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THE ENVIRONMENTAL IMPACT OF NANOPLASTICS: EVALUATION OF THEIR EFFECTS ON DIFFERENT AQUATIC SPECIES AND FIRST INSIGHTS INTO THEIR OCCURRENCE IN WILD ORGANISMS

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Abstract

Nanoplastics (NPs) are plastic items ranging in size between 1 and 1000 nm which originate from the environmental fragmentation of larger plastic items. The environmental issue of NPs is of significant concern. Despite the increasing research interest, there are still existing knowledge gaps that hamper the realization of a proper environmental risk assessment of NPs. The main limitations are related to the lack of methodologies for the identification and quantification of NP environmental levels and the absence of data regarding the effects induced by the multitude of NP polymers and NP shapes present in the environment. The present thesis addresses some of these gaps by providing new insights into the current lack of knowledge, particularly in the context of freshwater environment.

Regarding the effects of NPs, the available data are limited to those concerning engineered polystyrene (PS) nanospheres. To provide data to help establish whether different polymers exhibit comparable toxicity to PS, this thesis evaluated the sublethal effects of environmentally relevant concentrations of PS-NPs, polyethylene (PE), and polyvinyl chloride (PVC) NPs on the freshwater model species *Daphnia magna*. In contrast to PS, PVC increased the amount of reactive oxygen species and stimulated the swimming behaviour of *D. magna* after 48 hours of exposure. Conversely, PE has induced statistically significant effects only at the biochemical level, without affecting the higher level of biological organisation.

The effects of PVC-NP were subjected to further analysis with the objective of elucidating the role of polymer type in NP toxicity. This was achieved by evaluating the chronic effects resulting from a prolonged exposure of 21 days to PVC and PS NPs. The results demonstrated that chronic exposure to PVC induced morphological, physiological, and reproductive alterations. Of particular concern was the observed delay in reaching sexual maturity, which raises questions regarding the potential for threats to D. magna populations in real ecosystems.

Furthermore, since there is still a lack of evidence regarding the effects of plastic nanofibers in aquatic organisms, this thesis provides an evaluation of the impact of polypropylene (PP) micro- and nanofibres (MNFs) on zebrafish (*Danio rerio*), a freshwater model species. PP-NMFs were derived from the artificial photodegradation of surgical face masks through the application of UV radiation. Environmentally relevant concentrations of PP-MNFs induced morphological alterations in *D. rerio* individuals, with transcriptomic data suggesting neurodevelopment alterations and an energy stress state.

Based on the reported results, the importance of investigating the effect of different NP polymers and different NP shapes has been highlighted, suggesting that the exclusive use of PS nanospheres is insufficient for the comprehensive understanding of the risks to which organisms are subjected in the environment.

The present thesis also focused on the detection of NPs in the environment, through the validation and application of a methodology to detect NPs in chironomids (Diptera, Chironomidae) from glacial fed streams. Chironomids of the genus Diamesa are the primary and even exclusive insect colonizing

glacial environments. Since NP contamination has been recently discovered in these remote systems, the presence of NP in larvae of two chironomid species colonizing two Italian high-altitude glacier-fed streams was investigated.

A recently proposed analytical method developed by the Joint Research Centre (European Commission, Ispra, Italy) was applied and adapted to chironomids. The procedure comprises a combination of enzymatic and oxidative digestion followed by a purification step in ethanol to enable on-chip identification through Raman spectroscopic analysis. The results highlighted the presence of PS-NPs in one of the investigated samples: to the best of our knowledge, this is the first evidence of bioaccumulation of NP in alpine aquatic insects. Furthermore, the work highlighted that the confocal Raman spectroscopy is effective in the analysis of NPs in aquatic biota.

In conclusion, the present thesis has provided useful data that can be considered in future research for a better understanding of the environmental hazard of NPs.

Chapter1

General introduction: The Plastic Era

1.1. Plastic materials

The term ‘plastic’ refers to a wide range of synthetic and semi-synthetic organic polymers that are artificially produced by industrial processes involving petrochemical feedstocks and fossil fuels [1]. The addition of various additives (e.g., plasticisers, flame retardants, dyes, and blowing agents) during the production process enhances or softens the chemical and physical properties of the resulting material, thereby facilitating the creation of plastics with diverse characteristics and functions [2]. According to the International Union of Pure and Applied Chemistry, plastic can be therefore defined as a “polymeric material that may contain other substances to improve performance and/or reduce costs” [3].

Plastic is undoubtedly considered one of the greatest discoveries in human history, becoming an inseparable and integral part of our lives and a defining characteristic of modern society. The widespread use of plastics can be attributed to their distinctive physical and chemical properties, that confer to plastics their ability to be modified to meet specific technical requirements. Plastics are materials with advantageous safety and hygiene properties, with a unique resistance to chemicals, water, and impact that ensures durability and longevity. Furthermore, plastics can exhibit excellent thermal and electrical insulation properties, in addition to low production costs. Notwithstanding, their unique ability to combine with other materials are fundamental factors that contribute to plastic’s diffusion [4]. For these reasons, plastic is the ideal material for a wide range of application in packaging, building, construction, automotive, electrical, agricultural, sports, and leisure end-use markets [5]. In 1950, global plastic production was approximately 2 million tons: in the last seventy years, it exponentially increased reaching nearly 400 million tons in 2022. Over than 5,300 existing plastic polymers [6], around 45% of global production is dominated by three major polymer types: polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC). Besides, other highly commercially demanded plastics are polystyrene (PS), polyurethane (PU), and polyethylene terephthalate (PET).

1.2. Plastic pollution

The improper disposal of plastic can result in significant environmental contamination. Currently, 32% of plastic materials end up directly in the environment [7], with consequent pollution representing a major issue and challenge for ecosystems, governments, and citizens [8].

Natural forces, like wind, rainfall, and ocean currents, constantly influence the distribution of plastics in environments, transporting plastics within the lithosphere, hydrosphere, biosphere, and atmosphere

[9]. Plastic has been detected in every continent in every environment, from the deep ocean [10] to the top of Mount Everest [11]. Moreover, the occurrence of plastic within organisms, both at the bottom [12] and at the top of the food chain [13] is widely documented, with recent evidence even in human placenta [14] and human brain [15]. Concerning the historical deposition of plastic in core sediment, the observations at large temporal scales have revealed a vertical trend in plastic distribution, which can be attributed to the increasing global production of plastic over time [16]. In various regions worldwide, the observed pattern of plastic distribution in core samples is consistent the global plastic production, with higher concentrations over time of the most produced polymers (PE, PP, PVC, PET) [17-19]. The presence of plastic in the environment has been proposed as a significant feature of the Anthropocene, leading to the proposal of the term "Plasticene" to indicate a new stage of the geologic time scale of the Earth [20-22].

In recent years, to compound the already dramatic plastic pollution scenario, the COVID-19 global pandemic caused by the virus "SARS-CoV-2" caused an increase in the environmental concentration of plastic [23]. During the pandemic countries have introduced the obligation to use personal protective equipments (PPE), which are mainly face masks, to enter public areas. Since face masks are mainly made of plastic materials, this demand has resulted in an unprecedented rise in the global production of plastic [24]. The improper disposal of surgical face masks has been quantified in a number of plastic items that ended up in the environment exceeding 1.24 trillion [25].

The negative ecological impact of macroplastics, defined as those plastic items >5 mm, is evident, with well-documented effects (such as the ingestion, intestinal blockage, abrasion, and entanglement) on both aquatic and terrestrial species [26,27]. However, the issue of plastic pollution is not limited only to macroplastics. The environmental scenario is much more complex, as plastic items in the environment are exposed to different environmental reactions (through physical fragmentation or chemical degradation pathways) that over time weather, alter and fragmented plastic into smaller items [28,29]. Plastic materials are therefore categorized depending on the size of the particles: "large microplastic" are defined as any solid plastic particle with any dimension between 1 mm and 5 mm, "microplastic" as those with dimensions between 1 µm and 1000 µm, while "nanoplastics" are those items smaller than 1 µm [30]. Microplastics (MPs) and nanoplastics (NPs) are considered contaminants of emerging concern, as they are widely detected in the environment and can cause adverse ecological and human health impacts [2].

Since MPs and NPs are mainly originated from the environmental fragmentation of larger plastic items, they are considered contaminants with unique properties derived from plastic exceptional complexity and heterogeneity, in terms of shape, size, density, polymer type, and degradation state [31,32]. The heterogeneous transport pathways, highly depending on the characteristics of MPs and

NPs, hinder the prediction of these emerging contaminants' fate in the environment [33,34]. Moreover, the release of toxic additives present in the bulk plastic materials and the adsorption of contaminants in the environment introduce further complexity in the determination of MP and NP toxicity [35]. Furthermore, the size strongly influences the ingestion of MPs and NPs by a wide range of species [36,37]. The resulting complex scenario translates into a multitude of different possible adverse effects on the biota.

To date, various environmental risk assessments (ERA) have been proposed for MPs in the aquatic environment, highlighting an acceptable risk. However, no risk assessment framework has adequately considered the multidimensional nature of plastics [38]. Moreover, it is essential to underline that, despite the currently absence of risk evidence, plastic size distributions will gradually shift towards a greater proportion of smaller particles, increasing the current plastic concentrations and posing a potentially higher risk to the ecosystems.

Concerning the risk associated with NPs, the situation is even more challenging due to the current knowledge gaps in the determination of environmental concentrations and effects that hamper a rigorous scientific investigation for a proper NP-ERA assessment [39].

1.3. The environmental issue of nanoplastics

Even if NPs share with MPs some characteristics, like the high level of complexity and heterogeneity, the transition from the micro- to the nano- scale involves the onset of changes in several properties that profoundly differentiate NPs from MPs [40]. Compared to MPs, NPs, due to their size, are characterized by unpredictable transport pathways, major surface interactions with the surrounding particles and molecules, a higher bioavailability, and faster diffusion rates for the release of additives. Moreover, NPs can easily enter the aquatic food chain through ingestion, and, in contrast to MPs, potentially bioaccumulating in the tissues of organisms from lower to higher trophic levels [41]. For these reasons, NPs are considered potentially more hazardous than MPs [42].

On the other hand, although being in the nanometre size range, NPs are clearly distinguished from engineered nanomaterials (ENMs) (such as fullerenes, silver particles, and TiO_2 nanoparticles) that are defined as nanoscale anthropogenic materials that are designed for specific purposes or functions [43]. NPs, in contrast to ENMs, are unintentionally originated from the environmental fragmentation of larger items and are therefore characterized by a higher heterogeneity [40].

For these reasons, NPs are considered emerging contaminants with unique characteristics. The environmental issue of NP pollution started to receive attention relatively recently [44,45]. The NP-ERA is still in its infancy due to the current knowledge gaps in the determination of effects induced

on organisms and the quantification of the environmental concentrations. The existing knowledge gaps can be ascribed to the lack of reference materials able to reflect the complexity and unique characteristics of NPs [46,47].

1.3.1. Detection of nanoplastics

The determination of the environmental levels of NP pollution is essential to correctly evaluate the impacts of NP on organisms. However, the extraction and detection of NPs in the environment is still an emerging field. The nanometric size of NPs renders the common identification approaches applied for MPs, such as Micro-Fourier Transform Infrared (FTIR) and Raman Spectroscopy, unsuitable. On the other hand, the heterogeneity of NP polymers makes the application of techniques that are typically used for ENMs difficult [40]. For these reasons, the identification and quantification NPs in complex matrices require the development and adaptation of innovative and advanced analytical techniques.

The detection techniques were unable to quantify NPs in environmental matrices until 2019 [48]. The first pioneering study that qualitatively identified the occurrence of NPs in the environment was seven years ago, in the North Atlantic Subtropical Gyre [49]. Over the past year, research has made significant efforts towards the development of extraction methodologies. However, no suitable standardized methodology is currently yet available for the detection of NPs in environmental matrices [46]. To date, only few studies have successfully detected NPs in the environment, in particular seawater [50], freshwaters [51-53], snow [54,55], ice [56], air [57], sediments [58-60], tap water [61], and sea salt [62]. In the aquatic environment, the measured concentrations range from 0.1 to 500 µg/L. However, the disparate methodologies deployed by different studies pose a significant challenge to the effective evaluation and comparison of the available data, hindering efforts to understand the full extent of NP contamination.

1.3.2. Effects of nanoplastics

In 2015, NP pollution has been indicated as “probably the least known area of marine litter, but potentially also the most hazardous” [63]. The first pioneering effect studies were performed on aquatic species testing effect thresholds higher than NP environmentally relevant concentrations [64,65]. The NP number of studies has increased exponentially over the years [66], due to growing evidence indicating that the negative effects of plastic items are closely related to their smaller size [67,68]. The observed effects highlight the potential of NPs to be ingested and accumulated by various

aquatic and terrestrial animals, such as fish, crustaceans, and mammals [69]. In addition to ingestion, other potential routes for the take up of NPs by organisms are dermal absorption [70] and respiration [71]. Moreover, it has been recently shown that maternal transfer can act as an alternative pathway for the uptake of NPs in the offspring [72]. Once in the organisms, NPs can cross the tissues and be progressively bioaccumulated through the food chain, with potential major adverse effects on higher-level consumers [41]. However, the role of particle size on the biological accumulation of NPs remains poorly understood, with conflicting evidence suggesting either higher accumulation in smaller [73] or larger [74] particles.

A growing number of studies report the negative effects of NPs on the individual level [75], such as alteration in morphometric parameters [76], feeding rate [77], swimming behavior [78], and reproduction [79]. The toxicity mechanism of NPs is still not clearly understood. To date, the available evidence suggests the NPs can disrupt cellular membranes, generate reactive oxygen species, damage DNA, blockage the cell pores, disturbance to neurotransmitter levels, affect mitochondria, cause inflammation, and disrupt the endocrine system [41,80].

Despite the reported observations, the evaluation of NP effects on organisms is still in its infancy. To date, there is a lack of reference materials representative of the complexity of NPs that are formed through plastic degradation in the environment. The development of NP reference materials with a variety of shapes, sizes, polymer types, and chemical compositions is therefore essential for a proper NP-ERA [81]. To date, the only polymer that is easily synthesised at the nanoscale is PS as nanospheres [82]. Effective synthesises protocols are still not feasible for the other plastic polymers. For these reasons, almost all NP effect studies have been conducted by testing spherically engineered PS-NPs [83]. This represents a significant constraint as PS is only the fifth most prevalent polymer on the market [5], while the effects of the three most produced polymers (PE, PP, and PVC), from which the majority of NPs will be derived, remain largely unstudied (Figure 1.1).

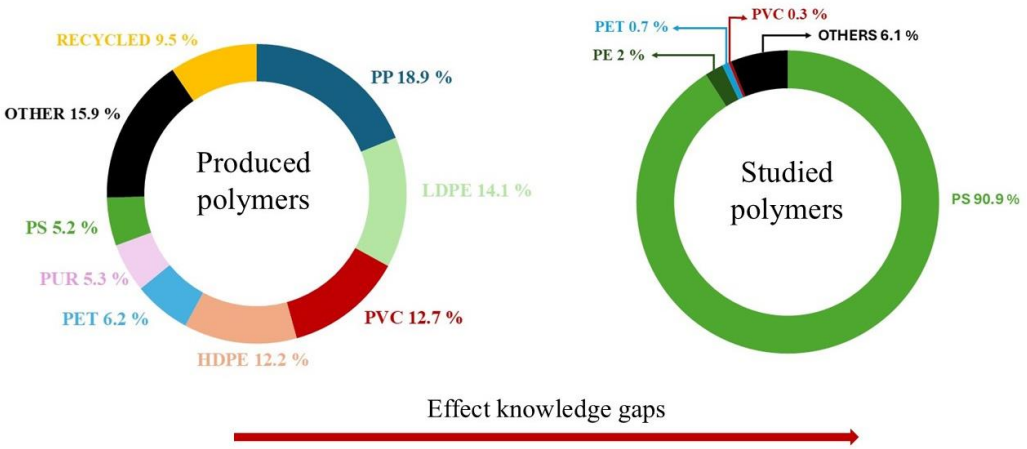

Figure 1. Representation of the different plastic polymer type globally produced types and number of publications that evaluated the toxicity of different NP polymers (data modified from: [5,83]).

Furthermore, since the shape of NPs plays a fundamental role in the onset of plastic toxicity [84], the exclusive use of nanospheres contributes to a deficiency in the environmental realism of the current NP studies, since the available evidence reveals that the predominant shapes of the plastic present in the environment are fibres and irregular fragments [85,86].

1.4. Developments of environmental legislations

The concern for plastic pollution has raised concerns among scientists, regulators, and citizens, highlighting the necessity of global and local governance strategies to mitigate pollution at international and regional levels [87]. In the last years, the global community has advanced regulatory efforts that have resulted in the implementation of national laws or regulations in numerous countries to control the widespread of plastic pollution. By July 2018, 127 countries had implemented legislation to minimize the source of plastics into the environment, by reducing the use of plastic bags [88]. Moreover, 27 countries had either banned or placed restrictions on the production of other specific items, like plastic straws. More recently, in March 2022, the United Nations Environmental Assembly (UNEA), the world's highest-level decision-making body on the environment, adopted measures to ban or restrict the use of MPs in cosmetic products through the resolution “End of plastic pollution: Towards an international legally binding instrument” [89].

In the last years, the European Union (EU), through the collaboration with the European Chemicals Agency (ECHA), demonstrated its commitment to reducing plastic pollution [90]. These endeavours have resulted in proposals for the imposition of limitations on the presence of MPs in a wide range of products, including cleansers, cosmetics, and fertilizers [91]. A particular tentative to reduce plastic pollution is represented by French legislation (Circular Economy Law 2020–105, Article 79) that requires that washing machines sold after 1 January 2025 must have a filter device for MPs to limit the release of MPs during laundry practice. The release of plastic fibers from textiles during laundry practice is an important source of MPs in the environment, with an estimated release of MPs of approximately one-third of primary MPs entering the ocean [92]. Nevertheless, the legislation has not yet been linked to compliance with a MP limit value in surface water or to a reduction in release by a specified percentage [90].

However, although progress has been made, the management of plastic pollution requires indispensable further efforts [93] It is indeed essential that also the nanometric component of plastic pollution, which is potentially the most hazardous, be included in existing regulatory frameworks. It is essential to underline that also the scientific community should provide more detailed and rigorous

evidence on the effects on organism and environmental concentrations of NPs. At the current state, the scientific knowledge on these two aspects of NP pollution is still limited. In the absence of robust scientific data on the potential risks associated with plastics, policymakers encounter significant challenges in formulating and developing precise and effective regulatory frameworks [94].

1.5. Thesis outline

The environmental issue of NPs is of significant concern. Despite the increasing research interest, there are still existing knowledge gaps that hamper the realization of a proper NP-ERA. In particular, the main limitations are related to the identification and quantification of NP environmental levels and to the evaluation of the possible adverse effects on organisms.
The present thesis addresses some of these gaps by providing new insights into the current lack of knowledge concerning the following topics:

- the role of the polymer type and polymer shape in the onset of NP toxicity in different aquatic species;
- the occurrence of NP in organisms of glacial fed streams of remote high mountain environments.

This thesis is composed of seven chapters. The present **Introduction** offers an overview of the state of the art of plastic pollution with a focus on NPs, providing the aims and motivations of the thesis. Thereafter, a collection of five papers (one published review, three research papers already published or submitted, and one in preparation) is presented, while the last chapter provides a synthesis of the overall findings and suggests further recommendations and perspectives.
In **Chapter 2**, a detailed review of the knowledge gaps that hamper the NP-ERA is provided. Firstly, the characteristics of plastic items in the environment are detailed described, followed by a summarization of the data necessary for a proper risk assessment. Subsequently, the review summarizes the progress made in the detection of NPs in biological samples. Furthermore, the evidence of the effects of NPs at relevant environmentally relevant concentrations on biota is explored, through a summarization of the available toxicity data. Finally, a proposal for a tiered framework for NP-ERA and future recommendations are presented. The reviewed literature revealed a critical issue concerning the almost exclusive use of polystyrene (PS) as polymer for evaluating the effects of NPs. This highlights the necessity to conduct exposure with other NP polymers to gain a more comprehensive understanding of the effects of NPs.

In this context, the **Chapter 3** reports a study conducted on engineered nanospheres of PS, polyethylene (PE), and polyvinyl chloride (PVC) to help establish whether different NP polymers exhibit comparable toxicity to PS, and whether PS can be used as a proxy for all other NP polymers. This study involved a collaboration with the Joint Research Centre of the European Commission (Ispra, VA, Italy), where the nanospheres of PE and PVC were synthetised. PE is the globally most produced polymer, constituting approximately 26% of the overall global plastic production, with a massive use for packaging and agriculture. On the other hand, PVC is the third largest globally produced polymer, displaying diverse applications, ranging from beverage bottles, pipes to footwear, and medical equipment. The effects induced by PS-, PE-, and PVC- NPs were investigated on *Daphnia magna*, a freshwater cladoceran considered an excellent model organism for ecotoxicological studies. The exposure bioassays were conducted in the laboratory of the Department of Earth and Environmental Sciences (DISAT) of the University of Milano-Bicocca. To provide useful data from a NP-ERA perspective, individuals were exposed to environmentally relevant levels of NPs. As the selected concentrations are far below those inducing mortality, the effects of NPs were evaluated considering biochemical and behavioral endpoints. Individuals were exposed to a short period of 48 hours, to obtain preliminary toxicity data on the role of the polymer type in the onset of NP hazard.

In **Chapter 4**, to better help elucidate the role of polymers in the onset of hazards to organisms, the chronic effects resulting from a 21-day exposure to PVC and PS nanospheres were evaluated. Chronic exposure more closely reflects the conditions experienced by *D. magna* in the environment than short-term exposure, allowing therefore to achieve useful and relevant data for a more comprehensive evaluation of NP toxicity. *D. magna* individuals were exposed to a low and environmentally realistic NP concentration: the NP harmful effects were evaluated by measuring alterations related to life-history traits of survival, growth, and reproduction.

The studies reported in Chapter 3 and Chapter 4 involved the use of plastic engineered nanospheres. However, the evidence in literature suggest that MPs and NPs are present in the environment mainly as fibers originated from the environmental fragmentation of larger plastic. To date, the knowledge of the potential effect on aquatic biota of plastic micro and nano fibers (MNFs) is still limited. In this perspective, in **Chapter 5** the effects of secondary polypropylene (PP) NMFs originated from the artificially weathering of nonwoven PP fabrics of surgical face masks were evaluated. The secondary MNFs were prepared in the laboratory of DISAT of the University of Milano-Bicocca, while the exposure bioassays were conducted at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of Porto (Portugal). The MNF effects were assessed on zebrafish (*Danio rerio*), a freshwater model organism for toxicity testing of chemicals. The biological scale level of NMF-

induced adverse effects was assessed through the evaluation of several apical endpoints (embryonic development, survival, growth, morphology, behaviour), by testing low (0.2 mg/L), medium (1 mg/L), and high (5 mg/L) environmentally MNF relevant levels. The resulting impact of NMFs on embryonic and larval developmental stages was determined by respectively short-term (up to 6 days) and median-term (up to 15 days) bioassays. Furthermore, insights into the mode of action of NMFs were provided through transcriptomics analyses.

Finally, **Chapter 6** presents a study conducted to assess if Raman spectroscopy could be used to detect NPs in organisms from glacial habitats. As previously reported in the Introduction, the detection and quantification of NPs in the environment is considered a challenging area of research. In this perspective, an analytical method developed by the Joint Research Centre has been applied and adapted to chironomids to investigate the presence of NPs in a remote alpine environment. The aim of the study was to investigate the presence of NPs in chironomid species colonizing high-altitude glacier-fed streams and provide useful data of a potential NP pollution of glacial environments. This part of the thesis was conducted in collaboration with the Joint Research Centre and the Science Museum (MUSE), section of Invertebrate Zoology and Hydrobiology, of Trento (Italy).

Lastly, in **Chapter 7** overall conclusions are drawn from the results described in the previous six chapters, and future recommendations for a proper NP-ERA are suggested.

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Chapter 2

Nanoplastics: Status and Knowledge Gaps in the Finalization of Environmental Risk Assessments

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Abstract

Nanoplastics (NPs) are particles ranging in size between 1 and 1000 nm, and they are a form of environmental contaminant of great ecotoxicological concern. Although NPs are widespread across ecosystems, they have only recently garnered growing attention from both the scientific community and regulatory bodies.

The present study reviews scientific literature related to the exposure and effects of NPs and identifies research gaps that impede the finalization of related environmental risk assessments (ERAs). Approximately 80 articles published between 2012 and 2021 were considered. Very few studies (eight articles) focused on the presence of NPs in biotic matrices, whereas the majority of the studies (62 articles) assessed the lethal and sublethal effects of NPs on aquatic and terrestrial organisms. Whilst many studies focused on nude NPs, only a few considered their association with different aggregates. Amongst NPs, the effects of polystyrene are the most extensively reported to date. Moreover, the effects of NPs on aquatic organisms are better characterized than those on terrestrial organisms. NP concentrations detected in water were close to or even higher than the sublethal levels for organisms. An ERA framework specifically tailored to NPs is proposed.

2.1. Introduction

2.1.1. The plastic era

The term ‘plastics’ is commonly used to describe a wide range of synthetic and semi-synthetic materials, with over 5300 existing polymers [1]. According to the International Organization for Standardization (ISO), plastics are defined as ‘materials that contain, as an essential ingredient, a high-molecular-weight polymer and that, at some stage in its processing into finished products, can be shaped by flow’ [2]. According to the International Union of Pure and Applied Chemistry (IUPAC), plastics are ‘polymeric materials that may contain other substances to improve performance and/or reduce costs’ [3].

Although it is considered a ‘modern’ material, the history of plastic began in the 19th century, with the patent for the first semi-synthetic plastic material by Alexander Parkes. In 1909, the first resin of synthetic origin was produced in the laboratory by the Belgian chemist Leo Baekeland, who patented it under the name of Bakelite. The discovery of this material, which for many years was the most widespread and used plastic, was followed by the invention of a process for the production of polyvinyl chloride (PVC) in 1912 and cellophane in 1913. Between the two World Wars, the plastics industry underwent a remarkable expansion with the invention of nylon, polystyrene (PS), polyethylene terephthalate (PET) (1938), and polypropylene (PP) (1951) as well as the discovery of stereospecific polymerization to form isotactic polypropylene (1954), for which Giulio Natta and Karl Ziegler jointly won the Nobel Prize in Chemistry in 1963 [1,4]. Since then, plastic has become an irreplaceable material in everyday life. Since the 1950s, global plastic production has grown rapidly, as shown in Figure 2.1. In 2020, nearly 370 million tons of plastic were produced globally, of which 55 million tons, due to the strong relationship between plastic production and demand, were produced in Europe alone. Plastic materials are widely used because of their excellent physical and chemical properties (e.g., malleability, durability, lightness, corrosion resistance, and electrical insulation), which render them ideal for a wide range of applications [5].

The most widely used plastic polymers (with an average global production of more than 5% of the total) are polypropylene (PP), polyethylene (PE), PVC, PET, polyurethane (PUR), and polystyrene/expandable polystyrene (PS/EPS) (Table 2.1). These plastics are principally employed in food packaging, automotive parts, and household goods. PVC and PUR are mainly used in the building and construction sector and in the automotive industry, whilst PET is principally utilized in drink bottles and textile applications [6]. PS is used in packaging and insulation materials. Together, these polymers represented over 80% of all plastics used in Europe in 2020.

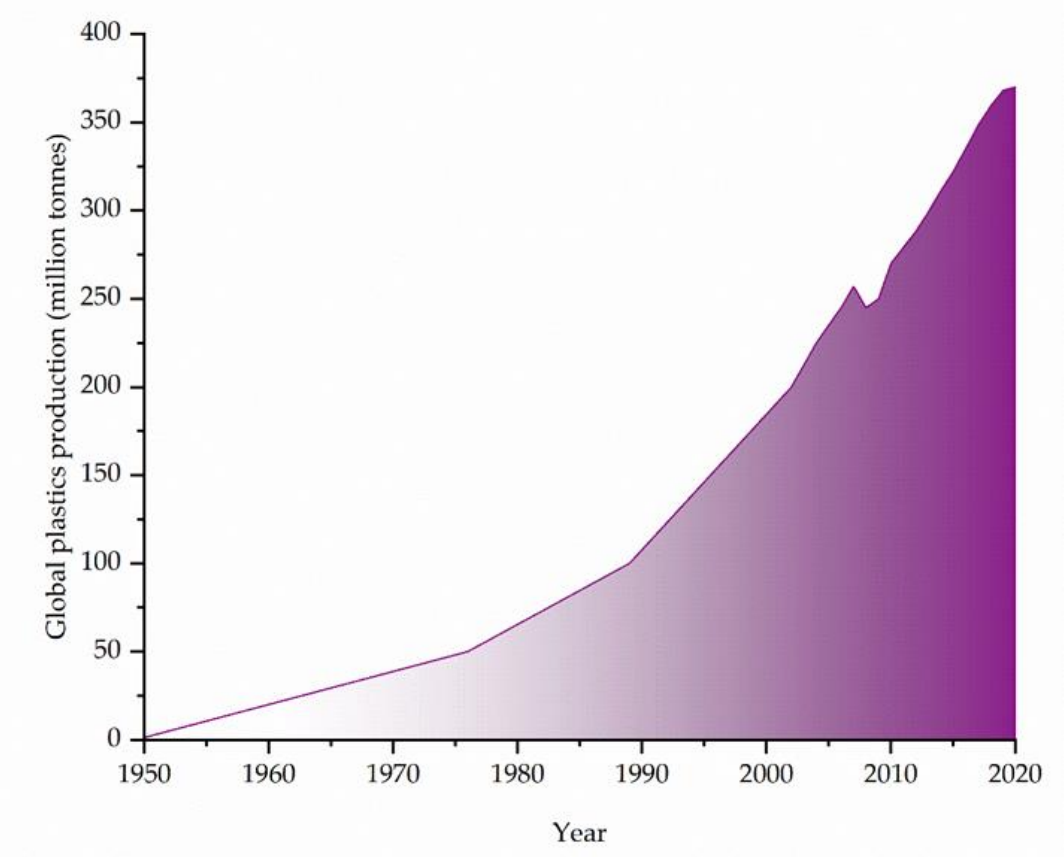

Figure 2.1. Global plastic production from 1950 to 2020 [5].

Table 2.1. European plastic demand in 2020. The column 'Mt' presents the annual demand of plastic expressed in million tons (total: 49.1 Mt). For further details, please refer to [5]. EPS: expandable polystyrene; PMMA: polymethylmethacrylate.

Plastic Polymer	Mt	%	End-Use Market
PE	14.9	30.3	Food packaging, bulding, construction
PP	9.7	19.7	Food packaging, automotive
PVC	4.7	9.6	Bulding, construction
PET	4.1	8.4	Food packaging
PUR	3.8	7.8	Bulding, construction, others
PS/EPS	3.0	6.1	Food packaging, bulding, construction
Other plastics (e.g., epoxy resins, PMMA)	8.9	18.1	Bulding, construction, automotive, others

Despite being designed to last for a long time, plastics are used only briefly, particularly in the packaging sector. According to the World Bank estimate, in 2016, the amount of plastic waste generated was 242 million metric tons in the world, of which 57 million metric tons were produced in Asia, 45 million metric tons in overall Europe, and 35 million metric tons in North America. As the global production in the same year was 336 million metric tons, approximately 70% of the produced plastic was not recycled [4] and possibly entered the environment.

2.2.2 Characteristics of Plastic Debris Present in the Environment

The physical and chemical properties of plastics make them a unique and excellent material. However, these properties do not allow decomposing organisms to digest plastic polymers (e.g., because of their rigid structure and high molecular weight) [7]. Nonetheless, once released into the environment, plastic materials are exposed to different environmental reactions (through physical fragmentation or chemical degradation pathways), which decrease their molecular weight and cause chemical changes [8]. Following these processes, plastics are fragmented into smaller particles [1,8]. The size of plastic debris plays a fundamental role in ecological dynamics, representing one of the main factors driving the environmental interaction between plastics and biota [9–11]. As pointed out by Mitrano et al. [12], size contributes to the characterisation of the physical and chemical properties of plastics, thus affecting their mobility, environmental and biodistribution, and interaction with organisms.

The classification of plastic debris based on size presents ambiguous terminology owing to the lack of universal definitions for different size ranges [9,13]. Typically, the classification of microplastics (MPs) as particles <5 mm is accepted [14]: the upper 5 mm limit is biologically significant because a wide range of small particles of this size (<5 mm) can be ingested by organisms [1]. However, the upper size limit of nanoplastics (NPs) remains controversial, and an arbitrary size cut-off of 100 or 1000 nm is selected. The upper 100 nm limit is based on the definition of nanomaterials proposed by the US National Nanotechnology Initiative [11]. NPs present different properties from classic nanomaterials because they are not intentionally produced in the nanometric size range. Furthermore, both in terms of methodologies required for their detection and their properties and toxic effects, plastic fragments are significantly different when they cross the size scale from micrometers to nanometers [11]. As proposed by Gigault et al. [13], NPs should, therefore, be considered as ‘the plastic particles that present colloidal behaviour within the size range of 1 to 1000 nm’. According to

this classification, which covers the entire nanometric size range in the definition of NPs, plastic particles are defined as macroplastics (>5 mm), MPs (1–5 mm), and NPs (1–1000 nm) (Figure 2.2).

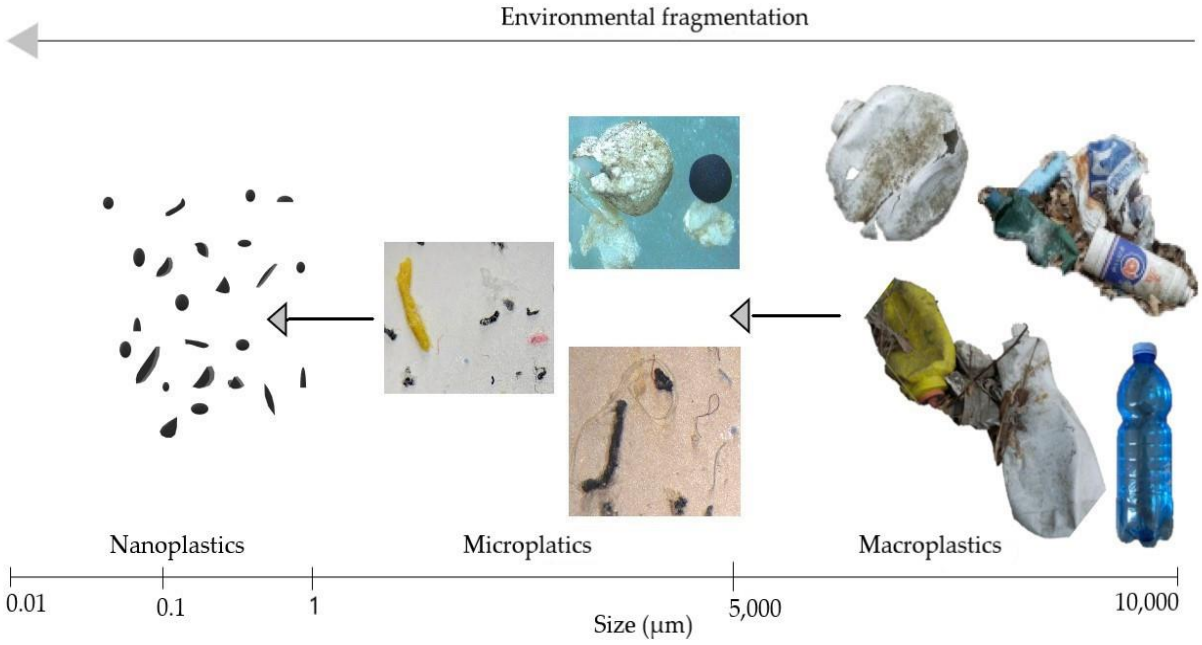

Figure 2.2. Graphical representation of plastic debris of macro, micro, and nano size (please note that the images representing plastic particles are not in scale).

2.1.3. Environmental Risk Assessment (ERA) of Plastic Debris

ERA is a rigorous process for quantifying how likely an ecosystem is to be impacted as a result of exposure to one or more environmental stressors, such as xenobiotics. Risk is assessed by comparing the exposure data with ecological effects to evaluate the likelihood of adverse impacts associated with exposure to a given stressor. Therefore, risk assessment depends on the environmental exposure of organisms to a certain contaminant, and its effects result from the degree of exposure [15].

Scientific interest in the potential toxic effects of NPs on living organisms has significantly grown in recent years [16], suggesting that these contaminants are potentially more harmful than MPs [11,17–20]. However, our understanding of NPs remains at early stages [21], and there is a paucity of data regarding both the environmental exposure and toxic effects of NPs on biota at realistic environmental concentrations [22,23]. This knowledge gap hampers the finalization of risk characterisation of NPs [12].

Even the risk of MPs is difficult to estimate owing to many knowledge gaps [20]. Indeed, although MPs have been studied for a longer time and much more data are available on them than NPs, only a

few articles on the risk assessment of MPs have been published. For instance, the pioneering studies by Burns and Boxall [24] and Everaert et al. [25] have characterized the joint risk of MPs in freshwater and marine systems. More recently, the risks associated with MPs in freshwater and marine waters have been assessed separately [26,27]. Furthermore, Jung et al. [28] proposed a marine MP risk assessment method that considers differences in size and shape. The outcomes of these assessments highlight an acceptable risk associated with the presence of MPs in water compartments, whereas environmentally relevant concentrations of MPs in soil pose a considerable risk to soil biota [29].

2.1.4. Aims of the Review

The present review aims to provide a useful reference for better understanding the phenomenon of NP pollution in the environment. This manuscript not only aims to summarize and analyze the most recent studies on NPs, but also proposes a new tailored ERA procedure scheme. Since current NP risk assessments prioritize the identification of environmental exposure and relative effects on biota [12,20,22,23], we discuss and summarize the following three aspects of these priorities in the present review:

(a) *Exposure assessment: detection of NPs in environmental samples*
Developing suitable and reliable analytical methods for quantifying the environmental occurrences of NPs is pivotal. As reported by Besseling et al. [30], the detection techniques were not capable of identifying and quantifying NPs in environmental matrices until 2019, and research is rapidly progressing towards the development of novel methodologies suitable for these purposes [31]. A possible approach for the detection of NP exposure in the environment is the use of biomonitors, which can overcome some analytical limitations in NP extraction due to the potential of these contaminants to bioaccumulate in organisms [32]. From this perspective, different approaches have been developed to extract and quantify NPs from biological samples. This review summarizes progress in the detection of NPs in biological samples, detailing the current status of the characterisation of these contaminants in biological samples.

(b) *Effects assessment: effects of NPs at the relevant environmental concentrations on biota*
Assessing the possible modes of action of NPs and quantifying their adverse effects are some of the main knowledge gaps in ERAs. As previously mentioned, ecotoxicological studies on NPs should be

performed at realistic environmental concentrations to determine effective thresholds for comparison with existing environmental levels. In this context, the present review analyses data on the adverse effects of NPs on biota at environmentally realistic concentrations ($\mu g \cdot L^{-1}$). Briefly, articles in literature are compared to summarize the different polymer types, sizes, concentrations, model organisms, and effects.

(c) *Proposal for the harmonization of NP ERA*

We provide suggestions and propose strategies for NP ERAs, highlighting the current knowledge gaps and critical points and emphasizing the priorities for harmonizing future studies.

2.2 Materials and Methods

Literature was searched using the key-words ‘nanoplastics’ combined with ‘detection’ and ‘biological samples’, ‘effect’, and ‘environmental concentration’ or ‘risk assessment’. The Minerva (University of Milan) and Prometeo (Milan-Bicocca University) datasets were used for this research, with data covering the timeframe until December 2021. Only peer-reviewed articles were included. As previously stated, there are no universal definitions of different range sizes. To harmonize the collected data, only studies reporting the analyses of NPs with dimensions within the range of 1–1000 nm were included. In the first section of this review, we focus on the detection of NPs in environmental samples. A total of 16 articles were selected: eight demonstrating the occurrence of NPs in abiotic matrices and remaining regarding the development of appropriate methodologies for NP extraction from biological samples. In the second section, we overview the effects of NPs on organisms. For this, a further criterion for literature selection was applied: the maximum concentration tested should be below 100 $\mu g \cdot L^{-1}$, representing an order of magnitude considered realistic in the environment. Finally, 62 articles were included.

2.3 Exposure Assessment: Detection of NPs in Environmental Samples

Very limited scientific literature (only eight papers) regarding the occurrence and concentration of NPs in the environment is available.

The detection of these contaminants in complex matrices is a real challenge. As such, the nanometric size of NPs renders the common extractive approaches applied for MPs unsuitable [33], and the heterogeneity of NP polymers makes the application of techniques that are typically used for

engineered nanomaterials (ENMs) difficult [11]. Contrary to ENMs, which present a uniform composition owing to their intentional production as nanoparticles, NPs contain a mixture of different polymer types, sizes, and shapes, because they are derived from the unintentional fragmentation of larger plastic items dispersed in the environment. Moreover, the presence of a large variety of additives in the plastic materials of origin hinders the identification of polymer [11,33,34]. Currently, there are no straightforward methodologies for the extraction, identification, and quantification of NPs in the environment [35]. To the best of our knowledge, only eight studies have successfully extracted these contaminants from abiotic field samples. These studies have documented the occurrence of nanoscale plastic particles in seawater [36], rivers [37], snow [38,39], air [40], soil [41], sand [42], and tap water [43]. As shown in Table 2.2, only a few studies have simultaneously reported the concentration and relative polymeric types of NPs present in samples [37,38,43]. The measured environmental concentrations of NPs are extremely low ($\mu g \cdot L^{-1}$), posing significant analytical challenges for their detection in the environment. A possible strategy to overcome some of these limitations is to consider the use of biomonitoring. Bioindicators are living organisms that provide information on environmental quality [44]. More precisely, exposure indicators (e.g., bioaccumulation of xenobiotics in tissues) are considered useful tools to obtain data on environmental pollutants [45].

Table 2.2 List of the studies that successfully detected NPs in environmental abiotic matrices (updated to December 2021). PA: polyamide; PO: polyolefins.

Samples	Concentration	Polymer Type	References
Seawater	-	PET, PS, PE, PVC	[36]
River water	1.92–2.82 μg/L	PS	[37]
Snow	46.5 μg/L	PET, PP	[38,39]
Air	-	PET, PS	[40]
Soil	-	PVC, PS, PE	[41]
Sand	-	PVC, PS	[42]
Tap water	1.67–2.08 μg/L	PVC, PS, PA, PO	[43]

Owing to their capacity to accumulate nanosized particles, organisms may accumulate higher concentrations of NPs in the biological matrix than in other complex matrices [32]. Following uptake, NPs, especially the small ones (<100 nm), can penetrate the biological membranes [46] and potentially bioaccumulate in tissues or organs, leading to their transfer along trophic chains. This assumption is supported by experimental evidence [47]. Briefly, Zhou et al. [47] reported for the first time that the concentration of NPs in aquatic organisms is approximately 1 $\mu g \cdot g^{-1}$. Therefore, we believe that the use of biomonitoring offers significant advantages. Specifically, once the most suitable biomonitor is selected for different matrices, the extraction methodologies for different organisms only require optimization at the digestion step. As with any novel approach to a challenging issue, biomonitoring presents critical points. Assuming that NP concentration can be measured in biomonitor tissues, composition of the medium to which organisms are exposed must be estimated. For classical contaminants, the conventional approach is to achieve medium exposure through partition coefficients (e.g., K_{ow}). However, the factors driving NP bioaccumulation and/or bioconcentration remain largely unknown. Therefore, the correct approach to calculating NP concentrations in the environmental matrix in which the organisms live remains unknown. Notwithstanding, a promising approach to model the mass balance of ingestion and loss processes of MPs has been recently reviewed [30], highlighting the possibility of modelling NP uptake and release in a similar manner. To calibrate and validate models, experimental determination of concentration is pivotal; consequently, a sound methodology is essential.

To the best of our knowledge, eight studies to date have proposed different methodologies to achieve this goal [32,47–53] (Table 2.3). All these studies utilized non-properly ‘environmental’ organisms, since all samples were collected from local markets [32,47,48] or directly from farms [49,50]. To test the efficiency of the proposed methodologies, tissue samples were spiked with nanoPS. Zhou et al. [47] verified the suitability of their method in *Tilapia* sp. and, as mentioned previously, successfully proved the environmental occurrence of NPs in three different species, at concentrations ranging from 0.09 to 0.78 $\mu g \cdot g^{-1}$.

The application of such methodologies can aid better comprehension of NP exposure in the environment. Nonetheless, the detection of NPs in biological samples is limited by certain analytical issues. From this perspective, a harmonized methodology of biological extraction is required to favor intercomparisons amongst studies. In the following sections, innovations reported in studies listed in Table 3 are elaborated, comparing different proposed extraction methods, and highlighting their strengths and critical points.

Table 2.3. List of studies that developed novel methodologies for the detection of NPs in biological samples.

Organism	Target	Polymer	Size (nm)	Ref.
Mollusk, Crustacean, Fish	Muscle	PS, PMMA	100	[47]
Mollusk (oyster) Mammal (C57BL/6 mice)	Whole organism Gut, liver, kidney	PS	70 70	[48]
Fish (*D. labrax*)	Muscle	PS	100	[49]
Bird (non-specified)	Eggshell	PS	60, 200, 600	[50]
Mammal (*R. norvegicus*)	Blood	PS	100, 200, 500	[51]
Tunicate (*R. ciona*)	Whole organism	PS	100	[32]
Mollusk (*M. galloprovincialis*)	Whole organism	PS	100, 500, 1000	[52]
Mollusk (*M. edulis*)	Stomach	PS	49	[53]

2.3.1 Digestion of Organic Matter in Biological Samples

Steps required for the extraction of NPs from biological samples can be summarized as follows: sampling, pre-treatment, digestion, preconcentration, separation, identification, and quantification [35,47].

Regarding sampling methods, please refer to a recently published review on this topic [54]. The complexity of the biological matrix makes the detection of NPs challenging. Plastic particles must be isolated and separated from the organic matter of biological samples, which could lead to interference in the subsequent steps of extraction. In this perspective, digestion is a key step which should be suitable to remove the organic matter, preserving, however, the properties and quantity of NPs

[33,55]. Two different approaches are commonly adopted: chemical degradation and enzymatic digestion [16,35]. The first approach involves the use of different chemicals for acid (e.g., HCl and HNO_3) or alkaline (e.g., NaOH and KOH) digestion, whilst the second utilizes specific enzymes (e.g., proteinase K, papain) that degrade the organic matrix. Chemical degradation enables efficient removal of the background matrix and is a cost-effective approach. However, acid treatment has recently been questioned because it tends to destroy and cause aggregation of NPs polymers [16,49]. Therefore, an alkaline approach, which is less invasive, is recommended [51]. Zhou et al. [47] obtained satisfactory results using this approach to digest the organic tissues of fish samples. When comparing the efficiency of two different alkaline reagents, tetramethylammonium hydroxide (TMAH) and sodium hydroxide (NaOH), TMAH showed a greater recovery without NP aggregation. In another recent study, Muhammad et al. [56] digested the gut and intestinal contents of insect larvae following the digestion protocol proposed by Zhou et al. [48].

Meanwhile, the enzymatic approach is considered a valid alternative to alkaline degradation. Although this approach incurs higher costs [54], it is non-destructive and avoids particle aggregation [57]. Correia and Loeschner [49] compared acid degradation (HNO_3) with enzymatic digestion (proteinase K) in muscle tissues of fish samples and demonstrated that the former approach led to NP aggregation and subsequent hindrance in the extractive steps, whilst the latter could overcome these analytical issues. Furthermore, a recently proposed enzymatic digestion protocol [57] has shown satisfactory results for NPs extraction from biological matrices [32,52].

2.3.2. Separation, Identification, and Quantification of NPs in Biological Samples

Following digestion, it is necessary to isolate and separate particles in the nanometric size range; identify their effective sizes, shapes, and concentrations, and confirm the effective plastic nature of the extracted polymers through chemical characterisation. Simultaneous achievement of these results remains a huge challenge because of the lack of an efficient methodology for the extraction, identification, and quantification of NPs. The main steps adopted in the proposed methodologies for the detection of NPs in biological samples are summarized in Table 2.4.

2.3.2.1. NP Precipitation

Recently, two different methodologies have been proposed to separate and isolate NPs present in biological samples [47,48] by exploiting the tendency of NPs to aggregate and co-precipitate with suspended materials. Typically, NPs tend to be dispersed in the medium, since they are dominated by

Brownian motion; however, under specific conditions of pH and organic matter concentration, they associate with the material present in the solution, with their consequent sedimentation [48,58].

Table 2.4. Methods for the detection of NPs in biological samples. TEM: transmission electron microscopy; SEM: scanning electron microscopy; py–GC–MS: pyrolysis–gas chromatography–mass spectrometry; CSE, coagulation-sedimentation extraction; AF4, asymmetrical flow-field fractionation; DAD, diode array detector; MALS, multiangle light scattering detector; CRM, confocal Raman spectroscopy; EDX, energy-dispersive X-ray; FIB, focused ion beam.

Digestion	Separation	Quantification	Identification	Ref.
Alkali (TMAH)	Ethanol-precipitation	TEM	py-GC-MS	[47]
Alkali (KOH)	CSE	-	py-GC-MS	[48]
Enzimatic (Proteinase K)	AF4	AF4-MALS	-	[49]
Acid (HCl) Alkali (TMAH)	AF4 Ultracentrifugation	AF4-MALS	-	[50]
Alkali (KOH)	AF4	AF4-DAD-MALS TEM	-	[51]
Enzimatic (Papain)	AF4, Ultrafiltration Chip trapping	AF4-DAD-MALS SEM	CRM SEM-EDX	[32]
Enzimatic (Papain) FIB	Microcavity-size selection	SEM	CRM SEM-EDX	[52]
Alkali (NaOH, KOH)	Centrifugation	Microplate fluorescence reader	-	[53]

Zhou et al. [47] utilized the high binding affinity of NPs to proteins to extract them from biological samples through ethanol precipitation. The authors successfully validated the effectiveness of the proposed method by spiking the digested muscle tissue samples from *Tilapia* sp. With 2 $\mu g \cdot g^{-1}$ of PS and PMMA of different sizes (50, 100, and 500 nm) and obtained high recovery rates for all polymer sizes. Moreover, using pyrolysis–gas chromatography–mass spectrometry (py–GC–MS), they reported excellent limits of detections (LODs) (0.03 and 0.09 $\mu g \cdot g^{-1}$ for 100 nm PS and PMMA, respectively). Further, by adopting this methodology for environmental samples, the authors succeeded in detecting nanoPS (0.093–0.785 $\mu g \cdot g^{-1}$) in three different aquatic species.

Gao et al. [48] proposed a protocol based on coagulation–sedimentation extraction (CSE) between NPs and diatomite. By spiking the tissue samples from oysters and mice with nanoPS (70 nm), microPS (2 µm), and diatomite (7 µm), the authors successfully isolated and extracted NPs. As opposed to MPs, NPs tend to bind diatomite and precipitate upon centrifugation; thus, NPs can be isolated from MPs and the remaining suspended material with high recovery rates (95%). Finally, the authors chemically characterized the extracted particles using py–GC–MS and obtained an LOD of 0.012 $\mu g \cdot g^{-1}$.

2.3.2.2. AF4 and Chip Trapping

Various studies have adopted an approach based on asymmetrical flow-field fractionation (AF4) to separate NPs from spiked biological samples [49–52]. AF4 allows the fractionation of nanoparticles based on their hydrodynamic size, which upon combination with detectors, such as diode array detector (DAD) and multiangle light scattering detector (MALS), provides additional information on particle size and concentration [31,35].

The first studies that adopted this approach in biological samples performed AF4-MALS to separate NPs from spiked digested fish tissue [49] and bird shell samples [50]. Luo et al. [51] recently proposed an AF4-DAD-MALS approach to separate and detect nanoPS (from 30 to 500 nm) in spiked blood samples of rats (*Rattus norvegicus*), demonstrating the feasibility of this methodology to separate and quantify nanoPS in biological samples, with satisfactory results for >100 nm PS.

In a recent study, Valsesia et al. [32] proposed a methodology for separating biological sample fractions with the same particle size. The authors spiked tunicate samples (*Ciona robusta*) with nanoPS (100 nm) and subjected them to AF4-DAD-MALS following enzymatic digestion. Subsequently, through ultrafiltration, the AF4-derived fractions were concentrated to a small volume and placed on a chip. Exploiting the tendency of nanoparticles to aggregate as the sample dries, NP clusters were grouped in a small area of the chip, achieving a sufficient signal for analysis using

confocal Raman spectroscopy (CRM). By adopting this procedure, the authors succeeded in the chemical characterisation of nanoPS, combined with the estimation of NP concentration using scanning electron microscopy (SEM) coupled with an energy-dispersive X-ray (EDX) analyzer. The proposed method yields concentration data that are comparable to environmental levels.

In another study, Valsesia et al. [52] proposed a similar methodology that involves drying a sample on a chip with arrays of cavities of different sizes. Owing to the peculiar structure of the chip surface, single nanoparticles fall into the cavities according to their size, allowing the formation of small NP aggregates. The precise positioning of NPs of a certain size in the corresponding nanocavities enables their examination using SEM and spectroscopy (CRM). By testing the methodology on mollusk tissues (*Mytilus galloprovincialis*) exposed to 100 nm nanoPS, the authors succeeded in isolating nanoPS using a chip with 300 nm pores on the surface and finally characterized the isolated particles using CRM.

Moraz and Breider [53], improving the previously proposed methods [59,60], proposed a fluorescence-based approach for the detection of nanoPS in biological tissues. The method uses a fluorescent probe (a particular molecular rotor) that can conjugate with NPs. However, owing to the complexity of the investigated biological matrix (*Mytilus edulis* tissues), the authors did not obtain satisfactory results; nonetheless, the methodology presents the feasibility of detecting and quantifying NPs in biological samples.

2.3.3. Comparison Amongst Different Approaches

By comparing the methodologies presented in the literature, attempts can be made to fill this gap for successful extraction, identification, and quantification of NPs in biological samples.

The extraction of NPs from biological samples requires an efficient digestion step that allows for the removal of organic matter without damaging or causing the agglomeration of NPs. From this perspective, enzymes (e.g., papain) and alkaline reagents (e.g., TMAH) appear to be the most promising. Once digestion is performed, several methodologies can be adopted. Currently, however, no straightforward approach that allows simultaneous collection of data on concentrations and polymer characterisation is available.

The precipitation approach, followed by py–GC–MS, is relatively simple, yields an excellent LOD (e.g., 0.03 $\mu g \cdot g^{-1}$ [47]) and a high recovery rate (>85% [47,48]), and allows for the detection of small NPs (e.g., nanoPS 70 nm, [48]). However, although the application of py–GC–MS allows for covering the entire nanometric range, it is a destructive method that makes it impossible to

simultaneously obtain information on the physical properties of individual particles [35]. Furthermore, because this methodology involves size-selective precipitation, handling real samples containing NPs of different sizes may be difficult. From this perspective, the use of AF4 as a separation step may be an effective alternative for obtaining fractions with NPs of the same size.

Furthermore, methodologies based on the evaporation of sample droplets on specific chip surfaces, followed by CRM analysis, are non-destructive. Using these methods, the number of particles can be directly counted through SEM and their polymeric type can be directly confirmed through Raman spectroscopy, with satisfactory LODs. However, these approaches require advanced instrumentation (e.g., functionalized chips) and additional analytical steps, such as an added purification step involving focused ion beam (FIB) [52], ultrafiltration [32], and EDX analysis, which may result in greater reproducibility.

Owing to the complexity of NP pollution, formulation of an extraction protocol for these contaminants from biological samples warrants effort. However, we believe that the scientific community is moving in the right direction, with significant progress being made towards better understanding the effective concentrations and levels of NPs in the environment.

2.4. Effect Assessment: Evidence from Laboratory Experiments

The number of ecotoxicological studies on the adverse effects of NPs has considerably increased in recent years. Whilst many of these studies have focused on aquatic biota, few studies have considered the effects on terrestrial organisms. Moreover, the currently available literature on the effects of NPs is limited to nanoPS. To determine the ecotoxicological risks of NPs, an assessment of exposure should be compared to an assessment of effect thresholds. Here, we present an overview of the effects of NPs at an individual level and at the lower levels of the biological structures and functions.

2.4.1. Lethal Effects on Aquatic Species

Only a few studies (11 articles to date [61–71]) have reported the classic toxicological endpoint of survival (EC_{50}: effect concentration at which 50% of the exposed organisms are affected) following exposure to NPs, and these studies focused almost exclusively on PS, which is currently the most studied polymer. Of note, the exposure times vary amongst different tests, and often, the age of the tested organisms is not reported, particularly for *Daphnia* sp. This may increase the uncertainty and variability observed in the collected data (Figure 2.3). The concentrations of NPs affecting individual

survival (EC_{50}) are similar for organisms belonging to different taxa and/or phyla (Anostraca, Copepoda, Echinodermata, Rotifera, and Chlorophyceae) and are representative of diverse aquatic ecosystems (marine or freshwater). In particular, for marine crustaceans (*Artemia franciscana*, *Artemia salina*, and *Tigriopus japonicus*), echinoderms (*Paracentrotus lividus*), rotifers (*Brachionus plicatilis*), and chlorophytes (the marine alga *Dunaliella tertiolecta* and the freshwater alga *Pseudokirchneriella subcapitata*), EC_{50} values range from 0.54 to 13.0 $mg \cdot L^{-1}$. Conversely, higher values have been reported for freshwater crustaceans (*Daphnia magna*, *Daphnia pulex*, and *Macrobrachium nipponense*), with EC_{50} between 15.7 and 80 $mg \cdot L^{-1}$ for cladocerans and a much higher value of 396 $mg \cdot L^{-1}$ for decapods (*Macrobrachium nipponense*).

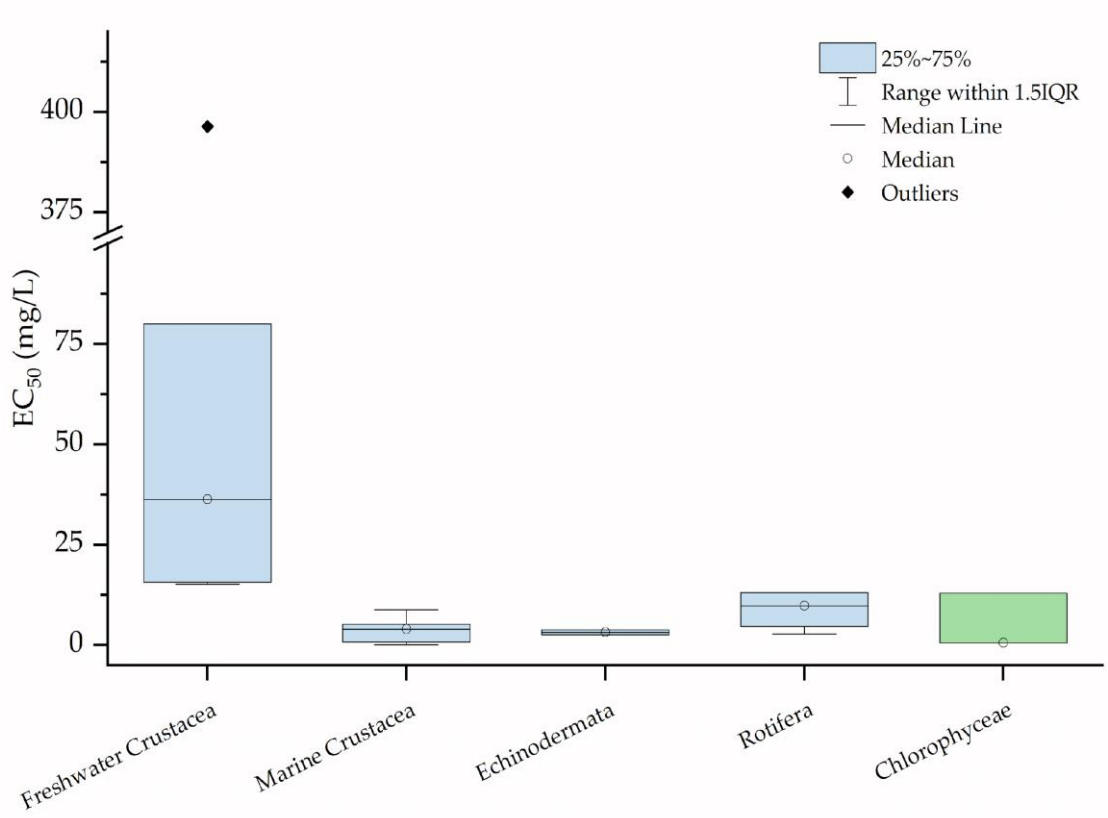

Figure 2.3. Lethal values (EC_{50}) expressed in $mg \cdot L^{-1}$ of PS for different freshwater and marine taxa.

These results indicate that cladocerans are less sensitive to nanoPS than other taxa. This is particularly relevant because *Daphnia* sp. is used as a model organism in toxicological screening. From an ecological perspective, these results may provide unclear information on the sensitivity of natural zooplankton communities [72] and a proper assessment factor (AF) should be considered in risk characterisation to protect the entire aquatic community.

To describe the sensitivity of a given number of species to a xenobiotic, the statistical distribution function of species sensitivity distribution (SSD) is commonly used for assessing the hazardous concentration for 5% of the species (HC_5) and safety concentration for 95% of the species in an ecosystem. Besseling et al. [30] built an SSD using both acute and chronic median lethal concentration (LC_{50}), EC_{50}, and the lowest observed effect concentration (LOEC) values available in the literature for aquatic organisms, derived an HC_5 of 5.4 $\mu g \cdot L^{-1}$ for PS, and suggested applying an assessment factor of 5 in ERAs. However, this HC_5 value involves several uncertainties and was extrapolated using limited available data obtained from marine, freshwater, and typical estuarine species.

Moreover, the LOEC values are usually considered controversial and inappropriate for this calculation because they can be influenced by study design [73,74]. Nevertheless, these values are currently acceptable because little data are available and SSD requires a specific number of points.

2.4.2. Sublethal Effects on Aquatic Species

NPs can penetrate biological membranes because of their small size, producing toxic effects at the cellular and molecular levels [75].

Amongst the studied organisms, the nematode *Caenorhabditis elegans* has emerged as a reliable toxicity model owing to its several practical and technical advantages [76]. We found 26 articles [77–102] reporting the sublethal effects of environmentally relevant concentrations ($\mu g \cdot L^{-1}$) of NPs on *Caenorhabditis elegans*. Of these, twenty-three studies examined the effects of nanoPS alone, whilst the other three considered the enhancement of the toxicity of other stressors in combination with exposure to nanoPS. However, the effects of NPs exposure in this taxon were limited to a few endpoints. Specifically, locomotion dysfunction was the most studied effect (17 of 23 articles). Many studies (16 of 23 articles) focused on reactive oxygen species (ROS) production to evaluate oxidative stress or intestinal damage. Almost all of these studies noted a decrease in locomotor activity and an increase in ROS production when *Caenorhabditis elegans* was exposed to an NP concentration of 1 $\mu g \cdot L^{-1}$ or higher. The only exception is the work of Zhao et al. [101], who noted the induction of these effects at concentrations exceeding 10 $\mu g \cdot L^{-1}$.

Five studies evaluated the potential of nanoPS to generate reproductive toxicity, whilst some studies focused on other molecular markers (i.e., neurotoxicity, metabolic toxicity, and so on).

Based on these observations, a concentration of 1 $\mu g \cdot L^{-1}$ can be assumed as a safety threshold for NPs to prevent the negative sublethal effects on wild organisms. Even at environmental

concentrations (see Section 2.3) far below those that cause acute effects (see Section 2.4.1), continuous exposure to sublethal concentrations may lead to ecologically relevant adverse effects. Behaviour depends on integrated processes at the subcellular, cellular, and organismal levels and is thus susceptible to disruption by nanoPS. A change in locomotor activity can induce a series of indirect effects at the higher levels of ecological hierarchy (i.e., the community), such as alteration of the prey–predator interactions or competition for food and space [103,104].

To date, research on the effects of NPs on aquatic organisms was mainly focused on marine ecosystems. Studies on marine crustaceans (*Amphibalanus amphitrite* and *Artemia franciscana*) have demonstrated the negative effects of NPs on enzyme activities (acetylcholinesterase, propionyl cholinesterase, and catalase), indicating possible neurotoxicity and oxidative stress induction [105]. Moreover, negative effects of PS on catalase and glutathione S-transferase activities have been observed in fish cell lines [106]. Exposure to nanoPS showed adverse effects on the development of *Mytilus edulis* larvae [107] as well as induced oxidative damage and behavioural alterations and reduced regenerative capacity in *Hediste diversicolor* [108,109]. All of the above-mentioned effects were observed even after exposure of the organisms to low concentrations of PS (1 $\mu g \cdot L^{-1}$). Fish appear to be less sensitive, as behavioural alterations of locomotor activity, swimming hypoactivity, and bradycardia were observed in *Danio rerio* only after exposure to nanoPS concentrations as high as 100 $\mu g \cdot L^{-1}$ [110].

Amongst freshwater organisms, studies conducted on *Daphnia pulex* have demonstrated the induction of cytochrome P450 enzymes [111] and adverse effects on growth, development, and reproduction in organisms exposed to 100 $\mu g \cdot L^{-1}$ nanoPS [66]. Moreover, Liu et al. [112] reported that chronic exposure to 1 $\mu g \cdot L^{-1}$ nanoPS produced toxic effects on second-generation offspring, inducing stress and reducing growth rate and reproduction.

A few studies have examined the effects of amino-modified nanoPS. For instance, Balbi et al. [113] highlighted malformations of D-veligers in *Mytilus galloprovincialis* at environmental concentrations (from 1 $\mu g \cdot L^{-1}$) of nanoPS. Other laboratory experiments at higher nanoPS concentrations demonstrated changes in lipid composition and increases in lipid reserves in the marine alga *Chaetoceros neogracile* (50 $\mu g \cdot L^{-1}$). Further, effects on enzymatic activities and embryo-larval toxicity in marine crustaceans and mollusks exposed to nanoPS concentrations of 100 $\mu g \cdot L^{-1}$ or higher have been reported [114,115]. In contrast, only nanoPS concentrations exceeding 20 $mg \cdot L^{-1}$ have been shown to negatively affect the growth and biofilm formation of marine bacteria [116].

In addition to PS, only a limited number of studies have evaluated the effects of PMMA. For instance, Brandts et al. [117,118] reported increased expression of genes associated with lipid metabolism and

antioxidant defense in marine fish exposed to environmentally realistic concentrations (1 $\mu g \cdot L^{-1}$) of PMMA. Booth et al. [119] observed significant toxicity in *Daphnia magna* exposed to poly(methyl methacrylate-co-stearyl methacrylate) copolymer, suggesting that surface chemistry plays an important role in ecotoxicity.

Of note, the majority of the toxicity studies used dialyzed particles without considering the presence of additives or other contaminants, which may pose more severe hazards to organisms than NPs themselves. Heinlaan et al. [120] assayed the toxicity of non-dialyzed nanoPS in *Daphnia magna*, *Raphidocelis subcapitata*, and *Vibrio fischeri* due to the presence of the biocidal additive NaN_3 and other surfactants in the suspension.

The coexistence of NPs with other particles or chemical substances is crucial in NP research. Thanks to their specific surface area and hydrophobicity, NPs can, indeed, interact with other particles [121] and act as a vector of toxic molecules to the biota, causing the so-called 'Trojan Horse' effect [122]. There remain knowledge gaps regarding the possible combined effects of contaminants and NPs. However, two studies on *Caenorhabditis elegans* have demonstrated the potential role of nanoPS in enhancing the toxicity of other chemicals. First, Dong et al. [77] demonstrated that PS could enhance the toxicity of TiO_2-NPs to decrease locomotor behaviour and induce intestinal ROS production. Second, Qu et al. [85] showed that nanoPS could increase the toxicity of the cyanobacterial toxin microcystin-LR to reduce brood size and locomotor behaviour and induce oxidative stress.

2.4.3 Lethal and Sublethal Effects on Soil Species

Soil constitutes an important reservoir of MPs and NPs [123]. The presence of plastic fragments has been recognized as one of the most important problems related to the quality of soil in agroecosystems [124], and the calculated risk for MPs has been assessed as unacceptable to soil biota [29]. Nevertheless, the problem of NP pollution in terrestrial environments has received less attention than in marine ecosystems. Identifying the effects and potential hazards on soil invertebrates is fundamental for preserving soil function. In addition to the nematode *Caenorhabditis elegans*, discussed in the previous section, which exhibits both aquatic and terrestrial free-living forms [125], few studies have investigated the effects of NPs on other terrestrial organisms.

As such, lethal effects on soil-dwelling organisms have been little investigated. Jiang et al. [126] observed significant changes in mortality in the earthworm *Eisenia fetida* exposed to NPs. Conversely, Zhu et al. [127] did not find any change in the mortality of soil oligochaete *Enchytraeus crypticus*.

Nevertheless, emerging evidence has confirmed the uptake of NPs and underscored their potential to cause detrimental effects following ingestion by soil organisms. Using realistic exposure experiments, Lahive et al. [128] verified the uptake of NPs by the earthworm *Lumbricus terrestris* and suggested the potential for long-term accumulation. The uptake of 100 nm PS was also confirmed in the earthworm *Eisenia fetida* after exposure for 14 days [126], with consequent histopathological changes in the intestinal tissue, induction of oxidative stress, and increase in the degree of DNA damage induced by low concentrations of PS (1 $mg \cdot kg^{-1}$ soil).

Furthermore, Zhu et al. [127] have highlighted the negative effects of NP exposure in soil organisms. The authors demonstrated a significant reduction in the weight and changes in the gut microbiome of the oligochaete *Enchytraeus crypticus* following exposure to 10% nanoPS (dry weight). However, they observed an increase in reproduction at lower NP concentrations.

Chae et al. [129] investigated the transfer of 28 nm PS particles from the soil to mung bean (*Vigna radiata*) plants and subsequently to snails (*Achatina fulica*) feeding on those plants in a terrestrial ecosystem. The authors noted a decrease in the root growth and nanoparticle accumulation in the leaves of mung bean plants. In addition, the authors highlighted a decrease in the growth rate as well as the feeding and foraging speeds of snails, which was associated with a decrease in the viability of gut microbiota and damage to the digestive tissues.

Additionally, the presence of additives used to produce plastics may damage soil organisms. Indeed, the biochemical and genetic toxicity of di-(2-ethylhexyl) phthalate (the most used additive in PVC production) has been demonstrated in the springtail *Folsomia candida* [130] and earthworm *Eisenia fetida* [64].

In the light of results reported by the above-mentioned studies, we cannot rule out the possible detrimental effects of NPs on terrestrial organisms and their potential transfer to organisms at higher trophic levels.

2.5. ERA Framework for NPs

Exposure to NPs is a major environmental concern. Based on evidence compiled to date, it can be emphasized that the measured nanoPS concentrations in river waters could produce sublethal effects in *Caenorhabditis elegans*. In fact, the concentrations (1.92–2.82 $\mu g \cdot L^{-1}$) measured by Zhou et al. [37] are higher than the proposed sublethal effective threshold of 1 $\mu g \cdot L^{-1}$, which has been proven to alter the locomotor ability and induce ROS production. The ubiquitous presence of NPs, their uptake by organisms, and their potential to act as vectors for toxicants and pathogens render their risk

assessments a priority on stakeholders' agendas at the global level. To the best of our knowledge, no framework for assessing the ecological risks of NPs has been adopted internationally. In principle, the current risk assessment paradigm for chemicals is applicable to nanomaterials and NPs. According to this classical scheme, the risk assessment process involves four steps: hazard identification, exposure assessment, effect assessment, and risk characterisation [30,131]. By combining the information on exposure levels (estimated or measured) with data on the expected effects, the final step of risk characterisation allows the establishment of whether the expected risk level could be considered acceptable. The risk quotient (RQ) is the ratio of potential exposure to a substance to the level at which no adverse effects are expected (RQ = exposure/toxicity). An RQ < 1 indicates an acceptable level of NP pollution, whereas an RQ > 1 indicates a reason for concern. The current regulatory ERA is based on a tiered and mostly deterministic approach to rapidly identify the substances of low concern. The lower tiers present highly conservative assumptions and high levels of uncertainty; therefore, AF is used to cover all uncertainties in the approach. As such, AF decreases with each step of the tiered approach as more data are available, implying that the uncertainty in assessment decreases with each step [132]. Indeed, higher tiers are characterized by a higher degree of complexity but a higher ecological realism.

With the collected data, an exercise has been conducted to characterize the risk associated with the presence of nanoPS in the aquatic medium. In particular:

$$RQ_{nanoPS} = \text{Exposure/Toxicity} = 1.92–2.82\ \mu g \cdot L^{-1}/(5.4\ \mu g \cdot L^{-1}/5) = 1.7–2.6$$

where the exposure data are from Table 2.2; the toxicity data (HC_5/AF) are from [30], and here above reported.

As the RQ is >1, it can be concluded that the current monitored concentrations of nanoPS in aquatic ecosystems pose an unacceptable risk for the wild communities.

The evidence of possible negative effects on both aquatic organisms and *Caenorhabditis elegans* highlights the urgent need to provide useful data for a sound risk assessment of nanoplastics in the environment.

In this light, we suggest adopting the conventional frameworks proposed by Besseling et al. [30] and Koelmans et al. [131] with some addenda to realize a more realistic risk assessment scheme tailored to the specificities of NPs. The proposed method is illustrated in Figure 2.4.

We suggest a tiered framework in which four different levels of complexity are foreseen and characterized as follows:

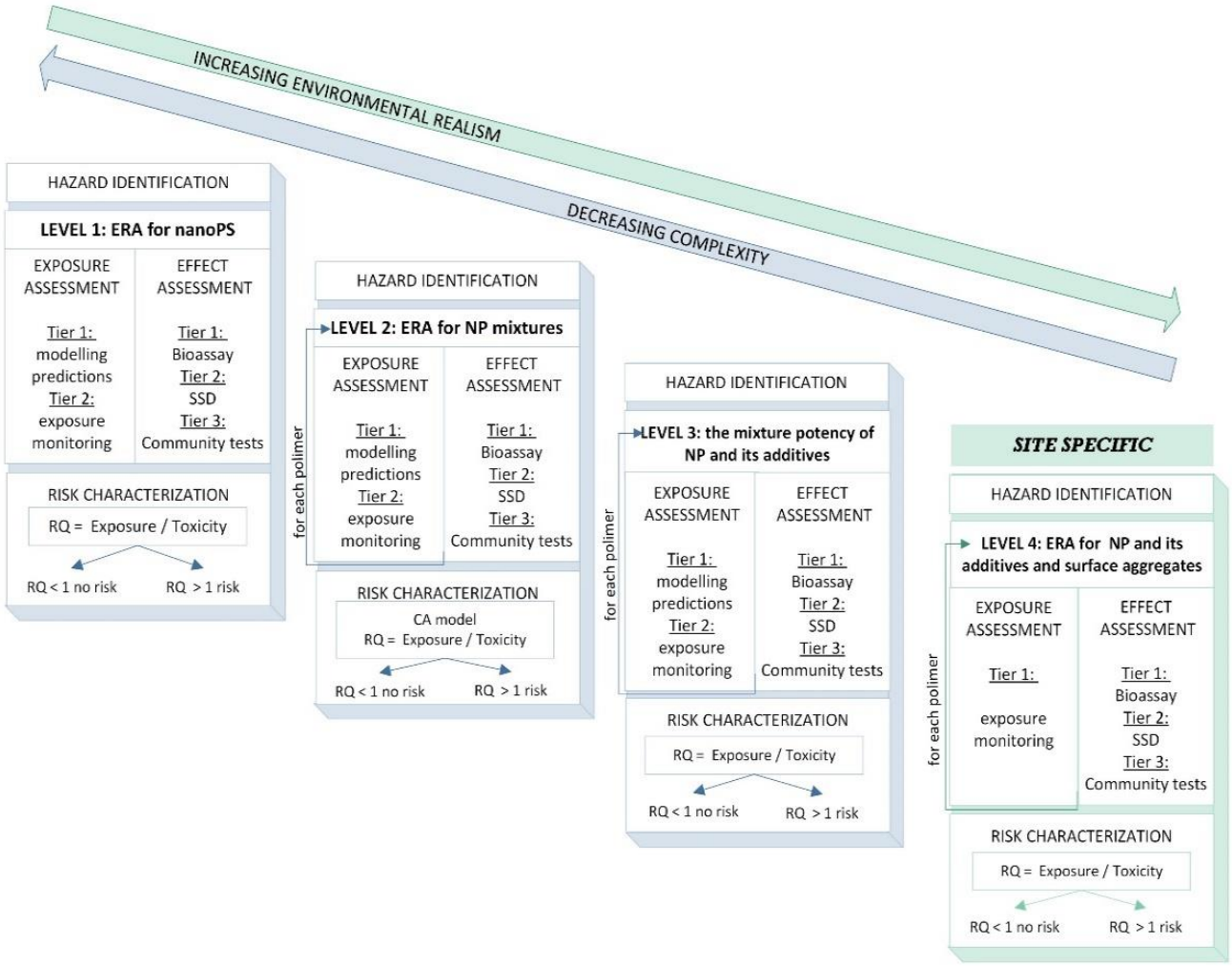

Figure 2.4. Tools for exposure and effect assessments as part of the general environmental risk assessment framework for NPs.

Level 1: ERA for NanoPS

At this stage, we propose considering the conventional ERA scheme with its tiers, as detailed by Besseling et al. [30]. This step, which is less environmentally realistic but more easily applicable, is based on the assumption that the potential risk to wild organisms is generated by exposure to a single polymer. As highlighted in earlier, data on the effects of exposure to many polymers are scarce. Indeed, the majority of the studies to date focused on PS. Therefore, PS is suggested as a proxy for all NPs. To maximize the risk and simplify the ERA procedure, all fragments are assumed to be of the same size. However, the dimension that should be considered to better characterize the risk is not yet fully understood.

At this level of ERA, the estimated and/or measured concentrations of a type of polymer are compared with the safety thresholds extrapolated from the toxicological data of PS, that is, from a precautionary point of view, and the risk is calculated as follows:

$$\mathrm{RQ} = \frac{\sum_{i=1}^{n} \mathrm{EC}}{\text{No effect} - \text{safety threshold for PS}}$$

where i = different NP polymers.

We are aware that these assumptions are simple; however, they are internationally agreed upon in the risk assessment of poorly characterized contaminants. In some cases, owing to the lack of data on the effects induced by a chemical compound, data from a similar compound may be used, assuming that the effects can be assessed fairly well without the need for further testing. All assessments are based on the 'read across' principle, in which toxicological data related to chemicals, species, compartments, or endpoints are translated into data-free scenarios. Such approaches are convenient, albeit rather uncertain. Therefore, these methods should be placed at the lowest level of the ERA framework [133].

Level 2: ERA for NP Mixtures

As all produced polymers are released in the environment, to achieve greater ecological realism, the risk generated by an individual polymer should be calculated and then the overall mixture toxicity assessed. The potential mixtures likely to be present in the environment are virtually infinite; therefore, experimental testing is not feasible. In this context, predictive approaches for estimating the effects of mixtures with known compositions are required. The concentration addiction model [134] has been suggested as the acceptable worst-case precautionary approach [135]. According to CA, the toxicity of a mixture of NPs can be described as the sum of the so-called toxic units (TUs) of all mixture components. TU is the ratio of the concentration of a plastic polymer to the toxicological endpoint (EC_{50}).

The mathematical CA concept for a multi-compound mixture can be expressed as follows:

$$\text{Mixture toxicity} = \sum_{i=2}^{n} \frac{\mathrm{ECi}}{\mathrm{ECx_i}}$$

where n is the number of compounds in the mixture causing x% of the total effect; Ci is the concentration of the ith component; and ECx_i is the concentration of the respective component that produces the same effect when applied individually.

Although CA is based on the assumption that all components of a mixture have the same mode of action, this concept also enables reliable assessment of the effects of mixtures of non-similarly acting chemicals.

Level 3. Mixture Potency of NPs and Their Additives

Plastics used in commercial goods are not composed of pure polymers and often contain organic additives and inorganic co-formulants in their mixture. In the light of evidence that co-formulants can enhance the effects of nude NPs or even act as sinergizants [120], the risk characterisation of plastic mixtures is essential. ERA should be performed with toxicological endpoints obtained by testing the true composition of plastics, that is, nude NPs and their additives.

Level 4: ERA for NPs with Their Additives and Surface Aggregates

In the environment, there exist types of plastics that differ not only in chemical composition, shape, and size but also in the composition of surface aggregates. Numerous environmental pollutants and microbial communities colonize NP surfaces. ERA at this level is extremely complex and feasible only with exposure data and site-specific effects. Level 4 should be applied in specific case studies related to a certain environmental reality, in which a high degree of knowledge and characterisation of NP contamination is required.

2.6. Conclusions and Research Recommendations

Data for robust risk characterisation are not available; however, with evidence accumulated to date, it can be emphasized that environmental concentrations of nanoPS induce unacceptable effects in diverse wild organisms. In this context, we propose a methodology for the ERA of NPs based on different tiers characterized by ever-increasing, deepening levels, complexity, and ecological realism. However, there remain substantial knowledge gaps related to NP exposure and toxicity fields, which hamper the finalization of ERA. Moreover, there is an urgent need for harmonization between different researchers and stakeholders both in terms of techniques and concepts. Firstly, the univocal definition of NPs is strongly suggested.In this light, we therefore suggest the following recommendations for the future research:

(1) An important aspect is the complexity of establishing an optimal analytical method for identifying and quantifying NPs in environmental matrices owing to their extremely low concentrations. The use of a biomonitor is a reliable strategy to overcome these limitations, although predicting NP concentrations in the environments in which these organisms live based on bioaccumulated levels remains a critical point. We therefore suggest applying the above reported methodologies on organisms belonging to different trophic levels, especially of terrestrial ecosystems.

(2) We support the need for an harmonization of the experimental procedures in NPs ecotoxicology studies. NPs often contain different additives that can leach out either in the exposure media or in the intestine after being ingested, which might lead to the increased toxicity to the studied organism. Cleaning the NPs suspension from additives is a fundamental step. Nevertheless, there are still no clear guidelines on how to clean these suspensions, and this involves issues of reproducibility and comparability among the different studies. Moreover, since it is difficult to obtain information about co-formulants included in marketed plastics, it would be beneficial if plastic producers would provide this information.

(3) The influence of size on the efficiency of internalisation and on toxicity is still scarcely investigated. Therefore, a better characterization of toxicity in the function of different particle sizes should be provided for a more comprehensive understanding of most hazardous particle dimensions.

(4) Another important knowledge gap is related to the toxicities of different polymers; indeed, information is mostly limited to nanoPS, whilst other NPs await further work.

(5) In addition, a limited number of ecotoxicological studies have been performed at realistic environmental concentrations.

(6) Furthermore, a vast majority of studies have focused on aquatic biota, although NP pollution extends to terrestrial environments, such as agroecosystems. These represent largely under-investigated sources of NP contamination, and the potential impact of NP pollution on terrestrial biota warrants thorough investigation

The foreseen increase in environmental NP concentrations underscores the need for the investigation of their impacts on wildlife by both the scientific community and regulatory bodies.

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Chapter 3

Sublethal effects induced by different plastic nano-sized particles in *Daphnia magna* at environmentally relevant concentrations

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Abstract

A growing number of studies have reported the toxic effects of nanoplastics (NPs) on organisms. However, the focus of these studies has almost exclusively been on the use of polystyrene (PS) nanospheres.

Herein, we aim to evaluate the sublethal effects on *Daphnia magna* juveniles of three different NP polymers: PS-NPs with an average size of 200 nm, polyethylene [PE] NPs and polyvinyl chloride [PVC] NPs with a size distribution between 50 and 350 nm and a comparable mean size. For each polymer, five environmentally relevant concentrations were tested (from 2.5 to 250 µg/L) for an exposure time of 48 h.

NP effects were assessed at the biochemical level by investigating the amount of reactive oxygen species (ROS) and the activity of the antioxidant enzyme catalase (CAT) and at the behavioral level by evaluating the swimming behavior (distance moved).

Our results highlight that exposure to PVC-NPs can have sublethal effects on *Daphnia magna* at the biochemical and behavioral levels. The potential role of particle size on the measured effects cannot be excluded as PVC and PE showed a wider size range distribution than PS, with particles displaying sizes from 50 to 350 nm. However, we infer that the chemical structure of PVC, which differs from that of PE of the same range size, concurs to explain the observed effects. Consequently, as PS seems not to be the most hazardous polymer, we suggest that the use of data on PS toxicity alone can lead to an underestimation of NP hazards.

3.1 Introduction

Nanoplastics (NPs) are particles sized between 1 and 1,000 nm (Gigault *et al.*, 2018; Hartmann *et al.*, 2019) and are mainly formed by the environmental fragmentation of large plastic items (Gigault *et al.*, 2021). Many ecotoxicological assessments utilize engineered nanometric plastics instead of naturally occurring ones in their bioassay. For enhanced terminological and experimental consistency, it is advised that the origin of plastics used in bioassay is explicitly stated. In comparison to primary particles present within commercial products, particles that enter ecosystems can deviate considerably in dimensions and physical-chemical characteristics (Lowry *et al.*, 2012; Mitrano *et al.*, 2015; Nowack & Mitrano, 2018). While several groups endeavor to create environmentally meaningful nanoparticles (Baudrimont *et al.*, 2020; McColley *et al.*, 2023), scrutinies of the ecotoxicological influence of such entities have usually investigated engineered plastic nanospheres (also referred to as primary NPs) as a substitute for the secondary plastic elements detected in the surroundings (Pikuda *et al.*, 2023).

Recently, NPs have generated increasing scientific interest (Cerasa *et al.*, 2021) owing to the unique properties that make them potentially more hazardous than microplastics (MPs) (Gigault *et al.*, 2021). Different studies have demonstrated that NPs can negatively affect organisms (Shen *et al.*, 2019) at the subindividual (Della Torre *et al.*, 2014; Corsi *et al.*, 2020; Liu H. *et al.*, 2020a,b) and individual levels (Mattsson *et al.*, 2014; Rist *et al.*, 2017; Qu *et al.*, 2019; Liu Z. *et al.*, 2019). However, the environmental risk assessment (ERA) of NPs (NP-ERA) is in its infancy.

The classical risk assessment framework requires the assessment of exposure and effect to determine whether the expected level of risk can be considered acceptable. The occurrence of NPs in ecosystems was confirmed in 2017 in ocean water (Ter Halle *et al.*, 2017); however, recently, only a few studies have verified their presence in other abiotic matrices and biota, suggesting that NPs are present in the environment at low levels (Materić *et al.*, 2021; Zhou *et al.*, 2021 a,b; Materić *et al.*, 2022a,b; Xu *et al.*, 2022; Li *et al.*, 2022). Comparing environmentally measured concentrations with data from ecotoxicological studies that tested similar concentrations, it was recently suggested that exposure to these environmental levels of NPs can be considered hazardous to aquatic ecosystems (Masseroni *et al.*, 2022). However, the lack of standardized test materials for NPs makes assessing the effect of environmentally relevant concentrations of NPs challenging (Koelmans *et al.*, 2022; Mitrano *et al.*, 2023), thus leading to knowledge gaps that hamper NP risk characterization. To date, almost all studies have tested the effects induced by polystyrene NPs (PS-NPs) (Masseroni *et al.*, 2022); this is because PS is the plastic polymer that is most easily processed into nanoparticles (Lehner *et al.*, 2019).

In the environment, NPs are present as a complex mixture of different polymers with varying sizes, shapes, surface functionalities, additive compositions, and degrees of weathering (Raynaud *et al.*, 2021); therefore, the toxicity data of PS-NPs cannot be considered a reliable proxy for all other polymers (Monikh *et al.*, 2022). There are very few effect assessment reports of other NP polymers whose presence on the market is very diffuse (e.g., polypropylene [PP], polyethylene [PE], and polyvinyl chloride [PVC]) (Kokalj *et al.*, 2021; Plastics Europe, 2022).

This study aims to address this gap by evaluating the sublethal effects of different NP polymers (PS, PE, and PVC) on the freshwater cladoceran *Daphnia magna*, setting the NP concentrations to environmentally relevant levels (lower than 250 µg/L), and the exposure time to 48 h. Even if this species is not the most sensitive one (Masseroni *et al.*, 2022), it is the most frequently used model species in ecotoxicology (Qiao *et al.*, 2022) and, as a filter-feeding zooplankton, it is representative of primary consumers of the freshwater food chain (Kukkola *et al.*, 2021). As the selected concentrations are far below those inducing mortality, we have evaluated the effects of NPs at the subindividual and individual levels of biological organization, considering biochemical and behavioral endpoints.

In summary, this study aims to provide crucial data that will help establish whether different NP polymers exhibit comparable toxicity to PS-NPs and whether PS-NPs can be used as a proxy for all other NP polymers.

3.2. Materials and methods

3.2.1. Procurement of nanoplastics

Three polymers (PS, PE, and PVC) that best represent the most common plastic polymers present on the market and in the environment (Plastics Europe, 2022) were selected for this study. PS-NPs (200-nm nominal diameter, 25 mg/mL) were purchased from Polysciences Europe GmbH, Germany. PE-NPs and PVC-NPs were procured from the European Commission's Joint Research Center in Ispra, Italy, where they were synthesized following the protocol proposed by Cassano *et al.* (Cassano *et al.*, 2021; Cassano *et al.*, 2023), with sizes ranging between 50 and 350 nm. As reported by the suppliers, the polymer density was 0.92 g/mL for PE (Sigma Aldrich, Milan, Italy), 1.4 g/mL for PVC (Sigma Aldrich, Milan, Italy), and 1.05 g/mL for PS (Polysciences Europe GmbH, Germany).

3.2.2. NP characterization and quality control

The three investigated solutions of PS-NPs, PE-NPs and PVC-NPs were well characterized before the exposure experiments.

NP shape: Transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Rome, Italy) and scanning electron microscopy (SEM, FEI Nova NanoLab 600 DualBeam, Thermo Fisher Scientific, Delft, The Netherlands) analyses were performed to check the morphology of the polymeric particles. In brief, stock suspensions were diluted in Milli-Q water and manually deposited on Formvar carbon-coated 200 mesh copper grids (Agar Scientific, London, UK) for TEM analysis at 120 kV, and on silicon wafer for SEM analysis.

Chemical characterization of NPs: To accomplish the chemical characterization of the NP polymers, Raman spectral analyses were performed using an alpha300 R confocal Raman microscope (WITec, GmbH, Berlin, Germany). In brief, stock suspensions were manually deposited (10 µL) on silicon wafer and analyzed by Raman microscope equipped with a 532 nm laser. Spectra were collected using the 50X objectives by averaging at least 10 spectra. The identification of the spectra was achieved by comparing the PS-NPs, PVC-NPs, and PE-NPs with the respective reference polymers.

NP concentration and size: Each polymer suspension was subjected to nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern Panalytical, Worcestershire, UK) to verify the nominal size and concentrations of the NPs. In brief, the NP suspensions were diluted with Milli-Q water to achieve a concentration within 10^8 particles/mL, and then each polymer suspension was analyzed in triplicate (three 60-s videos were recorded). The nominal concentration (particles/mL) of the stock solution of PS-NPs was provided by the supplier, whereas for PVC-NPs and PE-NPs this was calculated using the following formula:

$$Concentration = \frac{6W \times 10^{12}}{\rho \times \pi \times \varphi^3}$$

where W is the grams of polymer per mL in latex, ρ is the density of the polymer, and φ is the diameter in microns of latex particles.

As reported in recent studies (Pikuda *et al.*, 2019; Vaz *et al.*, 2021; Kelpsiene *et al.*, 2022), to avoid the unexpected effects of surfactants on organisms during ecotoxicology assays, it is essential to

purify the synthesized NPs by means of a washing procedure. The PS-NP solution, given the presence of residual surfactants in the suspension, was washed using gentle sonication and centrifugation (13,500 rpm for 13 min), and then, the pellet was resuspended in Milli-Q water. NTA was also carried out for the washed PS-NP solution to identify any changes in the solution characteristics occurring as a consequence of the washing process. For PVC- and PE-NP suspensions, this step was not required because the synthesis protocols involved the use of biocompatible sodium cholate surfactant (Cassano *et al.*, 2021; Cassano *et al.*, 2023), presents in the highest NP tested exposure at a concentration of 0.0625 mg/L. Data from leachate experiment support the lack of detrimental effect induced by sodium cholate (refer to paragraph 3.2.2: *Additional quality control for PVC-NPs in the exposure water*). Furthermore, the size distribution of the investigated particles was assessed through TEM imaging acquired at 120 KV. Images of 200 individual particles from each sample (PS-NPs, PVC-NPs, and PE-NPs) underwent manual analysis, with data being collected via ImageJ software. MinFeret (nm) size distribution, corresponding cumulative percent curve and Gauss fit (Xc; sigma) were obtained by OriginPro 2015 software. Mean and corresponding standard deviation of MinFeret (nm) were calculated by excel.

NP surface charge: To investigate the stability of the particles in suspension, their hydrodynamic size and ζ-potential were measured through dynamic light scattering (DLS) analyses using a Zetasizer Nano ZS instrument (Malvern Panalytical, Worcestershire, UK) equipped with a 633-nm helium–neon (HeNe) laser. Each polymer suspension was diluted to a concentration of 10^9 particles/mL and analyzed both in Milli-Q water and in the testing sample water. Three consecutive measurements (both for hydrodynamic size and ζ-potential) were carried out at T0 and T1 (48 h). Since it is plausible that the surface charge changes as soon as the plastic particles bind to molecules excreted by the daphnids, we have performed further DLS measurements to mimic the behavior of the investigated polymers in the exposure water. Briefly, we left the daphnids in the exposure water for 48 h, then removed the organisms from the NPs polymer suspension, which was analysed by DLS as described above.

NP ingestion: A preliminary test was performed to confirm the effective ingestion of NPs by *D. magna* juveniles. In brief, *D. magna* juveniles were exposed to 20-nm fluorescent PS-NPs (F8787 FluoSpheres, Invitrogen, Thermo Fisher Scientific, USA) at a concentration of 1,000 µg/L for 48 h. At the end of the exposure, the presence of NPs in the digestive tract and on the body surface was

investigated using point scanning confocal microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany).

Additional quality control for PVC-NPs in the exposure water: Since it is known that Cl^- and PVC monomers can induce toxicity in *D. magna* (Al-Malack *et al.*, 2000; Lithner *et al.*, 2012), we conducted further analyses on PVC-NPs leaching into the exposure water. In brief, PVC-NPs at the highest tested concentration (250 µg/L) were allowed to leach into the exposure water for 48 h. The release of Cl^- was investigated using ion chromatography (Thermo Scientific™ Dionex™, Waltham, MA, USA) and the Cl^- levels in the leachate were compared with those in pristine exposure water solution, as described by Nava *et al.* (Nava *et al.*, 2020). Finally, to exclude the role of leachate in influencing changes in the swimming behavior of *D. magna* individuals, the PVC-NP suspension was centrifuged (13,500 rpm for 15 min) and the supernatant (the water phase containing the leachate) was separated from the pellet (containing precipitated PVC-NPs); then individuals were exposed to the supernatant.

3.2.3. *Daphnia magna* population maintenance

The test organisms were derived from a single parthenogenetic female *D. magna* Straus, 1820, obtained from the Istituto Superiore di Sanità (Rome, Italy), and they were cultured by following Test Guideline 202 (OECD, 2004). In brief, 20 *D. magna* individuals were bred in a glass beaker filled with 500 mL of a 1:1 mixture of two commercial mineral water samples (San Benedetto®—conductivity: 428 µS/cm at 20 °C; pH: 7.55; constitution: 283-mg/L HCO^{-3}, 51.1-mg/L Ca^{2+}, and 29.9-mg/L Mg^{2+}; San Bernardo®—conductivity: 52 µS/cm at 20 °C; pH: 7.00; constitution: 32.3-mg/L HCO^{-3}, 48.6 mg/L Ca^{2+}, and 0.39-mg/L Mg^{2+}), also referred to as exposure water throughout this article. The beakers were placed in a thermostatic chamber under a 16:8 light–dark cycle at a controlled temperature of 20.0 °C ± 0.5 °C. The daphnids were fed three times a week with a suspension of the commercial green algae spirulina (*Arthrospira platensis)* (1.25 mg/mL) and yeast (*Saccharomyces cerevisiae)* (10 mg/mL). The culture medium was renewed three times a week.

3.2.4. *Daphnia magna* exposure assay

48-h exposure assays were conducted with the third-generation neonates of *D. magna* (less than 24-h old). Once solution conditions were confirmed, the different NP suspensions were diluted with the

exposure water to obtain solutions with a concentration of 250 µg/L; other subsequent concentrations (125, 25, 12.5, and 2.5 µg/L) were obtained via serial dilution. For each NP polymer, each concentration was tested to separate sets of 60 daphnid neonates. For each treatment, the samples were divided into 6 replicates, each containing 10 daphnids, and added into glass vessels containing 20 mL of the exposure solution. Control treatments (CTRL = treatment with the pristine exposure water) were performed in parallel. After the exposure, the organisms were collected for sublethal toxicity analysis.

3.2.5. Analyses of biochemical and behavioral biomarkers

For each NP treatment, the swimming activity of 30 daphnids was analyzed. Each individual daphnid was gently placed in a well plate containing 2 mL of exposure water. The plates were placed in a thermostatic chamber (20.0 °C ± 0.5 °C) equipped with a digital high-definition camera (Raspberry Pi 3 with Camera Module 2) set at a high resolution (1920 × 1080 pixels). After 5 minutes of acclimation, the swimming activity of the *D. magna* neonates was tracked through video tracking analysis using the software LoliTrack V4 (Loligo Systems, Denmark). The movement of each daphnid was recorded three consecutive times for 30 s (each video consisting of 750 frames at 25 frames/s). The distance traveled, expressed in mm, was chosen as the representative movement endpoint (Bownik, 2017). For the PVC-NP leachate experiment, a similar experimental set-up was used, with the different treatments (CTRL, leached PVC-NPs, and 250-µg/L PVC-NPs) being performed in parallel, each involving a total of 12 daphnids separated into 3 replicates of 4 individuals each. For all the investigated polymers, the 30 daphnids analyzed for behavioral analysis and the other 30 were frozen at −80 °C for subsequent biochemical analysis. For each treatment, three replicates (pools of 18–20 organisms) were utilized. The amount of reactive oxygen species (ROS) and the activity of catalase (CAT) were investigated using spectrofluorimetry and spectrophotometry, respectively, in accordance with the procedures reported by De Felice *et al.* (De Felice *et al.*, 2022).

3.2.6. Statistical analyses

The effects of NP exposure on the investigated endpoints were analyzed using one-way Analysis of Variance (ANOVA). Each endpoint was considered a dependent variable, while the treatments were considered the predictor. The normality of data was verified using the Shapiro–Wilk test, with the significance being set at a p-value < 0.05. When data were not normally distributed, they were log

transformed before being considered for ANOVA. Data not normally distributed even after log transformation were analyzed using the Kruskal–Wallis test. When ANOVA results were statistically significant, Dunnett's posthoc test was used to prove significant differences between the control and treatment groups. Conversely, Dunn's posthoc test was used for data analyzed using the Kruskal–Wallis test. Statistical analyses were performed using the R software (R Core Team, 2022).

3.3. Results and discussion

3.3.1. NP characterization and quality control

SEM, TEM, and Raman analyses confirmed the spherical shape and the chemical structure of the investigated polymers (Fig. 3.1)

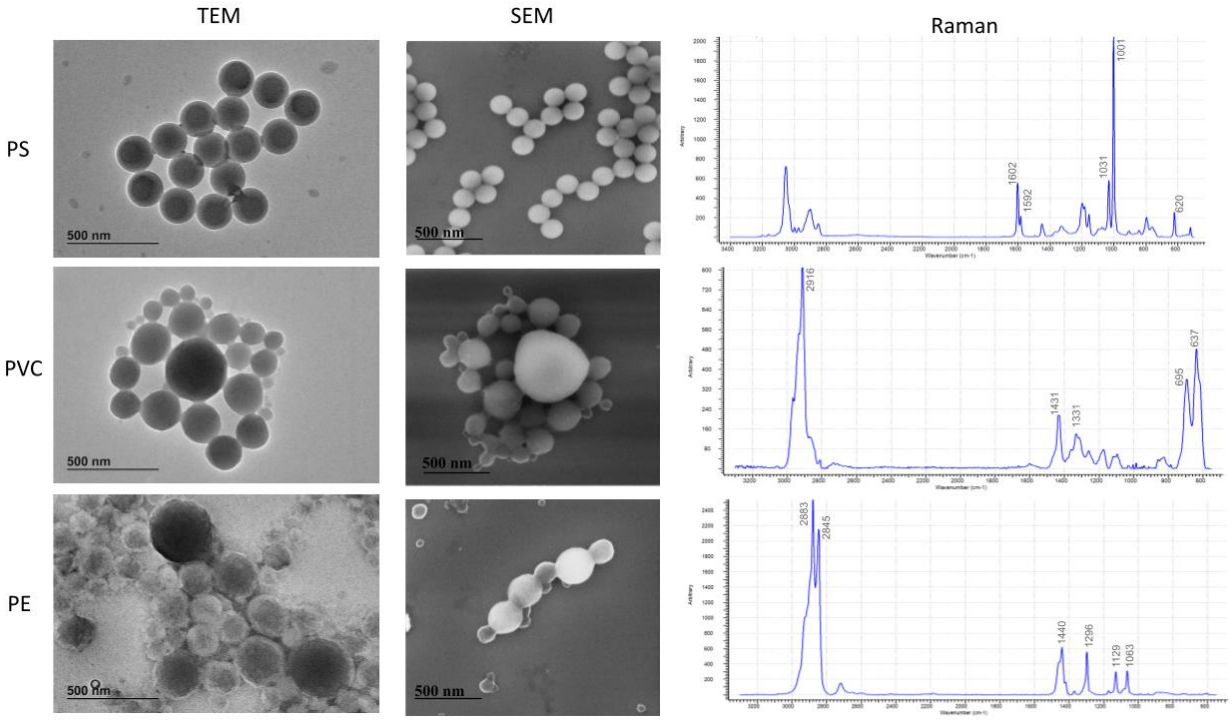

Fig. 3.1. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and Raman spectra of PS-NPs, PVC-NPs, and PE-NPs. The numbers in the Raman spectra specify the precise peaks utilized to identify PS, PVC, and PE polymers.

The NTA results revealed that the particle size and concentration were within 15% and 30%, respectively, of the nominal values (Table 3.1). PVC and PE show a higher standard deviation in size measurements as they were synthesized in a 50 to 350 nm size range (Cassano *et al.*, 2023). NTA

results for particle number concentration are highly dependent on particle scattering characteristics, image acquisition, and analysis settings (Filipe *et al.*, 2010; DeRose *et al.*, 2022). In our study, the measured values were considered acceptable as they were within 30% of the nominal values. NTA verified that the PS washing step neither altered the relative size and concentrations of PS-NPs nor caused aggregation or loss of material (Fig. S3.1).

Table 3.1. NTA results for the size and concentration of the tested NPs. Data are presented as mean values of three consecutive measurements ± standard deviation.

Solution conditions	PS	Washed PS	PVC	PE
Nominal size (nm)	200	200	200	200
Measured size (nm)	182 (± 32)	175 (± 30)	229 (± 65)	174 (± 74)
Nominal concentration (particles/mL)	5.68×10^{12}	5.68×10^{12}	3.46×10^{11}	5.31×10^{11}
Measured concentration (particles/mL)	$4.92 (\pm 0.02) \times 10^{12}$	$4.23 (\pm 0.10) \times 10^{12}$	$3.47 (\pm 0.12) \times 10^{11}$	$3.86 (\pm 0.19) \times 10^{11}$

DLS size intensity measurements of PS-NPs, PVC-NPS, and PE-NPs in both Milli-Q and exposure water are reported in Table 3.2 and in Figure S3.2. DLS size measurements in Milli-Q water were consistent with NTA size measurements. Polydispersity Index (PDI) results confirmed that PVC and PE particles exhibit a higher size distribution range than PS (Table 3.2). The ζ-potential values confirm the high stability of all the investigated polymers (Table 3.2). The ζ-potential is in fact considered a suitable indicator of NP surface charge (Bhattacharjee *et al.*, 2014): ζ-potential values higher than |30| mV indicate a stable behavior of particles within the solution, whereas values between |10| and |30| mV suggest incipient stability (Martin *et al.*, 2022). Moreover, surface charge is considered an important factor that can influence NP toxicity (Schwegmann *et al.*, 2010; Sukhanova *et al.*, 2018; Yu *et al.*, 2019). The surface charge and size of NPs were also measured in the exposure water (both in presence and absence of *D. magna*) since these parameters can be influenced by the pH, salt content of the medium the NPs are suspended in (Auguste *et al.*, 2020), and molecules excreted by *D. magna*. The ζ-potential and size values obtained for PS-NPs and PE-NPs showed that these parameters remained unaltered in the exposure water. In the case of PVC-NPs, slight changes in size and ζ-potential were detected in the exposure water as a function of time, with a decrease in the ζ-potential (from −31 to −25 mV) and an increase in the size (from 225 to 277 nm). The DLS measurements for NPs in the exposure medium with the presence of the molecules excreted by the

daphnids (Table S3.1) suggest that the different polymers exhibit smaller surface charge values and slightly larger sizes, but with modest variations in size and charge between the three investigated polymers.

Table 3.2. DLS measurements of the hydrodynamic size (nm), PDI, and ζ-potential (mV) of the NPs in Milli-Q water and exposure water at T0 and T1 (48 h). Data are presented as mean values of three consecutive measurements ± standard deviation.

		Exposure time	Size	PDI	ζ-potential
PS-NPs	Milli-Q water	T0	214 ± 49	0.028	−33 ± 6
		T1	218 ± 48	0.011	−33 ± 6
	Exposure water	T0	199 ± 44	0.028	−32 ± 6
		T1	200 ± 51	0.033	−32 ± 7
PE-NPs	Milli-Q water	T0	227 ± 84	0.112	−38 ± 5
		T1	231 ± 86	0.114	−34 ± 5
	Exposure water	T0	207 ± 75	0.124	−31 ± 6
		T1	212 ± 86	0.125	−29 ± 6
PVC-NPs	Milli-Q water	T0	245 ± 85	0.164	−37 ± 5
		T1	246 ± 93	0.115	−36 ± 5
	Exposure water	T0	224 ± 108	0.167	−32 ± 6
		T1	277 ± 163	0.244	−25 ± 4

The size distribution obtained by TEM measurement shows that PS present an average size of 184.5 (± 6.2) nm, while PVC and PE exhibit a distribution within the range of 50–350 nm, with an average size respectively of 116.7 (± 65.0) nm and 145.5 (±39.5) nm (Fig. S3.3).

The results of the preliminary test with fluorescent PS-NPs (Fig. S3.4) confirmed in our test conditions the presence of NPs in the digestive tract and on the body surface of *D. magna* juveniles. These data are in line with the evidence provided in the literature, showing that *D. magna* can ingest different types of plastic polymers (Zimmermann *et al.*, 2020) of both micrometric and nanometric size (Rist *et al.*, 2017; Canniff & Hoang, 2018; Vighi *et al.*, 2021; Jeyavani *et al.*, 2023).

The ion chromatography results highlighted the absence of any difference in the chlorine content between the control exposure water and the PVC-NP leachates.

3.3.2. Effect assessment: biochemical biomarkers

Test results indicated that exposure to different NP polymers invoked different biochemical responses in *D. magna*. The tests were considered valid as they fulfilled the criteria elucidated in OECD Test Guideline 202 because during the exposure time the recorded mortality of daphnids under the control and test conditions was below 10% (Table S3.2). The detailed results of the statistical analyses are reported in the supplementary data (Table S3.3), and ROS and CAT histograms are presented in Figure 3.2.

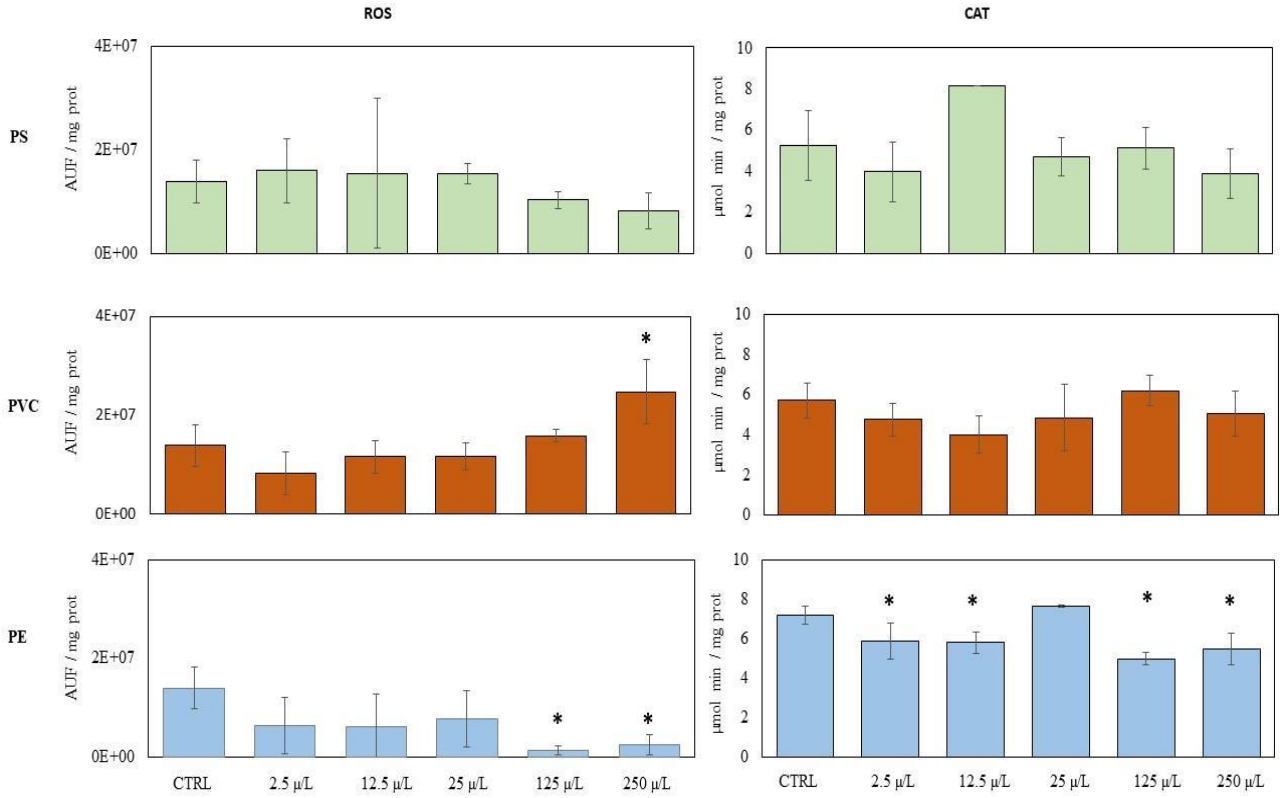

Fig. 3.2. ROS and CAT histograms measured after a 48-h exposure of *D. magna* to PS, PVC, and PE. * indicates statistically significant differences with respect to controls (p-value < 0.05).

3.3.2.1. PS-NP exposure

In the PS-NP treatment group, no statistically significant differences were observed both for ROS levels ($p = 0.33$) and CAT activity ($p = 0.0934$). The absence of PS-NP effects is consistent with results reported in the literature; in particular, in a recent study, *D. magna* individuals were exposed to PS-NPs (75 nm) for 21 d at concentrations comparable to those used in this study (50 and 500 µg/L), but they did not show signals of oxidative stress (De Felice *et al.*, 2022). That said, as the response of an organism to NP exposure is strongly influenced by several factors that vary from one study to another (e.g., particle size, concentration, exposure time, and the model species involved), a

straightforward comparison of our results with those reported in other studies is not possible. For example, the exposure of *D. pulex* to PS-NPs of 75 nm (100 µg/L) for 96 h resulted in the activation of antioxidant defense responses (Liu *et al.*, 2018). This trend was further confirmed for an exposure time of 21 d using similar experimental conditions (Liu *et al.*, 2020a), even at lower concentrations of PS-NPs (Liu *et al.*, 2020b).

3.3.2.2. PVC-NP exposure

In the case of the PVC-NP treatment groups, significant differences were recorded in ROS levels at the highest tested concentration (250 µg/L, $p = 0.003$) in comparison to the controls, whereas no significant variations were detected in the CAT activity ($p = 0.253$).

A recent study reported alterations in the antioxidant system of *D. magna* after PVC-MP exposure (Liu *et al.*, 2022). However, to the best of our knowledge, only one study has investigated the effects of PVC-NP exposure on *D. magna*, and this study established that PVC-NPs did not inflict any toxicity on the reproductive endpoints of the organism (Monikh *et al.*, 2022). Evidence of ROS overproduction induced by PVC-NP exposure has been reported only for human cell lines (Mahadevan *et al.*, 2022).

The PVC-NP surface charge has been considered an important factor influencing PVC toxicity (Weber *et al.*, 2022). In our study, we have observed that in the exposure scenario the measured surface charge of PVC presents modest differences when compared to those of PS and PE. This might indicate that the surface charge might not be the major vector of the observed effects.

Conversely, PVC-NP exposure was found to have no effects on the CAT activity in the daphnids. This dichotomy in the results of the two investigated biochemical endpoints could be attributed to the fact that 48 h of exposure, despite being enough to cause a surge in ROS levels, was not enough to induce the activation of the CAT activity. This result does not exclude that other proteins involved in the antioxidant machinery (for instance superoxide dismutase and glutathione peroxidase) might be induced in response to PVC-NP. Further analyses are therefore recommended, to assess the potential of this NP to impact on the antioxidant system and eventually induce oxidative damage.

3.3.2.3. PE-NP exposure

Our results revealed a sharp decline in the ROS content and CAT activity in daphnids for all the tested concentrations of PE-NPs (Fig. 3.2). With respect to ROS values, the two highest tested concentrations were statistically different from the control (125 µg/L, $p = 0.0008$; 250 µg/L, $p = 0.004$), a trend observed in the CAT histogram as well. The CAT activity results were statistically

significant for all concentrations, except 25 µg/L (2.5 µg/L, p = 0.046; 12.5 µg/L, p = 0.034; 25 µg/L, p = 0.754;125 µg/L, p = 0.001; 250 µg/L, p = 0.008). To date, the few studies that have evaluated the effects of PE-NPs on organisms (Baudrimont *et al.*, 2020; Ekvall *et al.*, 2022) have not considered oxidative stress endpoints. Alternatively, there have been studies that confirmed a PE-MP-mediated CAT activity decrease in oysters (Teng *et al.*, 2021) and fish (Espinosa *et al.*, 2019), suggesting that the polymer might hamper the smooth functioning of the antioxidant machinery. However, the authors of these studies suggest that an overproduction in ROS can overwhelm the antioxidant system, disrupting the activity of antioxidant enzymes such as CAT. Therefore, we would have expected an increase in ROS content and not a decrease. Further investigation of other oxidative stress biomarkers can provide useful information leading to a better understanding of the mechanism through which PE-NPs can induce an imbalance of the antioxidant machinery in *D. magna*.

3.3.3. Effect assessment: behavioral biomarkers

As in the case of biochemical biomarkers, treatment with different NPs of varying concentration invoked different swimming behavior from the *D. magna* juveniles (Fig. 3.3). The baseline swimming distance for the control groups was 77.7 (± 26.5) mm, which is consistent with values reported in the literature (Pikuda *et al.*, 2019; Bownik *et al.*, 2020). The results of statistical analyses are reported in the supplementary data (Table S3.3), and the results pertaining to the swimming distance are reported in detail in Table S4. As seen from the results of the PVC-NP leachate experiment (Fig. S3.5), the leachate did not influence the swimming performance.

3.3.3.1. PS-NP exposure

It was observed that exposure to PS-NPs did not lead to changes in the swimming behavior of juvenile daphnids (p = 0.496). A few previous studies provide evidence of behavioral changes seen after a 48-h exposure to PS-NPs, but these studies used PS-NP concentrations several orders of magnitude higher than those tested in this study (Pikuda *et al.*, 2019; Vaz *et al.*, 2021; Nogueira *et al.*, 2022). Behavioral changes (hopping frequency) were reported in a previous study (Pikuda *et al.*, 2022) for longer exposure times and a higher concentration of 50,000 µg/L. Considering both the shorter exposure time and the lower tested concentrations used in this study, the absence of effects observed strengthens the evidence that PS-NPs do not induce behavioral alteration in *D. magna* at environmentally relevant concentrations, for both long (De Felice *et al.*, 2022) and short exposure times.

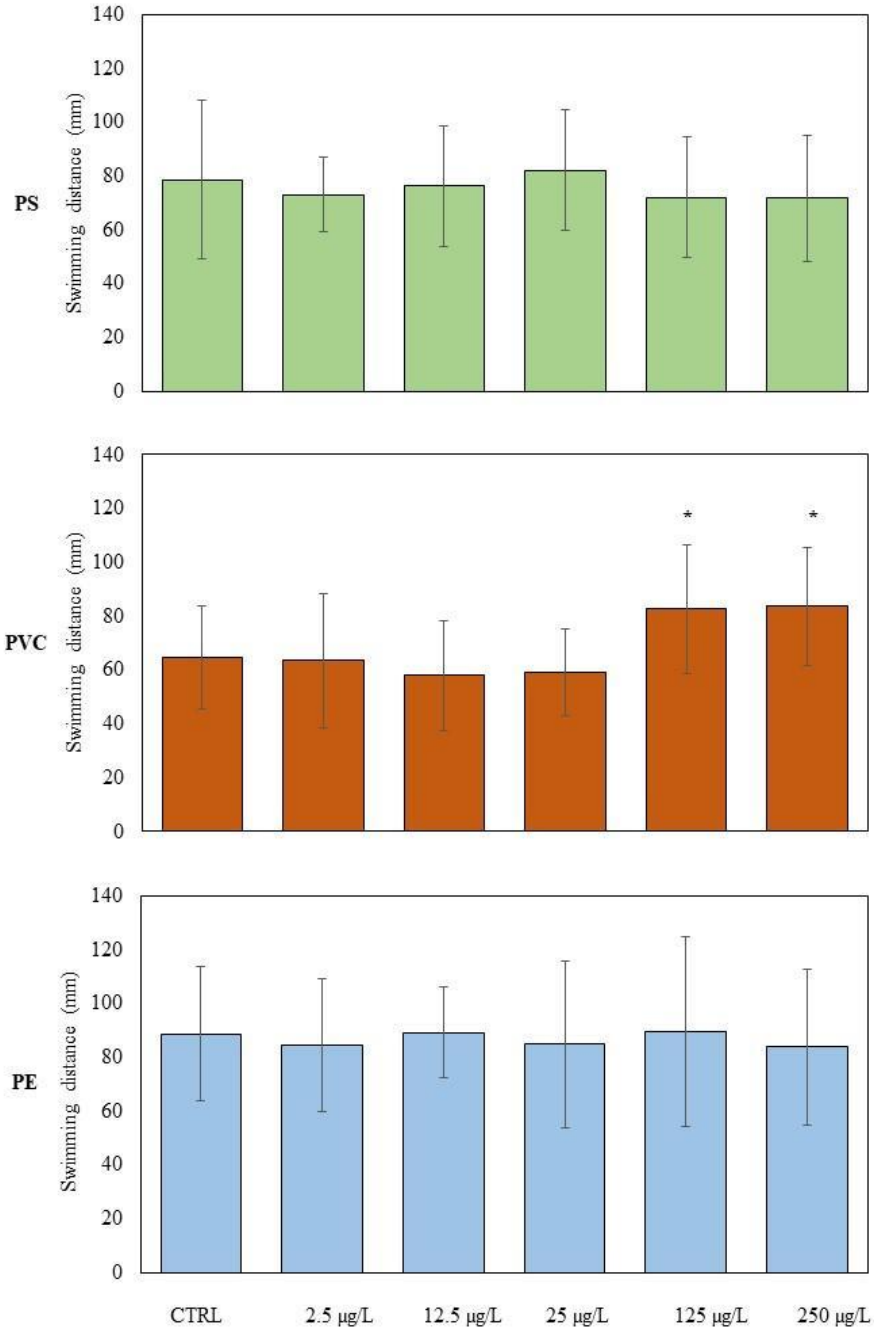

Fig. 3.3. Swimming behavior histograms showing the distance traveled by *D. magna* after a 48-h exposure to PS (a), PVC (b), and PE (c). * indicates statistically significant differences with respect to controls (p-value < 0.05).

3.3.3.2. PVC-NP exposure

The distances traveled by *D. magna* juveniles after treatment with 2.5-, 12.5-, and 25-µg/L PVC-NPs were very similar to the control values; however, at the two highest tested concentrations, the juveniles exhibited increased swimming activity (125 µg/L, p = 0.0335; 250 µg/L, p = 0.0138). The

PVC-NP leachate experiment further reinforced this stimulation in swimming activity caused by PVC-NPs ($p = 0.0198$), but, alternatively, it recorded no differences in the swimming activity between the leachate-treated juveniles and control group juveniles ($p = 0.9417$). Therefore, we conclude that the observed behavioral alterations are induced by PVC-NPs and not by the leachate released during the exposure.

The altered swimming behavior of daphnids draws attention to the potential hazards of this polymer to aquatic organisms. Behavioral alterations are considered ecologically relevant because they can potentially impact the ecosystem at a higher level (e.g., at the population and community levels) through a cascade of indirect effects (Saaristo *et al.*, 2018). In particular, stimulation of swimming activity can alter competition ability and prey–predator interactions, leading to increased encounter rates with predators or making individuals less adept at escaping from them (Saaristo *et al.*, 2018).

To the best of our knowledge, this is the first time that PVC-NP exposure–induced behavioral alterations in *D. magna* have been reported. This study has shown the adhesion of fluorescent-PS-NPs (20 nm) to the surface of the daphnid body and their presence in the digestive tract of *D. magna* juveniles (Fig. S3.4). Owing to the lack of fluorescent-PVC-NPs, the presence of PVC-NPs in the daphnids could not be verified, but daphnids exposed to PVC-NPs were found to retain reasonably similar behavior. In the literature, it is reported that the adhesion of nanoparticles (TiO_2 and SiO_2) to the external surface of *D. magna* can be considered a possible mode of inflicting physical nanoparticle toxicity (Dabrunz *et al.*, 2011). The coating of the body surface of organisms with nanoparticles can result in increased specific weight and, in turn, swimming burden, leading to changes in the swimming behavior aimed at mitigating the negative effects of nanoparticle adhesion (Wang *et al.*, 2021). In light of this, the changes in the swimming behavior observed after PVC-NP exposure could be ascribed to the physical properties and chemical structure of this polymer, which probably enhances its adhesion to the *D. magna* body surface as recently described by Yip *et al.* (Yip *et al.*, 2022). They report that PVC-NPs exhibit stronger surface interactions with the carapace of the acorn barnacle than PS-NPs. We are aware that this is a speculation and further investigations are required to better understand the observed effect, such as molecular changes in neurotoxicity biomarkers and SEM images of daphnids at the end of the exposure time.

Moreover, Weber *et al.* (Weber *et al.*, 2022) suggested that the density of PVC may be a contributory factor in its toxicity. The observations made in this study further corroborate this inference since PVC density (1.45 g/mL) is higher than those of PS (1.05 g/mL) and PE (0.92 g/mL), it may potentially have led to a higher burden on the body surface of *D. magna* juveniles, thus impacting their swimming behavior.

We underline that PVC-NPs (and PE-NPs) exhibit a higher size distribution than PS-NPs. The differences in size distribution, in particular the presence of particles smaller than 100 nm, could have played a role in the onset of the observed effects. In literature there is consensus on the importance of plastic size in causing hazard effects, with small plastic particles often tend to induce greater effect than large (Stock *et al.*, 2020; Pochelon *et al.*, 2021; Sendra *et al.*, 2021). Unfortunately, due to the analytical issues in isolating PVC and PE particles larger than 100 nm, we have not tested PVC-NPs in the exact size range of PS-NPs. Therefore, we cannot exclude that the observed differences could derive from differences in the size distribution. However, comparing PVC data with those of PE (refer to paragraph 3.3.2.), we infer that other factors, such as the surface chemistry of the different plastic polymers, might contribute rather than solely the size.

3.3.3.3. PE-NP exposure

PE-NP exposure did not induce behavioral alterations in the daphnids ($p = 0.808$). This result suggests that the modulation of ROS levels and CAT activity observed in *D. magna* exposed to this NP is not indicative of a condition of adverse sublethal effects that affects higher biological levels. It is plausible that, in contrast to PVC, an exposure time of 48 h was not enough for PE to induce behavioral alterations. Therefore, exposing *D. magna* to PE-NPs for longer periods of time (e.g., 21 days) could provide more effective information on the toxicity of this polymer on the organism.

By comparing the observed effects of different NP polymers with a similar particle size distribution, PE-NPs appear to be less toxic than PVC-NPs since at the individual level they don't induce changes in swimming behavior.

3.4 Conclusions

That different plastic particles induce different toxicity has been effectively demonstrated for MPs (Renzi *et al.*, 2019; Zimmermann *et al.*, 2020), whereas research is still in its infancy for NPs (Monikh *et al.*, 2022; Weber *et al.*, 2022; Li *et al.*, 2023). This study established that environmentally relevant NP concentrations could inflict sublethal toxicity on *D. magna* and that these effects could differ with the various polymers tested. It further confirmed that sublethal endpoints, both at the biochemical level (oxidative stress endpoints) and the individual behavioral level (swimming distance), can be sensitive to even low concentrations of the NP contaminant. This study also suggests that the onset of sublethal effects after PVC exposure can be ascribed to the chemical structure and physical properties of this polymer, which are different from those of PE. However, the direct comparison

between the toxicity of PS-NPs and the other two tested NP polymers is not straightforward due to the role of particle size on toxicity. Notwithstanding this, as PVC-NPs seem to induce a greater negative effect on *D. magna* than PE-NPs and given that they have particles of the same size, we have suggested that other factors might contribute to the toxicity rather than size only. The determination of the specific physical and chemical properties involved in the enhanced toxicity of PVC requires additional study. As the results in this study reveal that PS-NPs could not be the most hazardous NPs, it is crucial that the toxicity of other nanopolymers with different physicochemical properties be explored as well.

SUPPLEMENTARY MATERIAL

Table S3.1. DLS measurements of the hydrodynamic size (nm), PDI, and ζ-potential (mV) of the NPs in the exposure water in presence of *D. magna* excreted molecules at T0 and T1 (48 h). Data are presented as mean values of three consecutive measurements ± standard deviation.

	Exposure time	Size	PDI	ζ-potential
PS-NPs	T0	206± 61	0.069	-25± 7
	T1	218± 73	0.069	-23± 5
PE-NPs	T0	217± 89	0.193	-31± 8
	T1	244 ± 78	0.282	-16± 6
PVC-NPs	T0	234± 94	0.174	-28 ± 6
	T1	249± 100	0.172	-22 ± 7

Table S3.2. Mortality recorded for *D. magna* juveniles at the end of exposure time (n=60 for each tested concentration and relative control).

Treatment	Concentration (µg/L)	Mortality (%)
PS	2.5	5
	12.5	0
	25	5
	125	0
	250	1.6
	CTRL	5
PVC	2.5	6.6
	12.5	8.3
	25	1.6
	125	1.6
	250	6.6
	CTRL	3.3
PE	2.5	6.6
	12.5	10
	25	1.6
	125	3.3
	250	3.3
	CTRL	6.6

Table S3.3. Results of statistical analyses on the measured endpoints (ROS levels, CAT activity and swimming distance) for the different NPs exposures (PS, PVC, PE) on *D. magna.* * reports statistically significant differences (p-value < 0.05).

Endpoint	Treatment	F	Df	P
ROS	PS	1.24	5	0.33
	PVC	5.791	5	0.002*
	PE	14.818	5	0.011*
CAT	PS	2.522	5	0.0934
	PVC	1.528	5	0.253
	PE	10.02	5	0.0004*
Swimming distance	PS	0.88	5	0.496
	PVC	7.239	5	< 0.0001*
	PE	0.457	5	0.808

Table S3.4. Results of swimming distance recorded or the different NPs exposures (PS, PVC, PE) on *D. magna*. The mean swimming distance (± standard deviation) is expressed in mm.

Treatment	Concentration (ug/L)	Swimmming distance (mm)
PS	2.5	73.0 (± 13.7)
	12.5	76.1 (± 22.6)
	25	82.0 (± 22.3)
	125	71.8 (± 22.4)
	250	71.6 (± 23.3)
	CTRL	78.5 (± 29.3)
PVC	2.5	63.5 (± 25.0)
	12.5	57.9 (± 20.5)
	25	59.1 (± 16.9)
	125	82.6 (± 23.3)
	250	83.6 (± 21.9)
	CTRL	64.6 (± 18.9)
PE	2.5	84.6 (± 24.6)
	12.5	89.2 (± 16.8)
	25	84.7 (± 31.0)
	125	89.6 (± 35.1)
	250	83.7 (± 29.1)
	CTRL	88.6 (± 25.0)

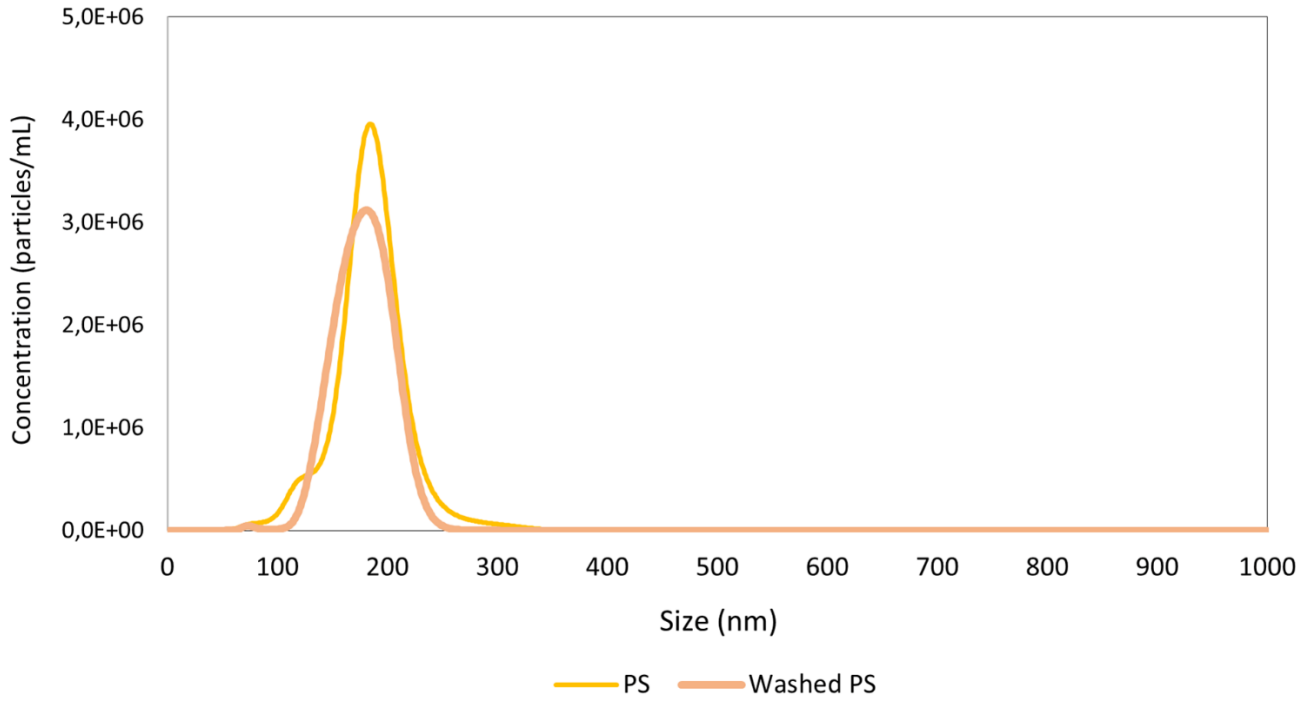

Figure S3.1. NTA analyses results for PS-NPs and washed PS-NPs.

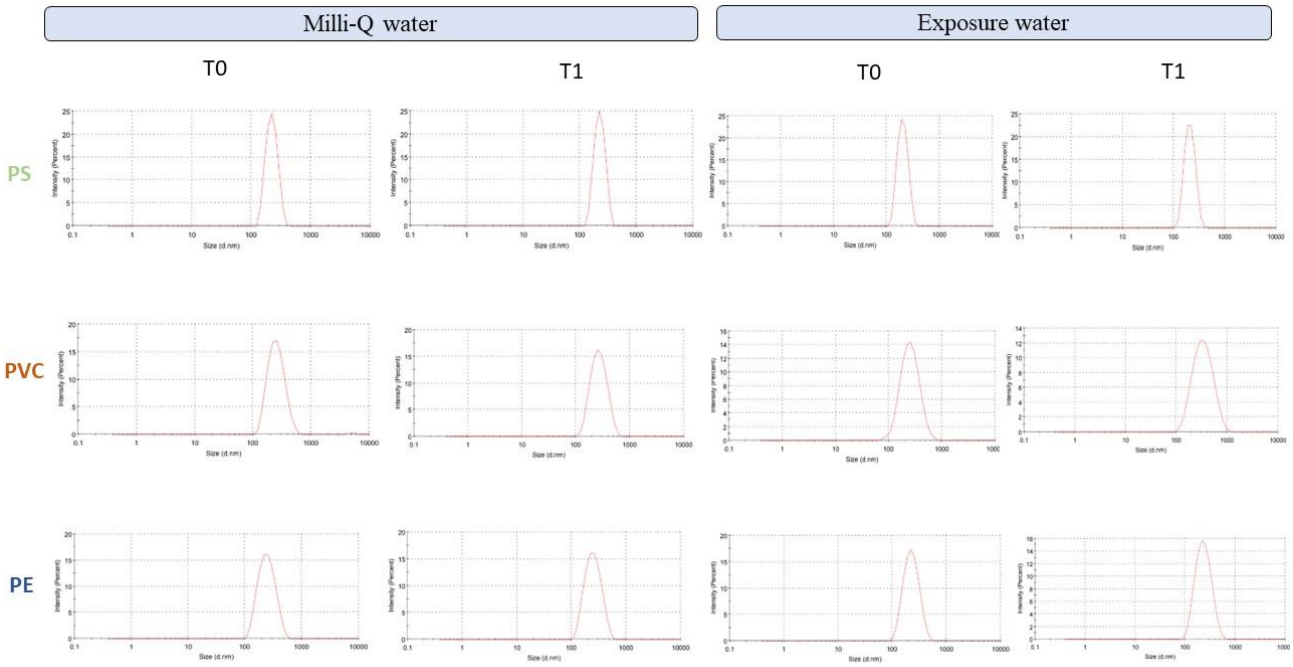

Figure S3.2 DLS intensity measurement of PS-NPs, PVC-NPs and PE-NPs at T0 and T1 in both Milli-Q and exposure waters.

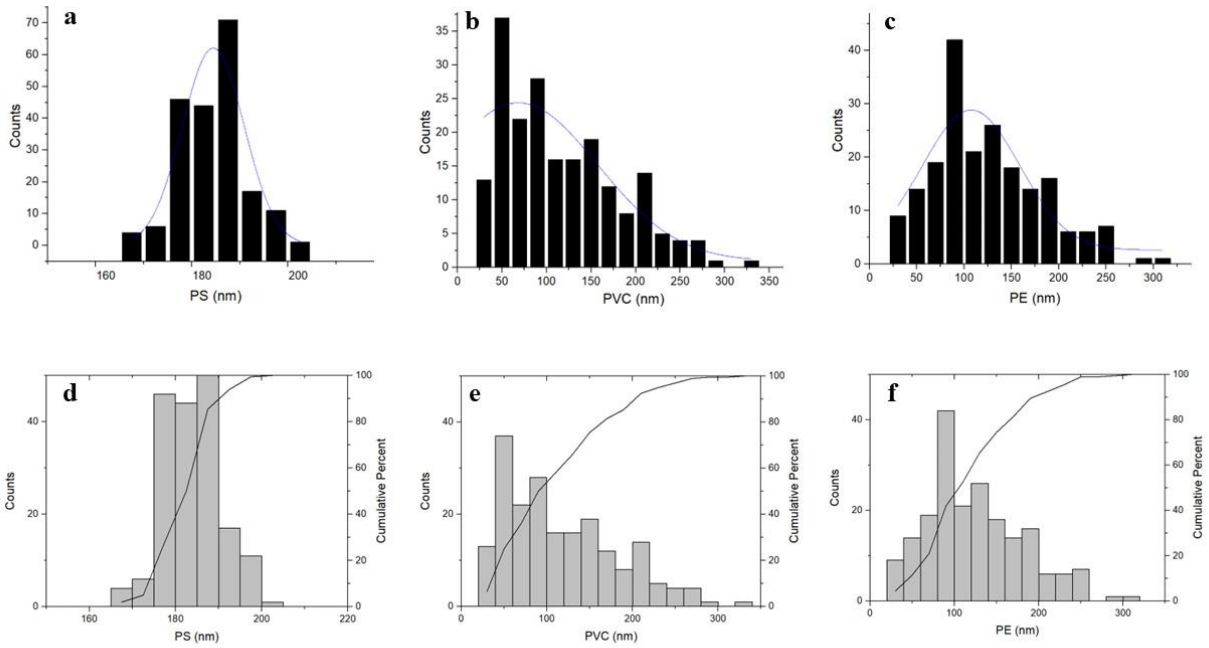

Figure S3.3: Histograms in the top row report the MinFeret (nm) size distribution and relative Gauss fit for PS-NPs (a), PVC-NPs (b), and PE-NPs (c), calculated through TEM measurements. The lower row reports the corresponding cumulative percent curves for PS-NPs (d), PVC-NPS (e), and PE-NPs (f).

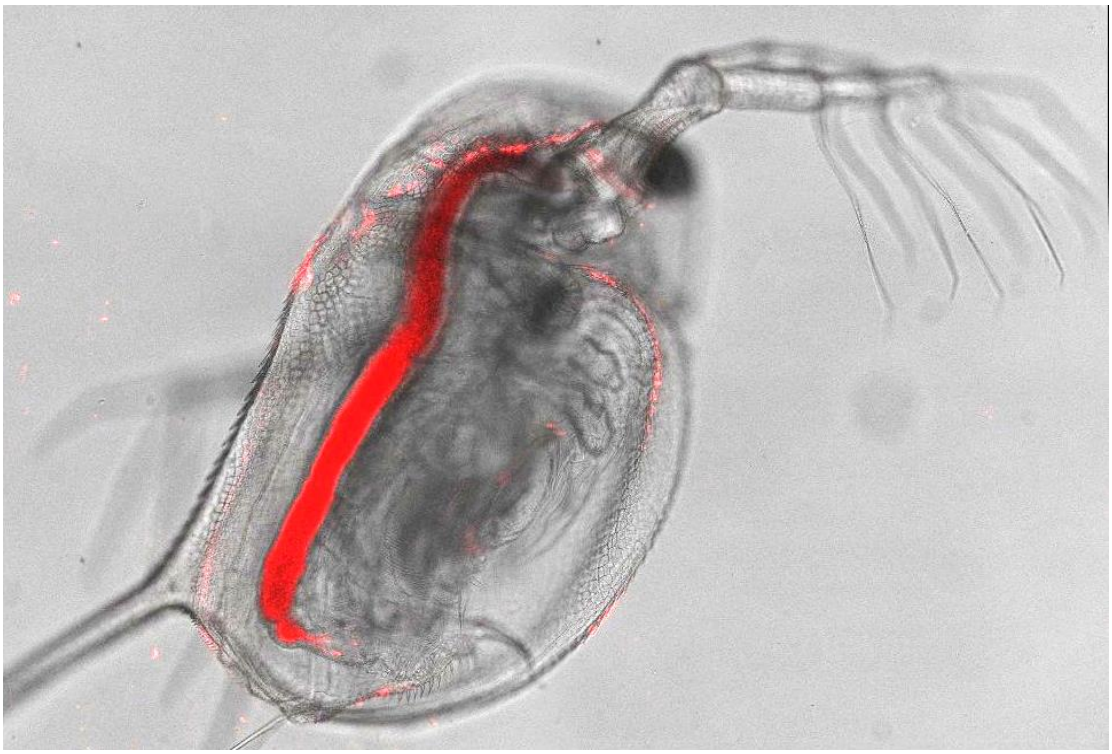

Figure S3.4. Confocal fluorescence microscopy image of PS-NPs ingestion by *D. magna*.

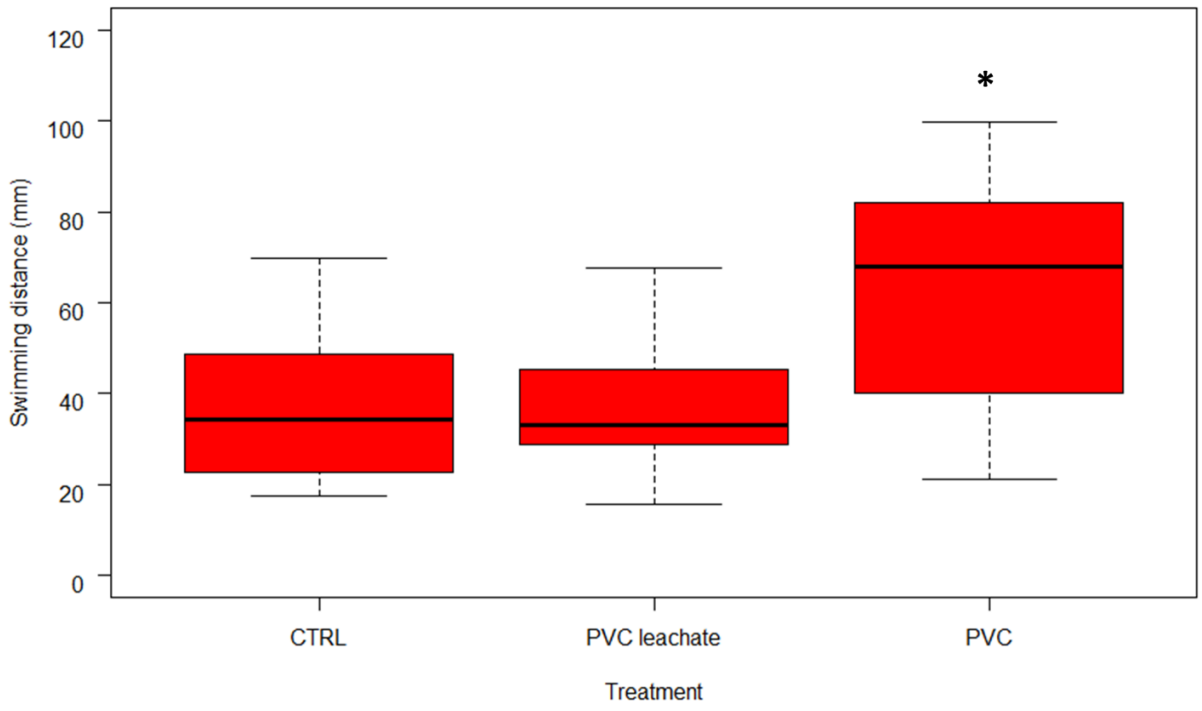

Figure S3.5. Boxplot of swimming activity (distance traveled) measured in *D. magna* individuals after the exposure to PVC-NPs leachate and PVC-NPs (250 µg/L) treatments . * indicates statistically significant differences with respect to controls (p-value < 0.05).

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Chapter 4

Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in *Daphnia magna*

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Abstract

The aim of this study was to evaluate the effects of chronic exposure (21 days) to an environmentally relevant concentration (10 µg/L) of two different nanoplastic (NP) polymers on the aquatic model organism *Daphnia magna*. This study examined the impact of exposure to 200 nm polystyrene nanoplastics (PS-NPs) and polyvinyl chloride nanoplastics (PVC-NPs), which had an average size similar to that of PS-NPs (ranging from 50 nm to 350 nm). The effects of polymer exposure on morphometric parameters, number of molts, swimming behaviour, and reproductive outcomes were evaluated.

The findings indicate that PVC exposure induced higher body dimensions, while both polymers resulted in an increase in molting behaviour. Moreover, exposure to PVC-NPs had a negative impact on the reproduction of *D. magna*, as evidenced by a delay in the day of the first brood, a reduction in the total number of offspring produced, and, consequently, a slower population growth rate. It is hypothesised that the ingestion of PVC-NPs by *D. magna* may have resulted in an impairment of ecdysone hormone functionality and that the increased moulting events potentially representing an adaptive response to the negative effects of PVC-NP adhesion to the organism's body surfaces. These two organisms' responses could concur to explain the observed effects.

This study identified the fitness impairments caused by exposure to PVC-NPs, which can lead to relevant ecological consequences. The comparative analysis of the effects induced by two types of polymers has revealed the generation of disparate hazards to *D. magna*. Furthermore, the chemical composition appears to be a pivotal factor in the onset of these effects. It can therefore be stated that PS is not a suitable standard for representing the toxicity of all plastics.

4.1. Introduction

Plastics are considered ubiquitous contaminants [1] because of their constant disposal into the environment, which is enhanced by their global production, reaching nearly 400 million tons in 2022 [2]. In freshwater ecosystems, municipal wastewater treatment plants contribute significantly to the presence of plastics in the environment, derived from industrial wastewater, domestic wastewater, and rainwater [3-5]. Microplastics (MPs) can be defined as plastics from 5 mm to 1 μm in diameter, whereas nanoplastics (NPs) are those ranging between 1 nm and 1000 nm [6,7]. The size of plastics strongly affects their interactions with biota and the potential hazards they pose to ecosystems [8], with small plastics generally having a greater impact than larger ones [9,10]

Scientific research has largely focused on MPs [11,12]. The available data on their environmental concentrations and effects on organisms have contributed to progress in assessing the risks of MPs [13]. In contrast, our understanding of the environmental concentrations and effects of NPs on biota is still limited [14-17]. The quantification of NPs in environmental matrices is currently hampered by methodological limitations, which present a significant challenge in assessing the environmental pollution levels of these particles [18,19]. Ecotoxicological studies investigating the hazards of NPs to aquatic organisms are scarce. The evaluation of the toxic effects of NPs has typically focused on engineered plastic nanospheres as substitutes for environmentally relevant NPs [20]. In this context, polystyrene (PS) is the only plastic polymer that can be easily synthesised in the nanometric size range [21]. Consequently, evaluation of the effects of other NP polymers is still in its infancy because of the lack of feasible synthesis protocols [22,23]. However, since a wide variety of plastic polymers are occurring in the environment [24,25], it is crucial to identify the specific hazard associated with each polymer to ensure appropriate risk assessment [26]. Moreover, the polymer type can influence plastic toxicity [27-29]. Consequently, the use of PS toxicity data as a proxy for all other NPs has recently been questioned [30].

From this perspective, polyvinyl chloride (PVC) is worth consideration because it is the third-most produced plastic polymer worldwide [2]. Furthermore, its presence in environmental matrices as NP has been recently confirmed [25,31], and it is considered a potential hazard for the environment [32-34].

The present study aimed to contribute to the production of evidence of the effects of PVC-NPs and PS-NPs on *Daphnia magna*, by evaluating the chronic effects resulting from a 21-day exposure to engineered PVC and PS nanospheres at environmentally relevant concentrations. The microcrustacean *Daphnia magna* is a freshwater species recommended by the Organization for

Economic Cooperation and Development (OECD) as a model organism for toxicity testing of chemicals [35] and it is also considered a suitable model for studying the toxicity of NPs [23,36]. Chronic exposure more closely reflects the conditions experienced by *D. magna* in the environment than short-term exposure [37] and allows the investigation of chronic effects that are considered essential for the correct evaluation of NP toxicity [38]. However, only a limited number of studies have investigated the chronic effects of NPs on organisms. Moreover, most of these studies have focused on PS-NPs, whereas the impacts of other polymers at the nanometric scale remain largely unexplored [20].

A multiple endpoint approach was used to assess the potentially harmful effects of *D. magna* exposure. For each individual, the life-history traits of survival, growth, and reproduction were monitored until the end of the experiment. According to the dynamic energy budget theory [39], certain contaminants can affect an organism's uptake of food and its use of energy and nutrients for maintenance, growth, juvenile development, and reproduction. In addition, we investigated the modulation of swimming behaviour, which is considered a sensitive biomarker [40]. This approach allows for a broad evaluation of the potential impact of NPs on *D. magna*. The evaluation and comparison of the chronic effects induced by PS-NPs and PVC-NPs can help elucidate the role of polymers in the onset of hazards to ecosystems, providing useful data for better characterisation of the ecological risk of NPs.

4.2 Materials and methods

4.2.1. Procurement and characterization of nanoplastics

The PS-NPs used in this study were monodisperse nanospheres (nominal diameter200 nm) supplied at a nominal concentration of 25 mg/mL (Polysciences Europe GmbH, Germany). PVC-NPs were procured from the European Commission’s Joint Research Centre (JRC, Ispra, Italy) and consisted of polydisperse nanospheres synthesised in a range of sizes between 50 and 350 nm at a concentration of 2 mg/mL, as described in the protocol proposed by Cassano et al. [41,42]. As reported by the suppliers, PS-NPs had a density of 1.05 g/mL, whereas that of PVC was 1.40 g/mL.

Both tested NP solutions were carefully characterised before the exposure experiments. A full and detailed description of the NP characterisation and quality control has been published in our previous work [29]. The spherical shape of the particles was confirmed by scanning electron microscopy and

transmission electron microscopy, and the chemical characterisation of the polymer was assessed using confocal Raman spectroscopy. The nominal concentrations and sizes of the NPs were verified through nanoparticle tracking analysis. Moreover, the hydrodynamic size and ζ-potential of NPs in the testing sample water were assessed before starting the exposure and after 48 h through dynamic light scattering (DLS). The size of the NP suspension was also analysed by transmission electron microscopy; PS-NPs exhibited an average size of approximately 200 nm, whereas PVC-NPs showed a wider size distribution ranging from 50 nm to 350 nm, with a mean size comparable to that of PS-NPs. The ingestion and presence of NPs on the body surfaces of exposed *D. magna* individuals were confirmed using a test with fluorescent PS-NPs. Finally, because the release of Cl^- from PVC solutions can induce toxicity in organisms [43], ion chromatography analyses were conducted on the PVC-NP leachate, which showed the absence of chlorine release by the PVC-NPs. Transmission electron microscopy, scanning electron microscopy, Raman spectroscopy, and DLS intensity measurements of PS-NPs (Fig. S4.1), and PVC-NPs (Fig. S4.2) are reported in the Supplementary Data.

4.2.2. *Daphnia magna* population maintenance

D. magna Straus (1820) individuals, obtained from ephippia purchased from Microbiotest (Daphtoxkit F, DM586), were cultured at the University of Milano-Bicocca (Italy) according to OECD Test Guideline No. 211 [35]. A detailed description of *D. magna* population maintenance was reported in our previous study [29].

4.2.3. *Daphnia magna* chronic exposure assay

The 21-day reproduction tests were conducted in accordance with the OECD Test Guideline No. 211 [35], using third-generation *D. magna* neonates less than 24 h old. Three treatments were simultaneously performed: PVC-NPs, PS-NPs, and a control treatment (CTRL) consisting of pristine water. Each treatment involved 12 replicates with one individual placed in a glass vessel filled with 25 mL of the exposure solution. For the treatments involving PVC-NPs and PS-NPs, a concentration of 10 µg/L of NPs was added to each vessel. The individuals were maintained under the same conditions as the breeding individuals and provided with ample food. The static renewal method was applied every 48 h in this study. Molts and newborns were monitored daily and released.

The population intrinsic growth rate (r_m, day^{-1}), was derived from the Euler–Lotka equation [44]:

$$\sum e^{-rx}\, l_{xm_x=1} \quad \text{(Eq. 4.1)}$$

where x is the age class (days; 0–n), l_x is the probability of survival at age x, and m_x is fecundity at age x.

At the end of the bioassays, morphometric and behavioural data were collected for each adult. Behavioural alterations were evaluated as detailed in our previous study [29] by measuring the distance travelled (mm), velocity (mm/s), acceleration (mm/s^2), and activity time (%).

Morphometric parameters were assessed after video recording. Individuals were photographed, and images were analysed using ImageJ software to measure body length and width. Body length was measured from the top of the head to the base of the spine [35], and body width was measured at the widest point (Fig. S4.3). To assess the vitality of the offspring, 15 individuals (less than 24 h old) were randomly selected from the first brood of the CTRL, PS-NP, and PVC-NP groups. Offspring behaviour was recorded in the same manner as that of adults, and their swimming distance travelled was measured as an indicator of vitality.

4.2.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the effects of NP exposure on the investigated endpoints. Each endpoint (body length, body width, number of molts, number and time of the first brood, number of broods, number of neonates, and swimming behaviour parameters) was considered a dependent variable, and the different treatments (CTRL, PS-NPs, and PVC-NPs) were used as predictors. The Shapiro–Wilk test was used to verify data normality. An ANOVA or t-test was used to analyse the effects of NP exposure on the investigated endpoints of the normally distributed data. If the data were not normally distributed, they were log-transformed before statistical analyses were conducted. In cases where data were not normally distributed after log transformation, the Kruskal–Wallis test was used. Statistical significance was set at $p < 0.05$. Tukey's or Dunn's post-hoc tests were used after ANOVA or Kruskal–Wallis tests, respectively, to detect significant differences between the treatments.

Pearson's or Spearman's correlation analysis was conducted to determine the correlation between different variables. Principal component analysis (PCA) was performed to integrate and highlight the associations among the investigated variables, with data scaled prior to analysis. Before proceeding

with the PCA, variables with a high correlation value ($r > 0.9$) were discarded. PCA was conducted on a data matrix comprising 36 samples (rows) and 6 variables (columns). All the statistical analyses were conducted using R software, implementing "ggplot2", "ggfortify", and "corrplot" packages in R version 4.3.2 [45].

4.3. Results and discussion

The tests met the requirements specified in the OECD Test Guideline No. 211 [35], as the mortality observed in the CTRL treatment was below 20%. The results of the ecotoxicological endpoints are presented in Table 4.1. According to the statistical test results, exposure to the different NP polymers elicited varying responses in *D. magna*.

Table 4.1. Morphological, physiological, survival, reproductive, behavioural, and population dynamic endpoints measured in *D. magna* individuals over 21 days of exposure to CTRL, PS-NP, and PVC-NP treatments. Data are reported as mean ± standard deviation. (*p < 0.05; **p < 0.01; and ***p < 0.001 vs. CTRL).

Endpoint	Unit of measure	CTRL	PS-NPs	PVC-NPs
Morphological	Length (mm)	2.9 ± 0.2	2.9 ± 0.2	3.2 ± 0.1**
	Width (mm)	1.9 ± 0.1	1.9 ± 0.2	2.1 ± 0.1*
Physiological	Number of molts	6.0 ± 1.1	6.9 ± 1.6 *	7.3 ± 1.1**
Survival	Number of dead	0	0	0
Reproductive	Number of broods	3.0 ± 0.8	2.2 ± 1.3	0.83 ± 0.8***
	Time of the 1st brood (day)	10.8 ± 1.4	14.7 ± 4.9	18.4 ± 5.1***
	Number of offspring per parent	27.5 ± 18.4	25.9 ± 16.2	10.9 ± 11.4**
Behavioural	Velocity (mm/s)	12.8 ± 3.6	12.8 ± 4.2	12.3 ± 3.1
	Acceleration (mm/s^2)	215 ± 45.9	206 ± 38.2	210 ± 37.4
	Distance (mm)	313 ± 103	299 ± 125.0	297 ± 89.0
	Activity (%)	79.6 ± 12.0	72.1 ± 11.7	79.0 ± 10.0
Population dynamic	Population growth rate (day^{-1})	0.41 ± 0.22	0.30 ± 0.18	0.10 ± 0.10***

4.3.1. Morphological and physiological endpoints

Regarding morphological traits, individuals exposed to PVC-NPs exhibited a higher average length and width than those exposed to CTRL and PS-NPs. Statistically significant differences in length were observed between individuals in the PVC-NP and CTRL groups ($p = 0.0029$) and between PVC-NP and PS-NPs groups ($p = 0.0260$). Statistically significant differences in body size were confirmed for width, with differences observed between PVC-NPs and CTRL ($p = 0.0203$) and PVC-NPs and PS-NPs ($p = 0.0209$). However, no alterations in the width/length ratio were detected, with a width/length ratio of 1.5 as reported by Chen et al. [46].

Regarding PS-NP results, the absence of effects on morphometric parameters is in line with the findings of Liu et al. [47] after a 21-day exposure at 1 µg/L of 75 nm PS-NPs. However, Besseling et al. [48] and Pikuda et al. [49] have shown the onset of morphometric alterations in *D. magna* in response to PS-NP exposure only at high tested concentrations (mg/L) and Heinlaan et al. [50] after multigenerational exposures at low concentrations (µg/L). To the best of our knowledge, there is no evidence in the literature on the dimensional changes in *D. magna* in response to exposure to PCV-NPs. Kim et al [51] reported morphological changes in the cladoceran *Moina macrocopa* when exposed to concentrations > 100 µg/L of a mixture of micro and nanosized PVC, resulting in a decrease in dimensions. In contrast, for PVC-MPs (>10 µm), alterations in morphometric parameters have generally been ascribed to the toxic chemicals present in the plastic bulk materials rather than to the polymer itself [27,33].

Considering the physiology of *D. magna*, both NP treatments increased the number of molts (Fig. 4.1).

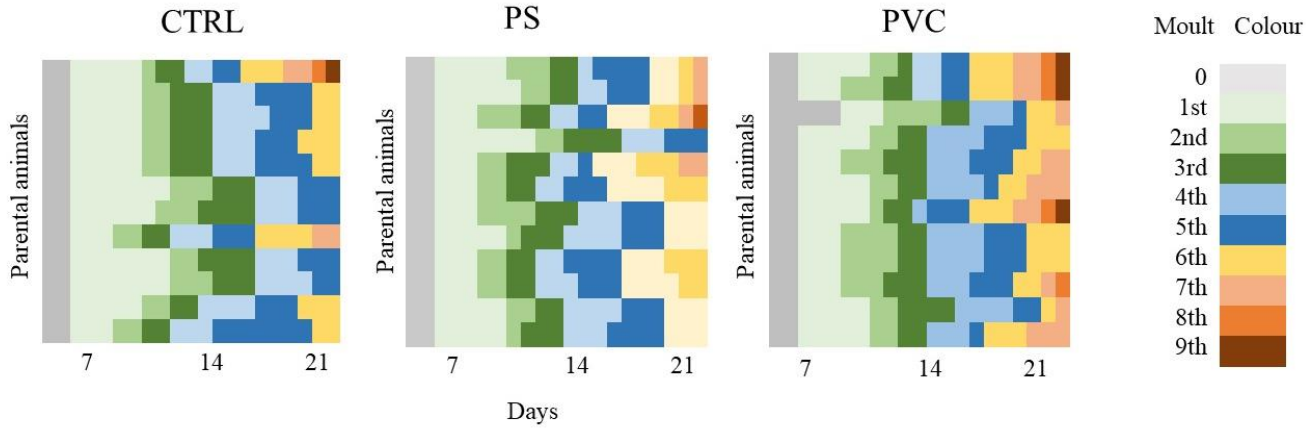

Figure 4.1. Graphical representation of the number of molts produced by *D. magna* (12 individuals per treatment) over a 21-day exposure to CTRL, PS-NPs, and PVC-NPs. Each row corresponds to a surviving parent animal.

The molting rate of CTRL individuals was lower (0.29 ± 0.05 molt/day) than those of PS (0.33 ± 0.06 molt/day) and PVC (0.35 ± 0.05molt/day). Statistical analysis showed that molting behaviour was significantly stimulated by both PVC-NP and PS-NPs treatments compared to CTRL ($p = 0.0022$ and $p = 0.0208$, respectively).

The CTRL treatment displayed a stepped curve in the molting cycle, with intermolt periods lasting three days, which is consistent with the normal behaviour reported by LeBlanc [52]. In contrast, the two NP treatments exhibited a continuum in the molt cycle, resulting in shorter intermolt periods (Fig. S4.4).

The present study showed that exposure to PS-NPs stimulated molting behaviour, which contrasts with previous findings. Rist et al. [53] found no alteration in molting behaviour after a 21-day exposure to 100 nm PS-NPs at 0.1, 0.5, and 1 mg/L, whereas Pikuda et al. [49] reported a decrease in the number of molts at 50 mg/L of 200 nm PS-NPs. The different particle sizes and concentrations tested may have contributed to the observed differences.

With regard to the effects of PVC exposure, in addition to the observed increase in body size, a stimulation of moulting activity was also observed. In arthropods, the functions of ecdysteroids, in particular ecdysone, are related to both growth and molting differentiation [54]. *D. magna* undergoes periodic exoskeletal exfoliation throughout its lifespan, accompanied by continuous molting and growth [55], with ecdysteroids mediating this process [56]. The observed effects of PVC-NPs on the body dimensions and molting behaviour of *D. magna* may therefore indicate a potential impairment of the hormonal functionality of ecdysone. Owing to the lack of studies conducted on PVC-NPs and *D. magna*, we considered it opportune to compare our data with the current evidence for PVC-MPs. A recent study showed that small-sized PVC-MPs (2 ± 1 μm) can cause an increase in the molting frequency of *D. magna* [57]. The authors suggested that the ingestion of small PVC-MPs might affect the expression of genes responsible for the molt cycle, specifically those related to ecdysone biosynthesis. However, Kim et al. [51] reported the opposite effect, with micro-nano PVC fragments causing an extension of the intermolt period in *M. macrocopa*.

In addition to NP ingestion, another factor that may have concurred to the observed change in the molting cycle of *D. magna* exposed to PVC is the difference in the physical and chemical properties between PVC and PS [29]. This difference may have increased the adhesion and burden of PVC on the body surfaces of *D. magna*, which could have stimulated the observed molting behaviour. Yip et al. [58] reported that PVC-NPs exhibited stronger surface interactions than PS-NPs, which supports our hypothesis. Moreover, it should be noted that the external surface of *D. magna* individuals may

have a higher load because of the higher density of PVC compared with PS. Interestingly, previous studies on nanomaterials have reported that the adhesion of TiO_2 and SiO_2 nanoparticles to the body surface of Daphnia can increase their specific weights. This can lead to alterations in the molting behaviour as a possible pathway for physical nanoparticle toxicity [59,60]. The authors proposed a causal relationship between the upregulation of genes related to molting (e.g., "*eip*") and the increase of ecdysone production, which accelerates the exfoliation of the exoskeleton to facilitates the shedding of the negative effects of nanoparticle adhesion. Similarly, the stimulation in molting behaviour for both NP treatments observed in the present study suggests that a possible pathway of NP toxicity could be related to biological surface coating. It is important to note that the main objective of this study was to evaluate the chronic effects of different NP polymers rather than to determine their specific mechanisms of action. Therefore, further analyses should be conducted to gain a better understanding of the role of physicochemical properties in the onset of the observed effects.

4.3.2. Reproductive endpoints

The effects on *D. magna* reproduction were assessed by monitoring the number of broods (Fig. 4.2), day of broods (Fig. S4.5), number of neonates produced in each brood (Fig. S4.6), and number of neonates produced over 21 days (Fig. 4.3).

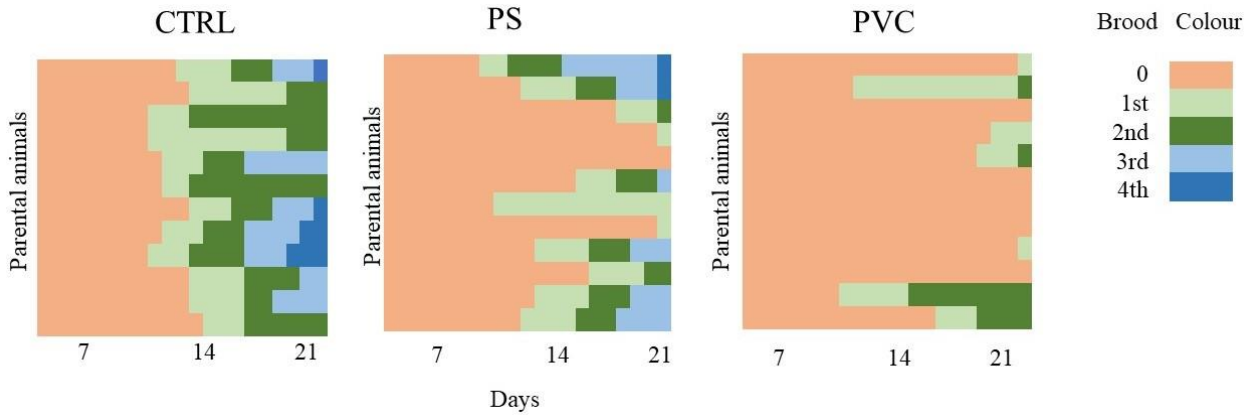

Figure 4.2. Graphical representation of the number of broods produced by *D. magna* (12 individuals per treatment) over a 21-day exposure to CTRL, PS-NPs, and PVC-NPs. Each row corresponds to a surviving parent animal.

The average number of broods generated under the CTRL treatment was 3.0 (± 0.8), whereas for PS-NPs and PVC-NPs, the averages were 2.2 (± 1.3) and 0.83 (± 0.8), respectively (Fig. 2). Statistical

analyses revealed a significant difference in the number of broods between PVC-NPs and CTRL ($p < 0.00001$), and between PVC-NPs and PS-NPs ($p = 0.0057$). In the CTRL group, the mean for the first brood was 10.8 days (± 1.4), whereas in the PS-NP and PVC-NP groups, the means were 14.7 (± 4.9) and 18.4 (± 5.1) days, respectively. The delay of the day of the first brood observed for the PVC-NP treatment was statistically different from that of CTRL ($p = 0.0007$), whereas no statistically significant difference was observed between CTRL and PS-NPs. No statistical differences were found among the treatments with respect to the timing of the second, third, and fourth broods (Fig. S4.5).

In terms of the number of offspring produced per parent animal, CTRL individuals had an average number of 27.5 (± 18.4) newborns per female, whereas parents exposed to PS-NPs and PVC-NPs produced 25.9 (± 16.2) and 10.9 (± 11.4) newborns, respectively. The average number of offspring in the PVC-NP group differed significantly from that in the CTRL ($p = 0.0089$) and PS-NP ($p = 0.0137$) groups. The cumulative curve of the number of offspring produced by the parent animals (Fig. 4.3) indicated a negative impact of NP treatments on *D. magna* reproduction. The number of offspring produced under each treatment was modelled by a second-degree polynomial regression, which provided a good fit for the CTRL ($R^2 = 0.9869$; $F = 413.2$; $p < 0.00001$), PS-NP ($R^2 = 0.9862$; $F = 393.4$; $p < 0.00001$), and PVC-NP ($R^2 = 0.9278$; $F = 70.65$; $p < 0.00001$) treatments.

In the CTRL treatment group, 50% of all offspring (n = 165 newborns) were produced by day 15. However, the PS-NP group produced 88 newborns by day 15, requiring three more days to reach 165 newborns. In contrast, PVC-NP parents produced only 11 % (n = 18) of the offspring produced under CTRL by day 15. Moreover, the PVC-NP group, in contrast to the PS-NP group, did not reach the 50% of the total offspring produced by the CTRL group; by the end of the test, they produced only 131 newborns in total. Notably, there was no statistically significant difference in the average number of neonates produced in each brood between the different treatments (Fig. S4.6). Therefore, the observed impairment in the fitness of *D. magna* under the PVC-NP treatment appeared to be primarily caused by a delay in the timing of the first brood, and a consequent decrease in the number of broods. PS-NP exposure resulted in an initial delay in newborn production without significantly affecting population growth. In contrast, the PVC-NP treatment impaired *D. magna* reproduction, leading to a significant reduction in the number of offspring produced.

At the current state of knowledge, the effects of NPs on reproduction in *D. magna* are still uncertain, with contrasting observations among different studies. Heinlaan et al. [50] reported the absence of an effect on the reproductive endpoints after multigenerational exposure to environmentally relevant concentrations of PS-NPs, whereas Monikh et al. [28] reported a decrease in the number of broods after a 21-day exposure.

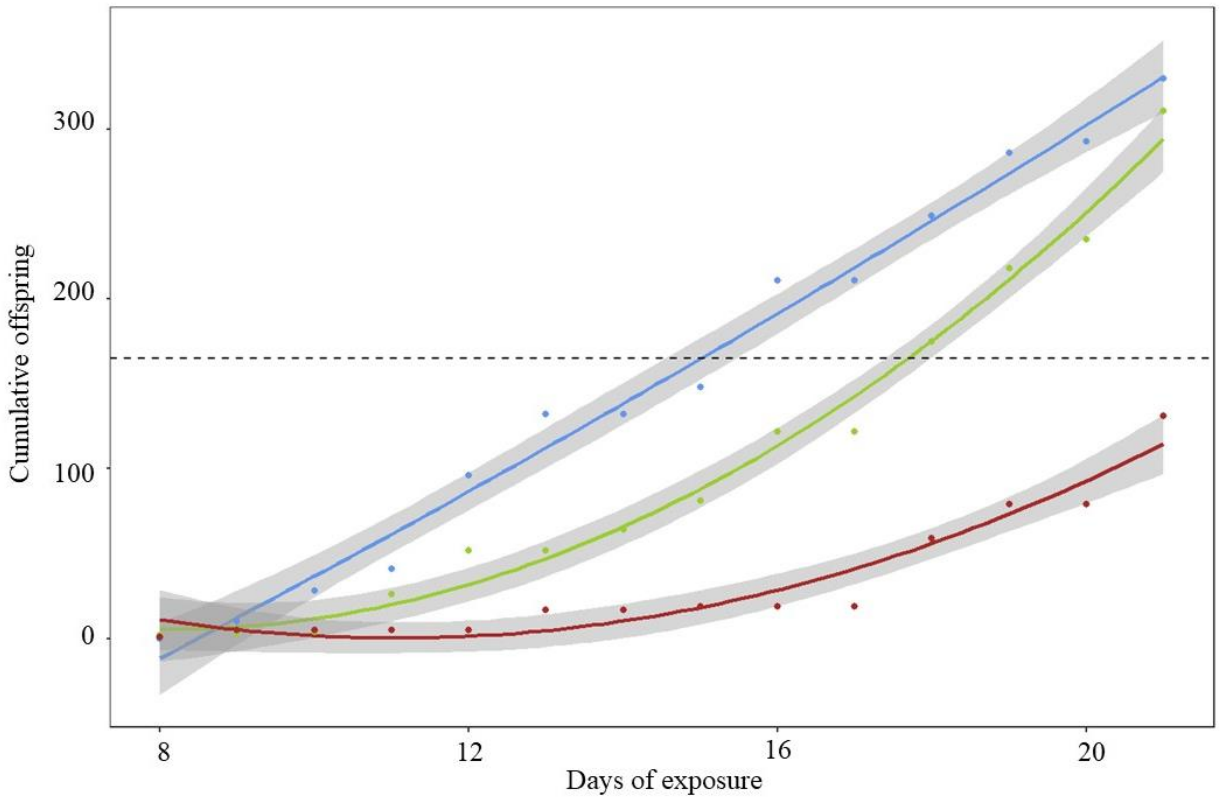

Figure 4.3. Cumulative offspring curves of the twelve *D. magna* parents over 21 days for the CTRL (blue), PS (green), and PVC (red) treatments, obtained through a second-grade polynomial regression of experimental data (coloured dots). The black dashed line indicates the 50% of the total offspring (n = 165) produced under the CTRL treatment. The shaded region represents the 95% confidence interval for the true value.

Although the absence of the effects induced by PVC NPs was recently reported [28], the results of other studies are consistent with those of the present study. Liu et al. [57] showed that small PVC-MPs (2 ± 1 μm) caused a delay in the day of the first brood and a decrease in the average number of offspring, whereas the exposure of the cladoceran *M. macrocopa* to PVC fragments (ranging from 0.2 μm to 20 μm) led to inhibitory effects on reproductive fitness and population structure [51]. This suggests that PVC may have a long-term impact on the health of cladoceran populations. Notwithstanding, the mechanism of PVC-NP toxicity in reproduction remains largely unknown. We retain plausibly that the increased molting behavior and body dimension observed in PVC-NP exposure treatments may have concurred to a reduction of energy nutrients available for embryo development, leading to the observed effects on *D. magna* reproduction. The molting process plays a fundamental role in determining the growth and development of *D. magna* [61-63], and its cost is considered a major life-history constraint that can lead to an insufficient amount of energy to be spent

for reproduction [64]. In this context, a recent article suggested that the increase in molting behavior and the decrease of offspring observed after exposure of *D. magna* to small PVC-MPs can be ascribed to alteration of energy metabolism [57].

4.3.3. Population dynamic endpoint

The population intrinsic growth rate (r_m), calculated using Eq. 1, is considered a suitable parameter for evaluating population health, because it is affected by the fecundity, lifespan, and developmental speed of organisms [65]. Figure 4.4 shows the calculated r_m values for the three groups. Statistical analysis revealed significant differences in the r_m values between PVC and CTRL ($p = 0.0003$), and between PVC and PS ($p = 0.0162$).

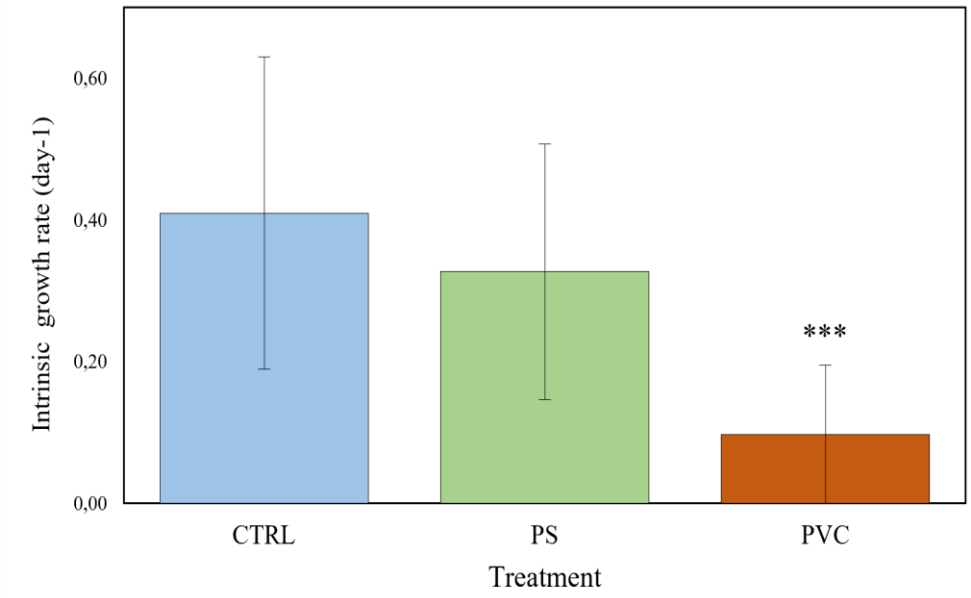

Figure 4.4. Intrinsic population growth rate of *D. magna* after a 21-day exposure to CTRL, PS-NP, and PVC-NP treatments. (***$p < 0.005$ vs. CTRL).

The r_m in the CTRL group (0.41 ± 0.22 days^{-1}) is in line with previous studies [66,67]. Regarding PS-NP exposure, our results showed a lower r_m (0.30 ± 0.10 days^{-1}) than that for the CTRL group. A similar reduction in r_m was reported for *D. magna* in response to 100 µg/L of PS-MPs ranging from 1 to 5 µm [68]. In contrast, the PVC-NP treatment resulted in a strong decrease in reproductive performance, which was confirmed by a very low r_m (0.10 ± 0.10 days^{-1}).

These results suggest that exposure to PVC-NPs had a greater effect on the sexual maturity and reproduction of *D. magna* than exposure to PS-NPs. This was demonstrated by a significant delay in

the day of the first brood, significant reduction in the average number of offspring produced, and sharp decrease in the intrinsic population growth rate.

4.3.4. Behavioural endpoints

Regarding behavioural traits, the evaluation of swimming behaviour did not reveal statistically significant differences among the different treatments (Table 4.1). In our previous study [29], we reported that a shorter exposure time (48 h) to a comparable concentration (12.5 µg/L) of the two tested NP polymers did not induce alteration in the swimming behaviour of *D. magna* juveniles. Regarding PS-NPs, the absence of swimming alterations is in line with the evidence reported in the literature after 21 days of exposure [69,70]. Regarding PVC-NPs, we previously showed that an exposure of 125 µg/L induced stimulation of swimming activity of *D. magna* juveniles [29]. The lack of effect observed in the present study could be attributed to the lower tested concentration (10 µg/L) and to the fact that *D. magna* juveniles are generally more sensitive to plastic exposure than adult individuals [71]. It is worth noting that the offspring vitality results shows that PVC individuals of the first brood moved less (69.5 ± 20.9 mm) than CTRL individuals (87.2 mm ± 23 mm) and those exposed to PS-NPs (77.9 ± 22.1 mm) (Fig. S4.7). Although statistical analyses did not reveal statistically significant differences, the observed trend suggests that the lower viability of first-brood PVC-exposed offspring. Individuals exposed to PVC-NPs exhibited increased size, significant stimulation of molting behavior, and a decline in the number of individuals produced. This suggests that the potential imbalance in the hormone functionality of ecdysone may have concurred in allocating more energy towards growth, with a subsequent reduction in reproductive investment. Consequently, the reduced viability of PVC-NP newborns could be ascribed to a decrease in energy resources allocated for reproduction by parental individuals.

4.3.5. Correlation among the different treatments and the investigated variables

Correlation analysis between the investigated parameters (Fig. 4.4a) showed that the number of broods was strongly negatively correlated with the time of the first brood ($r = -0.75$, $p < 0.001$), and positively correlated with the number of offspring ($r = 0.81$, $p < 0.001$). Surprisingly, the time of the first brood showed a positive correlation with the length of the individuals ($r = 0.65$, $p < 0.001$), indicating that the individuals with a delay in the time of the first brood have greater dimensions.

PCA yielded two principal components (PC1 and PC2) that collectively explained 71.5% of the total variation (Table S4.1). PC1 accounted for 47.6% of the total variance and exhibited positive loadings with time of the first brood (r = 0.85) and length of *D. magna* (r = 0.68), as well as negative loadings with the total number of offspring (r = -0-89) and number of broods (r = -0.93). PC2 accounted for 24.3% of the total variance, with all variables exhibiting positive loadings, particularly swimming distance (r = 0.73). The relationship between the two principal components and the analysed variables is illustrated in Figure 4.4b, and detailed PCA correlation results are presented in Table S4.2.

As shown in Figure 4.4b, organisms subjected to the CTRL and PVC-NP treatments were grouped into two distinct systems, whereas those from the PS-NP treatment group showed an overlapping distribution between these two groups. These results indicate that the negative effects of PVC-NPs are attributed to variables with high positive loadings with PC1, namely day of first brood, and body dimensions. Specifically, at the right end of the PC1 axis, a subgroup of the PCV-NP treatment was identified (dots surrounded by a triangle), corresponding to organisms that never reached sexual maturity. These were large organisms (Fig. 4.4b).

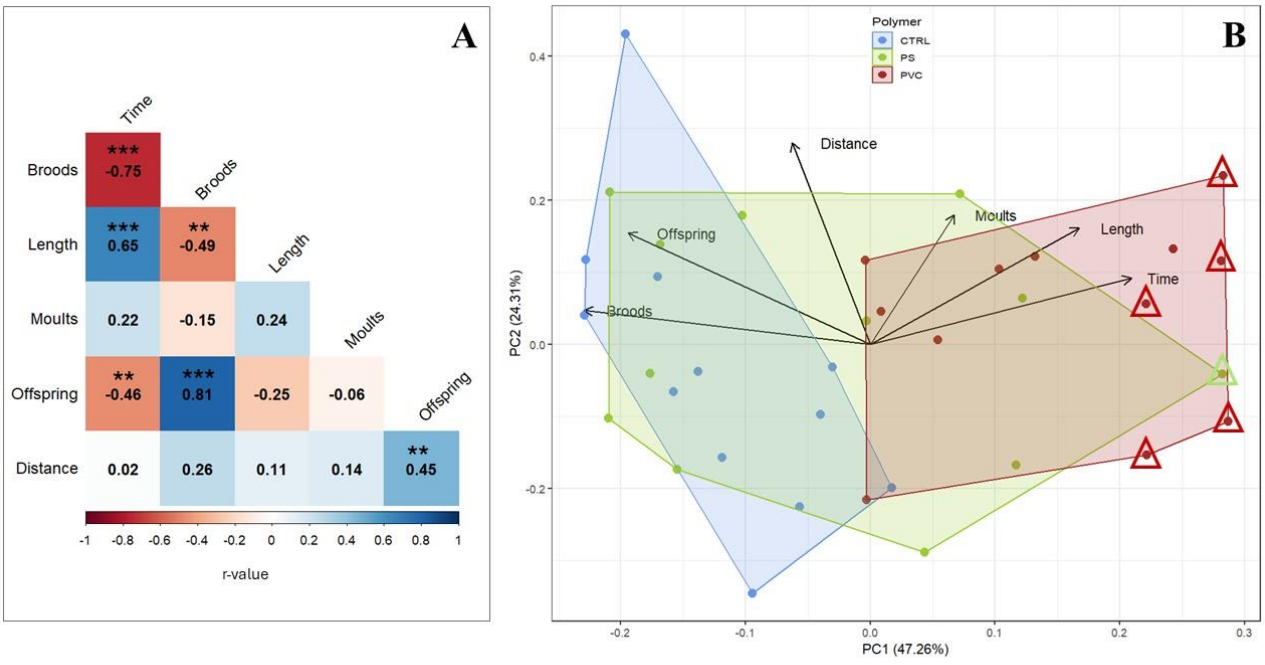

Figure 4.4. Correlation plot (A) of *D. magna* number of broods, number of offspring, swimming distance, number of molts, length and time to the first brood (***p < 0.001, **p < 0.01, *p < 0.05). Principal component analysis (B) of the effect of exposure conditions (CTRL, PS-NPs, and PVC-NPs) on *D. magna* based on the investigated parameters. Dots surrounded by triangles correspond to organisms that never reached sexual maturity.

4.4. Conclusions

The impact of NPs on organisms is complex because of their presence in the environment as a mixture of plastic polymers of varying chemical compositions, shapes and sizes. The data presented here provide new evidence on the role of polymer type in toxicity. The different effects observed for PS and PVC treatments suggest that the chemical composition of the two investigated polymers may have contributed to their onset. It has been hypothesised that the ingestion of PVC-NPs by *D. magna* individuals had impaired the hormonal functionality of the ecdysone, leading to increased molting activity and larger body dimensions. It has also been suggested that the adhesion of PVC-NPs to the body surfaces of *D. magna* may have concurred to the stimulation of molting activity, thereby accelerating exoskeleton exfoliation to facilitate the elimination of the negative effects of PVC-NP adhesion. Consequently, it can be hypothesised that the increase in molting behaviour and body dimensions observed in the PVC exposure treatments may have led to a reduction in the energy nutrients available for reproduction, thus contributing to the observed ecological fitness impairment, in particular the delay in reaching sexual maturity.

In addition to the contribution of chemical composition, the role of size differences of the particles used in this study cannot be excluded, as PCV-NPs used had a slightly different size distribution than the PS-NPs.

The data presented here provide new evidence on the role of nanoparticle polymer type in toxicity. This study observed the effects of PVC on various endpoints at both the individual and population levels. PVC-NPs affected the *D. magna* population molting behaviour, morphometric parameters, and reproductive fitness over a prolonged period. In particular, the correlation between higher dimensions and lack of reproduction suggests the impairment of hormonal systems linked to ecdysone. From an ecological perspective, the observed delay in reaching sexual maturity could have a significant effect on *D. magna* populations in real ecosystems. Therefore, further analyses are recommended to assess the potential toxicity of this polymer. Based on the evidence reported here, it can be concluded that PVC-NPs has a greater environmental impact than PS-NPs. This information is useful for assessing the environmental effects of the emerging pollutants.

It is recommended that future studies consider the potential toxic effects of other NP polymers with higher global production, including low/high-density polyethylene (L/HDPE) and polypropylene (PP). In addition to the engineered nanospheres, it would be useful to study the non-engineered ones, such as fibres and irregular fragments. Furthermore, new types of biodegradable plastics, such as

polylactic acid (PLA), represent a field that warrants further investigation. Moreover, it would be beneficial to investigate materials of greater complexity, such as products containing plastics, including tire-wear particles, cigarette butts, and clothing. It is also important to acknowledge the potential risks associated with the organic additives and inorganic co-formulants present in their mixtures.

Given that organisms in the environment are subjected to long-term exposure that far exceeds the 21 days generally foreseen by international guidelines, there is a clear need for multigenerational studies to provide data on NP effects that more closely reflect those occurring in natural environments. In this context, we endorse the proposal to update the current OECD No. 211 guideline to a duration of 28/30 days [72] to generate data that are more environmentally relevant. We also encourage the production of more ecologically relevant data regarding the toxicological direct and indirect effects induced by NPs at different levels of the bio/ecological hierarchical scale.

SUPPLEMENTARY MATERIALS

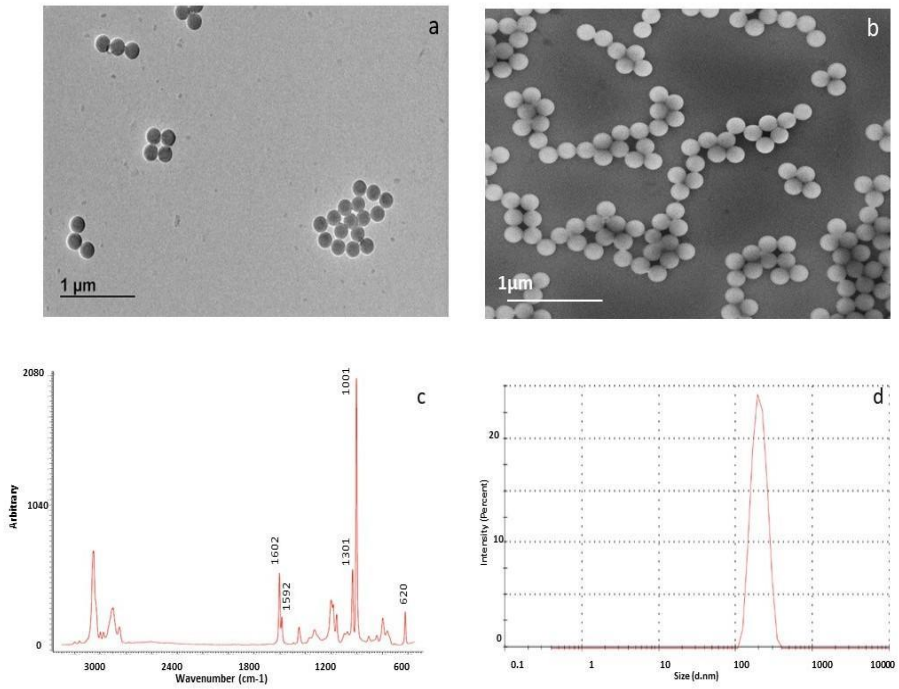

Figure S4.1. Transmission electron microscopy (a), scanning electron microscopy (b), Raman spectra (c), and DLS intensity measurement (d) of PS-NPs. The numbers in the Raman spectra specify the peaks used to identify PS.

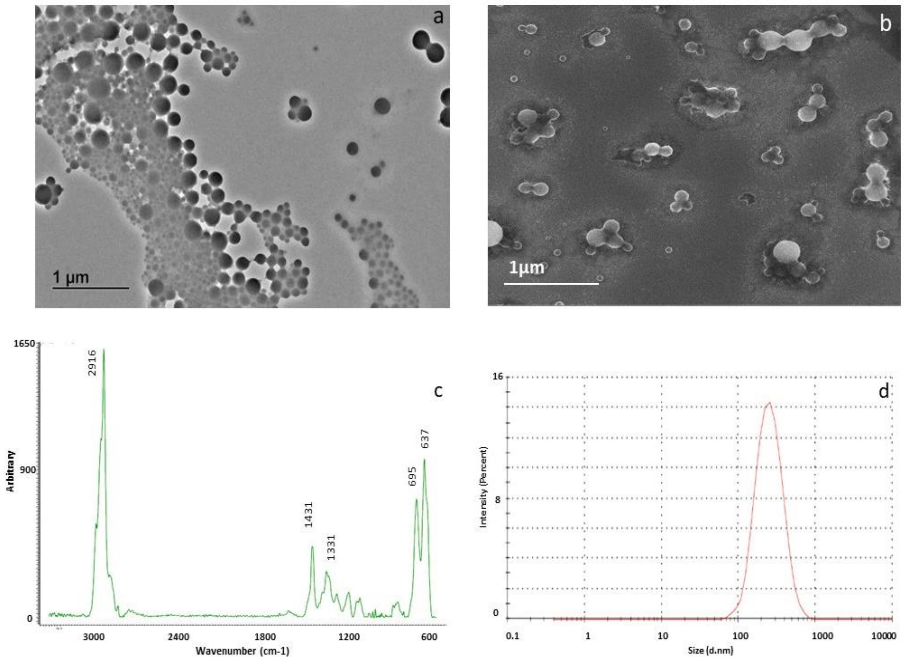

Figure S4.2. Transmission electron microscopy (a), scanning electron microscopy (b), Raman spectra (c), and DLS intensity measurement (d) of PVC-NPs. The numbers in the Raman spectra specify the peaks used to identify PVC.

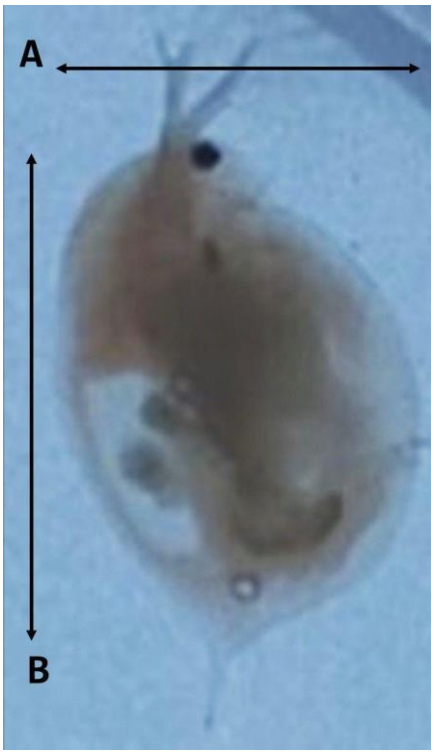

Figure S4.3. Measured characteristics of *Daphnia magna* morphology: A, body length; B, body width.

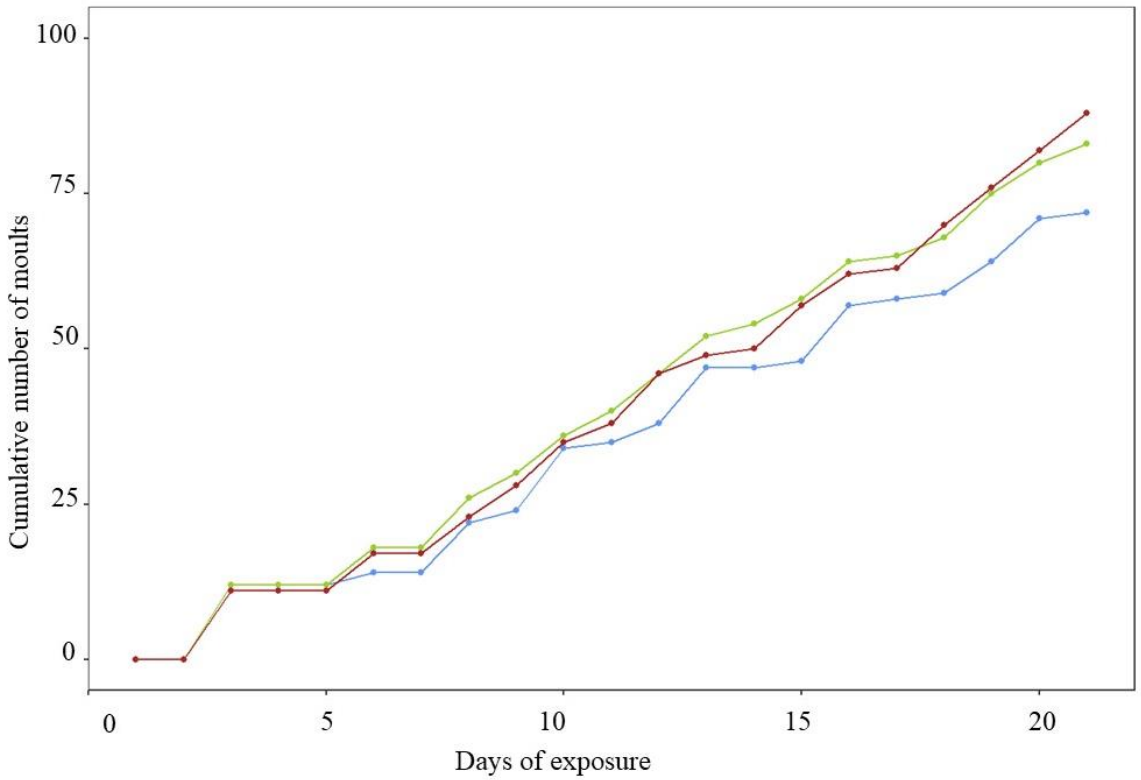

Figure S4.4. Cumulative curves of the number of molts in the 12 twelve *D. magna* parents over 21 days under the CTRL (blue), PS (green), and PVC (red) treatments.

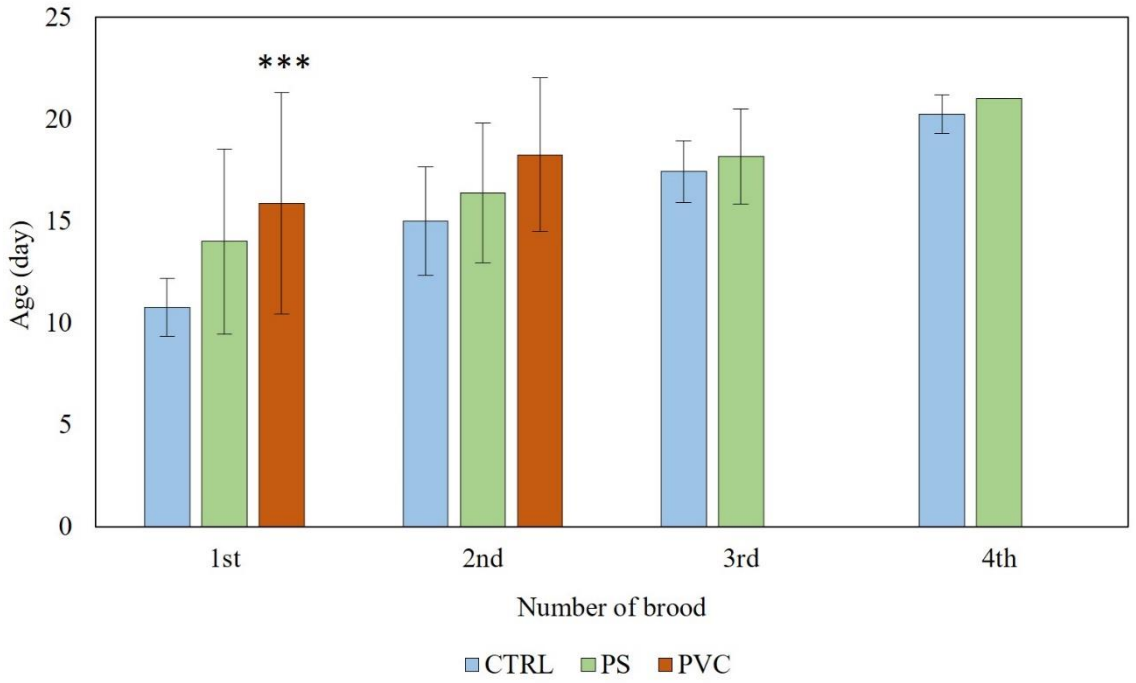

Figure S4.5. Histograms of the mean day (± standard deviation) of 1st, 2nd, 3rd, and 4th brood under the CTRL, PS-NP and PVC-NP treatments (***p < 0.005 vs. CTRL).

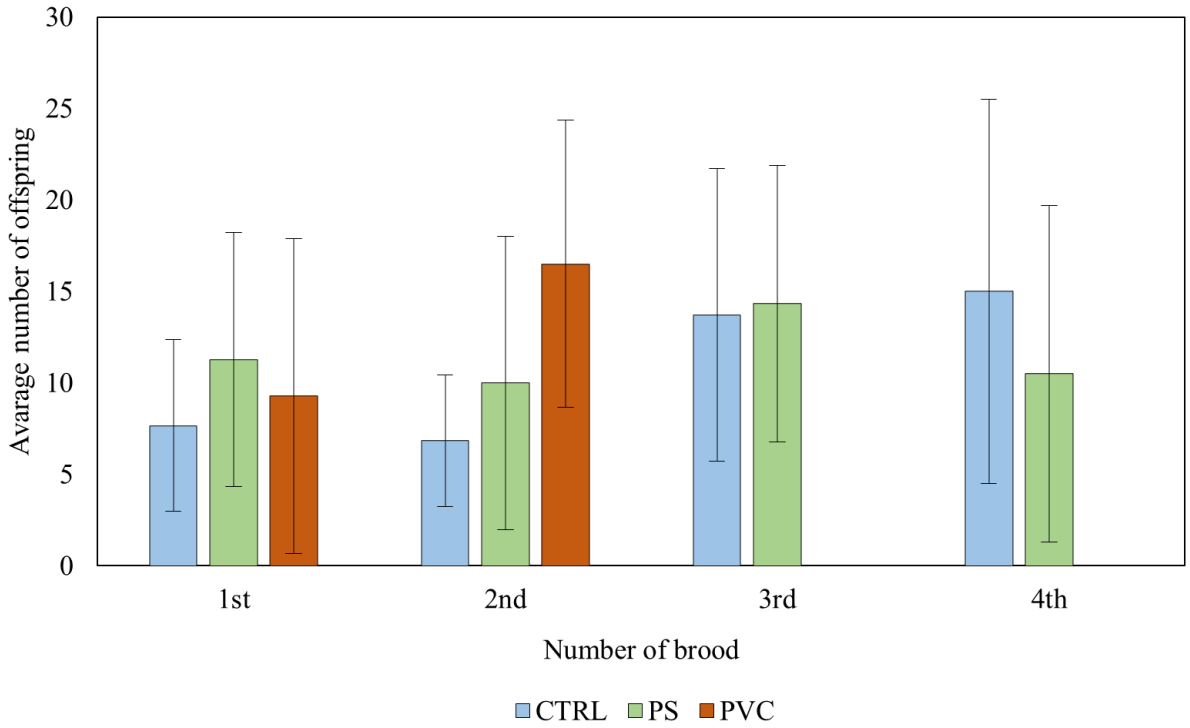

Figure S4.6. Histograms of the average number of offspring (± standard deviation) produced in each brood (1st, 2nd, 3rd, and 4th) under the CTRL, PS-NP, and PVC-NP treatments.

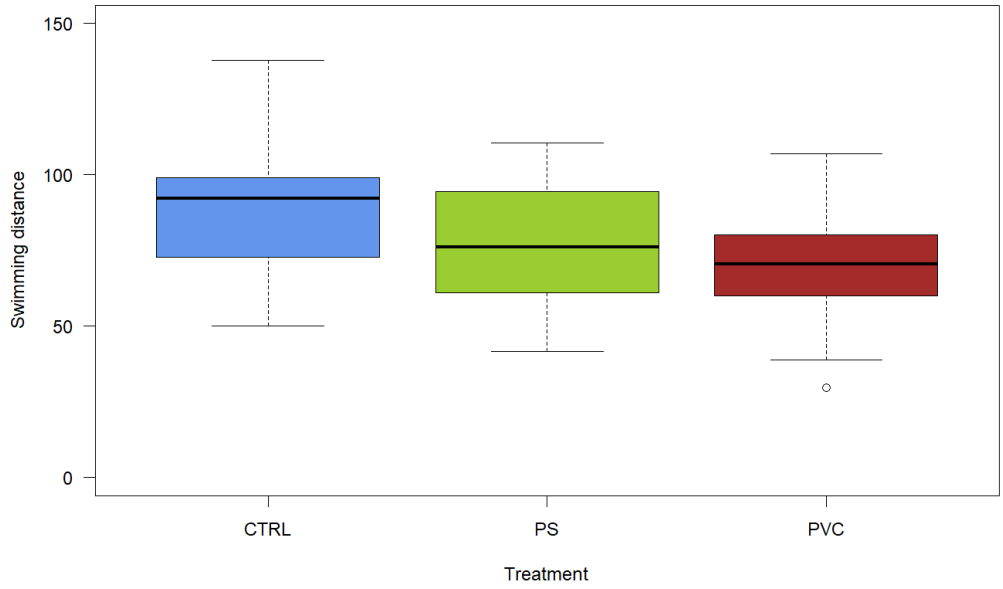

Figure S4.7. Boxplot of swimming activity (distance travelled, mm) measured in *D. magna* offspring from the first brood (< 24 h old) under the CTRL, PS-NP, and PVC-NP treatments.

Table S4.1. Proportion of the variance explained by each principal component (PC).

	PC1	PC2	PC3	PC4	PC5	PC6
Standard deviation	1.6839	1.2077	0.9103	0.69826	0.53492	0.32197
Proportion of variance	0.4726	0.2341	0.1381	0.08126	0.04769	0.01728
Cumulative proportion	0.4726	0.7157	0.8538	0.93503	0.98272	1.0000

Table S4.2. Correlations between the investigated variables and principal components (PC).

	PC1	PC2	PC3	PC4	PC5	PC6
Time of the first brood	0.8529344	0.2682673	-0.20613318	0.01877321	-0.37802358	-0.121615793
Number of broods	-0.9355443	0.1382783	0.04185366	-0.20692051	0.01152937	-0.246850623
Length	0.6820008	0.4725158	-0.23756818	-0.43800143	0.25162925	0.001593532
Number of molts	0.2748249	0.5241950	0.80573705	-0.01219955	-0.01807711	0.001749412
Number of offspring	-0.7916650	0.4512351	-0.11369102	-0.25630708	-0.25150752	0.166668709
Swimming distance	-0.2555722	0.8159727	-0.25649863	0.43210843	0.12731217	-0.012398872

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Chapter 5

Effects of nano- and micro- fibers derived from surgical face masks in *Danio rerio*

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Abstract

The massive use of surgical face masks during the COVID-19 pandemic has compounded the challenge of plastic waste. Surgical face masks are made of polypropylene (PP) and tend to release nano- and micro- fibers (NMFs).

The present study aims to provide insights into the impacts of NMFs in aquatic organisms by evaluating the effects of PP-NMFs derived from the artificial photodegradation of surgical face masks on the model species *Danio reri*o (zebrafish). The impact of NMFs on embryonic and larval developmental stages has been evaluated by investigating the effects of low (0.2 mg/L), medium (1 mg/L), and high (5 mg/L) NMF levels. Alterations in apical endpoints and transcriptomic analysis were investigated.

After 6 days, a significant reduction in the eye area was observed. The upregulation of genes related to the negative regulation of developmental processes could explain the observed alterations, while the downregulation of genes involved in energy-related metabolic processes suggests an energy stress state. Increased mortality occurred between 9 and 12 days, a period when zebrafish make the transition from endogenous to exogenous feeding, suggesting an impairment in foraging behaviour due to NMF exposure.

The presented findings demonstrate that environmental levels of NMFs may pose a hazard to aquatic organisms, suggesting the potential for an ecotoxicological risk associated with the improper disposal of surgical face masks.

5.1. Introduction

Since the 1950s, the wide production and use of plastic materials have strongly characterised anthropogenic activity, making plastic an important indicator of the Anthropocene, even leading to the proposal of a new definition of the current historical era as Plasticene (Muthulakshmi et al., 2023). The use of plastics has brought unimaginable benefits to our everyday lives, reaching a global plastic production of nearly 400 million tons in 2022 (Plastics Europe, 2023). Polypropylene (PP), polyethylene (PE), and polyvinyl chloride (PVC) are the three most produced plastic polymers, with packaging, building, and construction applications being the two largest world plastics markets (Plastics Europe, 2023). Furthermore, the use of plastics in the textiles industry is another important commercial application, mainly related to knitting and woven fabrics, household items, or technical textiles applications (de Oliveira et al., 2023). However, the positive aspects of plastic are counteracted by the environmental burden related to their improper disposal. Plastic materials are considered contaminants of emerging concern due to their ubiquitous presence in all terrestrial and aquatic environments and the threat they pose to ecosystems' health (Masseroni et al., 2022).

In recent years, the environmental challenge of plastic waste has been further compounded by the COVID-19 global pandemic caused by the virus "SARS-CoV-2" (Wang et al., 2023). During pandemic, to prevent and control the spread of SARS-CoV-2, countries have introduced the obligation to use personal protective equipment (PPE), which are mainly face masks, to enter public areas. Since face masks are produced using polymeric plastic materials, this demand has resulted in an unprecedented rise in the global production of plastic (Fadare et al., 2020). Face masks are predominantly manufactured from PP and comprise a three-layer structure. The inner layer comprises soft fibres, the middle layer is composed of melt-blown filter, while the outer layer is formed of non-woven fibres (Uddin et al., 2022). The improper disposal of surgical masks has raised a particular concern, as it has been quantified that more than 1.24 trillion of these plastic items ended up in the environment during pandemic (Idowu et al., 2023). Once in the environment, surgical masks tend to release nano- and micro- fibers (NMFs) (Chen et al., 2021), and a large amount of toxic compounds (e.g., benzene and 1,2-dichloropropane) (Huang et al., 2023a). Despite the wide distribution of NMFs in the aquatic ecosystem (Sharma et al., 2024), and their potential accumulation in aquatic organisms, the knowledge of their effects on aquatic biota is still limited (Ma et al., 2023).

In this context, zebrafish (*Danio rerio*) is a freshwater species recommended by the Organization for Economic Cooperation and Development (OECD) as a model organism for acute toxicity testing of chemicals (OECD, 2013) and it is also considered an appropriate model to investigate the toxicity of

microplastics (MPs) and nanoplastics (NPs) (Torres-Ruiz et al., 2021). Fish are one of the most sensitive groups to plastic ingestion, with MPs and NPs that can enter directly via ingestion and through the gills (Huang et al., 2021). To date, the few studies that have explored the effects of NMFs derived from COVID-19 face masks on this species suggest that these emerging contaminants can down-regulate the transcription of genes related to reproduction (Sendra et al., 2022), cause histopathological and behavioural alterations, (Kim et al., 2023) and induce oxidative stress, cell apoptosis and disruption of amino acid and lipid metabolism (Hu et al., 2024).

In the present study, we evaluated the effects of secondary PP-NMFs derived from the nonwoven PP fabrics of surgical face masks on *D. rerio* individuals. The aim is to provide new insights on the impacts of secondary NMFs in aquatic ecosystems by using a model species and specifically:

1) understanding the biological scale level of NMF-induced adverse effects, through the evaluation of several apical endpoints (embryonic development, survival, growth, morphology, behaviour);
2) providing insights into the mode of action of NMFs, through transcriptomics analyses;
3) assessing the concentration-dependent effects of NMFs, by testing low, medium, and high environmentally relevant levels;
4) determining the impact and toxicity of NMFs on embryonic and larval zebrafish developmental stage, by respectively short-term (up to 6 days) and median-term (up to 15 days) bioassays.

5.2 Materials and methods

5.2.1 Preparation and characterization of nano/micro fibers

In this study, in accordance with the definitions proposed by Hartmann et al. (2019), the term "microplastics" (MPs) is used to refer to plastic debris with a size range of 1 µm to 5 mm, while the term "nanoplastics" (NPs) to those smaller than 1 µm. The secondary NMFs used in this study derived from the photodegradation of PP melt-blown nonwoven fabrics, a plastic advanced material that is used in several technical applications and especially in PPE, such as the filtering layers that are commonly placed in surgical face masks. The effect of solar irradiation breaks the C–C and C–H bonds of PP in the face masks present in the environment, leading to chain scission and the subsequent release of NMFs (Saliu et al., 2021). To mimic this process, we submitted layers of nonwoven PP fabric cut into small pieces to artificial weathering by using a UV-A lamp (340 nm; 0,76 $W \cdot m^{-2} \cdot nm^{-1}$) at 65 °C for a total duration of 180 h. This part of the study was conducted in the laboratory of the

Department of Earth and Environmental Sciences (DISAT) of the University of Milano-Bicocca, following the procedure described in Saliu et al. (2021). For a detailed description of the morphological and chemical characterization of the UV-A treated fibers, please refer to Isa et al. (2023).

5.2.2. Preparation of nano/micro fiber solutions

The weathered PP-NMFs were used to prepare the stock solution for the toxicological bioassays. In brief, 30 mg of NMF dry powder were weighed using an analytical balance and then dispersed in 20 mL of dechlorinated tap water. Subsequently, the solution was subjected to repeated sonications to disperse the NMFs in the medium, using a bath sonicator (Bandelin Sonorex RK100H, GmbH & Co, Germany) operating at 35 ± 0.1 kHz for 10 s. The resulting cloudy solution was diluted up to 100 mL and left to decant for 30 minutes. This step allowed for the separation of larger fibers and fiber aggregates from the solution, which floated on the top of the solution as reported by Saliu et al. (2021). To eliminate this larger size fiber component, the middle 80 mL of the water column was transferred to a glass vessel. The remaining 20 mL were diluted once again up to 100 mL with dechlorinated tap water and then the procedure was repeated. The final solution was diluted with chlorine tap water to a final volume of 1 L. To assess the loss of material, the remaining 20 mL of the previous operation were filtered (glass microfiber paper, 1.2 µm pore size, Fisher brand) and then discarded. The filter was weighed prior and after the filtration and the difference in weight was subtracted from the initial 30 mg of NMFs to ascertain the final concentration of the stock solution.

It is emphasised that this procedure may result in a certain degree of uncertainty in the determination of the final concentration, nevertheless, it was considered necessary to eliminate those MFs of large sizes.

Once the concentration of the stock solution was established, serial dilutions were made to obtain the concentrations tested during the experiment: three concentrations were selected to reflect and investigate the effects of low, medium, and high NMF exposure levels in zebrafish. As reported by Sun et al. (2024), surgical face masks immersed in river water release plastic in a time-dependent manner, ranging from ~1 mg/L of plastic released on day one to ~5 mg/L on day 5, to ~70 mg/L after one month. For this reason, a concentration of 0.2 mg/L was selected to represent a low-level contamination, a concentration of 1 mg/L to reflect the amount of fiber released in one day from a single face mask, and a higher concentration of 5 mg/L to be indicative of a longer time of NMF release.

5.2.3. Characterization of nano/micro fiber solutions

Before running the ecotoxicological assays, an aliquot of each NMF solution (0.2, 1, 5 mg/L) was characterised by using dynamic light scattering (DLS) and Scanning Electron Microscopy (SEM) to determine the size and morphology of the fibers. The DLS experiments were conducted using a Malvern Zetasizer (Malvern Instruments, Malvern, UK), with a HeNe laser at a wavelength of 633 nm and a fixed scattering angle of 90°. Measurements were collected every 15 s at 25 °C for 25 min. Three independent measurements were conducted for each tested concentration.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analyses were performed to analyse the size, morphology, and elemental composition of the tested NMFs. An aliquot of each of the tested NMF solutions was evaporated, subjected to Au and Pd sputtering, and then analysed through a scanning electron microscope (Schottky, FEI Quanta 400 FEG ESEM) equipped with an energy-dispersive X-ray spectrometer (EDAX Genesis X4M). The average length and width of NMFs (n=35) was determined from SEM images using ImageJ v.151 software.

5.2.4. Experimental setup

Zebrafish (*Danio rerio*, AB strain) individuals originated from the facilities of Biotério de Organismos Aquáticos (BOGA), at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Matosinhos, Portugal). Handling of zebrafish was conducted in accordance with the European Directive 2010/63/EU and the Portuguese Law (Decreto Lei 113/2013), with procedures approved by the CIIMAR animal welfare body (ORBEA, Directive 2010/63/EU). The fertilisation and collection of zebrafish embryos were performed as described by Barros et al. (2023).

5.2.4.1. Short-term exposure experiment

Short toxicological zebrafish bioassays were carried out in accordance with the OECD guidelines for Fish Embryo Acute Toxicity (OECD, 2013), with slight modifications. Experiments were conducted in 24-wells plates (6 x 4). Eggs less than one hour post fertilisation (hpf) were washed with dechlorinated tap water and placed in 2 mL of dechlorinated water for the control treatment (CTRL) or in 2 mL of NMF solutions (0.2, 1, 5 mg/L), one egg per well. In the CTRL treatment, all 24 wells were filled with 2 mL of dechlorinated tap water. In the NMF treatments, not all wells were filled

with NMF solution, since 4 wells were filled with CTRL water to assess and exclude any possible effect of the plate in the observed effects.

The 24-well plates were incubated at 28 ± 1 °C for 144 h under a 14:10 h (light:dark) photoperiod. The exposure medium was daily renewed, with NMF solutions shaken and sonicated for 1 min prior to use. Following each medium renewal, the dead embryos and chorions were removed. The four individuals placed on the same column of the plates were considered as a single replicate. The bioassays were repeated three independent times, for a total of 108 individuals and 27 replicates for CTRL and 60 individuals and 15 replicates for each NMF treatment.

During the bioassays, the NMF toxicity was evaluated considering different endpoints by observing the individuals through an inverted microscope (Nikon Eclipse 5100T) equipped with a digital camera (Nikon D5-Fi2) and a microscope camera controller (Nikon's Digital Sight DS-U3). Embryo development, hatching rate, abnormalities, and mortality were monitored at 24-hour intervals (24, 48, 96, 120 and 144 hpf). The developmental stages were compared with those described by Kimmel et al. (1995) to highlight abnormalities in the eyes, head, yolk-sac, tail or onset of edema, haemorrhages, or alteration in pigmentation. At 48 hpf and 72 hpf, the heartbeat rate was measured for one embryo for each replicate using a stopwatch for 15 seconds. Additionally, the length (from the head to the end of the last somite) and the eye area were measured for each individual at 96 hpf and 144 hpf using NIS-Elements D 4.13.03 software. Furthermore, the sensorimotor reflexes were evaluated at 120 hpf following the procedures reported by Pinho et al. (2018). Briefly, individuals were gently touched with a micropipette tip in either the head or tail region to elicit either a positive (immediate swimming and reaction) or negative response (no movement upon touch). To prevent any influence on the positive reactions, each larva was only touched in the head or tail region. Half of the replicates were touched in the tail region, while the other half were touched in the head region.

5.2.4.2. Median-term exposure experiments

In the median exposure experiment, 20 fertilised eggs (< 1 hpf) were placed in a crystallising glass dish filled with 100 mL of either CTRL or NMF exposure medium. The bioassays were conducted in triplicate under identical conditions, resulting in a total of 60 individuals per treatment. Half of the exposure medium was replaced daily. The zebrafish larvae were provided with a commercial fish diet, Zebrafeed (Sparos, Portugal), *ad libitum*, three times a day, starting on day 6. The mortality and development abnormalities of the different treatments were monitored daily. On the 15th day, the fish were video recorded for 30 seconds and their swimming distance was assessed using Kinovea

software. Following the video recording, the subjects were anaesthetised with MS-222 (tricaine methanesulfonate, 0.25 mg/mL) prior to photography. Subsequently, the body length and eye area were quantified in accordance with the previously described short-term exposure methods.

5.2.4.3. RNA isolation and extraction and RNA-seq

RNA-seq analyses were performed to evaluate the effects of NMFs at the molecular level and to provide further insights into their mode of action. The effects were evaluated at 144 hpf and 15 dpf for CTRL and 0.2 mg/L treatments. The lowest NMF tested concentration (0.2 mg/L) was selected as it represents a pollution scenario comparable to environmental conditions. For each treatment, 3 replicates were subjected to RNA extraction. In the short-term exposure experiment, each replicate consisted of a pool of 8 individuals, while in the medium-term exposure experiment each replicate was composed of a number of 4 individuals. The RNA isolation was performed using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare), following the manufacturer's standard protocol. The quantification of RNA was conducted through the measurement of optical density with a Take3™ on a microplate reader (Biotek Synergy HT) coupled with Gen5 software (version 2.0). The purity of the extracted RNA was evaluated by measuring the ratio of absorbance at λ260/λ280 nm, while the integrity of the RNA was evaluated by electrophoresis on a 1.5% agarose gel. Thereafter, the RNA samples were stored at −80 °C. RNA-seq analyses were obtained at Novogene (United Kingdom). Samples were subjected to sequencing (Illumina Novaseq 6000 paired-end (2 × 150)) and subsequent bioinformatics analysis with parameters set to a false discovery rate (FDR), adjusted p-value (padj) < 0.05, and a Log2(FoldChange) > 0.

5.2.5 Statistical analysis

All the statistical analyses were conducted using R software version 4.3.2. To compare the results of the different replicates with each other, the measured results were normalised against the respective CTRL. Data was checked for normality and homogeneity of variances, using Shapiro-Wilk and Levene's tests. The effects of NMF exposure were investigated using one-way Analysis of Variance (ANOVA) when data were normally distributed and presented homogeneous variances; if not, Kruskal-Wallis test was adopted. Significance was set at a p-value < 0.05. If statistical tests resulted statistically significant, Dunnett's (after ANOVA) or Dunn's (after Kruskal-Wallis) post-hoc test was applied to evaluate significant differences between the CTRL and treatments.

5.3. Results and discussion

5.3.1. Characterization of nano/micro fiber solutions

DLS measurements can be expressed as number distributions, which emphasise the species with the highest number of particles, or as intensity distributions, which tend to accentuate the species with the largest scattering intensity (which often correspond to the larger particles). As the tested NMFs are obtained from the photodegradation of macroscopic fibers, which are inherently polydisperse, both the number distribution and intensity distribution results are presented here.

The DLS measurements of the tested NMF solutions expressed by number (Fig. 5.1) indicated the presence of a polydisperse particle distribution in all the three tested concentrations, characterised by an unimodal distribution, with the presence of one single peak (intensity > 99%) and an average hydrodynamic size of 271 nm (± 107 nm) in 0.2 mg/L, 265 nm (± 83 nm) in 1 mg/L, and 296 nm (± 82 nm) in 5 mg/L treatments. Conversely, the same analyses expressed as intensity distribution (Fig. S5.1) suggest a more heterogeneous dispersion, characterised by a bimodal distribution with two distinct peaks: one peak is in the nanometric size range (approximately 500 nm), while the other is in the micrometric size range (approximately 5 µm). These two distinct peaks are observed for all the tested concentrations of NMFs. The polydisperse distribution of the NMF solutions is highlighted by their polydisperse index (PDI), which is 0.792 (± 0.2), 0.837 (± 0.1), and 0.652 (± 0.05) respectively for 0.2 mg/L, 1 mg/L, 5 mg/L treatments.

The NMF solutions were further analysed by SEM analyses, which confirmed the presence of fibers in the form of micro and nano items. NMFs exhibited an average length of 26 µm (± 16 µm) and width of 2 µm (± 0.9 µm) (Fig. S5.2). The EDS analyses conducted on the measured fibers revealed that the elemental spectrum is dominated by the presence of carbon as the main element (Fig S5.3). The chemical characterization through µ-FTIR analyses of the investigated PP-NMFs is detailed in Isa et al. (2023).

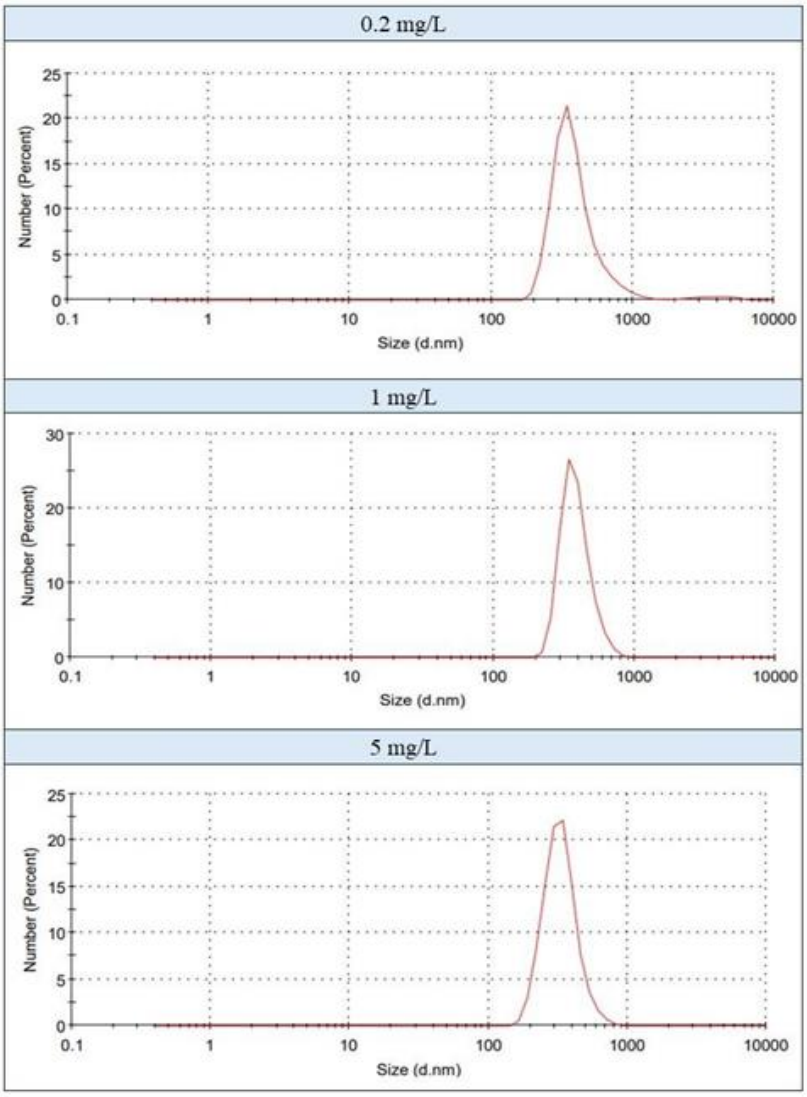

Fig. 5.1. DLS measurements of hydrodynamic size expressed as number distributions of the three investigated NMF concentrations (0.2 mg/L, 1 mg/L and 5 mg/L) in the exposure test water.

5.3.2. Short-term exposure

In the short-term exposure, the occurrence of adverse effects in *D. rerio* embryos/larvae was evaluated throughout the test, to assess whether the onset of NMF toxicity occurs early during embryonic development or later, during the first days as swimming larvae. Specifically, we evaluated alterations in embryonic development, morphometrics, behaviour, and gene expression. We retain that the evaluation of the effects induced by the exposure of *D. rerio* to environmentally relevant concentrations of NMFs could provide useful data on the possible environmental risk associated with PP-NMFs in the environment.

5.3.2.1. Validation criteria of the test

The tests were considered valid as they fulfilled the criteria outlined in OECD Test Guideline 236, as at 96 hpf embryo survival in the CTRL was higher than 90% and the hatching rate was of 100% (Table S5.1). At 144 hpf, there was an increase in mortality up to 12% (± 3.2) (Table S5.1).
The NMFs exhibited no acute toxicity, since the mortality rates, both at 96 hpf and 144hpf, were comparable to those observed in the CTRL group. The mortality results are in line with those reported in the literature, showing that exposure to NMFs released from face masks does not induce lethal effects in aquatic organisms at the range of tested concentrations (Oliveira et al. 2023).

5.3.2.2. NMF interaction with zebrafish

NMF interaction with zebrafish larvae was evaluated through the observation of individuals at the inverted microscope. A qualitative evaluation of the chorion adsorption of NMFs is provided in Fig. S5.4.
The lack of fluorescent NMFs hamper the gain of quantification of their distribution inside the body of organisms. Pictures taken at the chorion released after the hatching at 72 hpf suggest a concentration-dependent increase in the number of NMFs that have come into contact with zebrafish embryos (Fig. S5.4). A high number of large fibers is attached to the chorion surface of embryos at 5 mg/L treatment. Conversely, the amount of visible fibers decreased in the 1 mg/L, until being absent at the lowest concentration tested (0.2 mg/L) and in the CTRL treatment. Due to the resolution limit of the microscope (1.4 µm), it is not possible to obtain further information on the distribution of the nanometric component of the investigated fibers.
The chorion is the eggshell that surrounds the embryo until hatching, acting as a barrier to external substances. It presents a large number of pores with diameters of up to 500 nm, which allow the permeation of gases and nutrients (Duan et al., 2020). In this context, the size of plastics determines the accumulation patterns: NPs can pass through the chorion and enter the embryo, while MPs cannot penetrate (Torres-Ruiz et al., 2021). However, MPs can nevertheless be harmful, causing biological impacts due to their adhesion on the chorionic surface (Duan et al., 2020). It is therefore plausible that the nanometric component of the tested NMFs passed through the chorion, while the micrometer one, as visible in Fig. S5.4, was blocked.

5.3.2.3. Malformation and morphological alterations

The incidence of malformation (skeletal alteration and pericardial edema) at 144 hpf in the CTRL (9.3%) was in line with those recorded in NMF treatments (0.2 mg/L: 8.3%; 1 mg/L: 8.3%, 5 mg/L: 5%). The existing literature describes contrasting data on the impact of MPs and NPs on zebrafish embryo development. As recently reviewed by Torre-Ruiz et al. (2021), *D. rerio* embryos exposed to primary spherical polystyrene (PS) NPs have been observed to exhibit increased rates of malformation, despite differing studies that did not observe any developmental effects. However, when considering the effects of NMFs derived from nonwoven PP, the only available study that has evaluated embryo malformation reports results conflicting with our data, showing that exposure to NMFs can induce deformities (Hu et al., 2024). In this perspective, we emphasised that a comparison between different studies is challenging, due to the different processes adopted to obtain NMFs that involve differences in size and chemical structures of the investigated fibers.

Regarding morphological alterations, detailed results of the statistical analyses are reported in the supplementary data (Tab. S5.2), while Fig. 5.2 reports the results of eye area at 48 hpf, 96 hpf and 144 hpf. The results showed no statistically significant differences between the eye area of NMFs and CTRL treatments at 48 hpf ($p= 0.138$) and 96 hpf ($p=0.815$). In contrast, after 144 hpf a significant reduction (3%) in the eye area both in 0.2 mg/L ($p=0.020$) and 5 mg/L ($p=0.005$) treatments was recorded. Furthermore, although not statistically significant ($p=0.129$), a reduction trend (2%) in the eye area was observed also in 1 mg/L treatment.

Body length revealed less sensitivity to NMF exposure, as no significant differences were observed in any of the treatments at any of the measured times (Tab. S5.2). However, it is worth reporting that the ratio between the body length and the eye area was statistically different at 0.2 mg/L ($p=0.019$), 5 mg/L ($p<0.001$) and in the range of significance at 1 mg/L ($p=0.057$) (Fig. S5.5).

The decrease in the eye area in *D. rerio* individuals at 144 hpf draws concern on the possible hazard of NMFs to aquatic ecosystems, as it was recorded both at a high (5 mg/L) and environmentally relevant (0.2 mg/L) concentration. The eyes are an essential element in the perception of the surrounding environment (Oger et al. 2024), and their impairment can lead to damage to the entire organism, potentially resulting in the alteration of prey-predator interactions, and ultimately a lower individual survival (Huang et al., 2023b). The capacity of plastic exposure to reduce the eye size in zebrafish has been reported also by Torres-Ruiz et al. (2023), following a 72h exposure to 0.1, 0.5, and 5 mg/L of PS nanospheres of 25 nm. Similarly, Santos et al. (2024) demonstrated that larger PS-

NPs (300 nm) caused a significant reduction in eye size in zebrafish larvae following 144 h of exposure, even at lower concentrations (ng/L).

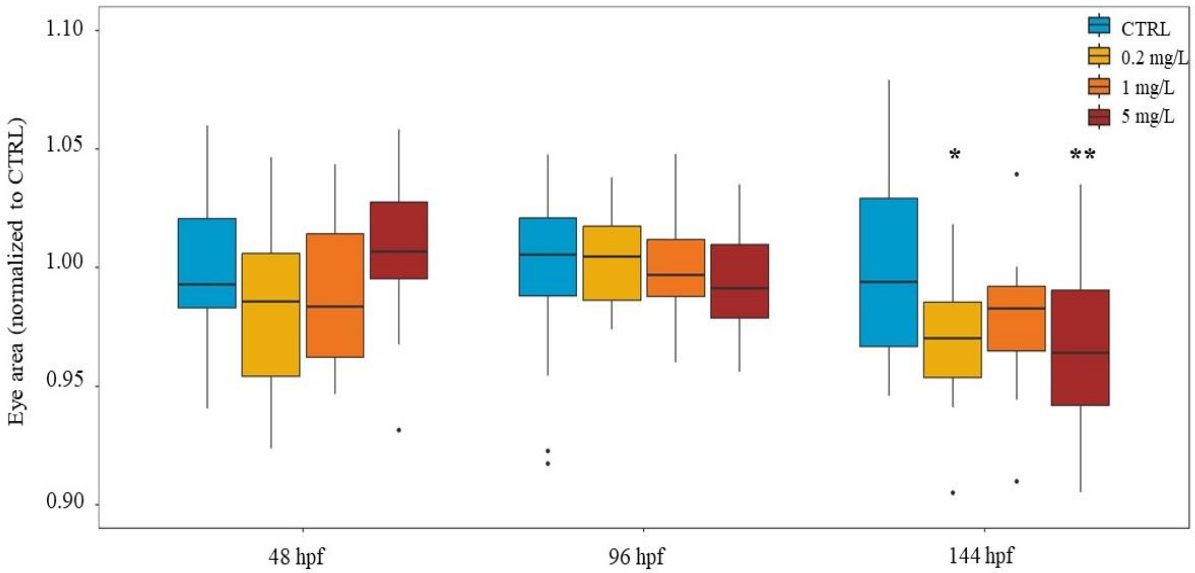

Figure 5.2. Boxplots represent the eye area (normalized to CTRL) measured in CTRL and NMF treatments (0.2 mg/L, 1 mg/L, 5 mg/L) at 48 hpf, 96 hpf, 144 hpf. Asterisks indicate statistically significant differences with respect to CTRL (*: $p < 0.05$; **: $p < 0.01$).

Furthermore, irregular fragments of nano/micro biodegradable plastic material (polyglycolic acid and polylactic acid, ranging from ~400 nm to ~4 µm) led to a decrease in the eye area of *D. rerio* at comparable concentrations to those tested in our study after 96 h of exposure (Luan et al., 2023).
To the best of our knowledge, we report in the present study for the first time the impairment of eye development in zebrafish induced by secondary photodegraded NMFs. Collectively, these findings corroborate the evidence that the eye area of zebrafish larvae is a sensitive endpoint for plastic pollution (Chen et al., 2024).
Concerning body length, the existing literature contains conflicting data regarding the effects of MPs and NPs on zebrafish body length. The absence of effects reported in the present study is in contrast with those of Hu et al. (2024), who observed a reduction in the body length of zebrafish larvae following 96 and 120 h of exposure to 1 and 10 µg/L of NMFs. A reduction in larval body length has been also observed following a 96-hours exposure to 10 mg/L of PS of varying sizes (80 nm, 200 nm, and 500 nm) (Chen et al., 2024). Conversely, in accordance with our results, Zhou et al. (2024) reported that at 120 hpf, the body length of individuals exposed to 10 mg/L of 100 nm, 500 nm, or 100 nm PS-NPs was not significantly different from the CTRL group. Moreover, Torres-Ruiz et al.

(2023) showed that body alterations did not occur after 72 hours of exposure to PSNPs (25 nm) at lower concentrations.

5.3.2.4. Effects on heartbeat and sensorimotor reflexes

In the current study, no alteration in heart rate was observed at 48 hpf (p=0.175) and 72 hpf (p=0.636) (Fig. S5.6). The lack of effect on heart rate after exposure to NMFs is consistent with that reported by Hu et al. (2024). As reviewed by Torres-Ruiz et al. (2021), even if plastic can have detrimental effects on heart rate, various studies have not observed alterations after MPs or NPs exposures. For example, Oger et al. (2024) recently reported that the heartbeat of zebrafish remained stable after 72h exposure to 1 mg/L of micro (5 um) or nano (250 nm) PS nanospheres.
Concerning sensorimotor reflexes, literature reports contrasting evidence regarding the impact of plastic exposure on zebrafish behaviour (Torres-Ruiz et al., 2021). In the present study, all individuals, whether stimulated on the tail or the head, exhibited a swimming response, suggesting that sensorimotor reflexes of zebrafish are not affected by exposure to NMFs.

5.3.2.5. NMFs dysregulated energy related metabolic pathways in zebrafish

An overview of the quality of sequencing reads is reported in Table S5.3A. The exploratory RNA-seq analysis on zebrafish individuals exposed to 0.2 mg/L of NMFs identified a total of 65 differentially expressed genes (DEGs). Among these, 52 were upregulated and 13 downregulated. The Gene Ontology (GO) enrichment analysis and the pathways highlighted by the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis are reported respectively in Figure 5.3A and Figure 5.3B. A detailed report of DEGs and significantly enriched KEGG pathways is reported in Table S5.4 and Table S5.5.
The downregulated genes are mainly involved in energy related metabolic processes, in particular "*acetyl-CoA biosynthetic process*", "*acetyl-CoA metabolic process*" (p= 0.043) (*pdha1a*) and "*thioester biosynthetic process*" (p=0.044) (*pdha1a*). Downregulated genes are also involved in the sulphur metabolism, such as "*sulphur compound metabolic process*", and "*iron-sulphur cluster assembly"* (p=0.043) (*pdha1a, fam96b*). The upregulated genes are instead involved in the "*negative regulation of developmental processes*" and in the "*negative regulation of multicellular organismal processes"* (*bcl11aa, vsx1, unc5b, srgap2*), with a significance level close to the threshold (p=0.050).

KEGGs analysis revealed the "*alanine, aspartate and glutamate metabolism*" (*nat8l*/*gad1a*/*got2a*) (p= 0.009) as the only upregulated pathway. Moreover, 8 down regulated pathways were found to be close to the significant threshold.

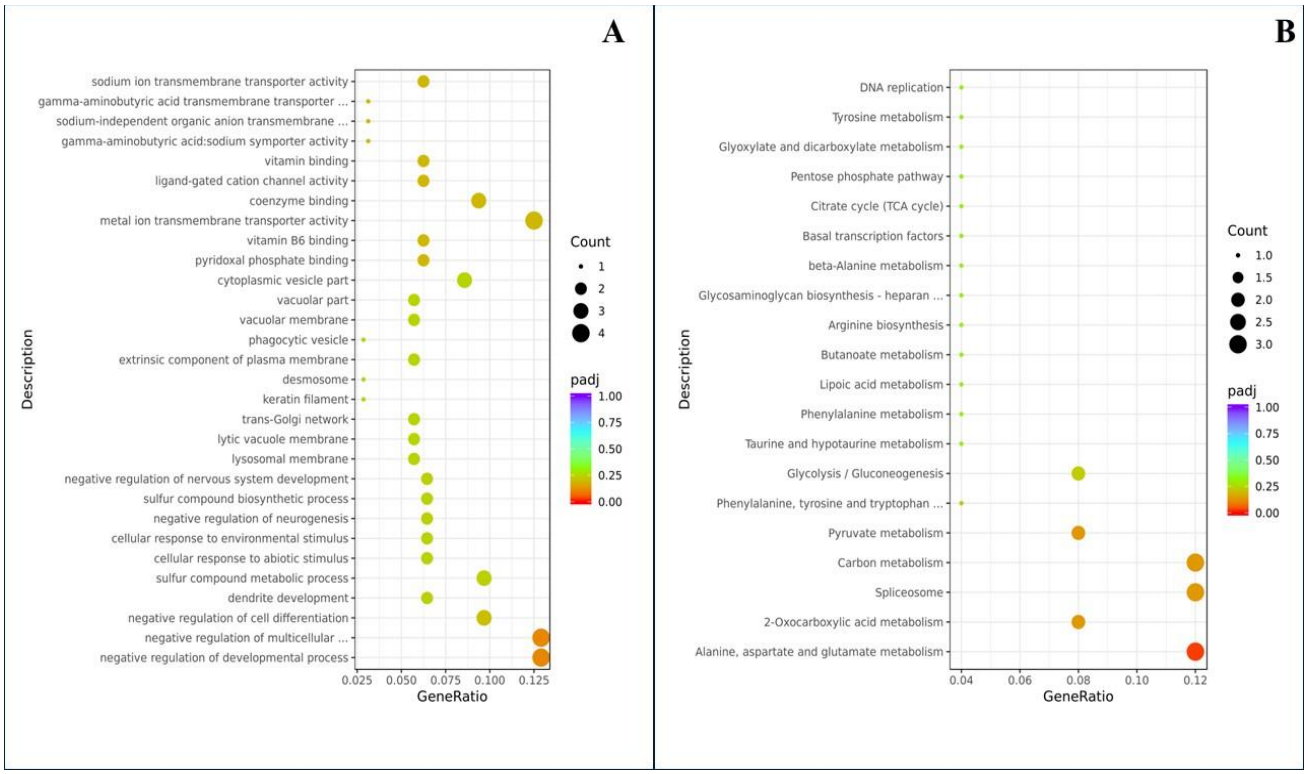

Figure 5.3. Gene Ontology (A) and KEGG pathways (B) after 6 days of NMF exposure (0.2 mg/L). The abscissa is the ratio of the number of differential genes linked with the GO term/ KEGG pathway to the total number of differential genes. The ordinate is GO term/KEGG Pathway. The size of a point represents the number of genes annotated to a specific GO term/ KEGG pathway. The colour from red to purple represents the significant level of the enrichment.

Given the short duration of the exposure (6 dpf) of the low and environmentally relevant concentration of tested NMFs (0.2 mg/L), we considered it appropriate also to examine the dysregulated pathways close to the significance threshold. The pathways are those referred to "*spliceosome*" (p=0.062) (*fus/plrg1*), "*lipoic acid metabolism*" (p= 0.069) (*pdha1a*), "*basal transcription factors"* (p=0.069) (*gtf2h5*), "*citrate (TCA) cycle*" (p= 0.069) (*pdha1a*), "*DNA replication*" (p=0.069) (*rnaseh2b*), "*2-Oxocarboxylic acid metabolism*" (p=0.069) (*pdha1a*), "*pyruvate metabolisms"* (p= 0.069) (*pdha1)*, and *"nucleotide excision repair*" (p=0.069) (*gtf2h5*).
Of particular interest for their biological functions are *fus/plrg1* genes. Both genes belong to the pathway referred to as "*spliceosome*" whose major functions are related to pre-mRNA splicing, a crucial step of gene expression in eukaryotic cells. However, it is noteworthy that in recent years new

spliceosome functions emerged as a novel player in neurodegenerative diseases and during neurodevelopmental stages (Jutzi et al., 2018). These results along with those of the upregulation of genes involved in "*negative regulation of developmental processes*" suggest a role of 0.2 mg/L NMFs in neurodevelopment and a possible impact on eye development, as indicated by the morphometric data on decreased eye area in *D. rerio* individuals at 144 hpf (*par. 5.3.2.3*, Table S5.4). Among the upregulated genes, *bcl11aa* is mainly distributed in the brain of larval zebrafish (Liu et al., 2019), and its alteration can lead to abnormal embryonic development, including microcephaly and structural brain abnormalities (Bagheri et al., 2016). Also, *vsx1* is expressed during zebrafish's early developmental stages (Xu et al. 2014), and its role in the regulation of the development of visual circuits is widely documented (Letelier et al., 2023). Furthermore, *vsx1* is involved in response to light stimulus and photoperiodism, and its alteration can lead to impairment in the correct function of the zebrafish eye (Shi et al., 2019).

To the best of our knowledge, the present study reports for the first time the reduction of eye size and the alteration of the expression of developmentally related genes. Since the capacity of NPs to penetrate the brain and eyes of *D. rerio* embryos was recently reported (Urani et al., 2024), the results suggest that NMF exposure can impact *D. rerio* development, with the consequent observed decrease in eye area. The observed effects in the gene expression and eye dimension occurred at the lowest tested concentration (0.2 mg/L), considered environmentally relevant, posing attention to the possible environmental hazard induced by the improper disposal of surgical face masks.

In addition to the potential impact on developmental processes, the transcriptomic analyses reveal the downregulation of genes related to energy metabolism, suggesting a possible disorder in energy metabolism induced by NMF exposure in *D. rerio* individuals. The observed downregulation of *pdha1a* is linked to pivotal processes of cellular respiration, in particular to "acetyl-CoA" and "pyruvate" metabolic processes (Table S5.4). Moreover, this gene is downregulated in the pathway of TCA cycle and pyruvate metabolism (Table S5.5). In energy metabolism, *pdha1a* is localized in the mitochondrial pyruvate dehydrogenase complex and plays a pivotal role in acetyl-CoA biosynthetic process from pyruvate before entering the TCA cycle. In this perspective, the decreased expression of *pdha1a* may be related to mitochondrial functions that could be affected by exposure to NMFs.

This suggestion is consistent with evidence in the literature indicating that plastic exposure can induce mitochondrial dysfunction and energy impairment in organisms. Bashirova et al. (2023) reported that 70 nm NPs of polyethylene terephthalate (PET) caused alterations in numerous metabolites associated with energy metabolism pathways in zebrafish embryos, while Kim et al. (2019a) found that exposure

to PS-NPs (50 nm, 200 nm) caused the perturbation of metabolites related to energy metabolism in TCA cycle in the nematode *C. elegans*. Moreover, Zhang et al. (2023) showed that 20 µm PS-MPs caused alterations in the TCA cycle of Asian clams. Since *pdha1a* in *D. rerio* is orthologous to the human gene *PDHA1*, it is worth noting that similar impairment in TCA cycle efficiency has been shown also in human cell lines exposed to 100 nm PS-NPs (Wang et al., 2022).

The downregulation of *pdha1a* and *fam96b* is also associated with the metabolism of sulphur compounds and iron-sulphur clusters (Table S5.4), strengthening the possible onset of an energy-stressed state in individuals exposed to NMFs. Cellular respiration is strongly dependent on the presence of iron-sulphur proteins, as they are integral components of mitochondrial electron transport chain protein complexes, and their inactivation has been correlated with impaired metabolism, growth and development (Curtis et al., 2022).

In addition, the observed upregulation of alanine, aspartate and glutamate metabolism gene pathways (Table S5.5), could potentially be another indicator of an energy stress state of zebrafish individuals, as the amino acids involved in this pathway play a pivotal role in the energy metabolism (Tiller and Stringer, 2023).

The downregulation of the TCA cycle and pyruvate metabolisms gene pathways, and the upregulation of alanine, aspartate, and glutamate metabolisms suggest inadequate ATP production. Due to the absence of alteration in apical endpoints, besides eye area, *D. rerio* individuals could potentially have adopted compensatory mechanisms to withstand the metabolic challenges posed by these environmental pollutants. However, chronic exposure to NMFs in the environment could exacerbate this impairment, possibly hampering the fish' energy production and potentially overall metabolic homeostasis.

5.3.3. Median-term exposure

In zebrafish larvae, the transition from endogenous feeding (obtaining nutrients from the yolk) to exogenous feeding (obtaining nutrients from foraging) typically occurs before 168 hpf (Lucore & Cunnaughton 2021).

In this perspective, we exposed zebrafish individuals to NMFs for a longer period of time, from 0 up to 15 days post fertilisation (dpf), to investigate the onset of hazards related to NMF ingestion. Specifically, we evaluated the onset of mortality and alteration in morphometrics, behaviour, and gene expression.

5.3.3.1. Mortality

After 15 days, the mortality rate of CTRL was 20% (±5%), which is consistent with the findings of Lucore and Connaughton (2021). In contrast, individuals exposed to NMFs exhibited higher mortality rates in all the tested concentrations (Fig. 5.4): the recorded mortality in 0.2 mg/L treatment was 50% (± 10%), while for 1 mg/L and 5 mg/L was respectively 38% (± 20%) and 48% (± 10%). These differences were in the close range of statistical significance, (p-value = 0.059).

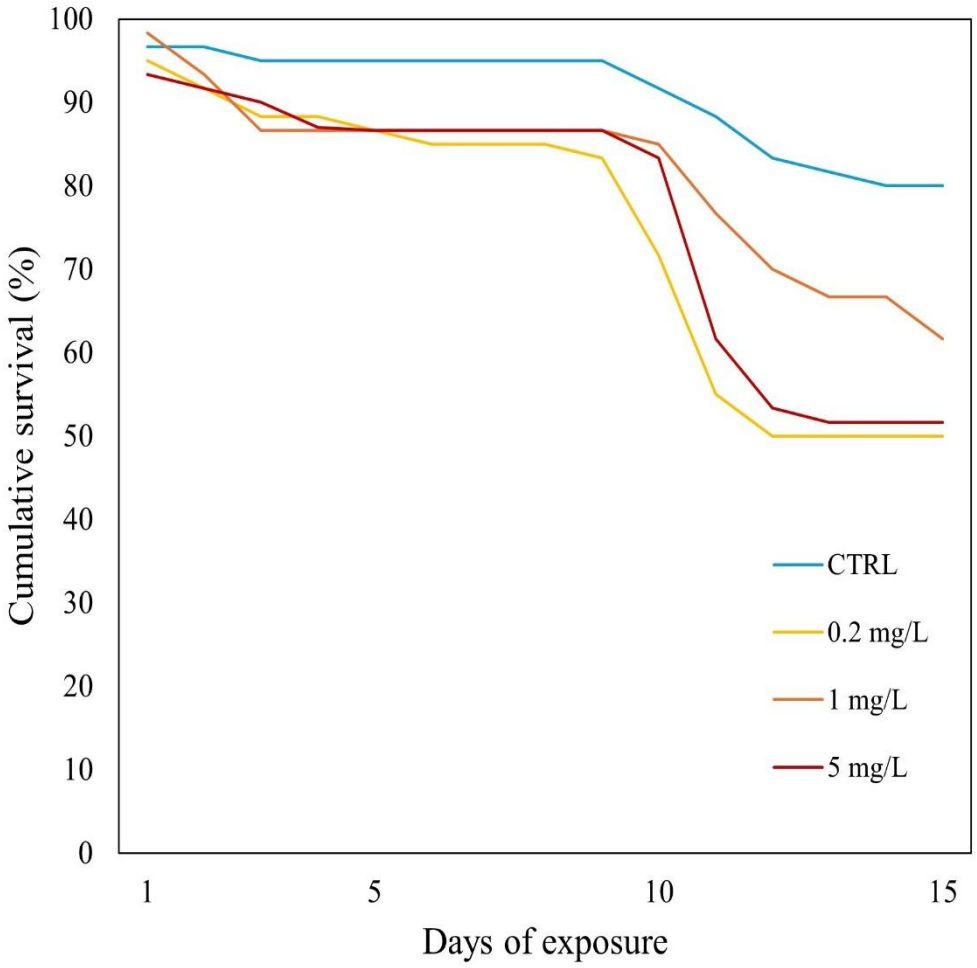

Figure 5.4. Survival curves in CTRL, 0.2 mg/L, 1 mg/L and 5 mg/L treatments (n= 3, twenty individuals per replicate) during the 15 days of the bioassays.

The increased mortality observed in NMF treatments mainly occurred between 9 and 12 dpf. Zebrafish larvae rely on a yolk sac for nutrition until 6-7 dpf. After this time, between 7 and 8 dpf, individuals that do not have external sources of nutrition die to irreversible starvation (Lucore and Connaughton, 2021). This phenomenon is called Point of No Return (PNR). In this context, it is interesting to note that individuals exposed to NMFs died in the period immediately following the

zebrafish PNR. This suggests a possible relationship between mortality and impaired feeding behaviour in individuals exposed to NMFs. Zebrafish cannot discriminate between food and plastic items when both are co-present (Kim et al., 2019b), and MP/NP ingestion can cause the blockage of the digestive tract, feeling of satiety and the consequent reduced feeding activity (Xin et al., 2024).
In the present study, we speculate that this impairment may also have occurred because of the exposure to secondary PP-NMFs, which may have contributed to the observed mortality. Further analyses are essential to investigate the possible toxicity mechanism and confirm this hypothesis.

5.3.3.2. Morphological and behavioural alterations

In contrast to the short exposure, morphometric parameters values settle between CTRL and NMF treatments, with no statistically significant differences observed in eye area ($p= 0.908$), body length ($p= 0.616$) and body/eye ratio ($p= 0.735$) Since in zebrafish both the body (Singleman and Holtzman, 2014) and the eyes (Collery et al., 2014) continue to growth throughout lifespan, the results of the median-term assays suggest that after the initial impairment of eyes recorded at 6 dpf, *D. rerio* individuals are able to cope with the damage caused by NMFs, and to return to a condition similar to CTRL ones. Alternatively, given the increased mortality reported here, these findings could also be associated with the fact that only the animals with the best fitness survived up to the end of the assay when the endpoints were recorded. Although these individuals showed no alteration in the individual endpoints measured, it is worth noting that the transcriptomic analysis results (*par. 5.3.3.3*) suggest the onset of a stress condition in individuals exposed to NMFs.
Regarding swimming behaviour, no alteration in travelled distances were recorded between CTRL and NMF treatments ($p= 0.292$) (Fig. S5.7).

5.3.3.3. Median-term exposure of NMFs dysregulated energy metabolism in zebrafish

Table S5.3B reports the quality of sequencing reads of median-term exposure experiments. The RNA-seq analysis identified a total of 325 differentially DEGs between CTRL and zebrafish individuals exposed to 0.2 mg/L of NMFs. Among these, 122 were upregulated and 203 downregulated. Figure 5.5A and Figure 5.5B respectively report the results of GO and KEGG enrichment analysis, while Table S5.6 and Table S5.7 report the detailed results of DEGs and significantly enriched KEGG pathways.

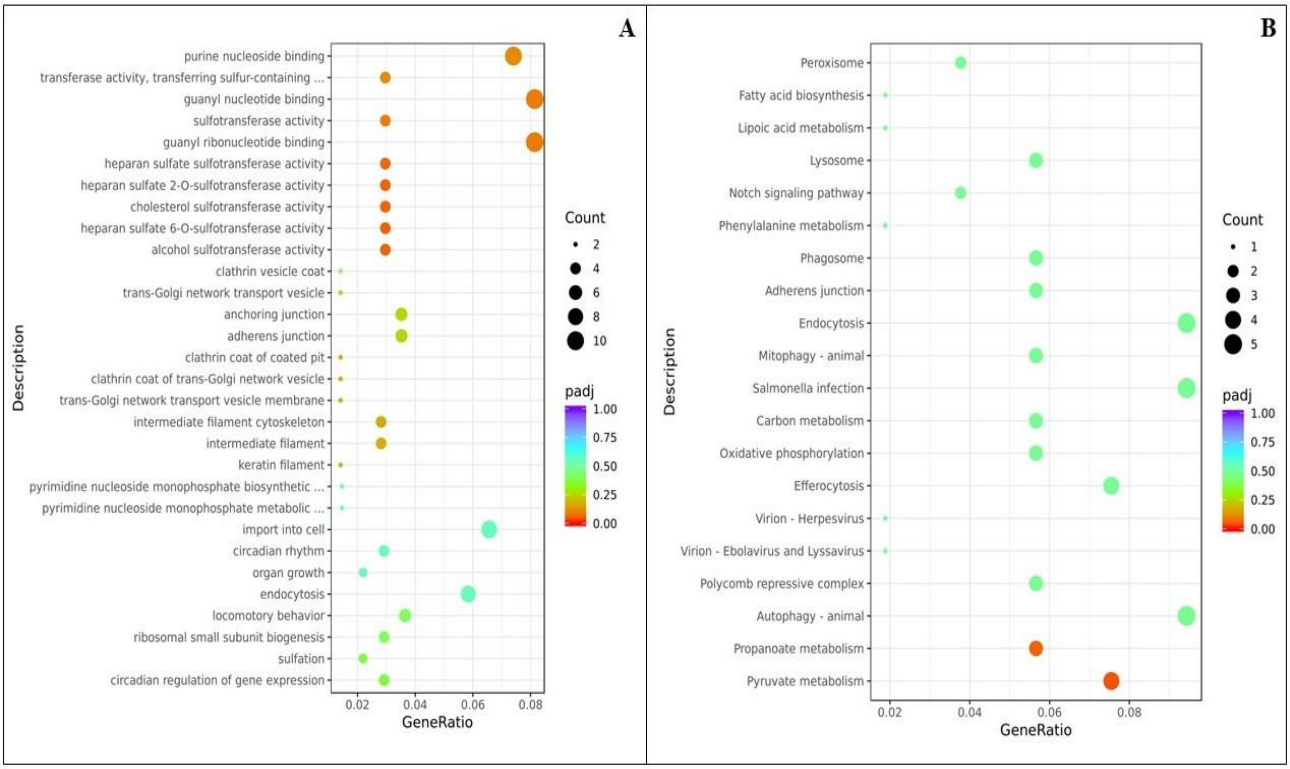

Figure 5.5. Gene Ontology (A) and KEGG pathways (B) after 15 days of NMF exposure (0.2 mg/L). The abscissa is the ratio of the number of differential genes linked with the GO term/ KEGG pathway to the total number of differential genes. The ordinate is GO term/KEGG Pathway. The size of a point represents the number of genes annotated to a specific GO term/ KEGG pathway. The colour from red to purple represents the significant level of the enrichment.

The downregulated genes are mainly related to sulfotransferase activity, in particular "*alcohol sulfotransferase*", "*heparan sulfate sulfotransferase*" and "*cholesterol sulfotransferase*" (p= 0.008) *(sult1st2/sult1st3/zgc:194879/sult1st6)* activities. Moreover, genes involved in binding activities, in particular those of "*guanyl ribonucleotide binding*" (p= 0.026), "*purine nucleoside binding*" (p= 0.048) and "*GTP binding*" (p= 0.048) (*rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/pde6h/rhoab/tuba1b/irge4/anxa6*) resulted to be downregulated. GO analysis revealed that the only genes that exhibited upregulation were those related to the process of "*endocytosis*" (p= 0.022) (*synj1/cltca/hspa8/rab5b/ldlrad3/elmo2/met*).

The KEGGs analysis revealed upregulation of energy related pathways, in particular those of "*pyruvate metabolism*" (p= 0.045) (*LOC103909985/acss1/acot12/me2*), while "*propanoate metabolism*" (p= 0.061) (*LOC103909985/acss1/ bckdha*) was found close to the significant threshold. No downregulated pathways were highlighted.

It is noteworthy that among the upregulated genes associated with endocytosis there is *Synj*, which plays an important function related to synaptic vesicle endocytosis (Fasano et al., 2018).

Defects in *Synj1* have been shown to disrupt the endolysosomal pathways in photoreceptor neurons of zebrafish (George et al., 2014), possibly leading to morphological and functional abnormalities (Holzhausen et al., 2009). From this perspective, the altered expression of *Synj* observed after 15 days of exposure to 0.2 mg/L of NMFs suggest that NMFs can cause imbalance in *D. rerio* neurodevelopment, confirming the observation recorded in the short-term exposure experiment ("*par 5.3.2.5"*).

Regarding the downregulated genes, we observed the alteration of genes associated with sulfotransferase activity. Sulfotransferases are essential catalysts in the sulphation of exogenous molecules, such as drugs and toxic chemicals (Nimmagadda et al., 2006) and represent an important line of defence against xenobiotics (Enyoh et al., 2023). Therefore, it can be inferred that the alterations of expression of several genes involved in sulfotransferase activity suggest an increase in defences against toxic compounds displayed by individuals exposed to plastic pollution, as proposed by Trotter et al. (2021).

Furthermore, the downregulation of several genes related to guanosine triphosphates (GTP) binding activities suggests an energetic stress state of individuals exposed to NMFs. GTP is in fact involved in a multitude of biological processes (Huang and Wang, 2020) and provides energy for protein synthesis, playing a crucial role in mitochondrial physiology (Thomson, 1998). A recent study has highlighted alterations in genes coding for GTP binding following exposure to PS-MPs in the nematode *Steinernema feltiae* (Li et al., 2024). To the best of our knowledge, the present study reports for the first time the alterations in the expression of GTP binding genes in *D. rerio* due to plastic exposure.

The upregulation of propanoate and pyruvate pathways revealed by KEGG analyses suggest that NMFs dysregulated the energetic metabolism in zebrafish, as observed in the short-term exposure. Propanoate and pyruvate metabolisms play a fundamental role in providing energy for TCA cycle (Huang et al., 2020), and their alteration, as recently shown by Hou et al. (2022), can induce an energy stress state in organisms. Given that the high mortality rates observed in all the tested concentrations can be associated with an imbalance in foraging activity, the alteration of energy-related pathways in surviving organisms could be indicative of imbalances incurred during the transition to active feeding. Due to the absence of alteration in the evaluated apical endpoints, the surviving zebrafish individuals may have adopted compensatory mechanisms to overcome these energy metabolic imbalances.

5.4. Conclusion

In the present study, PP-NMFs were obtained from surgical face masks through an accelerated weathering process. The application of UV radiation enabled the simulation of the effects of solar radiation, which in natural environments drives the fragmentation of plastics into smaller particles. The effects of the secondary NMFs were evaluated in *D. rerio*, a freshwater model species whose embryonic and larval development stages are susceptible to disruption by plastic pollution. When considering the existing literature, it is evident that the impact of NMFs on zebrafish is not entirely consistent among studies. This can be a consequence of the limited number of studies conducted so far and the use of various experimental designs. In particular, the different protocols adopted to obtain NMFs from surgical face mask provide fibres with different sizes and different chemical properties, thus limiting the comparison between different studies. In light of the aforementioned considerations, the present study showed that the PP-NMFs derived from the artificial photodegradation of the melt-blown nonwoven fabric of surgical facemasks can induce adverse effects on zebrafish. Due to their dimensions, these fibers can adhere to chorion surfaces, potentially penetrating it. After 6 days of exposure, morphological alterations were reported, with transcriptomic data suggesting a possible link with neurodevelopment alteration and an energy stress state. Prolonged exposure up to 15 days showed how ingestion of these plastics can further induce negative effects, leading to increased mortality and worsening energy imbalances. The findings of the present study can provide valuable data from a risk assessment perspective. The observed effect occurred at environmentally relevant concentrations, suggesting the potential for an ecotoxicological risk associated with the improper disposal of surgical face masks. In particular, the results point to the potential of NMFs to negatively impact not only zebrafish individuals, but also the ecological status of other aquatic species and populations. In fact, the high genomic and molecular similarities between zebrafish and other vertebrates, including humans, allow the interpretation of toxicity outcomes from exposure experiments in a broader context. However, given the current lack of studies conducted on this model species beyond 144 hpf, we consider particularly urgent an increase in the number of median/long-term exposure studies, to gain a better comprehension of the risk caused by the active ingestion of these particles.

Despite the end of the SARS-CoV-2 pandemic, the considerable quantity of masks that were discarded into the environment persist, resulting in the continuous release of NMFs. This will inevitably result in an increase in environmental concentrations of these emerging contaminants in the coming years. Given the circulation of micro- and nano-plastics in different environmental

matrices, we suggest closely monitoring the threat of NMF pollution not only in aquatic environments, but also to extend the evaluation of NMF toxicity to individuals of different trophic chains.

SUPPORTING MATERIALS

Table S5.1. Effects of NMFs on survival and hatching rate of *D. rerio* at 96 hpf and 144 hpf. Data are presented as mean values ± standard deviation.

Treatment	Mortality 96 hpf (%)	Hatching rate 96 hpf (%)	Mortality 144 hpf (%)
CTRL	7.4 (± 1.6)	100	12 (± 3.2)
0.2 mg/L	1.7 (± 2.9)	100	10 (± 5)
1 mg/L	10 (± 5)	100	15 (± 0)
5 mg/L	5 (±5)	100	6.7 (± 7.6)

Table S5.2. Results of statistical analyses on the measured morphometrics endpoints (eye area, body length, body length/eye area) normalized to CTRL for the different NMF exposures (0.2 mg/L, 1 mg/L, 5 mg/L) on *D. rerio.* Asterisks report statistically significant differences (*: $p < 0.05$; **: $p< 0.01$. ***: $p< 0.001$).

Time	Endpoint	Anova			Dunnett (p-value)		
		Df	F	P	0.2 mg/L	1 mg/L	5 mg/L
48 hpf	Eye area	3	1.901	0.138	/	/	/
96 hpf	Eye area	3	0.314	0.815	/	/	/
	Body length	3	0.426	0.735	/	/	/
	Ratio	3	0.684	0.565	/	/	/
144 hpf	Eye area	3	4.571	**0.005**	**0.020***	0.129	**0.005****
	Body length	3	1.546	0.211	/	/	/
	Ratio	3	5.881	**0.001**	**0.019***	0.056	**< 0.001 *****

Table S5.3. Summary of the data quality of sequenced samples at 144 hpf (A) and 15 dfp (B).

A	Sample	Clean reads	GC content	Q30
	CTRL1	3,83E+05	48.5%	93.8%
	CTRL2	4,23E+05	46.3%	93.9%
	CTRL3	4,12E+05	46.1%	93.3%
	0.2 mg/L 1	4,09E+05	46.6%	94.1%
	0.2 mg/L 2	4,16E+05	46.4%	94.2%
	0.2 mg/L 3	4,01+E05	47.1%	93.7%

B	Sample	Clean reads	GC content	Q30
	CTRL1	4,35E+07	48.1 %	94.7 %
	CTRL2	3,93E+07	48.8 %	95.5 %
	CTRL3	4,52E+07	49.2 %	95.5 %
	0.2 mg/L 1	3,84E+07	49.6 %	95.4 %
	0.2 mg/L 2	4,91E+07	49.7 %	94.4 %
	0.2 mg/L 3	4,77E+07	49.7 %	95.2 %

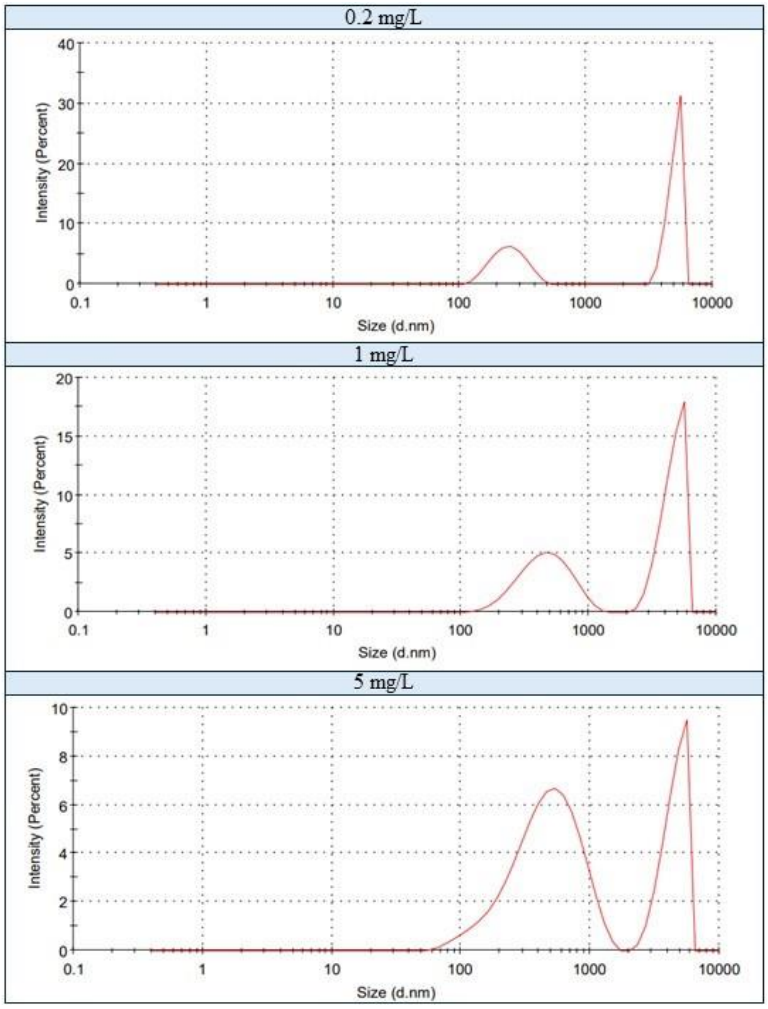

Figure S5.1. DLS measurements of hydrodynamic size expressed as intensity distributions of the three investigated NMFs concentrations (0.2 mg/L, 1 mg/L and 5 mg/L) in the exposure test water

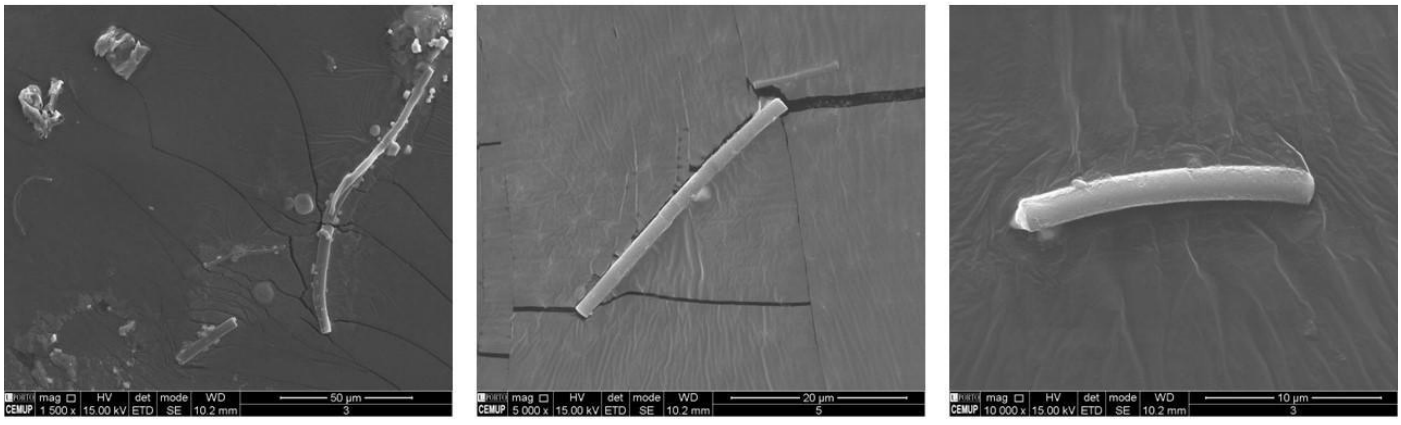

Figure. S5.2. Scanning electron microscopy (SEM) of the photodegraded NMFs.

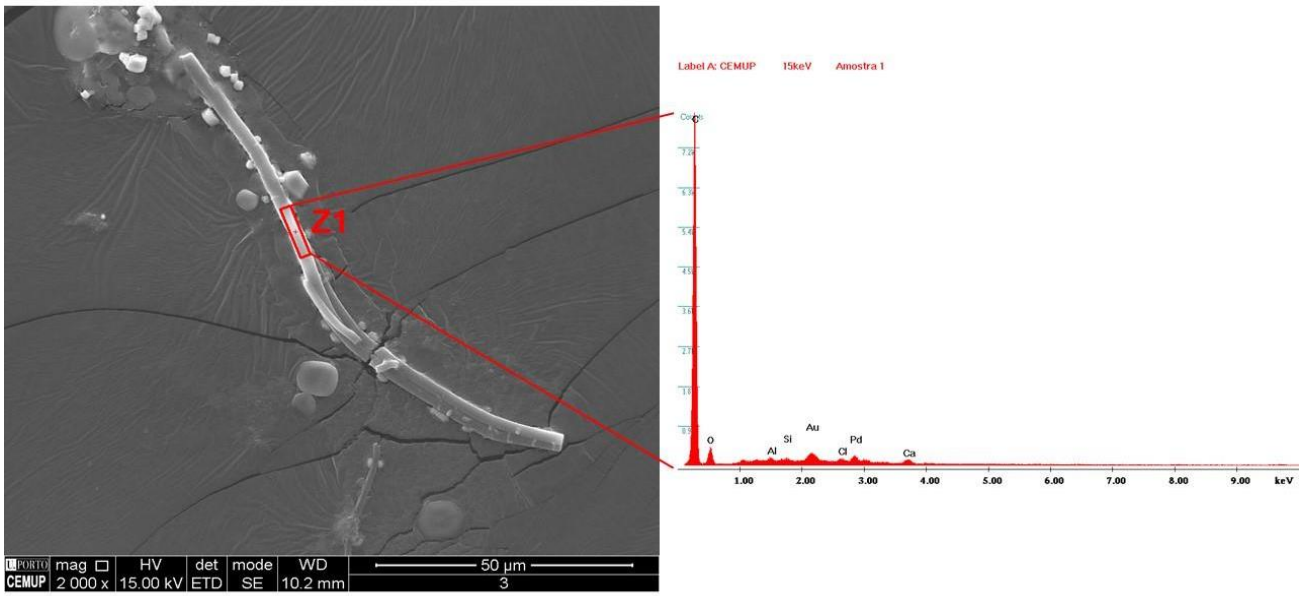

Figure. S5.3. Detail of a SEM image of a single microfiber (on the left) and its elemental composition investigated through energy dispersive X-ray spectroscopy (EDS) analyses. Gold (Au) and palladium (Pd) signals derived from the sample preparation, while oxygen (O) is from the atmosphere of the sample chamber. The spectrum of the samples exhibits C as expected for polypropylene.

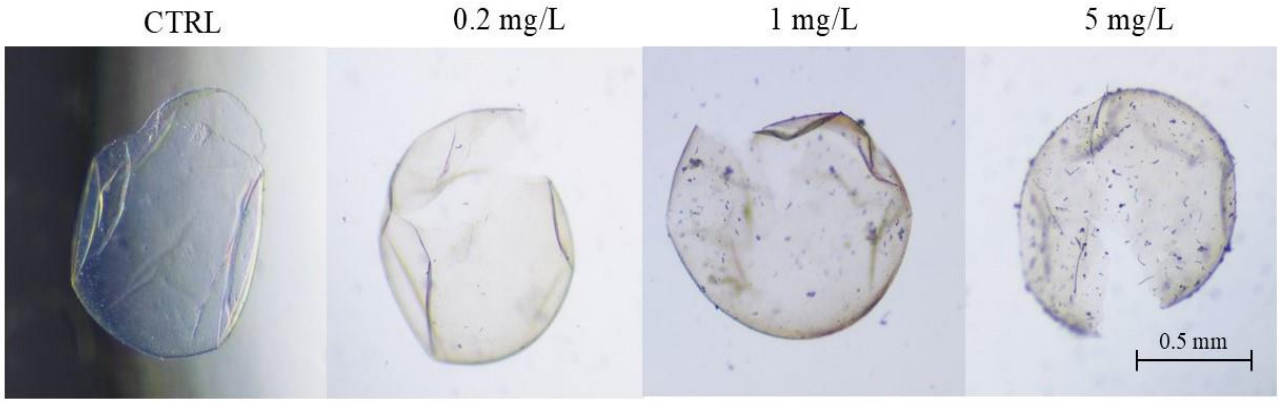

Figure. S5.4. Microscopy images of the hatched chorions at 72 hpf in the different treatments, showing a concentration-dependent increase in the number of NMFs adsorbed onto zebrafish chorion.

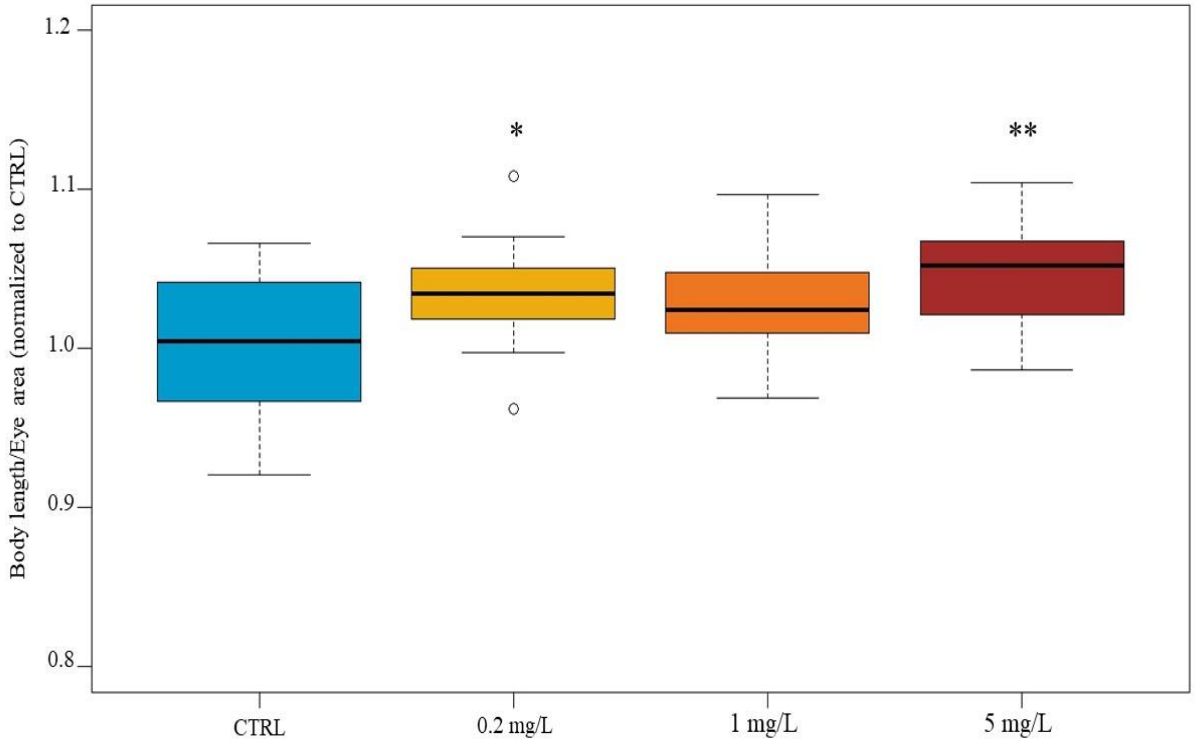

Figure S5.5. Boxplot of the body length/eye area (normalized to CTRL) measured in *D. rerio* individuals after the exposure to different concentrations (0.2 mg/L, 1 mg/L, 5 mg/L) of NMFs. Asterisks indicate statistically significant differences with respect to CTRL (*: $p < 0.05$; **: $p< 0.01$; ***: $p< 0.001$).

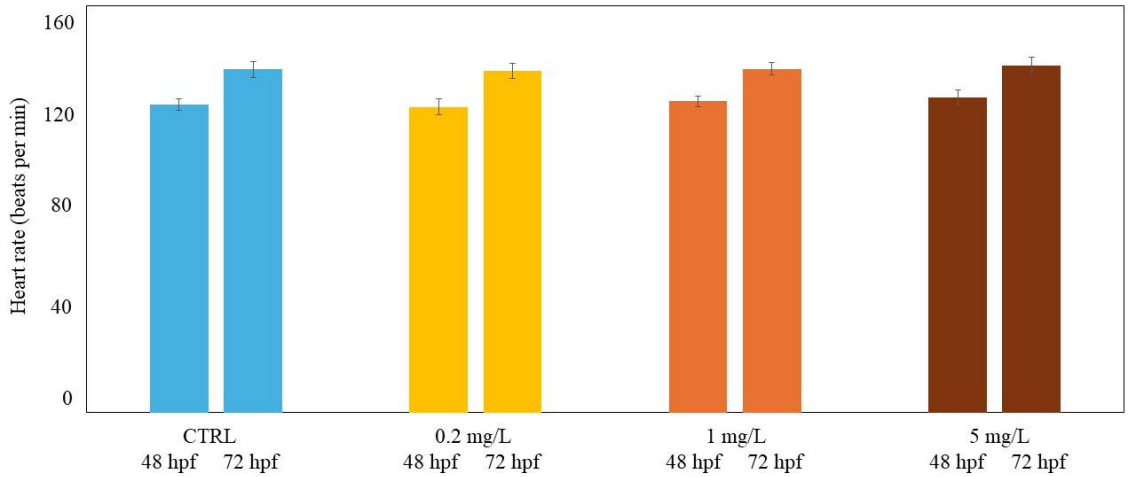

Figure S5.6. Histograms of heart rate measured in *D. rerio* individuals after the exposure to different concentrations (0.2 mg/L, 1 mg/L, 5 mg/L) of NMFs at 48 hpf and 72 hpf.

Table S5.4. Zebrafish differentially expressed genes following 6 days of exposure to 0.2 mg/L of MNFs. Parameters for differentially expressed genes set to: padj ≤ 0.05 and a |log2(FoldChange)| > 0.

	Category	GOID	Description	GeneRatio	BgRatio	pvalue	padj	geneID	geneName	Count
Down regulated	BP	GO:0006790	sulfur compound metabolic process	45414	201/15992	0.0015	0.0434	406702/436722	pdha1a/fam96b	2
	BP	GO:0006085	acetyl-CoA biosynthetic process	45413	10/15992	0.0031	0.0434	406702	pdha1a	1
	BP	GO:0051186	cofactor metabolic process	45414	324/15992	0.0039	0.0434	406702/436722	pdha1a/fam96b	2
	BP	GO:0006084	acetyl-CoA metabolic process	45413	19/15992	0.0059	0.0434	406702	pdha1a	1
	BP	GO:0016226	iron-sulfur cluster assembly	45413	19/15992	0.0059	0.0434	436722	fam96b	1
	BP	GO:0031163	metallo-sulfur cluster assembly	45413	19/15992	0.0059	0.0434	436722	fam96b	1
	BP	GO:0035384	thioester biosynthetic process	45413	26/15992	0.0081	0.0445	406702	pdha1a	1
	BP	GO:0071616	acyl-CoA biosynthetic process	45413	26/15992	0.0081	0.0445	406702	pdha1a	1
	BP	GO:0006090	pyruvate metabolic process	45413	55/15992	0.0170	0.0720	406702	pdha1a	1
	BP	GO:0006637	acyl-CoA metabolic process	45413	58/15992	0.0180	0.0720	406702	pdha1a	1
	BP	GO:0035383	thioester metabolic process	45413	58/15992	0.0180	0.0720	406702	pdha1a	1
Up regulated	BP	GO:0051093	negative regulation of developmental process	46113	186/15992	0.0002	0.0504	678650/30598/494130/556198	bcl11aa/vsx1/unc5b/srgap2	4
	BP	GO:0051241	negative regulation of multicellular organismal process	46113	208/15992	0.0003	0.0504	678650/30598/494130/556198	bcl11aa/vsx1/unc5b/srgap2	4

Table S5.5. Zebrafish significantly enriched KEGG pathways following 6 days of exposure to 0.2 mg/L of MNFs (p adj <0.05)

	KEGGID	Description	GeneRatio	BgRatio	pvalue	padj	geneID	geneName	keggID	Count
Down regulated	dre03040	Spliceosome	45445	165/8607	0.0052	0.0625	394058/100004402_1	fus/plrg1	dre:394058/dre:100004402	2
	dre00785	Lipoic acid metabolism	45444	26/8607	0.0179	0.0687	406702	pdha1a	dre:406702	1
	dre03022	Basal transcription factors	45444	43/8607	0.0296	0.0687	751713_1	gtf2h5	dre:751713	1
	dre00020	Citrate cycle (TCA cycle)	45444	45/8607	0.0309	0.0687	406702	pdha1a	dre:406702	1
	dre03030	DNA replication	45444	49/8607	0.0336	0.0687	393241	rnaseh2b	dre:393241	1
	dre01210	2-Oxocarboxylic acid metabolism	45444	50/8607	0.0343	0.0687	406702	pdha1a	dre:406702	1
	dre00620	Pyruvate metabolism	45444	64/8607	0.0438	0.0687	406702	pdha1a	dre:406702	1
	dre03420	Nucleotide excision repair	45444	67/8607	0.0458	0.0687	751713_1	gtf2h5	dre:751713	1
	dre00010	Glycolysis / Gluconeogenesis	45444	102/8607	0.0690	0.0920	406702	pdha1a	dre:406702	1
	dre03015	mRNA surveillance pathway	45444	117/8607	0.0788	0.0946	394058	fus	dre:394058	1
Up regulated	dre00250	Alanine, aspartate and glutamate metabolism	43525	56/8607	0.0002	0.0093	564754_1/100329827/406688	nat8l/gad1a/got2a	dre:564754/dre:100329827/dre:406688	3

Table S5.6. Zebrafish differentially expressed genes following 15 days of exposure to 0.2 mg/L of MNFs. Parameters for differentially expressed genes set to: padj ≤ 0.05 and a |log2(FoldChange)| > 0.

	Category	GOID	Description	GeneRatio	BgRatio	pvalue	padj	geneID	geneName	Count
Down regulated	MF	GO:0004027	alcohol sulfotransferase activity	31868	48/15795	0.0001	0.0079	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0017095	heparan sulfate 6-O-sulfotransferase activity	31868	48/15795	0.0001	0.0079	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0051922	cholesterol sulfotransferase activity	31868	48/15795	0.0001	0.0079	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0004394	heparan sulfate 2-O-sulfotransferase activity	31868	50/15795	0.0001	0.0079	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0034483	heparan sulfate sulfotransferase activity	31868	55/15795	0.0002	0.0092	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0008146	sulfotransferase activity	31868	68/15795	0.0005	0.0174	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0032561	guanyl ribonucleotide binding	31868	468/15795	0.0010	0.0265	436718/100007523/64263/100007552/393758/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/pde6h/rhoab/tuba1b/irge4/anxa6	9
	MF	GO:0016782	transferase activity, transferring sulfur-containing groups	31868	82/15795	0.0010	0.0265	791732/368270/100170782/436872	sult1st2/sult1st3/zgc:194879/sult1st6	4
	MF	GO:0019001	guanyl nucleotide binding	31868	480/15795	0.0012	0.0280	436718/100007523/64263/100007552/393758/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/pde6h/rhoab/tuba1b/irge4/anxa6	9
	MF	GO:0017017	MAP kinase tyrosine/serine/threonine phosphatase activity	31868	15/15795	0.0030	0.0478	450040/353314	dusp14/dusp6	2
	MF	GO:0001883	purine nucleoside binding	31868	453/15795	0.0034	0.0478	436718/100007523/64263/100007552/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/rhoab/tuba1b/irge4/anxa6	8
	MF	GO:0005525	GTP binding	31868	453/15795	0.0034	0.0478	436718/100007523/64263/100007552/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/rhoab/tuba1b/irge4/anxa6	8

	MF	GO:0032550	purine ribonucleoside binding	31868	453/15795	0.0034	0.0478	436718/100007523/64263/100007552/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/rhoab/tuba1b/irge4/anxa6	8
	MF	GO:0001882	nucleoside binding	31868	457/15795	0.0036	0.0478	436718/100007523/64263/100007552/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/rhoab/tuba1b/irge4/anxa6	8
	MF	GO:0032549	ribonucleoside binding	31868	457/15795	0.0036	0.0478	436718/100007523/64263/100007552/100006041/373080/100033386/353363	rhocb/si:dkey-79f11.10/gch2/si:dkey-79f11.7/rhoab/tuba1b/irge4/anxa6	8
	MF	GO:0008395	steroid hydroxylase activity	31868	60/15795	0.0044	0.0539	558541/492759/259306	cyp3c4/cyp3c3/cyp2ad2	3
	MF	GO:0033549	MAP kinase phosphatase activity	31868	19/15795	0.0048	0.0556	450040/353314	dusp14/dusp6	2
Up regulated	BP	GO:0006897	endocytosis	19176	289/16055	0.0033	0.0218	337236/323579/573376/405821/570705/100006857/100150664	synj1/cltca/hspa8/rab5b/ldlrad3/elmo2/met	7
	BP	GO:0098657	import into cell	19176	381/16055	0.0002	0.0613	337236/323579/573376/405821/570705/100006857/100150664	synj1/cltca/hspa8/rab5b/ldlrad3/elmo2/met	7

Table S5.7. Zebrafish significantly enriched KEGG pathways following 15 days of exposure to 0.2 mg/L of MNFs (p adj <0.05).

	KEGGID	Description	GeneRatio	BgRatio	pvalue	padj	geneID	geneName	keggID	Count
Up regulated	dre00620	Pyruvate metabolism	19450	67/8642	0.0007	0.0450	103909985/541435_1/100536873/445233	LOC103909985/acss1/acot12/me2	dre:559403/dre:541435/dre:100536873/dre:445233	4
	dre00640	Propanoate metabolism	19419	41/8642	0.0019	0.0610	554124/103909985/541435_1	bckdha/LOC103909985/acss1	dre:554124/dre:559403/dre:541435	3

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Chapter 6

Can Raman spectroscopy be used to detect nanoplastics in *Diamesa* larvae (Diptera Chironomidae) from glacial habitats?

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In preparation

Abstract

The detection and quantification of nanoplastics (NPs) in the environment is challenging mainly due to the size and the low concentrations of NPs, especially in natural ecosystems where their presence is estimated to be scares, such as headwaters. Our aim was to investigate the presence of NPs in IV-instar larvae of two chironomid species (*Diamesa zernyi* and *Diamesa tonsa*) colonizing two high-altitude glacier-fed streams (Mandrone and Amola streams, Trentino, NE-Italy).

We applied and adapted an analytical method developed by the Joint Research Centre (European Commission, Ispra, Italy) to quantity polystyrene NPs of 500 nm in size in marine (tunicates) invertebrates. The procedure comprised a combination of enzymatic and oxidative digestion followed by a purification step in ethanol to enable on-chip identification through Raman spectroscopic analysis. To validate the extraction procedure, three pools of 100 mg (wet weight) each of *D. zernyi* larvae from the Mandrone stream were spiked with NPs at two different theoretical concentrations (10^9 and 10^7 particles/mL) and then subjected to the entire extraction process. Quantification of the particles in the residual matrix was performed using Single Particle Extinction and Scattering (SPES) analysis.

The results demonstrate good recovery rates, respectively of 109 ± 28 % and 82 ± 12 % for the high and low concentration spiked samples. This methodology enabled the effective identification of plastic particles using confocal Raman spectroscopy.

Successively, NPs were searched for in three pools of 100 mg (wet weight) of non-spiked specimens of *D. tonsa* from the Amola stream, highlighting the presence of polystyrene particles. Although the low number of animal replicates from only one site analysed, the consideration of only one polymer, and the detection limits of the Raman spectroscopy in respect to the NPs size (500 nm), this represents the first evidence of accumulation by aquatic insect larvae and the potential environmental pollution of glacial streams by NPs for the Italian Alps.

6.1 Introduction

The European Alps are experiencing a decline in freshwater biodiversity due to climate change, particularly due to the retreat of glaciers caused by rising temperatures during the summer months and a reduction in snowfall during the winter [1]. However, climate change is not the only threat to alpine environments [2]. These remote and pristine systems are also contaminated by pollutants transported from the atmosphere to the glaciers and released in ice-melt waters [3-5]. The presence of organic, inorganic, and emerging contaminants has been detected in glacial habitats in the European Alps [6,7]. The role of glaciers as temporary sinks of atmospherically transported pollutants has been demonstrated by the higher contaminant concentrations in glacial communities compared to those from non-glacial catchments [4]. Furthermore, recent studies suggest the role of high-mountain ecosystems as potential indicators of plastic pollution [8,9]. Microplastics (MPs) can be defined as plastic items from 5 mm to 1 μm in size, while nanoplastics (NPs) as those in the size range below 1 μm [10,11]. The presence of MPs [12] and NPs [13] has been confirmed in remote mountain systems, due to the long-range transport pathways of these particles through the movement of air masses [14,15]. Specifically, Materić et al. [13] detected an average NP concentration of 46.5 ng/mL of melted surface snow in a remote territory at 3106 m a.s.l. in the Austrian Alps, with a prevalence of mainly by polypropylene (PP) and polyethylene terephthalate (PET).

In recent years, research has been focused on the evaluation of the environmental concentrations of MPs, thanks to well-known sampling protocols and analytical characterization techniques [16]. The available data have enabled advances in the ecological MPs risk assessment [17]. On the contrary, our knowledge of the environmental levels and relative hazards on biota of NPs is still at an early stage [18], due to the current methodological limitations and the lack of methods for the characterization and quantification of these particles in complex matrices [19,20]. The detection and quantification of NPs in the environment is challenging mainly due to the size and the low concentrations of NPs, with matrix effects and surface contamination that contribute to harsh difficulties in the analytical performances [21]. A possible solution to monitor NP pollution is the use of bioindicators, because of their capacity to take up and concentrate nanoscale particles enhancing their concentration [22].

The presence of NPs in high mountain matrices has been recently proved in the snow of Austrian alps [13,23] while, to the best of our knowledge, there is no information of the presence of NPs in organisms of high-mountain ecosystems. In this context, chironomids (Diptera Chironomidae) might

become valid plastic bioindicators in freshwater systems [24], being benthic organisms living in and feeding on sediments [25], apparently without selecting what is in the sediment and so ingesting plastic litter if present [26]. Specifically, chironomids of the genus *Diamesa* Meigen, 1935 are the main exclusive insect colonizing this habitat type [27].

According to findings of Materić et al. [13] we assumed that snow and ice on the glaciers feeding the streams that we sampled could be contaminated by MPs and NPs as well, mainly transported by the atmosphere. As a consequence, the ice- and snowmelt waters are expected to be contaminated as well, as we demonstrated for many other organic contaminants (such as pesticides, polycyclic aromatic hydrocarbons and synthetic fragrances) found in the same streams [2,28] Notwithstanding the hypothesized presence in consistent amount of MPs and NPs on the surface snow of the glaciers feeding the streams we sampled, according to Materić et al. [13] findings, we hypothesized to have a low level of contamination of stream water fed by snow and icemelt due the high discharge and current velocity and low deposition rate characterizing these habitats during summer. Dahms et al. [29] demonstrated that river sectors of increased flow do not allow MPs fragments to settle down to the sediment hindering their ingestion by benthic macroinvertebrates. This is specifically true for NPs, due to their smaller dimensions [30]. As a consequence, our hypothesis to test was to detect a low amount of NPs in the larvae of chironomids collected, in summer, in glacial streams. To this purpose, we applied and adapted an analytical method developed by the Joint Research Centre (Ispra, Italy) to quantity polystyrene NPs of 500 nm in size in marine (tunicates) [22].

6.2 Materials and methods

The analytical workflow used in this study is reported in Figure 6.1 and was designed for extracting NPs from chironomid larvae collected in a remote alpine environment. Various approaches were tested during the protocol development, with the most effective method being selected for application.

6.2.1. Study area

Larvae were collected in two glacier-fed streams, Mandrone and Amola, within 15 m downstream from the glacier snout at 2583 m. a.s.l. and 2557 m. a.s.l., respectively (Adamello-Presanella Mts, Trentino, Italy; 46 °N, 10°E).

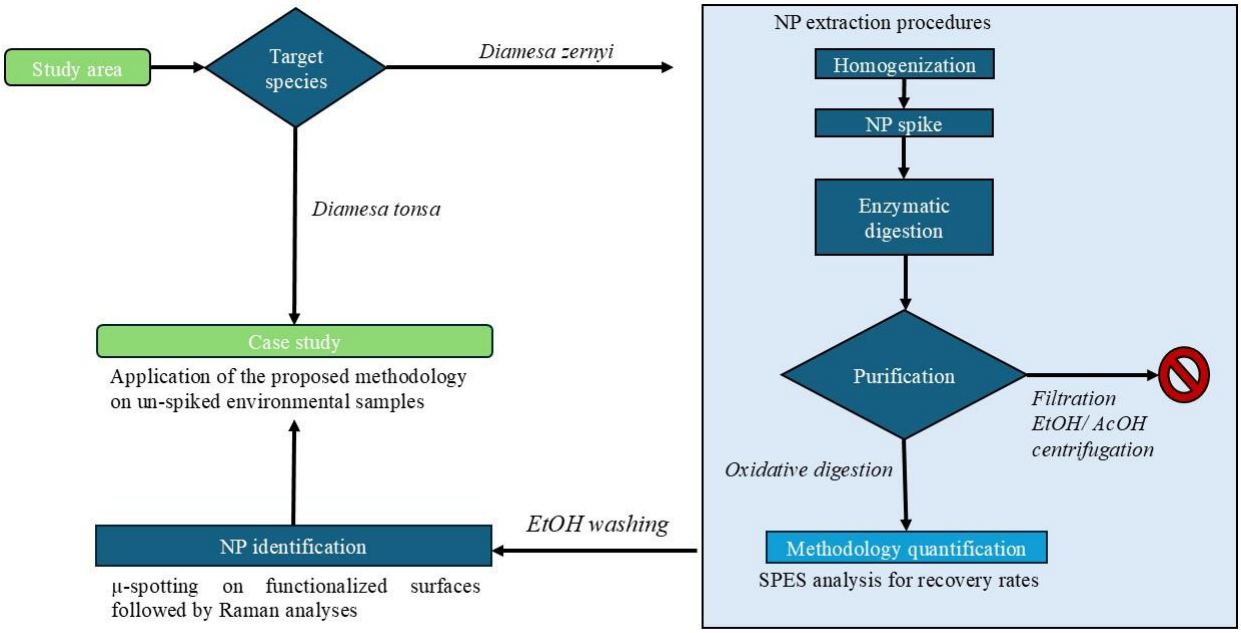

Figure 6.1. Analytical workflow representing the different steps performed in the development of an analytical method for the detection of nanoplastics in biological samples.

6.2.2. Target species and sampling activity

Among invertebrates, species of the genus *Diamesa* Meigen, 1835 (Diptera, Chironomidae, Diamesinae) represent the main and even the exclusive taxon colonizing the kryal habitats of uppermost sectors of glacier- fed streams [1] (Figure 6.2). In the present study, *Diamesa zernyi* Edwards, 1933 and *Diamesa tonsa* (Haliday, 1856) were selected as target species. *D. zernyi* larvae were stored in the MUSE collections at the beginning of the research, while *D. tonsa* was collected successively specifically for this work. *D. zernyi* I larvae were collected in August 2019 in the Mandrone glacier-fed stream to validate the extraction and isolation protocol. Successively, *D. tonsa* larvae were collected in the Amola glacier-fed stream in August 2022 to apply the developed methodology on non-spiked specimens. The two glacier-fed streams at the glacier front are cold (average summer water temperature 1-2°C), turbid and turbulent [1]. The two species have the same ecological valency, being cold-adapted and restricted to high mountain freshwaters [1,31]. Larvae were collected using a pond net (30 × 30 cm, mesh size 100 µm) (Scubla SNC, Italy) by kick sampling within 50 m downstream from the glacier front, according to Lencioni et al. [1]. In the field, the material was poured into a polypropylene vessel from which larvae were sorted with steel tweezers, with bare hands. Larvae were transferred in polypropylene bottles with stream water and transported

alive to the laboratory in a refrigerated bag. After species identification under the stereomicroscope (MZ 7.5; Leica Microsystems, Germany), according to Rossaro and Lencioni [32], larvae were weighted and stored for further analysis, partly freeze-dried at -20 °C (those collected in the Mandrone stream) and partly preserved in ethanol 90% (those collected in Amola). *D. zernyi* was the dominant species in Mandrone while *D. tonsa* prevailed in Amola. For this reason, we decided to analyse these two species for which about 4 g of fresh biomass was measured.

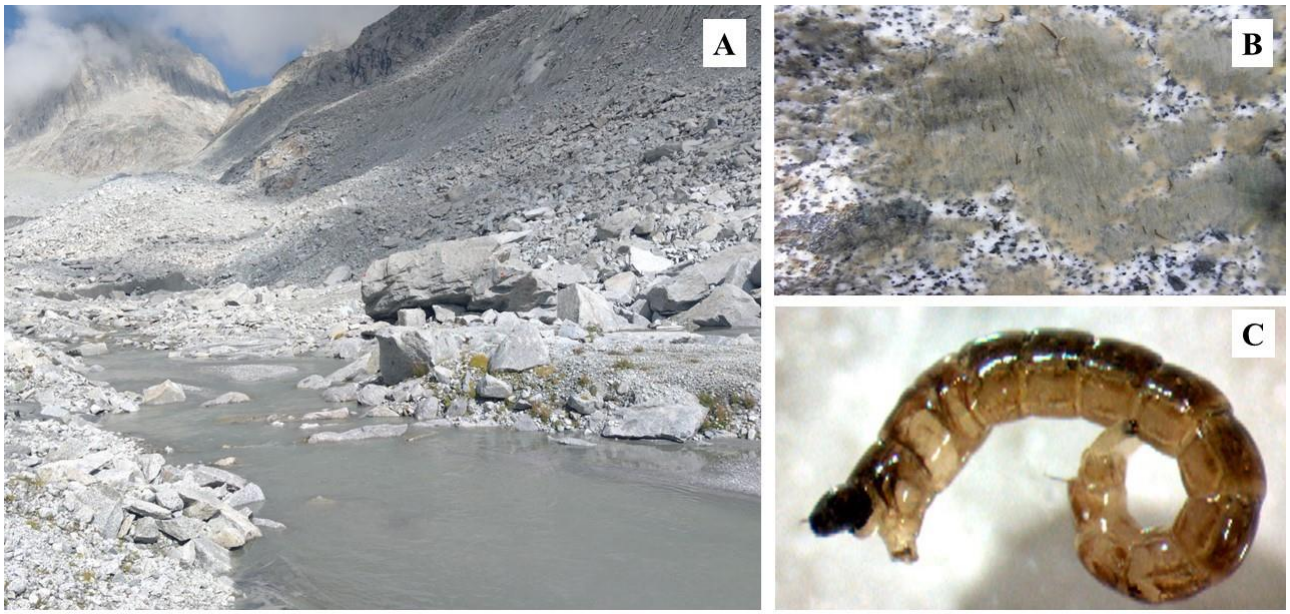

Figure 6.2. Amola glacier–fed stream and glacier front in 2022. (A). Chironomids attached to a stone in the sampling area. (B). A IV-instar larva of *Diamesa zernyi* collected in the Mandrone glacier-fed stream (C). Photos by ©V. Lencioni.

6.2.3. Methodologies

6.2.3.1. Procurement of nanoplastics and chemicals

Commercial polystyrene (PS) beads were selected as reference material for this study. PS-NPs of 500 nm nominal diameters were purchased from Polysciences Europe GmbH, Germany.

Sodium cacodylate buffer (50 mM) was prepared using sodium cacodylate trihydrate (Sigma Aldrich, CAS 6131-99-3) to stabilize pH during different enzymatic digestion steps.

For the enzymatic digestion, lipase from *Aspergillus niger* (Sigma Aldrich, 200UI, CAS 9001-62-1, powder), chitinase from *Streptomyces griseus* (Sigma Aldrich, 200UI; CAS 9001-06-3) and papain from *Carica papaya* (Roche, 10mg/mL, Basel, Switzerland, REF 1010814001) were used to digest

respectively lipids, chitin, and proteins. Prior to use, papain stock solution needs to be activated for 1 hour at 50°C in an activation buffer (1 mL) composed of 784 µL of sodium cacodylate solution (50mM), 11µL EDTA (100mM) (Sigma-Aldrich, CAS 10378-23-1), 55µL of L-cysteine solution (100 mM) (Sigma Aldrich, CAS 52891), and 150µL of papain stock solution.
The purifications steps employed ethanol (EtOH, 96% v/v, AnalaR NORMAPUR®, VWR International Srl, 20823.293), acetic acid (AcOH, 90%, Sigma Aldrich, 64-19-7) or hydrogen peroxide (H_20_2, 30% Sigma-Aldrich, STBK0464).

6.2.3.2. NPs extraction procedure in *D. zernyi*

Before extraction, all laboratory equipment and materials were rinsed with pre-filtered (0.22 µm) Milli-Q water. Additionally, wherever feasible, equipment manufactured from plastic was substituted with glass or metal parts.
Three pools of 100 mg (about 80 larvae) each of IV-instar freeze-dried specimens of *D. zernyi* were utilized to develop the extraction protocol. The specimens were first transferred into a falcon tube containing 50 mL of Milli-Q water and they were vortexed to eliminate any residual detritus from prior sampling. Afterward, the larvae were dried under fume hood and subsequently weighed using an analytical balance (Pioneer, Ohaus Europe, GmbH). For each test, 100 mg of larvae was processed. The samples were first homogenized with 1 mL of cacodylate buffer using a Potter homogenizer (IKA, Eurostar 40, IKA-Werke GmbH & Co. KG). The resulting sample was transferred to a 25 mL glass vial, and the mortar walls were rinsed with an additional cacodylate buffer to ensure complete transfer to the vials. At this point, a known amount of commercial 500 nm PS-NPs were added for the validation of the protocol.
The samples were digested using an enzymatic method, including modifications based on the protocol by Facchetti et al. [33]. In particular, the samples underwent three sequential enzymatic digestion steps, using lipase, chitinase, and papain. First, lipase was dissolved in Milli-Q water (2 mg/mL) and added to the samples, equating to 2 mg of lipase per gram of initial *D. tonsa* biomass. The mixture was incubated in an oven at 50°C for 3 hours with agitation. Subsequently, chitinase (1 mg/mL) was added, corresponding to 1 mg per gram of sample, and the mixture was incubated for 2 hours at 37°C with agitation. Finally, 1 mL of activated papain solution was added, corresponding to 1.5 g of papain for 1 g of initial sample. The papain digestion was allowed to proceed overnight at 50° C.

6.2.3.3 Purification step and recovery rate in *D. zernyi*

After enzymatic digestion, further steps were necessary to enable accurate identification of NPs through spectroscopic techniques. In this perspective, two different approaches to cleaning up the matrix were tested and the efficiency of each was evaluated by measuring the recovery rates through SPES (Single Particle Extinction and Scattering) analysis. The recovery rate was assessed by spiking the larval suspensions after the homogenization step, with PS-NPs of 500 nm. Two theoretical spiking concentrations were tested: 10^9 and 10^7 particles per milliliter. The samples were then subjected to enzymatic digestion and then to the two different purification processes. For each of the tested concentrations, recovery rates were measured in triplicate (100 mg of homogenized larvae for each replicate), and a negative control was included.

First tested approach: filtrations and washing steps in D. zernyi

The first approach involved, after enzymatic digestion, two filtration steps using nylon membranes (10 and 5 µm) followed by purification with ethanol (EtOH) and acetic acid (AcOH). After diluting the digested larval suspensions, the suspension was filtered (10 µm and 5 µm nylon filter, Millipore), aliquoted into 2 mL Eppendorf tubes and evaporated overnight at 60°C. Subsequently, the solid residue was washed with absolute ethanol, 90% acetic acid and Milli-Q water (centrif. 16,000 rcf x 5 min). The final pellets, filters and supernants were analyzed by SPES.

Second tested approach: oxidative digestion in D. zernyi

As an alternative method, the digested spiked larvae suspensions were transferred to 15 mL polypropylene tubes, and a double volume of H_2O_2 (30% v/v) was added with a final concentration of 20% v/v. The samples were then incubated in a heating block at 80°C, shaking (300 rpm) for 2 hours, to complete the digestion process. Samples were gently centrifuged at 2000 rcf for 30 s to remove any residue remaining from the digestion prior to SPES analysis.

6.2.3.4. Nanoplastics identification in biomatrix in D. *zernyi*

Samples with best recovery were analyzed by electron microscopy and Raman spectroscopy. A drop (1 µL) of the suspension was deposited directly onto a hydrophobic surface (PTFE, contact angles, CA, ~ 105°) and dried at room temperature. Subsequently, the same chip was analyzed by SEM (FEI NOVA 600, Dual Beam at 2 kV) and Renishaw (inVia™ confocal Raman microscope, equipped with

532 nm laser) to determine the chemical structure of the particles. The spectra of spiked samples were collected in static mode (mean value 1500 cm^{-1}) using the 50X objective by averaging at least 5 spectra and an accumulation time of 5 s. The identification of the spectra was obtained by comparing the digested nanoparticles with the reference spectrum of PS.
For TEM analysis, 3 µL of suspension were manually deposited on Cu-Formvar carbon coated grid. The samples were left to dry overnight in a desiccator and analysed by TEM (JEOL 2100 at 120 kV).

6.2.4. Detection of NPS in *D. tonsa*

Once the methodology to be used has been established, the present study applied the proposed methodology on three pools of 100 mg each of wild non-spiked specimens of *Diamesa tonsa*, with the aim to investigate the occurrence of NPs in a pristine high-altitude cryosphere environment. Specimens of *D. tonsa* were removed from their storage alcohol suspensions and placed in a falcon tube filled with 50 mL of Milli-Q water. Samples were then vortexed to remove any residual detritus from the sampling activity. Samples of *D. tonsa* were analyzed using the optimized methodology previously described. Raman spectroscopy was applied using the same mapping configuration as in the spiked tests. However, given that these samples were of unknown composition, the scan mode was switched from static to extended (600 - 3200 cm^{-1}) to cover a broader spectral range. While static scans are faster, they are limited in spectral range, whereas extended scans provide a more comprehensive analysis, although they require longer acquisition times. In addition, the default exposure time for the extended mode was set to 10 seconds, which is the minimum required for this range.

6.3. Results and discussion

6.3.1. The adaptation of the protocol to chironomids

The detailed results of recovery rates are reported in the Supplementary Material, Appendix A.
The SPES results demonstrate that the first approach is inefficient, with a total recovery rate of only 30.5% and just 17.2 ± 3.6 % recovery in the final pellet (Figure S6.1, S6.2, S6.3). On the other hand, the second approach, involving two digestive processes, resulted much more efficient. The measured

particle concentrations for the two spike levels were $6.8 \times 10^8 \pm 1.7 \times 10^8$ parts/mL and $5.1 \times 10^6 \pm 7.6 \times 10^5$ parts/mL, corresponding to a recovery of 109 ± 28 % and 82 ± 12 % (Figure S6.4).

To verify that PS-NPs maintained their morphological integrity and original dimensions throughout all extraction steps, they were processed in the same way as the spiked samples, but without the biological matrix. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) analysis showed that NPs were unaffected by enzymatic and oxidative digestion, retaining their size and morphology, while Raman spectroscopy revealed no significant changes in chemical composition (Figure S6.5).

For identification using confocal Raman spectroscopy, an aliquot of the extracted samples was deposited on substrates with varying contact angle (CA): hydrophilic (θ ~90°), hydrophobic (θ ~105°), and super-hydrophobic (θ ~135°). The CA of the substrate is critical in controlling droplet behavior, with higher angle values expected to inhibit diffusion and reduce the droplet footprint. Initial tests using PS500 in ultrapure water were conducted with prominent results. However, spotting the samples with spikes after both digestion protocols did not yield satisfactory results. The specific chemical composition of the larvae matrix prevented the observation of the Marangoni effect, which typically results in the suppression of droplet spreading. In addition, surface analysis with Raman spectroscopy was hampered by residual organic matter that caused signal saturation, making it impossible to obtain clear spectra of polystyrene.

To address this issue, an additional purification step involving ethanol was introduced into the extraction process before the Raman identification step. After gentle centrifugation (2000 rcf, 30 seconds), 400 µL of the supernatant was diluted tenfold with pure EtOH and purified using Amicon centrifugal filters (Amicon Ultra-4®, 100 kDa centrifugal filters, Millipore, Bedford, MA). The samples underwent two rounds of purification with EtOH, followed by two more rounds with Milli-Q water to remove any remaining alcohol.

An aliquot (1 uL) of the purified sample after both digestion steps was sputtered onto the three substrates. As can be seen in Figure S6, this change of substrate caused the particles to concentrate in a smaller area only when the hydrophobic surface was used, concentrating the particles in an area with a diameter of 0.48 ± 0.09 mm. This clearly improves the possibility of mapping and identifying the droplet's Raman spectral fingerprint and reduces analysis time. This effect is likely due to the role of ethanol in altering the chemical properties of the matrix, reducing its wettability on the hydrophobic surface.

Finally, all processed samples were analyzed by spectroscopic technique and, in parallel, by scanning electron microscope. A Renishaw inVia Confocal Raman Microscope equipped with 532 nm laser

excitation and white light imaging tool was used to create a montaged area for mapping. Raman data were collected across the entire sample surface. Spectral maps were generated at specimen points spaced non-uniformly, with a regular interval of 20 µm between points. This approach was chosen to balance the trade-off between analysis duration and the spectral resolution of the maps, optimizing both efficiency and detail in the detection of particles across the sample surface.

After the acquisition, chemical image maps were obtained for each sample. In the case of PS spiked samples, data was analyzed using a direct classical least-squares method incorporating the reference spectra (Figure 6.3). The specific conditions for collecting map data are shown in Table S6.1.

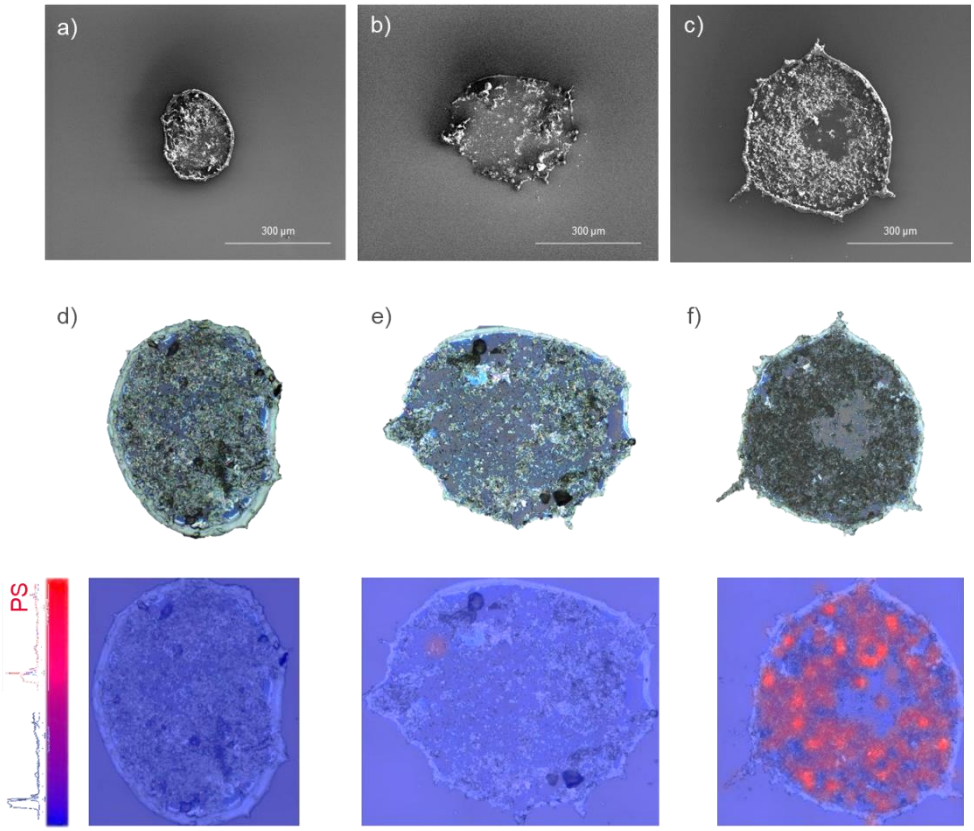

Figure 6.3. Scanning electron micrographs and white light montage with relative mapping with Raman images overlay of control (a, d), spike 10^7 particles/mL (b, e) and spike 10^9 particles/mL (c,f). The red dots in the Raman images (bottom) highlight if the PS is present in the sample.

Raman imaging confirmed the presence of PS-NPs, particularly in samples with higher PS concentrations. Microscopic surface scans revealed prominent aggregation in samples dosed at 10^9 particles/mL, while in samples with lower PS concentrations aggregation was mostly absent (Figure S6.7). Additionally, surface scanning by confocal Raman with at step size 20 µm resolution introduces a probabilistic limitation in detecting specimen nanoparticles or smaller aggregates (Figure S6.8).

6.3.2. Identification of NPs in *Diamesa tonsa* specimens from Amola glacial river

Initially, in non-spiked *D. tonsa* samples, no polymeric particles were detected in any of the environmental samples. However, by reducing the mapping step size from 20 µm to 10 µm and thereby increasing the spatial resolution, a PS signal was successfully detected in one of the larval samples (Figure 6.4).

This adjustment was critical for the identification of smaller or less abundant polymer particles within the samples. Nevertheless, this modification significantly increased the total scan time, necessitating the acquisition of smaller, more focused maps to manage time effectively while maintaining detection sensitivity.

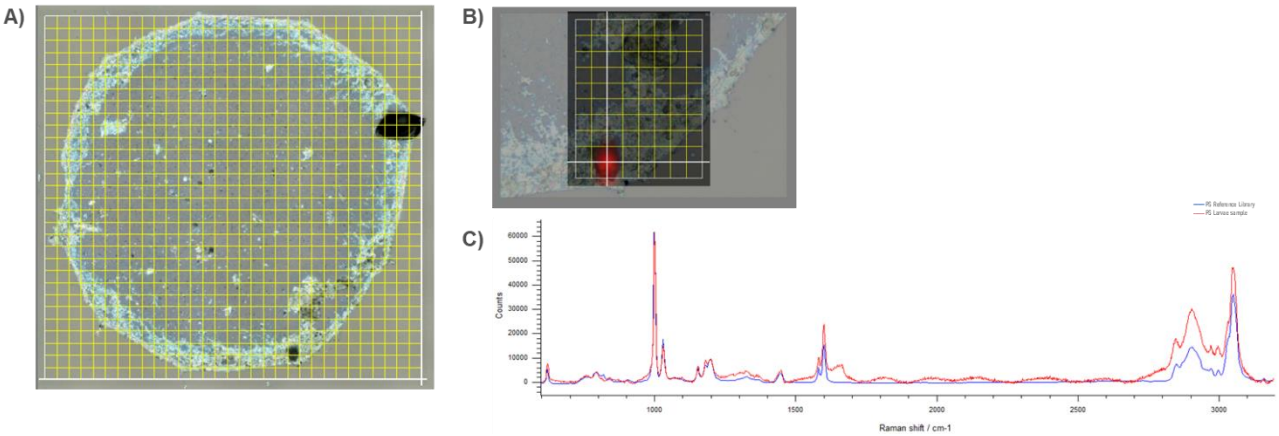

Figure 6.4. White light montage images of the entire area (20 µm step size, A), and of a small region of the same sample (10 µm step size, B). An overlay of the corresponding Raman image, where polystyrene particles (PS) were identified, is shown in panel B. The associated Raman spectrum (C) was acquired in extended mode (600-3200 cm^{-1}) with a 10 s exposure, 10% laser power and 3 accumulations.

6.3.3. What has been done and what is missing for a better identification of NPs in complex matrices

The presented methodology has managed to identify the occurrence of plastic in chironomid wild samples, proving efficient for both freeze-dried and preserved in ethanol samples.

As widely reported in the literature [18,21], enzymatic digestion is confirmed to be non-destructive to extract particles from complex matrices.

The subsequent combination of oxidative digestion with a final clean up with ethanol allowed suitable conditions for the subsequent NPs identification by spectroscopic techniques. The use of hydrophobic

surfaces enabled the concentration of the samples in a small area of the surface, allowing the identification of NP by Raman.

The proposed methodology has been demonstrated to be efficient at concentrations up to 10^7 particles/mL, showing, however, a probabilistic limitation in detecting specimen nanoparticles or smaller aggregates. To achieve precise identification of localized nanostructures at lower concentrations, a finer spatial resolution may be necessary. Moreover, it should be stressed that the methodology has only been tested on PS-NPs. Future applications will consist in testing the entire methodology on a range of NP polymers, to ascertain its efficiency in relation to the polymer type. Furthermore, the proposed methodology was effective on PS-NPs of 500 nm, but, since NPs are present in the environment in a wide range of size classes [34], it is necessary to evaluate its efficiency on different NP sizes. In light of the aforementioned considerations, the development of suitable reference materials for the validation of NP methodology extraction protocols, as recently emphasized by Mitrano et al. [35], result to be essential.

The presented approach provides advantages over other techniques that have been tested in the identification of NPs, such as pyrolysis gas chromatography combined with mass spectrometry (Pyr-GC-MS) [36,37]. In particular, as demonstrated by the reported results, this approach enables the chemical characterization of NPs while simultaneously offering visual information through microscopic analysis, such as shape and size data, that cannot be obtained via Pyr-GC-MS, a destructive technique. However, it is important to note that, unlike Pyr-GC-MS which is the most widely used methodology in the study of NPs [18], the presented methodology only provides qualitative identification of the presence of NPs. In this perspective, future application could involve a combination of spectroscopy and mass spectrometric techniques to gain a quantification of NP environmental concentrations in biota. Data of environmental levels of NPs are essential for a comprehensive assessment of the environmental risk associated with these emerging contaminants.

6.4. Conclusion

In this study, a protocol never applied before on insects was used for the detection of nanoplastic particles in insects from glacial habitats. The success of the procedure was not a foregone conclusion. Our work highlighted that: (i) the confocal Raman spectroscopy is effective in the analysis of nanoparticles in aquatic insects (biomatrices) based mainly on the high recovery rates obtained even at low concentrations; (ii) the protocol works on both freeze-dried and ethanol preserved larvae; (ii) the digestion protocol, combining enzymatic and oxidizing agents, offers a promising approach to

obtain complementary qualitative and quantitative information on nanoparticles using other analytical approaches, such as Pyr-GC-MS.

To the best of our knowledge, this is the first evidence of bioaccumulation of nanoplastics in alpine insects, suggesting chironomids as possible sentinels for plastic pollution in headwaters. The initial hypothesis of detecting a low amount of NP in chironomid larvae collected in glacial streams has been confirmed. However, an integrated approach should be developed, searching for different polymers (not only PS) of smaller size (< 500 nm) both in abiotic (e.g. glacial waters and sediments) and other abiotic (e.g. algae) matrices to assess the real level of contamination of such headwaters. Possible applications will include the quantification by Pyr-GC-MS, a current and robust approach used for the identification of microplastics in complex biological samples.

SUPPLEMENTARY MATERIAL

Appendix A: SPES results details

The SPES technique relies on light scattering to analyze, classify, and count individual particles in fluids by examining their optical properties. As particles pass through the focal area of a light beam, they scatter and transmit light, which is captured by a sensor located in the far field of the laser beam. This setup allows for the characterization of particle populations within the size range of 250 nm to 5 µm, providing details such as refractive index, size distribution, and concentration. This technique is particularly useful in recovery tests for tracking and quantifying target particle populations. SPES is a powerful technique used for characterizing individual particles, particularly in terms of their size and refractive index. Once the size range is selected knowing the refractive index, it can provide quantitative information on the number of particles (Figure S6.1). In order to have a proper concentration of selected particles, the matrix contribution of the negative control was subtracted from the 2D distribution of spiked samples, for both of the tested concentrations. The recovery calculation was based on the experimental spike values at both high and low concentrations, which were $6.2 \times 10^8 \pm 1.4 \times 10^7$ part/mL (mean ± SD) and $6.2 \times 10^6 \pm 1.2 \times 10^5$ part/mL, respectively.
The SPES results demonstrate that the first approach is inefficient, with a total recovery rate of only 30.5% and just 17.2 ± 3.6 % recovery in the final pellet (Figure S6.2). To gain insight into the factors contributing to the main loss of NPs, further experiments were conducted. The impact of acetic acid on the potential degradation of polystyrene nanoparticles (PS-NPs) was specifically investigated. PS suspensions of known concentration (10^7 particles/mL) were exposed to varying concentrations of AcOH (10%, 25%, 50%, and 100% v/v) to evaluate its effect on nanoparticle integrity. The SPES analyses highlighted a low chemical resistance of PS-NPs to the acid, in particular to the higher tested concentrations (Figure S6.3). Therefore, the present approach was found to be ineffective, as it led to unacceptable recovery rates and resulted to be destructive.

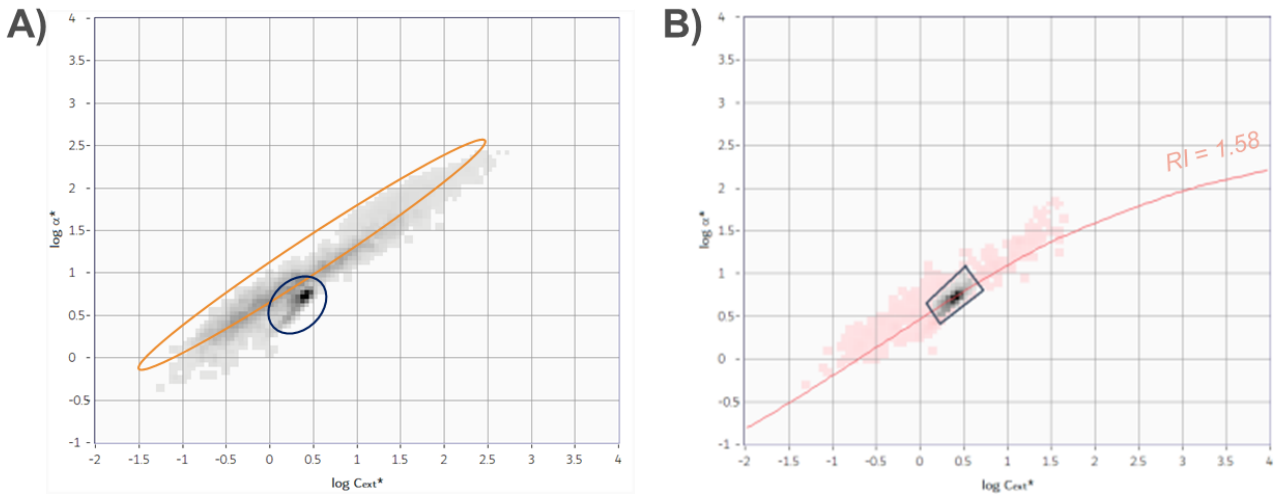

Figure S6.1. SPES raw data of a sample with PS500 spikes (left). In the 2D histogram, the single population of PS500 (RI = 1.58) in the heterogeneous sample can be selected (right).

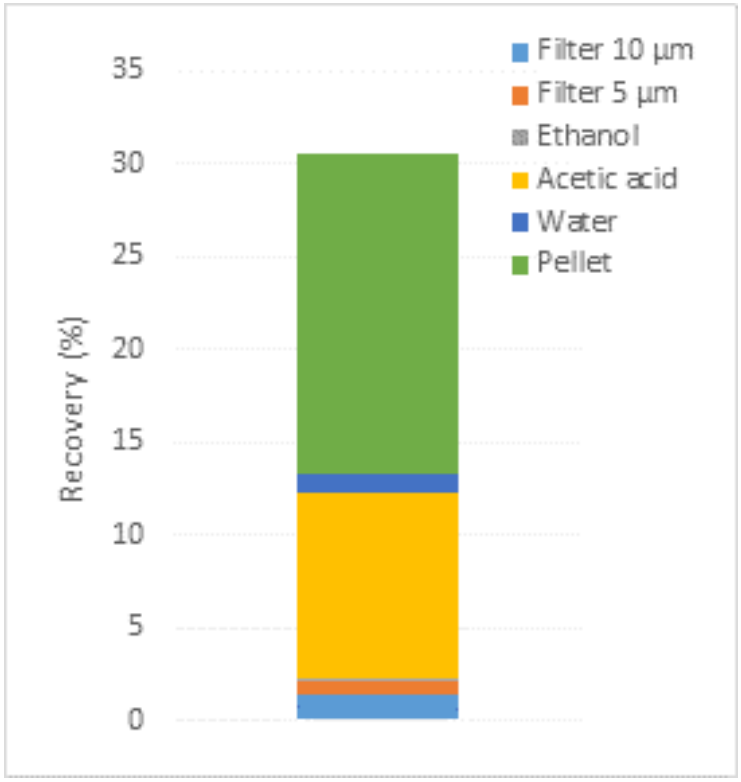

Figure S6.2. Recovery rates of the purification step with filtration and acetic acid purification, obtained through SPES analysis.

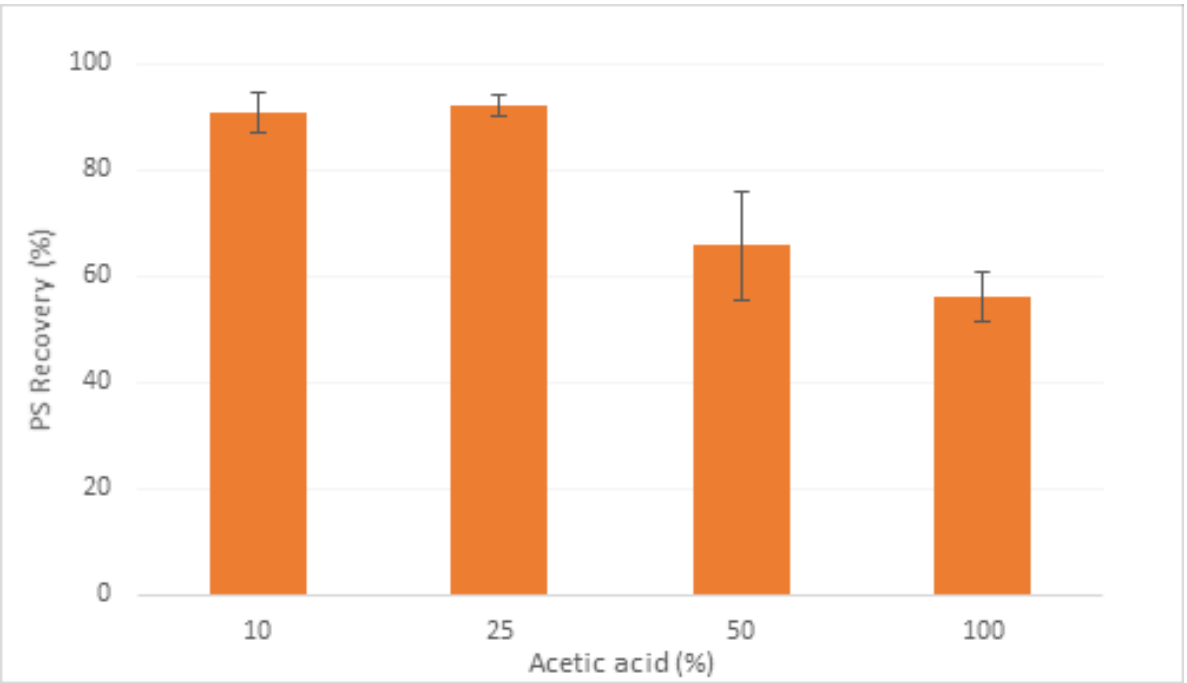

Figure S6.3. Recovery rates obtained through SPES analysis with four different concentrations of acetic acid.

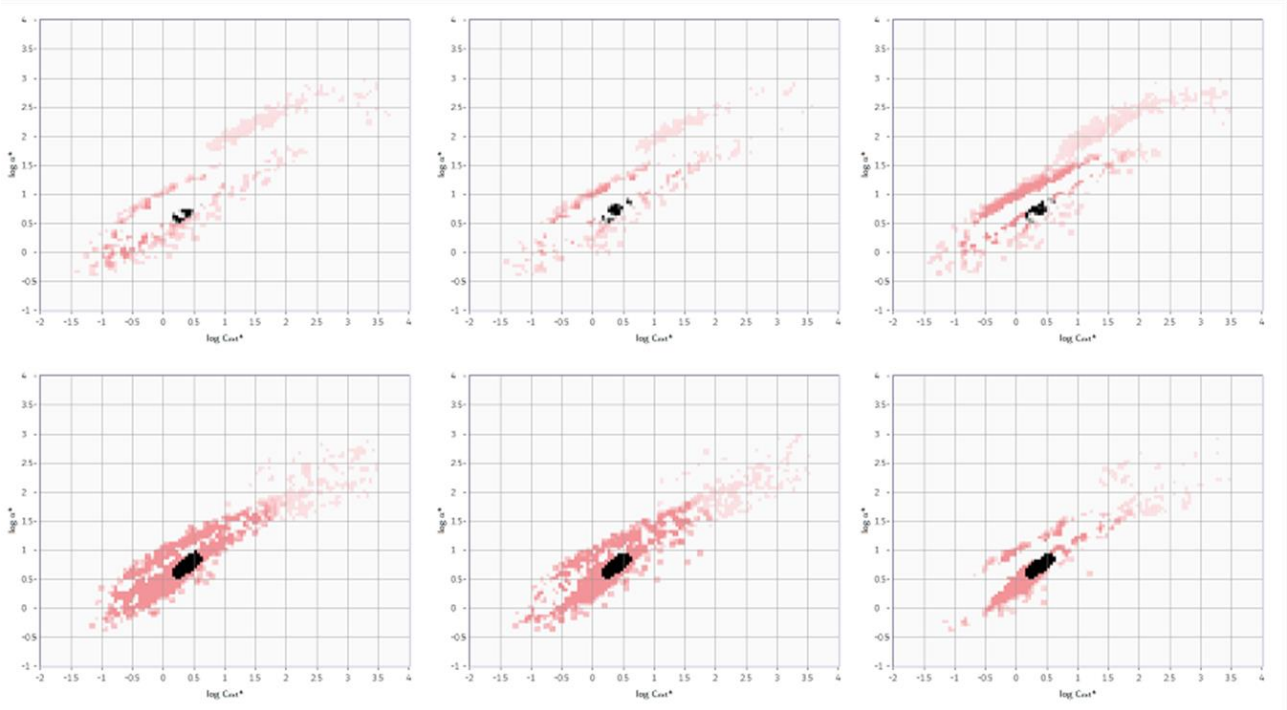

Figure S6.4. SPES raw data illustrating the PS500 spiked sample after subtracting the matrix contribution. The data is presented for triplicate samples spiked with polystyrene at 10^7 particles/mL (first line) and 10^9 particles/mL (second line).

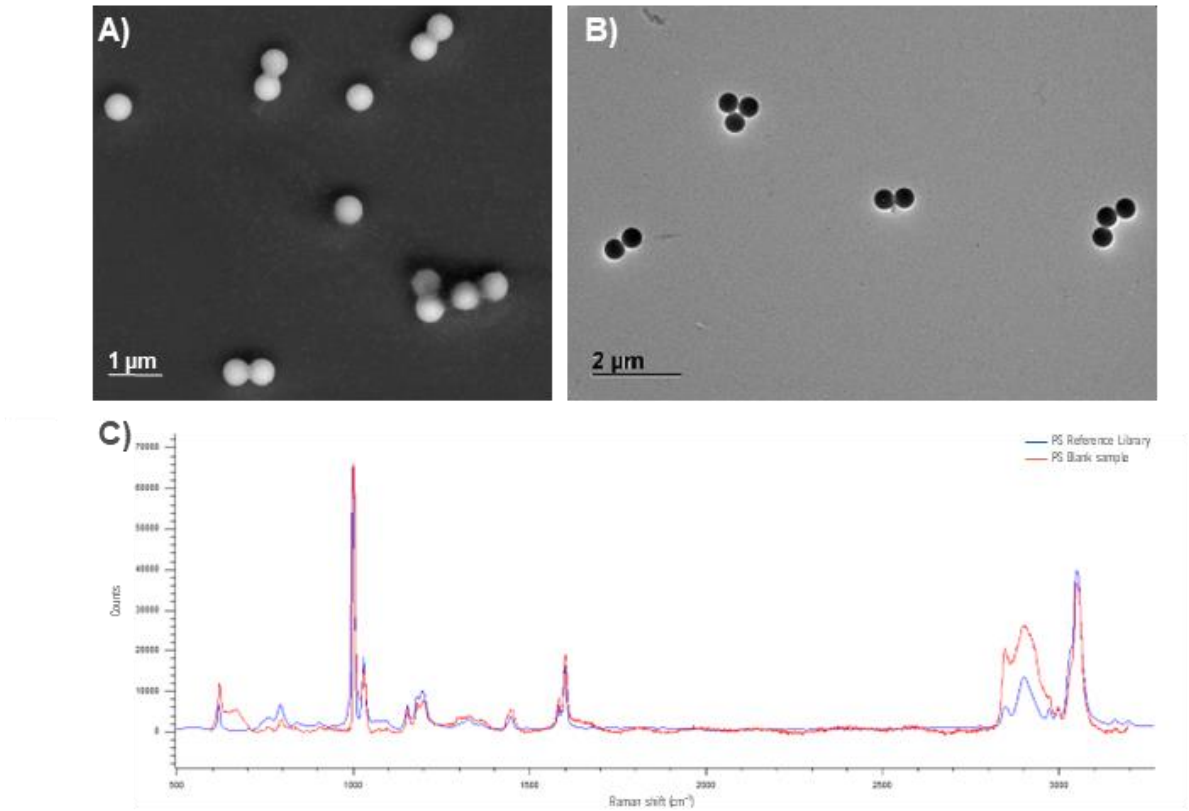

Figure S6.5. Characterization of PS-NPs 500 nm by SEM (A), TEM (B), and RAMAN (C) after digestion steps. Raman spectra: blue line = PS reference spectra, red line = PS after extraction protocol.

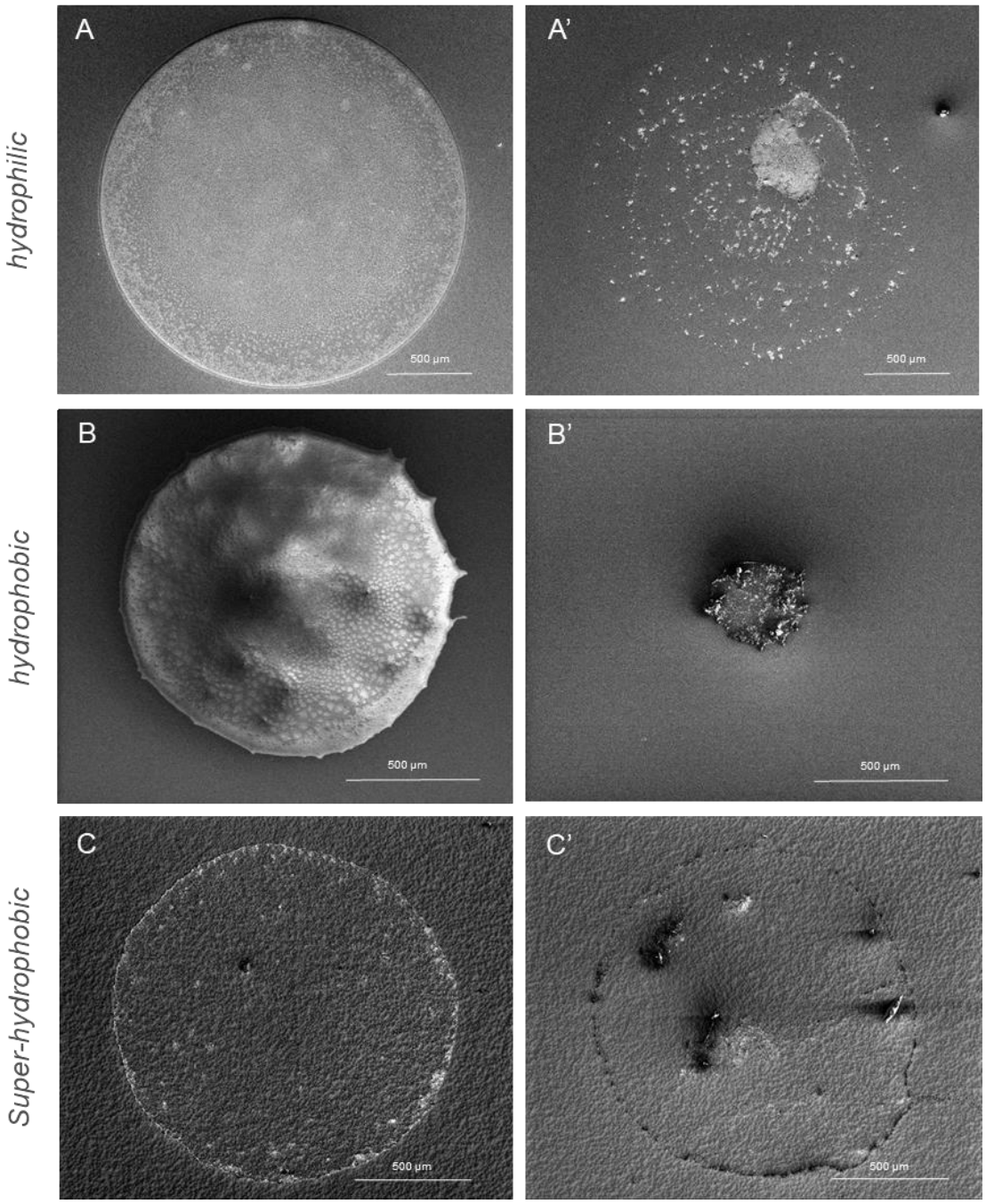

Figure S6.6. Scanning electron micrographs of processed sample (1 µL) of on hydrophilic (panel A), hydrophobic (panel B), and super-hydrophobic surface (panel C) after digestion (A, B, C) and the following purification step (A', B', C'). This comparison illustrates the influence of the hydrophobic properties of the surface and the impact of both processes on the characteristics of the sample and highlights the improvements achieved with purification.

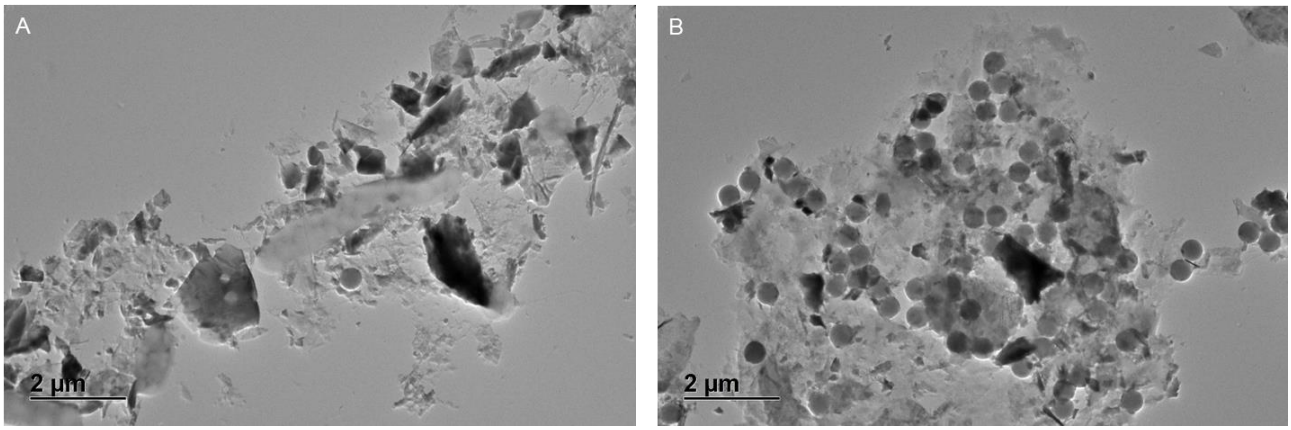

Figure S6.7. Transmission electron micrographs of spiked samples at low (A) and high (B) concentration levels.

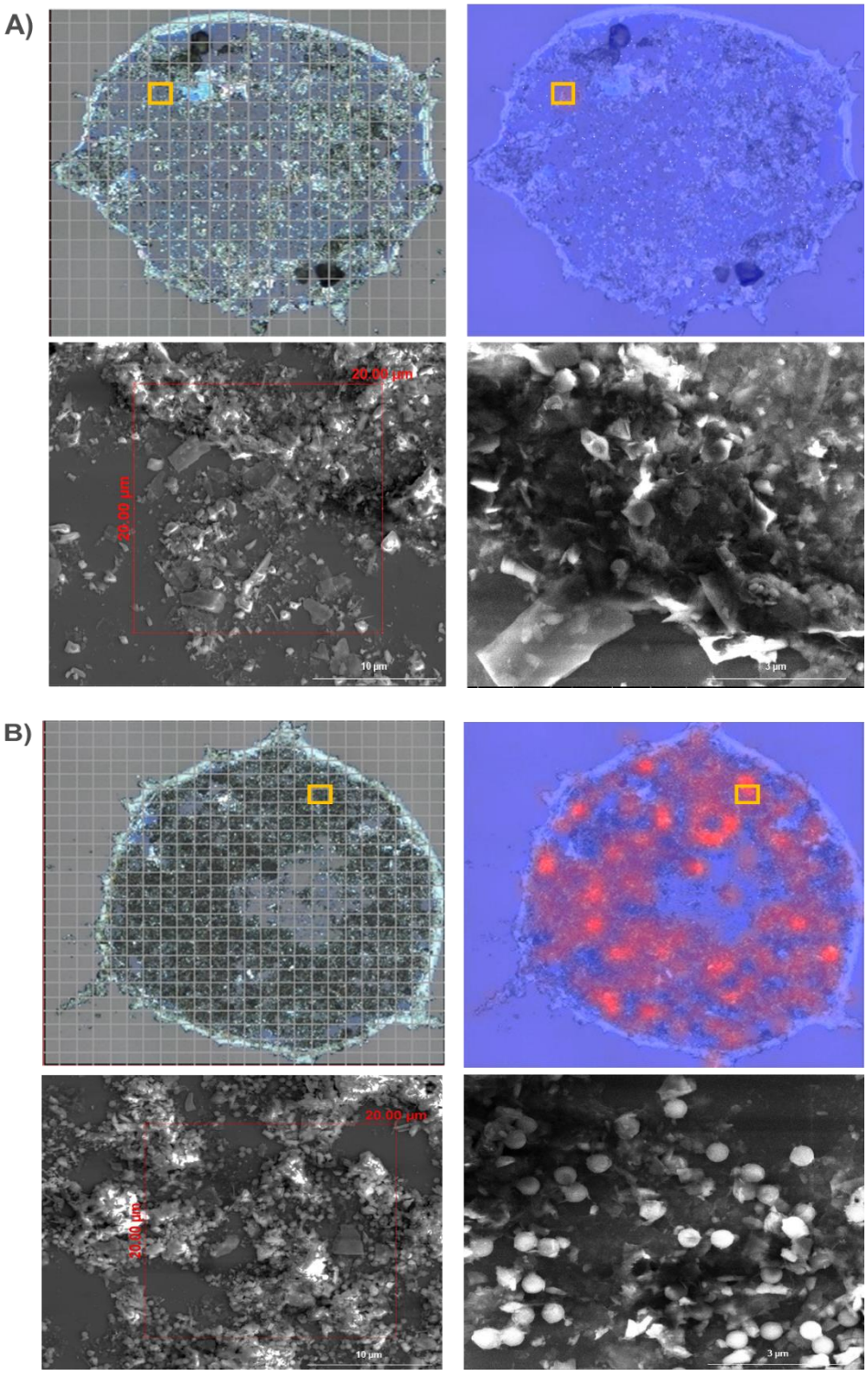

Figure S6.8. Above are the white light montage images of the entire sample area (step size 20 µm) alongside the corresponding Raman image. The yellow square highlights an example of a scanned area (20 x 20 µm) where polystyrene (PS) was detected. In the lower part of the figure, the corresponding scanning electron micrographs of that area confirm the variation in particle number between regions of low (A) and high (B) PS spiked concentration.

Table S6.1. The specific map data collection conditions used for Raman spectroscopy identification in the final samples.

Raman microscope	***Mapping setup***	
	Low spike level	*High spike level*
Wavelength	*532 nm*	
Laser power	*10 %*	
Accumulations	*5*	
Time	*5 sec*	
Mode	*Static / 1500 cm^{-1}*	
Lens	*50X*	
Step size	*20 µm*	
Area	*390 ± 14 µm (X) x 470 ± 174 µm (Y)*	*507 ± 83 µm (X) x 533 ± 31 µm (Y)*
Mapping time	*~ 4 h*	*~ 4.3 h*
N° of spectra (AVE)	*499*	*728*

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Chapter 7

Conclusions

7.1 Synthesis and future recommendations

The issue of plastic pollution represents a defining characteristic of the contemporary era. The extremely slow rate of degradation resulted in the accumulation of vast quantities of plastic debris in all the environmental systems, including remote and pristine areas. The dispersion of plastics in the environment results in the progressive fragmentation of these anthropic materials into microplastics (MPs) and nanoplastics (NPs), leading to an extremely heterogeneous pollution scenario. In environmental matrices, MPs and NPs are in fact present as a multitude of different polymers, exhibiting a wide range of different shapes and sizes. Additionally, plastic debris, depending on their persistence time in the environment, are characterized by different stages of degradation. Furthermore, the huge variety of toxic additives present within the bulk materials influences the overall toxicity of plastic items.

In this complex context, a comprehensive understanding of the level of plastic pollution is still not achieved. The environmental risk assessment of NP (NP-ERA) is still in its infancy. The smaller size of NPs poses a challenge in developing suitable protocols for their extraction from complex matrices. Furthermore, the lack of effective methodologies for their identification and quantification hinders the comprehensive establishment of environmental NP concentrations. Additionally, the absence of environmentally relevant reference materials capable to representing the inherent complexity of NPs hampers to ascertain the effects that these contaminants may induce on organisms. Currently, the effect available data originates from studies conducted with engineered polystyrene (PS) nanospheres, which do not permit the drawing of conclusions regarding the risk posed by the multitude of polymers and shapes present in the environment.

In this perspective, this thesis offered a valuable contribution to the field, by providing useful data that can improve our understanding of the potential effects of NPs on freshwater organisms. Furthermore, it proposes the application of a methodology that can be used for identifying NP contamination in organisms from glacial environments.

The present thesis, in **Chapter 2**, provided a comprehensive and detailed overview of the knowledge gaps that hamper the NP-ERA. In light of this, a tiered framework for NP-ERA, comprising different levels of increasing complexity and ecological realism, was proposed. Although robust risk characterisation data are not currently available, the review highlighted that, based on the currently available evidence, environmental concentrations of PS-NPs could induce unacceptable effects in diverse wild organisms.

Due to the heterogeneity of NP polymers present in the environment, it is imperative to employ approaches for estimating the effects of mixtures with known compositions in order to achieve greater

ecological realism. This entails calculating the risk posed by an individual polymer and then assessing the overall mixture toxicity. Data on the toxicity of the most prevalent NP polymers present in the environment is considered indispensable, as these are the polymers from which the majority of NPs will be originated.

In this context, the **Chapter 3** of this thesis reported the investigation of the effects of polyethylene (PE) and polyvinyl chloride (PVC) engineered nanospheres, representing respectively the first and third most produced plastic polymers. The aim was to provide crucial data to help to establish whether different NP polymers exhibit comparable toxicity to PS, and whether PS can be used as a proxy for all other NP polymers. The impact of these different NP polymers was assessed on the freshwater model organism *Daphnia magna*. A preliminary study was conducted over a short-term exposure of 48-hour to provide an initial toxicity screening. The onset of sublethal effects induced by environmentally relevant NP concentrations was evaluated through a multilevel approach. PE and PVC resulted to be more harmful than PS. Furthermore, the evidence suggested that PE is less toxic than PVC, as it did not induce alterations in swimming behaviour at the individual level.

Consequently, in **Chapter 4**, the effects of PVC-NP were further analysed to better elucidate the role of polymer type in NP toxicity, by evaluating the chronic effects resulting from a prolonged exposure of 21-day to PVC and PS NPs. A chronic exposure, compared to the 48-hour exposure of Chapter 3, represents a more environmentally realistic scenario. The study confirmed the role of the polymer type in the onset of adverse effects on *D. magna*. In particular, the observed delay in reaching sexual maturity induced by PVC-NPs raised concerns regarding the potential for significant threats to populations in real ecosystems.

In Chapters 3 and 4 it was examined the impact of engineered plastic nanospheres. However, given the different shapes of plastics that occur in the environment, it is crucial to consider also the role of form in the onset of toxicity. In this context, despite the evidence indicating that fibres are the most prevalent form of plastics in the environment, there is a current knowledge gap on the effects of plastic nanofibers in aquatica organism.

For this reason, an evaluation of the effects of polypropylene (PP) micro- and nanofibres (MNFs) is reported in **Chapter 5**. The massive use of surgical face masks during COVID-19 pandemic has compounded the challenge of plastic waste, as surgical face masks are made of PP and, once in the environment, tend to release NMFs. The effects of PP-NMFs were evaluated on zebrafish (*Danio rerio*), a freshwater model species. The reported findings (morphological alterations and transcriptomic data suggesting neurodevelopment alterations and an energy stress state) occurred at environmentally relevant concentrations, suggesting the potential for an ecotoxicological risk

associated with PP-MNFs. The findings, therefore, reinforced the necessity to conducts further studies to better understand the role shapes in NP toxicity.

Based on the reported results, the importance of investigate the effect of different NP polymer and different NP shapes has been highlighted, suggesting that the exclusive utilisation of PS nanospheres is insufficient for the comprehensive understanding of the risks to which organisms are subjected in the environment, potentially leading to an underestimation of the risk related to NPs.

After the collection of valuable data for a more comprehensive characterisation of the effects of NPs, the **Chapter 6** present a focus on the detection of NPs in the environment, through the application of a protocol never tested before on insects to detection of NPs in chironomids (Diptera, Chironomidae) from glacial habitats. Chironomids are recognised as valid MP bioindicators of freshwater systems; however, there is currently no information available regarding the presence of NPs in these organisms. Chironomids of the genus Diamesa Meigen, 1935 are the primary and even exclusive insect colonising glacial environments. These are remote systems where NP contamination has been recently discovered. In this context, the presence of NP larvae of two chironomid species (*Diamesa zernyi* and *Diamesa tonsa*) colonising two Italian high-altitude glacier-fed streams was investigated. A recently proposed analytical method developed by the Joint Research Centre (European Commission, Ispra, Italy) was applied and adapted to chironomids. The procedure comprised a combination of enzymatic and oxidative digestion followed by a purification step in ethanol to enable on-chip identification by Raman spectroscopic analysis. The results highlighted the presence of PS NPs in one of the investigated samples: to the best of our knowledge, this is the first evidence of bioaccumulation of NP in alpine insects, suggesting chironomids as possible sentinels for NP pollution in headwaters. The work highlighted that the confocal Raman spectroscopy is effective in the analysis of NPs in aquatic insects. Moreover, the proposed methodology offers a promising approach to obtain complementary qualitative and quantitative information on NPs using other analytical approaches.

In conclusion, the present thesis has provided useful data that can be considered in future research for a better understanding of the environmental contamination of NPs. However, there are still significant knowledge gaps that hinder the completion of the ERA, therefore some recommendations for future research are provided below:

a) need to increase the complexity and ecological realism in NP exposure test

It is strongly suggested that future investigations prioritise a comprehensive evaluation of the toxicological effects resulting from the combined effects of different NP polymers with other pollutants at environmentally relevant concentrations, for prolonged period of exposure, to simulate

and better illustrate the toxicity of NPs in authentic environmental scenarios. In this perspective, the development of NP reference materials is essential and requires further efforts.

b) use of an integrated and innovative approaches to establish the environmental level of NP pollution

Given the considerable complexity of establishing an optimal analytical method for identifying and quantifying NPs in environmental matrices, it is recommended that future research should adopt a more integrated approach, combining existing mass-based methods with spectroscopy techniques, in order to gain a better understanding of NP environmental concentrations in biota. Moreover, in addition to abiotic matrices, it is recommended that greater efforts be made in the selection of key bioindicator species, which could help to determine and monitor the environmental levels of NPs.

APPENDIX A

A.1. Peer reviewed publications

Masseroni A, Rizzi C, Urani C, Villa S. Nanoplastics: Status and Knowledge Gaps in the Finalization of Environmental Risk Assessments. *Toxics*, 2022, 10(5), 270; https://doi.org/10.3390/toxics10050270

Federico L, Masseroni A, Rizzi C, Villa S. Silent Contamination: The State of the Art, Knowledge Gaps, and a Preliminary Risk Assessment of Tire Particles in Urban Parks. *Toxics*, **2023**, 11(5), 445; https://doi.org/10.3390/toxics11050445

Masseroni A, Fossati M, Ponti J, Schirinzi G. Becchi A, Saliu F, Soler V, Collini M, Della Torre C, Villa S. Sublethal effects induced by different plastic nano-sized particles in *Daphnia magna* at environmentally relevant concentrations. *Environmental Pollution*, **2024**, 343, 123107; https://doi.org/10.1016/j.envpol.2023.123107

A.2. Submitted publications

Masseroni A, Federico L, Villa S. Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in *Daphnia magna*. *Under review to Helyon*.

Masseroni A, Ribeiro M, Becchi A, Saliu F, Granadeiro C, Villa S, Urani C, Santos M. Effects of micro and nano fibers derived from surgical face masks in *Danio rerio*. *Submitted*.

A.3. Publications in preparation

Masseroni A, Schirinzi G, Villa S, Pozzi S, Ponti J, Valsesia A, Lencioni V. Can Raman spectroscopy be used to detect nanoplastics in Diamesa larvae (Diptera Chironomidae) from glacial habitats?. In preparation as Short Communication for Environmental Toxicology and Chemistry

Schirinzi G, Masseroni A, Ponti J, Gilliland D, Valsesia A. Analytical approaches for on-chip detection of nanoplastics in complex biomatrices.

A.4. Conferences

[25/05/2022 – 26/05/2022] Online. Incontro Giovani Ricercatori in Ecologia e Scienze dei Sistemi Acquatici.
Online presentation: "Effects of different nanoplastics polymers on *Daphnia magna* at environmentally relevant concentrations".

[13/09/2022 – 15/09/2022] Siena, Italy. XXXI CONGRESSO S.It.E. ADATTAMENTI DEGLI ECOSISTEMI ALLE PRESSIONI DELL'ANTROPOCENE.
Poster presented: "Sub lethal effects induced by different nanoplastic polymers to *Daphnia magna*".

[28/09/2022 – 30/09/2022] Lido di Camaiore (Lucca), Italy.
10 a Edizione delle Giornate di Studio "Ricerca e Applicazione di Metodologie Ecotossicologiche".
Oral presentation: 'Sub-lethal effects induced by different nanoplastic polymers in *Daphnia magna*".

[30/04/2023 – 04/05/2023] Dublin, Ireland. SETAC Dublin - SETAC Europe 33rd Annual Meeting.
Posters presented: "Development of an analytical method for the detection of nanoplastics in biological samples: the case of the chironomid *Diamesa tonsa*" and "Sub-lethal effects induced by different nanoplastic polymers to *Daphnia magna*".

[05/05/2024 – 09/05/2024] Sevilla, Spain. SETAC Sevilla - SETAC Europe 34th Annual Meeting.
Poster presented: "Multilevel biological responses of *Daphnia magna* exposed to nano-size engineered polystyrene and polyvinyl chloride plastics"

[23/09/2024 – 26/09/2024]. Rome, Italy. XXXIII Congresso Nazionale S.I.T.E.
Oral presentation: "Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in *Daphnia magna*".

[07/10/2024 – 08/10/2024] Milan, Italy. SETAC ITALIAN LANGUAGE BRANCH 3rd Workshop.
Oral presentation: "Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in *Daphnia magna*".

A.5. Awards

"Valeria Matranga" award winner with the oral presentation: "Sub-lethal effects induced by different nanoplastic polymers in *Daphnia magna*" at "10 a Edizione delle Giornate di Studio Ricerca e Applicazione di Metodologie Ecotossicologiche" (28/09/2022 – 30/09/2022, Lido di Camaiore, Lucca, Italy)

"Best Young Italian Scientist Award" (3rd place) with the oral presentation: "Ecological fitness impairments induced by chronic exposure to polyvinyl chloride nanospheres in

Daphnia magna" at the "SETAC ITALIAN LANGUAGE BRANCH 3rd Workshop" (07/10/2024 – 08/10/2024, Milan, Italy)