that the plasmon resonance peak is influenced by the presence of algae. No significant differences between the actual concentration of AuNP alone and in the mixture with MP

were found ($F_{1,12} = 3.099$, $P = 0.104$) (Table 5-4). Moreover, the size of particles in the mixture is lower than when they are alone in the test medium. Significant deviations for actual concentrations in relative to nominal concentrations were found in the treatments.

Table 5-1: Nominal and actual concentrations of AuNP in the test medium with microalgae in treatments containing AuNP alone (AuNP) or in mixture with MP (AuNP+MP), diameter, percentage of spheres and deviation of actual concentrations from nominal ones at 0 h. The values are presented as the mean ± standard error of the mean. Each value is the average of three individual replicates. Nom.con. - Nominal concentration, Treat. - Treatment, Abs.-Absorbance, Dev. - Deviation, O.D.-Optical density, N - number of samples.

Nom. Con. (mg/l)	**Treat.**	**N**	**Actual Con. (mg/l) Mean (± SD)**	**Size (nm)**	**Dev. (%)**	**Spheres (%)**
5.00	AuNP	3	5.639 (±0.354)	5.467 (±0.364)	13	100
	AuNP + MP	3	5.965 (±0.484)	4.746 (±0.195)	19	88
1.00	AuNP	3	0.995 (±0.207)	5.089 (±1.838)	14	97
	AuNP + MP	3	1.299 (±0.051)	4.374 (±1.322)	30	85
0.20	AuNP	3	0.302 (±0.185)	4.330 (±0.693)	78	83
	AuNP + MP	3	0.347 (±0.161)	3.673 (±0.901)	83	81

Two-way ANOVA	Diameter			Spheres %		
	Factor	*F*	*P*	Factor	*F*	*P*
	Medium	$F_{(1,12)} = 3.099$	0.104	Medium	$F_{(1,12)} = 103.513$	0.000
	Conc.	$F_{(2,12)} = 24.337$	0.000	Conc.	$F_{(2,12)} = 35.213$	0.000
	Int.	$F_{(2,12)} = 0.427$	0.662	Int.	$F_{(2,12)} = 12.387$	0.001

5.3.4. Behaviour and concentrations of microplastics in test medium during the bioassay

The percentages of MP decay after 96 h of exposure, and the estimated exposure concentrations along the bioassay are indicated in Table 5-5. At 0 h, no significant differences between treatments with and without AuNP, and no significant interaction between the presence of AuNP and MP actual concentrations were found (Table 5-5). The MP decay during the bioassay in different treatments ranged from 13 % to 39 %. No significant differences in MP decay among MP concentrations nor between treatments with and without AuNP, and no significant interaction were found (Table 5-5). Moreover, no significant differences in the exposure concentrations of MP between treatments with and without AuNP, and no significant interaction between AuNP and MP concentrations were found (Table 5-5). Overall, these findings indicate that corresponding treatments with and without AuNP had comparable concentrations of MP, and that MP and AuNP do not interact significantly in the test medium used.

The total mean (± SD, N = 6) of the exposure concentrations of treatments with the same nominal concentration of MPs (0.2, 1 or 5 mg/l) were: 0.34 ± 0.02 mg/l; 0.88 ± 0.09 mg/l; and 3.9 ± 0.2 mg/l. Thus, the biological results were expressed in relation to 0.3, 0.9 and 4 mg/l of MPs. All these concentrations are ecologically relevant because they are lower than some found in environmental waters, such as mean concentration of 5.51 ± 9.09 mg/l (Lasee *et al.*, 2017).

Table 5-2. Mean and standard deviation (SD) of microplastics (MP) actual concentrations (MP actual conc) determined in test medium of treatments containing these particles at the begging (0 h) and at the end (96 h), percentages of decrease of MP after 96 h of exposure (MP decay), and estimated exposure concentrations of MP along the bioassay (MP EXP conc 0-96 h). The results of two-way ANOVA (2-ANOVA) investigating the effects of gold nanoparticles (AuNP) presence, MP concentrations, and the interaction between the factors (interaction) in the actual concentrations of MP at the beginning of the bioassay (0 h), decay of MP over 96 h, and MP exposure concentrations. Mix 1 - 5 mg/l AuNP + 5 mg/l MP. Mix 2 - 1 mg/l AuNP + 1 mg/l MP. Mix 3 - 0.2 mg/l AuNP + 0.2 mg/l MP. N – number of test medium samples analysed. Different letters after the mean indicate statistically significant differences (2-ANOVA + Tukey's test, p ≤ 0.05). Treat - Treatment.

Treat (nom. conc)	MP actual conc 0 h (mg/l)	MP actual conc 24 h (mg/l)	MP actual conc 48 h (mg/l)	MP actual conc 72 h (mg/l)	MP actual conc 96 h (mg/l)	MP decay 0-96 h (%)	MP EXP conc 0-96 h (mg/l)
5 mg/l MP	4.6 ± 0.2	4.37 ± 0.06	4.4 ± 0.2	3.8 ± 0.3	3.2 ± 0.1	31 ± 3	4.03 ± 0.05
1 mg/l MP	0.97 ± 0.05	0.92 ± 0.07	0.82 ± 0.03	0.8 ± 0.1	0.9 ± 0.2	13 ± 7	0.87 ± 0.05
0.2mg/l MP	0.4 ± 0.03	0.40 ± 0.03	0.32 ± 0.04	0.31 ± 0.06	0.31 ± 0.08	25 ± 3	0.34 ± 0.02

Mix 1	4.6 ± 0.2	4.3 ± 0.2	3.9 ± 0.6	3.3 ± 0.3	2.8 ± 0.1	39 ± 1	3.7 ± 0.2
Mix 2	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	27 ± 5	0.88 ± 0.07
Mix 3	0.45 ± 0.03	0.39 ± 0.02	0.34 ± 0.02	0.30 ± 0.02	0.29 ± 0.03	35 ± 3	0.35 ± 0.01

2-ANOVA				
Factor	**Level**	**N**	**Mean ± SD**	**ANOVA**
2-ANOVA MP actual concentrations at 0 h				
AuNP presence	No Yes	9 9	2 ± 2 2 ± 2	$F_{1, 12} = 0.252$, $p = 0.624$
MP nominal concentration (mg/l)	0.2 1 5	6 6 6	0.42 ± 0.04 a 1.00 ± 0.07 b 4.6 ± 0.2 c	$F_{2, 12} = 1889.688$, $p < 0.001$
Interaction				$F_{2, 12} = 0.129$, $p = 0.880$
2-ANOVA MP AuNP decay after 96 h				
AuNP presence	No Yes	9 9	23 ± 15 34 ± 8	$F_{1, 12} = 3.776$, $p = 0.076$
MP nominal concentration (mg/l)	0.2 1 5	6 6 6	30 ± 16 20 ± 12 35 ± 6	$F_{2, 12} = 2.468$, $p = 0.127$
Interaction				$F_{2, 12} = 0.078$, $p = 0.926$
2-ANOVA MP exposure concentrations along the bioassay				
AuNP presence	No Yes	9 9	2 ± 2 2 ± 2	$F_{1, 12} = 2.937$, $p = 0.112$
MP nominal concentration (mg/l)	0.2 1 5	6 6 6	0.34 ± 0.02 a 0.88 ± 0.09 b 3.9 ± 0.2 c	$F_{2, 12} = 1412.962$ $p < 0.001$
Interaction				$F_{2, 12} = 3.299$, $p = 0.072$

Moreover, significant differences in the mean percentages of decay among treatments with different MP concentrations were observed, indicating that the concentration of the particles affected the decay. Probably aggregation of MP reduced the MP concentration in the test medium and lead to sedimentation and sorption of MP to the beakers (Luís *et al.*, 2015; Fonte *et al.*, 2016; Long *et al.*, 2017; Prata *et al.*, 218). Moreover, reduction of MP concentrations in test medium may be occurred due to some interaction such as, interaction of microalgae cells with MP (Lagarde *et al.*, 2016; Long *et al.*, 2017; Prata *et al.,* 2018) and also interaction with AuNP as well as other factors that could influence the surface charge of particles in the test MP with connection onto the external surfaces of exposed organisms, potentially restricting their movement and providing another route for indirect ingestion of MP (i.e. trophic transfer) (Watts *et al.*, 2014; Rehse *et al.*, 2016).

5.3.5. Effects of gold nanoparticles, microplastics and their mixtures on *T. chuii*

The mean of *T. chuii* cells/ml at the beginning of the bioassay and after 24 h, 48 h, 72 h and 96 h exposure in different treatments of the bioassay are indicated in Table 5-6, and the means of the average specific growth rates per treatment after 96 h of exposure are indicated in figure 5-5A. For all the treatments, the mean of the number of cells was higher after 96 h than at the beginning of bioassay (Figure 5-5 A), indicating growth of the cultures in all the treatments. The mean (± SEM) of the average specific growth rate obtained in the control group was 1.01 ± 0.02 day^{-1} at 96 h (Fig. 2B) and compare with corresponding values from the literature for *T. chuii* (Prata *et al.,* 2018). Significant differences in the average specific growth rate among treatments were found (Kruskal-Wallis, $H_{10} = 30.649$, $p < 0.001$).

Table 5-3.: Number of cells in the bioassay recorded after 24 h, 48 h, 72 h and 96 h. (using the inoculated volume at 0h as recommended in the guideline). The values are the mean of three culture replicates per treatment with corresponding standard error of the mean within brackets. Citrate – treatment containing 28.86 ml of citrate. AuNP – gold nanoparticles. MP –microplastic.

Treatment	24 h	48 h	72 h	96 h
Control	29667 (±6506)	60667 (±2028)	177333 (±22040)	567111 (±64352)
Citrate	8444 (±3356)	53889 (±17516)	140778 (±26900)	499000 (±31840)
0.2 mg/l AuNP	13667 (±58936)	62556 (±7501	144889 (±19313)	415111 (±26312)
1 mg/l AuNP	15889 (±10222)	68000 (±14189)	126444 (±32005)	379333 (±16667)
5 mg/l AuNP	58667 (±6692)	44889 (±15837)	97333 (±60250)	243667 (±16001)
0.3 mg/l MP	16111 (±3238)	95889 (±26839)	87000 (±52391)	332333 (±20867)
0.9 mg/l MP	11111 (±1924)	57222 (±5581)	126444 (±22324)	286222 (±7784)
4 mg/l MP	12222 (±2546)	75333 (±3863)	126444 (±33201)	237000 (±47438)
0.2 mg/l AuNP + 0.3 mg/l MP	17222 (±4333)	80222 (±7767)	104222 (±26802)	76222 (±32539)
1 mg/l AuNP + 0.9 mg/l MP	15778 (±2009)	73000 (±9939)	160889 (±55544)	343556 (±26374)
5 mg/l AuNP + 4 mg/l MP	26333 (±1018)	45667 (±10905)	118778 (±38690)	191667 (±16554)

As shown in Figure 5-5, where the results of the Tukey's test carried out after a 1-ANOVA comparing all treatments ($F_{10, 32} = 35.810$, $p < 0.001$) are indicated, the treatment containing citrate alone did not cause significant differences on the average specific growth rate relative to the control group indicating that the tested concentration of this substance was not toxic to *T. chuii*. Thus, the significant reduction (21 %) of the average specific growth rate in the treatment containing 5 mg/l of AuNP alone was caused by the particles and not by the citrate that this treatment also contains.

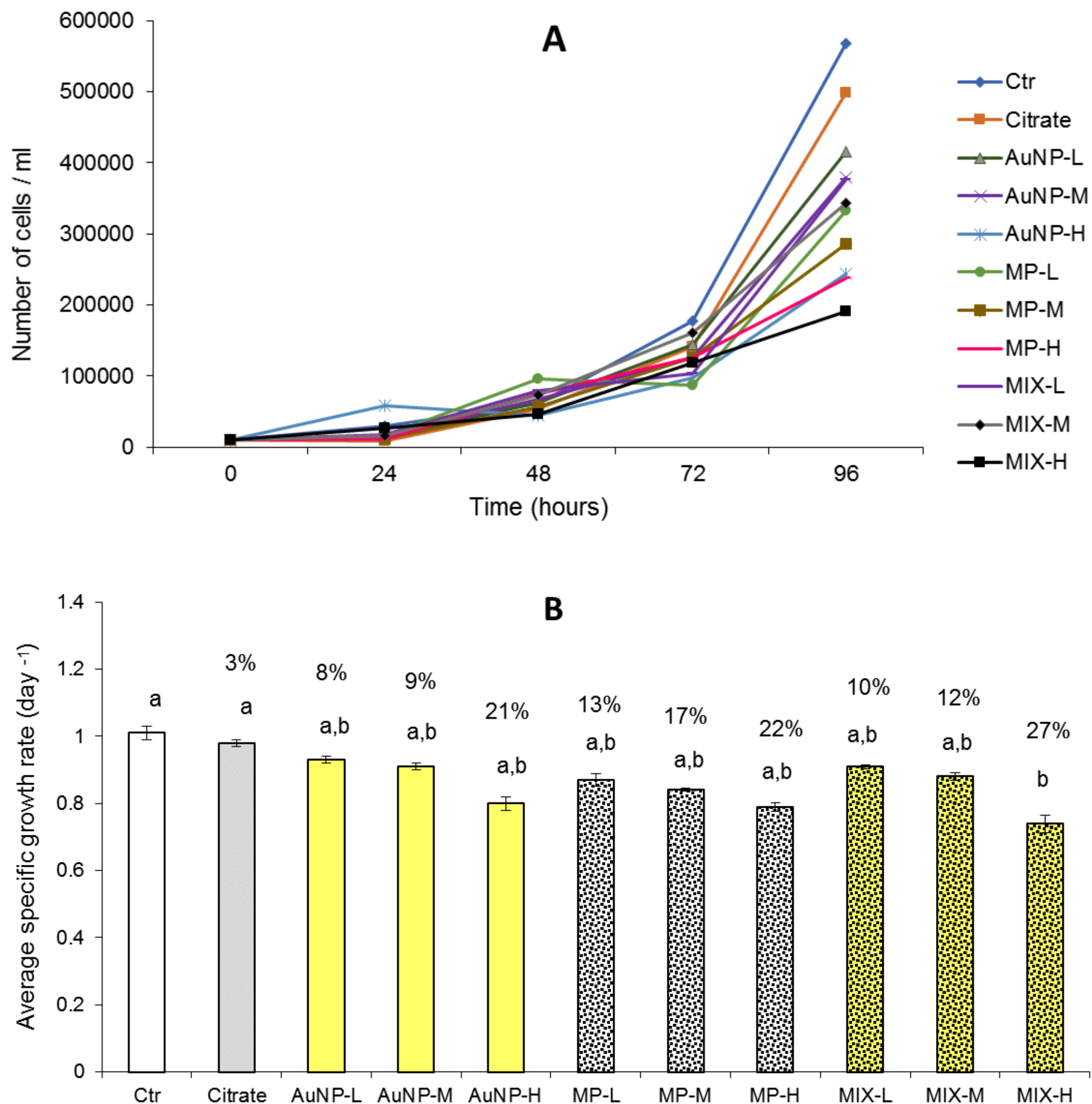


Figure 5-5: A: Growth rate of *Tetraselmiss chuii* cultures of different treatment during the assay (number of cells/ml). B: Average specific growth rate of *Tetraselmis chuii* cultures of different treatments. The values are the mean of 3 replicates per treatment with corresponding standard error of the mean. AuNP – gold nanoparticles. MP – microplastics. Ctr – control. Citrate – treatment containing 28.7 ml citrate; AuNP-L – treatment containing 0.2 mg/l of AuNP. AuNP-M – treatment containing 1 mg/l of AuNP. AuNP-H – treatment containing 5 mg/l of AuNP. MP-L – treatment containing 0.2 mg/l of MP. MP-M – treatment containing 1 mg/l of MP. MP-H – treatment containing

5 mg/l of MP. MIX-L – mixture containing 0.2 mg/l of AuNP and 0.2 mg/l of MP. MIX-M – mixture containing 1 mg/l of AuNP and 1 mg/l of MP. MIX-H – mixture containing 5 mg/l of AuNP and 5 mg/l of MP. Different letters indicate statistically significant differences (Tukey test, $p \leq 0.05$).

The highest concentrations of MP, when tested individually, slightly reduced the average specific growth rate (22 % of decrease) but with no significant differences in relation to the control group. No other significant differences were found. Therefore, based on the average specific growth rate, MP alone did not cause significant toxicity on *T. chuii* up to 4 mg/l (NOEC = 4 mg/l; LOEC > 4 mg/l), in good agreement with a previous study with the same type of MP and microalgae (Prata *et al.,* 2018) and other studies with distinct microplastics of comparable size and marine microalgae (Sjollema *et al.,* 2016). Moreover, in other previous studies, no significant effect of microplastics on microalgae were found (Bhattacharya *et al.*, 2010; Besseling *et al.*, 2014; Lagarde *et al.*, 2016; Sjollema *et al.*, 2016; Long *et al.*, 2017; Nolte *et al.*, 2017; Zhang *et al.*, 2017). Contradictory results can be obtained due to different physical properties of microplastics such as type, size, charge, composition of test medium, exposure time and concentration level (Nolte *et al.*, 2017; Sjollema *et al.*, 2016; Zhang *et al.*, 2017).

The highest concentration of AuNP alone reduced the average specific growth rate by 21 % but with no significant differences in relation to the control group. Thus, based on the average specific growth rate, the AuNP tested were not toxic to *T. chuii* up to 3 mg/l (NOEC = 3 mg/l; LOEC > 3 mg/l), in good agreement with previous studies that investigated the effects of comparable particles in other microalgae species. The difference between the toxicity effects of nanoparticles is due to the application of different coatings, the initial diameter of the nanoparticles, the ionic strength of the test medium, the type and the surface charge of particles (*Botha et al., 2015*). For example, ~ 10 mg/l of AuNP (~ 49 nm) did not cause significant reduction of *Scenedesmus subspicatus* growth (García-Cambero *et al.*, 2013), citrate-coated AuNP (~ 5 nm) up to 50 μM did not cause toxic effects (several endpoints) on *Chlamydomonas reinhardtii* (Behra *et al.*, 2015), and a 72 h EC_{10} equal to 9.9 mg/l (95 % CI: 4.3 – 23 mg/l) of AuNP (~ 51 nm) on *Pseudokirchneriella subcapitata* growth was reported (Hartmann *et al.*, 2013). However, toxic effects of AuNP on microalgae species in the range of concentrations tested were also reported, with several factors (*e.g.* species sensitivity, effect criteria, particles properties, experimental conditions) contributing to these apparently contradictory findings as discussed in the literature (Lapresta-Fernández *et al.*, 2012; Hartmann *et al.*, 2013; Van Hoecke *et al.*, 2013; Iswarya *et al.*, 2016; Moreno-Garrido *et al.*, 2015). Contradictory results can be obtained due to different physical properties of AuNP such as type, size, surface charge, coating, composition of test medium, exposure time and concentration level.

The mixtures containing the lowest and the intermediate concentrations of the tested substances caused no significant differences in relation to the control group (Figure 5-5 2B). However, the mixture containing the highest concentration of both AuNP and MPs caused significant reduction of the average specific growth rate (27 %) in relation to the control group (Figure 5-5 2B), indicating that it was toxic to *T. chuii*. The NOEC of the mixture was 0.3 mg/L AuNP + 0.9 mg/l MP. The LOEC of the mixture was 3 mg/L AuNP + 4 mg/l MPs. Some microplastics adsorb to microalgae cell wall (Bhattachayra *et al.*, 2010), a process that may contribute to their toxicity and facilitate the entry of other contaminants when microalgae are simultaneously exposed to microplastics and other contaminants (Prata *et al.*, 2018). Thus, the increased toxicity of the mixture relative to the effects caused by the substances tested alone may have been due to some damage in the cell wall induced by microplastic that facilitated the entrance of AuNP into the cells, resulting in increased toxicity. Because AuNP are also known to bind to microalgae cell wall (Renault *et al.*, 2008; Gilroy *et al.*, 2014), another possibility is that binding of both MP and AuNP to *T. chuii* cells wall occurred, interfering with cells mobility, uptake of nutrients and/or oxygen and CO_2 changes, and resulting in reduced population growth. Since, the adsorption of both types of particles depends on the availability of surface cell wall to bind, this may also explain the effects were not addictive.

5.4. Conclusions

The AuNP tested were found to increase their size and modify their shape over 96 h in the test medium used (F/2 Guillhard medium), likely due to the formation of particles aggregates that increased of size along time. In good agreement, during the bioassay, decrease of ~ 5 nm AuNP concentrations in test medium along time was found. The concentrations of MP were also found to decrease in test medium over 96 h. No significant differences in the decrease of both AuNP and MP concentrations between treatments containing the substances individually or in mixture were observed, indicating that the two types of particles did not interact significantly in test medium.

Both AuNP and MP when tested individually slightly decreased the average specific growth rate of *T. chuii* (up to 21 % and 22 % of reduction, respectively) but with no significant differences in relation to the control group. Therefore, the NOEC was 3 mg/l for ~ 5 nm AuNP and 4 mg/l for MP, whereas the LOEC values were > 3 mg/l and > 4 mg/l, respectively. The mixture containing 3 mg/l of ~ 5 nm AuNP and 4 mg/l of MPssignificantly decreased *T. chuii* average specific growth rate (27 %), thus, it was more toxic than the same concentrations of its components tested individually. The NOEC for the mixture was 0.3 mg/l AuNP + 0.9 mg/l MP, whereas the LOEC was 3 mg/l AuNP + 4 mg/l MPs. Overall, the

findings of the present study indicate that the AuNP and MP tested have a low toxicity to *T. chuii*, but their mixtures are more toxic.

Chapter VI:

Main Conclusions and Future Work Perspectives

6.1. Main Conclusions

International surveys and scientific studies indicate extensive contamination of the marine environment, from coasts to remote areas, by microplastics (Browne *et al.* 2010; Van Cauwenberghe *et al.* 2013; Eerkes-Medrano *et al.* 2015; Lusher *et al.*, 2015; Waller *et al.*, 2017) due to widely use of plastics in various industries. Contamination, accumulation and potential adverse effects of microplastics are therefore one of the newest emerging environmental issues. The massive presence of microplastics in urbanized rivers and coastal environments (Ivar do sul and Costa, 2014; Jambeck *et al.*, 2015; McCormick *et al.* 2016; Lebreton *et al.,* 2017) and the physicochemical properties of microplastics allow them to interact with a wide-range of marine organisms from algae and zooplankton species (Goldstein and Goodwin, 2013; Setälä *et al.*, 2014; Prata *et al.*, 2018) to fin whales (Fossi *et al.*, 2016). Microplastics can be a threat for ecosystems not only because of their effects on marine organisms but also because of their potential for changing the properties of other environmental contaminants (Thompson *et al.*, 2009; Fries and Zarfl, 2012; Brennecke et al., 2017). However, our knowledge and understanding of their ecotoxicological effects in marine environments is still limited.

In addition, nanometals are known as another serious threat to aquatic environments. Therefore, there are many concerns about nanometals due to their widespread use in biomedicine and otherapplications, and their ability to interact with other environmental pollutants in aquatic environment (Lapresta-Fernández *et al.,* 2012). Thus, their environmental impacts are difficult to study and predict particularly in marine and estuarine environments.

The review made in the Chaper II showed that field studies indicated a global contamination of marine water, sediments and organisms by microplastics. Moreover, laboratory studies showed a wide range of toxic effects caused by microplastics on marine organisms and trophic transfer of microplastics and associated chemicals. Limited modeling approaches have been used to model realistic environmental scenarios. Models generally indicate that contribution of plastics to accumulation of associated chemicals and transfer to the aquatic organisms is lower than from other sources (Koelmans *et al.*, 2016). In fact, some recent reviews suggest that the bioaccumulation of chemicals associated to microplastics is most likely overwhelmed by uptake through other pathways (GESAMP, 2015; Koelmans *et al.*, 2016). A limited number of studies investigating the effects of microplastics and other common contaminats in organisms exposed to mixtures. Several of these studies showed that the toxicity induced by the mixtures can be higher than the effects

induced by their components when tested individually, highlighting the need of more research on this topic.

The challenges of assessing the Environmental fate and toxicity of metallic nanoparticles in the marine environment, and their biological effects were reviewed in Chapter III. The main findings were that physicochemical properties of metallic nanoparticles (e.g. size, shape, degree of agglomeration and coating) and the characteristics of the environment (e.g. pH, temperature, salinity and ionic strength) should be taken into consideration when assessing the toxicity of such environmental contaminants. Furthermore, it is of crucial importance to understand how they are uptaken by organisms, how they are distributed inside the body, and their interactions with other environmental contaminants (Fabrega *et al.*, 2011; Schirmer *et al.*, 2013; Velzeboer, 2014; Peijnenburg *et al.*, 2015; Zhang *et al.*, 2016; Peng *et al.*, 2017). Data on biological effects show that metallic nanoparticles can be toxic to bacteria, algae, invertebrates, fish and mammals (Klain *et al.,* 2008). However, understanding their mechanisms of toxicity is complex (Oukarroum *et al.,* 2012a). Also, toxicity of nanometals can change from one material to another due to their differences in properties, synthesis methods, concentrations, solubility, coating and surface characteristics, and presence of additives. Thus, without a proper characterization of of nanometals in the particular medium to be tested, a general conclusion on their toxicity might be difficult.

In Chapter IV, the toxicity of a particular type of microplastics (polyethylene microspheres, 1-5 µm diameter) alone, copper alone, and binary mixtures of the tow substances to the marine microalgae *T. chuii* were investigated. The results indicated that the tested microplastics at concentrations up to 1.472 mg/l had no significant effects on *T. chuii* average specific growth rate but a slight decrease of the average specific growth rate with the microplastics concentration increase was found. These results suggest that significant effects may be induced at higher microplastic concentrations. In the range of concentrations tested, copper significantly inhibited the average specific growth rate of the microalgae with EC_{50} of 0.139 mg/l (95 % CI: 0.106 – 0.187 mg/l). The toxicity curves of copper in the presence and absence of 0.184 mg/l of microplastics were not significantly different, indicating that in the range of microplastics and copper concentrations and environmental conditions tested, microplastics did not modulate the toxicity of copper to *T. chuii*.

In Chapter V, the toxicity of another type of microplastics (1-5 µm diameter microespheres of unkown composition) alone, AuNP (~ 5 nm diameter) alone, and binary mixtures of the tow substances to the marine microalgae *T. chuii* were investigated. The results indicated that the tested microplastics (at concentrations up to 4 mg/l) and AuNP

(at concentrations up to 3 mg/l) had no significant effects on *T. chuii* average specific growth rate, despite slight reductions at the highest concentration tested. The mixture containing the highest concentrations of both microplastics and AuNP significantly reduced the average specic growth rate of *T. chuii* indicating a higher toxicity of the mixture relative to the corresponding concentrations of its components when tested alone. This study also evidencied changes in the behaviour and concentrations in the water column of microplastics and AuNP.

Overall, the results of Chapters IV and V highlighted the need of characterizing microplastics and AuNP behaviour and changes during toxicity bioassays, the relevance of further assessment of the toxicity mixtures of microplastics and other environmental contaminants to marine organisms and evidenced several challenges in toxicity testing with microplastics and nanomaterials and in the comparison of toxicity results. The findings of these studies are in agreement with findings of previous autors that tested different types of microplastics and AuNP in *T. chuii* or in other microalgae species in comparable conditions (*e.g.* Blasco *et al.*, 2012; Moreno-Garrido *et al.*, 2012; García-Cambero, 2013; Behra *et al.*, 2015; Sjollema *et al.*, 2016; Long *et al.*, 2017; Prata et al., 2018).

6.2. Future work perspectives

The work done in the scope of the present Thesis, highlighted the need of more research and technological improvements as further indicated.

Improvement and standardization of methods to detect and quantify very small particles, such as microplastics with size in the low micro and nano scales and nanomaterials, in complex environmental samples (e.g. environmental waters, soils and sediments) is needed. In fact, the information on the microplastics present in the environment, does not include very small particles of plastic due to lack of cost-effective technology to extract and quantify them at large scale (Andrady, 2017). In relation to nanomaterials, similar problems exist and the environmental contamination by the wide diversity of nanomaterials in use is largely unkown (Ray *et al.*, 2009; Geissen *et al.*, 2015).

Characterization and behaviour of microplastics and nanomaterials in complex matrixes (such as environmental waters, soils and sediments): this is crucial to assess and quantify the environmental contamination by these materials, to understand their bioavailability and interactions with abiotic and biotic components of ecosystems, and to assess their toxicity (Anderson *et al.*, 2016). This knowledge is crucial to their environmental and human risk assessment.

Ecotoxicological and ecological effects of microplastics and nanoparticles and the other chemicals that they may contain: considering the high diversity of microplastics and nanomaterials that are suspected to be present in the environment, more data on their effects on organisms, populations, communities, and ecosystem functioning and services is needed (Moore, 2006; Navarro *et al*., 2008; Wang *et al*., 2016; Green *et al*., 2017; Peng *et al*., 2017). A major aspect in relation to both environmental and human health is the potential biomagnification of such materials in food webs increasing the risks for top predators and humans consuming contaminated food (Ferreira *et al*., 2016).

Toxicity induced on wild species and humans exposed to mixtures of contaminants, including those mixtures having microplastics and/or nanomaterials; the toxicity of mixtures is still poorly understood (Gallo *et al*., 2018; Sá *et al*., 2018), and this knowledge is of fundamental importance to environmental and human risk assessment as in real scenarios exposure to mixtures rather than to individual substances occurs (Barboza *et al*., 2018b; Smith *et al*., 2018).

Finally, strategies and technologies to reduce the environmental pollution by microplastics and nanomaterials, should be developed, the existing ones improved, and implemented.

Chapter VII:

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