

Toxicity of Microplastics and Nanoplastics to Model Aquatic Organism, *Daphnia magna*

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To God

My maker, all I can say is THANK YOU.

To Tobi

You are my backbone, my crown, I love you.

To Everett and Amabel

You are my inspiration, my strength, my all.

To Dad and Mum, 'Bola, 'Bowale, 'Bayo and 'Joke,

Without you, I wouldn't be here. Thank you for loving me.

To every woman who pursued a PhD while raising a family,

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Abstract

Plastic pollution in the aquatic environment has become a major environmental concern over the past few decades. It is estimated that ~10 million metric tons of plastic waste ends up in the aquatic environment annually, accounting for up to 80% of debris in the natural waters. Some studies even predict that by 2050, there would be more plastic than fish in the ocean. Over time, these plastic debris break down into smaller size particles, i.e., microplastics and nanoplastics, due to various factors including mechanical friction, UV irradiation, etc. Several research studies have investigated the toxicity impacts of these microplastics and nanoplastics on aquatic organisms using a wide array of polymer types, particle shapes, particle sizes, and test concentrations. However, there are still significant knowledge gaps in understanding their impacts to aquatic invertebrates. Hence, the objective of this thesis is to address these gaps to advance our knowledge on the impacts of these particles on aquatic invertebrates.

In the first part of this thesis, existing scientific literature was critically reviewed to understand the status of research in this field and identify the knowledge gaps. The different factors that contribute to observed toxicity were identified. Based on the reviewed literature, it was recognized that there is no clear consensus on how to pre-treat commercially prepared microplastics and nanoplastics before using them for toxicity assessments; and the impacts of the preservatives present in these commercial formulations were not yet properly understood. Also, it was observed that current research studies use different dose metrics for their toxicity assessments. Furthermore, the critical review of literature showed that most studies use spherical microplastics and nanoplastics from these commercial formulations while only limited studies have investigated the impacts of irregularly shaped microplastics and nanoplastics which are a more realistic representation of the

plastic particles present in the natural environment. Hence, the impacts of these irregular-shaped particles, and their associated complexity is still unclear.

To address some of these gaps, the next part of the thesis investigated the impacts of a preservative, sodium azide, that is widely present in the commercial formulations of microplastics and nanoplastics. Acute toxicity experiments of as-purchased and dialyzed nanoplastics were used to understand the role of sodium azide in toxicity assessments. The results show that sodium azide contributes to the observed toxicity of commercial formulations of polystyrene nanoplastics. The results of the study also revealed that 20 nm and 200 nm polystyrene particles do not have any significant impacts on the acute survival of *Daphnia magna*. Further experiments were performed to investigate the sub-lethal impacts of the same particles upon chronic exposure, as well as the role of dose metrics in the test results. The experiments showed that in the absence of preservatives, the tested nanoplastics do not have significant impact on the survival of *Daphnia magna*, but can cause severe impacts on other historic traits, such as growth and reproduction.

Another knowledge gap that was addressed in the final part of this thesis is understanding the potential occurrence of non-spherical plastic particles in the aquatic environment, as well as their toxicity to *Daphnia magna*. In this case, single-use face masks were selected as a potential source of non-spherical plastic particles in the aquatic environment. First, the study investigated the release of microplastic fibres from single use face masks and the fate of the released microplastics in the environment. The results showed that face masks can be another major source of microplastics in the environment, and a significant portion of these microplastics may end up in natural waters. Jar test results estimate that ~4–47 million microplastic fibres released from the face masks can be released into natural waters per day after wastewater treatment in an urban environment (assuming a population of 4300 persons/km²). Finally, a comprehensive toxicity

assessment was conducted to assess the toxicity of these released plastics to *Daphnia magna*. The study found that the face masks release chemicals alongside the particles; hence, the properties and toxicity of these chemicals were also investigated using various chemical analysis techniques. It was observed that the impacts of these particles and chemicals vary based on the type of experiments, and the age of the exposed organisms. In the long term, the particles alone do not have significant impact on the survival of *Daphnia magna* while the chemicals cause significant impact to the survival of the organisms.

Overall, the findings of this thesis improved our understanding of the toxicity of micro- and nanoplastics. The experimental results also advance our knowledge by showing how different factors may impact the observed toxicity, and the importance of taking these factors into consideration when planning toxicity studies or interpreting the results.

Résumé

La pollution plastique du milieu aquatique est devenue une préoccupation environnementale majeure au cours des dernières décennies. On estime qu'environ 10 millions de tonnes métriques de déchets plastiques se retrouvent chaque année dans le milieu aquatique, représentant jusqu'à 80 % des débris dans les eaux naturelles. Certaines études prédisent même que d'ici 2050, il y aurait plus de plastique que de poissons dans l'océan. Au fil du temps, ces débris de plastique se fragmentent en particules de plus petite taille, i.e. en microplastiques et nanoplastiques, et ceci due à leur altération provoquer par les frottements mécaniques, l'irradiation UV, etc. Plusieurs recherches ont étudié la toxicité de ces particules sur les organismes aquatiques à l'aide d'un large éventail de types de polymères, de formes de particules, de tailles de particules et de concentrations de test. Cependant, il existe encore des lacunes importantes dans la compréhension de leurs impacts sur les invertébrés aquatiques. Ainsi, l'objectif de cette thèse a été de combler ce manque de connaissance et ainsi mieux comprendre les impacts de ce type de particules sur les invertébrés aquatiques.

Dans la première partie de cette thèse, la littérature scientifique existante a été examinée de manière critique pour comprendre l'état de la recherche dans ce domaine et identifier de potentiel verrous scientifiques. Les différents facteurs qui contribuent à la toxicité observée ont pu être identifiés. Sur la base de cet état de l'art, aucun consensus clair sur la manière de prétraiter les microplastiques et les nanoplastiques n'a été identifiée avant leur utilisation lors de test toxicologiques. Il est à noter que les impacts des conservateurs présents dans ces formulations commerciales n'étaient pas encore bien compris. De plus, les récentes études utilisent différentes mesures de dose pour leurs évaluations de la toxicité. L'état de l'art a montré que la plupart des études utilisent des

microplastiques et des nanoplastiques sphériques et que seulement un nombre limité de travaux utilisent des particules ayant une forme irrégulière, pourtant plus réaliste des particules de plastique présentes dans l'environnement. Par conséquent, les impacts de ces particules de forme irrégulière et leur complexité associée ne sont toujours pas clairs.

Dans la seconde partie de cette thèse l'impact d'un conservateur, l'azoture de sodium, largement présent dans les microplastiques et de nanoplastiques commerciaux. A cet effet, des nanoplastiques commerciaux ont été utilisés pour comprendre le rôle spécifique l'azide de sodium dans les évaluations de toxicité aiguë. Les résultats montrent que l'azoture de sodium contribue à la toxicité observée des nanoplastiques commerciaux de polystyrène. Les résultats de l'étude ont également révélé que les particules de polystyrène de 20 nm et 200 nm n'ont pas d'impact significatif sur la survie aiguë de *Daphnia magna*. D'autres expériences ont été réalisées pour étudier les impacts sublétaux des mêmes particules lors d'une exposition chronique, ainsi que le rôle des paramètres de dose dans les résultats des tests. Les expériences ont montré qu'en l'absence de conservateurs, les nanoplastiques testés n'ont pas d'impact significatif sur la survie de *Daphnia magna*, mais peuvent avoir de graves impacts sur d'autres caractéristiques, telles que la croissance et la reproduction.

Une autre lacune dans les connaissances qui a été abordée dans la dernière partie de cette thèse est la compréhension de la présence potentielle de particules de plastique non sphériques dans l'environnement aquatique, ainsi que leur toxicité pour *Daphnia magna*. Pour ce faire, les masques faciaux à usage unique ont été ciblés comme source potentielle de particules de plastique non sphériques dans le milieu aquatique. Dans un premier temps, le relargage de microfibres plastiques par ces masques ainsi que leur devenir environnemental ont été étudiés. Les résultats ont montré que les masques faciaux peuvent être une source majeure de microplastiques dans l'environnement,

et qu'une partie importante de ces microplastiques peut se retrouver dans les eaux naturelles. Les résultats indiquent que les masques faciaux libèreraient environ 4 à 47 millions de microfibrilles dans les eaux naturelles par jour et ce même après le traitement de ce type d'eaux usées urbaines (en supposant une population de 4300 personnes/km²). Enfin, l'évaluation complète de la toxicité des ces fibres plastiques a été menée avec les *Daphnia magna*. L'étude a révélé que les masques faciaux libèrent des produits chimiques en plus des particules. Les propriétés et la toxicité de ces produits chimiques ont également été étudiées à l'aide de diverses techniques d'analyse chimique. Il a été observé que les impacts de ces particules et produits chimiques varient en fonction du type d'expériences et de l'âge des organismes exposés. À long terme, les particules seules n'ont pas d'impact significatif sur la survie de *Daphnia magna* alors que les produits chimiques ont un impact significatif sur la survie des organismes.

Dans l'ensemble, les résultats de cette thèse ont amélioré notre compréhension de la toxicité des micro- et nanoplastiques. Les résultats expérimentaux ont également progressé nos connaissances en montrant comment différents facteurs peuvent influencer la toxicité observée, et l'importance de prendre ces facteurs en considération lors de la planification des études de toxicité ou de l'interprétation des résultats.

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List of Abbreviations

ABS	Acrylonitrile butadiene styrene
DLS	Dynamic light scattering
EAA	Ethylene acrylic acid
EPA	Environmental Protection Agency, USA
GIT	Gastro-intestinal tract
HDPE	High density polyethylene
ICP-OES	Inductively coupled plasma - optical emission spectrometry
LDPE	Low-density polyethylene
MHRW	Moderately hard reconstituted water
MPs	Microplastics
NPs	Nanoplastics
OECD	Organization for Economic Co-operation and Development
PDI	Polydispersity index
PE	Polyethylene
PET	Polyethylene terephthalate
PLA	Polylactic acid
PMAA	Polymethacrylic acid

PMMA Polymethyl methacrylate

PP Polypropylene

PPDO Polydioxanone

PS Polystyrene

PVA Polyvinyl alcohol

PVC Polyvinyl chloride

Chapter 1: Introduction

The accumulation of plastic debris in the aquatic environment has become a growing concern. About 10 million tons of plastics end up in the aquatic environment every year.^{1,2} The biological consequences of larger plastic debris (≥ 5 mm) commonly referred to as macroplastics have been extensively studied.^{3,4} However, the fragmentation of these larger debris into smaller pieces through chemical/mechanical stress or weathering⁵ as well as the commercial production of micro- or nano-sized plastics have led to the widespread distribution of microplastics and nanoplastics in freshwater and marine environments worldwide.⁶⁻¹⁰ Because of their small size, microplastics and nanoplastics can be easily ingested by smaller aquatic organisms resulting in uncertain consequences for their health both in the short term and long term.^{11,12}

Aquatic invertebrates such as *Daphnia magna* are vulnerable primary consumers of these particles.¹³ Yet, they are an important component of the ecosystem, and a disruption of their population due to microplastics and nanoplastics pollution may have significant impact on other key species.¹⁴

Furthermore, the particles ingested by these invertebrates may travel up the aquatic food chain, and even to humans through bioaccumulation or biomagnification.^{15,16} Ingestion of microplastics and nanoplastics has been reported for various aquatic invertebrates and the hazards can include obstruction of the digestive tract,¹⁷ impaired reproduction,¹⁸ reduced growth,¹⁹ and death.^{20,21}

Despite the numerous studies investigating the toxicity of these microplastics and nanoplastics to aquatic organisms, there are existing challenges and factors that confound the evaluation of the results of these studies. Most studies assessing the toxicity of these plastics use commercial

spherical shaped particles for their assessment. Some of the commercial formulations contain preservatives and surfactants which interfere with the observed toxicity results.²² Also, the studies use different dose metrics and there is not yet consensus on the dose metrics for conducting toxicity assessments with microplastics and nanoplastics.²³ Moreover, microplastics and nanoplastics in the environment are mostly irregularly shaped, as they are obtained from environmental weathering of larger plastic debris. Yet, only limited studies have investigated the toxicity of irregularly shaped plastics on aquatic invertebrates.²⁰

This work explores the toxicity of two different types of microplastics and nanoplastics on a model aquatic invertebrate, *Daphnia magna* (*D. magna*). *D. magna* is selected as a model organism because of various reasons such as its high sensitivity to environmental contaminants, ease of culturing in the laboratory, and large brood size as well as the availability of standardized guidelines for investigating different types of toxicity such as acute (short-term) and chronic (long-term) tests.²⁴⁻²⁶ The first plastic type is a commercial formulation of spherical polystyrene and nanoplastics, which is the most popular type of microplastics and nanoplastic used for toxicity assessments. Some of the attractive properties in this plastic type for laboratory toxicity studies include its availability in numerous sizes, narrow size distribution, fluorescent options and customizable surface properties e.g., functional groups. The second plastic type investigated in this study are the microplastics and nanoplastics released from single-use face masks. These face masks are an emerging contaminant in the environment following the popular global usage to curb the spread of the COVID-19 pandemic. Moreover, the particles are non-spherical and even release leached chemicals. Hence, investigating the toxicity of these particles may bring us closer to understanding the toxicity of non-spherical, non-polystyrene particles and the contribution of chemicals which are present in these plastic products.

1.1 Research Objectives

1. To study the acute toxicity of spherical polystyrene nanoplastics to the model aquatic invertebrate, *Daphnia magna*, and the role of preservatives present in the commercial formulations in the observed toxicity.
2. To investigate the chronic effects of spherical polystyrene nanoplastics to *Daphnia magna*, by examining different sublethal endpoints including survival, reproduction, growth, and behavioural endpoints, as well as the impact of dose metrics on nanoplastic toxicity.
3. To study the release of plastic particles from a selected commonly used plastic product (i.e., single-use face masks) and the fate of the released microplastics in conventional wastewater treatment process.
4. To assess the toxicity of plastic particles released from single-use face masks to *Daphnia magna*.

1.2 Thesis Organization

This thesis is presented in a manuscript-based format and comprises 5 manuscripts.

Chapter 2 provides an extensive literature review of existing studies that investigated the toxicity of microplastics and nanoplastics to *Daphnia magna*. The review revealed that commercial polystyrene beads are the most popular particle type for toxicity tests, while there are minimal studies on other particle shapes and polymer types.

In Chapter 3, the effect of the presence of sodium azide, a preservative in commercial formulations of polystyrene nanoplastics was investigated. 20 nm and 200 nm polystyrene nanoplastics were dialyzed to remove the sodium azide. The observed acute toxicity was compared with those in which the particles were not dialyzed. The results of this study confirmed that the preservatives present in commercial polystyrene preparations may be a confounding factor to the reported toxicity results.

In Chapter 4, the sublethal effects of dialyzed 20 nm and 200 nm polystyrene nanoplastics were examined. The 21-day chronic study investigated sublethal effects including survival, growth, reproduction, and behavioural changes. The toxicity effects of both particle sizes were compared using both mass concentration and particle count as dose metrics. The results did not find any significant difference in the toxicity observed from both dose metrics, at the tested concentrations. Moreover, there was no significant difference in the observed toxicity from low and high concentrations of the 20 nm particles.

In Chapter 5, face masks were selected as a potential source of microplastics to the aquatic environment. New and used face masks (obtained from the streets) were subjected to minimal mechanical friction and the release of microplastics was examined. The released microplastic fibres were used for a simulated conventional wastewater treatment process to investigate the fate of the released plastics once they are in the environment. X-ray photoelectron spectroscopy (XPS) and Fourier Transform Infrared Spectroscopy (FTIR) techniques were used to confirm the composition of the released microplastics. The results of this study showed that face masks release microplastics, and some of the plastics may end up in the aquatic environment.

In Chapter 6, a simulated weathering method was used to generate microplastics and leached chemicals from single-use face masks under UV irradiation and stirring. Subsequently, the released plastics and chemicals were used for a comprehensive toxicity assessment on *Daphnia magna*. The tests include acute toxicity on three age stages of *Daphnia magna*, as well as 21-day chronic toxicity. The plastic particles were characterized using FTIR, scanning electron microscope (SEM), optical photothermal IR spectroscopy (O-PTIR), and nanoparticle tracking analysis (NTA) techniques while the composition of the leached chemicals was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) and liquid chromatography mass spectrometry (LC-MS). The study found toxicity on the exposed organisms, although the severity of the toxicity varied based between the particles and chemicals and also depended on the age of the exposed organisms. The results suggest that microplastics and nanoplastics, as well as chemicals released from face masks into the aquatic environment may have serious implications on the organisms.

Chapter 7 provides a comprehensive discussion of the overall thesis findings in addressing the thesis objectives.

Chapter 8 provides a brief conclusion of the thesis and provides suggestions for future studies.

1.3 Contribution to Original Knowledge

The original contributions of this thesis towards understanding the toxicity of microplastics and nanoplastics are highlighted below:

Demonstrated the role of the preservatives present in commercial formulations of microplastics and nanoplastics in confounding the observed results of toxicity assessments.

Most of the toxicity assessments on aquatic invertebrates use commercial formulations of microplastics and nanoplastics, some of which contain preservatives. The effect of these preservatives on the observed toxicity in exposed organisms is unclear. This is the first study to investigate the effects of one such preservative (sodium azide) and found that its presence in the microplastic and nanoplastic suspensions introduce considerable additional toxicity to the test organism. This highlights the need for more careful consideration in the use of commercial microplastic and nanoplastic formulations for toxicity assessment studies.

Examined the role of dose metrics on the toxicity of nanoplastics to *Daphnia magna*. To date, there is no consensus on the appropriate dose metric for the toxicity assessment of microplastics and nanoplastics to aquatic invertebrates. Hence, different studies use different dose metrics for toxicity studies and the role of the employed dose metrics on the observed toxicity of microplastics or nanoplastics is unclear. This is the first study to assess the effect of dose metrics on the toxicity of microplastics and nanoplastics. The study tested 50 mg/L of 20 nm and 200 nm nanoplastics, as well as 0.1 mg/L of the 20 nm particles which has comparable particle number as the 50 mg/L 200 nm particles. The results showed no observable differences in the toxicity effects when mass concentration and particle counts were used in the chronic toxicity assessments of these nanoplastics to *Daphnia magna*. These findings set a foundation and call for more studies to understand the appropriate dose metrics for assessing the risks of microplastics and nanoplastics.

Demonstrated the effect of interaction with natural environment on the behaviour of microplastic fibres in wastewater treatment. Numerous studies have investigated the efficiency of wastewater treatment systems for the removal of microplastics and nanoplastics. These studies

are important to better understand the proportion of microplastics and nanoplastics that end up in the natural waters from wastewater effluents. However, most of these studies use pristine or primary microplastics and nanoplastics. This is the first study to use microplastics from masks previously exposed to the environment and demonstrated exposure of the plastics to complex (organic and inorganic) matrices in the environment improved the removal efficiency of the plastics during a simulated conventional wastewater treatment process, compared to pristine microplastics. This provides additional insight on the environmental relevance of using pristine versus non-pristine particles to understand the behaviour of microplastics and nanoplastics in the environment. The findings revealed that microplastics previously exposed to the environment show a higher removal efficiency in wastewater treatment compared to pristine particles.

Investigated the toxicity of the particles and chemicals released from an emerging plastic pollutant. Face masks are an emerging source of plastic pollution in the environment. This study is the first to conduct a comprehensive toxicity assessment of the different components of the face masks, as well as the role of the age of the test organism in the observed toxicity. The findings improve our understanding of the complexity of the toxicity mechanism of microplastics and nanoplastics, and the factors to be considered when interpreting the results of toxicity studies. The results show that the mechanism of toxicity from the face masks is influenced by both the released particles and leached chemicals, and that the observed toxicity by these different components of the face masks vary with the age of the exposed organism.

1.4 Contribution of the Authors

Three of the 5 manuscripts in this thesis have been published in peer-reviewed scientific journals, while the remaining two articles are being prepared for submission. In all the publications, the first author of each manuscript is the primary author of this thesis. The original contributions of the authors are summarized below.

1. “Toxicity of Microplastics and Nanoplastics to *Daphnia magna*: Current Status, Knowledge Gaps and Future Directions”, *Trends in Analytical Chemistry*, accepted.

Authors: Oluwadamilola Pikuda, Eva Roubeau Dumont, Qiqing Chen, Jun-Ray Macairan, Stacey A. Robinson, Dimitrios Berk, Nathalie Tufenkji

Contributions: OP conceptualized the idea, conducted preliminary review, and wrote the initial outline of the manuscript. OP, ERD, QC and JRM wrote the initial manuscript. All authors reviewed and edited the manuscript.

2. “Toxicity Assessments of Micro- and Nanoplastics Can Be Confounded by Preservatives in Commercial Formulations”, *Environmental Science & Technology Letters* (2018), 6(1), 21-25.

Authors: Oluwadamilola Pikuda, Elvis Genbo Xu, Dimitrios Berk, Nathalie Tufenkji

Contributions: OP, DB and NT conceptualized the idea, OP conducted the experiments, analysed the data, and drafted the initial manuscript. All co-authors reviewed and edited the manuscript.

3. “Sub-lethal effects of nanoplastics upon chronic exposure to *Daphnia magna*”, *Journal of Hazardous Materials Advances* (2022), 7, 100136.

Authors: Oluwadamilola Pikuda, Eva Roubeau Dumont, Sara Matthews, Elvis Genbo Xu, Dimitrios Berk, Nathalie Tufenkji

Contributions: OP, EGX, DB and NT conceptualized and designed the experiment. OP, SM and EGX conducted the experiments. OP and ERD conducted the statistical analysis. DB and NT supervised the project. OP wrote the initial draft of the manuscript. All co-authors reviewed and edited the manuscript.

4. “Fate of Microfibres from Single-Use Face Masks: Removal during Wastewater Treatment and Release in Natural Waters”, *Journal of Hazardous Materials* (2022), 438, 129408.

Authors: Oluwadamilola Pikuda, Mathieu Lapointe, Olubukola S. Alimi, Dimitrios Berk, Nathalie Tufenkji

Contributions: OP and NT conceptualized the experiments. OP, ML and OSA conducted the experiments. ML and OSA conducted XPS and FTIR analysis, respectively. DB and NT supervised the project. OP wrote the initial draft of the manuscript. All co-authors reviewed and edited the manuscript.

5. “Toxicity of particles and chemicals released from surgical face masks to the model aquatic organism, *Daphnia magna*”, submitted to *Environmental Science: Nano*.

Authors: Oluwadamilola Pikuda, Laura Hernandez, Dahyun Kyung, Qiqing Chen, Jun-Ray Macairan, Lan Liu, Xiaoyu Gao, Stephane Bayen, Subhasis Ghoshal, Dimitrios Berk, Nathalie Tufenkji

Contributions: OP, DB and NT designed the experiments, with consultation with SB and SG. OP prepared the experimental materials. OP conducted the toxicity experiments, with assistance from DK, and QC. LH characterized the particles using FTIR, O-PTIR, SEM and NTA. JRM imaged the *Daphnia magna* with light sheet microscopy. LL conducted the LC-MS analysis while XG supported the ICP-MS analysis. SB, SG, DB and NT supervised the project. OP wrote the initial draft of the manuscript. All co-authors reviewed and edited the manuscript.

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Chapter 2: Toxicity of Microplastics and Nanoplastics to *Daphnia magna*: Current Status, Knowledge Gaps and Future Directions

Connecting Text

Numerous studies have assessed the toxicity of microplastics and nanoplastics on aquatic invertebrates. However, the complex nature of the plastics, as well as the different test species and test types, cause difficulty in the comparison of results to fully understand the toxicity of microplastics and nanoplastics. In this chapter, a systematic review of the toxicity studies that have been conducted on a specific aquatic invertebrate, *Daphnia magna*, was conducted. The review covers the different types of toxicity tests conducted on *Daphnia magna* including acute toxicity, chronic toxicity, multi-generational tests, and the effects of co-contaminants. The chapter summarizes the findings of this review, identifies key research gaps, and provides recommendations on future research direction. The knowledge gaps identified in this chapter motivated the research objectives for other chapters in this thesis.

This Chapter is a review paper that has been accepted for publication in *Trends in Analytical Chemistry*: Oluwadamilola Pikuda, Eva Roubeau Dumont, Qiqing Chen, Jun-Ray Macairan, Stacey A. Robinson, Dimitrios Berk, Nathalie Tufenkji. “Toxicity of Microplastics and Nanoplastics to *Daphnia magna*: Current Status, Knowledge Gaps and Future Directions”, submitted.

Abstract

We conducted a systematic review of 124 published articles that investigated the toxicity of microplastics and nanoplastics to *Daphnia magna*. This review summarizes studies assessing acute, chronic, and multigenerational impacts, as well as the effects observed via leached chemicals from plastics and the role of plastics as contaminant vectors. Overall, observed toxicity varies across different polymer types, and shapes. One of the most visible findings is that targeted research synthesis of the acute toxicity tests found more toxicity in smaller-sized particles. Most studies use spherical plastics that are commercially available, especially polystyrene, while the use of irregular-shaped and/or secondary plastics is still emerging. Also, there are still various confounding factors that make the comparison of the observed results difficult. Future studies should focus on irregular-shaped particles, and other polymer types, besides polystyrene. More research efforts are needed to understand the impacts of environmental factors and complex matrices.

2.1 Introduction

Since the discovery of plastics in the 1900s¹, their durability and low cost have led to excessive usage and disposal of plastic products resulting in about 400 million metric tons of plastic waste annually.² Only ~9% of the waste is recycled while most of the remaining unrecycled plastics end up in landfills or in the ocean.³ Plastic debris was first observed in the oceans in the 1960s. At that time, they were considered biologically inert. Their effects in the marine environment were merely reported as reduced aesthetic values by the tourism industry, and seen as obstruction hazards for

shipping and energy production processes.⁴ Since then, plastic waste have been found in water bodies and published literature has consistently classified plastics to be the most abundant anthropogenic material in marine bodies.⁵⁻⁹ The task of quantifying plastic debris in the ocean is often complicated and unrealistic. Eriksen *et al.*¹⁰ used an oceanographic model analysis which predicts that at least 5.25 trillion plastic particles weighing over 250,000 tons are floating over the sea. In fact, it is estimated that roughly 10% of produced plastics end up in the ocean^{11,12} and other reports suggest that there may be more plastic than fish in the ocean by 2050¹³.

Marine plastic pollution stems from direct routes (e.g., release from wastewater treatment plants, run off from tourism, recreational and commercial fishing activities, waste from plastic production facilities) or indirect routes (e.g., infiltration and runoff from landfills).¹⁴ Based on their size, plastics debris has been commonly categorized as macroplastics, microplastics and nanoplastics. For the purpose of this review and in agreement with previous reports, we define microplastics as particles greater than 1000 nm and less than 5 mm, and nanoplastics as particles smaller than or equal to ~1000 nm in size.¹⁵⁻¹⁷ The exact quantity of microplastics and nanoplastics in the aquatic environment cannot be ascertained because they are too small and difficult to detect in complex natural environments. However, Gregory and Andrady¹⁸ and Thompson *et al.*^{19,20} are of the opinion that microplastics and nanoplastics have been accumulating in the oceans for at least four decades. Substantial evidence suggests the potential for higher particle concentrations of the microplastics and nanoplastics as the larger debris disintegrates into smaller micrometre and sub-micrometre sized particles.²¹⁻²⁴

Microplastics and nanoplastics are present in different types of water bodies including freshwaters, seawater, estuarine surface water, ocean surface and even marine sanctuaries,²⁵⁻²⁷ and are bioavailable to vertebrate and invertebrate aquatic organisms.²⁸⁻³¹ Also, the observed increase in

both the amount and occurrence of microplastics and nanoplastics in aquatic organisms supports the arguments that there is an increase in the presence of microplastics and nanoplastics in the aquatic environment.^{32,33} Aquatic invertebrates such as water fleas (*Daphnia*) are vulnerable primary consumers of small microplastics and nanoplastics in the aquatic ecosystem.^{34,35} These invertebrates are an important component of the ecosystem, and a disruption of their population due to stressors such as microplastic and nanoplastic pollution may have significant impact on other key species.³⁶⁻³⁸ Furthermore, the particles ingested by these invertebrates may result in bioaccumulation or biomagnification up the aquatic food chain, and even to humans.^{39,40} For example, Yu *et al.*⁴¹ found that zebrafish contain PE microplastics after feeding on water fleas which have been earlier exposed to PE microplastics (1 mg/L). Hence, there is a need for comprehensive knowledge on the potential health impacts of microplastics and nanoplastics to aquatic invertebrates.

2.1.1 *Daphnia magna* as a model organism for microplastic and nanoplastic toxicity studies

Daphnia magna (*D. magna*) is one of the most commonly used organisms to study the toxicity of contaminants to aquatic invertebrates. The first application of *D. magna* in ecotoxicology began in the 1930s through the works of Naumann (1934)⁴² and Anderson (1944)⁴³ who used the organism to assess the toxicity of contaminants found in industrial wastewater. This was closely followed by other similar works to investigate the toxicity of various compounds and contaminants.^{44,45} Since then, *D. magna* has been a popular model organism in examining the toxicological effects of environmental contaminants.⁴⁶⁻⁴⁸ *D. magna* has various desirable advantages including high sensitivity to environmental contaminants, ease of culturing in the

laboratory, parthenogenetic reproduction, short generation time, large brood size, as well as their extensive range of endpoints in toxicology studies.⁴⁹⁻⁵¹ In fact, highly reputable organizations such as the Organization for Economic Co-operation and Development (OECD), Environmental Protection Agency, USA (EPA) and Environment and Climate Change Canada have standardized guidelines for investigating the acute (short-term) and chronic (long-term) toxicity of contaminants on *D. magna*, (e.g. OECD 202,⁴⁶ OECD 211,⁵² EPA-821-R-02-012,⁵³ EPA-821-R-02-013,⁵⁴ EC Biological test method,⁵⁵ etc.). The acute tests (typically range from 48 h to 96 h) examine the mortality or immobilization of the organisms while the chronic tests (typically 21 days) include sub-lethal effects such as survival, growth, and reproduction.

The application of *D. magna* also allows extended examination of physiological or behavioral endpoints to understand contaminant effects at the subcellular, cellular, organ, and ecosystem levels.^{56,57} Examples of emerging indicators used in understanding these sublethal effects include swimming behavior parameters (e.g., swimming speed, swimming time, distance traveled, hopping frequency, vertical migration, and resting time⁵⁸⁻⁶¹), feeding rate, thoracic limb movement, filtration rate, heartbeat rate, and food ingestion rate.⁶²⁻⁶⁴ The transparent body of *D. magna* is another advantage of the organism, which allows to measure different physiological endpoints simultaneously using optical methods such as imaging, and video recording, as well as quantitative measurement of particle uptake using laser particle analysis, especially for studies using fluorescent-labelled microplastics.^{63,65-67} Recent chronic toxicity experiments have also been extended to include multigenerational studies using *D. magna* as the model organism.⁶⁸⁻⁷¹ This characteristic is of particular interest for ecotoxicological studies because it allows the investigation of long-term effects of contaminants on organisms. Notably, it also sheds light on

how they adapt to the contaminants' effects or other environmental stressors over extended periods.

2.1.2 Research objective and methodology

Several studies have investigated the effects of microplastics and nanoplastics on aquatic invertebrates, including *D. magna*. Yet, these existing studies target different types of toxicity tests using different toxicity indicators. Furthermore, the toxicity results are complicated due to the heterogeneity in plastics with researchers examining different polymer types, particle sizes, particle shapes, concentrations, and metrics. In addition, there are other confounding factors such as the presence of preservatives/additives in the tested particles and leaching of chemicals from the particles, which may further impact the toxicity results.⁷² Reviews have been conducted to investigate the toxicity of microplastics and nanoplastics on aquatic invertebrates.⁷³⁻⁷⁶ These reviews, however, combine the toxicity results across multiple species; hence they are limited in the extent of comparison that can be carried out to understand the broad impacts of microplastics and nanoplastics to the tested organisms.

The scope of this study has been limited to focus on *D. magna* due to its widespread use as a model organism for toxicity studies on microplastics and nanoplastics, and to allow for easier comparison of the toxicity data. This paper provides a comprehensive review of research publications that have used *D. magna* as a model organism to examine the acute, chronic, and multigenerational toxicity of microplastics and nanoplastics as well as the impacts of microplastics and nanoplastics as contaminant vectors.

We conducted an electronic search for research papers using the terms (Microplastic OR Nanoplastic) AND (Toxicity OR Exposure OR Effects) AND (*Daphnia magna* OR *D. magna*) on

the following databases: Google Scholar (scholar.google.com), Science Direct (sciencedirect.com), Toxicity of Microplastic Explorer (sccwrp.shinyapps.io/aq_mp_tox_shiny), and PubMed (pubmed.ncbi.nlm.nih.gov). The search strategies identified 110 publications. The reference lists of these 110 papers were consulted to identify any relevant papers that may have been omitted. This increased the total number of papers to 138, after the removal of duplicates.

Next, we established exclusion criteria to ensure that we focused on relevant papers within the scope of the review. Based on a preliminary review of the titles and abstract, we excluded studies that (1) were not written in English language; (2) investigated other *Daphnia* species (other than *D. magna*); (3) investigated other toxicity topics outside the scope of this paper (e.g., phototoxicity, feeding behavior, uptake and depuration, biochemical responses, and gene expression). No exclusion criteria were specified regarding the microplastics and nanoplastics due to the complexities in the properties of the tested plastics (e.g., polymer type, particle size, and concentration). However, given that *D. magna* are generally small sized organisms, none of the research papers found in the search results used particles larger than 5 mm in size. Also, no publication date criteria were set, however, the dates of the publications produced by the search were between 2011 and 2023. The search deadline was February 2023, and the reviewed papers included all published papers, including both indexed and non-indexed papers. The screening and selection process was conducted in accordance with the guidelines of Preferred Reporting Items for Systematic reviews and Meta Analyses (PRISMA) and the PRISMA flow diagram is shown in the Supporting Information (**Figure S2.1**).

After applying the exclusion criteria stated above, the total number of research papers was reduced to 124 papers. The full text of these 124 papers were read completely and summarized. No authors were contacted for additional information on their study as part of this review. Based on the

summaries, the papers were categorized according to the type of toxicity investigated. In certain cases, single papers covered more than one type of toxicity, and were added to multiple categories. We reviewed the papers assigned to the different categories, summarized the results, and derived conclusions where possible. Additional exclusion criteria were applied during a targeted research synthesis based on the types of toxicity being investigated and are discussed under the toxicity result sections, and the reasons for their exclusion were provided in the SI (where applicable). Finally, we derived conclusions, identified gaps, and proposed future directions towards understanding the impact of microplastics and nanoplastics to aquatic invertebrates.

2.2 Microplastics and Nanoplastics used in Toxicity Studies with *D. magna*

The physical and chemical properties of the particles used in investigating the impact of microplastics and nanoplastics to *D. magna* are very diverse. The shapes range from spherical beads to fibres, irregular shapes (fragments), films, and pellets, while the polymer types include polystyrene (PS), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polymethyl methacrylate (PMMA), ethylene acrylic acid (EAA), polyvinyl alcohol (PVA), polymethacrylic acid (PMAA), acrylonitrile butadiene styrene (ABS), polylactic acid (PLA), polydioxanone (PPDO), epoxy, rubber and Bakelite. For the purpose of this review, microplastics and nanoplastics are broadly categorized as **spherical** micro- and nanoplastics and **non-spherical** micro- and nanoplastics.

2.2.1 Spherical micro- and nanoplastics

Spherical micro- and nanoplastics are the most common type of microplastics and nanoplastics used for toxicity studies with *D. magna*, accounting for almost 70% of the studies included in this

review (**Figure 2.1**). They are obtained as dry powder or suspensions from different sources including commercial vendors and laboratory synthesis. In some cases, the suspensions were in deionized water or included additional preservatives or surfactants such as Tween 20 and sodium azide. While some studies washed the particles using centrifugation or dialysis to remove the added chemicals,⁷⁷⁻⁷⁹ others simply used the particles as purchased or did not provide any information about whether the particles were washed or not.⁸⁰⁻⁸³

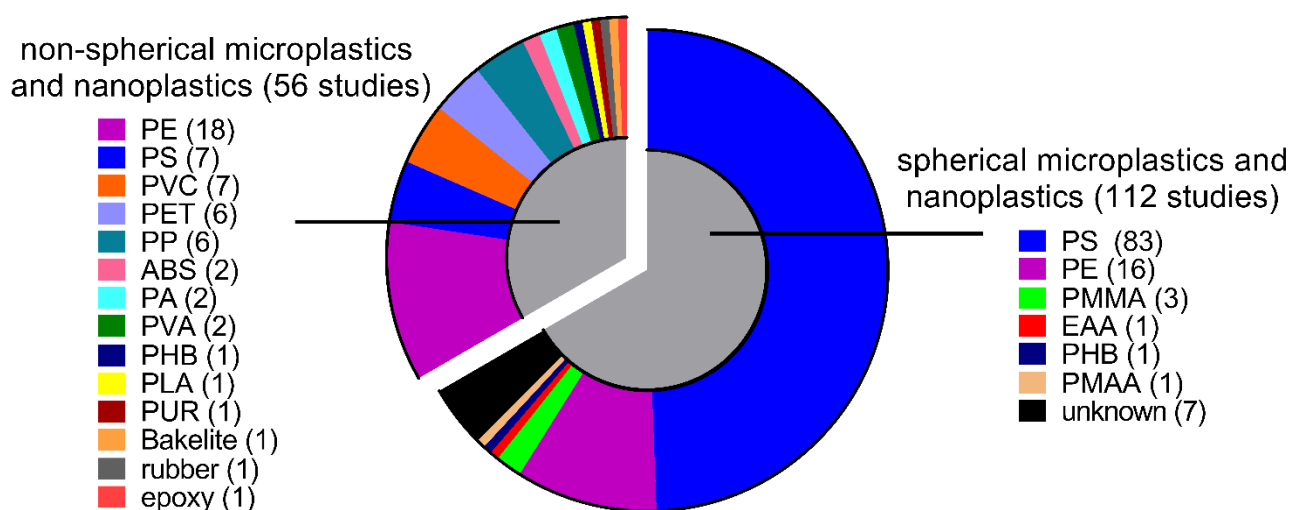


Figure 2.1. Summary of the type of plastic and polymers used in toxicity studies based on 168 studies reported in 124 papers (note that some papers investigated multiple plastic polymers and each polymer type was considered as a separate study, hence, the total number of studies = 168). Studies using different sizes, preparation methods, or test concentrations of the same polymers were counted as 1.

As shown in **Figures 2.1 and 2.2**, commercial PS spherical micro- and nanoplastics are the most popular type of spherical micro- and nanoplastics used in the toxicity studies. Other common types of spherical micro- and nanoplastics used in *D. magna* toxicity studies include PE and PMMA. However, there are fewer tests using these polymer types compared to PS. This is not surprising as PS is one of the most widely used plastic type due to its strength, stability, inexpensive cost, as well as high detection rate in natural waters.⁸⁴ Also, the commercial formulations of PS microplastics and nanoplastics are one of the only types of commercial particles that provide options of surface modification through the addition of functional groups (e.g., carboxyl, sulfate, amino). This is particularly important to understand the role of different functional groups in toxicity as these functional groups may be introduced to the surfaces of plastic particles upon exposure to environmental conditions such as UV irradiation and biological degradation.²² Hence, the use of functionalized particles can contribute to better understand the role of functional groups in the mechanism for toxicity. Another desirable property of the PS particles is the fluorescent-labelling option which allows visualization and tracking of particle uptake by the exposed organisms. Also, PS spherical micro- and nanoplastics are available in uniform sizes compared to particles of other polymer types. Hence, it is a convenient polymer type to compare the impacts of different sizes of particles although it may be argued that the uniform sizes of these particles do not mimic the shapes or sizes of particles found in the environment.

Overall, the tested sizes of spherical micro- and nanoplastics exposed to *D. magna* ranged from 20 nm to 150 μm and the concentrations ranged from 0.01 mg/L to 5000 mg/L. The most common test conditions include PS (20 nm - 150 μm ; 0.01 mg/L - 1000 mg/L), PE (1 μm - 100 μm , 100 mg/L - 5000 mg/L), and PMMA (0.1 μm , 0.01 mg/L - 1000 mg/L). In our review, we did not find any study that has investigated the toxicity impacts of PP or PVC spherical microplastics and

nanoplastics on *D. magna*. A summary of the polymer types, particle sizes and tested concentrations of the spherical micro- and nanoplastics that have been used in toxicity studies with *D. magna* is reported in **Figure 2.2**. The unit of concentrations used in **Figure 2.2** were reported as mg/L. Studies whose concentrations were reported in other units were converted to mg/L based on the calculation method described by Leusch *et al.*⁸⁵ and using the appropriate polymer densities, i.e., PS (1.055 g/cm³)⁸⁶, PE (0.93 g/cm³)⁸⁷, EAA (0.92 g/cm³)⁸⁸, and unknown polymer (1.30 g/cm³)^{89,90}.

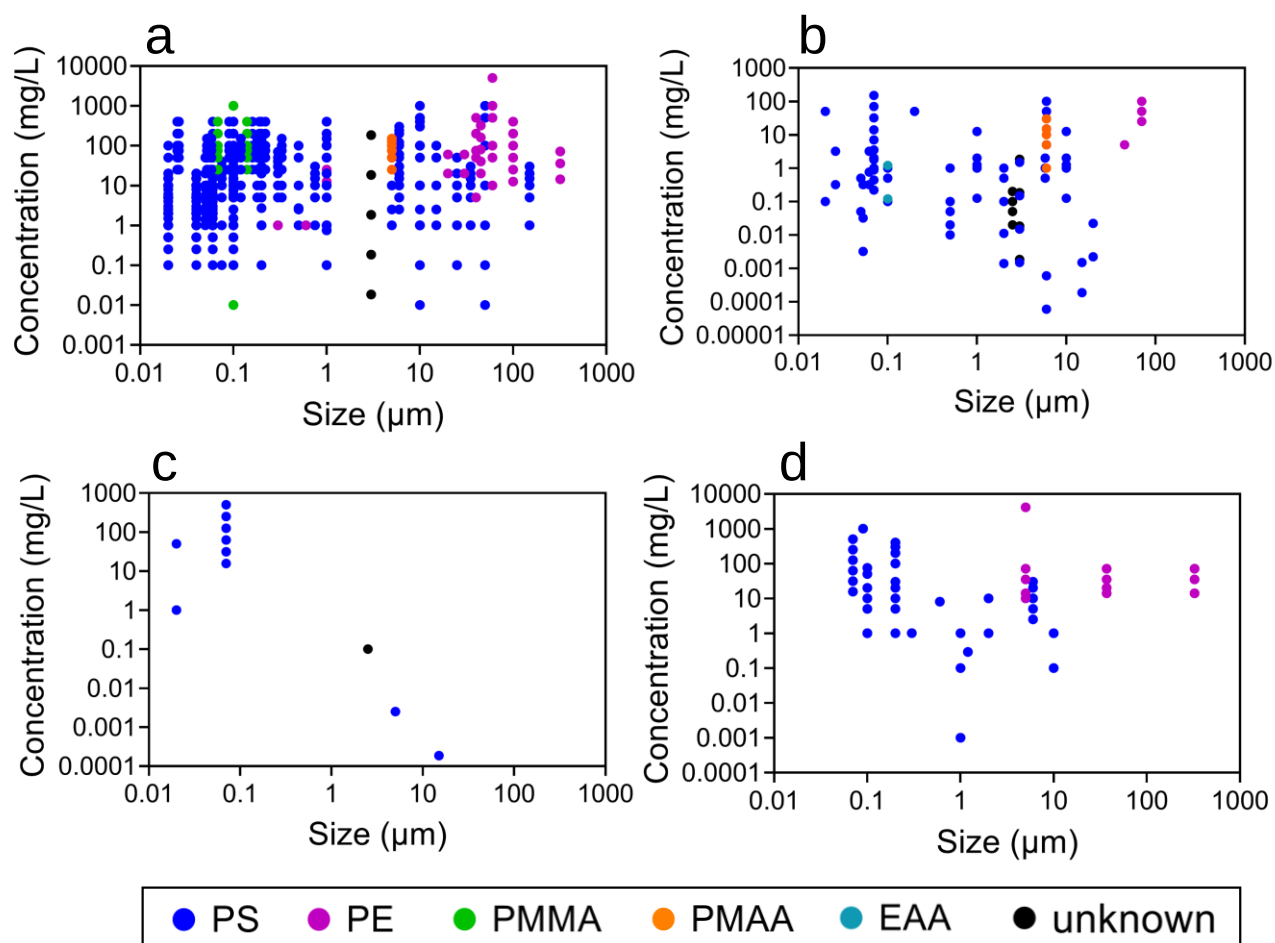


Figure 2.2. Polymer type, size (diameter), and concentration of spherical microplastics and nanoplastics used in (a) acute toxicity tests (n = 605); (b) chronic toxicity tests (n = 91); (c) multigenerational tests (n = 11); and (d) co-contaminant tests (n = 54). Each dot represents a test conducted on one particle type of a given size and concentration. The colour of the dots represents the polymer type. (Data provided by studies using mass concentration were used as is. For studies whose data were presented as particle number per volume, necessary conversions were carried out to derive the equivalent mass concentration).

2.2.2 Non-spherical microplastics and nanoplastics

Non-spherical micro- and nanoplastics used in the reviewed studies include fibres and fragments obtained directly from commercial vendors or by grinding larger particles. To mimic environmental weathering, some researchers exposed the spherical microplastics and nanoplastics to weathering conditions such as UV irradiation and stirring^{91,92} while others obtained microbeads from the filtration of personal cosmetic products such as facial scrubs.^{93,94} The rationale for the toxicity assays using such particles was either to assess (1) an increase of biological interaction and toxicity for weathered particles due to surface changes, or (2) an increased toxicity for non-spherical particles due to mechanical damages. The resulting particles obtained from these weathering processes or cosmetic products will be considered as non-spherical because, as shown in **Figures S2.2** and **S2.3**, the particle images from their characterization show that they appear as irregularly shaped fragments, not spheres.

These types of particles account for ~30% of the reviewed studies as shown in **Figure 2.1** and are mainly PE, PS, or PVC polymers. They typically are polydisperse in size within a given toxicity

test, and therefore, are not suitable for investigating the impacts of specific micro- or nanoparticle sizes. However, plastic fragments mimic the microplastics and nanoplastics generated from mechanical abrasion or UV-irradiation of larger debris in the environment, or waste particles released from plastic production factories.²⁴ For example, PE is commonly used for different types of plastic products and accounts for more than 30% of total plastic production annually.⁹⁵ Hence, testing the toxicity of irregular PE microplastics and nanoplastics is necessary for the assessment of this ubiquitous polymer type. Likewise, fibres are important to consider because they allow the assessment of the toxicity of fibres released from washing clothes and not removed by wastewater treatment.^{23,96} Overall, the most common types and concentrations of the non-spherical micro- and nanoplastics used for toxicity tests on *D. magna* include PS (0.1 mg/L - 50 mg/L), PE (10 mg/L - 100 mg/L), PET (12.5 mg/L - 100 mg/L), and PP (1 mg/L - 100 mg/L). Although some studies have used microplastics obtained from the natural environment⁹⁷ or exposed organisms to the natural marine environment,⁹⁸ no such studies have been conducted on *D. magna*.

2.3 Toxicity of Microplastics and Nanoplastics to *D. magna*

2.3.1 Acute toxicity

Acute toxicity is one of the most common methods of assessing the toxicity of plastics to *D. magna*. The experiments are typically conducted on neonates (≤ 24 h) using standardized laboratory protocols such as the OECD 202 with durations ranging from 48 h to 96 h. A few studies have modified the acute tests by conducting them on older organisms,⁹⁹⁻¹⁰¹ in the presence of food,¹⁰² or reducing the test duration.¹⁰³⁻¹⁰⁵ While the most common endpoints are mortality or immobility, some studies also explored other endpoints such as swimming behavior⁷⁷, food ingestion rate¹⁰⁶, and particle uptake¹⁰⁷.

We carried out a targeted research synthesis using the available data on the acute toxicity tests that have been conducted on *D. magna* using microplastics and nanoplastics. To ensure that similar test conditions were compared, the targeted research synthesis included results of studies that aligned with the minimum OECD 202 requirements for immobilization tests on *D. magna*. This includes tests with a minimum of 20 animals per condition and a valid control result (i.e., maximum of 10% immobility). Also, the targeted research synthesis only included studies which involved neonates (≤ 24 h) and were not fed during the duration of the tests. For studies extended to 72 h or 96 h, only the results at the end of 48 h were considered. In studies that included additional objectives such as co-exposure with other compounds, only the results of the acute toxicity of plastic particles alone were selected and used in the analysis provided in this section. The nominal size of the particles was used if it is provided for the study. Otherwise, the size obtained by the authors from their characterization tests were used. In cases where there is a size range of the particles, an average size was selected and used. When the same study investigated multiple polymer types, sizes and/or concentrations, each of these conditions were treated as separate tests in the analysis. Most studies reported the results of acute toxicity tests in terms of immobilization or mortality. In instances where the authors have reported both mortality and immobility, the latter findings were used. However, when no information on immobility was provided, the mortality results were considered. The selected studies including the polymer types, particle size, and tested concentration are shown in **Table 2.1**. This table also indicates whether the studies have used some washing methods such as dialysis or centrifugation to remove preservatives/surfactants from the plastic suspensions and labelled “unknown” if the information was not provided. A summary table for the excluded studies and the reason for the exclusion are provided in the Supporting Information (**Table S2.1**).

Table 2.1. Studies investigating the acute toxicity of microplastics and nanoplastics to *D. magna* (included in the targeted research synthesis). -COOH and -NH₂ means carboxylated and aminated functional groups, respectively. (f) means fluorescently labelled. Where applicable, circled numbers were used to distinguish particles that possess different properties within the same study.

Type of plastic	Shape	Sizes	Concentration	Source of plastic	Was the plastic washed?	Refs.
PS	spheres	5.8 µm	5, 10, 20, 50, 100, and 200 mg/L	BaseLine ChromTech Research Center, China	unknown	80
PS-COOH; PS-NH ₂	spheres	1 µm	0.1, 0.75, 1, 10, 20, and 50 mg/L	Nanjing Xfnano Materials Tech Co., China	unknown	108
PS-NH ₂	spheres	~100 nm	1, 10, 50, 100, 200, and 400 mg/L	Sigma Aldrich, US	yes (ultra-centrifugation)	79
①PS; ②PS-COOH; ③PS-n-NH ₂ ; ④PS-p-NH ₂	spheres	① 100 nm; ② 300 nm; ③ 50-100 nm; ④ 110 nm	① 1, 5, 10, 20, 50, and 75 mg/L; ② 5, 10, 20, 30, 40, 50, and 70 mg/L); ③ 0.1, 1, 5, 10, 15, 20, 30, and 40 mg/L; ④ 0 to 100 mg/L	①②③ Aladdin, China; ④ Thermo Scientific, China	unknown	109
PS	spheres	① 50 nm; ② 500 nm; ③ 5 µm; ④ 10 µm; ⑤ 15 µm	① 1, 2.5, 5, 10, 20, and 50 mg/L; ②③④⑤ 1, 2.5, 10, 50, and 100 mg/L	BaseLine ChromTech Research Center, China	unknown	110
PS (f)	spheres	6 µm	5, 15, 30, 60, 120, 240, and 300 mg/L	Polysciences, Germany	unknown	111
PS	spheres	10 µm and 50 µm	0.01, 0.1, 1, 10, 100, 500, and 1000 mg/L	BaseLine Chromtech Research Centre, China	unknown	112
PE	spheres	1-4 µm and 90-106 µm	12.5, 25, 50, 100, 200, and 400 mg/L	Cospheric, USA	clean, (vendor's declaration)	113
PS	spheres	① 1 µm; ②10 µm	① 1, 5, 10, 50, 100, and 150 mg/L; ② 1, 10, 100, 300, 400, and 500 mg/L	Tianjin Goose Technology Co., China	clean, (vendor's declaration)	114
PS-NH ₂	spheres	100 nm and 1 µm	1, 10, 50, 100, 200, and 400 mg/L	Sigma-Aldrich, USA	yes (ultra-centrifugation)	115
PS; PS-COOH	spheres	200 nm	1, 5, 10, 20, and 30 mg/L	Bangs Laboratories Inc., USA	unknown	116
PS	spheres	100 nm	1, 5, 10, 20, 50, and 75 mg/L	Aladdin, China	unknown	117

PS-NH ₂	spheres	① 20 nm; ② 40 nm; ③ 60nm; ④100 nm	①②③ 0.5, 1, 2, 5, 10, and 15 mg/L; ④ 1, 5, 10, 15, 20, and 30 mg/L	Thermofisher Scientific, USA	clean, (vendor's declaration)	118
PET	fibres	length: 60-1400 µm; width: 31-528 µm; diameter: 2-21.5 µm	12.5, 25, 50, and 100 mg/L	Milling of a PET fabric	yes (washed in deionized water)	107
PP	fibres	diameter: 21.2 ± 1.5 µm (inner layer), 22.1 ± 1.6 µm (outer layer), and 4.3 ± 2.2 µm middle layer). Length not provided	1, 10, and 100 mg/L	Cryo-milling of medical single-use face masks	unknown	119
PS-NH ₂	spheres	50 nm, 200 nm, and 500 nm	2.7 mg/L	Bangs Laboratories Inc., USA	yes (dialysis)	120
PET	fibres	diameter: 14 ± 3 µm; length: 366 ± 275 µm	20, 100, and 500 mg/L	Milling continuous PET filament (Goodfellow)	unknown	121
PE	fragments	1A, 1B, 1C, 1D, 2, 3 and 4 are ~200 µm, ~100 µm, ~50 µm, ~250 µm, ~250 µm, ~150 µm, and ~20 µm respectively.	100 mg/L	2 facial cleanser products, 1 plastic bag and 1 textile fleece	yes (washed in deionized water)	93
PE	spheres	40-48 µm	20, 40, 80, 160, and 320 mg/L	Sigma Aldrich, USA	unknown	83
PE	① spheres ② fragments	① 10-106 µm; ② 10-75 µm	10, 50, 100, 500, 1000, and 5000 mg/L	① Cospheric, USA; ② a plastic recycling company, Denmark	unknown	106
PS-COOH (f)	spheres	20 nm and 200 nm	0.1, 1, 5, 10, 20, 50, and 100 mg/L	ThermoFisher	yes (dialysis)	77
PE	fragments	180.5 ± 118.7 µm	10 and 100 mg/L	Commercial body scrub	yes (washed in deionized water)	122
PP; PE; PVC; PVC/PE	fragments	10-100 µm	50 mg/L	Grinding bulk plastic pieces	yes (washed in ethanol)	123
PS (aged)	spheres	0-1.5 µm; 10-60 µm; and 60-230 µm	1, 5, 10, 15, 20, and 30 mg/L	Guanbu Electromechanical Technology Co., Ltd., China, (aged under natural light for 180 days)	unknown	92
① PS-COOH; ②PS-NH ₂	spheres	200 nm	① 10, 50, 100, 125, and 150 mg/L; ② 10, 25, 50, 75, and 100 mg/L	Invitrogen (Thermofisher Scientific), USA	clean, (vendor's declaration)	124

pristine PS; aged PS	spheres	0.1-50 μm	0.1, 1, 5, 10, 20, and 50 mg/L	Dongguan Huachuang Plastic Chemical Co., Ltd, China. Aged samples were obtained through continuous stirring and UV light for 6 months.	unknown	91
PE	fragments	140.6 $\pm$ 80.0 μm	100 mg/L	facial scrub product	yes (washed in deionized water)	94
PS (f)	spheres	51 nm	2, 4, 6, 8, and 10 mg/L	Bangs Laboratories Inc., USA	unknown	81
PMMA (f)	spheres	86-125 nm	0.01, 0.1, 1, and 1000 mg/L	Synthesized in the laboratory (fluorescent and non-fluorescent)	unknown	125
PE	fragments	44.39 $\pm$ 11.16 μm	5, 10, 25, 50, 100, 125, and 250 mg/L	Cryo-milling of PE pellets (Sigma Aldrich, USA)	yes (rinsed with hexane, methanol, and deionized water)	126
① PMAA; ② PVA	① spheres; ② films and fibres	① 1.5-10 μm ② size not provided	① 25, 50, 75, 100, 125, and 150 mg/L; ② 25, 50, 75, 100, and 125 mg/L	Synthesized in the laboratory	unknown	127
PS (f)	spheres	6 μm	2.5, 5, 10, 20, and 30 mg/L	Polysciences, USA	unknown	128
PE	spheres	300-354 μm	14.3, 35.7, and 71.4 mg/L	Cospheric, USA	unknown	129
PS-NH ₂	spheres	① 20 nm; ② 40 nm; ③ 60 nm; ④ 100 nm	Media 1: ①②③ 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 15, and 20 mg/L; ④ 0.5, 1, 5, 6, 7.5, 8, 10, 12.5, 15, and 20 mg/L. Media 2: ①②③④ 0.5, 1, 1.5, 2, 2.5, 5, 10, and 20 mg/L	Invitrogen (Thermofisher Science), USA	unknown	130

A summary of the targeted research synthesis results is presented in **Figure 2.3** while the detailed results (including polymer types and references) are presented in the Supporting Information (**Table S2.2**). In both, all spherical micro- and nanoplastics were presented based on their size (diameter) and concentration, while non-spherical micro- and nanoplastics are presented in the last column of the table based on their concentration alone, as information on size range could not be easily presented due to the different shapes and size ranges. Details on the particle sizes for each test using non-spherical micro- and nanoplastics are presented in **Table S2.2**. Also, when necessary, the concentration of the particles and the diameter of the spherical particles were rounded up for a more concise number. For example, 5.8 μm was approximated to 6 μm while 1.4 mg/L concentration was approximated to 1.5 mg/L. There was a total of 505 tests including spherical micro- and nanoplastics and non-spherical micro- and nanoplastics across five major polymer types (**Figure 2.3** and **Table S2.2**). As shown in **Figure 2.3**, the toxicity data are categorized as insignificant, mild, or severe when the percentage of mortality or immobility are $\leq 20\%$; $>20\%$ and $\leq 50\%$; and $> 50\%$, respectively.

Concentration (mg/L)	Spherical beads (µm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
0.01																						
0.1																						
0.25																						
0.5																						
0.75																						
1.0																						
1.5																						
2.0																						
2.5																						
3.0																						
4.0																						
5.0																						
6.0																						
7.5																						
8.0																						
10																						
12																						
15																						

Concentration (mg/L)	Spherical beads (µm)																				Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100
20																					
25																					
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250																					
300																					
320																					
400																					
500																					
1000																					
5000																					

Figure 2.3. Targeted research synthesis of immobility or mortality observed on *D. magna* neonates (≤ 24 h) after 48 h exposure to microplastics and nanoplastics. Each colored cell represents a toxicity test conducted on one particle type of a given size and concentration. The spherical beads columns provide the particle diameter and the corresponding concentration of each toxicity test.

For the non-spherical or irregular-shaped plastics, the cells under the “other shapes” column present the concentration of each toxicity test. The percentage of dead or immobile neonates are represented by the cell colors. Blue cell color represents no significant acute toxicity ($\leq 20\%$), yellow cells represent mild acute toxicity ($> 20\%$ and $\leq 50\%$) and red cells represent severe acute toxicity ($> 50\%$), N = 505. Detailed information including the polymer types, functional groups (where available), presence of fluorescent dye, whether the particles were washed or not, references, as well as the sizes of the non-spherical particles are presented in the Supporting Information (**Table S2.2**).

Acute toxicity of spherical plastic particles

The size and concentration of the tested spherical micro- and nanoplastics for acute toxicity ranged from 20 nm - 150 μm and 0.01 mg/L to 5000 mg/L, respectively. However, the most commonly used concentrations and sizes were 10 mg/L, 50 mg/L and 100 mg/L; and 0.1 μm , 0.2 μm and 1 μm , respectively. The observed acute toxicity from the tested spherical micro- and nanoplastics varied due to various factors including their polymer type, size, and concentration. The results are summarized below according to the different plastic polymer types.

Polystyrene (PS): There are a total of 417 tests conducted on PS spherical micro- and nanoplastics to investigate acute toxicity to *D. magna* (**Figure 2.3** and **Table S2.2**).^{77,79-81,91,92,108-111,114-118,120,124}

The tests varied largely in the size of particles, functional groups and test concentrations. Due to the high number of tests in the category and for easier analysis and discussion, the tests are divided into the following size categories: (i) ≤ 100 nm; (ii) >100 nm and < 35 μm ; and (iii) ≥ 35 μm . Spherical PS (≤ 100 nm) were tested on *D. magna* between 0.1 mg/L and 400 mg/L in 269 tests.

Out of the 75 tests conducted at ≤ 1 mg/L in this size range, 84% did not observe significant acute toxicity while 11% and 5% showed mild and severe acute toxicity, respectively. The results were quite opposite for the 194 tests conducted > 1 mg/L. In those tests, 60% found severe acute toxicity while 17% found mild toxicity. Only 23% of the tests did not find significant toxicity at the end of the acute toxicity tests. At increased size range of the spherical micro- and nanoplastics (>100 nm and < 35 μ m), 129 acute tests have been conducted at concentrations ranging from 0.01 mg/L to 1000 mg/L. This size range accounts for $\sim 30\%$ of all spherical PS acute toxicity tests on *D. magna*. It includes 66 tests (< 20 mg/L), 49 tests (≥ 20 mg/L and ≤ 100 mg/L) and 14 tests (> 100 mg/L). 80% of tests conducted at < 20 mg/L found no toxicity while the remaining 20% reported mild acute toxicity to *D. magna*. When the test concentrations increased between 20 mg/L and 100 mg/L, 51% showed no significant toxicity while 14% and 35% observed mild and severe acute toxicity, respectively. At above 100 mg/L, the proportion of tests in this size category that showed severe acute toxicity increased, accounting for 64% while the remaining 36% showed no acute toxicity. 89% of 19 tests exposed to spherical PS (≥ 35 μ m) consistently did not show acute toxicity, with the 11% remaining which represents 2 tests exhibiting severe acute toxicity at 500 mg/L and 1000 mg/L^{92,112} (**Figure 2.3** and **Table S2.2**).

Polyethylene (PE): Twenty-six (26) tests were reported in four different studies to assess the acute toxicity of spherical PE to *D. magna* (**Figure 2.3** and **Table S2.2**).^{83,106,113,129} Two tests on spherical PE (10-100 μ m) conducted at 1000 mg/L and 5000 mg/L showed mild acute toxicity while the remaining 24 tests (92%) did not show significant acute toxicity to *D. magna*. The size and concentration ranges of the spherical PE tests which showed no acute toxicity are 10-100 μ m (10-500 mg/L)¹⁰⁶, 40-48 μ m (20-320 mg/L)⁸³, 1 μ m (12.5-400 mg/L)¹¹³, 100 μ m (12.5-400 mg/L)¹¹³, and 300-354 μ m (15-70 mg/L)¹²⁹.

Polymethyl methacrylate (PMMA): Eight acute toxicity tests were conducted on plain and functionalized PMMA plastic beads (85-125 nm).¹²⁵ The particles were synthesized in the laboratory and the tests were conducted at 0.01 mg/L, 0.1 mg/L, 1 mg/L, and 1000 mg/L.¹²⁵ All 8 tests showed no acute toxicity to *D. magna* (**Figure 2.3** and **Table S2.2**).

Polymethacrylic acid (PMAA): Six acute toxicity tests were conducted using PMAA particles (1.5 -10 μm) at concentrations ranging from 25 mg/L to 125 mg/L. Only one test, at 100 mg/L, showed mild toxicity while other concentrations do not cause any significant mortality after 48 h exposure.¹²⁷

Overall, **Figure 2.3** clearly shows more severe toxicity at lower particle sizes, at concentrations above 1 mg/L. This aligns with other reports that have shown that when the same concentration of particles was tested, nanoplastics showed higher acute toxicity than larger sized particles.^{82,110} Previous studies also suggested that nano-sized particles (≤ 100 nm) may have easier diffusion abilities into the cells and can more readily localize in small structures of organs and tissues through the circulation system of the organism while larger sized particles are egested without being retained in the intestinal tract.¹³¹ Hence, the impacts of microplastics may be limited to physical injuries in the intestinal tracts of animals while nanoplastics, just like other nanoparticles, may be more harmful due to their higher penetration ability.¹⁰⁹ This may explain the increased toxicity observed in the spherical nano-sized PS compared to larger sized particles of the same polymer type and concentration.⁸² In addition, Ma *et al.*¹¹⁰ showed no impact of the larger sized PS particles (500 nm - 15 μm) but observed severe immobility as well as visible physical damage with 50 nm PS particles at the same concentration. Specifically, there was damage to the thoracopods which play an important role in the mobility and filter-feeding of daphnids. Interestingly, Frankel *et al.*¹²⁰ reported that the aggregation of 50 nm PS-NH₂ nanoplastics does

not diminish their toxicity towards daphnids. It is suggested that when entering the organism, the aggregates rapidly break down into free 50 nm NH₂-functionalized PS beads that are inherently toxic.

The surface chemistry of the particle can also play a pivotal role in toxicity. Lin *et al.*¹⁰⁹ showed this connection by studying different types of functionalized PS particles. Interestingly, they noted that plain PS nanoplastics are more toxic than modified PS nanoplastics such as negatively charged carboxylated PS nanoplastics (PS-COOH), and positively charged aminated PS nanoplastics (PS-NH₂). Among functionalized PS particles, the NH₂-functionalized PS particles show higher toxicity than their COOH-functionalized counterpart.¹⁰⁸ Particularly on the acute exposure of *D. magna* resulting in the immobilization of the organisms, PS-NH₂ nanoplastics were found to be three times more toxic than PS-COOH nanoplastics.¹²⁴ This observation is due to the positively charged surface of PS-NH₂ nanoplastics that can readily interact with the overall negatively charged microorganisms. Nasser *et al.*¹³² also explored the role of secreted proteins that can coat the surface of PS nanoplastics, forming an eco-corona, and alter the toxicity of the particles. Eco-corona-coated nanoplastics had a lower EC₅₀ than their uncoated counterparts. However, it was noted that there was a greater retention for corona-coated nanoplastics in *D. magna* gut, affecting its ability to feed on algae.¹³² Subsequent studies could examine the long term effects of eco-corona-coated nanoplastics and how such bioaccumulation can impact toxicity assessments.

Minimal acute toxicity was observed during exposures to spherical PE. However, it is noteworthy that most of the tests used larger particles (e.g., 100 µm). At such sizes, it is uncertain whether the particles were bioavailable to be ingested by the daphnids.¹¹³ Also, it can be hypothesized that the observed severe toxicity at 1000 mg/L and 5000 mg/L may be due to hindrance in their movement followed by exhaustion and drowning, rather than the ingestion of the particles. The only study

that investigated PMMA nanoplastics (86-125 nm) found no toxicity at up to 1000 mg/L.¹²⁵ The results of the toxicity data from the different polymer types are incomparable due to the different size range of the exposed particles. Hence, there is need for more studies on other polymer types, particularly using smaller sized particles. Also, most of the studies did not provide information on whether they washed the commercial formulations to remove the chemicals that may be present. Hence, the extent of effects from these chemicals, if present, could not be confirmed.

Acute toxicity of non-spherical plastic particles

There was a total of 48 acute toxicity tests on non-spherical micro- and nanoplastics accounting for less than 10% of the total acute toxicity tests covered in this study (**Figure 2.3** and **Table S2.2**). The tests include fragments and fibres from PE, PP, PET, PVA, and PVC and the results of the acute toxicity upon exposure of daphnids to the particles from each polymer type is summarized below:

Polyethylene (PE): Six studies conducted a total of 24 tests on PE non-spherical micro- and nanoplastics at concentrations ranging from 10 mg/L to 5000 mg/L (**Figure 2.3** and **Table S2.2**).^{93,94,106,122,123,126} Different sizes of washed PE fragments ranging from 50 to 250 μm obtained from facial cleansers, as well as 150 μm and 20 μm sized fragments obtained from fleece textile and plastic bag, respectively were tested at 100 mg/L.^{75,76} No significant acute toxicity to *D. magna* was observed across all types of PE fragments and sizes. Also, Song *et al.*¹²⁶ did not observe significant toxicity upon exposing PE fragments ($44.39 \pm 11.16 \mu\text{m}$) at up to 250 mg/L while Renzi *et al.*¹²³ showed mild acute toxicity upon exposure of PE fragments (10-100 μm) obtained by grinding bulk plastic pieces at 50 mg/L. Also, at 10 mg/L, acute toxicity tests on PE fragments, 10-75 μm ¹⁰⁶ and $180.5 \pm 118.7 \mu\text{m}$ ¹²² did not show any significant acute toxicity^{106,122} while all

three tests conducted on PE fragments (10-75 μm) at ≥ 500 mg/L showed severe acute toxicity to *D. magna*¹⁰⁶.

Polypropylene (PP): There were a total of 10 tests on non-spherical PP to assess acute toxicity to *D. magna*.^{119,123} Kokalj *et al.*¹¹⁹ obtained PP fibres (diameter = 4-24 μm , length not provided) by milling medical masks. The tests were conducted on the three layers of the masks. Nine tests were conducted, at 1 mg/L, 10 mg/L, and 100 mg/L on the three layers of single-use face masks. All nine tests showed no acute toxicity to *D. magna*.¹¹⁹ Also, Renzi *et al.*¹²³ tested PP fragments (10-100 μm) obtained by grinding bulk particles. Although the source of the bulk particles was unspecified, exposures at 50 mg/L showed no significant acute toxicity to *D. magna*.

Polyethylene terephthalate (PET): Jemec *et al.*¹⁰⁷ and Tourinhoa *et al.*¹²¹ carried out a total of 7 tests to examine the acute toxicity of PET particles on *D. magna*. Jemec *et al.*¹⁰⁷ tested washed fibres obtained from a PET fleece material (length = 60-1400 μm , width = 30-530 μm , diameter = 2-21.5 μm) at 12.5 mg/L, 25 mg/L, 50 mg/L and 100 mg/L. In all 4 tests, the authors reported mild acute toxicity. In contrast, Tourinhoa *et al.*¹²¹ did not observe any immobility when fibres obtained from a continuous PET filament (length = 366 ± 275 μm , diameter = 14 ± 3 μm) were tested at 20 mg/L, 100 mg/L, and 500 mg/L.

Other polymer types: Only two studies were found in this review that tested other types of plastic for acute toxicity to *D. magna*. Renzi *et al.*¹²³ tested 50 mg/L of washed PVC particles (10-100 μm) and a mixture of PVC/PE particles (10-100 μm) for acute toxicity and found no significant immobility to *D. magna* at both conditions. Gökçe *et al.*¹²⁷ tested a mixture of PVA films and fibres (unknown size) at five different concentrations up to 125 mg/L. The study found severe toxicity at 50 mg/L, 75 mg/L, and 125 mg/L but no toxicity at 25 mg/L and 100 mg/L.¹²⁷

Only the PE studies that used unwashed particles and high concentrations (50-5000 mg/L) found acute toxicity to *D. magna*¹⁰⁶ while other studies using washed particles at various concentrations did not observe similar results. While some particles of the tested size ranges may not be ingested by *D. magna*, the chemicals present in the particle suspension may be responsible for the observed toxicity. Also, PET fibres ($14 \pm 3 \mu\text{m}$ in diameter, 20-500 mg/L) from filament showed no acute toxicity while those from a PET fleece material showed mild acute toxicity within similar particle size and concentration range.^{107,121} Tests on PP fibres showed no acute toxicity as well.^{119,123} It is important to note that there are only a few studies that investigated these particle shapes; hence, it is difficult to assess the role of the shape or polymer type. While our understanding of the impact of particle shape on toxicity is still in the early stages of development, some studies demonstrated that the shape of the microplastics may influence the impact on *D. magna*.^{101,133} For instance, Frydkjær *et al.*¹⁰⁶ using 10-75 μm PE fragments and 10-106 μm PE spheres demonstrated that irregularly shaped microplastics were far more likely to be retained in the gut of daphnids after 90 minutes of exposure. It is implied that the regular spherical shaped plastics possessed a smoother surface resulting in depuration. This is concerning as most plastics present in the aquatic ecosystem are irregularly shaped.

2.3.2 Chronic toxicity

Chronic toxicity studies target sublethal endpoints such as growth, development, behavior, reproduction, as well as survival to better understand effects of contaminants on population dynamics in the ecosystem. We reviewed 36 chronic studies of microplastics and nanoplastics on *D. magna*. Eighteen out of the 36 were focused solely on PS while an additional 2 studied PS along

with other polymer types. The number of studies on other polymers are as follows: PE (9 studies), PVC (4 studies), PET (2 studies), PA (1 study), PLA (1 study), EAA (1 study), PMAA (1 study) PVA (1 study), and PUR (1 study). Some studies only assessed a single polymer type while others investigated multiple polymers. Four studies used commercial particles with no information on the polymer type. In some cases, metrics varied from mass concentration (mg/L) to particle concentration (particles/mL); hence comparison of the results and synthesis of effect across studies was difficult for similar polymer type. Like the acute toxicity section, some studies assessed microplastic and nanoplastic toxicity in the presence of co-contaminants and/or other factors, such as temperature changes or UV, and in these situations only the results obtained from microplastic and nanoplastic exposure alone were reported in this section. A summary of the studies is presented in **Table 2.2**.

Table 2.2. Studies investigating the chronic toxicity of microplastics and nanoplastics to *D. magna*. -COOH and -NH₂ means carboxylated and aminated functional groups, respectively. (f) means fluorescently labelled. Where applicable, circled numbers were used to distinguish particles that possess different properties within the same study.

Type of plastic	Shape	Size	Concentration	Source of plastic	Was the plastic washed?	Duration of test	Target endpoints	Refs.
① unspecified (f), (1.30 g/cm ³); ② PE	① spheres; ② fragments	① 1-5 µm; ② 1-10 µm	10 ² , 10 ³ , 10 ⁴ , and 10 ⁵ particles/mL	① Cospheric, USA; ② Grinding PE spheres (850-1000 µm, Cospheric, USA)	unknown	21 days	reproduction (time to first brood, size of first brood, number of broods, size of first three broods, number of neonates); growth (terminal body length).	⁸⁹
① unspecified (f), (1.30 g/cm ³); ② PE	① spheres; ② fragments	① 1-5 µm; ② 2.6 ± 1.8 µm	10 ² , 10 ³ , 10 ⁴ , and 10 ⁵ particles/mL	① Cospheric, USA; ② Grinding PE spheres (1 mm, Cospheric, USA)	unknown	21 days	survival; growth; reproduction (time to first brood, time between broods, number of broods, number of neonates).	⁹⁰
PVC	fragments	① 2 ± 1 µm; ② 50 ± 10 µm	① 2.05 mg/L ② 4.97 mg/L	Guangzhou Huayu Trade Co., Ltd., China	clean, (vendor's declaration)	21 days	reproduction (time to first brood, size of first brood, number of broods, total number of neonates); molting (frequency of molting)	¹³⁴
①rigid PVC; ②flexible PVC	fragments	① 4-141 µm; ② 12-276 µm	4342 particles/L	① Nexans Research Center, Germany; ② modification of ① using diisononylphthalate, chalk, and stabilizer	unknown	25-31 days	survival; growth (body length, body width, tail spine length); reproduction (number of neonates in first six broods, time to first brood)	¹³⁵
unspecified (f), (1.30 g/cm ³)	spheres	1-5 µm	0.02 and 0.2 mg/L	Cospheric, USA	unknown	21 days	survival; growth (somatic growth); reproduction (time to first brood, number of broods, number of immobile neonates, number of aborted eggs)	¹³⁶
① virgin PA; ② weathered PA	fragments	0-180 µm	100 and 300 mg/L	① Abifor AG, Germany; ② UV-weathering of ① for 26 days	no	21 days	survival; reproduction (number of neonates)	¹³⁷

① PS; ② PET; ③ ABS	fragments	3.2-3.7 μm	① 7.3×10^4 particles/mL; ② 1.15×10^5 particles/mL; ③ 1.19×10^5 particles/mL	Grinding plastic products made of the corresponding materials	yes (centrifugation)	14 days	survival; growth (body length); reproduction (number of neonates)	⁷⁸
① PE; ② PP; ③ PS; ④ PVC	unclear	①② 50 nm; ③ 200 and 600 nm; ④ 200 nm	3×10^{10} particles/mL	CD-bioparticles	no	21 days	survival; reproduction (number of broods, number of neonates, number of neonates/brood)	⁸²
① PVC; ② PUR; ③ PLA	fragments	① $\leq 20 \mu\text{m}$; ②③ $\leq 40 \mu\text{m}$	10, 50, 100, and 500 mg/L	Grinding plastic products made of the corresponding materials	unknown	21 days	survival; growth (body length); reproduction (time to first brood, number of neonates)	¹³⁸
① virgin LDPE ② recycled LDPE	fragments	① $39.8 \pm 8.82 \mu\text{m}$; ② $205 \pm 144 \mu\text{m}$	1, 10, and 100 mg/L	① ICO Polymers, USA; ② cryomilling recycled LDPE granules/pellets (Estonia)	unknown	21 days	survival; growth (body length); reproduction (time to first brood, number of broods, number of neonates)	¹³⁹
PE (f)	spheres	63-75 μm	25, 50, and 100 mg/L	Cospheric, USA	unknown	21 days	survival; reproduction (number of neonates, average number of neonates/day)	⁶⁴
PE	① spheres; ② fragments	① 40-48 μm ; ② $17 \pm 3 \mu\text{m}$ and $34 \pm 13 \mu\text{m}$	5 mg/L	① Sigma Aldrich, USA; ② Grinding and cryomilling of PE pellets	no	21 days	survival; growth (body length of adults and neonates); reproduction (time to first brood, number of broods, number of neonates, number of neonates/brood)	¹⁴⁰
HDPE	fragments	90-200 nm	unspecified	Grinding bulk pieces	yes (filtration)	134 and 98 days	survival; reproduction (number of neonates)	¹⁴¹
PS (f)	spheres	100 nm and 2 μm	0.1, 0.5, and 1 mg/L	Phosphorex	unknown	21 days	survival; growth (body length); reproduction (time to first brood, number of broods, total number of neonates, number of neonates/brood); molting (number of molts)	¹⁴²
PS	fragments	$13.03 \pm 7.75 \mu\text{m}$	101.6 mg/L	Cryomilling larger particles	unknown	19 days	growth (body length, tail length, body width); reproduction (time to first brood, number of neonates per brood, sum of neonates from	⁹⁹

PS	spheres	5.8 µm	0.5, 1, and 2 mg/L	BaseLine ChromTech Research Center, China	unknown	21 days	first two broods); others (proteomic analysis) growth (body length); reproduction (time to first brood, number of neonates in first brood, number of broods, total number of neonates)	80
PS	① spheres; ② fragments; ③ fibres	① 6 µm, 20 µm; ② 5.7 µm, 17.7 µm; ③ diameter = 3 µm, length = 75.5 µm	500 and 5000 particles/L	① Polysciences, USA; ② wet milling; ③ electrospinning	unknown	21 days	survival; growth (body length); reproduction (number of neonates per brood, total number of neonates)	143
PS (f)	spheres	2 µm	1.39×10 ⁻³ and 1.11×10 ⁻² mg/L	Sigma Aldrich, UK	unknown	21 days	survival; growth (body length); reproduction (number of neonates)	102
PS (f)	spheres	1-5 µm	10 ² , 10 ³ , 10 ⁴ , and 10 ⁵ particles/mL	Cospheric, USA	unknown	21 days	growth (body length); others (total biomass, number of ephippia)	37
PS	spheres	0.07, 1, and 10 µm	1 and 2 mg/L	BaseLine ChromTech Research Centre, China	clean, (vendor's declaration)	27 days	survival; reproduction (net reproductive rate, generational time and intrinsic rate of population increase)	144
① PS-COOH; ② PS-NH ₂	spheres	① 26 and 62 nm; ② 53 nm	① (26 nm): 0.32 and 3.2 mg/L; ① (62 nm): 0.32, 0.76, 3.2, and 7.6 mg/L; ② 0.0032, 0.032, and 0.32 mg/L	Bangs Laboratories Inc., USA	yes (dialysis)	103 days	survival; reproduction (total number of neonates)	145
① PS; ② PS (f); ③ PS (f) and palmitic acid (PS(f)-PA))	spheres	① 90 ± 5 nm; ② 95 ± 4 nm; ③ 100 ± 4 nm	① 1.6-26.6 mg/L; ② 1.7-28.6 mg/L; ③ 0.4-7.7 mg/L	Synthesized in the laboratory	unknown	21 days	survival; growth (body length); reproduction (time to first brood, number of neonates/brood)	146
PS (f)	spheres	50 nm	0.05 and 0.5 mg/L	Fisher Scientific, USA	unknown	21 days	other (gene expression analysis, oxidative stress and energetic biomarkers, swimming activity)	147
PS (f)	spheres	1 µm and 10 µm	0.125, 1.25, and 12.5 mg/L	Sigma Aldrich, Italy	unknown	21 days	growth (body length); other (swimming activity, phototactic behaviour)	148

PS (f)	spheres	70 nm	0.22, 0.44, 0.88, 1.8, 3.5, 7, 14, 32, 70, and 150 mg/L	AVT-PCC, Wageningen UR.	unknown	21 days	survival; growth (body length of adults and neonates); reproduction (total number of neonates in first three broods, number of neonates/brood); other (malformations in neonates)	149
unspecified (f), (1.30 g/cm ³)	spheres	1-5 µm	0.05, 0.1, and 0.2 mg/L	Cospheric, USA	unknown	21 days	survival; growth (somatic growth); reproduction (time to first brood, size of first brood, number of broods, number of neonates, number of dead/immobile neonates, number of aborted eggs)	68
PS-COOH (f)	spheres	500 nm	1 mg/L	Sigma Aldrich, UK	unknown	until second brood	survival; growth (body length, growth rate); reproduction (size of brood, number of neonates); other (haemocyte counts)	150
PS (f)	spheres	6 µm	5, 15, 30, 50, and 100 mg/L	Polysciences, Germany	unknown	21 days	survival; growth (body length of adults and neonates); reproduction (time to first brood, number of neonates)	111
PS-COOH (f)	spheres	① 20 nm; ② 200 nm	① 0.1 and 50 mg/L; ② 50 mg/L	ThermoFisher	yes (dialysis)	21 days	survival, growth (body length); reproduction (time to first brood, number of neonates); other (time to first molt, number of molts, swimming activity, hopping frequency)	151
PS (f)	spheres	6 µm	5 mg/L	Polysciences, USA	unknown	21 days	survival; growth (adult body length, first neonate body length, neonate body length); reproduction (time to first brood, number of neonates)	128
PE	fragments	≤50 µm	10, 25, 50, 100, and 200 mg/L	Sigma Aldrich	yes (washed with methanol and deionized water)	10 days	survival	152
① PMAA; ② PVA	① spheres; ② films and fibres	① 1.5-10 µm ② size not provided	① 1, 5, 10, 15, and 30 mg/L; ② 2.5, 5, 10, 25, and 50 mg/L	Synthesized in the laboratory	unknown	21 days	survival; growth (body length, width length, spine length, specific growth rate); reproduction (number of	127

PS-COOH (f)	spheres	0.5 µm	0.01, 0.02, 0.05, and 0.1 mg/L	Sigma Aldrich	unknown	7 days	neonates); other (morphologic deformations) survival; reproduction (occurrence of egg clutches)	153
PE	fragments	44.39 ± 11.16 µm	4.5 mg/L	Cryo-milling of PE pellets (Sigma Aldrich, USA)	yes (rinsed with hexane, methanol, and deionized water)	17 days	survival; growth (adult body length, neonate body length); reproduction (number of neonates, embryonic development rate); other (phototactic behavior, oxidative stress analysis)	126
EAA	spheres	103 nm	2.3×10 ¹¹ and 2.3×10 ¹² particles/L	Dow Chemical Co., USA	no	21 days	survival; growth (body length); reproduction (number of neonates); other (transcriptomic responses)	88
PS-COOH	spheres	15 µm	100 and 800 particles/L	Sigma Aldrich, UK	unknown	21 days	survival; growth (body length); reproduction (number of neonates)	154

Exposure to microplastics and nanoplastics has been documented to affect population and individual size, trigger malformation and mortality at high concentrations, but those effects are commonly dependent on (1) polymer type¹³⁸, (2) particle size¹⁴⁰, (3) duration of exposure¹⁴⁵, and (4) concentration¹²⁷, with a broad range of variation across studies. We list and compare below the effects described in the reviewed studies by particle type, followed by the size of the particles. Most research articles using commercial particles did not wash the particles prior to exposures, hence the absence of washing was mentioned when it could introduce confounding effects regarding toxicity results.

Chronic toxicity of spherical plastic particles

Polystyrene (PS): Many studies have highlighted PS microplastic and nanoplastic toxicity to *D. magna* across a range of different endpoints, from standard apical endpoints such as survival, to molecular stress markers. For instance, Yin *et al.*⁸⁰ reported that 5.8 µm PS showed effects on reproduction after 21 days at concentrations as low as 2 mg/L, with a decrease in the number of broods and neonates. Jeong *et al.*¹²⁸ found a decrease in survival, reproduction, and adult body length in *D. magna* exposed to 5 mg/L PS (6 µm) for 21 days. Growth rate was decreased by accumulation of PS particles, in response to which mitochondrial biogenesis was enhanced through *PGC-1a* gene upregulation. They also observed decreased cellular reactive oxygen species levels compared to controls after 24 h, and decreased superoxide dismutase and catalase activities. Likewise, Schwarzer *et al.*¹⁴³ found that 6 µm PS inhibited growth and reproduction when the study tested 6 and 20 µm PS at two concentrations, 500 and 5000 particles/L. No significant effects were observed in the *D. magna* exposed to 20 µm particles and the effects at both concentrations

were comparable for both particle sizes. Another study from Bosker *et al.*³⁷ found a decrease of 26% in the population of adults after 21 days of exposure to 1 μm and 5 μm PS and decrease of total biomass of 21%. This study reported particle concentration rather than mass concentration (from 10^2 to 10^5 particles/mL). Similarly, Pikuda *et al.*¹⁵¹ exposed *D. magna* for 21 days to washed 200 nm and 20 nm PS for 21 days to assess whether different metrics (particle counts versus mass) impacted the observations. No mortality was observed, however exposures impacted growth, reproduction through the number of neonates, and molting, at 50 mg/L in both tested sizes, and concentrations as low as 0.1 mg/L in the 20 nm PS. Another study did not observe mortality upon exposure of 70 nm PS particles, however, the particles caused a significant decrease in the total number of neonates and brood from the first three broods, starting from 0.2 mg/L.¹⁴⁹ Behavioral effects have also been observed - exposure to 1 and 10 μm PS from concentrations ranging from 0.125 to 12.5 mg/L increased swimming activity and overall body size of *D. magna* adults after 21 days of exposure.¹⁴⁸ The phototactic behavior was impacted as well, suggesting potential long-term consequences upon exposure. A study from Aljaibachi *et al.*¹⁰² highlighted selective behavior from *D. magna* during a 21-day exposure to 2 μm PS, with a decrease of uptake in the presence of algae. De Felice *et al.*¹⁴⁷ went further into physiological and molecular endpoints, and exposed *D. magna* to very low concentrations of 50 nm PS beads (0.05 and 0.5 mg/L) to observe changes in genes involved in stress response. A slight modulation in the transcription of those genes was observed, notably genes involved in energy reserves, but no increase in oxidative stress markers was noted. Sadler *et al.*¹⁵⁰ observed an increase of hemocyte number after 21 days of exposure to 500 nm PS beads for 8 different clones, although the amplitude of the response was clone-dependent. The surface chemistry of PS particles also plays an important role in uptake and toxicity. For instance, Vicentini *et al.*¹⁴⁶ showed that ~90 nm particles functionalized with palmitic

acid were more taken up than non-functionalized particles after 21 days, with a lowest observed effect concentration of 3.8 ng/L, and were 6.9 times more toxic, with a decrease in growth and reproduction at concentrations below 30 mg/L. Similarly, Huang *et al.*¹⁵³ studied the impact of carboxylated PS (500 nm) at low concentrations (as low as 0.1 mg/L) and found no mortality despite a high body burden. Due to fluorescent labelling, they were able to positively correlate uptake with duration of exposure (7 days). They also observed a delayed sexual maturation in exposed individuals at concentrations as low as 0.01 mg/L, correlated with a strong decrease in offspring numbers starting at 0.02 mg/L. Some studies noted fewer effects with no impact on survival nor reproduction. Rist *et al.*¹⁴² observed uptake and retention times of 100 nm and 2 µm PS beads at concentrations ranging from 0.1 to 1 mg/L and observed a higher ingestion of the bigger particles at high concentration compared to the 100 nm sized ones, with no toxicological effects. They did observe a 21% decrease of the feeding rates with the smaller particles but no effects on reproduction. Prolonged exposures (103 days) showed that both carboxylated and aminated PS nanoplastics (washed) shortened the life-time of *D. magna*, with a decrease of almost three times in the aminated particles.¹⁴⁵ It is noteworthy that the studies that washed PS-COOH nanoplastics at 0.01 mg/L and 50 mg/L did not impact survival after 21 days chronic toxicity test¹⁵¹, while washed PS-COOH with comparable size range at concentrations ranging from 0.32-7.6 mg/L significantly reduce survival in longer term exposures (103 days) with the decrease in survival observable after about 30 days¹⁴⁵. This suggests that washed PS particles even at low concentrations may impact survival in daphnids after prolonged exposure.

Polyethylene (PE): Cannif *et al.*⁶⁴ studied PE beads with a size ranging from 63 to 75 µm and concentrations ranging from 25 to 100 mg/L. They did not observe toxic effects, despite a high

uptake and body burden. An *et al.*¹⁴⁰ also investigated 5 mg/L of 40-48 μm PE beads and found no significant effects on survival, growth and reproduction.

Other polymer types: Gökçe *et al.*¹²⁷ studied the effects of lab-generated 1.5 to 10 μm PMAA on *D. magna* for 21 days and found toxicity, with a LC_{50} of 14.8 mg/L, and a decrease in population was observed throughout exposure. These particles also influenced body length and width, but the effect did not follow a dose-response relationship. According to Coady *et al.*⁸⁸, no effects on survival, growth and reproduction were observed in *D. magna* after 21 days of exposure to 103 nm EAA (2.3×10^{11} and 2.3×10^{12} particles/L). However, genes involved in metabolism, reproduction and oxidative stress were upregulated at the highest concentration of ethylene acrylic acid copolymer, suggesting potential effects for concentrations above 2.3×10^{12} particles/L.

Unknown polymer type: Several researchers investigated the toxicity of spherical microplastics with no specific polymer identification (*i.e.*, absence of FTIR/Raman to identify polymer type, and no information from the provider). Although these studies do provide information on potential toxicity, the impossibility to link them to a polymer type decreases the relevance of such studies for mechanistic comprehension of microplastic and nanoplastic toxicity. These studies highlighted the need to include characterization of polymer type for comparison across studies. For instance, Guilhermino *et al.*⁶⁸ found that microplastics from 1 to 5 μm caused parental and juvenile mortality at 0.2 mg/L after 21 days of exposure, with a strong decrease in reproduction and offspring EC_{50} ranging from 0.101 to 0.146 mg/L particles. Another study found effects on reproduction with similar particle size and concentration after 14 days,¹³⁶ with the production of immobile juveniles, but no effects on mortality nor individual growth. Ogonowski *et al.*⁹⁰ used similar sized pristine particles (1-5 μm) from the same supplier to compare effects of primary and secondary microplastics. No effects on life-history traits were found after 21 days exposure on adults and

juveniles. Decrease of growth, fecundity, and survival in F₁ females were attributed to limited food availability due to the experimental design and the addition of plastic particles. A similar study by Jaikumar *et al.*⁸⁹ using the same particles from the same supplier found a decrease in neonates after 21 days of exposure to these microplastics, as well as a decrease in individuals' growth, but survival was not monitored.

Chronic toxicity of non-spherical plastic particles

Polystyrene (PS): Hiltunen *et al.*⁷⁸ generated 3.2 to 3.7 µm PS microplastic particles from a PS tray, and introduced the particles as 1% of *D. magna* diet. They did not find effects on survival, size, and reproduction after 14 days of exposure. Schwarzer *et al.*¹⁴³ generated two sizes of PS fragments (5.7 µm and 17.7 µm) and one size of PS fibres (diameter = 3 µm, length = 75.5 µm). The fibres did not show any significant effects to *D. magna* while the fragments impacted reproduction. Similarly, Trotter *et al.*⁹⁹ observed reduction in growth and reproduction when *D. magna* were exposed to ~100 mg/L of 13.03 ± 7.75 µm PS fragments.

Polyethylene (PE): Ogonowski *et al.*⁹⁰ found significant impacts on survival but slight effects on growth and reproduction upon 21-day chronic exposure to 2.6 ± 1.8 µm irregularly shaped PE particles. Another study exposing *D. magna* to 1-10 µm irregularly shaped PE particles observed reduced size of the first brood and the total number of neonates produced within the first three broods but not any significant impact on growth.⁸⁹ An *et al.*¹⁴⁰ demonstrated that PE fragments (~17 and ~34 µm) were internalized (*i.e.* ingested) to a greater extent than commercial microbeads of comparable size (~40 µm). Survival after 21 days was heavily impacted in a size-dependent manner, with a decrease of 20% and 60% for particle sizes of 17 and 34 µm, versus 90% for the

spherical particles. They also observed that the smaller fragment size reduced the feeding rate, growth, and reproduction of *D. magna*, likely due to a higher retention time. In contrast, studies investigating larger sized fragments observed less significant toxicity. Song *et al.*¹²⁶ assessed the influence of PE fragments (48 µm) on *D. magna* survival, reproduction and development. Interestingly, after 17 days of exposure to PE, they observed no change in growth, reproduction, and phototactic behavior. Concurring to this finding, Kokalj *et al.*¹³⁹ did not find any effects on growth, reproduction, and lipid content after 21 days of exposure to ~40 and ~200 µm low density PE (LDPE) fragments, with concentrations from 1 to 100 mg/L. Using two prolonged exposures (134 and 98 days), Ekvall *et al.*¹⁴¹ showed that high density PE (HDPE) 90 and 200 nm did not cause any toxicity to *D. magna*. However, a smaller fraction containing nanoparticles and oligomers with a size < 3 nm resulting from the breakdown of PE chains, and associated leached chemicals, decreased reproduction over time. To investigate further, they separated the small particles from the leached chemicals with sequential filtration, concentration, and dilution, and determined that toxicity was substantially reduced with only the small particles. They therefore showed that chemicals leaching from the plastic particles were highly toxic to *D. magna*, highlighting the potential long-term risk of additive leaching from plastics into the environment.

Polyvinyl chloride (PVC): A study aimed at comparing rigid PVC microplastics (4-141 µm) to flexible PVC microplastic (12-276 µm) containing 30% diisononylphthalate plasticizer.¹³⁵ Both PVC types did not impact survival, although the flexible microplastics reduced offspring number and increased body length. Similarly, in another study by Zimmerman *et al.*¹³⁸, flexible PVC particles reduced reproduction and the effect was mostly attributed to the same additive, diisononylphthalate, leaching from the particles. Indeed, separation of leached chemicals from the particles showed that toxicity could mostly be attributed to these chemicals because when

separated and used alone, they had similar effects on reproduction compared to the results in the presence of the combination of the particles and leached chemicals. Effects of PVC were studied further by Liu *et al.*¹³⁴, who observed disruption of detoxification enzymes after 21 days exposure to 2 µm PVC and a change in transcription activities of superoxide dismutase and catalase enzymes after 50 µm PVC particle exposure. Both sizes of PVC particles were shown to decrease the number of neonates, but the effect of potential additives was not investigated.

Other polymer types: Khosrovyan *et al.*¹³⁷ showed no effects on survival nor on reproduction of 180 µm PA, pristine or UV-weathered, after 21 days, despite proof of ingestion of particles by *D. magna* with a high body burden. Hiltunen *et al.*⁷⁸ generated PET and ABS microplastics with a size ranging between 3.2 to 3.7 µm from a shredded bottle and a toy brick, respectively. They did not find effects on survival, size, and reproduction after 14 days of exposure to $\sim 10^5$ particles/mL of both polymer types, but microplastic represented only 1% of *D. magna* diet, and concentrations may have been too low compared to other studies to observe an effect. Zimmerman *et al.*¹³⁸ reported slight effects on survival from exposure to irregular microparticles of 10-500 mg/L of PLA and PUR with sizes up to 40 µm after 21 days exposure. They showed that effects of PUR and PLA particles were due to the particles themselves, but not their associated chemicals, highlighting that polymer composition is also an important driver of potential toxicity. Furthermore, PLA had an overall higher effect on survival, highlighting the concern for bio-based and biodegradable plastics that can be as harmful as non-biodegradable polymers.¹³⁸ Exposure to a mixture of PVA films and fibres impacted survival, growth and reproduction.¹²⁷ The LC₅₀ for the 21-day study was found to be 24.4 mg/L and no neonates were born at concentrations higher than 5 mg/L, hence total progeny could not be assessed. However, they observed an increase in organisms' growth until the end of exposure, and malformations after the 10th day. The limited

availability of research articles on potential toxicity of these polymer types highlights a knowledge gap that needs to be addressed in future studies.

2.3.3 Multigenerational effects of microplastics and nanoplastics

Multigenerational effects of microplastics and nanoplastics have been examined in 9 studies compared to the 36 studies that assessed chronic toxicity.^{69-71,133,149,154-157} The details of the studies are summarized in **Table 2.3**. Similar to the single-generation chronic studies, 7 out of those 9 studies investigated effects of PS. Reported effects varied depending on multiple factors such as polymer type, particle size and exposure time, and ranged from no sensitivity¹⁵⁵ or a decrease in population or reproduction over time¹⁵⁶ to complete population extinction.⁶⁹ Milder effects such as malformation¹⁴⁹ and overall fitness decrease¹³³, as well as subsequent effects on non-exposed offspring have also been observed, which could indicate potential long-term effects, such as population weakening.

Besseling *et al.*¹⁴⁹ found that aged PS increased malformation rates up to 68% starting from 30 mg/L in the F₁ generation. Another study using the same duration of exposure with a similar particle size of ~73 nm observed impacts on the reproduction and age at first brood of F₁ and F₂ generations of *D. magna* after exposure to 6.62 mg/L and 13.24 mg/L PS.¹⁵⁶ Similar results were found for PS⁷¹ and PE microplastics⁷⁰. One study using commercial particles of unknown polymer type found a complete extinction of *D. magna* population after 2 generations upon 21 days exposure to 0.1 mg/L pristine microplastics ranging from 1 to 5 µm in size, however there are no information regarding additives potentially present in the commercial suspension that may influence toxicity results.⁶⁹ Schur *et al.*¹³³ showed that irregular ≤ 63 µm PS particles generated

from coffee lids decreased size and growth of *D. magna* across 4 generations at a concentration of 2000 particles/mL, and resulted in extinction within one generation at 10000 particles/mL. They also tested the fitness of neonates from each generation by exposing them to potassium dichromate but saw no significant effects - hence maternal exposure to PS particles had no impact on their offspring's fitness. Using the same particles, the authors conducted a second multigenerational study over 21 days investigating (1) effect of aged versus pristine particles and (2) potential toxicity mitigation using wastewater for PS incubation, with a broader range of concentrations (from 80 to 10000 particles/mL, Schur *et al.*¹⁵⁷). They observed that both particle types affected survival, reproduction, and organisms' length - with a concentration-dependent impact on survival in F₀-F₂, but not F₃. The main effect observed at the lowest concentration (80 particles/mL) was 25% mortality in the fourth generation. Similar to the earlier study, only 5% of the organisms survived when exposed to 10000 particles/mL. Interestingly, PS incubation in wastewater decreased overall mortality despite no observed changes in particle size, surface, and structure.

Other research on 20 nm PS particles reported opposite results, with no effects on offspring growth and reproduction despite proof of particle transmission to the next two generations at 50 mg/L.¹⁵⁵ Similarly, a mesocosm study over 12 weeks using 15 µm unwashed PS particles at 100 particles/mL saw weekly changes in *D. magna* abundance in the first half of the mesocosm experiment, but the population recovered and no effects were observed after 12 weeks, suggesting an acclimatization over time.¹⁵⁴

Most studies focused on apical and population endpoints, such as survival, number of broods and growth, as they are considered the most relevant to evaluate long-term effects. Only one study aimed at investigating epigenetics molecular biomarkers as a potential mechanism for multigenerational stress response upon microplastic exposure in *D. magna*.⁷⁰ The toxicity of 16.7

µm PE (4.35 mg/L) was studied for 21 days across 4 generations - direct exposure induced mortality, but the decrease in population was recovered after 3 generations. Growth and reproduction remained lower than the controls despite no DNA methylation observed across generations. This study highlights that mechanisms other than epigenetics may play key roles in long-term toxicity and response to microplastic exposure.^{69,70}

Table 2.3. Studies investigating the multigenerational toxicity of microplastics and nanoplastics to *D. magna*. -COOH means carboxylated functional group. (f) means fluorescently labelled.

Type of plastic	Shape	Sizes	Concentration	Source of plastic	Was the plastic washed	Duration of test	Number of generations	Targeted endpoints	Refs.
unspecified (f), (1.30 g/cm ³)	spheres	1-5 µm	0.1 mg/L	Cospheric, USA	unknown	21 days	F ₀ , F ₁ , F ₂ , F ₃	survival; growth (body length); reproduction (time to first brood, number of brood, number of neonates, number of dead/immobile neonates)	⁶⁹
PE	fragments	16.68 ± 7 .04 µm	4.35 mg/L	Cryomilling of PE pellets	yes (rinsed with hexane, methanol, and deionized water)	21 days	F ₀ , F ₁ , F ₂ , F ₃	survival; growth (somatic growth); reproduction (number of neonates); other (gut damage, global DNA methylation)	⁷⁰
PS-COOH (f)	spheres	15 µm	100 particles/L	Sigma Aldrich, UK	unknown	12 weeks	unspecified	survival; growth (body length); reproduction (number of neonates)	¹⁵⁴
PS	fragments	≤ 63 µm	80, 400, 2000, and 10000 particles/mL	PS lids of coffee-to-go cups	unknown	21 days	F ₀ , F ₁ , F ₂ , F ₃	survival; growth (body length of adults and neonates); reproduction (time to first brood, number of neonates)	¹⁵⁷
PS (f)	spheres	5 µm	2.5 µg/L	Da'e Scientific Co., Ltd, China	unknown	21 days	F ₀ , F ₁	survival; growth (body length); reproduction (time to first brood, number of neonates); other (population growth rate, swimming and feeding behavior)	⁷¹
PS (f)	spheres	20 nm	1 and 50 mg/L	ThermoFisher Scientific, Canada	yes (dialysis)	Until the production of first neonates (F ₁)	F ₀ , F ₁ , F ₂	survival; growth (body length); reproduction (number of neonates); others (swimming distance, heartbeat rate, appendage beat rate, tentacles beat rate, post-abdominal curling rate)	¹⁵⁵
PS	fragments	≤ 63 µm	400, 2000, and 10000 particles/mL	PS lids of coffee-to-go cups	unknown	21 days	F ₀ , F ₁ , F ₂ , F ₃	survival; growth (body length of adults and neonates);	¹³³

PS (f)	spheres	70 nm	0.22, 0.44, 0.88, 1.8, 3.5, 7, 14, 32, 70, and 150 mg/L	AVT-PCC, Wageningen UR.	unknown	21 days	F ₀ , F ₁	reproduction (time to first brood, number of neonates) survival; growth (body length of adults and neonates); reproduction (total number of neonates in first three broods, number of neonates/brood); other (malformations in neonates)	149
PS (f)	spheres	73 ± 7 nm	1.65, 3.31, 6.62, and 13.24 mg/L	Synthesized in the laboratory	unknown	21 days	F ₀ , F ₁ , F ₂	survival; growth (body length of neonates); reproduction (time to first brood, number of neonates, number of neonates/brood)	156

2.3.4 Impacts of co-contaminants on toxicity results

In polluted environments, *D. magna* is not only exposed to plastics, but also to co-existing contaminants when they accidentally ingest or encounter microplastics. The vector role of microplastics and the combined pollution exposure complicate their toxic effects on *D. magna*. Commonly found co-existing contaminants mainly include two categories: plastic additives released from the particles themselves and environmental pollutants sorbed onto the particles from the environment.¹⁵⁸

Impacts of plastic additives

Plastic additives primarily refer to chemicals that are incorporated into plastics during the manufacturing process to meet different product performance requirements. These chemicals include plasticizers, antioxidants, adhesives, antimicrobials, stabilizers, and flame retardants. Other chemicals, such as certain catalysts used during manufacturing and remaining oligomers, alongside non-intentionally added substances, can also be present at high concentrations and behave similarly to additives. **Table 2.4** presents a summary of studies which have investigated the impacts of plastic additives in the observed toxicity of *D. magna*.

Table 2.4. Studies investigating plastic additives and microplastics co-exposure to *Daphnia magna*. Where applicable, circled numbers were used to distinguish particles that possess different properties within the same study.

Plastic Type	Size and Shape	Exposure concentration	Type of exposure	Observed effects from leachates	Additives	Concentration of additive in leachate	Common percentage in plastic	Plastic concentrations in medium (solid to liquid ratio)	Additives leaching %	Mixture toxicity	Refs.
① rigid PVC ② flexible PVC	① 4-141 µm (fragments) ② 12-276 µm (fragments)	N/A	chronic (25-31 d)	Rigid PVC caused delay in time to first brood Flexible PVC increased body length and reduced reproduction Delayed reproduction, reduced body length	diisononyl phthalate (endocrine-disrupting chemical)	2.67 µg/L	N/A 30%	N/A 1:2000	N/A	synergistic	135
① PVC ② PUR ③ PLA	① ≤ 20 µm; ②③ ≤ 40 µm (fragments)	① 500 mg/L ②③ 0.2-500 mg/L	chronic (21 d)	Reduced the reproduction, reduced the somatic growth of daphnids	N/A	N/A	N/A	1:5000000 1:500000, 1:50000 3:5000, 1:10000, 1:2000	N/A	synergistic	138
PET	length: 60-1400 µm; width: 31-528 µm; diameter: 2-21.5 µm (fibres)	12.5, 25, 50, and 100 mg/L	acute (48 h)	Mortality increased	N/A	N/A	N/A	12.5, 25, 50, and 100 mg/L	N/A		107
PE	39.72 ± 14.45 µm (fragments)	20 mg/L	acute (48 h)	Mortality increased	benzophenone-3	10.27 ± 0.40%	5-10%	1:2000, 1:50000	41.1 ± 2.7%	synergistic	101
Mixture (HDPE, PET, PP,	≤ 17 µm	10% and 50% of the plastic leachate.	acute (48 h)	No mortality	Mixture: BPA confirmed	N/A	0.20%	1:50	N/A	independent	155

and nylon)		100% of the plastic leachate.	chronic (10 d)	Plastic leachate reduced the appendage curling rate but increased growth and reproduction. No effect on the heartbeat rate and swimming distance was observed.							
PA	15-20 µm (fragments)	200 mg/L	acute (48 h)	No leachate effect on acute exposure.	N/A	N/A	N/A	1:500	N/A	N/A	159
rubber	155.72 ±143.68 µm (fragments)	1000 mg/L 3.125%, 6.25%, 12.5%, and 25% of the sneaker leachate.	acute (24 h and 48 h)	sneakers (shoes) leachate showed immobility and mortality	benzothiazole, carbon disulfide, ethyl acetate, p-xylene, Zn, As, etc.	N/A	0.1%, 0.5%	1:1000; 1:500	N/A	synergistic	160
Bakelite	7.6 ± 3.5 µm (fragments)	15, 25, 30, 40, 50, 60, 75, and 100 mg/L	acute (24 h and 48 h)	Leachates had more severe adverse effects than microplastics alone Survival rate decreased, number of offspring decreased (F ₃), population growth rate decreased.	phenol and phenol-like compounds	N/A	0.1%, 1.0%	1:10000	N/A	synergistic	161
PE	17.35 ± 5.50 µm (fragments)	5 mg/L	multi-generational (F ₀ -F ₃)	Survival rate decreased, number of offspring decreased (F ₃), population growth rate decreased.	benzophenone-3	N/A	2.85 ± 0.16%	1:200000	N/A	synergistic	70
PP	diameter: 21.2 ± 1.5 µm (inner layer), 22.1 ± 1.6 µm (outer layer), and 4.3 ± 2.2 µm middle	1, 10, and 100 mg/L	acute (24 h and 48 h)	No effects on mobility and survival	mixture	N/A	N/A	1, 10, and 100 mg/L	N/A	N/A	119

	layer). Length not provided (fibres)											
PPDO, PE, PS	5 mm (pellets, films)	0.0001, 0.001, 0.01, 0.1, and 1 g plastic/L leachate	acute (48 h)	Elevated mortality occurred in biodegradable plastics (PPDO) compared to conventional plastics (PE and PS)	mixture	N/A	N/A	0.0001, 0.001, 0.01, 0.1, and 1 g plastic/L leachate	N/A	N/A	162	
PVC	2-3 mm (fragments)	10, 100, and 500 mg plastic/L leachate	acute (48 h); chronic (F ₀ -F ₁)	Low mortality occurred, decrease in population development	mixture	N/A	N/A	10, 100, and 500 mg plastic/L leachate	N/A	N/A	163	
PE	0.2-9.9 µm, 30-45 µm, 300-354 µm (spheres)	1, 2.5, and 5 mg in 70 mL of assay media with and without BACs.	acute (48 h)	Survival rate increased.	benzalkoni um chlorides: (BAC ₁₂ (C ₂₁ H ₃₈ NC l), BAC ₁₄ (C ₂₃ H ₄₂ NC l), BAC ₁₆ (C ₂₅ H ₄₆ NC l))	N/A	N/A	1:9	N/A	antagonistic	129	
PE	44.39 ± 11.16 µm (fragments)	4.5 mg/L	chronic (17 d)	Embryonic development inhibited, and offspring growth decreased.	benzophen one-3 (BP- 3)	(10.82 ± 1.20% w/w)	2.85 ± 0.16%	1:200	N/A	synergistic	126	
PP, HDPE, PVC, ABS, and epoxy	4 cm ² (square pieces)	250 g particle/L	acute (24 h and 48 h)	Leachates from plasticized PVC and epoxy had more severe effects compared to HDPE. No effects observed in leachates from PP,	N/A	N/A	N/A	1:4	N/A	N/A	164	

ABS, and rigid
PVC.

According to the studies reported in **Table 2.4**, there are already 104 kinds of additives or non-intentionally added substance that have been reported in the leachates of microplastics^{119,162,163} (**Figure 2.4**), which may represent only a fraction of the range of chemicals incorporated into plastics. When plastics are degraded and fragmented into smaller microplastics and nanoplastics, the higher specific surface area facilitates migration of plastic additives into the environment.^{17,158,165}

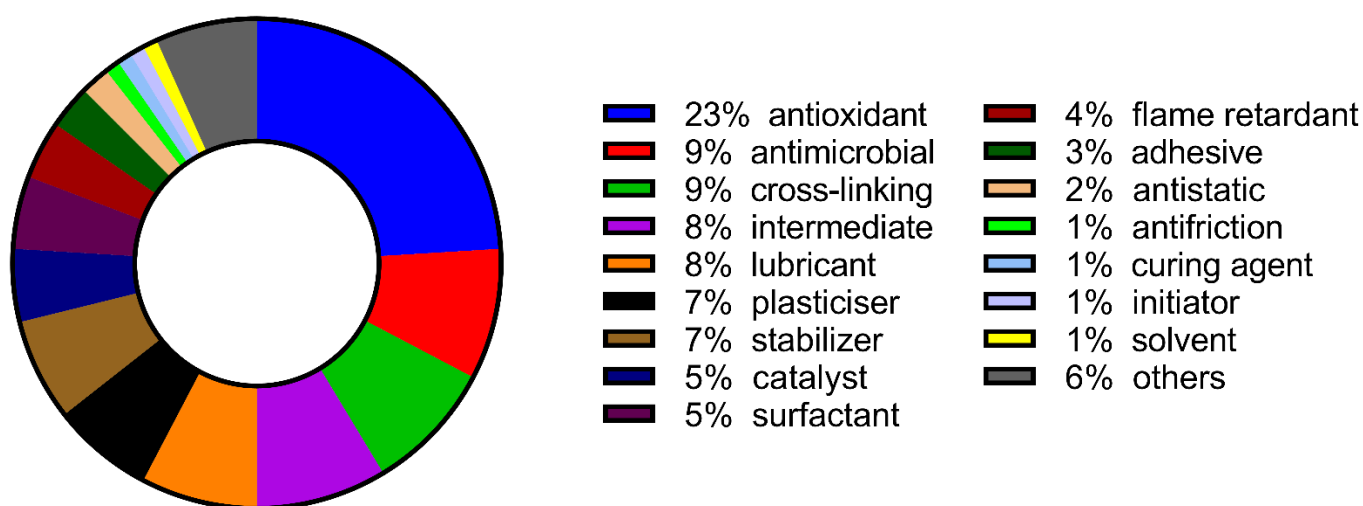


Figure 2.4. Compositional ratios of the various types of additives in plastic leachate mixtures. The analyzed data were obtained from the *D. magna* toxicity studies listed in **Table 2.4** which have (tentatively) quantified the additive compositions in the plastic leachates.

The leaching of additives makes the environmental behavior of microplastics very complex and its toxicological effects difficult to predict especially when a mixture of compounds is released. The composition of leached additives and their toxic effects have been studied extensively. The proportion of additives incorporated in plastics can be quite high and thus their leaching is

generally unavoidable. As shown in **Table 2.4**, the content of some commonly used additives in plastics ranged from 0.1-30% w/w while sometimes this percentage can be even higher, reaching 180% for plasticizers (more than the polymer itself) in soft rubber-like plastics, according to some manufacturers.^{70,101,126,135,160,161} Moreover, many plastic additives, such as phthalates, are in the form of non-covalent bonds, and therefore they can be easily released upon entering the environment.¹⁶⁶

Aquatic organisms in the natural environment, such as *D. magna*, can be exposed to plastic additives during both aqueous and dietary exposure to microplastics which subsequently may lead to toxicological effects. *D. magna* has been exposed to additives such as antioxidants, plasticizers, and stabilizers to evaluate their toxicological effects. In most cases, co-exposure of microplastics and additives usually leads to synergistic effects.^{72,135,155,160,161} For instance, in comparison to the additive benzophenone-3 alone, the presence of microplastics further enhanced the toxicity of benzophenone-3 by increasing the generation of reactive oxygen species and lipid peroxidation levels. This is partly due to the increased bioconcentration of benzophenone-3 in *D. magna* in the presence of microplastic, and because irregular microplastic fragments (20 mg/L, 39.72 ± 14.45 μm) caused physical damage which subsequently caused severe intestinal injury.¹⁰¹

The solid-to-liquid ratio (SLR) varies widely among different studies and can directly affect the concentrations of released additives (**Figure 2.5a**). Although there is no significant difference between the laboratory-simulated solid-to-liquid ratio (10^{-7} ~ 10^0) and the real environmental solid-to-liquid ratio (10^{-7} ~ 10^{-3}), the average solid-to-liquid ratio value in exposure studies is two orders of magnitude higher than that in the real environment. Hence, most results mainly reflect the potential toxicity of combined pollution of microplastics and leached additives in pollution hotspots. Also, we observed that, with the exception of bisphenol A (BPA), which was much

higher than those in the field, the co-exposed pollutant concentrations were roughly in the same range as those in the actual environment (**Figure 2.5b**).

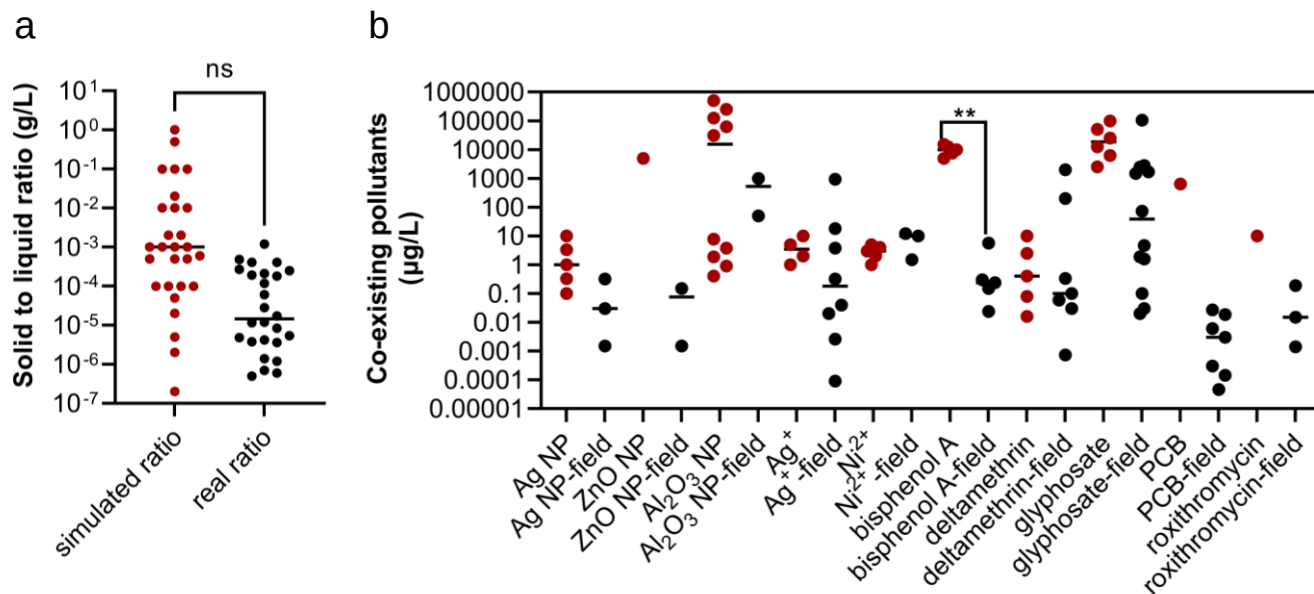


Figure 2.5. (a) Co-exposure with additives: the plastic weight to liquid volume ratio; Simulated ratios are used in laboratory simulation experiments, while real ratios are plastic mass to liquid volume ratios found in nature. (b) Co-exposure with environmental pollutants: the concentrations used in experiments (red dots) versus those measured in the field (black dots).

It should be noted that the reporting of the final aqueous concentrations and/or released percentages of additives is largely missing. Only very limited studies quantified the freely dissolved additive concentrations. Schrank *et al.*¹³⁵ reported the final aqueous freely dissolved concentration (2.67 $\mu\text{g/L}$) of diisononyl phthalate from PVC microplastics, which is important for the understanding of the joint toxicity of microplastics and additives. In addition to the fact that the quantification of additive concentrations was largely unknown in most studies, there are also two other issues that

require consideration. First, plastics often contain a variety of additives, and their composition is predicted by non-target screening, and identification of each compound is time consuming and sometimes very difficult due to a lack of standards. Second, as most researchers are unable to obtain the actual amounts of additives incorporated in plastics, the percentage of additives released is difficult to determine. Also, we noted that little attention has yet been paid to emerging flame retardants, such as organophosphates, and novel brominated flame retardants, which are used as substitutes for conventional flame retardants, *i.e.*, polychlorinated biphenyls and polybrominated diphenyl ethers. Moreover, some plasticizers can reach an extremely high incorporation rate (*i.e.*, 70%)¹⁶⁷, which also deserves more attention in the future.

Impacts of environmental pollutants.

In this review, the environmental pollutants on microplastics refer to the associated pollutants coming from the surrounding environment, which can sorb onto microplastics, and most have reached sorption-desorption equilibrium and are at relatively lower concentrations in comparison to plastic additives.

Several studies have employed laboratory simulation approaches to explore the impacts of co-exposure of environmental pollutants and microplastics or nanoplastics to *D. magna* (**Table 2.5**). However, most of these works utilized similar research methods and experimental designs to those for microplastic additives, and thus the results cannot well reflect the real accumulation and/or toxicity of these associated environmental pollutants. First, the tested concentrations of the environmental pollutants are considerably higher compared to those detected in the natural environment and no background trace contamination was taken into account.¹⁵⁹ Second, various

joint toxic action modes, such as synergistic,^{100,114,116,121,156,168} antagonistic,^{159,169} and independent,¹⁷⁰ have been reported for the co-exposure of microplastics and environmental pollutants; however, most of the studies do not reflect the actual scenario in the natural environment. Specifically, most laboratory-based studies adopted the non-equilibrium experimental design, only using pollutant-contaminated microplastics for exposure, without considering other ubiquitous environmental matrices (e.g., natural organic matter, food particles, and sediments), and only clean organisms were used for testing, which promotes the migration of pollutants from microplastics to *D. magna*.

Table 2.5. Studies investigating environmental pollutants and microplastics co-exposure to *Daphnia magna*. -COOH and -NH₂ means carboxylated and aminated functional group, respectively. (f) means fluorescently labelled. Where applicable, circled numbers were used to distinguish particles that possess different properties within the same study.

Type of test	Plastic Type	Size and shape	Exposure concentration	Co-exposed compound name and CAS number (if available)	Compound concentration	Mixture toxicity	Effects on <i>D. magna</i>	Refs.
acute (48 h)	PS; PS-COOH	~200 nm (spheres)	1, 5, 10, 20, and 30 mg/L	nickel	1, 2, 3, 4, and 5 mg/L	when combined with Ni, PS had a slight antagonistic effect on toxicity, whereas PS-COOH had a slight synergistic effect.	Higher immobilization toxicity observed in combined exposure group in comparison to microplastics alone	¹¹⁶
chronic (7 days)	PS	1 µm and 10 µm (spheres)	0.1 mg/L	roxithromycin	0.01 mg/L	synergistic effect	Glutathione peroxidase and glutathione S-transferase enzyme activities and malondialdehyde levels decreased in combined exposure group in comparison to microplastics alone	¹¹⁴
chronic (7 days)	① PE ② PET/PA	① 1-10 µm, average diameter of 2.09 µm (spheres); ② length = 10 µm; width = 2 µm (fibres)	① 2.2×10 ⁶ particles/mL (0.01 mg/mL) ② 10-30 particles/mL	① glyphosate acid; 2.5 mg/L ② a commercial formulation of Roundup: 47.5% n-(phosphonomethyl)-glycine-monosodium salt powder; ③ n-(phosphonomethyl)-glycine, monoisopropylamine salt solution		① glyphosate acid, but also with Roundup Gran: synergistic effect ; ② glyphosate-IPA salt: antagonistic effect	Higher mortality was observed in combination with glyphosate acid and Roundup Gran, but decreased when in combination with glyphosate-IPA salt	¹⁶⁸
acute (48 h)	PS	100 nm (spheres)	1-75 mg/L	polychlorinated biphenyl-18	640 µg/L	antagonistic effect (the addition of plastic could decrease the free-dissolved concentration of organic pollutants and the	Lethality decreased when polychlorinated biphenyl combined with low amounts of PS nanoplastics, whereas the lethality	¹¹⁷

						toxicity. Meanwhile, the excessive amounts of plastic could also cause toxic effect towards <i>D. magna</i>) antagonistic effect	was enhanced when combined with excessive amounts of PS nanoplastics.	
acute (48 h)	PA	15-20 µm (fragments)	200 mg/L	bisphenol A	5, 7.5, 10, 12.5, and 15mg/L		The combination of BPA and PA led to decreased immobilization.	159
acute (72 h)	PS (f)	1.2 ± 0.2 µm (spheres)	0.29 µg/mL	dimethoate, deltamethrin	0.156-5 mg/L for dimethoate, 0.016-10 µg/L for deltamethrin	no effects (no alteration in the acute toxicity of either deltamethrin or dimethoate to <i>D. magna</i> , regardless of the chemical binding capacity (log K _{ow}). Mortality and mobility impairment increased with concentration and time for both pesticides, however the concentrations at which detrimental effects occurred were not influenced by the presence of microplastics.	Pesticide toxicity was not affected by the presence of microplastics	170
acute (48 h)	PS (f)	~73 ± 7 nm (spheres)	15.6, 31.2, 62.5, 125, 250, and 500 mg/L	glyphosate	6.2, 12.5, 25, 50, 100, and 200 mg/L	synergistic effect	Increase in immobility and reactive oxygen species production and decrease in swimming activity for combined exposure.	156
acute (72 h)	① PS ② PE	① 300 and 600 nm (spheres) ② 300-9000 nm (spheres)	6.74 × 10 ¹⁰ particles/L	① Ag ⁺	① Ag ⁺ : 1, 2, 5 and 10 µg/L	① Ag-PS-NPD: synergistic effect ; ② Ag-PE-NPD: no effects. PE-NPD did not change the toxicity profile of 1 µg/L Ag ⁺ compared to the PS-NPD and the control. The presence of PS-NPD led to an increased toxicity of Ag ⁺	When the Ag ⁺ concentration in the exposure media increased the survival decreased.	100
acute (48 h) chronic (21 days)	PS (f)	~90 nm (spheres)	31.25, 62.5, 125, 250, and 500 mg/L 0.4, 0.9, 1.9, 3.8, and 7.7mg/L	palmitic acid	N/A	synergistic effect	PSNP/F-PA was almost 3 times more toxic than PSNP, led to reproduction inhibition and mortality following long-term exposure at lower concentrations. Palmitic acid induced increased immobility and reduced reproduction	146

acute (48 h - 96 h)	PET	diameter: 14 ± 3 µm; length: 366 ± 275 µm (fibres)	100 mg/L	① AgNP ② AgNO ₃	0.33 µg Ag/L	synergistic effect	Sublethal effects of cellular energy allocation was decreased ¹²¹
acute (8 h)	PS	0.1 µm (spheres)	1 mg/L	nanoparticles	5 mg/L	synergistic effect	Increased the ion-related toxicity (mortality) in the combined exposure group ¹⁷¹
acute (48 h)	PS-COOH, PS-NH ₂	200 nm (spheres)	10-400 mg/L	① alginate ② SRHA/Humic acid	2 mg/L	antagonistic effect	Significantly reduced the immobilization of both amidine and carboxyl PS nanoplastics ¹²⁴
acute (up to 72 h)	PS (f)	1 µm (spheres)	1820 particles/mL (10 ⁻⁹ g/ mL)	chromium	2 and 5 mg/L	synergistic effect	Mortality increased with the presence of microplastics. ¹⁷²
acute (24 h and 48 h)	PS (f)	6 µm (spheres)	10 mg/L		0.1-1.0 mg/L		Acute mortality increased with the presence of microplastics. ¹²⁸
chronic (21 days)			5 mg/L	chromium	10 and 100 µg/L	synergistic effect	Survival, growth, and reproduction decreased in co-exposure to 100 µg/L chromium
chronic (21 days)	PS-COOH (f)	2 µm (spheres)	1 and 10 mg/L	zinc	N/A	synergistic effect	Reproduction reduced, body length decreased, survival rate decreased. ¹⁷³
chronic (21 days)	PE	1-4 µm (spheres)	1 and 10 mg/L	pyrethroid insecticide (deltamethrin)	40 ng/L	synergistic effect	Increase in mortality and a reduction in longevity, number of neonates per surviving adult, number of broods, body length and cumulative number of molts ¹⁷⁴
acute (48 h)	PS	30 nm (spheres)	12.5, 25, 50, 100, and 200 mg/L	Wastewater effluent containing chemicals such as antibiotics, anti-inflammatories, contrast agents, antidepressants, beta-blockers, and several intermediate metabolites, etc.	N/A	antagonistic effect (the formation of PS-wastewater heteroaggregates was observed which could result in lower bioavailability of PS NPs and sorbed micropollutants, thus lowering toxicity.)	A decrease of toxicity when <i>D. magna</i> was exposed to PS-wastewater mixtures ¹⁷⁵

chronic (21 days)	unspecif ied (f), (1.30 g/cm ³)	1-5 µm (spheres)	0.05, 0.10, and 0.20 mg/L	lithium	0.02, 0.04, and 0.08 mg/L	The toxicological interaction between lithium and microplastic was antagonism at the lowest and the highest concentrations, and synergism at the medium concentration.	Significantly reduced the population fitness. Growth and reproduction decreased

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In a real co-exposure scenario of microplastics and environmental pollutants, the pollutants have (nearly) reached equilibrium, and other particle/matter migration routes for environmental pollutants should be considered besides the one mediated via microplastics. Koelmans *et al.*¹⁷⁷ verified that the contribution of environmental pollutants to bioaccumulation through microplastics is negligible and far below that of other natural matrix carriers in a quantitative modeling study. Recently, we also demonstrated that microplastic has negligible contribution to the bioaccumulation and toxicity of the co-existing environmental pollutants at environmentally relevant microplastic concentrations when compared with other natural particles.¹⁵⁸

Research on the toxic effects of microplastics and co-existing pollutants on *D. magna* is newly emerging. There is still a dearth of information on the conditions in which the presence of multiple pollutants can have synergistic adverse effects. We also need to pay more attention to additives and investigate their toxicity in microplastics independently of the coexisting environmental pollutants.

General toxicity endpoints (*i.e.*, immobilization, mortality, and reproduction) are commonly monitored in combined pollution exposure studies (**Figure 2.6**). Less attention has been paid to the specific effects of additives or environmental pollutants. For instance, the baseline toxicities of Ni, bisphenol A, and deltamethrin have been studied in the presence of microplastics, mainly focusing on the mortality, immobilization endpoints.^{100,170,178} However, some unique sublethal toxicities of these chemicals, such as the respiration function impairment caused by Ni,¹⁷⁹ the affected energy metabolism and neurotoxicity caused by bisphenol A and pesticides,^{178,180} can also be affected by the presence of microplastics but have not been emphasized yet.

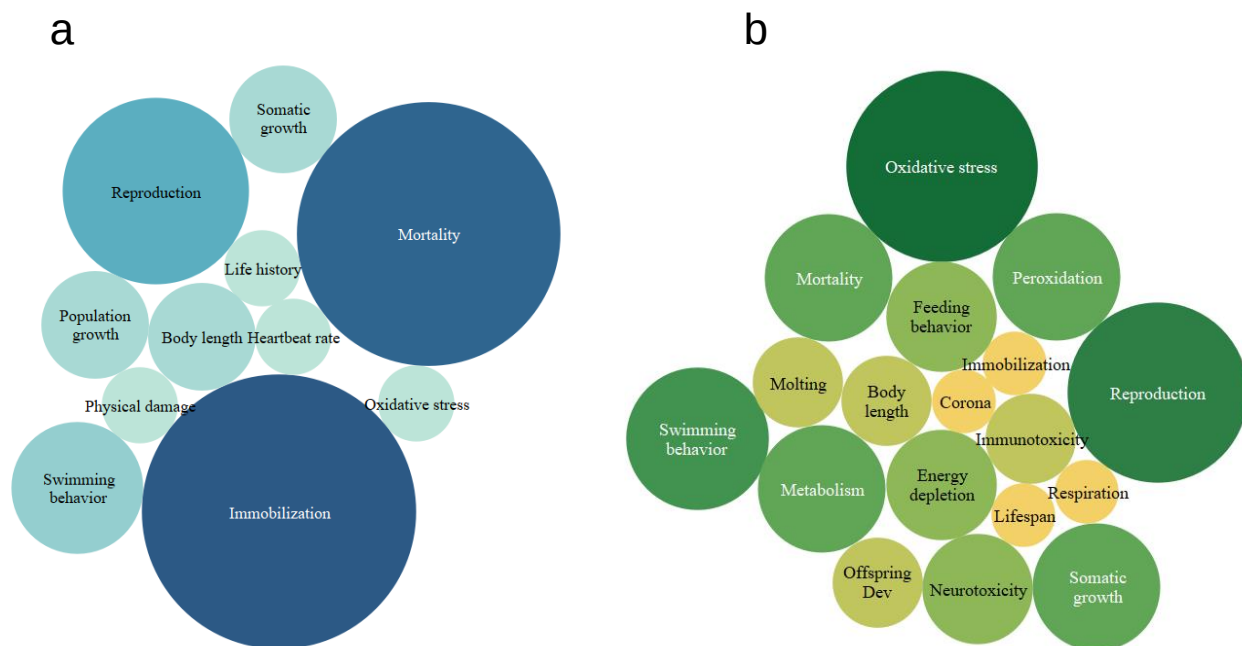


Figure 2.6. Different bioassay endpoints in exposure experiments of: (a) microplastics and co-existing pollutants (including both additives and environmental pollutants) and (b) co-existing pollutants alone.^{119,155,162} The size of each circle represents the relative number of studies incorporating the bioassay endpoints.

Additives and environmental pollutants have different sources, exposure concentrations and distinct environmental behaviors. For the co-exposure of microplastics and environmental pollutants, it is necessary to carry out studies under relative equilibrium scenarios at concentrations closer to environmentally relevant ones, and the influence of natural substances (e.g., food particles, natural organic matter, sediments) should be considered for reliable conclusions to be made.

Currently, around 75% of additive co-exposure studies focused on only a single additive. But a cocktail of various additives is present in microplastics, and thus the mixture toxicity becomes extremely complex. Only a few studies have explored the mixture toxicity of additives. For instance, Kokalj *et al.*¹¹⁹ and Dao *et al.*¹⁶³ analyzed the toxic effects of additive mixtures released from PP and PVC microplastics on the mortality and population development of *D. magna*. Recently, Gao *et al.*¹⁶² also developed the *D. magna* toxicity test into a rapid screening method for microplastic additive mixture toxicity analysis. More studies are needed in this area to explore the effects of additive mixtures.

2.4 Methodological Innovations and Limitations of the Study

This study employed an innovative approach to conduct a comprehensive review of the state of knowledge and available data for the toxicity of micro- and nanoplastics to *Daphnia magna*. First, the study conducted a rigorous screening using detailed inclusion and exclusion criteria to ensure unbiased selection of relevant papers and increase the degree of comparability of the toxicity results reviewed. Another methodological innovation is the analysis and discussion of different types of toxicity (i.e., acute, chronic, multigenerational, and co-contaminant studies) on a single organism, *Daphnia magna*. This approach allowed for a more comprehensive evaluation of the toxicity of the particles, capturing the different endpoints associated with each toxicity type. Also, the synthesized data from each type of toxicity enabled the identification of potential patterns or trends in the observed toxicities for the different plastic polymer types. Furthermore, for the first time, this study used a targeted research synthesis method to present a graphical representation of the acute toxicity of micro- and nanoplastics and the potential effects of the relationship between

particle size and exposure concentration which will improve our understanding of existing data in this area.

Despite these innovative methods, we recognize that the study may have a few limitations which are highlighted below:

Publication Bias: This review paper only assessed the results of published scientific papers. Hence, it may be biased by the fact that some studies showing insignificant toxicity (i.e., no effects studies) or positive effects to *Daphnia* may not have been submitted to peer-reviewed journals for publication.

Limited Long-Term Studies: Only very few studies investigated the multigenerational effects and even fewer studies investigated longer term effects beyond the 21-day duration that is typical for chronic studies. Considering the potential for prolonged exposure of micro- and nanoplastics in the natural environment, these short-term studies may not fully cover the environmental impacts of micro- and nanoplastics to the model organism, *Daphnia magna*.

Focus on a Single Specie: While *Daphnia magna* is commonly used as a model organism for micro- and nanoplastic toxicity studies and toxicity studies in general, it is essential to acknowledge that the toxicity results observed upon exposure to this organism may not fully represent the effects of the same particles on other aquatic invertebrates. Hence, readers are encouraged to exercise caution when extrapolating findings from this review to other species or organisms.

Lack of Real-World Complexity: The acute, chronic, and multigenerational experiments conducted in the reviewed papers employed simplified laboratory conditions in which the organisms were exposed to the particles, rearing media, and food for longer term studies. Also, the

co-contaminant studies limited co-exposure to single pollutants being investigated. Hence, the absence of other factors or materials that are present in the real environment such as fluctuating temperature, interaction with mixture of pollutants or contaminants, interaction with natural organic matter, etc. may limit the environmental relevance of the studies or the findings of this review.

2.5 Conclusions and Future Directions

Research findings show that there is no one factor that governs the toxicity of the particle to an organism. Larger microplastics (e.g., $> 35 \mu\text{m}$) do not have impacts towards *D. magna* at environmentally relevant concentrations - both in the short term and the long term while nanoplastics exhibit high toxicity levels not only on short term exposure but also in the long term with impacts on reproduction and gut integrity of the exposed organism. Existing data suggests that smaller sized particles may be more bioavailable to *D. magna*. It is unclear whether fragments are more toxic than their spherical counterparts as only a limited number of studies have explored this topic. Moreover, most of the studies using fragments either focused on short-term exposures or used large-sized particles that may not be ingested by the organisms.

Polymer type and tested concentrations may also play important roles. However, in this review, most of the studies used PS particles with a limited number of studies exploring the impact of other polymer types. Also, it is difficult to compare concentrations across different plastic types owing to differences in shapes, sizes, and densities and that several studies use different concentration metrics such as mass of particles or number of particles.

One of the common reasons for using spherical microplastics and nanoplastics was because of their potential release from personal cosmetic products. However, the studies which have obtained microplastics from these products found that the particles appear as fragments rather than spheres. Furthermore, most of these products are gradually being banned by various countries. Hence, although use of these “simple-shaped” particles is an important first step in understanding the isolated impacts of plastics before advancing into more complex parameters or environmental interactions, it is equally important to increase the focus on other shapes of microplastics and nanoplastics.

Finally, observed toxicity is not limited to isolated plastic; it can also arise from the presence of leached chemicals or additives. Based on our review, we have identified several gaps and suggested the following recommendations for future research to improve our understanding of the toxicity of microplastics and nanoplastics:

1. A vast majority of studies have focused on spherical particles, particularly PS. Future research should aim to explore the impacts of other polymer types. Also, there is a need for further studies to assess the impacts of particle shape, particularly the toxicity of irregular-shaped particles which may be more representative of the particles in the real environment.
2. Only limited studies carry out characterization to understand the properties of the tested particles. In fact, there are studies in which the polymer types were neither identified by the vendors nor verified by the researchers. Future toxicity assessments should include comprehensive characterization of the particles used.
3. Future studies are encouraged to use standard protocols and parameters including number of replicates, duration or exposure, and validity of controls to ensure that the results are

comparable, for future meta-analysis to further improve our understanding of the toxicity of microplastics and nanoplastics.

4. While an increasing number of studies are washing tested particles before toxicity exposure, most studies still use the particles or suspensions as purchased. Commercial preparations of microplastics and nanoplastics often contain additives which may confound toxicity results. To aid the comparability of toxicity data and to be able to separate the effects of solution additives from the particles and their leached chemicals, there is a need to wash commercial suspensions of particles used for toxicity exposures.
5. Although it could be argued that there is still limited data and technological methods to inform the actual concentration of micro- and nanoplastics in the aquatic environment, several studies have tested considerably high concentrations, up to thousands of mg/L. Hence, there is a need for more studies using advanced techniques to understand the existing range of concentrations of plastic particles in the environment to better inform the tested concentrations in future toxicity studies.
6. More research efforts are needed to understand the impacts of leached chemicals, additives, and co-contaminants, as well as other environmental factors such as natural organic matter, temperature, and pH.

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2.5 Supporting Information

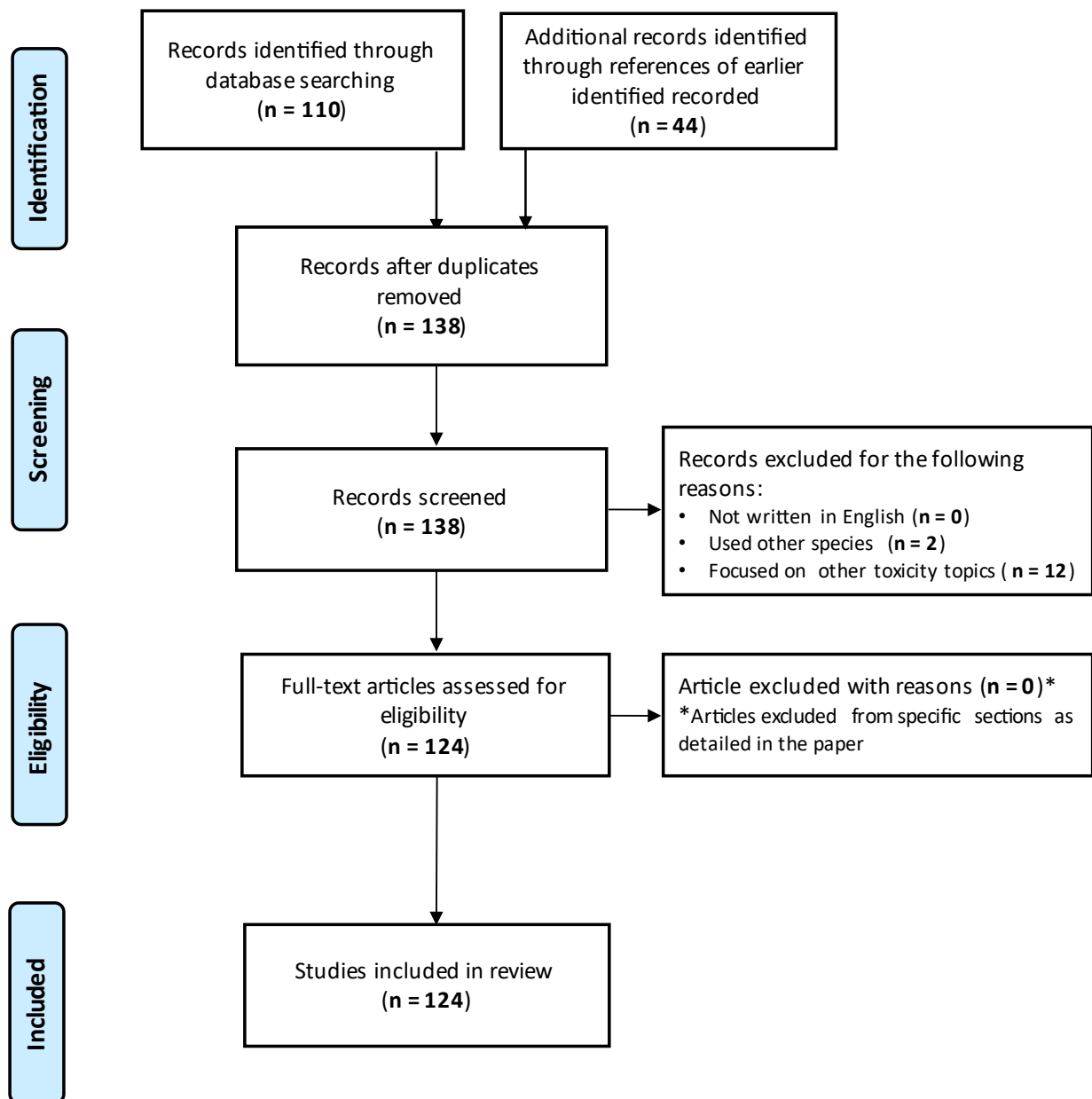


Figure S2.1. PRISMA flow diagram showing the screening and selection process. Initial electronic search was conducted using four different databases namely, Google Scholar (scholar.google.com), Science Direct (sciencedirect.com), Toxicity of Microplastic Explorer (sccwrp.shinyapps.io/aq_mp_tox_shiny), and PubMed (pubmed.ncbi.nlm.nih.gov). A total of 154 articles were first identified. 16 of them were duplicates while 14 were excluded for other reasons. A total of 124 articles were eligible and included in review.

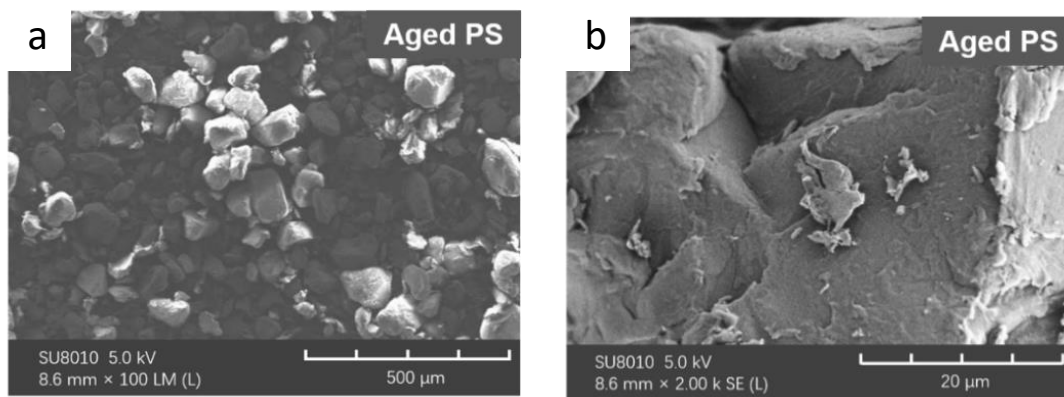


Figure S2.2. Scanning electron microscopy images of aged PS under (a) lower and (b) higher magnifications. Reproduced from Chen *et al.*¹ (with permission from Elsevier).

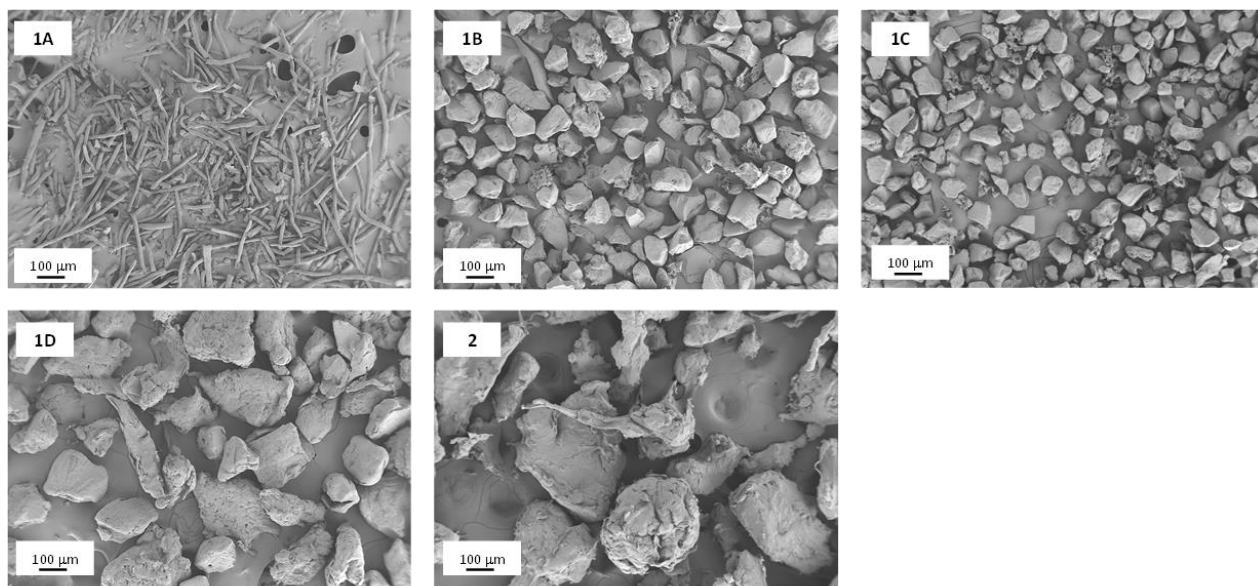


Figure S2.3. Scanning electron microscopy images of microplastics obtained from different facial cleansers. Reproduced from Kokalj *et al.*² (with permission from Elsevier).

Table S2.1. Studies investigating the acute toxicity of microplastics and nanoplastics to *D. magna* (excluded from the targeted research synthesis). -COOH, -HSO₄, and -NH₂ means carboxylated, sulphated, and aminated functional groups, respectively. (f) means fluorescently labelled. Where applicable, circled numbers were used to distinguish particles that possess different properties within the same study.

Type of plastic	Shape	Sizes	Concentration	Source of plastic	Was the plastic washed?	Reason for exclusion	Refs.
① PS-COOH; ② PS-HSO ₄ ; ③ PS-NH ₂ and ④ PMMA	spheres	① 26 nm, 60 nm, 90 nm, 160 nm, 190 nm, and 220 nm; ② 25 nm, 200 nm; ③ 52 nm, 53 nm, 57 nm, 58 nm, 120 nm, 180 nm, 330 nm; ④ 68 nm, and 140 nm	①②④ 25, 50, 75, 100, 200, and 400 mg/L; ③ 5, 10, 25, 50, 75, 100, and 150 mg/L	Bang laboratories, USA	yes (dialysis)	24 h exposure	³
①unspecified (f); ② PE	① spheres; ② fragments	① 1-5 µm; ② 1-10 µm	10 ³ , 10 ⁴ , 10 ⁵ , 10 ⁶ , and 10 ⁷ particles/mL	Cospheric LLC, USA	① unknown; (②yes (centrifugation) unknown	Concentration in particles/mL	⁴
PET	fragments	3-10 µm	0.1, 1, 10, 100, 1000, and 10000 mg/L	Goodfellow	unknown	96 h test, no data available for end of 48 h period	⁵
① PS; ② PS-COOH; ③ PS-NH ₂ ; ④ PS (f)	spheres	① 0.1 µm, 1 µm, and 10 µm; ②③④ 0.1 µm	1 mg/L	Tianjin Baseline Chromtech Research Centre, China	unknown	Adult organisms were used, and toxicity tests lasted for 8 h	⁶
① PS; ② PE	spheres	① 300 nm, 600 nm; ② 300-9000 nm	1 mg/L	① 300 nm (Thermo Fisher Scientific, the Netherlands); ① 600 nm (GmbH Forschungs, Germany); ② Cospheric LLC, USA	yes (PE particles washed in ethanol)	9-day old daphnia and 72 h tests	⁷
PS, PS-COOH and PS-NH ₂	spheres	50-100 nm	10 mg/L	Aladdin Industrial Co., China	unknown	Invalid control, 35% (at pH of 7.8)	⁸

PS-COOH (f) and PS-NH ₂	spheres	100 nm	10 mg/L	Obtained in the laboratory from a previous project	unknown	1–3-day old neonates and 24 h exposure	9
PS (f)	spheres	100 nm	10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/L	unavailable	unknown	24 h	10
PE	①spheres; ②fragments	① 25-50 µm; ② 40-48 µm	① 5, 50, 75, 200, and 500 mg/L; ② 0.05, 0.1, 0.5, 1, 5, 10, and 20 mg/L	① Sigma Aldrich, USA; ② prepared from PE pellets (Sigma Aldrich, USA).	yes (washed with hexane, methanol, and deionized water)	4-day old neonates	11
PE (f)	spheres	20 µm and 30 µm	20 and 60 mg/L	Cospheric	no	24 h	12
PS	spheres	26 nm and 100 nm	unavailable	Bang Laboratories, USA	yes (dialysis)	unavailable data for test concentrations	13
① PET; ② PP	fibres	① 120.49 ± 82.46 µm; ② 134.83 ± 88.70 µm	1000 and 2000 mg/L	Korea Institute of Industrial Technology, Korea	clean, (author's declaration)	3-day old neonates	14

Table S2.2. Targeted research synthesis of immobility or mortality observed on *D. magna* neonates (≤ 24 h) after 48 h exposure to MPs and NPs. Each colored cell represents a toxicity test conducted on one particle type of a given size and concentration. The “other shapes” column represents the non-spherical plastics. The spherical beads columns provide the particle diameter and concentration of each toxicity tests while the sizes of the non-spherical particles are provided within each cell (l = length; w = width; d = diameter; (s) means size not provided). The percentage of dead or immobile neonates are represented by the cell colors. Blue cell color means no significant acute toxicity ($\leq 20\%$), yellow cell means mild acute toxicity ($> 20\%$ and $\leq 50\%$) and red cell means severe acute toxicity ($> 50\%$). N = 505. (c) means that the particles were washed. (f) means fluorescently labelled. -COOH and -NH₂ means carboxylated and aminated functional groups, respectively.

Concentration (mg/L)	Spherical beads (μm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
0.01						PMMA ¹⁵ (f) PMMA-F ¹⁵ (f)							PS ¹⁶					PS ¹⁶				
0.1	PS-COOH ¹⁷ (c) (f) PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸	PS-n-NH ₂ ¹⁹	PMMA ¹⁵ (f) PMMA-F ¹⁵ (f) PS-NH ₂ ¹⁸	PS-COOH ¹⁷ (c) (f)			PS-NH ₂ ²⁰ PS-COOH ²⁰			PS ¹⁶		PS ¹ PS ¹ aged			PS ¹⁶				
0.25	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																
0.5	PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS-NH ₂ ²¹ PS-NH ₂ ²¹		PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹		PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸																

Concentration (mg/L)	Spherical beads (µm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
	PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸	PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸		PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸																		
0.75										PS-NH ₂ ²⁰ PS-COOH ²⁰												
1.0	PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-COOH ¹⁷ (c) (f) PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS ²²	PS-NH ₂ ²¹ PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS-n-NH ₂ ¹⁹	PS-NH ₂ ²³ (c) PS ¹⁹ PS-NH ₂ ²⁷ (c) PS ²⁹ PS-NH ₂ ²¹ PMMA ¹⁵ (f) PMMA-F ¹⁵ (f) PS-NH ₂ ¹⁸ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS ²⁴ PS-COOH ²⁴ PS-COOH ¹⁷ (c) (f)		PS ²²	PS-NH ₂ ²⁰ PS-NH ₂ ²⁷ (c) PS-COOH ²⁰ PS ²⁸ (c)	PS ²²		PS ²²	PS ²²	PS ¹ PS ¹ aged	PS aged ₂₅	PS ¹⁶			PS aged ₂₅	PP (I) ²⁶ d = 21.2 ± 1.5 µm PP (O) ²⁶ d = 22.1 ± 1.6 µm PP (M) ²⁶ d = 4.3 ± 2.2 µm	
1.5	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸	PS ³⁰ (c)	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																

Concentration (mg/L)	Spherical beads (µm)																						Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100		
2.0	PS-NH ₂ ²¹	PS-NH ₂ ²¹	PS ³¹ (f)	PS-NH ₂ ²¹																			
	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																			
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹																			
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹																			
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹																			
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹																			
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹																			
2.5	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸	PS ²²	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸	PS ³⁰ (c)		PS ²²		PS ²²	PS ³² (f)	PS ²²	PS ²²									
			PS ³⁰ (c)		PS-NH ₂ ²¹			PS ³⁰ (c)															
3.0	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																			
4.0	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸	PS ³¹ (f)	PS-NH ₂ ¹⁸																			
5.0	PS-NH ₂ ²¹	PS-NH ₂ ²¹	PS ²²	PS-NH ₂ ²¹	PS-n-NH ₂ ¹⁹	PS ²⁹	PS ²⁴	PS-COOH ¹⁹	PS ²⁸ (c)	PS ²⁵ aged	PS ³³ (f)	PS ³² (f)			PS ¹	PS aged ²⁵					PS aged ²⁵	PE ³⁴ d = 44.39 ± 11.16 µm	
	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ²¹	PS-COOH ²⁴				PS ³⁵												
	PS-COOH ¹⁷ (c) (f)	PS-NH ₂ ²¹		PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸	PS-NH ₂ ²¹																
	PS-NH ₂ ²¹	PS-NH ₂ ¹⁸		PS-NH ₂ ²¹		PS-NH ₂ ²¹																	
	PS-NH ₂ ¹⁸	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																	
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																	
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																	
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹	PS-NH ₂ ²¹																
	PS-NH ₂ ²¹			PS-NH ₂ ²¹		PS-NH ₂ ²¹	PS-NH ₂ ²¹																
	PS-NH ₂ ²¹			PS-NH ₂ ²¹		PS-NH ₂ ²¹	PS-NH ₂ ²¹																
6.0	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸	PS ³¹ (f)	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																	
7.5						PS-NH ₂ ¹⁸																	

Concentration (mg/L)	Spherical beads (µm)																						Other shapes																					
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100																							
8.0	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸	PS ³¹ (f)	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸																																						
10	PS-NH ₂ ²¹	PS-NH ₂ ²¹	PS ²²	PS-NH ₂ ²¹		PS ¹⁹	PS ²⁴	PS-COOH ¹⁹		PS-NH ₂ ²⁰		PS ³⁵	PS ²²		PS ¹								PP (I) ²⁶ d = 21.2 ± 1.5 µm																					
	PS-COOH ¹⁷ (c) (f)	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸	PS-COOH ²⁴																													PP (M) ²⁶ d = 4.3 ± 2.2 µm								
	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ²³ (c)	PS-COOH ¹⁷ (c) (f)			PS-COOH ²⁰			PS ¹⁶																								PP (O) ²⁶ d = 22.1 ± 1.6 µm							
	PS-NH ₂ ¹⁸	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²⁷ (c)				PS-NH ₂ ²⁷ (c)																												PE ³⁶ d = 10-75 µm						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹	PS ³¹ (f)	PS-NH ₂ ²¹	PS-n-NH ₂ ¹⁹	PS ²⁹	PS-NH ₂ ³⁷ (c)		PS ²²	PS ²⁸ (c)	PS ²²		PS ²⁸ (c)	PS ²²	PS ¹ aged	PS aged ₂₅		PS ¹⁶	PE ³⁶		PS aged ₂₅	PE ³⁸ (c) d = 180.5 ± 118.7 µm																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹	PS-COOH ³⁷ (c)			PS aged ₂₅													PE ³⁴ d = 44.39 ± 11.16 µm																					
	PS-NH ₂ ²¹	PS-NH ₂ ²¹				PS-NH ₂ ¹⁸																																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹				PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹					PS-NH ₂ ²¹																																						
						PS-NH ₂ ²¹																																						
PS-NH ₂ ²¹	PS-NH ₂ ²¹				PS-NH ₂ ¹⁸				PE ³⁹ (c)										PE ³⁹ (c)		PET ⁴⁰ l = 60-1400 µm; w = 31-528 µm; d = 2-21.5 µm																							
15	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹	PS-n-NH ₂ ¹⁹	PS-NH ₂ ¹⁸				PS aged ₂₅		PS ³³ (f)				PS aged ₂₅						PS aged ₂₅	PE ⁴¹																					
	PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹	PS-NH ₂ ²¹		PS-NH ₂ ²¹		PS-NH ₂ ²¹																																						
	PS-NH ₂ ²¹			PS-NH ₂ ²¹		PS-NH ₂ ²¹																																						
20		PS-NH ₂ ¹⁸	PS ²²	PS-NH ₂ ¹⁸	PS-n-NH ₂ ¹⁹	PS ¹⁹	PS ²⁴	PS-COOH ¹⁹		PS-NH ₂ ²⁰		PS ³⁵			PS ¹	PS aged ₂₅	PE ⁴²				PS aged ₂₅	PET ⁴³																						

Concentration (mg/L)	Spherical beads (μm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
	PS-COOH ¹⁷ (c) (f) PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸	PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸		PS-NH ₂ ¹⁸ PS-NH ₂ ¹⁸ PS ²⁹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS-COOH ²⁴ PS-COOH ¹⁷ (c) (f)			PS-COOH ²⁰ PS aged ₂₅		PS ³² (f)			PS ¹ aged							d = 14 ± 3 μm; l = 366 ± 275 μm
25							PS-NH ₂ ³⁷ (c)			PE ³⁹ (c)	PMAA ⁴⁴									PE ³⁹ (c)		PET ⁴⁰ l = 60-1400 μm; w = 31-528 μm; d = 2-21.5 μm PVA ⁴⁴ (s) PE ³⁴ d = 44.39 ± 11.16 μm
30					PS-η-NH ₂ ¹⁹	PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹ PS-NH ₂ ²¹	PS ²⁴ PS-COOH ²⁴	PS-COOH ¹⁹		PS aged ₂₅		PS ³³ (f) PS ³² (f)				PS aged ₂₅					PS aged ₂₅	
35																					PE ⁴¹	
40					PS-η-NH ₂ ¹³			PS-COOH ¹⁹									PE ⁴²					
50	PS-COOH ¹⁷ (c) (f)		PS ²²			PS-NH ₂ ²³ (c)	PS-COOH ¹⁷ (c) (f)	PS-COOH ¹⁹	PS ²²	PE ³⁹ (c) PS-COOH ²⁰	PS ²²	PS ³⁵	PS ²²	PS ²²	PS ¹ PS ¹ aged				PE ³⁶	PE ³⁹ (c)		PET ⁴⁰ l = 60-1400 μm; w = 31-528 μm; d = 2-21.5 μm PE ³⁶ d = 10-75 μm

Concentration (mg/L)	Spherical beads (µm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
						PS-NH ₂ ²⁷ (c)				PS-NH ₂ ²⁰												PP ⁴⁵ (c) d = 10-100 µm
						PS ²⁹				PS-NH ₂ ²⁷ (c)												PVC ⁴⁵ (c) d = 10-100 µm
																						PE ⁴⁵ (c) d = 10-100 µm
						PS ¹⁹	PS-NH ₂ ³⁷ (c)			PS ²⁸ (c)	PMAA ⁴⁴											PE ³⁴ d = 44.39 ± 11.16 µm
							PS-COOH ³⁷ (c)															PVA ⁴⁴ (s)
																						PVC/PE ⁴⁵ (c) d = 10-100 µm
60												PS ³³ (f)										
70								PS-COOH ¹⁹													PE ⁴¹	
75						PS ¹⁹	PS-NH ₂ ³⁷ (c)				PMAA ⁴⁴											PVA ⁴⁴ (s)
80						PS ²⁹												PE ⁴²				
100	PS-COOH ¹⁷ (c) (f)					PS-NH ₂ ²³ (c)	PS-COOH ¹⁷ (c) (f)		PS ²²	PE ³⁹ (c)	PS ²²		PS ²²						PS ¹⁶	PE ³⁶	PE ³⁹ (c)	PET ⁴⁰ l = 60-1400 µm; w = 31-528 µm; d = 2-21.5 µm
																						PP (I) ²⁶ d = 21.2 ± 1.5 µm
																						PP (M) ²⁶ d = 4.3 ± 2.2 µm
							PS-NH ₂ ³⁷ (c)															PP (O) ²⁶ d = 22.1 ± 1.6 µm
																						PET ⁴³ d = 14 ± 3 µm; l = 366 ± 275 µm
										PS ²⁸ (c)												PE ² (c) d = ~200 µm
											PMAA ⁴⁴											PE ² (c) d = ~100 µm
						PS-NH ₂ ²⁷ (c)																PE ² (c) d = ~50 µm
							PS-COOH ³⁷ (c)															PE ² (c) d = ~250 µm
																						PE ² (c) d = ~250 µm

Concentration (mg/L)	Spherical beads (µm)																					Other shapes	
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100		
										PS-NH ₂ ²⁷ (c)			PS ¹⁶										PE ² (c) d = ~150 µm
																							PE ² (c) d = ~20 µm
																							PE ⁴⁶ (c) d = 140.6 ± 80.0 µm
																							PE ³⁶ d = 10-75 µm
																							PE ³⁸ (c) d = 180.5 ± 118.7 µm
																							PVA ⁴⁴ (s)
																							PE ³⁴ d = 44.39 ± 11.16 µm
120												PS ³³ (f)											
125							PS-COOH ³⁷ (c)				PMAA ⁴⁴												PVA ⁴⁴ (s)
150							PS-COOH ³⁷ (c)			PS ²⁸ (c)	PMAA ⁴⁴												PE ³⁴ d = 44.39 ± 11.16 µm
160																	PE ⁴²						
200						PS-NH ₂ ²³ (c)				PE ³⁹ (c)		PS ³⁵								PE ³⁹ (c)			
						PS-NH ₂ ²⁷ (c)				PS-NH ₂ ²⁷ (c)													
240												PS ³³ (f)											
250																							PE ³⁴ d = 44.39 ± 11.16 µm
300												PS ³³ (f)	PS ²⁸ (c)										
320																	PE ⁴²						
400						PS-NH ₂ ²³ (c)				PE ³⁹ (c)			PS ²⁸ (c)							PE ³⁹ (c)			
						PS-NH ₂ ²⁷ (c)				PS-NH ₂ ²⁷ (c)													
500													PS ¹⁶	PS ²⁸ (c)				PS ¹⁶	PE ³⁶			PET ⁴³ d = 14 ± 3 µm; l = 366 ± 275 µm	
												PS ²⁸ (c)											

Concentration (mg/L)	Spherical beads (µm)																					Other shapes
	0.02	0.04	0.05	0.06	0.075	0.1	0.2	0.3	0.5	1	5	6	10	15	25	35	45	50	60	100	>100	
1000						PMMA ¹⁵ (f)							PS ¹⁶					PS ¹⁶	PE ³⁶			PE ³⁶ d = 10-75 µm
5000						PMMA- F ¹⁵ (f)													PE ³⁶			PE ³⁶ d = 10-75 µm

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Chapter 3: Toxicity Assessments of Micro- and Nanoplastics Can Be Confounded by Preservatives in Commercial Formulations

Connecting Text

The findings from the systematic review in Chapter 2 revealed that a large proportion of the toxicity studies on *Daphnia magna* use commercial polystyrene particles due to various advantages including narrow size ranges, and availability of fluorescent-labelling and functionalization options. However, most of these commercial particle suspensions contain additional chemicals in the form of preservatives or surfactants which may impact the result of the toxicity studies with the plastic particles. Some studies wash the particles using various methods such as dialysis and centrifugation while other studies use the commercial particles as purchased. Hence, the goal of this chapter is to investigate whether the chemicals present in these particle suspensions have any significant impact on the toxicity results obtained.

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Abstract

Micro- and nanoplastics derived from environmental degradation of larger plastic debris can be ingested and accumulated in aquatic organisms, raising increasing global ecological concerns. Toxicology studies on aquatic organisms predominantly use commercial formulations of micro- and nano-sized polystyrene particles as model plastics. However, many of these commercially available formulations contain different preservatives, antimicrobials, or surfactants such as sodium azide, Tween[®] 20, and sodium dodecyl sulfate, which may introduce artifacts in toxicity assessments. In this study, we carried out acute toxicity tests on *Daphnia magna*, using commercial 20 nm and 200 nm polystyrene nanoparticles (PS-NPs) containing 2 mM sodium azide as an antimicrobial preservative. The acute toxicity of non-dialyzed PS-NPs, dialyzed PS-NPs, and sodium azide alone was compared. The results reveal that the acute toxicity of the complete commercial formulation of PS-NPs was mainly associated with sodium azide and not the particles themselves. The dialyzed PS-NPs did not cause mortality but significantly disrupted the swimming behavior of *D. magna*. As commercial PS-NPs are commonly and increasingly used in plastic toxicity assessments, these results highlight the importance of considering the impacts of the suspension matrix.

3.1 Introduction

The ubiquitous presence of microplastics (MPs) in natural waters is a global concern.¹ Moreover, the likely presence of nanoplastics (NPs) that are too small to be seen with the naked eye, difficult to detect and to quantify is also of growing scientific interest.^{2,3} Recent studies show that MPs are bioavailable to vertebrate and invertebrate aquatic organisms.⁴⁻⁷ For example, Farrel and Nelson

found MPs in the stomachs, gills, and ovaries of crabs after feeding them with mussels that had been earlier fed with MPs.⁸ However, despite the prevalence of MPs and (likely) NPs in the aquatic environment, there is limited information available about the potential health impacts following ingestion.⁹⁻¹¹

Daphnia magna is a non-selective feeder¹² and dominant in freshwaters in nearly all continents and climate zones around the world. It has been widely used to investigate the toxicity of nanomaterials such as CuO, TiO₂, fullerene, ZnO, and Al₂O₃¹³⁻¹⁹, as it is one of the freshwater species recommended for toxicity studies by the U.S. Environmental Protection Agency (EPA).²⁰ Hence, it is an ideal model organism to test the toxicity of MPs and NPs upon ingestion. The effects of the uptake of MPs and NPs on *D. magna* have been fairly well documented.^{11,21-24} Polystyrene (PS) is the most common type of plastic used in *D. magna* toxicity assessments because of its commercial availability in the nano size range. However, suspensions of commercial polystyrene particles contain various preservatives or surfactants such as sodium azide, Tween[®] 20, sodium dodecyl sulfate (SDS), etc. These particles have been used in previous toxicity or exposure studies without conducting adequate control experiments to assess the effects of the additives,^{21,23-30} which can be toxic to different organisms at low concentrations.³¹⁻³⁴ Surprisingly, only few researchers washed the PS-NPs to eliminate the preservatives before their use in toxicity experiments (**Table S3.1**).^{22,35-37}

Although commercial formulations of PS-NPs have been shown to cause mortality upon short-term exposure,^{24,37} it is unknown whether the same effect would be observed for PS-NPs in the absence of the preservatives. This study aims to investigate the effect of the presence of sodium azide (a common additive in commercial formulations of PS) on the acute toxicity of PS-NPs to *D. magna* neonates. The acute toxicities of commercial PS-NPs containing sodium azide, sodium

azide alone, and dialyzed PS-NPs in which the sodium azide was removed were compared to identify the causative reagent of the observed effects. Furthermore, a swimming assay was conducted to assess the sublethal behavioral effects of the dialyzed PS-NPs.

3.2 Materials and Methods

3.2.1 Polystyrene Nanoparticles (PS-NPs)

Yellow-green fluorescently labeled carboxylated PS-NPs (20 nm, 505 nm excitation, 515 nm emission) and blue fluorescently labeled carboxylated PS-NPs (200 nm, 365 nm excitation, 415 nm emission) purchased from ThermoFisher were used for this study. These particles are widely used in plastic ecotoxicity studies for their ease of detection.^{11,25,27-29} The commercial PS-NP stock suspensions were supplied in deionized water containing 2 mM sodium azide.

The size of the particles was confirmed using Dynamic Light Scattering (DLS) (Zetasizer NanoZS, Malvern Instruments). The DLS measurements were performed in triplicate, each containing 13 runs of 10 s. Z-average (nm) and Polydispersity Index (PDI, dimensionless) were measured to verify the size of the PS-NPs. In this study, a single batch of PS-NPs of each nominal size was used to complete all experiments. To remove sodium azide, the stock suspension was dialyzed against deionized water for 5 days using Spectra7 dialysis membranes, MWCO 1000. The water was exchanged every 3 h in the first 12 h, and every 12 h for the remaining four days. The size of the particles before and after dialysis was verified using DLS. Fluorescence measurements of the suspension before and after dialysis were compared to confirm that the concentration did not significantly change as a result of dialysis (Infinite 200 PRO microplate reader, Tecan). To confirm the removal of sodium azide during dialysis, inductively coupled plasma - optical emission

spectrometry (ICP-OES) was used to measure the concentration of sodium ion in the non-dialyzed and dialyzed PS-NP suspensions. Sodium ion concentrations were measured instead of azide due to the methodological challenges in measuring the latter. The concentration of sodium in the final dialysis wash water was also measured. The sodium concentrations are reported in **Table S3.2**. Sodium azide powder ($\geq 99.5\%$) and all other chemicals for the preparation of Moderately Hard Reconstituted Water (MHRW) were purchased from Sigma-Aldrich.

3.2.2 Acute Toxicity Test

Daphnia magna used for this study was obtained from Environment and Climate Change Canada (Montreal) and maintained under controlled conditions in the laboratory for more than 1 year in MHRW at room temperature and a 16 h light, and 8 h dark cycle. They were fed daily with cultured green algae (*Chlamydomonas reinhardtii*) grown in an algae growth incubator (INFORS HT - Multitron Pro) following the method described by Le Faucheur *et al.*³⁸ The MHRW used for culturing *D. magna* and for the toxicity assessments was prepared following the EPA guidelines for acute toxicity studies.²⁰ *Daphnia* neonates (< 24 h old) were harvested and used for the acute toxicity tests.

A standard 48 h acute toxicity test was conducted in compliance with the OECD guidelines (OECD, Test 202).³⁹ *Daphnia* neonates were exposed to 20 nm and 200 nm PS-NPs at a series of concentrations (0.1, 1, 5, 10, 20, 50 and 100 ppm) and observed over 48 h. These concentrations were chosen to cover a range where no acute effects were observed until mortality was observed based on preliminary range finding tests. The concentrations are also within the range of previous toxicological studies using PS-NPs. The 100 ppm concentration is equivalent to 1.15×10^{15}

particles/mL and 1.7×10^{10} particles/mL for the 20 nm and 200 nm PS-NPs, respectively. 20 mL of PS-NP suspension in MHRW was prepared in 50 mL glass beakers. PS-NP suspensions were vortexed immediately before use. Five neonates were exposed in each beaker, and all exposures were carried out in triplicate. The animals were observed for mortality after 24 h and 48 h.

Three independent exposures were carried out with different objectives. The first investigated the effect of non-dialyzed 20 nm and 200 nm PS-NPs (containing 2 mM sodium azide) on *Daphnia* neonates, the second studied the effect of dialyzed 20 nm and 200 nm PS-NPs particles, and the third, the effect of sodium azide alone. The concentrations of sodium azide in the third exposure were the same as in the first exposure. Triplicated MHRW controls were carried out alongside each exposure.

3.2.3 Swimming Assay

After 48 h exposure, the swimming behavior of the animals exposed to 100 ppm dialyzed PS-NPs as well as control conditions was observed by microscopy (10× magnification, stereomicroscope, Fisher Scientific). The swimming behavior of neonates exposed to non-dialyzed PS-NPs could not be assessed as all neonates were dead at 100 ppm. The animals were placed individually in glass bottom culture dishes (14 mm diameter, Matsunami Glass, VWR) filled with 1 mL MHRW. The light source from the microscope was placed directly above the glass bottom culture dish. The neonates were allowed to adapt to the lighting environment for 3 min before the recordings were started. For each neonate, a recording of 60 s was made. Ten animals were randomly selected and recorded for each treatment. The video recordings were analyzed with Kinovea software (<https://www.kinovea.org/>). The swimming of the neonates over the 60 s period was traced and

the total distance covered was recorded. Comparisons between means of swimming distance among the experimental groups were achieved by one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey's multiple comparison test. The level of significance was set at $p \leq 0.05$.

3.2.4 Accumulation of PS-NPs

After the video recording, *Daphnia* neonates were returned into the same 50 mL glass beakers containing the PS-NPs for 1 h before being observed by fluorescence microscopy (IX71, Olympus) at 100 $\times$ magnification to verify the accumulation of PS-NPs. This 1 h waiting period was to ensure that the animals were not stressed before imaging.

3.3 Results and Discussion

3.3.1 PS-NP Characterization

The DLS sizes of the 20 nm and 200 nm PS-NPs were 33 ± 8 nm and 263 ± 9 nm, respectively. This suggests that a fraction of the particle population aggregated when initially suspended in the MHRW. Hereafter, we continue to refer to the particle suspensions by their nominal sizes. DLS measurements were also conducted 48 h after the suspensions were prepared to verify whether the particles aggregate over the period of exposure. The sizes of the 20 nm and 200 nm PS-NPs were 38 ± 16 nm and 278 ± 7 nm, respectively after the 48 h period (**Figure S3.1** in Supporting Information). This indicates that the particles remain mostly well dispersed over the 48 h exposure period. In addition, the total fluorescence of the PS-NP suspensions was similar before and after

dialysis (**Figure S3.2**), showing that most of the particles were not lost as a result of dialysis. No sodium ion was detected in the dialyzed PS-NP suspensions (**Table S3.2**).

3.3.2 Mortality Assessment

Exposure to non-dialyzed PS-NPs (*i.e.*, containing sodium azide) caused mortality starting from 10 ppm and reached 100% at 100 ppm for both 20 nm and 200 nm PS-NPs (**Figure 3.1**). The results showed significant mortality after 48 h exposure for both 20 nm and 200 nm PS-NPs with LC50 of 15.7 ppm (equivalent to 3.0×10^{14} particles/mL) and 30.4 ppm (equivalent to 1.6×10^{10} particles/mL), respectively (**Figure 3.1c, d**). Mortality was also observed for neonates exposed to sodium azide at the same concentration as that present in the non-dialyzed PS-NPs with LC50 of 13.4 ppm (**Figure 3.1e**). The mortality observed for sodium azide alone was very similar to that observed for the 20 nm PS-NPs at all concentrations and slightly different for the 200 nm PS-NPs between 5 ppm and 20 ppm (**Figure 3.1**). In contrast, dialyzed PS-NPs caused no mortality at all concentrations for both 20 nm and 200 nm PS-NPs (**Figure 3.1**). This agrees with some earlier studies exposing aquatic organisms to dialyzed PS-NPs.^{22,36} For instance, Mattsson *et al.*²² observed no mortality of *D. magna* exposed to dialyzed PS-NPs up to 24 h. Similarly, Cole and Galloway³⁶ did not observe any mortality when dialyzed PS-NPs were fed to Pacific oyster larvae. The observed survival of *D. magna* neonates upon exposure to dialyzed PS-NPs contradicts the results of Mattsson *et al.*³⁷ and Aljaibachi and Callaghan²⁴ who reported mortality when *Daphnia* was exposed to PS-NPs. In Aljaibachi and Callaghan's study²⁴, adult *Daphnia* were fed with 146 ppm MPs and mortality was observed within 72 h. However, the particles were not dialyzed but were rather used as purchased. Mattsson *et al.*³⁷ reported mortality when *Daphnia* was exposed to 75 ppm 52 nm PS-NPs for 24 h, but it is important to note that the particles used for the study were dialyzed for 24 h only, which is far shorter than the 5 days of dialysis used in the present study.

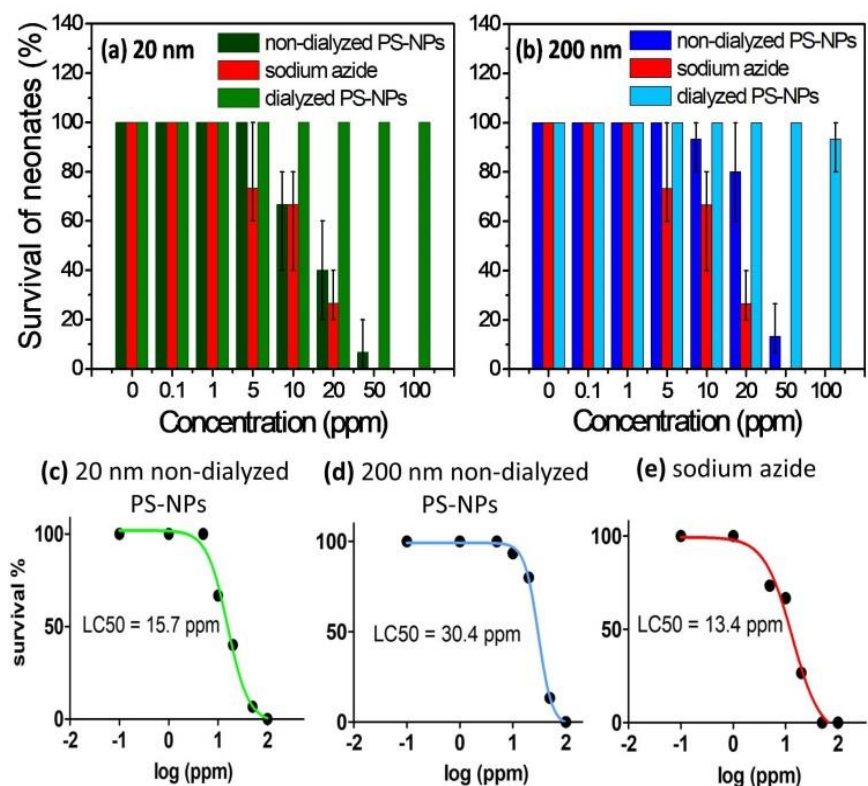


Figure 3.1. The mean survival percentage of *Daphnia* neonates at 48 h after exposure to (a) 20 nm non-dialyzed PS-NPs, equivalent concentration of sodium azide, and 20 nm dialyzed PS-NPs; (b) 200 nm non-dialyzed PS-NPs, equivalent concentration of sodium azide and 200 nm dialyzed PS-NPs. Dose response curves of (c) 20 nm non-dialyzed PS-NPs, (d) 200 nm non-dialyzed PS-NPs, and (e) equivalent concentrations of sodium azide.

3.3.3 Swimming Behavior of *Daphnia* Neonates

To reveal the sublethal effects of PS-NPs, the swimming behavior of the neonates after exposure to dialyzed PS-NPs as well as control neonates was assessed. The dialyzed 20 nm and 200 nm PS-NPs showed no acute toxicity at 100 ppm but significantly affected the swimming behavior of *Daphnia* when compared to the control neonates. The swimming behavior of neonates exposed to

non-dialyzed PS-NPs could not be assessed as all neonates were dead at this concentration. As shown in **Figure 3.2**, the swimming activity was low for the control neonates, but significantly higher for the neonates exposed to dialyzed PS-NPs. Neonates exposed to PS-NPs swam up to 40 cm in one min while the neonates from the control experiments barely moved up to 10 cm during the same time period. For the 200 nm PS-NPs, the animals demonstrated more activity during the 1 min period than the 20 nm PS-NPs suggesting that particle size can be an important factor for the toxicity of PS-NPs as suggested earlier by Mattsson *et al.*³⁷ *Daphnia* possessing a hyperactive swimming behavior and demonstrating longer swimming distance within a given period of time are more likely to be exposed to predators, and thus at higher ecological risk.⁴⁰ It is interesting to note that the observed hyperactive swimming behavior upon exposure to PS-NPs is similar to that noted with other nanomaterials such as nano-C₆₀ and the hydrogenated C₆₀-H_xC₇₀H_x,⁴⁰ suggesting possible shared toxicity mechanisms of these nanoparticles. Further studies are needed to investigate the molecular mechanisms of behavioral toxicity of different nanoparticles.

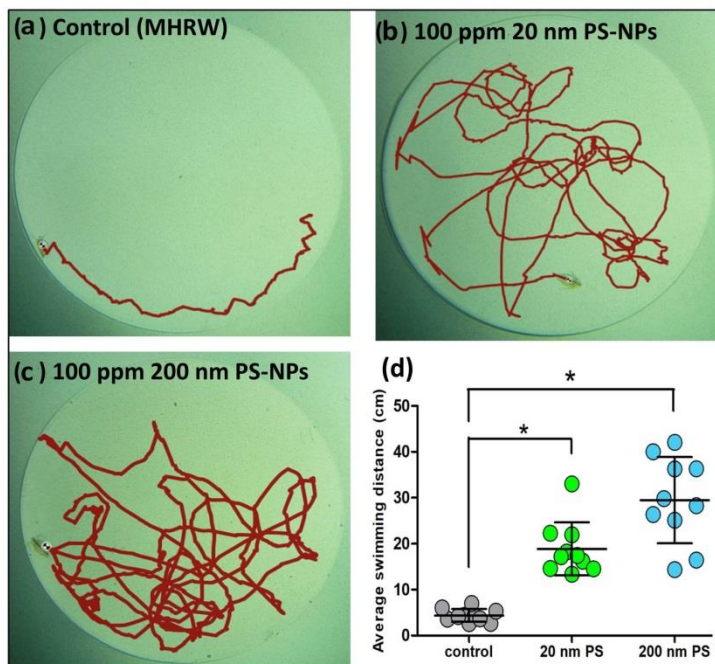


Figure 3.2. Representative swimming tracking of *Daphnia* neonates treated with dialyzed PS-NPs after 48 h exposure for (a) control (MHRW), (b) 100 ppm 20 nm PS-NPs, and (c) 100 ppm 200 nm PS-NPs, respectively. (d) Average swimming distance of neonates after 48 h exposure for control, 100 ppm dialyzed 20 nm PS-NPs and 100 ppm dialyzed 200 nm PS-NPs. Asterisk indicates that the measurement is statistically significantly different (p-value<0.05, one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test) from the control (n=10).

3.3.4 Ingestion and Accumulation in the Gastro-Intestinal Tract (GIT)

Fluorescence images of the neonates were taken after 48 h exposure to the PS-NPs to ascertain whether the neonates ingested the dialyzed PS-NPs even though no mortality was observed. The images show the uptake of both non-dialyzed and dialyzed PS-NPs in the gastrointestinal tract (GIT) of the animals (**Figure 3.3**). The images of the neonates exposed to 100 ppm of 20 nm and 200 nm dialyzed PS-NPs show significant accumulation of the particles in the GIT without any visible damage. In contrast, neonates exposed to the same concentration of non-dialyzed PS-NPs after 48 h show that the PS-NPs were not only ingested, but there was also visible rupture of the GIT (**Figure 3.3b** and **3.3d**). This indicates that the dialyzed PS-NPs were ingested by the neonates and significantly accumulated in the GIT even though no mortality was observed, and the visible rupture observed in the GIT of the neonates exposed to non-dialyzed PS-NPs is likely linked to the sodium azide, which may explain the high mortality observed in the sodium azide exposure.

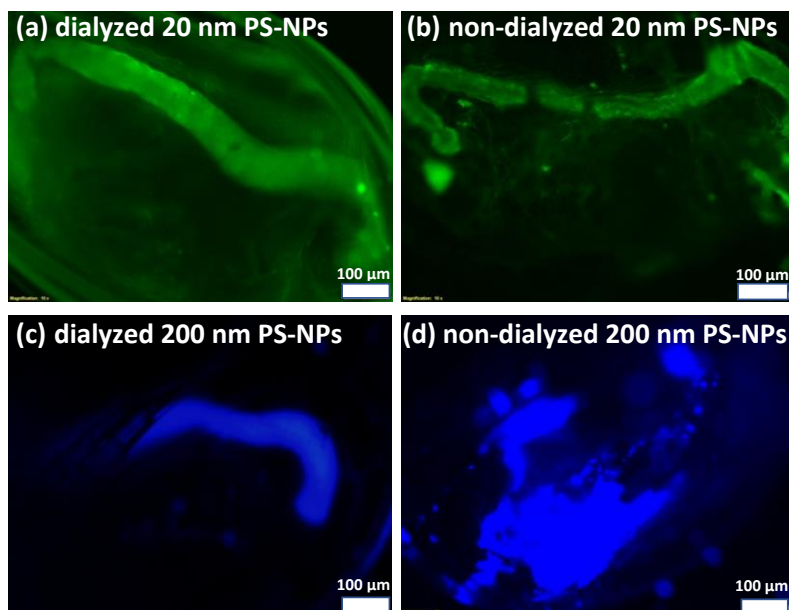


Figure 3.3. *Daphnia* neonates imaged by fluorescence microscopy after 48 h exposure to 100 ppm PS-NPs: (a) 20 nm dialyzed PS-NPs, (b) 20 nm non-dialyzed PS-NPs, (c) 200 nm dialyzed PS-NPs, and (d) 200 nm non-dialyzed PS-NPs.

In summary, this study is the first to show that the observed mortality of *D. magna* can be directly attributed to a preservative (in this case, sodium azide) present in a PS-NP suspension. After the removal of sodium azide by dialysis, the PS-NPs caused no mortality but significantly affected the mobility of the neonates. Interestingly, of the studies conducted using commercial stock suspensions of PS-NPs, a considerable number exhibit higher toxicity than those where the plastic particles were washed (**Table S3.1**). Even though stabilizers are very useful in the preparation and preservation of engineered nanoparticles, the presence of these stabilizers may be misleading and cause over- or underestimation of particle toxicity,³⁴ and there is no exemption for commercial micro- and nanoplastics. As the number of toxicology studies on micro- and nanoplastics

continues to increase, the results of this study call for a more careful assessment of the toxic effects of the additives in commercial particle formulations.

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3.4 Supporting Information

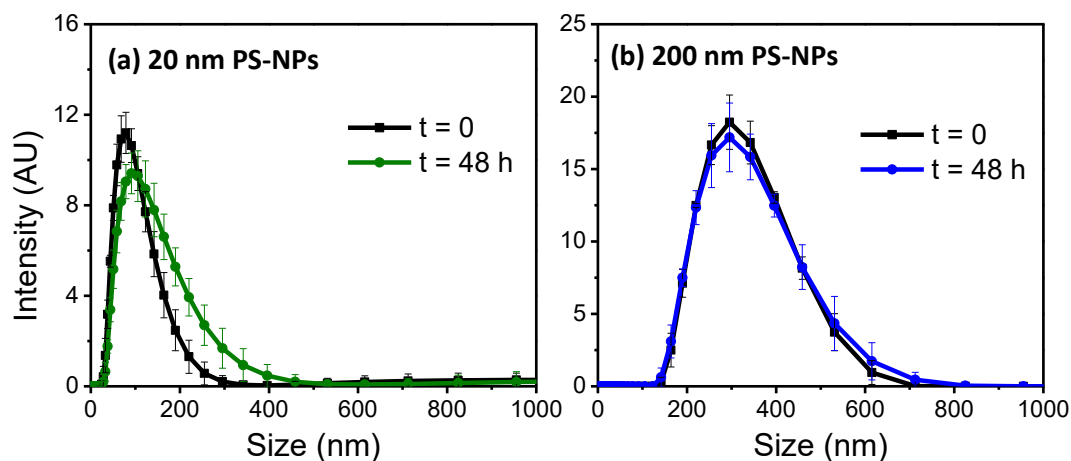


Figure S3.1. DLS measurements of PS-NPs suspended in MHRW at $t=0$ (upon sample preparation) and after 48 h at room temperature: (a) 20 nm non-dialyzed PS-NPs, (b) 200 nm non-dialyzed PS-NPs.

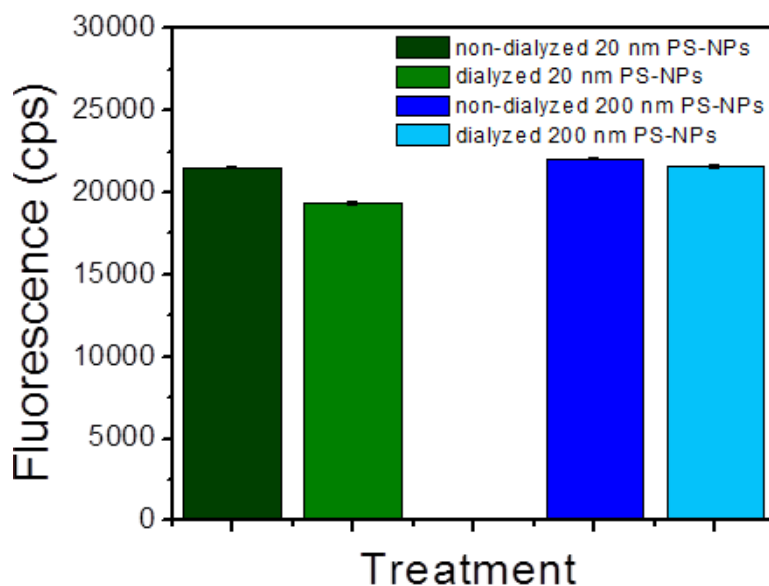


Figure S3.2. Fluorescence (counts per second) of non-dialyzed and dialyzed PS-NP suspensions.

Table S3.1. Studies on the toxicity of PS-NPs to various organisms.

Size of PS Particles	Dosage	Duration of Exposure	Additive/Preservative Present	Organism Studied	Were the Particles Washed?	Key Findings/Observations	Ref.
24 nm; 27 nm	100 ppm	24 h 61 days	Tween 20 or SDS ($\leq 0.1\%$), sodium azide ($\leq 0.09\%$)	zooplankton (<i>Daphnia magna</i>), crucian carp (<i>C. carassius</i>)	Yes (dialyzed; 3 days)	No mortality was observed in <i>D. magna</i> . However, behavioural difference in fish fed with <i>Daphnia</i> which were previously fed with PS-NPs.	[22]
24 nm	100 ppm	24 h 30 days	Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	zooplankton (<i>Daphnia magna</i>), crucian carp (<i>C. carassius</i>)	Yes (dialyzed; duration not specified)	Lipid metabolism and behaviour of top consumer is affected.	[35]
1 μm ; 10 μm	100 particles mL^{-1}	8 days	0.01% NP40 detergent, 0.02% sodium azide	Pacific oyster larvae	Yes (centrifuged and washed; duration not specified)	No significant effect on the growth of oyster larvae.	[36]
25 nm - 330 nm	150 ppm*	24 h 67 days	Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	zooplankton (<i>Daphnia magna</i>), crucian carp (<i>C. carassius</i>)	Yes (dialyzed; 24 h)	Brain tissue and behaviour of top consumer affected. Mortality after 24 h of exposure of <i>Daphnia magna</i> to PS-NPs.	[37]
52 nm	5 ppm	5 days	0.1% Tween 20; 2 mM sodium azide	<i>Daphnia galeata</i>	No	Mortality after 48 h of exposure. Abnormal embryo development and low hatching rate after 5 days of exposure.	[21]
100 nm; 2 μm	1 ppm	21 days	0.1% Tween 20; 20 mM sodium azide	<i>Daphnia magna</i>	No	Ingestion and egestion rates of PS-NPs by <i>Daphnia magna</i> are dependent on the presence of food.	[23]
2 μm	0.01 ppm*	21 days	Unnamed anionic surfactant	<i>Daphnia magna</i>	No	Mortality after 7 days of exposure to a mixture of MPs and algae.	[24]
1. 40 nm 2. 50 nm	100 ppm*	48 h	1. Azide-free 2. Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	brine shrimp larvae (<i>Artemia franciscana</i>)	No	No mortality reported but several sub-lethal effects were observed.	[25]
50 nm	4 ppm*	48 h	Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	sea urchin embryos (<i>Paracentrotus lividus</i>)	No	Disruption of sea urchin embryo (poorly developed skeleton rods, underdeveloped/poorly developed arms, poor symmetry upon exposure, etc.)	[26]
1. 40 nm 2. 50 nm	50 ppm*	48 h	1. 2 mM sodium azide. 2. Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	sea urchin embryos (<i>Paracentrotus lividus</i>)	No	Slight malformation in embryos after 48 h.	[27]
1. 40 nm 2. 50 nm	100 ppm*	72 h 14 days	1. 2 mM sodium azide. 2. Tween 20 or SDS ($\leq 0.1\%$); sodium azide ($\leq 0.09\%$)	marine planktonic species (<i>Dunaliella tertiolecta</i> ; <i>Artemia franciscana</i>)	No	No visible effects on microalgae and brine shrimp up to 50 ppm and 10 ppm, respectively.	[28]
20 nm	8 ppm*	2 h	None	algae (<i>Chlorella sp.</i> and <i>Scenedesmus sp.</i>)	No	Adsorption of nano-sized plastic beads hindered algal photosynthetic activities and promotion of ROS production.	[29]
50 nm; 500 nm; 6 μm	25 ppm*	96 h	Unnamed surfactant	marine copepod (<i>Tigriopus japonicas</i>)	No	Survived 96 h exposure at 300 ppm. Mortality was observed in two-generation chronic toxicity test.	[30]

* Maximum concentration tested

Table S3.2. Concentration of sodium ion in dialyzed and non-dialyzed PS-NP suspensions and final dialysis wash water.

	Conc. (mg/L)
¹ Blank	BDL
² Final wash water for 20 nm PS-NPs	BDL
² Final wash water for 200 nm PS-	BDL
³ Dialyzed 20 nm PS-NPs	BDL
³ Dialyzed 200 nm PS-NPs	BDL
⁴ Non-dialyzed 20 nm PS-NPs	1.509
⁴ Non-dialyzed 200 nm PS-NPs	1.164

¹ Filtered DI water; ² Final dialysis wash water; ³ 200 ppm of dialyzed PS-NPs;

⁴ 200 ppm of non-dialyzed PS-NPs; BDL: below detection limit.

Chapter 4: Sub-lethal Effects of Nanoplastics Upon Chronic

Exposure to *Daphnia magna*

Connecting Text

In Chapter 3, it was shown that the dialyzed polystyrene nanoplastics in the absence of preservatives, may not have a significant impact on the acute survival of *Daphnia magna*. It was observed, however, that the same particles may cause sublethal effects such as increasing swimming behavior. It is beyond doubt that plastics can last in the environment for more than 48 h, as in the case of acute toxicity tests. Also, the systematic review in Chapter 2 revealed that the toxicity tests with plastics presently use different types of dose metrics. So far, there have been no studies to assess the impacts of different dose metrics on the observed toxicity of nanoplastics. Hence, in this chapter, the sublethal effects of polystyrene nanoplastics on *Daphnia magna* as well as the effects of different dose metrics on the observed toxicity effects were investigated.

The findings from this chapter were published as: Oluwadamilola Pikuda, Eva Roubeau Dumont, Sara Matthews, Elvis Genbo Xu, Dimitrios Berk, and Nathalie Tufenkji. "Sub-lethal effects of nanoplastics upon chronic exposure to *Daphnia magna*." *Journal of Hazardous Materials Advances* 7 (2022): 100136.

Abstract

Studies of microplastic and nanoplastic toxicity on aquatic invertebrates predominantly focus on short-term effects such as mortality and immobility. This study investigated sublethal effects on *Daphnia magna* when exposed to dialyzed 20 nm and 200 nm carboxylated polystyrene nanoplastics for 21 days. The study tested the effects of 50 mg/L of both particle sizes and 0.1 mg/L of the 20 nm particles. Data results between the 50 mg/L for both particle sizes, which maintains the particle mass concentration constant, and between the 50 mg/L 200 nm and 0.1 mg/L 20 nm treatments, which maintains particle number concentration constant, allowed comparison of dose metrics. Exposures of *Daphnia magna* to 0.1 mg/L and 50 mg/L of 20 nm particles enabled the evaluation of the effects of particle mass concentration on the toxicity of the smaller particles. Chronic exposure to polystyrene nanoplastics impacted growth, molting and reproduction of *Daphnia magna* at both tested sizes, and at particle concentrations as low as 0.1 mg/L. Overall, the results of this study showed similar trends in several measured endpoints whether using the mass concentration or particle counts as dose metrics and change in particle concentration did not result in a significant difference in the observed endpoints for 20 nm particles.

4.1 Introduction

The continual accumulation of plastic wastes in aquatic environments has raised concerns about the potential toxicity to aquatic organisms. The biological consequences of larger plastic debris (≥ 5 mm) commonly referred to as macroplastics have been extensively studied.^{1, 2} However, their fragmentation into smaller pieces through chemical/mechanical stress or weathering^{3, 4}, as well as the commercial production of micro- or nano-sized plastics have led to the presence of

microplastics and nanoplastics in freshwater and marine environments worldwide.⁵⁻⁷ The potential toxicity of these microplastics and nanoplastics is of growing interest in the field of ecotoxicology especially for small-sized aquatic invertebrates since they are relatively smaller in size, with limited ability to move away from pollution sources compared to their vertebrate counterparts.⁸

Polystyrene is a commonly used plastic polymer particularly for its broad applications in packaging, electronics, and household appliances. The global polystyrene production volume was 10.4 million tons in 2018 with an estimated increase of about 2 % per year over the next few years.⁹ Several studies have investigated the effects of short-term exposures of polystyrene microplastics and nanoplastics to aquatic invertebrates such as *Daphnia magna*¹⁰, *Hyalella azteca*¹¹, *Gammarus pulex*¹², *Allorchestes compressa*¹³, *Diaphanosoma celebensis*¹⁴, etc. However, most of these toxicity studies were conducted over a short period (48 h or 96 h) and did not include their regular food nutrients such as algae which are an important component of the aquatic environment and can influence both uptake and sensitivity.¹⁵ Until now, only a few studies have investigated the possible long-term effects of microplastics and nanoplastics to aquatic invertebrates.¹⁶⁻²⁰

The importance of appropriate dose metric selection in environmental toxicology studies has been extensively documented for various nanoparticles and engineered nanomaterials.²¹⁻²³ While total mass continues to be a standard dose metric for nanoparticles such as heavy-metal based nanoparticles (e.g. ZnO, CuO, etc.), there is evidence of considerable variability in ecotoxicological effects for the same type of nanoparticles using different dose metrics.²⁴ Some studies have suggested that other parameters, such as particle number or total surface area, may be a more suitable dose metric for certain nanoparticles such as TiO₂, Ag, and carbon-based nanoparticles.^{22, 25-29} Although substantial data have been reported concerning the toxicity of microplastics and nanoplastics, to date, no study has investigated the role of dose metrics in the

assessment of the toxicity of nanoplastics. While several studies have used mass concentration as a dose metric,^{30, 31} others have investigated the toxicity of microplastics and nanoplastics using particle count as dose metric.^{11, 32} The growing presence of microplastics and nanoplastics in the aquatic environment, and increasing studies on their toxicity raise the need for the selection of appropriate dose metrics for the toxicity evaluation and risk assessment of plastic particles as it can influence observed toxicity.^{21, 33}

Thus, a study comparing the observed toxicity at different particle mass concentrations and different particle counts on a model species such as *D. magna* in the presence of food is of interest to lay the groundwork for more comprehensive investigations involving a wider range of particle sizes and numbers that may be present in natural aquatic environments. The results of this study will set a foundation for estimating the sensitivity of the sublethal effects in *Daphnia magna* to two different dose metrics through a 21-day chronic exposure to two different sizes of polystyrene nanoplastics. *Daphnia* species are commonly used for ecotoxicology studies due to their significant role in the aquatic food chain, easy manipulation and cultivation. *D. magna* has been particularly used for various plastic toxicity tests especially because of its sensitivity to pollutants, and its ability to ingest micro- and nano-sized particles between 20 nm and 70 μm .^{34, 35} In this study, toxicological endpoints such as survival, growth and reproduction as well as behavioral endpoints were observed as it has been demonstrated that *D. magna* can demonstrate behavioral changes as a result of nanoparticle exposure.^{36, 37} While environmental nanoplastics can have a wide range of sizes, shapes and composition, in this mechanistic study, model spherical polystyrene beads were used to allow for improved control of shape, size and dose metric.³⁸

4.2 Materials and Methods

4.2.1 Test Organism – *Daphnia magna*

D. magna used for this study was originally obtained from Environment and Climate Change Canada (Montreal, Quebec) in 2017 and has since then been continually cultured in the Biocolloids and Surfaces Laboratory, Chemical Engineering Department, McGill University. The *D. magna* cultures consisted of 250 mL polypropylene beakers each containing 2 daphnids in 100 mL of moderately hard rear water (MHRW). MHRW was prepared by mixing 96 mg L⁻¹ NaHCO₃, 60 mg L⁻¹ CaSO₄·2H₂O, 60 mg L⁻¹ MgSO₄, and 4 mg L⁻¹ KCl in well-aerated deionized water as recommended by the United States Environmental Protection Agency (USEPA).³⁹ The 100 mL MHRW media was changed daily and the daphnids were fed with 1 mL each of YCT mix (a mixture of yeast, cereal leaf, and trout chow; Arofish, USA) and cultured green algae (*Chlamydomonas reinhardtii*) after each media change. *Chlamydomonas reinhardtii* was originally obtained from the Wilkinson laboratory, Université de Montréal, Canada, and has since then been grown in-house in an algae growth incubator (INFORS HT - Multitron Pro) under fluorescent light and at 20 ± 1 °C. Artificial tris-phosphate culture media containing 14 mg L⁻¹ KH₂PO₄, 33 mg L⁻¹ K₂HPO₄, 94 mg L⁻¹ NaCl, 12.5 mg L⁻¹ CaCl₂·2H₂O, 25 mg L⁻¹ MgSO₄·7H₂O, and 600 mg L⁻¹ Tris buffer was prepared and autoclaved. A small quantity of algal seed was inoculated into 100 mL of the autoclaved culture media and allowed to grow in the incubator for 3 days before expansion into 500 mL of media. After another three days of growth in the 500 mL of media, the resulting algae was harvested and separated from the culture media by centrifugation and resuspended in MHRW. The cell numbers of the resulting algae suspension was measured as ~3.0 × 10⁶ cells/mL using a Multisizer 3 particle counter (Beckman Coulter).

The culture medium comprising of the MHRW, YCT and green algae was renewed daily, and the beakers were rinsed thoroughly with deionized water during each medium change. All offspring were discarded. Brood daphnids were discarded every 6 weeks and replaced with their neonates. The culture was maintained under controlled conditions in the laboratory at $20 \pm 2^{\circ}\text{C}$ and a 16 h light, and 8 h dark cycle measuring $20 \mu\text{Em}^{-2}\text{s}^{-1}$ at the water surface of the beakers. All chemicals used were purchased from Sigma-Aldrich at the highest purity available.

4.2.2 Test Materials – Particle Preparation and Characterization

Fluorescently labelled 20 nm (excitation/emission: 505 nm/515 nm) and 200 nm (excitation/emission: 365 nm/415 nm) carboxylate-modified polystyrene particles were purchased from ThermoFisher. The particle suspensions were first dialyzed to remove any additives and then characterized according to Pikuda *et al.*⁴⁰ The particle suspension was vortexed for 15 s immediately before use and the test concentrations were prepared by pipetting the required amount of the stock suspension into MHRW. The resulting mixture which comprises MHRW and polystyrene particles is henceforth referred to as the test suspension. The size of the 20 nm and 200 nm particles in the test suspensions was confirmed using Dynamic Light Scattering (DLS) (Zetasizer NanoZS, Malvern Instruments) before exposure to *Daphnia magna* and presented in the Supporting Information (**Figure S4.1**).

4.2.3 Test Conditions

Test beakers were covered with glass lids and kept at $20 \pm 2^\circ\text{C}$ under 16 h light and 8 h dark photoperiod. Water quality parameters such as dissolved oxygen, temperature and pH were monitored daily throughout the experiment. Dissolved oxygen was measured with a Fisherbrand™ Traceable™ Portable Dissolved Oxygen Meter Pen and was between 7–10 mg L⁻¹ over the duration of the experiment. The pH and temperature were also measured with a Fisher Scientific pH meter and an Accumet electrode and were between 7.2–8.2 and 20–22 °C, respectively. The experimental conditions were recorded during each medium change, before discarding the old medium and immediately after changing the medium, to confirm that the dissolved oxygen level did not drop, and the pH did not change significantly during the tests. The conditions throughout the 21-day toxicity test are summarized in **Table S4.1**.

4.2.4 Experimental Design

Four separate 21-day chronic toxicity tests were conducted using an adapted version of the OECD guidelines (OECD, Test 211) in the presence of food.⁴¹ The static renewal method was used for this study as it requires less stock of particles. Our previous study showed that up to 100 mg/L of both 20 nm and 200 nm polystyrene particles did not cause any acute toxicity to *D. magna*⁴⁰. Thus, in the first two treatments, a non-lethal concentration equal to 50 mg/L of both particle sizes was chosen to allow evaluation of exposures at equivalent particle mass (mass concentration). These treatments were referred to as 20 nm (50 mg/L) and 200 nm (50 mg/L) and were equivalent to 4.6×10^{12} particles mL⁻¹ and 8.5×10^9 particles mL⁻¹, respectively. To compare the effect of the two particle sizes at equivalent particle numbers, the number of particles for the 200 nm (50 mg/L)

particle treatment was calculated to be equivalent to 0.1 mg/L of the 20 nm particles. Thus, the third treatment used 0.1 mg/L of 20 nm particles and is referred to as 20 nm (0.1 mg/L). The final treatment was a control with no polystyrene particles.

The test animals were neonates (≤ 24 h old) from the third generation of a single parthenogenetic female. The neonates were raised in a 50 mL glass beaker with 20 mL of test medium. Ten replicates with one animal per beaker were used for each treatment group. Tests were conducted using freshly prepared MHRW, algae and YCT. A complete exchange of the test medium (MHRW, particle suspension, algae, YCT) was done three times per week. All beakers were rinsed with deionized water after each change of media. The light bulbs were spread evenly in the test chamber and the position of each beaker was changed randomly after each change of test medium to reduce spatial effects. Due to the nature of the organisms, feeding rations were adjusted depending on the age of the organisms.⁴² Detailed information on their feeding ration over the 21 days is presented in **Table S4.2**.

4.2.5 Observation Measurement and Analysis

The number of neonates, molts, immobility, and deaths of animals were recorded daily over the 21-day exposure. An animal was considered immobile if no mobility was observed after a gentle probing with a transfer pipette and dead if no heartbeat was observed after 15 s using a 10 $\times$ magnification objective mounted on a stereomicroscope (Fisher Stereomaster). All neonates, molts, and dead animals were removed and discarded daily. The presence of resting eggs (ephippia), an indicator of stress⁴¹, was also monitored and recorded.

The hopping frequency and swimming behaviour of each animal was measured three times per week by recording 1-min videos using a Samsung S6 edge smartphone mounted onto the stereomicroscope using the method described by Pikuda *et al.*⁴⁰ The measurements were taken immediately before transferring the daphnids into the fresh test suspension. Full-length images of the daphnids were also taken during each video recording using the same phone. The images and videos were analyzed with Kinovea software (<https://www.kinovea.org/>) using the methods described by Xu *et al.*¹⁸ The body lengths were measured from the top of the head to the base of the spine.

4.2.6 Statistical Analysis

All statistical analyses were performed using R studio Software with MASS and PGIRMESS packages.⁴³⁻⁴⁵ Homoscedasticity was checked using Bartlett test and normality was checked on ANOVA residuals, with log-transformation when normality assumption was not met with raw data. Group comparisons were performed using 2-way ANOVAs to assess interactions between time and treatment factors (*i.e.* particle concentration and size). Significance letters were obtained using TukeyHSD posthoc test to account for interactions. When no normality was found despite transformation, non-parametric test Kruskal-Wallis was performed followed by Kruskalmc post-hoc test to assess significant differences factor by factor. Statistical significance was set to P-value < 0.05. Box and whisker plots are used to show all data points. The boxplot outline corresponds to the upper quartile and lower quartile with a line at the median while the whiskers correspond to the minimum and maximum values. Although there were 10 neonates at the start of each toxicity test, the number of neonates with available results for data analysis varied between 7 and 10

depending on the target endpoint due to the deaths of neonates or other technical challenges during the experiments.

4.3 Results

The results of this study met the OECD Guideline 211 criteria as the mortality observed in the control experiment was below 20 %.⁴¹ No significant difference was observed in the mortality of animals exposed to the polystyrene particles in all test groups (**Table 4.1**). This is in agreement with the results of our previous study whereby up to 100 mg/L of dialyzed 20 nm and 200 nm polystyrene particles did not induce acute toxicity in *Daphnia magna*, as well as other chronic toxicity studies in which no significant mortality was observed after 21 day exposure.^{18, 40}

Table 4.1. Summary of statistical analyses following polystyrene particle exposure to *Daphnia magna* over 21 days, displaying effects of exposure on each endpoint. Data are reported as mean $\pm$ standard deviation where applicable. P-values of the treatment factor compared to the control are shown. Significance levels (^{ns}, *, **, ***, ****) obtained using TukeyHSD posthoc test are used to represent $P > 0.05$, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.0001$, respectively.

Measured exposure	Mortality (%)	Change in body length (mm)	Time to first molt (days)	Number of molts per animal	Time to first brood (days)	Number of neonates per animal
Control	10.0	2.4 $\pm$ 0.3	4.1 $\pm$ 0.3	5.8 $\pm$ 1.1	10.1 $\pm$ 1.2	33.2 $\pm$ 10.3
20 nm	30.0 ^{ns}	1.9 $\pm$ 0.1 ^{***}	5.6 $\pm$ 3.1 ^{ns}	4.2 $\pm$ 1.4 ^{ns}	12.1 $\pm$ 1.6 ^{ns}	33.6 $\pm$ 9.8 ^{ns}
(0.1 mg/L)						
20 nm	20.0 ^{ns}	1.7 $\pm$ 0.3 ^{****}	6.0 $\pm$ 2.4 ^{ns}	4.1 $\pm$ 1.3 ^{ns}	14.9 $\pm$ 3.1 ^{****}	26.1 $\pm$ 7.5 ^{ns}
(50 mg/L)						
200 nm	10.0 ^{ns}	2.0 $\pm$ 0.1 ^{**}	8.0 $\pm$ 2.8 ^{**}	3.6 $\pm$ 1.3 ^{**}	9.4 $\pm$ 0.5 ^{ns}	45.8 $\pm$ 8.0 [*]
(50 mg/L)						
P value	0.619196	0.000008	0.009521	0.007421	0.000001	0.001466

4.3.1. Effects of particle concentration on the toxicity of polystyrene nanoplastics

The overall effects of all treatments are shown in **Table 4.1** while **Figures 4.1-3** compare the observed effects based on particle mass concentration and particle number concentration. The average change in body lengths of animals exposed for 21 days was inhibited by 21 %, 28 % and 16 % for the 20 nm (0.1 mg/L), 20 nm (50 mg/L) and 200 nm (50 mg/L) concentrations respectively, compared to the controls (**Figure 4.1**). The growth of the neonates based on change

in body length was significantly inhibited by exposure to polystyrene particles in all treatments (1-way ANOVA, $F_{3,26} = 14.80$, $P < 0.0001$, **Table 4.1**, **Figure S4.2**), and the same sensitivity pattern was observed between the two dose metrics (**Figure 4.1**).

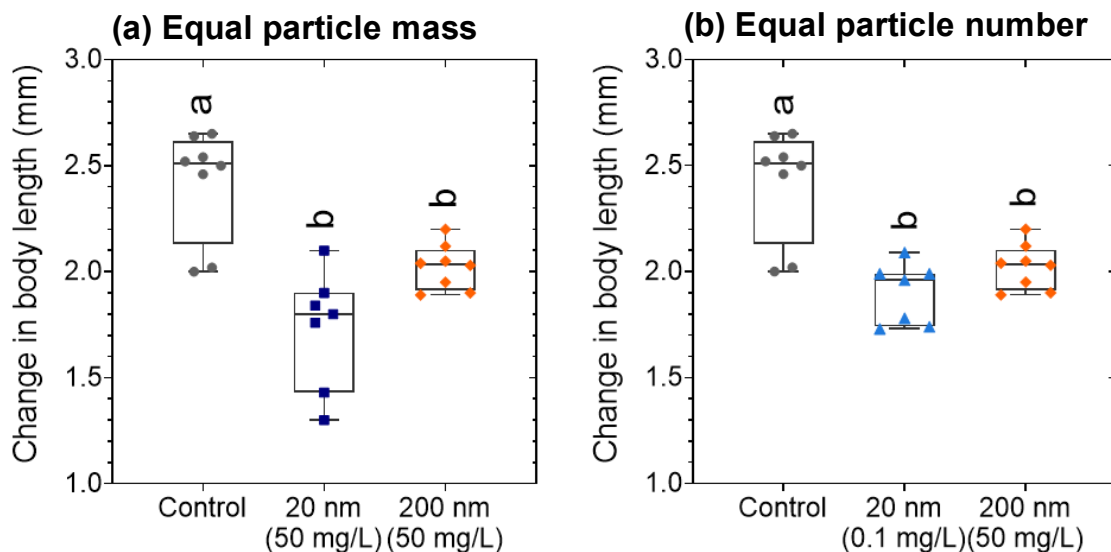


Figure 4.1. Change in body length over the 21-day period of exposure of *Daphnia magna* neonates to polystyrene particles for: (a) equal particle mass concentration of 20 nm (50 mg/L) and 200 nm (50 mg/L) particles; (b) equal particle count of 20 nm (0.1 mg/L) and 200 nm (50 mg/L) particles. $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

Molting behaviour was also significantly affected by exposure to polystyrene particles. On average, control animals molted within 4.1 days while the treated animals first molted between 5.6 and 8 days, as shown in **Table 4.1**. Based on the statistical analysis of all treatments, the time to

first molt and average number of molts were significantly affected by the 200 nm (50 mg/L) treatment ($(F_{3,34} = 4.0849, P = 0.009521)$ and $(F_{3,30} = 4.82, P = 0.007421)$, respectively, 1-way ANOVA, **Table 4.1**). There was no difference in terms of sensitivity between the two dose metrics for both time to first molt (**Figure 4.2a-b, Table S4.3**) and average number of molts (**Figure S4.3a-b, Table S4.3**).

The average time to first brood was significantly impacted by polystyrene particle exposure (1-way ANOVA, $F_{3,32} = 16.617, P < 0.0001$, **Table 4.1**). It was delayed by 4.8 days in the 20 nm (50 mg/L) treatment, compared to the control (**Table 4.1**). The average time to first brood was significantly higher in the 20 nm (0.1 ppm) and 20 nm (50 ppm) treatments compared to 200 nm (50 ppm) (Tukey HSD, $P = 0.0215$ and $P < 0.0001$, respectively). The average sum of neonates produced by the daphnids was significantly influenced by particle exposure (1-way ANOVA, $F_{3,28} = 6.728, P = 0.001466$, **Table 4.1**). It changed by +38 % for the 200 nm (50 mg/L) treatment compared to the control (**Table 4.1**). The results showed similar trends in time to first brood whether using the mass concentration or particle counts as dose metrics (**Figure 4.2c-d, Table S4.3**). The trend was also followed for the number of neonates in **Figure S4.3c-d**.

The effect of plastic exposure for 21 days on the hopping frequency was not the same for the two dose metrics, as the 20 nm (50 mg/L) group was significantly different from both the control and 200 nm (50 mg/L) treatments at equivalent mass concentration (2-way ANOVA, $F_{4,50} = 2.637, P = 0.0447$, **Figure 4.3a, Table S4.3**), but no significant difference was observed at equal particle numbers (**Figure 4.3b, Table S4.3**). No significant difference was observed in swimming behaviour based on both dose metrics (**Figure S4.4a-b**).

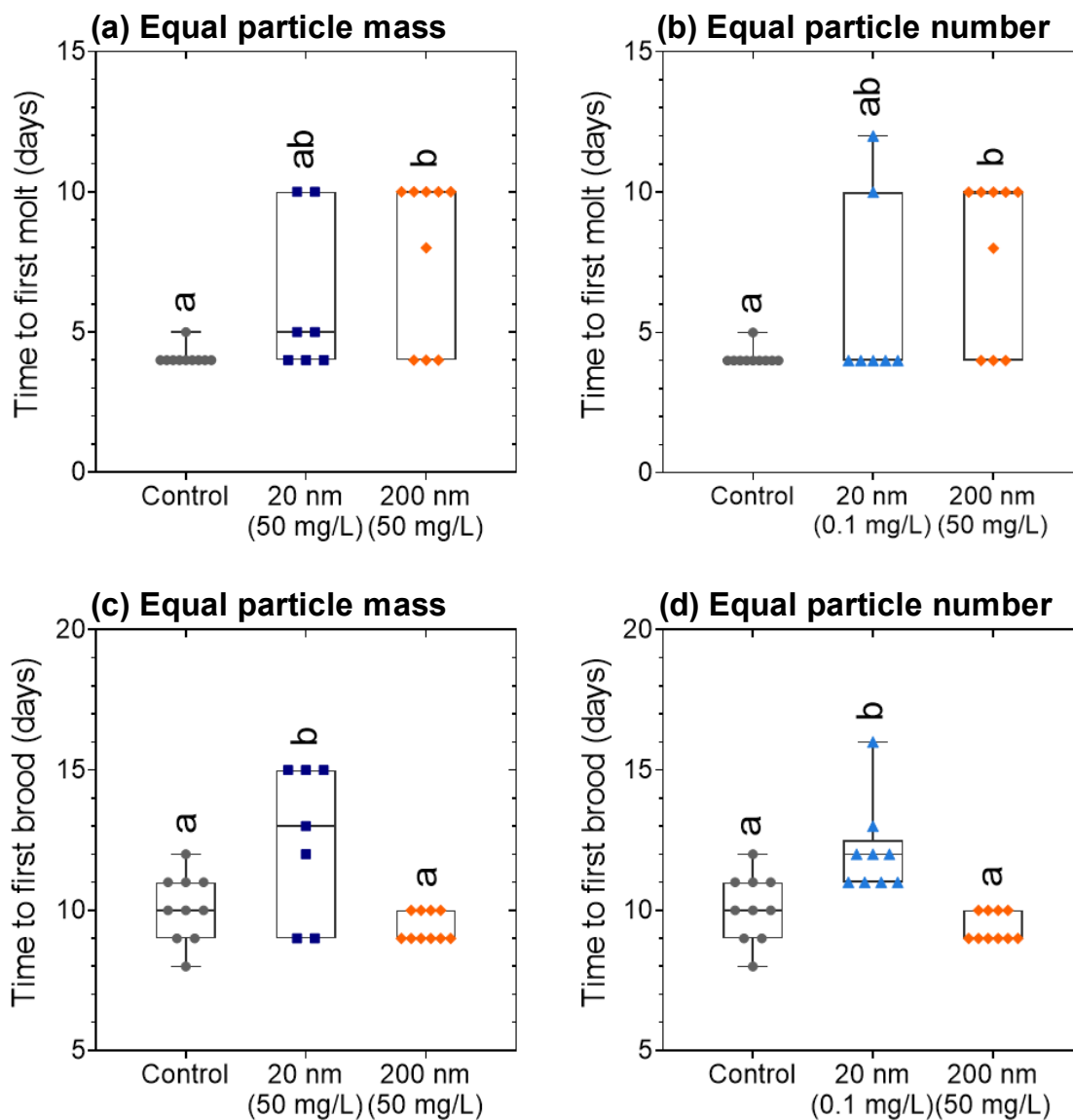


Figure 4.2. Molting and reproduction behaviour of *Daphnia magna* during the 21-day period of exposure to polystyrene particles. (a) time to first molt at equal particle mass concentration of 20 nm and 200 nm particles, (b) time to first molt at equal particle counts of 20 nm and 200 nm particles, (c) time to first brood at equal particle mass concentration of 20 nm and 200 nm particles, (d) time to first brood at equal particle counts of 20 nm and 200 nm particles. $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

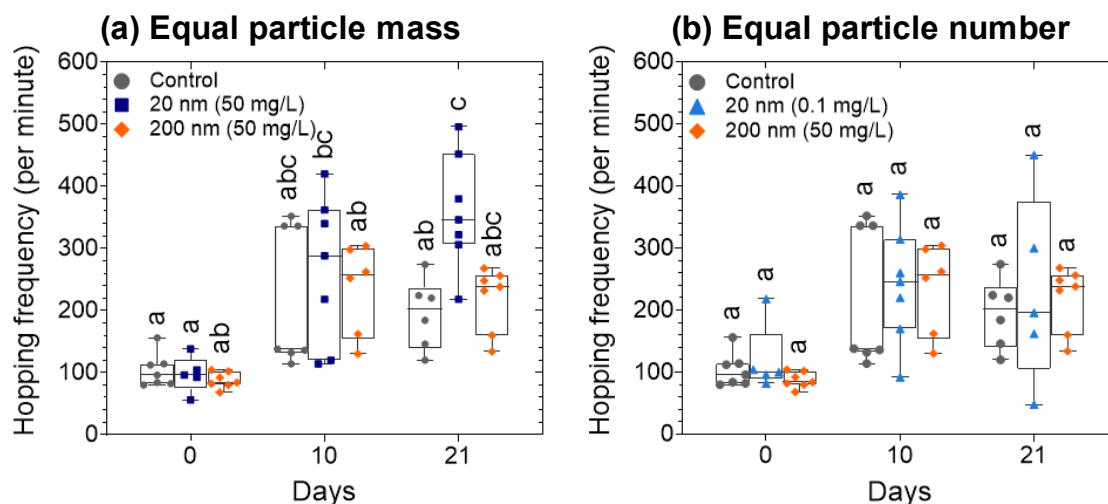


Figure 4.3. Hopping frequency of *Daphnia magna* during the 21-day period of exposure to polystyrene particles. (a) at equal particle mass concentration of 20 nm and 200 nm particles, (b) at equal particle counts of 20 nm and 200 nm particles. $n \geq 7$, 2-way ANOVA, $P < 0.05$. Different letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

4.3.2 Effects of mass concentration on the toxicity of 20 nm polystyrene nanoplastics

Figure 4.4 compares the toxicity endpoints observed for only the 20 nm particles at low (0.1 mg/L) and high (50 mg/L) concentrations. There was a significant effect in the growth of animals upon particle exposure at both concentrations compared to controls (1-way ANOVA, $F_{2,19} = 19.23$, $P < 0.001$), but no significant dose-effect relationship (**Figure 4.4a**). No significant effect of treatments was observed in the time to first molt of the animals (**Figure 4.4b**). However, the time to first brood was significantly impacted at the higher particle concentration (1-way ANOVA, $F_{2,20} = 9.967$, $P = 0.009$, **Figure 4.4c**). The hopping frequency was significantly impacted by 20 nm

particle exposure (1-way ANOVA, $F_{2,46} = 21.723$, $P < 0.001$), with animals exposed to 50 mg/L demonstrating a higher hopping frequency over time compared to the control (**Figure 4.4d**). Significantly increased swimming behaviour was also observed at 50 mg/L at day 10. However, this effect disappeared by day 21 (**Figure S4.4c**).

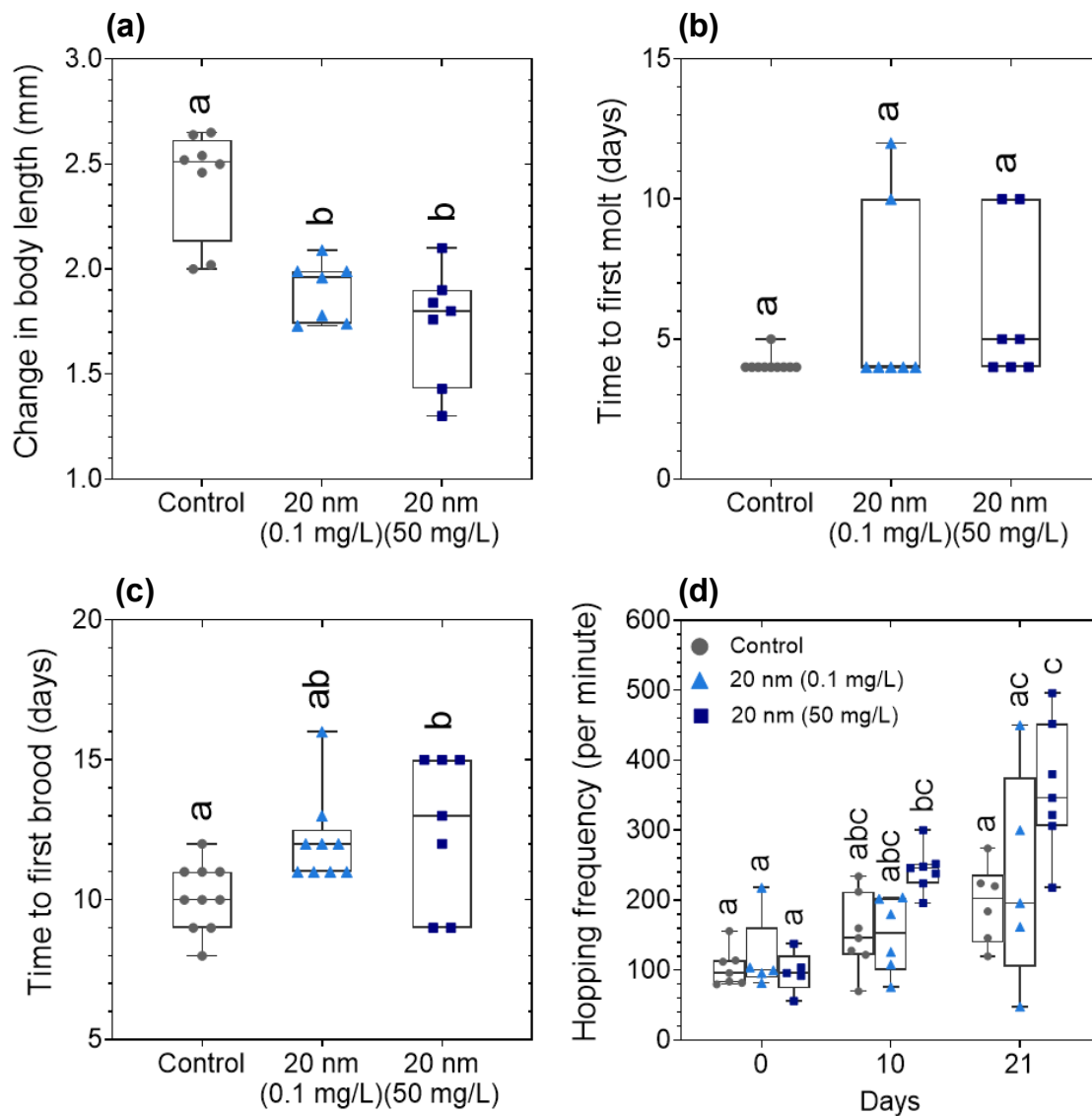


Figure 4.4. Comparison of the observed sublethal endpoints upon the chronic exposure of *Daphnia magna* to 20 nm polystyrene particles at 0.1 mg/L and 50 mg/L. (a) change in body length, (b) time to first molt, (c) time to first brood, (d) hopping frequency. $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

4.4 Discussion

The results of this study provided insights into the sublethal effects of long-term exposure of *D. magna* to polystyrene nanoplastics, as well as the possible influence of the selection of dose metrics on observed toxicity. No significant mortality was observed upon exposure of the daphnids to the particles during the 21-day period (**Table 4.1**). While reports on the effects of chronic exposure of plastic particles to invertebrates, especially *Daphnia*, are still limited, this is consistent with other studies which have earlier suggested that polystyrene microplastics or nanoplastics do not cause mortality to *D. magna* upon chronic exposure.^{18, 46} This observation for washed microplastics and nanoplastics is similar to the results of exposure to some other nanoparticles. For example, previous studies showed that metal oxide nanoparticles such as ceria (CeO₂) and alumina (Al₂O₃) caused little to no mortality to *D. magna* after 21 days of exposure at concentrations as high as 2000 mg/L.⁴⁷ However, exposure to Ag and La nanoparticles even at low concentrations (as low as 80 µg/L) resulted in mortality up to 100% in *D. magna* after 21 days of exposure.^{48, 49} The difference in the observed mortality of polystyrene nanoplastics compared to other nanoparticles which cause mortality may be associated with a number of factors involving different modes of action, such as nanoparticle dissolution, induced oxidative stress or organism-dependent cellular uptake.⁵⁰ For example, Wang *et al.* showed that the observed toxicity of ZnO and CuO nanoparticles originated from the release of metal ions.⁵¹ Zimmerman *et al.* reported that the observed toxicity of certain microplastics such as polyvinyl chloride may be due to the chemicals present in the materials.⁵² This may explain why there was no significant mortality observed upon exposure to virgin polystyrene nanoplastics which do not contain any additives or plasticizers, and hence, are less likely to leach toxicants.

Polystyrene nanoplastics inhibited growth in the exposed animals at both sizes and irrespective of the concentration (**Figure 4.1, Table 4.1**). Moreover, there was significant delay in reproduction upon exposure to the 20 nm particles, while exposure to the 200 nm particles resulted in delay in molting and reduced number of molting during the 21 day period (**Figure 4.2, Figure S4.3**). This is the first study to link the exposure of polystyrene nanoparticles to the molting behaviour of *D. magna*, although exposure to 100 nm TiO₂ nanoparticles also resulted in delayed molting.⁵³ Only a few studies have shown growth or reproduction inhibition effects of microplastics or nanoplastics on *D. magna* upon chronic exposure.^{18, 28, 54} The size of the nanoplastics used in the study were significantly smaller than the average size of the regular algae which serves as the main food for *Daphnia magna*. In fact, other studies have reported higher depuration rate and lower body burden upon chronic exposure of *Daphnia magna*⁵⁵ and *Drosophila melanogaster*⁵⁶ to polystyrene nanoplastics in the presence of food. While it is not clear whether the ingestion of nanoplastics may block the gut of the *Daphnia magna*, it may cause other feeding-related impacts such as reduction in the absorption of nutrients, increased feeding rate due to continuous hunger, etc. Enserink *et al.*⁵⁷ and Karimi *et al.*⁴⁷ suggested that reduced nutrient intake following the consumption of a mixture of food and plastic particles compared to feeding on the same volume or mass of nutrients such as algae and YCT only may be responsible for inhibition in growth and reproduction. Other reports have indicated that the maximum rate of growth and reproduction of daphnids may require these nutrients, and in their absence, the organism may need more time to accumulate the nutrients required for molting, growth and reproduction.^{58, 59} Porcella *et al.*⁶⁰ previously reported that the molting rate of *D. magna* was largely dependent on its accumulation of calcium and could be affected by both physiological and environmental variables. The increase in the production of neonates for the 200 nm particles may be due to hormesis, an adaptive response to low-level stress, as suggested by Xu *et al.*¹⁸ who also

reported increase in reproduction upon exposure to secondary nanoplastics. Similar behaviour has also been observed upon exposure of *D. magna* to metal nanoparticles such as 150 nm f-SiO₂.⁴⁷ Studies on molecular responses have indicated that exposure of *Daphnia pulex* to nanoplastics can stimulate various stress defense and breeding adjustment mechanisms such as production of antioxidants as part of its “hormesis effect”, which may result in increased number of neonates.⁶¹ Also, multigenerational studies observed significant changes in the transcription of reproduction-related genes upon exposure of *Daphnia pulex* to nanoplastics.⁶¹

In general, no significant difference was observed in toxicity endpoints when either a particle mass or particle count basis was used as dose metric (**Figure 4.1-2**). However, a significant difference in the hopping frequency was observed after 21 days when *D. magna* were exposed to 50 mg/L of 20 nm particles (**Figure 4.3**). For a given mass concentration of particles, the smaller sized particles are present in much greater numbers compared to the larger particles. The increased hopping frequency observed upon exposure of *Daphnia* to 50 mg/L of the 20 nm particles could be due to the considerable increase in number of particles present at that concentration (4.6×10^{12} particles mL⁻¹), compared to the 8.5×10^9 particles mL⁻¹ present in both 20 nm (0.1 mg/L) and 200 nm (50 mg/L) treatments. However, no significant difference was observed in the hopping frequency at equal particle numbers of 200 nm (50 mg/L) and 20 nm (0.1 mg/L). This suggests that a higher particle count of the polystyrene particles may introduce stress to the animals without necessarily causing changes to other toxicity endpoints. Also, the swimming behaviour for the 20 nm (50 mg/L) particles was slightly higher between day 10 and 17 but appeared to normalize at day 21 (**Figure S4.4**). No significant difference was observed in the swimming behaviour of *Daphnia* exposed to equal particle counts of 200 nm (50 mg/L) and 20 nm (0.1 mg/L) particles. Movement behaviour such as hopping frequency and swimming distance is important regarding

survival as it is related to predation rate, as increased movement increases the visibility of animals to predators and vice versa.^{40, 62, 63} De Felice *et al.* observed increased swimming activity upon exposure of *D. magna* to 12.5 mg L⁻¹ polystyrene particles.²⁸ Besides polystyrene particles, other studies on the effects of carbon-based and metal-based nanoparticles such as fullerenes (nano-C₆₀ and C₆₀H_xC₇₀H_x), nano-Ag and nano-Ti have shown drastic changes in hopping frequency and swimming behaviour.^{36, 64}

The observed toxicity of 20 nm polystyrene nanoplastics at a high and low concentration was compared. While exposure to 20 nm particles resulted in a significant effect on growth, reproduction, and molting endpoints relative to the control (**Figure 4.1, Figure 4.2(c-d) and Figure S4.3(a-b), respectively**), there was no significant difference when two concentrations, 50 mg/L and 0.1 mg/L, were compared (**Figure 4.4**). Previous studies have used high concentrations of polystyrene particles, up to 400 mg/L, for toxicity studies with *Daphnia magna*.⁶⁵ Only a few studies have compared the long-term effects of same type and size of polystyrene particles at different concentrations.^{19, 20, 55, 66} This report suggests that there are no significant differences in the toxicity endpoints between high and low concentrations of 20 nm polystyrene nanoplastics. Similarly, Rist *et al.* showed that an increase in concentration did not cause a significant difference in the time to first brood, number of broods and number of neonates per brood when *D. magna* was exposed to 100 nm polystyrene particles between 0.1 and 1 mg/L.⁵⁵ Also, Kelpsiene *et al.* did not see any significant difference in reproduction output of *D. magna* upon exposure to polystyrene particles between 0.0032 and 0.32 mg/L in chronic toxicity studies over the same 21-day period.¹⁹ The dose-response of organisms upon exposure to nanoplastics is not linear, hence, the saturable uptake of the particles may occur at a concentration below the two tested concentrations.⁶⁷ Hence, the number of particles at the low concentration (0.1 mg/L) may be high enough to cause the

observed impacts. This may explain why there is no significant difference at the two tested concentrations for the 20 nm nanoplastics. Also, deleterious effects reduce over time for sub-lethal exposures, particularly in the presence of food.^{55, 68} Thus, it is possible that the effect of the higher concentration reduces over the exposure period. However, there is a need for more studies to investigate the effects of varying concentrations using more sensitive endpoints such as molecular and biochemical responses. Advances in imaging techniques will also enable localization of nanoplastics in exposed organisms for improved understanding of transport and uptake mechanisms.⁶⁹

Assessing the toxicity of microplastics and nanoplastics is complex, partly due to the spherical shape of commercial plastic formulations which may not mimic most of the particles found in the environment, as well as due to the absence of various salts, organic molecules, pollutants, and other factors which may be present in natural environments and may interact with the particles.⁷⁰ Furthermore, due to difficulties in determining the concentration of microplastics and nanoplastics in environmental waters, there are debates in the scientific community about the discrepancy between true concentrations of these particles in the aquatic environment and the tested concentrations in scientific studies.^{70, 71} The higher concentrations tested in this study may be considered representative of pollution “hotspots” where plastic litter breaks down, thereby releasing a large number of nanoplastics.

Polymer type, size and shape are critical parameters to understand the toxicity of nanoparticles.⁷²⁻

⁷⁴ While most toxicity studies on polystyrene microplastics and nanoplastics traditionally use mass as a standard dose metric, the appropriateness of this approach has been questioned. Kennedy *et al.* showed that mass as a dose metric is not adequate for particle-organism interactions when investigating exposure to small-sized particles.²⁵ Particle number concentration has been

previously reported to be more relevant than mass as a dose metric for particle-organism interactions such as translocation of nanoparticles such as Au.²⁶ However, this has been debated as being less predictive in comparison to surface area.²³ Wittmaack *et al.* investigated the dose metrics that are most suitable for the study of the exposure of TiO₂ nanoparticles to mice. They reported that all tested dose metrics worked effectively with the exception of particle-size based surface area while the particle number was the most effective.⁷⁵ Other studies have shown that the size of the particles plays an important role in which dose metric will be most appropriate for toxicity studies.⁷⁶ Oberdörster *et al.* believes that the appropriate dose metric of each type of particle is distinct and dependent on several factors such as charge, coating, crystallinity, porosity, etc.²³

While we only tested two levels of particle number or particle mass concentration, our experimental results show that the choice of dose metric did not impact the key conclusions. Surface area may be an important dose metric in assessing the toxicity of microplastics and nanoplastics that was not investigated in this study. Therefore, more studies are needed with a higher number of test concentrations to understand the role of dose metrics on other size ranges of polystyrene nanoplastics as well as other types of plastics. The effects of dose metrics such as surface area, mass concentration and particle number on leaching of soluble chemicals from plastic particles, as well as the adsorption of organic pollutants on the plastic particles, should also be studied. As the naturally weathered plastic particles in the environment are not pristine commercially formulated spheres, our future work will emphasize the effects of irregularly shaped microplastics and nanoplastics formed from the breakdown of larger particles that are more prominent in natural waters.

4.5 Conclusion

The results of this study showed that exposure to 20 nm and 200 nm polystyrene particles had effects on growth, reproduction, and molting behaviour of daphnids. The observed effects on most endpoints were similar for both mass and particle count dose metrics. Furthermore, exposure of *Daphnia magna* to 50 mg/L of 20 nm particles did not show any significant difference on the toxicity endpoints observed when compared to exposure to 0.1 mg/L of the same particle size, suggesting that polystyrene nanoplastics may cause significant toxicity even at low and environmentally relevant concentrations. The observed changes in hopping frequency and swimming behaviour suggest that higher particle counts may introduce additional sub-lethal stress to the animals, thereby causing long-term effects on the population of *D. magna*.

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4.6 Supporting Information

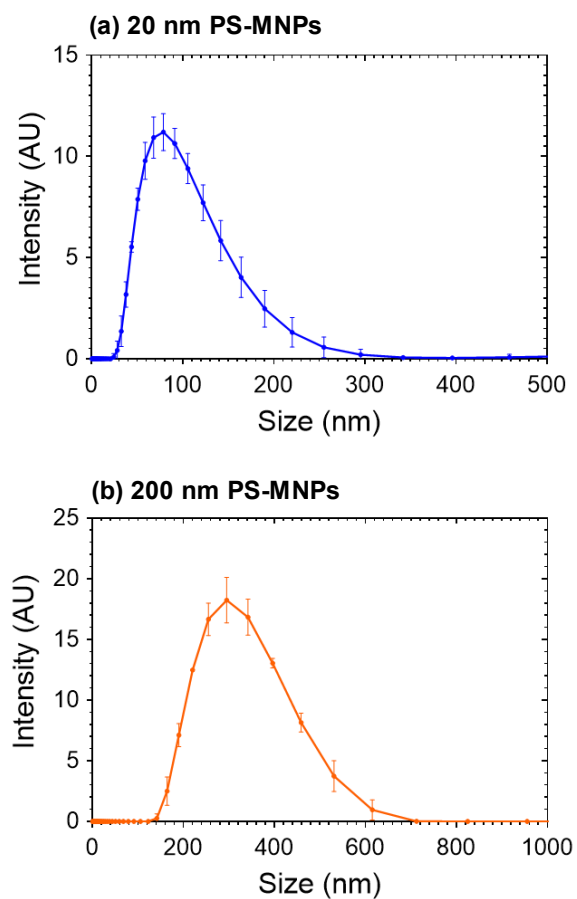


Figure S4.1. DLS measurements of nanoplastics suspended in MHRW before exposure to *Daphnia magna*: (a) 20 nm particles, (b) 200 nm particles.

Table S4.1. Water quality parameters throughout the 21-day chronic toxicity study.

	Day 2	Day 4	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 21
Dissolved oxygen (mg L⁻¹)									
Control	7.3 ± 0.0	9.4 ± 0.1	7.5 ± 0.1	9.6 ± 0.2	9.4 ± 0.2	8.9 ± 0.1	9.1 ± 0.1	9.2 ± 0.1	9.3 ± 0.1
20 nm (0.1 ppm)	7.0 ± 0.1	8.9 ± 0.1	6.9 ± 0.0	9.0 ± 0.1	9.2 ± 0.2	9.0 ± 0.1	9.2 ± 0.3	9.0 ± 0.1	8.7 ± 0.1
20 nm (50 ppm)	7.0 ± 0.1	8.9 ± 0.0	7.3 ± 0.2	9.1 ± 0.0	9.1 ± 0.1	9.0 ± 0.1	9.4 ± 0.1	8.9 ± 0.1	8.9 ± 0.1
200 nm (50 ppm)	7.3 ± 0.1	9.3 ± 0.1	7.5 ± 0.0	9.6 ± 0.1	9.2 ± 0.1	9.0 ± 0.1	9.3 ± 0.1	9.2 ± 0.1	9.2 ± 0.1
pH									
Control	7.3 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.0	7.7 ± 0.0	7.8 ± 0.1	8.1 ± 0.0
20 nm (0.1 ppm)	7.6 ± 0.1	7.8 ± 0.0	7.8 ± 0.1	7.7 ± 0.0	7.7 ± 0.0	7.9 ± 0.0	7.5 ± 0.0	7.8 ± 0.0	7.9 ± 0.2
20 nm (50 ppm)	7.6 ± 0.0	7.8 ± 0.0	7.7 ± 0.1	7.7 ± 0.0	7.8 ± 0.0	7.9 ± 0.0	7.4 ± 0.0	7.9 ± 0.0	8.0 ± 0.0
200 nm (50 ppm)	7.4 ± 0.2	7.8 ± 0.0	7.8 ± 0.1	7.7 ± 0.1	7.8 ± 0.0	7.9 ± 0.0	7.6 ± 0.1	7.7 ± 0.0	8.1 ± 0.0

Temperature

Control	20.3 ± 0.1	21.1 ± 0.0	21.3 ± 0.1	21.5 ± 0.0	21.1 ± 0.1	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0
20 nm (0.1 ppm)	20.4 ± 0.1	21.2 ± 0.1	21.3 ± 0.0	21.4 ± 0.1	20.8 ± 0.0	21.2 ± 0.0	21.1 ± 0.0	21.2 ± 0.1	21.2 ± 0.1
20 nm (50 ppm)	20.5 ± 0.1	21.2 ± 0.0	21.4 ± 0.0	21.4 ± 0.1	21.0 ± 0.0	21.2 ± 0.0	21.1 ± 0.0	21.2 ± 0.0	21.2 ± 0.0
200 nm (50 ppm)	20.3 ± 0.0	21.2 ± 0.0	21.5 ± 0.1	21.5 ± 0.1	20.9 ± 0.1	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0	21.2 ± 0.0

Table S4.2. Feeding rations (per test beaker) of *C. reinhardtii* and YCT to *D. magna* during a 21-day chronic exposure to polystyrene particles. ⁴²

Day	Algae	YCT
0	0.3 mL	0.3 mL
2	0.3 mL	0.3 mL
4	0.6 mL	0.6 mL
6	0.6 mL	0.6 mL
8	0.6 mL	0.6 mL
10	1 mL	1 mL
13	1 mL	1 mL
17	1 mL	1 mL
21	*	*

*Organisms were not fed on the last day

Table S4.3. Summary table of the P-values and significance levels (*, **, ***) for factorial ANOVAs testing the effects of the concentrations (*i.e.* particle concentrations or mass concentrations, 1-way), days (*i.e.* duration of exposure, 2-ways), and the interactive effect between concentrations and days for two particle sizes, 20 and 200 nm, with two levels of particle concentration for 20 nm. Kruskal-Wallis test was applied when normality assumption was not met for 1-way ANOVAs. *F* = Fischer calculated values, followed by the degree of freedom and residual degree of freedom. χ^2 = Chi square value from Kruskal-Wallis followed by the degree of freedom. Significance was set to 0.05.

Endpoint	Factor(s)	Mass concentration (Control, 20 nm (50 ppm), and 200 nm (50ppm))	Particle concentration (Control, 20 nm (0.1 ppm), and 200 nm (50 ppm))
Physiology			
- Length	Concentration	$\chi^2_{2} = 4.55, P = 0.1026$	$\chi^2_{2} = 8.38, P = 0.0151$ *
	Days	$\chi^2_{9} = 255.92, P < 2.2^{-16}$ ***	$\chi^2_{9} = 256.16, P < 2.2^{-16}$ ***
- Change in length	Concentration	$F_{2, 20} = 19.25, P = 2.18^{-05}$ ***	$\chi^2_{2} = 11.21, P = 0.003679$
Behavior			
- Hopping frequency	Concentration	$F_{2, 50} = 4.401, P = 0.0174$ *	$F_{2, 51} = 0.313, P = 0.7327$
	Days	$F_{2, 50} = 19.5253, P = 5.41^{-07}$ ***	$F_{2, 51} = 9.683, P = 2.72^{-04}$ ***
	Concentration:Days	$F_{4, 50} = 2.637, P = 0.0447$ *	$F_{4, 51} = 0.455, P = 0.7682$
- Swimming distance	Concentration	$F_{2, 71} = 6.207, P = 0.0033$ **	$F_{2, 71} = 4.132, P = 0.0201$ *
	Days	$F_{3, 71} = 5.362, P = 0.0022$ **	$F_{3, 71} = 0.065, P = 0.9784$

	Concentration:Days	$F_{6, 71} = 2.839, P = 0.0155 *$	$F_{6, 71} = 0.561, P = 0.7599$
Reproduction			
- Neonates per animal	Concentration	$F_{2, 22} = 10.36, P = 0.0007 ***$	$F_{2, 22} = 5.056, P = 0.0156 *$
- Time to first brood	Concentration	$\chi^2_2 = 15.836, P = 0.0004 ***$	$F_{2, 22} = 11.14, P = 0.0005 ***$
Moult			
- Number of molts	Concentration	$F_{2, 23} = 7.142, P = 0.0039 **$	$F_{2, 24} = 7.141, P = 0.0037 **$
- Time to first molt	Concentration	$F_{2, 22} = 5.731, P = 0.0099 **$	$F_{2, 22} = 4.67, P = 0.0204 *$

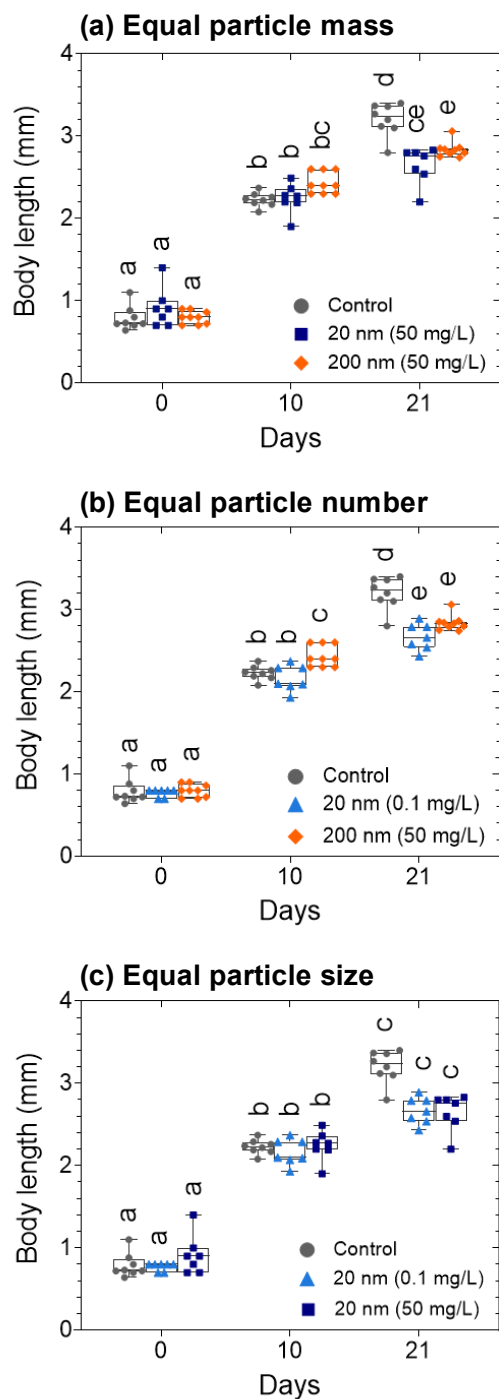


Figure S4.2. Body length of *Daphnia magna* at the end of the 21-day period of exposure to polystyrene nanoplastics. (a) control and equal particle mass concentration of 20 nm and 200 nm particles, (b) control and equal particle counts of 20 nm and 200 nm particles, (c) control, 0.1 ppm and 50 ppm of 20 nm particles (equal particle size). $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different

letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

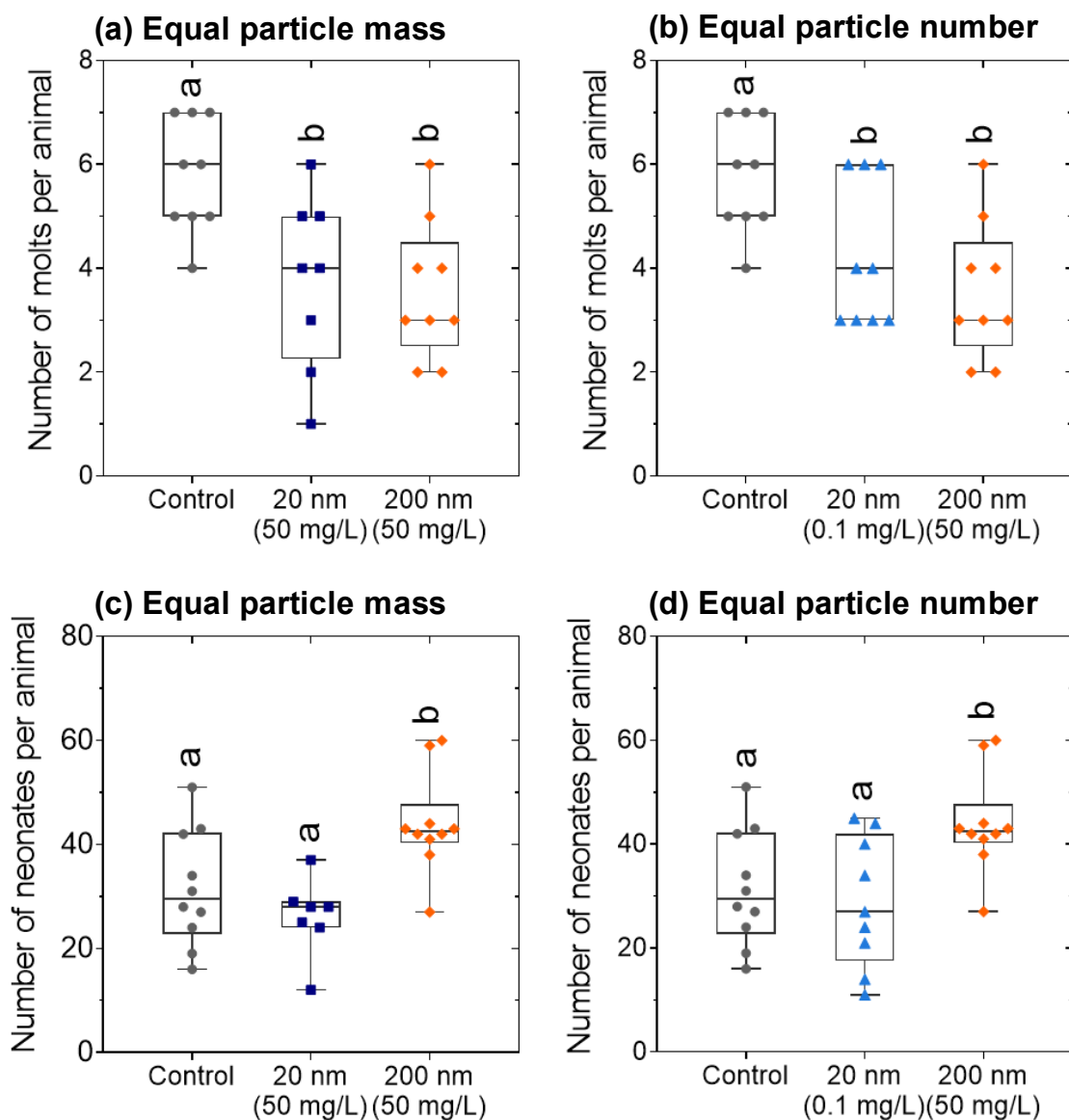


Figure S4.3. Reproduction behaviour of *Daphnia magna* during the 21-day period of exposure to polystyrene nanoplastics. (a) number of molts per animal at equal particle mass concentration of 20 nm and 200 nm particles, (b) number of molts per animal at equal particle counts of 20 nm and

200 nm particles, (c) number of neonates per animal at equal particle mass concentration of 20 nm and 200 nm particles, (d) number of neonates per animal at equal particle counts of 20 nm and 200 nm particles. $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

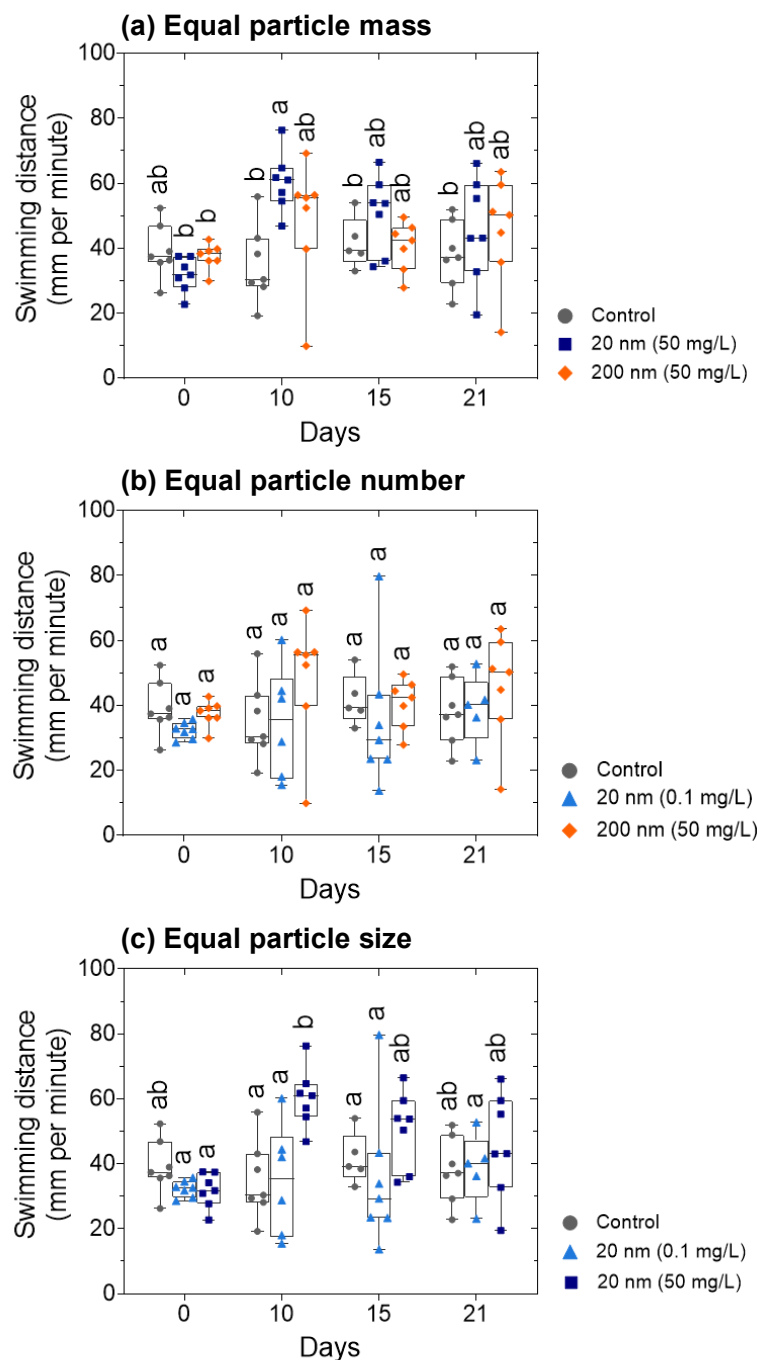


Figure S4.4. Swimming behaviour of *Daphnia magna* during the 21-day period of exposure to polystyrene nanoplastics. (a) control and equal particle mass concentration of 20 nm and 200 nm particles, (b) control and equal particle counts of 20 nm and 200 nm particles, (c) control, 0.1 ppm and 50 ppm of 20 nm particles (equal particle size). $n \geq 7$, 1-way ANOVA, $P < 0.05$. Different

letters represent significant difference at $P < 0.05$, while the same letter indicates no significant difference.

Chapter 5: Fate of Microfibres from Single-Use Face Masks: Release to the Environment and Removal During Wastewater Treatment

Connecting Text

In Chapter 2, we found that studies using irregular shaped particles for toxicity are rare as most studies use spherical plastic beads in their toxicity assessments. While the most common sources of spherical microplastics are anthropogenic and regulated by most countries, a considerable portion of the non-spherical microplastics and nanoplastics in the environment is produced from environmental degradation and fragmentation of larger plastic debris. There are also limited studies to investigate the fate of microplastics from real plastic products and their potential concentration in natural aquatic systems. The goal of this chapter is to select a sample of real-life source of microplastics and investigate the release of microplastics from the selected plastic product and their fate in the aquatic environment. The COVID-19 pandemic has motivated the need to investigate single-use face masks as another potential source of microplastics, following its global usage to curb the spread of the pandemic. This study developed a novel approach to assess the release of microplastics from face masks. The chapter also examined the fate of these microplastics during wastewater treatment.

The findings from this chapter were published as: Pikuda, Oluwadamilola, Mathieu Lapointe, Olubukola S. Alimi, Dimitrios Berk, and Nathalie Tufenkji. "Fate of microfibres from single-use

face masks: Release to the environment and removal during wastewater treatment." *Journal of Hazardous Materials* 438 (2022): 129408.

Abstract

Single-use face masks can release microfibres upon exposure to environmental conditions. This study investigates the number of microfibres released in the presence and absence of UV irradiation and mechanical friction and the removal of the released microfibres in a simulated conventional wastewater treatment process. UV exposure results in a four-fold increase in the number of microfibres released from new masks and used masks resulting in ~2400 microfibres/mask and ~1100 microfibres/mask, respectively. Application of mechanical friction to the UV-exposed new and used masks further increases the number of released microfibres per mask. In a simulated coagulation/flocculation process, the removals of microfibers originating from new masks and used masks are 79% and 91%, respectively. XPS analysis reveals that the silica content of the used masks is 240% higher than that of new masks, which could explain the higher removal efficiency of microfibers from used masks. FTIR analysis of the masks after UV exposure shows carbonyl indices of 0.73 ± 0.70 and 0.27 ± 0.10 for the microfibres from used and new masks, respectively. Based on available data, we estimate that 4–47 million polypropylene microfibres can be released into natural waters per day after wastewater treatment in an urban environment.

5.1 Introduction

The COVID-19 outbreak has triggered an increase in the production, consumption and disposal of personal protective equipment, including single-use face masks.^{1, 2} The global use of single-use face masks has been estimated to be more than 1.5 trillion per year.³ While masks used by medical or healthcare professionals are managed through a regulated waste disposal system, those used by the general public are primarily disposed as domestic waste or non-recyclable items. Also, several studies report littering of the environment due to improper disposal of face masks.^{4, 5}

Single-use face masks are commonly produced from three layers of polypropylene, the outer and inner layers consisting of non-woven fabric and a middle layer of melt-blown filter fabric. Although these face masks are critical to curbing the spread of the COVID-19 pandemic, it is equally pertinent to understand the potential implications associated with the uncontrolled disposal of these masks to the environment, especially to natural waters.^{6, 7} Plastic waste in the environment can break down into smaller fragments due to (photo)oxidation, temperature variations, and mechanical stresses.^{8, 9}

Recent studies aimed at understanding the breakdown of single-use face masks in marine environments report the release of hundreds of microfibrils per new mask when submerged in deionized water under low mechanical stress.¹⁰⁻¹² Other studies reported the release of up to millions of microfibrils from a single mask when exposed to single or multiple intense stressors such as UV irradiation, mixing with sand, vigorous mechanical shaking in water, hand-washing, blending in kitchen choppers, etc.¹³⁻¹⁷ A considerable portion of single-use face masks are used and discarded in urban settings where they are exposed to wear and tear on roadways and sidewalks

but less likely to be completely submerged in high salinity waters; the release and fate of mask microfibres in these environments are not understood.

Up to 80% of plastic debris in the marine aquatic environment originates from land sources such as poorly managed landfills, windborne waste, stormwater runoff, untreated sewage, industrial plastic production facilities.^{18, 19} In many urban environments, plastic litter such as face masks and their released fibres are likely to be introduced into the combined sewer system via stormwater runoff (or directly released to natural waters for separated sewers). While the separated sewers release the runoffs directly into natural waters, the combined sewer system removes solid particles such as microplastics through wastewater treatment plants (WWTP) before releasing the effluents into natural waters. In fact, microplastics, including microfibres, have been shown to be effectively removed in WWTP.^{20, 21} However, the unremoved microfibres could still account as a significant source of millions of microfibres in the water environment daily.²² This explains why WWTP have also been identified as an important point source for microplastics, especially microfibres, into waterbodies.²³ While the removal of microfibres and microplastics during wastewater treatment has been investigated²⁴⁻²⁷, there is still limited information regarding the fate of polypropylene microfibres released from face masks in conventional wastewater treatment.

Estimates of weathered plastics in the natural environment have been reported using environmental samples²⁸ and model predictions²⁹. However, the reports from environmental sampling studies vary largely and are difficult to harmonize due to methodological differences such as collection method, and the depth of water column sampled.³⁰ The concentration of light-weight microplastics such as polypropylene may decrease with depth in the natural waters. Hence, there may be more plastics floating on water surfaces than reported in sampling studies.³¹ Face masks have low density and have a high likelihood of floating on water surfaces. Hence, it may be difficult to

estimate accurately the presence of the face mask fibres in the natural environment through conventional sampling methods. Yet, there is a need for environmentally relevant studies to investigate the release of microfibrils from face masks through laboratory tests that feature mechanical stress as well as the combined influence of UV irradiation and exposure to extreme temperatures.

The first part of this work aims to study the release of microfibrils from new and used single-use face masks under weathering conditions more relevant to urban settings; namely, when the face mask is exposed to friction but not completely submerged in water. Used single-use face masks were collected from the streets in Montreal (Canada) to compare the release of microfibrils from the used masks with those from new masks. The masks were exposed to UV irradiation while floating on a water surface to assess the release of microfibrils and their size distribution. The second phase of this work aims to investigate the properties of the released microfibrils and the efficiency of a conventional physicochemical treatment (i.e., coagulation/flocculation) for the removal of the released microfibrils from municipal wastewaters. Coagulation/flocculation is a very popular method for both water treatment and wastewater treatment applications.^{32, 33} Bench-scale coagulation/flocculation tests (commonly known as jar tests) were used to evaluate the removal of mask microfibrils from a synthetic wastewater via conventional coagulation/flocculation treatment. To study the microfibril properties and their interactions with coagulants, the microfibrils were characterized using microscopy, Fourier-transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). We hypothesize that the combination of different weathering processes would accelerate the release of microfibrils and impact the removal of the microfibrils during wastewater treatment. Finally, we used existing data and the results of the first two sections to project an estimate of the number of face mask

microfibres that may be released into natural waters from face mask pollution in an urban environment.

5.2 Materials and Methods

5.2.1 Source of Face Masks

New three-ply single-use non-medical disposable protective face masks were purchased from JCJZ, USA. Used face masks were collected from the streets in the Cote St. Luc/Notre-Dame-de-Grace Area, Montreal Island, Quebec, Canada. Photos were taken showing the environment where the used masks were collected (**Figure S5.1**). The new masks were used as purchased and the used face masks were used as collected. The three layers of the 3-ply masks are referred to as inner layer, middle layer and outer layer as shown in **Figure S5.2**. This study focused on only the inner and outer layers because both layers are made of non-woven fabric and have direct exposure to environmental stressors.

5.2.2 Weathering of Face Masks

In an urban setting, face mask litter is often found on the street where it can be wetted by precipitation or floating in a puddle. A custom-built weathering chamber was set up to weather single-use face masks floating on a water surface to capture released microfibres. The chamber could hold six 1.5 L rectangular glass dishes (20 cm×15 cm, height of 7 cm), each placed on a magnetic stirrer. Each dish was filled with 1 L of filtered deionized water and a glass stir bar. In each of the glass dishes, a single mask was placed with either the outer or the inner layer contacting

the water surface such that released microfibres would be collected in the water inside the dish. The dishes were covered with clean glass sheets to minimize the risk of contamination from the air. All stirrers were set to 200 rpm to gently stir the water in each dish.

The chamber was equipped with four UV lamps (ReptiSun® 10.0 Mini Compact Fluorescent) with 10 % UVB and 30 % UVA output ($\lambda=280\text{--}400\text{ nm}$). The distance between the UV lamps and the top of the water surface in the glass dishes was 20 cm. The UV irradiation received by the face masks during the weathering process was $9.8 \pm 0.2\text{ W/m}^2$, measured using an ultraviolet meter (Apogee Instruments, MU-200). The weathering experiment was carried out for 10 days. For “dark” conditions, the UV lamps were turned off and the chamber was covered in aluminum foil and fabric to prevent light from penetrating. A picture of the weathering chamber is shown in **Figure S5.3**.

Twelve treatments were carried out as shown in **Table 5.1**. The treatments were labelled to indicate the type of mask used, the side of the face mask that was in contact with water during weathering, as well as the weathering condition. For example, “new mask, outer layer, UV”. For the treatments that indicate “both sides”, the side of the face mask that was in contact with water was switched after 5 days. Hence, both the outer layer and inner layer were in contact with water for 5 days each. For the treatments labelled as “new mask, both sides, rubbed, UV” and “used mask, both sides, rubbed, UV”, the face masks were rubbed for 2 min daily throughout the weathering period by applying a pressure between $\sim 31\text{--}47\text{ kPa}$. The method is described in the Supporting Information and schematically represented in **Figure S5.4**. Control glass dishes with only deionized water were also exposed to the same conditions to take into account airborne contamination. The temperature in the chamber with UV irradiation was $\sim 25\text{ }^{\circ}\text{C}$ while the temperature in the dark chamber was $\sim 20\text{ }^{\circ}\text{C}$. Three dishes were set up for each treatment, including the control.

Table 5.1. Summary of the experimental treatments.

Treatments	Label	Number and type of masks	Condition	Layer of mask in contact with water	Method
control 1	control, dark	no mask*	in dark	-	floating on water
control 2	control, UV	no mask*	under UV	-	floating on water
1	new mask, inner layer, dark	3 new masks	in dark	inner layer	floating on water
2	used mask, inner layer, dark	3 used masks			
3	new mask, outer layer, dark	3 new masks	in dark	outer layer	floating on water
4	used mask, outer layer, dark	3 used masks			
5	new mask, inner layer, UV	3 new masks	under UV	inner layer	floating on water
6	used mask, inner layer, UV	3 used masks			
7	new mask, outer layer, UV	3 new masks	under UV	outer layer	floating on water
8	used mask, outer layer, UV	3 used masks			
9	new mask, both sides, UV	3 new masks	under UV	both sides	floating on water
10	used mask, both sides, UV	3 used masks			
11	new mask, both sides, rubbed, UV	3 new masks	under UV	both sides	floating on water and rubbed with fingers
12	used mask, both sides, rubbed, UV	3 used masks			

* control 1 and control 2 were performed in triplicate.

At the end of the 10-day weathering period, the masks were removed using stainless steel tweezers, and all the water in each glass dish was filtered using a 20 µm nylon screen (Pentair Aquatic Eco-Systems, USA) in a laminar flow biological safety cabinet. Microfibres collected on the screens

were counted using a stereomicroscope (Fisher Stereomaster). The counting was repeated three times for each sample. To estimate the size distribution of the microfibres, 100 microfibres from each of the treatments were randomly selected and imaged using a stereomicroscope (10× magnification, Fisher Stereomaster). The length of the microfibres was estimated from the images using the built-in freehand drawing tool in ImageJ to trace the length of each microfibre. The lengths of the microfibres were measured to scale and recorded.

5.2.3 Tests to Simulate Wastewater Treatment

Classic jar test experiments were conducted using ~200 microfibres from three different treatments: (i) “new mask, outer layer, dark”, (ii) “new mask, outer layer, UV”, and (iii) “used mask, outer layer, UV”. The microfibres were obtained by manual counting of filtered samples on a screen, and suspended in separate 500 mL glass beakers containing 200 mL of synthetic wastewater prepared using the OECD protocol³⁴ for each of these treatments. Although both the inner and outer layers could be directly exposed to solar irradiation when littering urban settings, this study focused on the outer layer after preliminary tests showed no significant difference in the removal of fibers originating from either layer (**Figure S5.5**).

In addition to these 3 treatments, a 4th treatment in which microfibres were obtained through a more aggressive method was also tested. For this 4th treatment, one new single-use face mask was cut using steel scissors to separate the three layers. The outer layer was ground in a blender (Nutri Ninja® Pro) for 3 min and filtered to retain only microfibres (**Figure S5.6**). The microfibres obtained from this additional process are referred to as “new mask, outer layer, blended, dark” microfibres. From this treatment, ~200 microfibres were used for wastewater treatment

experiments. The length distribution of these microfibrils was measured using 100 microfibrils that were randomly selected and imaged using the same approach described above.

The composition of the synthetic wastewater is shown in **Table S5.1**. SiO₂ particles (1-5 µm, S5631, Sigma-Aldrich) were added to reach a total suspended solids concentration of 100 ± 5 mg/L. The microfibre-containing wastewater was treated using a conventional alum coagulation process.²¹ Aluminum sulfate (commonly known as alum, ALS, Kemira, Canada) was used as the coagulant while an anionic high molecular weight polyacrylamide (PAM, A-100, Kemira) was used as the flocculant. The concentrations of alum and PAM in the test system were 100 mg/L (9.1 mg Al/L) and 0.6 mg/L, respectively. These concentrations were determined based on preliminary tests to achieve the desired turbidity for wastewater treatment. All chemical reagents used were analytical grade and obtained from Sigma-Aldrich (Canada). All stock solutions were stored in the dark at 4 °C.

Jar tests were performed in a flocculation tester (JLT6, VELP Scientifica) equipped with six 500 mL beakers and 6 stirring rods. Microfibre-containing synthetic wastewater suspensions for all four treatments were mixed at 90 rpm for 1 min before the addition of alum at the same mixing speed. After the addition of alum, the stirring of the suspension was continued for 2 min (coagulation period). The total volume of PAM was equally divided into two parts. The first portion of PAM was added to initiate flocculation, and continued stirring for 2 min. The second portion of PAM was added immediately and also stirred for 2 min. The total duration of the coagulation and flocculation was 7 min. This sequence is especially important to enhance the regrowth of flocs, and thus increase the floc size during flocculation.^{35, 36} Flocculation was subsequently followed by a 3 min settling period. All experiments were repeated in triplicate.

At the end of the settling period, 100 mL samples were withdrawn from each beaker with minimal disturbance and filtered using a 20 μm nylon screen (Pentair Aquatic Eco-Systems, USA). The number of microfibres in each sample was counted using a stereomicroscope (Fisher Stereomaster) and microfibre removal was calculated by comparing the number of microfibres present in the settled water to the original spiked number (~ 200). The experiments were conducted at room temperature. Samples were collected after coagulation for pH measurements. The recorded pH values of all test beakers were 7.1 ± 0.2 . The flocs were collected at the end of settling and an aliquot was removed and placed onto a glass slide. The glass slide was dried at 50 $^{\circ}\text{C}$ for 6 h before imaging the flocs with a stereomicroscope to observe the clustering of microfibres within the floc.

5.2.4 Microfibre Characterization

Microfibres from the four treatments that were used for the simulated wastewater tests were further characterized using Fourier-transform infrared (FT-IR) and X-ray photoelectron spectroscopy (XPS), and microscopy. For the used masks, the microfibres were obtained through random selection from a mixture of microfibres released from different face masks. A FTIR spectrometer (Spectrum II, PerkinElmer) was used in attenuated total reflectance mode with a single-bounce diamond. 10 random microfibres were analyzed in the region 400–4000 cm^{-1} with 32 scans at 4 cm^{-1} resolution for each treatment. The carbonyl index is used to characterize changes in the oxidation of polyolefins, hence, it was calculated using the specified area under band (SAUB) method³⁷ as the ratio of the integrated band under the carbonyl region (1550–1800 cm^{-1}) and a reference peak band (CH_2) which does not change in polypropylene after weathering (2885–2940 cm^{-1}).³⁸ Peak analysis and corrections were done using the Spectragryph software which allows

the selection of baseline limits. The composition of microfibres was assessed in duplicate by X-ray photoelectron spectroscopy (XPS, K-Alpha X-ray Photoelectron Spectrometer, Thermo Fisher Scientific). Images of the microfibres were obtained using a wide-zoom stereomicroscope (SZX16, Olympus).

5.2.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 9.2.0 for Windows 64-bit (GraphPad Software, La Jolla California USA). Normality test was performed on the raw data, followed by transformation when normality was not achieved with the raw data. The differences between different samples or tests were investigated and transformed to significance letters using TukeyHSD posthoc test. When no normality was achieved despite transformation, Kruskal-Wallis non-parametric test was performed followed by Dunn post-hoc test to assess significant differences between tests. Size distribution was analyzed using Chi-square test. The test results use a 95% confidence ($p\text{-value} \leq 0.05$) interval to show any significant difference between the test conditions.

5.3 Results

5.3.1 Face Masks Release Microfibres while Floating on Water

Both the used and new masks release microfibres when gently floating on the water surface for ten days whether exposed to UV irradiation or in the dark. There was significant difference in the number of microfibres released under the different conditions (one-way ANOVA, $F_{11, 24} = 44.19$, $p < 0.0001$). The outer and inner layers of the new face masks released an average of ~650

microfibres/mask when kept in the dark, compared to the average of ~300 microfibres/mask released from used masks exposed to the same conditions. When masks were exposed to UV irradiation, the number of microfibres released from each layer increased significantly to an average of ~2400 microfibres/mask and ~1100 microfibres/mask, for new and used masks, respectively. For both the new and used masks, no significant difference was observed between the number of microfibres released from the outer and inner layers when exposed to similar conditions (**Figure 5.1a**). The control dishes contained 6 ± 4 microfibres which suggests minimal contamination during the weathering experiments.

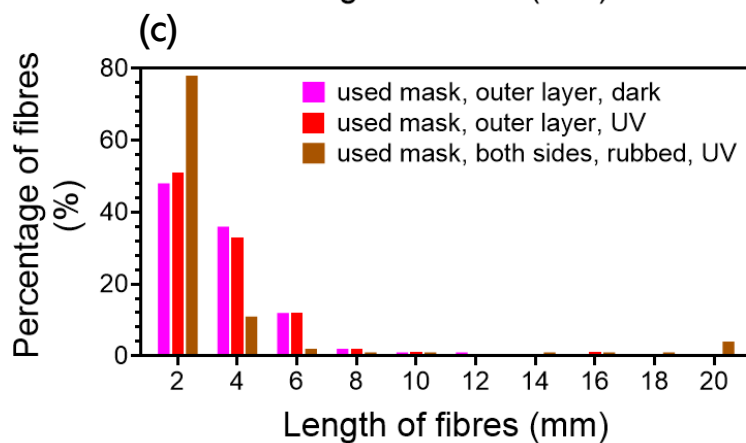
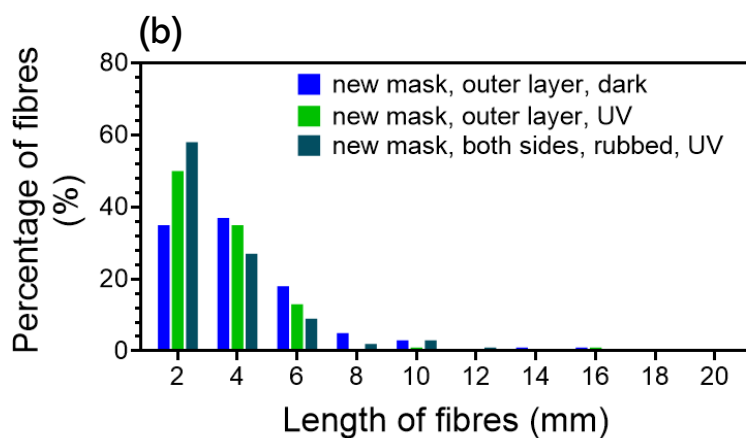
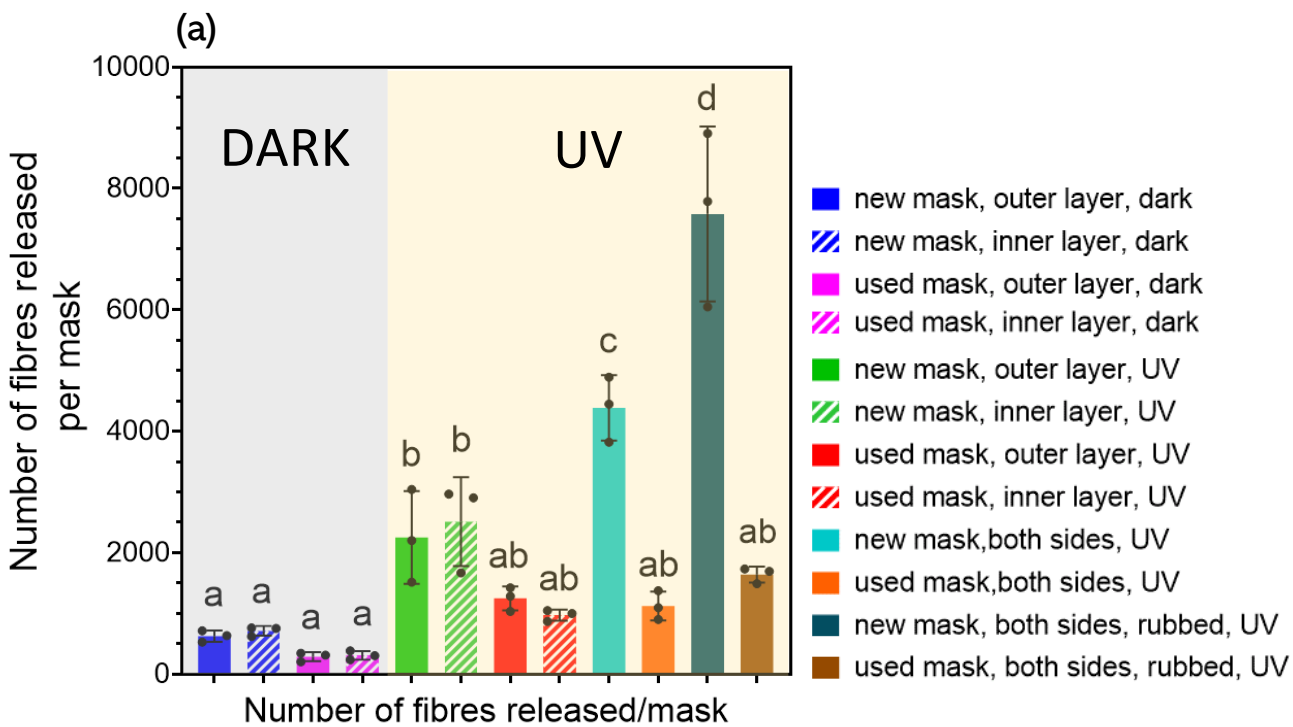


Figure 5.1. (a) Number of microfibrils released from the single-use face masks under different weathering conditions, bars represent mean $\pm$ standard deviation (SD), “●” represents individual values, and letters of significance show statistical difference between tests (n=3); (b) length distribution of released microfibrils from new masks per treatment, based on the measured length of 100 randomly selected microfibrils (average lengths of the fibrils released from the outer layer (dark), outer layer (UV) and both sides (rubbed, UV) treatments are 3.3 ± 2.5 mm, 2.5 ± 2.0 mm and 2.3 ± 1.9 mm, respectively); (c) length distribution of released microfibrils from used masks per treatment, based on the measured length of 100 randomly selected microfibrils (average lengths of the fibrils released from the outer layer (dark), outer layer (UV) and both sides (rubbed, UV) are 2.5 ± 1.9 mm, 2.5 ± 1.9 mm and 2.6 ± 4.3 mm, respectively). Panels (b) and (c) show the length distribution of 6 out of 12 treatments since there was no significant difference in the length distribution between the inner and outer layers, as shown in **Figure S5.7a-b**.

When the new masks were flipped with the inner and outer layer floating on water for 5 days each under UV irradiation, the number of microfibrils released from the mask increased by more than 70% compared to treatments that targeted only one layer (either inner layer or outer layer) floating on water for 10 days. This suggests that the inner and outer layers of the new face masks released more microfibrils within the first 5 days of the weathering experiment and the number of microfibrils released per day tends to decrease over time. The introduction of moderate mechanical friction by rubbing the mask material between two fingers significantly increased the number of microfibrils released by the new masks to ~ 7600 microfibrils/mask. However, mechanical abrasion does not cause a statistically significant increase in the number of microfibrils released from the used masks, with only ~ 1640 microfibrils/mask.

The average length of the microfibres released from both sides of the face masks after UV irradiation was measured for the new and used masks. In this work, we focused on the fraction of microfibres retained on a 20 μm screen; thus, we did observe some fibres that were longer than the 5 mm cutoff length typically used to define microfibers. The longest fibre measured across the treatments that were investigated for size distribution was 19.9 mm while the shortest microfibre was 0.2 mm (**Figure 5.1b, 5.1c and 5.2b**). **Figure 5.2b-c** shows that the average length of the released microfibres is less than 5 mm. Also, for the new masks, the sizes of the released microfibres are significantly lower for UV-irradiated conditions compared to when the face masks were weathered in dark conditions ($\chi^2(2, N = 100) = 9.631, p < 0.05$). Overall, used masks released more smaller-sized microfibres than the new masks ($\chi^2(2, N = 100) = 18.03, p < 0.0001$, **Figure 5.1b-c**). The rubbing treatment increased the percentage of smaller-sized microfibres in the used masks ($\chi^2(2, N = 100) = 8.730, p < 0.05$). No significant difference was observed in the length of microfibres released by the inner and outer layers for both new and used face masks (**Figure S5.7**).

5.3.2 Removal of Microfibres during Wastewater Treatment

Using a conventional coagulation/flocculation process to remove microfibres from wastewater, we observe a removal efficiency of 85% for pristine microfibres obtained by blending of the masks (outer layer). This was significantly different from the removal efficiency observed for microfibres released from the new masks and used masks (one-way ANOVA, $F_{3,8} = 10.53, p = 0.0038$, **Figure 5.2a**). The average lengths of the microfibres used for the jar tests are shown in **Figure 5.2b**. The microfibres from the blending process have a narrower size distribution (**Figure 5.2b, Figure S5.7c**) which may be responsible for the difference in removal efficiency compared to microfibres from the “new mask, outer layer, dark” condition which have mostly smaller-sized microfibres.

Microfibrils released from the new masks in the dark or under UV showed no significant difference with removal efficiencies of 74% and 79%, respectively. However, microfibrils from the used masks exposed to UV have a significantly higher removal efficiency of 91% compared to new masks exposed to similar treatment. The image in **Figure 5.2c** shows a representative floc formed with an individual microfibril released from a new mask entrapped within the floc recovered after coagulation-flocculation.

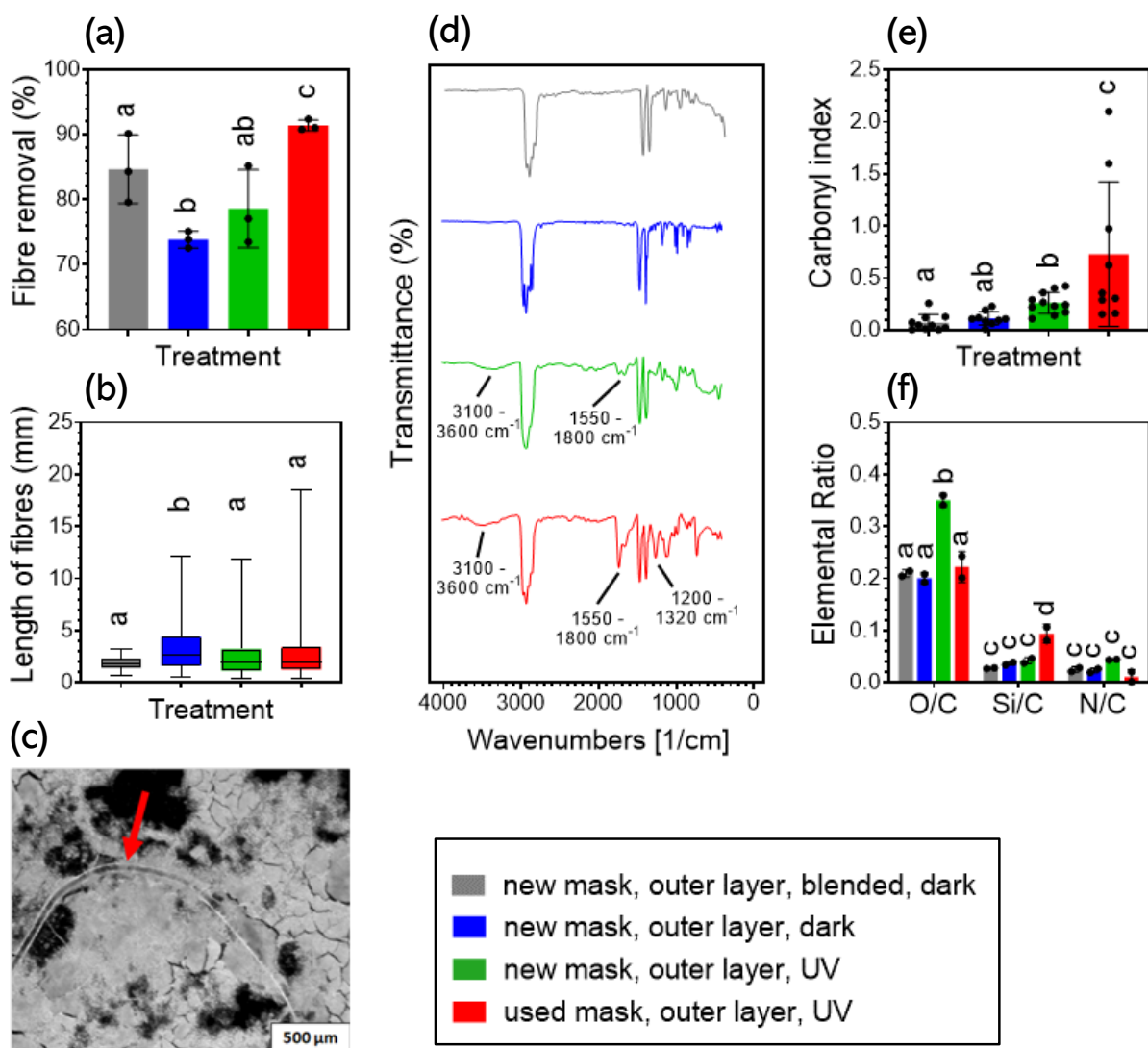


Figure 5.2. (a) Removal efficiency of microfibres released from masks exposed to different treatments (n = 3); (b) size distribution of the microfibres from the treatments used for the jar tests (n=100); (c) representative microscope image of dried floc showing entrapped microfibre after coagulation (scale bar = 500 μm); (d) FTIR spectra of microfibres released from masks exposed to different treatments; (e) Carbonyl index of microfibres from the selected conditions determined by FTIR analysis (n $\geq$ 9); (f) XPS analysis showing the elemental content of microfibres for different mask treatments (n=2);. “●” represents individual measurements of different microfibres while letters of significance show statistical difference between tests. Bar graphs in (a), (e), and (f) represent mean $\pm$ standard deviation (SD), while box plot in (b) represents mean and min/max values.

5.3.3 Microfibre Characterization

The microfibres obtained by blending as well as the released microfibres were confirmed to be polypropylene by FTIR spectroscopy. The FTIR spectra of the analysed microfibres show strong peaks at approximately 2950 cm^{-1} (CH_3 asymmetric stretching), 2917 cm^{-1} (CH_2 asymmetric stretching), 2870 cm^{-1} (CH_3 stretching), 2838 cm^{-1} (CH_2 symmetric stretching), 1456 cm^{-1} (CH_2 symmetric bending), 1376 cm^{-1} (CH_3 symmetric bending), and 1166, 998, 973, 842 and 808 cm^{-1} (isotactic polypropylene bands) which are all standard peaks of polypropylene.^{39, 40} In addition, the built-in Perkin-Elmer standard polymer library database returned matches ranging from 86-93 % of polypropylene for all analyzed microfibres. There were no notable differences in the FTIR spectra between the blended fibres and the “new mask, outer layer, dark” fibres (**Figure 5.2d**). However, C=O (1550-1800 cm^{-1}) and O-H/N-H (3100 – 3600 cm^{-1}) peaks were observed in both

new and used mask microfibres after UV weathering compared to microfibres from new face masks that were kept in the dark. Although microfibres from UV-irradiated used masks show similar oxidation peaks as the UV-irradiated new masks, the used mask microfibres showed additional differences in the spectra in the region 1200-1320 cm^{-1} (C-N/C-O bond)⁴¹ that were absent or weaker in the other treatments (**Figure 5.2d, Figure S5.8**).

The extent of oxidation was quantified by calculating the carbonyl index and as shown in **Figure 5.2e**, the method for microfiber release did not influence the carbonyl indices for the masks kept in the dark, with low values of 0.07 ± 0.08 and 0.11 ± 0.06 for blended and non-blended samples, respectively.⁴² In contrast, the carbonyl index of the microfibres after UV irradiation was 0.27 ± 0.10 , showing statistical difference from the blended microfibres ($p < 0.0001$), but no statistically significant difference from the “new mask, outer layer, dark” microfibres. The carbonyl index of microfibres from the used masks was 0.73 ± 0.70 , which is significantly higher than the indexes of the microfibres from the other conditions ($p < 0.0001$).

Similar to FT-IR analysis, XPS analysis in **Figure 5.2f** showed no significant difference in the atomic percentage (C, O, Si, N) of the microfibres obtained by blending compared to those released while floating on water in the dark. This further confirms that the difference in the removal efficiency observed between these two treatments is more likely due to differences in the microfibre and fiber size distribution rather than their surface chemistry. After UV weathering, microfibres from both new and used masks showed a significant increase in the atomic ratio of oxygen to carbon. Also, the Si atomic % in microfibres from used masks was 240% higher than that of microfibres from the new masks.

Comparing the images of microfibres from the new masks with those from the used masks shows the attachment of solids from environmental exposure (**Figure 5.3, Figure S5.9**). Polypropylene

has a relatively low density ranging between 0.895 and 0.92 g/cm³. Thus, polypropylene microfibres are unlikely to settle if not incorporated into a floc structure. The addition of solid matter, such as silica, to the microfibres may increase their relative density and settling rates.

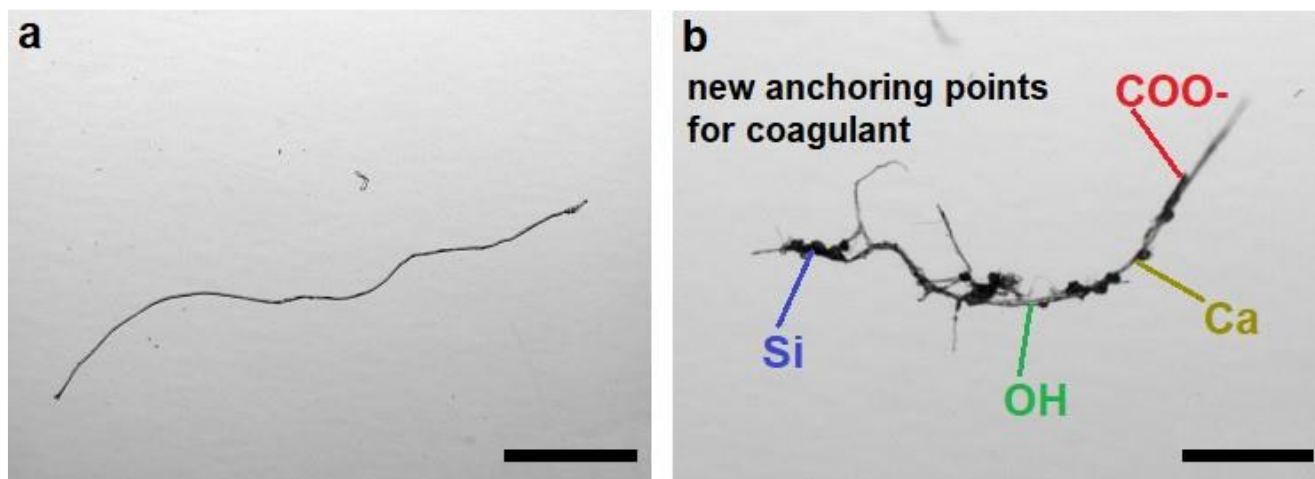


Figure 5.3. Images of microfibres released from a single-use face mask taken using a stereomicroscope (scale bar = 500 μ m). (a) representative microfiber from a new mask, (b) representative microfiber from a used mask; and expected interaction between aluminum hydroxides (produced during alum hydrolysis) and COO⁻/OH groups of Si and Ca-coated microfibres.

5.3.4 Projected Occurrence of Polypropylene Microfibres in Natural Aquatic Environments

A combination of the results of this study and previous studies was used to estimate the number of polypropylene microfibres that may end up in the natural environment compared to anthropogenic (microplastic) particles. It is estimated that roughly 90 masks/km² are inappropriately discarded in

urban areas.⁴³ In major urban cities, there are two major pathways for the transport of mask-released microfibrils into natural aquatic environments. In the first scenario, we can assume that the city uses a separated sewer system in which all released polypropylene microfibrils are discharged directly into surface waters without passing through the wastewater treatment plant. In this case, and assuming that the number of microfibrils reported in this study are released from the face masks over a period of 10 days, up to 4.74×10^7 polypropylene microfibrils may be released directly into the natural environment each day (calculation details are in the Supporting Information). In a second scenario, we consider a city that uses a combined sewer system in which polypropylene microfibrils from urban run-offs are treated in the wastewater treatment plant before being released into natural waters. As approximately 91% of weathered microfibrils are expected to be removed in conventional wastewater treatment, the number of polypropylene microfibrils released into natural waters after treatment can be estimated as 4.27×10^6 microfibrils/day. Various reports have shown the release of up to billions of microfibrils from face masks per day under vigorous weathering conditions.¹⁶ Hence, it is possible that most of the microfibrils are released within the first day of the weathering exposure. Considering this scenario, up to 4.7×10^8 and 4.3×10^7 microfibrils/day may be released into natural waters via a separated and combined sewer system, respectively.

5.4 Discussion

5.4.1 Number of Released Microfibres

The number of microfibres released in dark conditions agrees with reports from other studies that were conducted under low mechanical stress.¹⁰⁻¹² The considerable increase in microfibre release when exposed to UV irradiation is in agreement with the findings of Saliu *et al.* after exposing single-use face masks to UV irradiation,¹³ and the notion that photooxidation through UV irradiation is a critical factor in the degradation of floating plastic materials on water surfaces.^{44, 45} It also agrees with previous studies that suggest that UV irradiation enhances the release of microfibres from the melt-blown nonwoven textile, through the weakening of mechanical strength, followed by the breakage of microfibres, and a slight increase in temperature.⁴⁶ The microfibre release in the presence of mechanical abrasion is similar to previous results following the abrasion of polyester textiles resulting in increased formation of microfibres.⁴⁷ Also, Shen *et al.*¹⁵ reported increase in the number of microfibres released from single-use face masks after multiple cycles of washing and drying. Using new masks, Saliu *et al.*¹³ observed an average release of 135000 microfibres/mask that can be explained by their more intense weathering procedure, and Chen *et al.*¹¹ reported that previously worn masks release a significantly higher number of microfibres compared to new masks. Vigorous shaking of face masks in sand has also been shown to increase the release rate of microfibres.¹⁴ Our results suggest that weakly bound microfibres in the mask material may have already been released to the environment before the used masks were collected. This would explain why the mechanical friction increased the number of microfibres released from the new masks while the same treatment did not cause any significant increase in the release of microfibres from the used masks. Also, this is in agreement with previous reports that show that

the release of microfibers and fibres from textiles such as acrylic, polyester, and polyester–cotton blend textile, polyester fleece blankets, etc. during washing reaches an initial spike before declining with increasing number of washes.^{48, 49}

5.4.2 Size Distribution of Released Microfibres

The high variability in length of the released microfibres is similar to the observed lengths of microfibres released from clothing textiles.⁵⁰ **Figure 5.1b-c** shows that the average length of the released microfibres is less than 5 mm, in agreement with previous studies exposing masks to low mechanical stress.¹¹ Chen *et al.*¹¹ reported no significant difference in the size distribution of microfibers from new masks compared to masks that had been worn for one day when both are exposed to agitation in deionized water at 120 rpm for 24 h. On the contrary, our study shows significant difference in the sizes of the released microfibres from new and used masks. This may suggest that the environmental weathering may have weakened the masks, thereby causing more degradation of the fibres. This agrees with reports that suggest that longer fibres will be subjected to further degradation into small sizes over time.^{51, 52} Also, Saliu *et al.* reported the release of microfibres as short as 25 μm from masks submerged in artificial seawater at higher temperatures of 50-65 °C followed by 24 h of vigorous stirring.¹³ An average of 135000 microfibres/mask (between 25-500 μm in length) was detected compared to the average of 2400 microfibres/mask (between 200-19900 μm in length) in this study conducted at room temperature. Others have also reported even smaller, nano-sized, particles when the face masks are subjected to rigorous shaking.¹⁶ These studies suggest that increased temperature or stirring may also result in further

degradation of the fibres and the release of smaller-sized microfibrils, and hence, motivate the need to further examine the environmental fate and impacts of smaller microfibrils.

5.4.3 Microfibre Removal Efficiency and Characterization

While no other studies report the fate of microfibrils released from face masks during conventional coagulation/flocculation wastewater treatment, our results can nonetheless be compared to data obtained for other types of microfibrils. Using similar coagulation/flocculation conditions as this study, Lapointe *et al.*,²¹ also reported a high removal efficiency (up to 95%) for polyester microfibrils. Also, Vardar *et al.* reported up to 92% removal efficiency of microfibrils from water samples collected from the Ambarli WWTP, Istanbul, Turkey, that serves approximately 2 million people.⁵³ Electrocoagulation wastewater treatment of polypropylene microfibrils from aged face masks has also shown high removal efficiency of 98.4%.⁵⁴

The increased removal efficiency in the used masks may be due to accumulation of Si-based particles on the used masks when lying on the road surface before they were collected for this study. The higher Si content in the used mask microfibrils and the greater removal of Si-coated microfibrils may suggest that the coagulant has higher affinities with Si-based particles than the polypropylene backbone. In the microfibrils from the used masks, N atomic % is lower compared to the new masks and there was a presence of an average of 0.64% Ca which is absent in microfibrils from other tested conditions. In addition, the significantly higher carbonyl index of the microfibrils from the used masks compared to the other three conditions suggests that these fibres were oxidized with additional -COOH sites leading to a possible favorable interaction with the

coagulant species. This may explain the high removal efficiencies observed for fibers from the used masks.

5.4.4 Projected Occurrence of Microfibres

Municipal wastewater effluent is an important source of microplastics. The city of Toronto treats approximately 1200×10^6 L/day of wastewater⁵⁵, while another study shows that 13.3 microplastics per liter of treated water from the same city could be released to the natural environment.⁵⁶ Based on these data, the number of microplastics released into the natural aquatic environment is estimated as 1.6×10^{10} microplastics/day (calculation details are in the Supporting Information). Comparing the number of microfibres released from face masks and the reported estimate of microplastics in municipal wastewater effluent, it is estimated that face masks may account for up to 0.03 – 0.3% and 0.3 – 3% of the microplastics in the natural environment for a combined sewer system and separated sewer system, respectively.

5.5 Conclusion

This study shows that microfibres can be released even under mild environmental conditions as used herein and that photodegradation through UV irradiation significantly increases the number of microfibres released by single-use face masks. As a result, polypropylene microfibres from face masks can potentially contribute to the general plastic mass discharged in natural waters, especially for cities having separated sewer systems, which are a bypass from the water treatment facilities for polypropylene microfibres and several other anthropogenic stressors.⁵⁷

Several environmental factors are not accounted for by the simulated weathering in this study, particularly wind, temperature cycling, and exposure to environmental components such as sand.⁴⁵
⁵⁸ Also, real-life weathering, mechanical abrasion from walking or driving on the masks, as well as the presence of sand and gravel may significantly increase the number of fibres released.⁴⁵ However, based on this study, there are certain concerns about the potential fate of single-use face masks in the environment, especially for cities with separated sewer systems.

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5.6 Supporting Information

Calculations of projected future presence of polypropylene microfibres in natural aquatic environments assuming the release of microfibres occurs over 10 days

Ammendolia *et al.*¹ found 290 plastic personal protective equipment (PPE) items/km² in Toronto (Canada) and 31 % of the items were single-use face masks. Based on the report, the number of face masks discarded per person is estimated as follows:

Estimated number of discarded PPE items/km² (in Toronto) = 290 plastic items/km²

Number of discarded face masks = 31 % of 290 plastic items/km²

Number of discarded face masks = 90 discarded face masks/km²

Population density in Toronto, Canada = 4300 persons/km²

In 1 km² land area number of discarded masks per person = $\frac{90 \text{ discarded face masks}}{4300 \text{ persons}}$

$$= 0.021 \frac{\text{discarded face mask}}{\text{person}}$$

From the results of this study, assuming that an average mask stays on the streets for approximately 10 days before being removed by waste management services and considering the population of 2.97 million people in Toronto (2019)², the average number of microfibrs released per day can be estimated as follows:

Average number of PP microfibrs released per new mask = 7,600 in 10 days = 760 microfibrs/mask/day.

$$\begin{aligned} \text{Total PP microfibrs released per day} &= \frac{760 \text{ PP fibres}}{\text{mask} \times \text{day}} \times \frac{0.021 \text{ mask}}{\text{person}} \times 2,970,000 \text{ persons} \\ &\approx \frac{47,400,000 \text{ PP fibres}}{\text{day}} \end{aligned}$$

Grbić *et al.* estimated that the Toronto WWTP effluents release 13.3 microplastics/L.³ The estimated number of microplastics released into the natural environment per day is calculated below based on the volume of wastewater treated/day in Toronto (1,200,000,000 L/day).⁴

Total number of microplastics released from the Toronto WWTP effluent per day =

$$\begin{aligned} &= \frac{13.3 \text{ microplastics}}{\text{L}} \times \frac{1,200,000,000 \text{ L}}{\text{day}} \\ &\approx \frac{16,000,000,000 \text{ microplastics}}{\text{day}} \end{aligned}$$

There are two likely scenarios for the introduction of PP microfibres into the environment:

Scenario 1: Separated sewer system in which all PP microfibres released are discharged directly into the river without passing through the WWTP.

To compare the estimated number of PP microfibres released from the face masks to the number of microplastics released from WWTP effluent daily, the number of microfibres released in both cases is divided as follows:

Percentage of PP microfibres with respect to microplastics =

$$\frac{47,400,000 \text{ PP microfibres}}{16,000,000,000 \text{ microplastics}} \times 100 \% = 0.296 \%$$

This implies that based on the above calculations, the number of microfibres released from single-use face masks into the natural environment may account for ~ 0.3% of the estimated number of microplastics released into the natural environment daily.

Scenario 2: Combined sewer system in which PP microfibres from run-offs are treated in a WWTP before being released into the river. In this case, we assume the 91% removal efficiency measured in this study. Therefore, the ratio of microfibres released into the natural water environment is calculated as follows:

Percentage of PP microfibres released after coagulation treatment = $(100 - 91)\% = 9\%$

Number of PP microfibres released into the natural environment after coagulation treatment =

$$= 9\% \times \frac{47,400,000 \text{ PP microfibres}}{\text{day}}$$

$$\approx \frac{4,270,000 \text{ PP microfibres}}{\text{day}}$$

Thus, only 9 % of the released microfibres calculated above end up in the natural environment =

$$= 9\% \times 0.296\% = 0.03\%$$

Calculations of projected future presence of polypropylene microfibres in natural aquatic environments assuming the release of the microfibres occurs within the first day of weathering

Using the same approach, the number of PP microfibres that may end up in the natural environment was estimated assuming the microfibers are released within the first day of weathering or environmental exposure.

Average number of PP microfibres released per new mask = 7,600 in 24 hours = 7,600 microfibres/mask/day.

$$\text{Total PP microfibres released per day} = \frac{7,600 \text{ PP microfibres}}{\text{mask} \times \text{day}} \times \frac{0.021 \text{ mask}}{\text{person}}$$

$$\times 2,970,000 \text{ persons}$$

$$\approx \frac{474,000,000 \text{ PP microfibres}}{\text{day}}$$

Scenario 1:

Percentage of PP microfibres with respect to microplastics =

$$\frac{474,000,000 \text{ PP microfibres}}{16,000,000,000 \text{ microplastics}} \times 100 \% = 2.96 \%$$

This implies that the microfibres released from single-use face masks into the natural environment may account for up to 3% of the estimated number of microplastics released into the natural environment daily.

Scenario 2:

Number of PP microfibres released into the natural environment after coagulation treatment =

$$= 9\% \times \frac{474,000,000 \text{ PP microfibres}}{\text{day}}$$

$$= \frac{42,700,000 \text{ PP microfibres}}{\text{day}}$$

Thus, only 9 % of the released microfibres calculated above end up in the natural environment =

$$= 9\% \times 2.96\% = 0.27\%$$



Figure S5.1. Pictures of 18 used face masks picked up on May 19, 2021 between 9 am and 3 pm in the Cote St. Luc/Notre Dame de Grace Area, Montreal Island, Quebec, Canada.

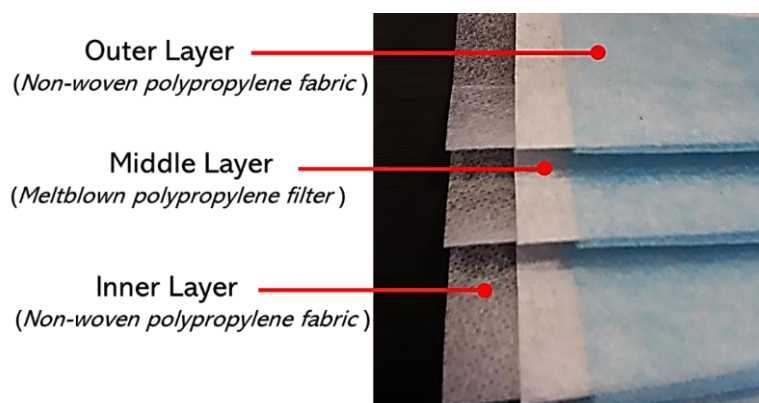


Figure S5.2. Composition of single-use surgical face masks.

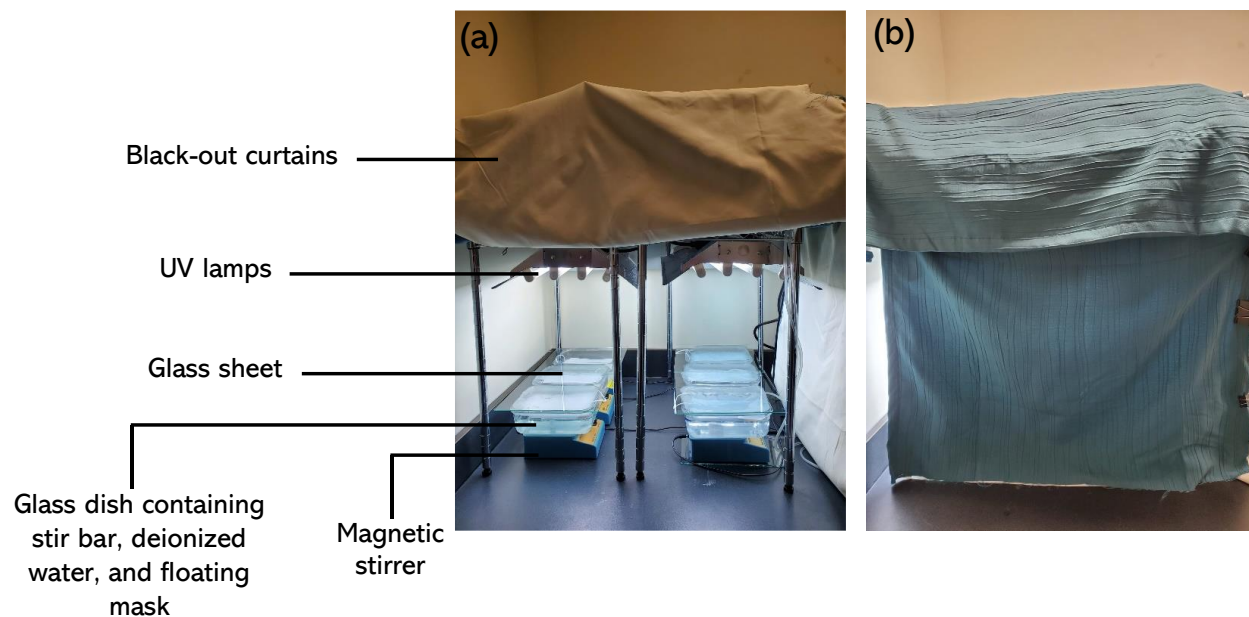


Figure S5.3. Custom-built weathering chamber (a) with the UV lamps turned on (the lamps are turned off for the dark experiments); (b) black-out curtains are used to cover the chamber during all weathering experiments.

Rubbing of the face masks

The face masks were rubbed as shown in **Figure S5.4**. First, the mask is folded into two. Then, constant pressure was applied with two fingers from each hand starting at the edges of the masks. The fingers were moved inwards, outwards, upwards and downwards for a total of two minutes daily throughout the weathering experiment. Similar pressure was applied to a weigh balance to measure the pressure applied to the face masks during the rubbing process.

Mass after pressure is applied to weigh balance = $1000 - 1500\text{ g} = 1\text{ kg} - 1.5\text{ kg}$

$$\text{Force} = \text{mass} \times \text{acceleration} = \text{mass} \times 9.81 \text{ m/s}^2 = 9.81 \text{ N} - 14.715 \text{ N}$$

The force was provided by the total of four fingertips of approximately 1cm diameter each.

$$\text{Total area} = 4\pi r^2 = 4 \times \pi \times 0.5^2 = 3.14 \text{ cm}^2 = 0.000314 \text{ m}^2$$

$$\text{Pressure applied on face mask} = \frac{\text{Force}}{\text{Area}} = 31,242 \text{ N/m}^2 - 46,863 \text{ N/m}^2$$

$$\text{Pressure applied on face mask} = 31.2 \text{ kPa} - 46.9 \text{ kPa}$$



Figure S5.4. Illustration of how the face masks were rubbed.

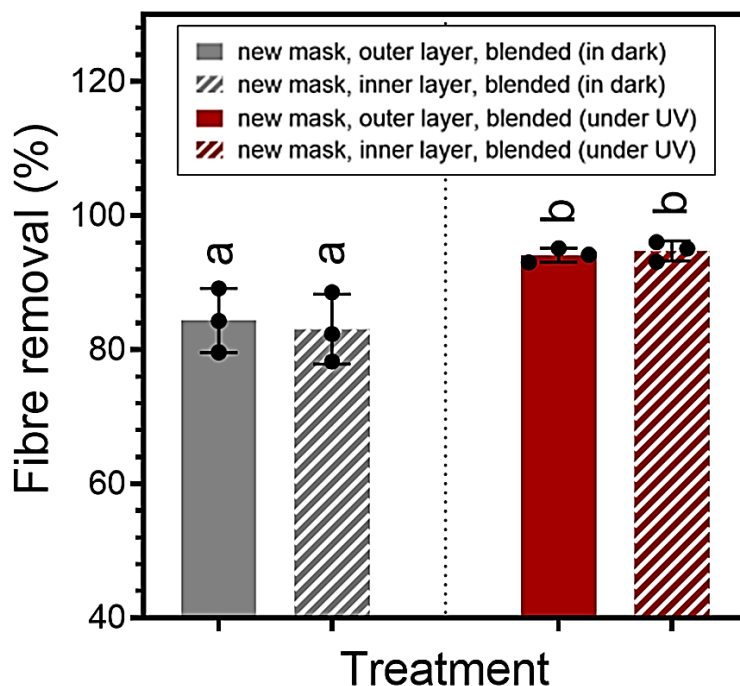


Figure S5.5. Comparison between the removal efficiency of microfibres from the outer and inner layers of blended masks, in dark and under UV conditions (n = 3).

Filtration of the blended new face masks to obtain microfibres

The fibre suspension obtained after blending was filtered using the filtration and fibre collection process shown schematically in **Figure S5.6**. The 3 mm mesh was a stainless-steel wire mesh (T-fal strainer, Canadian Tire), while the 1 mm mesh was a lightweight-particle-filtering stainless steel wire cloth (catalogue number – 9238T29, McMaster-Carr). Retentate 2 (containing microfibres with a size range of 1-3 mm) was used as “new masks, outer layer, blended, dark” for the jar tests.

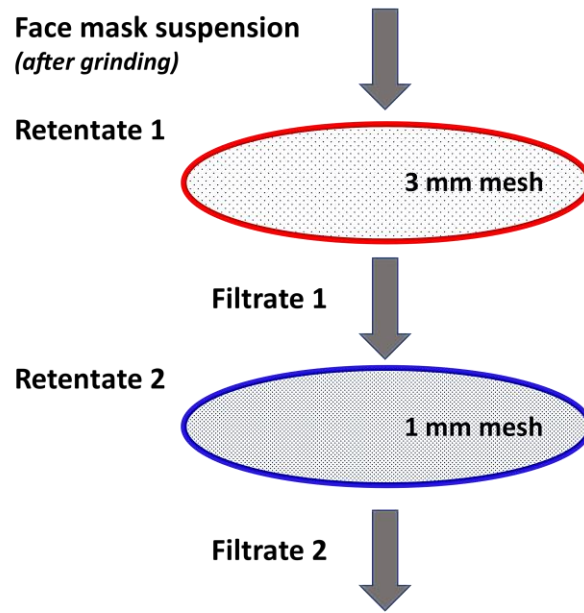


Figure S5.6. Schematic description of the filtration and collection process of microfibres for the coagulation/flocculation test.

Table S5.1. Composition of synthetic wastewater (per 200 mL).

Composition	Mass (mg)
Peptone	32.0
meat extract	22.0
Urea	6.0
anhydrous dipotassium hydrogen phosphate	5.6
sodium chloride	1.4
calcium chloride dihydrate	0.8
magnesium sulphate heptahydrate	0.4
1-5 μm SiO_2 particles	20

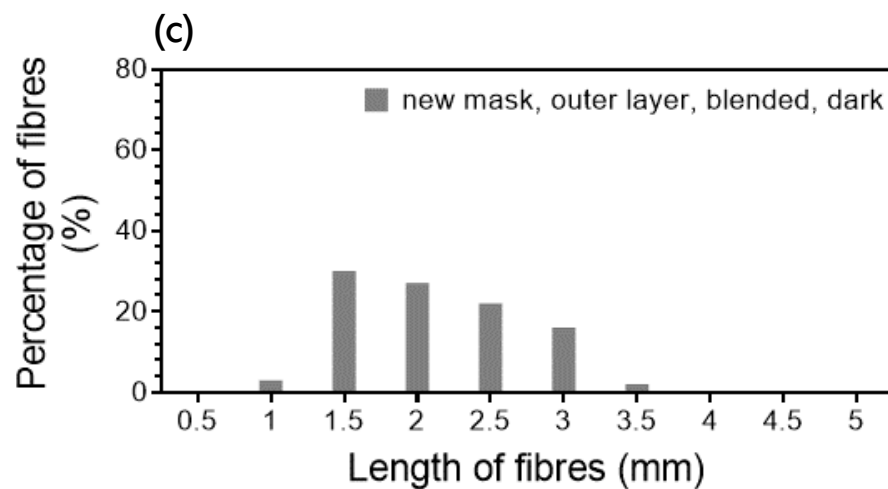
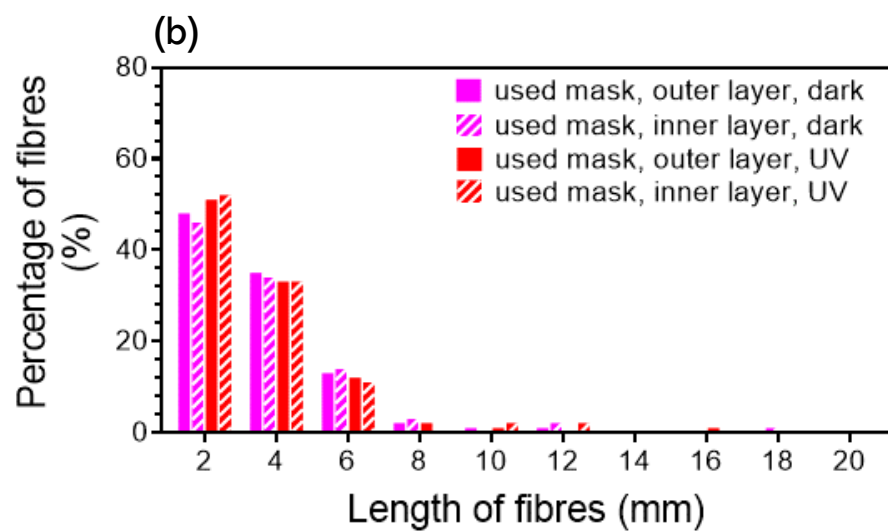
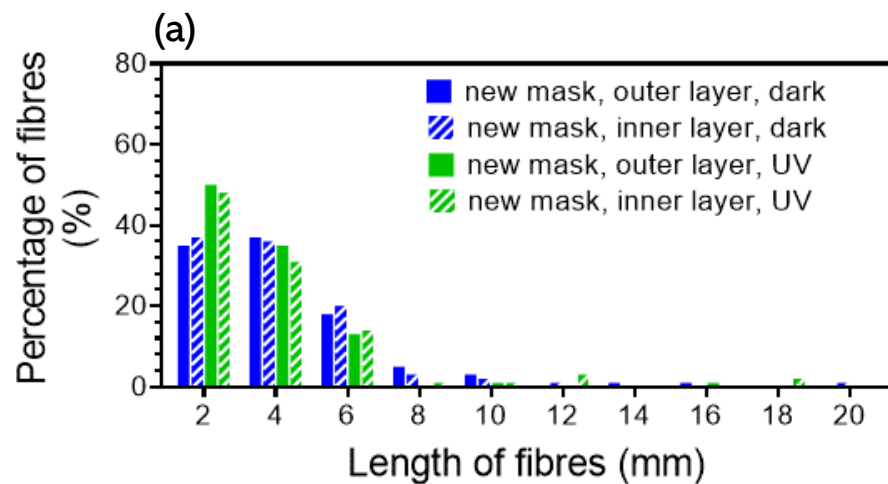


Figure S5.7. Length distribution of released microfibrils based on the measured length of 100 randomly selected microfibrils: (a) new mask, (b) used mask, (c) blended mask.

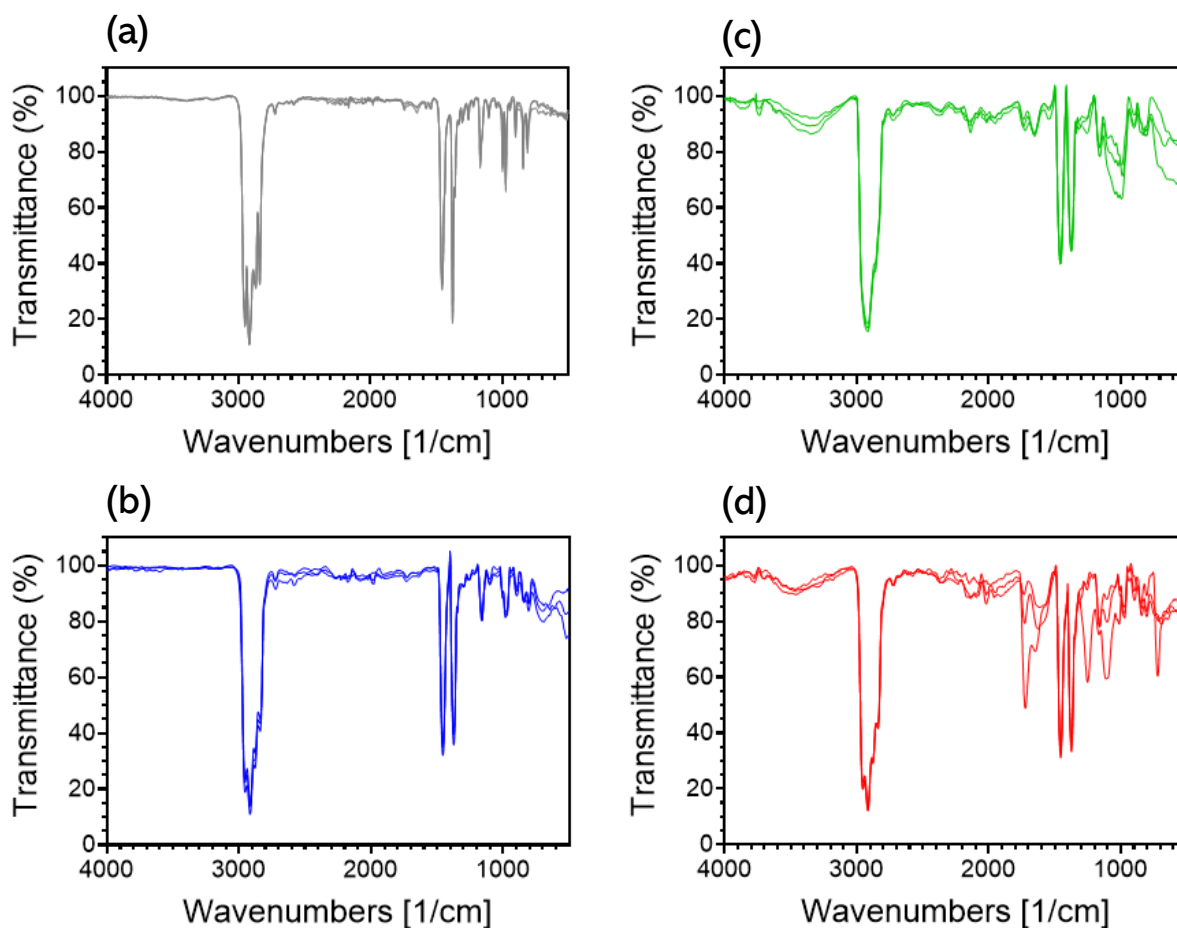


Figure S5.8. FTIR spectra of three separate microfibrils released from masks exposed to different treatments. (a) new mask, outer layer, blended, dark, (b) new mask, outer layer, dark, (c) new mask, outer layer, UV, and (d) used mask, outer layer, UV.

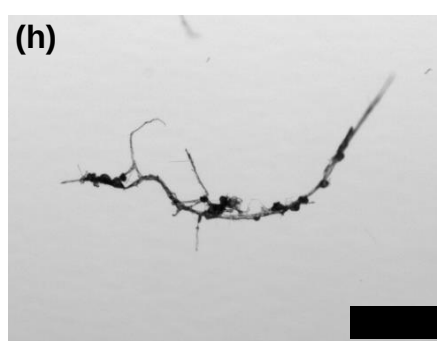
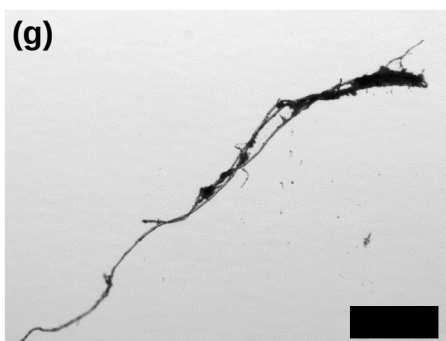
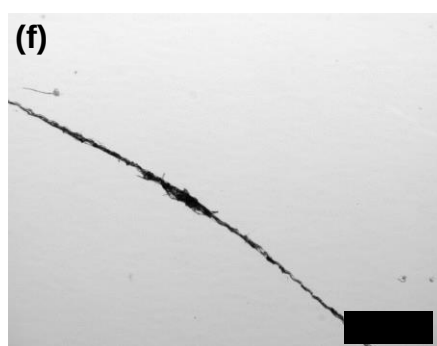
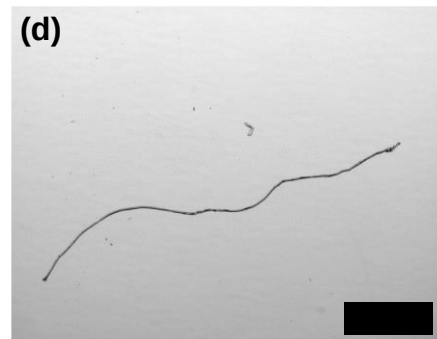
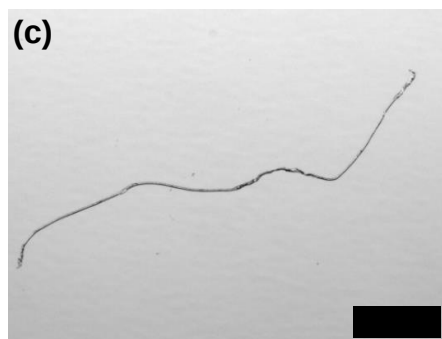
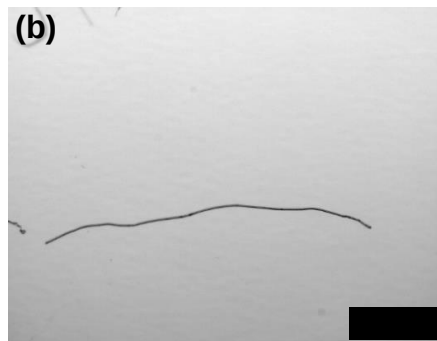
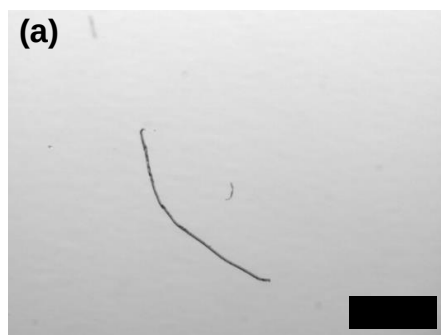


Figure S5.9. Image of microfibres released from a single-use face mask taken using a stereomicroscope (scale bars = 500 μm). (a-d) are from new masks, images (e-f) are for microfibers from used masks.

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Chapter 6: Toxicity of particles and chemicals released from surgical face masks to the model aquatic organism, *Daphnia magna*

Connecting Text

The systematic review of the literature in Chapter 2 revealed that more than 70% of micro- and nanoplastics used in toxicity studies on *Daphnia magna* are commercial spherical particles. In fact, more than half of these studies use spherical polystyrene microplastics and nanoplastics. Yet, there are other sources of plastics in the environment which are rarely investigated. Chapter 5 showed that single-use face masks are a potential source of non-polystyrene non-spherical microplastics and nanoplastics in the aquatic environment. Hence, the next research question was: “what are the impacts of these released plastic particles once they get into natural waters?”. The chapter addresses that question by assessing the potential toxicity of the released microplastics and nanoplastics to *Daphnia magna*. The toxicity of the chemicals, particles, and the synergistic toxicity of both was extensively studied using *Daphnia magna* as a model organism. The properties of the released particles and the composition of the leached chemicals were also investigated.

The findings from this chapter are currently under review as the following manuscript: Oluwadamilola Pikuda, Laura Hernandez, Dahyun Kyung, Qiqing Chen, Jun-Ray Macairan, Lan Liu, Xiaoyu Gao, Stephane Bayen, Subhasis Ghoshal, Dimitrios Berk, Nathalie Tufenkji. “Toxicity of particles and chemicals released from surgical face masks to the model aquatic organism, *Daphnia magna*”, *Environmental Science: Nano*.

Abstract

The use of single-use face masks during the COVID-19 pandemic has resulted in indiscriminate disposal of face masks, some of which end up in the aquatic environment. These masks release microplastics and plastic-associated chemicals when exposed to environmental conditions. However, there is still limited knowledge of their impact on aquatic organisms. We subjected face masks to a simulated weathering process and separated the resulting leachate into three fractions: the first fraction contained a mixture of leached dissolved chemicals and released particles, the second fraction contained dissolved chemicals alone, and the third fraction contained particles alone. These fractions were then used to conduct acute and chronic toxicity tests on *Daphnia magna*. Complementary spectroscopy techniques were used to confirm that the particles consisted of polypropylene while SEM indicated that the particle sizes were mostly in the nano-range. ICP-MS analysis detected Pb, Zn, Mn, and Cr in the face mask leachates while LC-MS found mono-isobutyl phthalic acid in the fraction containing particles alone. Both the acute and chronic tests revealed that the chemicals present in the masks affected the survival of younger daphnids. The particles alone fraction only affected the juveniles during acute exposures. In chronic exposures, the particles did not impact survival, but inhibited growth and reproduction. This study shows that the released microplastics and chemicals may have different and possibly joint toxicity impacts on *Daphnia magna*, which may also depend on the life stage of the exposed organisms.

6.1 Introduction

Since plastics were first developed in the 1900s,¹ they have become an indispensable material for the production of common consumer products largely due to their durability, versatility, and

affordability. These desirable properties once again served their purpose in the production of single-use face masks during the COVID-19 pandemic to limit the spread of the SARS-CoV-2 virus. Although single-use face masks were primarily worn by public health professionals before the pandemic, the production and use of single-use masks has seen significant expansion following the onset of the COVID-19 pandemic. In fact, more than 1.2 trillion face masks have been used globally since the beginning of the pandemic,² representing a mass of 4.6 million tons assuming that each single-use face mask weighs 3.5 g.³ It is expected that these masks will continue to be used for the foreseeable future to mitigate the spread of the coronavirus and other infectious agents.⁴

The indiscriminate disposal of billions of masks has resulted in widespread environmental contamination of both terrestrial and aquatic environments worldwide.^{5, 6} It is estimated that approximately 1.6 billion face masks ended up in the oceans in 2020.⁷ Given the global scale of consumption and high level of indiscriminate disposal of these face masks, freshwater and marine environments will likely be subjected to more plastic pollution from their emerging debris in the near future.^{8, 9} Studies have shown that face masks release microplastics and leach chemicals when exposed to environmental conditions.^{10, 11} Furthermore, Pikuda *et al.*¹² reported that plastic particles released from face masks in the urban environment may also end up in natural waters. Hence, the increasing accumulation of these masks in water bodies, paired with their degradation over time, would worsen the already existing plastic pollution problem and have potentially toxic impacts, especially on smaller aquatic invertebrates.

Various studies have previously shown that the particles and chemicals released during the gradual fragmentation and leaching of different types of plastic debris in the natural aquatic environment can cause toxicity to aquatic invertebrates.^{13, 14} To date, only a few studies have investigated the

potential toxicity of the particles and chemicals released from face masks to aquatic invertebrates. One study reported that chronic exposure to the particles alone significantly impacted reproduction in *Tigriopus japonicus*¹⁵ while another study showed that exposure to degraded face masks may impact genes associated with reproduction in *Danio rerio*. Using *Lumbriculus variegatus*, Chomiak *et al.*¹⁶ showed that chemicals and particles released from face masks may impact their short-term behaviour and long-term population, respectively. Nonetheless, there is not sufficient data available yet to fully understand the ecological impacts of face mask pollution on aquatic invertebrates.

This study aims to advance our knowledge on the potential toxicity of particles and chemicals released from single-use face masks by using *Daphnia magna* (*D. magna*) as a model organism to carry out acute and chronic toxicity experiments. *D. magna* is a popular model organism for toxicity studies due to its sensitivity to pollutants, its significant role in the aquatic ecosystem, and the existence of standard protocols to conduct ecotoxicity tests. Through these experiments, this study seeks to investigate the effects of the exposure of *D. magna* to the separate impacts of the particles and chemicals released from single-use face masks, as well as the combined impacts of the mixture of the chemicals and particles.

New single-use face masks were exposed to laboratory-simulated weathering. The generated chemicals and particles were collected at the end of the weathering process and used for experiments to assess acute and chronic toxicity to *D. magna*. The study also includes a detailed analysis and characterization of the released chemicals and particles, as well as observation of particle uptake by daphnids using light sheet microscopy. We hypothesized that the combination of the characterization techniques and chemical analysis would provide useful insights into understanding the toxicity observed in *D. magna*, and the chemical analysis would help to identify

the potential compounds leached from the face masks that may be responsible for the observed toxicity.

6.2 Materials and Methods

6.2.1 Simulated Face Mask Weathering

Fifty single-use disposable surgical face masks purchased from JCJZ, USA, were exposed to an accelerated weathering process to obtain leached chemicals and plastic particles. The hemmed edges and ear loops were separated using ceramic scissors, and the remaining mask materials were cut into squares approximately 1 cm² in size using the same scissors. The face masks were not washed before this experiment. To accelerate the weathering process, the small pieces were blended in a Nutri Ninja® Pro blender using moderately hard rear water (which will henceforth be referred to as rear water, **RW**). The RW was prepared with Optima LC/MS grade water (Fisher Scientific) using the required salts (96 mg L⁻¹ NaHCO₃, 60 mg L⁻¹ CaSO₄·2H₂O, 60 mg L⁻¹ MgSO₄, and 4 mg L⁻¹ KCl).¹⁷ RW was used as a blending and leaching medium as it is the same medium used for culturing the test organism (*D. magna*). Mask squares were blended (in batches) at medium speed for 5 min. The total volume of RW used for the blending process was 5 L. The starting concentration of 33 g of face mask per liter of RW is equivalent to a liquid-to-solid ratio (L/S) of 30.

A custom-made weathering chamber equipped with six 24 W UV lamps (7000 K, Arcadia T5 D3 Desert UVB) with 12 % UVB and 30 % UVA output (λ =280–400 nm) was designed as described in Pikuda *et al.*¹² to conduct simulated weathering of the masks. The distance between the UV lamps and the top of the face mask suspension surface in the glass beakers was 15 cm. The UV

irradiation received by the face mask squares during the weathering process was $10.2 \pm 0.2 \text{ W/m}^2$, measured using a UV meter (Apogee Instruments, MU-200). The blended face masks along with the ear loops and hemmed edges were kept in a clean 5 L glass beaker, covered with a clean glass sheet, and kept under UV irradiation and stirring at 300 rpm. In addition, a 5 L beaker of freshly prepared RW without any face mask pieces was also blended and weathered under stirring and UV irradiation in the same chamber to serve as a procedural control. This procedural control is subsequently referred to as weathered rear water (**WRW**). Prior to the weathering, the glassware was rinsed three times with deionized water and acetone. The weathering process was carried out at room temperature ($23 \pm 2 \text{ }^\circ\text{C}$) for six weeks. The flexible nose strips of 30 face masks were also removed and weathered separately, under similar conditions (i.e., same concentration as the original suspension of face masks).

At the end of the weathering, large solid chunks from the weathered mask suspension were removed using stainless-steel tongs, and the remaining suspension was filtered using a 38 μm sieve (3"-Diameter Sieve, Full Height Stainless Steel, No. 400, Cole-Parmer). The filtrate, which contains both microplastic particles and leached chemicals, was divided into the three fractions to be used for the toxicity tests as follows: 2500 mL of the initial filtrate was set aside and used for the toxicity experiments as is and is henceforth referred to as **Fraction 1 (F1)**. To prepare separate fractions for the particles and dissolved leached chemicals, the remaining 2500 mL from the initial filtrate was further filtered using an Amicon stirred cell (Millipore Sigma #UFSC05001) with a 500 kDa regenerated cellulose ultrafiltration membrane (Microdyn Nadir, PM UC500, 47 mm, Sterlitech). This filtration step was conducted in batches of 50 mL due to the volume capacity of the stirred cell. For each batch, 50 mL of the initial filtrate was added into the Amicon stirred cell, the cell was stirred, and the pressure was increased to 70 psi. The steps were repeated until all the

2500 mL had been filtered. The filtrate was collected and herein termed **Fraction 2 (F2)**. After the last batch, the pressure was released. The final volume of the suspension (i.e., retained particles) in the cell after the filtration was ~10 mL. These particles were washed with water (LC-MS grade) to remove residual chemicals. To wash the particles, water was added to the stirred cell to increase the volume to 50 mL, pressure was applied to the cell, and the volume was reduced to ~10 mL. The filtrate was discarded. The addition of water was repeated for a total of three rinses. After three rinses, the resulting ~10 mL of particles was collected and diluted in WRW to achieve the initial volume of 2500 mL and is henceforth termed **Fraction 3 (F3)**. The WRW was also filtered through the 38 μm sieve as part of the procedural control. The separation and filtration method used to generate these three fractions (F1 – F3) is similar to the process described by Roubeau Dumont *et al.*¹⁸ and is schematically represented in **Figure S6.1**. The mass concentration of the three face mask fractions was determined using a microbalance (Sartorius S4 Electronic Ultramicro Balance). The detailed procedure is provided in the Supporting Information (Text S6.1).

6.2.2 Particle Characterization

F3 was characterized using scanning electron microscopy (SEM), nanoparticle tracking analysis (NTA), Fourier Transform Infrared spectroscopy (FTIR), and Raman spectroscopy. To prepare the particles for SEM analysis, a polycarbonate filter (Sterlitech #PCT00147100, pore size 10 nm) was attached to an SEM stud (Ted Pella # 16084-1) using carbon tape (Ted Pella #16111) in a laminar flow cabinet. Subsequently, 100 μL of F3 was deposited onto the polycarbonate filter and left to dry for 48 h. The deposited particles were then coated with a 3 nm layer of platinum (Leica Microsystems EM ACE600 Sputter Coater) and imaged using an FEI Quanta 450 environmental

SEM (5 kV, spot size 2.5). The particle size distribution was determined using ImageJ software. For each image, the background was subtracted, and the threshold was adjusted to select only the particles. The particles were then counted by using the automatic function in the software. The Ferret diameter was taken as the diameter of the particles, which is the longest distance between two points on the particle.

The number of particles smaller than $\sim 1\ \mu\text{m}$ in F3 was determined using an NTA LM14 instrument with 532 nm green laser, NanoSight Ltd. Briefly, 1 mL of the sample was introduced into the analysis chamber. The laser was turned on to illuminate the particles and a 60-sec video was recorded at 355 shutter aperture and 1 gain. The video was then processed with the NanoSight software; a successful acquisition had over 200 particles per run recorded. Each acquisition was repeated three times, cleaning the chamber with acetone and water between acquisitions, and adding a new batch of 1 mL samples. Using the same procedure, NTA analyses of F1 and F2, as well as the controls, were also conducted for comparison.

A benchtop ATR-FTIR (Spectrum II FTIR Spectrometer with a single-bounce diamond in attenuated total reflection mode, PerkinElmer) was used to confirm the composition of particles from the different layers of the face mask (i.e., inner layer, middle layer, and outer layer), including microparticles and sub-micron particles. To separate the microparticles from the sub-micron particles, 5 mL of F3 were filtered through a Whatman binder-free glass microfibre filter (Fisher Scientific 09-873H, $1.5\ \mu\text{m}$). The retentate (microparticles) were carefully taken with metallic tweezers and placed on the FTIR for analysis. The filtrate (sub-micron particles) was drop-casted onto aluminum foil until a white film was observed—this white film was analyzed by FTIR spectroscopy. The FTIR spectra were taken from 500 to $4,000\ \text{cm}^{-1}$ with a spectral resolution of $8\ \text{cm}^{-1}$ with the co-addition of 16 scans.

The composition of individual particles in F3 was determined using an optical photothermal IR microscope (O-PTIR, Photothermal Spectroscopy Corp.). One drop ($\sim 100\ \mu\text{L}$) of F3 was deposited onto a calcium fluoride window (Crystran, Raman grade, 13 mm diameter). A piece of each of the three layers of a face mask was adhered to a glass slide as a reference. Raman spectra were acquired over 101 to $4116\ \text{cm}^{-1}$, whereas IR spectra were acquired in two segments: 940 to $1800\ \text{cm}^{-1}$ and 2690 to $2998\ \text{cm}^{-1}$. The spectra were obtained at a resolution of 600 lines per mm, an integration time of 3 sec, 10 averages, 41% of the probe power, and a confocal pinhole opening of $20\ \mu\text{m}$.

6.2.3 Chemical Analysis

The chemical composition of the three fractions was investigated using inductively coupled plasma-mass spectrometry (ICP-MS) and liquid chromatography-mass spectrometry (LC-MS). The two controls, as well as LC-MS water, were also analyzed for comparison. 20 mL and 10 mL of all six samples were used for ICP-MS and LC-MS analysis, respectively.

For the ICP-MS analysis, a two-step acid digestion was carried out using HNO_3 and H_2O_2 .¹⁹ After the digestion, the samples were filtered using $0.22\ \mu\text{m}$ PTFE syringe filters and stored in 15 mL centrifuge tubes. Samples were prepared in duplicates. The presence of 6 metals namely ^{52}Cr , ^{55}Mn , ^{59}Co , ^{60}Ni , ^{63}Cu , and ^{66}Zn , as well as 5 metalloids namely ^{75}As , ^{98}Mo , ^{114}Cd , ^{208}Pb , and ^{121}Sb were quantified using ICP-MS (NexION 300, quadrupole ICP-MS, PerkinElmer). These elements were selected based on previous studies analyzing similar face masks.^{11, 20, 21} A linear calibration range of 20 ppt to 10 ppb was prepared for all the elements ($R^2 > 0.9999$). The calibration samples were prepared by diluting a multielement standard purchased from SCP Science (Quality Standard 4). The solution obtained after weathering of the nose strips was also analyzed using a similar protocol.

For the LC-MS analysis, the samples were also filtered using 0.22 μm PTFE syringe filters and acidified using methanol (LC-MS grade). Agilent 1290 Infinity II LC system coupled to the 6545 Q-TOF-MS was applied for data collection (Agilent Technologies). The LC separation was performed on a Poroshell 120 EC-C18 analytical column (Agilent Technologies; 2.7 μm $\times$ 3 mm $\times$ 100 mm) connected to a Poroshell 120 EC-C18 guard column (Agilent Technologies; 2.7 μm $\times$ 3 mm $\times$ 5 mm). The mobile phase A was HPLC water, and the mobile phase B was an acetonitrile/methanol mixture (50:50 v/v), with ammonium acetate (5 mM) added to both mobile phases A and B. Samples were kept at 4°C in the multisampler compartment. HPLC parameters for both ion modes were as follows: flow rate = 0.3 mL.min⁻¹, injection volume = 20 μL , and column temperature = 30°C. The elution gradient was: 5% B (0 to 0.5 min), linear increase to 100% B (0.5 to 8 min), 100% B (8-13 min), decrease to 5% B (13 to 13.5 min) and finally 5% B (13.5 to 15 min) with 1.5 min post-column run. MS conditions were: drying gas temperature = 175°C, drying gas flow rate = 10 L/min, sheath gas temperature = 375°C, sheath gas flow rate = 12 L/min, nebulizer pressure = 30 psi, capillary voltage = 4000 V, fragmentor voltage = 125 V, skimmer voltage = 50 V, and nozzle voltage = 1000 V (ESI+) or at 2000 (ESI-). Full scan MS data were recorded at a mass-to-charge ratio (m/z) range from 100 to 1700 with a scan rate of 2 spectra/sec and were collected using both centroid and profile modes. Two reference ions (m/z at 121.0508 and 922.0098 for ESI+, 112.9856, and 1033.9881 for ESI-) were used in each ion mode for automatic mass recalibration during data acquisition. Suspect screening was performed using the software MassHunter Profinder B.10.00 from Agilent Technologies. Data treatment was conducted in the “Batch Targeted Feature Extraction” mode (RT tolerance $\pm$ 0.05 min, mass tolerance $\pm$ 20 ppm, absolute height threshold $\geq$ 20000 counts, score $\geq$ 80) for full scan MS data, and the resulting MS information was screened with the library *Extractables & Leachables PCDL*

(Agilent Technologies). Among the tentatively identified molecular features from the full MS data, only those with the most intense signals as well as those with signals significantly higher than in the blanks and controls were selected for the comparison of the targeted MS/MS fragments with the MS/MS library or for SIRUS structure prediction.²² Authentic standards were finally used to confirm the tentatively identified molecular features based on the retention time and targeted MS/MS fragments.

6.2.4 Toxicity Tests on *Daphnia magna*

The *D. magna* strain used in the experiments was originally obtained from Environment and Climate Change Canada (Montreal, Quebec) and cultured in an environmental chamber (Panasonic MLR-352H-PA Environmental Chamber). The *D. magna* cultures were fed and maintained as described in Pikuda *et al.*²³ Toxicity tests were conducted on the three fractions and two controls. The solutions for these 5 treatments were kept in clean glass bottles, covered with glass sheets, wrapped in aluminum foil, and stored in the dark before the toxicity experiments. Oxygen was bubbled into all five bottles for 48 h before each toxicity experiment.

Acute toxicity tests were conducted on neonates (≤ 24 h old), juveniles (7 days old), and adults (15 days old) for 48 h following OECD Test Guideline 202.²⁴ The tests were conducted using 15 mL of solution in 50 mL glass beakers, stirred for 5 min immediately before use. The animals used in these tests were selected from the third generation of a single parthenogenetic female. Five neonates were exposed in each beaker, and exposures were performed in ten replicates for all five treatments. Test beakers were covered with glass lids and kept at $20 \pm 2^\circ\text{C}$ under a 16 h light and 8 h dark photoperiod. Mortality or immobility was recorded after 48 h. An animal was considered immobile if no mobility was observed after a gentle probing with a transfer pipette and dead if no

heartbeat was observed after 15 s using a 10× magnification objective mounted on a stereomicroscope (Fisher Stereomaster). The swimming behaviour of the *D. magna* was observed in the surviving animals at the end of the 48 h tests. The animals were placed in 1 mL clean RW and the swimming speed was measured by recording 1-min videos of the surviving animals using a Samsung S10+ smartphone mounted onto the stereomicroscope using the procedure described by Pikuda *et al.*²⁵ The videos were analyzed with Kinovea software (<https://www.kinovea.org/>)²⁶ and the swimming speed was recorded. Temperature (°C), pH, and dissolved oxygen (mg/L) were measured at the beginning and the end of the exposures. The temperature, pH, and DO were 20–21 °C, 7–8, and 7–9 mg/L, respectively, which are within the acceptable range for the tests. The test conditions did not change significantly throughout all the tests.

The chronic toxicity test (21 days) was conducted on neonates (≤ 24 h old) following OECD Test Guideline 211²⁷ using the static renewal method. Each neonate was placed in 10 mL test medium in a 50 mL glass beaker. Ten replicates with one animal per beaker were used for each treatment group. The neonates were fed green algae (*Chlamydomonas reinhardtii*) and YCT mix (a mixture of yeast, cereal leaf, and trout chow) during the test. The green algae were prepared according to our previous work²³ while the YCT was purchased from Arofish, USA. Using a Multisizer 3 particle counter (Beckman Coulter), the concentration of the resulting algae suspension was measured as $\sim 5.0 \times 10^5$ cells/mL. Throughout the tests, a complete exchange of the test medium, algae, and YCT was done three times a week. The feeding ration of the neonates was adjusted to match their age as described in earlier studies.²³ Detailed information on their feeding rate throughout the test duration is presented in **Table S6.1**. The test organisms were monitored daily, and the number of offspring, immobility, and deaths were recorded. All offspring and dead organisms were removed and discarded daily. The presence of ephippia was also monitored. The

swimming speed of each surviving animal and the heart rate of the organisms was also recorded. After the video recording, two sets of images of the surviving daphnids were taken at the end of the experiment: one set using the same Samsung S10+ smartphone phone mounted onto the stereomicroscope, and the second set using a wide-zoom stereomicroscope (SZX16, Olympus). The first set of images was used to assess the physical appearance of the daphnids while the second set of images was analysed using Kinovea to measure the body lengths (top of the head to the base of the spine) of the organisms. Additionally, at each medium change, water conditions were measured before discarding the old medium and immediately after changing the medium to confirm that the DO level did not drop and that the pH and temperature did not change significantly during the tests. The test conditions throughout the 21-day toxicity test align with the acceptable range for toxicity tests, with the results being similar to those for the acute toxicity tests.

6.2.5 Uptake of Face Mask Particles by *Daphnia magna*

The ingested plastic particles were imaged using light sheet microscopy using a method that was previously reported.²⁸ At the end of the toxicity experiments, the exposed daphnids were stored in 10% neutral-buffered formalin for 24-48 h at 4 °C. Following fixation, samples were washed with water and stained with a solution consisting of 1 part 1 mg/mL meso-tetraphenylchlorin (TPC) (Toronto Research Chemical T310540) dissolved in tetrahydrofuran and 3 parts water by dropwise addition onto the sample. To reduce the tissue-bound dye in the organisms, the daphnids were kept in a tissue-clearing solution (adapted from Hama *et al.*²⁹) for 7 days.²⁸ A detailed description of the procedure of the sample preparation and imaging is provided in the Supporting Information (Text S6.2).

6.2.6 *Quality Assurance and Quality control (QA/QC) procedures*

Several QA/QC protocols were applied throughout the experiments. The face masks used were selected randomly from two boxes of single-use face masks. The ceramic scissors used for cutting as well as the glass beakers used for storage were rinsed in LCMS-grade water and subsequently in ethanol before handling the face masks. All activities were carried out in a laminar flow biological safety cabinet and the surfaces were wiped down with ethanol before use to minimize dust. Only cotton laboratory coats and colored latex gloves were worn during the experiments. To avoid contamination, tools used for this experiment were not exposed to any other materials throughout the duration of the weathering and toxicity experiments. WRW was used as an analytical control during the particle characterization and chemical analysis. Fresh RW was blended following a similar procedure as the face masks and stirred under UV irradiation in the same weathering chamber. The beakers containing the control and face mask samples were tightly covered with glass sheets or aluminum foil to avoid external contaminants. The resulting WRW was also analyzed, and the results were compared to those of fresh RW. The face mask fractions were vigorously stirred before any experiments or analysis and all tests or analysis were carried out in triplicates. Standard PerkinElmer spectral library was used to validate the composition of the face mask particles.

6.2.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 9.2.0 for Windows 64-bit (GraphPad Software, USA). Normality tests were carried out on the raw data, and when the data sets did not pass the normality test the raw data were log-transformed to normalize the data and increase the homoscedasticity. When the analysis compared only two conditions, parametric t-test with Welch's correction was used to investigate significant differences between the two conditions if the data passed the normality test, while the non-parametric Mann-Whitney test (also known as Wilcoxon test) was used when the data failed normality tests despite transformation. When the analysis involved more than two conditions, one-way analysis of variance (ANOVA) followed by TukeyHSD posthoc test was used to test differences between the treatments if the data passed the normality test. In cases when normality was not achieved despite the transformation for more than two groups of data, Kruskal-Wallis non-parametric test was performed followed by Dunn post-hoc to assess significant differences between the treatments. In all cases, statistical significance was set to $P < 0.05$ and the statistical significance between the treatments was investigated and reported as letters of significance. All toxicity graphs are presented as box plots (mean $\pm$ standard deviation).

6.3 Results and Discussion

6.3.1 Particle Characterization

NTA showed that the particle concentration was $3.3 \times 10^8 \pm 6.2 \times 10^7$ particles/mL in F1 and $2.4 \times 10^8 \pm 5.31 \times 10^7$ particles/mL in F3, which shows that the washing process resulted in a ~29% loss of particles. The results of the NTA analysis for F2, RW and WRW controls were below the detection limit of the instrument. SEM images (**Figure 6.1a-b**, **Figure S6.2**) showed the presence

of small-sized irregular-shaped fragments and fewer, longer fibres. Kokalj *et al.*³⁰ observed fibres from the inner and outer layers of the face masks, and irregularly shaped fragments from the middle layer. Since our study did not separate the different mask layers, this may explain why we found a mixture of these two types of particle shapes. Also, the results show that ~99% of the particles were below 2 μm in size while ~91% were below 1 μm (**Figure 6.1c**), suggesting that most of the particles are in the nano-range.

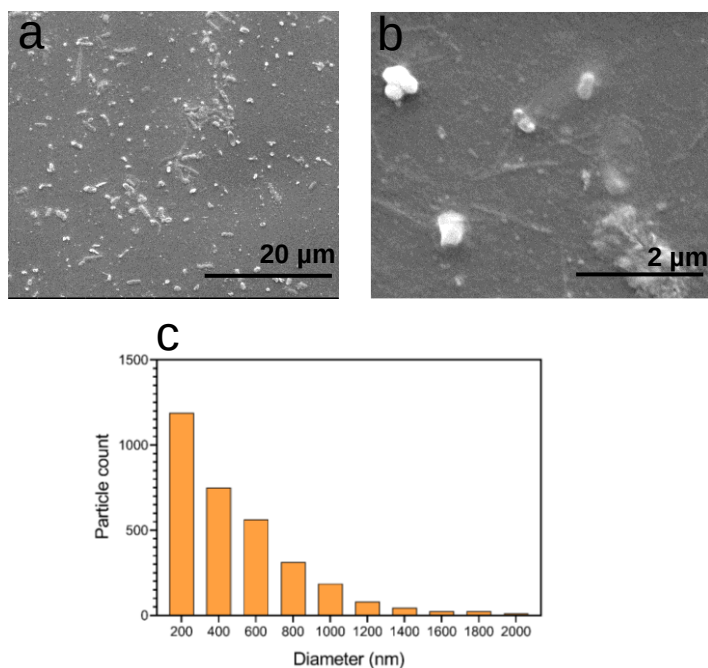


Figure 6.1. SEM images of face mask particles in F3 at (a) low magnification (2,500×) and (b) high magnification (30,000×), and (c) a histogram showing the Feret diameter of the particles observed by SEM (the total number of particles counted and analyzed was 3217).

The ATR-FTIR spectra (**Figure S6.3**) yielded a match between the three layers of the mask (inner layer, middle layer, and outer layer) and F3 (micrometer-sized particles and submicron particles). A spectral search in the PerkinElmer spectral library matched with polypropylene in all analyzed

samples. Details of the characteristic peaks identified and the ATR-FTIR spectra are provided in Supporting Information **Text S6.3**. Additionally, a piece of the face mask (inner layer) and randomly selected particles from F3 were characterized simultaneously by obtaining both Raman and IR spectra using O-PTIR. Representative spectra of both samples and a microscope image of a fibre particle analyzed with the O-PTIR are shown in **Figure 6.2a-c**. The characteristic peaks for polypropylene found in the ATR-FTIR spectra were also found in the IR spectra obtained using O-PTIR for both the face mask and the single particles that were analyzed (**Figure 6.2a**).^{31, 32} The characteristic peaks obtained from the Raman shift also agree with the IR spectra obtained from the O-PTIR as well as the ATR-FTIR spectra (**Figure 6.2b**, details provided in the Supporting Information **Text S6.3**). In addition to the presence of all the characteristic peaks of polypropylene, the face mask particles had additional peaks, most likely due to degradation during the weathering process or due to contact with the salts present in the RW.

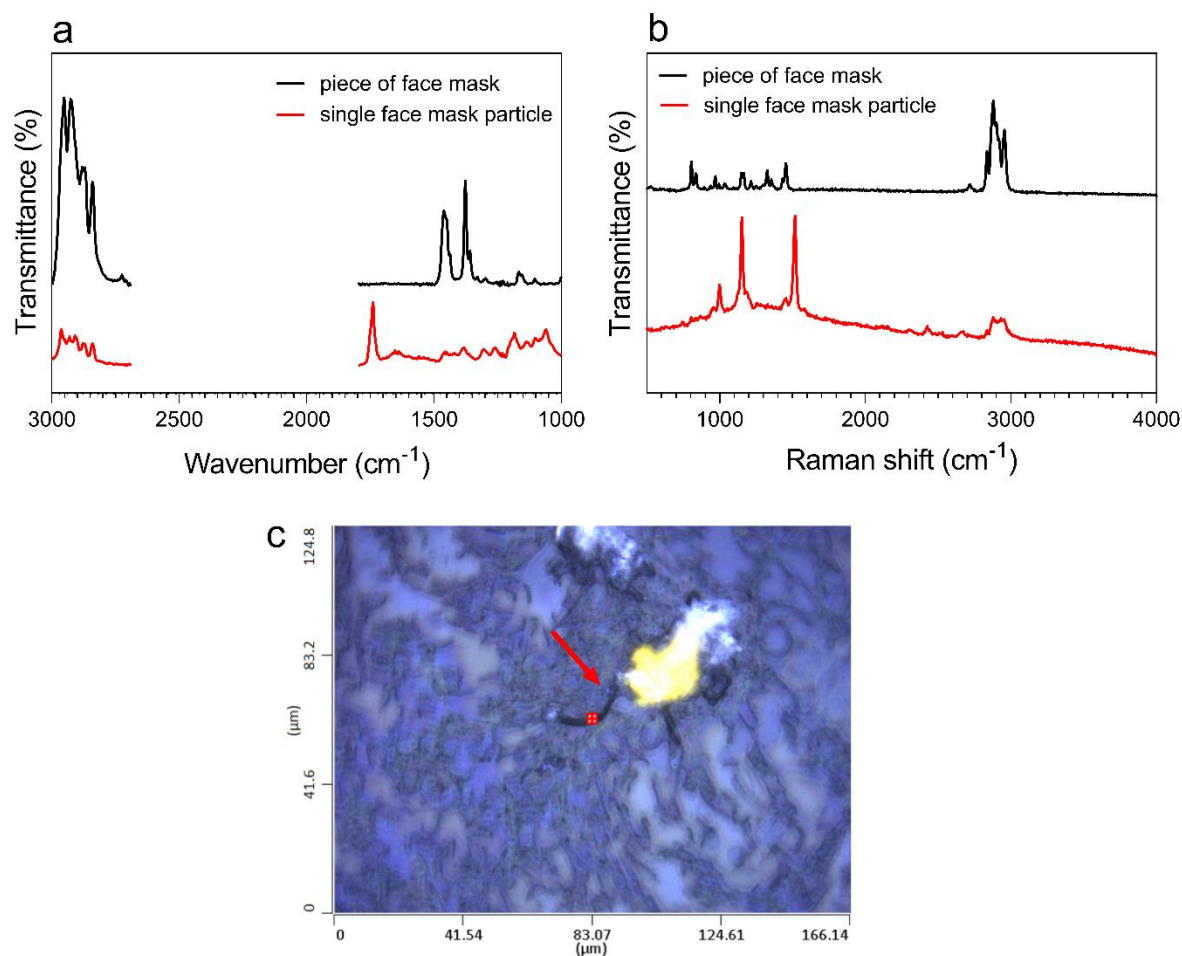


Figure 6.2. O-PTIR spectra for the inner layer of a face mask and a single fibre from F3. (a) IR spectra, (b) Raman shift, and (c) microscope image of the single fibre analyzed with O-PTIR; fibre dimensions are approximately 1.3 μm thick and 24 μm long.

6.3.2 Chemical Analysis

ICP-MS Analysis

As shown in **Table 6.1**, the most abundant metal or metalloid in the weathered face mask samples was Zn with a concentration of 84.54 ± 2.81 $\mu\text{g/L}$ in F1, which was mostly in soluble form, with 67.25 ± 0.61 $\mu\text{g/L}$ in F2 compared to 20.78 ± 1.36 $\mu\text{g/L}$ in F3. Others such as Cr, Mn, and Pb were

also identified as significantly higher in F1 with concentrations of $2.32 \pm 0.10 \mu\text{g/L}$, $2.78 \pm 0.06 \mu\text{g/L}$, and $1.11 \pm 0.01 \mu\text{g/L}$, respectively. The concentrations of Cr in F2 and F3 were similar to those in the controls. In contrast, Mn in F2 and F3 were higher compared to the controls with $1.26 \pm 0.33 \mu\text{g/L}$ and $1.76 \pm 0.94 \mu\text{g/L}$, respectively. Also, it is interesting to note that the particulate fraction of Pb showed a higher concentration of $0.51 \pm 0.01 \mu\text{g/L}$ than its soluble fraction of $0.37 \pm 0.02 \mu\text{g/L}$. Other tested metals or metalloids did not show any significant difference in concentration compared to those in the control samples. A separate analysis of weathered face mask nose strips also found $0.37 \pm 0.06 \mu\text{g/L}$ and $0.98 \pm 0.41 \mu\text{g/L}$ of Ni and Cu, respectively.

Similar metals and metalloids have also been detected in single-use face masks as reported by other studies.^{11, 21} Liu *et al.*³ sourced six different brands of single-use face masks and found Zn, Mn, and Pb in all 6 tested masks and Cr in 5 out of 6 mask samples. The concentration of Zn reported by Liu *et al.*³ is comparable with what was found in this study. The authors found up to $18 \mu\text{g/L}$ of Zn upon soaking 2 face masks per litre for 2 weeks while this study found $84 \mu\text{g/L}$ of Zn upon exposure of 10 face masks per litre (ca. 5 times higher). Hence, it can be estimated that each face mask releases $\sim 8.5 \mu\text{g}$ of Zn. The continued accumulation of face masks in the aquatic environment may lead to increased concentration and bioavailability of these metals and metalloids to aquatic organisms.

Table 6.1. Detected metal and metalloid concentrations in different fractions of face mask suspension ($\mu\text{g/L}$). The measurements in bold represent those with significantly high concentrations compared to the controls.

Sample	Zn	Cr	Mn	Pb
LC-MS water	14.11 ± 10.37	1.14 ± 0.23	0.21 ± 0.04	0.10 ± 0.02
RW	18.27 ± 1.75	1.41 ± 0.08	0.29 ± 0.00	0.11 ± 0.01
WRW	15.36 ± 4.87	1.43 ± 0.04	0.41 ± 0.00	0.26 ± 0.06
F1	84.54 ± 2.81	2.32 ± 0.10	2.78 ± 0.06	1.11 ± 0.01
F2	67.25 ± 0.61	1.98 ± 0.09	1.26 ± 0.33	0.37 ± 0.02
F3	20.78 ± 1.36	1.93 ± 0.04	1.76 ± 0.94	0.51 ± 0.01
nose strips	24.29 ± 3.19	0.42 ± 0.01	0.98 ± 0.67	0.14 ± 0.03

LC-MS Analysis

Non-targeted screening of the LC-MS data resulted in a total of 1141 and 213 molecular features from the ESI+ and ESI-, respectively, in the face mask samples. A compact grouping among six sample groups was observed in the principal component analysis (PCA) plots depicting the feature intensities in both ion modes, indicating satisfactory data quality (Supporting Information **Figure S6.5**). Venn diagrams also indicated that F1, F2, and F3 share common chemical features as well as other unique features (**Figure 6.3, Figure S6.4a-b**). As identification of all these molecular features would be extremely challenging and time-consuming, further investigation was limited to molecular features with significantly higher intensities (fold change 10.0) in the face mask samples than that in the controls. Upon suspect screening, 16 features under positive ion mode with tentative compound names assigned (**Table S6.2**) were selected for further targeted MS/MS fragmentation. Only 4 compounds were either with matched MS/MS fragments to MS/MS library spectra or with relatively high SIRIUS prediction scores. These four include mono-isobutyl

phthalic acid ($C_{12}H_{14}O_4$, RT = 5.8 min, m/z 223.0965, SIRIUS prediction 78%, MS/MS matched with that of MiBP in the database), mono-n-butyl phthalate ($C_{12}H_{14}O_4$, RT = 5.8 min, m/z 223.0965, SIRIUS prediction 78%), phthalic acid ($C_8H_6O_4$, RT = 5.8 min, m/z 167.0339, SIRIUS prediction 92%), and tetraethylene glycol monomethylether ($C_9H_{20}O_5$, RT = 4.4 min, m/z 209.1384, SIRIUS prediction 90%). By comparing retention time and fragmentation of these four authentic standards, only the identity of MiBP was confirmed in the face mask samples. However, it is important to note that molecular feature with m/z 167.0339 has the same retention time as MiBP standard and the same fragment pattern as phthalic acid standard. The different retention times of this feature from phthalic acid standard indicated that this feature could be the in-source fragment (phthalic acid moiety) of MiBP. This also indicated that the detected molecular features included some in-source fragments. Also, MiBP is a major hydrolysis product of dibutyl phthalate, which is one of the phthalates most commonly exposed to humans, and has been considered a sufficient indicator of the presence of DiBP.^{33, 34} Hence, the detected MiBP may have metabolized from DiBP during the sample preparation process or due to the presence of salts in the RW.³³ The concentrations of mono-isobutyl phthalic acid were quantified ($R^2 > 0.9995$) as $490 \pm 6 \mu\text{g/L}$ and $206 \pm 7 \mu\text{g/L}$ (average, $n=3$) for F1 and F3, respectively. The concentration in the controls and F2 was $< 2.5 \mu\text{g/L}$. Based on the initial number of face masks used to generate the mixtures, it can be estimated that each face mask releases $\sim 50 \mu\text{g}$ of MiBP.

Although MiBP is the only confirmed organic compound in the face mask samples, the similarities in the molecular features of MiBP and other phthalates such as mono-n-butyl phthalate, phthalic acid, and dibutyl phthalate cannot be ruled out. Phthalates are commonly added to plastics as plasticizers to improve their hardness and flexibility. However, since they are additives, they do not bind permanently to the products but can easily transport to their immediate environment.³⁵

There are limited studies on the impact of MiBP on invertebrates such as *D. magna*.³⁶ Overall, similar phthalates such as di(2-ethylhexyl)phthalate (DEHP) and di-n-butyl phthalate (DnBP) cause significant inhibition of reproduction in different species of fishes as well as in humans.³⁷⁻³⁹

Furthermore, the LC-MS results (as shown in the Venn diagrams, **Figures 6.3** and **S6.4**) show the complex nature of the chemical matrix of face masks. **Figure S6.4 a** and **b** show the features present in ESI+ and ESI-, respectively. After combining the identified normalized features in ESI+ and ESI- and removing the features that are common to F1, F2, and F3, the resulting number of features are presented in **Figure 6.3**. There are 399 features common to both F1 and F2 which is noticeably higher compared to the 30 features common to F1 and F3, and 16 features common in F2 and F3 ($\chi^2(2, N = 100) = 5.99, p < 0.05$). This agrees with the findings of the PCA plots as shown in **Figure S6.5**. In both ESI+ and ESI- modes in **Figure S6.5a** and **S5b** respectively, F1 and F2 are relatively closer compared to F1 and F3 or F2 and F3. Overall, the Venn diagrams (**Figure 6.3** and **S6.4**) show that there are many more chemicals in F1 and F2 compared to F3, while both the Venn diagrams (**Figure S6.4**) and PCA plots (**Figure S6.5**) show that F1 and F2 show comparable composition compared to F3. Confirming the identity of all the compounds in these fractions remains challenging due to the unavailability of their information in the database or due to the presence of these compounds at low concentrations. Also, there may be several molecular features whose peaks interfere with one another, thus hindering their detection in the analysis. Still, the complexity of the present features should be accounted for when interpreting toxicity results, as interactions among individual components may also cause toxicity to the organisms.

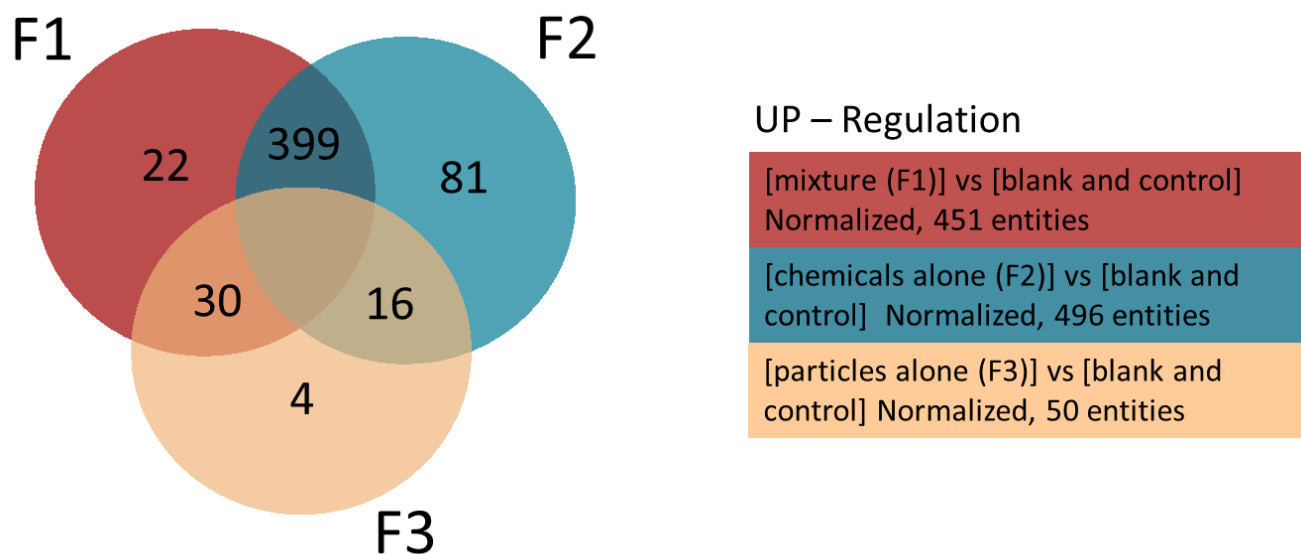


Figure 6.3. Venn diagram showing the molecular features of the different face mask fractions. Features with intensities higher than in "blank and controls" (fold change =10) were selected as UP regulation to make the Venn diagram. The ESI+ and ESI- features were combined and the features common to all three fractions were removed. Detailed ESI+ and ESI- features are presented in **Figure S6.4**.

6.3.3 Toxicity Tests

Preliminary statistical analysis of the toxicity results was conducted using either RW or WRW as statistical controls to investigate the effects of the choice of control in the statistical results. No significant difference was observed in the statistical significance results when either of the two controls was used. Thus, for the reported toxicity results in this paper, WRW was considered as the control for all statistical analysis. All results for RW are presented in **Table S6.3**.

Acute Toxicity

The results of the WRW control for the acute toxicity experiments met the validity requirements stated in the OECD 201 Guideline. The survival rates in WRW were 96%, 100%, and 98% for the tests on the neonates, juveniles, and adults, respectively (**Figure 6.4a-c**). The average survival of the neonates exposed to the three fractions was 64%, 44%, and 90% for the F1, F2, and F3 treatments, respectively, which showed statistically significant differences compared to the procedural control, WRW (Kruskal-Wallis test, $H(4) = 28.42$, $P < 0.0001$, **Figure 6.4a**). F3 did not have any significant impacts on the survival of the neonates, while F1 and F2 treatments resulted in a significantly lower survival rate compared to the controls (Dunn's test post-hoc, $P = 0.0119$ and $P < 0.0001$, respectively, **Figure 6.4a**). The survival rate of the juveniles was 76%, 80%, and 82% for the F1, F2, and F3 treatments, respectively. The survival of the juveniles was significantly affected by exposure to all three face mask treatments (Kruskal-Wallis test, $H(4) = 17.15$, $P = 0.0007$, **Figure 6.4b**); however, there was no difference in survival when comparing exposure to the three different fractions. Exposure of the adult *D. magna* to the three fractions resulted in 88% (F1), 96% (F2), and 84% (F3) survival. Statistically, no significant difference was observed in the acute survival in the three fractions compared to the control (**Figure 6.4c**). The EC_{50} values for all three acute tests could not be determined because almost all observed mortality were less than 50%.

Overall, the acute exposure to the three face mask fractions had dissimilar effects on different life stages of *D. magna*. In the neonates, in agreement with previous studies,³⁰ we observed that acute exposure to particles alone did not impact the survival of neonates. However, exposure to the mixture and dissolved chemicals alone caused significant mortality in neonates. Juveniles showed significant mortality in the presence of all three fractions while the same fractions did not have any impact on the adults. This result aligns with previous studies reporting that younger or smaller

organisms may be more susceptible to leached chemicals compared to their adult counterparts.^{40,}

⁴¹ Various studies have shown that *D. magna* is very sensitive to metals and metalloids, and some have reported their toxicity effects on *D. magna*.⁴²⁻⁴⁵ It has been reported that Zn, which is the most abundant metal in the face mask samples, ranked as one of the most toxic metals to *D. magna*, after Hg, Ag, and Cu.^{46, 47} Hoang *et al.*⁴¹ found that *D. magna* juveniles were more sensitive and had higher mortality upon exposure to Zn compared to adults. The concentrations of each of the metals and metalloids detected in the face mask samples are significantly lower than their reported EC₅₀ values after 48 h exposure to *D. magna*.^{42, 48, 49} However, the impact of the combination of these metals and metalloids in a co-exposure as in the case of the face mask samples is not yet fully understood. To date, no studies have assessed the toxicity of face mask particles and leached chemicals on multiple life stages of *D. magna*; however, studies exposing soil invertebrates (i.e., springtails) to face mask particles also show higher toxicity in juveniles compared to adults.⁵⁰

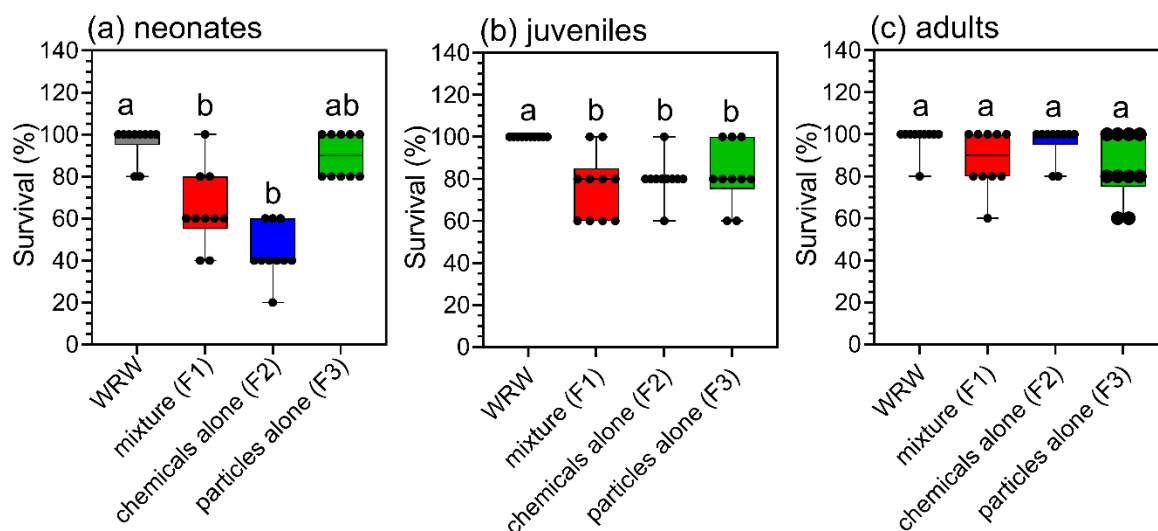


Figure 6.4. Survival of different life stages of *D. magna* upon 48 h acute exposure to different face mask fractions: (a) neonates, (b) juveniles, and (c) adults. Each test comprises 5 animals per treatment and all tests were conducted in 10 replicates. N = 50, $P < 0.05$. In each figure, the letters

of statistical significance are presented above the box plot for each treatment. Letter “a” at the top of the WRW represents the statistical control. Other treatments labelled “b” are significantly different from the control while those labelled “a” or “ab” do not show statistical significance compared to the control.

A notable difference in swimming behaviour was observed in exposed animals in all three life stages. The average swimming speed of the neonates exposed to the three fractions was 414 ± 71 mm/min, 270 ± 120 mm/min, and 273 ± 78 mm/min for the F1, F2, and F3 treatments, respectively, compared to 429 ± 124 mm/min in WRW. A significantly lower swimming speed was observed in the neonates exposed to F2 and F3 (1-way ANOVA, $F_{3,36} = 6.584$, $P = 0.0012$, **Figure 6.5a**). The swimming speed in the juveniles was 116 ± 57 mm/min, 164 ± 69 mm/min, and 115 ± 69 mm/min for the F1, F2, and F3 treatments, respectively, while the swimming speed in adults was 206 ± 51 mm/min, 140 ± 60 mm/min, and 137 ± 62 mm/min for the F1, F2, and F3 treatments, respectively. The swimming speeds in the WRW for the juveniles and adults were 281 ± 42 mm/min and 370 ± 65 mm/min respectively. The swimming speed also significantly decreased upon exposure to all three face mask fractions in the juveniles (1-way ANOVA, $F_{3,36} = 17.61$, $P < 0.0001$, **Figure 6.5b**) and adults ($F_{3,36} = 29.79$, $P < 0.0001$, 1-way ANOVA, **Figure 6.5c**). Swimming behaviour is becoming increasingly used as an ecotoxicity indicator for both acute tests²⁵ and chronic tests²³. Vaz *et al.*⁵¹ and Vicentini *et al.*⁵² also reported reduced swimming speed in *D. magna* upon acute exposure to polystyrene nanoparticles, while similar effects have been observed upon acute exposure of *D. magna* to titanium dioxide nanoparticles⁵³ and carbon-based nanomaterials⁵⁴. Vicentini *et al.*⁵² suggested that the reduced swimming behaviour may be due to accumulation of the particles in the organism, which may reduce their mobility. Other studies have

also shown that the presence of chemical contaminants such as phthalates, as found in this study, induce stress and swimming difficulties in *D. magna* as found in juveniles and adults.⁵⁵ However, it is unclear why the neonates exposed to F1 did not demonstrate decreased swimming speeds compared to their F2 and F3 counterparts, as well as the juvenile and adult animals.

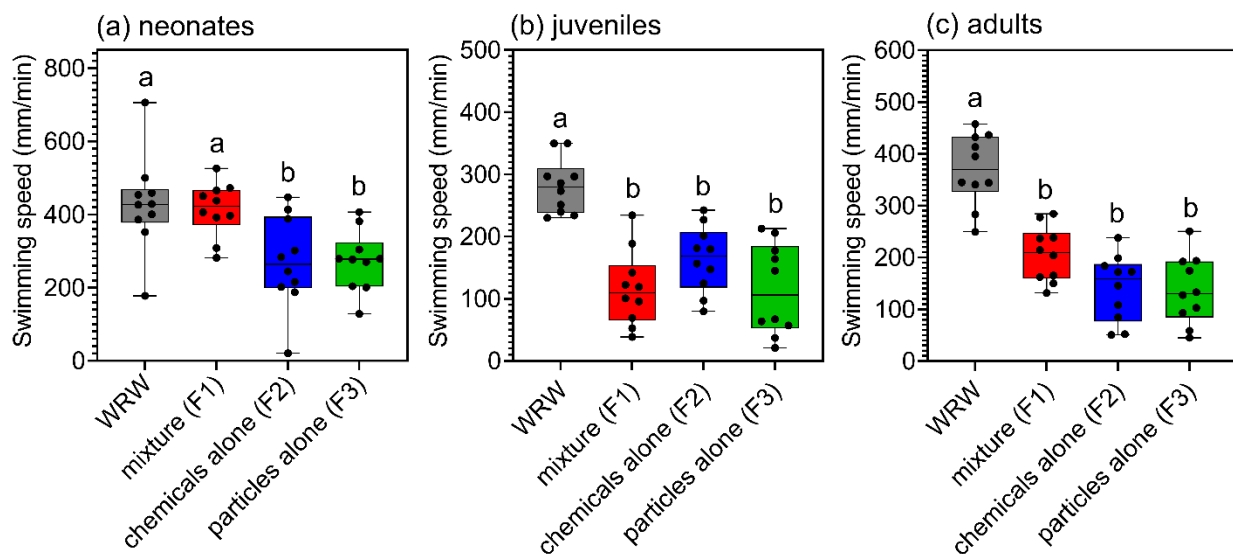


Figure 6.5. Swimming speeds of different life stages of *D. magna* at the end of the 48 h acute exposure to face mask leachates: (a) neonates, (b) juveniles, and (c) adults. $N = 10$, $P < 0.05$. In each figure, the letters of statistical significance are presented above the box plot for each treatment. Letter “a” at the top of the WRW represents the statistical control. Other treatments labelled “b” are significantly different from the control while those labelled “a” do not show statistical significance compared to the control.

Chronic Toxicity

Chronic exposure to the three face mask fractions significantly impacted the survival of the exposed daphnids (Kruskal-Wallis test, $H(4) = 52.28$, $P < 0.0001$, **Figure 6.6a**). 70% of neonates

exposed to F1 died within the first 48 h of exposure, and all exposed organisms were dead by Day 19. Similarly, all ten daphnids exposed to F2 were dead by Day 16. In the case of daphnids exposed to F3, all ten daphnids were alive by Day 20, three of which died by Day 21. There was no significant difference between the survival of the daphnids exposed to F3 versus the control. However, the survival was significantly lower in the daphnids exposed to F1 and F2 compared to F3 (Dunn's test post-hoc, $P < 0.0001$ and $P = 0.0001$, respectively). Higher mortality of neonates was observed upon exposure to F1 in the chronic toxicity tests compared to acute tests. The presence of food (algae and YCT) in the chronic tests may increase the biomagnification and the assimilation efficiency of chemicals by the exposed organisms.^{56, 57} The mortality in the WRW was 10%, which meets the validity requirement for the OECD Test 211.

Since all the daphnids exposed to F1 and F2 were dead by the end of the chronic exposure, their growth, reproduction, and behavioural endpoints could not be measured. However, these parameters were assessed for F3 and compared to WRW. The results show that the growth, heart rate, and swimming behaviour were significantly affected by exposure to the particles alone (**Figure 6.6b-d**). The average body length of the daphnids exposed to F3 was 1.8 ± 0.2 mm, which was significantly lower compared to the 2.8 ± 0.6 mm of the daphnids exposed to the WRW condition (t-test, $t(10.01) = 4.493$, $P = 0.0012$, **Figure 6.6b**). Daphnids exposed to F3 did not reproduce throughout the 21 days while the daphnids in the WRW treatment produced an average total of 68 ± 10 neonates within the 21-day chronic toxicity experiment. The inhibited reproduction may be due to the presence of phthalates such as MiBP, as has been reported of other phthalates found in plastic materials such as polypropylene tubes.⁵⁸ Comparable to the 20 µg/L of Zn found in F3, chronic exposure to 30 µg/L and 60 µg/L of Zn also caused a significant decrease in the reproduction of *D. magna* through potential accumulation in cells or tissues that are important for

vitellogenin synthesis, which provides egg yolk protein for invertebrates⁵⁹ and impacts the gene expression in *D. magna*⁶⁰.

The average heart rate of the daphnids exposed to F3 was 611 ± 68 counts/min, which is significantly higher compared to the 499 ± 29 counts/min recorded for the WRW treatment (Mann–Whitney test, $U = 8.5$, $P = 0.0118$, **Figure 6.6c**). In addition, the average swimming speed for the daphnids exposed to F3 was 503 ± 64 mm/min, which was significantly higher compared to 390 ± 47 mm/min recorded for WRW (t -test, $t(9.792) = 2.28$, $P = 0.0463$, **Figure 6.6d**).

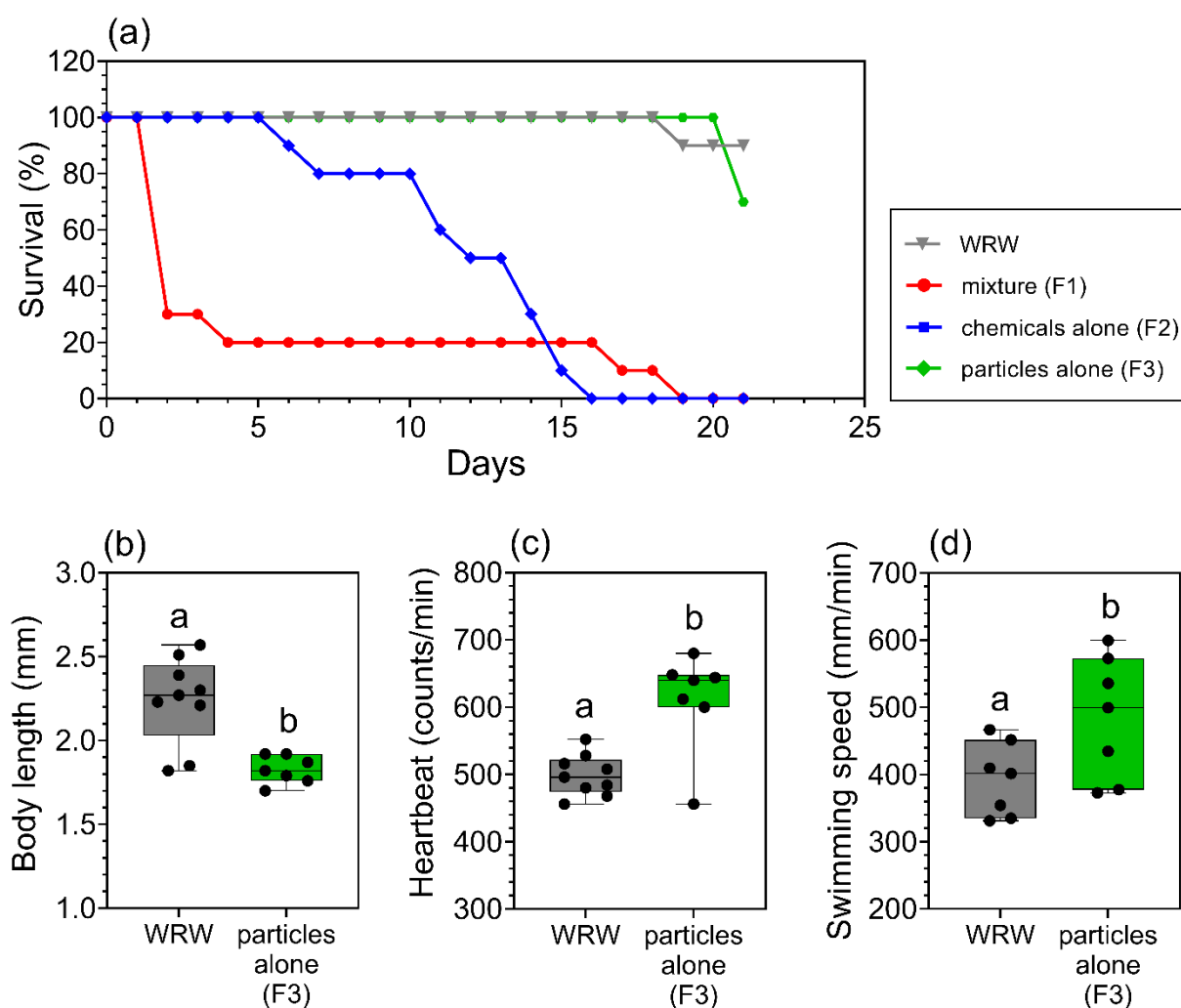


Figure 6.6. Observed impacts on *D. magna* after 21-day exposure to control and face mask fractions. (a) survival, $n = 10$ daphnids per treatment, (b) body length, (c) heartbeat, and (d) swimming speed. $N \geq 7$ $P < 0.05$. In figures b-d, the letters of statistical significance are presented above the box plot for the WRW and F3 treatment. Letter “a” at the top of the WRW represents the statistical control while letter “b” above the F3 treatment indicates statistical significance compared to the control.

Furthermore, images of the daphnids exposed to F3 show that there were no eggs or developing neonates in the egg chambers compared to the images of the daphnids exposed to the RW and WRW conditions (Representative images of the daphnids after the 21-day chronic test are shown in **Figure 6.7** and **Figure S6.6**). The images also show slight discoloration and visibly smaller size after the 21-day test. The average body surface areas of the daphnids were significantly different among the groups, measuring $2.8 \pm 0.6 \text{ mm}^2$ and $1.8 \pm 0.63 \text{ mm}^2$, respectively, for the WRW and F3 (t-test, $t(10.64) = 3.893$, $P = 0.0027$).

The behavioural effects of contaminants to aquatic invertebrates depend on the level or duration of exposure and may include no effect, stimulation, acclimation or adaptation, toxic effect, and sometimes readjustment.^{61, 62} The changes in behaviour upon contamination may also be influenced by changes in the physical and developmental needs of the organisms, e.g., need for food, search for protection, or preparation for reproduction.⁶¹ While acute exposure to the particles alone temporarily induces stress,⁵¹⁻⁵⁴ it can be speculated that as the organisms become aware of the presence of the particles either through accumulation in their digestive tracts or hindrance to their movements, they may attempt to swim away from the contaminated environment, particularly after prolonged exposure as in the case of the chronic tests, hence leading to increased heart rate

and swimming speed.⁶³ Assessment of the behavioural effects of chronic microplastic exposure to *Daphnia magna* is still scarce; however, increased swimming activity has also been reported upon chronic exposure of zebrafish to polystyrene microplastics.⁶⁴ Also, the discoloration of the exposed organisms after chronic exposure may be due to possible oxidative stress or ROS production.^{65, 66}

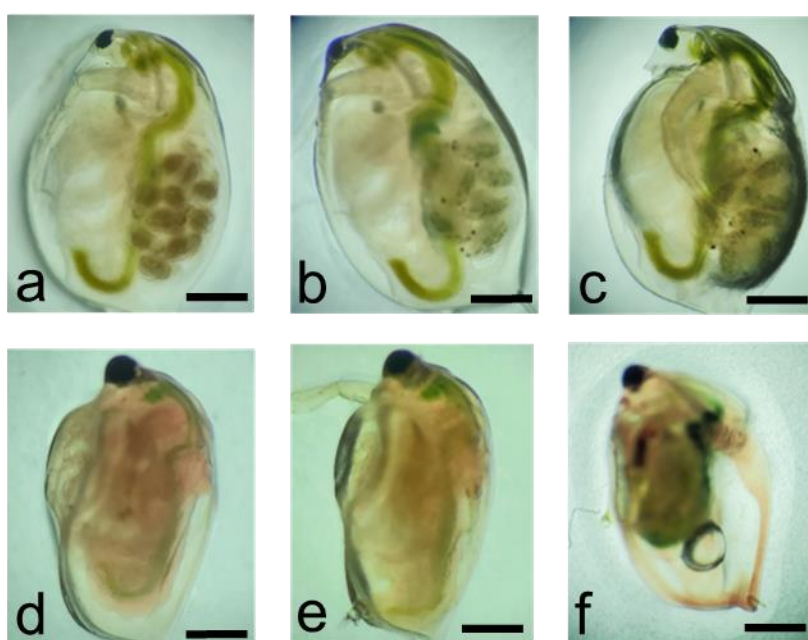


Figure 6.7. Images of *D. magna* after 21-day exposure to: (a-c) WRW; (d-f) F3. Scale bar = 500 µm.

3.5. Uptake of Particles upon Exposure to *Daphnia magna*

Light sheet images were taken at the end of the acute and chronic tests to investigate the ingestion of the particles by the daphnids (**Figure 6.8**). The images show minimal uptake of the particles by the neonates after acute exposure to F1 and F3. In contrast, juveniles and adults exposed to F1 and

F3 show significant uptake of the particles in the gastrointestinal tract after the acute tests. As expected, no presence of particles was found in the animals exposed to F2. While the neonates may not have ingested the particles during the acute tests, we hypothesize that this may be partly due to the small mouth size of neonates, which may also explain why there is no significant mortality upon exposure to the particles alone while the toxicity from F1 and F2 may be from the presence of chemicals in both fractions (**Figure 6.3**). The larger mouth size of the juveniles and adults may have allowed more uptake of the particles compared to the smaller mouth size of the neonates. Also, although there was a visible accumulation of particles in both the juveniles and the adults, previous studies have suggested that the observed toxicity of microplastics to *D. magna* depends on the age of the exposed organism, and may be more significant in younger organisms.⁶⁷ These reasons may explain why there was no significant impact on the survival of the adults upon exposure to all three fractions, compared to the observed impacts on the neonates and juveniles.

At the end of the chronic toxicity test, there were no surviving organisms from the F1 and F2 treatment groups. Hence, no images were taken of the daphnids in these groups. However, images of animals exposed to F3 also show significant uptake of the particles. It can be assumed that the particles were ingested as the organisms grew into juveniles and eventually adults.

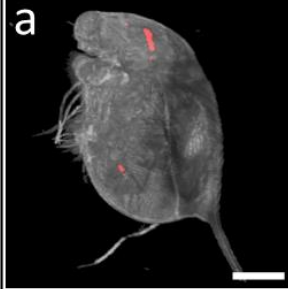
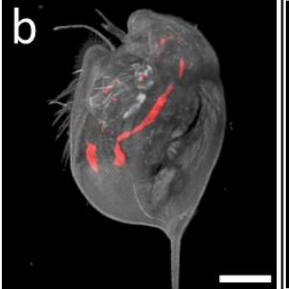
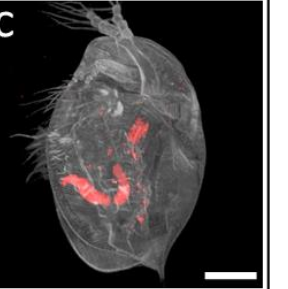
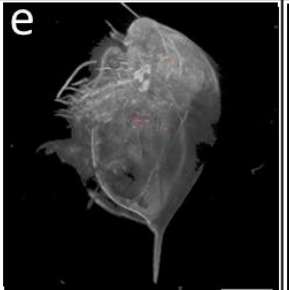
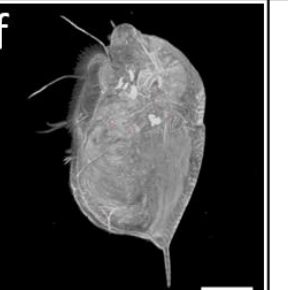
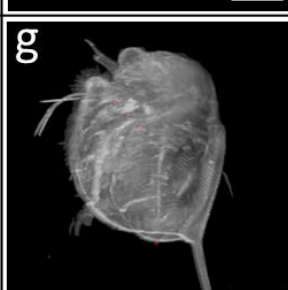
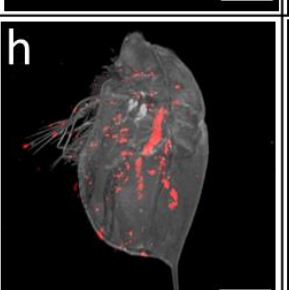
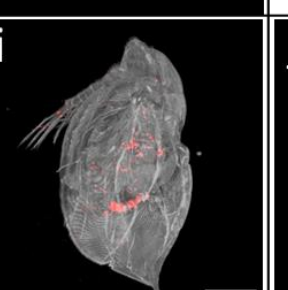
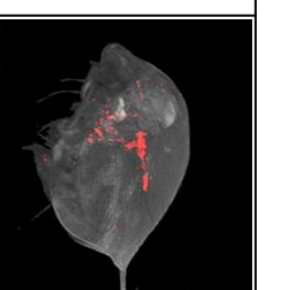
	Acute exposure (neonates)	Acute exposure (juveniles)	Acute exposure (adults)	Chronic exposure
Mixture (F1)				not available
Chemicals alone (F2)				not available
Particles alone (F3)				

Figure 6.8. Images of *D. magna* after acute and chronic exposures to face mask fractions. Scale bars: (a-c) = 350 μm ; (d-f) = 500 μm ; (g-i) = 650 μm ; (j) = 750 μm . Red coloration in (a), (b), (c), (h), (i) and (j) represents face mask plastic particles. For the chronic test, there are no images for *D. magna* exposed to F1 and F2 treatments because all exposed organisms were dead by the end of the experiment.

6.4 Conclusions

This study sheds light on the properties and toxicity effects of particles and chemicals obtained from single-use face masks after simulated weathering. The results showed that nanoplastics and chemicals released from single-use face masks may have different toxicity impacts on *D. magna*, and the observed impacts may depend on the life stage of the exposed organism. Younger organisms are more vulnerable to the released chemicals compared to their older counterparts. Furthermore, prolonged exposure to the particles alone in the presence of food may not impact survival but may affect life-history traits by causing slowed growth, inhibited reproduction, and other stress-related behavioural changes. The results suggest that the mechanism of toxicity is very complex, it is different for the different components of face masks and also depends on the life stage of the exposed organisms.

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6.5 Supporting Information

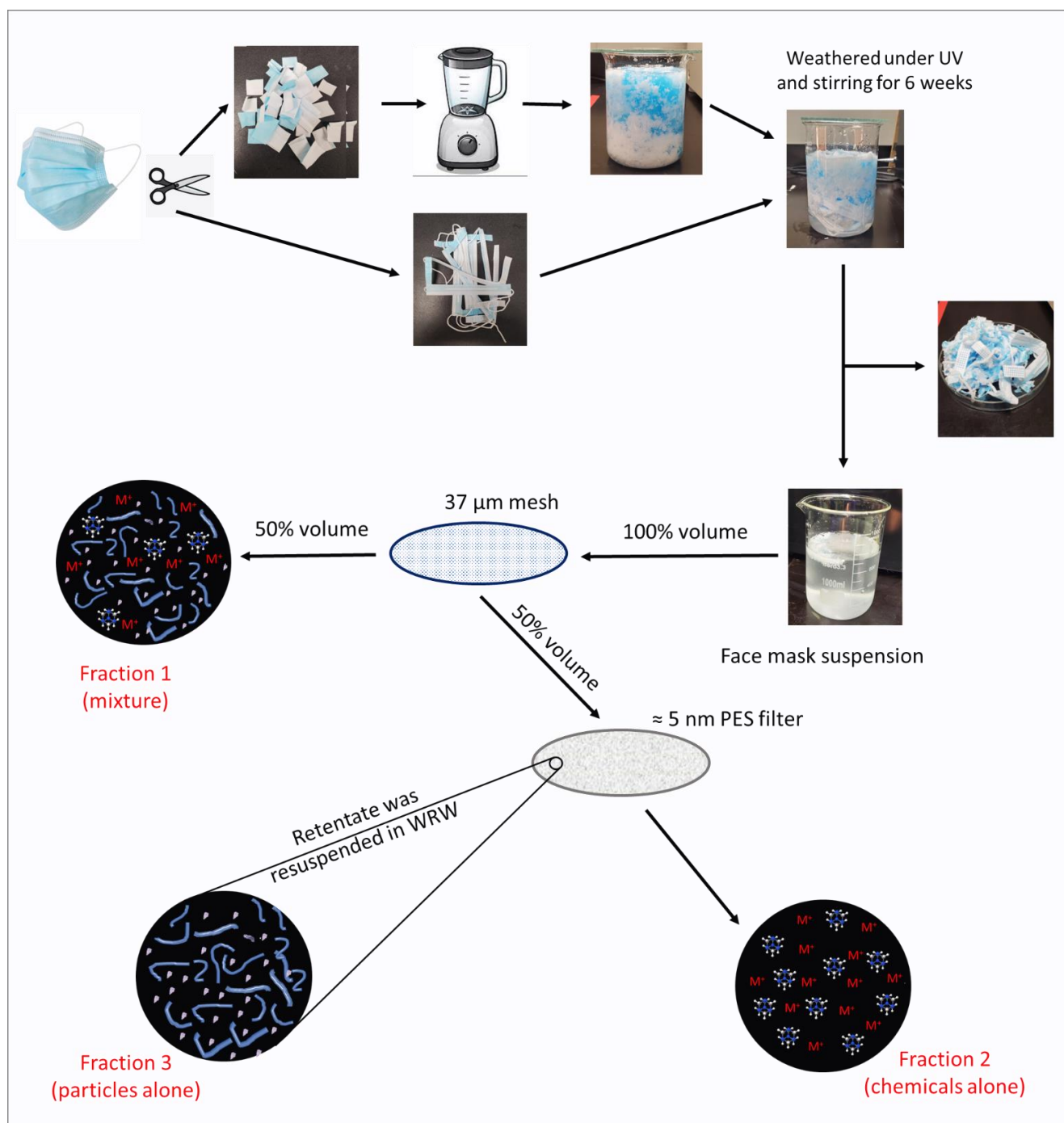


Figure S6.1. Schematic description of the preparation of the three face masks fractions from single-use face masks.

Text S6.1: Determination of the mass concentration of the face mask fractions.

The mass concentration of the three face mask fractions was determined using a microbalance (Sartorius S4 Electronic Ultramicro Balance) as described in the Supporting Information. The concentration of the salts in the two controls (RW and WRW) was also calculated to serve as reference points. Fifty antistatic weighing boats were rinsed with deionized water and dried at 40 °C for 3 days. 500 µL of each fraction and control was added to 10 weighing boats and dried at 40 °C for 14 days. The final masses of the boats were recorded after drying, and the difference in mass before and after drying was considered as the total mass of the material contained per fraction. The average mass of the 10 replicates was calculated to determine the total mass in each of the fractions and controls. The concentration of the procedural control (i.e., WRW) was subtracted from the concentration of the three face mask fractions and the difference was used to estimate the concentration of the face mask material in each of the three fractions. The RW and WRW controls show an average mass concentration of 224.4 ± 6.7 mg/L and 230.8 ± 3.6 mg/L, representing the salts added in the preparation. Upon subtracting the concentration of the salts in the procedural control (i.e., WRW), the concentration of F1, F2, and F3 were 94 ± 1.3 mg/L, 45.1 ± 1.3 mg/L, and 32.2 ± 4.0 mg/L, respectively.

Table S6.1. Detailed information of the feeding rate of *C. reinhardtii* and YCT to *D. magna* during a 21-day chronic exposure to face mask fraction.¹

Day	Algae (per test beaker)	YCT (per test beaker)
0	0.5 mL	0.5 mL
2	0.5 mL	0.5 mL
4	1.0 mL	1.0 mL
6	1.0 mL	1.0 mL
8	1.0 mL	1.0 mL
10	1.5 mL	1.5 mL
13	1.5 mL	1.5 mL
17	1.5 mL	1.5 mL
21	*	*

*Organisms were not fed on the last day

Text S6.2: Lightsheet sample preparation and imaging procedure for *D. magna*

The clearing solution consisted of 550 g/L urea, 225 g/L sorbitol, and 50 g/L Triton-X100 in water. The daphnids were then imaged using a Zeiss lightsheet microscope as previously reported.² Samples were mounted in 1.5 % low melting temperature agarose gel made from ScaleS4 mounting solution (240 g/L urea, 400 g/L sorbitol, 100 g/L glycerol, 150 g/L dimethyl sulfoxide in water) as described by Hama *et al.*³ in 1 mL syringes. The refractive index was measured with a refractometer and adjusted to 1.44 by adding glycerol or water as needed. Image acquisitions were carried out with a 5× 0.16 NA objective for detection and 5× 0.1 NA objectives for illumination. Autofluorescence from tissue was detected using a 405 nm laser for excitation and a 420 nm to 470 nm bandpass filter for emission. The signal from TPC was detected using a 405 nm excitation laser and a 660 nm long-pass filter for emission.

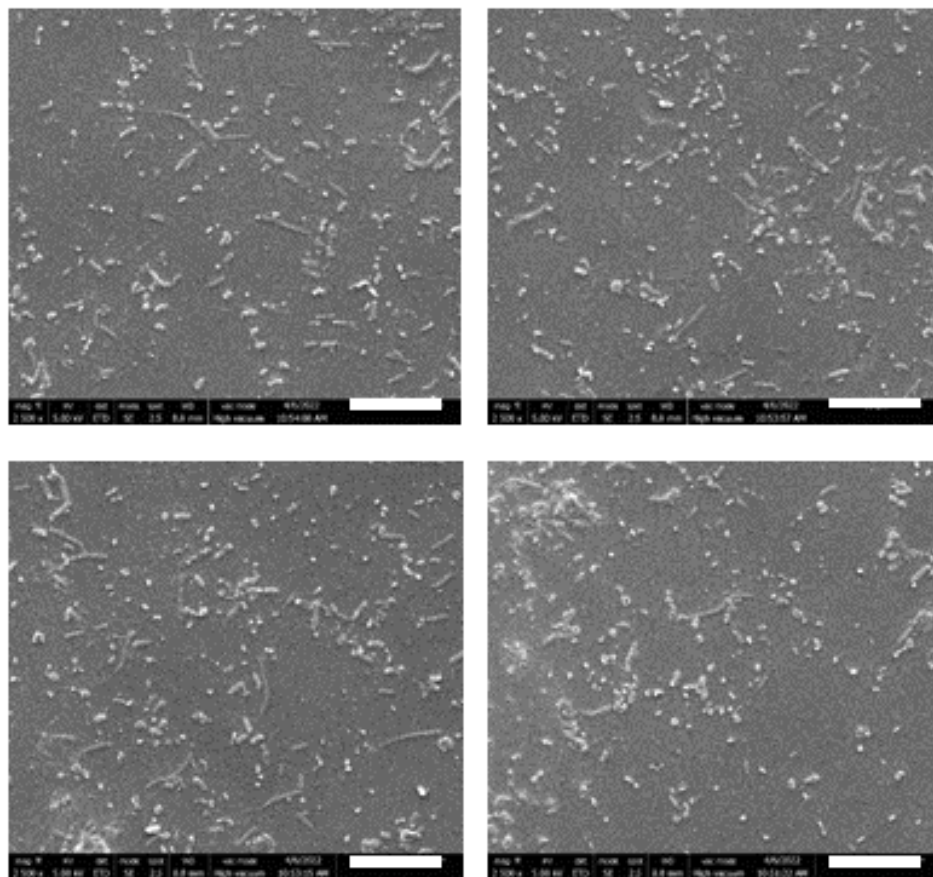


Figure S6.2. Additional SEM images of face mask particles in the particle alone fraction at low magnification (2,500×).

Text S6.3: Detailed Summary of ATR-FTIR and Raman Results

ATR-FTIR spectra found characteristic peaks of polypropylene at 2956 cm^{-1} (stretching CH_3 asymmetric), 2926 cm^{-1} (stretching CH_2 asymmetric), 2878 cm^{-1} (stretching CH_3 symmetric), 1458 cm^{-1} and 1380 cm^{-1} (bending CH_3 symmetric), 1168 cm^{-1} (rocking CH_3 , wagging CH), and 978 cm^{-1} (stretching CC backbone). Other additional peaks at 996 cm^{-1} (rocking CH_3 , stretching CC backbone), 840 cm^{-1} (rocking CH_3 , rocking CH), and 808 cm^{-1} (stretching CC backbone) that are

characteristic of polypropylene were also found in the ATR-FTIR spectra (**Figure S6.3**) of all analyzed samples.

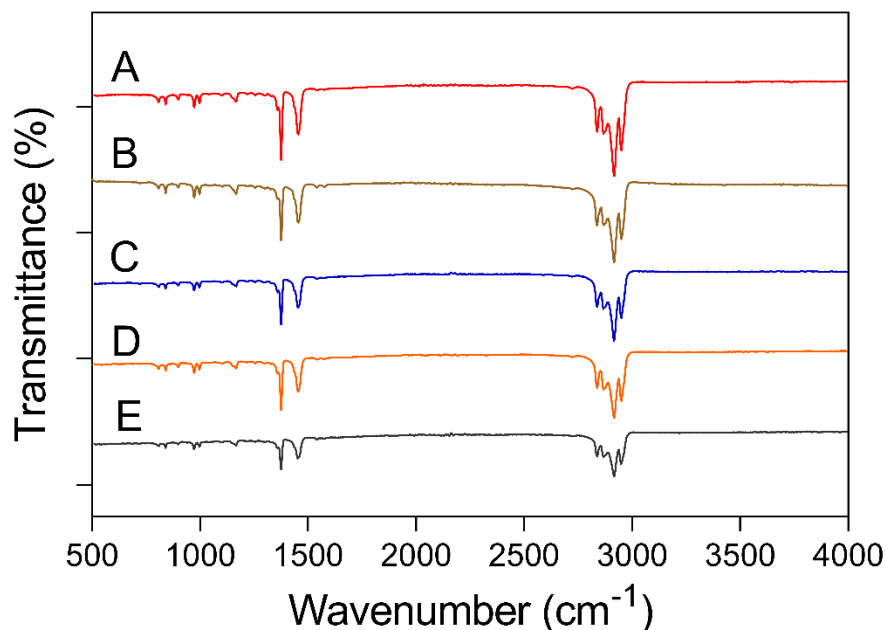


Figure S6.3. ATR-FTIR spectra for the (A) inner, (B) middle, and (C) outer layers of a face mask, as well as (D) microparticles and (E) sub-micron particles from the particles alone fraction.

In the case of the Raman shift obtained using the O-PTIR (**Figure 6.2b**) for both the piece of face mask and the single particles, characteristic peaks were identified at 2924 cm^{-1} (stretching CH_3 asymmetric), 2924 cm^{-1} (stretching CH_2 asymmetric), 2901 cm^{-1} (stretching CH), 2874 cm^{-1} and 2835 cm^{-1} (stretching CH_2 symmetric), 1454 cm^{-1} and 1431 cm^{-1} (bending CH_3 asymmetric), 1358 cm^{-1} (bending CH_3 symmetric), 1327 cm^{-1} (bending CH , twisting CH_2), 1296 cm^{-1} (wagging CH_2 , twisting CH_2 , bending CH), 1214 cm^{-1} (twisting CH_2 , bending CH , stretching CC backbone), 1164 cm^{-1} (rocking CH_3 , bending CH , stretching CC backbone), 1152 cm^{-1} (bending CH , rocking CH_3 ,

stretching CC backbone), 1040 cm^{-1} (bending CH, stretching CC backbone), 998 cm^{-1} (rocking CH_3 , bending CH, wagging CH_2), 971 cm^{-1} and 936 cm^{-1} (rocking CH_3 , stretching CC backbone), 835 cm^{-1} (rocking CH_3 , stretching CC backbone, rocking CH_2), 804 cm^{-1} (stretching CC backbone, rocking CH_2), 452 cm^{-1} and 312 cm^{-1} (wagging CH_2), and 394 cm^{-1} (wagging CH_2 , bending CH).

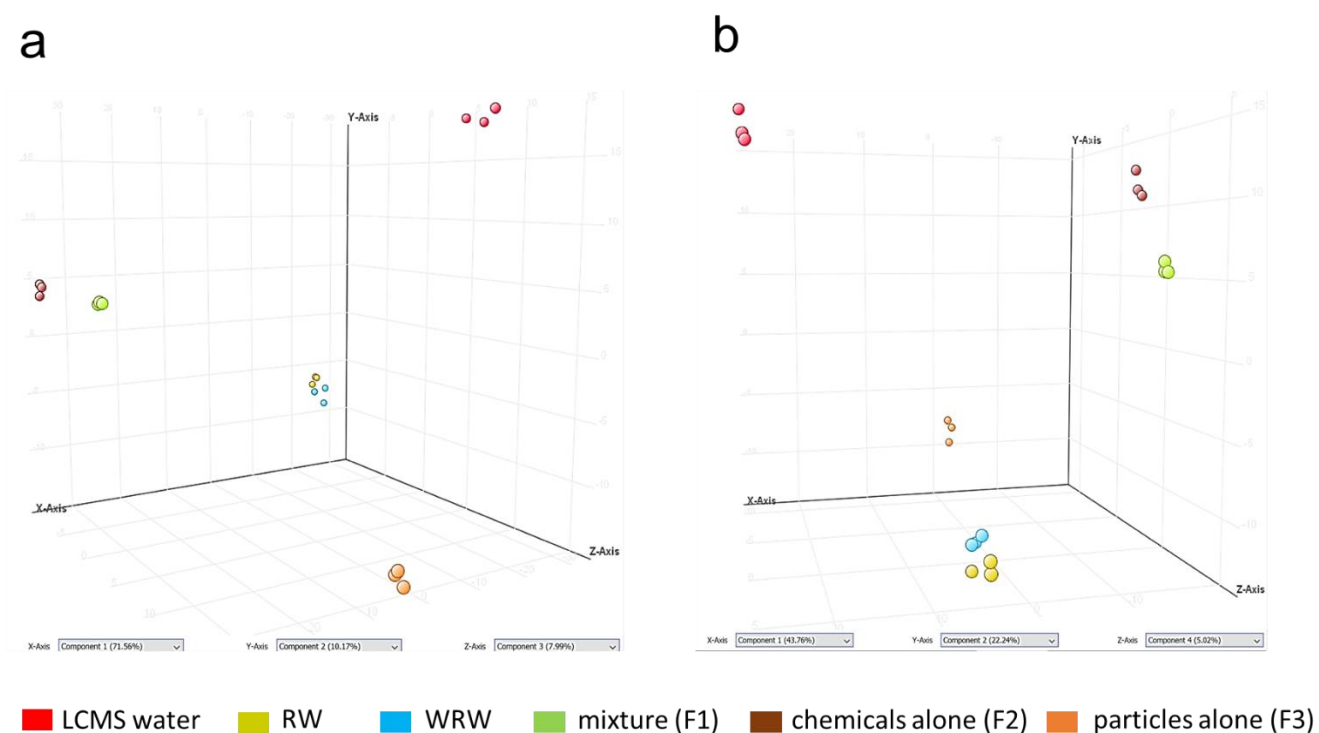


Figure S6.4. Principal component analysis (PCA) of the intensities of molecular features detected in all analyzed samples in (a) ESI+, PCA components 1, 2, and 3; (b) ESI-, PCA components 1, 2, and 4.

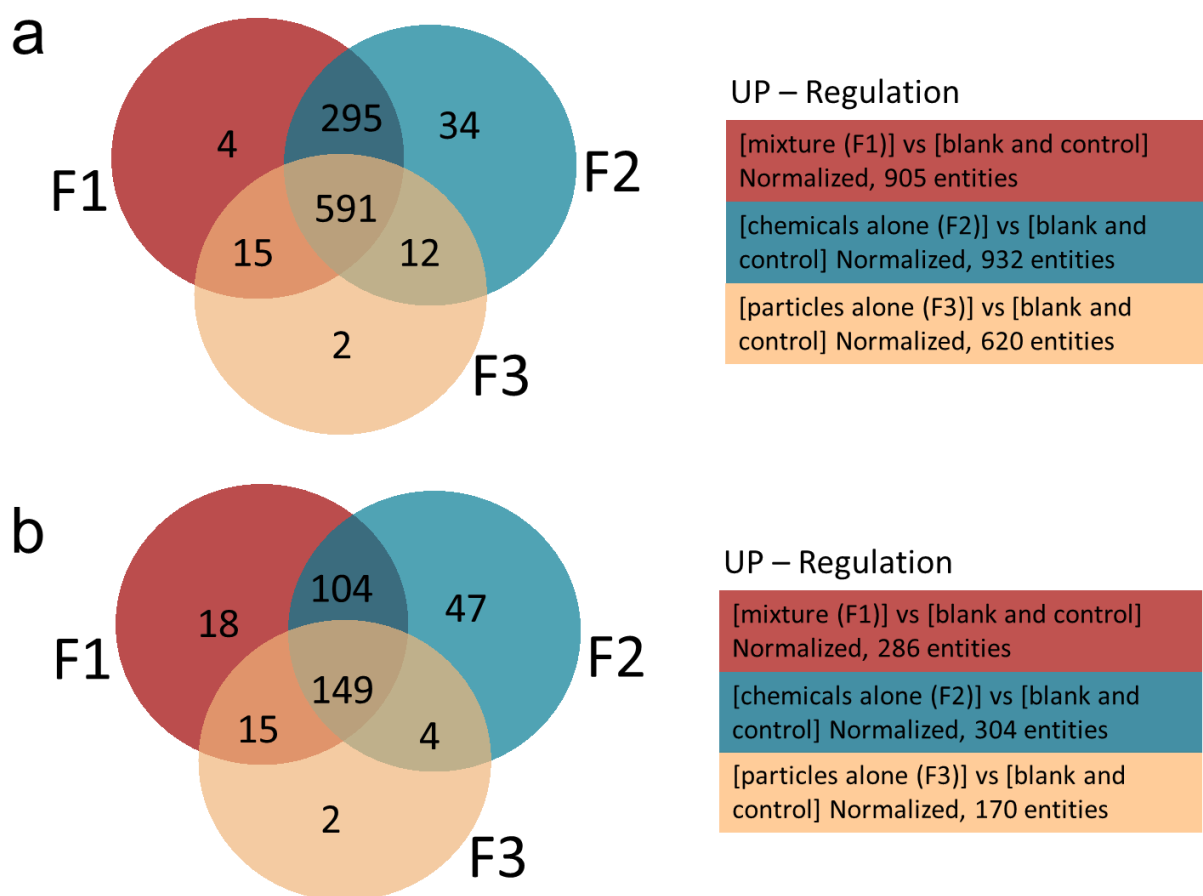


Figure S6.5. Venn diagrams showing the molecular features of the different face mask fractions. (a) ESI+ (b) ESI-. In each group, features with intensities higher than in "blank and controls" (fold change =10) were selected as UP regulation to make the Venn diagram.

Table S6.2. Selected features from screening face mask samples. Suggested molecular formulas are based on accurate mass and isotopic pattern.

m/z	RT (min)	Suggested Molecular formula	Suspected identity	*Identification level
158.1539	2.659	C ₉ H ₁₉ NO	N'N-Dibutylformamide	4
227.1754	4.018	C ₁₂ H ₂₂ N ₂ O ₂	Caprolactam cyclic dimer	4
223.0965	5.876	C ₁₂ H ₁₄ O ₄	Monoisobutyl phthalic acid (MiBP)	1
135.1016	4.22	C ₆ H ₁₄ O ₃	Diethylene glycol ethyl ether, Dipropylene glycol, Polypropylene glycol isomers.	4
209.1384	4.366	C ₉ H ₂₀ O ₅	Other iomer of tetraethyleneglycol monomethylether	4
251.1278	5.61	C ₁₄ H ₁₈ O ₄	Diisopropyl phthalate, Dipropyl phthalate, Monohexyl phthalate isomers	4
679.5117	5.02	C ₃₆ H ₆₆ N ₆ O ₆	Caprolactam tetramer	4
311.2064	5.426	C ₁₄ H ₃₀ O ₇	Hexaethyleneglycol dimethylether	4
205.1223	5.6	C ₁₃ H ₁₆ O ₂	1-Hydroxycyclohexyl phenyl ketone (Irgacure 184)	
167.0339	5.865	C ₈ H ₆ O ₄	Phthalic acid (in-source fragment of MiBP)	1 (in-source fragment of MiBP)
369.206	6.25	C ₂₃ H ₂₈ O ₄	Benzyl isooctyl phthlate or Benzyl 2-ethylhexyl phthalate	4
415.2115	8.937	C ₂₄ H ₃₀ O ₆	DMDBA / Bis (3,4-dimethyl-dibenzylidene sorbitol)	4
167.0339	5.865	C ₈ H ₆ O ₄	Phthalic acid	1 (in source fragment of MiBP)

*Identification level: Level 1 for confirmed identity by authentic standards. Level 4 indicates MS full scan with isotope pattern.

Table S6.3. Toxicity data for the RW control.

Tests	Measured endpoints	Results
Acute exposure (neonates)	Survival	98%
Acute exposure (neonates)	Swimming speed	357 ± 64 mm/min
Acute exposure (juveniles)	Survival	100%
Acute exposure (juveniles)	Swimming speed	240 ± 56 mm/min
Acute exposure (adults)	Survival	98%
Acute exposure (adults)	Swimming speed	389 ± 66 mm/min
Chronic exposure	Survival	90%
Chronic exposure	Number of neonates	63 ± 8
Chronic exposure	Body length	2.6 ± 0.6 mm
Chronic exposure	Heartbeat	472 ± 46 counts/min
Chronic exposure	Swimming speed	370 ± 34 mm/min

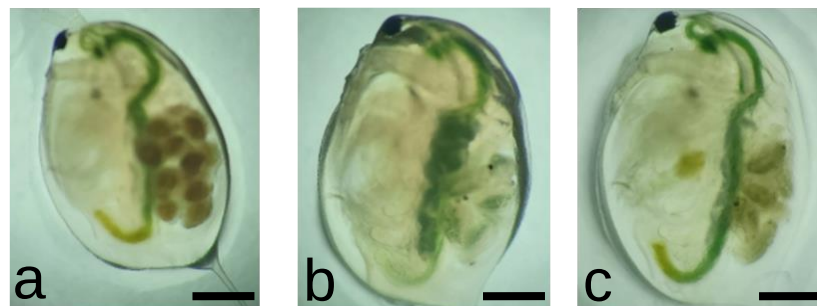


Figure S6.6. Images of *D. magna* after 21-day exposure to RW. Scale bar = 500 µm.

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Chapter 7: Discussion of Key Findings

This chapter is a comprehensive discussion of the key findings in Chapters 2 to 6. While each of those chapters addressed specific research questions and can be understood as standalone studies, this chapter aims to provide an overarching discussion of the key findings of this thesis and the connection between the findings of the manuscript chapters.

7.1 What factors influence the observed toxicity of microplastics and nanoplastics to *Daphnia magna*?

As highlighted in Chapter 1, this thesis aims to provide insights to advance our knowledge on some of the factors that influence the observed toxicity upon the exposure of *Daphnia magna* to microplastics and nanoplastics. While there are several factors that may contribute to toxicity, this section only discusses the factors that were covered by the findings in Chapters 2-6 of this thesis.

7.1.1 Presence of added or leached chemicals.

This thesis found that the presence of chemicals either added to microplastic and nanoplastic suspensions as a preservative or leached from the plastic particles significantly affect the observed results from toxicity assessments of *Daphnia magna*.

Chapter 2 showed that non-dialyzed 20 nm and 200 nm polystyrene nanoplastics (*i.e.*, containing sodium azide) caused severe mortality reaching 100% at 100 ppm to neonates upon 48 h acute exposure (**Figure 3.1**). When neonates were exposed to sodium azide only at the same concentration as that present in the non-dialyzed nanoplastics, the observed mortality was similar

(**Figure 3.1**). In contrast, dialyzed nanoplastics caused no mortality for both 20 nm and 200 nm nanoplastics at up to 100 ppm (**Figure 3.1**).

Chapter 6 examined the toxicity from different fractions of single-use face masks namely: mixture of particles and chemicals, particles alone, and chemicals alone to *Daphnia magna*. The results showed that the average survival of the neonates exposed to the mixture, particles alone, and chemicals alone were 64%, 90%, and 44% respectively after 48 h acute toxicity (**Figure 6.4**). Statistically, there was no significant effect on survival for the organisms exposed to the particles alone fraction compared to the controls. However, the organisms have significant mortality when exposed to the mixture and the leached chemicals alone.

The findings from these two chapters is in agreement with previous studies which reported that microplastics and nanoplastics alone do not impact the survival of neonates.¹ In both Chapters 2 and 6, exposure to the chemicals alone showed similar toxicity to those observed when the neonates were exposed to a mixture of particles and chemicals. The sodium azide in the nanoplastics used in Chapter 2 was added to the nanoplastic suspension as a preservative. However, the chemicals present in the mixture and chemicals alone fraction of the face masks in Chapter 6 were added during the manufacturing process. Unlike the results in Chapter 2 where it was clear that the toxicity was from a specific compound, i.e., sodium azide, the leached chemicals from the face masks are generally more complex in composition as shown by the chemical analysis (**Table 6.1 and Figure 6.3**). Thus, it is more complicated to understand the source of the observed toxicity in the neonates for such plastics. Nonetheless, the findings from these two chapters show the need to pay attention to the chemical matrixes of microplastics and nanoplastics used in toxicity assessments, especially when interpreting observed toxicity.

7.1.2 Type of tests and the measured endpoints

The findings of this thesis reveal that the effects observed by exposing *Daphnia magna* to microplastic and nanoplastics depend on the type of toxicity tests conducted and the measured endpoints.

Chapter 2 revealed that the dialyzed PS-NPs did not impact the survival of *Daphnia magna* neonates at concentrations up to 100 ppm for both 20 nm and 200 nm polystyrene nanoplastics upon acute exposure (**Figure 3.1**). However, the same acute exposure significantly affected the swimming behavior of the neonates when compared to the controls. Neonates exposed to polystyrene nanoplastics swam up to 40 cm in one min compared to an average of 10 cm per minute observed in the control organisms. Similar hyperactive swimming behavior has been observed in other nanomaterials such as nano-C₆₀ and the hydrogenated C₆₀-H_xC₇₀H_x.²

In Chapter 3, the same dialyzed nanoplastics used in Chapter 2 were exposed to chronic toxicity with several sublethal endpoints such as survival, growth, reproduction. Similar to the acute toxicity results in Chapter 2, the findings in Chapter 3 revealed that the particles do not impact the survival of *Daphnia magna*. However, the chronic test found that both the 20 nm and 200 nm plastics inhibited growth in the exposed organisms (**Figure 4.1, Table 4.1**). Also, exposure to the 20 nm particles delayed reproduction, while exposure to the 200 nm particles resulted in delay in molting and reduced number of molts during the 21 day period (**Figure 4.2, Figure S4.3**).

In Chapter 6, the acute and chronic toxicity of the different face mask fractions were investigated. The particles alone did not have any significant impacts on the survival and swimming behaviour of the neonates after acute exposure. However, the same particles inhibited growth and

reproduction, as well as increased swimming speed, increased heart rate, and discoloration upon chronic exposure to contaminants.

While survival is an important parameter in toxicity assessments and is the most common endpoint for acute toxicity tests, the findings from this thesis consistently show that the microplastics and nanoplastics may not have significant impact on the survival of the exposed organisms due to acute toxicity as shown in Chapters 2 and 6, but they may impact other toxicity endpoints such as swimming behaviour (**Figure 3.2, 6.5**) and heartrate (**Figure 6.6**). The findings from Chapters 3 and 6 also buttress the importance of assessing long-term impacts using sub-lethal endpoints such as growth and reproduction. While it is increasingly common for research studies to find that microplastics and nanoplastics may not have negative impacts on the survival of *Daphnia magna* (in the absence of leached or added chemicals), these two chapters showed that the particles significantly impact other life-history and behavioural traits.

7.1.3 Particle concentration

Chapter 4 compared the effects of chronic exposure to low and high concentrations of 20 nm polystyrene particles at 0.1 mg/L and 50 mg/L respectively. Exposure to both concentrations affect the growth and molting behaviour of the exposed organisms. However, there was no significant difference when the toxicity effects of the two concentrations were compared. This agrees with other reports that increase in concentration does not impact the observed chronic toxicity of *Daphnia magna*.^{3,4} Also, the results of the Chapter showed that concentrations as low as 0.1 mg/L of nanoplastics cause significant toxicity to *Daphnia magna*, hence the need for more studies at lower concentrations.

7.1.4 Age of test organism

Only a few studies have investigated the role of the life stage of test organisms in nanoplastic toxicity assessments. Chapter 6 revealed that the age of the exposed *Daphnia magna* significantly influences the observed toxicity upon exposure to microplastics and nanoplastics. The experimental results from the acute toxicity tests in Chapter 6 were statistically analyzed to compare the effects of the face mask fractions to different life stages of *Daphnia magna* (**Figure 7.1**). The survival percentage after 48 h acute exposure to the particles alone fraction was 90%, 82% and 84% survival for the neonates, juveniles, and adults respectively, representing statistical significance compared to the control (1-way ANOVA, $F_{3,56} = 11.39$, $P < 0.0001$).

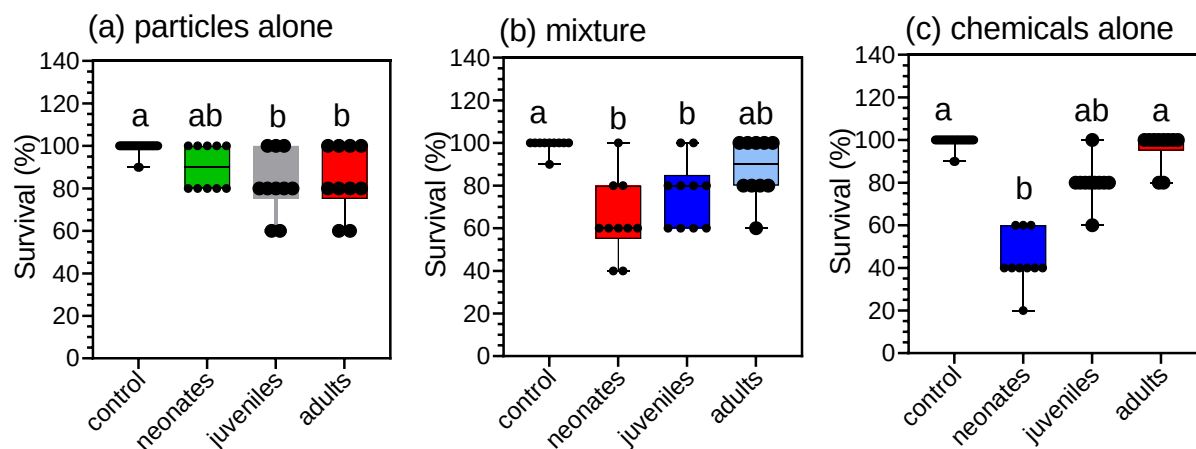


Figure 7.1. Survival of different face mask fractions upon 48 h acute exposure to different life stages of *Daphnia magna*: (a) particles alone, (b) mixture, and (c) chemicals alone. Each test comprises 5 animals per treatment and all tests were conducted in 10 replicates. The controls used in this analysis includes all the controls from the exposure to each of the three life stages. $N = 60$, 1-way ANOVA, $P < 0.05$. In each figure, box plots with different letters (a and b) above them

have a significant difference at $P < 0.05$, while box plots with the same letter above them have no significant difference.

The results showed that upon exposure to the particles alone, the survival in the juveniles and adults were statistically lower compared to the control (Tukey HSD, $P < 0.0001$ and $P = 0.0004$, respectively). One possible explanation for this result is the minimal uptake of the particles by the neonates after acute exposure compared to the significant uptake of the particles in the gastrointestinal tract of the juveniles and adults after the tests. Moreover, the LC-MS analysis found the presence of MIBP in the particles. Hence, it is possible that the quantity of particles, and the resulting concentration of MIBP ingested may be linked to the observed differences in the toxicity of the particles for the different life stages of *Daphnia magna*.

In contrast, acute exposure to the mixture of particles and chemicals found more toxicity in the neonates and juveniles than the adults. The results showed 64%, 76% and 88% survival for the neonates, juveniles, and adults respectively, representing statistical significance compared to the control (1-way ANOVA, $F_{3,56} = 28.53$, $P < 0.0001$). Further statistical analysis showed that the survival in the neonates and juveniles were significantly lower compared to the control (Tukey HSD, $P < 0.0001$ and $P = 0.0043$, respectively). This result aligns with previous studies reporting that younger organisms may be more susceptible to leached chemicals compared to their older counterparts.^{5,6}

Overall, this chapter presents that there is no single factor that is responsible for the observed toxicity of microplastics and nanoplastics to *Daphnia magna*. Hence, there is a need for comprehensive consideration of these factors when assessing the toxicity of microplastics and nanoplastics.

7.2 Future directions

Even though numerous studies have been conducted to investigate the toxicity of microplastics and nanoplastics to aquatic invertebrates, there is a need for more consideration regarding the type of plastics used in the studies, and how these plastics help our understanding of the toxicity of the plastics in the real environment. Key points for future research directions are summarized below.

- Although there are multiple benefits of the use of commercial formulations of microplastics and nanoplastics for toxicity studies, there is also a need to pay attention to the presence of preservatives or surfactants which may confound the observed toxicity effects. Commercial plastics should be thoroughly washed to ensure the removal of the additives before their usage for toxicity tests.
- The role of dose metrics in the observed toxicity needs to be further explored. To date, little is known about the role of dose metrics in the risk assessment of microplastics and nanoplastics. Different studies employ different dose metrics and there is no consensus of the appropriate dose metrics for the studies. Previous studies on other types of nanoparticles critically examined this topic before determining the appropriate dose metric for certain nanoparticles. This evaluation is still missing in the case of microplastics and nanoplastics.
- The use of irregular-shaped particles for toxicity tests needs further attention. To date, most studies use pristine spherical particles for toxicity tests, while a large proportion of plastic particles in the natural environment are generated through breakdown from larger debris, hence, they are hardly spherical. It has been reported that the shape of the particles plays a significant role in the toxicity effects observed, hence, more studies should use particles whose shapes represent those that exist in the aquatic environment. Also, the composition

of microplastics and nanoplastics in the environment is less simple as in the pristine spherical particles obtained commercially - they comprise chemicals and additives which are subsequently released into the natural environment. Hence, more realistic studies to account for the impacts of these leached chemicals and additives are crucial. Similarly, it is widely known that plastics function as vectors for organic compounds. There is a need for more studies to investigate how this impacts the observed toxicity of the plastic particles.

- While most toxicity studies focus on acute and chronic tests, which last for up to ~96 h and ~21 days respectively, it is unarguable that plastics last in the natural environment for a longer period. Thus, more multi-generational studies will help in further understanding the impacts of exposure to these particles over an extended period, typically spanning multiple generations of offspring from the originally exposed organisms.
- Ultimately, these plastics can remain in the environment for thousands of years, continuously breaking down and releasing microplastics and nanoplastics before completely degrading. There is an urgent need for more relevant studies and improved knowledge transfer to regulatory agencies and other stakeholders such as plastics manufacturers/users towards the implementation of adequate waste management practices, hence, minimizing the mass of plastic wastes that end up in the natural waters to produce these microplastics and nanoplastics.

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Chapter 8: Conclusions

The overall goal of this thesis is to better understand the toxicity impacts of microplastics and nanoplastics on aquatic invertebrates. This thesis assessed the implications of using commercial formulations of spherical microplastics and nanoplastics for toxicity assessments on *Daphnia magna*. Further, the thesis aimed to identify and address other existing gaps to advance our knowledge on the toxicity impacts of microplastics and nanoplastics to *Daphnia magna*.

The review of existing literature revealed that most microplastic and nanoplastic studies use particles obtained from commercial formulations. The use of commercial formulations of microplastics and nanoplastics for toxicity assessments is an effective approach to understand the impacts of specific properties of the particles to aquatic organisms, e.g., the impacts of specific polymers, specific sizes of particles. It also offers additional benefits through the availability of customizable properties such as surface functional groups, fluorescence, etc. However, one of the limitations of these particles is the presence of preservatives or surfactants in the solution which may introduce additional toxicity, leading to over-estimation of the observed toxicity from the particles. This study is the first report to investigate the role of a common preservative, sodium azide, in the toxicity assessment of commercial polystyrene nanoplastics. Following the removal of the sodium azide from 20 nm and 200 nm polystyrene suspensions through dialysis, no mortality was observed upon acute exposure of the dialysed particles to *Daphnia magna* neonates. In contrast, significant mortality was observed when the organisms were exposed to non-dialysed particles. This result further demonstrates that the dialysed particles significantly affected the mobility of the exposed *Daphnia magna*, hence calling for more studies on the longer term and sub-lethal impacts of the particles in the absence of sodium azide (or other additives).

Chronic toxicity tests were conducted on *Daphnia magna* using dialyzed 20 nm and 200 nm polystyrene nanoplastics. The results showed that exposure to the dialyzed particles did not have significant impact on survival, but it indeed affected growth, reproduction and molting behavior. The 20 nm particles were exposed at high and low concentrations, i.e., 50 mg/L and 0.1 mg/L, respectively. The observed effects at the 50 mg/L were comparable to those at 0.1 mg/L, which suggests that even low concentrations of polystyrene nanoplastics may have significant impacts on the exposed organisms. The study also revealed that ingestion of higher loads of the particles by the organisms may introduce additional sub-lethal stress and other long-term impacts to *Daphnia magna*. Furthermore, the effects of mass concentration and particle count as dose metrics were compared. However, the observed effects were similar between the two dose metrics.

Following the assessment of both short-term and long-term exposure to nanoplastics obtained from commercial formulations, this thesis further conducted a two-part study to examine the fate and impacts of microplastics and nanoplastics from a selected commonly used plastic product, namely, single-use face masks. This selection was inspired by the several reports showing the indiscriminate disposal of single-use face masks during the COVID-19 pandemic. The results of the study showed that plastic microfibrils can be released from the face masks under low mechanical stress such as floating on moving water, and the introduction of UV irradiation leads to a significant increase in the number of microfibrils released by the face masks. Jar tests (also known as coagulation and flocculation tests) were conducted on the released microfibrils. It was shown that a significant number of the released plastics may escape wastewater treatment processes, ending up in the natural waters and contributing to the mass of plastics currently in the waters. Additionally, the study found that microfibrils released from face masks which have been previously exposed to the natural environment show higher removal efficiency in the jar tests due

to the presence of additional matter coated on the fibres. Hence, it may be hypothesized that the use of pristine particles for such tests may not capture the complexity of the particle properties after their exposure to the natural environment.

Finally, for the second part of the face mask study, a simulated weathering was used to generate microplastics and nanoplastics from the face masks for toxicity tests. Inadvertently, leached chemicals were also released from the masks and their impacts were also examined. Detailed characterization of the particles identified them as polypropylene, with sizes mostly below 1 μm . Chemical analysis of the released chemicals using LC-MS and ICP-MS reveals the presence of various heavy metals such as Zn, Pb, etc. as well as organic compounds, such as phthalates. Acute exposures found that the younger organisms may be more sensitive to the released chemicals than their older counterparts. In the chronic tests, particles alone had limited impact on the survival, but caused significant impact on reproduction, growth as well as behavioral stress such as heart rate and swimming speed.

Although numerous studies exist investigating the impact of microplastics and nanoplastics on *Daphnia magna*, the findings of this thesis showed that most of the studies use commercial formulations whose results may be confounded by the presence of preservatives. Understanding the toxicity from commonly used plastic products may be more complex than the simplified results obtained from spherical pristine particles. More research studies are needed to better understand the toxicity from microplastics and nanoplastics from plastic consumer goods.

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