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Ecotoxicological aspects of microplastic PET (Polyethylene Thereftalate) in freshwater using biochemical biomarkers from *Danio rerio*

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Orientadora: Prof. Dra. Renata Fracácio Francisco
Coorientadora: Prof. Dra. Cleoni dos Santos Carvalho

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“What we know is a drop, what we don’t know is an ocean.”

— Isaac Newton

Resumo

Os microplásticos (MPs) representam contaminantes emergentes que têm atraído extensa atenção da investigação devido à sua prevalência tanto em ambientes naturais como urbanos. Entre estes ambientes, os ecossistemas de água doce foram significativamente impactados. No entanto, atualmente faltam níveis de concentração seguros estabelecidos para MPs. Assim, através de uma revisão bibliográfica da abundância e distribuição de MPs nestes ecossistemas, bem como da sua relação com os estudos ecotoxicológicos mais recentes, foi possível fazer uma análise de risco e propor níveis seguros de MPs para ambientes de água doce (PNEC: 0,0027 mg L^{-1} ou 2,61 partículas L^{-1}). Isto foi estimado para 7 tipos de MPs (PE, PS, PVC, PA6 e PES, PET e copolímero EEA) que foram baseados na revisão ecotoxicológica da pesquisa. Posteriormente, reconhecendo lacunas na literatura relativas a tipos específicos de MP, o presente estudo concentrou-se na investigação da ecotoxicidade de MPs de tereftalato de polietileno (PET-MPs). Com base nessas informações, foram realizados dois experimentos crônicos de 21 dias com *Danio rerio* para estudar sua ecotoxicidade isolada e misturada com cádmio (Cd). O primeiro teste consiste na comparação dos diferentes tamanhos do PET-MP isolado (300 - 450 µm; 53 - 75 µm); e o segundo da sua mistura (53 - 75 µm) com Cd. Concentrações ambientalmente relevantes de MPs (983 partículas. L^{-1} e 330 partículas. L^{-1}) e Cd (10 ug L^{-1}) foram utilizadas durante os testes de laboratório. Análises bioquímicas foram realizadas para observar a toxicidade em termos de estresse oxidativo (proteínas carboniladas), metabolismo (glicose e triglicerídeos) e atividade enzimática (malato desidrogenase e lactato desidrogenase). Especificamente, no caso do experimento de diferentes tamanhos e concentrações de PET-MP, os níveis de glicose, CP, MDH e LDH exibiram que partículas maiores têm efeitos mais pronunciados em comparação com as menores, e concentrações mais altas produziram similarmente maior impacto. No segundo experimento envolvendo a combinação de PET-MPs e Cd, foi observada toxicidade aumentada em termos de níveis de estresse oxidativo e atividade enzimática como resultado das análises de CP, LDH e MDH.

Palavras-chave: Microplásticos, Água doce, Ecotoxicidade, Análise de risco, Análise bioquímica.

Abstract

Microplastics (MPs) represent emerging contaminants that have garnered extensive research attention due to their prevalence in both natural and urban settings. Among these environments, freshwater ecosystems have been significantly impacted. However, established safe concentration levels for MPs are currently lacking. Thus, by a bibliographical review of the abundance and distribution of MPs in these ecosystems, as well as their relationship with the most recent ecotoxicological studies, it was possible to make a risk analysis and to propose safe MPs levels for freshwater environments (PNEC: 0.0027 mg L^{-1} or 2.61 particles L^{-1}). This was estimated for 7 types of MPs (PE, PS, PVC, PA6, and PES, PET, and EEA copolymer) that were founded on the ecotoxicology review of the survey. Subsequently, recognizing gaps in the literature pertaining to specific MP types, the present study focused on investigating the ecotoxicity of Polyethylene terephthalate MPs (PET-MPs). Based on this information, two 21-day chronic experiments were carried out with *Danio rerio* to study its ecotoxicity isolated and mixed with cadmium (Cd). The first test consists of the comparison of the different sizes of isolated PET-MP (300 - 450 µm; 53 - 75 µm); and the second of its mixture (53 - 75 µm) with Cd. Environmentally relevant concentrations of MP (983 particles. L^{-1} and 330 particles. L^{-1}) and Cd (10 ug L^{-1}) were used during laboratory tests. Biochemical analyzes were performed to observe toxicity in terms of oxidative stress (carbonylated proteins), metabolism (glucose and triglycerides), and enzymatic activity (malate dehydrogenase and lactate dehydrogenase). Specifically, in the case of the experiment of different sizes and concentrations of PET- MP, Glucose, CP, MDH, and LDH levels exhibited that larger particles have more pronounced effects compared to smaller ones, and higher concentrations similarly yielded greater impact. In the second experiment involving the combination of PET-MPs and Cd, heightened toxicity was observed in terms of oxidative stress levels and enzymatic activity as a result of CP, LDH, and MDH analyses.

Keywords: Microplastics, Freshwater, Ecotoxicity, Risk analysis, Biochemical analysis.

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List of Acronyms

ABNT: Associação Brasileira de Normas Técnicas

AF: Assessment Factor

BSA: Bovine serum albumin

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CEUA: Comissão de Ética no Uso de Animais

Cd: Cadmium

CONAMA: Environmental National Council of Brazil

CP: Carbonylated protein

DNPH: Dinitrophenylhydrazine

EC_{50} Effect Concentration of 50% of the organism

ICTS: Instituto de Ciência e Tecnologia - Câmpus de Sorocaba

IFPS: Federal Institute of São Paulo

HONEC: Highest Observed No Effect Concentration

LC_{50} Lethal Concentration of 50% the organism

LDH: Lactate dehydrogenase

LOEC: Lowest Observed Effect Concentration

MEC: measured environmental concentrations)

MDH: Malate dehydrogenase

MPs: Microplastics

NPs: Nanoplastics

PE: Polyethylene

PES: Polyester

PET: Polyethylene Terephthalate

PET-MPs:	Polyethylene Terephthalate microplastics
PNEC:	Predicted no-effect concentration
PP:	Polypropylene
PS:	Polystyrene
PUR:	Polyurethane
PVC:	Polyvinylchloride
PA:	Polyamide
OECD:	Organization for Economic Co-operation and Development
RQ:	Risk Quotient
SEM:	Scanning electron microscopies
SSD:	Species Sensitivity Distribution
UNESP:	Universidade Estadual Paulista "Júlio de Mesquita Filho"
UFSCar:	Universidade Federal de São Carlos

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Introduction

The modern era has witnessed the prolific production and introduction of diverse plastic polymers into the environment. Notable examples include polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polystyrene (PS), and polyurethane (PUR). The production of these polymers escalated significantly, increasing by twentyfold between 1964 and 2015, amounting to an estimated 322 million metric tons. Alarmingly, projections suggest that this figure could double by 2035 and quadruple by the 2050s (STAP, 2018).

Ritchie & Roser (2018) estimated that there were more than 5 trillion plastic particles in the world's surface waters in 2018. Bringing concern abroad about the degradation of these compounds that are made through small pieces of plastic, characterized as microplastics (MPs) or nanoplastics (NPs) (depending on their size) (Nkwachukwu et al., 2013). These residues remain in environments available to interact with every life form, triggering ecotoxicological repercussions in different ecosystems (Bajt et al., 2021).

The effects of MPs extend to a wide variety of aquatic organisms, not only due to the physical damage caused by their ingestion but also due to their toxicity or bioaccumulation of certain associated chemicals (Horton & Dixon, 2018), including negative effects on the rates of growth, survival and fertility, feeding inhibition, genotoxicity, inflammation, and tumor promotion (Pico et al., 2019; GESAMP, 2015). All these effects represent a great risk for this type of organism and unfortunately is quite difficult to assess and/or quantify, due to the diversity of existing types, properties, sizes, formats, ecotoxicological biomarkers, and impact assessment methods for MPs (Koelmans et al., 2022). Another relevant fact to consider is that most of the risk assessments have been carried out for marine environments, leaving aside freshwater environments, despite the fact that the quantity of MPs in this medium is almost the same as the one on the marine environment (Peng et al., 2017; Li et al., 2018). That is why it is extremely important to give greater emphasis to this type of risk analysis, for the sake of understanding what really happens in these ecosystems and thus be able to establish a safe level of MPs for the protection of the species that inhabit it.

Ecotoxicological studies carried out with MPs have mainly focused on PS, hence there is a great gap in the ecotoxicological effects produced by other types of MPs such as PET (Bashirova et al. 2023). PET is a widely used type of plastic worlwide, and in Europe it represents a percentage of 7.1% of all plastics used (Plastics Europe, 2022). Currently, it already constitutes potential risks to human health, since some studies such as that of Harvey and Watts (2018), demonstrated the existence of PET-MPs in human feces. Due to these alarming data, it is also essential to study what happens to them in aquatic environments and what possible effects they may cause on the organisms that inhabit them.

Another important factor is the MPs joint exposure with other xenobiotics, since it is presumed that it could increase their bioavailability, as well as their toxicity (Zhang et al. 2020). This is a matter of great concern since the coexistence of MPs and other compounds has already been occurring in the environment for many years (Yang et al. 2022). Some factors, such as the type of surface, and the size vary its ability to adsorb other contaminants, such as metallic and organic compounds and some pharmaceuticals (Zhang et al., 2022). In the case of heavy metals, they represent a greater risk because they easily can be adsorbed from MPs (Zhou et al., 2019). A realistic scenario of this phenomenon was investigated by Deng et al., 2020, who studied the interaction of heavy metals with MPs in mangrove sediments, determining that the most abundant MP in the study area was PET and due to the characteristics of its surface it was able to adsorb heavy metals such as Cr, Zn, Pb, and Cd.

Biomarkers are defined as quantitative measures of changes in the biological system related to the toxic effects of some chemicals that exist in nature (Torres et al. 2008). Within the wide variety of them, biochemical biomarkers allow us to understand aspects of the chemical environment that cannot be made just by quantifying pollutants, such as bioavailability, interactions between other chemicals, long-term effects, and others. (Venturino & Pechen, 2005). To date, and according to various biochemical analyzes carried out in ecotoxicological trials, it has been shown that MPs, depending on their size and dose, can induce oxidative damage (increased lipid peroxidation and DNA strand breaks), alter the antioxidant system and metabolism (Prokić et al., 2019).

So, in view of what was previously described, this research had two main objectives: 1) Contribute with data on abundance, distribution and ecotoxicological aspects of MPs, as well as establish possible safe environmental concentration for microplastics in freshwater; 2) To evaluate the potential effects of PET secondary MPs at different particle sizes, doses and combination with cadmium, measured with biochemical biomarkers of metabolism, enzymatic activity, and oxidative stress of *Danio rerio*.

In order to facilitate the understanding of this work, they were divided into the following chapters:

Chapter 1 – Review article: ***Microplastics in Freshwater Ecosystems: Occurrence, Ecotoxicological Aspects, and Ecological Risk Analysis.*** Planned to be submitted in the periodic “Ecotoxicology”. The first chapter aims to collect data from the literature on MPs found in freshwater ecosystems around the world, discussing the types, size, abundance, and distribution, in addition to the bibliographic survey on the tests carried out with different types of MPs and different groups of organisms and a final Risk analysis that includes the predicted no effect concentration (PNEC) and the risk quotient (RQ).

Chapter 2 – Research article: ***Ecotoxicological studies of secondary microplastic (PET) in freshwaters using biochemical biomarkers from Danio rerio: emphasis on granulometric conditions, environmental concentrations and co-exposure with cadmium***. Intend to be published in the periodic “Environmental Pollution”. This work has two main objectives, evaluated through biochemical biomarkers of metabolism (total proteins, glucose and triglycerides), oxidative stress (protein carbonylation) and enzymatic activity (lactate dehydrogenase and malate dehydrogenase) throughout two different experiments: (1) To determinate if there is ecotoxicological codependency between different sizes (53-75 µm, 350-400 µm) and environmentally relevant concentrations (330 particles. L^{-1} and 938 particles. L^{-1}) of the isolated PET-MP; (2) To establish if the effects of PET-MPs are potentiated with the addition of Cd at theoretically concentration safe levels for the protection of aquatic life, based on CONAMA 357 legislacion (10 µg. L^{-1}).

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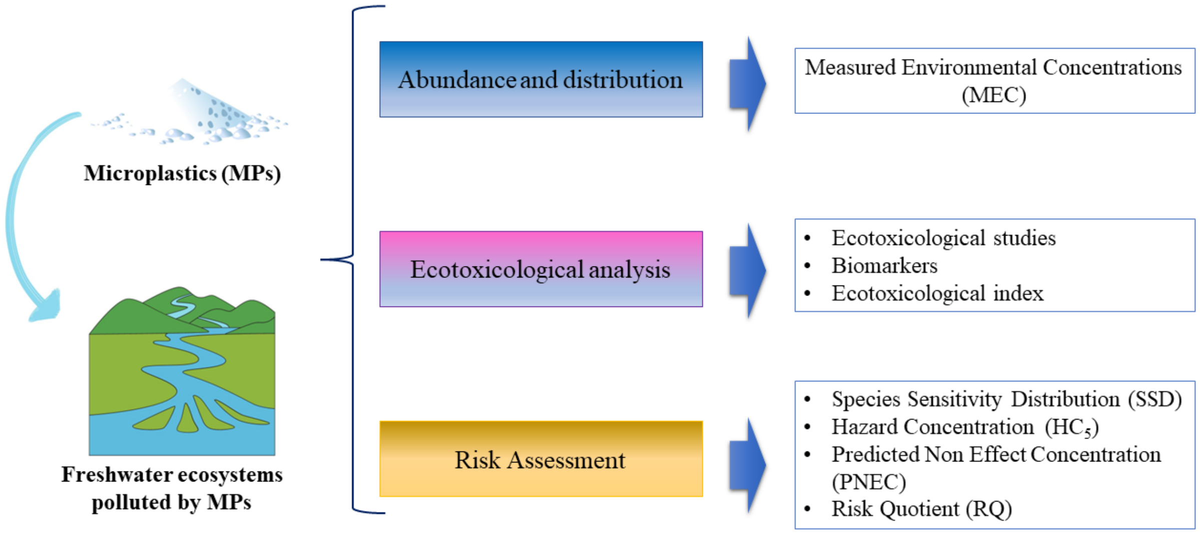


ABSTRACT

Ecotoxicological studies of Microplastics (MPs) carried out on a laboratory scale, do not always represent the real and existing conditions in the environment (types, sizes, shapes, and concentrations). This review will focus on studies regarding the abundance of MPs in freshwaters around the world based on their morphology, granulometry, and type of polymer, as well as ecotoxicological studies of MPs from different freshwater organisms, making a critical analysis of the obtained data and relating it with observed on studies of the distribution of MPs around the world. Finally, based on the ecotoxicological data, we carried out different ecological risk analyses. We estimated the predicted non-effect concentration (PNEC) from the HC_5 value (Hazardous concentration for 5% of the most sensitive species), which was calculated from species sensitivity distribution curves (SSDs). Afterward, the Risk Quotient (RQ) was calculated, for 18 countries, using the data from the MEC (measured environmental concentrations) and the PNEC. In the case of the PNEC of all analyzed MPs, a value of 0.0027 mg L^{-1} or 2.61 particles L^{-1} was estimated for 7 types of them (PE, PS, PVC, PA6, and PES, PET, and EEA copolymer). Regarding the RQ, and considering all MEC data worldwide, an RQ of 0.094 was obtained, which would assume that MPs, in global terms, have a low-risk potential. However, by separating RQ values from different countries, we found that the risk values fluctuate very much. The maximum value was 22.06 in Malaysia and the minimum was 0.000008 in Australia.

Keywords: Microplastics, Freshwater toxicology, Predicted non-effect concentration, Species sensitivity distribution, Risk assessment.

INTRODUCTION

The unbridled consumption of plastics makes them ubiquitous in the modern world and generates waste that is released into environmental compartments, by punctual or diffuse sources of pollution, where they remain represent a risk to biota (Olivatto et al., 2018). In recent years, the global plastic production has grown significantly in quantitative terms (Bajt, 2021). Comparing the years 1950 and 2017, there is an accretion of almost 20 000% in the estimated average production, since it was 1.5 million and 300 million tons, each year, respectively (Plastics Europe, 2019). Considering this high worldwide plastic production and consumption there is a growing concern about how its residues are disposed in the environment, since plastics can be persistent, and their natural degradation is slow and complex (Hahladaki et al., 2018; Alabi et al., 2019; Chen et al., 2021). This plastic degradation process may occur mainly through physical mechanical actions (variations in the structure of the plastic) or chemical actions (changes at the level of molecules) (Chamas et al., 2020, Tiwari et al. 2020), but in some cases could be by microorganisms (Jaiswal et al., 2020). Whatever form of degradation, there is a small plastic fragment generated commonly called microplastic (MP) that represents a worldwide problem due to its risks to the structure and functions of ecosystems (Li et al., 2020).

According to Frias & Nash (2018), MPs can be defined as any particle with a size that range from 1 μm to 5 mm with a variable shape (regular or irregular). According to MPs' origin, they can be classified into primary and secondary, where primary MPs are manufacturing plastics that are produced intentionally in microscopic size for different uses in the industry; while secondary MPs are originated from the degradation of larger plastics, usually discarded in the environment (Olivatto et al., 2018).

MPs have been detected in all ecosystems, in fact, in 2018, there were more than 5 trillion plastic particles in the surface waters around the world, according to estimative (Ritchie & Roser, 2018). Freshwater ecosystems are direct recipients of these polymers, coming from multiple primary sources, such as industrial facilities and landfills (Guimarães et al., 2021). Since rivers contribute to the transport of approximately 80% of the solid waste present in the ocean, the study of MPs in these environments makes sense as a consequence of their ecotoxicological potential (Alimi et al 2018; Van Cauwenberghe et al 2014).

Within this framework, research studies have documented the extensive ecotoxicological repercussions of these compounds across a broad spectrum of organisms. The deleterious impacts encompass a range of outcomes, with ingestion resulting in obstruction of gills and intestines, internal tissue lacerations due to sharp edges, and the accumulation of associated chemicals that induce various biomarkers. Notably, these biomarkers include adverse effects on growth and fertility rates, which in turn can jeopardize subsequent generations (Horton & Dixon, 2018; Pico et al., 2019).

In order to mitigate the impact on the biota, it becomes imperative to assess the potential risks posed by these plastic polymers. The underlying principle of risk analysis is to evaluate the extent of harm, considering the exposure of a community to a substance present within the environment (Adam et al., 2021). This analysis is conducted using concentrations that facilitate the estimation of the Predicted No-Effect Concentration (PNEC)—the concentration of a new pollutant in a specific organism where no adverse effects are anticipated as a result of exposure (US EPA, 2012). The calculation of the risk assessment index typically relies on data derived from ecotoxicity tests (Peake et al., 2016). This index can be determined using either an assessment factor approach or, when an adequate body of data exists in the scientific literature, through statistical extrapolation techniques (ECHA, 2008).

In the literature review on risk analysis records for MPs in surface waters, only two studies were found. One of these studies belongs to Burns and Boxall (2018), that analyzed the MPs in water columns and sediments from freshwater and marine environments. The paper did not separate compartments to estimates no observed effect concentration (NOEC) and the lowest concentration observed effect (LOEC). Results were based in endpoints such as mortality, growth, and reproduction of different test organisms, In addition, these authors obtain a sensitive species distribution (SSD) and, as a result, they obtained the HC_5 value (Hazardous concentration for 5% of the most sensitive species) of 6.4×10^4 particles. L^{-1}. However, they were unable to propose a PNEC due to limited data and because they mixed data from two different aquatic ecosystems (freshwater and marine water). The second study was from Adam et al.

(2018), and they performed a risk analysis study of MPs in freshwater, calculating the PNEC from the fifth percentile of the SSD, as well as risk characterization indices (RCR) in North America, Europe, and Asia, establishing an average safe concentration of 8.0 $\times 10^{-2}$ µg. L^{-1} or 9.5 × 10^5 particles. m^{-3} of MPs worldwide.

Although the literature has reported risk assessment studies of MPs in aquatic ecosystems, there are few that establish a safe value (e.g. such as PNEC, HC_5) for biota in freshwater environments. Hence, it is interesting to estimate a PNEC value for MPs considering main features such as size, type, and shape. In this context, the aim of this study is 1) to carry out a critical analysis of the abundance of MPs in freshwater ecosystems and the exposure of different organisms to different types of MPs, in different contexts such as exposure time, shapes, concentrations and sizes, based on EC_{50} (Effect Concentration of 50% of the organism), LC_{50} (Lethal Concentration of 50% the organism), LOEC (Lowest Observed Effect Concentration), HONEC (Highest Observed No Effect Concentration); 2) Calculate the RQ (Risk Quotient) and PNEC values based on the SSD and HC_5 analysis, in order to be able to mark out the dimension of the impact of MPs in surface freshwaters.

MATERIALS AND METHODS

Distribution and abundance of MPs

The bibliographic review was carried out with data regarding MPs distributed on freshwaters. The keywords used were microplastics, freshwater, distribution, abundance, ecotoxicological test, LC_{50}, EC_{50}, LOEC, NOEC, in Scopus, Web of Science, Google Scholar, and PubMed databases from January 1st of 2015 to October 1st of 2022. Studies that did not present statistical data were excluded. Considering that the concentrations obtained in the literature were expressed in different units (particle L^{-1}, µg L^{-1}, mg L^{-1}), all data obtained were converted to mg L^{-1}, using the method by Connors et al. (2017). In cases where the study did not report the density of the micro-polymer used, densities for conversion of particles to mg were obtained from Ji-Horng Plastic Co., Ltd.

Firstly, the abundance and distribution of different MPs in the world were analyzed by considering type, concentration, size, and shape. Data were separated by continents: Africa, Asia, Europe, America, and Oceania. For the unification of units (particles. L^{-1}) was followed the methodology of Yang et al. (2022), especially those with a value of particles per surface unit (ex: particles. Km^{-2}), and the value of the trawl net opening used in the sampling of MPs was considered. Data from estuarine ecosystems and from sediments were disregarded since the focus of this review is on freshwater ecosystems. The average in some studies was not available, because of the several sampling points within the entire watershed, due to the great variation in the abundance of the different sectors.

Ecotoxicological data analysis

Similarly, for ecotoxicological data, the same characteristics (type, concentration, size, and shape) were related. Then, NOEC values were estimated, because usually this value is not always obtained in ecotoxicological studies, but it can be rated using the HONEC, LOEC, LC_{50}, or EC_{50} value. Each of them has its own weighting factor (AF), as shown in Table 1 proposed by Adam et al. (2018) and adapted following the protocol of the European Chemicals Agency (2008).

Table 1. Assessment factors to obtain NOEC values, according to Adam et al. (2018).

Type of dose descriptor	AF Value
LC25-50	10
EC25-50	
LC10-20	2
EC10-20	
LOEC	
HONEC	1

AF: Assessment Factor

In some studies that presented two types of ecotoxicological tests: acute and chronic, NOEC was preferably calculated from the chronic data. In this case, if the study presented LOEC and HONEC values, the last was used, because, according to Table 1, the AF value is smaller.

In conclusion, all the values that were obtained from more restrictive dose descriptors, were used to achieve the objective of the most realistic PNEC. This also corroborates the method used by Jung et al. (2020), who selected the EC_{10} or LOEC value to elaborate the SSD whenever several toxicity values were available for a species.

Subsequently, the protocol of the European Chemicals Agency (2008) was followed to estimate the PNEC values for MPs from the SSD curves. It was made with NOEC values of different species, using the following endpoints: growth, reproduction, and mortality considered ecologically relevant biomarkers (OECD, 2011). In the present review, the ETX 2.0 program was used to plot the SSD curve. The distribution curves were statistically validated by the Anderson-Darling fit test. This test can be used as a criterion to prove whether a normal distribution rightly describes a comprehensive data set (NIST, 2012). As a result, 4 SSD curves were obtained: 2 representing two different types of polymers (PS and PP) since the number of data allow to elaborate these curves; and the remaining 2, delineate the two units (mg. L^{-1}, particles. L^{-1}) derive from the combination of all the types, shapes, and size of MPs. The HC_5 value was determined in the same program, and then the PNEC value was calculated by dividing the HC_5 by a weighting factor of 5 (ECHA, 2008).

Risk Assessment

The risk quotient (RQ) was obtained according to equation 1, considering the concentrations of MPs measured in freshwater environments or MEC (measured environmental concentrations) and the obtained PNEC. The MEC concentration was obtained through a compilation of information in the literature from the year 2015 on all the sources of information described above.

$$RQ = MEC / PNEC \quad (1)$$

According to Nika et al. (2020), an RQ<1 indicates a low potential risk to the aquatic environment, and RQ≥1 indicates a possible ecological threat, being the same criterion considered for the present work.

RESULTS AND DISCUSSION

Microplastics in freshwater bodies: emphasis on type, concentration, and form.

Firstly, we analyzed the number of studies regarding MPs toxicity published throughout the years, and the number of studies per continent (Fig 1). Figure 1A shows the number of publications found on MPs in water samples from continental water bodies according to their year of publication, divided by continent.

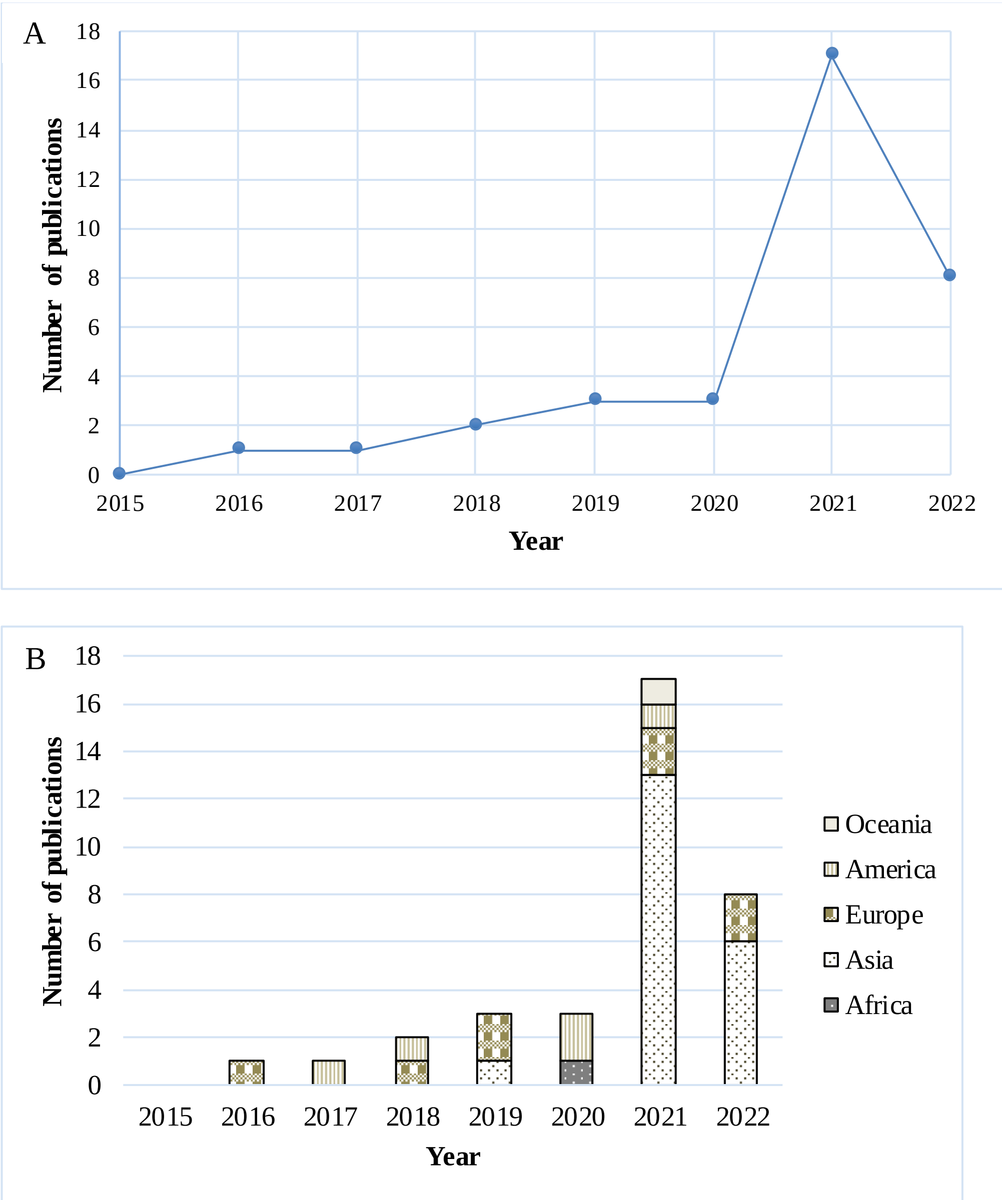


Figure 1. (A) Number of publications of microplastics studies in freshwater ecosystems, considering just water samples, and (B) number of publications per zone.

Figure 1A shows a tendency to study the abundance of MPs. For example, 2021 was a highly productive year for work on MPs in freshwater ecosystems, in total 17 articles were found that contained information on MPs in water samples around the world. The year 2022 also has a good amount of data, which is certainly lower, but it must be considered that it was only searched until October 1st, 2022, and possibly will be published later or in the remaining months of 2022. In the case of 2020, it was a year in which the number of publications decreased, possibly due to the COVID-19 impacts, as also reported in the study by Yang et al. (2022).

Figure 1B, shows the continents and the articles produced each year since 2016. The Asian region, with 17 publications in total, the majority (13) being published in the year 2021, followed by Europe, the only continent that published an article in 2016 and with 8 publications in total. America is the third continent with 5 works distributed in North America and South America. Central America still does not contain publications on MPs in freshwater. Finally, Africa and Oceania only contain one publication each, being the continents with less information on the abundance of MPs in freshwater samples.

Table 2 includes data collected from 2015 to 2022 on the abundance of MPs concentrations in freshwater bodies around the world, including samples of surface water and sediments, as well as the most common types of MPs, formats, and granulometry identified in these studies. Data were separated by continents: Africa, Asia, Europe, America, and Oceania. In some cases, the number of registered MPs presents both the abundance of data and its average, but it wasn't always the case. The average in some studies was not available, because of the several sampling points within the entire watershed, which made it difficult to obtain average values for the authors, due to the great variation in the abundance of the different sectors.

In an effort to visually comprehend the current state of microplastic pollution in freshwater bodies, a global distribution map depicting the maximum concentrations of microplastics (MPs) was generated using the aggregated data (Table 1). This map, represented as Figure 2, provides an insightful comparison across various geographic zones, highlighting the regions that currently exhibit the highest levels of microplastic contamination. By presenting this information spatially, the map contributes to a clearer understanding of the global distribution patterns of microplastics, facilitating informed assessments of the areas most affected by this ecological concern.

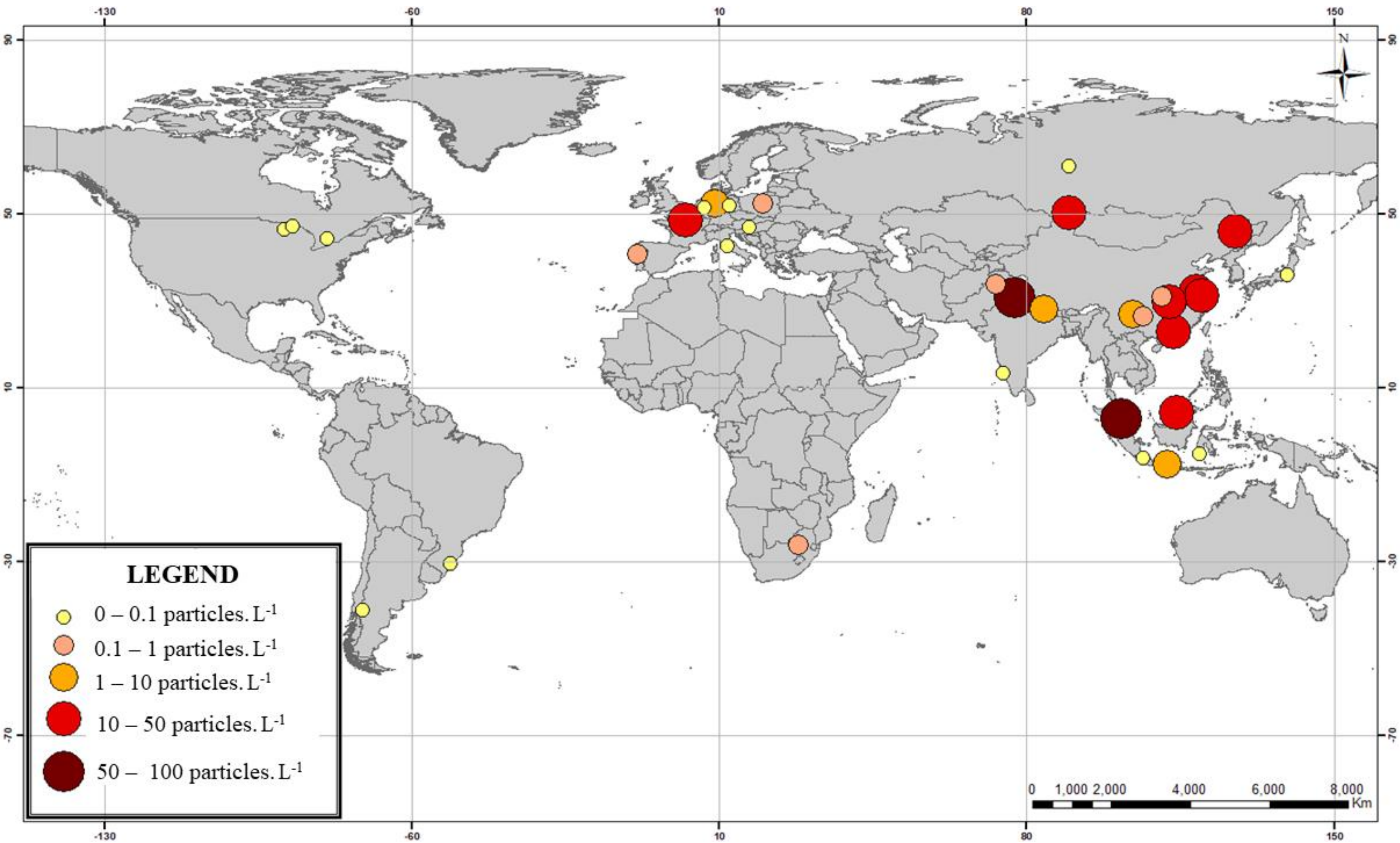


Figure 2. Worldwide distribution of peak concentrations of microplastics found in freshwater ecosystems

Table 2. Abundance, distribution, size, and shape of different types of microplastics found in water samples around the world.

Zone	Ubication	Type	Shape	Tamanho (µm)	Mean Concentrations (particles. L^{-1})	Range of concentration (particles. L^{-1})	Autor
Africa	Braamfontein Spruit, South Africa	Unknown	Fragments, fibers, beads, angular	<5000	0.705	0.160 to 2.080	Dahms et al., 2020
Asia	Songhua River, China	PE, PET, PS	Fibers, fragments	<500	5.720 ± 4.020	1.090 to 15.970	Tang et al., 2021
	Baram River, Borneo Island	PE, PET, silicon polymer, nitrile, PS	Fragments, fibers, films, pellets, and foams	300–1000	Unknown	9.300 to 18.000	Choong et al., 2021
	Renuka Lake, India	PE, PS, PP	Fibers, fragments, foams, films, beads	<200	21.000 ± 13.000	2.000 to 64.000	Ajay et al., 2021
	Tallo River, Indonesia	PE, PP, Poly(styrene:butadiene), PES, Rayon	Fragments, fibers, films, pellets	<450	Unknown	0 to 0.003	Wicaksono et al., 2021
	Taihu Basin (lakes), China	PVC, PE, PS, PP	Fragments, fibers, film, pellets	100–200	5.990	1.700 to 8.500	Zhang, et al., 2021
	Taihu Basin (river), China	PVC, PE, PS, PP	Fragments, fibers, film, pellets	<100	4.870	1.800 to 18.200	Zhang, et al., 2021
	Brantas River, Indonesia	PE, PVC, PC, PA	Fragment, fiber, film, pellets	N.D.	Unknown	0.133 to 5.467	Buwono et al., 2021
	Yenisei River, Russia	Unknown	Fibers, fragments, films	300 - 1000	Unknown	0.001 to 0.005	Frank et al., 2021
	Pearl River, China	POE, PET, PUA	Fragments, fibers	10–100	8.140 ± 4.530	4.000 to 16.330	Mai et al., 2021
	Siberia Lakes, Russia	Unknown	Films, fragments, fibers, foam,and pellets.	0.01-0.96	11.000 ± 7.000	4.000 to 26.000	Malygina et al., 2021
	Qinhuai river, China	PP, PE	Fragments, fibers	100 - 500	Unknown	1.467–20.567	Yan et al., 2021
	Yangtze River Basin, China	PP, PE, PS, PET, PES	Fibers,fragments, film, pellet, others	20 - 333	2.343	0 - 15.600	Zhang et al., 2021

	Arakawa River, Japan	PE, PP, PS	Fragment	500 - 5000	1.8 *10-3	0 to 0.0047	Sankoda & Yamada, 2021, 2021
	Langat River, Malaysia,	PET, HDPE, LDPE, EV, PVC, PS	Fibers,fragments, film, beads	<53	45.860 ± 24.760	4.390 to 90.000	Chen et al., 2021
	Xijin Wetland Park, China	Rayon, PES, PE, CS, PP, LDPE, HP, LDSBS, AC, PPCO, CP, PS, N-viny pyrrolione/vine/vinyl acetate copolymer, HDPE, PAM, PEU, SAN, PAA, PH	Fiber, film, chip, pellet, and foam	50 - 4000	0.431 ± 0.015	0.150 - 1.067	Yuan et al., 2022
	Lake Phewa, Nepal	Unknown	Fibers, flms, fragments, foam	200-5000	2.240 ±1.020	0.400 to 8.000	Malla-Pradhan et al., 2022
	Rawal Lake, Pakistan	PE, PP	Film, fiber, foam and granules	45 - 5000	Unknown	0.700 to 2.300	Bashir and Hashmi, 2022
	Caohai Lake, China	Unknown	Fibers, pellets, fragments and films	0 - 5000	Unknown	4.600 to 10.100	Zhang et al., 2022
	Lake Manipal, India	PET, CS	Fibers, films, fragments, pellets	300 - 5000	0.423 ± 0.250	0.136 to 0.936	Warrier et al., 2022
	Cisadane River, Indonesia	PE, PS, PP, PU, PET, CS, nylon, PA, PES, polybutadiene	Fragment, foam, fiber, and granule	<300 - >1000	0.0447 ± 0.024	0.013 to 0.113	Sulistyowati et al., 2022
	Yangtze River Basin, China	PP, PE, and polycarbonate (PC)	Fibers, fragments	<5000	0.780 ± 0.429	0.240 to 1.800	Li et al., 2019
Europe	Elbe River, Germany	PE, PP	Fibers, fragments, beads, foils	150–5000	0.006	0 to 0.013	Scherer et al., 2022
	Carpathian basin, Hungary	PE, PP, PS, PTFE, PAC, PES	Unknown	<300	0.014 ± 0.009	0.004 to 0.032	Bordós et al., 2019
	Lake Bolsena and Lake Chiusi, Italy	Unknown	Fragments, fibers	500 - 1000	0.003	8.2×10^{-4} to 4.42×10^{-3}	Fischer et al., 2016
	Seine River, Paris	PP, PE, PES	Fibers	1960 - 5410	2.600	1.900 - 5.500	Treilles, et al., 2022

	Seine River, Paris	PP, PE, PES	Fragments	115 - 248	15.500	9.300 - 26.500	Treilles, et al., 2022
	the Weser River, Germany	(acrylates/PUR/varnish), PP	Fragments, fibers foam, pellet	11–500	Unknown	0.00023 to 9.7	Roscher et al., 2021
	Vistula River, poland	PP, PS, PE, PA	Fibers, beads, fragments	50 - 5000	2.020 ± 0.280	1.60 to 2.550	Sekudewicz et al., 2021
	Rhine River, Germany	PS	Beads	286 - 954	Unknown	3×10^{-5} to 9.2×10^{-3}	Mani et al., 2019
	Antuã River, Portugal	PE, PP, PS, PET, PVA, EVA, PTFE, PMMA, PEA, SBR, CS	Fragments, pellets, films, foam and fibers	55 - 5000	Unknown	0.058–1.265	Rodrigues et al., 2018
North America	Lake Superior, US	PVC, PP, PE, PET, CPE, PS, PDMS.	Fibers, fragments, films, Beads, foams	<333	2.64×10^{-4}	0 - 7.85×10^{-4}	Hendrickson et al., 2018
	Lake Otawa, Canada	Unknown	Fibers, fragments, beads	<100	0.100	0.050 - 0.240	Vermaire et al., 2017
	Lake Simcoe in Ontario, Canada	PU, PES	Fibers, fragments and foams	<125	0.250 ± 0.220	0 to 0.710	Felismino et al., 2021
South America	Patagonia lakes, Argentina	PET, PU, PP, PS	Fiber, film, fragment, foam, rubber, and pellets	200 - 400	9×10^{-4}	3×10^{-4} to 1.9×10^{-4}	Alfonso et al., 2020
	Lake Guaíba, Brazil	PP, PE, PTFE, PA, PU, PS	Fragment, fibre, bead	100–250	0.037	0.012 to 0.061	Bertoldi et al., 2020
Oceania	Onkaparinga River Australia	iPP, HDPE and PE, PA	Unknown	<25	1.69×10^{-5}	Unknown	Hayes et al., 2021

The different types of microplastics have been abbreviated as follows: Polystyrene (PS), Polyethylene (PE), Polyurethane Acrylate (PUA), Polyurethane (PU), Isotactic polypropylene (iPP) Polyvinyl chloride (PVC), Polyester (PES), Polyurethanes (PUR), Polyethylene Terephthalate (PET), Polyamide (PA), Polypropylene (PP), polyacrylonitrile (PAN), Polyolefin elastomer (POE), ethylene-vinyl acetate (EV), polycarbonate (PC), polytetrafluoroethylene(PTFE), polyacrylate (PAC), polyvinyl acetate (PVA), ethylene-vinyl acetate (EVA), polymethylmethacrylate (PMMA), poly(ethylacrylate) (PEA), styrene butadiene rubber (SBR), cellulose (CS), expanded polystyrene (EPS), polypropylene homopolymer (HP), plastic wrap, low density poly(styrene-block-butadiene-block-styrene) (LDSBS), acrylic (AC), polypropylene copolymer (PPCO), polypropylene-polyethylene copolymer (CP), polystyrene (PS), N-viny pyrrolione/vine/vinyl acetate copolymer, high density polyethylene (HDPE), polyacrylamide (PAM), polyetherurethane (PEU), poly (styrene:acrylonitrile) (SAN), plastic bag, poly (acrylic acid) (PAA), polyethylene homopolymer (PH), ethylene-vinyl acetate (EV)

Overall, and visually expressed in Figure 2, the highest values were found in Malaysia, with a maximum value of 90 particles. L^{-1} in the Langat River (Chen et al., 2021) and Renuka Lagoon, India with a maximum value of 64 particles. L^{-1} (Ajay et al., 2021).

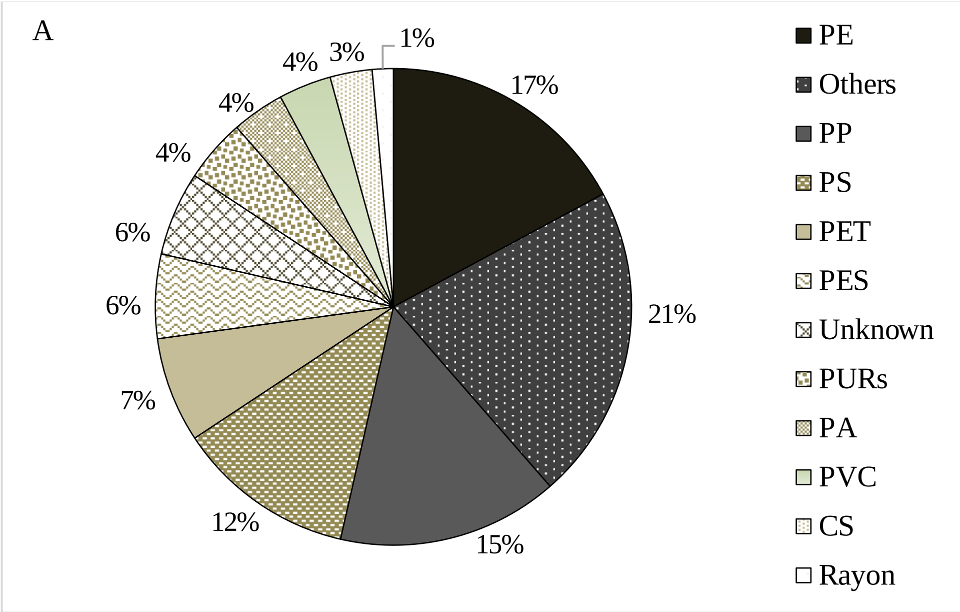
The concentrations or range of MPs found in the surface waters of the world are highly variable in each studied area. The continent with the most data on the occurrence and abundance of MPs is Asia, with China standing out as the country with the most data, with studies from the same basins being found, but by different authors such as Zhang et al. (2021) (0 – 15.6 particles. L^{-1}) and Li et al. (2019) (0.24 - 1.8 particles. L^{-1}) who studied the Yangtze River Basin in different years, having the possibility to compare those values.

On the American continent, the north zone has only one more work than South America, as well as the amount recorded in terms of surface water with a maximum value of 0,710 particles. L^{-1} from Lake Simcoe in Canada (Felismino et al., 2021). In the southern part, the maximum value was found in the Guaiba lagoon, Brazil, with 0.061 particles. L^{-1} (Bertoldi et al., 2020).

On the European continent, the highest value for surface water was recorded in the Seine River, Francia, with 26,500 particles. L^{-1} (Treilles, et al., 2022).

In Africa and Oceania, only one study was registered for surface waters. In the first case with an abundance of 0.16 particles. L^{-1} up to 2.08 particles. L^{-1} in the Braamfontein Spruit, South Africa (Dahms et al., 2020). In the second, it recorded only an average in the Onkaparinga River, Australia, with a value of 1.69×10^{-5} particles. L^{-1} (Hayes et al., 2021).

For a better analysis of the types and formats of MPs found in aquatic ecosystems around the world, figures 3A and 3B were created, considering the data presented in table 2.



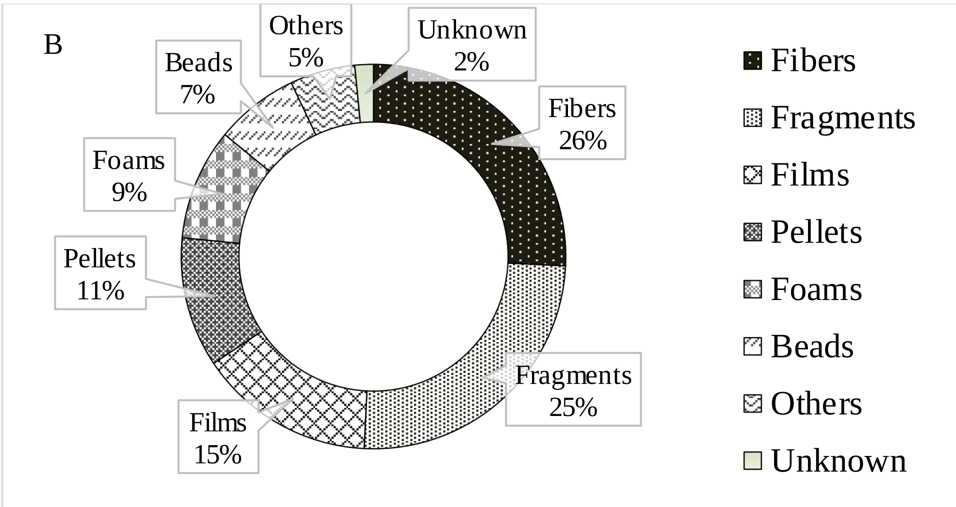


Figure 3. Percentage of types (A) and formats (B) of microplastics in freshwater ecosystems worldwide.

The most common types of MPs found in the world were: PE, PS, PP, and PET, which is in line with what was recorded in other review studies on the abundance of MPs in the world as stated by Li et al. (2020), a review study in which it was determined that PP, PE, PS, and PET correspond to 75% of the total amount of MPs in freshwater ecosystems. Speaking specifically of the most abundant type, PE, is one of the most used plastics in the world, being present in cosmetics, clothes, medicines, paints, medical devices, and electronics, among others, having a low recycling rate. (Kik et al., 2020).

Something to consider is that although the category “Others” was represented by MPs that were registered only once, it was the category that represented most of the total, with an abundance of 21%. In this group, it can be found isolated propylene (iPP), polyacrylonitrile (PAN), polyolefin elastomer (POE), ethylene vinyl acetate (EV), polyacrylate (PAC), polyvinyl acetate (PVA), ethylene vinyl acetate (EVA), polymethylmethacrylate (PMMA), poly(ethylacrylate) (PEA), styrene-butadiene rubber (SBR), cellulose (CS), expanded polystyrene (EPS), polypropylene homopolymer (HP), plastic film, low-density poly(styrene-block-butadiene-block-styrene) (LDSBS), acrylic (AC), polypropylene copolymer (PPCO), polypropylene-polyethylene copolymer (CP), polystyrene (PS), N-vinylpyrrolidone/vine/vinyl acetate copolymer, high-density polyethylene (HDPE), polyacrylamide (PAM), polyurethane (PEU), poly(styrene: acrylonitrile) (SAN), plastic bag, poly(acrylic acid) (PAA), polyethylene homopolymer (PH), ethylene vinyl acetate (EV). These MPs tend to be rare or used infrequently. However, in some cases these types of micropolymers tend to be the most abundant in the studied aquatic ecosystem, for example, the iPP, described in the study by Hayes et al. (2021), was the most abundant, and therefore recommend considering these polymers for future ecotoxicological studies, aiming at the protection of aquatic life.

Another group that is an uncommon type of microplastic, but which also shares considerable percentages, according to what is described in the studies, is that of Polyurethanes (PURs), registered, for example, by Roscher et al., (2021), Mai et al., (2021) and Alfonso et al., (2020), indicating that they should be studied considering impacts on biota.

According to Figure 3B, the most frequent type of shape are fibers (26%) followed by fragments (25%), films (15%), pellets (11%), while other groups with less occurrence are foams (9%) and spheres (7%), followed by other shapes (5%) that could not be identified in the previous groups, such as Dahms et al., 2020, who recorded angular shapes, and Zhang et al., 2021, who recorded other shapes, but do not specify which ones.

Fiber-shape MPs have typically been produced during the manufacture of synthetic clothing, ropes, fishing lines, and other textile products. (Choong et al., 2021). Its synthetic origin is attributed to synthetic fibers made from polymers of petrochemical origin such as PES, PA, PP, and those derived by chemical modification of natural polymers such as cellulose acetate (Zhang et al., 2021).

Foam and films, in terms of their origin, are often derived from sources such as clothing trimmings, plastic bags, food packaging, and synthetic textile materials (Sang et al., 2021). Another noteworthy point is that microplastics taking the form of foam have predominantly originated from materials like polystyrene packaging boxes and floral foam plastics (Trestrail et al., 2020).

Ecotoxicological aspects

As of now, the comprehensive review has yielded a substantial corpus of research encompassing 76 studies. Within this wealth of information, a total of 103 distinct ecotoxicological data points pertaining to various types of MPs have been extracted. As can be seen in Figure 4, 9 groups of organisms were recorded: fish, crustaceans, algae, mollusks, insects, amphibians, annelids, platyhelminths, and cnidarians. The families most used in ecotoxicological studies are fish (43%) and crustaceans (26%), followed by mollusks (10%), algae (9%), and amphibians (4%). The groups of organisms with just one study are in the group of others (8%), within which only one work was found on the following group of organisms: insects, amphibians, annelids, and platyhelminths. This is probably owing to these animals are rarely used at the laboratory level for ecotoxicological tests, either because of the high maintenance cost, low representativeness, or indefinite life cycle. However, in the future, should be considered exploring more aquatic species from these groups, because as with more data, a better comparison can be made between them, as well as a better analysis in contemplation to have greater protection for the biota.

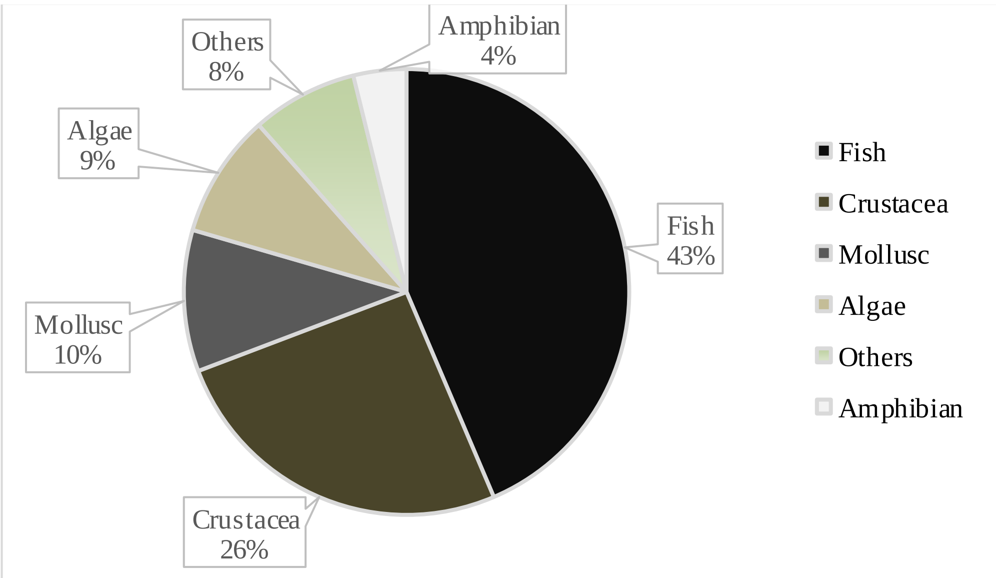


Figure 4. Percentage of groups of organisms used in ecotoxicological studies of Microplastics.

Within the specie analysis, it was found that inside the fish group, *Danio rerio* is the most used in all studies, as well as the microcrustacean *Daphnia magna*. In the case of *D. rerio*, this species is widely used in ecotoxicological studies due to its small size, ease of reproduction, short life cycle, and economic maintenance, but mainly in studies with MPs it presents greater advantages, especially when studying fluorescent MPs, as in the case of embryos and larvae, they are easier to observe as they are naturally transparent (Bhagat, 2020).

Considering the types of MPs used in the ecotoxicological tests, the following were found: Polystyrene (PS), Polyethylene (PE), Polyvinyl chloride (PVC), Polyester (PES), Polyethylene Terephthalate (PET), Polyamide (PA), Polypropylene (PP), mixing of different types of MPs (Mixture), MPs not specified in the studies (Unknown) and other unusual types (Others). As already mentioned, Li et al. (2020) report that polypropylene (PP), polyethylene (PE), polystyrene (PS), and polyethylene terephthalate (PET), represent almost 3/4 of the contamination, owing to the high production and use of these two types of plastic products, which also coincides with the data analysis of this research. However, this is not reflected in the same way in ecotoxicological studies (Figure 5). While PS and PE have garnered substantial attention with 36 and 30 data points respectively, the limited availability of data for PP and PET is striking, with just 5 and 2 data points respectively. This discrepancy in data distribution underscores the importance of aligning ecotoxicological studies with the ecological reality, where certain types of MPs are more abundant than others. To achieve a more comprehensive understanding of the potential effects of MPs on tested organisms, it's recommended that researchers prioritize the study of MPs that are more prevalent in natural environments.

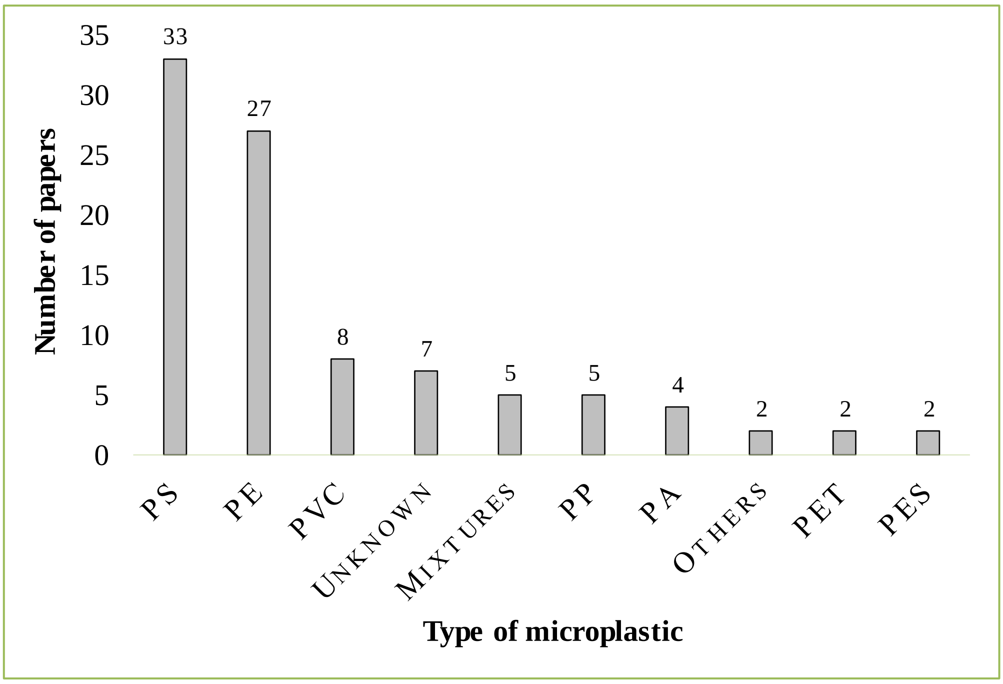


Figure 5. Types of microplastics found in ecotoxicological studies of surface water and numbers of related articles. The different types of PMs have been abbreviated as follows: Polystyrene (PS), Polyethylene (PE), Polyvinylchloride (PVC), Polypropylene (PP), Polyamide (PA), Polyester (PES), Polyethylene Terephthalate (PET).

As mentioned above, in a more realistic environment, MPs of all types are present in freshwater systems. This very reality underscores the significance of evaluating the toxicity of MP mixtures. Again, this is not reflected in the number of data obtained, as only 5 data on MPs mixtures were found.

In terms of mixture ecotoxicity, Limonta et al. (2021) explored the toxicity of PS+PE at a concentration of 50% of each, causing significant damage to the kidneys of *D. rerio* at a concentration of 0.1 and 1 mg. L^{-1}. In another study, was tested two types of MPs mixtures on *D. magna*, one with the most used and common MPs found in aquatic environments (PA+ PC+ PET+ PVC) and another with the less common ones (ABS + PVC, POM + SAN) (Imhof et al., 2017). However, the concentration used in this study (290 particles. mL^{-1} as the total value of the mixture of all MPs), based on high environmentally relevant concentrations, did not cause significant effects on growth, genetic and reproductive biomarkers (number of neonates).

Indeed, the limited availability of ecotoxicological data concerning MPs in mixtures can be attributed to their relatively recent recognition as environmental pollutants, and that is why the different effects and individual concentrations are still being discussed, and clearly, their combination has not been taken into account. However, because mixtures are an environmental reality they should be investigated in terms of exposure to biota. In this context, there are many aspects to be clarified, for example, if there is a difference when carrying out studies at the same concentration of every MP in the mixture, in relation to different concentrations of each one. Plus, if the mixtures are more or less toxic in relation to the isolated MPs, which mixtures are more toxic or which species are more sensitive to these mixtures, among other approaches? At the moment, these are questions that cannot be resolved and that should be the focus of future studies.

Another relevant fact to be discussed is that some of the surveyed studies did not record the type of microplastic, which difficulted the risk analysis (SSD, PNEC, and RQ) because they could not be included. It should be highlighted that the paucity of this information precludes knowing what type of microplastic is causing the reported effects in these studies, and at the same time grinds a creation of a solid database to discuss and compare the data with other studies.

In the current study, the classification of microplastic origin was executed through a clear set of criteria. When a study indicated that the microplastic was procured from a commercial entity, it was categorized as primary. On the other hand, if the microplastic was generated from the breakdown of larger plastic materials, it was classified as secondary. This categorization process aligns with the framework established by Rillig et al. (2012), ensuring consistency and comparability with existing literature and research practices. As a result of classification, 70 primary and 24 secondary MPs data were reported. This means that most of the tested MPs were acquired in laboratories, and few were prepared from larger (secondary) plastics, which does not demonstrate a realistic approach.

However, some primary MPs are found in daily-use products for personal care and beauty, such as toothpaste and sunscreens, but especially in most facial cleansers, which are known to contain polyethylene MPs (Fendall et al., 2019). Based on that, studies of primary MPs should be focused on these daily-use products as they represent a remarkable source of MPs' pollution. Our research identified one paper that carried out ecotoxicological tests considering this approach. Wang et al. (2022), tested PE MPs obtained from a beauty product on the mollusk *Biomphalaria glabrata*. The results do not record significant effects for survival and growth attributable to exposure to the environmentally relevant primary MP concentration but represent important new data to take into consideration.

Another relevant aspect that was considered in addition to the origin of MPs, was their shape, divided into 3 types: spherical, fragmented, fibrous, and granular. Figure 6 shows the amount of data collected according to the form and origin.

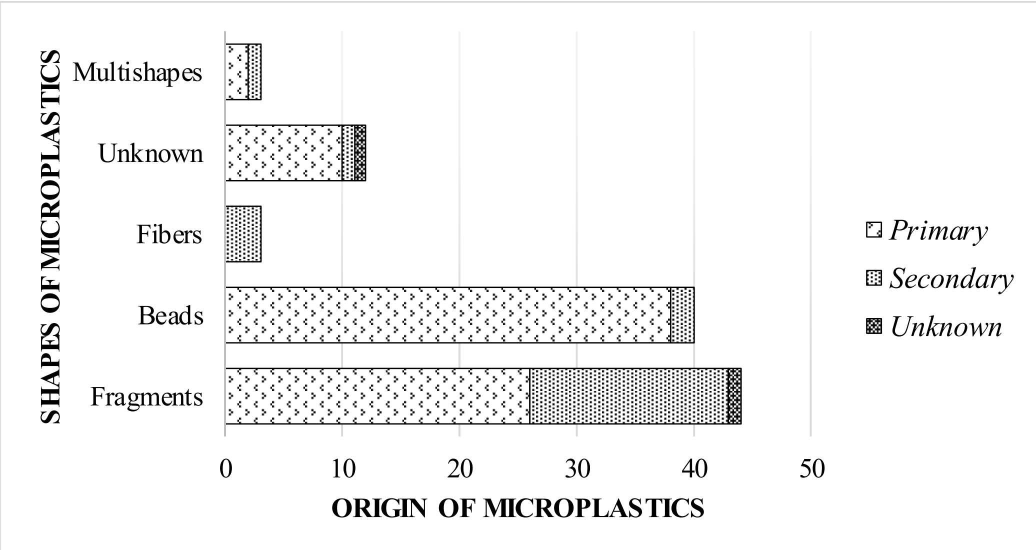


Figure 6. MPs shapes used in ecotoxicological studies, according to their origin.

Among the various shapes utilized for ecotoxicological tests, fragments and spheres emerged as the most prevalent, with 44 and 40 studies respectively. The popularity of using fragments is in line with their significant occurrence in nature, as illustrated by Figure 3B, which is why their wide use in ecotoxicological tests is representative. In relation to the origin, almost half of the tests were made with primary MPs, but it is important to encourage more studies with secondary. On the other hand, spheres draw attention, because when reviewing the data available in Figure 3B, this form only corresponds to 7% of that found in freshwater environments, but when testing this kind of MPs with organisms they are a high fraction of the total data. Fibers have been rarely addressed in ecotoxicological studies but should be encouraged as they are one of the most common forms in surface freshwater systems (Figure 3B). In a recent study, 70% of the MPs found ingested by zooplankton were microfibers (Bucci et al., 2020).

The essence of ecotoxicological tests lies in examining the relationship between concentrations of substances and their corresponding effects on the biota, with the overarching goal of assessing potential compromises to the organisms (Silva, 2017). In this comprehensive review, this crucial information was succinctly consolidated within Table 3. The data within this table was thoughtfully categorized based on the prevailing biomarkers that are commonly employed. The data delineates instances of chronic and/or acute exposure to various types of microplastics (MPs), shedding light on the intricate interactions and potential impacts on aquatic organisms. Furthermore, was calculated the NOEC from LOEC or EC_{50} / LC_{50} or LC_{10} values. Color of MPs was not taken into consideration because was not mentioned in many of the studies.

Table 3. NOEC values obtained from ecotoxicological studies of different MPs in species from different tropics, considering shape and size.

Class	Type of MP	Specie	Shape	Origin	Size mean or range (µm)	Type of test	Duration (days)	Concentration (mg/L)	Endpoint	Dose descriptor (D.D)	Value of DD (mg/L)	NOEC (mg/L)	Reference
Fish	PS	*Danio rerio*	Spherical	Primary	1	Chronic	5	0.1, 1	Behavior, reproduction, growth	LOEC	0.10	0.050	Qiang and Cheng, 2019
					1	Chronic	21	0.01, 0.1, 1	Biochemical and gonads histological analysis, gene expression	HONEC	0.01	0.010	Qiang and Cheng, 2021
					3–12	Chronic	21	10	Molecular, metabolomic, biochemical analyses.	EC 50	10.00	0.100	Dimitriadi et al., 2021
					0.10 - 0.12	Chronic	35	0.01, 0.1	Gene expression, histological changes	LOEC	0.01	0.005	Umamaheswari, Bhattacharjee et al., 2021
					0.05 - 0.5	Acute	3	0.1, 1, 10	Biochemical analysis, Growth, gene expression.	HONEC	0.10	0.100	Gu et al., 2020
					1	Chronic	21	10, 100, 1000	Growth, mortality, gene expression	HONEC	10.00	10.000	Qiang et al., 2020
					0.10–0.12	Chronic	35	10, 100	Histological analysis, Gene expression, Biochemical analysis	LOEC	10.00	5.000	Umamaheswari et al., 2021
			Fragments	Virgin	28.5-15.4	Acute	4	10.28, 102.8, 1028, 12.5, 25, 50, 100	Mortality, malformations, growth, heart rate	HONEC	10.28	10.280	Prata et al., 2022
				Secondary	35.8-15.1	Acute	4	10.28, 102.8, 1028, 12.5, 25, 50, 100	Mortality, malformations, growth, heart rate	HONEC	102.80	102.800	Prata et al., 2022
				Secondary	150	Acute	5	0.075, 75	Biochemical and cytotoxic analysis.	LOEC	0.08	0.038	Guimarães et al. 2020
		Gobiocypris rarus	Spherical	Primary	1.00	Chronic	28	0.2	Gene expression, biochemical analysis	LOEC	0.20	0.100	Wang et al., 2021
		Oreochromis niloticus	Spherical	Primary	0.1	Chronic	14	1, 10, 100	Biochemical and metabolomic response	LOEC	1.00	0.500	Ding et al., 2018
		Oryzias latipes	Spherical	Primary	2	Chronic	21d	0.044	Survival, growth, reproduction	HONEC	0.04	0.044	Assas et al., 2020
	PE	*Danio rerio*	Spherical	Primary	10–600	Acute	4	0.1, 1,10	Behavior, gene expression	HONEC	0.10	0.100	Mak, et al.,2019

				4	Chronic	7	0.01, 0.1, 1	Gene expression, biochemical and metabolomic analysis	HONEC	0.01	0.010	Zhao et al., 2021
				10–45	Chronic	10	20	Gene analysis, microbiome composition	LOEC	20.00	10.000	Kurchaba et al., 2020
				45–53	Chronic	10	58	Biochemical analysis, gene expression	LOEC	58.00	29.000	Xue et al., 2021
		Fragments	Primary	38.26± 15.64	Acute	6	6.2, 12.5, 25, 50 100	Neurotoxicity, cardiotoxicity, teratogenic effects, and external morphotoxicity	HONEC	6.20	6.200	Malafaia et al., 2020
		Unknown	Primary	10-45	Chronic	14	5, 20	Survival, growth, reproduction, metabolism and gene analysis	LOEC	5.00	2.500	LeMoine et al., 2018
	Carassius carassius	Fragments	Secondary	<848.37	Chronic	30	6.38, 12.18, and 22.33	Growth, liver damage, and gut microbiome	LOEC	6.38	3.190	Hu et al., 2022
	Clarias gariepinus	Fragments	Primary	50 - 500	Acute	4	500, 1000, 1500, and 2000	Survival, respiratory rate, and behavior	HONEC	1500.00	1500.000	Tongo and Erhunmwunse, 2022
	Cyprinus carpio	Multishapes	Primary	8–12	Chronic	60	1.5, 4.5	Biochemical, Metabolomic, Histopathological, gene analysis, behavior	LOEC	1.50	0.750	Chen et al., 2022
	Pimephales promelas	Spherical	Primary	425–500	Chronic	30	10, 20	Growth	HONEC	20.000	20.000	Malinich et al., 2018
Unknown	*Danio rerio*	Spherical	Primary	1--5	Chronic	14	2	Mortality and biochemicals analysis	LOEC	2.00	1.000	Santos et al., 2021
				175	Chronic	5	20	Immune toxicity, biochemical and metabolomic analysis	LOEC	20.00	10.000	Qin et al., 2021
	Oreochromis niloticus	Fragments	Primary	0.10	Chronic	15	1, 10, 100	Biochemical and gene analysis	LOEC	1.00	0.500	Hamed et al. 2020
				> 0.1	Chronic	15	1, 10, 100	Biochemical and hematological parameters	LOEC	1.00	0.500	Hamed et al. 2019
				< 0.1	Chronic	15	10	Hematological, biochemical parameters, hormonal levels, reproduction	LOEC	10.00	5.000	Ismail et al., 2021
PA	*Danio rerio*	Fragments	Primary	>180	Acute	2	30, 330	Growth, Morphological analysis	HONEC	330.00	330.000	Khosrovyan et al., 2020

					19.75 - 24.3	Chronic	10	1, 10, and 20	Growth, biochemical analysis	HONEC	1.00	1.000	Zhang et al., 2022
					6.37	Chronic	7	5, 20, and 50	Gene analysis, toxicity and immune analysis	HONEC	5.00	5.000	Zhang et al., 2020
	PP	*Danio rerio*	Fragments	Primary	115.8-50.2	Acute	4	12.5, 25, 50, 100,538.8,5388,53889	Mortality, malformations, growth, heart rate	HONEC	538.80	538.800	Prata et al., 2022
				Secondary	148.2-94.2	Acute	4	12.5, 25, 50, 100,538.8,5388,53889	Mortality, malformations, body length, heart rate	NOEC	12.50	12.500	Prata et al., 2022
	PVC	*Cyprinus carpio*	Spherical	Primary	100–200	Chronic	60	0.045, 0.091, 0.136	Growth, Biochemical, histological analysis	LOEC	0.05	0.023	Xia et al., 2020
		Carassius auratus	Fragments	Primary	0.1 - 1000	Chronic	21	0.1, 0.5	Gene expression	LOEC	0.10	0.050	Romano et al., 2020
	PS+PE	*Danio rerio*	Fragments	Primary	25 -90	Chronic	20	0.1, 1	Neurotoxicity, biochemical analysis, gene expression and histological analysis	LOEC	0.10	0.050	Limonta et al., 2021
Crustacea	PS	*Daphnia magna*	Spherical	Primary	1	Acute	2	0.1, 1, 10,100, 300, 400, 500, 600	Survival	LC50	87.83	8.783	Zhang et al., 2019
					10	Acute	3	0.1, 1, 10,100, 300, 400, 500, 600	Survival	LC50	291.69	29.169	Zhang et al., 2019
					1 --5	Chronic	21	0.05, 0.5, 5 and 50	Growth	HONEC	50.000	50.000	Bosker et al.,2019
					6 ± 1.0	Chronic And Acute	A:5/C:15	A: 5, 15, 30, 60, 120, 240, 300, C: 5, 30 and 100	Survival, growth, reproduction	HONEC	5.000	5.000	Eltemsah & Bøhn, 2019
					2	Chronic	21	1	Growth, biochemical and gen analysis	LOEC	1.00	0.500	Lee et al., 2021
					1, 10	Chronic	21	125, 1250, 12500	Behavior and reproduction	LOEC	125.00	62.500	De Felice et al., 2019
			Fragments	Secondary	<63	Chronic	21	0.0014, 0.03, 0.15	Survival, growth, and reproduction	LOEC	0.001	0.007	Schür et al., 2020
					≤63	Chronic	21	0.001, 0.005, 0.03, 0.15	Survival, reproduction, growth	HONEC	0.001	0.001	Schur et al., 2021
			Unknown	Secondary	<20	Chronic	19	101.6	Reproduction, growth	LOEC	101.60	50.800	Trotter, Wilde, Brehm et al., 2021

PE	*Daphnia magna*	Spherical	Primary	40-48	Acute	4	20; 40; 80; 160, 320	Mobility, growth	HONEC	320.00	320.000	Castro et al., 2020
			Secondary	37.24 ± 11.76	Acute	2	0-500	Biochemical analysis	EC50	323.00	32.300	Na et al., 2021
		Granules	Primary	39.8 ± 8.82,	Chronic	21	1, 10, 100	Survival, reproduction, biochemical analysis	HONEC	100.00	100.000	Heinlaan et al., 2021
			Secondary	205 ±144	Chronic	21	1, 10, 100	Survival, reproduction, biochemical analysis	HONEC	100.00	10.000	Heinlaan et al., 2021
		Fragments	Primary	37.05 ± 3.96	Acute	2	0-20	Oxidative stress, Bioconcentration analysis	EC50	3.900	0.390	Na et al., 2021
			Secondary	10–100	Acute	4	50	Mortality, immobilization	LOEC	50.000	25.000	Renzi et al, 2019
				1--5	Chronic	21	0.05, 0.5, 5 and 52	Reproductive effects	LOEC	0.050	0.025	Jaikumar et al., 2019
	Ceriodaphnia dubia	Spherical	Primary	1--4	Chronic And Acute	8	0.0625 - 2	Survival, growth, swimming behavior, reproduction	LOEC	0.063	0.031	Ziajahromi et al., 2017
		Fragments	Secondary	1--5	Chronic	7	0.05, 0.5, 5 and 55	Reproduction, growth	LOEC	0.0500	0.025	Jaikumar et al., 2019
	Daphnia pulex	Fragments	Secondary	1--5	Chronic	21	0.05, 0.5, 5 and 53	Reproduction, growth	LOEC	0.05	0.025	Jaikumar et al., 2019
	Hyalella azteca	Unknown	Primary	10--27	Acute	10	0-242	Mortality	LC50	115.000	11.500	Au et al.,2015
PVC	*Daphnia magna*	Unknown	Primary	2 ± 1	Chronic	21	2.05	Mortality, reproduction, gene analysis	LOEC	2.050	1.025	Liu et al., 2022
				50 ± 10	Chronic And Acute	21	4.97	Mortality, reproduction, genes analysis	LOEC	4.970	2.490	Liu et al., 2022
				4 - 276	Chronic	31	88585	Growth and hatching rate	LOEC	88585.000	44292.500	Laforsch et al., 2019
		Fragments	Secondary	10–100	Acute	4	50	Mortality, immobilization	LOEC	50.000	25.000	Renzi et al, 2019
Unknown	*Daphnia magna*	Spherical	Primary	1-5	Chronic	21	0.05, 0.5, 5, 50	Reproduction	LOEC	0.050	0.025	Jaikumar et al., 2019
	Daphnia pulex	Spherical	Primary	1-5	Chronic	21	0.05, 0.5, 5, 50	Reproduction	LOEC	0.050	0.025	Jaikumar et al., 2019
	Ceriodaphnia dubia	Spherical	Primary	1-5	Chronic	7	0.05, 0.5, 5, 50	Reproduction	LOEC	0.050	0.025	Jaikumar et al., 2019
PET	*Gammarus pulex*	Fragments	Secondary	<150	Chronic	48	0.001,0.01, 0.08, 0.8, 8	Survival, reproduction, biochemical analysis	HONEC	8.000	8.000	Weber et al., 2018

	PES	*Ceriodaphnia dubia*	Fibers	Secondary	280	Chronic And Acute	8	0.03125- 1	Survival, growth, behavior, reproduction, morphology	LOEC	0.032	0.016	Ziajahromi et al., 2017
	PP	*Daphnia magna*	Fragments	Secondary	10–100	Acute	4	50	Mortality, immobilization	LOEC	50.000	25.000	Renzi et al, 2019
	EEA copolymer	*Daphnia magna*	Spherical	Primary	0.103	Chronic	21	460, 4600	Survival, growth, reproduction, transcriptomic profile, gene expression analysis	HONEC	4600.000	4600.000	Coady et al., 2020
	PE+PES	*Ceriodaphnia dubia*	Spherical + fiber	Primary + secondary	1-280	Acute	2	0.23, 0.45, 0.92, 1.85, 3.7, 7.4	Survival	LC10	1.85	0.925	Ziajahromi et al., 2017
	PVC+PE	*Daphnia magna*	Fragments	Secondary	10–100	Acute	4	50	Mortality, immobilization	LOEC	50.00	25.000	Renzi et al, 2019
	PA+PC+ PET+ PVC	*Daphnia magna*	Fragments	Primary	~40		2	290 particles/mL**	Growth, reproduction, gene expression analysis	HONEC	290 particles/mL**	290 particles/mL**	Imhof et al., 2017
	ABS + PVC, POM + SAN	*Daphnia magna*	Fragments	Primary	~40	Chronic	2	290 particles/mL**	Growth, reproduction, gene expression analysis	NOEC	290 particles/mL**	290 particles/mL**	Imhof et al., 2017
Algae	PS	*Chlamydomonas reinhardtii*	Spherical	Secondary	93.6	Chronic	4	50, 250, 500	Growth, Biochemical analysis	LOEC	50.000	25.000	Yang et al., 2021
			Multishapes	Primary	0.3–0.6	Chronic	10	5, 25, 50, 100	Growth, photosynthetic activity	LOEC	5.000	2.500	Li et al, 2020
		Raphidocelis subcapitata	Fragments	Weathered	35.8-15.1	Acute	3	10.28, 102.8, 1028, 12.5, 25, 50, 100	Growth, toxin production	LOEC	10.280	5.140	Prata et al., 2022
				Virgin	28.5-15.4	Acute	3	10.28, 102.8, 1028, 12.5, 25, 50, 100	Growth, toxin production	LOEC	10.280	5.140	Prata et al., 2022
		Microcystis aeruginosa	Unknown	Primary	3.00	Acute	4	A: 10, 25, 50, 100, and 200	Growth, toxin production and biochemical analysis	EC 50	200.00	20.000	Mbedzi et al.,2020
		Euglena gracilis.	Spherical	Primary	5 - 0.1	Acute	4	0.5, 1, 10, 50	oxidative stress, genotoxicity	HONEC	0.500	0.500	Xiao et al, 2020
	PE	*Microcystis aeruginosa*	Spherical	Primary	212-300	Chronic	28	0.25, 0.5, 0.2	Growth, Photosynthetic activity	LOEC	0.250	0.125	Sanchez-Fortún et al., 2021
			Unknown	Primary	3.00	Acute	4	10, 25, 50, 100, and 200	growth, toxin production and oxidative stress	EC50	25.000	2.500	Mbedzi et al.,2020
		Scenedesmus armatus	Spherical	Primary	212-300	Chronic	28	0.25, 0.5, 0.1	Growth, Photosynthetic activity	LOEC	0.250	0.125	Sanchez-Fortún et al., 2021
	PP	*Raphidocelis subcapitata*	Fragments	Primary	115.8-50.2	Acute	3	12.5, 25, 50, 100, 538.8,5388,53889,	Growth, cytotoxicity	LOEC	12.500	6.250	Prata et al., 2022
				Secondary	148.2-94.2	Acute	3	12.5, 25, 50, 100, 538.8,5388,53889,	Growth, increased toxicity	LOEC	12.500	6.250	Prata et al., 2022

	PVC	*Microcystis aeruginosa*	Unknown	Primary	3.00	Acute	4	10, 25, 50, 100, and 200	Growth, biochemical analysis, cytotoxicity	EC50	50.000	5.000	Mbedzi et al.,2020
	PA	*Microcystis aeruginosa*	Fragments	Primary	1–3	Chronic	30	25, 50, and 100	Growth, gene and biochemical analysis, photosynthetic activity	LOEC	25.000	12.500	Zheng et al., 2022
Mollusc	PS	*Corbicula fluminea*	Spherical	Primary	4	Chronic	42	0.01, 0.10, 1, 10	Behavior and antioxidant analysis	HONEC	1.000	1.000	Fu et al., 2022
			Fragments	Primary	179	Chronic	28	3.2	Histopathology, gene expression, mortality	LOEC	3.200	1.600	Rochman et al., 2017
				Unknown	<200	Chronic	7	2	Biochemical and neurotoxicity analysis	LOEC	2.000	1.000	Parra et al., 2021
		Dreissena polymorpha	Fragments	Secondary	≤63	Chronic and Acute	A: 7/ C: 42	0.0007, 0.02, 0.48, 11.99	Mortality, metabolic and biochemical analysis	NOEC	0.480	0.480	Weber et al., 2021
	PE	*Corbicula fluminea*	Fragments	Primary	209	Chronic	28	2.8	Survival, behavior, gene expression, mortality	LOEC	2.800	1.400	Rochman et al., 2017
		Corbicula javanicus	Fragments	Secondary	1 - 500	Acute	1	0.01, 0.1, 1	Biochemical analysis	HONEC	0.010	0.010	Esterhuizen et al., 2023
		Biomphalaria glabrata	Fragments	Secondary	83 -789	Chronic	21	0.5	Survival and growth	HONEC	0.500	0.500	Wang et al., 2022
		Dreissena bugensis	Spherical	Primary	167.11	Acute	1	100, 400, 800	Survival, filtration rate and reproduction	LOEC	100.000	50.000	Pedersen et al., 2020
	PES	*Corbicula javanicus*	Fibers	Secondary	100–250	Acute	1	102.5	Biochemical analysis	LOEC	102.500	51.250	Esterhuizen et al., 2022
	PET	*Corbicula fluminea*	Fragments	Primary	198	Chronic	28	4.1	Histopathology, gene expression, mortality	LOEC	4.100	2.050	Rochman et al., 2017
	PVC	*Corbicula fluminea*	Fragments	Primary	169	Chronic	28	4.1	Histopathology, gene expression, mortality	LOEC	4.100	2.100	Rochman et al., 2017
Insect	PS	*Chironomus riparius*	Fragments	Primary	38.9 ± 28.6	Chronic	1.5	1, 10	Gene expression	LOEC	1.000	0.500	Carrasco-Navarro et al., 2021
	Tire rubber MPs	*Chironomus riparius*	Fragments	Secondary	82.3 ± 40	Chronic	1.5	1, 10	Gene expression	LOEC	1.000	0.500	Carrasco-Navarro et al., 2021
Amphibian	PS	*Xenopus laevis*	Unknown	Primary	3.00	Acute	1	0.000125, 0.00125, 0.0125	Growth, swimming activity.	HONEC	0.013	0.013	De Felice et al., 2018
	PE	*Physalaemus cuvieri*	Fragments	Primary	35.46 ± 18.17	Chronic	7	60	Histopathological analysis	LOEC	60.000	30.000	Araújo et al., 2020
	PES	Xenopus laevis	Fibers	Secondary	200-800	Acute	4	0.001,0.01,0.05	Malformations at the gut level	EC50	0.060	0.006	Bacchetta et al., 2021

Annelids	PE	*Allonais inaequalis*	Fragments	Primary	40 - 48	Chronic	10	20, 40, 80, 160, 320	Mortality, Reproduction,	HONEC	320.000	320.000	Castro et al., 2020
Platyhelminthe	PS	*Dugesia japonica*	Unknown	Unknown	1.00	Chronic	7	0.01	Biochemical analysis, gene expression	LOEC	0.010	0.005	Shang et al., 2022
Cnidarian	PE	*Hydra attenuata*	Fragments	Secondary	<400	Chronic	4	10, 20, 40, 80	Growth	HONEC	40.000	40.000	Murphy and Quinn, 2017
Macrophyte	PS	*Utricularia vulgaris*	Unknown	Primary	1-5	Acute	7	15, 70 and 140	Growth	HONEC	70.000	70.000	Yu et al, 2020
Rotifer	PE	*Brachionus plicalitis*	Spherical	Primary	10-22	Acute	1	500, 1000, 5000, 10000, 100000	Mortality	LC50	764.000	76.400	Berber and Yurtsever, 2018

The different types of MPs are abbreviated as follows: Polystyrene (PS), Polyethylene (PE), Polyvinyl chloride (PVC), Polypro pylene (PP), Polyamide (PA), Polyester (PES), Polyethylene Terephthalate (PET), Polycarbonate (PC)

Table 3 shows that particularly in the case of PS, all studies reviewed for this type used only this *D. magna* and *D. rerio*. Furthermore, it's worth noting that crustaceans exhibited a wider range of microplastic (MP) types and their various mixtures compared to other taxonomic groups. This diversity in exposure scenarios underlines the relevance of considering the specific sensitivities and responses of crustaceans in relation to different MP types and combinations. The broader array of MP types and mixtures encountered by crustaceans may contribute to variations in their ecological responses, making them a crucial focal point for understanding the multifaceted impacts of microplastics in aquatic ecosystems. From the algae group, *Microcystis aureginosa* stands out as the most used specie, as well as *Corbicula fluminea* from the mollusks. In the case of insects, only 2 studies were found with the species *Chironomus riparus*.

The type of test (chronic or acute) varied according to the specie, as well as the biomarker used. However, some studies do not present the most used biomarkers according to the type of test, for example, acute studies whose objective is not necessarily to find a LC_{50}/EC_{50}, but to see other biological responses, as Gu et al. (2020), who performed acute tests in *D. rerio* with PS. However, he did not find LC_{50}/EC_{50}, but analyzed biochemical biomarkers, morphology, and genetic analysis.

The NOEC values listed in Table 1 exhibit substantial variability across different taxonomic groups. This variability is influenced by a range of factors, including the type of microplastics (MPs), their shape, and granulometry.. Na et al., (2021) made a comparison of the same PE MPs but with different formats, a spherical one was primary and the other was obtained by processing the same MP, obtaining fragments, and being secondary. In the end, they concluded that the shape of the MP, influenced the toxicity of *D. magna*, since the EC_{50} of the PE fragments was 80 times more toxic than the PE in the spherical form, at the same concentration of 323 mg. L^{-1}. This result indicates that not just the shape may affect toxicity, but also the origin.

Considering the analysis of biomarkers and the ecotoxicological effects caused by MPs, it is observed in summary form in figure 7A, that the biomarkers were classified as follows: growth (20%), survival (16%), biochemical analysis (15%), gene expression analysis (13%), reproduction (12%), others (7%), behavior (7%), histological analysis (4%), metabolomic analysis (2%), heart analysis (2%), immune responses (1%), and blood analysis (1%). The “others” category includes teratogenic effects, microbiome analysis, and increased toxicity.

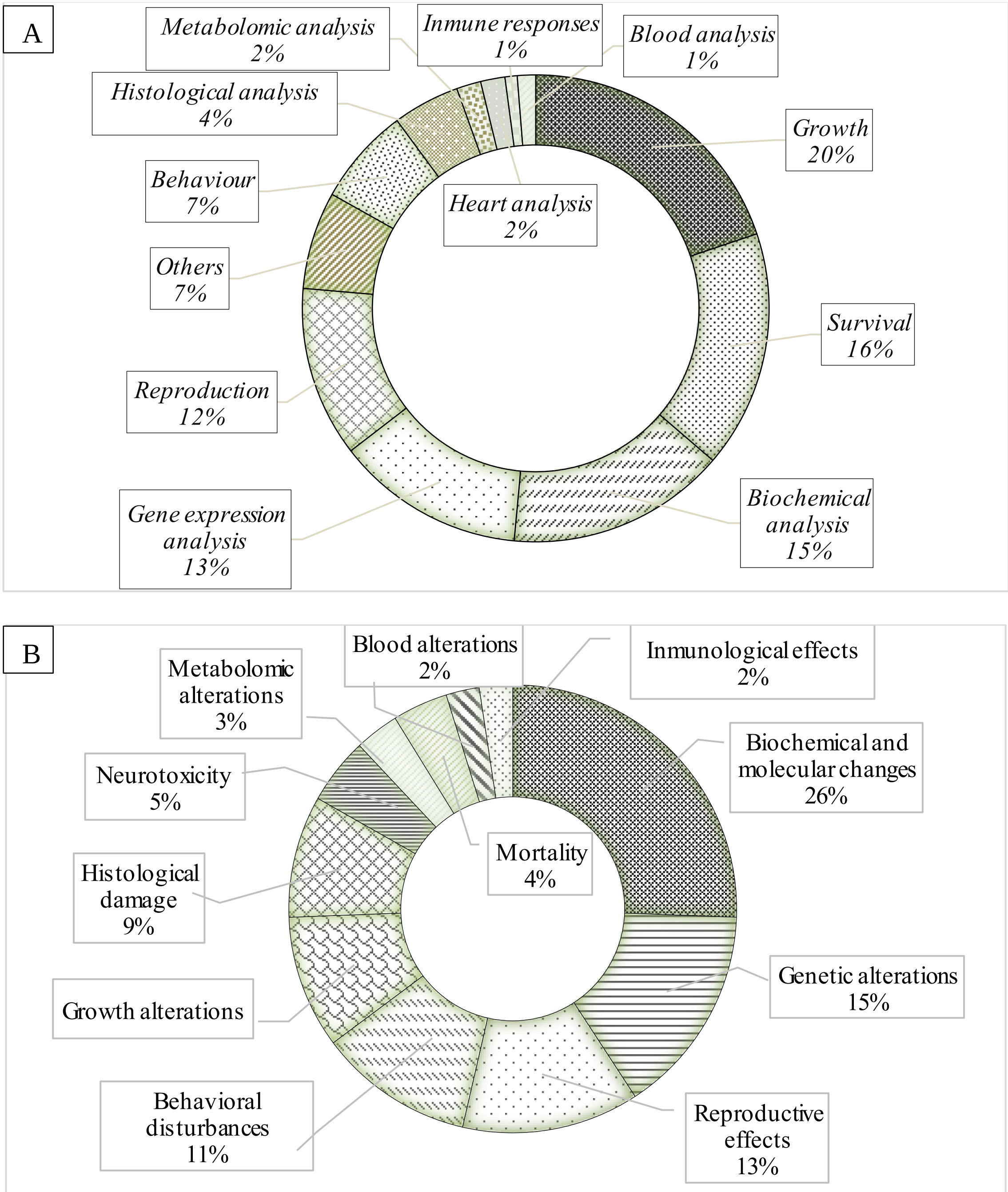


Figure 7. (A) Biomarkers analyzed in freshwater organisms exposed to MPs in laboratory experiments; (B) Ecotoxicological effects reported in freshwater organisms exposed to MPs in the laboratory

Figure 7. (A) Biomarkers analyzed in freshwater organisms exposed to MPs in laboratory experiments; (B) Ecotoxicological effects reported in freshwater organisms exposed to MPs in the laboratory

In the case of the effects found (Figure 7B), they were classified into the following categories: Biochemical and molecular changes (26%), Genetic alterations (19%), reproductive effects (14%), Behavioral disturbances (11%), growth alterations (10%), histological damage (9%), neurotoxicity (5%), mortality (4%), metabolomic alterations (3%), blood alterations (2%), immunological effects (2%).

The results obtained from the research highlight that a range of biomarkers and parameters have been extensively employed for evaluating the impact of microplastics (MPs) on aquatic organisms in freshwater environments. Notably, growth, survival, biochemical analysis, gene expression analysis, and reproduction are among the most frequently utilized biomarkers and metrics in these studies. These parameters serve as essential indicators to comprehend the potential effects of MPs on various aspects of the organisms' physiology, behavior, and overall health. Conversely, the observed effects encompass a broad array of outcomes. Predominantly, these include biochemical and molecular changes, genetic alterations, reproductive effects, behavioral disturbances, and growth alterations. These multifaceted impacts collectively underline the complexity and far-reaching consequences that MPs can impart on the delicate ecosystems of freshwater environments. By investigating and documenting these effects, researchers can gain a comprehensive understanding of the ecological implications and potential risks associated with the prevalence of microplastics in aquatic habitats.

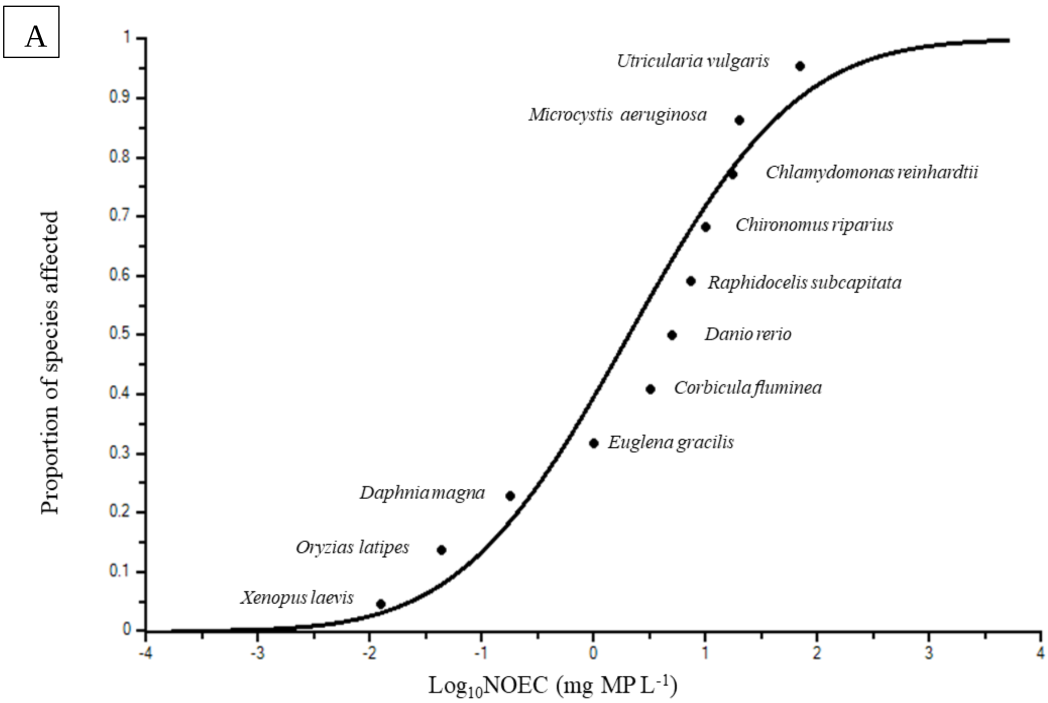
It can be observed that the most used biomarkers apparently demonstrate a relationship with their respective effects, such as biochemical analysis and biochemical and molecular changes, as well as genetic alterations. In contrast, it is also observed in Figure 7 that there are biomarkers that did not always generate effects, for example, the case of growth, which was the most used biomarker in most of the studies. However, according to Figure 7B, the same percentage of alterations in growth was not so significant. Such information does not mean that one specific biomarker should discard another, it is necessary, depending on the species and life cycle, to test all the possible biomarkers to understand the effects of MPs in a specific specie. For example, the use of the growth biomarker for algae proved to be fundamental to demonstrate the toxic effect, because it is necessary to estimate the EC_{50} (OECD, 2011).

Given the above, it is expected that, when ecotoxicological studies are developed, the type of biomarker reflects the effects caused by exposure to the pollutant. In this sense, exploring the different types of biomarkers for MPs studies is recommended, since the larger the database, the better and more careful the risk analysis will be to protect, in the future, the exposed biota. In this context, it would be important to enable the use of biomarkers that safely reflect the significant effects of exposure to this group of pollutants.

Risk Analysis

PNEC estimation

The data acquired through the literature review (referenced in Table 1) and detailed within the methodology section served as the foundation for constructing distinct Species Sensitivity Distributions (SSDs). These distributions were instrumental in identifying the most vulnerable species and subsequently estimating the Predicted No-Effect Concentration (PNEC) values. Figure 8 shows a comparison between two types of MPs (PS and PE), which were those with more data according to the literature and, therefore, due to the amount of data greater than 10, it was possible to perform the SSD.



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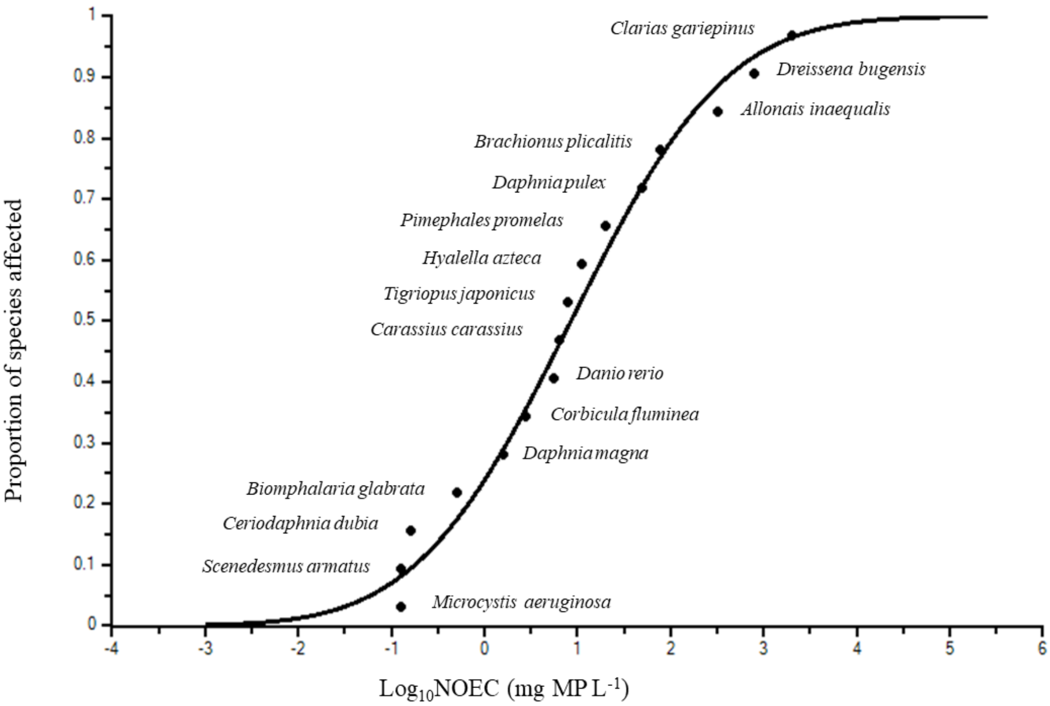

Figure 8. Species sensitivity distribution (SSD; black line) based on chronic no observed effect concentrations (NOECs) of all forms of microplastics for (A) Polystyrene (PS) and (B) Polyethylene (PE). Endpoints included: lethality, growth, and reproduction

As depicted in Figure 8A, PS SSD represents 11 NOEC values obtained from 19 data from ecotoxicological studies (Table 4), preferably considering values from chronic studies (HONEC, NOEC). The curve covers 5 taxonomic classes (fish, algae, rotifers, crustaceans, and amphibians) with 11 different species for different types of granulometry and formats of this type of MPs. In comparison, Figure 8B, the SSD of PE, also obtained with the same criteria as the PS SSD, shows 16 data of NOEC values obtained from data found in the literature for this type of microplastic (Table 5) different covering 6 taxonomic classes (fish, algae, rotifers, crustaceans, mollusks, and annelids).

Table 4. Estimated NOEC (mg. L^{-1}) of aquatic organisms exposed to PS microplastics used for SSD analysis, obtained from Table 3.

Taxonomical Group	Especie	NOEC (mg. L^{-1})	Referencia
Amphibian	*Xenopus laevis*	0.0125	De Felice et al., 2019
Fish	*Oryzias latipes*	0.044	Assas et al., 2020

Crustacean	*Daphnia magna**	0.42	Jaikumar et al.,2019, Eltemsah & Bøhn, 2019, De Felice et al., 2019, Schur et al., 2021
Euglenide	*Euglena gracilis.*	1	Xiao et al, 2020
Mollusc	*Corbicula fluminea*	3.2	Rochman et al., 2017
Fish	*Danio rerio**	5	Qiang and Cheng, 2019, Qiang et al., 2020, Prata et al., 2022
Crustacean	*Raphidocelis subcapitata*	7.27	Prata et al., 2022
Insect	*Chironomus riparius*	10	Carrasco-Navarro et al., 2021
Algae	*Chlamydomonas reinhardtii**	17.33	Yang et al., 2021, Li et al, 2020
Algae	*Microcystis aeruginosa*	20	Zheng et al.,2021
Macrophyte	*Utricularia vulgaris*	70	Yu et al, 2020

* The geometric mean was calculated for species with more than one data available in the literature.

Table 5. Estimated NOEC (mg. L^{-1}) of aquatic organisms exposed to PE microplastics used for SSD analysis, obtained from Table 3.

Grupo de Organismo	Especie	NOEC (mg. L^{-1})	Referencia
Algae	*Microcystis aeruginosa*	0.125	Sanchez-Fortún et al., 2021
Algae	*Scenedesmus armatus*	0.125	Sanchez-Fortún et al., 2021
Crustacean	*Ceriodaphnia dubia*	0.16*	Ziajahromi et al., 2017, Jaikumar et al., 2019
Mollusc	*Biomphalaria glabrata*	0.5	Wang et al., 2022
Crustacean	*Daphnia magna*	1.58*	Jemec Kokalj et al., 2021, Jaikumar et al., 2019
Mollusc	*Corbicula fluminea*	2.8	Li et al., 2017
Fish	*Danio rerio*	5.57*	Malafaia et al., 2020, LeMoine et al., 2018
Fish	*Carassius carassius*	6.38	Hu et al., 2022
Crustacean	*Tigriopus japonicus*	7.98	Yu et al., 2020
Crustacean	*Hyalella azteca*	11.15	Au et al.,2015
Fish	*Pimephales promelas*	20	Malinich et al., 2018

Crustacean	*Daphnia pulex*	50	Jaikumar et al., 2019
Rotifer	*Brachionus plicalitis*	76.4	Berber and Yurtsever, 2018
Annelid	*Allonais inaequalis*	320	Castro et al., 2020
Mollusc	*Dreissena bugensis*	800	Pedersen et al., 2020
Fish	*Clarias gariepinus*	2000	Tongo and Erhunmwunse, 2022

* The geometric mean was calculated for species with more than one data available in the literature.

According to the SSD curves (Fig. 8) constructed based on literature data, *Xenopulus laevis* was the most sensitive organism with a NOEC value of 0.0125 mg. L^{-1} (Table 6) for PS and *M. aureginosa* with the same NOEC value of 0.0125 mg. L^{-1} (Table 7) for PE. Apparently, the two types of MPs are similar in terms of possible safe values for freshwater species. However, the sensitivity of the same species to these contaminants varies, *M. aureginosa* does not have the same sensitivity for PS (20 mg. L^{-1}) as for PE (0.0125 mg. L^{-1}), being 160 times greater than that obtained in PE. Possibly, the difference is because in the first case the study by Zheng et al., (2021) an acute test was performed, with higher concentrations (10 -200 mg. L^{-1}) with this species, in comparison with the second case, in which Sanchez-Fortún et al., (2021), performed a 28-day chronic test and with lower concentrations (0.25, 0.5, 1 mg. L^{-1}).

Granulometry should be considered as well, because in the first case, for PS, they worked with a diameter of 3µm, and in the second (PE), with a range of 212 µm -300 µm. Therefore, it is important to carry out new studies comparing granulometry in different types of MPs and in different species to compare if there is a difference in toxicity. Another recommendation to consider is to carry out larger studies with the same species, to have more points of comparison since it was not possible to compare data from *X. laevis* with the two types of MPs since no ecotoxicological studies of this species were found.

For the other SSD (Figure 9), the initial idea was to compare acute data with chronic ecotoxicological data to determine whether the sensitivity of the species varies depending on the duration of the tests and the concentrations obtained from them. However, it was not possible because the curve did not pass the Anderson Darling test of normal logaritmization, due to a lack of EC_{50} and LC_{50} data. Therefore, a comparison of NOEC values for all types of MPs in both units, mg. L^{-1} and particles. L^{-1} was made instead (Figure 9A, 9B). Figures 9A and 9B show 23 NOEC values of different species obtained from 53 data obtained from the literature summarized in tables S5 and S6, covering 9 groups of organisms (fish, algae, rotifers, crustaceans, mollusks, insects, macrophytes, annelids, and amphibians) and 7 types of MPs (PE, PS, PVC, PA 6 and PES, PET and EEA copolymer).

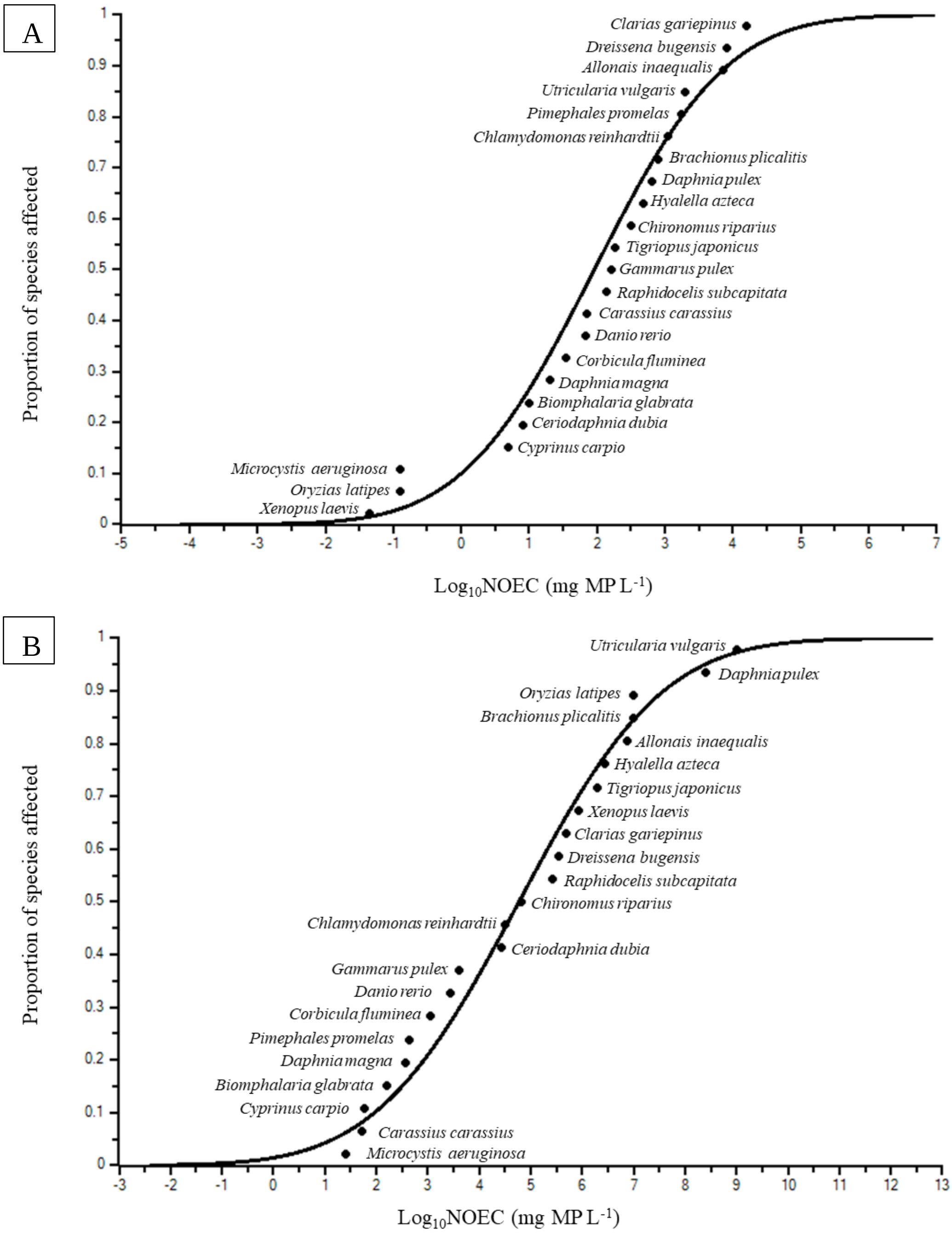

Figure 9. Species sensitivity distribution (SSD; black line) based on chronic no observed effect concentrations (NOECs) of all forms and types of microplastics, data expressed in (A) mg. L^{-1} and (B) particles. L^{-1}. Endpoints included lethality, growth, and reproduction.

Table 6. Estimated NOEC (mg. L^{-1}) of aquatic organisms exposed to microplastics used for SSD analysis, obtained from Table 3

Grup organism	Especie	NOEC (mg. L^{-1})	Referencia
Amphibian	*Xenopus laevis*	0.0125	De Felice et al., 2018
Fish	*Oryzias latipes*	0.044	Assas et al., 2020
Algae	*Microcystis aeruginosa*	0.125	Sanchez-Fortún et al., 2021
Fish	*Cyprinus carpio*	0.136	Xia et al., 2020
Crustacean	*Ceriodaphnia dubia**	0.1842	Ziajahromi et al., 2017 and Jaikumar et al., 2019
Mollusc	*Biomphalaria glabrata*	0.5	Wang et al., 2022
Crustacean	*Daphnia magna**	0.69	Bosker et al.,2019, Eltemsah & Bøhn, 2019, De Felice et al., 2019, Schur et al., 2021, Trotter et al., 2021, Jemec Kokalj et al., 2021, Jaikumar et al., 2019, Liu et al., 2022, Coady et al., 2020
Mollusc	*Corbicula fluminea*	3.52	Rochman et al., 2017
Fish	*Danio rerio**	4.741	Qiang and Cheng, 2019, Qiang et al., 2020, Prata et al., 2022, Malafaia et al., 2020, LeMoine et al., 2018, Santos et al., 2021, Zhang et al., 2022, Prata et al., 2022
Fish	*Carassius carassius*	6.38	Hu et al., 2022
Algae	*Raphidocelis subcapitata*	7.269	Prata et al., 2022
Crustacean	*Gammarus pulex*	8	Weber et al., 2018
Crustacean	*Tigriopus japonicus*	8.289	Yu et al., 2020
Insect	*Chironomus riparius*	10	Carrasco-Navarro et al., 2021
Crustacean	*Hyalella azteca*	11.15	Au et al.,2015
Crustacean	*Daphnia pulex*	15.811	Jaikumar et al., 2019
Rotifer	*Brachionus plicalitis*	16.4	Berber and Yurtsever, 2018
Algae	*Chlamydomonas reinhardtii**	17.33	Yang et al., 2021 and Li et al, 2020
Fish	*Pimephales promelas*	20	Malinich et al., 2018
Macrophyte	*Utricularia vulgaris*	70	Yu et al, 2020
Annelid	*Allonais inaequalis*	320	Castro et al., 2020
Mollusc	*Dreissena bugensis*	800	Pedersen et al., 2020
Fish	*Clarias gariepinus*	2000	Tongo and Erhunmwunse, 2022

* For species with more than one data available in the literature, the geometric mean was calculated

Table 7. Estimated NOEC (particle. L^{-1}) of aquatic organisms exposed to microplastics used for SSD analysis, obtained from Table 3.

Group of organism	Especie	NOEC (particles. L^{-1})	Referencia
Algae	*Microcystis aeruginosa*	1.57E+01	Sanchez-Fortún et al., 2021
Fish	*Carassius carassius*	2.51E+01	Hu et al., 2022
Fish	*Cyprinus carpio*	5.31E+01	Xia et al., 2020
Mollusc	*Biomphalaria glabrata*	5.99E+01	Wang et al., 2022
Crustacean	*Daphnia magna**	3.62E+02	Bosker et al.,2019, Eltemsah & Bøhn, 2019, De Felice et al., 2019, Schur et al., 2021, Trotter et al., 2021, Jemec Kokalj et al., 2021, Jaikumar et al., 2019, Liu et al., 2022, Coady et al., 2020

Fish	*Pimephales promelas*	4.41E+02	Malinich et al., 2018
Mollusc	*Corbicula fluminea*	1.12E+03	Rochman et al., 2017
Fish	*Danio rerio**	2.68E+03	Qiang and Cheng, 2019, Qiang et al., 2020, Prata et al., 2022, Malafaia et al., 2020, LeMoine et al., 2018, Santos et al., 2021, Zhang et al., 2022, Prata et al., 2022
Crustacean	*Gammarus pulex*	4.12E+03	Weber et al., 2018
Crustacean	*Ceriodaphnia dubia**	2.68E+04	Ziajahromi et al., 2017 and Jaikumar et al., 2019
Algae	*Chlamydomonas reinhardtii**	3.15E+04	Yang et al., 2021 and Li et al, 2020
Insect	*Chironomus riparius*	6.62E+04	Carrasco-Navarro et al., 2021
Algae	*Raphidocelis subcapitata*	2.61E+05	Prata et al., 2022
Mollusc	*Dreissena bugensis*	3.45E+05	Pedersen et al., 2020
Fish	*Clarias gariepinus*	5.03E+05	Tongo and Erhunmwunse, 2022
Amphibian	*Xenopus laevis*	8.42E+05	De Felice et al., 2018
Crustacean	*Tigriopus japonicus*	1.94E+06	Yu et al., 2020
Crustacean	*Hyalella azteca*	2.80E+06	Au et al.,2015
Annelid	*Allonais inaequalis*	7.55E+06	Castro et al., 2020
Rotifer	*Brachionus plicalitis*	9.77E+06	Berber and Yurtsever, 2018
Fish	*Oryzias latipes*	1.00E+07	Assas et al., 2020
Crustacean	*Daphnia pulex*	2.54E+08	Jaikumar et al., 2019
Macrophyte	*Utricularia vulgaris*	1.02E+09	Yu et al, 2020

* For species with more than one data available in the literature, the geometric mean was calculated

Figure 9 shows that the sensitive species vary according to the units, in the first case (figure 9A) *X. Laevis* was the most sensitive organism for MPs in mg. L^{-1} and *M. aeureginosa* for particles. L^{-1}. This is curious since the most sensitive species in Figure 9A (NOEC value of 0.0125 mg. L^{-1}) turns out to be one of the least sensitive in the Figure 9B (NOEC value of 8.42×10^4 particles. L^{-1}), just for the transformation of the units. This seems to be because the MPs tested were one of the smallest particle sizes tested, so when transforming a weight unit (mg) into a quantity unit (number of particles), the smaller the size, the greater the number of particles obtained. This brings up the discussion on the importance of describing both units in ecotoxicological tests for MPs. The present study followed the method of Connors et al. (2017) assuming that all particles were spherical and obtaining an average of the particle diameter, as studies often presented size ranges and not an average. In addition, some studies do not always show the density of the tested MPs, and this value was estimated from the data of Ji-Horng Plastic Co., Ltd.

It is noteworthy that although the species most used in ecotoxicological tests were *D. rerio* and *D. magna*, according to Table 1, they were not the most sensitive species in any of the cases (Fig 8 and 9). This could be because some newly tested species presented little data or only one study, which does not allow obtaining a more precise average or at least comparing values. Such information should be considered in the future, carrying out studies with the aforementioned species, such as *X. Laevis*, for example, to have a more solid database and verify whether these species are really the most sensitive or not. Mainly because ecotoxicological studies aim to propose values and establish regulations that protect all aquatic species based on the response of the most sensitive species.

Once the SSD curve was elaborated, it was possible to estimate the HC_5 and subsequent PNEC values, which are shown in table 8.

Table 8. Hazardous concentration 5% (HC5) of PS (mg. L^{-1}), PE (mg. L^{-1}), all MP data expressed in mg. L^{-1}, particles. L^{-1} and the predicted no-effect concentration (PNEC) derived from the species sensitivity distribution (Figure 9).

Type of MP	Parameter	Central Tendency	Lower Limit	Upper Limit
PS (mg. L^{-1})	HC_5	2.00E-02	9.50E-03	1.20E-01
PE (mg. L^{-1})	HC_5	5.38E-02	4.22E-03	2.83E-01
MP Total (mg. L^{-1})	HC_5	1.38E-02	1.79E-03	6.04E-02
	PNEC	**2.76E-03**	3.58E-04	1.21E-02
MP Total (particles. L^{-1})	HC_5	1.30E+01	4.63E-01	1.37E+02
	PNEC	**2.61E+00**	9.27E-02	2.74E+01

Highlighted data are the average values of PNEC.

For PS, and PE the HC_5 was: 0.002 mg. L^{-1}., 0.053 mg. L^{-1}, respectively. The estimated PNEC for all the MPs in freshwater was 0.002 mg. L^{-1} or 2.61 particles. L^{-1}. Studies such as Adam et al (2018), acquired values of 4.2 x 10^{-5} mg. L^{-1} or 714 particles. L^{-1}. Comparatively, the data obtained in the present review proved to be more restrictive in the units of particles. L^{-1}, but it is the opposite in mg. L^{-1}. If the data obtained for freshwater were compared with data obtained in studies of marine ecosystems, the value of 12 particles. L^{-1} from Jung et al. (2021), still would be above the value obtained in the present work. This must be considered, as marine ecosystems actually receive whatever freshwater ecosystems discharge into them.

Risk Quotient

As a final part of the risk analysis, the RQ was estimated, which corresponds to the division between the MEC and the PNEC values previously obtained, using the value expressed in particles. L^{-1}, Table 9, shows the RQ values obtained for each continent and the estimated value considering data from all MPs quantities found in the world.

Table 9. Risk quotient of Microplastics worldwide and in different continents

Country	MEC (particles. L^{-1})	RQ
Argentina	0.00090	0.00035
Australia	0.00002	0.00001
Brazil	0.03655	0.01403
Canada*	0.15811	0.06067
China*	2.39144	0.91767
France*	6.34823	2.43600
Germany	0.00557	0.00214
Hungary	0.01379	0.00529
India*	2.98044	1.14368
Italy	0.00300	0.00115
Indonesia	0.04470	0.01715
Japan	0.00180	0.00069
Malaysia	45.86000	17.59785
Nepal	2.24000	0.85955
Poland	2.02000	0.77513
Russia	11.00000	4.22103
South Africa	0.70500	0.27053
US	0.00020	0.00008
Worlwide*	0.24742	0.09494

*Geometric mean was calculated for places with more than one available literature data .

A risk map was created according to the averages and after the data were normalized as shown in figure 10.

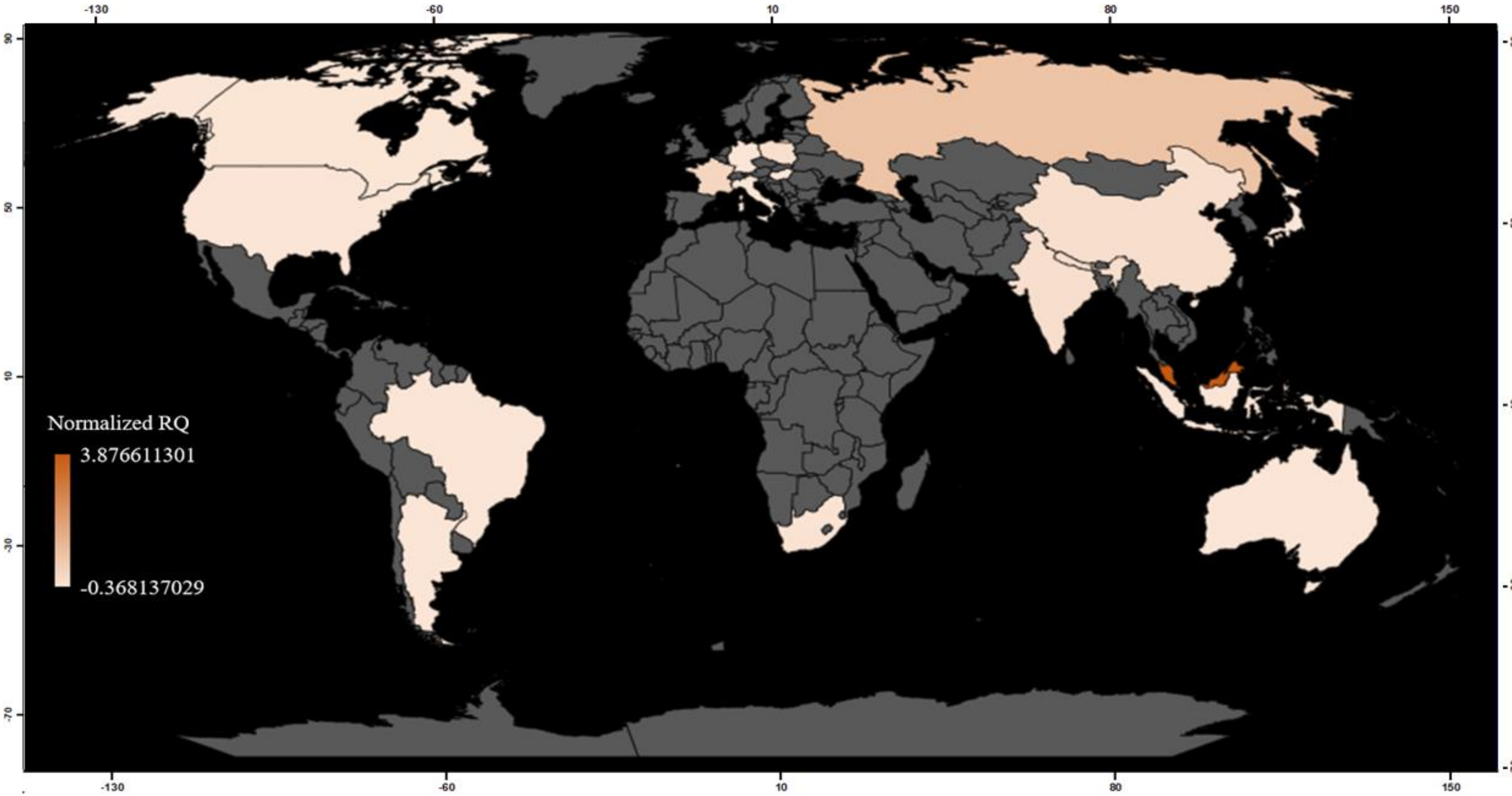

Figure 10. Map of ecotoxicological risk zones for microplastics, where the RQ values (risk quotient) were obtained from the average of the MPs values found in freshwater ecosystems in the world and after that, normalized (Z score), where observed the greatest risks in the darkest shades of green, and the risk decreases, as indicated by the lighter shade of green.

Figure 10 shows that the area with the highest ecotoxicological risk, with a value of 17.598, was in the Langat River in Malaysia, considering that any value greater than 1 already represents a potential risk to the ecosystem (Nika et al., 2020). In terms of pollution, this river already had high levels of heavy metals since 1985, as it is located in the most populous and fastest developing state in Malaysia (Ahmed et al., 2022), with the main sources of pollution being the disposal of domestic sewage, and industrial (Juahir et al., 2011), especially those from the clothing and textile industries in the area (Chen et al., 2021). Another zone that also poses a great potential risk is Russia, as its RQ greater than 1 (4.221) must be taken into account. Similarly, France and India still represent an ecological threat.

These data can be used in the future to compare other studies that carry out similar analyzes and to finally establish regulations that include the regulation of this emerging pollutant.

CONCLUSIONS

It is a fact that MPs are widely found in the environment. Results exposed that different types and shapes of MPs can be found in many freshwater ecosystems worldwide. Apparently, developing countries registered more data on the presence of MPs, as well as the abundance and types.

Ecotoxicological information reflects the existence of a solid database on the effects of MPs on a wide variety of species. However, exists a lack of ecotoxicological studies with certain types of MPs, which, do not reflect what happens in aquatic ecosystems. PP and PET are polymers that are found in greater abundance in aquatic ecosystems, but this does not happen in ecotoxicological studies. Another aspect is the paucity of studies with fibers and, in general, MPs of secondary origin. Certainly, is simpler to get the MPs from companies, than produce them on a laboratory scale.

In terms of the proposed PNEC for MPs from freshwater, this study focused on including all types of MPs, as well as shapes and sizes from 0.1 to 5000 µm. It was intended to know what would happen if all the data were processed and analyzed, and how the result would change. This was thought of in previous works it was common to separate these characteristics (size and type).

For future trends and based on the results, this should be considered:

a) Relate the number of MPs to different factors in each country, such as the amount of population, the GDP (Gross Domestic Product), etc. to understand the abundance and to estimate the possible sources.

b) Make more studies of MPs in freshwater ecosystems to achieve more accurate results in risk analyses. The fact of only having one data per continent causes a little difficulty when estimating the risk quotient.
c) Less common species used in ecotoxicological tests, should not be left aside, as they also represent an important part of freshwater ecosystems and there is a need to compare results with the common ones to see if they could be more sensitive and grant better protection to other species.
d) Testing with the most common types of MPs should continue as it is vitally important to compare the results of different species, to see the behavior of each type of MPs, and if the toxicity changes between species or between types.
e) Conduct more studies with fibers and MPs from the secondary origin since MPs are certainly of secondary origin in nature.
f) Both units, mass and numerical should be mandatorily placed in future studies for better data unification.
g) If possible, perform future risk studies separating the origin of MPs with the aim of comparing values, in order to assert with greater certainty the importance of each of these factors in the ecotoxicity of the different MPs. (PNEC)

In contemplation of protecting freshwater ecosystems, this review could be used as one of the bases for future regulations, because although the world average value of RQ does not represent an environmental risk, it has zones in the world that present values that are above the proposed PNEC values and there is a need for regulating this new pollutant.

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Chapter 2

Ecotoxicological studies of secondary microplastic (PET) in freshwaters using biochemical biomarkers from Danio rerio: emphasis on granulometric conditions, environmental concentrations and co-exposure with cadmium.

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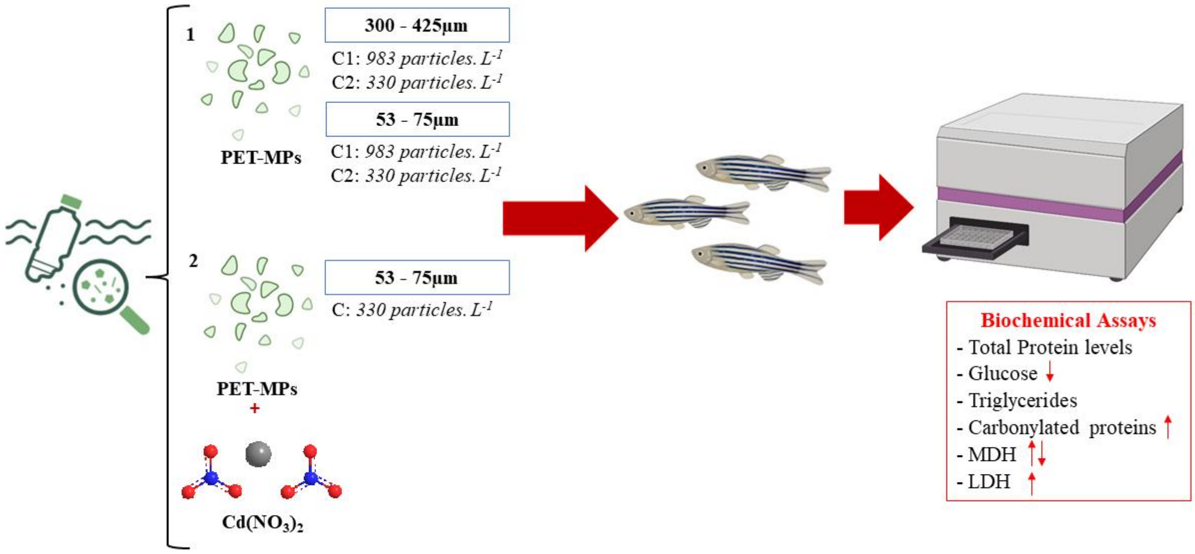
GRAPHICAL ABSTRACT



ABSTRACT

The escalating presence of microplastics (MPs) within aquatic environments has ignited substantial concern due to the manifold adverse effects they impose on biota. A prevailing discourse revolves around the potential influence of factors like type, size, and concurrent exposure to other contaminants on the toxicity of MPs. Amidst this discourse, Cadmium (Cd), a naturally occurring metal, has garnered particular attention. Although naturally existing, numerous industrial practices have led to elevated concentrations of Cd within certain aquatic ecosystems. This phenomenon underlines the significance of assessing the interplay between microplastics and contaminants like Cd. Here, two 21-day experiments with adult *Danio rerio* were carried out to (1) Verify the effects of different particle sizes of Polyethylene Terephthalate (PET-MPs) (300-425 µm and 53-75 µm), at two different environmentally relevant concentrations: 983 particles. L^{-1} and 330 particles. L^{-1}, and (2) demonstrate the possible effects of Cd (10 µg. L^{-1}), 53 µm PET-MP (330 particles. L^{-1}) and their mixture (PET-MP + Cd), on a set biochemical indicator: total proteins, carbonylated protein (CP), glucose, triglycerides, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activity. There was a significant increase in CP levels, and a decrease in glucose and LDH levels compared to the control in both concentrations for the largest size (300-425 µm). In the experiment with PET-MPs co-exposed to Cd, the CP levels showed a significant increase in the PET-MP + Cd group compared to the control and PET-MP alone, evidencing oxidative stress because of the addition of Cd. LDH levels decreased significantly when Cd was added to the PET-MP. MDH of the Cd group showed a significant increase when compared to the control group and PET-MP alone. This study has achieved a groundbreaking milestone by unveiling the toxicity of PET-MPs on *D. rerio*. This investigation has illuminated the interplay of various factors, including size, concentration, and co-exposure with Cd, in shaping the toxicological impact of PET-MPs. The combined effects of these variables have been elucidated for the first time through this comprehensive research effort.

Keywords: Zebrafish, microplastics, metal, metabolism, oxidative stress, enzymatic activity.

INTRODUCTION

Microplastics are particles derived from the degradation of plastic materials that are usually less than 5 mm and larger than 1 µm in diameter (Wagner et al., 2014). The increasing concentrations of microplastic in marine and freshwater environments observed in recent decades became of global concern because they are important emergent pollutants (Zhang et al. 2022; Pratiwi, 2023). Capable of causing mechanical lesions in the gill epithelium and in the digestive tract; disturbs behavioral, metabolic, and neurological functions; reduction in intestinal microbiota; inhibition of growth and reproduction; genotoxicity; neurotoxicity, and oxidative malfunctions, affecting a variety of organisms at different trophic levels (Triebskorn et al., 2019; Huang et al., 2021; Li et al. 2021; GESAMP, 2015, Horton & Dixon, 2018).

MPs also have the potential to act as vectors and to increase the toxicity of other organic and inorganic pollutants, such as heavy metals (Wen et al., 2018). Cadmium (Cd), for instance, is frequently used in plastic additives (Lu et al., 2018; Massos and Turner, 2017). Cd alone tends to bioaccumulate in the liver, digestive tract, and gills of fish (Hilal & Ismail, 2008), but when in combination with MPs, it can produce other effects (Chen et al. 2022), including the reduction in the activity of acetylcholinesterase (AChE) and gamma glutamyl-transferase (GGT) (Banaee et al., 2019); alterations in the chorion membrane and growth; oxidative damages, and apoptosis in the earliest life stages of the zebrafish, *D. rerio* (Zhang et al. 2020; Yang et al. 2022). The adsorption capacity of other contaminants increases with the reduction of MPs particle sizes (Wagner et al. 2014). The capacity to adsorb xenobiotics, for example, can increase 10 to 100 times depending on the sizes of MP particles (Acosta-Coley et al., 2019). There are also some shreds of evidence about the variation of the effects depending on the size of MPs (Zhou et al., 2023). Devi et al. (2023), reported that nanoplastics (NPs) caused more damage than MPs in the liver of zebrafish. In the case of algae, the effects decrease when the particle is larger, but this varies when the concentration increases, enhancing the growth inhibition effects for example (Wang et al., 2019).

Among the various types of plastic polymers, Polyethylene Terephthalate (PET) has important properties, such as high electrical and solvent resistance, high melting point, and low production costs, reason why it has been extensively used in the construction of capacitors, decorative coatings, magnetic tapes, packages, and PET bottles (Abbasi et al., 2020; Pencik et al., 2023). Although PETs represent 7.7% of global industrial plastic production (Plastics Europe, 2022), they can be responsible for up to 60% of the amount of MP from aquatic environments (Sait et al., 2021). However, few ecotoxicological studies have been carried out with this polymer in freshwater species, but they have already reported alterations such as liver damage, growth and survival rate, and oxidative stress (Queiroz et al. 2022; Pencik et al. 2023; Yang et al. 2022).

This study leverages adult zebrafish (*D. rerio)* as the chosen test organism to enhance our understanding of the ecotoxicological consequences stemming from the interaction between microplastics (MPs) and Cadmium (Cd). Notably, this research represents the inaugural instance of employing secondary PET particles for this purpose. The approach taken involves the incorporation of an expanded array of biochemical markers, thereby enabling a comprehensive exploration of the potential impacts resulting from varying doses and particle sizes of PET-MPs in isolation and in conjunction with Cd. With chronic toxicity tests (21d) the metabolism level, enzymatic activity variations, and the oxidate stress was analized.

METHODOLOGY

MPs Preparation and Chemicals

The MPs (microplastics) were produced from PET raw bottles material (density: 1.78 g.cm^{-3}) purchased from Coca-Cola LTDa, at the Federal Institute of São Paulo – IFSP, Sertãozinho Campus. They were obtained by physical methods, mainly using a metallographic sander that was passed over the PET raw material. Next, the pieces obtained were sieved in strainers of different mesh sizes until obtaining the desired dimensions of 300-425 µm and 53-75 µm. These PET-MPs were kept in a contaminant-free hermetic vial for further analysis and use for ecotoxicological experiments. Analytical tetrahydrate grade cadmium nitrate (99.9% purity), was purchased from Vetec Quimica Fina LDTA. and used as the cadmium nominal concentrations. Then, a stock solution of 100 mg/L was prepared with distilled water.

Analysis of microplastics

The sizes of the PET-MPs particles were first confirmed using a LEICA DM3000 LED optical microscope. Later, PET-MPs were counted to transform them from number units (particles. L^{-1}) to mass units (mg. L^{-1}) using also a LEICA DM3000 LED optical microscope in two Laboratories from (1) Instituto de Ciência e Tecnologia – Sorocaba - UNESP and (2) Instituto Federal São Paulo, Campus Sertãozinho, to guarantee the accuracy of the particle count. Results can be observed in Appendix A. Morphological characteristics were analyzed using two different scanning electron microscopies (SEM). HITACHI-TM3000 microscope was used for comparing both sizes (300-425 µm and 53-75 µm). ZEISS Microscope model EVO MA 10 was used to see more detail about the surface of the PET-MPs. To make it possible, a PET sample was coated with gold by using a sputtering Cressington Sputter Coater 108, in contemplation of making it conductive and able to be observed in the microscope. Finally, to ensure that the experiments were carried out with PET-MPs further analyses were made with Raman Espectometry detector (iRaman -R) at Universidad Nacional de San Agustin -Peru, using a mixed sample of both sizes of the PET-MPs.

Zebrafish maintenance and exposure

Adult zebrafish (*D. rerio*) were acquired at a local pet store and kept under standard laboratory conditions at the Instituto de Ciência e Tecnologia - Câmpus de Sorocaba - UNESP facilities. During the acclimatization period, adult zebrafish were maintained at 26 ± 1 °C under a 12:12 h photoperiod (light: dark), with dechlorinated, aerated, and activated charcoal-filtered water (pH 7.4 ± 0.2) (Appendix A). Adults were fed with TETRAMIN 98% protein chow twice daily. All the conditions followed the OECD guideline 203 on toxicity tests.

Subsequently, two experiments were conducted, one to verify the toxicity of each different PET-MP size, and another to verify the toxicity of PET-MP in association with Cd. Adult zebrafish were randomly placed in 2.5 L glass tanks for both cases. Each aquarium contained 4 fishes and 2L of test solution. Five treatments were carried out: control (prepared water without any contaminants), two MP concentrations of 300-425 µm (983 particles. L^{-1}; 330 particles. L^{-1}), and two MP concentrations of 53-75 µm (983 particles. L^{-1}; 330 particles. L^{-1}). Four replicates were performed for each treatment, but a total of 50 fish were used for biochemical analysis (Appendix B). PET-MPs concentrations were selected based on environmentally relevant concentrations and toxicity to aquatic organisms according to Azizi et al. (2020), who established a value of 983 particles. L^{-1} as the average number of MPs at the influent of wastewater treatment plants, based on 407 studies. The value of 330 particles. L^{-1} was based on a mean of MPs concentrations of the Sinos River, Brazil, where untreated water is thrown into this ecosystem (Ferraz et al., 2020).

Afterward, four treatments were carried out in the second PET-MP + Cd experiment: control (prepared water without any contaminants), one PET- MP concentration of 330 particles. L^{-1} from the size of 53-75 µm, one Cd concentration (10 µg. L^{-1}), and finally the association of both MPs of 53-75 µm + Cd (10 µg. L^{-1}). Four replicates were made and a total of 32 fish were used in this experiment (Appendix C). To prepare the aquariums for Cd treatment, an appropriate volume of Cd stock solution was added to the culture water and the final Cd concentration in the test solution was 10 µg/L. After that, for the PET-MP + Cd condition, this test solution was incubated on a shaker at 270 rpm for a period of 90 min at room temperature (20 ± 1 °C). Since there are no studies about Cd adsorption onto PET-MPs, it was based on another type of similar polymer: high-density polyethylene (HDPE) MPs. According to the batch adsorption experiment of Wang et al. (2019), at 90 min the equilibrium time is reached by the HDPE-MPs to adsorb Cd. In this experiment, the Cd dose was chosen according to the safe water concentration for the protection of aquatic life described by the Environmental National Council of Brazil (CONAMA 357, 2005) to see if the described level was truly achieving this objective.

These chronic experiments lasted 21 days. To maintain the concentration and avoid variations in the parameters of each aquarium, the test solution in each tank of all the conditions in the two experiments was renewed every 2d.

Biochemical assays

Tissue Samples

The head of all of the used zebrafish individuals was separated from the rest of the body, and the remaining part including organs and muscles tissues was homogenized (N = 8 per group in the PET-MP + Cd experiment, and N=10 in the PET-MPS isolated experiment) in Saline phosphate buffer (containing NaCl 58.44 g.mol^{-1}, KCl 74.56 g.mol^{-1}, Na_2HPO_4 ($7H_2O$) 268.07 g.mol^{-1} and KH_2PO_4 136.09 g.mol^{-1}, pH 7.2 in a Teflon tissue homogenizer (Potter-Elvehjem Teflon tissue) at -4 °C. The homogenate was centrifuged at 10,500 g (Hettich Universal

320 R) for 20 min at 4 °C. Then, the supernatant was collected and immediately stored at -40 °C for biochemical determinations. All biochemical assays were performed in the Microplate reader - Spectrofluorimeter, Synergy HTX Reader (BioTek Microplate Reading spectrophotometer) at 25 °C.

Total protein content

The quantification of the total protein concentration of the homogenate was determined according to the methodology of Bradford (1976), which uses bovine serum albumin (BSA) as a standard at 595 nm. Lectures on the Microplate reader were made in duplicate for the case of the experiment of the co-exposition of Cd with PET-MPs, but in the case of the PET-MPs isolated experiment, were made in triplicate.

Triglycerides and Glucose Content

The quantification of triglycerides and glucose content was conducted using Labtest Kits, specifically Liquiform Kit No. 87 for triglycerides and Liquiform Kit No. 133 for glucose. Subsequent measurements were taken at a wavelength of 505 nm. This process involved triplicate readings for both experiments, thereby ensuring the accuracy and consistency of the obtained results.

Carbonylated Protein

Carbonylated Protein (CP) levels were measured according to the methods described by Levine (1994) which is based on the reaction of carbonylated proteins using 2,4-dinitrophenylhydrazine (DNPH) and the molar extinction coefficient, 22 mM^{-1} cm^{-1} at 370 nm, and it was used for the calculation of the final concentration of CP levels expressed as nmol per mg of protein. The Cd + PET-MP experiment was made in duplicate, but the PET-MPs alone was made in triplicate.

Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activity

LDH activity was determined according to Bergmeyer (1974) using sodium pyruvate as substrate and $NaDH+H^+$ + KCl as buffer solution. LDH activity was expressed as mU per mg protein per minute at 340 nm. The same was performed for the MDH activity, following the Childress and Somero (1979) methodology. In this case, oxaloacetate was used as substrate and $NaDH+H^+$ + $MgCl_2$ as buffer solution. MDH quantification was performed by following NaDH oxidation at 340 nm.

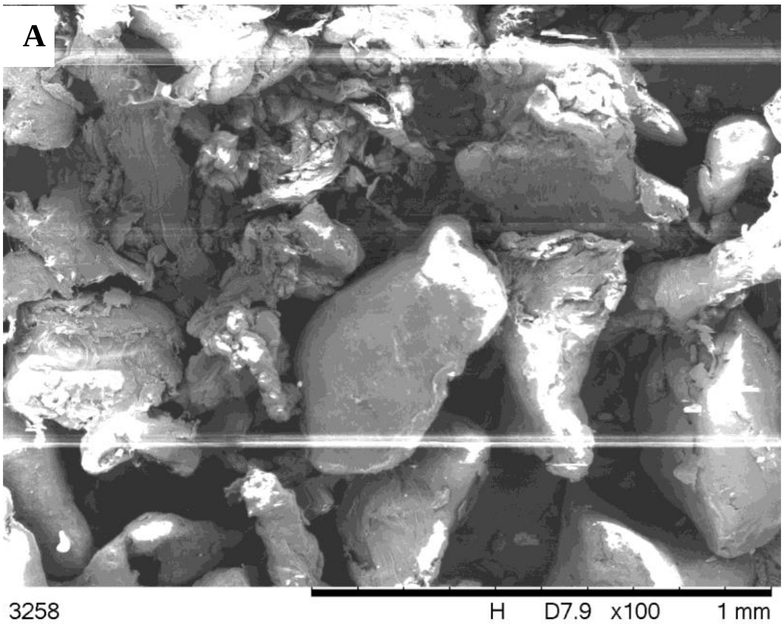
Statistical Analysis

SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA, USA) was used to examine data normality using the Kolmogorov-Smirnov test. After verifying the normal distribution, a one-way analysis of variance (ANOVA) with Tukey's post-hoc multiple comparison test was used to establish differences between the control and treatments. Differences at $p<0.05$ were considered as statistically significant. When normal distributions were not found, Krustall-Wallis test and Dunn's post-hoc multiple comparison test were performed.

RESULTS

Microplastics Analysis

Figure 1 illustrates the size and configuration of the PET-MPs employed in the experiment. The outcomes validate that the particles fell within the size ranges of 300-425 µm and 53-75 µm. Moreover, their shapes were characterized by irregularities, resembling the natural patterns observed in various contexts.



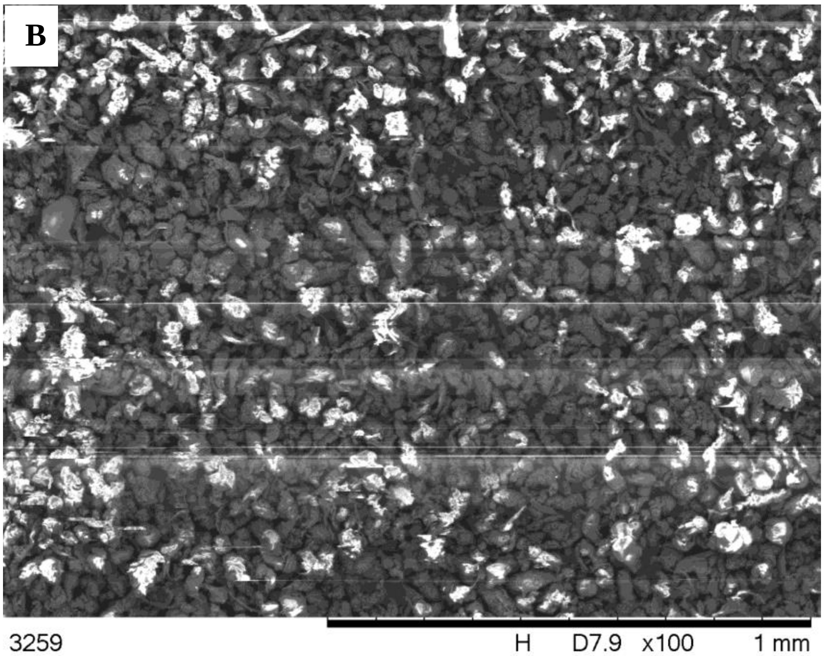


Figure 1. Polyethylene Terephthalate microplastics (PET-MPs) used in this study. (A) shows a 300-425 µm size and (B) shows a 53-75 µm size.

Figure 2 provides an enhanced depiction of the surface characteristics, offering a more intricate view. Notably, the PET-MPs exhibit a notably expansive surface area. Furthermore, the surface is marked by noticeable roughness, a distinctive feature of the material that was employed for the ecotoxicological investigations.

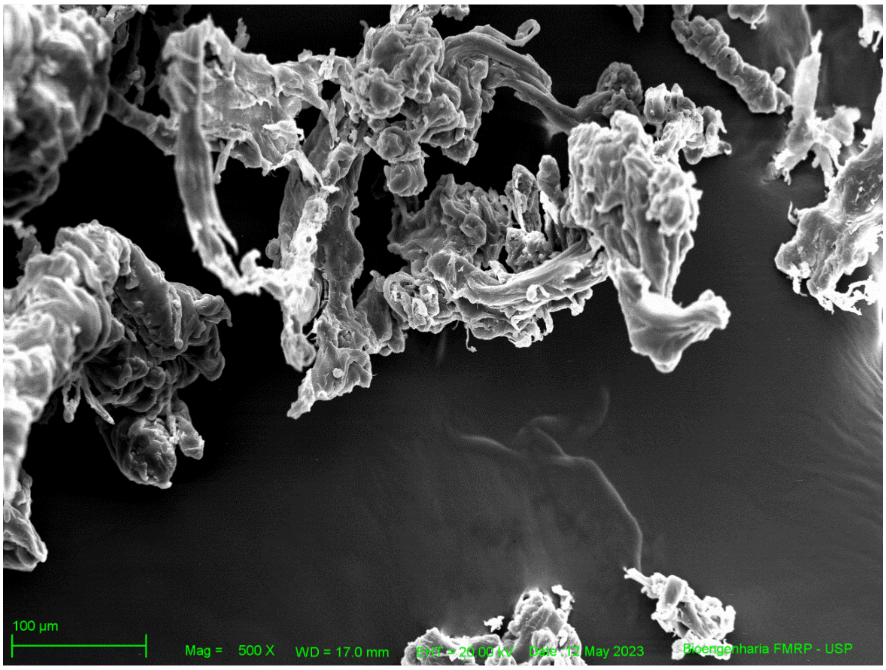


Figure 2. Polyethylene Terephthalate microplastics (PET-MPs) surface used in this study. The image represents a particle from a 300-425µm.

Raman espectometry as depicted in Figure 3, has yielded significant insights. Notably, the analysis reveals that the PET-MPs samples with varying granulometries exhibit a striking 96.62% similarity with the pure PET pattern. This finding serves as compelling validation for the utilization of this specific polymer, namely PET-MPs, across all experimental contexts.

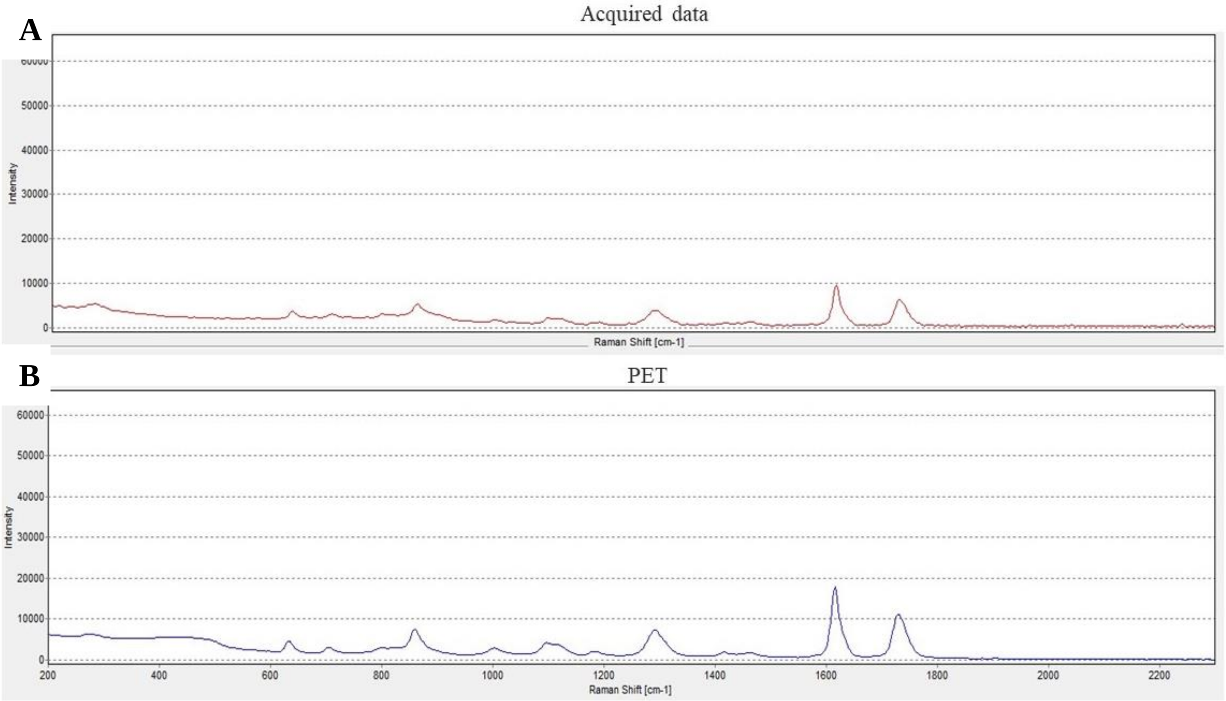


Figure 3. (A)Raman spectra for Polyethylene Terephthalate microplastics (PET-MPs) samples and (B) Polyethylene Terephthalate pure pattern. Match data with and HQI of 96.62%

Biochemical parameters

All the biochemical parameters in both experiments are shown in Table 1 and Table 2.

Table 1. Changes in biochemical parameters in zebrafish (*D. rerio*) exposed to different sizes and concentrations of PET-MPs. The number of individuals per treatment was 10.

Biochemical Parameters	**Treatments**				
	Control	**PET-MP 1-1**	**PET-MP 1-2**	**PET-MP 2-1**	**PET-MP 2-2**
Proteins (mg/L)	24.666±2.987	22.002±3.451	22.763±2.837	23.638±2.476	22.435±2.074
Glucose (mg/L)	13.566±3.119[a]	7.344±1.734[abc]	12.093±2.389[b]	7.915±1.836[ab]	11.211±2.194[c]
Triglycerides (mg/L)	64.613±12.591	61.192±11.208	49.590±11.908	53.174±9.493	59.434±10.996
CP (nmoles/mg protein)	2.788±0.314[a]	5.596±0.941[ab]	5.561±0.847[ab]	4.780±0.719[a]	3.659±0.746[b]
LDH (mU/mg protein)	0.050±0.009[a]	0.015±0.001[abc]	0.066±0.020[b]	0.035±0.010[bc]	0.070±0.020[c]
MDH (mU/mg protein)	0.004±0.003	0.004±0.001	0.003±0.001	0.005±0.001	0.004±0.001

Results are shown as mean ± standard deviation (S.D.). Lowercase letters represent significant differences between different experimental groups. Acronyms of PET-MPs represent different sizes and concentrations, PET-MP 1-1: 300-425µm and C: 983 particles. L^{-1}; PET-MP 1-2: 300-425µm and C:330 particles. L^{-1}; PET-MP 2-1: 53-75µm and C: 983 particles. L^{-1} and PET-MP 2-2: 53-75 µm and C: 330 particles. L^{-1}

Table 2. Changes in biochemical parameters in zebrafish (*D. rerio*) exposed to PET-MPs in combination with cadmium. The number of individuals per treatment was 8.

Biochemical Parameters	**Treatments**			
	Control	**Cd**	**PET-MP 2-2**	**PET-MP 2-2+ Cd**
Proteins (mg/L)	16.554±2.673	16.801±1.703	16.272±2.539	16.794±1.883
Glucose (mg/L)	7.631±1.554	7.335±5.728	5.379±5.728	8.491±5.728
Triglycerides (mg/L)	27.117±1.554	26.293±1.578	35.603±1.106	35.233±2.615
CP (nmoles/mg protein)	2.333±0.555[a]	2.622±0.607	1.700±0.343[a]	3.508±0.714[a]
LDH (mU/mg protein)	0.037±0.031	0.006±0.002	0.066±0.010[a]	0.001±0.000[a]
MDH (mU/mg protein)	0.007±0.002[a]	0.019±0.004[a]	0.009±0.003[a]	0.013±0.003

Results are shown as mean ± standard deviation (S.D.). Lowercase letters represent significant differences ($p < 0.05$) between different experimental groups. Acronyms of

PET-MPs represent different sizes and concentrations, PET-MP 2-2: 53-75 µm and C: 330 particles. L^{-1}, Cd: Cadmium, PET-MP 2-2+ Cd: 53-75 µm, and C: 330 particles. L^{-1} in combination of Cadmium.

Metabolites on isolated PET-MPs and co-exposed with Cd

Protein, glucose, and triglyceride levels of PET-MP isolated and for the mixture of PET-MP + Cd are shown in Figure 3.

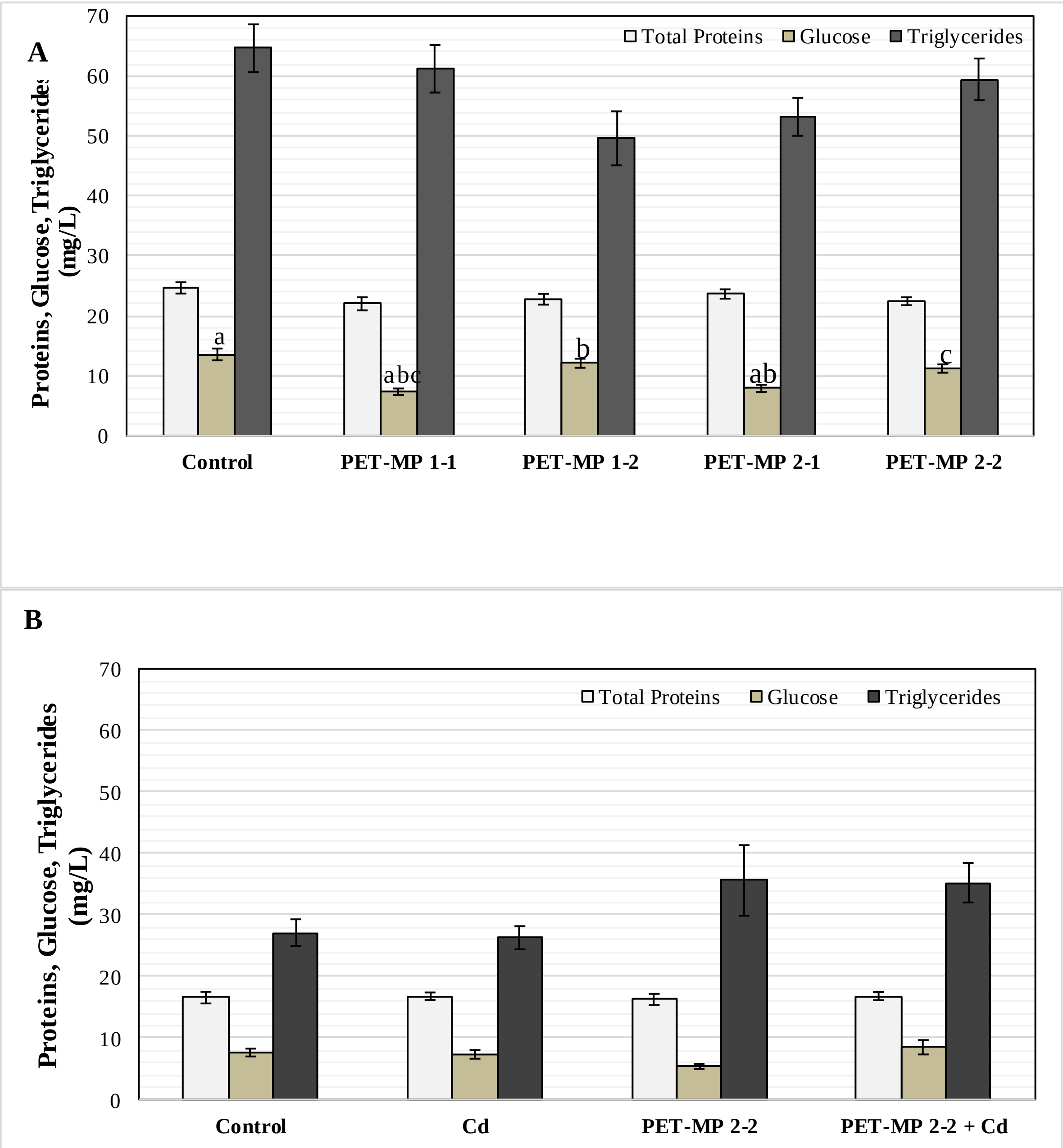


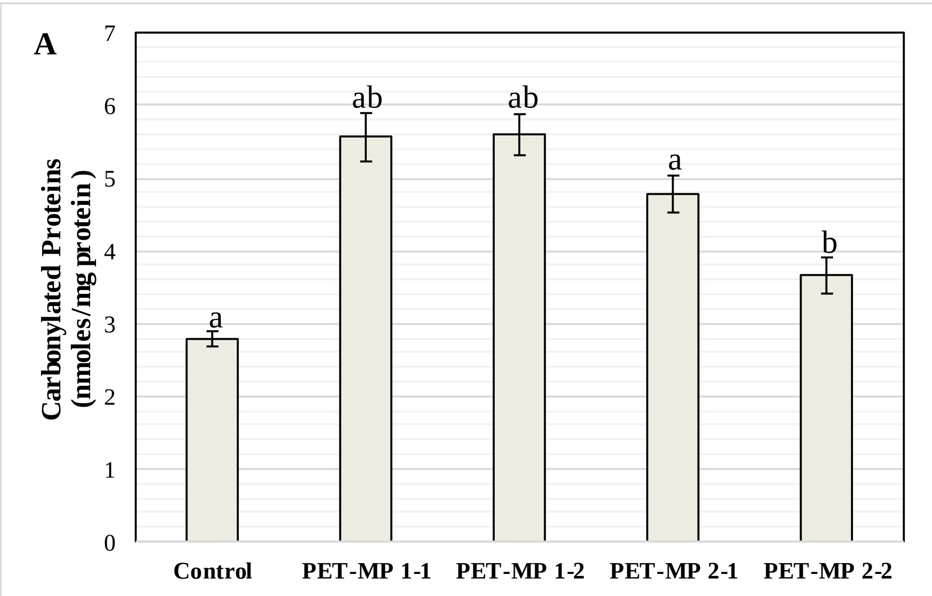
Figure 4. Parameters related to the metabolites of *D. rerio* adults exposed to PET-MP isolated (A) and in combination with Cd (B). Acronyms of Figure A from PET-MPs represent different sizes and concentrations, PET-MP 1-1: 300-425 µm and C: 983 particles. L^{-1}; PET-MP 1-2: 300-425 µm and C:330 particles. L^{-1}; PET-MP 2-1: 53-75µm and C: 983 particles. L^{-1} and PET-MP 2-2: 53-75 µm and C: 330 particles. L^{-1}. Each letter

means significant differences between PET-MPs concentrations within each condition ($p < 0.05$). Results are shown as mean ± standard deviation (S.D.).

In Figure 4A, the glucose level data from the PET-MPs experiment illustrate noteworthy variations among the groups. Specifically, groups PET-MPs 1-1 and PET-MPs 2-1 displayed a significant decrease in glucose levels compared to the control group. Additionally, a significant increase was observed in the PET-MPs 1-2 group in comparison to PET-MPs 1-1. This trend was mirrored in the PET-MPs 2-2 and PET-MPs 2-1 groups as well. In contrast, Figure 4B unveils a distinct pattern. Within the tested groups, no statistically significant differences were noted in the levels of protein, glucose, or triglycerides.

Carbonylated Protein levels on isolated PET-MPs and co-exposed with Cd

CP levels were affected in different ways in both experiments: PET-MP isolated and the mixture of PET-MP + Cd as shown in Figure 5.



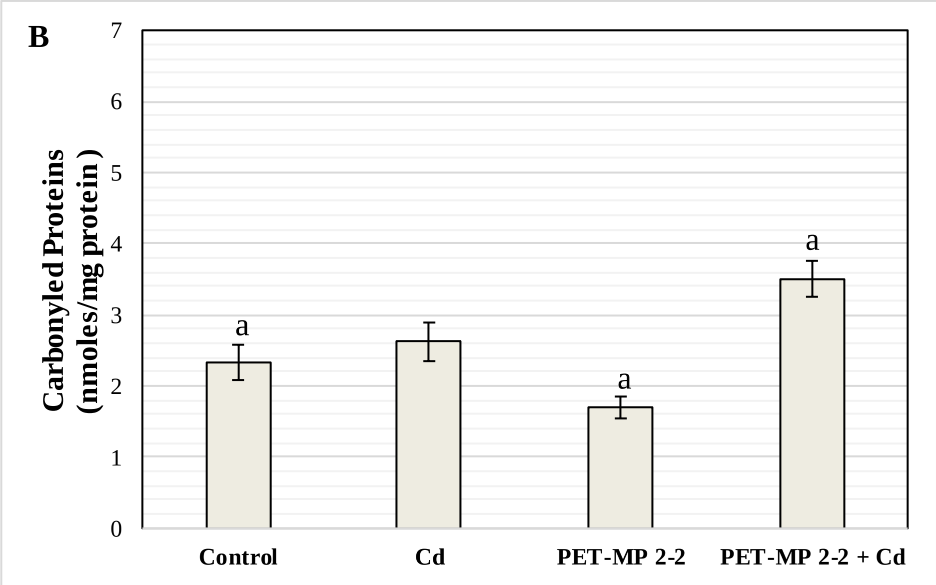


Figure 5. Parameters related to oxidative stress levels of adult *D. rerio* exposed to PET-MP isolated (A) and in combination with Cd (B). Acronyms of Figure A from PET-MPs represent different sizes and concentrations, PET-MP 1-1: 300-425 µm and C: 983 particles. L^{-1}; PET-MP 1-2: 300-425 µm and C:330 particles. L^{-1}; PET-MP 2-1: 53-75µm and C: 983 particles. L^{-1} and PET-MP 2-2: 53-75 µm and C: 330 particles. L^{-1}. Each letter means significant differences between PET-MPs concentrations within each condition ($p < 0.05$). Results are shown as mean ± standard deviation (S.D.).

In Figure 5A, focusing on the isolated PET-MPs experiment, an intriguing pattern emerges concerning carbonylated protein (CP) levels. Notably, CP levels exhibited a substantial increase in groups PET-MP 1-1, PET-MP 1-2, and PET-MP 2-1 in comparison to the control group. A distinct trend became apparent regarding the size of the PET-MPs. This size-based influence on CP levels is further highlighted by the significant elevation in CP levels observed in PET-MPs 2-2, in contrast to both the PET-MP 1-1 and PET-MP 1-2 groups.

Shifting to Figure 5B, an important observation comes to light. When PET-MPs were combined with Cd, CP levels exhibited a marked increase. This increase is noteworthy when juxtaposed with both the control group and the isolated PET-MP group. This finding underscores the distinct impact of the introduction of Cd on CP levels within this experimental context.

Enzymatic activity on isolated PET-MPs and co-exposed with Cd

The LDH activity presented significant variations for both experiments: isolated PET-MPs and the experiment of PET-MPs co-exposed to Cd. In the case of MDH activity, there were significant changes in the experiment of PET-MPs co-exposed to Cd. All results are shown in Figure 6.

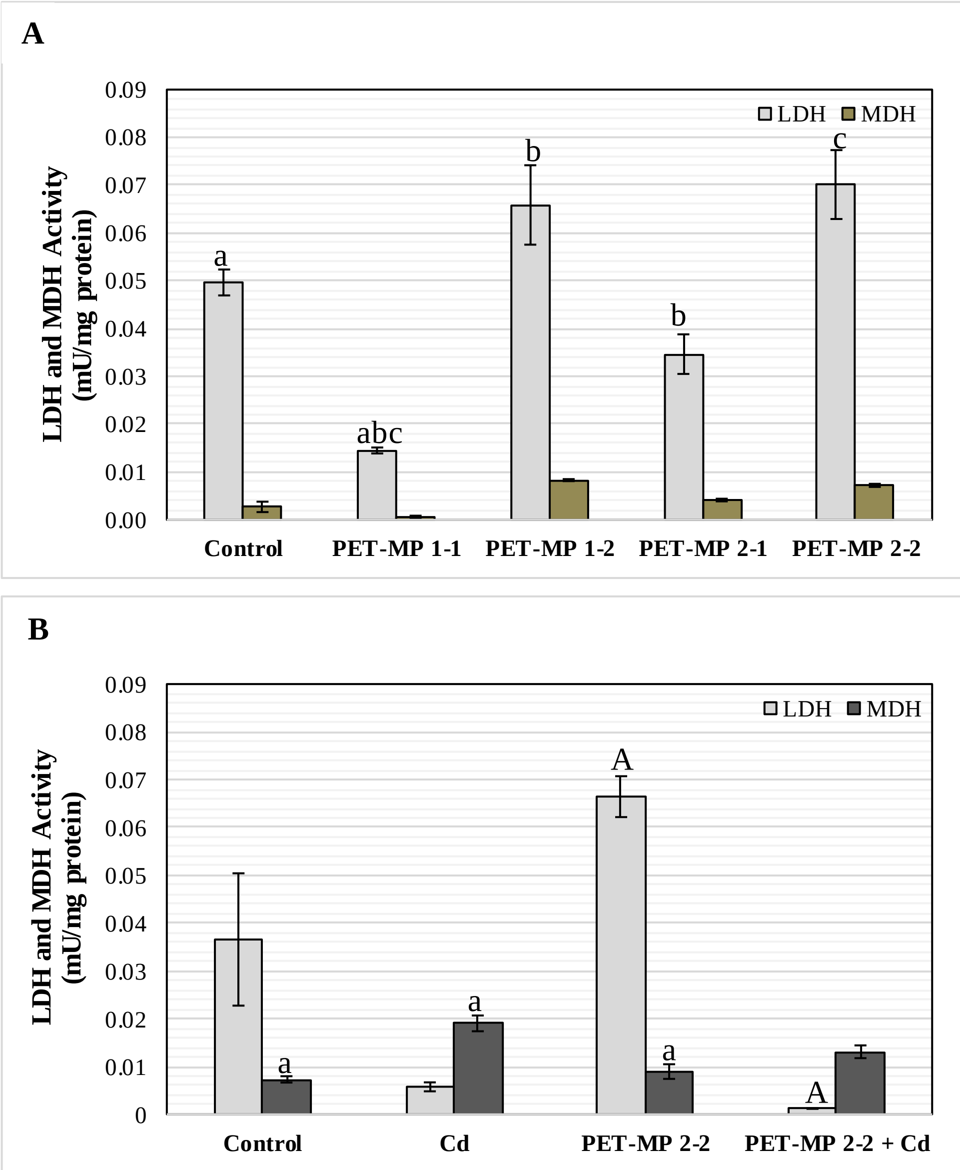


Figure 6. Parameters related to the enzymatic activity of adult *D. rerio* exposed to PET-MP isolated (A) and in combination with Cd (B). Acronyms of Figure A from PET-MPs represent different sizes and concentrations, PET-MP 1-1: 300-425µm and C:983 particles. L^{-1}; PET-MP 1-2: 300-425µm and C:330 particles. L^{-1}; PET-MP 2-1: 75-53µm and C: 983 particles. L^{-1} and PET-MP 2-2: 75-53 µm and C: 330 particles. L^{-1}. Each letter means significant differences between PET-MPs concentrations within each condition ($p < 0.05$). In Figure 6B, lowercase letters represent significant differences for MDH activity and upper case for LDH activity. Results are shown as mean ± standard deviation (S.D.).

Figure 6A illustrates the dynamics of lactate dehydrogenase (LDH) activity within the PET-MP 1-1 treatment. Notably, a significant reduction in LDH activity is evident when compared to the control group. Additionally, the groups featuring higher concentrations, namely PET-MP 1-1 and PET-MP 2-1, exhibit a noteworthy decrease in LDH activity compared to the groups with lower concentrations, represented by PET-MP 1-2 and PET-MP 2-2.

Figure 6B presents a distinct scenario, specifically for the isolated PET-MPs experiment. The levels of malate dehydrogenase (MDH) activity, however, do not exhibit statistically significant differences among the groups.

Transitioning to the experiment involving the co-exposure of PET-MPs with Cd, intriguing outcomes emerge. LDH content displays a significant decrease within the Cd and PET-MPs mixture group, when contrasted with the isolated PET-MPs group. In terms of MDH activity, the Cd treatment signifies a noteworthy increase compared to the control group, alongside a significant decrease relative to the isolated PET-MPs group. These findings unveil the intricate interplay between Cd and PET-MPs in shaping LDH and MDH activities.

DISCUSSION

This study has pioneered a significant revelation, uncovering that the impact of secondary PET-MPs exposure can be intensified when combined with a heavy metal such as cadmium. Although mortality was not observed in the exposed groups, discernible alterations in biochemical parameters were evident.

The initial focus lies on the morphological analysis results (Figure 1) of the secondary PET-MPs. A comparative examination against various studies reveals a striking resemblance between these particles and those encountered in freshwater environments (Shen et al., 2021; Ding et al., 2019; Zhao et al., 2018). Notably, fragments and fibers emerge as the most prevalent forms, corroborating with findings by Yan et al. (2022). This alignment between morphological analyses and ecotoxicological tests underscores the realistic representation of the processes unfolding within freshwater ecosystems.

An equally noteworthy factor is the size and surface roughness (Figure 2), which may confer an enhanced capacity for pollutant adsorption. This feature becomes particularly relevant in the context of the second experiment, where it aids in the increased adsorption of Cd by smaller MPs (Wang et al., 2019). The composition of the PET-MPs underscores a high degree of purity, as evidenced by the absence of detected inorganic compounds.

The outcomes of this study provide a comprehensive understanding of the intricate interplay involving secondary PET-MPs, exposure to heavy metals like cadmium, and their subsequent repercussions on freshwater ecosystems. Beyond enhancing our grasp of these complex ecological dynamics, this research emphasizes the potential risks inherent in such interactions. Essential biomarkers such as glucose, triglyceride, and protein levels serve as crucial parameters for assessing the health status of fish, contributing to a comprehensive evaluation of their physiological well-being (Öner et al., 2007). This study contributes significantly to our knowledge of these ecological relationships and their implications for aquatic organisms.

When examining the scenario involving the combination of Cd with PET-MPs, no notable alterations were observed. Conversely, in the isolated PET-MPs experiment, it was primarily glucose that exhibited significant variations across the treatments. Figure 4A illuminates an interesting trend in glucose levels. Notably, as the concentration decreased, glucose levels demonstrated a tendency to revert back to concentrations similar to the control group. This pattern implies a dose-dependent relationship, suggesting that glucose levels are intricately linked to the administered dosage. Glucose transport is fundamental to supplying energy to the metabolism, so constant glucose levels are essential for key metabolic processes for its regulation and control (Brown, 2000) and it is also important to highlight that oxidative stress is closely related to low glucose levels since its effect is potentiated (Bashirova et al. 2023). These results are similar to those obtained by Bashirova et al. (2023), who also described a decrease in glucose levels in *D. rerio* embryos, which were exposed to different concentrations (5-200 mg. L^{-1}) of PET NPs (70 ± 5 nm). However, they differ from what was obtained by Banaee et al., 2019, and Hamed et al., 2019, who obtained an increase in glucose compared to the control in *Cyprinus carpio* and *Oreochromis niloticus* respectively. This may be because both studies worked with other types of MPs. Unfortunately, to date, there are no more studies of PET-MPs to compare glucose levels, but the results suggest that there are indications of effects on energy metabolism due to the reduction in glucose levels.

Stress proteins make an estimate of whether the effective concentration of pollutant induces metabolic alterations in an organism (Torres et al., 2008). Protein carbonylation is a form of protein oxidation widely used to determine oxidative stress (Yan & Forster, 2011; Sorolla et al., 2008). This oxidative damage is irreversible and often leads to a loss of protein function, resulting from disease (Donne et al., 2006). For the first experiment, CP levels increased significantly in all sizes and concentrations of PET-MPs with respect to the control (Figure 5B). These results show similarity with that obtained by Kaloyianni et al. (2021) in their study with *D. rerio* and *Perca fluviatilis* exposed to Polystyrene MPs (PS MPs). Banaei et al., (2022) also recorded the same effect testing PE MPs in *Cyprinus carpio*. Another interesting result is that for the largest granulometry, the levels of CP of *D. rerio* were higher than those from the smaller granulometry, indicating codependency of the toxicity of the PET-MPs based on the size. Li et al. 2021, recorded that oxidative stress depended on size and type, when *Eisenia fetida* was exposed to Polypropylene (PP) and Polyethylene (PE) MPs, and particularly in the case of PP, those with smaller granulometry (8–125μm), had the greatest effect. When the PET-MPs were co-exposed with Cd, the CP levels increased, these results demonstrate that the addition of the metal Cd to the PET-MPs increases the levels of oxidative stress. The combination of other stressors can also trigger further oxidative damage (Rodrigues et al., 2021). Similar responses were obtained by Yang et al., (2022) and Chen et al., (2022). The last authors indicate that Cd concentrations are also important parameters to consider, since when the Cd concentration is low, MPs potentiate its toxicity, but when the concentration of Cd is high, MPs can reduce its bioaccumulation and toxicity.

Enzymes are present in all cells of the body, and their release indicates damage at the cell membrane level (Hameed et al., 2019; Banaee et al., 2014). LDH, under aerobic conditions of cellular energy production, catalyzes the transformation of pyruvate to lactate and is one of the enzymes that is used as a biomarker of cell death and injury (Prokic et al., 2019; Legrand et al., 1992). Figure 6A shows variations from the control of LDH release when *D. rerio* was exposed to a larger size and higher concentration of PET-MPs. Additionally, unlike the glucose levels (Figure 6A), the lower concentrations of PET-MPs of both sizes, the greater release of this enzyme, which also makes it codependent with concentration. These changes in the activity of this enzyme indicate the state of liver function (Yancheva et al., 2015) and suggest that there is possible damage at the tissue level of this organ by exposure to PET-MPs alone. The literature shows variable results regarding MPs and LDH activity. While Banaee et al. (2019) and Barboza et al., (2018) show increases in LDH activity at higher concentrations than those used in this study in *C. carpio* and *Dicentrarchus labrax*; others did not register any alteration in LDH levels for the same specie *C. carpio* (Nematdoost and Banaee, 2017), as well as for others such as *D. rerio* and *Clarias gariepinus* (Limonta et al., 2021; Karami et al., 2016), suggesting that in some conditions LDH is more sensitive to indicate ecotoxicity by MPs. In the second experiment (Figure 6B), a drop in LDH levels could only be observed when the PET-MPs were mixed with Cd, and this is closely related, since its induction may have contributed to the oxidative stress (Figure 4B) since this enzyme is important for the maintenance of the cellular redox balance (Barboza et al., 2018). Similar results were obtained when exposing *Corbicula fluminea* to the mixture of Cd and PS-MPs (Parra et al., 2021). However, it differs from the results found by Banaee et al. 2019, which describe an increase in LDH enzymes when Cd is mixed with PE-MPs. Curiously, there are also other studies with other metals such as mercury, where increases in LDH levels were also recorded, indicating a possible potentiation of negative effects on living organisms such as *Dicentrarchus labrax* (Barboza et al., 2018).

Malate dehydrogenase (MDH) is an important part of the tricarboxylic acid cycle, which helps prevent hydrogen peroxide damage to cells, through the action of oxaloacetate (Vijayaraghavan et al., 2020). The experiment focusing solely on PET-MPs did not yield significant alterations in MDH levels. However, the response shifted significantly when MDH was exposed to Cd. Remarkably, it exhibited the highest activity in the presence of Cd. Intriguingly, in the context of exposure to the mixture of Cd and PET-MPs, no distinct differences were observed. Yet, when exposed exclusively to PET-MPs, a notable increase in MDH release was evident. It is known that the increase in MDH levels increases ATP in the mitochondria, meaning greater cellular efforts to compensate for the energy deficit, and that its release is linked to resistance to oxidative damage by H_2O (Kim et al., 2002). The results coincide with those obtained by Casalino et al. (2000), who exposed male Wistar rats to 2.5 mg of Cd/kg of body weight, in which they concluded that the increase in MDH levels due to Cd reduces the activity glycolytic. In the case of treatment with PET-MPs, it is possible that the MDH levels are due to the fact that PET-MPs can change the fractal properties of the cytoplasmic space, modulating enzymatic activity (Auclair et al., 2020).

CONCLUSIONS:

The conducted experiments have effectively highlighted that concentrations of secondary PET-MPs, which are commonly found in natural environments, possess the capacity to induce significant changes in aquatic organisms, specifically D. rerio. These effects were substantiated through a thorough biochemical examination encompassing Glucose, CP, MDH, and LDH analyses. This investigation shed light on the toxic interplay between size and concentration, revealing a codependent relationship that exacerbates the overall impact.

Furthermore, an intriguing dynamic emerged when PET-MPs were combined with Cd. This conjunction resulted in an amplified toxicity, manifesting through elevated oxidative stress levels and increased enzymatic activity. This was clearly evidenced by the analyses of CP, LDH, and MDH, which displayed heightened values in comparison to the isolated PET-MPs scenario. These findings underscore the intricate interactions at play and the potential for synergistic effects when different pollutants coexist, ultimately influencing the health and wellbeing of aquatic organisms.

Ethical Statement

All experimental procedures undertaken in this study received the official permission of the Ethics Committee for the Use of Animals at the Sorocaba Institute of Sciences and Technology - UNESP. The conducted experiments adhered to the guidelines stipulated in the approved protocol with the reference number 01A/2022 (as outlined in Appendix C). This rigorous adherence to ethical standards underscores the responsible and conscientious approach taken in the research, ensuring the well-being and ethical treatment of the animals involved.

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Chapter 3

Final Considerations and Future Perspectives

The foundation of this research was built upon a series of intricate questions that underscored the complexity of the challenges posed by MPs. These inquiries were not easily answered, questions such as, "Can we accurately quantify the risk posed by microplastics in our surroundings?" and "Is there a well-defined safe threshold to safeguard aquatic life?" exemplify the uncertainty that looms over this subject. Additionally, the exploration extended to the behavior of different types of MPs—do they all elicit the same effects across various species? The concurrent presence of other pollutants alongside MPs, along with the potential for intensification of ecotoxicological impacts due to MPs' size, format, and origin, further deepened the complexity of the matter. It was within these gaps, borne from the limitations of existing literature, that the two chapters of this study found their genesis. The research endeavors to shed light on these intricate questions and contribute to a more comprehensive understanding of the multifaceted challenges presented by microplastics in freshwater ecosystems.

The first chapter grapples with the absence of established safe levels for MPs and embarks on a daunting task: conducting a risk analysis for these particles. This undertaking is not without its challenges, primarily stemming from the unique nature of microplastics as pollutants. Unlike conventional molecules, microplastics exhibit a remarkable degree of complexity owing to their diverse array of characteristics—ranging from types, densities, physicochemical properties, and surface area, to sizes and origins.This research decided to first collect information about what is found naturally in the freshwater environments, and from this, relate it to ecotoxicology, and verify if what is being tested is what really causes problems in nature. Different parameters were considered, such as size, format, origin, and type of MPs. Throughout our search, it was indeed verified that most of the ecotoxicological studies do not test what is more common to be found in nature. Although each type, format, and size of MPs is important, greater efforts should be put into studying those gaps that would allow the establishment of regulations in the future. Secondly, the present work proposed a safe level, based on all the sizes, types, and formats that could be found in ecotoxicological studies. All of this was elaborated based in the fact that freshwater, MPs of different characteristics will be in constant movement due to the dynamics of these ecosystems, representing a risk for organism from all the different depths (benthic and pelagic).

In the second chapter, another notable blank space emerged, partly due to the comprehensive exploration undertaken in the first chapter: the paucity of PET studies with freshwater species such as *D. rerio*. This may be because the production of MPs for ecotoxicological studies is not very common, since it is usual to work with MPs of the primary type, which are easily acquired from factories. However, they did not experience the processes, that normally MPs go through in nature. Also, in addition to choosing the PET for the development of an ecotoxicological study, it was compared different sizes, and concentrations, to see if the effects varied or not, since in some studies certain correlations between sizes, formats, and concentrations were observed.

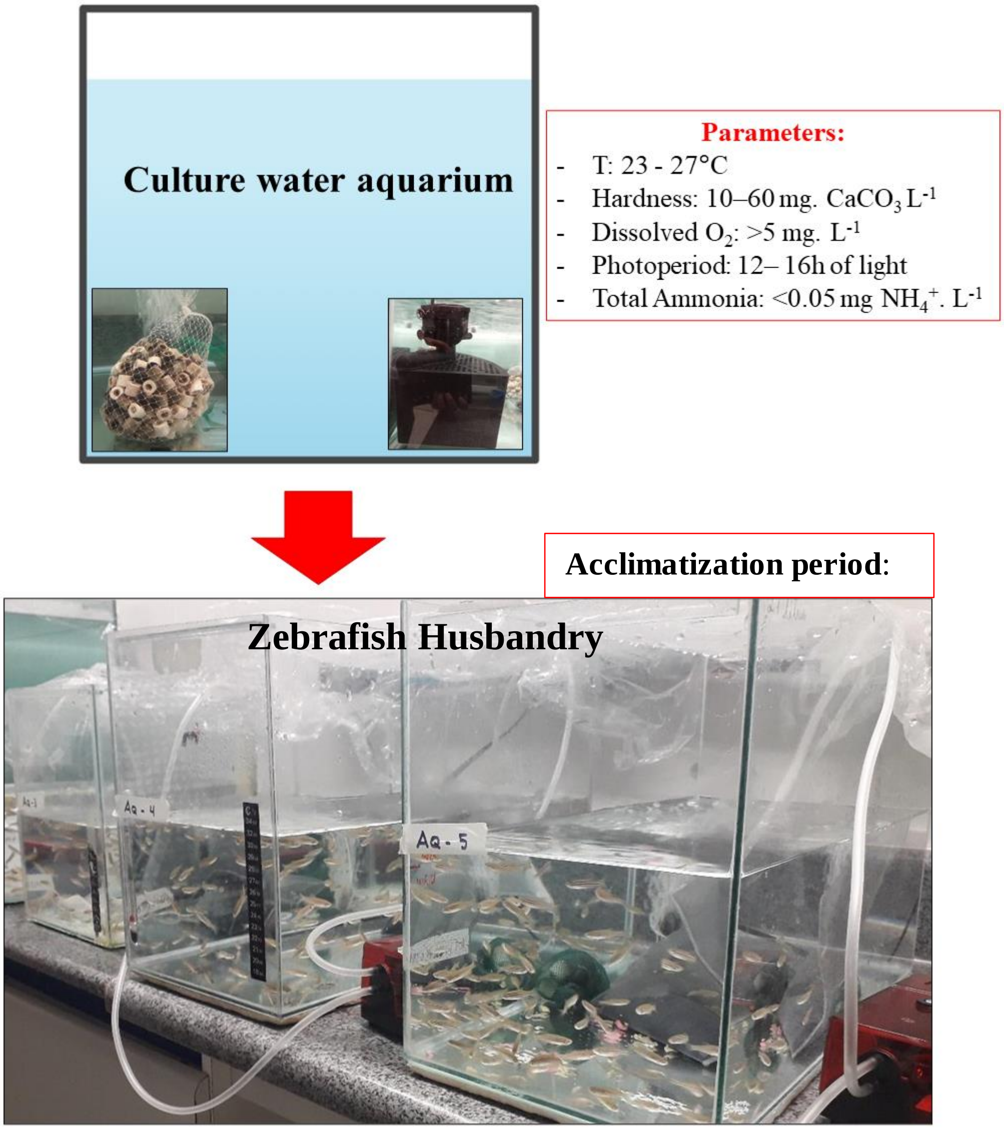
Another particularity that was observed, in the case of some studies, was the association with other pollutants. Greater effects were seen than those obtained when some type of MPs was tested alone. For this reason, it was decided to test a metal in a dose that is theoretically safe for aquatic life (10 µg. L^{-1}), as this is established in Brazilian regulations (CONAMA 357). Thus, the outcomes of this research effectively validated the trends observed through the analysis of biochemical biomarkers associated with oxidative stress and enzymatic activity, even with safe concentration levels. Results confirmed that both the size and the concentrations are factors to consider when talking about the ecotoxicity of PET-MPs and is one more piece of evidence, as well as those studies carried out with other types of MPs, in which this codependency is demonstrated.

As already mentioned, MPs are complex substances, therefore, based on what has been obtained, it is imperative that future research in the field of ecotoxicology focus on amassing a more extensive dataset regarding the ecotoxicological impacts of various types of MPs, including those that have not yet undergone thorough investigation.The different shapes should not be neglected either, fibers are another abundant MP shape in nature, important to be further analyzed in the future. In addition, more environmental concentrations should be tested, since each aquatic environment presents its own levels of MPs. Unfortunately, as microplastic contamination escalates due to the continuous and increasing utilization of plastic materials, understanding the effects of MPs at varying concentrations becomes even more crucial, that is why the scientific community can shed light on the intricate relationships between microplastics and aquatic ecosystems, paving the way for more informed environmental management strategies in the face of mounting plastic pollution.

APPENDIX A

Standard Operating Procedures of the LATHIS Laboratory

A. Preparation of culture water (reconstituted) for D. rerio Husbandry.



ABNT NBR 15088 (2011)

B. NaCl sensitivity test with D. rerio.

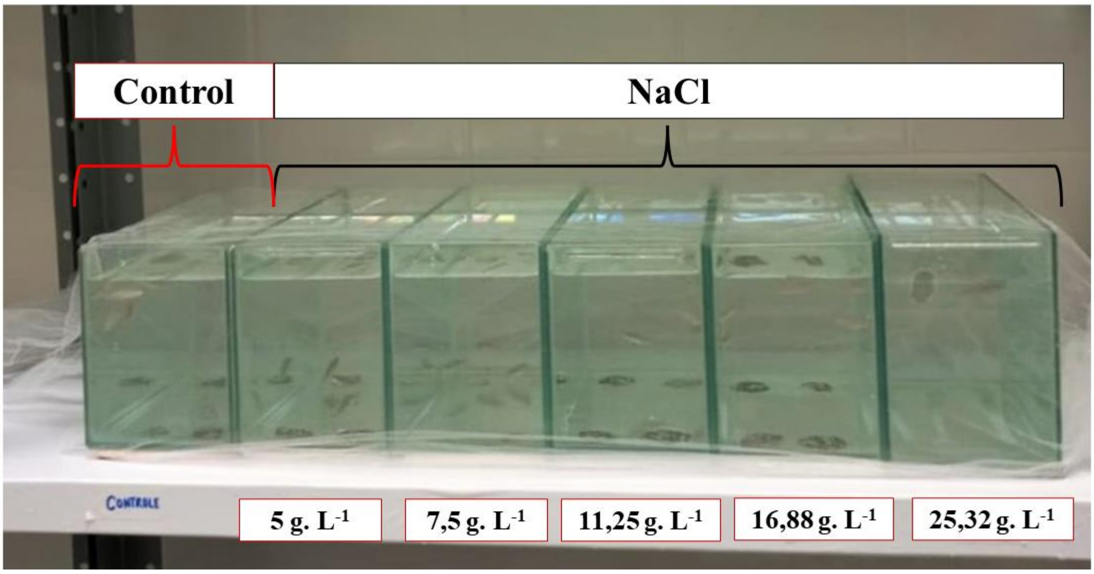


Figure II-1. NaCl sensitivity test with *D. rerio*

Table II-1. Sensitivity test results of *D. rerio* exposed to 5 concentrations of NaCl after 48h.

Dose (g. L^{-1})	**Exposed organisms**	**N° of dead fishes in 48h**
Control (0)	10	0
5	10	0
7.5	10	0
11.25	10	8
16.88	10	10
25.22	10	10

Table II-2. $LC(I)_{50,\ 48h}$ (g. L^{-1}) of *D. rerio* exposed to 5 concentrations of NaCl.

LC(I)$_{\mathbf{50,\ 48h}}$ (g. L^{-1})	***Upper Limit*** (g. L^{-1})	***Lowere Limit*** (g. L^{-1})
9.96	11.04	8.99

APPENDIX B

Supplementary Material from Chapter 2

A. PET microplastics quantification.

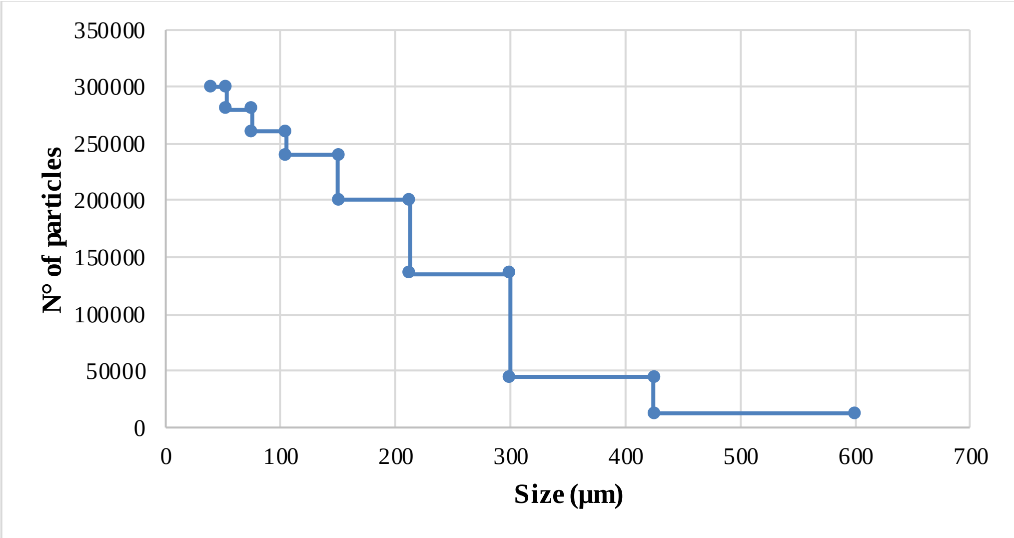


Figure III-1. N° of particles. g^{-1} of each particle size of PET-MPs.

Table III-1. Different units from tested sizes of PET-MPs, elaborated with the microplastics quantification from Figure III-1.

Size	Concentration	
	Particles. L^{-1}	mg. L^{-1}
300-425 µm	983	22
	330	7
53-75 µm	983	3
	330	1

B. PET-MPs isolated experiment.

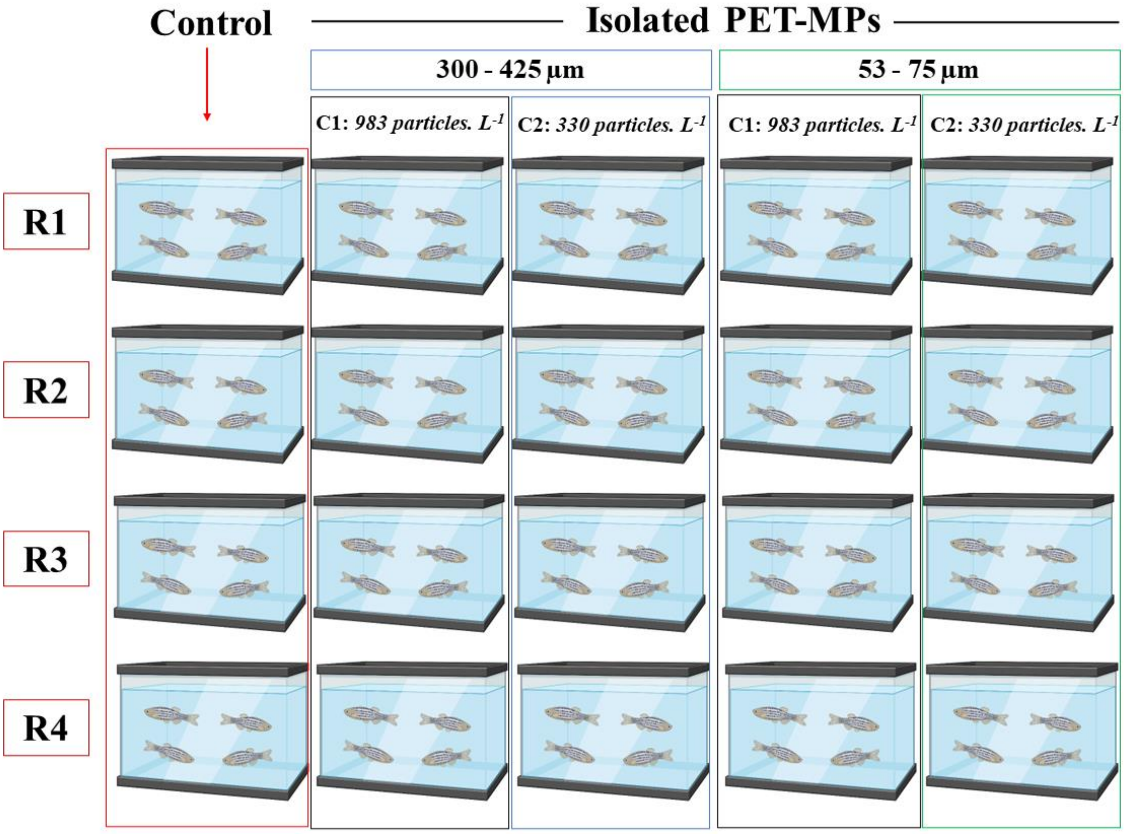


Figure III-2. Experimental design of the PET-MPs isolated assay.

C. PET-MPs and Cd experiment

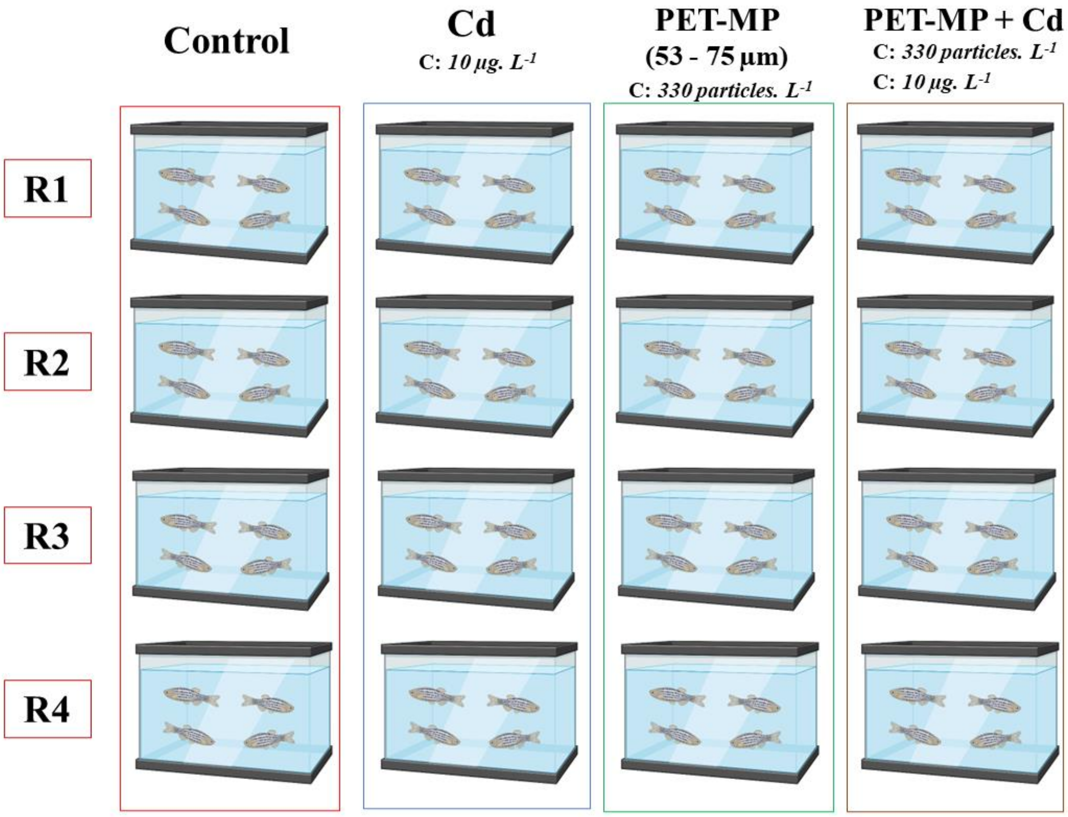


Figure III-3. Experimental design of the PET-MPs + Cd assay.

APPENDIX C

Protocol N°01A/2022 approved for the Ethics Committee for the Use of Animals of the Sorocaba Institute of Sciences and Technology - UNESP

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Câmpus de Sorocaba

CERTIFICADO

CEUA ICTS– Comissão de Ética no Uso de Animais do Instituto de Ciência e Tecnologia de Sorocaba - UNESP

CERTIFICAMOS, que o protocolo registrado sob **o n° 01A/2022**, intitulado:- *"Aspectos ecotoxicológicos do microplástico PET (Tereftalato de Polietileno) em águas doces utilizando-se biomarcadores histológicos e bioquímicos de Danio rerio"* (**sub projeto vinculado ao protocolo 01/2022, aprovado por este conselho em reunião ordinária realizada em 30/08/2022**), sob a responsabilidade da **Profa. Dra Renata Fracacio Francisco** (**UNESP ICTS**), tendo como colaboradores:- **Profa. Dra. Cleoni dos Santos Carvalho** (**UFSCar – Campus Sorocaba-SP**) e **Mestranda Leslie Lissethe Morales Espinoza** (**Programa de Pós Graduação em Ciências Ambientais – PPGCA – Unesp ICTS**), e que envolve a utilização de animais pertencentes ao filo Chordata subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei n° 11.794, de 8 de outubro de 2008, do Decreto n° 6.899 de 15 de julho de 2009 e com as Normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADO** pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA – ICTS – CAMPUS DE Sorocaba-UNESP), em reunião de 30/08/2022.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da Autorização	Outubro/2022 a outubro/2024
Espécie/linhagem/raça	*Danio rerio*
N° de Animais	84 fêmeas; 84 machos = 168 total
Peso/Idade	Aproximadamente 3 meses
Sexo	Machos e fêmeas
Origem	Aquisição comercial
Compromisso/Relatório(s)	Parcial:- Envio para a CEUA:- outubro/2023 Final:- Envio para a CEUA:- outubro/2024

Sorocaba, 01 de setembro de 2022

Darllan Collins da Cunha e Silva:22397267845 Assinado de forma digital por Darllan Collins da Cunha e Silva:22397267845 Dados: 2022.09.01 17:32:11 -03'00'

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