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Physical and chemical toxicity of microplastics on marine organisms

David Leistenschneider

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École doctorale des Sciences et de l'environnement (ED₁₂₉)

Physical and chemical toxicity of microplastics on marine organisms

Thèse préparée et soutenue par
David Leistenschneider

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"Nous sommes comme des nains sur des épaules de géants. Nous voyons mieux et plus loin qu'eux, non que notre vue soit plus perçante ou notre taille plus élevée, mais parce que nous sommes portés et soulevés par leur stature gigantesque."

Bernard de Chartres XII^{ème} siècle

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Abstract (EN)

Due to the ubiquitous presence of plastic in the environment, it has drawn the attention of governments, civilians and the scientific community. Indeed, plastic is a peculiar pollutant since it encompasses three types of toxicity: chemical, physical and biological. In this PhD thesis, we will only focus on the first two.

Plastic chemical toxicity can be divided into two categories: leaching and adsorption. The aim of the first chapter was to better decipher the toxicity kinetics of plastic leaching and determine the origin of toxicity. A wide variety of chemicals (i.e. 16,000) are present in plastic and can be leached into the environment. Most of the articles studying leaching performed leaching time up to 1 month. Therefore, the toxicity of leachates, up to 8 months, was determined through 2 standardized toxicity tests (bacteria and sea urchin). The origin of toxicity was evaluated through an inorganic analysis of the leachates. The oxodegradable polyethylene showed the highest toxicity. For the first time we determined the toxicity kinetics (i.e. exponential decay) between the EC_{50} values and leaching time. The inorganic element analysis only partially explained the observed toxicity.

The second chapter focuses on the adsorption of pollutants on plastic surfaces, a topic predominantly studied in laboratory conditions. Thus, plastic pellets were immersed along 9 European rivers along the river-to-sea continuum. After a month of immersion, pollutants adsorbed on plastic surface were extracted and their subsequent toxicity analyzed through two standardized tests (bacteria and oysters). Moreover, the organic and inorganic compounds adsorbed were examined through chemical analyzes. Extracted adsorbed pollutant induced toxicity for bacteria but not for oysters. The number of toxic sites increased along the river-to-sea continuum with a decrease at the estuary. Moreover, the inorganic analysis on the extracted elements indicated a spatial trend between the different sites. The inorganic element analysis on pellets indicate clearly a difference between the control and immersed material. The organic chemical analysis showed an important adsorption on plastic surfaces with up to 83 different chemicals.

The last chapter aims to better understand the physical toxicity of plastic, allowing a holistic evaluation of chemical and physical toxicity. Mediterranean mussels were exposed to custom-made microplastics at environmentally relevant concentrations for 100 days.

Special care was taken to ensure that the shape was representative of the environmental pollution. Toxic effects at the individual level (growth, condition index, survival rate) were examined. No effects were observed on the toxicological parameters tested.

Abstract (FR)

En raison de l'omniprésence du plastique dans l'environnement, on constate un intérêt croissant de la part des gouvernements, des civils et de la communauté scientifique. Le plastique est un polluant particulier du fait qu'il englobe 3 types de toxicité : chimique, physique et biologique. Cette thèse de doctorat se concentre spécifiquement sur les deux premiers aspects.

La toxicité chimique du plastique peut être catégorisée en deux types : la lixiviation et l'adsorption. Le premier chapitre de cette étude vise à mieux comprendre les cinétiques de toxicité des lixiviats de plastique et à identifier l'origine de la toxicité via une analyse chimique. Le plastique est composé d'une multitude de produits chimiques (plus de 16,000) qui peuvent être relâchés dans l'environnement. Les études sur la lixiviation ont généralement porté sur des périodes allant jusqu'à un mois maximum. C'est pourquoi des lixiviats de différents temps, jusqu'à 8 mois, ont été réalisés. La toxicité a été mesurée à travers deux tests standardisés avec comme modèle biologique des bactéries et des embryons d'oursins. L'origine de cette toxicité a été examinée à travers une analyse inorganique des lixiviats ainsi que des plastiques avant et après la lixiviation. Le plastique identifié comme le plus toxique est le polyéthylène oxodégradable. Pour la première fois, la cinétique de toxicité des lixiviats a été décrite. Elle est caractérisée par une décroissance exponentielle entre les valeurs de CE50 et le temps de lixiviation. L'analyse des éléments inorganiques n'a fourni qu'une explication partielle de la toxicité observée.

Le deuxième chapitre se concentre sur l'adsorption des polluants à la surface des plastiques, un sujet largement étudié en laboratoire concentré sur l'environnement marin. C'est pourquoi des granulés de plastique ont été immergés le long de 9 fleuves européens, le long du continuum fleuve-mer. Après un mois d'immersion, les polluants adsorbés ont été extraits et soumis à une analyse de leur toxicité à travers deux tests standardisés (bactéries et embryons d'huîtres). Des analyses chimiques des composés organiques et inorganiques adsorbés ont également été réalisées. Les résultats ont induit une toxicité chez les bactéries, mais pas chez les huîtres. Une augmentation du nombre de sites toxiques le long du continuum fleuve-mer, suivie d'une diminution à l'estuaire a été observée. De plus, on observe une différence entre les différents sites indépendamment

des fleuves des éléments inorganique sur les extraits de DMSO. L'analyse chimique des éléments inorganiques a mis en évidence des différences claires entre les granulés immergés et les granulés contrôle, tandis que l'analyse organique a révélé une adsorption importante (jusqu'à 83 produits chimiques différents) à la surface des plastiques.

Enfin, le dernier chapitre vise à explorer la toxicité physique du plastique pour compléter l'évaluation de sa toxicité chimique et physique. Des moules méditerranéennes ont été exposées à des microplastiques conçus pour refléter les concentrations environnementales pendant 100 jours. La forme des microplastiques a aussi été sélectionnée pour être représentative de la pollution environnementale. Les effets sur la croissance, l'indice de condition et le taux de survie des moules ont été examinés. Aucun effet n'a été observé sur les paramètres toxicologiques étudiés.

Table of content

STATE OF THE ART 1

1. GENERAL INTRODUCTION	1
2. A CRITICAL REVIEW ON THE EVALUATION OF TOXICITY AND ECOLOGICAL RISK ASSESSMENT OF PLASTICS IN THE MARINE ENVIRONMENT	10
3. PRESENTATION OF THE THESIS OBJECTIVES AND ORGANIZATION	42
REFERENCES.....	2

CHAPTER 1 – TOXICITY OF PLASTIC LEACHING ON BACTERIA AND SEA URCHIN20

FOREWORD	20
MANUSCRIPT TITLE: CRITICAL IMPACT OF LEACHING TIME ON MARINE TOXICITY TESTS FOR PLASTICS	22
ABSTRACT.....	23
1. INTRODUCTION	24
2. MATERIALS & METHODS	27
3. RESULTS	31
4. DISCUSSION	40
5. CONCLUSION	44
REFERENCES.....	45
SUPPLEMENTARY DATA 1.1 FTIR RESULTS OF PE-OXO (A), PE-BUBBLE (B), PE-PURE (C), PE-RECYCLED (D).....	51
SUPPLEMENTARY DATA 1.2 INORGANIC ELEMENT ANALYSIS ON THE LEACHATES AND VIRGIN/LEACHED PLASTICS...	53

CHAPTER 2 – TOXICITY OF ADSORBED POLLUTANTS ON PLASTIC SURFACE54

FOREWORD	54
MANUSCRIPT TITLE: ENVIRONMENTAL POLLUTION ADSORBED ON POLYETHYLENE MICROPLASTIC AND ITS RELATED TOXICITY: A CASE STUDY ALONG 9 EUROPEAN RIVERS	56
ABSTRACT.....	57
1. INTRODUCTION	58
2. MATERIALS AND METHODS.....	60
3. RESULTS.....	64
4. DISCUSSION.....	71
5. CONCLUSION	74

REFERENCES	75
SUPPLEMENTARY DATA 2.1 CONCENTRATION (MG/L) OF INORGANIC ELEMENTS IN THE PELLETS	80
SUPPLEMENTARY DATA 2.2 CONCENTRATION (MG/L) OF INORGANIC ELEMENTS IN THE DMSO EXTRACTS	81
SUPPLEMENTARY DATA 2.3 NUMBER OF ORGANIC ELEMENTS PRESENT IN THE DMSO EXTRACTS	82
SUPPLEMENTARY DATA 2.4 PERCENTAGE OF OYSTER MALFORMED LARVAE AFTER EXPOSITION TO THE DMSO EXTRACTS	83

CHAPTER 3 – PHYSICAL TOXICITY OF PLASTIC 84

FOREWORD	84
MANUSCRIPT TITLE: LIMITED IMPACT OF CHRONIC EXPOSURE TO REALISTIC CONCENTRATIONS OF MICROPLASTICS ON THE GROWTH AND CONDITION INDEX OF THE MUSSEL (<i>MYTILUS GALLOPROVINCIALIS</i>)	86
ABSTRACT	87
1. INTRODUCTION	88
2. MATERIALS AND METHODS.....	91
3. RESULTS.....	95
4. DISCUSSION.....	97
5. CONCLUSION	100
REFERENCES	102
SUPPLEMENTARY DATA 3.1 PARTICLE DISTRIBUTION OF PE-OXO (A), PE-PURE (B) AND DIATOMITE (C) IN FUNCTION OF THE SIZE. PERCENTAGE WRITTEN REPRESENT THE NUMBER OF PARTICLES OF THE DESIRED SIZE CLASS	108

CONCLUSIONS AND PERSPECTIVES..... 111

CONCLUSIONS AND PERSPECTIVES OF THE CHAPTER 1.....	111
CONCLUSIONS AND PERSPECTIVES OF THE CHAPTER 2.....	114
CONCLUSIONS AND PERSPECTIVES OF THE CHAPTER 3.....	116
BROADER PERSPECTIVE AFTER THE THESIS	118
REFERENCES	122

BIBLIOGRAPHY 127

List of figures

Figure 1: Description of the physical, chemical and biological toxic effects of plastics on a model organism (mussel) credit: Plastic At Sea.....	3
Figure 2: Denomination used for every plastic sizes (adapted from (GESAMP, 2015))	6
Figure 3: Origin and fate of plastic debris in the marine environment (M. J. Mercier et al., 2023).....	8
Figure 4: Graphical abstract of the review Leistenschneider & al. 2023.....	12
Figure 5: Compilation of the observed effects of plastic toxicity on marine organisms described at the molecular, cellular, tissue, individual and population levels in the plankton, nekton and benthic species.	16
Figure 6: Compilation of the MPs' characteristics in toxicity experiments: chemical composition, size, shape, and biological colonization (= plastisphere). PE = Polyethylene; PVC = Polyvinyl Chloride; PS = Polystyrene;  = Microplastic (1-1000 µm);  = Nanoplastic (< 1 µm);  = Regular shape;  = Irregular shape;  = Fibers. 22	
Figure 7: Range of MPs concentration (particle/L) used in the reviewed articles. When needed, an approximation of number of particle/L was calculated from data initially expressed mg/L (see conversion formula in the text).....	23
Figure 8: Repartition of experiments duration in the reviewed articles	26
Figure 9: Characteristic of the plastic used in the reviewed articles: size (a), shape (b), presence of additives and adsorbed pollutants (c) or polymer composition (d). Chart (e) and (f) decomposes the polymer composition in function of size class. PE = Polyethylene; PLA = Polylactic acid; PMMA = Polymethylmethacrylate; PP = Polypropylene; PS = Polystyrene; PVC = Polyvinyl chloride; B-Plastic sizes used in experimental studies. SMP = Small microplastic (1-1000µm); NP = Nanoplastic (1-1000nm) and N/A = leachates (dissolved).	27
Figure 10: Graphical representation of the PhD thesis objectives	43
Figure 11: Principal component analysis on the ICP results of the leachates (A) and the virgin/leached plastics (B). Only the metals and other toxic trait elements are presented. Cu = Copper; Co = Cobalt; Cr = Chromium; Fe = Iron; Mn = Manganese; Ni = Nickel; Pb = Lead; Ti = Titanium; Zn = Zinc. SW = Seawater.	33
Figure 12: Means of bioluminescence inhibition of <i>Aliivibrio fischeri</i> for all PE-oxo leachates. Toxicity values are expressed in percentage of bioluminescence inhibition in function of the plastic concentration in the leachate(g/L). The curve represents the drc model and the shaded areas indicate the confidence interval at 95%.	34
Figure 13: Means of the embryotoxicity values of leachates on <i>Paracentrotus lividus</i> larvae. Toxicity values are expressed in percentage of malformed larvae in function of the plastic concentration in the leachate (g/L). The curve represents the drc model and the error bars indicate the standard deviation.....	36
Figure 14: EC50 (expressed in g/L of plastic) in function of the leaching time (expressed in months). Grey area represents leachates of 1 and 10 days, which did not induce any toxicity.	37
Figure 15: Impact of plastic leaching on the larvae of <i>Paracentrotus lividus</i> after 1 day (A) and 10 days (B) at the concentration 10g/L. The letters indicate the statistical differences between the different conditions by	

leaching time. The box represents the interquartile range, the line the median. The red triangle represents the mean value. SW = Seawater; * = significant difference between 1 and 10 days of leaching..... 38

Figure 16: Graphical representation of the rivers sampled by the mission Tara Microplastiques 2019..... 60

Figure 17: Principal component analysis on the ICP results for different metals and trace metals in the mineralized plastic pellets classified by rivers. Cr = Chromium; Cu = Copper; Fe = Iron; Mn = Manganese; Ni = Nickel; Pb = Lead; Ti = Titanium; Zn = Zinc; Ctrl_PE = Control Polyethylene (not immersed)..... 65

Figure 18: PCA on the ICP results of metals and trace metals on the DMSO extracts classified by sites. Cu = Copper, Fe = Iron, Mn = Manganese, Pb = Lead, Ti = Titanium, Zn = Zinc. Sampling sites are described as Site 1 (marine), Site 2 (estuarine), Site 3 (intermediate salinity), Site 4 and Site 5 (downstream and upstream of the first heavily populated city close to the estuary). Ctrl_PE = Control Polyethylene (not immersed) 66

Figure 19: PCA on the sum of the LCMS results for each organic pollutant category on DMSO extracts classified by river. ED = Endocrine disruptors; Pharma = Pharmaceuticals; Phenol = Phenol & Bisphenol; PHA = Polyhydroxy acids, Polychlorinated Biphenyls and Polybrominated Diphenyl Ethers; Antimicro = Antimicrobial; PFAS = per- and polyfluoroalkyl substances; ExtrHaz = Extremely Hazardous substances; WatCont = Water Contaminants; Additives = Plastic additives; Control Polyethylene (not immersed). 68

Figure 20: Microtox results on 1% organic extracts of PE pellets immersed for one month at different locations of 9 European river-sea continuum. The results are expressed as the percentage of the relative bioluminescence inhibition compared to control water of the marine bacteria *Aliivibrio fischeri* exposed for 30 min to 1% of the extract (Mean +/- SD, n=3). Asterisks represent a significant difference with the virgin polymer DMSO extract. This extract is represented by the red dotted line with the standard deviation being the gray area. 69

Figure 21: Graphical representation of the percentage of significantly toxicity observed on the sampling sites for all combined rivers. n = total number of sampling sites with data available 70

Figure 22: Graphical representation of the experimental set up for microplastics exposition to mussels. A = Plasticells®; B = Dichotomies; C= Lifting pump; D = Stirring pump; E = Bubbler..... 93

Figure 23: Survival percentage by condition in function the experiment times (days). SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable..... 95

Figure 24: Condition index results for each condition separated by the exposition time. Box plots correspond to median, 1st and 3rd quartile and 95% extrema; red triangles correspond to the mean. * = Statistical difference; SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable..... 96

Figure 25: Mussel shell growth between each experimental condition after 50 and 100 days. Box plots correspond to median, 1st and 3rd quartile and 95% extrema; red triangles correspond to the mean. SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable. 97

Figure 26: Life cycle analysis of plastic materials (Life Cycle Approach to Plastic Pollution) 120

Figure 27: Life cycle analysis of plastic materials, along with the solutions and stakeholders needed for mitigating the environmental impact of plastics. SPP = Sustainable Public Procurement (Life Cycle Approach to Plastic Pollution) 121

State of the art

1. General introduction

1.1 What is toxicity?

“Everything is poison, nothing is poison, it is the dose that makes the poison”.

Paracelsus

In the book “Environmental toxicology: current developments”, toxicology is defined as the science of the study qualitative and quantitative effects on biology induced by chemical or physical agents (Rose, 1998). A more precise definition would add also the biological agents, including microbes and parasites (Garcia-Arredondo, 2016). Since this thesis focus concerns the impact on marine organisms, the field of concern can be described as environmental toxicology or more precisely as aquatic toxicology (Curtis D. Klaassen and Watkins, 2010).

Toxicity is described as the elucidation of the causality chain of interactions and their time course (exposure) between biological models and chemical, physical or biological agents. In other words, the aim is to determine the exposure-responses consisting of dose and time-responses. From those dose and time-responses, practical thresholds to evaluate the agent safety and allow an efficient comparison with other agents are determined (Rose, 1998).

When evaluating a particular agent, one of the main parameters influencing toxicity is the dose (i.e. concentration) used. Indeed, 500 years ago, a famous scientist (Paracelsus) already exposed a quantitative causality link between dose and impact on the organism. Later, a mathematical formula was introduced to modelized these interactions through a dose response model. Nowadays, a great variety of dose response models exist (Altshuler, 1981). This model allows to determine one key value that has become a standard statistic for the evaluation and comparison of a contaminant which is the EC_{50} (Noel et al., 2018). The EC_{50} is defined as the contaminant’s dose that will induce 50% of a specific biological effect under specific conditions.

Another critical parameter that influences greatly the response is the exposure time. Indeed, the duration of exposure has a critical impact on the consequent toxicity. Therefore, two main denominations in function of the exposure time are used: acute and chronic. Acute toxicity tests are short term tests that measure the effects at relatively high concentrations whereas chronic toxicity tests are long term tests with relatively lower concentrations (United States Environmental Protection Agency, 1994). An intermediate exposure time is often describes as subchronic (Rose, 1998). For acute toxicity tests, defined exposure times varies in the literature, corresponding either up to 96h (Newman, 2015; United States Environmental Protection Agency, 1994), 7 days (ASTM E2455-22), or up to 14 days (Curtis D. Klaassen and Watkins, 2010; Rose, 1998). Subchronic exposure time was described as between 7-28 days (ASTM E2455-22) or between 14 and 90 days (Curtis D. Klaassen and Watkins, 2010; Rose, 1998). Finally, chronic tests have also been described as more than 28 days (ASTM E2455-22), 90 days (Rose, 1998), or between 6 months and 2 years (Curtis D. Klaassen and Watkins, 2010). However, instead of having arbitrary exposure time for all species, a more relevant way would be to define them in function of the tested organism's life span (Blasco et al., 2016).

Moreover, the model species tested also influence greatly the toxicity response. Indeed, the organism sensitivity varies greatly between species, phenotypes, life history stages (Taddei et al., 2021). This is closely link to the body size of the organism (Taddei et al., 2021). Generally, the larger the body size the higher the resistance against stress (Reinikainen et al., 1998). Moreover, the life stage of the model organism plays also a role in the sensitivity. Generally, early life stages are more sensitive than adults (Hutchinson et al., 1998; Ringwood, 1990).

Toxicology was mainly focused on the anthropogenic chemicals, as shown in the book "Essentials of toxicology", where toxicology is defined as the impact of (only) chemicals on living organisms (Curtis D. Klaassen and Watkins, 2010). However, plastic is a peculiar contaminant that encompasses the 3 types of toxicity (i.e. physical, chemical and biological) (Figure 1) (Leistenschneider et al., 2023). Details are presented in the up-coming review. Most chemicals dissolve into water (Yaws et al., 2005) until a certain point when reaching their solubility values. However, plastic peculiarity also lies in the fact it does not dissolve into water and is hydrophobic (Kim et al., 2015). Biases come in play when the contaminant

tested does not dissolve into water since there is no homogenous repartition of the toxicant.

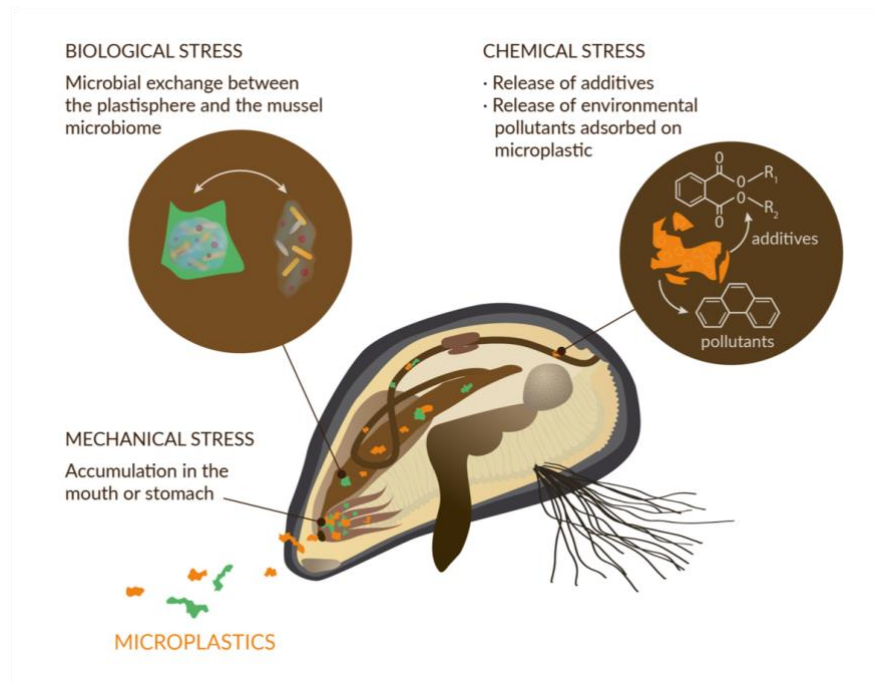


Figure 1: Description of the physical, chemical and biological toxic effects of plastics on a model organism (mussel) credit: Plastic At Sea.

1.2 What are plastics and plastic pollution?

The origin of the term plastic means capable of being shaped or molded. Therefore, plastic can have different meaning depending on the field (e.g. physical properties, behavior of materials, adaptability of organs). Here the term “plastic” is used to define a synthetic material made from a wide range of organic polymers to which different plastic chemicals are added (Wagner et al., 2024). The former definition of plastic was resumed to a polymer to which additives are added (GESAMP, 2015). However, when considering the full cycle of plastic production, only mentioning additives leads to the omission of starting substances, processing aids and non-intentionally added substances (NIAS).

Plastic followed an exponential growth in the common use since the 1950s thanks to numerous advantages (e.g. lightweight, low production cost, durability, ease of processing) compared to the material that were used prior to plastic (Andrady, 2011a). Therefore, an overconsumption of these polymers was made and nowadays plastics are present in a vast number of applications (e.g. packaging, construction, electronics, agriculture and scientific research). The plastic production reached 430 million tons in 2022 (UNEP, 2023b), that

combined with a poor waste management, lead to an important pollution of the environment. This is especially true in the marine environment since it is the final receptacle of land-based pollution (Tharpe, 1989). Plastic pollution is ubiquitous in the environment with its presence in all water compartment (surface, water column, deep sea and sediment) (Erni-Cassola et al., 2019) including the arctic waters (Lusher et al., 2015), but also in water and snow samples at the top of mount Everest (Napper et al., 2020). To conceptualize the magnitude of plastic pollution, a study evaluated (through a mathematical model coupled with environmental data of marine surfaces) the number of plastic debris in the oceans and estimated that there are more plastic items in the oceans than there are stars in our galaxy (Van Sebille et al., 2015).

This high number of plastic items is mostly linked to the degradation of larger plastic items that enter the environment. Indeed, plastic items undergo weathering degradation that happen at different rate in function of the location. For example, a much faster rate of degradation is observed on beaches compared to floating debris. Sunlight/UV radiation and mechanical abrasion are the most efficient cause of plastic degradation. However, pH, salinity, temperature and presence of a biofilm are also factors that influence degradation (Dimassi et al., 2022). Most experiments are only focused on the primary stages of degradation. Therefore, further studies are needed to better understand their degrading potential in the marine environment (Dimassi et al., 2022). Nevertheless, this mechanism is extremely slow and may take centuries or even millennium until total degradation of plastic especially in the marine environment (Chamas et al., 2020).

1.3 Levels of plastic pollution in the environment

To perform a relevant toxicity test, a precise characterization of plastic pollution (i.e. concentration, type, size, shape and state) in the environment is crucial. Therefore, a literature review of these important parameters is disclosed below. Since, this thesis is focused on microplastics (MPs), the following information are focused on this plastic size.

1.3.1 Plastic concentration at sea

There are large variations in the concentration of MPs in the marine environment, depending on the geographical locations. The different concentrations at different

geographical location are impacted by several factors, the most important are: water currents, wind, rain, season of the year, waste management (Chen et al., 2020; Zhang et al., 2021a). Another important factor is related to the type of measurement. Depending on sampling location, there are 3 main types of unit used: surface water (number of particles/m² or L), water column (number of particles/m³ or L) or sediment (number of particles/kg). The highest concentration recovered at sea surface was equal to 10² MPs/L (Song et al., 2014). However, most concentrations recovered in water column or deep sea were inferior to 1 MPs/L. Concentrations recovered in intertidal sediments were around 10⁰ and 10¹ MPs/kg (Erni-Cassola et al., 2019).

1.3.2 Polymer types encountered at sea

The most common polymers recovered in the marine environment are Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polyesters, Polyamides and acrylics (PP&A) (Erni-Cassola et al., 2019). They possess different repartition throughout the different zones studied.

At sea surface, more than 50% of plastic recovered was PE, followed by PP at 25% (Erni-Cassola et al., 2019). Since those two polymers possess a density lower than seawater, they are positively buoyant and will therefore float. In addition, they are the most produced polymers and represent together almost 40% of plastic production in 2022 (Plastics Europe, 2023). It is interesting to notice that some high-density polymers were also recovered in large proportions on the sea surface. For example, along China's coasts 74% of the plastics found were composed of PET, one of the most common Polyester (Qu et al., 2018a). This can be explained through the huge textile industry present in China (You et al., 2009).

In the water column, the vast majority of MPs were PP&A, which have a negative buoyancy. A small proportion of PE, PP, PS (ranged by proportion) were also present. However, in the Atlantic Ocean, PE, PP and PS represented 52% of the total plastic types in the water column (Enders et al., 2015), thus illustrating important disparities between the studies. In the deep sea, more than 75% of the total plastics belong to PP&A (Erni-Cassola et al., 2019).

In the sediments, the most common type of plastics is PP&A which is logical due to their high density. In addition, a consequent percentage of PE was found in the intertidal

sediments and PP was recovered in almost the same proportions as PE but in the subtidal sediments (Erni-Cassola et al., 2019).

As shown above, there is heterogeneity between studies. This can be due to the different geographical locations, affected by environmental factors like presence of manufacturers, tourists or water currents near the areas of sampling. The majority of the studies were performed at the sea surface (17 articles) or on the intertidal sediments (13), whereas studies in the water column or deep sea were limited (4 and 3, respectively) (Erni-Cassola et al., 2019). This can lead to possible misinterpretation linked to factors independent from the plastic types. Even though, a clear tendency has been observed where the positively buoyant plastics were mostly found at the surface and where the negatively buoyant plastics were mostly found in the water column and even more (in proportion) in the deep sea.

1.3.3 Size, shape and state

Plastics have been characterized into different denomination due to their size. Figure 2 indicates the denomination that will be used along this manuscript.

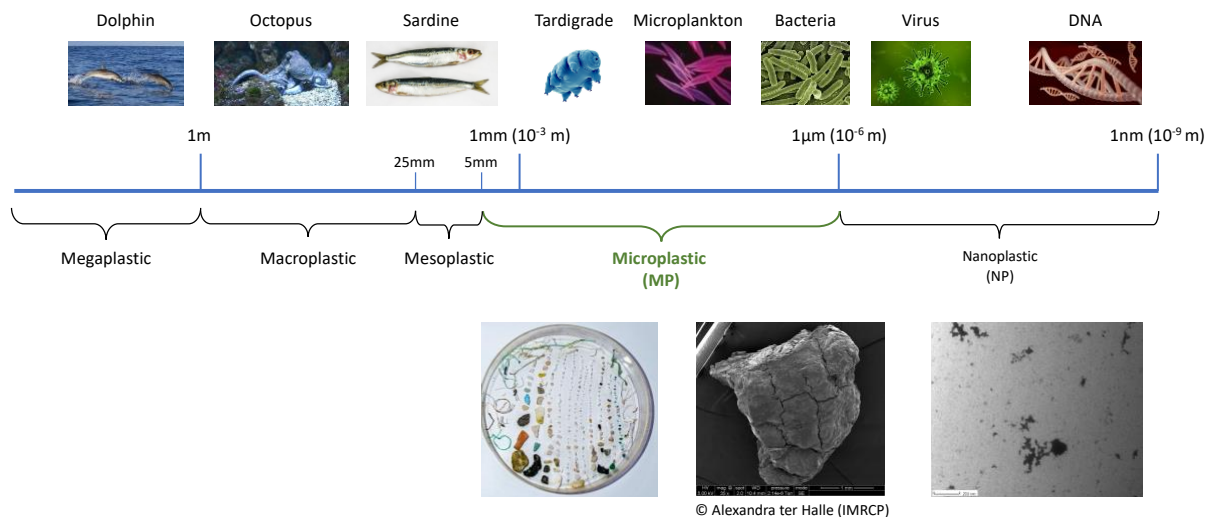


Figure 2: Denomination used for every plastic sizes (adapted from (GESAMP, 2015))

The plastic size recovered depends greatly on the type of equipment used. The most used equipment for surface sampling are the neuston nets (mesh size 333 µm) (Mu et al., 2019). Therefore, collecting and measuring the quantity of MPs (size <333 µm) or even nanoplastics is technically more complicated than using already existing equipments. Similarly, the water column and the deep sea are less studied due to technical complexities.

However, through degradation the number of particles increases, de facto the smaller the particle the higher the number (Bergmann et al., 2019; Erni-Cassola et al., 2017; Lorenz et al., 2019).

Microplastics also occur in different shapes. Some MPs are manufactured to be of a size inferior to 5 mm. They are named primary MPs and can either be industrial “scrubbers” used to blast clean surfaces, or micro-beads in cosmetic formulation, or plastic powders used in molding or even nanoplastics which are used in numerous industrial processes. In addition, resin pellets, which are the primary material for fabricating plastics products, are also considered as primary MPs. They usually possess a spherical or cylindrical shape. On the other hand, the secondary MPs are the result of the degradation of larger plastic products over time and possess irregular shapes (GESAMP, 2015).

Since most of the plastic that arrives at sea comes from land, plastic is most of the time already aged before reaching the marine environment. Furthermore, its travel across marine waters increases its aging and degradation. During this ageing plastic colonization happens quickly: in less than 24h for the earliest colonizer (Oberbeckmann et al., 2015) indicating that nearly all of the plastic located in the marine environment possess a biofilm. De facto, the vast majority of environmental MPs do not possess a virgin state and in addition are covered by a biofilm called the “Plastisphere” (Zettler et al., 2013).

1.3.4 Accurate estimation of plastic concentration depends on sampling constraints

Even though the results found in the literature are supposed to represent an accurate value of the MPs concentration encountered in the environment, several limits can be brought to light.

One important bias is a high concentration variability due to the complex currents present in the ocean (Law and Thompson, 2014). Therefore, results must be taken with hindsight, especially the one obtained near oceanic gyres (Law et al., 2010).

The majority of studies focused on environmental exposition study the seawater surface (Erni-Cassola et al., 2019). Since most of plastic waste is composed of buoyant plastics (Jambeck et al., 2015) it could be considered an easy and relevant way to evaluate plastic pollution. However, it has been demonstrated that as plastics stay at sea, their buoyancy decreases due to biofouling (Song et al., 2014). Indeed, plastics like other substrates are colonized by a series of organisms (i.e. diatoms, hydroids, ectocarpales, barnacles,

bryozans, etc.) right after their entry in marine environments (Andrady, 2011b). For small microplastics with a surface area to volume ratios below 100 (i.e. films, fragments and filaments with diameter $<50\ \mu\text{m}$) microbial biofilms alone are sufficient to cause sinking (Amaral-Zettler et al., 2021). Concerning larger debris, a decrease in hydrophobicity without changing its buoyancy favors its sinking just below the surface (M. J. Mercier et al., 2023). Therefore, plastics at the seawater surface only represent a fraction of the total MPs present at sea (Figure 3).

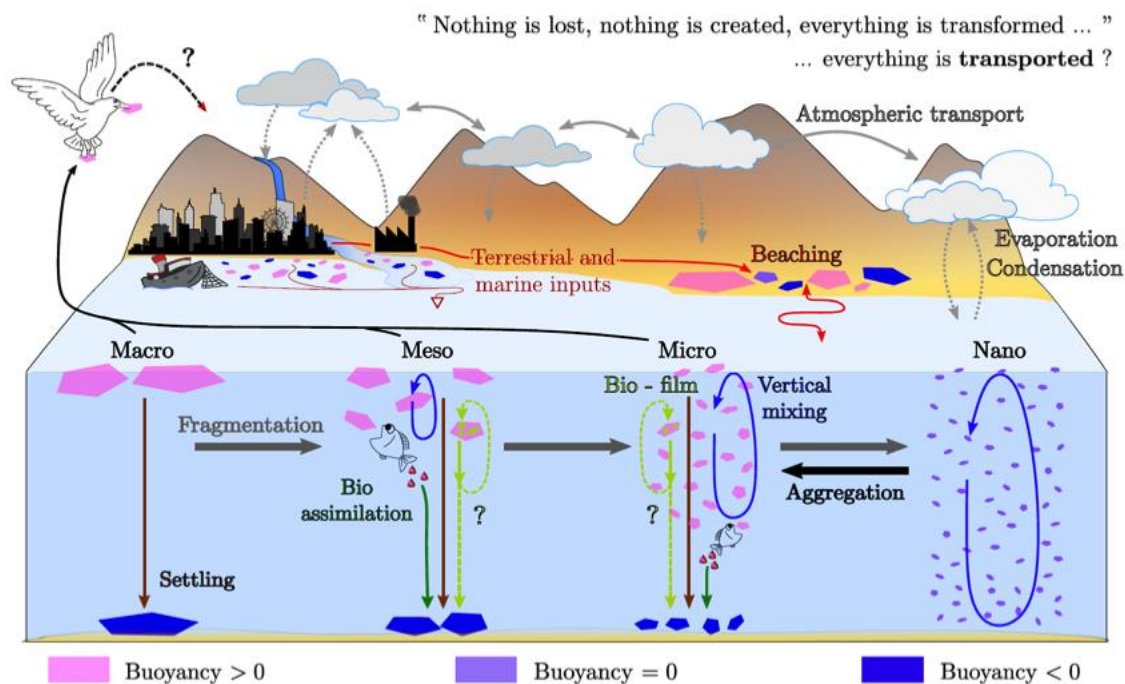


Figure 3: Origin and fate of plastic debris in the marine environment (M. J. Mercier et al., 2023)

Furthermore, the mesh size of the trawls used for plastic sampling may play an important role, where most of the surface surveys used a manta trawl. The mesh size was usually $333\ \mu\text{m}$ (Collignon et al., 2012; Law et al., 2010; Moore et al., 2005), resulting in missing a non-negligible portion of MPs. The proportion of MPs with a size $< 10\ \mu\text{m}$ is unknown and might even be more important than MPs with a size $> 10\ \mu\text{m}$. It has been demonstrated that, in terms of number, a vertical distribution exists and that more than 64% of MP were $< 40\ \mu\text{m}$, and that less than 1% of MP had a size higher than $300\ \mu\text{m}$ (Enders et al., 2015). Thus, the results obtained with a mesh size of $333\ \mu\text{m}$ represent only the “tip of the iceberg”. In addition, the actual standard for sampling the water surface has

been proven to have some limits. Indeed, a recent study disclosed that for a 1 mm mesh selectivity some MP, between 1 mm & 3 mm, still passed through the net (Tokai et al., 2021). This enlightens on the limits of the actual sampling methods.

2. A critical review on the evaluation of toxicity and ecological risk assessment of plastics in the marine environment

Foreword

Plastic pollution has become a crucial problem notably through its known toxicity. Even though plastic exists since the 1950's, it is only recently that plastic pollution is considered as a worldwide problem. In addition, plastic is a peculiar pollutant that encompasses 3 types of toxicity, i.e. physical, chemical and biological. In the last decade, a wide number of articles analyzed plastic toxicity and several reviews listed the different observed effects. However, few of them really focused on the biases of the current toxicity tests and the related standards.

Therefore, we decided to present a critical review of the plastic toxicity ongoing literature to identify the limits of the actual toxicity tests on marine organisms. To ensure a high-level and multidisciplinary overview of the current literature, this work was performed by a large list of co-authors that involved several research units affiliated to CNRS, ANSES, IRD, IFREMER, several Universities (Sorbonne Université, Université de Toulouse) and the SAS Plastic At Sea. This review paper is not simply a critique of the common limitations of toxicity tests found in the literature, but rather aims to optimize the representativeness of plastic toxicity tests.

First a review of 50 recent articles analyzing the toxic and ecotoxicity of plastic was performed. Second, biases between the toxicity tests and the environmental pollution were also disclosed. Finally, plastic risk assessment in relation with current standards, initiatives and laws towards plastic pollution was described.

Manuscript Title: A critical review on the evaluation of toxicity and ecological risk assessment of plastics in the marine environment

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Highlights:

- Recurrent toxic effects of plastic debris seen from molecular to population levels
- Tested conditions (concentration, type, size, shape) lack environmental relevancy
- Environmental studies on plastic debris are scarce
- Actual toxicity standards are not adapted to plastic

Status: Published (20/06/2023)

Graphical abstract

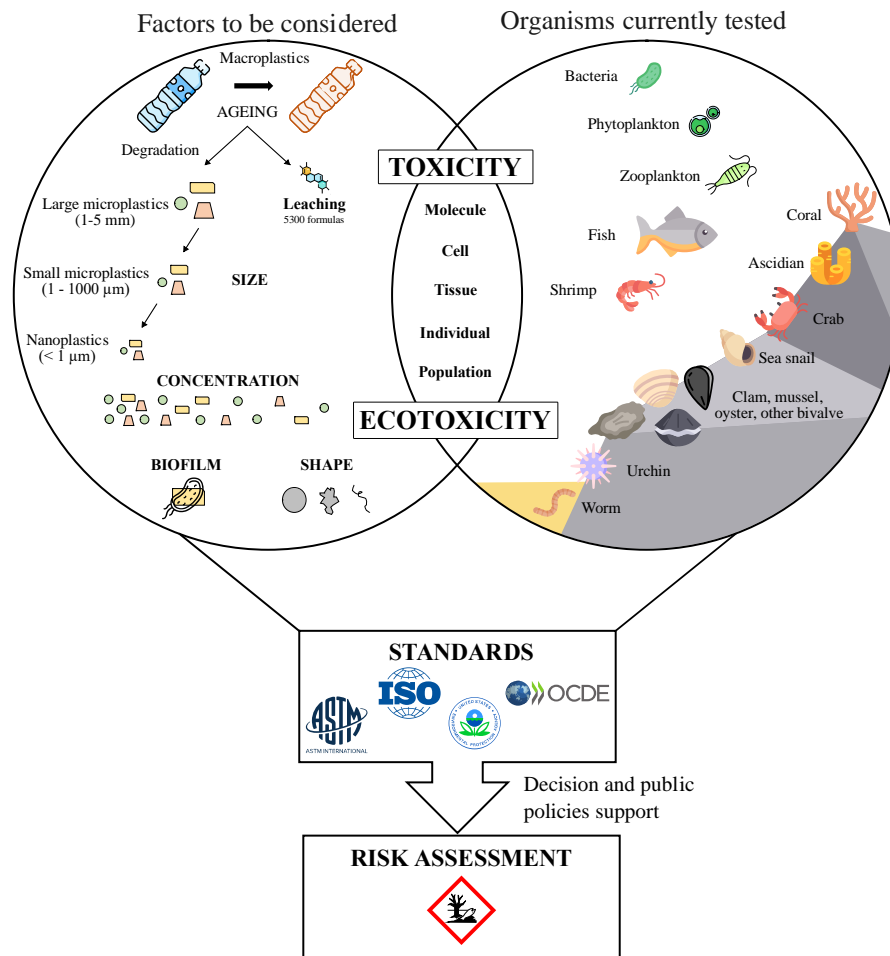


Figure 4: Graphical abstract of the review Leistenschneider & al. 2023

Abstract

The increasing production of plastics together with the insufficient waste management has led to massive pollution by plastic debris in the marine environment. Contrary to other known pollutants, plastic has the potential to induce three types of toxic effects: physical (e.g. intestinal injuries), chemical (e.g. leaching of toxic additives) and biological (e.g. transfer of pathogenic microorganisms). This critical review questions our capability to give an effective ecological risk assessment, based on an ever-growing number of scientific articles in the last two decades acknowledging toxic effects at all levels of biological integration, from the molecular to the population level. Numerous biases in terms of concentration, size, shape, composition and microbial colonization revealed how toxicity and ecotoxicity tests are still not adapted to this peculiar pollutant. Suggestions to improve

the relevance of plastic toxicity studies and standards are disclosed with a view to support future appropriate legislation.

Keywords: plastic debris, microplastics, nanoplastics, ecotoxicity, standards, quality assessment

2.1 Introduction

Plastic refers to a man-made material composed of polymers to which additives are supplemented to confer specific properties to the material (GESAMP, 2015). It is used in a wide variety of sectors, from packaging to electronics but also through construction, farming or transport (Geyer et al., 2017). This ubiquity is based on its low production costs and great variety of properties (e.g., lightweight, resilience, resistance to corrosion, ease of processing), explaining its use for a wide range of applications. Therefore, the plastic production followed an exponential increase since the 1950s. It almost doubled in the last twenty years, going from 234 to 460 millions of tons/year (OCDE, 2022).

The increase of plastic use leads to a significant waste production and thus to an important pollution all around the world (Bergmann et al., 2019), and especially in the oceans which are the final receptacle of mismanaged land-based wastes (Tharpe, 1989). Through different natural processes (light, heat, mechanical impact or biodegradation), plastics are fragmented in microplastics (MPs) (<5mm) that are subcategorized in 3 size classes: large microplastics (LMPs) (1-5 mm), small microplastics (SMPs) (1-1000µm) and nanoplastics (NPs) (< 1 µm) (Van Cauwenberghe et al., 2015). MPs are, in terms of number, the most dominant size-class of plastics in the oceans (Eriksen et al., 2014). In fact, according to a mathematical model, there are more MPs in the oceans than stars in the Milky Way (Van Sebille et al., 2015). The roots of the plastic issue lie in the dissonance between its single-use and one of its key features: durability. Its omnipresence is a growing concern for the entire marine ecosystem and represents physical, chemical and biological threats. The mechanical hazard corresponds to, for example, an obstruction or injury of feeding organs (GESAMP, 2015). Plastic also induces chemical toxicity through the release of additives or the sorption of environmental hydrophobic pollutants (Hermabessiere et al., 2017). Possible transfer from pathogenic strains from the microbial life living on plastics (so-called *plastisphere*) to an organism upon ingestion constitutes a biological threat (Bowley et al., 2021; Kirstein et al., 2016). The research interest on the toxic impacts of plastic has intensified in the last decade. Toxicity, defined as the potential for biological, chemical or physical stressors to affect an organism (Rose, 1998), is more studied on plastics than ecotoxicity, referring to the potential effects of stressors on an ecosystem, probably due to the higher level of complexity in the evaluation (Man et al., 2014). This

research effort is, however, necessary for an effective ecological risk assessment (ERA), which supports public policies (Curtis D. Klaassen and Watkins, 2010). ERA is defined as the assessment of the severity (nature and magnitude) and the probability of effects to nonhuman organisms, populations and ecosystems) (Suter, 2016). Contrary to other pollutants, no concentration threshold is indicated for the current seawater quality assessment, enlightening the lack of efficient standards to evaluate plastic toxicity. Indeed, the actual standards are mostly adapted to chemical toxicity that require dissolvable products, which is not compatible to plastic. We provided here some recommendations towards a better environmental relevance for future toxicity tests. Because standards are crucial for public policies and regulatory organizations, their limits and key points for their improvement are also disclosed.

The objective is not to produce an exhaustive list of toxic effects observed, since other reviews already treated this aspect (Guzzetti et al., 2018; Peng et al., 2020). In this review, we give a balanced critical overview of the literature on plastic toxicity in the marine environment. To ensure a base level of quality assurance, only peer-reviewed articles were selected. From the 87 articles reviewed, we selected 50 articles for this analysis. The selection criteria were a minimum of 20 citations (median of 86 citations, except for articles published after 2022) together with recent publication (96% were published in the last decade). We used common databases (ISI Web of Knowledge, Elsevier and Google Scholar) with search terms including: plastic, microplastic, synthetic polymers, toxicity, marine organisms. The following information was retrieved: species, type of plastic, size, shape, concentration, single and/or multiple exposure, duration of the test, endpoints and observed effects. The endpoints were classified in different levels of biological integration according to (Galloway et al., 2017). Even though a consequent literature study was performed, the studies retrieved might not be fully representative of the entirety of the published articles.

2.2 Evidence of microplastic toxicity on marine organisms at the molecular, cellular, organ, individual and population levels.

A compilation of the effects of MPs toxicity on marine organisms at the molecular, cellular, organ, individual and population levels is summarized in Figure 5. The most studied effects were first at the population (54 tests), individual (44 tests) and molecular (39 tests) levels, followed by tests at the cellular (22 tests) and organ levels (13 tests).

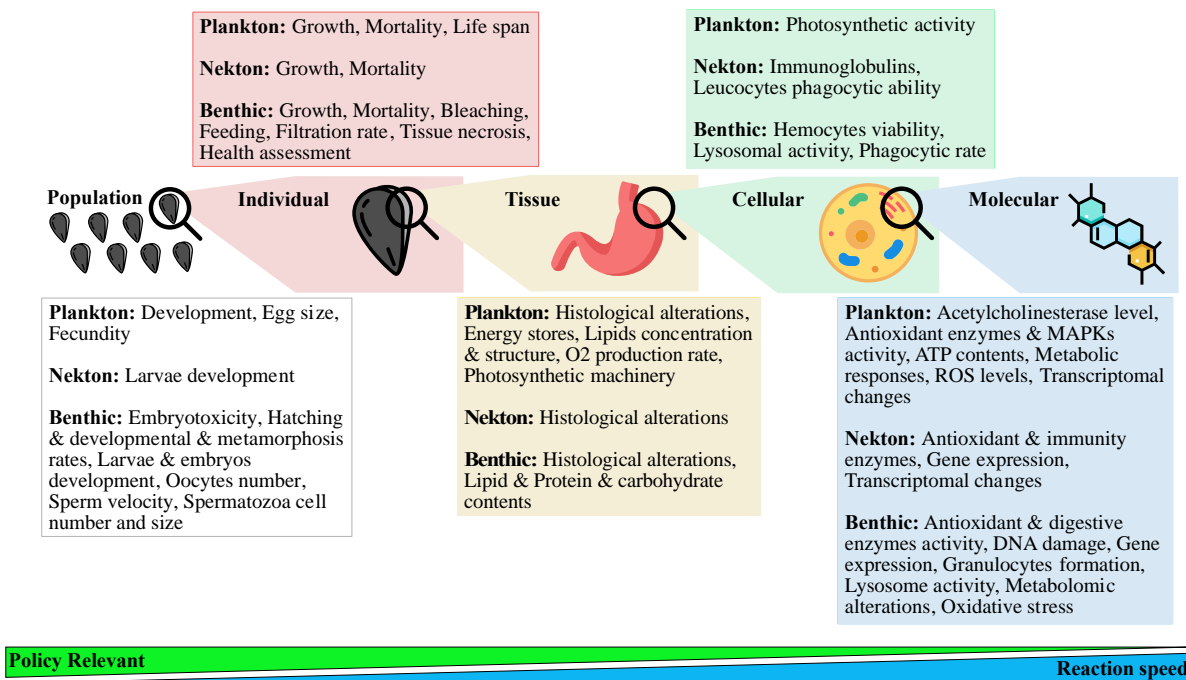


Figure 5: Compilation of the observed effects of plastic toxicity on marine organisms described at the molecular, cellular, tissue, individual and population levels in the plankton, nekton and benthic species.

2.2.1 Toxic effects at the molecular level

The evaluation of toxicity at the molecular level aims to decipher subtle impacts of plastic pollution on organisms through stress mechanisms involving gene expression, enzymatic activities, oxidative stress and metabolomic alterations. For instance, impact of MPs exposure on gene expression was observed on several marine organisms, from bacteria, with a decrease in transcription of genes associated with carbon fixation or cell wall transport (Tetu et al., 2019), to fish, for genes related to lipid, steroid oxidation and inflammatory response (Brandts et al., 2018; Espinosa et al., 2017, 2019; Mazurais et al., 2015; Zhang et al., 2021c). Enzymatic activities were also modified in many species, from

plankton (antioxidant and neurotransmitter enzymes) (Jeong et al., 2016, 2017; Jeyavani et al., 2022; Zhang et al., 2021b) to bivalves (antioxidant and digestive enzymes, lysozyme) (Trestrail et al., 2021; Wang et al., 2020b) and fish (antioxidant and immunity enzymes) (Brandts et al., 2018; Espinosa et al., 2019). Oxidative stress was observed on plankton (Jeong et al., 2016, 2017; Li et al., 2022; Zhang et al., 2021b), worms (Browne et al., 2013), and bivalves with an increase of ROS content and broken DNA strands (Avio et al., 2015; Huang et al., 2021; Sun et al., 2021). Metabolomic alterations after MPs exposure were also identified in microalgae (glycerophospholipids, carbohydrates, amino acids and ATP content), bivalves (hemolymph proteome) (Green et al., 2019) and fish (lipids, serum composition) (Espinosa et al., 2017; Zhang et al., 2021c).

2.2.2 Toxic effects at the cellular level

A large number of endpoints are available on cells, the smallest unit of life, encompassing the membrane stability, phagocytic response, hemocytes viability and mitochondrial metabolism. In the literature, MPs exposure led to the modification of not only the cell content of plankton (lipids and pigments) (González-Fernández et al., 2020; Guo et al., 2020b; Zhang et al., 2021b) and bivalves (lipids, proteins and carbohydrates) (Bour et al., 2018) but also the cell structure of diatom (thylakoid and lipid structure) (González-Fernández et al., 2020), worms (lipid droplets, secretory vesicles) (Browne et al., 2013) and bivalves (lysosomal membrane stability) (Avio et al., 2015). In many cases, immune cells were also affected, such as fish's leucocytes, immunoglobulin production and phagocytosis activity (Espinosa et al., 2019). In addition, hemocytes' viability and granulocytes' number in bivalves were negatively impacted (Avio et al., 2015; Sikdokur et al., 2020). Cell functioning was impacted for planktonic organisms (González-Fernández et al., 2020; Li et al., 2022; Tetu et al., 2019) and zooxanthellae corals (Reichert et al., 2019) through a reduction of photosynthetic efficiency. At last, microplastics also modifies the mitochondrial metabolism of mussels (Shang et al., 2021).

2.2.3 Toxic effects on tissues

Scientific articles at the tissue level focused on the effects of MPs on the histopathology, energy reserves and metabolism demand. After MPs exposure, histopathological alterations were observed on microcrustacean juveniles (eradication of the basal lamina

and epithelial layer) (Jeyavani et al., 2022), and on fish (histological alterations) (Espinosa et al., 2019; Pedà et al., 2016). Toxic effects on tissue functions were also observed on bivalves (epithelial deteriorations, hemolymph infiltrations in gills, reduction of cilia) (Sikdokur et al., 2020; Xia et al., 2020).

2.2.4 Toxic effects at the individual level

Toxic effect at the individual level has been classically evaluated by health assessment, survival and growth of individuals. Impacts of MPs exposure on health were observed on several organisms, from bleaching and tissue necrosis for corals (Reichert et al., 2018, 2019) to feeding disruption for worms (Browne et al., 2013) and bivalves (Sikdokur et al., 2020). Survival of plankton (Heinlaan et al., 2020; Jeyavani et al., 2022; Lee et al., 2013) and fish at different developmental stages (Brandts et al., 2018; Naidoo and Glassom, 2019) were affected, with a large increase in mortality. The growths of many species were also impacted, from plankton (Guo et al., 2020b; Jeong et al., 2016; Li et al., 2022; Tetu et al., 2019; Zhang et al., 2021b) to fish (Naidoo and Glassom, 2019) and benthic organisms such as ascidians (Messinetti et al., 2018), sea snails (Lo and Chan, 2018) and corals (Chapron et al., 2018; Mouchi et al., 2019; Reichert et al., 2019).

2.2.5 Toxic effects at the population level

Toxic effects at the population level are more ecologically relevant, classically used for decision making and support to public policy. Behavioral changes were observed on corals (polyp activity and prey capture rate) (Chapron et al., 2018; Mouchi et al., 2019) and mollusks (number and tenacity of byssal threads) (Green et al., 2019). In addition, swimming activity was impacted for microalgae (Zhang et al., 2021b), microcrustacean (Jeyavani et al., 2022) and bivalve larvae (Bringer et al., 2020b). Population recruitment of copepods and rotifers was shown to be troubled (Jeong et al., 2016, 2017) and benthic organisms such as bivalves (Bringer et al., 2020b; González-Fernández et al., 2018; Ke et al., 2019; Luan et al., 2019; Sussarellu et al., 2016) and sea urchins (Della Torre et al., 2014; Kaposi et al., 2014; Martínez-Gómez et al., 2017; Messinetti et al., 2018; Nobre et al., 2015; Trifuoggi et al., 2019) also displayed several signs of alteration of their fecundity (low hatching rate, sperm velocity or fertilization rate, small gamete number or diameter) and larval development (larval malformation, low larval growth or metamorphosis rate) after

MPs exposure. The severity of these effects at the reproductive level is of main concern, since reproduction ensures the continuity of species and prevents their disappearance. Impacts on fertility, fecundity, recruitment and offspring development of a species can have consequences at the population level (Galloway et al., 2017; Sussarellu et al., 2016), but also for other species with which they interact and for the ecosystem.

2.3 Ecotoxicity of plastics

Evaluating *in situ* effects of plastics on organisms is challenging due to the tampering of the marine environment with numerous chemical and trash (Alava, 2019), but also the existence of other sources of stressors (e.g. ocean warming and acidification, habitat degradation, diseases). Therefore, the origin of the toxicity assessed might not be directly linked to plastics, even if they are present in the organisms according to their size.

2.3.1 Ecotoxicity of macroplastics

Compared to MPs, fewer laboratory experiments studied the physical impact of macroplastics (Chapron et al., 2018; Mouchi et al., 2019). Since macroplastics are usually afflicting big size animals, the experiment set up is more complex and it is challenging to produce a comparable natural physical control with same sizes (Backhaus and Wagner, 2020). Moreover, as regulations on manipulations of living beings in laboratory are more and more restrictive, setting up experiments is laborious. Field studies demonstrated an evident impact of macroplastics on the marine wildlife. Significant effects linked to entanglement have been described since 1997 for birds, turtles and marine mammals (Laist, 1997). With the increase of plastic pollution, the number of marine species of these three last animal groups with known entanglement increased from 20.5% in 1997 to 30% in 2015 (Kühn et al., 2015). Physical impact included also smothering, which can induce deleterious effects on marine vegetation (Uhrin and Schellinger, 2011) and corals (Yoshikawa and Asoh, 2004), through shading effect or crushing due to weight. Corals were up to 89% more prone to disease when in contact with plastic waste (< 50mm) (Lamb et al., 2018). Ingestion of macroplastics was also a rising concern, with a clear increasing of ingestion percentage from 33% in 1997 (Laist, 1997) to 44% in 2015 (Kühn et al., 2015) for bird, turtle and mammal species. Even though direct mortality was probably not the most relevant

outcome of ingestion, it led to a partial blockage or damage of the digestive tract that contributed to poor nutrition and dehydration (Auman et al., 1997). Evidence of fibrosis was disclosed in a recent field studies on seabirds (Charlton-Howard et al., 2023). Interestingly, other natural particles such as pumices did not exert similar effects.

2.3.2 Ecotoxicity of MPs

A few experiments mimicked the impact of MPs on the biodiversity and ecosystem functioning, mainly on bivalve and lugworm habitats. Those experiments in controlled mesocosm conditions resulted in a higher filtration rate for oysters (*Ostrea edulis*) but a lower filtration rate for mussels (*Mytilus edulis*) when exposed to Polyethylene (PE) and Polylactic acid (PLA) (Green, 2016; Green et al., 2017). While for mussels, only the filtration differed from the control, for oysters the primary productivity of microphytobenthos (lower cyanobacteria biomass), the porewater nutrients (increase of ammonium) and the invertebrates and macrofaunal assemblages were impacted. Likewise, in a similar experiment set up with lugworms (*Arenicola marina*), the microphytobenthos was altered upon exposure of PE, PLA and Polyvinyl chloride (PVC) (Green et al., 2016). In addition, an increase in O₂ consumption by the lugworm and the bioturbation was reported, with a dose-dependent reduction in number of surface casts (Green et al., 2016).

2.3.3 Transfer along the trophic chain

The ingestion of plastics by marine biota has been demonstrated in laboratory experiments (Jeong et al., 2017; Kaposi et al., 2014) and also in the environment (Wesch et al., 2016). The residence time of MPs in the gut was closely linked to the size, shape (Gray and Weinstein, 2017), roughness (Mazurais et al., 2015), and of course the species (Botterell et al., 2020). Despite the evidence of MPs being ingested, a question subsists: do MPs manage to rise along the food web? A semi-systematic review underlined that MPs did not biomagnify along the marine food web and that there is currently no risk to human health when considering the current literature (Walkinshaw et al., 2020). However, few articles showed that NPs were transferred from preys to predators. For instance, trophic transfer from mussels to crabs has been demonstrated experimentally (Farrell and Nelson, 2013). NPs were observed in the stomach, hepatopancreas, gills but also in the ovary of mussels. The number of NPs in crab's hemolymph increased just after ingesting the

contaminated mussels. Another study showed that NPs could be transferred from algae exposed to polystyrene (PS) NPs to herbivores (*Daphnia magna*) and fish (Crucian carp), thus causing behavioral changes such as slower movement and less hunting but also disturbance in the lipid metabolism for the top consumer (Cedervall et al., 2012). Even though a trophic transfer is present, no biomagnification of SMPs has been observed (Walkinshaw et al., 2020). For example, the effect of SMPs exposure on beach hoppers found no behavioral change (Tosetto et al., 2017).

2.4 Plastic characteristics (concentration, duration of exposure, size, shape, chemical composition and biological colonization) as crucial factors for comparable toxicity tests.

Plastic characteristics used in the current literature were gathered and summarized in Figure 6, in order to evaluate the relevancy of actual toxicity studies.

Species	Type				Size		Shape			Joint contamination				Biological colonization	Nb articles
	PE	PVC	PS	Others	●	●	●	✱	~	Environ-mental	Additive	Pollutant	∅		
Bacteria	✓	✓		✓		✓	✓						✓		==
Phytoplankton	✓	✓	✓	✓	✓	✓	✓	✓			✓	✓	✓		===
Zooplankton	✓		✓		✓	✓	✓	✓				✓	✓		≠
Fish	✓	✓		✓	✓	✓	✓	✓		✓	✓		✓		≠
Shrimp			✓		✓		✓						✓		-
Coral	✓				✓		✓	✓					✓	✓	===
Ascidian			✓		✓		✓						✓		-
Crab					✓				✓				✓		-
Sea snail			✓		✓		✓						✓		-
Clam	✓		✓	✓	✓	✓	✓					✓	✓		===
Mussel	✓		✓	✓	✓		✓	✓				✓	✓		≠
Oyster	✓		✓	✓	✓	✓	✓						✓		===
Other bivalves	✓			✓	✓		✓	✓			✓				==
Urchin	✓		✓	✓	✓	✓	✓	✓		✓			✓		≠
Worm	✓	✓		✓	✓		✓	✓		✓	✓	✓			==

Figure 6: Compilation of the MPs' characteristics in toxicity experiments: chemical composition, size, shape, and biological colonization (= plastisphere). PE = Polyethylene; PVC = Polyvinyl Chloride; PS = Polystyrene; ● = Microplastic (1-1000 μm); ● = Nanoplastic (< 1 μm); ● = Regular shape; ✱ = Irregular shape; ~ = Fibers.

2.4.1 Plastic concentrations used in the toxicity tests

A comparison of MPs concentrations used in the literature enlightened that toxicity tests are generally far to be representative of environmental concentrations, which themselves are heterogenous in function of the location, meteorological parameters and time (Figure 7). Most studies (94%) used concentrations 10 to 10¹⁴ times higher than the

highest concentration measured in surface seawaters (150 particles/L, > 0.75µm) (Song et al., 2014), although this concentration can be mitigated by sampling biases.

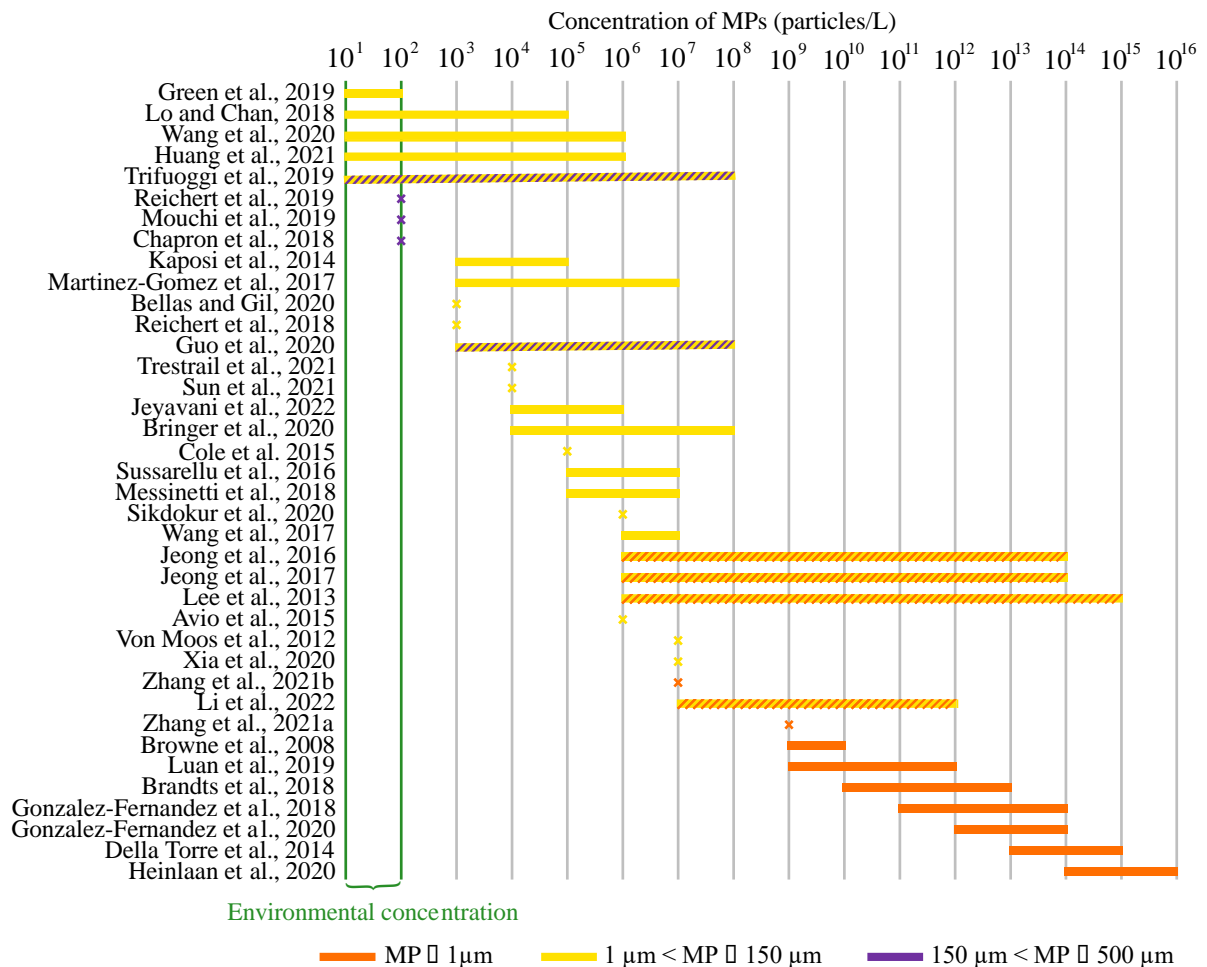


Figure 7: Range of MPs concentration (particle/L) used in the reviewed articles. When needed, an approximation of number of particle/L was calculated from data initially expressed mg/L (see conversion formula in the text)

Quantification of MPs were generally performed by using manta nets with 333 µm mesh size (Collignon et al., 2012; Law et al., 2010; Moore et al., 2005) , thus missing the non-negligible portion of small MPs and NPs. Sampling were mostly performed at the sea surface or sub-surface, leaving the deeper part of oceans poorly attended (Erni-Cassola et al., 2019). Other environmental factors such as the proximity of the coast or water currents present in the ocean were shown to induce a high variability of MPs and NPs concentration (Law et al., 2010; Law and Thompson, 2014). Methodological developments were necessary to assess small MPs and NPs invisible by eyes that need further field studies both in the water column and benthic environments (Cai et al., 2021).The mean and median

concentrations used in these studies were equal to 4×10^8 and 10^6 particles/L for SMPs, the latter being 10^3 higher than the highest concentration recovered in the environment. For NPs the mean value was equal to 3×10^{14} particles/L and the median to 10^{12} particles/L. It must be noticed that the concentration of MPs reported in the marine environment varies significantly depending on the geographical location and it has generally been estimated to MPs larger than $333 \mu\text{m}$ (*i.e.*, manta net mesh size), which underestimates the real concentration of MPs. Indeed, the environmental MPs concentration measured with a $100 \mu\text{m}$ manta net is 2.5 times higher than using a classical $333 \mu\text{m}$ net, and 10-fold greater than a $500 \mu\text{m}$ net (Lindeque et al., 2020). Another study underlined that SMPs that are poorly identified by classical manta sampling may represent similar weight but contain 10^2 to 10^5 more particles/L than LMPs (Poulain et al., 2019). Moreover, in surface waters, 86% of MPs were $< 100 \mu\text{m}$ in the North Sea (Lorenz et al., 2019). Therefore, some high concentrations in those articles may be more environmentally realistic than firstly thought. Another drawback for an effective comparison with environmental concentration is the unit of measure. Indeed, the unit of measure used in most toxicity studies is mg/L, which is convenient for the preparation of MPs solution by weighting. However, environmental concentrations units are, in majority, expressed as number of particles per m^2 for surface waters, per m^3 or per L in the water column, or per kg for sediment. Amongst the selected experimental studies, only a few expressed concentrations in particles/L (Chapron et al., 2018; Cole et al., 2015; Kaposi et al., 2014; Lo and Chan, 2018; Mouchi et al., 2019; Reichert et al., 2018; Wang et al., 2020b). To make these studies comparable, we propose that authors also provide information on the number of particles per liter or per gram of sediment, which can bring more information than only weighting that is very size dependent. Using the measure in weight per unit of volume may have severe drawback. Indeed, we calculated that a solution with 1 mg/L of perfectly spherical MPs with a diameter of $500 \mu\text{m}$ will contain 15.3 particles/L whereas a solution with the same concentration with a diameter of $1 \mu\text{m}$ will contain 1.91×10^9 MPs/L, thus increasing greatly the bioaccessibility. A formula : $MPs/L = \frac{(\text{weight}(\frac{\text{mg}}{\text{L}}) \times 3)}{4\pi \times \text{radius}(\mu\text{m})^3 \times 10^{-12} \times \text{density}(\text{g.cm}^3) \times 10^3}$, has been elaborated to link the number of plastic particles to their weight per unit of volume, assuming that particles were all spherical. Because of possible biases of this assumption,

we propose that authors provide information both in the number of particles (using laser granulometry for instance) and weight per unit of volume when running toxicity tests on MPs.

2.4.2 Duration of exposure

Another critical parameter in toxicity tests is the duration of exposure. We distinguish between acute tests, which are short-term tests with usually, high concentrations of pollutants, and chronic tests, which are long-term tests with relatively lower concentrations (United States Environmental Protection Agency, 1994). We included an intermediate term “subchronic”. These terms are closely related to the life span of the species tested and were adapted from (Blasco et al., 2016). For bacteria and algae, a toxicity test was considered chronic when the experiment lasted a complete life cycle. Subchronic was between half and a full life cycle, whereas acute was determined for toxicity tests with a duration of less than half of a life cycle. However, for every other organism with longer life expectancies, we adapted the duration from the standard ASTM E2455-22 for freshwater mussels which determines an acute, subchronic and chronic toxicity tests with duration of <7days, between 7 and 28 days and >28 days, respectively.

We observed an almost even repartition of the duration of the experiment in the literature, with a slight dominance of acute toxicity tests. Indeed, 40% concerned acute toxicity tests, 27% mid-term toxicity tests and 33% chronic tests (Figure 8). The median of the minimum and maximum concentrations (only in MPs/L) used in the different toxicity tests was calculated in function of the duration. Acute toxicity tests used higher concentrations (median min and max: 10^5 - 10^8 MPs/L) than mid-term (median min and max: 10^5 - 10^6 MPs/L) that were higher than chronic toxicity test (median min and max: 10^3 - 10^6 MPs/L).

Acute tests allow to determine the lethal dose (LD50) or the effect concentration (NOEC and LOEC) with small set-ups and a high number of replicates. Moreover, various parameters (e.g. concentration, size, shape) can be tested at low costs. Even though, chronic experiments are limited concerning the beforementioned assets, they are more representative of environmental conditions where organisms are continuously exposed to a relatively low plastic concentration. Both of these tests' duration are needed and can be complementary. Indeed, with the vast quantity of different plastic types and additives

acute toxicity experiments fit perfectly to assess quickly the impact of a wide variety of plastic. After this first categorization a more focused chronic study could be performed to analyze in depth the impact of previously determined plastics.

We recommend that preference should be given to a combination of acute and chronic toxicity tests that consider several life stages and sensitivity of the organisms. The size also plays a decisive role on the chosen concentrations since a higher bioaccessibility is generally associated with smaller size (see section 2.4.1).

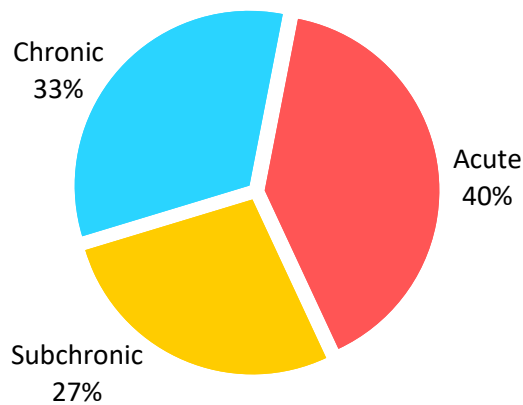


Figure 8: Repartition of experiments duration in the reviewed articles

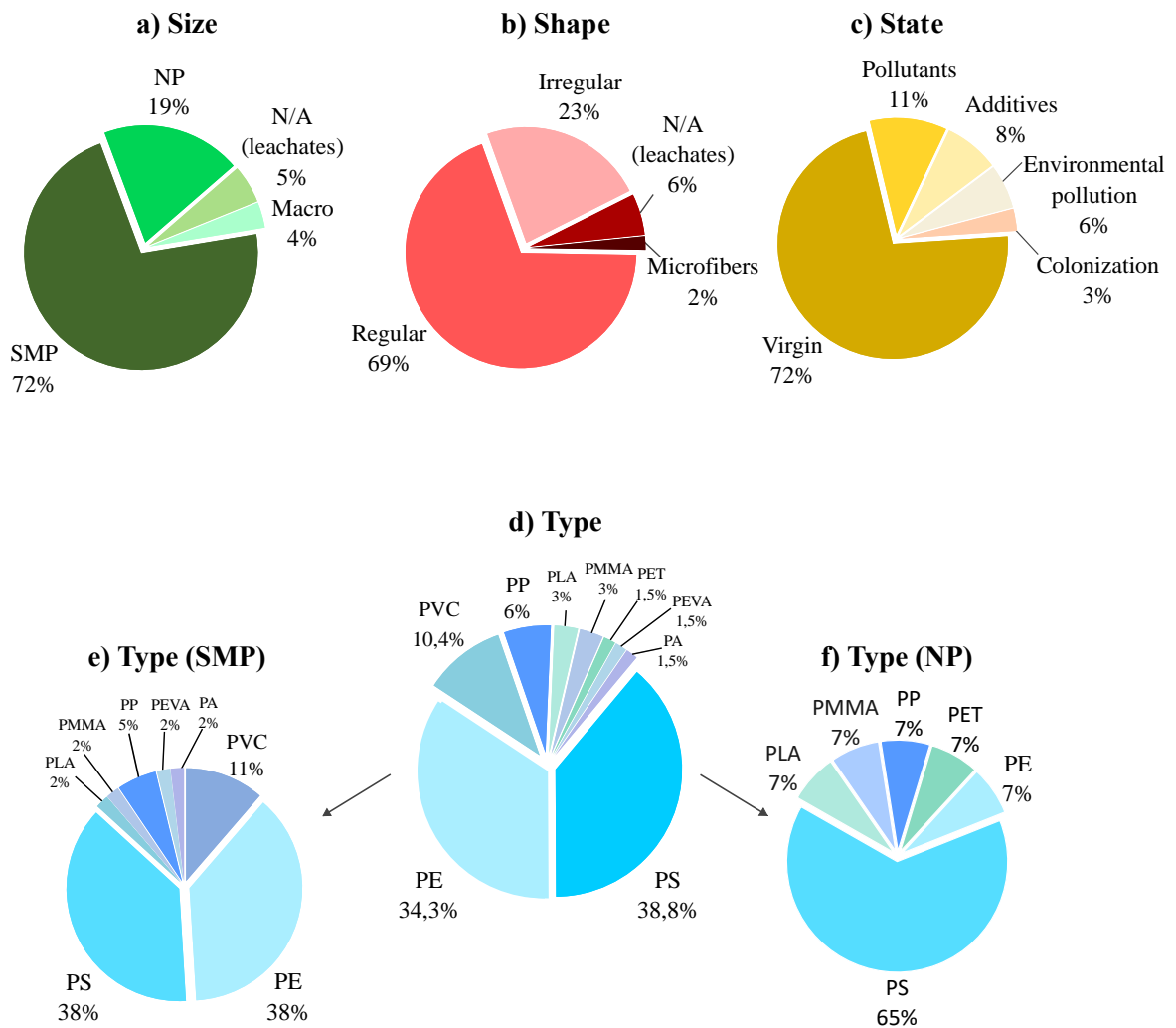


Figure 9: Characteristic of the plastic used in the reviewed articles: size (a), shape (b), presence of additives and adsorbed pollutants (c) or polymer composition (d). Chart (e) and (f) decomposes the polymer composition in function of size class. PE = Polyethylene; PLA = Polylactic acid; PMMA = Polymethylmethacrylate; PP = Polypropylene; PS = Polystyrene; PVC = Polyvinyl chloride; B-Plastic sizes used in experimental studies. SMP = Small microplastic (1-1000µm); NP = Nanoplastic (1-1000nm) and N/A = leachates (dissolved).

2.4.3 Range of plastic sizes used in toxicity tests

SMPs represent the majority of the tested microplastics, as they were used in 72% of the selected of studies for this review (Figure 9a). Nanoplastics (NPs) were used in 19% of the selected articles, whereas only 3 studies used leachates and 2 others used macroplastics.

Several studies enlightened the importance of plastic size in relation to ingestion rate, transit and the resulting potential toxicity on organisms. For example, the increase of abnormal larvae of oysters (*Crassostrea gigas*) was much greater with 4-13 μm compared to 25 μm size SMPs (Bringer et al., 2020b). The impact on protein content in sediment-dwelling bivalves was also significantly higher for large SMPs (125-500 μm) compared to smaller SMPs (6 and 25 μm) (Bour et al., 2018). The same tendency was observed in NPs, which were shown to be differentially ingested at a dispersed (< 1 μm) or aggregated (> 100 μm) state in mussels (*M. edulis*) and oysters (*Crassostrea virginica*) (Ward and Kach, 2009). NPs with a size of 26 nm induced toxicity for the bacteria *Vibrio fischeri*, whereas no effect was observed with NPs of 100 nm size (Heinlaan et al., 2020). Likewise, 50 nm-size NPs increased the mortality of copepods but did not affect their fecundity, whereas 6 μm -size SMPs had no impact on their mortality but had an effect on their fecundity (Jeong et al., 2017; Lee et al., 2013). These results enlightened the crucial role played by the size of the plastic debris in relation to the size of the organisms that would greatly influence the toxicity outcomes. It must be noted that the decrease in particle size did not result in an increase of toxicity. In fact, the opposite was observed in a literature review, where a higher concentration of smaller particles was required to induce an effect (Iwan Jones et al., 2019). We recommend to fill the gap of knowledge on NPs in further toxicity tests since they are the most abundant type of plastic in the marine environment in terms of particle numbers (Lindeque et al., 2020; Poulain et al., 2019) and also because the smaller the size, the greater is the potential for uptake by organisms. As they are mostly derived from the degradation processes of MPs, we also recommend to use in priority NPs obtained from MPs by grinding rather than commercial particles (El Hadri et al., 2020). The presence of NPs together with its eco-corona is also recommended in toxicity tests in order to fit with natural conditions (ter Halle and Ghiglione, 2021).

2.4.4 Plastics shape used in toxicity tests

Distinction was generally made between primary MPs, purposefully manufactured in small size, and secondary MPs that result from the weathering and breakdown of larger plastic items. Primary MPs usually possess a spherical or cylindrical shape (*i.e.*, regular shape), whereas secondary MPs present various irregular shapes (GESAMP, 2015). The majority of the reviewed articles used MPs of uniform shape for toxicity tests (Figure 9b).

However, spherical primary MPs represent a negligible part of the total MP pollution all over the world (Cózar et al., 2015; Kanhai et al., 2018; Nel and Froneman, 2015; Qu et al., 2018a). Those results highlight that the use of uniform shape is not the most representative of the environmental MP pollution. The shape influences the ingestion of MPs depending on the species (Botterell et al., 2020), which is probably linked to prey selectivity. The shape also influences the toxicity: irregular fragments were shown to induce higher toxic effects on *Daphnia magna* (Frydkjær et al., 2017; Na et al., 2021; Renzi et al., 2019). In addition, secondary MPs tended to provoke more intestinal injuries than primary ones (Mazurais et al., 2015). The shape plays a role in plastic toxicity (Wright et al., 2013) and since the environmental shapes of plastics are mostly fibers or irregular ones, we recommend using those shapes in relation with the model species used (what is preferentially ingested) and the experiment goal. For example, true-to-life MP and NP resulting from the cryogrinding degradation of plastic goods is gaining interest (Walkinshaw et al., 2023; Zimmermann et al., 2020).

2.4.5 Polymer composition of plastics used in toxicity tests

The mostly used polymer types in toxicity tests were PS, PE and PVC, with 39%, 34% and 10% of the reviewed articles, respectively (Figure 9d). A similar repartition of polymer composition was observed for SMPs in toxicity tests (Figure 9e). However, in the NPs toxicity tests, there was an important predominance of PS, because standardized PS nanospheres are commercially available with a great variety of sizes and functionality (Figure 9f). PE (including low and high density) is the most commercially produced polymer and constitutes the major source of plastic pollution on Earth (Geyer et al., 2017). PVC occupies an important fraction of the toxicity studies because standardized microbeads are commercially available, although its presence in the marine environment is low compared to other plastics (Erni-Cassola et al., 2019). This review analysis indicates that there is a gap between the polymer types used in the toxicity studies and their respective representativeness in the environment. For example, PP has only been used in 6% of the selected toxicity tests, whereas it is the second most abundant polymer at the sea surface (Erni-Cassola et al., 2019). Another concern is the lack of studies using polyesters (PES), polyamides and acrylics, which are among the most abundant polymers in the water column and in sediments (Erni-Cassola et al., 2019). This lack of studies is probably because

those polymers are a complex material to study. Indeed, fibers are difficult to obtain and were poorly quantified in the environment, even if a recent study started to tackle this issue (Walkinshaw et al., 2023).

It is noteworthy that the proportion of polymer types within the plastic litters sampled in the environment was rather stable. Even if local disparities exist, notably in coastal zones, the effects of the watershed and local activities (such as industries, tourism, wastewater treatment plants or water currents closed to the sampling areas) were of major importance in the observed plastic pollution. By instance, we emphasize here the need to broaden the scope of polymer types used in toxicity tests, and especially for PP, PES, Polyamide and acrylics that suffer from a severe lack of studies compared to their omnipresence in the environment.

Heterogeneous results were observed when comparing the toxicity of different plastic types (Espinosa et al., 2019; Guo et al., 2020b), or the same effect was observed, regardless of the polymer composition (Guo et al., 2020b; Trifuoggi et al., 2019). The mortality of *Vibrio fischeri* was only linked to the presence of additives (Heinlaan et al., 2020), whereas a material specific toxicity was observed for *Daphnia magna* (Zimmermann et al., 2020). Those results indicate that plastic toxicity is closely linked to its chemical composition as a whole, i.e. polymer and additive.

2.4.6 Plastic additives and adsorbed pollutants as part of plastic toxicity

Most of selected articles (>72%) used pristine MPs and do not take into account the possible adsorption of pollutants (e.g., PCBs, organochloride pesticides, PAHs, heavy metals, biotoxins) (Abd-Aziz et al., 2019; Tavelli et al., 2022) (Figure 9c). This is probably because reproducing an environmental pollution is complicated since no homogeneous concentrations of pollutants are present in the environment. Some authors underlined that a pre-incubation of pristine plastics in the natural environment before the tests would be a more realistic situation, because it would take into account the possible leaching of plastic additives together with the possible adsorption of environmental pollutants on plastics (Pedà et al., 2016). Another option would be to test the toxicity of plastic collected in the natural environment, even if such approach would need a large number of samples to counterbalance the variation due to local environmental conditions and to the various history of the plastics (Naidoo and Glassom, 2019; Nobre et al., 2015). The studies

evaluating the impact of plastic additives were performed in laboratory conditions, through plastic leaching (Ke et al., 2019; Tetu et al., 2019). Other studies tested the impact of adsorbed pollutants by adding one selected product (hydrocarbon, pesticide or metal) together with plastics (Guo et al., 2020b). It is difficult to consider that these laboratory experiments fully mimic the wide range of combination between plastic additives and adsorbed pollutants encountered in the environment. In any case, the part of hydrophobic organic chemicals held by MPs could be negligible compared to the part brought by natural particles which are much more numerous in nature (Koelmans et al., 2016) leaving this question under debate and calling for further *in situ* exploration.

2.4.6.1 Toxic impact of plastic coupled with additives

Plastics are generally produced with a range of chemical additives such as plasticizers, flame retardants, antioxidants and other stabilizers, pro-oxidants, surfactants, inorganic fillers or pigments, thus resulting in more than 5300 grades of synthetic polymers for plastics in commerce (Wagner and Lambert, 2018), (Murphy, 2001). Opposite effects were observed when MPs were co-exposed with additives. Triclosan had a significant impact on feeding and survival of lugworms (*A. marina*) when coupled with PVC particles, as compared to the additive alone. However, the effects of polybrominated diphenyl ethers (PBDE-47) were similar whether PVC particles were present or not (Browne et al., 2013). Scallops (*Chlamys farreri*) displayed a significant decrease of their phagocytic rate when PS microparticles were added to decabromodiphenyl ether (BDE-209) (Xia et al., 2020). On the other hand, the toxicity of triphenyl phosphate was decreased when coupled with PS particles (Zhang et al., 2021c).

The leaching of additives from plastic is linked to several factors ranging from the polymer type, texture, and strength of its bond with the additives, to the physicochemical properties of the additives themselves as well as the exposure media/surrounding environment characteristics. Laboratory analyzes on leaching additives suffer from methodological differences (e.g. leaching period, initial state of plastics, temperature or presence of light) hindering comparisons between the studies (Gunaalan et al., 2020). Moreover, the exact composition of plastic is usually not accessible and since a wide variety of additives are used, the comprehensive analysis of leachates is challenging (Gunaalan et al., 2020). Many additives were already recognized as endocrine disruptors (Darbre, 2020)

or “harmful for aquatic organism” or “causing long-term adverse effect in the aquatic environment” (Cherif Lahimer et al., 2017). Their ubiquitous presence in marine waters (Hermabessiere et al., 2017) could indicate a desorption into the environment. Nevertheless, those compounds have many sources, e.g. Polychlorinated Biphenyls (PCBs) are used for dielectric or adhesives substances (Wolska et al., 2014) and Polycyclic Aromatic Hydrocarbons (PAHs) can be introduced via urban runoff of oil spillage (Arias et al., 2010). Even though, the leaching of additives from plastics was proven and resulted in toxicity (Ke et al., 2019; Tetu et al., 2019), its overall impact on the marine ecosystem is yet to be determined. The “coho salmon case” is an exemplary demonstration that linked chemical signatures of tires in urban runoff and freshwater samples and abnormal mortalities of *Oncorhynchus kisutch* over decades in western North America (Tian et al., 2020).

2.4.6.2 Toxic impact of plastic coupled with environmental pollution

Few studies assessed the toxicity after pre-incubation of plastics in a marine environment, in order to evaluate the possible effects of the release of additives in the environment or the possible effects of adsorption of various and unknown pollutants on plastics. They showed higher toxicity for pristine MPs. Indeed, glassfish (*Ambassis dussumieri*) exposed to virgin and environmentally polluted MPs lead to the same growth decrease in mass, length, and body depth, but survival probability was lower for virgin rather than environmentally pre-incubated MPs (Naidoo and Glassom, 2019). Pristine plastic also led to more severe histopathological alterations in European seabass (*Dicentrarchus labrax*) than environmentally pre-incubated plastics for the first two month, even though it became similar after three months of exposure (Pedà et al., 2016). Higher toxicological effect (abnormal larvae development) was also found when comparing pristine to environmentally pre-exposed plastics for sea urchins (*Lytechinus variegatus*) (Nobre et al., 2015). These studies concluded that the leaching of additives might be a factor leading to a higher toxicity of the pristine compared to environmentally pre-incubated MPs.

2.4.6.3 Toxic impact of plastic particles coupled with chemical pollutant

Another set of studies evaluated the impact of other chemical contaminants (hydrocarbons, pesticides, metals) added before (test of adsorption on plastics) or during the plastic exposure (co-exposition). The sorption of pollutants on plastic particles has been

well documented, and the use of plastic waste was even suggested as a potential sustainable approach in remediating environmental pollution (Abd-Aziz et al., 2019).

The combination of PS and PE MPs with pyrene resulted in an increased frequency of micronuclei in hemolymph cells of mussels (*Mytilus galloprovincialis*) (Avio et al., 2015). An increase of toxicity, by addition of chlorpyrifos with PE MPs, was found on copepods (*Acartia tonsa*), when compared to the exposition of solely the pollutant (Bellas and Gil, 2020). Co-exposure of PS MPs and tetrabromobisphenol A on two microalgae was shown to be more toxic than single exposure, suggesting a synergistic effect (Zhang et al., 2021b).

Although adsorbed pollutants on plastic sometimes increased its toxic effect on marine organisms, decreased toxicity was also observed in other experiments. The combination of PVC together with phenanthrene and nonylphenol polluted sand was less toxic for lugworms (*Arenicola marina*) than solely exposed to the polluted sand (Browne et al., 2013). Another study showed that mercury pre-sorbed on PE particles was poorly transferred on clams (*Ruditapes philippinarum*) compared to mercury alone (Sikdokur et al., 2020). In addition, the phenanthrene stress induced on diatoms was minimized by the addition of MPs (Guo et al., 2020b) and several types of MPs decreased sulfamethoxazole (SMX) toxicity on the microalgae *Skeletonema costatum* (Li et al., 2022). However, two studies suffered from methodological limits. Due to lugworms' diet (sand), a higher desorption effect from polluted sand rather than MPs did not imply a negligible vector role of MPs (Browne et al., 2013). Moreover, the particle size was too big to be ingested by microalgae and since plastics act as sponge for pollutants, they could have reduced the pollutant accessibility (Guo et al., 2020b). The laboratory concentrations of pyrene and phenanthrene adsorbed on MPs were environmentally relevant for plastics located on beaches (Frias et al., 2010). However, when comparing with plastics recovered in marine waters, only phenanthrene is representative of concentration recovered in the environment (Bouhroum et al., 2019). However, representativeness towards environmental concentrations is unknown for these studies (Bellas and Gil, 2020; Sikdokur et al., 2020) since the quantity of pollutants pre-sorbed on plastics was not measured. The impact of pollutants adsorbed on plastics compared to the contamination through other media is challenging due to the unit difference: weight/L for environmental concentrations and weight/g for surface plastic concentration.

These contradictory results prevent from making any clear conclusions on the impact of adsorbed pollutants on plastics and further analysis are needed to better understand the potential impact of the combination between chemical pollutants and plastics. Nevertheless, the hypotheses under which MPs act as vectors for chemicals has been severely questioned. Indeed, the bioaccumulation flux of hydrophobic organic pollutants from ingested MPs was found negligible compared to its bioaccumulation through preys (Koelmans et al., 2016).

2.4.7 Taking into account the biofilms growing on plastics in toxicity tests

A growing body of literature described the microorganisms living on plastic debris (so-called plastisphere), including putative animal or human pathogens (Jacquin et al., 2019). The plastisphere is involved in the plastic debris buoyancy, which influence its bioavailability and its palatability. When a MP together with its biofilm is ingested, a transfer of microorganisms to the host microbiome has been described for several species (Fackelmann and Sommer, 2019; Lear et al., 2021). To date, only a few toxicological studies used a pre-incubation step of plastic pieces in the marine environment (Chapron et al., 2018; Mouchi et al., 2019), which would be more realistic considering the omnipresence of microorganisms on their surface (Jacquin et al., 2019). Moreover, several studies indicated that the plastisphere eased up the ingestion of MPs for some organisms. For example, copepods (*Eucalanus pileatus* and *Schizopera* sp.) did not consume any pristine MP particles but were differentially attracted by MPs covered by a biofilm (Paffenhöfer and Van Sant, 1985), (Dahms et al., 2007). Copepods chemically selected their food using long-range (particle capture) and short-range (particle ingestion) chemoreceptors at their mouth, thus explaining their ability to detect the nutritional values of the biofilm covering the MPs (Paffenhöfer and Van Sant, 1985). Similarly, example of oysters (*C. virginica*) ingested ten times more MPs with biofilm than pristine ones, in accordance to their preferential ingestion of organic compared to inorganic materials (Ecol Ser et al., 1983), (Fabra et al., 2021). Predators such as fish may also ingest MPs accidentally when attacking the plastic-fouling organisms (Carson, 2013). The role of the plastisphere in plastic debris bioavailability and overall toxicity might also be overlooked when considering its importance in contaminants sorption kinetics on plastics (Rummel et al., 2017). Indeed, the adsorption of persistent organic pollutants (POPs), heavy metals and other contaminants

were enhanced through the presence of a plastisphere on plastic (Bhagwat et al., 2021; Wang et al., 2020c).

We recommend to consider the role of the plastisphere in further toxicity analysis for more realistic experimental conditions, by incubating any plastic debris for at least one month in natural conditions. This time period has been shown to be sufficient for the development of a mature biofilm in the natural environment (Odobel et al., 2021). In addition, a characterization of the plastisphere is important in order to understand the role of the (at least) dominant species.

2.5 Evaluation of toxicity risk assessment

2.5.1 Regional, national and international initiatives to face plastic pollution

In the last decade, increasing international initiatives, law and policies denoted a growing political and societal concern on plastic litters in the environment (UNEP, 2018), the last initiative being from the G20, G7 and UNEA process, supporting the set-up of an international treaty, under negotiation (UNEP, 2022). Numerous bans of single-use plastics (mainly plastic bags) entered in force in all the continents. Contrary to usual norm pattern dynamic, it emerges from the South to the North (Clapp and Swanston, 2009). Africa is the continent where the largest number of countries (36 countries) instituted a prohibition of production and use of plastic bags (Hira et al., 2022). In Asia, 4 countries, including India and China, introduced a ban on single-use plastic bags with in particular Bangladesh which implemented a ban since 2002. Several countries in Oceania imposed a national ban of plastic bags and only local bans have been enforced in Australia (Nielsen et al., 2019). A list of single-use plastic items were banned in the European Union markets since 2021 (bags, cotton bud sticks, cutlery, plates, straws, stirrers, cups, beverage containers made of expanded polystyrene, exfoliating rinse-off cosmetic products, and all products made of oxo-degradable plastics) (European Parliament and Council, 2019). Recently, France aims to achieve the end of the marketing of single-use plastic packaging by 2040 (MINISTÈRE DE LA TRANSITION ÉCOLOGIQUE, 2021). In North America, a recent national ban is planned to be enforced gradually in Canada (2023-2025) for 6 single-use plastics (check out bags, cutlery, flexible straw, food service ware, ring carrier, stir stick and straw) (Canadian

Government, 2022). In the United States, several states and cities instituted bans, however 11 states enforced countermeasures prohibiting local regulation on plastics bags (Nielsen et al., 2019). Columbia, Chile, Panama, Bahamas, Haiti, Belize are the only countries of Central and South America that implemented national bans. In addition, several local bans were established in Argentina (Mendoza, Buenos Aires) and Brazil (Sao Paulo, Belo Horizonte, Rio de Janeiro) (Nielsen et al., 2019). It is noteworthy that the majority of bans were limited to thin plastic bags (from $<20\mu\text{m}$ to $<100\mu\text{m}$, depending on the country), meaning thicker plastic bags are still allowed (UNEP, 2018). Overall, these initiatives are used as a precautionary principle, based on (i) the overwhelming presence of single-use plastics in the environment, (ii) their ingestion by animals all along the trophic chain and (iii) their potential toxic effect observed on various animals under laboratory conditions.

Considering the difficulties of testing the large variety of composition of the targeted plastic items, none of these initiatives were based on relevant evaluation of ecological risk assessment (ERA). For example, in the case of plastic bags that have been banned in several countries, the exposition of marine animals has been proven because of their dispersion all over the world's Oceans (Galgani et al., 2000; Jamieson and Onda, 2022). Even though scientific articles analyzing plastic bags toxicity were published (Balestri et al., 2017; Green et al., 2015; Ke et al., 2019; Sarker et al., 2020), no thorough ERA has been conducted. Most of the impact of plastic bags have been proven for digestive tract obstruction and entanglement on turtles, sharks but also on large mammals such as seals or whales (De Stephanis et al., 2013; Denuncio et al., 2017; Fernández and Anastasopoulou, 2019; Mrosovsky et al., 2009). This contributed to growing media coverage and public awareness. Another study showed an increase of cold corals polyp activity but decreased prey capture rates after partial covering of living polyps (~50%) by plastic bags that acted as physical barriers for food supply (Chapron et al., 2018). Further studies are still needed to test more indirect toxicological effects, given the large variety of chemical composition of plastic bags that are generally based on PE but with a large variety of additives (Hahladakis et al., 2018). The toxic impact of plastic bags additives was analyzed through leachates. However, the different leaching procedures (e.g., leaching time, $T^{\circ}\text{C}$, agitation speed, light, shape and state of oxidation of plastic) make laborious comparison between the few articles available. As previously explained, there is a very large number of plastic composition and it is very

difficult to tests them all. The clear labelling and listing plastic additive content would greatly facilitate the establishment of a relevant strategy for of ERA. Additionally, the reduction of the number of plastic additives, for example by removing in priority the substances supposedly the more potentially toxic, will allow to significantly reduce the multitude of possible formulation and facilitate ERA processes.

Finally, most of the current legislation leave the door open to biosourced and/or biodegradable plastic bags, except for oxodegradable plastics that have been banned in Europe (European Parliament and Council, 2019). Despite the fact that several studies underlined the limits of current standards to mimic the fate of so-called “biodegradable plastics” in environmental conditions (Napper and Thompson, 2019), most toxicity tests on biodegradable plastics only concerned the polymers alone and do not yet take into account the toxicity of additives and degradation by-products (Paul-Pont et al., 2023).

Considering the large variety of composition of plastics and widespread dispersion in the Oceans, a more holistic view of plastic pollution is emerging by diverse stakeholders at the regional, national and international levels. There is an urgent need for further studies on accurate ERA measurements to support the current and future government measures and to increase their scope by being more realistic on the potential impact of plastic litters in the marine environment.

2.5.2 Plastic marine litters in the seawater quality assessment

In the last few years, plastic litter was selected as a criterion for water quality assessment in several countries. This was the case for the Canadian Water Quality Guidelines in 1999 for the Protection of Aquatic Life (Canadian Council of Ministers of the Environment, 1999), the European amendment in 2019 to the Marine Strategy Framework Directive (European Parliament and Council, 2008) and the United States amendment « Beaches Environmental Assessment and Coastal Health Act » in 2020 (not mandatory) to improve the quality of coastal recreation waters (United States Congress, 2000). Contrary to other chemical pollutants, none of these guidelines gave threshold and they focused only on macroplastics, not on MPs. Considering the size range among MPs may lead to a large variability of behavior and toxicity, it may be relevant to consider specific sizes ranges that remains to be clarified for toxicity/ecotoxicity as done for air particles. Other guidelines on water quality assessment omit plastic, as the Australian and New Zealand

Guidelines for Fresh and Marine Water Quality or the ASEAN Marine Water Quality Management Guidelines and Monitoring Manual (Asean, 2008). Adding plastic in the water quality assessment with a specific monitoring is a step further to better evaluate plastic pollution. Data on the temporal and spatial dynamics of MP concentration are needed for ERA. Another critical aspect of an effective ERA is missing: the development and standardization of toxicity studies (Gouin et al., 2019). Unfortunately, this coincides with the vast majority of European projects concerning marine litter being focused on “Monitoring” whereas “Risk Assessment” projects were underrepresented (Maes et al., 2019). We listed below three main aspects that should be taken into consideration for further improvement to include plastics in seawater quality assessment:

- *Plastic: a peculiar pollutant.* As explained above, plastic encompasses 3 levels of toxicity: physical, chemical and biological, making plastic a peculiar pollutant that should be assessed accordingly. Indeed, the existing frameworks for assessing environmental risks of pollutants, which are used in regulatory contexts worldwide, are yet to be applied to marine MPs. Such a generic ERA is composed of an exposure assessment, an effect assessment and a risk characterization and objectively determines the risk of a contaminant to marine ecosystems (Everaert et al., 2018).
- *Regulation on chemical toxicity.* The presence of harmful chemicals on commercial products is regulated by the “Registration, Evaluation and authorization of chemicals” (REACH) in the European market (Rudén and Hansson, 2010), by the “Toxic substances control act” (TSCA) in the US (Krimsky, 2017) and by the Canadian Environmental Protection Act (CEPA) in Canada (Scott, 2009). Additives such as bisphenol-A or phthalates have been banned in EU and North America through these regulations ((Conti et al., 2021)). Concerning plastics, the TSCA excluded completely all polymers because “they do not present an unreasonable risk of injury for human health or the environment” (US EPA and Office of Pollution Prevention and Toxics, 2014). On the other hand, REACH covers, in theory, monomers and polymers. However, there are in practice no requirements for their registration and evaluation “... until those that need to be registered due to the risks posed to human

health or the environment can be selected in a practicable and cost-efficient way on the basis of sound technical and valid scientific criteria” (European Parliament and Council, 2006b). The CEPA covers also in theory polymers, however without any standardized toxicity tests there is no possibility to determine the toxicity of a plastic.

- *Limits of actual toxicity standards.* In order to assess risks with the goal of setting risk reduction targets in a global approach of decision support, ERA tools such as standardized bioassays are essential. Numerous standardized toxicity tests already exist for the marine environment: EPA (1004.0 to 1008.0, 2019.0), ISO (5430:2023, 10253, 11348, 14380, 14669, 16712, 17244, 19820, 20666), OECD (203, 210, 210), ASTM (E1367-03, E1611-21, E1562-22, E2122-22, E729-23, E1191-03A(2023)e1, E724-21, E1218-21, E1022, E1192). These standards focus on chemical toxicity, but do not consider a physical or biological pollution. New standards are needed for an effective ERA of the physical effects of plastics, by using different sizes and concentrations. Very few initiatives have been putted also in standardizing the biological effect of plastic pollution, including the transport of invasive or pathogen species.
- *Evaluation of chemical toxicity.* Even though chemical toxicity of plastics could be assessed using already available standards, another adjustment is still needed: the standardization of leaching of additives. No standard exists on the leaching time, presence or absence of light/UV radiation, temperature. Other key methodological points are the plastic size class that should be introduced in the leachate and at which state (pristine or pre-weathered), as well as their specific shape (using of pre-grinding to reduce the specific surface difference, for example) or state of polymer oxidation. A special care to the laboratory equipment is needed in order to reduce cross contamination of additives (Hermabessiere et al., 2020). Glassware is strongly recommended for leachates formation.

- *Evaluation of physical toxicity.* The ideal way to observe MP physical toxicity would be through chronic experiments and using either irregular sized MPs or fibers which are the most recovered in the environment. Moreover, plastics should undergo a bacterial colonization of at least several weeks in the marine environment (Jacquin et al., 2019) and plastic sizes should be coherent with the species tested in terms of bioavailability and ingestion rate. In addition of a negative control, a “particulate control” with a natural particle such as smectites, diatomites or kaoline mimicking mineral particle in the environmental water is recommended. The objective is to decipher specific physical injuries related to plastic.

2.6 Conclusion

The omnipresence of MPs in marine waters makes a vast range of biota susceptible to MPs exposure, with a variety of adverse effects at different trophic levels of the marine food web and from molecules to population levels. Gaps concerning the quantification of exposure to large and small MPs as well as NPs in the water column and in benthic environments still needs to be addressed for relevant ERA. Moreover, methods to evaluate the hazardous effects of NPs and the potential difficulties of their identification in organs and tissues are still under development. In addition, knowledge about toxic effects suffers from non-negligible methodological biases that limit an effective ecological risk assessment of plastic in the marine environment. To tighten the gap between the environment and laboratory experiments, we mentioned that special cares are needed in further studies by considering the plastic type, size, shape, state of oxidation, concentration and colonization by marine microorganisms to better fit to environmental conditions and gaining into exhaustivity and therefore complexity. Public policies including seawater quality assessment concerning plastics are still in their infancy. The lack of scientific knowledge on the chemical, but also physical and biological aspects associated with plastic pollution, hinders the development of new standards that are more representative of the fate of plastics in the marine environmental conditions. With the development and analysis of growing datasets on acute and chronic exposure across discrete organisms in various environments, we will be able to transition from baseline and monitoring to an effective

ecological risk assessment of plastic pollution in the marine environment. These goals are critical, as we move forward towards a sustainable future of improved human and ocean health.

3. Presentation of the thesis objectives and organization

Plastic pollution is nowadays a major environmental and societal problem. Toxic effects are observed at all levels of biological integration (cellular to population) all over the world. In addition, the peculiarity of plastic complexifies an holistic analysis since its impact encompasses physical, chemical and biological toxicity. This PhD thesis focuses on the chemical and physical toxicity of microplastics (MPs) and does not consider the effect of organisms associated to plastics (including pathogens). We decided to focus on the toxicological effects of different polyethylene(PE)-based MPs which is the most produced polymer and consequently the most recovered in the marine environment (Erni-Cassola et al., 2019; Plastics Europe, 2023).

The objective of this PhD thesis was to better decipher the chemical and physical impact of plastic on marine organisms. Plastic chemical toxicity either originates from the leaching of plastics chemicals or through the adsorption of pollutants on plastic surfaces. The first chapter is focused on understanding the toxicity kinetics of plastic chemical leaching and to determine the origin of toxicity. The second chapter is focused sorption of pollutants on plastic surfaces. The objective was to better grasp the sorption capacity of plastic in the marine and freshwater environment and the toxicity of the adsorbed pollutants. The objective of the third chapter was to better understand the physical impact of MPs through ingestion. Thus, a filter-feeding organism was exposed to MPs at environmentally relevant concentrations and shape.

A schematic presentation of the three chapters of this PhD thesis is depicted as follows (Figure 10):

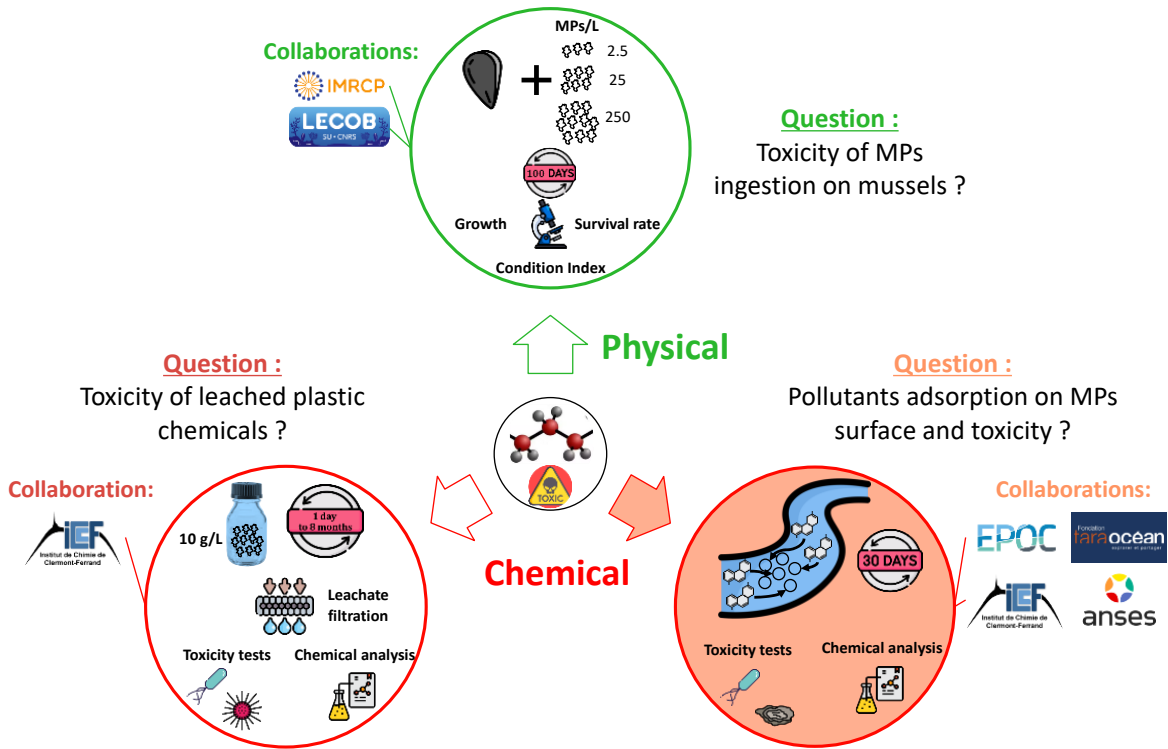


Figure 10: Graphical representation of the PhD thesis objectives

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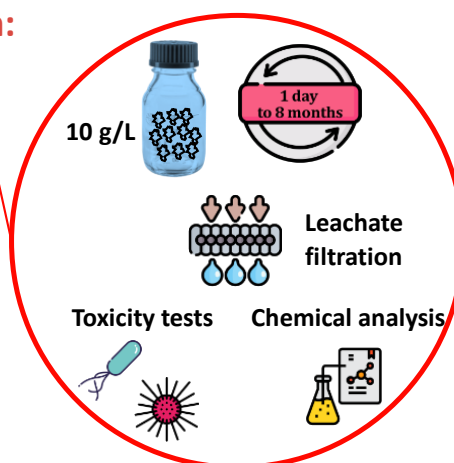
Chapter 1 – Toxicity of plastic leaching on bacteria and sea urchin

Foreword

Question :

Toxicity of leached plastic chemicals ?

Collaboration:



Context: Chemical toxicity of plastics can either be due to leaching or pollutant adsorption. Here, we focused on the effect of leaching, which is defined as the migration of plastic chemicals (including additives and non-intentionally added substances-NIAS) from the plastic to the surrounding medium. Most studies evaluating the toxicity of plastic leaching used leaching time between 1 and 10 days and none lasted more than a month. In this chapter, we focused on the toxicity kinetics as a function of the leaching time from days to months.

Methods: Four types of PE-based plastics were grinded at a similar size (100 μm – 2 mm). Then leachates of 1 day, 10 days, 1 month, 2 months, 4 months and 8 months were performed. After filtration, leachates were tested by using two standardized toxicity tests (bacteria and sea urchin larvae). Another non-standardized biometric measurement (i.e. larvae size) was performed. Furthermore, an inorganic element analysis through an

Induced Coupled Plasma Optical Emission Spectrometry (ICP-OES) was realized on the leachates and on the virgin/leached MPs.

Results: The most toxic polymer was the oxodegradable PE-based plastic. We found that leaching time played a critical role on the observed toxicity, with a minimum of one month of leaching that was necessary to detect toxicity. We observed for the first time the toxicity kinetic (i.e. exponential decay) in function of the leaching time. The inorganic chemical analysis partially explained the toxicity, suggesting that other parameters (e.g. organic chemicals, cocktail effect) not considered in this study were also involved. Finally, we found that the sea urchin larvae test was more sensitive than the bacteria toxicity test in our experimental conditions.

This chapter introduced the chemical toxicity of plastics, by focusing on the leaching of plastic chemicals. The next chapter will focus on the second aspect of chemical toxicity: adsorption of pollutants.

Manuscript Title: Critical impact of leaching time on marine toxicity tests for plastics

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Highlights:

- Leaching time plays a critical role in the release of toxic compounds from plastics
- Exponential decay of EC₅₀ values with increasing leaching time
- Partial explanation of the toxicity origin through elemental analysis
- Sea urchin larval size and development were more sensitive than Microtox[®] tests

Status: In preparation

Abstract

Plastics are peculiar pollutants that can induce 3 types of toxicity: physical, biological and chemical. In this study, we focused on their chemical toxicity through a leaching experiment. Most studies evaluating the toxicity of plastic leaching had leaching time between 1 and 10 days and none lasted more than a month. Thus, the influence of leaching time on toxicity was tested after 1 day, 10 days, 1 month, 2 months, 4 months and 8 months. Four plastic films made of the same polymer (polyethylene, PE) were analyzed: PE without any additives (PE-pure), recycled PE from plastics recovered at sea (PE-recycled), bubble wrap (PE-bubble) and oxo-degradable (PE-oxo). Leachates were tested with two standardized tests: a bioluminescent bacterium (*Aliivibrio fischeri*, Microtox[®], ISO 11348-3) and sea urchin larvae development (*Paracentrotus lividus*, ASTM E1563). In addition, a non-standardized test on sea urchin larvae size was performed. Chemical analyzes of the leachates, virgin and leached materials were characterized by Induced Coupled Plasma (ICP) analysis. Standard tests showed toxic effects after one month of leaching for PE-oxo and after eight months for PE-pure (only detected by the embryotoxicity test), but no effects were observed for PE-bubble and PE-recycled leachates. Interestingly, sea urchin larvae size tests revealed that toxicity was already detectable before 10 days. Toxic effect could be explained by the presence of zinc in the PE-pure leachates, but ICP analysis could not explain the plastic toxicity for PE-oxo. Direct exponential decay relationship was found between EC₅₀ and the leaching times. Under our conditions, larvae size tests were more sensitives than the embryotoxicity tests which were in turn more sensitives than the *A. fischeri* tests.

Keywords: Plastic leaching, Metal additives, Oxo-degradable, Recycled plastic, Bubble-wrap, Standardized toxicity tests, Marine models

1. Introduction

The constant increase of plastic production coupled with a poor waste management lead to an overall microplastic estimated concentration of 24.4 trillion pieces ($8.2 \times 10^4 \sim 57.8 \times 10^4$ tons) in the world upper ocean (Isobe et al., 2021). Plastic pollution has such an impact on the environment that it is nowadays considered as a marker of the Anthropocene epoch (Rangel-Buitrago and Neal, 2023). This overwhelming pollution impacts the whole marine food web at all levels of biological integration (i.e., molecular to population level) (Leistenschneider et al., 2023). Plastic toxicity encompasses the three types of stressors that may affect an organism, i.e. physical, chemical or biological stressors (Rose, 1998). This peculiar pollutant impacts physically organisms through entanglement, ingestion or smothering (GESAMP, 2015). It acts also as biological stressor due to the possible presence of pathogenic bacteria on its surface (Bowley et al., 2021; Kirstein et al., 2016). Finally, plastic impacts wildlife chemically through the release of its chemical or the sorption of chemical pollutants from the environment (Hermabessiere et al., 2017; Prajapati et al., 2022).

Chemical additives are an essential component of plastics and confer different properties. They can either be used as fillers, pigments, plasticizers, stabilizers or flame retardant (Murphy, 2001). Additives quantities vary depending on the polymer type and plastic use. For example, Polyvinyl chloride (PVC), which accounts for 10% of global plastic production (Geyer et al., 2017), uses 73% of additives production in volume (Murphy, 2001). Polyolefins, which accounts for 48% of global plastic production (Geyer et al., 2017), generally use 10% of the overall additive production in volume (Murphy, 2001). Based on the worldwide plastic pollution it was estimated that up to 917 tons of plastic additives are released in the environment each year (Suhrhoff and Scholz-Böttcher, 2016). The migration of chemical additives is facilitated, since the vast majority of additives are not chemically bound to the plastic polymer (Hahladakis et al., 2018). Beside these intentionally added additives, a set of non-intentional added substances (NIAS) are also present in plastics, including side products, breakdown products and contaminants (Geueke, 2018). Most of these remain unknown (Geueke, 2018) and represent a consequent threat since they can represent half of the compounds that leach out of plastics (UNEP, 2023a). Recently, the

number of plastic chemicals was evaluated to more than 16,000 additives (NIAS included), of which 66% do not have any hazard information. Moreover, from the 4,200 plastic chemicals of concern more than 3,600 are unregulated (Wagner et al., 2024).

Common chemical additives such as phthalates have been showed to display a role in reproductive toxicity and DNA damage in animals and humans (Sedha et al., 2021). Moreover, an important and repeated exposure to Bisphenol A (BPA) induces genotoxicity, mutagenicity and carcinogenicity in animals and humans (Tarafdar et al., 2022). Since these additive toxicities are well documented, they were partially banned in the European Union and North America. For example, phthalates have been forbidden in children's toys and BPA in food containers (i.e. bottles) (Conti et al., 2021). Numerous other additives were also shown to leach from plastic in water, such as brominated flame retardants (Kim et al., 2006), formaldehyde (Mutsuga et al., 2006), benzene and other volatile compounds (Skjevrak et al., 2003). In these studies, the measured concentrations in the leaching medium were most of the time lower than the toxicity test guideline limit values. However, there is no guideline on plastics leachate preparation, which can be greatly influenced by leaching time, T°C, agitation speed, light, shape and oxidation state of plastic (Hansen et al., 2013). Moreover, most toxicity tests were based on the effect of one chemical additive alone, which is not representative of the mixture of additives ('cocktail effect') that are commonly found in commercial plastics (Hahladakis et al., 2018). To date, most of the studies proceed to relatively short leaching periods (between 1 and 3 days). Few studies performed longer leaching periods of 14 (Capolupo et al., 2020) or 28 days (Chae et al., 2020). No studies have investigated leaching times longer than a month (Almeda et al., 2023). The plastic shape differed from powder to films, bags or under their sold shape (e.g. pipes, CD, toys), making comparison between studies difficult. Several review papers underlined the lack of standard toxicity test for plastics and a disparity compared to the ones made with water-soluble contaminants (Leistenschneider et al., 2023).

The aim of this study was to evaluate the impact of leaching time from different plastic types on the toxicity response of marine organisms. The experiments were performed with 4 plastics with similar polymer types (low density polyethylene, LDPE) but different additive compositions. Different plastic leaching periods (1 day, 10 days, 1 month, 2 months, 4 months and 8 months) were performed in seawater and tested for putative toxicity by

comparing two standardized marine toxicity tests. The first test evaluated the toxicity through the diminution of *Aliivibrio fischeri* (formerly *Vibrio fischeri*) bioluminescence. The second toxicity test consisted in evaluating the embryotoxicity of plastic leachates on sea urchin embryos (*Paracentrotus lividus*) coupled with pluteus size measurement. Finally, to better comprehend the origin of the toxicity, an Inductively Coupled Plasma (ICP) element analysis was performed on the leachates and on the virgin/leached plastics.

2. Materials & Methods

2.1 Testing materials and cryogrinding

Tests were performed on four plastic types made of similar polymer composition and similar form of films with similar thickness of 50 μm . The first two plastics were manufactured for plastic bag usage (kindly provided by Symphony Environmental Technology), including “PE-pure” composed of Low-Density Polyethylene (LDPE) without any additives added on the initial plastic pellets and “PE-oxo” formed at 99.84% of PE-pure together with 0.10% of Cobalt and 0.06% of Manganese. The third plastic, called “PE-bubble”, was commercially available in the form of plastic bubble wrap for packaging, which was composed of LDPE with unknown additives (kindly provided by BBA emballages, <https://www.bbaemballages.com/rouleau-film-bulles>). The fourth plastic is the recycling product of marine debris 100% made of LDPE, called “PE-recycled” (kindly provided by Waste Free Oceans, <https://www.wastefreeoceans.org/>). Films were obtained from pellets after 10 min pressing with a temperature of 140°C (LDPE melting point) and a pressure of 210 bar. The LDPE nature of each polymer was verified by Fourier Transform Infrared (FTIR) analysis. Spectra of film samples were recorded in attenuated total reflection mode (ATR) using a Cary 630 spectrometer (Agilent technologies) equipped with a diamond crystal (refractive index = 2.5). Spectra were recorded with 32 scans at a resolution of 4 cm^{-1} and were compared with a commercial database (Hummel polymers library) (Supplementary Data 1.1).

Cryogrinding was performed with the IKA A11 coupled with a cutting blade A11.2, as previously described (Mika et al., 2020). Briefly, the films were pre-cut in small pieces and then added to the grinder filled with liquid nitrogen. In total, 3 minutes of grinding were performed, with an addition of liquid nitrogen after each minute of grinding. The plastic was then sieved under different inox mesh: 5 mm, then 2 mm followed by 100 μm . The selected powder size for the leaching experiment was between 2 mm and 100 μm .

2.2 Leaching procedure

The leaching was performed in sterilized seawater (SSW) from the Banyuls Bay (NW Mediterranean Sea) after filtration at 0,2 μm (PC filter, Millipore) and autoclaving at 110°C.

Two grams of grinded plastic were added to 200 mL of SSW in 250 mL autoclaved bottles (equivalent to 10 g/L). This concentration was in accordance to the Dutch standards intended to test leaching of landfilled waste materials with particle size below 4 mm (NEN, 1995), which is 5 times lower than the American standard (US EPA, 1994) and 10 times lower than the European standard (CEN, 2004).

Different leaching times were performed: 1 day, 10 days, 1 month, 2 months, 4 months and 8 months. The leachates were mixed during the entire experiment with a rotating speed of 120 rpm with an IKA agitator (KS 501 digital, IKA Werke) at room temperature (18-20°C). The bottles were inclined in order to maximize the contact between plastic and the SSW leaching medium. A control was realized for each sampling time with SSW only. At the end of each leaching period, all leachates were filtered to 3 µm (PC, Millipore) in order to remove particles and then stored at -80°C. Before each experiment, the pH of each leachate was verified and adjusted if necessary to a value of 7-8. The salinity was also adjusted if necessary between 39-41 g/L for the embryotoxicity test and at 31 g/L for the Microtox® assay.

2.3 Measurement of metals in virgin/leached plastic and leachates

The metal and trace element content was determined for each virgin and leached polymer. Preliminary mineralization was carried out by microwave-assisted acid digestion. 200 mg of sample were weighed out in a PTFE vessels and 10 ml of concentrated nitric acid were added. The sample were digested using a Multiwave 5000 microwave system (Anton Paar) for 1 hour following a temperature ramp from 90 to 240 °C. After cooling, the digesta were transferred to 50 ml volumetric flask and diluted precisely to 50 ml with ultrapure water prior to analysis. Metal and trace element content of leachates and mineralized samples was measured on 10 ml aliquot. Concentrations of trace elements were determined by Inductively Coupled Plasma Optical Emission spectrometry using 5800 ICP OES Agilent system. The ICP system was calibrated, using multielement standard solutions containing: Aluminum (Al), Boron (B), Barium (Ba), Bismuth (Bi), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Gallium (Ga), Potassium (K), Lithium (Li), Magnesium (Mg), Manganese (Mn), Sodium (Na), Nickel (Ni), Lead (Pb),

Selenium (Se), Strontium (Sr), Tellurium (Te), Titanium (Ti), Zinc (Zn). The category corresponding to metals and other toxic trace elements was defined as: Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Ti and Zn.

2.4 Microtox® toxicity test

We used Microtox® test based on the bioluminescence of bacteria *Aliivibrio fischeri* (Beijerinck, 1889), in accordance with the ISO 11348-3 standard procedure, as previously described (Gómez et al., 2023). The positive control was performed using 3,5 dichlorophenol. All dilution (samples and control) was performed with artificial seawater (ASW) at 31 g/L since the Mediterranean seawater possesses a salinity too high for *A. fischeri* (40 g/L) as indicated in the standard. Dilutions with ASW are prepared in vials and incubated at 15°C. The highest concentration tested was 8 g/L. Lyophilized bacteria (Microtox® Acute Reagent, Modern Water Inc.) were resuspended in 1 mL of distilled water at 4°C. After waiting 10 minutes, 10 µl of suspended bacteria were added to ASW and were incubated 15 min at 15°C. The initial luminescence (without any sample; I_0) was measured through the Microtox LX incorporated vial reader (Microtox LX, Modern Water® Inc.), directly followed by the introduction of the different leachates, following manufacturer instructions. The luminescence is then measured 15 and 30 min after. The presented results are based on the luminescence after 30min of exposition. The percentages of bioluminescence inhibition are expressed as a percentage of bioluminescence decrease relative to control water.

2.5 Embryotoxicity in sea urchin

The embryotoxicity test on sea urchin was performed following the standard ASTM E1563, as previously described (DeForest and Schlekat, 2013). The sea urchins, *Paracentrotus lividus* (Lamarck, 1816), were sampled in Banyuls bay by snorkeling and further released just after the induction of the gametes. As soon as the production of gametes was induced, females were put on beakers filled with seawater and males were left dry. If the gametes were not released after 1 min of shaking, 1 mL of acetylcholine at 0,2 M was injected. Gametes of at least 10 males and 10 females were recovered and

counted. The eggs fertilization was realized with a sperm:egg ratio of 2500:1. After 30 min, the efficiency of fertilization was verified through binocular magnifier. If the fertilization was > 90%, the embryos were exposed to different concentrations of leachates in 24-well plates. Each concentration of leachates was performed in triplicates with around 50 embryos per replicate. Two-fold serial dilutions were performed with SSW. In parallel, cobalt was tested in the same conditions. After a 72h exposition, the larval development was blocked with 50 μ l of formaldehyde 1%. Immediately after, the larval development was observed through a widefield inverted microscope, and pictures were taken. The toxicity of the leachates, as well as that of pure cobalt, was evaluated using this method.

Concerning embryotoxicity measurements, results were measured in each replicate as the percentage of embryological success. Pluteus were considered well-formed if their four arms were fully developed and had the same morphology than control larvae. Abnormal larvae and undeveloped eggs were counted separately.

In addition, larvae size was measured in conditions where no impact was observed in embryotoxicity (i.e. leaching of 1 and 10 days). The total size of 20 larvae (Carboni et al., 2012) was measured in each replicate using the software ImageJ® (Abràmoff et al., 2004).

2.6 Statistical analyzes

All statistical analyzes were performed using R software (R Core Team, 2021). Embryotoxicity and bioluminescence results were analyzed with the “drc” package (Ritz et al., 2015). The “drm” function was used, with a symmetric model named Four-parameter log-logistic (LL.4), to display the curves model and the EC₅₀. To evaluate the precision of the model applied compared to the original data the root-mean-squared-error (RSME) was calculated. The formula used for the RSME is described as follows:

$$\sqrt{\frac{\sum_{i=0}^n (obs_i - pred_i)^2}{n}}; \text{ where } n = \text{number of values; } obs_i = \text{actual observation values; } pred_i =$$

predicted values by the model.

The normality of the data was tested through a Kolmogorov-Smirnov ($n > 50$) test followed by a homoscedasticity test with the Bartlett test. To determine statistical differences with the control we performed a Kruskal-Wallis test, for non-normally distributed data, followed by a Dunn test. The selected p-value was not adjusted since we

simply compare a specific site of a river to a control. The p-values were considered statistically significant when they were <0.05 . The first concentration to be statistically different from the control was defined as the Lowest Observed Effect Concentration (LOEC). The highest concentration before observing a statistical difference from the control was determined as the No Observed Effect Concentration (NOEC). When the NOEC, LOEC and EC_{50} values were beyond the range of tested dosage (> 10 g/L or 8 g/L) were reported as “ > 10 ” for the larvae development and “ > 8 ” for the bioluminescence inhibition test.

After testing the normality and homoscedasticity, significant differences in pluteus size were evaluated through an Anova test followed by a Tukey post hoc test.

3. Results

3.1 Chemical analysis of plastics and leachates

An induced coupled plasma (ICP) analysis of inorganic elements in the leachates, virgin and leached-materials was performed. We mainly focused on the quantitative analysis of metals and other trace elements with potential toxicity such as cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), titanium (Ti) and zinc (Zn).

Concerning the virgin materials, PE-recycled is the polymer containing the highest number of metals and other toxic trace elements (i.e. 8) (Table 1). Other polymers were composed of 4 (i.e. PE-oxo) or 5 (i.e. PE-pure and PE-bubble) metals. Inorganic elements clearly differed in number and type within plastics made from a single polymer. After the polymer leaching, we either observed a decrease or the same concentration in the quantity of metals compared to the virgin materials. Interestingly, the decrease in elements concentration between the leached and virgin plastic did not always result in an increase in the leachate concentration. Indeed, despite the decrease of elements concentration in the leached PE-recycled, none were measured in the 8 months leachate. PE-pure and PE-bubble showed a leaching of Zn of 0,09 mg/L and 0,01 mg/L (Supplementary Data 1.2), respectively. Concerning PE-oxo, the increase of leaching time showed a slight increase in leachate concentration going from 0,01 mg/L after 1-2 months to 0,02 mg/L of Co after 4

to 8 months. Mn was not detected after 1 month of leaching but found at a concentration of 0,01 mg/L until 8 months.

Analyzed material	Name	Number of elements detected	Metal and other toxic trace elements detected
Plastic	PE-oxo	4	Co, Mn, Fe, Zn
Plastic	PE-oxo_leached 1 month	4	
Plastic	PE-oxo_leached 2 months	4	
Plastic	PE-oxo_leached 4 months	4	
Plastic	PE-oxo_leached 8 months	4	
Leachates	PE-oxo_1 month	2	Co, Ni
Leachates	PE-oxo_2 months	2	Co, Mn
Leachates	PE-oxo_4 months	2	
Leachates	PE-oxo_8 months	3	Co, Mn, Zn
Plastic	PE-pure	5	Cr, Fe, Mn, Ti, Zn
Plastic	PE-pure_leached 8 months	3	Fe, Ti, Zn
Leachates	PE-pure_8 months	1	Zn
Plastic	PE-recycled	8	Cr, Cu, Fe, Mn, Ni, Ti, Pb, Zn
Plastic	PE-recycled_leached 8 months	8	
Leachates	PE-recycled_8 months	0	
Plastic	PE-bubble	5	Cr, Fe, Ni, Ti, Zn
Plastic	PE-bubble_leached 8 months	5	
Leachates	PE-bubble_8 months	1	Zn
Leachates	SW_1 month	0	
Leachates	SW_2 months	0	
Leachates	SW_4 months	0	
Leachates	SW_8 months	1	Zn

Table 1: Number and abbreviation of metals or other potentially toxic trace elements detected in the leachates, virgin and leached-plastics. SW = Seawater, Co = cobalt, Cr = chromium, Cu = copper, Fe = iron, Mn = manganese, Ni = nickel, Pb = lead, Ti = titanium, Zn = zinc

In addition, a Principal Component Analysis (PCA) describing metals and other toxic trait elements was performed on the leachates and materials. The leachates factor explained at 78.2% the differences and the materials factor explained at 91.7% the database of the PCA (Figure 11). In both cases, PE-oxo showed to be different from other leachates or other plastic due to the presence of Co and Mn. PE-pure leachate differentiates itself from the others due to its Zn concentration. PE-recycled plastic showed a difference in concentration of Cu, Cr, Fe, Pb, Ni. PE-bubble material differentiates itself due through its Zn concentration, that was not found in the leachates.

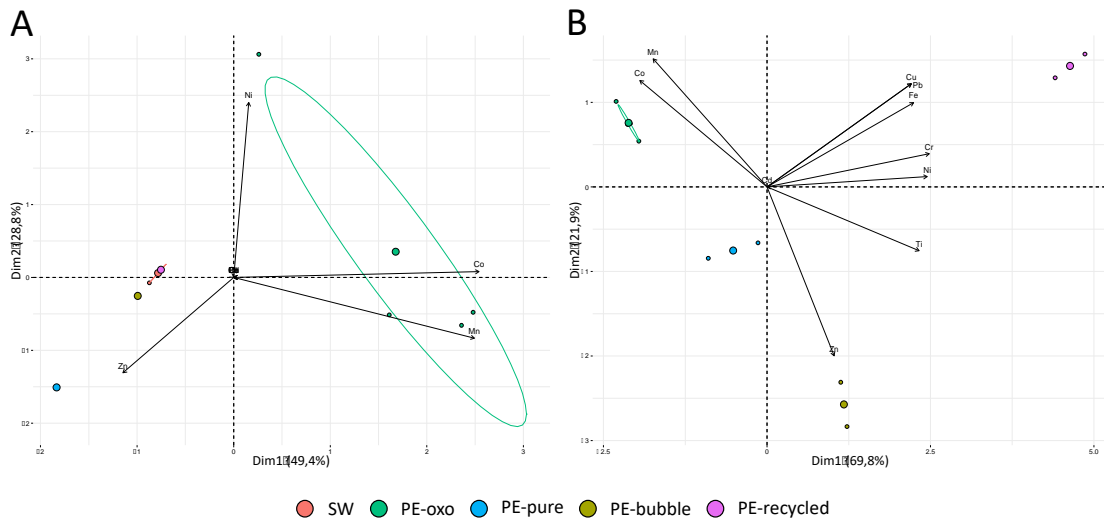


Figure 11: Principal component analysis on the ICP results of the leachates (A) and the virgin/leached plastics (B). Only the metals and other toxic trait elements are presented. Cu = Copper; Co = Cobalt; Cr = Chromium; Fe = Iron; Mn = Manganese; Ni = Nickel; Pb = Lead; Ti = Titanium; Zn = Zinc. SW = Seawater.

3.2 Effect of different leachate types, concentrations and times on the bioluminescent activity of the bacteria *A. fischeri*

PE-pure, PE-recycled and PE-bubble's leachates did not exert any toxicity whatever the duration of the leachate production. PE-oxo induced toxic effects statistically different from the control after only 1 month of leaching. Toxicity values are expressed as a percentage of bioluminescence decrease relative to control water. Toxicity of PE-oxo increased significantly with leaching time (Figure 12). The NOEC and LOEC after 1 and 2 months of PE-oxo leaching were similar and equal to 5 and 8 g/L, respectively (Table 2). Until 2 months, no EC_{50} was determined since the values never reached 50% of toxicity. After 4 months the NOEC and LOEC reached 3.33 and 5 g/L, respectively. We observed a significant diminution of the NOEC and LOEC after 8 months with values equal to 0.42 and 0.63 g/L, respectively. Moreover, we also observed more than a 10-fold diminution of the EC_{50} between 4 and 8 months of leaching.

	NOEC (g/L)	LOEC (g/L)	EC ₅₀ (g/L)	RMSE
PE-bubble 8 months	> 8	> 8	> 8	N/A
PE-recycled 8 months	> 8	> 8	> 8	N/A
PE-pure 8 months	> 8	> 8	> 8	N/A
PE-oxo 1 day	> 8	> 8	> 8	N/A
PE-oxo 10 days	> 8	> 8	> 8	N/A
PE-oxo 1 months	5	8	> 8	N/A
PE-oxo 2 months	5	8	> 8	N/A
PE-oxo 4 months	3.33	5	7.86	1.82
PE-oxo 8 months	0.42	0.63	0.61	3.74

Table 2: NOEC, LOEC, EC₅₀ values of plastic leaching (g/L) on the bioluminescence inhibition of Aliivibrio fischeri. The root mean square error (RMSE) was also calculated when EC₅₀ was obtained. N/A = not assessed.

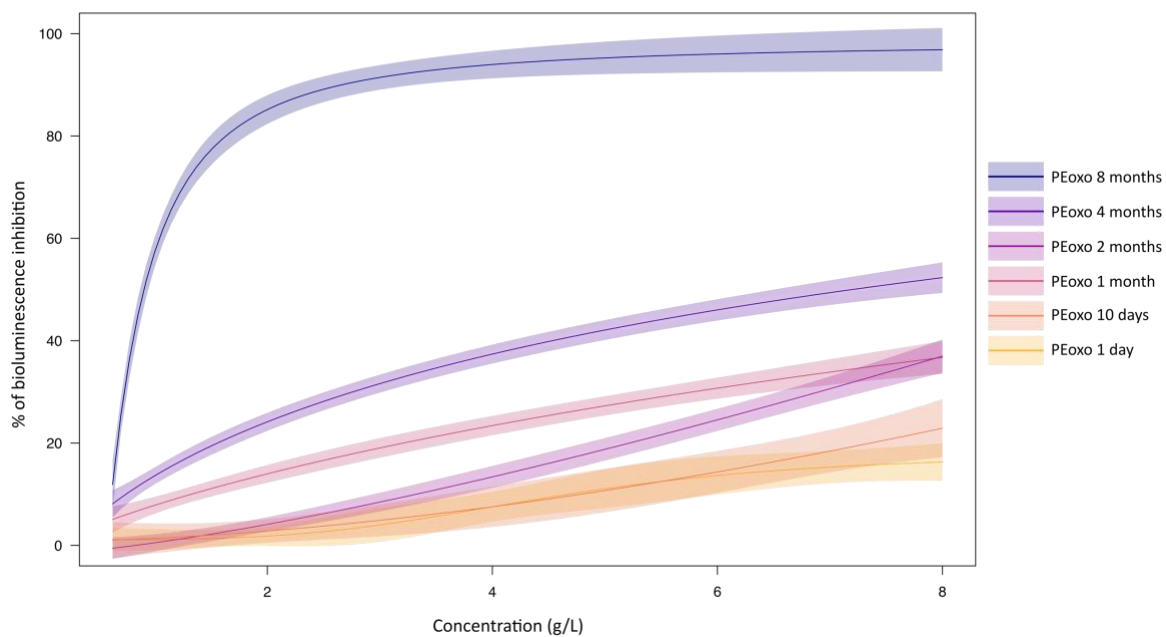


Figure 12: Means of bioluminescence inhibition of Aliivibrio fischeri for all PE-oxo leachates. Toxicity values are expressed in percentage of bioluminescence inhibition in function of the plastic concentration in the leachate(g/L). The curve represents the drc model and the shaded areas indicate the confidence interval at 95%.

3.3 Effect of different leachate types, concentrations and times on the sea urchin larvae development

3.3.1 Embryotoxicity tests

Embryotoxicity tests showed congruent results with the Microtox® tests, with no detected toxic effect for PE-recycled and PE-bubble leachates, whereas PE-oxo leachates induced toxicity. Furthermore, PE-pure induced toxic effects after 8 months.

PE-pure induced toxicity reached 83% of malformed larvae at 10 g/L, leading to an extrapolation of more than 100% at 20 g/L, allowing the estimation of the EC₅₀ at a value of 7.95 g/L.

Considering PE-oxo, no toxicity was observed after 1 and 10 days of leaching (Table 3). First signs of toxicity were observed after 1 months of leaching with NOEC and LOEC equal to 5 and 6 g/L, respectively. After 2 and 4 months of leaching, similar NOEC (i.e. 1.5 g/L) and LOEC (i.e. 2 g/L) were obtained. After 8 months a notable decrease in the NOEC and LOEC values were observed: 0.25 and 0.3125 g/L, respectively. The increase in toxicity was correlated to an increasing leaching time (Figure 13). This resulted in a clear decrease in the EC₅₀ values from 1 to 8 months of leaching, that ranged from 7.9 g/L to 0.27 g/L, respectively (Table 3). When plotted with the leaching time, these EC₅₀ values followed an exponential decay with a $R^2 = 0.94$ (Figure 14). To compare this result, we tested cobalt toxicity on larvae development. The NOEC and LOEC were estimated at 1 and 5mg/L, respectively, and the EC₅₀ was evaluated at 2,68mg/L.

	NOEC (g/L)	LOEC (g/L)	EC ₅₀ (g/L)	RMSE
PE-recycled 8 months	> 10	> 10	> 10	N/A
PE-bubble 8 months	> 10	> 10	> 10	N/A
PE-pure 8 months	5	6	7.95	3.29
PE-oxo 1 day	> 10	> 10	> 10	N/A
PE-oxo 10 days	> 10	> 10	> 10	N/A
PE-oxo 1 month	5	6	7.91	5.22
PE-oxo 2 months	1.5	2	2.30	2.87
PE-oxo 4 months	1.5	2	1.60	0.56
PE-oxo 8 months	0.25	0.3125	0.27	1.68
Cobalt (values in mg/L)	1	5	2.68	1.52

Table 3: NOEC, LOEC, EC₅₀ values of plastic leaching, expressed in g/L, and on Cobalt, expressed in mg/L, on the embryotoxicity of *Paracentrotus Lividus*. The root mean square error (RMSE) was also calculated when the EC₅₀ was obtained. N/A = not assessed.

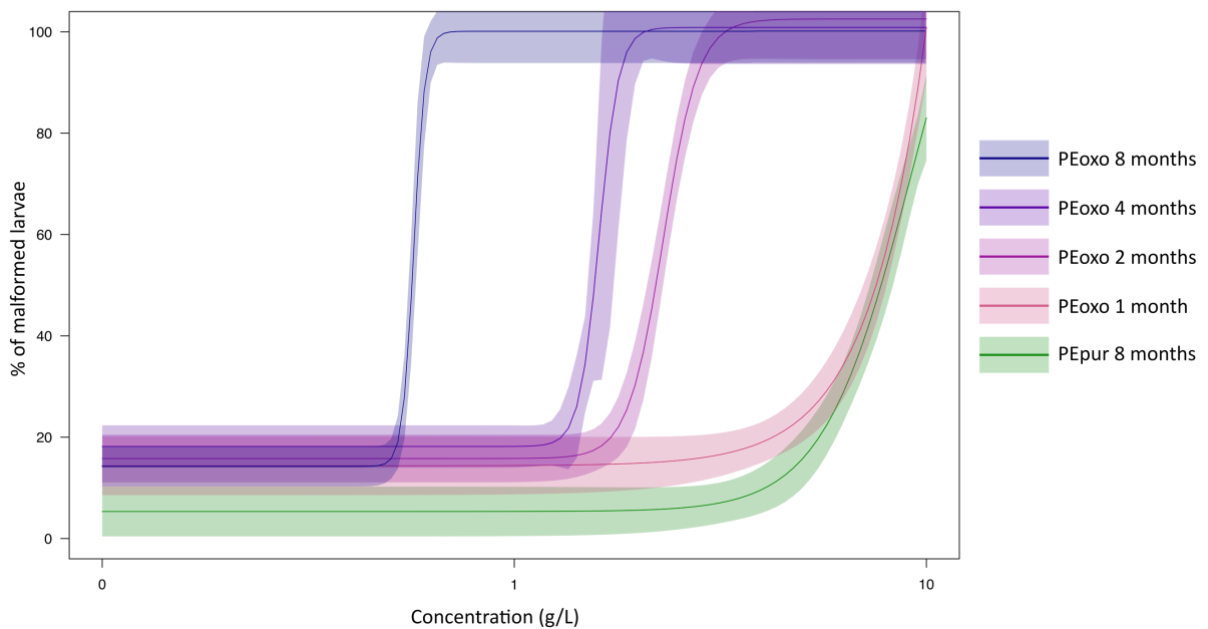


Figure 13: Means of the embryotoxicity values of leachates on *Paracentrotus lividus* larvae. Toxicity values are expressed in percentage of malformed larvae in function of the plastic concentration in the leachate (g/L). The curve represents the drc model and the error bars indicate the standard deviation

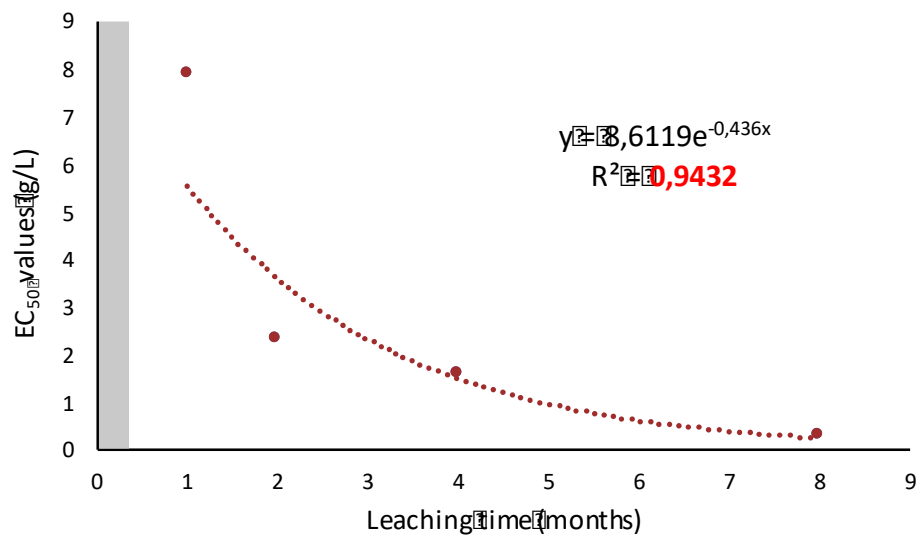


Figure 14: EC₅₀ (expressed in g/L of plastic) in function of the leaching time (expressed in months). Grey area represents leachates of 1 and 10 days, which did not induce any toxicity.

3.3.2 Influence of different leachate types, concentrations and durations on sea urchin larvae sizes

The toxic impacts of the different leachates were also measured through the whole larvae size for leaching times that did not exert any toxicity in terms of larval development (mortality and malformation) (i.e. 1 day and 10 days). After one month of leaching no well-formed larvae were observed in the PE-oxo leachate at the highest concentration (i.e. 10 g/L). Therefore, the larvae size could not be measured. This more sensitive biometric index was performed in order to visualize potential basal effects before malformation and mortality, the two criteria of the embryotoxicity test. After a day of leaching, only PE-recycled was not significantly different from the control water (Figure 15-A). On the other hand, PE-pure, PE-bubble and PE-oxo were significantly different from the control ($p < 0.0001$) and PE-recycled ($p < 0.001$). After 10 days of leaching, significantly higher pluteus sizes were observed in the control compared to all plastic leachates ($p < 10^{-8}$) (Figure 15-B). However, statistical differences after 10 days were observed between the control and the group of PE-recycled and PE-pure leachates, and the group of PE-bubble and PE-oxo with significant decrease of the larval size ($p < 0.01$). Effects of the leachates on pluteus sizes also increased with the leaching period. Indeed, PE-recycled which was not significantly different from the control after 1 day showed a significant difference after 10 days.

Moreover, significant differences in size were observed between 1 and 10 days for PE-recycled ($p < 0.01$), PE-oxo ($p < 0.01$) and PE-bubble ($p < 0.01$).

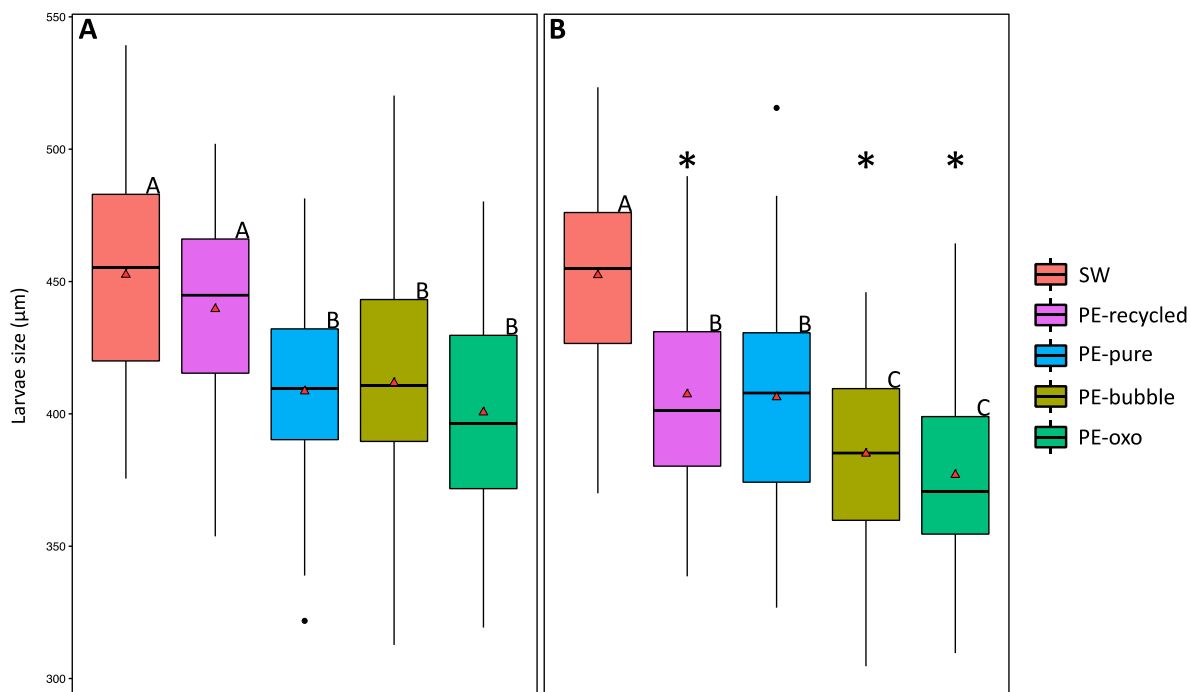


Figure 15: Impact of plastic leaching on the larvae of *Paracentrotus lividus* after 1 day (A) and 10 days (B) at the concentration 10g/L. The letters indicate the statistical differences between the different conditions by leaching time. The box represents the interquartile range, the line the median. The red triangle represents the mean value. SW = Seawater; * = significant difference between 1 and 10 days of leaching.

3.4 Comparison between EC_{50} relative to measured metal concentration and EC_{50} of the literature

Based on the results of the ICP analysis and the standardized toxicity tests, an EC_{50} relative to the metal concentration was calculated and compared to the literature (Table 4). Co and Mn concentrations reported in the PE-oxo leachates at all leaching time was at least 10^2 times inferior to the EC_{50} or LOEC observed in the literature. Concerning the embryotoxicity test, the EC_{50} relative to the Zn concentration of PE-pure leachates corresponds to the EC_{50} of Zn found in the literature. We did not obtain an EC_{50} value for PE-pure leachates with *A. fischeri*. Nevertheless, even at the highest concentration of Zn in the leachates, the EC_{50} of Zn in the literature was 10^2 times lower.

Metals	Organisms	Leachates	EC ₅₀ (relative to metal concentration) (mg/L)	EC ₅₀ literature (mg/L)	LOEC literature (mg/L)	Reference
Co	<i>Paracentrotus lividus</i>	PE-oxo 1 month	7.91*10 ⁻³	2.6831	5	This study
		PE-oxo 2 months	2.30*10 ⁻³			
		PE-oxo 4 months	3.19*10 ⁻³			
		PE-oxo 8 months	5.48*10 ⁻⁴			
	<i>Aliivibrio fischeri</i>	PE-oxo 4 months	1.63*10 ⁻²	N/A	3.8	(Fulladosa et al., 2005)
		PE-oxo 8 months	1.22*10 ⁻³			
Mn	<i>Paracentrotus lividus</i>	PE-oxo 2 months	2.30*10 ⁻³	8.85	N/A	(Pinsino et al., 2010)
		PE-oxo 4 months	1.60*10 ⁻³			
		PE-oxo 8 months	2.74*10 ⁻⁴			
	<i>Aliivibrio fischeri</i>	PE-oxo 4 months	8.17*10 ⁻³	351.35	N/A	(Teodorovic et al., 2009)
		PE-oxo 8 months	6.08*10 ⁻⁴			
Zn	<i>Paracentrotus lividus</i>	PE-pure 8 months	7.16*10 ⁻²	0.049-0.389 (mean: 0.14; median 0.071)	N/A	(Dermeche et al., 2012; Dinnel et al., 1989; Hillips et al., 1998; Novelli et al., 2003; Radenac et al., 2001)
		PE-oxo 8 months	2.74*10 ⁻⁴			
	<i>Aliivibrio fischeri</i>	PE-pure 8 months	9*10 ⁻² #	1.48-4.64 (mean: 2.5; median 2.155)	N/A	(Mowat and Bundy, 2002; Teodorovic et al., 2009; Tsiridis et al., 2006; Utgikar et al., 2004)
		PE-oxo 8 months	6.08*10 ⁻⁴			

Table 4: EC₅₀ values of this study expressed as the metal concentration measured in the leachates. The range, mean and median of EC₅₀ of each metal in the literature. Co = Cobalt; Mn = Manganese; Zn = Zinc; # = maximum concentration since no EC₅₀ value; N/A =not available.

4. Discussion

4.1 Plastic of same polymer type but different chemical additive composition showed different toxicity

FTIR analysis confirmed that the 4 plastic types (PE-pure, PE-bubble, PE-recycled, PE-oxo) were all identified as low-density PE. The ICP analysis indicated different elemental composition for each material, thus suggesting that the different toxic effects between the 4 plastic types may be related to the plastic chemicals rather than the polymer composition.

PE-pure was supposed to be a plastic with few plastic chemicals that may potentially induce toxicity. However, we observed a clear positive toxic response after 8 months of leaching on *P. lividus* tests. This result is consistent with previous studies that showed toxicity of PE-based materials, with abnormal development of *P. lividus* obtained only after 30 days of leaching (Martínez-Gómez et al., 2017). Toxic effect was also found in another sea urchin's species (*Lytechinus variegatus*) after only 24h of leaching (Nobre et al., 2015), but with very high concentration of pellets introduced in the leachates (i.e. 250 g/L). None of these studies described the chemical composition of the leachate, making the origin of the toxicity unknown. In our study, a consequent leaching of Zn (0.09 mg/L) was detected in the PE-pure leachate that may be responsible of part or total of the toxicity response, rejecting the hypothesis of a pure polymer *sensu stricto*. The EC₅₀ relative to the Zn toxicity was equal to 0.072mg/L for *P.lividus*, which is coherent with other studies with the same species. Indeed, previous EC₅₀ values with only a Zn exposition on *P. lividus* development ranged from 0.049 mg/L (Novelli et al., 2003) to 0.093 mg/L (Radenac et al., 2001). It must be noted that a higher value for Zn EC₅₀ (i.e. 0,389 mg/L) was also disclosed for *P.lividus* in another study (Dermeche et al., 2012). Nevertheless, similar values were obtained with a similar larvae development test with another sea urchin specie (i.e. 0.023 to 0.107 mg/L) (Dinnel et al., 1989; Hillips et al., 1998). Moreover, we also could have predicted the absence of toxicity for *A. fischeri* since the EC₅₀ values of Zn range from 1.47 to 4.64 mg/L (Mowat and Bundy, 2002; Teodorovic et al., 2009; Tsiridis et al., 2006; Utgikar et al., 2004) which are 10² times superior to the concentration recovered in the leachates.

We could not detect any toxicity on PE-bubble samples, even if a slight leaching of Zn (i.e. 0,01 mg/L) was detected in the leachate. The lower concentration of Zn (and

potentially other not detected chemical compounds) in the PE-bubble leachates compared to the high concentration in the PE-pure leachates may then explain the difference in the toxicity measurements between the two plastic types.

Previous studies suggested that recycled plastics were even more toxic than virgin plastics (Hocevar, 2022), the latter being composed of a wider variety of chemicals due to mixing of plastics from different origin during the recycling process (Brosché et al., 2021; Chibwe et al., 2023). Moreover, through the recycling an increased number of non-intentionally added substances (NIAS) may have been introduced in the material. These substances can originate either from side products, breakdown product or contaminants (Geueke, 2018) and can represent more than half of the leached substances (UNEP, 2023a). This assumption was confirmed here with the ICP elemental analysis, where the initial PE-recycled contained the higher number of putative toxic elements. However, despite the decrease of pollutant concentration between the virgin and leached plastic, none of them were detected after 8 months of leaching. PE-recycled was the only polymer with no detected element in the leachates, whereas all tested virgin plastics leached at least one element. As expected, PE-recycled leachates did not show any toxicity even after 8 months of leaching.

PE-oxo was the most toxic polymer in our study, with significant toxic effects appearing after only 1 month and increasing until the end of the 8 months leaching on the two standardized toxicity tests. These results are consistent with other tests on PE-oxo (additives not disclosed) which was also found toxic, for *A. fischeri*, after 3 weeks of leaching at a concentration of 100 g/L (Schiavo et al., 2020). However, solely based on the ICP analysis of leachates composition we could not have inferred the toxicity of PE-oxo. Indeed, based on the cobalt LOEC calculated in this study on sea urchins' larvae, concentrations in PE-oxo leachates are 10^2 times lower. Moreover, the cobalt LOEC found in the literature for *A. fischeri* was 10^2 times superior (i.e. 3.8 mg/L) (Fulladosa et al., 2005) than the concentration recovered in the leachates. Similarly, Mn concentrations are clearly not high enough to induce toxicity. Indeed, concentrations differences are 10^4 times higher for *A. fischeri* (i.e. 351.35 mg/L) (Teodorovic et al., 2009) and 10^2 times higher for sea urchins (i.e. 8.85 mg/L) (Pinsino et al., 2010). Moreover, no distinct link between the elements concentration over time relates to the increasing toxicity. Indeed, the same concentration

of Co and Mn are observed after 4 and 8 months of leaching whereas the toxicity increased greatly.

We demonstrated here that we could not infer efficiently the toxicity solely based on the leachate or plastic material chemical analysis. It must be noted that there is a wide variety of plastic chemicals, and since the majority have not been screened, they cannot be detected (UNEP, 2023a). Due to the broad number of possible plastic chemicals, quantifying the leached substances without knowing which ones to target is challenging. More transparency concerning the plastic chemical composition is needed for an effective evaluation of the leached substances. Moreover, we did not observe a correlation between the increase of toxicity over leaching time and the concentrations of metals detected. This is probably due to a lack of screening of the real origin of toxicity for PE-oxo or to a “cocktail effect” made by the multiplicity of the contaminants released at low concentrations in the leachate, with a synergistic effect increasing the toxicity (González-Pleiter et al., 2013).

4.2 Long-term leaching tested on different species with different sensitivity is crucial for plastic toxicity test

Plastic fate in the environment is to wander in marine waters for decades or centuries (WWF, 2023). Throughout this time, the desorption and further diffusion of plastic chemicals are influenced by a wide variety of factors, such as its chemical size, polymer state of degradation, but also temperature and medium (Hahladakis et al., 2018). Indeed, the smaller the size and molecular weight of the chemical, the greater the migration process. Moreover, an increase in chemicals leaching is observed when the temperature and percentage of amorphous regions in the polymer are high. A high solubility of the chemical and its low concentration in the leaching medium increases also the migration process (Hansen et al., 2013). The migration process is also influenced by the specific surface that is linked to the size and shape used (Song et al., 2023). Plastic chemical leaching has been showed to occur over time. Several studies have described the migration of additives from plastic to water which is governed by Fick’s law (Bridson et al., 2021). The release of organotin compounds from PVC (Chen et al., 2019) and phthalates from PE and PVC (Fikarová et al., 2019) follow a first order kinetics. Leaching has also been shown to

follow pseudo-first order or second-order kinetics (Bridson et al., 2021). In this study we clearly established that an increase of leaching time is linked to an increase in toxicity. Furthermore, it is the first time the kinetics of leaching time relative to plastic toxicity were described. Indeed, EC₅₀ values of PE-oxo followed an exponential decay with the increased leaching time. This kinetic is unfortunately only based on 4 values. Therefore, additional data are needed in order to confirm this trend. As explained above, this toxicity trend may change in function of the polymer state, types of chemicals and leaching medium.

Another critical aspect in the evaluation of toxicity is the sensitivity of the tested organism. Between the two standardized toxicity tests, the sea urchin test showed higher sensitivity. Indeed, EC₅₀ were obtained on the embryotoxicity test after a month of leaching whereas it took 4 months for *A. fischeri* to attain 50% of toxicity. In addition, PE-pure showed toxicity after 8 months of leaching for the embryotoxicity test, whereas none was observed through the *A. fischeri* test. This difference in sensitivity between *P. lividus* and *A. fischeri* was already observed for other chemical pollutants (Maranho et al., 2015). The larvae size depicted an even higher sensitivity with effects observed after only 1 and 10 days of leaching. Though not standardized, larvae size has been used in several articles to detect a slight delay in the larval development that is not considered as a malformation in the embryotoxicity test (Martínez-Gómez et al., 2017; Messinetti et al., 2018; Oliviero et al., 2019). It allowed the shortest leaching period to impact a measurable biometric parameter. Unfortunately, the higher the sensitivity, the more challenging and time consuming. Indeed, larvae size measurement requires taking pictures of numerous larvae and measuring their size whereas the embryotoxicity requires to count the number of well- and malformed larvae. *A. fischeri* toxicity test is the less challenging and time-consuming thanks to the Microtox lx analyzer since no snorkeling is needed and the test results are obtained after an hour and a half. However, it is performed on a single one bacterial strain, which is not representative of biological diversity.

5. Conclusion

This study aimed to better decipher the toxicity kinetics of plastic leaching in close relationship with the plastic chemicals migration process. We observed a difference in metals and toxic traits elements composition between the 4 different plastics. The chemical analysis by ICP allowed us to understand partially the origin of toxicity. Indeed, one polymer toxicity was explained whereas the most toxic polymer (i.e. PE-oxo) did not contain toxic concentration of the different elements measured. Our inorganic elemental analysis was limited and considering the wide variety of different possible plastic chemicals (i.e. >16,000) it is complicated to have a clear overview of all the chemicals present in plastics and subsequently their leachates. Furthermore, plastic chemicals composition is not specified by manufacturers which hinders the possibility to better grasp the origin of toxicity. Especially when leached components can also originate from non-intentionally added substances. We also displayed, for the first time, the toxicity kinetics in function of plastic leaching time. We observed an exponential decay of EC_{50} with the increasing leaching time. The standardized tests showed significant toxicity only after a month of leaching. The embryotoxicity test allowed the EC_{50} estimation after 1 month, but 4 months were required for the bioluminescent test on *Aliivibrio fischeri*. Therefore, we insist on using long-term leaching in order to efficiently evaluate plastic chemical toxicity. We also insisted on the need of using the most sensitive species in order to reduce the leaching time.

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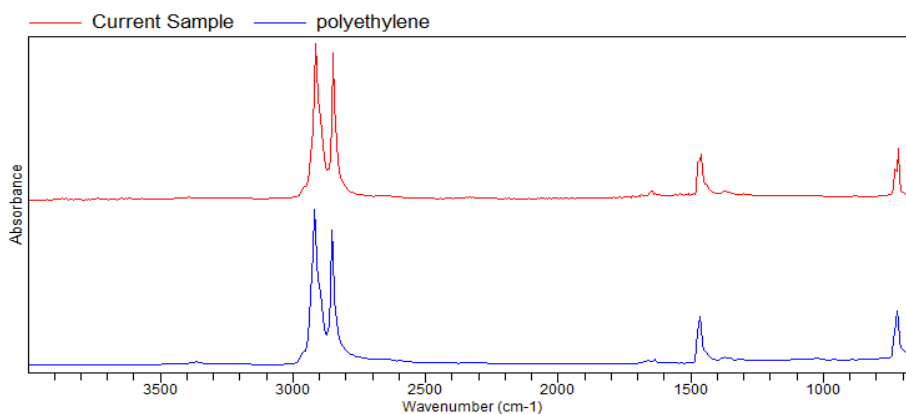
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Supplementary Data 1.1 FTIR results of PE-oxo (A), PE-bubble (B), PE-pure (C), PE-recycled (D)

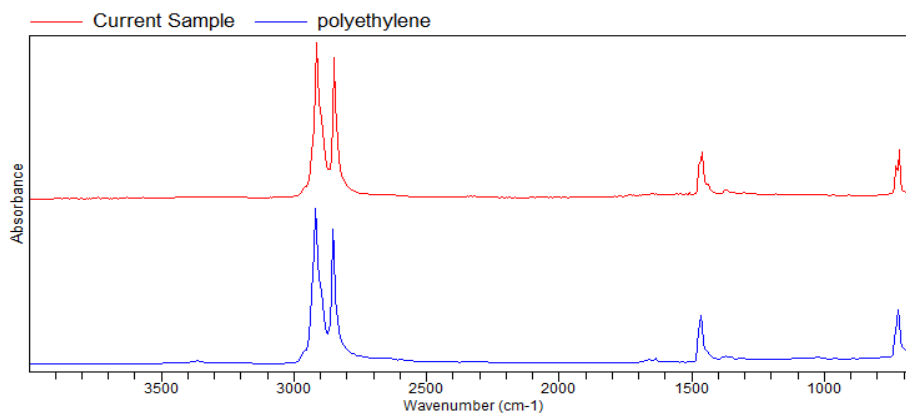
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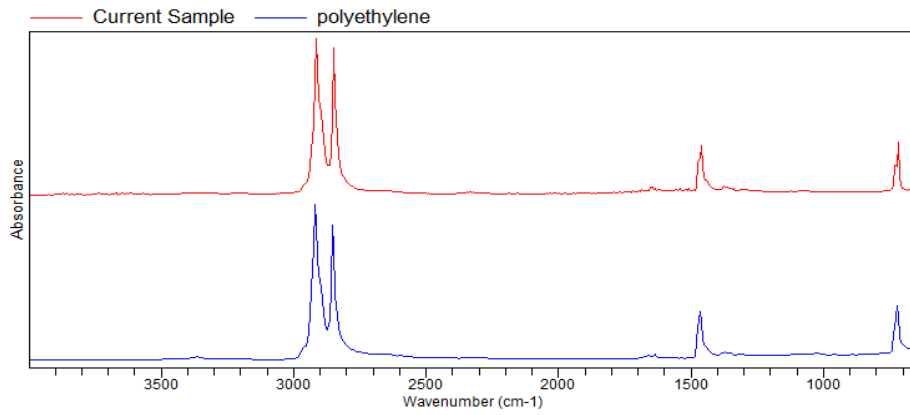
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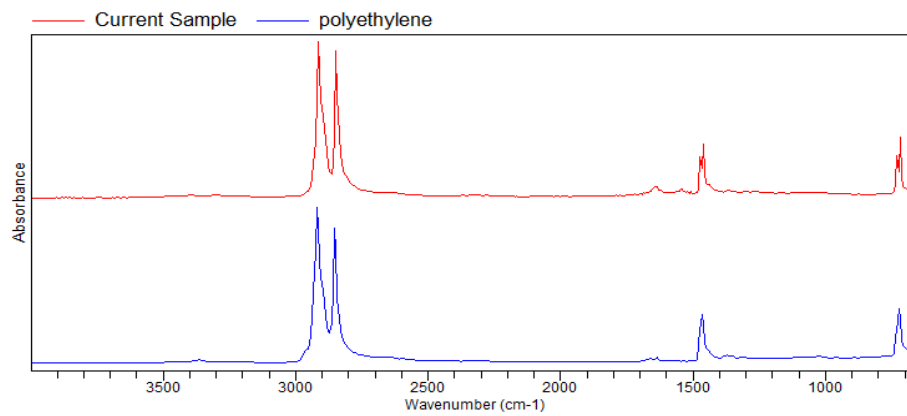
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D

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Supplementary Data 1.2 Inorganic element analysis on the leachates and virgin/leached plastics

	Al (mg/L)	B (mg/L)	Ba (mg/L)	Be (mg/L)	Bi (mg/L)	Cd (mg/L)	Co (mg/L)	Cr (mg/L)	Cu (mg/L)	Fe (mg/L)	Ga (mg/L)	Li (mg/L)	Mg (mg/L)	Mn (mg/L)	Ni (mg/L)	Pb (mg/L)	Se (mg/L)	Sr (mg/L)	Te (mg/L)	Ti (mg/L)	Zn (mg/L)	
PE-oxo 1 month	0	3.71	0.01	0	0.02	0	0.01	0	0	0	0	0.35	289.21	0	0.01	0	0	0	6.87	-0.01	0	0
Seawater 1 month	0	3.33	0.01	0	0.01	0	0	0	0	0	0	0.33	279.83	0	-0.01	0	0	0	7	-0.01	0	0
PE-oxo 2 months	0	3.34	0.01	0	0	0	0.01	0	0	0	0	0.34	279.86	0.01	0	0	0	0	6.73	-0.01	0	0
Seawater 2 months	0	3.47	0.01	0	0.02	0	0	0	0	0	0	0.34	282.16	0	0	0	0.01	0	6.91	-0.01	0	0
PE-oxo 4 months	0	3.44	0.01	0	0.02	0	0.02	0	0	0	0	0.35	283.27	0.01	0	0	0.01	0	6.85	-0.01	0	0
Seawater 4 months	0	3.38	0.01	0	0.04	0	0	0	0	0	0	0.34	282.57	0	0	0	0	0	6.92	-0.01	0	0
PE-pure 8 months	0	3.51	0.01	0	0.05	0	0.02	0	0	0	0	0.36	283.5	0.01	0	0	0.01	0	7.14	-0.01	0	0.01
Seawater 8 months	0	3.18	0.01	0	0.08	0	0	0	0	0	0	0.32	277.5	0	0	0	0	0	6.46	-0.01	0	0.09
PE-recycled 8 months	0.01	3.47	0.01	0	0.05	0	0	0	0	0	0	0.35	284.05	0	0	0	0	0	6.75	-0.01	0	0
PE-bubble 8 months	0.01	3.39	0.01	0	0.08	0	0	0	0	0	0	0.34	278.77	0	0	0	0.01	0	7.33	-0.01	0	0.02
Seawater 8 months	0	3.09	0.01	0	0.07	0	0	0	0	0	0	0.31	277.71	0	0	0	0	0	6.12	-0.01	0	0.01
PE-oxo	0.06	0.01	0	0	0	0	0.28	0	0	0	0	0	0.65	0.16	0	0	0	0	0	0	0	0.02
PE-pure	0.08	0	0	0	0	0	0	0.01	0	0	0	0	0.04	0.71	0	0	0	0	0	0	0	0.02
PE-recycled	2.24	0.01	0.03	0	0	0	0	0.06	0.29	1.66	0.01	0	0.43	0.03	0.02	0.1	0	0.01	0	0	0.05	0.08
PE-bubble	0.2	0	0	0	0	0	0	0.02	0	0.13	0	0	0.2	0	0.01	0	0	0	0.01	0	0.17	0.35
PE-pure 8 months	0.66	0	0	0	0.02	0	0	0	0	0.03	0	0	2.1	0	0	0	0	0	0.01	0	0.01	0.01
PE-recycled 8 months	2.11	0.01	0.03	0	0.02	0	0	0.05	0.26	1.56	0	0	2.08	0.02	0.02	0.09	0	0.02	0	0.19	0.07	
PE-bubble 8 months	0.85	0.01	0	0	0.01	0	0	0.02	0	0.11	0	0	5.73	0.13	0.01	0	0.01	0.04	0.01	0.19	0.24	
PE-bubble 1 month	1.4	0.02	0	0	-0.01	0	0.24	0	0	0.04	0	0	6.53	0.13	0	0	0	0.04	0.01	0	0.01	
PE-oxo 1 month	5.51	0.01	0	0	0	0	0	0	0	0.04	0	0	5.53	0.13	0	0	0	0.03	0	0	0.01	
PE-oxo 2 months	2.29	0.02	0	0	0	0	0.21	0	0	0.04	0	0	8.42	0.12	0	0	0	0.05	0	0	0.02	
PE-oxo 4 months	0.09	0.01	0	0	0.02	0	0.25	0	0	0.04	0	0	2.51	0.14	0	0	0	0.01	0.01	0	0.02	

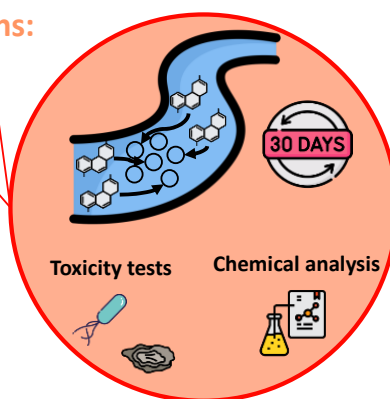
Chapter 2 – Toxicity of adsorbed pollutants on plastic surface

Foreword

Question :

Pollutants adsorption on MPs surface and toxicity ?

Collaborations:



Context: The previous chapter (chapter 1) showed that the leaching of plastic chemicals induce toxicity for bacteria and sea urchin embryo. In this chapter, we focused on another aspect of plastic chemical toxicity, namely the adsorption of pollutants on plastic surfaces. Plastic has already been described as a sponge for pollutants. Many studies were performed in laboratory conditions and mostly focused on the marine environment. The originality of our study was to evaluate the sorption of contaminants and their putative toxicity along the river-to-sea continuum.

Methods: PE-based plastic pellets were immersed along nine European river-to-sea continuum during the pan-European Microplastics 2019 expedition. After one month of immersion, plastic pellets were removed and stored (-80°C) without any prior cleaning (presence of biofilm). The adsorbed pollutant on plastic surface were recovered through a DMSO extraction and an organic element analysis was performed through a liquid chromatography mass spectroscopy (LC/MS) analysis. Moreover, inorganic analyzes

through Induced Coupled Plasma (ICP) were realized on the DMSO extracts and the PE pellets. The DMSO extract toxicity was further evaluated through two standardized toxicity tests (bacteria and oysters).

Results: The adsorbed pollutant induced toxicity for the bacteria test (Microtox®), whereas no toxicity was observed through the oyster embryotoxicity tests. An increase in toxicity (independent of the river) was observed along the rivers with a decrease at the estuary. One month incubation in natural environment was sufficient for the adsorption of up to 83 different organic chemicals. A high variability of the identified organic chemicals was observed between the different rivers and sites. The ICP analysis clearly indicated a difference between the control PE-based plastics and the immersed PE-based plastics. Moreover, the inorganic analysis on the DMSO extract depicted a spatial trend between the different sites along the river-to-sea continuum.

This chapter (chapter 2) ended the exploration of the chemical toxicity in this PhD thesis (both on leaching and adsorption of contaminants), before exploring another aspect of plastic toxicity in the next chapter (chapter 4): the physical impacts of plastics.

Manuscript Title: Environmental pollution adsorbed on polyethylene microplastic and its related toxicity: A case study along 9 European rivers

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Highlights:

- Immersion of plastic pellets for 1 month in 9 European river-to-sea continuum
- Up to 83 organic pollutants sorbed on pellets after 1 month incubation
- Spatial trend of the composition of inorganic elements in function of sites
- Increase of toxicity effects along the rivers, with a decrease at the estuary

Status: In preparation

Abstract

Plastic contamination of freshwater and marine ecosystems is a global ecological problem of increasing scientific concern. Plastics can adsorb persistent organic pollutants and trace metals from aquatic environments, with potential toxicity on a wide range of aquatic species. Here, we characterized the contaminant sorption and their toxicity levels, after sorption on polyethylene pellets along nine European river-to-sea continuum. After 1 month of immersion, we observed a high diversity of sorbed organic contaminants for several rivers such as pharmaceuticals, pesticides, antimicrobials, per- and polyfluoroalkyl substances (PFAS). Interestingly, a high variability of pollutant types was observed across and along the nine European rivers, suggesting both the influences of local anthropogenic activities and environmental physico-chemical characteristics. Trace metals analyzes also showed a clear difference between the control and river-incubated pellets. DMSO extracts from the river-incubated pellets displayed distinct chemical profiles and toxicity levels (standardized tests using bacteria) according to the sites. An increase of toxicity levels was observed from upstream to downstream, together with a decrease at the estuary. Our study highlighted that along rivers, plastic pellets adsorbed several pollutants from the surrounding waters that can lead to an increasing toxicity for aquatic organisms.

Key-words: microplastics, environmental pollutants, adsorption, European rivers, toxicity

1. Introduction

The contamination of aquatic ecosystems with plastic, and especially with microplastic, is a global ecological problem of increasing scientific concern (Leistenschneider et al., 2023). The small size of MPs enables its ingestion by aquatic organisms at different levels of the food chain and with different feeding strategies (Bergmann et al., 2015). In particular, the negative impact of plastic debris on living organisms can be related to the sheer mechanical damage induced by MPs in the gastrointestinal tracts and gills of ingesting organisms, and to leached substances, some of which have been proven to be toxic, carcinogenic, or endocrine-disrupting (Palmer and Herat, 2021). Furthermore, MPs can adsorb persistent organic pollutants (POPs, e.g. polychlorinated biphenyls (PCBs)), polycyclic aromatic hydrocarbons (PAHs)), or toxic metals from aquatic environments (Koelmans et al., 2016). Several studies underlined the modification of absorptive properties of new and aged plastics after immersion in aquatic ecosystems. For example, virgin polyethylene pellets acquire a net negative charge surface through the rapid adsorption of organic matter together with the biofilm formation which can enhance the adsorption of metal ions when immersed in aquatic environments (Richard et al., 2019).

Primary MPs are defined as originally manufactured to small size, whereas secondary MPs are broken down from larger plastic items (GESAMP, 2015). Virgin resin pellets or spherules (2 to 5 mm in diameter and usually in granular form) are primary MPs that constitute the raw material for manufacturing plastic products. It has been found in surface water samples and on beaches (Fernandino et al., 2015). They end up in the aquatic environment through various pathways such as losses during production, transport, and accidental spillage into the water due to handling and plastic processing plants (Karlsson et al., 2018). Numerous studies shed light on the physical and chemical hazards and even acute toxicity of ingested plastic pellets on various organisms including zooplankton (Cole et al., 2013; Setälä et al., 2014), bivalves and other macro-sized invertebrates (Bringer et al., 2020a; Browne et al., 2008; Graham and Thompson, 2009; Gray and Weinstein, 2017) as well as fish (Batel et al., 2016; Cormier et al., 2022b; Pannetier et al., 2019; Rochman et al., 2013b). Furthermore, some other studies have shown that aqueous extracts (leachates) or organic extracts of plastics immersed in an aquatic environment or stranded on beaches

could be toxic for many marine species from bacteria to fishes (Birnstiel et al., 2022; Cormier et al., 2021). This toxicity may not only be linked to additives and non-intentionally added substances present in the raw material, but also to pollutants and degradation products (Cormier et al., 2022a), which are added to the plastic during its ageing in the environment. Indeed, important accumulation of persistent organic pollutant on plastic surface was observed with concentrations 10^5 - 10^6 higher than in the surrounding environment (Mato et al., 2001). Current research on the adsorption behavior of MPs are mainly performed under laboratory conditions (Fu et al., 2021). The few environmental studies spend more attention to the marine environment than to the freshwater environment (Ashton et al., 2010; Fotopoulou and Karapanagioti, 2012; Karapanagioti and Klontza, 2008). Plastic waste found in rivers is probably less affected by the weathering and chemical changes compared to plastic waste found in the ocean, which has been floating or washed up for a longer time. As a result, estuaries can be seen as areas where newly introduced plastics from rivers mix with older and more altered ones from various locations in the ocean.

Given the efficiency of pollutants sorption on plastics surface (Abd-Aziz et al., 2019) and the lack of environmental studies (Fu et al., 2021), the aim of this study was to characterize the pollutant sorption on plastic surface in the river-to-sea continuum. Low density polyethylene (LDPE) plastic pellets were immersed for one month in nine European rivers (i.e. Thames, Elbe, Rhine, Seine, Loire, Garonne, Ebro, Rhône, and Tiber) along the river-to-sea continuum. The nature and concentrations of the adsorbed organic and inorganic pollutants as well as the toxicity of organic extracts of plastics pellets were characterized. The toxicity was evaluated through two standardized toxicity tests (i.e. ISO 11348-3 on marine bacteria and ISO 17244 on embryotoxicity of oysters).

2. Materials and methods

2.1 Sample immersion and preparation

2.1.1 Immersion of plastic pellets and sampling

The mission *Tara* Microplastics was conducted to investigate plastic pollution along nine major rivers in Europe: Thames (THA), Elbe (ELB), Rhine (RHI), Seine (SEI), Loire (LOI), Garonne (GAR), Ebro (EBR), Rhône (RHO) and Tiber (TIB) (Figure 16). An extensive suite of sampling protocols was applied from two to five sites on each river along a salinity gradient from the estuary to downstream and upstream of the first heavily populated city (Ghiglione et al., 2023). To measure pollutant sorption and toxicity of weathered plastic in aquatic environments, cages containing pristine plastic pellets of low-density polyethylene (LDPE, 2 mm diameter pellets, SABIC®, United Kingdom) were immersed in the flow of the river, at less than 1 m deep near the Riverbank at two to five sites along the river one month prior to sampling. After one month the plastic pellets were removed from the cages, immediately transferred in liquid nitrogen, and conserved at -80°C when back to the laboratory.



Figure 16: Graphical representation of the rivers sampled by the mission *Tara* Microplastics 2019

2.1.2 Plastic extracts

The DMSO extraction protocol allows the extraction of organic and metallic pollutants present on the surface of the plastic samples and in the biofilm, as previously described (Pannetier et al., 2019). Briefly, the glassware and utensils were carefully cleaned with hexane (3 x 1 ml) and then dried to remove traces of organic compounds. Two grams of uncleaned and freshly thawed plastic pellets were transferred to an 8 mL amber glass tube. Then, 4 mL of pure DMSO (Sigma, D8418) was added to each tube (weight/volume ratio 1:2). The tubes were fixed horizontally on an orbital shaker (KS 501 Digital, IKA Werke) and subjected to shaking for 16 hours at 250 rpm and 20°C. Three independent extractions were carried out for each sample as well as an extraction blank for each extraction series (with 2 g of unexposed original pellets). Immediately after the end of stirring, DMSO was collected with a glass Pasteur pipette and then placed in a glass hemolysis tube. Each extract was centrifuged at 2000 g for 5 min (20°C) to pellet the particles. The supernatant was collected with a glass Pasteur pipette and aliquoted into several 4 mL amber glass tubes then stored at -20°C.

2.2 Chemical characterization

2.2.1 Quantification of inorganic elements

The detection of metals and other trace elements was performed on DMSO extracts and polyethylene pellets. DMSO extracts were diluted to 1/10 before analysis while the PE pellets were freeze dried and mineralized by microwave-assisted acid digestion. 200 mg of sample were weighed and placed in a PTFE vessel containing 10 ml of concentrated nitric acid. The sample were digested using a Multiwave 5000 microwave system (Anton Paar) for 1 hour following a temperature ramp from 90 to 240°C. After cooling, the residues were transferred to 50 ml volumetric flask and diluted precisely to 50 ml with ultrapure water for analysis. Concentrations of trace elements were determined by Inductively Coupled Plasma (ICP) Optical Emission spectrometry using 5800 ICP OES Agilent system. The ICP system was calibrated, using multielement standard solutions containing : Aluminum (Al), Boron (B), Barium (Ba), Bismuth (Bi), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Gallium (Ga), Potassium (K), Lithium (Li), Magnesium (Mg), Manganese (Mn), Sodium (Na), Nickel (Ni), Lead (Pb), Selenium (Se), Strontium (Sr),

Tellurium (Te), Titanium (Ti), Zinc (Zn). The category corresponding to metals and other toxic trace elements was defined as: Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Ti and Zn.

2.2.2 LC/MS analysis of DMSO extracts

3 ml of DMSO extracts were dried down and resuspended in 200 μ l of acetonitrile. LC/MS analyzes were performed with an Ultimate 3000 RSLC chromatographic system (ThermoScientific) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray (ESI) source. The chromatographic separations were performed using a Kinetex EVO C18 column (2.1 x 100 mm; 1.7 μ m-Phenomenex) operating at 30°C and an injection volume of 5 μ l. The flow rate was fixed at 0.45 mL/min with 0.1% of formic acid in water (A) and 0.1% of formic acid in acetonitrile (B) for mobile phases at the following gradient: initial, 95% A; 0–7.5 min linear, 1% A linear; 7.5–8.5 min, 1% A; 8.5–9 min, 95% A linear, 9–11 min 95% A following by washing and reconditioning of the column. The mass spectrometer was operated in positive ion mode with capillary voltage at 3.2 kV and –3 kV, respectively and a capillary temperature set at 320°C. The detection was performed with full scan from m/z 50 to 750 using a resolution set at 70 000 at m/z 200. MS raw data were processed using Xcalibur Software (version 4.1) and python scripts. High resolution mass spectrometry was used to determine monoisotopic mass of ionized compounds. The m/z values with a peak intensity strictly greater than 10^4 were annotated by putative matches with a compiled database (34400 compounds) from EPA CompTox Chemical databases.

2.3 Toxicity assays

2.3.1 Microtox® assays

The Microtox® is a standardized acute toxicity assay based on the bioluminescent inhibition of the marine bacteria *Aliivibrio fischeri* (Beijerinck, 1889). The bioassay was carried out on serial 1/2 dilutions of plastic extracts from 1% to 0.125% in 20 g/L NaCl solution as described in the ISO 11348 guidelines. Each assay was run in duplicate on each triplicate of plastic extracts. After 30 min of exposure, the bioluminescence of the bacteria culture was measured using the Microtox 500 analyzer (Modern Water) and compared to

the negative control (NaCl 20 g/L). A positive control (200 mg/L potassium dichromate) was run with each set of samples.

2.3.2 Oyster embryo-larval assay

Only PE extracts samples that indicated a significant toxicity with the Microtox® assay were tested with the oyster embryo-larval assay. Mature oysters, *Magallana gigas* (Thunberg, 1793) were obtained from France Naissain (Bouin, France). The detailed protocol for the oyster embryo-larval assay was described in (Gamain et al., 2016). Gametes were obtained by thermal stimulation of mature oysters and in filtered seawater and sieved at 50 µm (sperms) or 100 µm (oocytes) to eliminate debris and feces. The number and mobility of sperm as well as the number of oocytes were checked at 100X magnification (LEICA DME). Oocytes from a single female were fertilized with sperm from a single male using a ratio of 1:10 (egg:sperm) to prevent polyspermy. After 20 min of incubation at room temperature, fertilized embryos were counted at 100X magnification. Around 500 embryos were then transferred to a well (24-well microplates) filled with 2 mL of filtered seawater (control) or diluted plastic extracts. Serial ½ dilutions of plastic extracts in filtered seawater from 1 to 0.125% were tested. The microplates were then incubated at 24 °C for 24 h in the dark. After incubation, 25 µl of 1% buffered formalin were added and the percentage of abnormal oyster larvae was recorded at 100X under inverted microscope (Nikon eclipse TS100/TS100-F). One hundred individuals per well were directly examined and the number of abnormal D-shell larvae and developmental arrests was recorded according to the criteria described in (His et al., 1999). For the test to be validated, less than 20% of abnormal larvae are required in the control. In this experiment, four replicates were performed for each condition.

2.4 Statistical analyzes

The toxicity results were analyzed using R software (R Core Team, 2021). The data of each toxicity test was first tested for its normality (i.e. Kolmogorov-Smirnov) and homoscedasticity (i.e. Bartlett's test). Non-normal distribution was observed for the Microtox® test and a normal distribution was recorded for the embryotoxicity of oysters. To determine statistical differences with the control we performed a Kruskal-Wallis test,

for non-normally distributed data, followed by a Dunn test. The selected p-value was not adjusted since we simply compare a specific site of a river to a control. For the normally distributed values, we performed an ANOVA followed by a t-test. The p-values were considered statistically significant when they were <0.05 .

Principal components analyzes were performed on R through the function “PCA” of the “FactoMineR” package (Lê et al., 2008). Statistical differences were performed using the function “pairwise.adonis2” from the same package.

3. Results

3.1 Chemical profile

3.1.1 Quantification of inorganic elements from plastic pellets

Analysis of inorganic elements on the mineralized pellet revealed the presence of macro elements characteristic of marine and freshwater environments, as well as metals and other trace elements with potential toxicity such as cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), titanium (Ti) and zinc (Zn) (Supplementary data 2.1). Fe, Mn, Ti and Zn were found in all sample sites, but no traces of Co and Cd could be detected. Depending on sampling sites, the total number of elements ranged from 6 to 20 with a number of toxic elements ranging between 2 to 8. On average, the rivers contained between 13 and 19 elements, including 4 to 6 toxic elements. The most contaminated samples were the RHI4, TIB3 and ELB3 with, respectively 8, 6 and 6 potential toxic elements detected with concentration ranging from 0.01 to 3.73 mg/L. The mineralized pellet incubated in the different rivers clearly showed a different chemical profile than the control indicating a noticeable sponge effect in all tested rivers when plotted on a principal component analysis (PCA) (Figure 17).

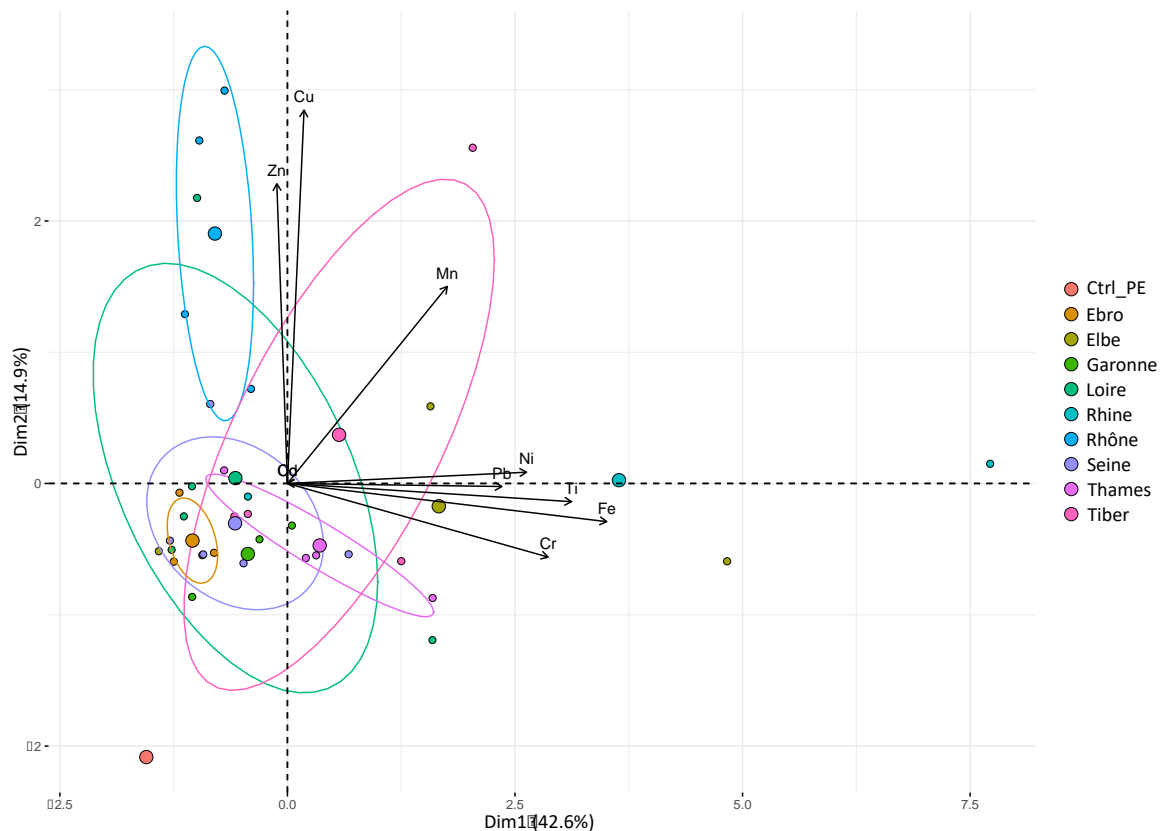


Figure 17: Principal component analysis on the ICP results for different metals and trace metals in the mineralized plastic pellets classified by rivers. Cr = Chromium; Cu = Copper; Fe = Iron; Mn = Manganese; Ni = Nickel; Pb = Lead; Ti = Titanium; Zn = Zinc; Ctrl_PE = Control Polyethylene (not immersed).

3.1.2 Quantification of inorganic elements from the DMSO extracts

Comparatively, analysis of DMSO extracts showed the presence of a total number of elements ranging from 8 to 17 with a specific number of toxic elements ranging between 2 and 5 (Supplementary Data 2.2). Only Ti was found in all sample sites, Fe occurred in 24 over 35 samples and no traces of Co, Cr, Cd and Ni could be detected. On average, the rivers contained between 13 and 18 elements, including 2 to 5 toxic elements (Cu, Fe, Mn, Pb, Ti, Zn). The most contaminated samples were the ELB1, RHI4, RHI5, TIB4, RHO2 and RHO4 with 5 potential toxic elements detected with concentration ranging from 0.01 to 47.56 mg/L. Through the PCA we observed a clear difference between the different sites (Figure 18). Indeed, a spatial trend was observed with a significant difference between S1-S2 and S4-S5. Moreover, S3-S4 were significantly different from S5.

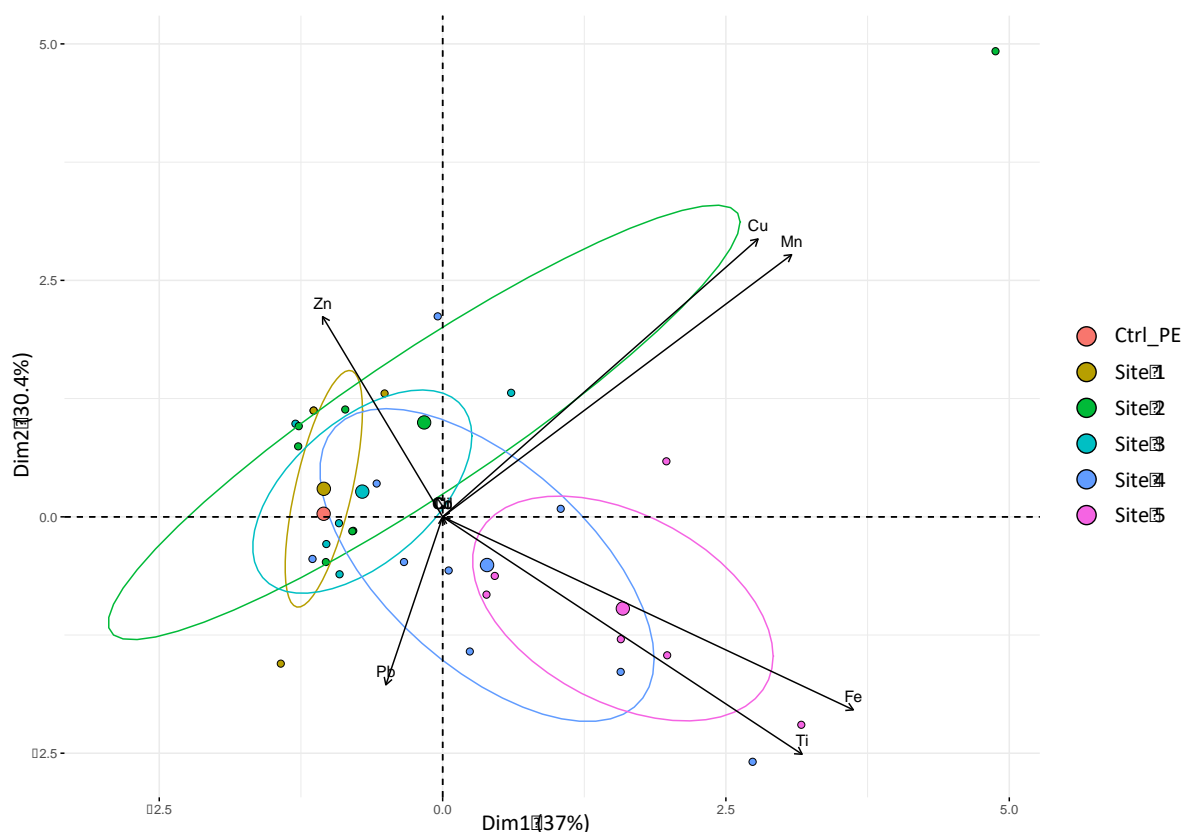


Figure 18: PCA on the ICP results of metals and trace metals on the DMSO extracts classified by sites. Cu = Copper, Fe = Iron, Mn = Manganese, Pb = Lead, Ti = Titanium, Zn = Zinc. Sampling sites are described as Site 1 (marine), Site 2 (estuarine), Site 3 (intermediate salinity), Site 4 and Site 5 (downstream and upstream of the first heavily populated city close to the estuary). Ctrl_PE = Control Polyethylene (not immersed)

3.1.3 Semi quantitative analysis of organic contaminants from the DMSO extract

Analysis of organic contaminants in DMSO extracts revealed the presence of numerous families of compounds with potential toxicity. Antimicrobials, pesticides, polyaromatic hydrocarbons (PAH), Polychlorinated biphenyl (PCB), Polybrominated diphenyl ethers (PBD), polyfluorinated substances (PFAS) as well as polymer additives were the compound families most frequently found in at least 31 of the 34 sampling sites analyzed (Supplementary Data 2.3). It must be noted that the organic contaminants were not measured for site ELB4. To a lesser extent, endocrine disruptors, pharmaceuticals and phenols were detected in 27, 23 and 18 sampling sites, respectively. Compounds such as tert-Butylhydroquinone, Di-p-methylbenzylidenesorbitol (plastic additives), 3,3,4,4-Tetrafluorocyclobutane-1,2-dione (PFAS), Quinoline (PAH) or nitrobenzene (endocrine disruptor) were found most frequently in the different sites. Depending on the sampling

site, the total number of compounds varied from 8 to 83. The most contaminated rivers were the Elbe, Rhône, Thames and Tiber, with an average of 47, 41, 38 and 37 compounds detected, respectively. The Ebro and Rhine were slightly less heavily contaminated, with 34 and 33 compounds detected, respectively. The least contaminated rivers were the Seine, Loire and Garonne, with 27, 17 and 13 compounds detected, respectively. The most contaminated samples (excluding plastic additives) were EBR2 (53 compounds), ELB3 (34), THA5 (33) RHO2 (30) and TIB3 (28). Different contamination profiles emerged according to river and sampling sites. The Ebro, Elbe and Rhône rivers were more likely to be contaminated by pharmaceuticals (EBR2: 11 compounds; ELB1: 8; RHO2: 10), pesticides (EBR2: 24; ELB1: 9; ELB3: 14; RHO1: 15; RHO2: 12) or antimicrobials (ELB3:6, RHO2:3). Thames and Tiber contained a highest number of PFAS (THA5: 9; TIB3: 7; TIB4: 8) and antimicrobials (THA5: 4; TIB3: 5).

The LC/MS analysis resulted in semi-quantitative values. For the PCA analysis those values were added within each category (e.g. Endocrine disruptors, PFAS, Pesticides) in order to obtain a more readable PCA (Figure 19). The same spectral window was selected, however the values were not normalized. The data normalization would have required an individual standard for each molecule with the same physico-chemical conditions as our DMSO extract. The objective was to obtain a readable visual representation due to a wide variety of different chemicals (i.e. total of 211 chemicals detected). These results are approximate and should be treated accordingly. No spatial trend between the different sites were observed, however significant differences between rivers were observed. Indeed, LOI was significantly different from ELB and TIB. Moreover, GAR was significantly different from THA.

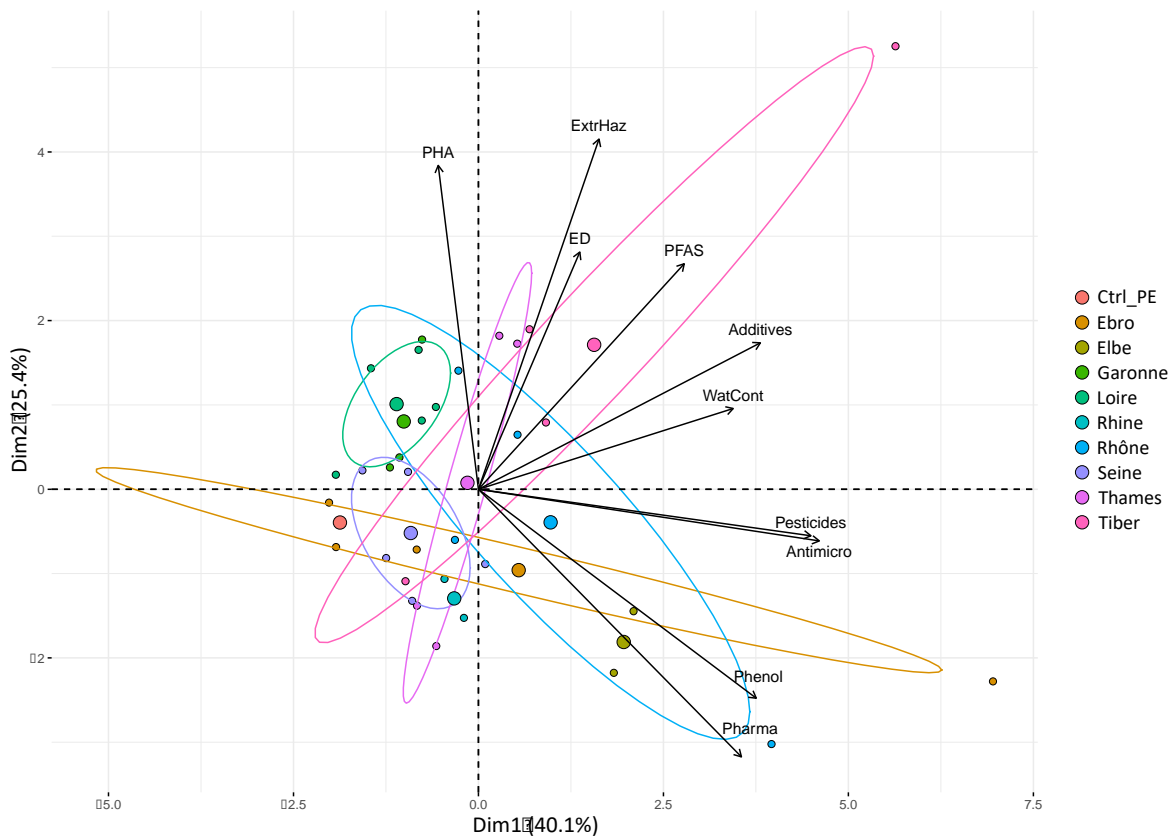


Figure 19: PCA on the sum of the LCMS results for each organic pollutant category on DMSO extracts classified by river. ED = Endocrine disruptors; Pharma = Pharmaceuticals; Phenol = Phenol & Bisphenol; PHA = Polyhydroxy acids, Polychlorinated Biphenyls and Polybrominated Diphenyl Ethers; Antimicro = Antimicrobial; PFAS = per- and polyfluoroalkyl substances; ExtrHaz = Extremely Hazardous substances; WatCont = Water Contaminants; Additives = Plastic additives; Control Polyethylene (not immersed).

3.2 Toxicity profile

3.2.1 Microtox® assay

PE pellet 1% extracts relative bioluminescence inhibition varied from 0.3% to 52.9% of toxicity (Figure 20). From the 34 sites analyzed, 10 displayed a toxicity that was significantly different from the control virgin PE. The two most toxic samples reaching 50% of toxicity were located at EBR2 and TIB3. Sites showing a toxicity between 30 and 40% were located at RHI3-4 and RHO4. RHO2, ELB1, LOI3, SEI2 and THA2 also induced a significant effect but ranging from 15 to 30% inhibition.

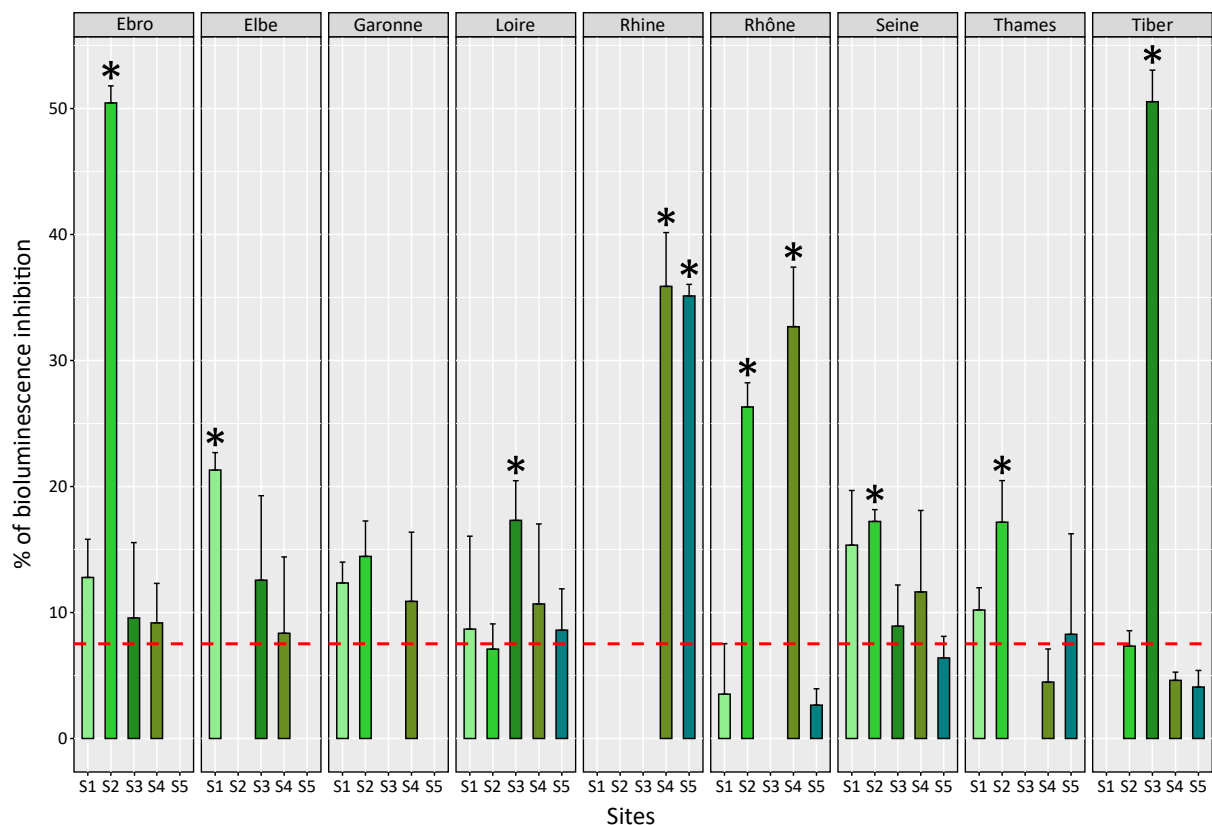


Figure 20: Microtox results on 1% organic extracts of PE pellets immersed for one month at different locations of 9 European river-sea continuum. The results are expressed as the percentage of the relative bioluminescence inhibition compared to control water of the marine bacteria *Aliivibrio fischeri* exposed for 30 min to 1% of the extract (Mean +/- SD, n=3). Asterisks represent a significant difference with the virgin polymer DMSO extract. This extract is represented by the red dotted line with the standard deviation being the gray area.

In addition, a spatial trend was observed between the different sites all rivers combined. Indeed, the percentage of significantly toxic sites increases along the river-to sea continuum starting at 17% for S5 and reaching 57% for S2 (Figure 21). We then observe a decrease of toxicity at the estuary with only 14% of all the sites that are significantly toxic.

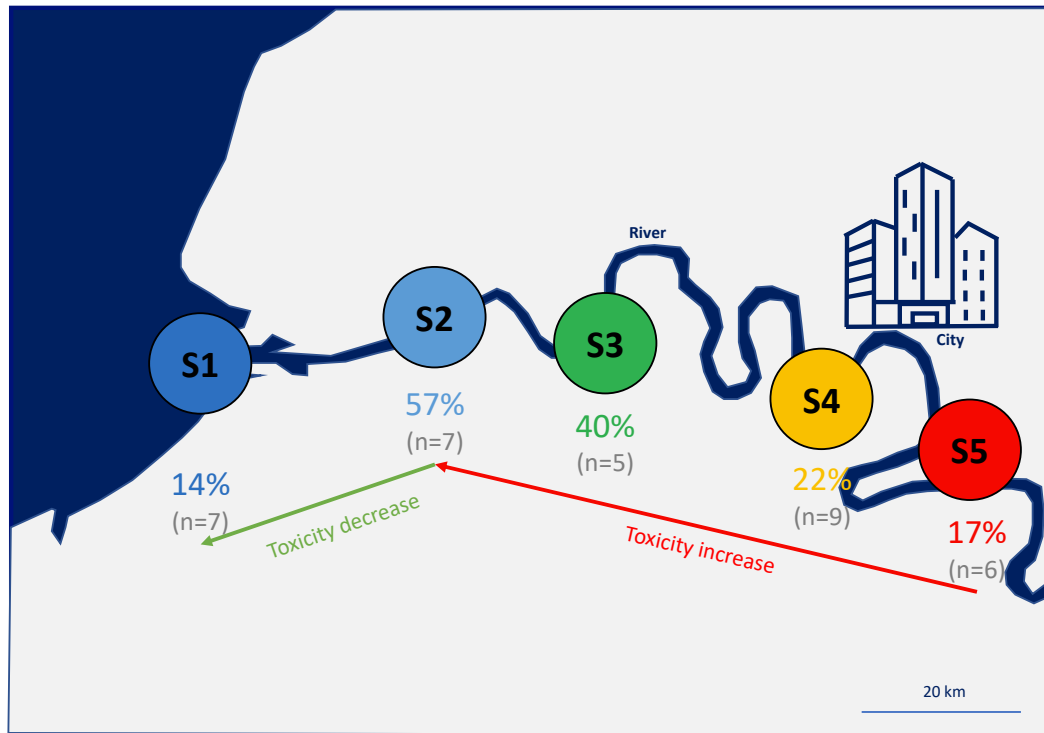


Figure 21: Graphical representation of the percentage of significantly toxicity observed on the sampling sites for all combined rivers. n = total number of sampling sites with data available

3.2.2 Oyster embryo-larval assay

Oyster embryo-larval assay was performed on DMSO extracts of PE at a maximum concentration of 1% only for sites that showed a significant difference in the Microtox® test. Developmental defects (arrest of development and malformations) were recorded after 24 h of exposure using three different pairs of mature oysters. The percentage of malformed D-larvae varied between 18.6% to 42% (Supplementary Data 2.4). No samples showed any statistical difference with the control DMSO with virgin PE.

4. Discussion

4.1 Level of sorbed pollution on plastic pellets according to the sampling location

A non-targeted approach implementing a variety of analytical methods was used to assess the presence of a wide range of contaminants on plastic pellets. Organic contaminants were identified by querying a targeted collection of EPA Comptox databases, focusing on toxic products (Williams et al., 2021). Inorganic elements were analyzed through ICP. The level and profile of contamination was assessed on PE-based plastic pellets incubated for one month in various European rivers and on DMSO extracts of these pellets also used for toxicity testing. Various contaminant profiles have been observed in the different rivers suggesting that the organic and inorganic contaminants originated mainly from the surrounding environment and from the watershed. Moreover, the LC/MS and ICP analyzes displayed many substances that were detected in the samples but not in control PE. The ICP on mineralized material clearly confirms the difference in trace metal elements composition between the incubated and virgin PE. Indeed, one month incubation was enough to detect, in all collected samples, a noticeable adsorption of pollutants. The number of detected contaminants reached up to 83 organic compounds and up to 19 inorganic elements in total. From the 33 analyzed sites more than 84% (i.e. 28) had a higher number of detected organic pollutants than the control pellets. The high content of pharmaceuticals, pesticides or antimicrobials in Ebro, Elbe and Rhône rivers contrasted with the high number of PFAS detected in Thames and Tiber. Moreover, a spatial trend was observed for the metals and toxic trace element with significant differences between sites (regardless of the rivers). This study highlights the numerous chemicals that are adsorbed on plastic surfaces as well as the anthropogenic pollution in rivers. The variability observed can be explained by the sorption capacity of plastic materials can be modified by plastic weathering, biofilm presence (Richard et al., 2019) or the chemical properties of contaminants (Fred-Ahmadu et al., 2020; Liu et al., 2020), as well as environmental conditions such as pH (Vujić et al., 2023), salinity, dissolved and particulate organic matter (Yu et al., 2019).

We underline that, through a DMSO extraction of the adsorbed pollutants on plastic surface, toxicity to *A. fischeri* was observed. These results confirmed high levels of pollution by organic and inorganic chemicals in estuarine and fresh-waters along major rivers in Europe. Such contamination has been reported in both freshwater (Schulze et al., 2019) and seawater (Avellan et al., 2022), and comes from different anthropogenic sources such as sewage, industrial (PAHs, PCBs, metal trace elements), agricultural wastes (pesticides, fertilizers) (Mushtaq et al., 2020) or waste water treatment plant (Gros et al., 2007). Moreover, it highlights also the strong interactions occurring between plastics and contaminants which have been previously reported in other aquatic systems such as estuaries (Bakir et al., 2014), sea water (Rochman et al., 2013a; Teuten et al., 2009) and more recently freshwater (Avazzadeh Samani and Meunier, 2023). These interactions may reflect the absorption and/or adsorption of environmental contaminants. Between the two adhesion processes, it was disclosed that the partition ratio is closely linked to the chemicals concentration of the surrounding environment (Hartmann et al., 2017). Therefore, we can infer that most of the adhesion of chemicals is through adsorption. Several studies emphasized the role of plastic as a vector of organic contaminants (Hartmann et al., 2017; Teuten et al., 2009) or metal trace elements (Bradney et al., 2019). Nevertheless, recently the role of microplastics as vectors of organic contaminants has been questioned due to the relatively low abundance of plastic particles in the environment compared to natural organic and inorganic particles (Koelmans et al., 2023).

4.2 Toxicity coupled with the chemical analysis and its spatial trend

Toxicity to *A. fischeri* was reported from the PE pellets DMSO extracts. From the inorganic composition of DMSO extract perspective, EBR2 differentiates itself from other samples through its high concentration of Copper and Manganese. However, the exposition concentrations, respectively 0.01 mg/L and 0.02 mg/L, were at least ten times lower than the lowest observed effect concentration (LOEC) of copper and manganese for *A. fischeri* (Fulladosa et al., 2005; Teodorovic et al., 2009). EBR2 sample also indicated the highest number of different organic contaminants (i.e. 83 chemicals) and differentiates itself from other samples by having the highest number of pesticides and additives. TIB3,

which is the second most polluted site, contains a high number of PAH/PBDE/PCB, PFAS, endocrine disruptors and additives. Other toxic samples displayed a difference from the other sites and the control with notable presence of hydroquinone and norethindrone for, respectively, SEI2 and RHO2. Based on the analyzed chemicals, the other toxic sites (i.e. ELB1, LOI3, RHI4-5, RHO4 and TAM2) did not show any significant difference with other samples. In addition, some discrepancy is observed where some samples did not induce any toxicity but the chemical analysis indicates a potential toxicity. For instance, LOI1 did not induce any toxicity on *A. fischeri*, however the final concentration of Pb (i.e. 1.1 mg/L) was much higher than a previously estimated LOEC (Fulladosa et al., 2005). Moreover, an increase in the number of detected pollutants does not necessarily mean an increase in toxicity. Indeed, ELB3 DMSO extract is the second sample with the highest number of organic contaminants (i.e. 56) but did not induce any toxicity for *A. fischeri* and LOI3 which had one of the lowest numbers in contaminant diversity (i.e. 14 chemicals) induced toxicity for the same species. These results enlighten the fact that even though the chemical analysis can sometimes be a relevant indicator of toxicity it is complicated to extrapolate the toxicity of a complex mixture of chemicals based solely on the chemical analysis. Moreover, even though a wide variety of pollutants was analyzed, we are aware that there may be some pollutants that we have not tested and that are at the origin of non-explained toxicity. In addition, since the chemical results are closely linked to the database used and no standard database exists, comparisons between different studies are complicated. Another bias lies between the chemical groups. Indeed, since there is a discrepancy in the number of chemicals known for each chemical group, the likelihood of finding pollutants of a well-known category is increased. For example, the number of known additives is higher than the number of endocrine disruptors since one is produced and used for plastic production whereas the other one is discovered over time.

We also observed a spatial trend for toxicity with an increase in the number of significant toxic sites from upstream to downstream of rivers. This trend was followed until reaching the estuary where the number of toxic sites decreased. This could be explained by the dilution effect of the river entering the ocean/sea and thus a decrease of pollutant sorption on the surface of the pellets. A similar trend of increasing concentration of organophosphates pesticides along Sarno river was observed (Montuori et al., 2015).

However, the concentration increased until the estuary. In addition, we were expecting an increase in toxicity between before (i.e. site 5) and after a major city (i.e. site 4) (Köck-Schulmeyer et al., 2021). A significant difference between those two sites was only observed for the Rhône river.

5. Conclusion

Plastic pollution became a global concern that combines 3 types of toxicity: physical, biological and chemical. Chemical toxicity can be categorized into two main categories: leaching or adsorption of substances. In this study, we focused on a poorly studied aspect of contaminants adsorption along the river-to-sea continuum. We highlighted the fact that rivers are polluted with a wide variety of different pollutants that can adsorb on plastic surfaces. Indeed, the inorganic analysis performed directly on plastic differentiate the virgin and weathered PE pellets. Distribution of metals and other toxic trace elements on the surface of PE pellets clearly described a spatial trend with significant differences along the river. 84% of our samples displayed a higher number of organic contaminants than the virgin PE. Moreover, the toxicity of adsorbed pollutants increased along the river with a decrease at the estuary probably due to a dilution effect. This supports the hypothesis of anthropogenic micropollutants that can sorb on plastics along the river causing this increase in toxicity. A one-month immersion of PE-based plastic pellets in rivers was sufficient for the adsorption of up to 83 organic contaminants on plastic surface. Our analysis focused on the plastic with its biofilm which has been shown to play a role in pollutants adsorption. However, no extraction was performed when the biofilm was removed. Therefore, more research is needed to fully understand the role of biofilm in pollutant adsorption.

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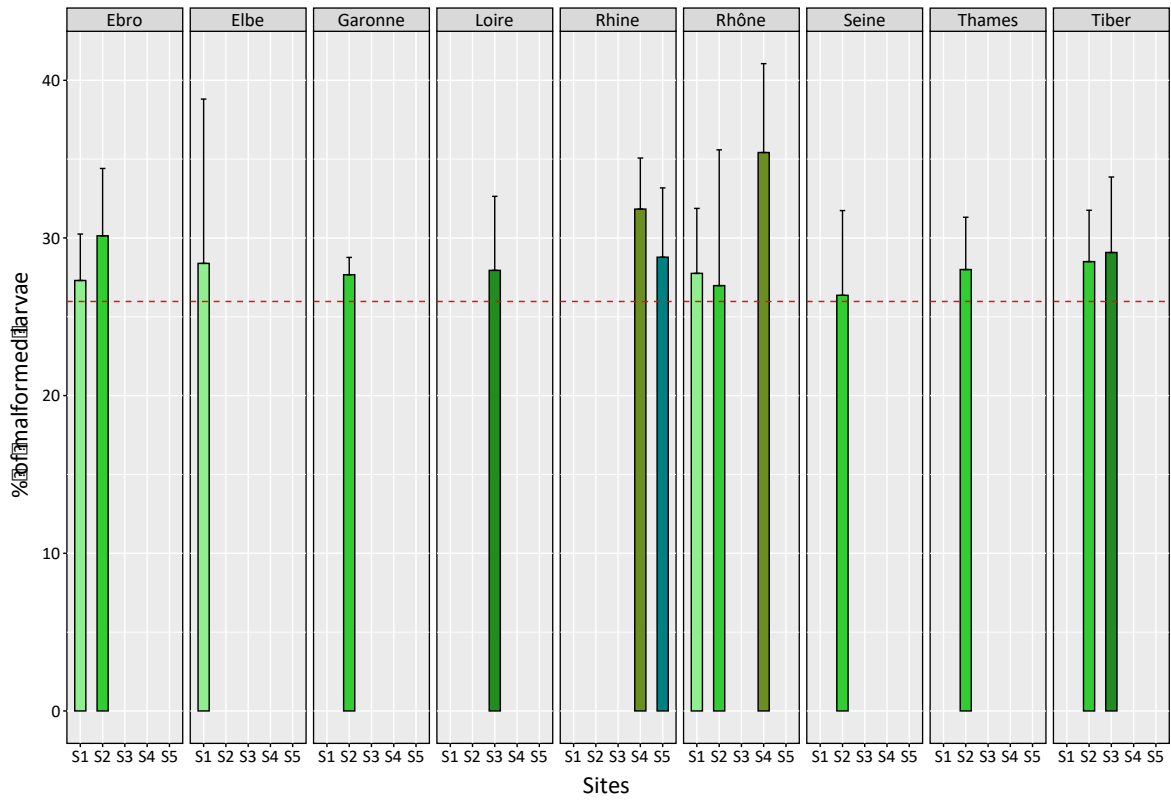
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Supplementary data 2.3 Number of organic elements present in the DMSO extracts

Name	PE	EBR1	EBR2	EBR3	EBR4	ELB1	ELB3	GAR1	GAR2	GAR4	LO1	LO2	LO3	LO4	LO5	RH4	RH5	TAM1	TAM2	TAM4	TAM5	TIB2	TIB3	TIB4	TIB5	SE1	SE2	SE3	SE4	SE5	RHO1	RHO2	RHO4	RHO5
Endocrine disruptors	2	1	1	0	1	1	1	1	2	2	0	0	1	3	3	1	1	1	0	2	3	2	1	2	1	1	0	1	1	1	0	2	1	
Pharmaceuticals	0	2	11	0	0	8	6	0	0	0	1	0	0	1	0	2	4	8	6	2	3	1	3	0	4	7	3	4	1	0	5	10	4	1
Phenol & Bisphenol	0	1	3	0	0	1	3	0	0	0	1	0	0	0	0	3	3	1	2	1	0	1	1	1	2	1	0	0	0	0	2	1	1	2
PHA, PDBE, PCB	1	0	1	1	1	1	3	1	4	1	2	1	2	1	1	0	1	1	1	3	7	2	3	1	0	1	1	1	3	4	1	1	1	2
Antimicrobial	0	2	6	1	1	2	3	1	1	1	1	1	1	1	1	2	2	1	1	3	4	1	5	1	3	2	1	3	2	1	3	3	3	2
PFAS	1	1	4	3	2	1	1	1	1	1	3	3	2	0	1	3	3	2	1	2	9	2	7	8	1	2	1	1	1	1	1	1	2	2
Extremely Hazardous Substances	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	1	0	1	1
Water Contaminants	1	1	3	0	0	2	3	1	2	2	1	0	1	2	2	1	1	1	1	1	2	3	1	2	2	3	0	3	2	1	0	1	2	2
Pesticides	2	7	24	1	0	9	14	1	2	0	2	2	3	3	2	2	6	11	10	3	4	2	6	2	3	11	5	3	1	3	15	12	5	2
Plastic Additives	7	11	30	10	4	12	22	2	7	4	9	6	3	9	7	13	17	11	13	10	17	14	27	10	19	17	9	13	7	19	17	26	8	
TOTAL DETECTED	14	26	83	16	9	37	56	8	20	11	21	13	14	20	17	28	38	38	35	28	50	29	55	27	35	45	21	29	19	45	47	47	23	

Supplementary Data 2.4 Percentage of oyster malformed larvae after exposition to the DMSO extracts



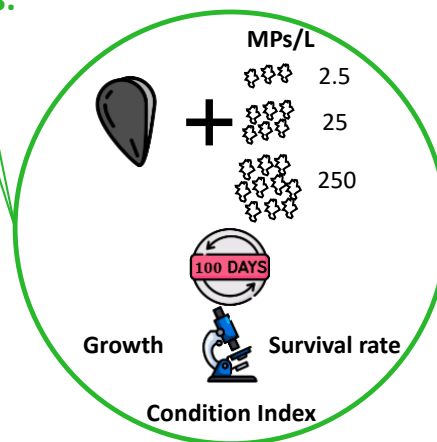
Chapter 3 – Physical toxicity of plastic

Foreword

Question:

Toxicity of MPs ingestion on mussels ?

Collaborations:



Context: In the two previous chapters, we observed that plastics can induce toxicity due to the leaching of plastic chemicals or through the adsorption of pollutants on plastic surfaces. In this chapter, we evaluated the physical toxicity of microplastics (MPs). As explained in our review paper (see State of the art), previous studies on physical toxicity of MPs generally used a uniform shape with concentrations higher than the environmental ones. Here, the originality of this chapter is the use of custom-made MPs (irregular shape), which were tested at environmental concentrations for a chronic toxicity test (100 days).

Methods: Two types of PE-based MPs were selected together with a particle control (i.e. diatomite). Custom-made MPs were specifically manufactured for this experiment. Several filtrations were needed to select the size class of 3-25 μm , which is the common size of mussel food. The experiment was realized in a new patented system design by Plastic At Sea that allowed a homogeneous repartition of MPs compared to a classical aquarium. Before exposing the mussels to MPs, the shells were marked by calcein, allowing to

measure the growth under the microscope at sampling time. In addition, the condition index was measured.

Results: Through environmentally relevant concentrations and shape of two types of polyethylene, we found no effects on growth, condition index and survival rate on chronic tests using mussel standard tests and under our experimental conditions.

Manuscript Title: Limited impact of chronic exposure to realistic concentrations of microplastics on the growth and condition index of the mussel (*Mytilus galloprovincialis*)

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Highlights:

- Chronic exposition (100 days) at environmentally relevant concentrations
- Realistic shape of microplastics and a particle control was used
- Size of microplastics adapted for mussel ingestion
- No effects observed on the growth and condition index

Status: In preparation

Abstract

Plastic pollution has emerged as a critical environmental issue, with its increasing presence in marine ecosystems posing significant threats to marine organisms. Three main types of physical effects exist: smothering, entanglement and ingestion. In this study we will focus on the ingestion of microplastics by mussel. A filter-feeding organism was selected for its filtration capacity and therefore its important exposition to microplastics in the environment. A chronic experiment was performed for 100 days with two polyethylene-based plastics. Most studies evaluating plastic chemical toxicity use microplastic of regular shape which are not representative of the environment. Thus, plastics were cryogrinded to obtain irregular shapes. Moreover, the size of microplastics were the same as mussel food to ensure their ingestion by mussels. Three environmentally relevant concentrations (i.e. 2.5, 25 and 250 MPs/L) were selected for the exposition to enable a more accurate transposition to natural conditions. In addition, a particle control was used (i.e. diatomite), to observe if the toxic impact is linked to the material or to the presence of particles. The toxicological parameters selected were at the individual level with the growth, condition index and survival rate. No toxic effects were observed for either of these toxic endpoints.

Keywords: Microplastic, Mussel, Chronic toxicity, health status, Particle control

1. Introduction

The increase of primary plastic production since the early 1950's together with inefficient waste management have led to a consequent pollution of the marine environment (Pourebrahimi and Pirooz, 2023). Plastic is a peculiar pollutant that can induce three types of toxicity: physical (e.g. intestinal injuries), chemical (e.g. leaching or adsorption of toxic chemicals) and biological (e.g. pathogen transfers) (Leistenschneider et al., 2023). Here we focused mainly on the physical impact of plastic on marine bivalves, with possibly a low chemical effect due to the low concentrations. Depending on plastic types and the size of species ingesting it, three main types of physical effects have been described : smothering, entanglement and ingestion (Bruemmer et al., 2023). Smothering can prevent filter feeders from feeding (Bergmann and Klages, 2012; Chapron et al., 2018), but also can overshadow, crush marine vegetation (Uhrin and Schellinger, 2011) or even turn sediments to anoxic conditions (Green et al., 2015). Entanglement concern intruding of plastic around an organism which can cause wounds, skin infections and restricted movements (Barreiros and Raykov, 2014). In the worst case entangled organisms won't be able to acquire food and avoid predators or become so exhausted they drown (Laist, 1997). Ingestion is the consumption of plastics by organisms and is the most difficult physical effect to observe in natural condition (Bruemmer et al., 2023). An increasing number of ingestions between 1997 and 2015 was described for birds, turtles and mammals (Kühn et al., 2015). Field studies on plastic ingestion indicated that intentional ingestion is linked to the foraging strategy, plastic color (Schuyler et al., 2014; Tourinho et al., 2010), and the age of the species (young seals are more prone to plastic ingestion) (Greg Hofmeyr et al., 2006). Plastic has been found in goose barnacles (Goldstein and Goodwin, 2013), whales (Baulch and Perry, 2014) and mussels (Cauwenberghe et al., 2014). Unintentional ingestion has a known impact on filter-feeding organisms, which filter large quantities of water for nutrition. This study focuses on the ingestion of plastics by the filter-feeding mussels.

Mussels have been systematically used for biomonitoring since 1975 with the beginning of the "Mussel Watch" program (Goldberg, 1975). This is primarily due to their capacity to filter large volume of water, and to bioaccumulate pollutants (Goldberg et al., 1978), but also to their large geographical distribution and sedentary lifestyle (Baralla et al., 2021).

The species *Mytilus galloprovincialis* is distributed throughout the Mediterranean Sea as an endemic species and further introduced into the northeast Atlantic, with additional populations in California, Japan, South Africa, Australasia and Chile (Daguin and Borsa, 2000; Hilbish et al., 2000). Commercial size is commonly about > 50 mm (Carver and Mallet, 1990). Mussels can filter between 12 and 43.2 L/day/individual (Boromthanasarat, 1986). Although non-food elements can be ejected before passing through the digestive system, MPs were found in mussels recovered in the environment (Van Cauwenberghe and Janssen, 2014). The most retained particles by mussels are between 4 µm and 8 µm (Boromthanasarat, 1986) but mussels can absorb particles in the 3-25 µm size range (Moltschaniwskyj, 2005). Experimental studies also indicate the transfer of MPs to different organs of mussels (e.g. gills, stomach, digestive gland) (Li et al., 2021). Moreover, they can cause secondary ingestion of MPs to higher trophic levels (e.g. crab), since they occupy a significant position in trophic transfer (Farrell and Nelson, 2013).

The most recovered plastic items in the marine environment are MPs of irregular shape, originating from the fragmentation of macrodebris (Duis and Coors, 2016). They are mostly composed of PE, which represents more than 25% of the world's plastic production (Plastics Europe, 2022) and is therefore the most recovered plastic type in the marine environment (Erni-Cassola et al., 2019). Reservations have been expressed about the representativeness of toxicity tests using commercially available virgin primary MPs with a regular form (Leistenschneider et al. 2023). The concentration range of MPs (particle/L) was also a matter of debate, with most toxicity studies used concentrations from 10 to 10¹⁴ times higher than the highest concentration measured in surface seawaters (150 particles/L, > 0.75 µm) (Song et al., 2014). Finally, for bivalves, most of the toxicity experiments were acute or subchronic, whereas chronic tests are still in the minority (Leistenschneider et al., 2023). A standardized test for freshwater mussels determines an acute, subchronic and chronic toxicity tests for duration of < 7 days, between 7 and 28 days and > 28 days, respectively (ASTM E2455–2). Chronic toxicity tests enable the observation of toxic effects that would have otherwise gone unnoticed. For example, mussel byssus production was impacted only after 36 weeks of exposure (Hamm and Lenz, 2021).

The objective of this study was to better understand the toxic physical effects of longer chronic MPs exposition at environmental concentrations on marine mussels (100 days). We

compared two types of MPs made of PE with different chemical composition, including oxodegradable PE with known additives (Cobalt and Manganese), with controls made of naturally occurring particle (diatomite) of similar size ($4 < d < 25$ mm). Tested concentration ranged from a low (2.5 MPs/L) to the highest concentration ($2.5 \cdot 10^2$ MPs/L) found in the marine environment (Song et al., 2014). A total of 128 mussels (*Mytilus galloprovincialis*) originated from the Banyuls Bay (NW Mediterranean Sea) were acclimated and placed in Plasticell® aquarium to observe health status corresponding to toxicity endpoints such as survival, growth and condition index, together with the presence of microplastics in the mussel tissues.

2. Materials and Methods

2.1 Plastic types and manufacturing of MPs

We used two PE-based plastic types provided by Symphony Environmental company that were under the form of films (thickness = 50 μm). One PE was called “pure” (i.e. without any chemicals added after the PE provider) and the other was made of the same PE (99.84%) but additivated with Cobalt (0.10%) and Manganese (0.06%) (each known as d2w prodegradant produced by Symphony Environmental) to form an oxodegradable PE. Diatomite, a naturally occurring particle that consists of fossilized skeletal remains of siliceous algae, was used as a control particle in parallel with seawater only. A special care to the particle size was taken. To obtain particles between 3 and 25 μm , a cryogrinding (6775 Freezer/Mill® Cryogenic Grinder) was performed on plastic films pre-cut in the capsule. The program used was composed of 10 min of pre-cooling followed by 1 min of run time with 15 strokes/second and a cool time of two minutes. This cycle was repeated 15 times. The powder recovered was introduced in an aluminum foil before filtration. The plastic powder was successively filtered with 99% EtOH (pre-filtered with 0.1 μm PTFE filters) through 4 filters of 200 μm , 100 μm , 50 μm and 25 μm . The powder left on the filters was recuperated after each filtration and stored in glass petri dishes. Every material was rinsed off with pre-filtered EtOH. Diatomite was not grinded and directly filtered. The powders were then analyzed through a granulometer in triplicates. The quantity of plastic introduced in the system was calculated from the granulometry measurement in 70% ethanol.

2.2 Mussels acclimatization and experimental set up

The species used for this experiment is the Mediterranean mussel: *Mytilus galloprovincialis*. Around 200 mussels of 40 and 50 mm in size were sampled in the Banyuls bay (NW Mediterranean Sea, 42.482028 N, 3.133223 E). Mussel’s shells were brushed with a toothbrush to remove all the epiphytes. Mussels were then acclimatized in a semi-open aquarium with seawater filtered at 20 μm . The acclimatization period lasted 11 days. During this period the mussels were fed every day with a mix of two algae *Tetraselmis sp.* and *Isochrysis galbana*. 128 mussels were measured thanks to a caliper before introducing

them in the experimental set-up, with a change of water two times per week with seawater directly sampled in the Banyuls bay and filtered at 1 μm . The experiments lasted between December and March 2023 where MPs were added after each water change.

This experimental set-up was built in order to ensure the exposure of mussels to microplastics. Indeed, any plastics with a density weaker than water will float, e.g. PE. Since mussels are benthic organisms, they would be placed at the bottom in a classical aquarium whereas plastics remain at the surface. Therefore, an innovative system was used to ensure that plastics are in contact with the mussels (Plasticell[®], patent BT8167PC00 pending, Plastic At Sea, Figure 22). One structure is composed of 8 Plasticells[®] (i.e. small glass aquarium) (Figure 22-A) allowing 16 mussels per system. The aquariums were filled up to 50 L.

Eight mussels were removed of their Plasticell[®] and directly dissected after 50 and 100 days. The tissue was stored in an aluminum foil, froze in liquid nitrogen and then stored at -80°C before the lyophilization. The shells were stored at room temperature. The lyophilization was performed for 48h at -93°C with a pressure of 0.014 mbar.

Several parameters were measured in the retention tank to ensure the water quality during the experiment including the temperature ($T^{\circ}\text{C}$), pH, salinity, ammonium (NH_4^+), nitrogen dioxide (NO_2), nitrate (NO_3^-), dissolved oxygen (DO). The $T^{\circ}\text{C}$, pH and salinity were measured daily whereas NH_4^+ , NO_2 , NO_3^- and DO were weekly measured. During the whole experiment, $T^{\circ}\text{C}$ was maintained at $17 \pm 1^{\circ}\text{C}$, pH at $7.5 \pm 0,5$ and salinity at 40 g/L. The values of the other parameters were: $< 0.05 \text{ mg/L}$ for NH_4^+ , $< 0.01 \text{ mg/L}$ for NO_2 , $< 0.5 \text{ mg/L}$ for NO_3^- and $> 10 \text{ mg/L}$ for DO.

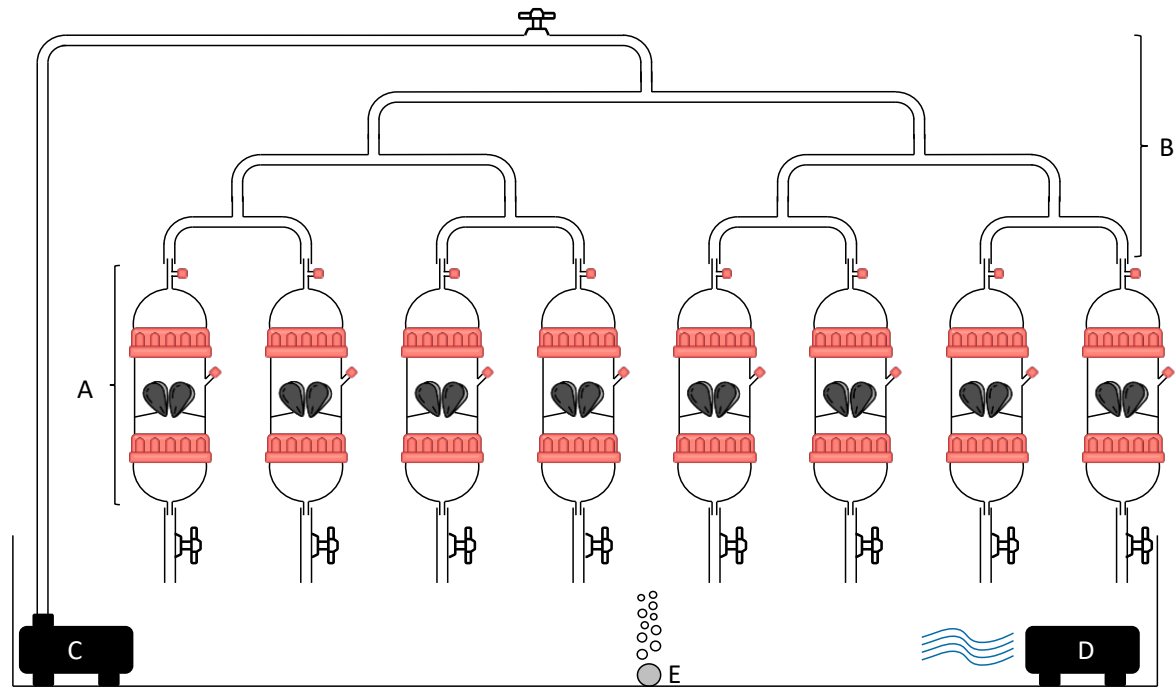


Figure 22: Graphical representation of the experimental set up for microplastics exposition to mussels. A = Plasticells®; B = Dichotomies; C = Lifting pump; D = Stirring pump; E = Bubbler.

2.3 Condition index

Some mortalities were observed randomly. The survival rate was measured daily per condition during the experiment. The condition index (CI) is an indicator of physiological responses to stress on somatic growth and is relevant for evaluating the commercial quality of bivalves (Sasikumar and Krishnakumar, 2011). Several different methods exist to measure the CI. Here, one of the most robust and repeatable formula was used (Davenport and Chen, 1987). The formula used was $\frac{\text{Dry tissue weight}}{\text{Shell weight}} \times 100$. The dry tissue was weighted after lyophilization.

2.4 Growth

The growth measurements were performed through a sclerochronological analysis. Mussels were incubated with a calcein solution (150 mg/L; Na₂CO₃ 1.048 g/L) for 2 hours at the start of the experiment before placing them in the exposure system. This incubation allows a growth mark deposition at the growing edge (Lartaud et al., 2017).

After the 100 days of exposure to plastics one valve from mussel shells was included in SODY 33 resin in order to have a basement for the cutting step (Mouchi et al., 2019). The shells were cut along the maximum growth axis using a Buehler Isomet low-speed diamond saw and polished with two successive circles of abrasion (120 & 240). Then, shells sections were mounted on slides with an Araldite 2020 resin, cut at 500 μm and polished with Al_2O_3 powder (at 3, 1 and 0.3 μm respectively) to obtain a shell thickness of < 100 μm for microscope observations. The fluorescent labelling was observed under a fluorescence microscope (Olympus BX-UCB) exciting at 460-490 nm. A picture was taken and the distance was calculated through ImageJ® (Abràmoff et al., 2004), by measuring the distance between the bright green fluorescent calcein mark and the growing tip.

2.5 Statistical analyzes

Statistical analyzes were performed using the R software (R Core Team, 2021). First the outliers were determined with the grubbs test and removed from the data set (Komstra, 2022). All data were tested for homogeneity and normality using the Bartlett's and Kolmogorov-Smirnov. If the data was not normally distributed, a Kruskal-Wallis analysis was performed followed by a Dunn test with an *fdr* correction. On the other hand, if the data was normally distributed, an Anova followed by a Tukey test was performed.

3. Results

3.1 Granulometry

The granulometry of each grinding was measured and results obtained allowed to determine which quantity was introduced into the system. The grinding and filtration allowed to have 99% of the particles (in number) of diatomite that are in the desired size class. (i.e. 3-25 μm) (Supplementary Data 3.1). Smaller proportion of 96 and 94% were recorded, respectively, for PE-oxo and PE-pure. From these results, we adapted the quantity of particles added for each material in order to obtain a final concentration of 250, 25 and 2.5 MPs/L. 250 MPs/L with a diameter of 25 μm is equivalent to 2 $\mu\text{g/L}$.

3.2 Mortality rate

The total mortality rate reached 18%, corresponding to 23 deaths for 128 individuals after a 100-day experiment. Although survival reached higher rates for mussels exposed to PE-oxo at all concentrations (>87%) compared to mussels exposed to PE-pure, diatomite and seawater (75%), no statistical differences were observed among the different conditions (Figure 23).

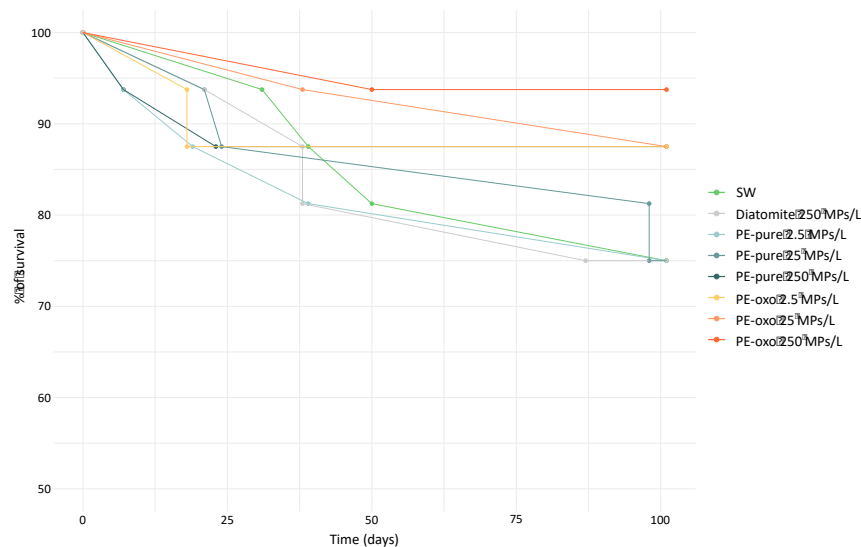


Figure 23: Survival percentage by condition in function the experiment times (days). SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable.

3.3 Condition Index

After 50 days of exposure, the mean condition index (CI) was 4.7 ± 1 and did not show any significant difference between the conditions (KW test, $p > 0.05$) (Figure 24). After 100 days of exposure, only one statistical difference was observed, between PE-oxo at 25 and 250 MPs/L.

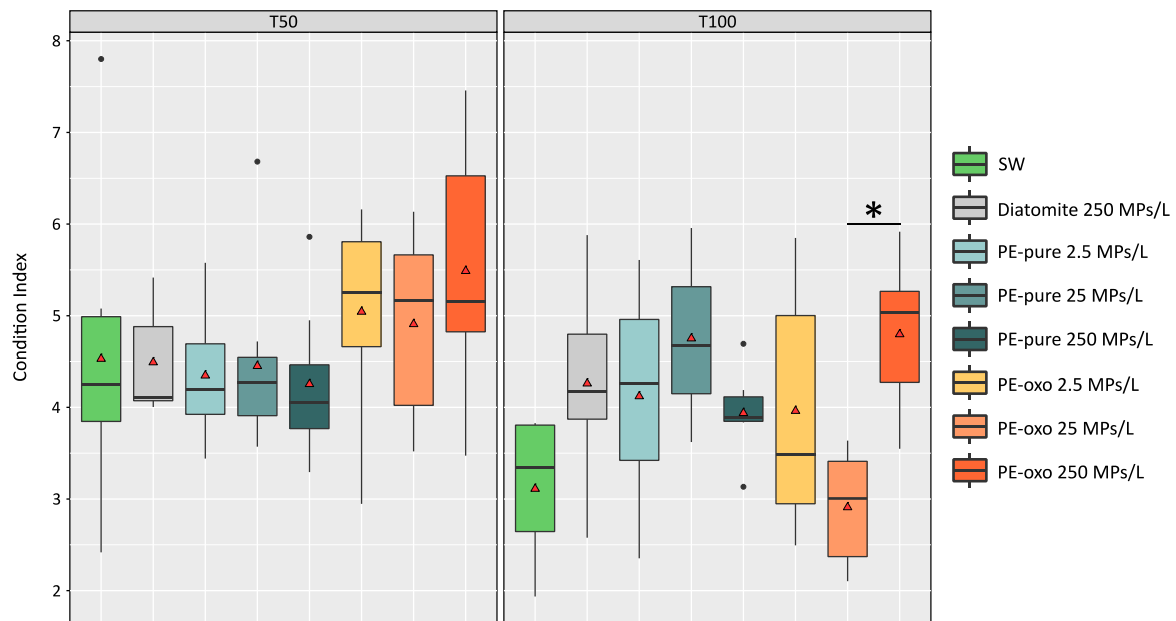


Figure 24: Condition index results for each condition separated by the exposition time. Box plots correspond to median, 1st and 3rd quartile and 95% extrema; red triangles correspond to the mean. * = Statistical difference; SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable.

3.4 Shell growth

The shell growth was measured through a mark and recapture approach. The values obtained were then extrapolated to the growth in mm per year (Figure 25). The mean shell growth rate was 2.23 ± 1.71 mm/year and no significant difference was observed between conditions, both after 50 and 100 days.

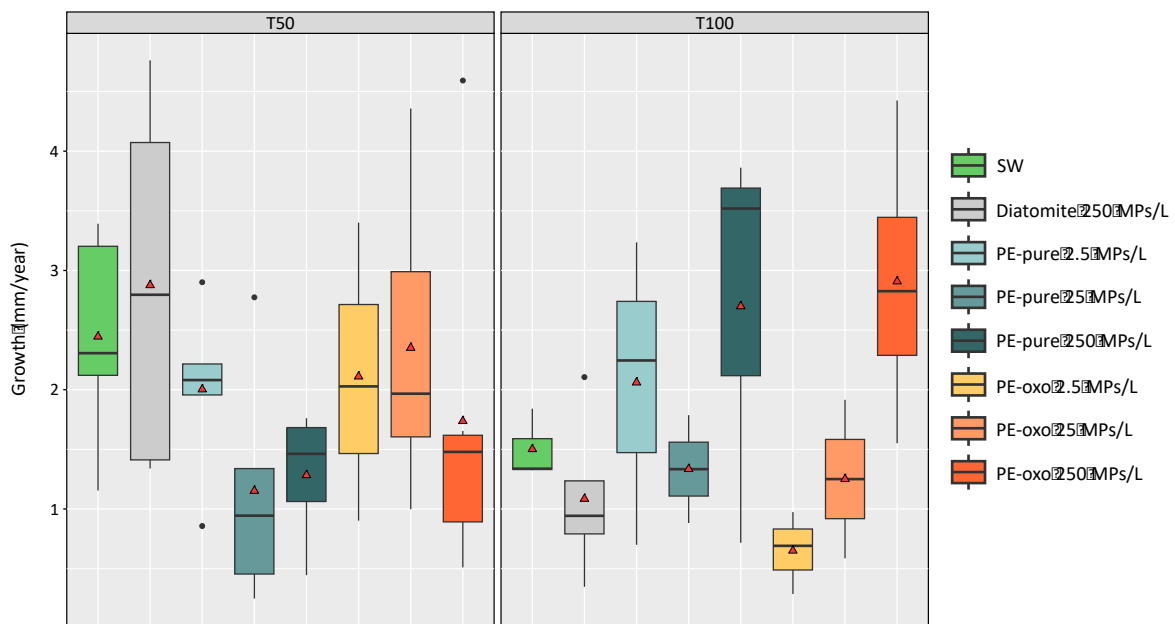


Figure 25: Mussel shell growth between each experimental condition after 50 and 100 days. Box plots correspond to median, 1st and 3rd quartile and 95% extrema; red triangles correspond to the mean. SW = Seawater; PE-pure = Polyethylene pure; PE-oxo = Polyethylene oxodegradable.

4. Discussion

In this study we evaluated the chronic toxicity of PE-based (pure and oxodegradable) MPs on the marine mussel *Mytilus galloprovincialis* through environmentally relevant concentrations. No statistical differences with the mussels maintained under control conditions or exposed to particle control (i.e. diatomite) was observed for the shell growth, condition index (CI) and mortality rate, during the whole experiment.

Comparison between MPs toxicity studies is not always possible, as authors used different units to describe the experimental exposition: weight or number of MPs/L. Here, the concentration of MPs in weight/L was converted into a number of MPs/L according to a previous study (Leistenschneider et al., 2023).

Our results indicated that a 100-day chronic exposure to 250 MPs/L (equivalent to 2 µg/L) of irregularly shaped microplastics had no impact on the growth and condition index of the mussel *M. galloprovincialis*. Similar results were found on the blue mussels, that show no clear effect on growth and condition index for chronic exposure (10.5 months) to PVC and PS, even at concentrations reaching 10⁶ MPs/L (Hamm and Lenz, 2021). No effect was also observed on the global energy budget of *M. edulis* exposed to PS particles during

14 days (Van Cauwenberghe et al., 2015), which supports the stability of the condition index in our experiment. Only for non-environmentally relevant concentrations (i.e. $> 10^2$ MPs/L), Hamm and Lenz (Hamm and Lenz, 2021) showed a decrease in byssus production (after 36 weeks) and on the superoxide dismutase (SOD) enzyme. It must be noted that SOD was also impacted after a 10 MPs/L exposure in the gills. Another chronic study (32 days) using microfibers exposure (made of PET-based plastics) at environmentally relevant concentration on *M. galloprovincialis* displayed effects on the relative estradiol and testosterone (only for males) levels (Choi et al., 2022). Most of studies analyzing the subchronic or acute toxic effects of MPs on mussels have used concentrations from 10^2 to 10^8 MPs/L, which are higher than those found in the environment (Leistenschneider et al., 2023). It is therefore questionable whether these studies can be accurately transposed to natural conditions. At these concentrations, toxic effects have been observed at the molecular and cellular levels in subchronic tests on mussels, with distinct and sometimes inverse impacts depending on the experimental conditions. For example, granulocytes/hyalinocytes ratio (G/H) were affected in mussels after 7 days of exposition (with 10^7 MPs/L; (Pittura et al., 2018)), whereas another study did not find any effects on G/H ratio (with 10^8 MPs/L; (Cole et al., 2020)). Similarly, the lysosomal membrane stability was impacted the study of Sun et al. (Sun et al., 2023) at 10^3 MPs/L, but not in Cole et al. (Cole et al., 2020), while bivalves were exposed to 10^8 MPs/L. The importance of MP concentration exposure was also highlighted when effects on ROS production were significantly different from control when mussels were exposed to 10^3 MPs/L, but not to 10^2 MPs/L (Sun et al., 2023). In acute tests, SOD, catalase (CAT) or other antioxidant responses were observed after 48h or 96h (with 10^4 MPs/L (Wei et al., 2021); 10^5 - 10^6 MPs/L in (Magara et al., 2018)). Most of the observed toxic effects (e.g. biochemical and immunological biomarkers, ROS production, gene expression, steroid hormones) were found at high MP concentrations. Indeed an increase of micronuclei, DNA fragmentation and decrease in lysosomal membrane stability were observed after a day of exposure at 10^7 MPs/L (Romdhani et al., 2022).

Between the two different polymers tested, the only difference lies in the additives composing the plastic. Addition of Cobalt and Manganese prooxidants made the difference between PE-oxo- and PE-based MPs. The prooxidant principal task is to break down the

large polymer molecular weight chain into smaller fragments, so the microorganisms can process and convert it to CO₂ and biomass (Fontanella et al., 2013). In 2019, the EU imposed a ban on the production of oxo-degradable plastics because of the production of persistent microplastics in the environment (European Parliament and Council, 2019). Previous studies underlined the toxicity of Cobalt on early developmental stages of mussels (Saidov and Kosevich, 2019). Moreover, PE-oxo was shown to induce toxic effects on *Aliivibrio fischeri* and sea urchin larvae (see chapter 1). This was not the case in our study, probably because the concentration used in the leaching experiment was more than 10⁴ higher than the one used in this experiment, and because the leaching period was shorter in our condition (3/4 days when changing the water twice a week in our study as compared to 1 month in the previous study). Another explanation could be the difference in the model organism and its developmental stage. We used adult mussels that were probably less sensitive than *A. fischeri* or sea urchin larvae (Gergs et al., 2015; Hutchinson et al., 1998). Although it is known that leaching from plastic in digestive fluids can occur (Guo et al., 2020a; Kühn et al., 2020), no toxicity was found on mussel organisms after realistic contact with PE- or PE-oxo-based MPs under environmentally relevant conditions.

Another originality in our study was to consider the shape of the MPs. Most of the previous studies used regular shape whereas irregular fragments are, together with fibers, are the most representative type of shape in the marine environment (Cózar et al., 2015; Kanhai et al., 2018; Qu et al., 2018b). Regular shape MPs are readily available in the market. Therefore, they are easier to experiment rather than cryogrinding and filtrating MPs (while avoiding contamination) and finally measure their granulometry, as we performed in this experiment. Plastic shape was shown to induce differential toxicity in mussels, with PE-based plastic fibers inducing higher toxicity on *M. galloprovincialis* than circular fragments (Rangaswamy et al., 2024). Toxicity tests on other aquatic organisms between regular and irregular microplastics have shown a higher toxicity for irregular fragments (Gray and Weinstein, 2017; Na et al., 2021; Renzi et al., 2019). However, no effect of irregular shape MPs under environmentally relevant conditions were observed.

Mussel growth and condition index have been widely used for biological studies, but also for commercial purposes as it is a measure of “fatness” and “marketability” of commercially exploited species (Okumuş and Stirling, 1998). In Europe, the production and

harvesting of bivalve mollusks is regulated by the European Commission (EC) directive 79/923 (European Commission, 1979), which defines the classification of the waters in which the mussels grow. The commercialization is regulated by the EC regulation 2022/158 (European Commission, 2022) which states the safety standards for live mollusk sale. No information about plastic pollution in water is given in those directives, since very few studies are available so far on the toxic effect of MPs on mussels under natural conditions. By exposing mussels to MPs at relevant environmental concentrations, we agree with previous study showing that the growth and condition index of mussels were not affected by PE-based MPs exposition, as previously showed on PVC and PS-based MPs (Hamm and Lenz, 2021).

5. Conclusion

This study focused on the physical impact of plastics at environmentally relevant concentrations on the Mediterranean mussel. This chronic toxicity test (100 days) was performed with custom-made microplastics of irregular shape and a particle control (diatomite). Since most studies evaluating plastic toxicity used concentrations much higher than the one measured in the environment, it is difficult to infer the possible effective impact of microplastics in the environment. Contrary to this study, most toxicity studies use spherical shapes which are not representative of environmental pollution. We aimed therefore, to perform a toxicity test more representative allowing an easier transposition to the natural conditions. We also considered the possibility of chemical leaching even though this study is mainly focused on the physical impact. Based on previous studies, the leaching of plastic chemicals in the medium could not have induce toxicity due to relatively low concentrations ($> 10^4$ times lower) and leaching time (3 days). Since plastic chemicals also leach directly in the digestive system of organisms we could have expected a higher toxicity for PE-oxo. However, since no effects were observed on shell growth, condition index or survival rates, the leaching inside the tested organism was, with our experimental conditions, too low to induce toxicity. The parameters tested within this study, widely used in biological experiments, are essential for commercial purposes as a measure of 'fatness'

and 'marketability'. Under our experimental conditions, we did not observe any effects of environmentally relevant plastic pollution on mussel farming.

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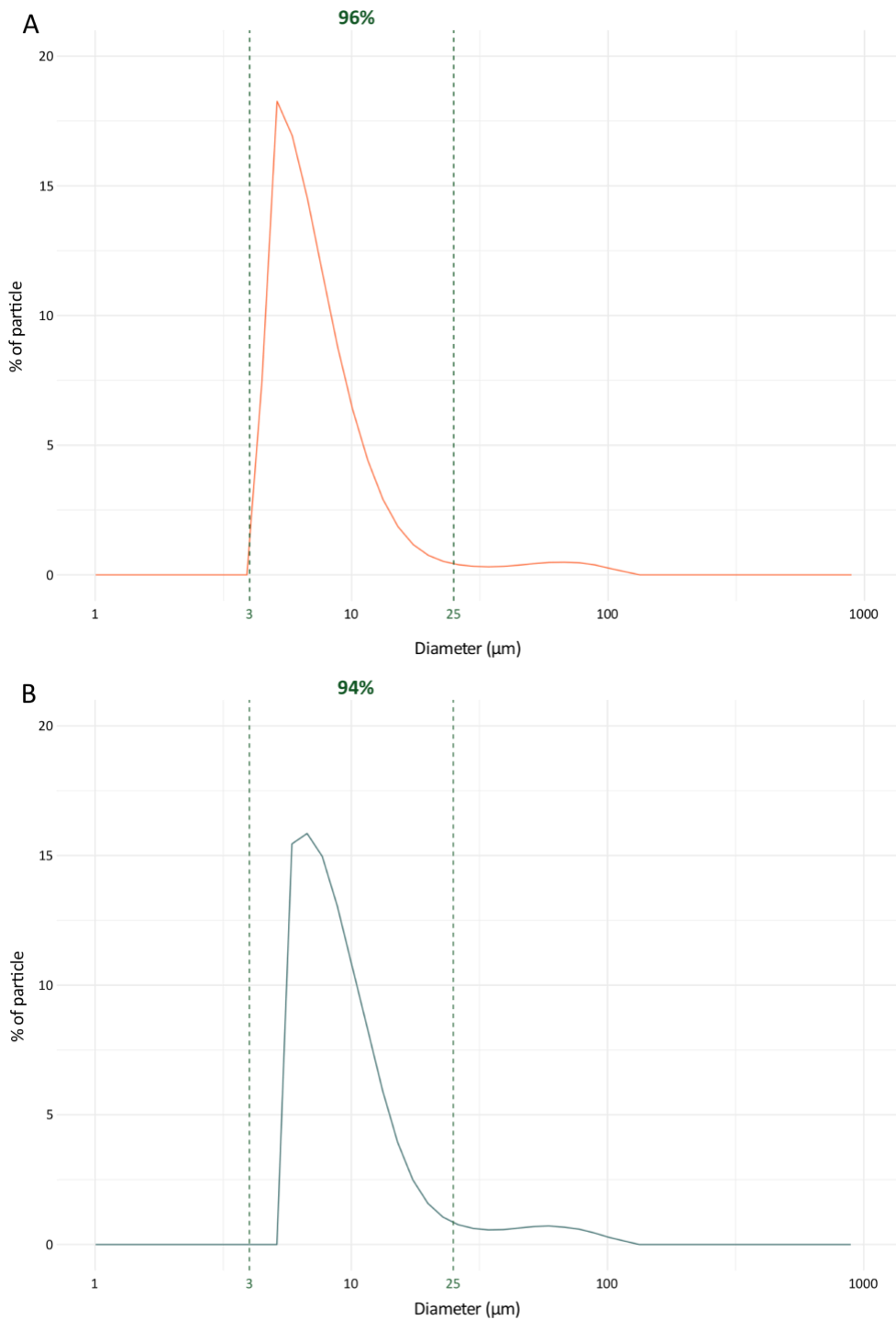
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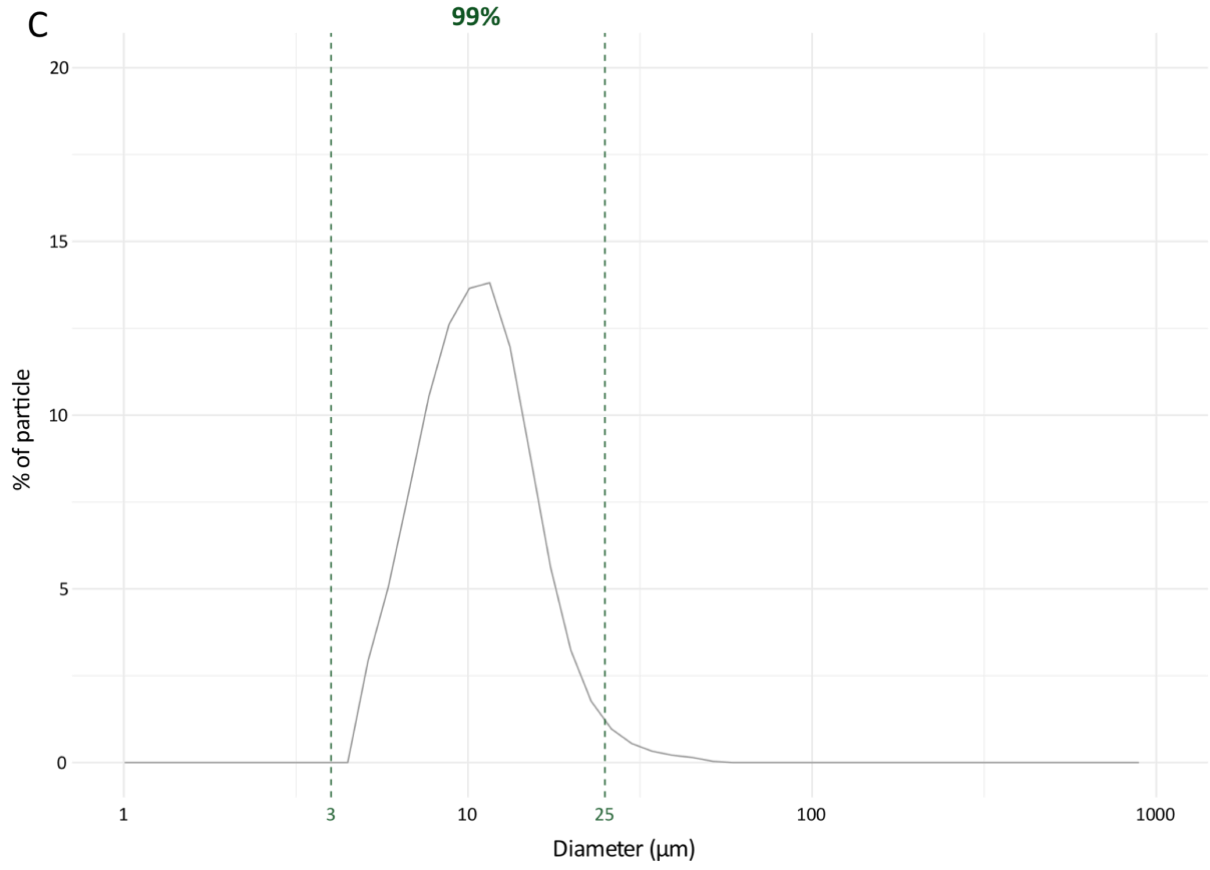
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Supplementary data 3.1 Particle distribution of PE-oxo (A), PE-pure (B) and Diatomite (C) in function of the size. Percentage written represent the number of particles of the desired size class





Conclusions and perspectives

This PhD thesis is focused on the chemical and physical toxicity effects of microplastics on marine organisms. Here, we described the main conclusions and perspectives for each corresponding chapter. Finally, we proposed a broader perspective on this PhD thesis in the last section.

Conclusions and perspectives of the Chapter 1

Conclusions of the chapter 1

The objective of the chapter 1 was to better decipher the toxic impact of leaching of plastic chemicals on marine bacteria (*Aliivibrio fischeri*, Microtox® test) and sea urchin (*Paracentrotus lividus*). Since no leaching study has extended the leaching time beyond 1 month (Almeda et al., 2023), we performed a long-term experiment for several months under natural conditions (1 day, 10 days, 1 month, 2 months, 4 months and 8 months). The objective was to better decipher the kinetics of plastic leachate toxicity in relation to plastic chemicals leaching process. The leaching was tested on 4 PE-based microplastics with different plastic chemical composition. Inorganic element analysis was conducted on the leachates and virgin/leached microplastics in order to determine the chemical origin of the toxicity.

PE-oxo-based plastics were found to be the most toxic, with effects observed after 1 month for the Microtox® test and for the larvae development test. PE-pure (without plastic chemicals added) induced toxicity after 8 months of leaching only on the embryotoxicity test. Our results emphasized the importance of long-term leaching experiments under natural conditions to accurately assess plastic chemical toxicity and the need to use several species with different sensitivity for toxicity tests. For the first time, we demonstrated the kinetics of toxicity in relation to plastic leaching time, observing an exponential decrease of EC₅₀ with increased leaching time. The ICP analysis on inorganic elements enlightened the differences in metals composition and toxic trace elements among the four different

plastics tested. The chemical analysis partially explained the source of toxicity for one polymer (i.e. PE-pure), while the most toxic polymer (i.e. PE-oxo) did not contain toxic concentrations of the measured elements. Our analysis of inorganic elements was limited, and given the wide variety of possible plastic chemicals (i.e. over 16,000 in all the known commercial plastic products), it is challenging to obtain a comprehensive overview of all chemicals present in plastics and their leachates. Furthermore, manufacturers never specify the exact chemical composition of the plastic products they put on the market, making it difficult to identify the source of toxicity.

Perspectives of the chapter 1

Perspective 1 - Chemical analyzes of organic pollutants

A chemical analysis was performed on the inorganic elements present in the plastic and the leachates. However, this analysis was limited and further analyzes could be realized to better comprehend the origin of the toxicity. For example, the measurement of organic contaminants in plastics and leachates is a relevant perspective. The most common method for analyzing organic chemicals present in plastic are through gas/liquid chromatography coupled with mass spectroscopy (GC/MS or LC/MS). These two method possess numerous advantages such as the ability to identify and quantify with sensitivity, specificity, reproducibility and versality (Bridson et al., 2021). Ideally several techniques should be used in order to detect a maximum the leached substances. Indeed, some chemicals will be detected by LC/MS but not by GC/MS and vice versa (Bridson et al., 2021). Moreover, there exist different databases of chemicals which will also influence the obtained results since different chemicals are referenced (EPA; European Union). Anyhow, a better understanding of the chemical composition of the leachates will allow to better comprehend the origins of toxicity.

Perspective 2 - Validation of the toxicity kinetics related to leaching kinetics

Further experiments are needed to confirm the exponential decay of toxicity with the increasing leaching time. The leaching is the result of a wide number of factors depending on the chemicals, plastic polymer and leaching medium.

Indeed, leaching time will vary since the migration rate is clearly dependent on the chemicals present. For example, most of the additives are not chemically bound to the polymer, whereas others reactive organic additives (such as flame retardants) are polymerized with the plastic molecules (Hahladakis et al., 2018) and will therefore migrate more slowly. Moreover, small molecules with low boiling points, high vapor pressure and molecular weight lower than 600 g/mol will tend to migrate faster. Finally, the solubility of chemicals in plastics also plays a role in the migration (Hansen et al., 2013).

The polymer type plays also a role in the leaching. It is generally considered that migration occurs faster in amorphous regions than crystalline ones due to the space between the monomers. Moreover, the less degradable the polymer is the slower the leaching will occur, since a higher degradation leads to a higher migration (Hansen et al., 2013).

The environment will also play a role such as the temperature, UV or salinity (Suhrhoﬀ and Scholz-Böttcher, 2016). Moreover, the solubility of the chemical in the leaching medium also influence its migration (Hansen et al., 2013).

Even though the leaching time will vary in function of the polymer type, type of plastic chemicals and surrounding environment, it can be stipulated that the observed kinetic will be conserved since migration of plastic chemicals will decrease with time as the concentration of “said” substance in plastic decreases (Hansen et al., 2013).

Perspective 3 - Different species and exposure time

In this PhD thesis, the toxicity tests were performed on bacteria and during the early life development of sea urchins. The impact on other species would be relevant since different effects may be found on different species due to their respective sensitivity (Taddei et al., 2021). Thus, testing leachates on different organisms will allow a better understanding of their ecotoxicological impact. Moreover, chronic toxicity tests are more representative of plastic environmental exposition. Therefore, performing long term experiments of those leachates with different species will allow a more relevant evaluation of threshold concentrations that will induce toxicity in the environment. Ideally, those toxicity tests should be performed on adult invertebrates and vertebrates.

Conclusions and perspectives of the Chapter 2

Conclusions of the chapter 2

Another chemical impact of plastic was considered in this PhD thesis, through the adsorption of pollutants on plastic surfaces. Most studies evaluated the adsorption of plastic in laboratory conditions, whereas studies on environmental adsorption are scarce (Fu et al., 2021; Wang et al., 2020a). Our experimental setup consisted in PE pellets immersed along 9 European river-to-sea continuum along a salinity gradient. After 1 month immersion, the adsorbed pollutants were extracted with DMSO and their toxicity was evaluated on marine bacteria (*Aliivibrio fischeri*) and oysters (*Magallana gigas*). In addition, an inorganic element analysis on the pellets and DMSO extracts coupled to an organic chemical analysis were performed. These analyzes were realized to understand the origin of toxicity but also the extent of plastic sponge effect.

In this chapter, we tackled the adsorption of pollutant on plastic surface. The chemical and toxicological analyzes were focused on plastics with their biofilms, which has shown to play a role in pollutant adsorption (Richard et al., 2019). Our findings highlighted that rivers were polluted with a wide variety of pollutants that adsorbed on the surface of plastics. Indeed, 1 month of incubation was sufficient for the adsorption of up to 83 organic contaminants at the plastic surfaces. 84% of samples exhibited a higher number of organic contaminants compared to virgin PE-based plastic. Inorganic analysis performed directly on plastics revealed significant differences between virgin and incubated PE-based pellets. The DMSO extraction of inorganic pollutants displayed a clear spatial trend, with significant variations along the rivers. Additionally, the toxicity induced by adsorbed pollutants increased along the river, with a decrease at the estuary probably linked to a dilution effect. This supports the hypothesis that anthropogenic pollutants added along the river contribute to increase toxicity.

Perspectives of the chapter 2

Perspective 4 - Environmental adsorption with other plastics

This chapter was focused on the pollutant adsorption of plastics made of only one polymer type: polyethylene. A perspective would be to immerse other plastics in order to identify their environmental adsorption capacity. Indeed, it has been showed that different plastics possess different adsorption capacity (Enyoh et al., 2021). In order to have a relevant comparison between the different tests, several key parameters must be considered. Particle size is crucial, since the specific surface area will impact the adsorption capacity. Usually, the smaller the particle size the higher the sorption capacity (Zhang et al., 2019). However, in some studies an opposite tendency was observed, especially at the nanoscale, where the aggregation of nanoplastics reduced the specific surface area and therefore the adsorption (Wang et al., 2019). Similar sizes of microplastics must be chosen in order to eliminate this bias. Moreover, the adsorption is linked to the degradation state, with an increasing adsorption when the degradation state increases (Wang et al., 2020a). Therefore, when testing different plastics, similar degradation states must be used. Several environmental parameters must also be taken into account since they influence pollutant adsorption: pH value, ionic strength and dissolved organic matter (Wang et al., 2020a). Therefore, immersion of plastics in the environment must be performed at the same place and time.

Perspective 5 - Impact of biofilm

We analyzed the adsorption of pollutants on plastic surface with the biofilm, state in which MPs are in the environment. Biofilm has been shown to increase adsorption of organic contaminants and metals (Bhagwat et al., 2021; Richard et al., 2019). (Bhagwat et al., 2021) compared the increase in adsorption of the 6 months immersed plastic with a virgin control. Since the weathering has an impact on pollutants adsorption, it may have played a role in adsorption. To better characterize the role of biofilm in pollutant adsorption, removing the biofilm and measuring the metals on the immersed polymer and in the biofilm will allow to better localize the higher zones of adsorption. Special care when removing the biofilm is needed since the ion-exchange mechanism is more loose in the biofilm rather than on the polymer (Kurniawan et al., 2012). A method development would

be needed in order to be able to remove the biofilm from the polymer without removing the adsorbed pollutants (using soft but efficient enzymatic lysis, for example).

Perspective 6 - Leaching in simulated body fluid

We observed a wide variety of different pollutants adsorbed on plastic surface. The extraction method used allowed us to retrieve and analyze this pollution. However, it is not representative at all of their environmental impact since we forced the extraction of contaminants in a solvent (i.e. DMSO). Therefore, an experiment analyzing, from immersed plastics, its leaching in digestive fluids of animals would allow to better grasp the impact observed in the environment. This leaching can also be performed in simulated body fluid. Pollutant adsorption gained importance in research studies since they have been shown to accumulate at concentrations 10^5 - 10^6 higher than the surrounding environment (Mato et al., 2001). However, its potential toxicity in the environment was challenged by disclosing that the flux of hydrophobic organic contaminants bioaccumulated from natural prey overwhelms the flux from ingested microplastic for most habitats (Koelmans et al., 2016). Therefore, understanding the leaching kinetics of adsorbed pollutant on plastic surface inside organism will allow a better evaluation of its environmental impact.

Conclusions and perspectives of the Chapter 3

Conclusions of the chapter 3

Microplastics also impact organisms through physical toxicity. Most of the studies so far used concentrations and shapes that were not environmentally relevant (Leistenschneider et al., 2023). In this PhD thesis, mussels (*Mytilus galloprovincialis*) were exposed to custom-made microplastics (irregular shape) at environmentally relevant concentrations (2.5, 25 and 250 MPs/L). The toxicity test lasted for 100 days with three different concentrations of plastics. In addition, a particle control was realized in order to see if the effects are linked to the plastic in itself or simply to the presence of particle. The monitored parameters were performed at the individual level. Our aim was to realize a more representative experimental design allowing an easier transposition to the natural conditions. The difference between the two plastics (PE- and PE-oxo-based) was in the added chemicals (here prooxidants for PE-oxo). Therefore, a possible leaching could have increased the

toxicity of PE-oxo compared to PE-pure. Based on the results of chapter 1, the leaching into the medium could not have induced toxicity due to the low concentrations and leaching time. We could have expected a toxicity through the leaching of prooxidant in the digestive system. However, since no effects were observed on growth, condition index or survival rate, the leaching inside the tested organism was too low to induce toxicity under our experimental conditions. Those results indicated that the actual plastic pollution does not present a danger on the “fatness” and “marketability” parameters classically used by mussel farmers.

Perspectives of the chapter 3

Perspective 7 - Quantity of ingested microplastics

After an exposition to MPs for 100 days, an analysis of the number of MPs ingested by the mussels would be relevant. Since 3 concentrations were realized for each plastic type in our study, it would be interesting to observe if there are different levels of ingestion. To quantify MPs quantity in mussels a digestion step is necessary. The most common methodological approach for tissue digestion is 10% aqueous solution of hydrogen peroxide for 24h (Di Fiore et al., 2024). Then, one of the most basic method is through a Nile red staining which helps the identification and counting of MPs in biota (Nalbone et al., 2021). Nile red does not successfully stains all plastics, however it works perfectly for the polymer used in this study: PE (Nel et al., 2021). The most common method for identifying MPs is through Fourier-transform infrared (FTIR) spectroscopy. In the context of this study, the μ -ATR-FTIR would be the most relevant method since it can detect particles smaller than 20 μ m (Ding et al., 2018). μ -Raman spectroscopy, is another possible method that is even more precise and can detect plastic particles down to 0.5 μ m (Di Fiore et al., 2024).

Perspective 8 - Analyzes at lower level of biological integration

The toxicity parameters used in this study concern the individual level. Performing analyzes at lower levels of biological integration could maybe indicate some impact of MPs at environmentally relevant concentrations. An example of test could be the analysis of heat shock proteins (HSP). This can be performed through a protein extraction followed by

an enzyme-linked immunosorbent assay (ELISA) (Hamer et al., 2004). For this purpose, a western blot is needed to confirm the primary antibody specificity. Other marker such as stress related enzyme (e.g. superoxide dismutase, catalase and glutathione peroxidase) that play a fundamental role in the total defense mechanism of biological organism could also be analyzed (Ighodaro and Akinloye, 2018).

Perspective 9 - Exposition of MPs coupled with a biofilm

As explained above, the vast majority of plastics in the environment are colonized by a wide variety of organism. The so-called “plastisphere” plays a role on the physical impact of plastic since it increases its palatability and will maybe lead to higher ingestion rates (M. J. Mercier et al., 2023). Indeed, oysters ingested ten times more MPs with biofilm than the virgin MPs (Fabra et al., 2021). It has been shown that the formation of a mature biofilm that mimics natural conditions needed at least one month of colonization of the pristine plastic under seawater conditions (Jacquin et al., 2019). Moreover, an impact on the gut microbiome of mussels may be observed (Lear et al., 2021). This question is still unanswered since other studies did not see an impact of the plastisphere on the gut microbiome of mussels (Collins et al., 2023)

Broader perspective after the thesis

Perspective 10 - Standardization and implementation in a life cycle assessment (LCA)

Standards can be broken down into 4 types (Grisoni, 1997):

- Fundamental standards: definition and vocabulary
- Methodological standards: uniformed protocol to evaluate the toxicity
- Specification standards: threshold based on uniformed protocol and *in situ* effects (if available)
- Organization standards: structural changes needed to reduce the toxicity levels

As a broader perspective and according to the PhD thesis topic, we propose directions for improvement of methodological standards. The negative impact of plastic on marine

species is well documented in the scientific literature. For example, the European commission acknowledged the importance of plastic in the evaluation of water's quality, even if no threshold has been declared (European Parliament and Council, 2019). This is due to the fact that no standardized method exists to evaluate plastic toxicity. Standards are essential in order to efficiently apply directives and lead to continuous improvement in the reduction of a given product impact (Ml'kva et al., 2016). Robust and repeatable methods of measurement and analysis are crucial for the effective implementation of international directives or regulations. To date, due to the absence of standards, there are no specific regulations on plastics under the European regulation "REACH", which aims to improve the protection of human health and the environment (European Parliament and Council, 2006a).

Numerous methodological standards for evaluating the toxicity on marine organisms already exist. However, those standards are not fitted for plastic. Compared to a wide variety of other pollutants, plastic is not defined as a molecule but as a broad range of different polymers with different plastic chemicals. Moreover, its size, shape and state vary over time and impact organisms in different ways. Most of ISO and EPA standards specified that the pollutant has to be dissolved in water, which is not the case for plastics particles. Plastic leachates can be tested through those standards. Nonetheless, the problem lies in the absence of standards for leachates preparation. Leachates toxicity from an identical plastic can greatly vary in function of several parameters: leaching time, particle size, temperature or pre-weathering. Therefore, a leaching standard is crucial to evaluate efficiently the chemical impact of plastics.

A recent standard, ISO 5430:2023, focuses on the ecotoxicity of plastic used in the marine environment. However, this standard only addresses biodegradable plastics and the ecotoxicity of their degradation products. It is relevant to ensure that the soluble intermediates of biodegradable plastics introduced in the marine environment are not toxic. Nonetheless we still lack standards on non-biodegradable plastics that represent the overwhelming majority of the actual pollution.

In parallel of the standardization of toxicity test for plastics, the number of plastic chemicals such as starting substances, processing aids, additives and non-intentionally added substances is colossal. There is a crucial need to follow the increase of tests

performed on the 16,325 plastic chemicals found in commercial products. We propose the development of a standardized database to list the current scientific advances that would give information on the plastic chemicals tested (organic chemicals particularly) and on the tested organisms. Such information would be very useful once more transparency on plastic composition is recommended in the Plastic International Treaty, which is still under negotiation in 2024. Moreover, the results of chemical analysis are closely linked to the database used. Thus, a uniform database for chemical identification would also be valuable for a homogeneous comparison between studies.

Life cycle analysis (LCA) for plastics is essential for environmental policies and industry to attain a more sustainable future with an evaluation of the product from “cradle to grave”(Ayres, 1995) (Figure 26).



Figure 26: Life cycle analysis of plastic materials (Life Cycle Approach to Plastic Pollution)

ISO 14040 defines the 4 basics for conducting an LCA analysis:

1. Goal scope and definitions
2. Life cycle inventory (mass and energy balances and inputs/outputs of the system)
3. Life cycle impact assessment (LCIA)
4. Life cycle interpretation

Determining LCIA can be realized through midpoint or endpoint methods. Midpoint methods can be defined as the environmental changes whereas the endpoint methods are focused on the results. For plastics, a midpoint method would be the increase of plastic concentration in the environment whereas the endpoint method will be the toxic impact

on organism (Alhazmi et al., 2021). Some examples of typical categories of LCIA for plastics are the ecotoxicity potential, global warming potential (due to CO₂ emissions) or abiotic depletion of fossil fuels. In order to evaluate efficiently those categories standardized indicators are needed (Antelava et al., 2019). For example, the ecotoxicity potential is usually normalized and expressed as 1,4-dichlorobenzene (DB) equivalents/kg emission (Alhazmi et al., 2021). For a relevant normalization, efficient methodological standards are crucial and will allow a more efficient LCA. Nevertheless, the big picture shows us that a great variety of other factors come into place when evaluating plastic products (Figure 27).

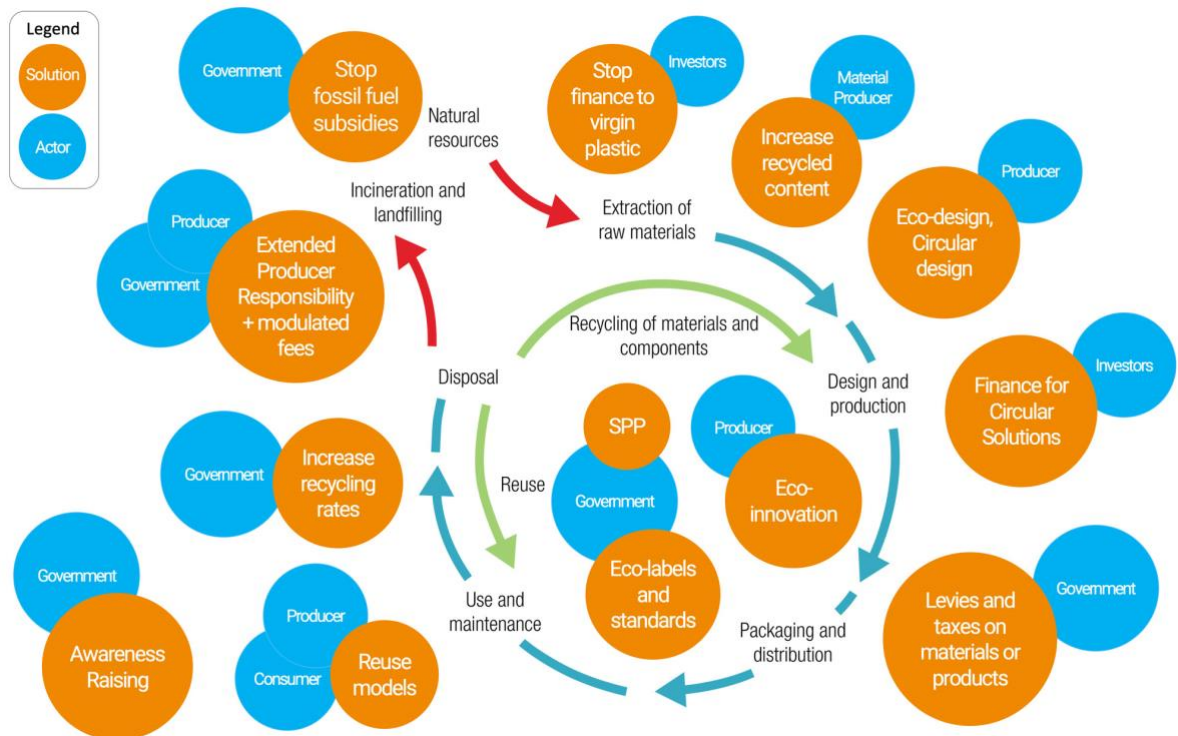


Figure 27: Life cycle analysis of plastic materials, along with the solutions and stakeholders needed for mitigating the environmental impact of plastics. SPP = Sustainable Public Procurement (Life Cycle Approach to Plastic Pollution)

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