Improving the Realism and the Relevance of Microplastic Effect Tests

Vera Nyangoma de Ruijter

Propositions

1. Microplastic effect studies require an environmentally diverse mixture of microplastics.
   (this thesis)
2. Microplastic effect studies require that particle effects are disentangled form chemical effects.
   (this thesis)
3. To ask the right scientific questions, practicality ought not be considered.
4. Sometimes, plastic is just quite fantastic.
5. Rather than finding eco-friendly alternatives, society needs to focus on preventing littering and mismanaged waste.
6. Environmental protection is a luxury that only developed countries are able to afford.

Propositions belonging to the thesis, entitled

Improving the realism and the relevance of microplastic effect tests

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Thesis

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Summary

Microplastics, which are ubiquitous in the environment, are prone to ingestion and known to exert adverse effects on broad range of organisms. In this thesis, I studied the effects of microplastics on aquatic species, more specifically on freshwater and marine benthic macroinvertebrates. These organisms are an important part of the ecosystem and are likely to come into contact with relatively high concentrations of microplastics, as sediment functions as a sink for the particles. To inform environmental risk assessment, the following research questions were defined.

- What is known about the effects of microplastics on aquatic organisms?
- How can we appropriately test microplastics on aquatic organisms?
- What is the quality of studies testing microplastics on aquatic organisms?
- What are the effects of microplastics on freshwater and marine benthic macroinvertebrates?
- How are organisms exposed to microplastics?
- What part of the MP continuum is bioavailable to organisms?
- What are key factors influencing the adverse effect of microplastics?

Chapter 1 starts with a general introduction to the topic of microplastics effects. We discuss the shortcomings of the current methods for environmental risk assessment and stipulate what improvements are needed. This chapter outlines our aims in more detail, placing them in a broader context.

The scientific field of microplastics research is a relatively young one, since the last decade the amount of studies being published have exploded. There is now a general consensus that microplastics studies need improvements and standardisation. Hence, in **Chapter 2** we review 105 microplastic effect studies on aquatic biota and provide a systematic overview of the study characteristics to answer the following questions; What type of microplastics do researchers use? Which organisms are tested? For how long? Do they find a negative effects? How are these effects explained? Moreover, we developed a new tool that consists of 20 quality criteria. We gather scientific consensus on what is the appropriate way to 1) characterize microplastics, 2) design an experiment, 3) design an experiment so that it is applicable to risk assessment and 4) increase the environmental relevance of microplastics experiments. We then retrospectively use this tool to assess the quality of microplastics effects studies and we find that no study has

managed to incorporated these 20 quality criteria. Additionally, we find that the weight of evidence for the effect mechanisms is highest for inhibition of food assimilation and/or decreased nutritional value of food, or more commonly phrased as “food dilution”.

In **Chapter 3** we continue with these 20 quality criteria (**Chapter 2**) and apply them to our own laboratory studies. Here we test 16 different freshwater and marine benthic macroinvertebrates and expose them to our home made Environmentally Relevant Microplastics (ERMP) for 28 days. We select organisms in such a way that a wide range of feeding traits is covered. Our results show adverse effects, more specifically reduced growth of the freshwater amphipod *Gammarus pulex*, and reduced growth and reproduction of the freshwater worm *L. variegatus*. Interestingly, we also find positive effects, namely for the marine and freshwater clams *Cerastoderma edule* and *Sphaerium corneum*. However for most species we do not find an effect. Exposure assessment of organism is one of the 20 quality criteria, which is addressed in **Chapter 4**.

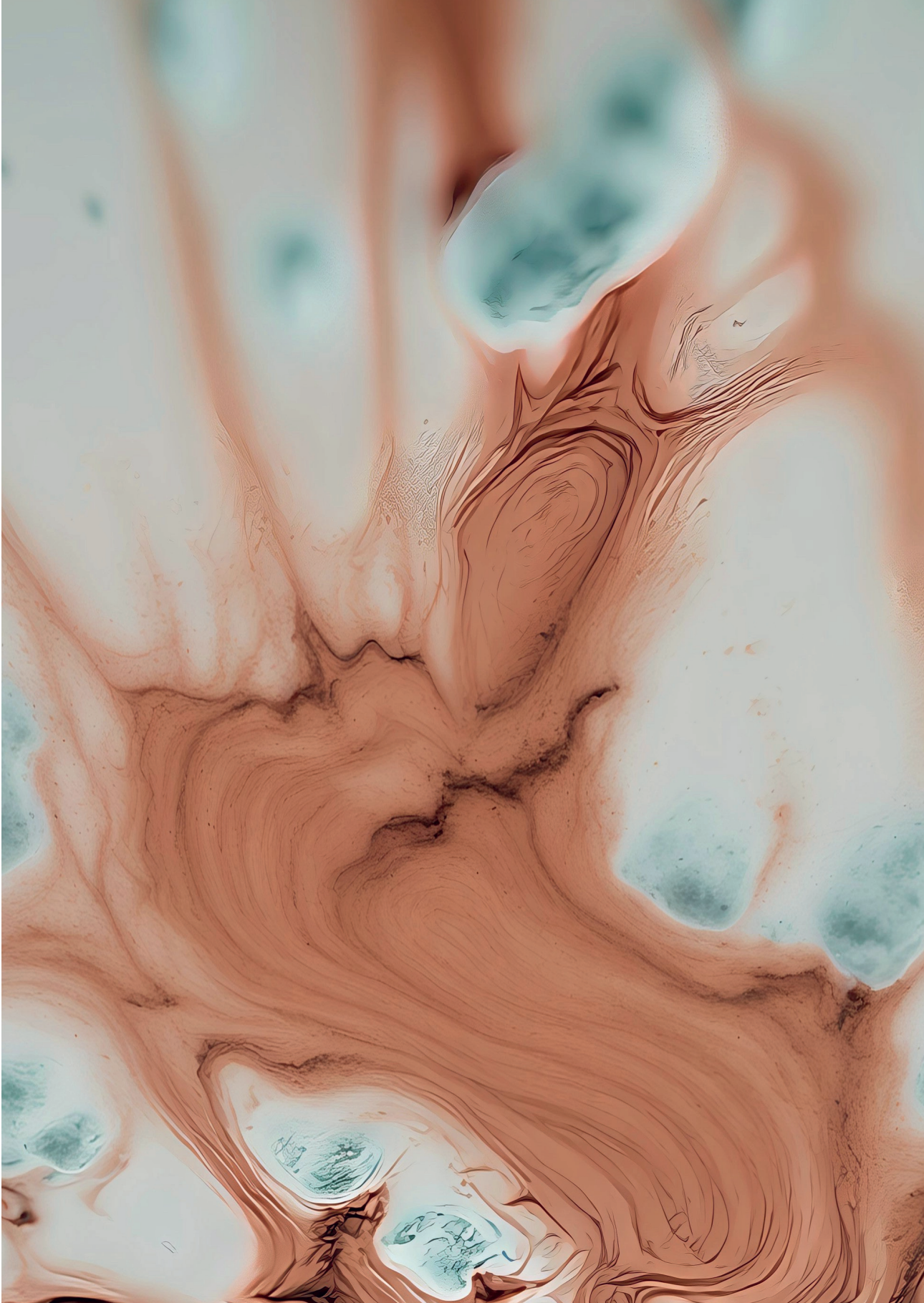
In **Chapter 4**, we analyse the uptake of ERMP in 11 different freshwater and marine benthic macroinvertebrates previously exposed to ERMP (**Chapter 3**). Measuring the uptake of microplastics by organisms enables us to causally link exposure to previous adverse effects detected. Moreover, by knowing the actual biological availability of microplastics for aquatic organisms, we can better inform risk assessment. Additionally, this can give us insight to the intricacies of species-specific responses to microplastics. In this study we detect on average approximately 8 particles/organism in the tissue samples of *L. variegatus*, indicating that indeed microplastics were ingested causing reduced growth and reproduction. Moreover, we find that overall the smaller range of ERMP is ingested by most organisms ranging from 5.5 to 27.7 µm and that organisms have preferences for certain polymer types. Additionally, we find that overall ERMP detected in gut water samples far exceeds that of tissue samples, indicating organisms are able to egest most ingested microplastics quite quickly. Finally we find similar size distributions in the tissue samples of the two epibenthic species *A. aquaticus* and H. azteca, which are both sediment grazers/scavengers. Additionally, similarities were detected among the freshwater and marine clams *C. fluminalis* and *L. balthica*, which are both facultative deposit feeders.

In **Chapter 5**, we elucidate on some of the key factors determining the adverse effects. Why do we see adverse effects? For this we continue experiments with the

sensitive species *L. variegatus* (**Chapter 3**). We hypothesize, that whatever the effect mechanism is, physical effects of particles should be equal for particles of the same shape and size, yet made from different materials. Hence, we design a polydisperse Environmentally Relevant microplastic mixture (ERMP) and a similarly diverse Mineral Microsolids (ERMS) mixture. Consequently, we test these two mixtures for their 28-day chronic effects on the reproduction and, growth of *L. variegatus* at two different organic matter (OM) levels (average and enriched). For both mixtures, no effects were found, which is consistent with the assumption regarding the comparability in potential harmlessness or harmfulness of the particles. Moreover, we observed no differences in growth or reproduction between ERMP and ERMS at particle concentrations of up to 10% (v/v). In contrast, an organisms exposed to sediment enriched with OM content showed a 30% increase in growth and a 20% increase in reproduction.

Finally, in **Chapter 6**, I synthesize the knowledge accumulated in MP effect testing over the years. This includes comparisons of my test results with those of other researchers, comparing the test guidelines that I developed with guidelines available from others, and providing a comprehensive overview of the knowledge on risk assessment at both the single species and community levels. Furthermore, I review the preceding chapters, evaluating their contributions to the scientific field and their relevance to our overall objectives. More specifically, I discuss standard test materials, characteristics of MP, and mechanisms explaining their effects. Finally, I offer recommendations for future research directions.

Contents



Chapter 1

Introduction

1.1 Effects of microplastic on aquatic organisms

Innovative solutions are being developed by organisations such as Ocean Clean up, Waste Free Ocean and Cleans Sea Solutions to remove plastic debris from the seas. Additionally, other organisations are focusing on preventing mismanaged plastic waste from reaching the seas by engineering contraptions such as The Great bubble barrier and Bandalong Litter Trap. Moreover, there are specific vacuums cleaners designed such as the Nurdle and the Hoola one to clean beaches from plastic debris. Despite these efforts, the amount of plastic waste in the environment has doubled since 2000, indicating an urgent need for improving in waste management practices, especially in emerging economic countries (OECD, 2022).

Through the abrasion by water, sand and UV light (Andrady, 2017; Onink et al., 2022), plastic debris will eventually break down into a heterogeneous mixture of particle types, shapes, and sizes, referred to as microplastics, typically defined as particles 1 µm to 5 mm in size (Arthur et al., 2009; Frias and Nash, 2019; Hartmann et al., 2019; Rochman et al., 2019; Thompson et al., 2009; Verschoor, 2015). As a consequence, microplastics have been detected worldwide, from remote marine to coastal zone and estuarine areas, as well as in freshwater lakes and rivers (Besseling et al., 2019; Burns and Boxall, 2018; Triebskorn et al., 2019; Van Cauwenberghe et al., 2015). Their ubiquity in aquatic systems coupled with their small size have resulted in concerns regarding their effects on aquatic biota for which ingestion has been observed in a wide variety of organisms (De Sá et al., 2018; Gouin, 2020; Xu et al., 2020). There are many studies that show that microplastic could potentially lead to negative impacts on aquatic ecosystems, albeit it at relatively high concentrations (Lenz et al., 2016; SAPEA, 2019).

External or internal exposure to microplastics has been suggested to cause adverse effects on a wide range of species, endpoints and levels of biological organization (Anbumani and Kakkar, 2018; Bucci et al., 2020; De Sá et al., 2018; Foley et al., 2018; Guzzetti et al., 2018; Van Cauwenberghe et al., 2015). Experimental work has suggested that effects of microplastics can occur at sub-organismal level (e.g., inflammation, oxidative stress, alteration in metabolism and gut microbiome, reduced lysosomal stability in the digestive gland, alteration of the genetic expression and enzymatic activity (Choi et al., 2018; Détrée and Gallardo-Escárate, 2017; Green et al., 2016; Qiao et al., 2019; Von Moos et al., 2012), at the individual level (e.g., survival, reproduction, growth, feeding, emergence, morphology), (Cole

et al., 2013; Jabeen et al., 2018; Murphy and Quinn, 2018; Redondo-Hasselerharm et al., 2018b; Zhang et al., 2019), at the population level (e.g., abundance) (Bosker et al., 2019) or at the community level (e.g., biodiversity, species composition) (Green, 2016; Redondo-Hasselerharm et al., 2020). While there is a slight bias, as these endpoints are more often studied, most of the negative effects reported include reproduction, oxidative stress, mortality and growth (Bucci et al., 2020; Foley et al., 2018; Thornton Hampton et al., 2022c).

Although adverse effects are usually detected at relatively high concentrations of microplastics, these concentrations have already been found in some microplastics hotspot sites such as the Mediterranean, the Yellow Sea, and several highly polluted freshwater sites. In the future it is likely that more places will follow as plastic consumption continues increase (Adam et al., 2019; Coffin et al., 2022b; Everaert et al., 2020; Everaert et al., 2018; Koelmans et al., 2020). Preliminary risk assessments couple these findings and warn us about the risk of microplastics, however what do we know of the quality of these studies? A risk assessment is only as good as its data input. So far, the testing of microplastics on aquatic organisms potentially at risk, have not been performed using a standardized method or any guidelines (Burns and Boxall, 2018; Connors et al., 2017). Moreover, there are just as many studies reporting no effects as there are studies reporting effects (Bucci et al., 2020). This can in part be explained by the lack of standardization of methods used such as the difference in exposure time, concentration and selected endpoint, but also due to the heterogeneity of microplastics differing in size, shape and morphology (Bucci et al., 2020; Kögel et al., 2020; Kooi and Koelmans, 2019). Additionally, there are many more factors that could explain the differences that we observe, such as for instance the selection of test species. Which raises the question whether some species might be more sensitive than others. Elucidating the effect mechanisms underlying adverse effects and the key factors triggering them could more accurately provide an understanding of the ecological risk associated with microplastics. Nevertheless, our current understanding of this aspect remains limited.

1.2 Environmental risk assessment

At what exposure concentrations of microplastics do we see negative effects? And what concentrations of microplastics do we measure in the environment? Comparing the answers to these questions is called Environmental Risk Assessment

(ERA). When microplastic concentrations in the environment exceed the exposure concentration for negative effects, we speak of risk. One way to make this analysis is by building species sensitivity distributions (SSD). An SSD is a cumulative distribution of collected ecotoxicological data from a wide range of species that are negatively affected by a toxicant (Figure 1). From this distribution, a microplastic concentration can be defined for which the risks are considered acceptable to the environment (Figure 1). Typically, this is defined as the Hazardous Concentration for 5% of species (HC5), representing the concentration at which 5% of the species in the distribution are expected to experience adverse effects from microplastics (Posthuma et al., 2001)(Figure1).

It is important that selected test species are representative for the ecosystem of interest (Posthuma et al., 2001). In this thesis the focus will be on the benthic environment, as sediments are expected to be a long-term sink for microplastics, (Besseling et al., 2017; Cózar et al., 2014; Morét-Ferguson et al., 2010). It is likely that sensitivity of organisms can be explained by their feeding behaviour, as effects of microplastics often occur after ingestion (Scherer et al., 2017; Wesch et al., 2016). Therefore benthic marine and freshwater species used in this thesis have been selected on the following traits: filter feeders, sediment/deposit feeder, sediment grazer/scavengers, as well as facultative deposit feeders. For instance, organisms with a more selective feeding strategy such as the sediment grazer/scavenger *Gammarus pulex* could avoid ingesting microplastics, whereas the non-selective feeding strategy such as that of the sediment feeder *Lumbriculus variegatus* makes such a selection less likely. This difference might indeed result in different sensitivities of organisms to microplastics.

Finally, it is crucial is that the SSD only consists of data that are of sufficient quality, from studies that used a standardized test systems, analytical tools and methods, and that enable the application of dose−response relationships relating environmental exposure to effect threshold concentrations (Klimisch et al., 1997; Moermond et al., 2016). Microplastic-science is a relatively young field and the past decade there has been an tremendous increase in effect studies. The available data however, will have to be assessed for quality through Quality Assurance & Quality Control (QA/QC) and to achieve this, it is essential to develop a framework criteria.

1.3 How to properly test microplastics?

Many researchers have acknowledged the necessity for improvements and standardization in methodologies within the field of microplastic research (Bucci et al., 2020; Burns and Boxall, 2018; Connors et al., 2017; Cowger et al., 2020; Everaert et al., 2020; Gouin et al., 2019; Karami, 2017). Nonetheless, how to effectively assess microplastics in a manner that can inform ecological risk assessments (ERAs) remains a challenge. Despite the lack of standardization of methodologies in most microplastics effect studies, there is a consensus among researchers on several key challenges. Ideally, researchers would 1) characterize their particles thoroughly with respect to shape, size and morphology, ensuring that the data is reproducible (Burns and Boxall, 2018; Connors et al., 2017; Karami, 2017; Paul-Pont et al., 2018), 2) carefully design their experiments making sure that the effect thresholds can be derived with statistical rigor (Burns and Boxall, 2018; Connors et al., 2017; Paul-Pont et al., 2018), 3) focus on ecologically relevant endpoints such as mortality, growth, and reproduction. Although exploring other endpoints on nonstandard organisms can give valuable insights, traditional endpoints are more useful from a risk assessment point of view (Burns and Boxall, 2018; Connors et al., 2017), 4) Enhancing the ecological relevance of tests could greatly improve our understanding of the environmental risks posed by microplastics. For instance, by using weathered microplastics instead of pristine microplastics and including environmentally relevant concentrations while testing (Burns and Boxall, 2018; Connors et al., 2017).

Addressing the complexity of microplastics as a diverse contaminants, varying in size, shape, and polymer type is essential (Bucci et al., 2020; Koelmans et al., 2020; Phuong et al., 2016). The most tested polymer for instance is polystyrene (PS) in the form of a sphere. Although PS is one of the most commonly detected polymers, the shape category spheres, only represents a fraction of the microplastics detected in the environment (Phuong et al., 2016). Overall, most of the effect studies so far have tested a very small part of the microplastic continuum (Phuong et al., 2016). Some researchers found for instance that a fibre is more toxic than a sphere, possibly due to longer gut retention times of fibres compared to spheres (Au et al., 2015; Jabeen et al., 2018). Another study showed that while both types of microplastics were ingested by the sheepshead minnow, the irregular microplastics decreased swimming behaviour more than spherical microplastics (Choi et al., 2018). While these studies have given us valuable insights on the differences in

toxicity and allow us to speculate about the mechanisms behind adverse effects, it is less informative from a risk assessment point of view. The X-axis of the SSD now represents both spheres, fibres and fragments with the unit of microplastics/ml, while in fact they are different stressors. It is as if you are comparing bananas (fibres) and apples (spheres)(Figure 1).

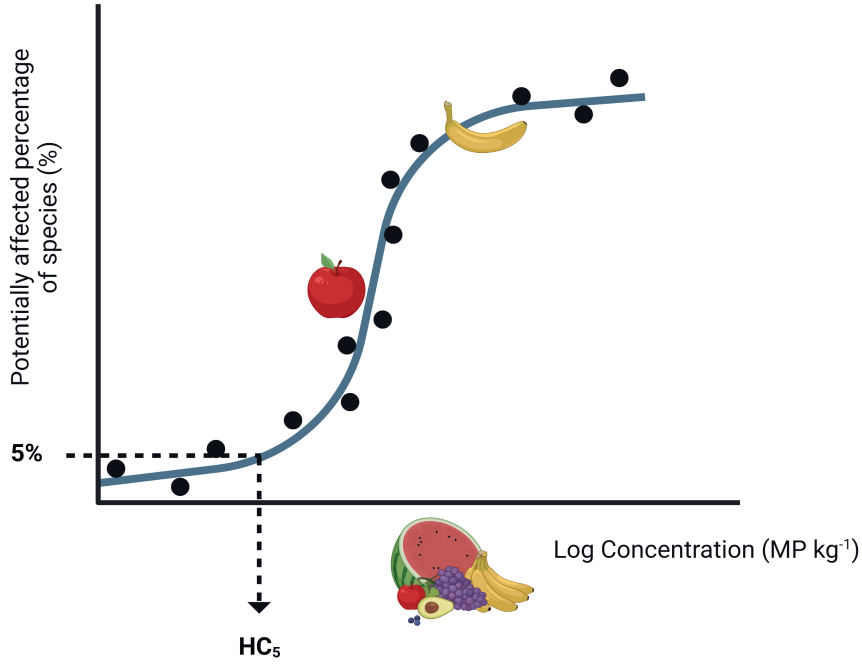


Figure 1. Species Sensitivity Distribution for microplastics in which the different data points relate to different stressors, such as a data point measured for fibers (bananas) and one for spheres (apples). This results in apples to oranges comparisons. Ideally, effect studies would test microplastics in their full diversity (the fruit basket on the x-axis).

Elegant conceptual solutions exist to convert these bananas and apples to the fruit baskets we find in the environment. So called 'alignment methods', that use mathematical probability density functions (PDFs) (Koelmans et al., 2020). These PDFs however, contain parameters that are inherently uncertain, which implies that data alignment introduces an extra uncertainty that better be avoided (Figure 1). Ideally we would test microplastics in their complexity that we find in the environment, i.e. testing whole 'fruit baskets'. While studies have been done testing different polymers types separately (Imhof and Laforsch, 2016), so far there is a limited number of studies that have investigated the impact of microplastics

with a realistic level of diversity (Phuong et al., 2016; Thornton Hampton et al., 2022a; Triebskorn et al., 2019). Finally, it is important that we assess what part of the 'fruit basket' is bioavailable in the environment for each test species. This means that the actual exposure must be measured under laboratory conditions.

1.4 Mechanisms and important factors explaining adverse effects

Although during my thesis I studied a wide variety of 16 different species of freshwater and marine benthic macroinvertebrates, it is not possible or desirable from a practical or ethical viewpoint to test every known aquatic species. Understanding why some species are more sensitive than others and unravelling the mechanism and key factors behind adverse effects, may add considerable value in assessing environmental risk. That is, we would be able to better identify the most vulnerable species, more accurately make predictions regarding risk, and mitigations strategies could be adapted accordingly.

Currently, comparing the importance of different factors on the effects of microplastic particle proves challenging due to significant variations in experimental setups. Nevertheless, when reviewing effect studies most crucial determinants of microplastics toxicity include concentration, particle size, and exposure duration. Additional noteworthy co-factors such as species and food availability have also been underlined (Kögel et al., 2020). These factors should be considered in a new series of exposure experiments, ideally using a systematic approach and testing a wide range of species.

To date, numerous studies have hypothesized that elevated microplastic concentrations can impede gut function, leading to the inhibition of food assimilation, and/or a decreased nutritional value, ultimately resulting in reduced growth (Lee et al., 2013; Straub et al., 2017; Ziajahromi et al., 2018). Some studies attribute the effects to specific properties of the polymer composition, such as the presence of functional surface groups (Au et al., 2015; Blarer and Burkhardt-Holm, 2016), while other studies link microplastics effects to the leaching of chemical additives, plasticizers or other hydrophobic organic pollutants (Gardon et al., 2018; Green, 2016; Imhof et al., 2017; Leung and Chan, 2018; Sussarellu et al., 2016). The overall understanding of the mechanisms underlying these effects is currently limited and predominantly speculative. Therefore, more carefully designed experiments are needed to enhance our understanding of microplastic-induced effects.

Furthermore, elucidating on effect mechanisms will provide essential information on what the most important ecologically relevant metrics (ERM) for microplastics are and enable us to better quantify exposure and effects more accurately (Koelmans et al., 2017; Koelmans et al., 2022b; Koelmans et al., 2020). For instance, if the mechanisms explaining toxicity is food dilution, it would make sense to measure volume (Koelmans et al., 2020; Thornton Hampton et al., 2022b). However if oxidative stress is a more likely effect mechanism, surface area of microplastics would be a more appropriate metric (Thornton Hampton et al., 2022b).

As natural particles are more abundant than microplastics in the environment, and organisms have evolved various species-specific traits to interact with particles, these should also be included in microplastic effect studies. Without taking natural particles into account, the observed adverse effects represent a system-dependent artefact that does not lend itself to risk assessment purposes (Gerdes et al., 2019; Koelmans et al., 2016; Ogonowski et al., 2018; Triebskorn et al., 2019; Yap et al., 2020). Moreover, including natural particles in carefully designed experiments can provide insight into the specific causes of microplastic effects and places the magnitude of the effects in a broader context. Until recently, studies have generally indicated that MPs have a slightly more negative impact than natural particles (Gerdes et al., 2019; Ogonowski et al., 2018; Ogonowski et al., 2016; Schür et al., 2020; Yap et al., 2020). However, these studies mainly tested monodisperse particles and a polydisperse mixture therefore remains to be tested.

1.5 Thesis outline, aim and overview

The four main objectives of this thesis are as follows (figure 2):

1) Develop QA/QC criteria for conducting microplastic effect studies, including a protocol for preparing environmentally realistic microplastic particles (ERMP);

2) Apply these QA/QC criteria in testing 16 different freshwater and marine benthic macroinvertebrates species;

3) Measure the bioavailable microplastics in these 16 different freshwater and marine benthic macroinvertebrates species;

4) Explore the factors and mechanisms underlying the adverse effects of microplastics, with a focus on food abundance and material type.

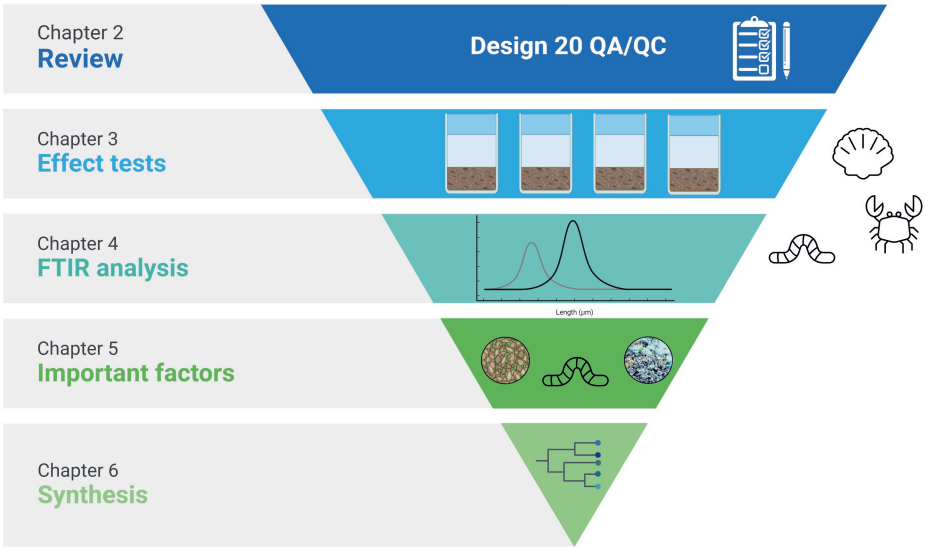


Figure 2. Schematic overview of the chapters of this thesis

Many researchers acknowledge that research methods for microplastic testing need to improve in order to better inform risk assessment and regulators. In **Chapter 2**, we develop 20 QA/QC criteria that we believe should be incorporated in microplastic effect studies in the context of risk assessment (Figure 2). Consequently, we reviewed 105 microplastic effect studies with aquatic biota to assess what the quality of studies are regarding particle characterization,

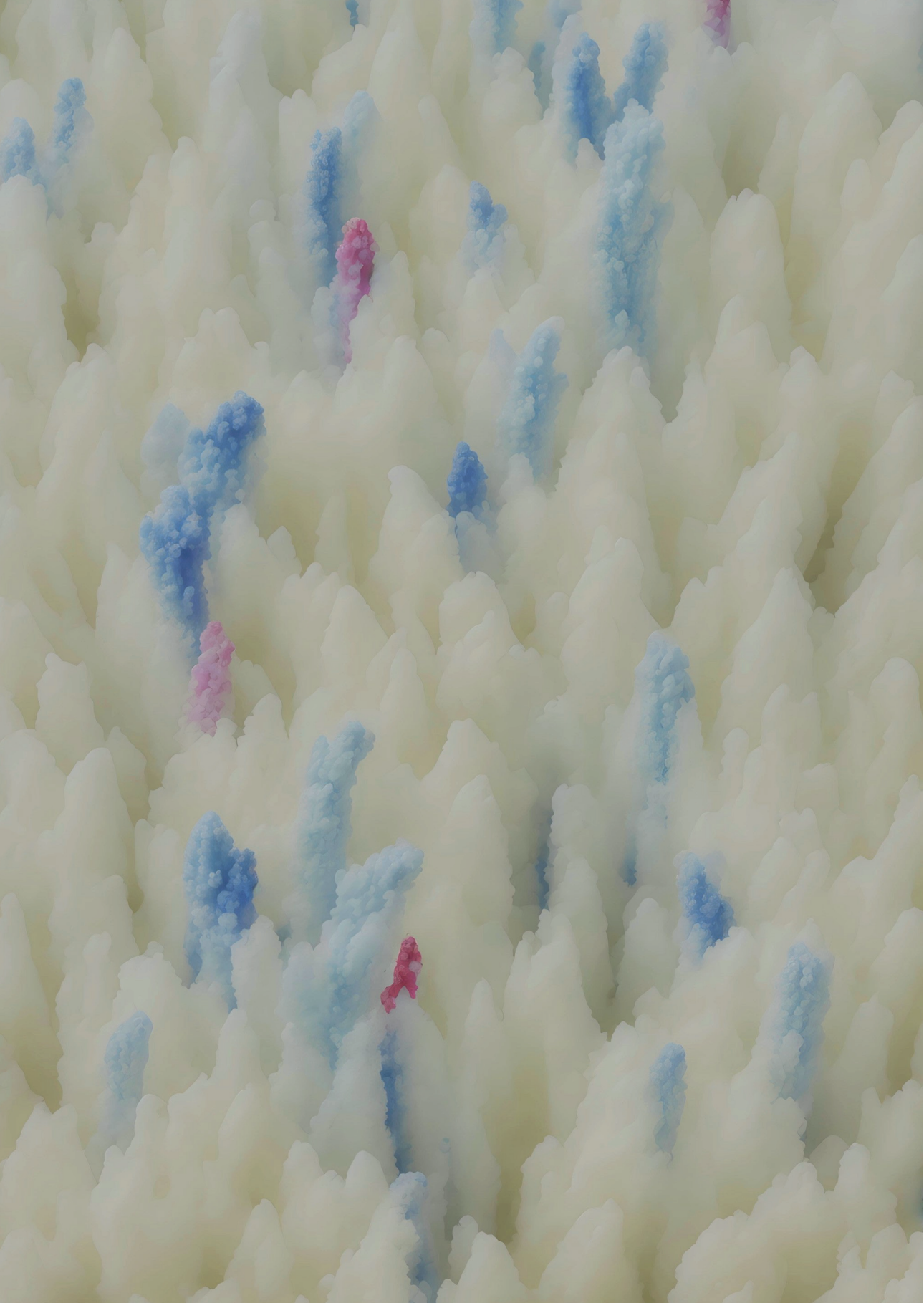
experimental design, applicability for risk assessment and ecological relevance. Additionally, we provide a systematic overview of studies characteristics and assess the literature for the weight of evidence for the mechanisms explaining adverse effects.

In **Chapter 3** we apply the 20 QA/QC criteria developed in **Chapter 2** and test 16 different freshwater and marine macroinvertebrate benthic species accordingly. The selected freshwater species include: *Gammarus pulex*, *Hyalella azteca*, *Asellus aquaticus*, *Sphaerium corneum*, *Corbicula fluminalis*, *Potamopyrgus antipodarum*, *Tubifex* spp., *Lumbriculus variegatus*, and *Chironomus riparius*. All test species are connected to the benthic environment, but differ in their feeding and living behaviour. The selected marine species include: *Alitta virens*, *Limecola balthica*, *Corophium volutator*, *Arenicola marina*, *Cerastoderma edule*, *Porcellana platycheles*, and *Mytilus edulis*. Organisms were exposed to an environmentally relevant mixture of microplastics (ERMP) for 28 days whereafter their growth, mortality or reproduction was recorded. Key features of this study are that ERMP was characterized extensively, contamination was minimized, exposure concentrations were homogeneous and verified, natural particles were included, biofouling of microplastics was allowed to increase environmental relevance, environmentally relevant concentrations were used, and six replicated doses were used to enable dose−response modelling to detect and report effect thresholds. Effect data were analysed using dose−response models, and generalized linear mix models were applied to explore differences in effects between marine versus freshwater species and between feeding traits.

In **Chapter 4** we verify the exposure of 11 different freshwater and marine benthic macroinvertebrates, previously tested in **Chapter 3**. We analyse the uptake and egestion of microplastics and define the bioavailable part of ERMP. Additionally, we study species-specific responses to microplastics, describing ingested particles as a continuum and quantifying them through continuous size distributions. Furthermore, we implemented a novel approach for blank correction in microplastics detection. This approach combines visual inspection, with the Wasserstein statistic, which distinguishes between cumulative size distributions. Subsequently, we applied traditional blank correction techniques, subtracting counts per size category and polymer type.

Whether microplastics cause different effects than inert natural particles, and how to create relevant test materials, are key questions in microplastics research. In **Chapter 5** we prepared Environmentally Relevant Microplastic (ERMP) and Mineral Microparticle (ERMS) mixtures with similar levels of environmentally realistic polydispersity and tested their 28-day chronic effects on the reproduction and, growth of *Lumbriculus variegatus* at two organic matter (OM) contents (average and enriched). Additionally, *L*. *variegatus* was exposed to ERMP and ERMS to study the particle egestion for 14 days at two different organic matter (OM) food levels.

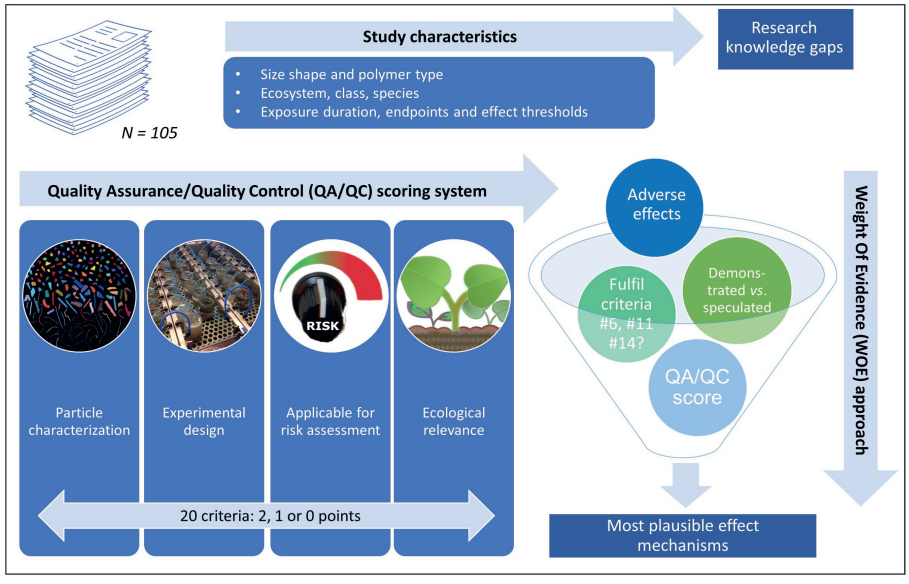
In **Chapter 6** I provide a synthesis of my work in the context of recent literature and international research developments. I provide a comprehensive overview of the knowledge on risk assessment at both the single species and community levels. Furthermore, I offer recommendations for future research directions. Finally, a step-by-step protocol on how to create polydisperse test materials is included.



Chapter 2

Quality criteria for microplastic effect studies in the context of risk assessment: A critical review

Vera N. de Ruijter, Paula E. Redondo-Hasselerharm, Todd Gouin, Albert A. Koelmans



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Abstract

In the literature, there is widespread consensus that methods in plastic research need improvement. Current limitations in quality assurance and harmonization prevent progress in our understanding of what the true effects of microplastic in the environment are. Following the recent development of quality assessment methods for studies reporting concentrations in biota and water samples, we propose a method to assess the quality of microplastic effect studies. We reviewed 105 microplastic effect studies with aquatic biota, provided a systematic overview of their characteristics, developed 20 quality criteria in four main criteria categories (particle characterization, experimental design, applicability in risk assessment, and ecological relevance), propose a protocol for future effect studies with particles, and, finally, used all the information to define the weight of evidence with respect to demonstrated effect mechanisms. On average, studies scored 44.6% (range 20-77.5%) of the maximum score. No study scored positively on all criteria, reconfirming the urgent need for better quality assurance. Most urgent recommendations for improvement relate to avoiding and verifying background contamination, and to improving the environmental relevance of exposure conditions. The majority of the studies (86.7%) evaluated on particle characteristics properly, nonetheless it should be underlined that by failing to provide characteristics of the particles, an entire experiment can become irreproducible. Studies addressed environmentally realistic polymer types fairly well; however, there was a mismatch between sizes tested and those targeted when analysing microplastic in environmental samples. In far too many instances, studies suggest and speculate mechanisms that are poorly supported by the design and reporting of data in the study. This represents a problem for decision-makers and needs to be minimized in future research. In their papers, authors frame 10 effects mechanisms as ‘suggested’, whereas 7 of them are framed as ‘demonstrated’. When accounting for the quality of the studies according to our assessment, three of these mechanisms remained. These are *inhibition of food assimilation and/or decreased nutritional value of food*, *internal physical damage* and *external physical damage*. We recommend that risk assessment addresses these mechanisms with higher priority.

1. Introduction

In the last decade, the body of literature addressing the occurrence and impacts of plastic debris has substantially increased (SAPEA, 2019). Particular attention has been given to microplastic particles (MP), generally defined as plastic particles 1 µm - 5 mm (Arthur et al., 2009; ECHA, 2019; Hartmann et al., 2019; Rochman et al., 2019; Thompson et al., 2009; Verschoor, 2015) which have been detected at a wide range of concentrations in various aquatic systems, from remote marine to coastal zone and estuarine areas, as well as in freshwater lakes and rivers (Besseling et al., 2019; Burns and Boxall, 2018; Triebskorn et al., 2019; Van Cauwenberghe et al., 2015). Their ubiquity in aquatic systems and their small size has resulted in concerns regarding their effects on aquatic biota for which ingestion has been observed at all levels of biological organization (De Sá et al., 2018; Gouin, 2020; Xu et al., 2020).

Characterizing and quantifying the environmental fate and transport of MP requires insight into the influence of various environmental processes and pathways (Besseling et al., 2017; Besseling et al., 2019; Kooi et al., 2017). The release of MP into the environment can occur either directly, such as via primary emissions from products during their manufacture and consumer-use life cycle, or alternatively, can be generated from the degradation and fragmentation of mismanaged plastic waste, commonly referred to as secondary MP, which results in a heterogeneous mixture of particle types, shapes, and sizes released to the environment (Kooi and Koelmans, 2019). It is generally agreed that secondary sources represent the dominant source of MP (Jambeck et al., 2015). Primary sources are estimated to contribute between 15 and 31% of all plastic in the environment (Boucher and Friot, 2017).

To assess the ecological risk associated with exposure to MP, there is a need to develop robust toxicological dose-response relationships, which can effectively relate environmentally relevant exposures with effects (O'Connor et al., 2020). Because of the heterogeneous presence of MP in the environment of varying concentrations of shapes, sizes, and polymer composition, there is a need to better understand effect mechanisms and the key factors triggering them. For instance, effects observed following exposure to MP on an organism can either be initiated due to sorption of the particles on the external surface of the organism or due to other mechanisms of action being triggered following their ingestion (De Sá et al., 2018.) Effects following exposure to MP, both external and internal, have been

assessed in laboratory studies for a wide range of species (Cole et al., 2013; Mateos-Cardenas et al., 2019; Murphy and Quinn, 2018; Redondo-Hasselerharm et al., 2018b). The ingestion and/or adsorption of MPs has been suggested to cause adverse effects on toxicological endpoints at various levels of biological organization, generally observed in laboratory test systems at relatively high exposure concentrations (Cole et al., 2013; Jabeen et al., 2018; Murphy and Quinn, 2018; Redondo-Hasselerharm et al., 2018b; Zhang et al., 2017). Furthermore, experimental work has suggested that effects of MPs can occur at the community level (e.g. biodiversity, species composition), (Green, 2016; Redondo-Hasselerharm et al., 2020) population level (e.g., abundance), (Bosker et al., 2019) individual level (e.g. survival, reproduction, growth, feeding, emergence, embryonic development, mobility, and physiology), (Cole et al., 2013; Redondo-Hasselerharm et al., 2018b; Silva et al., 2019; Zhang et al., 2017) or sub-organismal level (e.g., inflammation, reduced lysosomal stability in the digestive gland, reduced antioxidant capacity, DNA damage, neurotoxicity, oxidative damage, gut dysbiosis and alteration of the genetic expression, the ionic exchange and enzymatic activity)(Burns and Boxall, 2018; Jabeen et al., 2018; Jin et al., 2019; Ogonowski et al., 2018; Prokić et al., 2019; Ribeiro et al., 2017; SAPEA, 2019; Sussarellu et al., 2016). Several studies have speculated that elevated MP concentrations can cause physical damage (i.e., blockage of food passage), leading to a feeling of satiation and a reduced feeding(Lee et al., 2013; Straub et al., 2017; Ziajahromi et al., 2018). Some studies have attributed the effects to specific properties of the polymer composition, such as the availability of functional surface groups (Au et al., 2015; Blarer and Burkhardt-Holm, 2016), while other studies have assigned effects of MP to the leaching of chemical additives and plasticizers or other hydrophobic organic pollutants (Gardon et al., 2018; Green, 2016; Imhof et al., 2017; Leung and Chan, 2018; Sussarellu et al., 2016). A limitation identified for studies testing ecotoxicological effects, however, is a lack of consistency and standardization of test methods necessary to characterize dose-response relationships for specific endpoints. Particularly problematic is the need for standard methods in relation to the dosing of particulates, such as MP, an issue that can result in ambiguous results and considerable speculation regarding the proposed mechanisms of action representative of ecologically relevant exposures (ECETOC, 2018; O'Connor et al., 2020). Consequently, the weight of the evidence supportive of a quantitative risk assessment for MP remains unclear. Recent reviews have discussed the evidence regarding the occurrence of MP effects and the underlying effect mechanisms

(Bucci et al., 2020; Foley et al., 2018; Kögel et al., 2020). However, in their evaluations of the literature, the quality of studies was not taken into account, possibly leading to biased assessments. While these reviews underline that the quality of effect studies should improve, and call for more ecologically and environmentally relevant exposure systems in order to better assess the effect of MP on the environment, we argue that the quality of studies should be assessed first, in order to be able to discard unreliable data.

A fundamental element of assessing ecological risk is the availability of a suite of standardized test systems and analytical tools and methods, which enable the application of dose-response relationships relating environmental exposure to effect threshold concentrations that are consistent and of sufficient quality (Klimisch et al., 1997; Moermond et al., 2016). This also applies to the relatively young field of MP risk assessment, where many studies have emphasized the need to improve the quality of data needed to inform risks assessment(s)(Burns and Boxall, 2018; Connors et al., 2017; Hermsen et al., 2018; Jahnke et al., 2017; Koelmans et al., 2019; Paul-Pont et al., 2018; Rummel et al., 2017; Wesch et al., 2016). Efforts to assess the quality of data emerging from studies reporting on exposure concentrations of MPs in biota and in surface and drinking water, adopting methods similar to the existing Klimisch and CRED approaches (Klimisch et al., 1997; Moermond et al., 2016), have recently been developed and applied(Hermsen et al., 2018; Koelmans et al., 2019). Whereas these systems and aspects of these systems start to be adopted and recommended in the literature (Feng et al., 2019; Markic et al., 2019; Michida et al., 2019; Ogonowski et al., 2019; Slootmaekers et al., 2019; Su et al., 2019; Su et al., 2020; WHO, 2019), currently, a similar evaluation method for assessing the quality of MP effect studies is lacking.

The aim of the present study is to critically review the literature reporting on ecotoxicological effects of MP on aquatic biota, emphasizing quality assurance aspects of studies, and assessing the weight of the evidence (WOE) the studies provide with respect to the effect mechanisms that they report. This is done by first developing a quantitative evaluation method for effect studies and methods employed to assess effects of MP on aquatic biota. The evaluation method is subsequently applied retrospectively to the reviewed studies. Average scores per evaluation criterion are used to prioritize and provide guidance with respect to the analytical and test system protocol that would benefit most from refinement.

Based on our analysis, a guidance protocol for testing ecotoxicological effects of MP for aquatic species is provided. Demonstrated and suggested effect mechanisms reported in the reviewed papers are summarized and discussed, with the results of the quality evaluation applied as a method to assess the overall weight of evidence regarding probable ecologically relevant effects of MP.

2. Methods

2.1 Literature search

Literature was retrieved from the database from the systematic review underlying the SAPEA report.[1] In addition, an extensive literature search accessing the Natural Science Collection database available at ProQuest® was performed for ecotoxicological effect studies with MP until November 2019. The following search strings were used: (effect OR impact OR endpoint OR toxicity) AND (growth OR feeding OR consumption OR survival OR mortality OR behavior OR behaviour OR stress OR response(s) OR activity OR reproduction OR inhibition) AND (microplastic(s) OR microbead OR polyethylene (PE) OR polystyrene (PS) OR polyamide (PA) OR polypropylene (PP) OR polyvinyl chloride (PVC)) AND (aquatic OR freshwater OR marine OR estuarine) NOT (chemicals OR additives). Studies were only included when at least one type of MP tested had a diameter between 1 μm and 5 mm. To enable interpretation of particle effects, studies explicitly aiming to study effects of plastic-associated chemicals, or aiming to solely study accumulation, ingestion and/or egestion of MP were excluded from the analysis.

2.2 Assessment of general study characteristics

A total of 10 characteristics were extracted from each paper and summarized (see Table S1 in the Supporting Information): Size, Shape, Polymer type, Ecosystem (fresh, marine, estuarine), Taxonomy categories (Class, Species), Exposure duration, Endpoints studied, Endpoints affected and Effect threshold when reported (as either LCx, ECx, LOEC or NOEC). When a size range was used, the upper and lower size ranges are noted, however, if an average size was provided together with the range, the average is also recorded. In instances when the average was not given, it is assumed that the particles are uniformly distributed between the upper and lower size limit and that the average can be estimated accordingly. For shapes, the terms “beads” and “spheres” are assumed to be the same and are combined in

a single category. As the definition of “irregular” is ambiguous and could include any non-regular shape, it is included as a separate category.

For the analysis of the taxonomic groups we followed De Sá et al. (2018), where classes polychaeta and clitellata are combined in the category “annelida”, classes bivalvia and gastropoda are combined in the category “mollusca”, classes anthozoa and hydrozoa are combined in the category “cnidaria”, classes branchiopoda, hexanauplia and monogononta are combined in the category “small crustacea”, class malacostraca is renamed “large crustacea” and class actinopterygii is renamed “fish”.[12] Additionally, classes gammaproteobacteria and cyanophyceae are combined in the category “bacteria”, classes bacillariophyceae, chlorophyceae, trebouxiophyceae, dinophyceae and mediophyceae are combined in the category “microalgae” and class liliopsida is renamed “macrophyte”.

2.3 Quantitative quality assessment

All of the 105 reviewed studies are evaluated based on 20 Quality assurance/Quality control (QA/QC) criteria in the following categories: particle characterization, experimental design, applicability for risk assessment, and ecological relevance. These categories are consistent with the principles of sound ecotoxicology proposed by Harris et al. (2014), which represent fundamental elements for ensuring quality and reproducibility and are thus critical when designing, applying and reporting ecotoxicological effect studies for MP. A summary of the 20 QA/QC criteria is shown in Table 1 and a detailed motivation for each criterion is provided in the Supporting Information (see methods continued). Building on the methods developed by both Hermsen et al. (2018) and Koelmans et al., (2019), each criterion is assigned a score of either 2 (adequate), 1 (adequate with restrictions) or 0 (inadequate) points (see Table S2) (Hermsen et al., 2018; Koelmans et al., 2019). All studies collated as part of this literature review are independently assessed by three of the authors, with scores subsequently tabulated and discussed to reach consensus, sometimes leading to adjustments of the original formulation of a criterion to decrease potential ambiguities. The scores per individual study are provided in the Supporting Information (see Table S3).

Table 1. Summary of specific guidance proposed towards the adoption of Standardized Protocol for testing the effects of MP in aquatic test systems for the purposes of strengthening the quality of data generated with respect to quality assurance/quality control (QA/QC) criteria. A detailed motivation for each criterion is provided as Supporting Information (see 'Methods Continued).

GUIDANCE TO INCREASE THE TECHNICAL QUALITY OF EFFECT TESTS (1 – 12)		
Particle characterization		
1.	Particle size	Size is a crucial factor explaining effects of MP and thus should be reported. If a range of sizes is used; a full (i.e. ≥ 10 bins) size distribution is measured and reported. If a single size is used, that size is measured with an indication of measurement error and reported.
2.	Particle shape	Shape is a crucial factor explaining effects of MP and thus should be measured and reported. Shapes are measured with high resolution picture and reported.
3.	Polymer type	Polymer type can be a factor explaining effects of MP and thus should be reported. Polymer identity confirmed with e.g. FTIR, Raman spectroscopy or similar methods.
4.	Source of MP	Specification on where MP stock or solution is bought and/or how it is self-made maximizes reproducibility and thus should be reported. The origin and/or production of MP in own laboratory is reported in detail.
5.	Data reporting	Unambiguous units are required to ensure reproducibility of the experiment and to make it possible to compare data across experiments. MP concentrations are reported as mass as well as number concentration.
Experimental design		
6.	Chemical purity	In order to test particle toxicity, the toxicity of other chemicals in solution or mixture should be ruled out. This includes additives present in MPs, chemicals associated with food particles and surfactants (e.g. Tween). Chemical effects other than from the polymer or solution/mixtures are ruled out. MPs are cleaned with organic solvent.
7.	Laboratory preparation	MP contamination arising from the laboratory (air, water and materials) should be minimized. - All materials used (equipment, tools, work surfaces and clothing) should be free of MP. All materials used are thoroughly washed with high quality water (e.g. Milli-Q water). - Measures are taken to prevent MP contamination from air. - Cotton lab coats were used to avoid microfiber contamination.
8.	Verification of background contamination	MP contamination of the exposure systems in the laboratory should be assessed. Level of contamination evaluated and quantified, e.g. with FTIR, Raman or similar method.
9.	Verification of exposure	Not only the nominal concentration should be mentioned. The exposure concentration should be measured. Measurement of exposure concentration and evidence that at least 80% of the nominal concentration throughout the test is maintained.
10.	Homogeneity of exposure	Verification of homogeneity is crucial for the MP characterization and the assessment of bioavailability.

		- Water as medium: Picture or measurement of MP in water that demonstrated well mixed or dispersion in solution. - Sediment as medium: Description of method used to obtain homogenous exposure.
11.	Exposure assessment	Exposure of the organism to MP should be verified by measurement. Exposure of the organism to MP is measured quantitatively with e.g. FTIR or Raman. In case MPs are ingested additionally a digestion step is included (see criteria 9 and 10 Hermsen, Mintenig, Besseling, & Koelmans, 2018)
12.	Replication	For statistical rigor in detecting effect thresholds (e.g., EC_{50} or EC_{10}), sufficient replicates should be tested. 3 or more replicates
GUIDANCE TO INCREASE THE APPLICABILITY IN ECOLOGICAL RISK ASSESSMENT (13-20)		
Applicable for Risk assessment		
13.	Endpoints	Endpoints should be considered that inform ecologically relevant population level risk assessment and clearly reported. Endpoints taken at the community (e.g. bacteria and algae) or individual level (e.g. survival, mortality, growth, development, reproduction).
14.	Presence of natural (food) particles	The exposure conditions should be environmentally relevant. Natural particles (at least food) are added to avoid force feeding of MP. Criterion not applicable to algae or bacteria and hence these studies receive 2 points.
15.	Reporting of effect thresholds	To enable PEC/PNEC types of comparisons, the effect threshold should be assessed with error of uncertainty using dose- response relationships. Effect thresholds are reported as $L(E)C_x$ with error or uncertainty intervals.
16.	Quality of dose-response relationship	For statistical rigor in detecting effect thresholds (e.g., EC_{50}, EC_{10}), sufficient doses should be tested, including a treatment control, covering the full shape of the effect curve and emphasizing the slope for parameter estimation. Multiple doses, at least 6, including a treatment control.
Ecological relevance		
17.	Concentration range tested	Concentrations should be motivated (with a reference in the appropriate unit) from measured environmental concentrations (MEC). More than 1 environmentally relevant concentration was used within the range tested.
18.	Aging and biofouling	Aging and biofouling is what occurs in the environment and could affect the uptake of MP; therefore, it is crucial to consider this for an ecological relevant experiment. MP particles should have undergone process to make them more environmentally realistic, accounting for biofouling. Additionally, pictures of altered particles are provided.
19.	Diversity of MP tested	In the environment, MPs have a wide variety of shapes and sizes. This needs to be taken into account for environmentally relevant effect assessment. A wide range of sizes (order of magnitude), shapes and densities is used, thereby approaching the diversity of environmental microplastic.
20.	Exposure time	It is crucial to use appropriate exposure times to allow for the detection of adverse effects. - Bacteria and phytoplankton: 1 week or longer - Zooplankton: 21 days or longer - Benthic invertebrates: 28 days or longer - Fish: 3 months or longer

- Macrophytes: 28 days or longer

Consistent with the approach adopted in previous method evaluation papers (Hermsen et al., 2018; Koelmans et al., 2019), we emphasize that the scores assigned for each study should not be perceived as a judgement indicative of the relative value of a study, i.e. a paper scoring low on a certain criterion could still provide valuable and reliable information regarding other potential insights. Problem formulation is therefore an important element to understand, in that depending on the purpose of an effect study the results may or may not help to inform the decision-making process with respect to assessing risk. A WOE may be assembled, for instance, regarding an effect mechanism, but the mechanism may not necessarily be ecologically relevant (see SI, criterion 13 Endpoints, p11, methods continued). The primary objective of the evaluation criteria developed and applied in this study is directed at providing insight regarding those aspects of MP ecotoxicological effect studies that could be improved in future studies in order to better inform the application of a quantitative environmental risk assessment. The evaluation criteria, however, also provide the opportunity to assess the current WOE of effect mechanisms.

2.4 Analysis of perceived versus demonstrated mechanisms explaining adverse effects

Authors' conclusions with respect to observed adverse effects and the mechanisms explaining them are summarized in the Supporting Information (see Table S3). In instances where the discussion and conclusions included ambiguous terms, such as, 'may', 'could', 'can', 'would', 'postulate', 'suggest', 'might', 'potentially', 'most likely', 'imply' the reported mechanisms are classified under the category 'suggested'. If the discussion and/or conclusion used more definitive terminology, such as, 'demonstrate', 'observe', 'indicate', 'induce', 'provide', and 'evidence', the reported mechanisms are classified under the category 'demonstrated'. When a combination of both ambiguous and definitive terminology are used in the same sentence to describe an effect mechanism, the mechanism is considered as 'suggested'. Terms that imply a mechanism to be either 'demonstrated' or 'speculated' are reported in italic, whereas key words indicating the mechanism category are reported in bold. Finally, in addition to classifying effect mechanisms

as either ‘‘suggested’ or ‘demonstrated’, specific categories based on the modes of actions proposed by authors are recorded and numbered accordingly.

3. Results and discussion

3.1. Study characteristics

3.2.1 Characteristics of the tested microplastics: size, shape, polymer type

Size - A total of 178 different MP sizes have been tested in the 105 reviewed papers. The cumulative distribution illustrates that about 75% of studies tested the effects for MP <100 μm, or ‘small MPs’ (Koelmans et al., 2019; WHO, 2019) (Figure 1), with approximately 30% of MP having sizes <10 µm. Of the 178 sizes tested, 58.4% corresponded to a size range, while 41.6% consisted of one size only. Moreover, 16.3% of the tested MP included a size range greater than one order of magnitude.

Species-specific traits, such as size selective ingestion of MP have been demonstrated for aquatic organisms (Cole et al., 2013; Lee et al., 2013; Redondo-Hasselerharm et al., 2018a; Scherer et al., 2017). Size selectivity can potentially help understanding effect mechanisms that influence the toxicological response of an organism. Mechanistic insight, however, can only be demonstrated when an appropriate range of particles sizes is used. Therefore, when evaluating the effects of MP of only one size, the most detrimental sizes for a specific species may not be included in the analysis, resulting in an underestimation of actual effects across the MP size range. Furthermore, it can be assumed that effects of MP of a certain size will differ in the presence of other sizes of MP, since there can be complex particle-particle interactions that may influence exposure as well as complex organism-particle interactions that can be difficult to account for when limiting testing to one size or narrow size range distributions. The observation that effects testing of MP to date is dominated by particles < 100 μm (Figure 1) implies that comparisons between MP sizes used in effect studies and sizes of MP found in the environment are difficult to be made, particularly since the detection of MP <100 μm represents an ongoing analytical challenge (Koelmans et al., 2019). Nevertheless, we recommend the use of MP size distributions that are appropriate for the species being tested, which can potentially add greater insight between adverse effects and organism-particle interactions.

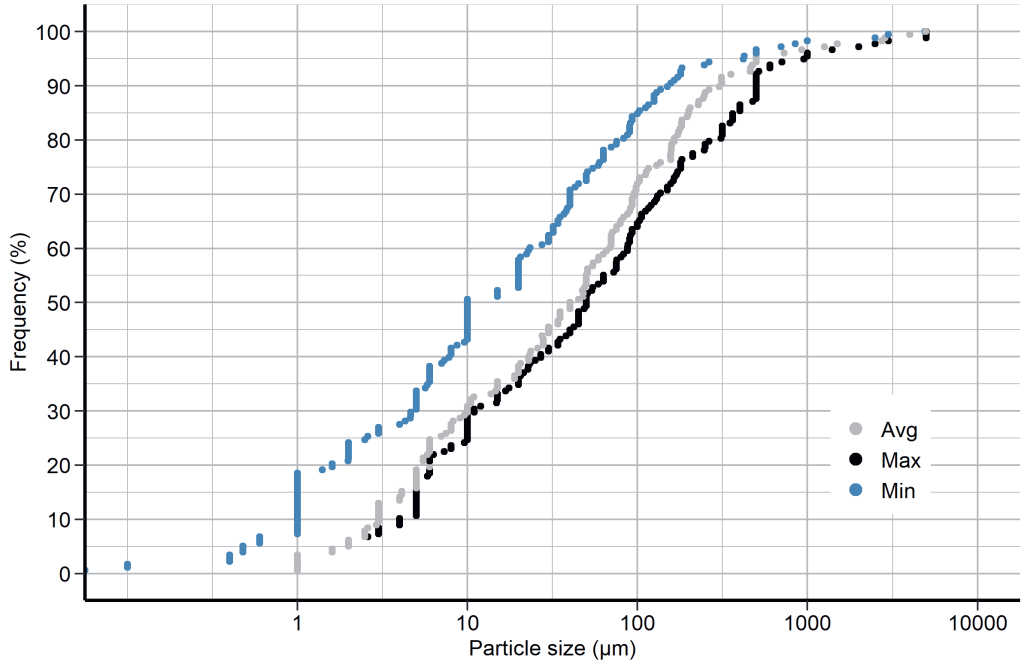

Figure 1. Cumulative frequency distributions for MP particle sizes used in effect tests for aquatic biota. The majority of studies tested a size range, which implies that separate cumulative distributions can be plotted for the minimum (Min), the maximum (Max) and the average size tested across studies.

Shape - The shapes of MP reported in the 105 studies are dominated by spheres/beads, followed by irregular MP, fragments and fibres (Figure 2A). We assume that most of the studies reporting the use of irregular MP have tested either fragments, films, foams and sheets, or a combination thereof. Consequently, characterizing MP into distinct categories includes a subjective, qualitative, element that is difficult to enable differentiation, but which could result in greater refinement of shapes divided into more categories that would provide opportunities for better mechanistic understanding.

When comparing the shapes used in different effect studies with those shapes commonly observed in environmental samples, there is considerable inconsistency. While 58.1% of effect studies have tested MP spheres/beads, this category only represents 6.5% of the MP detected in water and sediment samples (Kooi and Koelmans, 2019). In contrast, only 8.1% of the tested MP in effects studies were fibres, although they are the most abundant shape category detected in water and sediment, typically representing about half of MP detected (Kooi and Koelmans,

2019). Therefore, the use of fibres in effects studies represents a significant opportunity for advancing quantitative data for the purposes of assessing environmental risks.

An important factor to consider in future studies is how the shape of MP might influence their ingestion and egestion by aquatic organisms (Au et al., 2015; Frydkjær et al., 2017; Gray and Weinstein, 2017), which can potentially influence their relative toxicity. Thus, the use of shapes representative of those detected in the environment has the potential to benefit both the ecological relevance and mechanistic understanding of risks associated with MP commonly encountered in the environment.

Polymer type – The most common polymer types used in the 105 effect studies reviewed were PS and PE. Together they represent 62.3% of the MP types tested (Figure 2B). The use of these two polymers is relatively consistent with the polymer types typically observed in the environment, whereby the three most commonly detected polymers in surface waters are PS, PE, and PP (Koelmans et al., 2019). In effect studies, however, the inclusion of PP is limited to only 5.5% of MPs tested. Given that the polymer type can influence the fate of MP in both the test system and ecosystem, depending on its density, surface chemistry, degree of crystallinity, and presence of chemical additives and plasticizers, it is important to include as much detail as possible with respect to the polymer composition (Kooi et al., 2017; O'Connor et al., 2020). Consistent with the need to advance the effects testing and mechanistic understanding of MP with respect to size and shape, as discussed above, there is also a need to strengthen understanding of the influence that the polymer composition may represent towards an observed adverse effect on various species. Insight regarding the relationships between size, shape and polymer composition is important for advancing environmental risk assessment and helping to inform the decision-making process.

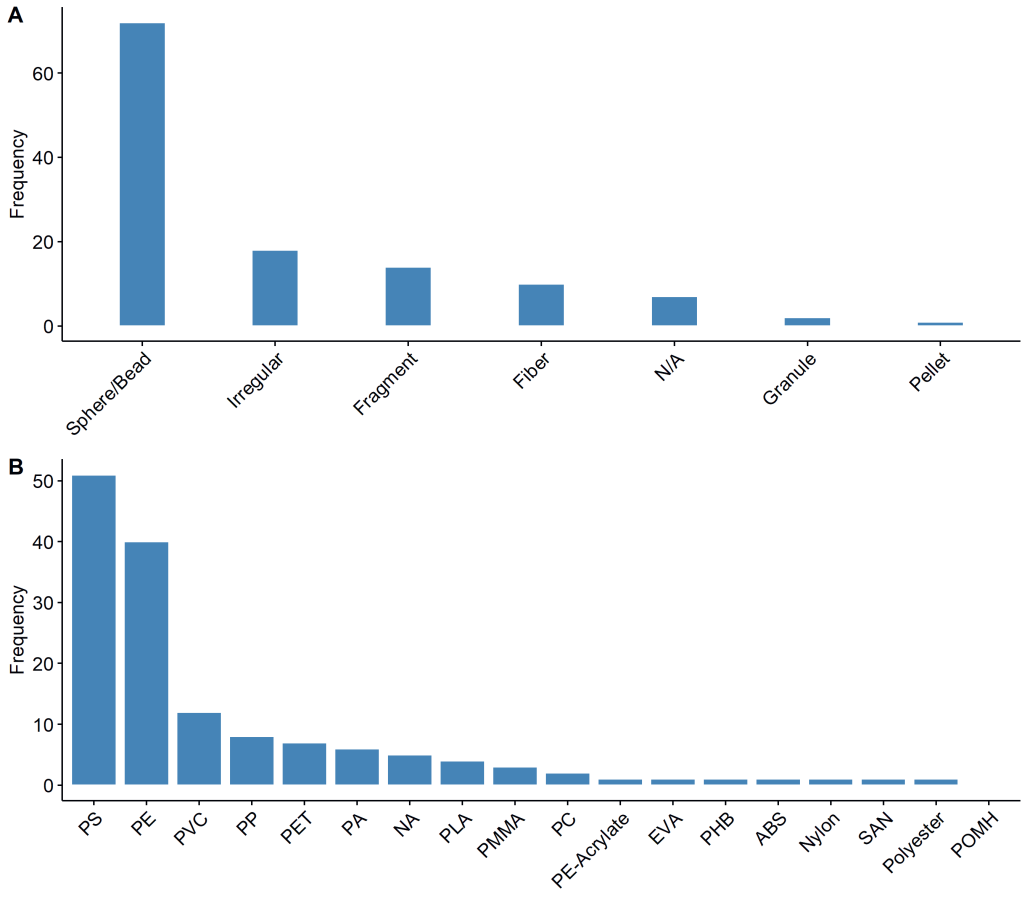

Figure 2. Number of studies reporting a particular shape (A) or polymer type (B) for the microplastics used in the exposure tests (from a total of 124 records for shapes and 145 records for polymer types). PS = polystyrene, PE = polyethylene, PVC = polyvinyl chloride, PP = polypropylene, PET = terephthalate, PA = polyamide, N/A = not analysed, PLA = polylactic acid, PMMA = poly(methyl methacrylate), PC = polycarbonate, PE-Acrylate = polyethylene-Acrylate, EVA = ethylene-vinyl acetate, PHB = Polyhydroxybutyrate, ABS = Acrylonitrile butadiene styrene, SAN = Styrene acrylonitrile resin and POMH = Polyoxymethylene - Homopolymer

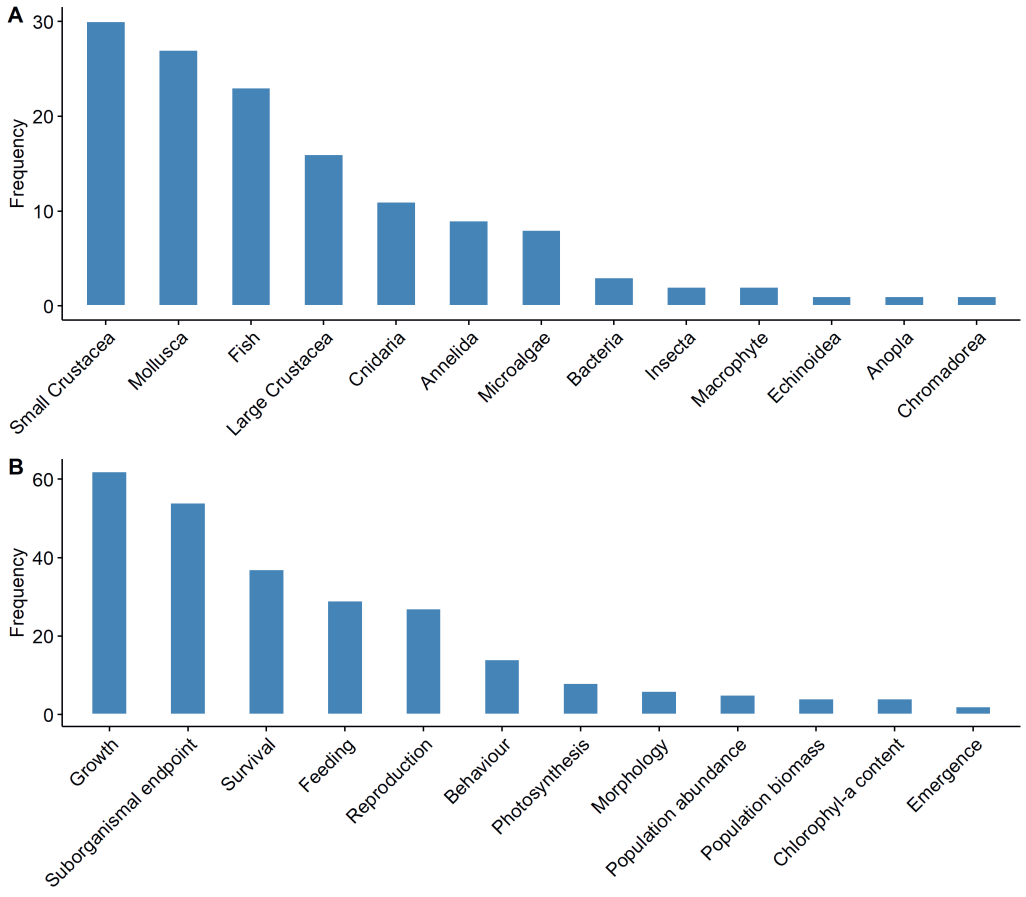

Figure 3. Number of studies evaluating the effects of MP on organisms of a certain taxonomic group (A) and on a particular endpoint (B) (from a total of 134 records for organisms and 252 records for endpoints).

3.2.2. Exposed organisms, exposure duration, endpoints studied, and effect thresholds reported.

The organisms tested in the 105 studies evaluated consist of 52.4% marine, 42.9% freshwater and 4.8% estuarine species. The most abundant organisms studied are small crustaceans (which belong to the zooplankton category), followed by molluscs and fish (Figure 3A). The most common exposure durations used were; 24 h, 96 h, 240 h (10 days), 336 h (14 days), 504 h (21 days) and 672 h (28 days) (see Figure S2 Supporting Information). The exposure durations generally correspond to the recommended exposure durations of standard ecotoxicity test guidelines for chemicals, implying that exposure durations are also closely linked to standard

effect endpoints, such as mortality, growth, and reproduction. However, there is literature indicating that effects of MP can be time dependent (Chapron et al., 2018; Redondo-Hasselerharm et al., 2020; Schrank et al., 2019) and standard test protocol guidelines applicable for chemicals may not be applicable for the effect testing of MPs. Nevertheless, chronic effects testing of MP adopting longer study durations does not appear to be well represented, with only 18% of studies using an exposure time > 28 d, and < 2% (i.e. 2 papers) with exposure times above three months. Consequently, it is recommended that future effects testing include greater emphasis on assessing longer term effects.

Effects of MP on growth are observed to be the most often studied (25.4%), followed by sub-organismal endpoints (21.4%), survival (14.7%), feeding (11.5%), and reproduction (9.9%) (Figure 3B). Population-level endpoints correspond to only <4% of the total endpoints studied. From the 105 papers, only about 10% reported effect thresholds (as either LC_x, EC_x, LOEC or NOEC). Of all the studies providing effect thresholds, 33.3% report them as number concentration (i.e., particles/L), 50% as weight concentration (i.e., mg/L), and 16.7% in both units. In order to assess the environmental risks of MP, effect thresholds are fundamental, preferably in both units, which will also further enable comparisons between studies for use in developing quantitative WOE with respect to effects and risks.

3.2. Quality assessment

The results of the scoring based on the quantitative quality assessment proposed in this study imply that substantial improvements can be made in how MP effect studies are designed and conducted (Figures 4 and 5). As previously stated, the scores obtained should not be interpreted as an absolute value of judgment, but as a guide for identifying and prioritizing study-design components that would benefit most in improvement for the purposes of assessing environmental risks. Consequently, we suggest that those studies with relatively high scores represent the most reliable and useful in the context of risk assessment (Figure 5). Individual studies, however, often had other objectives, which were not necessarily consistent with information needed to support an assessment of risk. It is important, therefore, to assess each of the specific criteria and to compare them with other studies rather than simply evaluating the studies based on how they rank on their total score. The first subset of criteria (criteria 1 – 12) enable the evaluation of the general technical quality of an effect test study. Here, the average score across all

studies is 11.3 (range 5 to 18), of a maximum possible score of 24. In this first subset there are no studies for which positive scores on all quality criteria is assigned. The second set of criteria (criteria 13 - 20) relates to the relevance of the papers for their use in environmental risk assessment. For these criteria, the average score across all studies evaluated is 6.6 (range 0 to 14) of a maximum potential score of 16. Again, no studies had positive scores for each of the ecological relevance quality criteria defined. Finally, the total scores combine both the technical quality and ecological relevance evaluation criteria, whereby the total score can be used as part of a quantitative WOE approach in the context of risk assessment. The average total score is 17.8 (range 8 to 31), from a maximum possible score of 40, indicating that results from effect studies assessing MP are often not fully reliable and/or reproducible. All studies included in this review were assigned a criterion value of 0 in at least one criterion, implying that important QA/QC criteria are consistently poorly addressed in the design and reporting of MP effect studies. With respect to the general technical quality of the effect studies evaluated, 34.8% of the criteria in studies are assigned a value of 0, whereas 50.1% of studies receive the same poor quality score with respect to their ecological relevance. Average scores per criterion ranged from 0.06 to 1.79 (Figure 4). Those criteria that are typically evaluated high across all studies include the reporting of the source of the MP, the use of replicates, reporting on ecologically relevant endpoints and the inclusion of food particles within the test study. A more detailed evaluation of each category is provided below.

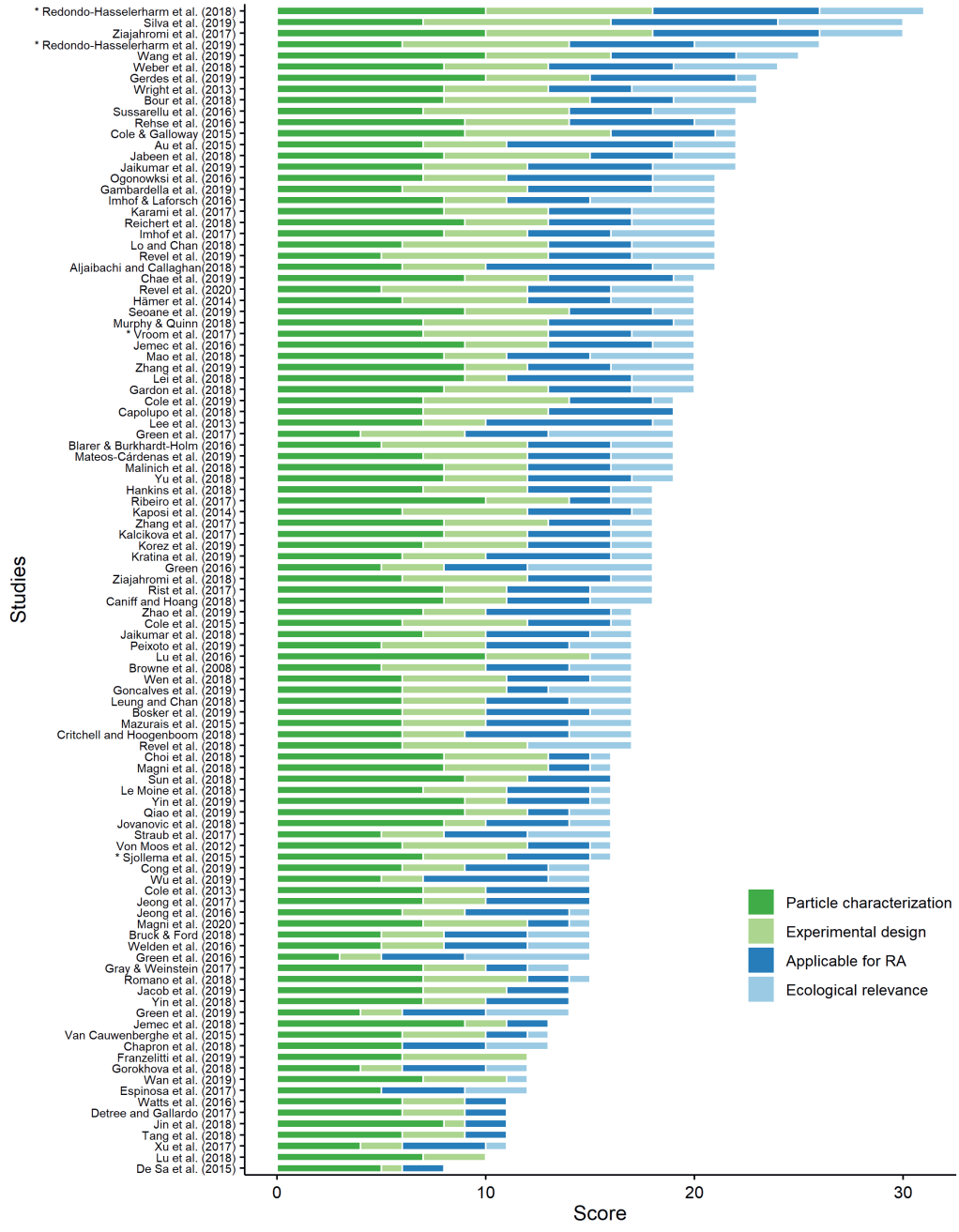

Figure 5. QA/QC quantitative system scores from *n = 105* studies. Scores per study with categories "particle characterization", "experimental design", "applicable for RA" and "ecological relevance". *Studies with involvement of 1 or more of the authors of the present paper. See Table S3 in the Supporting Information for the detailed scores.

Particle characterization - The category with the highest average score is "particle characterization" (Figure 4). Overall, the majority of studies evaluated are observed to provide satisfactory reporting on particle characteristics (scores >1). Only a limited number of studies (13.3%) fail to report on either one of these specifics. Improvements, however, are suggested, such as related to efforts towards the confirmation of size, shape and polymer type, as opposed to simply relying on information from the manufacturer. Nonetheless, by failing to provide characteristics of the particles, an entire experiment can become irreproducible. Lastly, it should be noted that approximately 60.0% of studies either don't report a concentration or limit reporting to a mass or number concentration, which further complicates comparison across studies. It is thus suggested that with relatively limited resource towards addressing the shortcomings identified, substantial improvements can be realized within this quality criteria category.

Experimental design - As a general observation, the majority of studies scored poorly within the category of experimental design (Figure 4). Concern is particularly apparent with respect to the quality evaluation criteria of "laboratory preparation" and "verification of background contamination", with average scores of 0.18 and 0.06, respectively. While MP are often said to be ubiquitous in the environment, including indoor (laboratory) air (SAPEA, 2019),[1] only 3.8% of the reviewed studies thoroughly report how they minimized potential contamination arising from air, water and all materials used during the experiment. Additionally, only 4.8% of the reviewed papers verified the background contamination (visually).

Only a few, 6.7%, of the evaluated studies included a protocol specifically used to pre-clean MP with an organic solvent. Additionally, 20% of studies took measures to ensure chemical purity. For instance, Karami et al. (2017) and Romano et al. (2018) measured certain chemical contaminants associated with the MP, however, this still does not exclude chemical effects from experimental results. Some studies include a solvent control, but do not account for chemical contaminants that might be present in the MP themselves. Importantly, the majority of studies (73.3%) do not mention the potential for chemical contaminants influencing observed adverse effects, making it difficult to disentangle particle toxicity from a potential chemical toxicity.

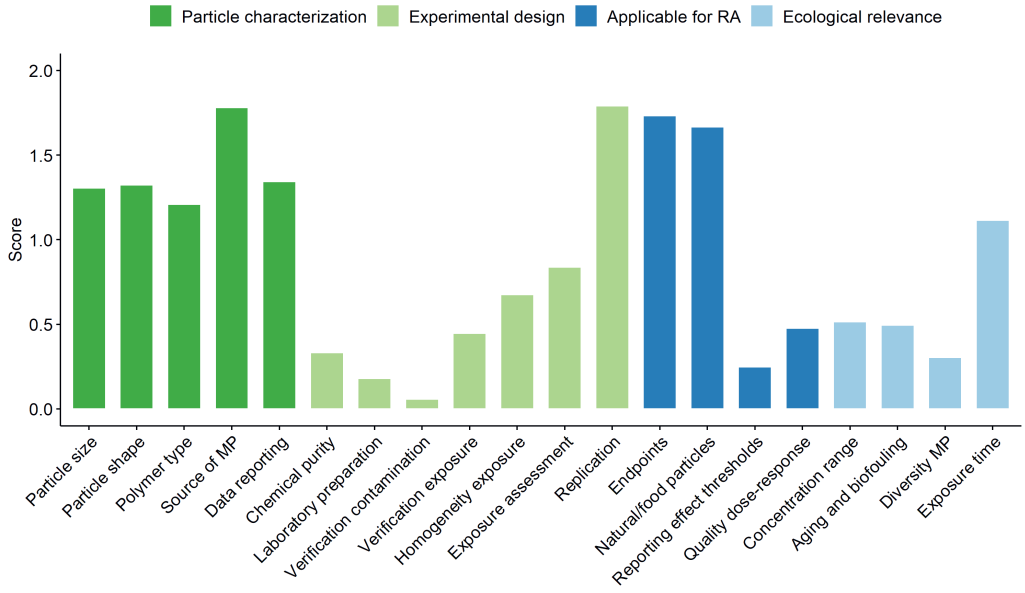

Figure 4. QA/QC quantitative system scores from *n=105* studies. Average scores per criterion with categories "particle characterization", "experimental design", "applicable for RA" and "ecological relevance". Each study is assigned a criterion value of either 2 (adequate), 1 (adequate with restrictions) or 0 (inadequate) points, for each of the 20 criteria.

The criteria "verification of exposure" and "homogeneity of exposure" also are observed to score low, with average scores of 0.45 and 0.68 (n = 105), respectively. These criteria are critical for enabling the reproducibility of study results, which further increase the uncertainty associated with reported effect thresholds. Finally, the criterion "exposure assessment" (average value of 0.84) is generally unsatisfactory in the studies evaluated. While most studies (78.1%) include a description verifying that MP have been ingested by test organisms, verification is often (72.4%) demonstrated in either a separate experiment, qualitatively, visually or without a digestion step.

While it is acknowledged that the resources needed to address the shortcomings identified with the criteria falling under the category of 'experimental design' are likely to be high, failing to address the various criterion results in studies with greater uncertainties and which thus fail to add value to broader scientific understanding as well as for strengthening opportunities to assess environmental risk. It is therefore prudent to carefully consider experimental design in future

effect studies, with the development and application of standard test protocols applicable to MP identified as an urgent need to better guide researchers.

Applicability to risk assessment – An important implication of data reported from ecotoxicity effects studies is their role in assessing environmental risks. Consequently, suggestions for improvement made under this category are perceived to have implications for the regulatory decision-making process. Results from the studies evaluated under the criteria related to applicability for risk assessment imply the need for improvements to "reporting of effect thresholds" and "quality of dose-response relationship", where average scores of 0.25 and 0.48 were assessed, respectively. As mentioned above, a limited number of studies (10.5%) are observed to explicitly report on effect thresholds with an indication of error. Moreover, only 30.5% of the 105 studies include a sufficient number of concentration doses to ensure statistical rigor in detecting these effect thresholds. The majority (86.7%) of reported endpoints for MP effects, however, are informative to the risk assessment process, with 84.8% including a source of food to avoid the artefact of force-feeding MP to test organisms.

Ecological relevance – Apart from the criterion "exposure time', which shows an average score of 1.11 and was thus evaluated as satisfactory among the 105 studies, all other criteria in this category score low. The criterion "diversity of MP", with an average of 0.30, is of particular concern. Only 33% of the studies included at least one environmentally realistic concentration, raising concerns regarding the relationship between laboratory-based observations of adverse effects and ecological risks. Most studies (71.4%) assessed the effects of MP using a single MP type or MP with a limited range of characteristics. Only one study used a mixture in their experiment representative of environmental exposure (Imhof and Laforsch, 2016). Only two studies included the influence of biofouling when assessing the effects of MP, subsequently characterizing the microbiology of the biofilm (Gorokhova et al., 2018; Imhof and Laforsch, 2016).

3.3. WOE for mechanisms explaining adverse effects of microplastic on aquatic biota

Currently, the knowledge on effect mechanisms for MP is limited and there is a need to increase mechanistic understanding of toxicological modes-of-action (Gouin et al., 2019; Jeong and Choi, 2019; Koelmans et al., 2017). Criterion #11 "exposure assessment of organism" aims at improving the strategic design of effect

testing that might enable results to differentiate between intrinsic physicochemical properties of the MP themselves and how those interact with species-specific biological and physiological traits to influence an observed adverse effect (see Supporting Information, methods continued). Acknowledging that MP represent a complex mixture of particles (shape, size and type), incorporating strategies that enable effect-assessment to move from a 'substance-based' approach to a 'mechanism-based' approach may add considerable value in assessing environmental risk, not just for MP but for any other particle-stressor organisms may encounter (Gouin et al., 2019; Jeong and Choi, 2019). Knowledge on effect mechanisms will enhance the strategic application of species sensitivity distributions for distinct categories of effects. Finally, advancing scientific understanding of particle effect mechanisms, such as those associated with exposure to MP, will aid in the development of effect models (Kong and Koelmans, 2019).

Given the importance of advancing the scientific weight-of-evidence with respect to the effect mechanisms following exposure to MP, each of the 105 studies is reviewed with respect to the mechanisms that authors used to explain the adverse effects they observed. The analysis is based on four considerations. Firstly, we verified whether authors refer to the mechanisms they described using terms such as 'suggested' versus 'demonstrated' (see Table S3 in the Supporting Information). If authors themselves described a mechanism as 'demonstrated', the WOE is perceived to be stronger. Secondly, the frequency of reporting certain mechanisms was assessed (Table 2). The more often a mechanism is reported in the literature, the stronger the perceived WOE can be considered to be, in that consistency between studies in relation to observed effect mechanisms is assumed. Third, the relative strength of the WOE supportive of an effect mechanism is further scrutinized based on the criteria #6 "chemical purity", #14 "addition of food" and most importantly #11 "exposure assessment of organism". While all 20 criteria are crucial in order to ensure quality and reproducibility of data from effect studies, the latter three criteria are specifically important in order to successfully assess the mechanisms behind adverse effects. Fourth and finally, the scores from the QA/QC assessment are used to assess the relative credibility of effect mechanisms reported.

Table 2. Tiered weight of evidence (WOE) approach for effect mechanisms reported in 105 studies, by number of studies that (a) frame a mechanism as 'suggested', (b) frame a mechanism as 'demonstrated', (c) fulfil the three quality assurance criteria (score > 0) considered most relevant to identify effect mechanisms (#6, #11, #14), and (d) average score according to QA/QC of studies that fulfilled those three quality assurance criteria.

#	Description of mechanism explaining adverse effect	Suggested	Demonstrated	Number of studies that fulfil criteria #6, #11 and #14[c]	Average score of studies that fulfil criteria #6, 11 and 14 QA/QC[d]
1	Inhibited food assimilation and/or decreased nutritional value	32	9	5	21.4
2	Internal physical damage	20	7	3	21.0
3	External physical damage	8	4	2	24.0
4	Oxidative stress	6	8	1	16.0
5	Disturbance of essential processes that affect physiology	8	3	0	-
6	Adjustment of energy metabolism to cope with MP	1	2	0	-
7	Microbial imbalance	2	1	0	-
8	Leaching additives or chemicals	14	0	-	-
9	(Cellular) stress	8	0	-	-
10	Effects of surface properties	2	0	-	-
	Total	100	34	11	

Suggested versus demonstrated mechanisms for adverse effects - From the 105 studies evaluated in this review, 10 separate effect mechanisms are identified as 'suggested', whereas 7 mechanisms are identified to be 'demonstrated', the latter including: 1) inhibited food assimilation and/or decreased nutritional value 2) internal physical damage, 3) external physical damage, 4) oxidative stress, 5) disturbance of essential processes that affect physiology, 6) adjustment of energy metabolism to cope with MP and 7) microbial imbalance (Table 2). Three additional

mechanisms are reported as speculated only: 8) leaching of additives or chemicals, 9) (cellular) stress and effects of 10) surface properties. While 100 times studies describe an effect mechanisms as “suggested”, only 34 times studies describe an effect mechanism as “demonstrated”. The most frequently suggested mechanisms are “inhibited food assimilation and/or decreased nutritional value” and “internal physical damage” with a frequency of 32 and 20 suggested occurrences, respectively. However, it is notable that only 9 and 7 studies have reported these mechanisms as demonstrated, respectively.

1) Inhibited food assimilation and/or decreased nutritional value - Within the studies that report on “inhibited food assimilation and/or decreased nutritional value ” as demonstrated, there are 5 studies that meet the crucial criteria “chemical purity”, “addition of food” and “exposure assessment of organism” and have therefore reliably concluded on the demonstrated effect explaining the adverse effect, scoring 21.4 points QA/QC on average (Blarer and Burkhardt-Holm, 2016; Cole and Galloway, 2015; Gardon et al., 2018; Murphy and Quinn, 2018; Redondo-Hasselerharm et al., 2018b) For instance, (Blarer and Burkhardt-Holm, 2016) visually quantified the presence of PA fibres in the digestive tract of *Gammarus fossarum* and showed inhibition of food assimilation.

2) Internal physical damage - Of the 7 studies that report on the demonstrated mechanism of “internal physical damage”, there are 3 studies that also comply with the aforementioned crucial criteria (#6, #11 and #14) (Qiao et al., 2019; Redondo-Hasselerharm et al., 2018b; Von Moos et al., 2012). The studies by Redondo-Hasselerharm et al. (2018b), Qiao et al. (2019) and Von Moos et al. (2012) are assigned a score of 31, 16 and 16 in the QA/QC assessment, respectively. Wang, Y. et al. (2019), scored relatively high with 25 points. Moreover, they were able to verify the exposure of MP to organisms, and also avoided potential system-dependent artefacts by including a protocol for adding food during their experiments. However, they do not include measures to ensure chemical purity, resulting in some caution when interpreting the mechanism as ‘demonstrated’.

3) External physical damage - Although not one of the most often speculated (8 times), the mechanism “external damage,” is concluded to be demonstrated in 4 studies (Jabeen et al., 2018; Kalčíková et al., 2017; Zhao et al., 2019; Ziajahromi et al., 2017). Among these, there are 2 studies that fulfilled the crucial criteria (#6, #11 and #14). The one with the highest QA/QC score is Ziajahromi et al. (2017) with 30

points, who observed malformations on the carapace of *Ceriodaphnia dubia*. Additionally, with a score of 18, Kalčíková et al. (2017) showed that microbeads with sharp edges affected the root growth and reduced viability of root cells of *Lemna minor*. This study qualitatively assessed the adsorption of MP onto root surface and took measures to ensure chemical purity.

4) Oxidative stress - Oxidative stress has frequently been framed as a demonstrated mechanism for the effects observed (8 times). There is, however only one study that complied with the three criteria crucial to reliably assess a demonstrated mechanism (i.e. #6, #11, #14). Qiao et al. (2019) observed inflammation and oxidative stress in the gut of *Danio rerio*. Besides qualitatively assessing MP in the gut, they also took measures to ensure chemical purity, and fish were fed daily. This study however, scored relatively low on QA/QC (16 points), rendering the results less reliable. Oxidative stress is a molecular mechanism and can be defined as an imbalance in the production of free radicals and the ability of organisms to deal with them (Prokić et al., 2019). As oxidative stress is also an endpoint, it is likely that it has often been considered as demonstrated. Moreover, oxidative stress is one of the most commonly measured biomarkers (Jeong and Choi, 2019; Revel et al., 2019). It is, however, not clear if oxidative stress is a response to another MP toxicity mechanism or that the MP toxicity directly works at the molecular level (Gouin et al., 2019; Ogonowski et al., 2018). Elucidating on this aspect will aid in choosing relevant endpoints to use within risk assessment frameworks (Gouin et al., 2019).

5) Disturbance of essential processes that affect physiology - The mechanism "disturbance of essential processes that affect physiology" is claimed to be demonstrated 3 times (Détrée and Gallardo-Escárate, 2017; Green et al., 2019; Seoane et al., 2019). No studies, however, comply with the criteria to credibly ascertain the demonstrated mechanism.

6) Adjustment of energy metabolism to cope with MP – While the mechanism "adjustment of energy metabolism to cope with MP" is suggested once, it is reported as 'demonstrated' two times (Seoane et al., 2019; Watts et al., 2016). Seoane et al. (2019) showed that MP caused a slight decrease in the growth rate of the marine diatom *Chaetoceros neogracile*, but also a significant decrease in the esterase activity and the lipid reserves of MP-exposed cells. While scoring relatively well on the overall QA/QC scores (20 points), this study did not take any measures

to ensure chemical purity, rendering the result less reliable. Additionally Watts et al. (2016) showed that Crabs were able to overcome minor effects on ion exchange by minor physiological regulation, however did not meet criteria #6 and #14.

7) Microbial imbalance - Two studies speculate that adverse effects are caused by microbial activity or the presence of bacteria on the MP (Green, 2016; Leung and Chan, 2018). Additionally there is one study by Jin et al. (2018) that has framed this mechanism as demonstrated. However, no measures were taken to ensure chemical purity or assess MP exposure to the organisms.

8) Leaching of additives or chemicals - 'In 14 studies, leaching of additive or adsorbed chemicals from MP was speculated to be an explanation for the observed effect of MP; however, this mechanism has never been framed as demonstrated. Demonstrating this mechanism can be achieved by simply washing MP with organic solvent thoroughly and repeatedly, subsequently enabling to distinguish particle from chemical toxicity of MP. Interestingly, Cole et al. (2019) only suggested that leaching of chemicals could have played a role, i.e., not claiming the mechanism to be demonstrated. However, they received maximum score of 2 on this criterion (#6), meaning that in our view they adequately addressed the issue actually rendering the mechanism to be demonstrated.

9) Cellular stress - As "cellular stress" is a broad term, hard to specify and hence not easily measurable, it is likely that for this reason it has never been framed as a demonstrated mechanism.

10) Effects of surface properties - Only two studies speculate that adverse effects measured in their studies are due to the surface properties of MP (Au et al., 2015; Blarer and Burkhardt-Holm, 2016). No study, however, claims to have demonstrated an effect of surface properties.

Overall final WOE assessment of mechanisms explaining adverse effects of MP - When comparing the demonstrated mechanisms according to studies it is apparent that "inhibited food assimilation and/or decreased nutritional value" has been demonstrated most often with relatively high overall QA/QC scores (average = 21.4). Most importantly 5 out of 9 studies comply with the crucial criteria to reliably assess a mechanism, making it a plausible mechanism to explain adverse effects with high overall WOE.

Additionally, the mechanism “internal physical damage” has a relatively high overall WOE. Of the 7 studies that managed to demonstrate this mechanism, 3 fulfilled the crucial criteria (#6, #11, #14) with an average score of 21.0 points. While the mechanism “external physical damage” has been demonstrated less often, effects have been measured with higher reliability than for other demonstrated mechanisms. The 2 out of 4 studies that comply with the crucial criteria to reliably assess a mechanism, score an average of 24 QA/QC points, thus also making it a plausible and high WOE mechanism explaining adverse effects.

4. Perspective and outlook

Research on the effects of MPs on biota in aquatic and other environmental compartments is a relatively new discipline in the environmental sciences. As a result, approaches to assess these effects vary widely across research groups, with both the nature of effects testing and analytical methods developing rapidly over time. Here, we evaluate the quality of 105 studies that report on the ecotoxicological effects of MPs for aquatic biota. The evaluation includes studies of organisms at various functional groups, such as phytoplankton, macrophytes, zooplankton, benthic invertebrates, and fish. The evaluation criteria developed as part of the evaluation can be used as guidance towards best practices to assess exposure, effects and effect threshold concentrations for MPs, and can provide a quantitative quality assessment of studies reporting adverse effects of MPs on aquatic organisms. Lastly, we summarize and discuss the characteristics of the tests that have been performed thus far (e.g., particle size ranges, concentrations, polymer types, particle shapes, species, endpoints, test duration) in order to detect knowledge gaps within effect studies, and use information gained from the review of the literature to assess the WOE with respect to the effect mechanisms most likely influenced by exposure to MPs.

When adopting strict quality criteria, an overall lack of reliability is observed in the studies evaluated in this review, particularly for how data from available effect studies can be used to help inform the risk assessment process. This is partly related to technical shortcomings in the experimental design, such as not ensuring chemical purity, prevention and verification of MP contamination in the laboratory, and partly to limitations in the relevance of studies, for instance when studies do not use ecologically relevant particles or testing conditions. This implies that based on the current state-of-the-science, the WOE for ecological effects is very limited

and the environmental risk of MPs is difficult to assess. The lack of clear evidence for ecological effects in nature due to relatively poor-quality effects studies available for the risk assessment process is worrying, particularly given concerns raised by the public and decision-makers to provide a quantitative assessment of the risks for MPs. The purpose of the present study is therefore to provide timely guidance on best practices needed to improve and standardize effects testing protocols. This includes the need for access to standardized test methods using reference MPs that can be used between research groups in an effort to strengthen both replication and inter- and intra-laboratory reproducibility. We recommend that at least one of these reference materials is an environmentally realistic mixture of particles, i.e. having a realistic range of sizes, shapes, densities and ages. This way, organisms themselves select the fraction from the mixture that is bioavailable and relevant for them. This would mimic the situation in nature better than tests with single type materials. The adoption of standardized test methods and use of environmentally relevant reference materials would help reduce uncertainties inherent in the effects data and strengthen both environmental risk assessment and mechanistic understanding of the ecotoxicity of MP.

Based on our review of study characteristics, it appears that particle type 'fibres' and polymer type 'polypropylene' are understudied in effect studies. Ideally, the MP tested should be as realistic as possible, thus representing a broad range of sizes, shapes, densities and polymer types. The ecological relevance of tests should be increased by extending exposure times, as chronic tests are rarely performed. In order for effect tests to be more informative for risk assessment, the reporting of thresholds effect concentrations should be made more accurate and explicit, preferably as either LC_x, EC_x, LOEC or NOEC values, with the use of both mass and particle unit concentrations.

Based on the evaluation of the WOE pertaining to effect mechanisms associated with exposure to MPs, we observe that the WOE is strongest for the mechanisms related to 'inhibition of food assimilation and/or decreased nutritional value', 'internal physical damage' and for the mechanism 'external physical damage'. To increase the WOE of ecological effects and effect mechanisms we recommend that the guidance provided in this evaluation study be used to develop studies that explore the mechanistic nature of both MPs and generic particle effects on aquatic organisms more broadly.

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Supporting information

Table S1, containing study characteristics and Table S3, containing the scores and mechanisms per author can be found online: https://pubs.acs.org/doi/10.1021/acs.est.0c03057.

Methods continued - Detailed motivation for each criterion used in the quality evaluation.

Particle characterization

Criterion 1. Particle size. Species-specific physiological and behavioural traits can strongly influence the relative size of particles ingested by an organism, including MP (Cole et al., 2013; Lee et al., 2013; Redondo-Hasselerharm et al., 2018b; Scherer et al., 2017). Size selectivity depends on the morphology and feeding strategy of a species, which determines the upper size limit for the food they can ingest, as well as for the ingestible size of MP (Burns, 1968; Jâms et al., 2020; Kooi and Koelmans, 2019). For instance, in a study assessing the ingestion of MP by seven Cladocera species, the maximum size of MP ingested increased proportionally with the body size (Burns, 1968). The upper size limit will differ between species at varying trophic levels, but can also show significant variation within species depending on their developmental stage (Au et al., 2015; Scherer et al., 2017). Based on species traits, size preferences have been demonstrated for a few organisms, being some MP sizes ingested in higher quantities than others (Browne et al., 2008; Jâms et al., 2020; Redondo-Hasselerharm et al., 2018b). Particle shape and polymer identity also affect the probability of MP to be encountered and ingested, thereby affecting the bioavailability of MP (Scherer et al., 2017). Furthermore, the residence time of MP in the body of the organisms has also been related with the size of the particles (Gray and Weinstein, 2017). The relative relationship between the ingestion and retention of MP can result in decreased nutritional value and/or physical obstruction in the digestive tract, which have been proposed as two of the mechanisms underlying observed adverse effects for organisms exposed to MP (Redondo-Hasselerharm et al., 2018b) (Au et al., 2015; Blarer and Burkhardt-Holm, 2016; Lee et al., 2013; Wright et al., 2013). As the ingestion and effects of MP can be size-dependent, the size distribution of the MP selected in an effect study can

directly influence the occurrence and severity of the effects observed and therefore requires analytical characterization. Consequently, studies that report the full particle size distribution of the tested MP are assigned a criterion value of 2. The distribution, however, should be provided with sufficient resolution, ideally with 10 bins or more. If only one size is reported instead of a range, a study receives 2 points when the size reported is supported by analytical characterization and reported with a measurement error. MP sizes should ideally be characterized analytically using dynamic light scattering or laser diffraction methods or alternatively estimated using high resolution microscopy of the MP with a scale in combination with imaging analysis software. When the particle size/sizes are reported but not supported by analytical characterization, based on information provided in material safety data sheets or size separation using sieves, a study is assigned a criterion value of 1. Finally, studies that did not report the size of the MP used in their experiments are assigned a criterion value of 0.

Criterion 2. Particle shape. For several species, selective ingestion, gut retention, and effects of MP have been found to depend on their shape (Au et al., 2015; Frydkjær et al., 2017; Gray and Weinstein, 2017). For instance, fibres were more lethal than spheres for the amphipod *Hyalella azteca* (Au et al., 2015). Authors report that fibres resulted in longer gut retention times, speculating that fibres may have aggregated in the gut (Au et al., 2015). Additionally, Piarulli et al. (2020) showed that MP analysed in six different benthic invertebrate species collected from salt marshes, were mostly fibres (98.5%) (Piarulli et al., 2020). MP fragments are also reported to be associated with longer gut retention times in the cladoceran *Daphnia magna* in comparison to spherical MP (Frydkjær et al., 2017). It has been suggested that the rounded shape of spherical MP facilitates their transport through the digestive system of organisms, resulting in less severe effects than for other shapes of MP (Au et al., 2015). Given several observations reporting on the relative influence of the shape of MP on effect endpoints, the evaluation criterion related to characterizing MP shape is seen as an important factor when interpreting ecotoxicological effects data. The shapes of MP have been defined in many ways, such as e.g., fragment, fiber, film, foam, pellet, sphere, line, bead, flake, sheet and granule (Hartmann et al., 2019; Koelmans et al., 2019; Rochman et al., 2019). Different shape categories can be found even within these categories; for instance, MP fragments can be further characterized as rounded circular or edgy rectangular shapes (Redondo-Hasselerharm et al., 2018b). Further complicating shape

characterization is the observation that the dimensions of MP vary along continuous scales and therefore do not lend themselves well to discrete categories of characterization (Kooi and Koelmans, 2019). Consequently, we consider the term "irregular MP" as an ambiguous definition of the shape, as it includes the potential to reflect several shape categories. Moreover, for a complete characterization of the shape, it is necessary to include at least one high-resolution photo illustrating each of the shapes included in the MP tested. Therefore, studies that provide an image obtained from a high-resolution microscope of the MP tested are assigned a criterion value of 2. Studies that limit the reporting of the shape of MP to the definitions of Rochman et al. (2019) or their synonyms (sphere vs. bead), based on the information obtained from material safety data sheets but without a visual confirmation by the authors are assigned a criterion value of 1. Finally, studies that do not report the shape of the MP used or reported shapes that did not fall within the definitions described by Rochman et al. (2019), are assigned a criterion value of 0.

Criterion 3. Polymer type. The fate, bioavailability, uptake and thus potential effects of MP can be also influenced by the composition of the polymer representing the MP, which determines the density of the particles in aqueous systems (Kooi et al., 2017; O'Connor et al., 2020). In a sterile system without potential biofouling of the particles and in the absence of agitation, positively buoyant MP will float on the water surface, while negatively buoyant MP will remain in the water column until they sink to the bottom of the system (Kooi et al., 2017). The fate of the MP in the water column thus influences their bioavailability and therefore the polymer type, as a proxy for density, needs to be characterized and reported. Additionally, knowing the polymer type will allow comparisons with field data on the occurrence, abundance and physical properties of the same polymer type, and possibly linking it with certain products and product emissions. Currently, elaborate techniques for polymer identification are available and widely applied in MP research, such as ATR-FTIR, micro-FTIR, Raman spectroscopy, pyrolysis GC-MS or similar methods (Shim et al., 2017). For studies that analytically characterize the polymer type using one of these methods, a criterion score of 2 is assigned. When the polymer type is reported following the information given in the material safety data sheet and not confirmed by the authors, the study is assigned a criterion value of 1. Finally, studies that did not report the polymer type of the MP used are assigned a criterion value of 0.

Criterion 4. Source of MP. Reporting the source of where the MP were obtained is essential in order to better interpret the data the MP relate to, and to strengthen data reproducibility in future studies. Some studies, for instance, use in-house manufactured MP, following ad-hoc procedures which may not lend themselves well to reproducibility. In these instances it is imperative that detailed descriptions of the protocol used in producing the MP is provided (e.g., Korez et al., 2019). Results of effect studies on MP published to date show a wide variety of responses for different organisms (Burns and Boxall, 2018). Even for the same species, different results can be obtained, which could be attributed to differences in the source(s) of MP (Weber et al., 2018; Wright et al., 2013). Therefore, when MP are purchased from a commonly available supplier and where specifics of the provider is provided in the main text or in the supporting information, a study is assigned a criterion value of 2, as this scenario lends itself best to reproducibility. For those studies where MP are prepared in-house using commercially available plastic products, we also assign a criterion value of 2 when the name of that plastic product is provided as well as a detailed protocol for the preparation or extraction of the MP. For instance, Jemec Kokalj et al. (2018), extracted MP from a facial cleanser and made MP from a plastic bag. Polymers were characterized using FTIR, particle size distributions were measured by laser diffraction, and images of the MP were taken with a field emission scanning electron microscope. However, they do not provide the name of the facial cleanser nor the precedence of the plastic bag. Consequently, when the information given on a MP source is incomplete and thus not fully reproducible, a criterion value of 1 is assigned. Finally, studies that do not provide any information on the source of the MP are assigned a criterion value of 0.

Criterion 5. Data reporting. It is widely acknowledged that inconsistency in how concentrations are reported make it difficult to compare between effects studies (Burns and Boxall, 2018; Connors et al., 2017; Van Cauwenberghe et al., 2015). Concentrations of MP can be presented as a particle number concentration, like the number of MP particles per L or per Kg of sediment, food or weight of the organism; or mass concentration, like grams of MP per L or per Kg of sediment, food or weight of the organism (Besseling et al., 2019). Some studies quantify the number of MP in a specific volume or weight using a hemocytometer, a flow cytometer or a coulter counter (Cole and Galloway, 2015; Ogonowski et al., 2016; Sussarellu et al., 2016; Ziajahromi et al., 2018). Other studies estimate the number

of MP manually using a stereomicroscope combined with image analysis software, applicable for MP (Ogonowski et al., 2016; Wang, Y. et al., 2019; Ziajahromi et al., 2018). Moreover, some studies convert mass concentrations to number concentrations or vice versa based on assumptions that correlate the size of a particle to its volume, for which MP characteristics such as size distribution, shape and density are required (Gerdes et al., 2019; Redondo-Hasselerharm et al., 2018b; Rehse et al., 2016). A few other studies make reference to the conversion provided by the supplier of the MP (Jeong et al., 2017; Lee et al., 2013; Sjollema et al., 2016). Thus, the reporting and conversion of concentrations between particle number and mass concentration units can be done using a variety of methods, and should be clearly described in the study in order to facilitate comparisons across studies. Since the units of concentration represent a fundamental parameter to assess risk, which compares environmental concentrations to effect threshold concentrations, consistency in units is therefore of paramount importance (Besseling et al., 2019; Koelmans et al., 2017). Studies that report concentrations in particle number as well as in mass concentrations are thus assigned a criterion value of 2, as they provide the greatest opportunity to compare between studies and for use in assessing environmental risk. Studies that limit the reporting of concentrations to only either particle number or mass concentrations, are assigned a criterion value of 1. Finally, studies where concentrations of MP are not reported receive a criterion value of 0.

Experimental design

Criterion 6. Chemical purity. Studies that aimed to investigate the interactive effects of MP and chemicals are not included in this study but are reviewed elsewhere.[22,38,39] Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and organochlorine pesticides are ubiquitous in the environment and will partition into any organic carbon, including MP (Koelmans et al., 2017). Experiments measuring the partitioning behaviour between MP and organic chemicals are relevant for determining sorption/desorption coefficients and/or sorption kinetics. However, from the perspective of assessing risk it is more relevant to evaluate the toxicity of plastic-associated chemicals in the absence of MP (Koelmans et al., 2017). Assessing the adverse effects of the chemical stressor in the absence of the MP individually first, can provide an effective strategy for developing more complex test systems aimed at assessing multiple chemical and non-chemical stressors, and help address the immediate challenges of assessing the

environmental risks of MP themselves (Koelmans et al., 2017). This reasoning also applies for the diversity of chemical additives and plasticizers commonly associated with plastic (Zimmermann et al., 2019). Moreover, disentangling the effect assessment associated with chemical stressors from the non-chemical particle stressor can strengthen overall understanding of the mechanisms that influence MP toxicity. For instance, studies by Martínez-Gómez et al. (2017) and Pikuda et al. (2018) have shown that the toxicity from leachates derived from additives are more harmful than the inert polymer material, highlighting the importance of washing MP before the start of an experiment, if insight regarding the effects associated with the particles themselves is the main objective. Otherwise, the chemical stressor overwhelms the effects that might be associated with the particles, preventing the ability to distinguish between the two. Additionally, the artificiality of an exposing test organisms to MP containing chemical additives within a closed system represents a worst-case scenario that is not representative of an environmentally relevant exposure. In the environment, organic chemicals, including POPs, chemical additives and plasticizers are widely dispersed as a consequence of their use in manufacturing and consumer products, and partition into all environmental media, resulting in various exposure pathways to exist. Consequently, assessing chemical exposure requires an understanding of the multimedia behaviour of organic chemicals, whereby exposure via MP likely represents a negligible pathway as compared to other sources (Bakir et al., 2016; Koelmans et al., 2016). Therefore, in order to disentangle the effects associated with the particle stressor from confounding chemical effects, the toxicity of background chemicals should be minimized. This includes minimizing exposure to chemical additives and plasticizer that might be present in MP, but also chemicals associated with food particle surfactants (e.g., Tween) and markers (fluorescence). Minimizing chemical exposures in MP effects studies, however, represents a major challenge. For instance, a recent study by Cole et al. (2019) extensively measured chemicals in MP, and reports that a wide variety of unknown chemicals are used in MP, making it nearly impossible to confirm conclusively that all relevant chemicals have been assessed. Therefore, it is preferred to repeatedly wash the particles with an organic solvent(s) in an effort to minimize effects associated with a chemical-associated contaminant. It is notable, however, that this could have the undesired effect of altering the properties of the particles themselves, consequently care is required with respect to which organic solvents are used as well as the conditions of cleaning. Alternatively, several studies have demonstrated that it is possible to

minimize the influence of the chemical stressor by providing evidence that the mass of chemical in the test system is at an exposure that remains below a chemical toxicity (e.g.,Bellingeri et al., 2019; Redondo-Hasselerharm et al., 2020; van Weert et al., 2019). In summary, studies that report the inclusion of methods to thoroughly clean MP by washing with an organic solvent are assigned a criterion value of 2, since the observations of adverse effects could be more confidently allocated to a particle-associated effect. If a certificate from the manufacturer was used or measurements were taken to subsequently use a control for the chemicals or the toxicity of chemicals was calculated based on L_{50} or EC_{50} from literature, the study is assigned a criterion value of 1. Finally, studies that did not address the potential influence of a chemical stressor on observed adverse effects when testing MP are assigned a criterion value of 0.

Criterion 7. Laboratory preparation. The importance of preventing contamination when testing MP is emphasized in several recent papers and critical reviews (Hanvey et al., 2017; Hermsen et al., 2018; Koelmans et al., 2019; Prata et al., 2019; Vandermeersch et al., 2015; Wesch et al., 2016). Catarino et al. (2018) for instance, quantified atmospheric fall-out within households, which, when rescaled to the surface area of a representative experimental test system of e.g. 20 x 25 cm^2, would imply a flux of 8333 particles per test system per day. The amount of natural fall-out of MP likely differs between locations within a laboratory and among laboratories. Catarino et al. (2018), emphasized the need to account for atmospheric deposition during experiments, even in instances where relatively high concentrations are tested. We argue, therefore, that the uncertainty related to contamination with MP during MP effects studies, also requires care in mitigating the potential for deposition and with respect to characterizing and quantifying the nature of the contaminants. This is because the nature of the MP-contaminants may be significantly different than those used in the test system, in that they may contain chemical additives that can strongly influence observed effects, negating test results. This is particularly relevant to the control test-system, meant to have zero MP concentration, or very low dosed systems, for which greater sensitivity would be anticipated due to the influence of MP-contaminants. Some studies thoroughly report measures taken to prevent MP-contaminates, such as wearing cotton lab coats, rinsing of equipment, covering the test systems or avoiding the use of plastic materials during the experiment (Revel et al., 2019; Revel et al., 2020; Silva et al., 2019). Consequently, a criterion value of 2 is assigned for those studies

adopting measures aimed at avoiding contamination from air, water and all materials used during the experiment. Studies adopting limited measures are assigned a criterion value of 1. Finally, studies that do not report the use of any measure to prevent contamination are assigned a criterion value of 0.

Criterion 8. Verification of background contamination. Whereas the previous criterion focuses on the measures taken to mitigate background MP-contaminants, the present criterion evaluates the extent to which studies verify that such measures are successful or alternatively that the adoption of taking no action to reduce background contamination is needed because the potential for MP-contaminants is demonstrated to be minimal. In this case, verification implies the use of methods that characterize and analytically measure MP concentrations in exposure systems. A study by Welden and Cowie (2016), for instance, observed a fiber in the foregut of one of their control animals, underlining the importance of including method verification in MP effects test studies. A few studies, on the other hand, have limited verification of background contamination to the reporting of visual observations (Revel et al., 2019; Silva et al., 2019; Ziajahromi et al., 2017). Visual inspection, however, is generally considered inaccurate, as there is a high probability of missing small and transparent MP (Hermsen et al., 2018; Koelmans et al., 2019). Moreover, reliance on the use of visual observations is susceptible to false positives (Shim et al., 2017). Based on these considerations, a criterion value of 2 is assigned to studies measuring background contamination with analytical detection methods, such as by FTIR or Raman. For studies that limit the verification of background MP-contaminants to a visual inspection, a criterion value of 1 is assigned. Finally, for studies that do not report on background contamination of MP, a criterion value of 0 is assigned.

Criterion 9. Verification of exposure. In order to obtain accurate dose-effect relationships, exposure concentrations in the test systems must be quantified. Test concentrations are typically prepared by adding particles to the test medium, occasionally followed by dilution and homogenization steps. There are several reasons why the actual exposure concentration can deviate from the nominal concentration estimated from the initial preparation. First, human error can occur in the initial calculations or laboratory manipulations of glass ware and equipment can lead to deviations in the concentration. Secondly, the test system itself can influence exposure, whereby particles can stick to container walls and/or become unevenly distributed across test systems when homogenization is insufficient.

Actual concentrations can also be higher than nominal concentrations due to background MP-contaminants, as discussed in the previous criterion (Catarino et al., 2018; Foekema et al., 2013). These factors can propagate and substantially influence initial estimates of test concentrations. Furthermore, the dynamic behaviour of the particles themselves can cause significant changes in exposure during the test. While less important for sediment-test systems, the behaviour of particles in aqueous test systems can result in settling, floating or aggregation of the particles, changing the actual exposure conditions over time (Besseling et al., 2017). Fundamentally, the exposure of the stressor in an ecotoxicity test system should be constant over time and reproducible for each test. Demonstrating consistency in the exposure concentrations for the duration of the test is thus important to develop accurate dose-effect relationships, and the quantification of the exposure concentration should therefore be verified. A criterion value of 2 is assigned to studies that verify the exposure concentration of MP and ensure that at least 80% of the nominal concentration is maintained throughout the test (Benfenati, 2011; OECD, 2014). Studies that measure the exposure concentration, but without verifying that at least 80% of the nominal concentration is maintained throughout the test are assigned a criterion value of 1. Studies that only report the nominal concentration or limit the verification of the concentration to the stock solution are assigned a criterion value of 0.

Criterion 10. Homogeneity of exposure. The previous criterion evaluates the extent to which the exposure concentration is verified. However, unlike the fate of dissolved chemicals in ecotoxicological effect testing, solid particles are prone to inhomogeneity of exposure as they tend to settle or float depending on a variety of factors, such as the difference in their density compared to that of the medium they are dispersed in (Galloway et al., 2017; Hartmann et al., 2015; Hjorth et al., 2017; Karami, 2017; Monikh et al., 2018; Petersen et al., 2014; Rist et al., 2017; Rist et al., 2018). Therefore, especially for aqueous test systems, MP that have a higher density than water may settle when the dispersion is not well mixed, whereas buoyant particles may tend to reside at the surface of the test system only. Presence of air pockets or biofilm layers may change over time and influence exposure as a result of settling or causing differences in particle-particle interactions and settling velocities as a function of time, thus questioning the assumption of exposure homogeneity. These inhomogeneities can strongly influence the bioavailability and thus the exposure of the particles, resulting in a

lack of control and reproducibility of test results. Methods for addressing heterogeneity in test systems assessing particle stressors include the use of ultrasonic agitation, and other physical mixing techniques (circular, wrist action shaking, plankton wheels) prior or during exposure, or by simply reporting the absence of such problems based on visual observations (Cole et al., 2019; Gambardella et al., 2019; Gerdes et al., 2019; Magni et al., 2019; Qiao et al., 2019; Tang et al., 2018).

Natural sediments are comprised of a mixture of particles with densities spanning a wide range, as compared to that of the solid polymeric particles that have been tested. MP mixed in sediment are 'held' in the sediment matrix and progressively encapsulated when biofilms form and test particles form hetero-aggregates and –agglomerates with the natural particles in the sediment matrix. This implies that exposure in effects test systems of MP mixed in sediment are homogeneously distributed. Many studies have recognized the need for homogeneity and have described in detail how MP were mixed in the exposure medium and sometimes also how homogeneity of exposure was verified (Bour et al., 2018; Redondo-Hasselerharm et al., 2020). For aqueous exposures, a criterion value of 2 is assigned to studies that verify that MP were homogeneously distributed through the use of microscopy photos and/or apply analytical tools to demonstrate that the MP were well mixed or dispersed in the solution. In instances where the method used to generate a homogeneous exposure is described but not verified, a criterion value of 1 is assigned. Effect testing of MP in sediment test systems, for which the verification of homogeneity is deemed to be not crucial, results in a criterion score of 2 for all studies that describe the method by which the MP are homogeneously mixed with the sediment, in detail. Studies that do not address the issue of homogeneity, or that observed an inhomogeneous exposure, are assigned a criterion value of 0.

Criterion 11. Exposure assessment of organisms. To be able to understand and interpret effect data, it is important to be able to causally link an observed effect to actual exposure data. The question 'what is an organism exposed to?' however can have different answers for different organisms, particles and/or test conditions. The metric used to quantify the effect should be ecologically relevant and should be the same as the one used to quantify exposure (Koelmans et al., 2017). Microplastics can have multiple of such environmentally relevant metrics (ERMs). They can be characterized on the basis of known species- and particle-specific

effect mechanisms. Hence, it is the actual effect mechanism which defines how microplastic particles and test organisms interact and how actual exposure should be assessed. Exposure then can be seen as accumulation at the receptor site, i.e. where the interaction takes place, and which is considered as the target for the microplastic effect under consideration. We illustrate the principle with three examples. For instance, one of the more well understood effect mechanisms, is the deterioration of food quality due to the dilution of nutritious food particles caused by an elevated exposure to low-caloric, non-digestible MP that are co-ingested with food (Redondo-Hasselerharm et al., 2018b; Wright et al., 2013). Therefore, for a study that would ascribe observed effects to this mechanism, demonstrating ingestion would be a crucial criterion. Instead, studies that ascribe suborganismal effects to damage at the cell level (Détrée and Gallardo-Escárate, 2017; Franzellitti et al., 2019; Goncalves et al., 2019; Qiao et al., 2019) should ideally demonstrate systemic uptake and/or penetration of MPs and should demonstrate that these cells are reached. As a final example, studies that explain growth inhibition in algal cultures from a decrease in photosynthesis, should verify the presence of MPs at or in between algal cells in the culture (Seoane et al., 2019; Zhang et al., 2017). A detailed overview and analysis of such reported effect mechanisms is provided in section 3.3 of his review. In the majority of instances, effects related to the ingestion of MP are reported as the most relevant exposure pathway, implying that the quality criteria to detect and quantify MP ingested by biota are of critical importance. Exposure due to translocation and cell penetration also requires detection and quantification of MP in biota tissue and is thus also important in defining the quality criteria. These criteria have been reported in a previous study (Hermsen et al., 2018), for which criteria related to tissue digestion, particle detection and polymer identification are all applicable. For adverse effects influenced by external exposure of MP, i.e. from MP just being present in water or sediment, as in the example for algae, criteria for the analysis and quantification of MP in water are most relevant. It is widely understood that visual sorting of MP is insufficient to detect the small and often light-coloured MP against a background of e.g., animal tissue. Therefore, following the QA/QC criteria suggested by Hermsen et al. (2018), a criterion value of 2 is assigned to studies that report the detection of MP quantitatively using e.g., FTIR or Raman imaging, to support statements of MP ingestion and/or penetration into cells of biological tissues that have been appropriately digested and filtered. Studies demonstrating exposure of organisms to MP based on qualitative or visual observation, or citing results from a

separate experiment, or in the absence of a digestion step, are assigned a criterion value of 1. Studies that do not report data on exposure, are assigned a criterion value of 0.

Criterion 12. Replication. In every effect assessment, an adequate experimental design requires a sufficient number of replicates in order to ensure statistically reliable results (Hanson et al., 2017; Moermond et al., 2016). Studies should therefore clearly explain the degree of replication of each treatment (Hanson et al., 2017).[81] Some studies, however, fail to report on the use of replicates in their experimental design (Jemec Kokalj et al., 2018; Welden and Cowie, 2016) while other studies report the use of replicates, but which are not actual replicates but better characterized as pseudo-replicates (Chapron et al., 2018; Jovanovic, 2017; Rist et al., 2017). For instance, Jovanović et al. (2018) considered as replicates the 15 fish exposed to MP in the same tank. As each replicate should be an independent experimental unit, with the experimental unit here being the tank, the exposure of all fish via the same tank should thus be better defined as multiple measurements taken one experimental unit (Calfee and Piontkowski, 1984). In contrast to soluble chemicals, which can be homogenously distributed in the test system, the severity of the effects detected in MP studies can be attributed to the relative extent of bioavailability of the particles and the probability of encountering them in the test system. Therefore, in the case of MP, it is especially important to have several replicates to compensate for the uncertainties associated with the potential for inhomogeneous exposure associated with the test system. Studies were assigned a value of 2 when they included results from a minimum of three replicates. A criterion value of 1 is assigned to studies using only two replicates. Finally, studies that do not include any replicates or do not report the number of replicates used are assigned a criterion value of 0.

Applicable to risk assessment

Criterion 13. Endpoints. Effect studies with MP use a wide variety of endpoints, sometimes even within studies. We argue that when data from such studies are to be used in ecological risk assessment, the ecological relevance of the selected endpoint represents an important criterion to consider. From a risk assessment perspective, endpoints such as survival, growth and reproduction are considered ecologically relevant, because these endpoints directly relate to a population-level effect. These endpoints are preferred over e.g. suborganismal or behavioural

endpoints, which are generally less relevant in assessing population-level responses, unless there is a clear demonstrated causal relationship between these responses and a higher level effect e.g. population effect (Connors et al., 2017; Posthuma et al., 2001). For instance, de Sa et al. (2015) speculated that reduced food intake caused by the ingestion of MPs adversely affects both the individual and population-level fitness of a species. The endpoints studied, however, are attributed to the predatory performance and efficiency of the species, which does not necessarily translate to an ecologically relevant population level effect. Whereas it has been suggested that suborganismal endpoints such as biomarkers can be representative of early warning signals and are thus more sensitive indicators than the traditional endpoints used in risk assessment (Forbes et al., 2006; Revel et al., 2019), they can also be perceived as being susceptible to type I and II error, due to under-replication and pseudo-replication in ecotoxicological bioassays, which could lead to false alarms or undetected effects (Forbes et al., 2006; Tincani et al., 2017). Moreover, there is no evidence that suborganismal endpoints are more sensitive than endpoints taken at higher organismal level responses, particularly for MP effects studies. Additionally it is possible that effects seen at the suborganismal level merely resemble reaction to decreased nutritional intake (Ogonowski et al., 2018). Furthermore, endpoints at these suborganismal levels are not likely to be useful predictors since they have complicated time- or dose-dependent responses, which makes it difficult to extrapolate correlations to higher levels of biological organization (Forbes et al., 2006). Still, in carefully controlled studies e.g. biomarkers can be useful for elucidating mechanisms of toxic action (Forbes et al., 2006). In summary, a criterion value of 2 is assigned to studies where endpoints at either community or individual level of biological organization (e.g. survival, growth, development or reproduction) are used. If suborganismal endpoints are used, for which a causal relationship with effects on higher levels of biological organization is demonstrated, a criterion value of 1 is assigned to the study. Finally, studies that use endpoints that cannot be unambiguously linked to a threat at the individual or population level are assigned a criterion value of 0.

Criterion 14. Presence of natural (food) particles. It is important to note that the natural environment is not free of particles and that organisms have adapted various species-specific traits in relation to strategies for interacting with particles. While MP are ubiquitous in the aquatic environment, the amount of natural particles is typically greater than the concentrations of MP that have been reported

in the environment (Koelmans et al., 2016; Triebskorn et al., 2019). Therefore, when designing an experiment meant to simulate natural conditions it is important to consider the response of organisms to both naturally occurring particles as well as a MP-stressor exposure (Connors et al., 2017; Ogonowski et al., 2018). Exposure to naturally occurring particulates, for instance, can represent an important food source to an organism or may otherwise form part of their natural habitat, such as sediment or suspended solids (Redondo-Hasselerharm et al., 2020; Redondo-Hasselerharm et al., 2018b). The inclusion of food and other particulates is needed because ecotoxicological effects of MP on organisms has been demonstrated to be influenced by the presence of naturally occurring particulates (Aljaibachi and Callaghan, 2018; Critchell and Hoogenboom, 2018; Rist et al., 2017; Scherer et al., 2017). Observations that the co-exposure of both naturally occurring particulates and MP can mitigate toxicity implies the relative importance of a species ability to selectively feed and therefore reduce the risks associated with MP under environmentally relevant conditions (Tincani et al., 2017). We argue that without taking natural (food) particles into account, the observed adverse effects represent a system-dependent artefact that does not lend itself to risk assessment purposes. An exception, however, is made for algal studies, as their food source are nutrients and light, and therefore the addition of other naturally occurring particles is less likely to influence adverse effects (Wu et al., 2019).

It is further noted that there are several studies that adopt standard test protocol guidelines for acute toxicity testing, which are applicable to soluble chemicals (Gambardella et al., 2017; Jemec et al., 2016; Jemec Kokalj et al., 2018; Rehse et al., 2016). In such experiments the test guidance is not to feed the organisms, which is logical when testing soluble chemicals as the food particles may influence the bioavailability of the test chemical and the presence of food does not represent a limiting factor due to the short duration of the acute study. However, this guidance is not applicable to experiments aimed at assessing the acute response of MP, because the adverse effects can also potentially be influenced by the presence of food particles (Ogonowski et al., 2018). Therefore, when natural particles (at least food) are added to avoid an exposure that might be perceived as analogous to 'force feeding' the organisms with MP, a criterion value of 2 is assigned to the study. Studies that add food, but in which the food is not optimally available to the organisms are assigned a criterion value of 1. Finally, studies that do not include any naturally occurring or food particles are assigned a criterion value of 0.

Criterion 15. Reporting of effect thresholds. To date, the majority of effect studies report adverse effects for MP at a single or limited number of test concentrations (Blarer and Burkhardt-Holm, 2016; Cole et al., 2015; de Sa et al., 2015; Green, 2016; Kaposi et al., 2014). These observations are beneficial in demonstrating the potential adverse effects that MP can have on biota. It remains unclear, however, the threshold concentration above which the adverse effect initiates. For the purposes of risk assessment, where the ratio of exposure concentrations to that of effect threshold concentrations are derived, accurate estimates of effect threshold concentrations, such as derived from dose-response relationships in the form of $L(E)C_x$, (or the generally less preferred NOEC or LOEC) (Green et al., 2013; Jager, 2012; Landis and Chapman, 2011), are required. Given the paucity of dose-response threshold effects data for MP, the need for effect threshold concentrations to help inform the risk assessment process has been widely recognized (Besseling et al., 2019; Burns and Boxall, 2018; SAPEA, 2019). Therefore, given the relative importance of this criterion regarding applicability in risk assessment, effect studies aiming at reporting effect thresholds are assigned the greatest value. To be effective it is notable that effect threshold concentrations must be accompanied with estimates of error or uncertainty, in order to evaluate that differences in exposure concentrations are statistically meaningful. Based on this reasoning, we assign a criterion value of 2 to studies that report threshold effects data using $L(E)C_x$ derived from dose-response relationship modelling, with error data (95% confidence interval, standard error or standard deviation). If other metrics like NOEC or LOEC are used, or when no error data are provided, the data are still considered useful and a criterion value of 1 is assigned. Studies that do not explicitly provide data on threshold concentrations for the reported effects are assigned a criterion value of 0.

Criterion 16. Quality of the dose-response relationship - Effect threshold concentrations, such as EC_{50} or LC_{50}, are typically obtained by fitting a logit or probit model to dose-response data (Lenz et al., 2016), in which EC_{50} or LC_{50} is a model parameter. This implies that the statistical significance of the resulting EC_{50} or LC_{50} value depends on the quality of the fit to the data, and on the number of parameters fitted, compared to the number of data points in the dose-response relationship. In standard ecotoxicity test systems it is generally suggested to assess effects using a minimum of six different exposure dose concentrations, including the control, to obtain an accurate EC_{50} or LC_{50} value (Ritz, 2010). Ideally, the

exposure concentrations used are representative of the full range of effects, i.e. from low effect to near-maximum effect, such that an EC_{50} or LC_{50} value can be derived without extrapolation. Intuitively, replication of test results at each exposure concentration will also contribute to more accurate EC_{50} or LC_{50} values. Since replication is already covered by criterion 12, only the number of exposure concentrations used in an effect study is evaluated under this criterion. Studies that use the recommended minimum of six exposure dose concentrations or more, including a treatment control (zero microplastic concentration), are assigned a criterion value of 2, and a criterion value of 1 if five different concentrations are used. For studies reporting dose-response relationships using less than five concentrations, a criterion value of 0 is assigned.

Criterion 17. Concentration range tested. Recent studies have drawn attention to the need to better define ecologically relevant concentration ranges for effect testing of MP (Lenz et al., 2016; Van Cauwenberghe et al., 2015). As previously discussed, studies reporting adverse effects for MP often use unrealistically high exposure concentrations, which has resulted in suggestions for future studies to assess effects using lower, more environmentally relevant, concentrations (Koelmans et al., 2017; Lenz et al., 2016). However, if studies limit assessing effects to low concentrations, it is possible that derivation of effect threshold concentrations may not be possible. Consequently, we argue that studies must follow standard principles adopted in assessing the risks of chemicals, such as through the use of quantitative dose-effect relationships to obtain an assessment of effect threshold endpoints typical of ecotoxicology (i.e., EC_{50} or LC_{50}) with sufficient quality. To meet this requirement, effect testing can include both high and low concentrations, as long as the results are used to quantitatively derive the appropriate threshold values. For example, if an effect observed in an ecotoxicity test system occurs only at concentrations that exceed environmentally relevant exposure concentrations by several orders of magnitude, the end result would be supportive of demonstrating low risk. Nevertheless, there can also be strong arguments that support the use of environmentally realistic, low concentrations in ecotoxicity effects tests. This is because the reported effects occurring at high concentrations may be linked to an effect associated with a decrease in food quality, resulting from either the ingestion of inert non-digestible particles or due to an overwhelming number of particles in the test system that results in a decreased potential for the test organisms to find food particles. This type of effects

occurs with any type of particle of low nutritional value and may be perceived as an artefact of the test system design, not an effect that is intrinsic to the MP themselves (Connors et al., 2017; Gerdes et al., 2019) and is therefore better understood as a non-specific particle effect. This exposure scenario typically results in the test organisms suffering from starvation prior to any other modes of action that the MP may cause – effects that might occur at lower concentrations following a chronic exposure (Bosker et al., 2019; Redondo-Hasselerharm et al., 2020). In other words, at environmentally relevant concentrations, it is unlikely that food dilution represents a mechanism of ecological significance, but that more subtle effect mechanisms (related to behaviour, avoidance, reproduction, particle toxicity) are likely of greater relevance to assess and for which long term chronic effects testing would be beneficial. For this reason, some studies intentionally assess the effects associated with lower test concentrations (Redondo-Hasselerharm et al., 2018b; Wang, Y. et al., 2019). In summary, environmentally relevant concentrations should be given priority for effects testing of MP, which forms the basis of a legitimate criterion for the ecological relevance associated with chronic ecotoxicity test system design. Note that exposure duration is evaluated below, in a separate criterion, and only the ecological relevance of the concentration is evaluated under this criterion. Thus, studies that use two or more environmentally realistic concentrations in the exposure concentration doses tested, supported by credible literature data, are assigned a criterion value of 2. If the test system uses only a single environmentally relevant concentration, supported by credible literature data, a criterion value of 1 is assigned. Studies that acknowledge that concentrations are far above environmentally relevant concentrations, or that do not evaluate their exposure concentrations with environmental monitoring data, are assigned a criterion value of 0.

Criterion 18. Aging and biofouling. Under environmentally relevant conditions, MP undergo abiotic and biotic processes that alter their shape, size, structure and eventually their bioavailability (Jahnke et al., 2017). Vroom et al. (2017) demonstrate that the aging of MP promotes their ingestion by marine zooplankton. As the surface of MP functions as a substrate for biofilm to grow, ingestion of biofouled MP potentially represents an additional energy source for test organisms (Canniff and Hoang, 2018). This implies that ecotoxicity tests that assess pristine particles may potentially underestimate the ingestion rates that may occur in the environment, whereby the potential to ingest aged and biofouled particles may be

higher. Since MP undergo both aging and biofouling in the environment, it would thus be beneficial to consider how such processes influence ecotoxicity results and would further strengthen aims directed at ecological relevance. Consequently, studies that include aging of MP to make them more environmentally realistic and also characterized the MP for aging and biofouling, for instance by scanning electron microscope (SEM), are assigned a criterion value of 2. Studies that have only aged the MP but do not characterize them (e.g.,Zettler et al., 2013) are assigned a criterion value of 1. Finally, studies that limit testing to only the use of pristine MP and/or conditions that prevent the formation of a biofilm are assigned a criterion value of 0.

Criterion 19. Diversity of MP tested: To date, most studies assessing the effects of MP limit observations to a relatively small sub-set of all possible characteristics. For instance, studies testing MP based on a single or limited range of particle sizes, shapes and polymeric type may provide valuable information on how specific particle characteristics influence uptake and effects, but under ecologically relevant conditions, organisms will encounter a wide variety of characteristics, of which size, shape and density often are considered the most important properties influence the transport, fate and bioavailability of MP (Andrady, 2011; Kooi and Koelmans, 2019; SAPEA, 2019). Species-specific biological and behavioural traits can also play an important factor in determining which properties of MP found in the environment will most likely result in an exposure for the individuals of a species (Redondo-Hasselerharm et al., 2018b; SAPEA, 2019). The ecotoxicological effects related to the properties of the relevant fraction of MP for a species, may also be influenced by the presence of either other MP or of naturally occurring particles. Simulating species-specific responses to exposures of environmentally relevant heterogeneous mixtures of both MP and naturally occurring particles represents a significant challenge in MP effects testing. Recently, Kooi and Koelmans (2019) reviewed the ranges and distributions of the characteristics of environmentally relevant MP and observed relative similarity across datasets taken from different locations, with respect to their physicochemical characteristics. Given the recent awareness associated with this criterion, we suggest that future studies adopt the use of distributions in physicochemical properties of MP as a standard approach to enable better environmental realism in MP effects testing. Consequently, studies that use MP with a range of sizes, shapes and densities in one mixture exposure, and which attempts to simulate the diversity of environmental MP, are assigned a

criterion value of 2. If the diversity related to only one or two of the physicochemical characteristics and/or a limited distribution, a criterion value of 1 is assigned. Studies that limited effect testing to a single type of MP were assigned a criterion value of 0.

Criterion 20. Exposure time. Standard test protocol guidelines for the ecotoxicity testing of chemicals recommend the application of defined exposure times for each of the endpoints assessed. While these guidelines are also routinely adopted in the effects testing of MP, some studies highlight the need for longer exposure times, due to the detection of time-dependent effects (Bosker et al., 2019; Chapron et al., 2018; Redondo-Hasselerharm et al., 2020; Rehse et al., 2016; Schrank et al., 2019; Von Moos et al., 2012; Zhang et al., 2019). For instance, the effects of MP on the freshwater coral *Lophelia pertusa* differed between exposure times of 7, 20 and 47 days. While the coral growth rate decreased over time, effects on capture prey and polyp activity disappeared after 47 days, revealing that both positive and adverse effects of MP can differ with time (Chapron et al., 2018). Furthermore, observations for the marine mussel *Mytilus edulis*, report the formation of granulocytomas and the destabilization of the lysosomal membrane increased significantly with longer exposure times when exposed to MP (Von Moos et al., 2012). Moreover, adverse effects of MP on the growth of the cladoceran *Daphnia magna* were only found after 25-31 days of exposure (Schrank et al., 2019). For *D. magna*, another study demonstrated that their immobilization increased over time when exposed to MP (Rehse et al., 2016). Generational effects following exposure to MP have also been reported, as in the case of the copepod *Tigriopus japonicus* (Zhang et al., 2019). Therefore, the importance of exposure duration, which can influence the detection of adverse effects that might differ between chemicals and MP is emphasized within this evaluation criterion. Exposure duration is of particular importance for endpoints that seem to be time-dependent, such as growth, reproduction and long term community effects (Foley et al., 2018; Redondo-Hasselerharm et al., 2020). Additionally, increasing the exposure time can be perceived as adding greater environmental relevance to the effect study, explaining the logic for why this criterion is in the ecological relevance category. Thus, for studies that include a minimum exposure time of 7 days for bacteria or phytoplankton, 21 days for zooplankton, 28 days for benthic invertebrates, macrophytes or fish larvae and 3 months for adult fish, the study is assigned a criterion value of 2. For studies that use an exposure time between 1 and 7 days for bacteria or phytoplankton, between

4 and 21 days for zooplankton, between 7 and 28 days for benthic invertebrates, macrophytes or fish larvae and between 1 and 3 months for adult fish, a criterion value of 1 is assigned. Finally, studies that use substantially shorter exposure times, specifically <1d for bacteria and phytoplankton, 4 days for zooplankton, 7 days for benthic invertebrates, macrophytes or fish larvae and 1 month for adult fish, are assigned a criterion value of 0, except in instances where multigenerational studies are performed, where a criterion value of 1 is assigned

Table S2. Explanation of the quantitative scoring system proposed to evaluate the studies testing the effects of MP on aquatic biota using the (QA/QC) criteria**.** The purpose of the quantitative scoring system criteria is to assess the quality of the papers and to give guidance for appropriate methods for MP particle studies in the future. The first subset of criteria (criteria 1 – 12) relates to the general technical quality of effect tests. The second set of criteria (criteria 13-20) relates to relevance of the papers to be used in risk assessment. For each criterion a score of either 2 (adequate), 1 (adequate with restrictions) or 0 (inadequate) points were assigned, which are explained below.

CRITERIA RELATING TO THE TECHNICAL QUALITY OF EFFECT TESTS (1 – 12)				
Criterion	**Explanation**	**Score 2**	**Score 1**	**Score 0**
Particle characterization				
1. Particle size	Size is a crucial factor explaining effects of MP and thus should be reported.	- If a range of sizes is used; a full (i.e. ≥ 10 bins) size distribution is measured and reported. - If a single size is used, that size is measured with an indication of measurement error and reported.	If particle size/sizes are reported but not measured.	No information on size reported.
2. Particle shape	Shape is a crucial factor explaining effects of MP and thus should be measured and reported.	Shapes are measured with high resolution picture and reported.	Particle shapes are reported but not measured.	No information on particle shape is reported.
3. Polymer type	Polymer type can be a factor explaining effects of MP and thus should be reported.	Polymer identity confirmed with e.g. FTIR, Raman spectroscopy or similar methods.	Polymer type provided with certificate or as provided by manufacturer.	No information on polymer identity is reported.

4. Source of MP	Specification on where MP stock or solution is bought and/or how it is self-made maximizes reproducibility and thus should be reported.	The origin and/or production of MP in own laboratory is reported in detail.	The information given on MP source is incomplete and hence not fully reproducible.	No information on MP source reported.
5. Data reporting	Unambiguous units are required to ensure reproducibility of the experiment and to make it possible to compare data across experiments.	MP concentrations are reported as mass as well as number concentration.	MP concentrations are reported as mass or as number concentration.	MP concentrations are not reported.
Experimental design				
6. Chemical purity	In order to test particle toxicity, the toxicity of other chemicals in solution or mixture should be ruled out. This includes additives present in MP, chemicals associated with food particles and surfactants (e.g. Tween).	Chemical effects other than from the polymer or solution/mixtures are ruled out. MP are cleaned with organic solvent.	- Chemicals are analyzed or studies relied on manufacturer certificate. - Controls are used or calculations are made with values from literature (i.e., LC_{50} or EC_{50}) to rule out toxicity of chemical impurities.	Not mentioned.
7. Laboratory preparation	MP contamination arising from the laboratory (air, water and materials) should be minimized. All materials used (equipment, tools, work surfaces and clothing) should be free of MP.	- All materials used are thoroughly washed with high quality water (e.g. Milli-Q water). - Measures are taken to prevent MP contamination from air. - Cotton lab coats were used to avoid microfiber contamination.	Only part of the measures under 2 are taken to avoid MP contamination.	Not mentioned.
8. Verification of background contamination	Assessment of MP contamination of the exposure systems in the laboratory.	Level of contamination evaluated and quantified, e.g. with FTIR, Raman or similar method.	Contamination visually inspected.	No verification of contamination.

9. Verification of exposure	Not only the nominal concentration should be mentioned. The exposure concentration should be measured.	Measurement of exposure concentration and evidence that at least 80% of the nominal concentration throughout the test is maintained.	Measurement of exposure concentration, however no evidence that at least 80% of the nominal concentration throughout the test is maintained.	No verification of exposure concentration.
10. Homogeneity of exposure	Verification of homogeneity throughout the entire exposure system is crucial for the MP characterization and the assessment of bioavailability.	Water as medium: Picture or measurement of MP in water that demonstrated well mixed or dispersion in solution. Sediment as medium: Description of method used to obtain homogenous exposure.	Water as medium: Description of the method used to obtain homogeneous exposure. Sediment as medium:	Not mentioned or exposure is not homogenous.
11. Exposure assessment	Exposure of the organism to MP should be verified by measurement.	Exposure of the organism to MP is measured quantitatively with e.g. FTIR or Raman. In case MP are ingested additionally an digestion step is included (see Hermsen et al., 2018).[51]	Exposure of the organism to MP is demonstrated qualitatively, visually, in a separate experiment or without digestion step.	No measurement of exposure of MP to organism.
12. Replication	For statistical rigor in detecting effect thresholds (e.g., EC_{50} or EC_{10}), sufficient replicates should be tested.	3 or more replicates.	2 replicates.	No replicates.

CRITERIA APPLICABLE TO ECOLOGICAL RISK ASSESSMENT (13-20)				
Criterion	**Explanation**	**Score 2**	**Score 1**	**Score 0**
Applicable to Risk assessment				

13. Endpoints	Endpoints should be considered that inform ecologically relevant population level risk assessment and clearly reported.	Endpoints taken at the community (e.g. bacteria and algae) or individual level (e.g. survival, mortality, growth, development, reproduction).	Suborganismal responses as long as a causal relationship with the endpoints mentioned under '2' has been demonstrated.	Endpoints cannot be unambiguously linked to a threat on the population or individual level.
14. Presence of natural (food) particles	The exposure conditions should be environmentally relevant.	Natural particles (at least food) are added to avoid force feeding of MP. Criterion not applicable to algae or bacteria and hence these studies receive 2 points.	Food is not optimally available.	No food or natural particles are added to avoid force feeding of MP.
15. Reporting of effect thresholds	To enable PEC/PNEC types of comparisons, the effect threshold should be assessed with error of uncertainty using dose- response relationships.	Effect thresholds are reported as L(E)Cx with error or uncertainty intervals.	Effect thresholds are reported as LOEC or NOEC, or as L(E)Cx value without error or confidence interval.	Effect thresholds are not reported explicitly with L(E)Cx, LOEC or NOEC or not possible to derive effect threshold.
16. Quality of dose-response relationship	For statistical rigor in detecting effect thresholds (e.g., EC_{50}, EC_{10}), sufficient doses should be tested, including a treatment control, covering the full shape of the effect curve and emphasizing the slope for parameter estimation.	Multiple doses, at least 6, including a treatment control.	Multiple doses, at least 5, including a treatment control.	Less than 5 doses.
Ecological relevance				
17. Concentration range tested	Concentrations should be motivated (with a reference in the appropriate unit) from measured environmental	More than 1 environmentally relevant concentration was used within the range tested.	At least 1 environmentally relevant concentration was used within the range tested.	- No relevant concentrations were used. - No comparison with MEC.

	concentrations (MEC).			
18. Aging and biofouling	Aging, weathering and biofouling is what occurs in the environment and could affect the uptake of MP; therefore, it is crucial to consider this for an ecological relevant experiment.	MP particles have undergone process to make them environmentally realistic by accounting for biofouling. In addition, pictures of altered particles are provided.	MP particles have undergone process to make them environmentally realistic, accounting for aging, weathering and/or biofouling, however they have not been characterized.	Pristine MP used and/or conditions were as such that it was not possible to form a biofilm during the exposure time.
19. Diversity of MP tested	In the environment, MP have a wide variety of shapes and sizes. This needs to be taken into account for environmentally relevant effect assessment.	A wide range of sizes (order of magnitude), shapes and densities are used, thereby approaching the diversity of environmental MP.	Diversity relates to only 1 or 2 of the characteristics (e.g. only a wide size range) and/or spans a part of the characteristics range only.	Only a single type of particles is tested (i.e. single size, shape and density).
20. Exposure time	It is crucial to use appropriate exposure times to allow for the detection of adverse effects.	Bacteria and phytoplankton ≥ 1 week Zooplankton ≥ 21 d Benthic invertebrates ≥ 28 d Fish ≥ 3 months Macrophytes ≥ 28 d	Bacteria, phytoplankton: 1 – 7 d Zooplankton: 4 – 21 d Benthic invertebrates: 7 - 28 d Fish: 1 - 3 months Macrophytes: 7 - 28 d	Bacteria, phytoplankton < 1 d Zooplankton < 4 d Benthic invertebrates < 7 d Fish < 1 month Macrophytes: < 7 d

Chapter 3

Microplastic effects tests should use a standard heterogeneous mixture: Multifarious impacts among sixteen benthic invertebrate species detected, under ecologically relevant test conditions

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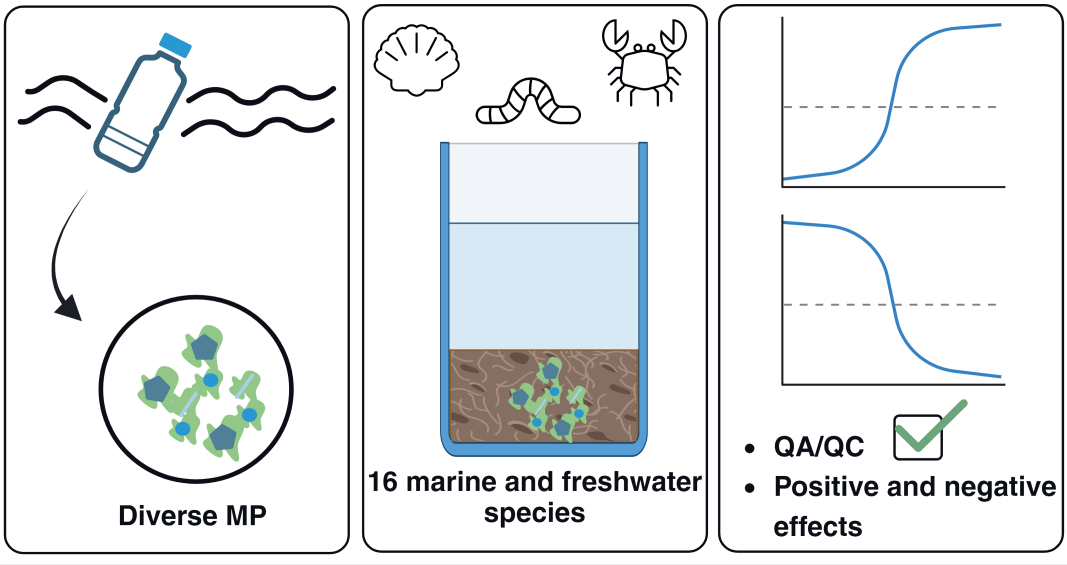

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Abstract

Microplastics require a risk assessment framework that takes their multidimensionality into account while exclusively considering robust data. Therefore, effect tests should use a diverse, environmentally relevant microplastics (ERMP) standard material that adheres to high-quality requirements. In this study, we provide chronic dose-effect relationships and effect thresholds for sixteen benthic species exposed to ERMP. The ERMP was created from plastic items collected from natural sources and cryogenically milled to represent the diversity of microplastics. The test design met twenty previously published quality assurance and quality control (QA/QC) criteria. Adverse effect thresholds (EC_{10}) were determined at ERMP concentrations of 0.11 ± 0.17% sediment dry weight (*Gammarus pulex*, growth), 0.49 ± 0.68% sediment dry weight (*Lumbriculus variegatus*, growth), and 1.90 ± 1.08% sediment dry weight (*Lumbriculus variegatus*, reproduction). A positive effect of microplastics, such as decreased mortality, was observed for *Cerastoderma edule* (EC_{10} = 0.021 ± 0.027% sediment dry weight) and *Sphaerium corneum* (EC_{10} = 7.67 ± 3.41% sediment dry weight), respectively. Several of these laboratory-based single-species effect thresholds for ERMP occurred at concentrations lower than those found in the environment. For other species, no significant effects were detected up to an ERMP dose of 10% dry weight.

1. Introduction

In recent years, research into microplastics has increased significantly due to growing concerns within the public, political, and scientific community. It is well known now that microplastics can be found anywhere and could potentially lead to negative impacts on aquatic ecosystems (de Ruijter et al., 2020; Koelmans et al., 2022b; SAPEA, 2019). Although sediments are considered a sink for microplastics, most research to date has focused on pelagic exposure scenarios. Moreover, not much is known about differences between habitats (marine versus freshwater) or about the implications of species traits on the toxicity of microplastic. Prospective risk assessments show that it is only a matter of time until the risk of adverse effects on the ecosystem function will increase and that this is already the case for some hotspot sites such as the Mediterranean, the Yellow Sea, and several highly polluted freshwater sites (Adam et al., 2019; Coffin et al., 2022b; Everaert et al., 2020; Koelmans et al., 2020). The results of these risk assessments should however be regarded as preliminary since they were not performed in a regulatory context, and the ecotoxicological data used is surrounded with uncertainties (de Ruijter et al., 2020; Everaert et al., 2018; Koelmans et al., 2020). Many researchers acknowledge that methodologies in plastic research need improvement and harmonization (Burns and Boxall, 2018; Connors et al., 2017; Cowger et al., 2020; de Ruijter et al., 2020; Everaert et al., 2020; Gouin et al., 2019; Karami, 2017). Moreover, there is an urgent need to deal with the complexity of microplastics as a diverse contaminant, varying in size, shape and polymer type and to elucidate the underlying mechanisms of effects to explain conflicting results (Bucci et al., 2020; Paul-Pont et al., 2018). Recently developed Species Sensitivity Distributions (SSDs) contain ecotoxicological data that do not test microplastics in their diversity as one would encounter in the aquatic environment (Adam et al., 2019; Besseling et al., 2019; Everaert et al., 2020; Everaert et al., 2018). Because datapoints in these SSDs relate to different particle types, results from such SSDs are fundamentally flawed. Rescaling methods have been developed that solve the problem of this misalignment (Koelmans et al., 2020; Kooi et al., 2021) and these have been applied to derive management thresholds for the first time in a regulatory context (Coffin et al., 2022a; Mehinto et al., 2022). While elegant and efficient, and can be considered the best option at the moment, they use several approaches and assumptions that introduce new uncertainties (Koelmans et al., 2020). In addition, they often have to use relatively poor-quality input data (Mehinto et al., 2022), a

problem that cannot be completely solved by scaling or alignment methods. We propose that to increase the realism of risk assessments, exposure, and effect threshold data should be used that meet the highest QA/QC standards, and that relate to environmentally relevant standard microplastic mixtures that reflect the full multidimensionality of the material (Koelmans et al., 2022b; Kooi et al., 2021). More specifically, for effect tests, microplastic mixtures used should provide a valid representation of the distribution of polymer types, shapes, and sizes as found in nature (de Ruijter et al., 2020), so that as little extrapolation via rescaling as possible is needed.

This study aimed to provide threshold effect concentrations for a range of benthic species with different feeding traits and habitats, for risk assessment purposes. To achieve this aim, we first prepared an environmentally relevant microplastic (ERMP) mixture, and then tested 16 invertebrate species using that mixture while fulfilling strict QA/QC criteria (de Ruijter et al., 2020). For instance, key features of the quality assurance were that ERMP was characterized extensively; contamination was minimized, exposure concentrations were homogenous and verified, natural particles were included, allowed for biofouling of MP to increase environmental relevance (Aljaibachi and Callaghan, 2018; Amariei et al., 2022), use of environmentally relevant concentrations, and six replicated doses to enable dose-response modelling to detect and report of effect thresholds. Effects data were analysed using dose-response models, and generalised linear mix models were applied to explore differences in effects between marine versus freshwater species, and between feeding traits.

2. Material and Methods

2.1. Quality assurance and quality control (QA/QC)

Test design, materials, handling of materials, control of background contamination, and exposure conditions fulfilled the twenty QA/QC criteria as defined by de Ruijter et al (2020). A summary of how these criteria were met is provided as Supporting information (Table S1). Additionally, a detailed description of how the criteria verification of exposure concentration and background contamination were met, is provided as Supporting information (Material & Methods, page S2, S3).

2.2. Preparation of environmentally relevant microplastics

Microplastic particles with varying polymer types, sizes, shapes, and colours were created in the laboratory. In brief, naturally aged macroplastics were collected from the river banks of the National Park Biesbosch (The Netherlands), analysed with ATR-FTIR to determine the polymer identity, and subsequently cryogenically milled (Figure S1, S2, S3, S4). Sieve fractions of the milling product were combined in such a way that the final mixture demonstrably resembles ERMP occurring in sediments (Figure S5) (de Ruijter et al., 2020; Kooi and Koelmans, 2019). The result was an ERMP standard test mixture, with polymer weight percentages: of irregular PE fragments (34%), irregular PP fragments (15.9%), PP fibres (10.5%), irregular PET fragments (20.6%), and irregular PS fragments (19%). These proportions are similar to those found in the environment.(Kooi and Koelmans, 2019) Moreover, the ERMP test mixture had a size range between 9 to 5386 µm and a power law slope α (α = 3.28 ± 0.02) (Figure S5) equal to the slope found for microplastic mixtures in freshwater sediments (α = 3.25 ± 0.19)(Kooi et al., 2021). A detailed description of the preparation and characterization of the ERMP test mixture is provided as Supporting information (Material & Methods, Page S4, S5, Figure S5, S6).

2.3. Test organisms

A total of sixteen benthic invertebrate species, comprising nine freshwater and seven marine species, were tested in this study. Freshwater species selected were *Gammarus pulex*, *Hyalella azteca*, *Asellus aquaticus*, *Sphaerium corneum*, *Corbicula fluminalis*, *Potamopyrgus antipodarum*, *Tubifex spp.*, *Lumbriculus variegatus,* and *Chironomus riparius*. Organisms were selected to differ in their feeding and living behaviour, however, are all connected to the benthic environment (Table S2). Marine species selected were *Alitta Virens*, *Limecola balthica*, *Corophium volutator*, *Arenicola marina*, *Cerastoderma edule*, *Porcellana platycheles,* and *Mytilus edulis* (Table S3). A detailed description of the origin and collection of the test organisms is provided as Supporting information (Material & Methods, Page S6).

2.4. Sediment

Clean freshwater sediments were collected from the experimental field station of Wageningen University (the Sinderhoeve, Renkum, The Netherlands). Marine sediments were collected from Oesterput and Roelshoek (Eastern Scheldt, The Netherlands) and mixed with a ratio of 1:4 (Redondo-Hasselerharm et al., 2018b).

For a detailed description of the collection and preparation of the sediments, see Supporting information (Material & Methods, Page S7).

2.5. Experimental setup

The systematic testing approach in this study is similar to the one followed by Redondo-Hasselerharm *et al.* (2018), with a few adjustments (Redondo-Hasselerharm et al., 2018b). Sediment-microplastics mixtures were added to sediment at the concentrations 0, 0.1, 0.3, 1.0, 2.5, 5.0, and 10.0 % dry weight (d.w.). This translated to a particle concentration of 5.9×10^6, 1.8×10^7, 5.9×10^7, 1.5×10^8, 2.9×10^8 and 5.9×10^8/kg d.w. sediment, respectively. In total 17 chronic, single-species bioassays were performed. The systematic testing approach in this study is similar to the one followed by Redondo-Hasselerharm *et al.* (2018), however, in the previous study the sediment that originated from a noncontaminated ditch in Veenkampen (Wageningen, the Netherlands), had a TOM content of 31.6% ± 3.5 (n=4) (Redondo-Hasselerharm et al., 2018b). While environmentally relevant, this high TOM content could mask the adverse effect of microplastics. After all, negative effects observed in aquatic organisms have been explained by the inhibition of food assimilation and/or decreased nutritional value of food, more commonly referred to as “food dilution” (de Ruijter et al., 2020). Hence we chose sediment with a lower, more common TOM content. To verify comparability between our testing approaches and implement a positive control, we repeated the previous experiment by Redondo-Hasselerharm *et al.* (2018) with *G. pulex.* Once with the lower TOM content sediment and PS fragments as used by Redondo-Hasselerharm *et al.* (2018), and once with the lower TOM content and with ERMP instead of PS fragments. For experiments 1 and 2, experimental units were made by either adding PS fragments or ERMP without PS fragments to sediment (Table S2, 3) in the following concentrations 0, 0.5, 1, 3, 5, 10 and 20 weight %. For experiments 3 through 17, ERMP including PS fragments (Table S2, 3) were added to the sediment in the following concentrations 0, 0.1, 0.3, 1.0, 2.5, 5.0, and 10.0 weight %. Concentrations ranging from environmentally relevant (0 to 1.0%)(Lenaker et al., 2019; Redondo-Hasselerharm et al., 2023; Xia et al., 2021) to high concentrations (2.5-20%) were included to cover criteria related to relevance as well as to statistical rigour in finding an effect threshold (de Ruijter et al., 2020).

Sediment-microplastic mixtures were manually homogenized with a stainless steel spoon. Consequently, Dutch Standard Water (DSW) or filtered seawater was gently added at a 3:1 water-to-sediment ratio. Experimental units were made in quadruplicate and four blanks (containing only DSW or seawater) were added to measure background microplastic contamination, e.g. from air fallout. Systems were randomized and left to acclimatize to allow for biofilm formation for two weeks before adding the organisms (Amariei et al., 2022; Diepens et al., 2015; Lobelle and Cunliffe, 2011; Ramsperger et al., 2020; Vroom et al., 2017). Experimental units received 11 to 22 organisms depending on the size of the organisms (Table S2, S3). Exposure lasted 28 days. Dissolved oxygen, pH, temperature, conductivity/salinity, and NH_3 concentrations were measured twice a week and DSW and seawater were refreshed periodically (Table S5, S4). For a detailed description of the experimental design, the reader is referred to the Supporting information (Material & Methods, Page S8).

2.6. Endpoints mortality, reproduction, growth, feeding rate, emergence, and development rate

After 28 days organisms were sieved, counted, and transferred to clean DSW or seawater to clear their gut for 24 hours. Consequently, organisms were rinsed with milli-Q water and microplastic particles in their gut contents, and body tissues were stored separately at -20°C for later microplastic analysis. Findings related to microplastic ingestion will be detailed in an companion paper. Organisms were photographed and either their length was measured using ImageJ (Schneider et al., 2012) or they were weighed per replica. The emergence of *Chironomus riparius* was counted daily. The feeding rate (mg d.w. leaf/organism/d) of *G. pulex* was calculated from the loss of poplar leaves (Equation S1). An overview of endpoints measured per organism is provided as Supporting information (Table S2, S3).

2.7. Data analysis

Selection of the best-fitting dose-response models, and detection of statistically significant EC_{50} threshold effect concentrations, was done using the dose response curve (DRC) package in R (Ritz et al., 2015). The significance of ERMP dose-dependence ($p_{noEffect}$) was assessed using a log-likelihood ratio test using the best-fit dose-response model compared to a linear regression model with a slope of 0, representing absence of dose-dependency.

To explore if the effects of the ERMP concentration on mortality were different for the freshwater versus marine species tested, a Generalized Linear Mixed model (GLMM) was used. Similarly, a GLMM was used to explore if the effects of ERMP on mortality were different for the feeding traits (filter feeders, sediment/deposit feeders, sediment grazers, and facultative deposit feeders). All statistical analyses and graphs were performed in R Studio (Team, 2021). For a detailed description of the statistical approach the reader is referred to the Supporting information (Material & Methods, Page S9, S10)

3. Results and discussion

3.1. Mortality, emergence and reproduction

The mortality in the controls was 20.0 % on average across all tests, ranging from 0.0 % (*P. platycheles* and *C. fluminalis*) to 75.0 % (*C. edule*) with a median mortality of 9.5% (Table S2, S3). The mean total of *C. riparius* that did not emerge was 40%. Additionally *L. variegatus* had a mean reproduction factor of 2.9, which is more than acceptable (OECD, 2007). Some marine species were never tested before, making control mortality less predictable and in some cases less ideal in the context of effect testing for regulatory purposes. Error in replicated treatments was fairly high in some cases. We observed a cascading phenomenon in the test systems of *Cerastoderma edule*, *Porcellana platycheles*, *Arenicola marina,* and *Mytilus edulis*, implying that the death and decay of only 1 individual resulted in a decline in water quality, which in turn resulted in the death of more individuals. Note that the effect thresholds derived from effect tests with high mortalities in the controls are not fit for regulatory purposes. Nevertheless, sediments were non-toxic, water quality parameters were good (Table S5, S6), and 80% of all individuals tested survived 28 d of exposure, illustrating that test conditions generally were sufficient.

There was no statistically significant effect threshold found for mortality of *G. pulex* after chronic exposure to ERMP without PS fragments with concentrations up to 20% d.w. (Figure1, Table S7). There were no statistically significant effect thresholds found for the marine and freshwater species *H. azteca*, *A. aquaticus*, *C. fluminalis*, *P. Antipodarum*, *Tubifex spp., A. Virens*, *L. balthica*, *C. volutator*, *A. marina, P. platycheles* or *M. edulis after* chronic exposure to ERMP with concentrations up to 10% d.w. in sediment (Figure 1, 2) (Table S7, S8). Statistically significant effect thresholds were found for mortality for the species *G. pulex* after chronic exposure

to PS, however the dose-response curve was not significant (Figure 1, Table S7). Moreover, a significant effect threshold was found after chronic exposure to ERMP with concentrations up to 10% d.w. in sediment for the emergence of *C. riparius*, however the dose-response curve was not significant (Figure 1, Table S7). A significant dose-response curve, however, was found for the reproduction of *L. variegatus* (p = 0.009; Table S7) (Figure 1). Dose-response curve fitting yielded a significant EC_{50} value of 2.51 ± 0.44 % (p = 0.002) for *L. variegatus* (Figure1, Table S7, Table 1).

We emphasize that it is challenging to compare these results with previous literature data, because, unlike the present study, previous data were obtained with microplastics that are not environmentally relevant, and previous effect tests often failed to meet sufficient QA/QC criteria. In this sense, the current data can be considered the first of its kind, and can only be compared with previous test results with caution.

Nevertheless, our results do not completely contradict previous microplastic experiments with benthic macroinvertebrates in terms of mortality (Imhof and Laforsch, 2016; Mateos-Cardenas et al., 2019; Redondo-Hasselerharm et al., 2018b; Weber et al., 2018). For instance, Imhof and Laforsch (2016) tested an environmentally relevant mixture of irregular PA, PET, PC, PS, and PVC particles ranging from 4.64 to 602 µm and did not find any effects on morphological or life-history parameters of the mud snail *Potamopyrgus antipodarum* (Imhof and Laforsch, 2016). Additionally, Redondo-Hasselerharm *et al.* (2018) tested a wide size range of irregular PS fragments on *G. pulex*, *H. azteca*, *A. aquaticus*, and *Tubifex spp*. with a dose up to 40% in sediment d.w. and reported no effects on mortality. Interestingly, they also reported no effects on the reproduction factor of *L. variegatus*, whereas we detected effects in the present study. Several factors may explain the difference. First of all, our present study used a more realistic microplastic mixture with a more diverse polymer composition, whereas also shapes were more diverse, e.g. including fibres and irregularly shaped PE, PP, and PET fragments ranging from 9 to 5386 µm. The inclusion of fibres associated with longer gut retention time in the current study may increase the impact of microplastics, as is seen also in other studies (Au et al., 2015; Ziajahromi et al., 2017). Second, our present study used sediment with a lower organic matter content (6.8 ± 0.42%) compared to the sediment used by Redondo-Hasselerharm *et al.* (2018) (31.6 ± 3.5%). This suggests that the inclusion of naturally occurring

organic particles can lower the impact of MP significantly or even mask adverse effects, as is also observed in other studies (Aljaibachi and Callaghan, 2018; Amariei et al., 2022; Rist et al., 2017; Scherer et al., 2017).

However, these explanations of observed differences in adverse effects remain speculative and the ingestion of ERMP by the organisms as well as effects at different food quality levels need further study to be conclusive. Nevertheless, the present study underlines the importance of testing a set of diverse microplastics to make accurate predictions on the population level. For instance, Silva *et al*. (2021), did not find any adverse effects on the reproduction of *L. variegatus* after long-term exposure to solely irregular-shaped PE, suggesting no negative impacts on *L. variegatus* population fitness (Silva, C. J. et al., 2021). Yet using a diverse suite of environmentally relevant microplastics we show that the organism *L. variegatus* is likely to be affected at the population level (EC_{10} = 1.90 ± 1.08 %, p = 8.1×10^{-6}) (Table 1).

Remarkably, a significant *positive* effect on survival was found for the marine clam *C. edule* (EC_{50} = 1.01 ± 0.45 %, p = 0.003) and the freshwater clam *S. corneum* (EC_{50} = 9.88 ± 0.68 %, p = 2.0×10^{-16}) (Table S7, 8) (Table 1). Note that mortality in the controls was high for *C. edule* (75%), meaning that the control, unlike those for the other species, was not representative of a habitat with optimal conditions. It does however indicate that with increasing concentration of ERMP in the sediment, the mortality decreased, and habitat quality for these species increased with concentration given the test conditions. *C. edule* as well as *S. corneum* inhabit the surface of sediments, burrowing to a depth up to 8 cm (McMahon and Wilson, 1981). A possible explanation is that the sediment was too compact for these species, causing resistance for burrowing in the top sediment layer. The microplastic amendments may have loosened sediment consolidation, reducing the energy required for digging. It remains unclear to what extent this effect would be detectable in nature where sediment top layers are more dynamic due to bioturbation and wind or wave-induced pressure gradients. Moreover, on an intertidal flat in nature, *C. edule* would be able to move to a location with more optimal sediment.

3.2. Growth and feeding activity

The growth of marine and freshwater organisms was assessed using length after 28 days and length of the population at the start. For the worm species *Tubifex spp.*,

L. variegatus, A. virens, and *A. marina*, growth is expressed in weight (mg d.w.) and showed relatively high variability among replicates (Figure 3, 4). As for the other organisms *G. pulex*, *H. azteca*, *A. aquaticus*, *S. corneum*, *C. fluminalis*, *P. Antipodarum, C. riparius, C. volutator*, *L. balthica*, *C. edule, P. platycheles,* and *M. edulis*, growth is expressed in length (mm) and variability among replicates was relatively low (Figure 3, 4).

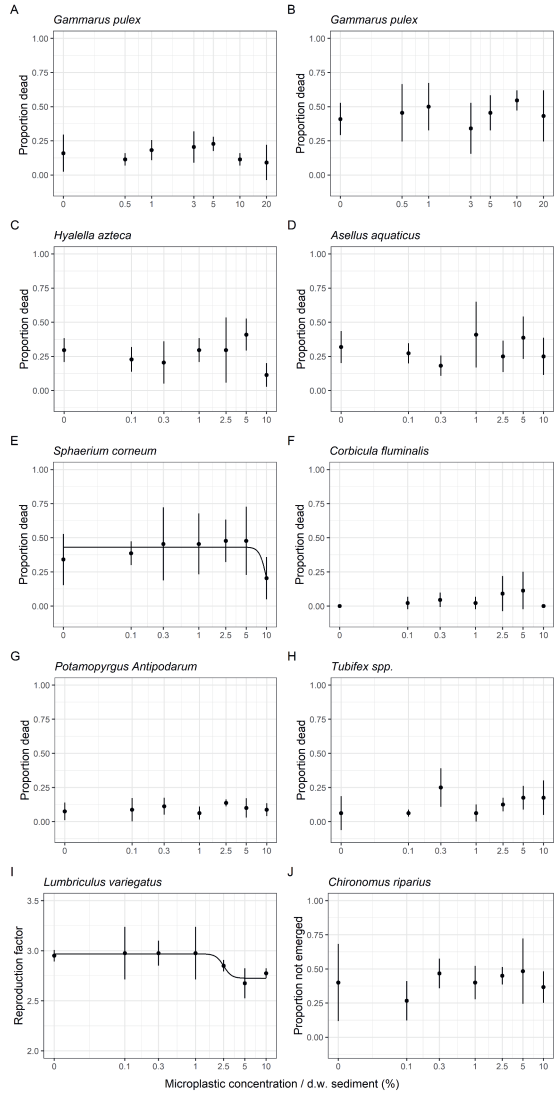

Figure 1 Mean mortality (± s.d.) expressed in proportion (dead/start population) of *G. pulex* exposed to PS (A), *G. pulex* exposed to ERMP (B) with concentrations up to 20% in d.w. sediment. *H. azteca* (C), *A. aquaticus* (D), *S. corneum* (E), *C. fluminalis* (F), *P. antipodaru*m, (G), *Tubifex spp.* (H) exposed to ERMP + PS with concentrations up to 10% in d.w. sediment. Mean reproduction factor (± s.d.) of *L. variegatus* (I) exposed to ERMP + PS and mean total emergence (± s.d.) of *C. riparius* (J) exposed to ERMP + PS. Exposure time for all freshwater species was 28 days. A three and a four parameter log-logistic dose response model were plotted for *S. corneum* (E) and *L. variegatus* (I), respectively. Concentrations are on a log scale. The zero concentration has been converted to 0.01 to allow plotting on the log scale.

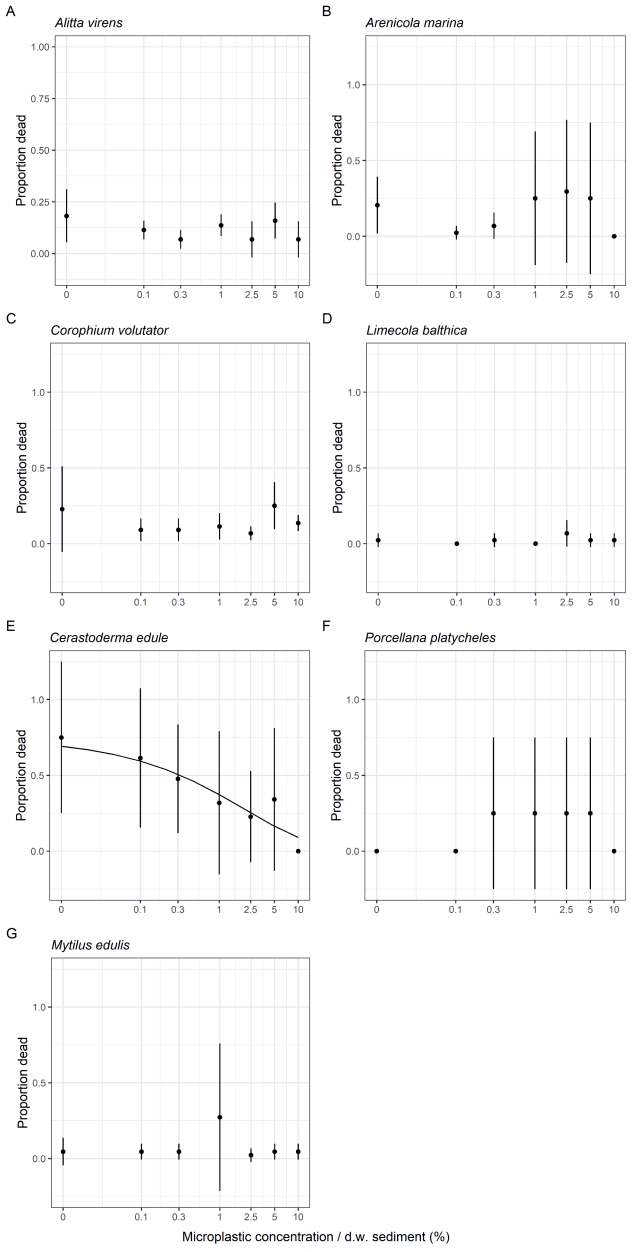

Figure 2 Mean mortality (± s.d.) expressed in proportion (dead/start population) of *A. virens* (A), *A. marina* (B), *C. volutator* (C), *L. balthica* (D), *C. edule* (E), *P. platycheles* (F), *M. edulis* (G) exposed to ERMP + PS with concentrations up to 20% in d.w. sediment. Exposure time for all marine species was 28 days. A three-parameter Weibull (type 1) model was plotted for *C. edule* (E). Note that concentrations are on a log scale, additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

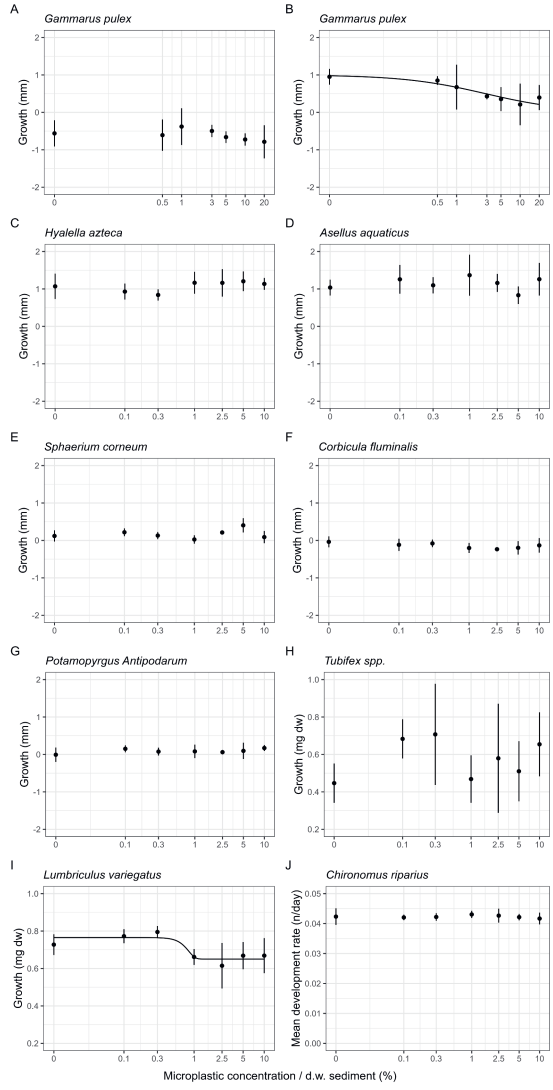

Figure 3 Mean growth (± s.d.) expressed in length (mm) of *G. pulex* exposed to PS (A), *G. pulex* exposed to ERMP (B) with concentrations up to 20% in d.w. sediment. Mean growth (± s.d.) expressed in mm or mg of *H. azteca* (C), *A. aquaticus* (D), *S. corneum* (E), *C. fluminalis* (F), *P. antipodaru*m, (G), *Tubifex spp.* (H) and *L. variegatus* (I) exposed to ERMP + PS with concentrations up to 10% in d.w. sediment. Mean development rate (± s.d.) of *C. riparius* (J) exposed to ERMP + PS with concentrations up to 10% in d.w. sediment. Exposure time for all freshwater species was 28 days. A two parameter log-logistic dose response model were plotted for *G. pulex* exposed to ERMP -PS (B), respectively. A four-parameter Weibull (type 1) model was plotted for *L. variegatus* (I). Note that concentrations are on a log scale, additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

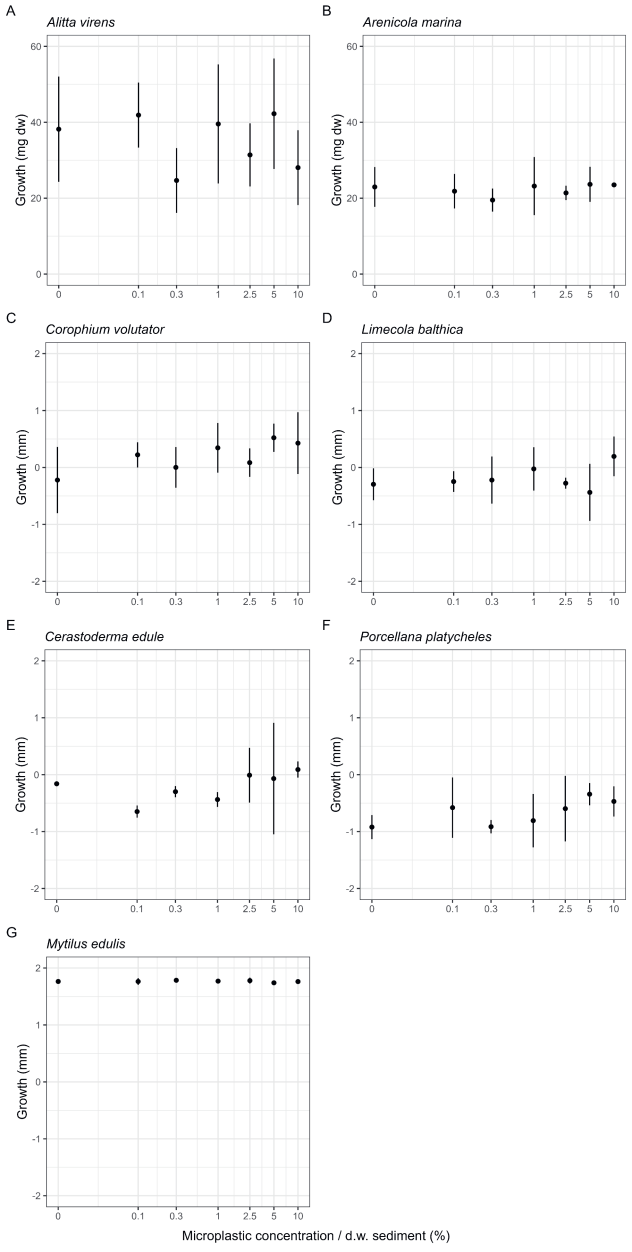

Figure 4 Mean growth (± s.d.) expressed in either length (mm) or weight (mg) of *A. virens* (A), *A. marina* (B), *C. volutator* (C), *L. balthica* (D), *C. edule* (E), *P. platycheles* (F), *s* (G) exposed to ERMP + PS with concentrations up to 20% in d.w. sediment. No statistically significant effect thresholds were found. Exposure time for all marine species was 28 days. Note that concentrations are on a log scale, additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

For the marine and freshwater species *H. azteca*, *A. aquaticus*, *S. corneum*, *C. fluminalis*, *P. Antipodarum*, *Tubifex spp., C. riparius, A. Virens*, *A. marina, C. volutator*, *C. edule, P. platycheles* or *M. edulis* no statistically significant effect thresholds were found after chronic exposure to ERMP with concentrations up to 10% d.w. in sediment for growth (Figure 3, 4) (Table S9, S10). For *L. balthica* a significant effect threshold was found ($p = 0.002$), however the dose response curve was not significant as was also apparent by visual inspection ($p_{noEffect} = 0.126$). Additionally, for *G. pulex*, exposed to PS fragments, a significant effect threshold was found ($p = 0.006$), however the dose response curve was not significant ($p_{noEffect} = 0.332$). For *G. pulex*, a reduction in growth was observed after chronic exposure to ERMP without PS-fragments sediment ($EC_{50} = 2.85 \pm 1.39$ %, $p = 0.050$) with concentrations up to 20% in d.w. (Table S9; Figure 3; Table 1). Note that this effect threshold is not fit for regulatory purposes as the control mortality was too high (40.9%). Additionally, for *L. variegatus* a statistically significant adverse effect was found for growth ($EC_{50} = 0.77 \pm 0.14$ %; $p = 0.007$; $p_{noEffect} = 2.32 \times 10^{3}$; Table S9; Figure 3; Table 1). Finally, no effect threshold was found for feeding rate of *G. pulex* after 28-day exposure to PS fragments. For the feeding rate of *G. pulex* exposed to ERMP without PS fragments a significant effect threshold was found ($p = 2.16 \times 10^{-16}$). However, the effect threshold concentration was not physically realistic, moreover the dose-response curve was not significant ($p_{noEffect} = 0.980$) as was also apparent by visual inspection (Table S9, Figure S8).

Our findings of *G. pulex* exposed to ERMP without PS are in accordance with the study done by Redondo-Hasselerharm et al. (2018b). Also here a reduction of growth on *G. pulex* was reported after exposure to PS, with a similar effect threshold (EC_{50} =3.57%). As discussed previously, almost identical systematic approaches were used, however, in the present study we used a more commonly occurring sediment with a lower TOM content and an even higher diversity of microplastic particles. The masking hypothesis previously mentioned does however not seem to apply to *G. pulex* from the study by Redondo-Hasselerharm et al. (2018b), suggesting a certain species-specific trait. Of all the species tested and considering the results of the various tests done with *G. pulex*, this species seems to be the most sensitive to adverse effects of microplastic (Table 1).

For *L. variegatus* a significant negative effect was found for growth ($EC_{50} = 0.77 \pm 0.14$ %, $p = 0.007$). This is in contrast to the finding reported by other studies (Redondo-Hasselerharm et al., 2018b; Silva, C. J. et al., 2021). For instance, Silva *et*

al. (2021) reported on sub-organismal effects such as depletion of energy reserves of *L. variegatus*, however, no adverse effects were found on biomass when exposed to only irregularly-shaped PE. The differences in effects are likely due to the different types, shapes, sizes and aging processes of the microplastics used. Again, to be conclusive about the latter, the ingestion of ERMP by *L. variegatus* would need to be studied.

No differences on feeding activity in the studies done for *G. pulex* (exp 1 and 2) were found after 28 day exposure to ERMP. This is similar to the results found in previous chronic exposure studies (Redondo-Hasselerharm et al., 2018b; Weber et al., 2018). Although testing solely PS and PET respectively, these studies also did not find any effect on the feeding rate of *G. pulex*. These results show that the reduction in growth cannot be explained by the reduction in food uptake as this stays constant throughout treatment concentrations (Redondo-Hasselerharm et al., 2018b). Moreover, Straub *et al*. (2017) showed that after chronic exposure to polymethylmethacrylate (PMMA) and polyhydroxy butyrate (PHB) in *Gammarus fossarum,* the assimilation efficiency and wet weight gain decreased, however the feeding rate was not affected. It is likely that the decrease in growth can be explained by the mechanism inhibition of food assimilation and/or decreased nutritional value of food, but also obstruction in the gut (de Ruijter et al., 2020; Straub et al., 2017).

Table 1. Overview of detected EC_{50} and EC_{10} (± S.E.) threshold effect concentrations [a)]

Organism	Aquatic environment	Endpoint	Adverse effect	Type of drc [a] fitted	EC_{10} ± S.E.%	EC_{10} microplastics / kg dw sediment	EC_{50} ± S.E.%	EC_{50} microplastics / kg dw sediment	p-value effect threshold- [b)]	p-value log-likelihood ratio test
Gammarus pulex (ERMP-PS) [c)]	Freshwater	Growth	yes	LL.2	0.11 ± 0.17	6.48E+06	2.85 ± 1.39	1.68E+08	**0.050***	**6.28E-04*****
Sphaerium corneum	Freshwater	Mortality	no	LL.3	7.67 ± 3.41	4.52E+08	9.88 ± 0.68	5.82E+08	**2.0e-16 *****	**0.013***
Lumbriculus variegatus	Freshwater	Reproduction factor	yes	LL.4	1.90 ± 1.08	1.12E+08	2.51 ± 0.44	1.48E+08	**0.002****	**0.009****
Lumbriculus variegatus	Freshwater	Growth	yes	W1.4	0.49 ± 0.68	2.88E+07	0.77 ± 0.14	4.53E+07	**0.007 ****	**2.32E-03****
Cerastoderma edule	Marine	Mortality	no	W1.3	0.021 ± 0.027	1.24E+06	1.01 ± 0.45	5.95E+07	**0.003 ****	**3.22E-15*****

[a)] The dose-response curve package in R provides the following models: Weibull type I model (W1.x) and log logistic (LL.x), with x giving the number of parameters fitted.

[b)] Significant findings ($p < 0.05$) are highlighted in bold. Significance codes: <0.0001 '***', < 0.001, '**' < 0.01, '*' < 0.05.

[c)] For *G. pulex* exposed to ERMP-PS a high mortality of 40.9% was found in the control. The effect thresholds for this species therefore is fit for regulatory purposes.

3.3. Different responses to microplastic exposure for groups of species with different feeding traits.

We also tested whether the response of species to an increasing dose of microplastics is different for groups of species with different feeding traits. We distinguished four types of main feeding traits: filter feeders, sediment/deposit feeders, sediment grazers and facultative deposit feeders (Table S12). For mortality, no significant differences were found, except for the group of species feeding in the water column (filter feeders). Increasing exposure to microplastics has a greater effect on organisms exposed to water than on organisms in any of the other feeding guilds (GLMM, $p < 3.90 \times 10^{-5}$; Table S13a-d). Here too *Cerastoderma edule* falls within the deviant group, a species whose mortality decreases with increasing concentration. Again, we can only speculate about possible explanations for the aberrant exposure from the aqueous phase. One possible explanation is that species that feed on the water above the sediment have a higher general sensitivity to particulate matter than species that are naturally more closely associated with sediment because that is their natural habitat.

4. Limitations and recommendations for future work

Here we have provided 17 dose-response relationships consisting of 5 effect threshold concentrations for 16 freshwater and marine benthic macroinvertebrates species using an environmentally relevant mixture of microplastic particles, demonstrating that this can be done under the strictest methods of quality assurance. Nevertheless, some species (*Gammarus pulex*, *Hyalella azteca*, *Asellus aquaticus*, *Sphaerium corneum* and *Cerastoderma edule*) exhibited high mortality rates in their control groups, rendering these results less suitable for regulatory considerations. Although these results have given us valuable insight, we suggest that these particular species be re-evaluated in future studies.

Earlier studies did not test such a wide range of species under the same conditions, were less reliable, and/or were less fit for purpose concerning risk assessment (de Ruijter et al., 2020). Rescaling and alignment methods

have been developed to correct for differences among studies that target different microplastic size ranges, densities, or shape categories (Koelmans et al., 2020; Kooi et al., 2021). However, the present effect data would require no or only a very limited level of corrections because microplastic particles were tested that were already close to those occurring in (aquatic) environmental samples. This enables direct use of the present data in risk assessment, without the need for mathematical alignments. Furthermore, for the present microplastic mixture used, probability density functions (PDFs) for shape, size, and density were provided nevertheless, thus still allowing alignments to microplastic exposure conditions that would deviate from those represented by our tested mixture. We propose that PDFs are always provided when microplastic particles are used in tests so that users can translate test results into other conditions. Examples of how the results of these types of complex exposures can be applied to risk assessments have been outlined in recent literature (Mehinto et al., 2022; Redondo-Hasselerharm et al., 2023).

We did not find statistically significant effects on mortality or emergence for the freshwater and marine species *G. pulex*, *H. azteca*, *A. aquaticus*, *C. fluminalis*, *P. Antipodarum*, *Tubifex spp., C. riparius, A. Virens*, *L. balthica*, *C. volutator*, *A. marina, P. platycheles* or *M. edulis* (Table S7, S8). Additionally, chronic exposure to ERMP with concentrations up to 10% d.w. in sediment caused no significant effect on growth for the marine and freshwater species *H. azteca*, *A. aquaticus*, *S. corneum*, *C. fluminalis*, *P. Antipodarum*, *Tubifex spp., C. riparius, A. Virens*, *A. marina, C. volutator*, *L. balthica* , *C. edule, P. platycheles or M. edulis* (Table S9, Table S10). Ideally, Species Sensitivity Distributions used for ecological risk assessment contain data on endpoints that are directly relevant to the population level, like reproduction, mortality, and growth. Here, we only considered such relevant endpoints, which implies that it is legitimate to speculate on implications for risks of microplastic in aquatic sediments based on our data. We detected number concentration effect thresholds EC_{10} for adverse effects for two freshwater species; 6.48×10^{6} #/kg (growth of *G. pulex* exposed to ERMP without PS) and

2.88×10^7 and 1.12×10^8 #/kg (growth and reproduction of *L. variegatus*). When comparing these effect threshold concentrations to rescaled measured environmental concentrations (MEC) for Liangfeng River sediments in China of 2.21×10^8 #/kg d.w. (Redondo-Hasselerharm et al., 2023; Xia et al., 2021) or for Menomonee river sediments in the USA of 1.73×10^8 #/kg (Lenaker et al., 2019; Redondo-Hasselerharm et al., 2023), it becomes apparent that risks would be indicated for *G. pulex* and *L. variegatus* and sufficiently similar species if our test conditions would be sufficiently representative of *in situ* exposure conditions.

Interestingly, we detected positive effects of microplastic on habitat quality for two species; *C. edule* and *S. corneum*, besides negative effects for several other species. The simultaneous existence of effect mechanisms that lead to a decline of the population density for some species and to a lesser decrease in population density for some other species has crucial implications for understanding the effects of microplastic on the community level. Previously, an analysis of community effects after long-term exposure to nano- and microplastic has revealed that the abundance of some species decreased whereas the abundance of other species increased, indicating either direct or indirect positive effects on the species level (Redondo-Hasselerharm et al., 2020). Another example of this is that the increase in microplastic concentrations in the oceans has reduced substrate limitation for oviposition for the pelagic insect *Halobates sericeus* (Goldstein et al., 2012), which also constitutes a positive effect on the population level. Additionally, Canniff and Hoang (2018)), showed in an algal experiment that microplastics enhanced the growth of *Raphidocelis subcapitata* and suggested that when ingested by *Daphnids* could provide a possible food source. Recently, Amariei et al. (2022) reported a negative effect of microplastic ingestion by *Daphnids* due to food dilution, however, a positive effect on the population was observed when that same microplastic was carrying a nutritious biofilm, which is the default situation in nature. Obviously, these observations should not be taken as a reason to scale down the problem of microplastic particles in the environment.

However, a thorough understanding of multifarious effects including negative as well as positive population effects is required to be able to understand and model secondary effects on the food web and ecosystem level (Kong and Koelmans, 2019).

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Supporting Information

The supporting information can be found online at:
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Chapter 4

Trait-based bioavailability of microplastics for benthic macroinvertebrates

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Manuscript in preparation

Abstract

Since effect and bioaccumulation tests have so far mainly used monodisperse microplastics while natural microplastics are diverse, there is no accurate understanding of the actual biological availability of microplastics. Therefore, risk assessments based on that test data are questionable. In this study, we investigate the species-specific uptake of an environmentally realistic microplastic mixture for 11 species. The species specificity is demonstrated by describing the ingested particles as a continuum, quantified through continuous size distributions. Differences are statistically tested, both among species data and in comparison to distributions for the controls. The quantity and type of ingested particles are determined based on a polymer and size-specific blank correction procedure. The results indicate that adverse effects like reduced growth and reproduction, previously observed in *Lumbriculus variegatus* after exposure to environmentally relevant microplastics (ERMP), can be causally linked, as microplastics have been detected in the species' whole body tissue samples. We discovered that only a select portion of ERMP is bioavailable for *Asellus aquaticus*, *Corbicula fluminalis*, *Corophium volutator*, *Hyalella azteca*, *Lumbriculus variegatus*, *Limecola balthica*, *Mytilus edulis* and *Tubifex* spp.. More specifically, the majority of particles detected in organisms ranged from 5.5 to 27.7 µm, but differed in polymer composition, with 5.5 µm also being the method's lower size detection limit. The abundance of egested ERMP significantly exceeded that retained in the tissue samples, indicating organisms can egest most ingested microplastics within a 24 h depuration period. The lowest number of ERMP was found in tissue and egestion samples of *Chironomus riparius* and *Potamopyrgus antipodarum* respectively, averaging <1 particle/organism. The highest egested ERMP count was for *M. edulis*, at 49.9 particles/organism, primarily polystyrene. Similar size distributions were found in whole body samples amphipods *A. aquaticus* and *H. azteca*, which are both sediment grazers/scavengers. Also, similarities were noted between the size distributions of freshwater and marine clams *C. fluminalis* and *L. balthica*, both facultative deposit feeders. The particle size and species-specific ingestion patterns highlighted here offer a critical foundation for understanding the relevance of effect mechanisms like particle volume-based food dilution.

1. Introduction

A large portion of microplastics research has concentrated on the biomonitoring of microplastics across a broad range of freshwater and marine organisms (Gouin, 2020; Hermsen et al., 2018; Wesch et al., 2016). Meanwhile, laboratory studies have primarily examined the ingestion of microplastics, often using monodisperse particles that may not realistically represent environmental conditions (de Ruijter et al., 2020). For ecotoxicological studies no standard protocols have been widely accepted to identify microplastics. While most ecotoxicological studies (78.1%) include a description verifying that microplastics have been ingested by test organisms, verification is often (72.4%) demonstrated in either a separate experiment, assessed qualitatively, visually, or without a digestion step (de Ruijter et al., 2020). The number of studies that qualitatively assesses and thus causally links observed effect to actual exposure data is limited (de Ruijter et al., 2020). However to be able to understand and interpret effect data in the context of hypothesized effect mechanisms, it is important to determine which fraction of the diverse environmental mixture of particles is available for organisms. For instance, when observing effects like a reduction in growth or survival, it is relevant to measure the ingested microplastic particles to determine if the observed effect is due to the mechanism of food dilution caused by the co-ingestion of low-caloric, nondigestible microplastics. Furthermore, it is essential that the metric used to quantify the effect is ecologically relevant and identical to the metric used for quantifying exposure. Microplastics, given their species- and particle-specific effect mechanisms, may have several such environmentally relevant metrics (ERMs). Research indicates that in scenarios of food dilution, the volume of ingested particles serves as the ERM (Koelmans et al., 2022b; Thornton Hampton et al., 2022b). Conversely, in instances where effects are triggered by translocation, the collective surface area of bio accessible particles becomes the ERM (Koelmans et al., 2022b). Thus, it is the effect mechanism that dictates the interaction between microplastic particles and organisms, as well as the proper method for assessing actual exposure (Koelmans et al., 2022b).

Effect studies often focus on narrow segments of the microplastics continuum, usually examining monodisperse pristine particles that inadequately represent the heterogeneous mixture found in natural environments (de Ruijter et al., 2020). This segmented approach, poses challenges in translating research findings into meaningful insights for risk assessment. Although alignment methods have been

developed and applied to address this issue (Koelmans et al., 2023a; Koelmans et al., 2020; Kooi et al., 2021; Redondo-Hasselerharm et al., 2023; Redondo-Hasselerharm et al., 2024), they involve complex calculations that introduce additional uncertainties. Ideally, effect studies should therefore examine microplastics that closely mirror the environmental continuum in terms of size, shape, and polymer type (Koelmans et al., 2022b; Koelmans et al., 2020). By testing a representative mixture of microplastics, researchers can more accurately determine the bioavailable fraction, acknowledging that the total substance concentration often lacks toxicological relevance (Posthuma et al., 2001). This comprehensive approach not only enhances the ecological relevance of effect studies but could also facilitates a more nuanced understanding of the environmental impact of microplastic pollution by unravelling the intricacies of species-specific responses to microplastics. For instance, non-selective feeders such as sediment feeders might struggle to avoid microplastics due to their feeding strategy, rendering them more susceptible to ingestion. Conversely, these organisms may have adaptations to cope with the presence of non-nutritious particles in their environment, potentially reducing the adverse effects of microplastic exposure.

In a previous study by de Ruijter et al., (2023), a total of 16 benthic marine and freshwater species with diverse feeding habits – including filter feeders, sediment/deposit feeders sediment grazers/scavengers, and facultative deposit feeders-, were exposed to environmentally relevant microplastics (ERMP) to quantify effect thresholds (**Chapter 3**). Subsequently, the present additional study was initiated that involved collecting and storing samples for microplastic analysis from the biota tissues and the egestion samples for each species.

In this study, we aim to measure the bioavailable fraction of ERMP mixture for 11 selected freshwater and marine benthic species with diverse feeding traits. To achieve this aim, we employed whole-body digestions using enzymatic purification protocols modified from previous studies (Löder et al., 2017; Pan et al., 2021). Additionally, we adhered to strict quality assurance and quality control (QA/QC) criteria, following Hermsen et al. (2018).

2. Material & Methods

2.1. Environmentally relevant microplastic (ERMP) mixture

Samples selected for the detection of ERMP originated from single-species bioassays with organisms that had been exposed to sediment-microplastic mixtures of up to 10.0 % dw sediment (5.9×10^8/kg of dw sediment). For details, the reader is referred to de Ruijter et al. (2023; **Chapter 3**). The ERMP mixture consisted of particles of varying polymer type, size, shape and colour, and was created in the laboratory in proportions that match those occurring in the environment (Kooi and Koelmans, 2019; Kooi et al., 2021). The weight distribution of polymers used to create the microplastic mix consisted of irregular polyethylene (PE) fragments (34%), irregular polypropylene (PP) fragments (15.9%), PP fibres (10.5%), irregular Polyethylene terephthalate (PET) fragments (20.6%), and irregular polystyrene (PS) fragments (19%). Moreover, the ERMP test mixture had a size range between 9 and 5386 µm and a power law slope α (α = 3.28 ± 0.02) equal to the slope found for microplastic mixtures in freshwater sediments (α = 3.25 ± 0.19)(Kooi et al., 2021). A detailed description of the preparation and characterization of the ERMP test mixture is provided in (de Ruijter et al., 2023; **Chapter 3**).

2.2. Sample preparation enzymatic purification

After exposure (see de Ruijter et al., 2023), all organisms were placed in clean water to enable depuration of their gut for 24 hours. Subsequently, both egestion samples and the whole body tissues, hereafter referred to as “tissue samples”, of organism samples were stored separately at −20 °C for later microplastic analysis. For each of the organisms samples (n=3) from the highest exposure concentration were selected. The amount of organisms per samples varied from 7 to 55 individuals (Table S1). The tested species included the freshwater species *Asellus aquaticus*, *Hyalella azteca*, *Corbicula fluminalis*, *Tubifex* spp., *Lumbriculus variegatus*, *Chironomus riparius*, *Potamopyrgus antipodarum*, *Sphaerium corneum*, and the marine species *Limecola balthica*, *Mytilus edulis*, and *Corophium volutator*.

The tissue or dissected soft tissue of *A. aquaticus*, *H. azteca*, *C. fluminalis*, *Tubifex* spp., *L. variegatus*, *C. riparius*, *L. balthica* and *C. volutator,* and the egestion samples of *A. aquaticus*, *H. azteca*, *C. fluminalis*, *P. antipodarum*, *Tubifex* spp., *S. corneum*, *M. edulis*, *L. balthica* and *C. volutator* were digested following an enzymatic purification protocol modified from previous studies (Löder et al., 2017)(Table S2).

Generally the digestion treatments began and ended with a hydrogen peroxide step and involved the following process: the sample was immersed in the appropriate solution, loosely covered with aluminium foil, and placed in drying chamber (Binder, GmbH) at either 37 or 40 °C. When transitioning from one treatment to the next, the sample was filtered using a 10 µm aluminium filter and rinsed thoroughly with milli-Q water and ethanol. Afterwards, the aluminium filter was placed into a new clean glass beaker and submerged in a new liquid. Depending on the class of organisms, different enzymes were used: protease A-01 (EC 3.4.21.62, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany), chitinase (EC 3.2.1.14, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) and lipase FE-01 (EC 3.1.1.3, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany)(Löder et al., 2017)(see Table 1). Crustaceans, consisting of *A. aquaticus*, *H. azteca*, *C. volutator* and *C. riparius*, which possess a chitin exoskeleton, were treated with chitinase. *Tubifex* spp. and *L. variegatus* underwent digestion with protease. After dissection, the shellfish species *C. fluminalis*, *P. antipodarum*, *S. corneum*, *L. balthica* and *M. edulis* were digested with both protease and lipase. For the egestion samples, only hydrogen peroxide (30%) was used (Table 1). Additionally, buffers such as tris(hydroxymethyl)-aminomethane (Tris), HCl 1 M buffer (pH5) and sodium acetate (NaOAc) 1 M buffer (pH9) were added to optimize enzyme activity. The digestion treatments and conditions were chosen based on evidence from previous work that microplastics would not be affected (Löder et al., 2017). For the final step of sample preparation, the solution was filtered on to an Anodisc filter (diameter 25 mm, pore size 0.02 µm, Whatman, U.K.). The Anodisc filter was then transferred to a glass Petri dish and placed in a closed drying chamber (Binder, GmbH) at 37 °C for at least two days.

2.3. Microplastic identification

Anodisc filters were placed on Calcium fluoride (CaF2) crystal windows and were analysed using an FTIR microscope (Cary 620/670 Agilent). The samples were analysed in transmission mode using a 15x objective lens and an FPA detector. The size limit, determined by the pixel size of the FTIR imaging, was 5.5 µm. Subsequently, FTIR data were analysed with siMPle, and MPAPP software (Primpke et al., 2020; Primpke et al., 2019; Primpke et al., 2017; Primpke et al., 2018). The threshold values for identifying polymer types underwent manual spectral evaluation based on a subset of samples. Specifically, the spectral threshold for polyester was optimized from 600 to 1050 cm^{-1}. Additionally, an organism-specific

threshold was optimized from 600 to 1100 cm^{-1} for polystyrene in the samples of *L. balthica.* In some cases, FTIR imaging included the outer edges of the Anodisc filter, which consists of polypropylene. Using the software Origin, these corners were excluded from further analysis.

2.4. Quality assurance and quality control (QA/QC) standards

All materials, equipment and laboratory surfaces were thoroughly rinsed with milli – Q and ethanol. All solutions used were filtered through a 10 µm aluminium filter and then transferred to glass bottles. Furthermore, all handling of samples took place in a laminar flow cabinet to minimize contamination risks. To further prevent contamination, 100% cotton lab coats were utilized. Each sample included a pre-processing digestion step, and was subsequently analysed using Fourier transform infrared (FTIR) spectroscopy to determine width, length and polymer identification. Moreover, negative (n = 14) and positive controls (n = 6) were used either in parallel with or prior to the procedures, respectively. The positive controls were spiked with green fluorescence polyethylene beads of approximately 90 µm, and had a recovery of 75.8 ± 18.4 (n = 6).

2.5. Data analysis

The data sets originating from the MPAPP software were cleaned by removing spectral hits for non-microplastic materials such as animal fur, plant fibres, sand, chitin, charcoal and coal. The data was checked for normality through visual inspection with a qqplot and with the formal statistical shapiro Wilkinson test. Given that the data were not normally distributed, the two-sample Wasserstein test was used to determine if two distributions were likely to originate from the same probability distribution (Vaserstein, 1969). Our reasoning is that if the distribution significantly differs between the blank and the sample, or among samples, or between the ERMP mix to which the organisms have been exposed and what is recovered, then species-specific uptake has been demonstrated. Moreover, even if the distribution difference is not significant, there can still be uptake if the amount of particles absorbed significantly deviates from the amount in the blank. Therefore, we tested the differences between the blanks and the treatments both in terms of the cumulative distribution of particle sizes, and in terms of the abundance of particles per polymer and size category (see below). The Wasserstein test calculates the area between two empirical cumulative distributions functions (ECDFs) and provides a measure of dissimilarity between distributions (equation 1).

The p-value was calculated by randomly resampling from the combined dataset of the two samples with the same size using the function *wass_test* from the R package "*twosamples*", where bootstraps were set at 10,000 (Dowd, 2020; Vaserstein, 1969). The Wasserstein distance is then calculated between the ECDFs of the original samples and the bootstrapped ECDFs generated from the resampled datasets. The two-sample Wasserstein tests were performed for comparing particle size distributions between blanks (controls), ERMP and ERMP found in tissue and egestion samples per organism, as well as among samples from different organisms. All two- sample Wasserstein tests were performed for the mixtures of ERMP and at the individual polymer levels of PE, PP, PET and PS. To decrease the false discovery rate (FDR), the Benjamini-Hochberg (BH) procedure was used to adjust p-values (Benjamini and Hochberg, 1995; Jafari and Ansari-Pour, 2019).

$$wass = \int_{-\infty}^{\infty} |\hat{F}(x) - \hat{E}(x)| dx \quad (1)$$

After visualizing and inspecting data, blank corrections were made based on polymertype and size. For this, datasets were first corrected for unequal sample sizes, as the sample effort needs to be comparable. This was done by cloning datasets with the least common multiple (LCM) to keep datasets "integer". Consequently, for each polymer and both tissue and egestion sample types were set at the same number of midpoints at 50, where after the amount of particles detected in blanks were subtracted from samples for each size and polymertype (Figure S1, S2, S3, S4).

3. Results & Discussion

Analysing microplastic size distributions from the perspective of the organism could offer essential insights. The likelihood of microplastic ingestion varies with the size of the organism's mouth opening, the organism's size and its feeding traits. Therefore, particles of certain sizes are more likely to be ingested. As particles approach or exceed the maximum ingestible size, their probability of being ingested decreases. Although overall the amount of particles will decrease when particle size increases, feeding traits can influence the selection of microplastics from the ERMP microplastic continuum, leading to unique size distributions.

3.1. Bioavailability of ERMP as a whole as measured in tissue samples

Here we focused on the selectivity of the uptake of microplastic particles based on their size, excluding polymer type considerations. The ERMP mixture showed a distinct size distribution compared to the (negative) controls (W = 57.57, p = 0.002), suggesting the ERMP mixture's detectability in tissue samples. Significant differences in size distributions in the tissue samples, when compared to the ERMP mixture, were observed for *A. aquaticus* (W = 40.76, p = 0.033), *C. fluminalis* (W = 72.22, p = < 0.001), *L. variegatus* (W = 67.78, p = < 0.0001), *L. balthica* (W = 78.96, p = 0.042) and *Tubifex* spp. (W = 47.81, p = 0.001), indicating that only a part of the ERMP mixture is bioavailable for these organisms (Figure 1; Table S3). The size range of the ERMP mixture spans from 9 to 5386 µm, with particles predominantly concentrated around a median of 68 µm, and an average size of 87.3 µm. In contrast, these organisms typically showed a median ingestible size of 5.5 µm. Interestingly, the minimum size of previously characterized ERMP particles is larger than that of the particles detected in the tissue, which could be explained by the fragmentation of particles by the organisms after ingestion (Mateos-Cárdenas et al., 2020).

The microplastics detected in *C. volutator* (W = 9.81, p > 0.05) and *H. azteca* (W = 12.71, p > 0.05) exhibited no significant differences in size distributions compared to the controls, suggesting that a part of the microplastics detected here might be attributable to contamination and were not a result of ingestion. However, significant differences in microplastic size distributions compared to the control were observed in *A. aquaticus* (W = 19.96, p = 0.002), *C. fluminalis* (W = 18.09, p = < 0.0001), *C. riparius* (W = 28.85, p = < 0.0001), *L. variegatus* (W = 15.33, p = < 0.0001), *L. balthica* (W = 21.39, p = 0.008) and *Tubifex* spp. (W = 13.37, p = 0.011), indicating that the presence of particles in these instances is not likely due to contamination (Figure 1; Table S3).

Both freshwater worms, i.e., *L. variegatus* and *Tubifex* spp. (W = 23.98, p = < 0.0001) showed significant different size distributions of microplastics detected in their tissue samples (figure 1; table S3). Although *Tubifex* spp. is a smaller worm, relatively more larger particles were detected than in *L. variegatus*. Particles of up to approximately 140 µm where detected in *Tubifex spp.*, which aligns with the previously reported maximum of 130 µm (Juget, 1979). In accordance with the previously reported ingestible size, most particles ingested by *L. variegatus* are

smaller than 250 µm (Silva, C. J. et al., 2021). There is however, also a relatively small number of particles that exceeds 1000 µm, indicating that some microplastics found were more likely to have been attached to the outside of the body (figure 1).

Interestingly, *L. balthica* and *C. fluminalis* (W = 7.43, $p = p > 0.05$), which are marine and freshwater clams respectively, displayed similar size distributions, indicating that their shared trait as facultative deposit feeders may influence the size distribution of the ingested material. Furthermore, the deposit feeder *L. variegatus* also showed similar size distributions of microplastics to both the marine and freshwater clams (W = 11.18, $p > 0.05$) (W = 5.40, $p > 0.05$) (Figure 1; Table S3). No significant differences in size distributions were detected between *C. volutator* and *C. riparius* (W = 25.32, $p > 0.05$), both of which are crustaceans. The ingested ERMP particles detected in *C. riparius* exceeded the previously reported maximum ingestible size of 135 µm. ERMP particles detected in *H. azteca* and *A. aquaticus*, both sediment grazers/scavengers, showed similar size distributions (W = 11.37, $p > 0.05$), with two peaks present at 5.5 and approximately 50 µm (Figure 1; Table S3). For both species, the maximum ingestible sizes were exceeded, consisting of 140 µm for *A. aquaticus* and 112 µm for *H. azteca (Moore, 1975; Redondo-Hasselerharm et al., 2023; Schmitz and Scherrey, 1983).*

3.2. Bioavailability of polymer-specific ERMP particles as detected in tissue samples

In addition to analysing the ERMP mix as a whole, we also looked at the uptake per polymer, namely PET, PE, PP, and PS. No significant differences were detected in the size distributions of PET particles between controls and organisms *Tubifex* spp., *C. volutator*, *H. azteca* or *A. aquaticus*. Most observations of PET were detected in the controls, where 62 particles were detected. In contrast, only 3, 1, 1 , 32 and 5 PET particles were detected in *A. aquaticus, C. fluminalis*, *C. riparius*, *C. volutator* and *H. azteca*, respectively (Table S4). This indicates that the PET particles detected in these samples are likely due to contamination (Figure 2; Table S4). Moreover, no PET particles were detected in *L. variegatus*, *L. balthica*, *C. fluminalis* and *C. riparius.* Additionally, there were no observed differences in the size distributions of PET particles among the organisms (Figure 2; Table S4).

No significant differences were detected in the size distributions of PE particles between controls and organisms *Tubifex* spp., *L. balthica*, *C. riparius*, *C. volutator*, *C. fluminalis*, *H. azteca* or *A. aquaticus*. With 177 PE particles detected in the

controls and 91, 5, 57, 5, 24, 17 and 56 respectively in the organisms, it is likely that these can be attributed to contamination (Figure 2; Table S5). The only significantly different size distributions of PE particles from the control were detected for the organism *L. variegatus* (W = 12.28, p = 0.014). Although the shapes of the size distributions appear similar, most of the 952 PE particles detected in *L. variegatus* were centered at the 5.5 µm size, compared to 177 PE particles in the control. Moreover, differences in size distributions were detected between *L. variegatus* and *A. aquaticus* (W = 25.14, p = 0.012), and *L. variegatus* and *C. riparius* (W = 29.80, p > 0.002), and *L. variegatus* and *Tubifex* spp. (W = 23.73, p < 0.0001). No differences were detected in the size distributions of detected PE particles among the other organisms (Figure 2; Table S5).

No significant differences were detected in the size distributions of PP particles detected in controls and organisms *Tubifex* spp., *L. variegatus*, *L. balthica*, *C. fluminalis*, *C. volutator*, *H. azteca* or *A. aquaticus*. This suggests that at least part of the PE particle detected in these samples likely results from contamination (Figure 2; Table S6). The only significant difference in size distributions for PE particles compared to the control was detected for the organism *C. riparius* (W = 60.65, p < 0.004), where the size distributions shows a distinct peak around 300 µm, while usually the maximum ingestible size is found to be around 135 µm (Redondo-Hasselerharm et al., 2023; Silva, Carlos JM et al., 2021). No differences were detected in the size distributions of PP particles detected among the other organisms (Figure 2; Table S6).

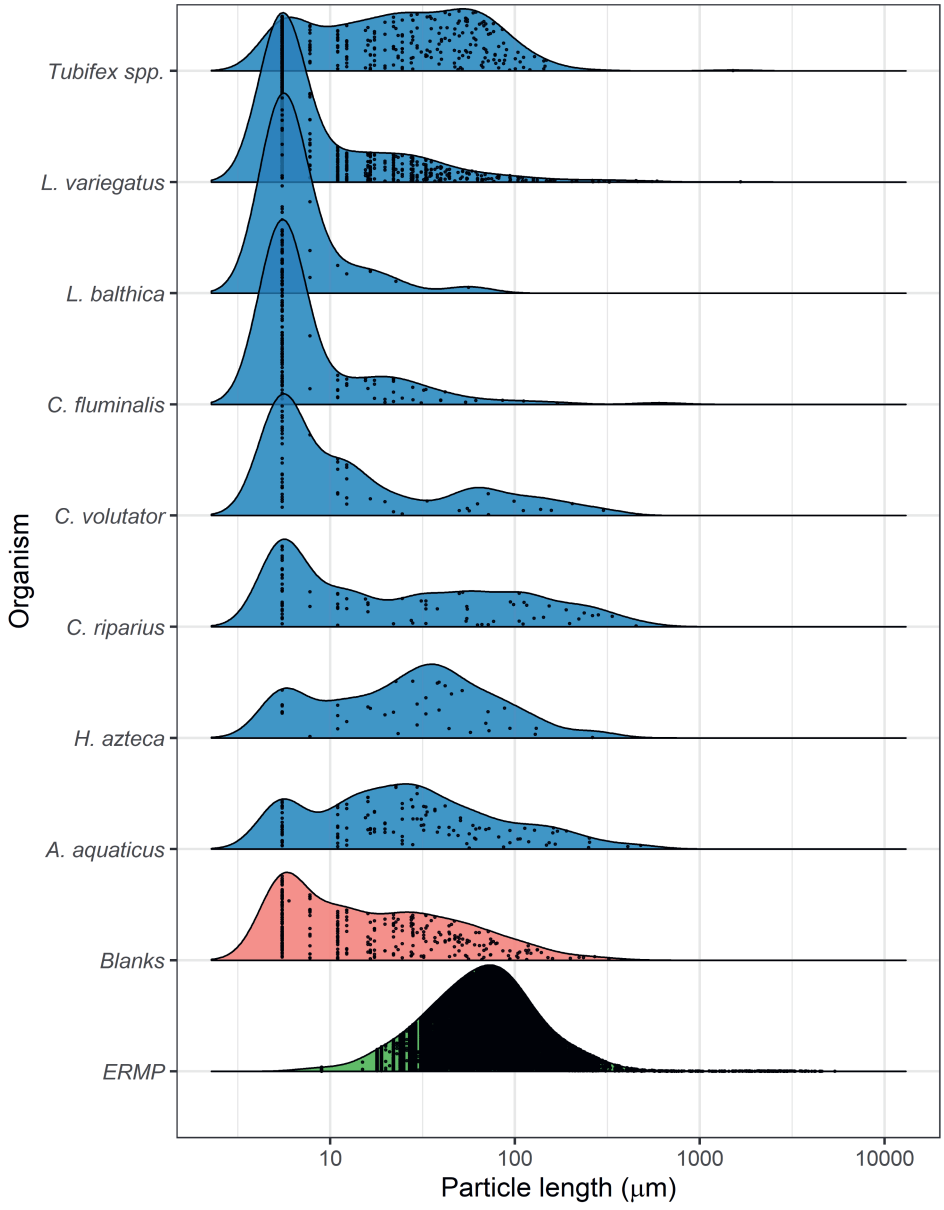


Figure 1. Density plots for ERMP detected in the whole body tissue or dissected soft tissue of organisms *A. aquaticus* (n = 3, observations = 123), *H. azteca* (n = 3, observations = 39), *C. riparius* (n = 3, observations = 78), *C. volutator* (n = 2, observations = 53), *C. fluminalis* (n = 3, observations = 151), *L. balthica* (n = 3, observations = 38), *L. variegatus* (n = 2, observations = 1046), and *Tubifex* spp. (n = 3, observations = 259) are depicted in blue. Additionally, ERMP (n = 48262) is depicted in green and ERMP detected in negative controls/ blanks are depicted in pink (n = 5, observations = 323).

No significant differences were detected in the size distributions of PS particles between controls and the organisms *C. volutator*, *H. azteca*, *A. aquaticus* or *L. variegatus*. Moreover, only a single PS particle was detected in *C. volutator*, *H. azteca*, or *L. variegatus* and 13 particles in *A. aquaticus*, suggesting that the presence of PS particles in these samples is likely due to contamination (Figure 2; Table S7). Significant differences in size distributions were detected between controls and *C. fluminalis* ($W = 47.90$, $p < 0.0001$), controls and *L. balthica* ($W = 46.46$, $p < 0.0001$) and controls and *Tubifex spp.* ($W = 20.67$ $p < 0.026$). These findings indicate that the detected PS particles are not likely attributed to contamination. Moreover, only 18 PS particles were detected in the control, with most being approximately 90 µm in size. Both the freshwater and marine clam species *C. fluminalis* and *L. balthica* exhibited similar size distributions ($W = 1.45$, $p > 0.05$), with PS particles primarily detected at 5.5 µm. Additionally, these organisms showed significantly different size distributions compared to other organisms such as *A. aquaticus* ($W = 68.36$, $p < 0.0001$) ($W = 66.92$, $p < 0.0001$) and *Tubifex* spp. ($W = 29.30$, $p < 0.0001$) ($W = 27.86$, $p < 0.0001$) (Figure 2; Table S7). A significant difference in size distributions was also detected between *A. aquaticus* and *Tubifex* spp. ($W = 47.12$, $p < 0.005$) where *A. aquaticus* also showed a small peak at approximately 500 µm. This is not in line with previous observations by Moore (1975), who identified the maximum ingestible size at approximately 140 µm. Despite thorough rinsing of organisms after exposure and before storage, it is possible that PS particles remained attached to the exterior of the organisms. Finally, no PS particles were detected in *C. riparius*.

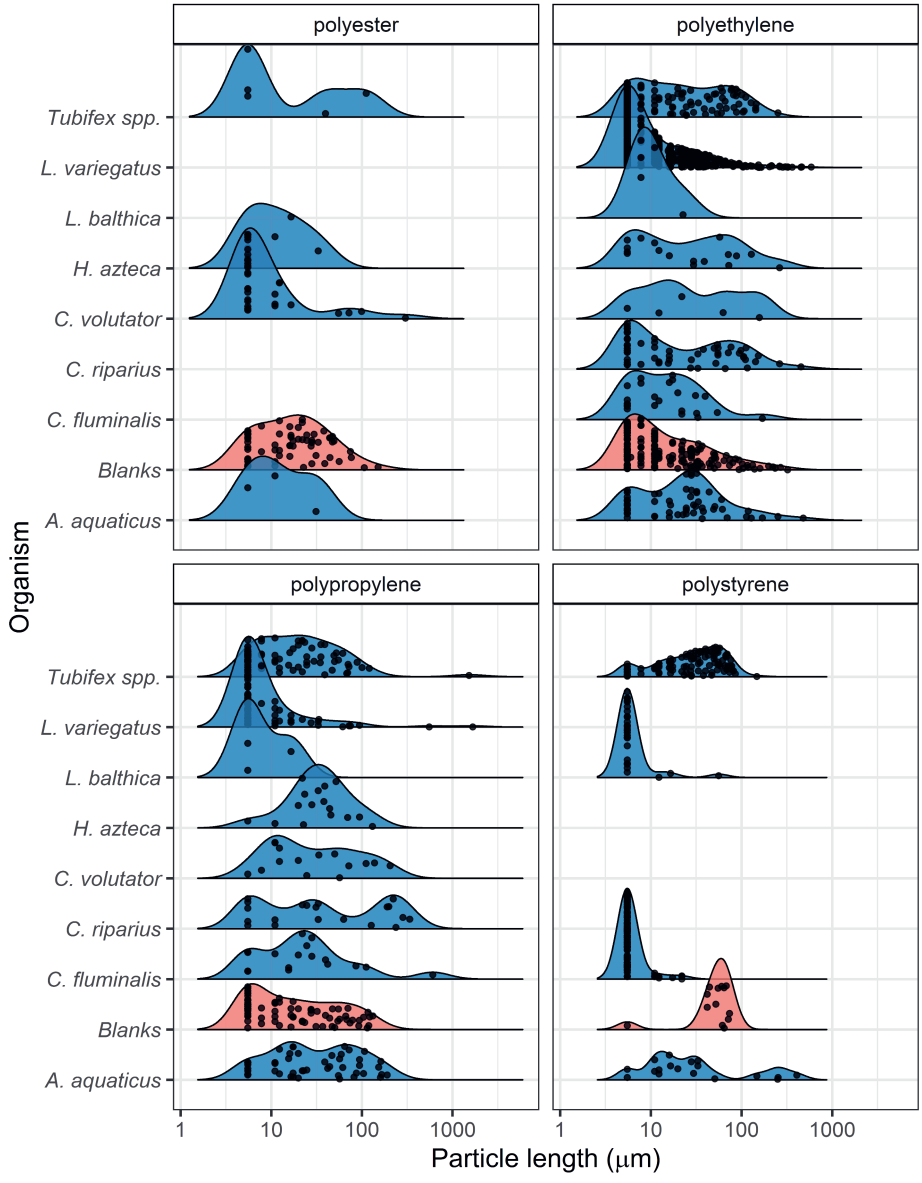


Figure 2. Density plots for ERMP detected in the whole body tissue or dissected soft tissue of organisms *A. aquaticus* (n = 3, observations = 123), *H. azteca* (n = 3, observations = 39), *C. riparius* (n = 3, observations = 78), *C. volutator* (n = 2, observations = 53), *C. fluminalis* (n = 3, observations = 151), *L. balthica* (n = 3, observations = 38), *L. variegatus* (n = 2, observations = 1046), and *Tubifex* spp. (n = 3, observations = 259) are depicted in blue. Additionally ERMP detected in negative controls/ blanks (n = 9, observations = 323)

3.3. *Bioavailability of ERMP as a whole as measured in egestion samples*

The ERMP mixture shows a size distribution that is distinctly different from that of the controls (W = 52.39, p = 0.005), suggesting that the ERMP mixture can be detected in the egestion samples. Moreover, significant differences in size distributions between ERMP and organisms were observed for *A. aquaticus* (W = 45.00, p = 0.004), *C. fluminalis* (W = 51.07, p = 0.005), *C. volutator* (W = 54.10, p = 0.043), *H. azteca* (W = 54.59, p = 0.022), *L. balthica* (W = 53.42, p < 0.001), *M. edulis* (W = 46.94, p < 0.0001), and *Tubifex* spp. (W = 53.11, p < 0.001). Thus indicating that only a part of the ERMP mixture is bioavailable to these organisms (Figure 3; Table S8). Only for the organism *P. antipodarum*, no significant difference in size distribution was detected compared to ERMP mixture (W 63.71, p > 0.05) (Figure 3; Table S8). Interestingly, particles larger than the previously reported size of 72 µm (Aberle et al., 2005), were detected in the egestion samples with the largest particle observed measuring approximately 250 µm.

The microplastic size distributions of *C. fluminalis* (W = 10.60, p > 0.05, obs = 210), *C. volutator* (W = 5.34, p > 0.05, obs = 81) *H. azteca* (W = 6.43, p > 0.05, obs = 104), *P. antipodarum* (W = 11.81, p > 0.05, obs = 48), and *S. corneum* (W = 12.89, p > 0.05, obs = 635), showed no significant differences from the controls (obs = 191) and it is likely that at least a part of the microplastics detected here can be attributed to contamination (Figure 3; Table S8). However, *A. aquaticus* (W = 36.57, p = 0.001), *L. balthica* (W = 13.89, p = 0.005), *M. edulis* (W = 10.43, p = 0.046) and *Tubifex* spp. (W = 33.77, p = 0.030) exhibited significantly different size distributions to the control. This indicates that it is not likely that the measured particles here can be attributed to contamination.

A. aquaticus (W = 36.57, p = 0.001) showed a distinct size distribution from almost all other organisms except *Tubifex spp*, with a second peak at approximately 60 µm (Figure 3; Table S8). Here, egested particles up to the mm range have been detected, exceeding the previously reported maximum ingested particles size of 140 µm (Moore, 1975). While no significant differences in size distributions were found between the marine clams *M. edulis* and *L. balthica* (W =7.93, p > 0.05), they significantly differ from the freshwater clam *S. corneum* (W = 13.47, p = 0.002) and (W = 20.29, p = 0.031), respectively. However, the freshwater clam *C. fluminalis* is similar to all these three marine and freshwater clams, with W =7.06, p > 0.05, W =9.06, p > 0.05, W =11.57, p > 0.05, respectively (Figure 3; Table S8).

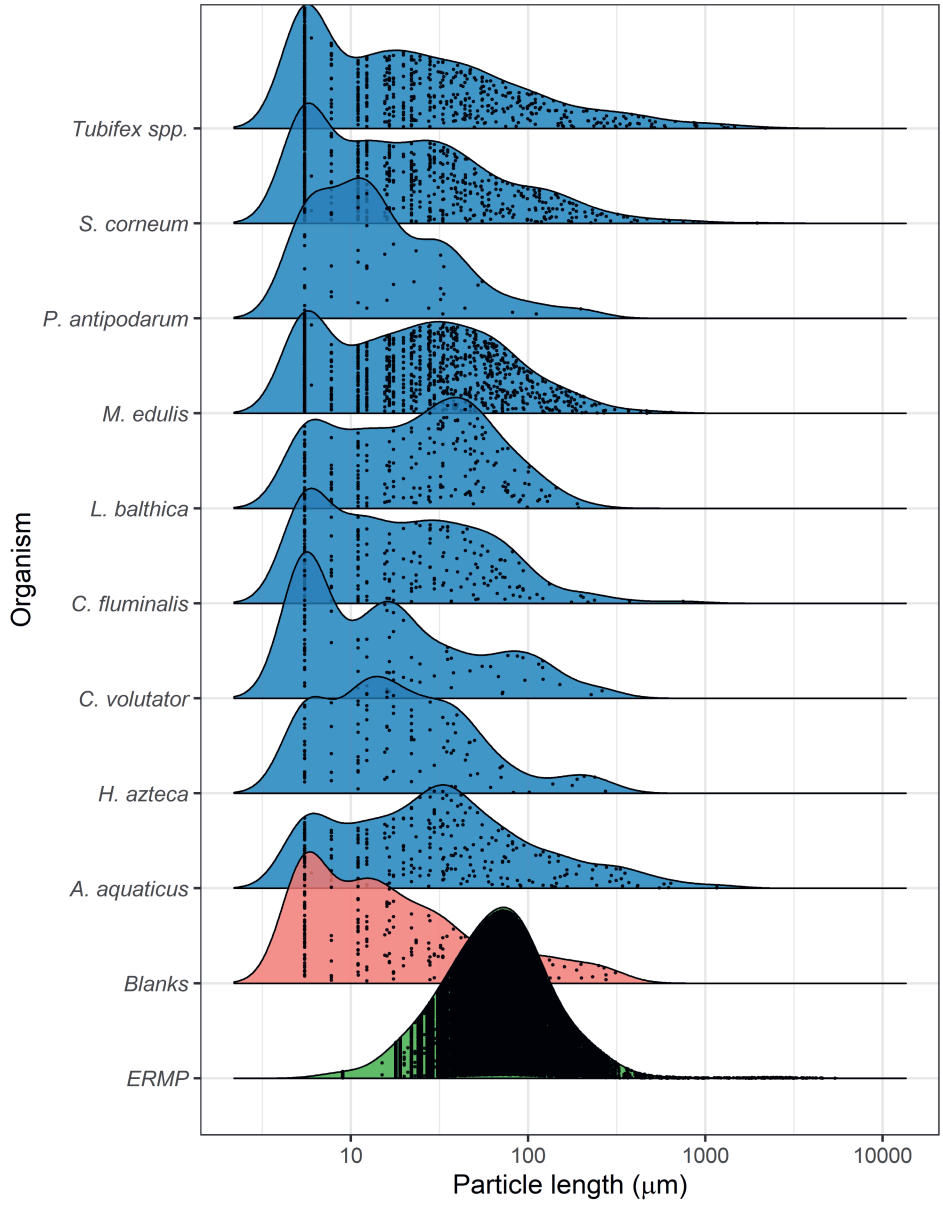


Figure 3. Density plots of ERMP size distributions in egestion samples for organisms *A. aquaticus* (n = 3, observations = 269), *H. azteca* (n = 3, observations = 104), *C. volutator* (n = 3, observations = 81), *C. fluminalis* (n = 3, observations = 210), *L. balthica* (n = 2, observations = 223), *M. edulis* (n = 2, observations = 1086), *P. antipodarum* (n = 2, observations = 48), *S. corneum* (n = 3, observations = 635) and *Tubifex* spp (n = 3, observations = 629). Additionally, ERMP is depicted in green and ERMP detected in negative controls/ blanks are depicted in pink (n = 9, observations = 128).

3.4. Bioavailability of polymer-specific ERMP particles as detected in egestion samples

No significant differences were detected in the size distributions of detected PET particles between the controls and the organisms *Tubifex* spp., *S. corneum*, *M. edulis*, *C. fluminalis*, *C. volutator*, or *A. aquaticus* (W = 26.10; W = 16.65; W = 58.33; W = 8.48; W = 24.17; W = 34.56, $p > 0.05$), indicating that the PET particle detected in these samples are at least partly due to contamination (Figure 4; Table S9). Moreover, no differences were detected in the size distributions of PET particles among the other organisms (Figure 4; Table S9). Only 14 PET particles were detected in the blanks, whereas the highest numbers of particles were detected in *Tubifex spp*. with 41 observations and *C. fluminalis* with 35 particles. The particles detected in *S. corneum*, *M. edulis*, *C. volutator*, and *A. aquaticus* where all less than 14 (Table S9). Additionally, no PET particles were detected in *P. antipodarum, L. balthica*, and *H. azteca.*

No significant differences were observed in the size distributions of detected PE particles between the controls and the organisms *Tubifex* spp., *S. corneum*, *P. antipodarum, M. edulis, L. balthica*, *C. fluminalis*, *C. volutator* or *H. azteca* (W = 9.96; W = 7.87; W = 11.68; W = 12.01; W = 13.44; W = 15.72, W = 6.67, W = 7.67, W = $p > 0.05$), indicating that the PE particle detected in these samples are likely due to contamination (Figure 4; Table S10). However, *A. aquaticus* shows a significantly distinct size distribution compared to the control, *L. balthica*, *M. edulis* and *Tubifex spp.* (W = 45.86, $p = 0.001$)(W = 42.55, $p = 0.001$)(W = 37.80, $p < 0.0001$)(W = 35.99, $p = 0.008$), respectively (Figure 4; Table S10).

No significant differences were detected in the size distributions of detected PP particles between the controls and organisms *Tubifex* spp., *S. corneum*, *P. antipodarum, M. edulis*, *C. volutator*, *H. azteca* or *A. aquaticus* (W = 78.42; W = 82.97; W = 56.17; W = 34.32; W = 43.07; W = 49.03, W = 52.44, $p > 0.05$), indicating that some of the PE particles detected in these samples could be due to contamination (Figure 4; Table S11). However the organisms in the blanks only contained 7 PP particles, whereas *Tubifex* spp., *S. corneum*, *P. antipodarum, M. edulis*, *C. volutator*, *H. azteca* or *A. aquaticus*, contained 269, 73, 4, 192, 14, 51, 62 particles, respectively. Significant differences were detected in the size distributions of PP particles between the controls and organisms *C. fluminalis* and *L. balthica*, (W = 49.62, $p = 0.047$)(W = 47.13, $p = 0.050$), indicating that PP particles

cannot be attributed to contamination. Additionally, size distributions of *A. aquaticus* were significant different from the organisms *C. fluminalis*, *H. azteca* and *L. balthica* (W = 69.20, p = 0.005) (W = 65.57, p = 0.047) (W = 66.99, p = 0.050). Moreover, size distributions of the freshwater clam *C. fluminalis* were significant different from marine and freshwater clam *M. edulis* and *S. corneum* (W = 32.19, p = 0.050) (W = 89.14, p = 0.050), respectively (Figure 4; Table S11).

Only one PS particle was detected in the control of the egestion samples, indicating that the majority of PS particles detected in samples cannot be attributed to contamination. No significant differences in size distributions were detected for PS particles, besides for *M. edulis* and *Tubifex* spp. (W = 23.56, p < 0.0001) (Figure 4; Table S12).

3.5. Microplastic abundance microplastics per organism after adjusting for background contamination

Most particles were detected in the egestion samples of *M. edulis,* with an average of 49.9 particles/organism (Figure 5; Table S13). This amount is almost four times lower than the highest detected in *Asellidae* collected from the Dommel river in the Netherlands, at 195 particles/organism (Pan et al., 2021), but comparable to what Li et al. (2015) detected in the commercial marine bivalve *P. yessoensis*, which had an average of 57.2 particles/organism. Contrastingly, for environmental samples, researchers typically report 0-10 particles/organism (Gouin, 2020). While no tissue samples were analysed, it has been reported that *M. edulis* is able to egest a considerable amount of microplastics > 60% from its gut in 9 h (Woods et al., 2018). Most particles detected in the egestion samples of *M. edulis* were polystyrene, amounting to an average of 23.8 particles/organism, with polyethylene also being significant at 16.8 particles/ organism. Most of the particles are approximately 28 µm size (Figure 5; Table S13).

The lowest microplastic abundance (PE and PP) was observed in the tissue samples of *C. riparius* with an average of 0.3 particles/organism (Figure 5; Table S13). Despite exposure to exceedingly higher concentrations of microplastics (5.9×10^8 particles/kg of dw sediment) in the lab, this result is in accordance with findings by Pan et al. (2021) Pan et al, 2021, who found a range of 0.0 to 0.4 microplastics/organism in environmental samples collected in the Netherlands, indicating there might be a limit to ingestible number of particles.

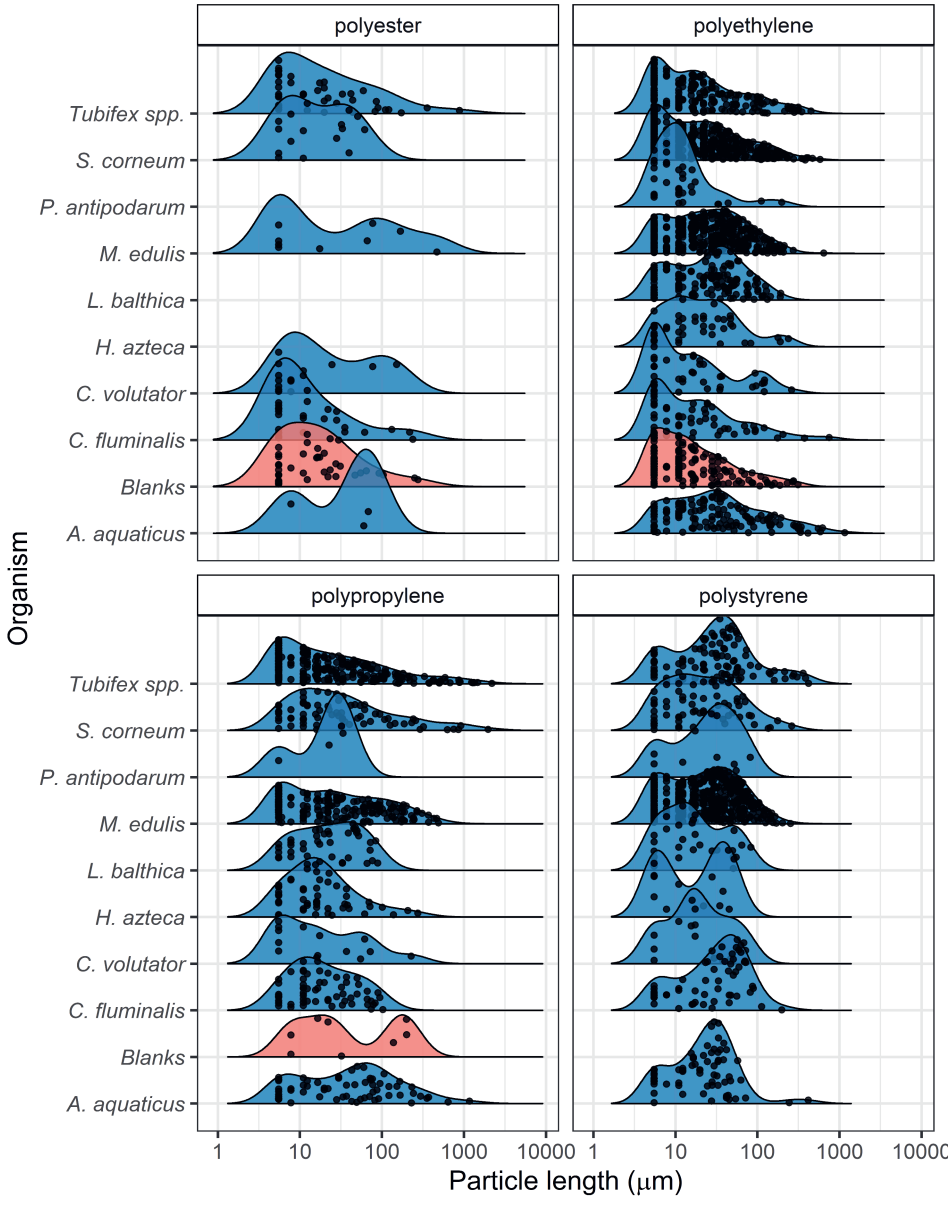


Figure 4. Size distributions of microplastics polyester, polyethylene, polypropylene and polystyrene in egestion samples for organisms *A. aquaticus* (n = 3, observations = 269), *H. azteca* (n = 3, observations = 104), *C. volutator* (n = 3, observations = 81), *C. fluminalis* (n = 3, observations = 210), *L. balthica* (n = 2, observations = 223), *M. edulis* (n = 2, observations = 1086), *P. antipodarum* (n = 2, observations = 48), *S. corneum* (n = 3, observations = 635) and *Tubifex* spp (n = 3, observations = 629). Additionally, ERMP detected in negative controls/ blanks are depicted in pink (n = 9, observations = 128).

For most organisms, specifically, *A. aquaticus*, *H. azteca*, *C. fluminalis*, *Tubifex* spp., and *C. volutator,* where both tissue and egestion samples were analysed, most microplastics were detected in the egestion samples (Figure 5; Table S13). This suggests that particles can easily be egested. For instance, in *A. aquaticus* an average of 6.6 microplastics/organism was detected in the egestion samples, while tissue samples revealed an average of 1.0 microplastics/organism (Figure 5; Table S13). Moreover, the *Tubifex* spp. tissue samples revealed an average of 3 particles/organism, while egestion samples contained an average of 10.6 particles/organism were detected, indicating that the majority of particles were egested (Figure 5; Table S13). Most particles that were retained and egested were detected in the 22.4 -27.7 µm size range (Figure S3, S4). While PP and PE particles were more prevalent in the egestion samples, PS particles were the most retained (Figure 5; Table S13).

Interestingly, the highest abundance of ERMP in tissue samples was observed in *L. variegatus* with an average of 8.6 microplastics per organism (Figure 5; Table S13). Of all organisms analysed this was the only organism where adverse effects, specifically reduced reproduction and growth, were detected after a 28-day exposure to ERMP, with EC_{50} values of 1.48×10^8 and 4.53×10^7 particles/kg of dry weight sediment [de Ruijter 2023]. Most particles detected in *L. variegatus* ranged from 5.5 to 22.4 µm. Furthermore, particles consisted mainly of PE with an average of 8.0 particles/organism. A small quantity of PP and PS was also detected, with on average 0.62 and 0.01 particles/organisms, respectively (Figure S3, S4).

Previously, a positive effect was detected for *S. corneum* after 28 day exposure to ERMP (de Ruijter et al., 2023; **Chapter 3**). That is, with increasing ERMP in the sediment, mortality decreased with an EC_{50} of 5.82×10^8 particles/kg of dw sediment. Although tissue samples were not analysed, the egestion samples of *S. corneum* indicated a relatively high abundance with an average of 21.8 egested particles/organism (Figure 5; Table S13). Most of these particles fell withing the size range of 5.5 to 22.4 µm (Figure S3, S4). Moreover, the particles were mainly composed of PE with an average of 16.7 particles/organism. Additionally, small amounts of PET, PP and PS were found, with averages of 0.2, 2.6 and 2.5 particles/organisms, respectively (Figure 5; Table S13).

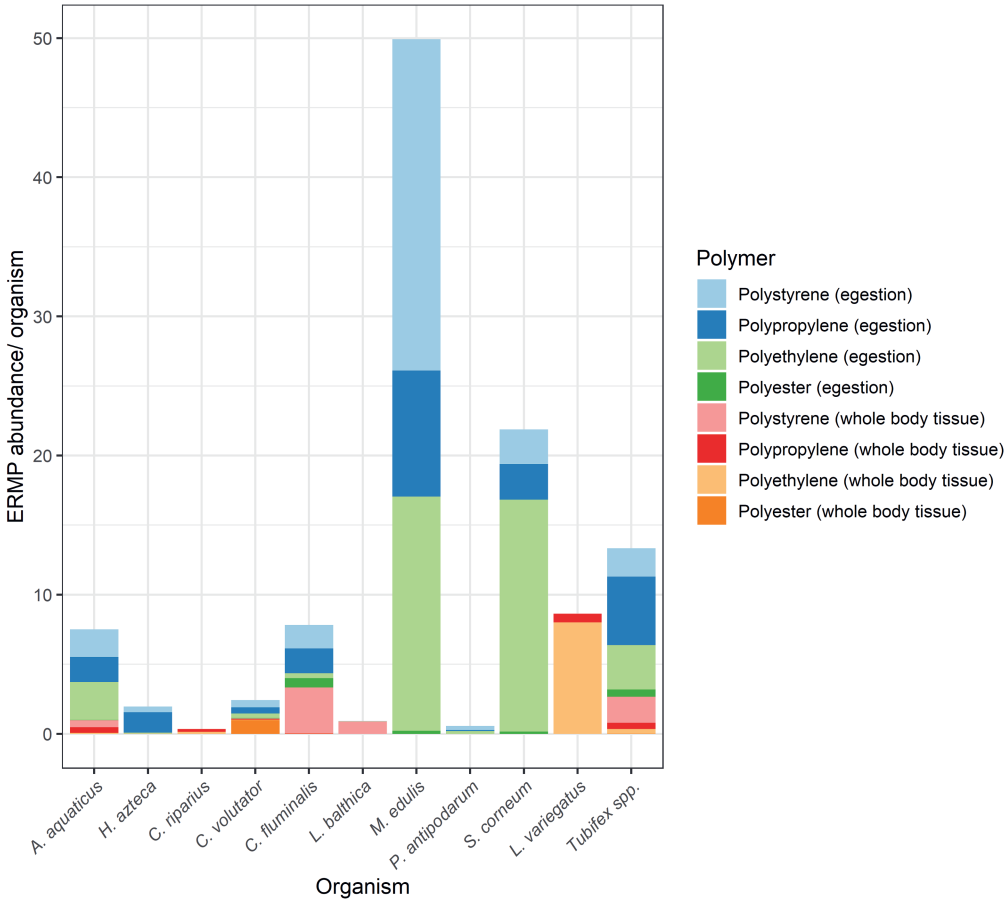


Figure 5. The average ERMP abundance per organism after correction for contamination. Note that for the organisms *A. aquaticus*, *H. azteca*, *C. fluminalis*, *Tubifex* spp., and *C. volutator,* both whole body tissue and egestion samples were analysed. For *C. riparius* and *L. balthica* only tissue samples were analysed. For *M. edulis*, *P. antipodarum* and *S. corneum* only egestion samples were analysed.

4. General discussion and conclusion

In this study we have directly and indirectly, verified the exposure of ERMP in 11 different marine and freshwaters benthic species previously exposed to ERMP for 28 days. Notably, the negative effects previously detected, specifically the reduction in reproduction and growth in *L. variegatus*, have now been causally linked because ERMP has been detected in the tissue samples of this freshwater worm. Our analysis has primarily focused on particle length, however ideally other ERM such as volume and surface area should be studied in order to determine the mechanism behind the observed effects. For instance, analysing the volume of particles relative to exposure concentration could provide more valuable insights into the "food dilution" mechanism.

Although the organisms were exposed to exceedingly high concentrations (10.0 % dw sediment, 5.9×10^8 particles/kg of dw sediment), the findings of this study were either comparable to or smaller than those observed in environmental samples, indicating a limit to the amount of particles that can be ingested by organisms. Overall we observed that organisms ingested only specific parts of the ERMP mixture, usually those in the smaller size range of 5.5 to 27.7 µm. Additionally, it was apparent that the uptake of specific polymer types is species-specific. Whether this is due to differences in density or the inherent shapes of the polymer types remains to be studied. Moreover, we find that overall, ERMP detected in egestion samples far exceeds that in the tissue samples, indicating that organisms can easily egest most ingested microplastics after only 24 h depuration in clean water. Furthermore, we see that the size distributions of ERMP are species-specific, showing distinct differences and similarities. For instance, similar size distributions were detected in the tissue samples of the crustaceans *A. aquaticus* and *H. azteca*, which are both sediment grazers/scavengers. Additionally, similarities in the size distributions of microplastics were detected among the freshwater and marine clams *C. fluminalis*, *M. edulis* and *L. balthica*. However, the freshwater clam *S. corneum* only showed a similar size distribution of microplastics to its freshwater counterpart *C. fluminalis*. Interestingly, no similarities were detected between the worms *L. variegatus* and *Tubifex spp.*, demonstrating that the size of the organisms is also an important factor in explaining the size distribution of ingested microplastics.

In this study, we implemented a novel approach to blank correction for microplastics detection. Here we introduced a combination of visual inspection, the Wasserstein statistic that distinguishes between the cumulative size distributions, consequently followed by a traditional blank correction by subtraction, per size bin and per polymer type. After thorough inspection and data analysis we determined that blank correction was most effectively accomplished by considering both size and polymer type. This is a different finding than that of Dawson et al. (2023). In their study, multiple blank correction methods were tested and they concluded that polymer specific correction was not advisable. We consider it fundamentally incorrect, however, to correct particles for particles of a completely different type, such as a different polymer, and therefore used a particle-specific blank correction approach. At this moment, the question of how to properly conduct blank corrections for such diverse particles as microplastics is a subject of discussion among various research groups. Although not the primary goal of this study, we contribute to this with our new approach.

The particle size-specific, polymer-specific, and species-specific ingestion patterns that we demonstrate in our study provide an essential basis for understanding the significance of effect mechanisms, such as those based on the volume of particles, like the food dilution hypothesis. Additionally, the specific ingestion patterns are important for establishing risk profiles for certain types of microplastics, as defined by their size or polymer type. This brings particle-specific risk assessments within reach and also offers a course of action for environmental managers and policymakers to address the emissions that lead to these specific particle types.

Supporting information

Table S1. Overview of organisms analysed with FTIR

Organism		Sample type	Replicate	Survival	Sum
Name	**Code**				
A. aquaticus	AA	tissue	1	11	
A. aquaticus	AA	tissue	2	11	
A. aquaticus	AA	tissue	3	11	33
H. azteca	HA	tissue	1	11	
H. azteca	HA	tissue	2	11	
H. azteca	HA	tissue	3	11	33
C. riparius	CR	tissue	1	10	
C. riparius	CR	tissue	2	10	
C. riparius	CR	tissue	3	11	31
C. volutator	CV	tissue	1	9	
C. volutator	CV	tissue	2	8	17
C. fluminalis	CF	tissue	2	11	
C. fluminalis	CF	tissue	3	11	
C. fluminalis	CF	tissue	4	11	33
L. balthica	LB	tissue	1	10	
L. balthica	LB	tissue	2	11	
L. balthica	LB	tissue	3	11	32
L. variegatus	LV	tissue	1	55	
L. variegatus	LV	tissue	3	55	110
Tubifex spp.	TSP	tissue	1	16	
Tubifex spp.	TSP	tissue	2	16	
Tubifex spp.	TSP	tissue	3	20	52
A. aquaticus	AA	egestion	1	11	
A. aquaticus	AA	egestion	2	11	
A. aquaticus	AA	egestion	3	11	33
H. azteca	HA	egestion	1	11	
H. azteca	HA	egestion	2	11	
H. azteca	HA	egestion	3	11	33
C. volutator	CV	egestion	1	9	
C. volutator	CV	egestion	2	8	

Organism		Sample type	Replicate	Survival	Sum
Name	**Code**				
C. volutator	CV	egestion	3	10	27
C. fluminalis	CF	egestion	2	11	
C. fluminalis	CF	egestion	3	11	
C. fluminalis	CF	egestion	4	11	33
L. balthica	LB	egestion	2	11	
L. balthica	LB	egestion	3	11	22
M. edulis	ME	egestion	1	10	
M. edulis	ME	egestion	2	11	21
P. antipodarum	PO	egestion	1	17	
P. antipodarum	PO	egestion	2	18	35
S. corneum	SC	egestion	1	11	
S. corneum	SC	egestion	2	8	
S. corneum	SC	egestion	3	7	26
Tubifex spp.	TSP	egestion	1	16	
Tubifex spp.	TSP	egestion	2	16	
Tubifex spp.	TSP	egestion	3	20	52

Table S2. Overview of the digestion treatments and conditions applied for different benthic invertebrates

Treatments	Annelida	Insecta/Crustacea	Molluscs/Bivalves	Egestion
10-15 ml H_2O_2	24h, 37℃	24h, 37℃	24h, 37℃	48h, 37℃
1 ml Chitinase + 20 ml NaOAc buffer	-	5 days, 37℃	-	
5 ml Protease + 25 ml Tris HCL buffer	24h, 40℃	24h, 40℃	24h, 40℃	
1 ml Lipase + 20 ml Tris HCL buffer			24h, 40℃	
10-15 ml H_2O_2	24h, 37℃	24 h days, 37℃	24h, 37℃	

Table S3. Wasserstein statistics comparing size distributions of detected ERMP in tissue samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	123	323	19.96	0.0002	**0.0019**
AA	CF	123	151	35.57	0.0000	**0.0000**
AA	CR	123	78	14.28	0.3172	0.3465
AA	CV	123	151	16.78	0.2046	0.2303
AA	HA	123	39	11.37	0.7310	0.7354
AA	LB	123	38	41.34	0.0013	**0.0032**
AA	LV	123	38	32.78	0.0000	**0.0003**
AA	TSP	123	259	18.04	0.0773	0.1144
AA	ERMP	123	48262	40.76	0.0184	**0.0330**
Blank	CF	323	151	18.09	0.0000	**0.0000**
Blank	CR	323	78	28.85	0.0000	**0.0000**
Blank	CV	323	151	9.81	0.1810	0.2155
Blank	HA	323	39	12.71	0.0905	0.1208
Blank	LB	323	38	21.39	0.0031	**0.0083**
Blank	LV	323	38	15.33	0.0002	**0.0000**
Blank	TSP	323	259	13.37	0.0068	**0.0109**
Blank	ERMP	323	48262	57.57	0.0000	**0.0000**
CF	CR	151	78	44.72	0.0000	**0.0000**
CF	CV	151	151	21.39	0.0126	**0.0215**
CF	HA	151	39	30.75	0.0012	**0.0019**
CF	LB	151	38	7.43	0.4056	0.4278
CF	LV	151	38	5.40	0.5526	0.5666
CF	TSP	151	259	27.31	0.0000	**0.0000**
CF	ERMP	151	48262	72.22	0.0004	**0.0003**
CR	CV	78	151	25.32	0.0707	0.1137
CR	HA	78	39	23.35	0.1560	0.2016
CR	LB	78	38	50.14	0.0008	**0.0000**
CR	LV	78	38	41.75	0.0001	**0.0012**
CR	TSP	78	259	30.10	0.0158	**0.0272**
CR	ERMP	78	48262	39.64	0.0511	0.0781
CV	HA	53	39	14.41	0.3057	0.3405
CV	LB	53	38	24.82	0.0046	**0.0083**
CV	LV	53	38	18.07	0.0656	0.0995

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
CV	TSP	53	259	20.58	0.1652	0.2154
CV	ERMP	53	48262	54.60	0.0376	0.0651
HA	LB	39	38	33.67	0.0000	**0.0000**
HA	LV	39	38	27.63	0.0385	0.0665
HA	TSP	39	259	11.44	0.6604	0.6739
HA	ERMP	39	48262	45.43	0.0870	0.1156
LB	LV	38	38	11.18	0.1988	0.2261
LB	TSP	38	259	32.55	0.1264	0.1648
LB	ERMP	38	48262	78.96	0.0270	**0.0421**
LV	TSP	1046	259	23.98	0.0000	**0.0000**
LV	ERMP	1046	48262	67.78	0.0000	**0.0000**
TSP	ERMP	259	48262	47.81	0.0001	**0.0012**

Table S4. Wasserstein statistics comparing size distributions of detected PET in tissue samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	3	62	9.40	0.8724	1.0000
AA	CF	3	1	45.62	0.2529	0.7443
AA	CR	3	1	10.37	1.0000	1.0000
AA	CV	3	32	18.50	0.3443	0.8066
AA	HA	3	5	3.06	1.0000	1.0000
AA	TSP	3	5	20.57	0.8209	1.0000
Blank	CF	62	1	42.07	0.0780	0.7443
Blank	CR	62	1	19.17	0.5276	0.9840
Blank	CV	62	32	15.86	0.0484	0.7443
Blank	HA	62	5	10.37	0.5678	0.9840
Blank	TSP	62	5	16.25	0.2273	0.7443
CF	CR	1	1	55.99	1.0000	1.0000
CF	CV	1	1	56.92	0.1182	0.7443
CF	HA	1	5	47.19	0.1672	0.7443
CF	TSP	1	5	47.94	0.3305	0.8066
CR	CV	1	32	17.11	1.0000	1.0000
CR	HA	1	5	8.80	1.0000	1.0000

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
CR	TSP	1	5	28.01	1.0000	1.0000
CV	HA	32	5	16.46	0.6286	1.0000
CV	TSP	32	5	22.81	0.2246	0.7443
HA	TSP	5	5	21.41	0.5309	0.9840

Table S5. Wasserstein statistics comparing size distributions of detected PE in tissue samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	56	177	14.02	0.1352	0.3348
AA	CF	56	24	19.48	0.3614	0.5527
AA	CR	56	57	11.95	0.6228	0.8101
AA	CV	56	5	26.11	0.6165	0.8075
AA	HA	56	17	16.40	0.7673	0.8328
AA	LB	56	5	32.67	0.2335	0.4233
AA	LV	56	952	25.14	0.0016	**0.0120**
AA	TSP	56	91	15.00	0.1975	0.3958
Blank	CF	177	24	7.97	0.7579	0.8328
Blank	CR	177	57	18.64	0.0299	0.1337
Blank	CV	177	5	28.33	0.1538	0.3608
Blank	HA	177	17	21.74	0.0799	0.3132
Blank	LB	177	5	19.08	0.3781	0.5527
Blank	LV	177	952	12.28	0.0011	**0.0144**
Blank	TSP	177	91	13.84	0.0195	0.1308
CF	CR	24	57	24.69	0.1604	0.3608
CF	CV	24	5	29.59	0.0866	0.3132
CF	HA	24	17	26.45	0.1084	0.3132
CF	LB	24	5	13.38	0.3869	0.5527
CF	LV	24	952	9.92	0.2575	0.4517
CF	TSP	24	91	17.11	0.0950	0.3132
CR	CV	57	5	19.74	0.8609	0.9106
CR	HA	57	17	12.09	0.9620	0.9903
CR	LB	57	5	37.72	0.2452	0.4363
CR	LV	57	952	29.80	0.0001	**0.0018**

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
CR	TSP	57	91	11.85	0.4339	0.5916
CV	HA	5	17	18.43	0.9915	0.9913
CV	LB	5	5	41.51	0.1291	0.3348
CV	LV	5	952	38.66	0.0587	0.2844
CV	TSP	5	91	15.68	0.7041	0.8145
HA	LB	17	5	39.58	0.2665	0.4544
HA	LV	17	952	33.98	0.0150	0.1210
HA	TSP	17	91	11.37	0.6751	0.8145
LB	LV	5	952	10.27	0.6560	0.8101
LB	TSP	5	91	28.63	0.1687	0.3608
LV	TSP	952	91	23.73	0.0000	**0.0000**

Table S6. Wasserstein statistics comparing size distributions of detected PP in tissue samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	46	71	18.59	0.0217	0.1591
AA	CF	46	17	38.24	0.1588	0.3405
AA	CR	46	20	45.69	0.0241	0.1591
AA	CV	46	15	7.80	0.9769	0.9752
AA	HA	46	16	16.34	0.3176	0.4171
AA	LB	46	3	41.22	0.1489	0.3405
AA	LV	46	93	55.46	0.0132	0.1332
AA	TSP	46	58	42.12	0.0790	0.3405
Blank	CF	71	17	33.93	0.1613	0.3405
Blank	CR	71	20	60.65	0.0002	**0.0036**
Blank	CV	71	15	19.53	0.1092	0.3405
Blank	HA	71	16	11.67	0.2825	0.4171
Blank	LB	71	3	22.63	0.3322	0.4171
Blank	LV	71	93	39.52	0.0051	0.0684
Blank	TSP	71	58	29.09	0.3099	0.4171
CF	CR	17	20	62.63	0.1484	0.3405
CF	CV	17	15	36.10	0.8138	0.8646
CF	HA	17	16	38.76	0.6337	0.6903

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
CF	LB	17	3	53.75	0.2999	0.4171
CF	LV	17	93	51.29	0.1702	0.3464
CF	TSP	17	58	42.27	0.5536	0.6063
CR	CV	20	15	42.99	0.2117	0.4090
CR	HA	20	16	59.12	0.0440	0.2592
CR	LB	20	3	83.28	0.2255	0.4090
CR	LV	20	93	91.22	0.0488	0.2895
CR	TSP	20	58	79.59	0.1062	0.3405
CV	HA	15	16	18.95	0.4243	0.5138
CV	LB	15	3	42.16	0.2655	0.4171
CV	LV	15	93	55.77	0.1447	0.3405
CV	TSP	15	58	42.51	0.2696	0.4171
HA	LB	16	3	33.17	0.0941	0.3405
HA	LV	16	93	49.98	0.1536	0.3405
HA	TSP	16	58	36.07	0.3636	0.4330
LB	LV	3	93	28.22	0.8530	0.8863
LB	TSP	3	58	44.95	0.2555	0.4171
LV	TSP	93	58	26.53	0.5122	0.6035

Table S7. Wasserstein statistics comparing size distributions of detected PS in tissue samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	18	13	65.67	0.0283	0.0687
AA	CF	18	109	68.36	0.0000	**0.0000**
AA	CV	18	1	69.20	0.4294	0.5480
AA	HA	18	1	67.37	0.4159	0.5480
AA	LB	18	30	66.92	0.0000	**0.0000**
AA	LV	18	1	88.54	0.2603	0.4048
AA	TSP	18	105	47.12	0.0012	**0.0052**
Blank	CF	13	109	47.90	0.0000	**0.0000**
Blank	CV	13	1	48.74	0.1459	0.2608
Blank	HA	13	1	17.95	0.4170	0.5480
Blank	LB	13	30	46.46	0.0000	**0.0000**

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
Blank	LV	13	1	27.52	0.1367	0.2608
Blank	TSP	13	105	20.67	0.0076	**0.0256**
CF	CV	109	1	0.84	1.0000	1.0000
CF	HA	109	1	35.55	0.0077	**0.0302**
CF	LB	109	30	1.45	0.2422	0.3778
CF	LV	109	1	75.42	0.0079	**0.0314**
CF	TSP	109	105	29.30	0.0000	**0.0000**
CV	HA	1	1	36.39	1.0000	1.0000
CV	LB	1	30	2.28	1.0000	1.0000
CV	LV	1	1	76.26	1.0000	1.0000
CV	TSP	1	105	30.14	0.3008	0.4341
HA	LB	1	30	35.05	0.0588	0.1312
HA	LV	1	1	39.88	1.0000	1.0000
HA	TSP	1	105	21.73	0.7166	0.8719
LB	LV	30	1	73.98	0.0324	0.0768
LB	TSP	30	105	27.86	0.0000	**0.0000**
LV	TSP	1	105	47.46	0.0380	0.0836

Table S8. Wasserstein statistics comparing size distributions of detected ERMP in egestion samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	269	191	36.57	0.0000	**0.0012**
AA	CF	269	210	34.87	0.0004	**0.0007**
AA	CV	269	81	38.36	0.0165	**0.0368**
AA	HA	269	104	38.86	0.0054	**0.0165**
AA	LB	269	223	37.69	0.0000	**0.0000**
AA	ME	269	1086	31.21	0.0000	**0.0000**
AA	PO	269	48	47.98	0.0214	**0.0453**
AA	SC	269	635	26.39	0.0028	**0.0110**
AA	TSP	269	629	15.49	0.3532	0.4034
AA	ERMP	269	48262	45.00	0.004	**0.0035**
Blank	CF	191	210	10.60	0.1316	0.1998
Blank	CV	191	81	5.34	0.7439	0.7538

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
Blank	HA	191	104	6.43	0.4786	0.5031
Blank	LB	191	223	13.98	0.0014	**0.0051**
Blank	ME	191	1086	10.43	0.0211	**0.0463**
Blank	PO	191	48	11.81	0.2042	0.2715
Blank	SC	191	635	12.89	0.2508	0.3262
Blank	TSP	191	629	33.77	0.0102	**0.0288**
Blank	ERMP	191	48262	52.39	0.0006	**0.0051**
CF	CV	210	81	8.47	0.6745	0.6912
CF	HA	210	104	8.01	0.6291	0.6499
CF	LB	210	223	9.06	0.1074	0.1566
CF	ME	210	1086	7.06	0.1864	0.2457
CF	PO	210	48	13.22	0.3183	0.3937
CF	SC	210	635	11.57	0.3348	0.3973
CF	TSP	210	629	32.43	0.0088	**0.0272**
CF	ERMP	210	48262	51.07	0.0006	**0.0050**
CV	HA	81	104	7.33	0.4603	0.4975
CV	LB	81	223	10.91	0.0267	**0.0493**
CV	ME	81	1086	8.47	0.3324	0.3973
CV	PO	81	48	10.52	0.2539	0.3279
CV	SC	81	635	14.67	0.4066	0.4686
CV	TSP	81	629	35.56	0.0866	0.1361
CV	ERMP	81	48262	54.10	0.0161	**0.0429**
HA	LB	104	223	10.03	0.0330	0.0566
HA	ME	104	1086	9.62	0.1329	0.2001
HA	PO	104	48	9.12	0.3430	0.3973
HA	SC	104	635	16.04	0.2616	0.3262
HA	TSP	104	629	37.07	0.0414	0.0612
HA	ERMP	104	48262	54.59	0.0080	**0.0223**
LB	ME	223	1086	7.93	0.0567	0.1013
LB	PO	223	48	11.91	0.0293	0.0524
LB	SC	223	635	20.29	0.0112	**0.0307**
LB	TSP	223	629	39.50	0.0006	**0.0051**
LB	ERMP	223	48262	53.42	0.0002	**0.0007**
ME	PO	1086	48	16.83	0.0544	0.0911
ME	SC	1086	635	13.47	0.0006	**0.0016**

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
ME	TSP	1086	629	31.67	0.0000	**0.0000**
ME	ERMP	1086	48262	46.94	0.0000	**0.0000**
PO	SC	48	635	24.36	0.1569	0.2001
PO	TSP	48	629	45.49	0.0819	0.1361
PO	ERMP	48	48262	63.71	0.0295	0.0566
SC	TSP	635	629	21.23	0.0103	**0.0272**
SC	ERMP	635	48262	45.42	0.0000	**0.0000**
TSP	ERMP	629	48262	53.11	0.0000	**0.0000**

Table S9. Wasserstein statistics comparing size distributions of detected PET in egestion samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	3	14	34.56	0.1844	0.9009
AA	CF	3	35	38.75	0.1790	0.9009
AA	CV	3	6	29.77	0.6742	1.0000
AA	LB	3	2	26.02	0.3965	0.9009
AA	ME	3	9	68.78	0.7206	1.0000
AA	PO	3	1	32.31	0.7516	1.0000
AA	SC	3	16	22.99	0.0872	0.7776
AA	TSP	3	41	52.28	0.3789	0.9009
Blank	CF	14	35	8.48	0.7456	1.0000
Blank	CV	14	6	24.17	0.5462	1.0000
Blank	LB	14	2	21.18	0.9349	1.0000
Blank	ME	14	9	58.33	0.0730	0.7776
Blank	PO	14	1	27.89	0.6987	1.0000
Blank	SC	14	16	16.65	0.3676	0.9009
Blank	TSP	14	41	26.10	0.5958	1.0000
CF	CV	35	6	26.49	0.2470	0.9009
CF	LB	35	2	20.68	0.8945	1.0000
CF	ME	35	9	62.52	0.0501	0.7776
CF	PO	35	1	28.55	0.2428	0.9009
CF	SC	35	16	18.00	0.2480	0.9009
CF	TSP	35	41	31.71	0.3599	0.9009

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
CV	LB	6	2	29.70	1.0000	1.0000
CV	ME	6	9	47.58	0.9330	1.0000
CV	PO	6	1	38.06	1.0000	1.0000
CV	SC	6	16	24.92	0.1471	0.9009
CV	TSP	6	41	34.38	0.9434	1.0000
LB	ME	2	9	73.43	0.7865	1.0000
LB	PO	2	1	13.75	1.0000	1.0000
LB	SC	2	16	6.36	1.0000	1.0000
LB	TSP	2	41	44.35	0.9412	1.0000
ME	PO	9	1	84.79	1.0000	1.0000
ME	SC	9	16	70.00	0.0365	0.7776
ME	TSP	9	41	52.97	0.3902	0.9009
PO	SC	1	16	16.21	0.8217	1.0000
PO	TSP	1	41	53.16	0.7436	1.0000
SC	TSP	16	41	39.26	0.4042	0.9009

Table S10. Wasserstein statistics comparing size distributions of detected PET in egestion samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	138	136	45.86	0.0001	**0.0011**
AA	CF	138	58	33.34	0.1589	0.3250
AA	CV	138	47	46.13	0.0338	0.0951
AA	HA	138	40	41.69	0.0878	0.2077
AA	LB	138	153	41.55	0.0001	**0.0011**
AA	ME	138	385	37.80	0.0000	**0.0000**
AA	PO	138	33	57.17	0.0302	0.0951
AA	SC	138	481	38.65	0.0000	**0.0000**
AA	TSP	138	213	35.99	0.0009	**0.0081**
Blank	CF	136	58	15.72	0.2843	0.4412
Blank	CV	136	47	6.67	0.7459	0.7629
Blank	HA	136	40	7.67	0.6792	0.7277
Blank	LB	136	153	13.44	0.0109	0.0554
Blank	ME	136	385	12.01	0.0259	0.0951

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
Blank	PO	136	33	11.68	0.3181	0.4604
Blank	SC	136	481	7.87	0.3097	0.4604
Blank	TSP	136	213	9.96	0.2072	0.3586
CF	CV	58	47	17.79	0.6391	0.7015
CF	HA	58	40	17.84	0.7233	0.7569
CF	LB	58	153	24.54	0.0123	0.0554
CF	ME	58	385	20.30	0.0329	0.0951
CF	PO	58	33	26.99	0.2410	0.4017
CF	SC	58	481	12.65	0.3274	0.4604
CF	TSP	58	213	11.85	0.6071	0.7005
CV	HA	47	40	12.46	0.3572	0.4871
CV	LB	47	153	13.23	0.0548	0.1451
CV	ME	47	385	13.11	0.1835	0.3418
CV	PO	47	33	13.59	0.2532	0.4069
CV	SC	47	481	9.36	0.6230	0.7009
CV	TSP	47	213	10.85	0.4939	0.6310
HA	LB	40	153	10.67	0.1790	0.3418
HA	ME	40	385	9.99	0.4120	0.5453
HA	PO	40	33	15.57	0.1899	0.3418
HA	SC	40	481	8.79	0.7735	0.7735
HA	TSP	40	213	11.24	0.5305	0.6420
LB	ME	153	385	5.60	0.5421	0.6420
LB	PO	153	33	17.98	0.0114	0.0554
LB	SC	153	481	13.09	0.0215	0.0880
LB	TSP	153	213	16.70	0.0075	0.0554
ME	PO	385	33	19.83	0.0689	0.1723
ME	SC	385	481	9.32	0.0297	0.0951
ME	TSP	385	213	13.16	0.0112	0.0554
PO	SC	33	481	18.96	0.1219	0.2612
PO	TSP	33	213	21.53	0.0923	0.2077
SC	TSP	481	213	5.89	0.5048	0.6310

Table S11. Wasserstein statistics comparing size distributions of detected PP in egestion samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	62	7	52.44	0.5865	0.6588
AA	CF	62	61	69.20	0.0000	**0.0045**
AA	CV	62	14	59.06	0.3075	0.5479
AA	HA	62	51	65.57	0.0020	**0.0465**
AA	LB	62	40	66.99	0.0067	**0.0495**
AA	ME	62	192	38.26	0.0280	0.0900
AA	PO	62	4	75.68	0.3137	0.5479
AA	SC	62	73	42.64	0.5729	0.6494
AA	TSP	62	269	38.47	0.3768	0.5990
Blank	CF	7	61	49.62	0.0042	**0.0465**
Blank	CV	7	14	43.07	0.2142	0.4663
Blank	HA	7	51	49.03	0.0290	0.0900
Blank	LB	7	40	47.13	0.0102	**0.0495**
Blank	ME	7	192	34.32	0.3898	0.5990
Blank	PO	7	4	56.17	0.3617	0.5990
Blank	SC	7	73	82.97	0.5223	0.6345
Blank	TSP	7	269	78.42	0.3600	0.5990
CF	CV	61	14	13.88	0.2874	0.5479
CF	HA	61	51	11.04	0.1953	0.4583
CF	LB	61	40	4.34	0.6298	0.6634
CF	ME	61	192	32.19	0.0048	**0.0495**
CF	PO	61	4	13.45	0.4505	0.6303
CF	SC	61	73	89.14	0.0054	**0.0484**
CF	TSP	61	269	69.75	0.0316	0.1017
CV	HA	14	51	10.78	0.8371	0.8346
CV	LB	14	40	15.48	0.2954	0.5479
CV	ME	14	192	21.39	0.6195	0.6588
CV	PO	14	4	25.81	0.4923	0.6345
CV	SC	14	73	79.14	0.3959	0.6068
CV	TSP	14	269	58.35	0.5653	0.6447
HA	LB	51	40	14.11	0.1362	0.3029
HA	ME	51	192	28.31	0.0302	0.0900
HA	PO	51	4	19.44	0.5464	0.6447

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
HA	SC	51	73	85.65	0.0201	0.0900
HA	TSP	51	269	66.13	0.0591	0.1578
LB	ME	40	192	30.84	0.0278	0.0900
LB	PO	40	4	12.09	0.5256	0.6345
LB	SC	40	73	87.16	0.0493	0.1229
LB	TSP	40	269	70.78	0.0738	0.1812
ME	PO	192	4	41.99	0.4307	0.6239
ME	SC	192	73	59.37	0.0086	0.0529
ME	TSP	192	269	45.68	0.0123	0.0740
PO	SC	4	73	97.97	0.4979	0.6345
PO	TSP	4	269	81.62	0.5196	0.6345
SC	TSP	73	269	27.58	0.7314	0.7515

Table S12. Wasserstein statistics comparing size distributions of detected PS in egestion samples

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
AA	Blank	66	1	98.46	0.0454	0.2554
AA	CF	66	56	15.18	0.1001	0.2897
AA	CV	66	14	12.92	0.7007	0.8504
AA	HA	66	13	12.52	0.7577	0.8543
AA	LB	66	28	13.51	0.3417	0.5803
AA	ME	66	500	11.76	0.0200	0.2554
AA	PO	66	10	13.08	0.7840	0.8637
AA	SC	66	65	11.45	0.3605	0.5955
AA	TSP	66	106	21.69	0.0660	0.2787
Blank	CF	1	56	83.48	0.0349	0.2554
Blank	CV	1	14	94.02	0.0682	0.2787
Blank	HA	1	13	96.65	0.0690	0.2787
Blank	LB	1	28	95.99	0.0374	0.2554
Blank	ME	1	500	89.86	0.0274	0.2554
Blank	PO	1	10	87.99	0.0952	0.2897
Blank	SC	1	65	91.76	0.0765	0.2787

Comparison		Observations		Wasserstein		Benjamini - Hochberg
Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Statistic**	**P value**	**P value**
Blank	TSP	1	106	94.78	0.1260	0.2992
CF	CV	56	14	13.33	0.2866	0.5371
CF	HA	56	13	15.95	0.1641	0.3508
CF	LB	56	28	15.30	0.0345	0.2554
CF	ME	56	500	8.20	0.1057	0.2897
CF	PO	56	10	7.70	0.9144	0.9165
CF	SC	56	65	10.88	0.2196	0.4308
CF	TSP	56	106	19.18	0.1245	0.2992
CV	HA	14	13	7.01	0.5446	0.7183
CV	LB	14	28	4.18	0.8912	0.9165
CV	ME	14	500	7.58	0.7009	0.8504
CV	PO	14	10	7.24	0.7368	0.8504
CV	SC	14	65	10.75	0.7398	0.8504
CV	TSP	14	106	29.58	0.2046	0.4308
HA	LB	13	28	6.33	0.5492	0.7183
HA	ME	13	500	9.03	0.5380	0.7183
HA	PO	13	10	9.16	0.5077	0.7183
HA	SC	13	65	13.32	0.5225	0.7183
HA	TSP	13	106	32.20	0.1523	0.3508
LB	ME	28	500	8.27	0.2932	0.5423
LB	PO	28	10	8.11	0.4959	0.7183
LB	SC	28	65	11.89	0.3033	0.5423
LB	TSP	28	106	31.55	0.0449	0.2554
ME	PO	500	10	6.92	0.9139	0.9165
ME	SC	500	65	5.10	0.4846	0.7183
ME	TSP	500	106	23.56	0.0000	**0.0000**
PO	SC	10	65	10.88	0.8694	0.9165
PO	TSP	10	106	24.07	0.5920	0.7588
SC	TSP	65	106	20.06	0.0812	0.2787

Table S13. Microplastic abundance per organism

Organism		Sample type	PET	PE	PP	PS	Sum
Name	**Code**						
A. aquaticus	AA	egestion	0.03	2.73	1.79	2.00	6.55

Organism		Sample type	PET	PE	PP	PS	Sum
Name	**Code**						
A. aquaticus	AA	tissue	0.00	0.07	0.40	0.50	0.97
H. azteca	HA	egestion	0.00	0.08	1.46	0.39	1.94
H. azteca	HA	tissue	0.00	0.01	0.00	0.00	0.01
C. riparius	CR	tissue	0.00	0.14	0.19	0.00	0.34
C. volutator	CV	egestion	0.04	0.32	0.43	0.52	1.31
C. volutator	CV	tissue	0.98	0.04	0.06	0.04	1.11
C. fluminalis	CF	egestion	0.68	0.34	1.79	1.69	4.49
C. fluminalis	CF	tissue	0.00	0.01	0.03	3.28	3.33
L. balthica	LB	egestion	0.00	0.01	0.01	0.01	0.04
L. balthica	LB	tissue	0.00	0.00	0.00	0.89	0.89
M. edulis	ME	egestion	0.21	16.84	9.06	23.80	49.91
P. antipodarum	PO	egestion	0.00	0.19	0.08	0.29	0.56
S. corneum	SC	egestion	0.17	16.67	2.55	2.49	21.87
L. variegatus	LV	tissue	0.00	8.01	0.62	0.01	8.63
Tubifex spp.	TSP	egestion	0.53	3.18	4.93	2.03	10.67
Tubifex spp.	TSP	tissue	0.02	0.32	0.45	1.87	2.66

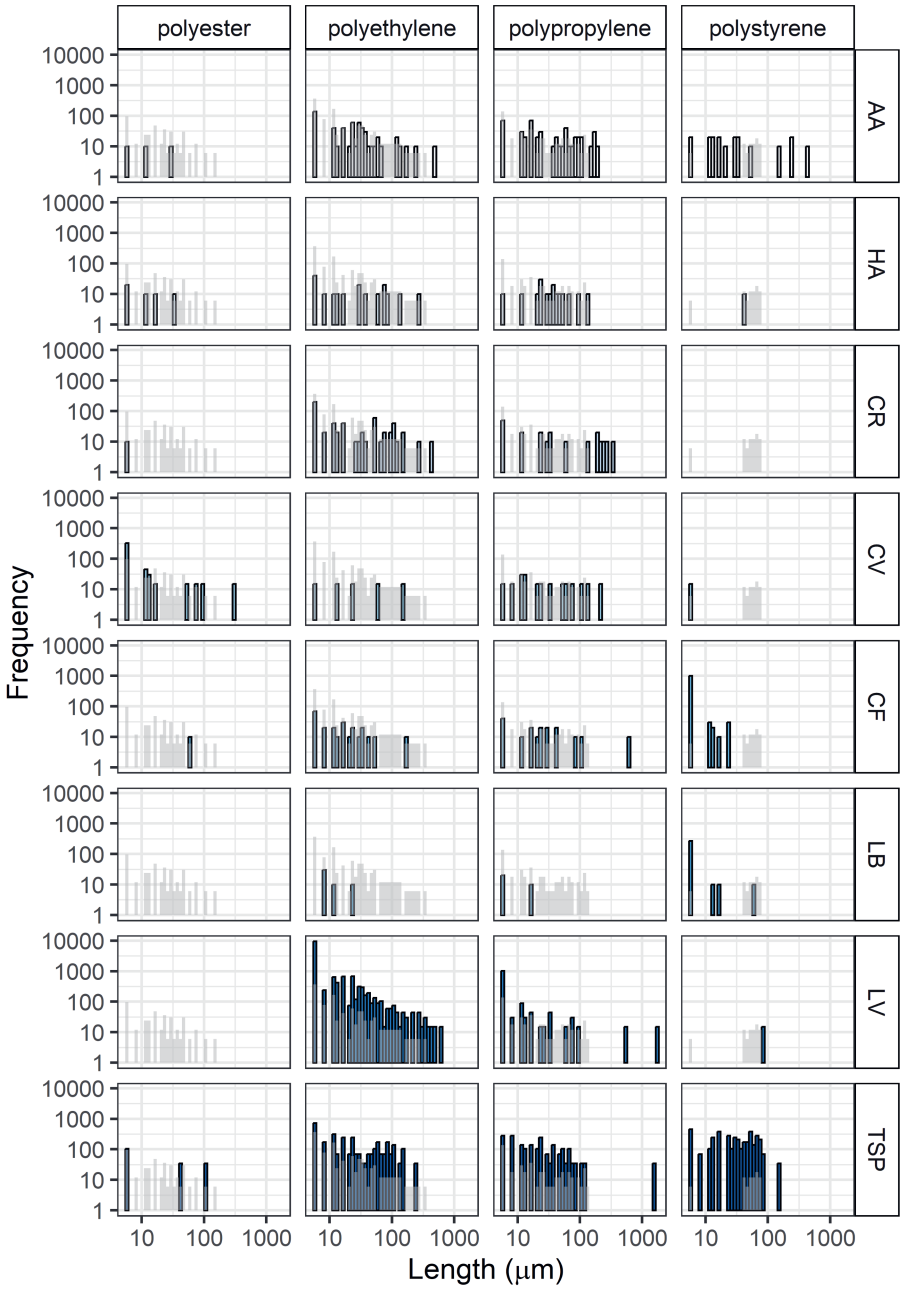


Figure S1. Histogram of ERMP retention samples. In grey the controls are depicted. Unequal sample effort has been corrected. 50 bins were selected for correction of contamination.

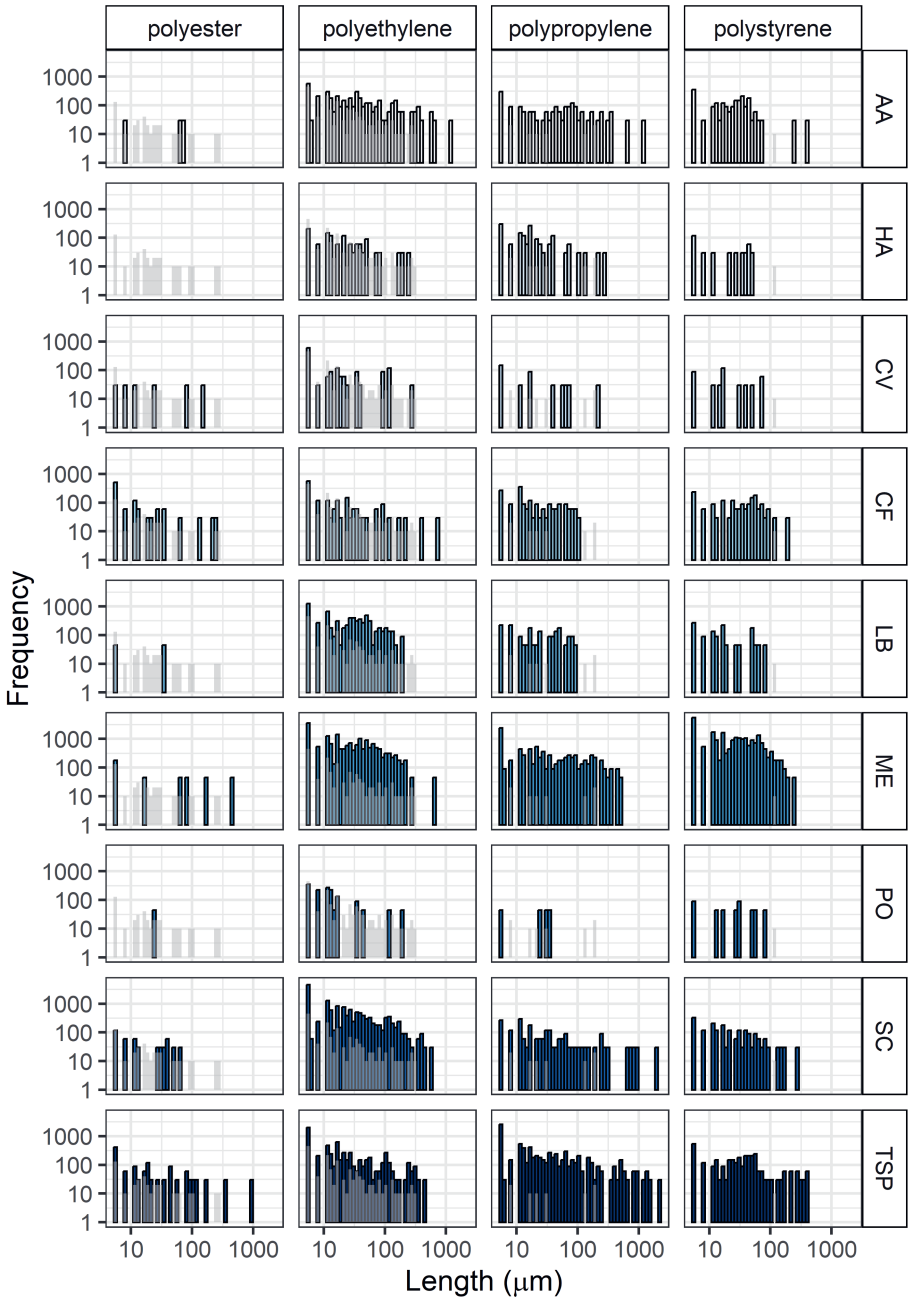


Figure S2. Histogram of ERMP detected in egestion samples. In grey the controls are depicted . Unequal sample effort has been corrected. 50 bins were selected for correction of contamination.

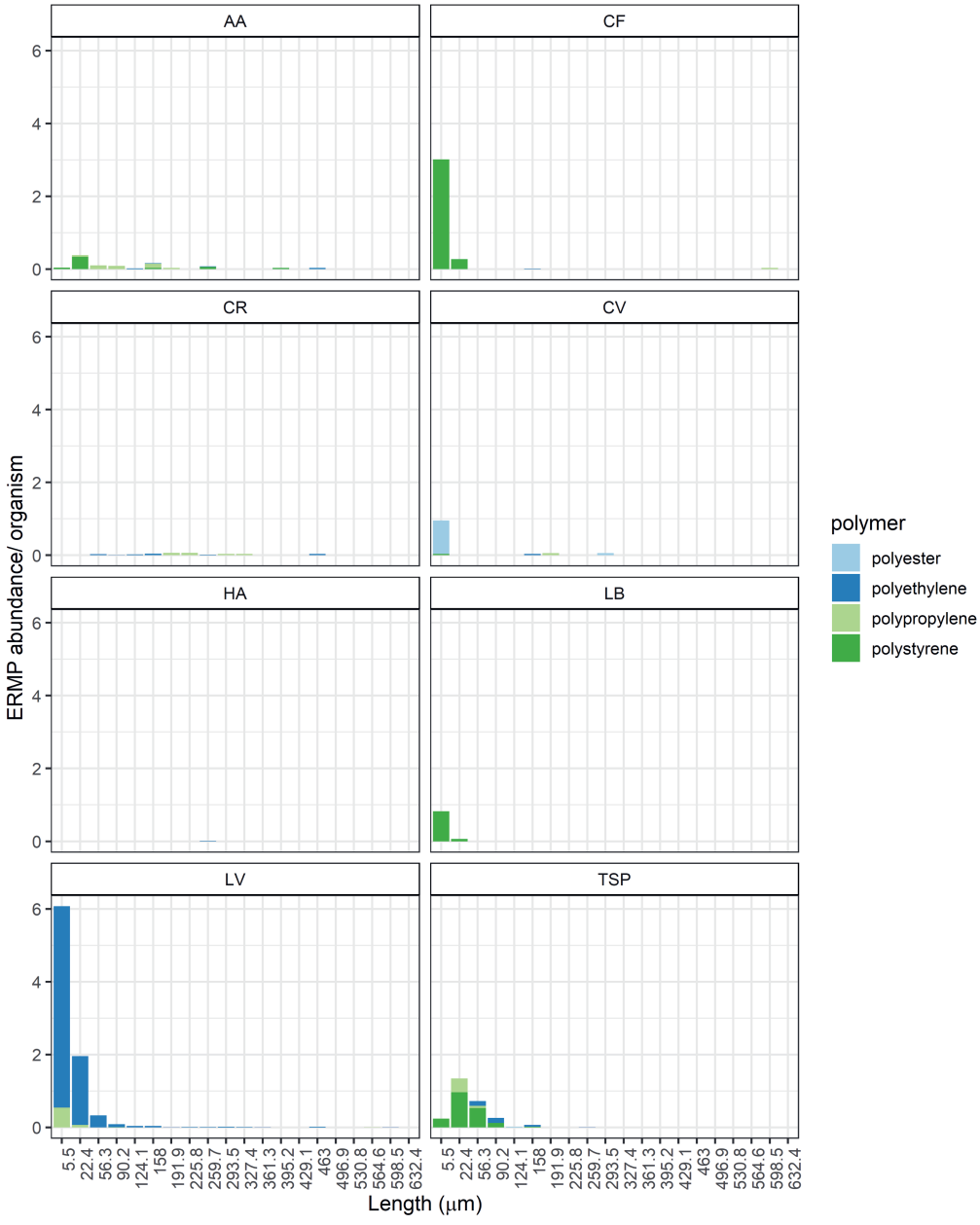


Figure S3. ERMP found in whole body tissue samples after blank correction. Note that particles > 650 µm were excluded, as 0.01 < counts were detected above this size range.

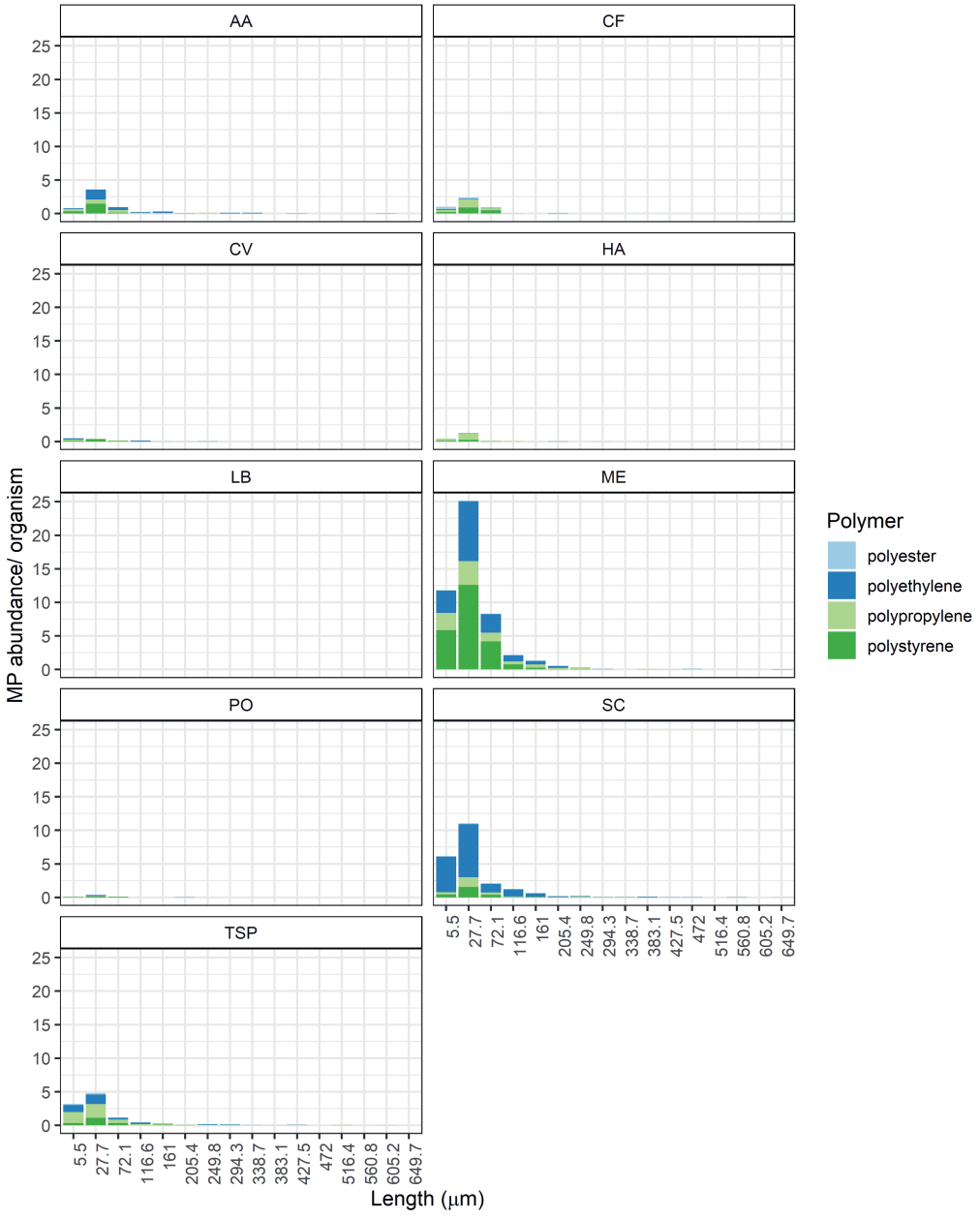
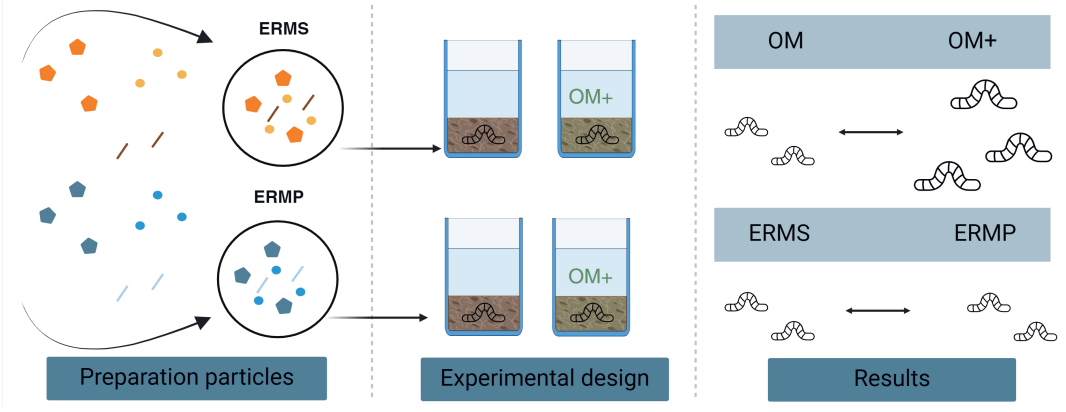


Figure S4. ERMP found in egestion samples after blank correction. Note that particles > 650 µm were excluded, as 0.01 < counts were detected above this size range.

Chapter 5

Microplastics versus natural mineral particles. How to create and test them while maintaining environmental relevance

Vera N. de Ruijter, Xinyi Xie and Albert A. Koelmans



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Abstract

Whether microplastics cause different effects than inert natural particles, and how to create relevant testing materials, are key questions in microplastics research. We prepared Environmentally Relevant Microplastic (ERMP) and Mineral Microparticle (ERMS) mixtures with similar levels of polydispersity and tested their 28-day chronic effects on the reproduction and growth of *Lumbriculus variegatus* at two different organic matter (OM) contents (average and enriched). Additionally *L. variegatus* was exposed to ERMP and ERMS to study the particle egestion for 14 days. We observed no differences in growth or reproduction between ERMP and ERMS at particle concentrations of up to 10% (v/v). In contrast, organisms exposed to enriched OM content increased their growth with 30% and increased reproduction of 20%. Only when *L. variegatus* was exposed to ERMP with an enriched OM content, a decrease in reproduction was detected with an effect threshold EC_{50} of 13.68 ± 5.54%, which however does not represent an environmentally realistic exposure concentration. After 14 days of exposure to 5% ERMP, the egestion of faecal pellets was higher compared to exposure to 5% ERMS, suggesting that in order to acquire the same amount of nutrition, *L. variegatus* is spending more energy. With this study, we demonstrate that refinements in the manufacturing of environmentally diverse particle mixtures can contribute to a more realistic testing of particle effects.

1. Introduction

Microplastics (MP) are ubiquitous in the aquatic environment today (Besseling et al., 2019; Burns and Boxall, 2018; Van Cauwenberghe et al., 2015). Consequently, they can be ingested by organisms and experimental work has shown that this ingestion could lead to potential negative effects (Foley et al., 2018). Many different kinds of negative effects have been reported including physiological stress, cell death, aberrant development, altered lipid metabolism, and intestinal damage (Kögel et al., 2020). However, most of the reported negative effects involve reduced body and population growth, or a decrease in energy levels (Kögel et al., 2020). While numerous effects have been documented, an almost equal number of studies have found no significant effect (Bucci et al., 2020). This inconsistency is likely attributable to variability across taxa, and differences in experimental design (Bucci et al., 2020; Foley et al., 2018; Kögel et al., 2020).

In order to more accurately assess the ecological risk associated with microplastics, a better understanding of the mechanisms underlying their effects and the key factors triggering these effects is needed (de Ruijter et al., 2020) (Bucci et al., 2020). Currently, it is difficult to compare the impact of various factors on the toxicity of microplastic particles, because experimental setups vary significantly. Furthermore, microplastic constitute a diverse suite of contaminants (Kooi and Koelmans, 2019; Lambert et al., 2017; Rochman et al., 2019; SAPEA, 2019), and in laboratory tests, different researchers often use particles that differ substantially from one another, and exhibit only a limited degree of polydispersity (de Ruijter et al., 2020). Nevertheless, it has been shown that the most important factors determining the toxicity of microplastics include concentration, particle size, shape and exposure duration (Bucci et al., 2020; de Ruijter et al., 2020; Kögel et al., 2020; Ogonowski et al., 2023; Thornton Hampton et al., 2022). Additionally, important cofactors such as species traits and food abundance and quality have been highlighted (de Ruijter et al., 2023). Much less is known about the mechanisms underlying adverse effects. Most studies are unable to demonstrate the hypothesized effect mechanisms. For invertebrate aquatic species, the strongest evidence exists for “inhibited food assimilation and/or decreased nutritional value”(de Ruijter et al., 2020; Thornton Hampton et al., 2022), more commonly referred to as ‘food dilution’. This would imply that any inert material causing the same dilution would have a similar effect on the organism.

Testing microplastics with an environmentally relevant degree of polydispersity (ERMP) is a challenge in itself. It is necessary to first determine what the relevant properties of ERMP are (Kooi et al., 2021). For effects related to 'food dilution', the relevant property is the volume of the ingested polydisperse ERMP mixture (Koelmans et al., 2020), which should contain all size classes of natural ERMP in the correct proportions. The same applies to translocation-based effect mechanisms, such as inflammation and oxidative stress, for which the reactive surface of the translocated particles is important (Kooi et al., 2021; Mehinto et al., 2022). Realism in these cases can only be achieved with an environmentally relevant degree of polydispersity. As a positive particle control, or to differentiate between effects caused by particle shape or particle material type, an inert non-polymer test material with the same specifications must be synthesized in the laboratory. To investigate whether the inert non-polymer material causes the same effects, if any, this test material must also meet those morphological specifications. The need to include positive particle controls in effect tests, as well as to investigate differences in material type, while keeping particles otherwise as similar as possible, is an often-mentioned research priority (Gerdes et al., 2019; Koelmans et al., 2022b; Ogonowski et al., 2018; Ogonowski et al., 2023). Several studies have been conducted comparing effects of MP with non-polymer test materials (Gerdes et al., 2019; Ogonowski et al., 2016; Schür et al., 2020; Yap et al., 2020). However, these studies used monodisperse or only slightly polydisperse particles. We are not aware of studies specifically aimed at keeping toxicologically relevant characteristics such as size and volume approximately equal, and also equal to the polydispersity observed in nature.

Recently, we detected a significant difference in the effects of ERMP on *L. variegatus* in sediments with varying organic matter (OM) content (de Ruijter et al., 2023; Redondo-Hasselerharm et al., 2018). While Redondo-Hasselerharm et al. (2018) found no effect on the growth or reproduction of *L. variegatus* in sediments with an OM content of 32% (Redondo-Hasselerharm et al., 2018), de Ruijter et al. reported negative effects on the growth and reproduction of *L. variegatus* (growth: EC_{50} = 0.77 ± 0.29 % d.w.; reproduction factor: EC_{50} = 2.51 ± 0.44 % d.w) when the OM content in the sediment was 4.5 times lower (de Ruijter et al., 2023). This suggests that the OM content is an important factor affecting the effects of microplastics.

The aim of this study is twofold. First, we aim to compare the effects of two different inert materials, i.e., environmentally relevant microplastic (ERMP), and environmentally relevant mineral microsolids (ERMS), while ensuring their particle size distribution is as similar as possible, on the benthic invertebrate species *Lumbriculus variegatus*. Secondly, we aim to introduce a method and provide a protocol for preparing polydisperse mixtures of microplastics or mineral particles based on a pre-known particle size distribution.

To achieve this goal, we utilized the model species *Lumbriculus variegatus* to compare the effects of ERMP on growth and reproduction, with those of an environmentally relevant mixture of ERMS. We conducted chronic dose-response tests with both particle types at two levels of sediment OM, following the QA/QC criteria proposed by de Ruijter et al. (2020) The ERMS consisted of a mixture of various clay, silt and sand fractions with the same polydispersity as the ERMP mixture, and the preparation of both mixtures was considered a secondary objective of the study. This approach allows us to contextualize the effects of ERMP in relation to natural particles.

Similarities in - or absence of differences in the effects between ERMP and ERMS are examined in the context of known effect mechanisms, such as food dilution (Koelmans et al., 2017; Koelmans et al., 2020). Given that the implications of an effect mechanism like food dilution are closely related to food availability, the egestion of faecal pellets was studied for 14 days.

2. Material & Methods

2.1. Preparation of environmentally realistic microplastic particles (ERMP)

Environmentally realistic microplastic particles were designed to closely mimic the properties of microplastics found in the environment including size, shape and polymer distributions (Kooi et al, 2021), and were manufactured as follows. Plastic granulates and flakes consisting of PE, PP, PET, PS and PA were all cryogenically milled under the same conditions, specifically at -50 °C at the industrial grinding company Netzch Lohnmatecknik GmbH (Bobingen, Germany). Our objective was to grind the plastic granulates and flakes into the following size distribution: D_{10} = 20 µm, D_{50} = 80 µm, D_{90} = 500 µm (Table S1), however it is important to note that each polymer type has a unique size distribution (Figure S1). This variation arises because, by using the same grinding energy for all polymers, relative differences in

polymer strengths and resistances are preserved, which also affect the relative size distributions of individual polymers in the environment.

The polymer identity was confirmed using ATR-FTIR. To verify size and shape distributions, we captured high resolution pictures (n>100 particles) for each polymer type and size class using an Olympus SZX10 stereomicroscope (Figure S5.1 -S5.9). These images were then analysed with ImageJ to determine for major- and minor axis (Schneider et al., 2012). In total nine classes of polydisperse particles were identified, with lengths ranging from 3 to 145 µm (PA), 5 to 185 µm (PS), 5 to 229 µm (PET), 9 to 348 µm (PP), 15 to 589 µm (PE_1), 7 to 590 µm (PE_2), 3477 to 5000 µm (PE_{big}), 2858 to 5000 µm (PET_{big}), and 4371 to 5000 µm (PP_{big}) (Table S1). To ensure that the particles remained within the standard maximum size for microplastics, they were sieved twice through a 5 mm sieve. However, it should be noted that a few particles longer than 5 mm were found, likely having passed the sieve perpendicular to their longitudinal axis. Nevertheless, based on particle number this represented a negligible amount of microplastic contamination within the microplastic mixture. The density of each polymer type, as well as the final mixture was measured using a gas pycnometer (Ultrapyc1200, Quantachrome Instruments) for powders and porous materials. The polymers PA, PS, PE, PP and PET showed densities of 1.16 ± 0.001, 1.10 ± 0.001, 0.97 ± 0.002, 0.93 ± 0.001 and 1.30 ± 0.015 g cm^{-3} respectively (Table S1). The proportions at which the different polydisperse polymer powders had to be mixed to obtain an ERMP mixture were based on an a priori *in silico* design, which is detailed in the next section. Finally the ERMP mixture had a density of 1.00 ± 0.006 g cm^{-3}.

2.2. Designing ERMP from polydisperse polymer particles with a limited size range

Because we know the particle size and polydispersity (the slope of the power law) for each particle class, we can simulate the properties of any mixture made from these 9 classes *in silico*. This allows us to optimize how the classes should be blended to closely match the power law exponent measured for ERMP in the environment. To create a size distribution similar to that found in the freshwater environment (Kooi et al, 2021), we calculated the required proportions of the nine polydisperse polymer powders a priori using equation 1, taking into account the upper ($UL;\ \mu m$) and lower size limit ($LL;\ \mu m$) of each size class and a mean power law exponent parameter of 3.25 ± 0.19 (α) (Kooi et al., 2021).

$$A = \int_{LL}^{UL} C\, x^{-\alpha}\, dx = \frac{UL^{1-\alpha} - LL^{1-\alpha}}{1-\alpha} \quad (1)$$

To obtain an accurate particle size distribution based on ImageJ analysis of microscope pictures, multiple pictures were combined until a number of >100 particles was obtained (Figure S5.1 -S5.9). For each polymer type, we determined the actual lower limit (LL) and upper limit (UL) through image analysis (Table 1, column 'LL', 'UL'). In some cases, a size class had varying sizes and multiple magnifications were required to capture all the particles within the sample. The smaller particles captured with a higher magnification were, in fact, a subsample and were rescaled by multiplying the whole unique dataset by the difference in magnification factor. Consequently, for each size class the amount of particles per dataset was calculated (Table 1, column 'Observed Image Count'). Using the major axis diameter (length) (φ_l) and the minor axis diameter (width) (φ_s) and the polymer density (ρ), we calculated the mass per unique dataset (Table 1, column "observed mass") using equation 2 (Tanoiri et al., 2021).

$$M_{Simon} = \frac{4}{3}\left(\frac{\varphi_l}{2}\right)\left(\frac{\varphi_s}{2}\right)\left(\frac{0.67\varphi_s}{2}\right)\pi\rho \quad (2)$$

We targeted an average number of particles in each size fraction for the LL and UL values using Equation 1, where α = 3.25 represents the power-law slope of the targeted particle size distribution. Based on these values, a relative targeted number concentration (%) was calculated (Table 1, column 'targeted particle (%)'). Since the observed and targeted relative fractions vary for each polymer, a multiplication factor (MF) was computed (MF = targeted% /observed%). For example, for PA, the targeted percentage was 58.4, while the observed percentage was 17%, resulting in an MF of 58.4 / 17 = 3.51. By applying this multiplication factor to the observed weights per polymer fraction, we derived a corrected weight for each polymer fraction. These weights are low and the weight of a mixture made from these polymer fractions is too low for experimental use. However, the calculated weight fractions for each polymer (Table 1, under the column 'targeted weight%') can be applied to achieve any desired total weight for the final mixture.

Table 1. Design table: How to design a polydisperse mixture with environmentally relevant microplastics.

Polymer	LL (µm)	UL (µm)	Targeted alpha	Avg number	Targeted particles (%)	Observed mass (g)	Observed ImageJ count	Observed particle (%)	Required factor	Required mass (g)	Recipe mass (%)
PA	2.8	145	3.25	0.0428	58.4	8.34E-05	1638	17	3.51	2.93E-04	11.34
PS	5.2	185	3.25	0.0107	14.7	2.53E-04	1763	18	0.82	2.07E-04	8.02
PET	5.5	229	3.25	0.0097	13.2	9.55E-04	3374	34	0.39	3.68E-04	14.23
PE	6.8	590	3.25	0.0060	8.2	1.14E-03	899	9	0.90	1.02E-03	39.46
PP	9.2	348	3.25	0.0030	4.1	3.26E-04	976	10	0.41	1.34E-04	5.20
PE	14.7	589	3.25	0.0011	1.4	1.53E-03	818	8	0.17	2.65E-04	10.26
PET_big	1749	5000	3.25	0.0000	2.8E-05	1.48E+01	164	2	0.00	2.47E-04	9.56
PE_big	3843	5000	3.25	0.0000	2.3E-06	1.39E+01	112	1	0.00	2.86E-05	1.11
PP_big	3881	5000	3.25	0.0000	2.2E-06	1.03E+01	105	1	0.00	2.15E-05	0.83

To verify the mean power law exponent parameter for the final mixture, the required proportions were adjusted in R by multiplying the datasets accordingly. Subsequently, power law distributions for the separate polymers and the final mixtures were fitted using maximum likelihood estimation (Clauset et al., 2009; Newman, 2005) and n = 100 bootstraps as previously designed by Kooi et al. (2021). All calculations were performed using the R package poweRlaw (Gillespie, 2014).

Finally, based on the design, we created in the laboratory the physical ERMP mix with varying polymer type, size, shape and colours, in mass proportions corresponding to those occurring in the freshwater sediment environment (PE > PET > PP > PS > PA), and a natural polydispersity characterized by an average exponent parameter of 3.57 ± 0.10 (Figure S1, S2, S6)(Kooi and Koelmans, 2019). Our research questions concern the particle effects of microplastics, rather than particle-associated chemicals. Therefore, to eliminate any additives present in the plastic, the microplastics were washed with methanol three times and mixed on a shaker table for at least two hours per wash, with a final overnight wash (de Ruijter et al., 2020; Koelmans et al., 2017).

2.3. Preparation of environmentally relevant micro-solids (ERMS) particles

Similar to the environmentally relevant polydisperse microplastic mixture, a mixture of inert non-polymer particles was designed and created from nine classes of mineral particles (clay, fine sand, coarse sand, very coarse sand and fine gravel) (Table S2). High resolution pictures (n>100 particles) were taken per size class with an Olympus SZX10 stereomicroscope (Figure S5.10 -S5.18). The particle properties of these nine classes were determined using ImageJ and exhibited unique size distributions (Figure S4). Kaolin particles ranging from 5 to 39 µm were purchased from Sigma Aldrich. Very fine sand to coarse sand with grain sizes ranging from 14 to 413 µm (Zilverzand), 16 to 533 µm (Ophoogzand), 16 to 353 µm (Speelzand), and 42 to 787 µm (Brekerzand), were collected from Karwei construction shop (Wageningen, the Netherlands). Very coarse sand particles ranging from 86 to 907 µm (RayherHobby) and 22 to 876 µm (MICADecoration) were ordered from Shoppartners b.v.. Finally, very fine gravel with particles ranging from 12 to 4047 µm (NurzurDekoration) and 50 to 5071 (Eurosand) were ordered from hobby shop Boetiek Chloë and Dutch QualityProducts respectively (Table S2).

The mineral-based ERMS mixture was made to closely match the size distribution of ERMP and followed the same design procedure. A mean exponent parameter of

2.37 ± 0.03 was obtained (Figure S3, S4, S7). Although no additives or sorbed chemicals were expected for these mineral particles, they underwent the same methanol washing treatment as the ERMP mixture (de Ruijter et al., 2020; Koelmans et al., 2017). The density of the sand and clay as well as the final mixture was measured using a gas pycnometer (Ultrapyc1200, Quantachrome Instruments) for powders and porous materials (Table S2). The particles from Eurosand, MICAdecoration, Rayherhobby, Nurzurdekoration, Brekerzand, Speelzand, Ophoogzand. Zilverzand and Kaolin had densities of 2.69 ± 0.004, 2.68 ± 0.004, 2.64 ± 0.003, 2.86 ± 0.003, 2.64 ± 0.006, 2.64 ± 0.003, 2.64 ± 0.015, 2.64 ± 0.012 and 2.71 ± 0.055 g cm^{-3} respectively. Finally the ERMS mixture has a density of 2.68 ± 0.004 g cm^{-3}.

2.4. Sediment

Clean freshwater sediments were collected from the experimental field station of Wageningen University (the Sinderhoeve, Renkum, The Netherlands). Subsequently, sediments were sieved through a 2 mm sieve and stored at -20°C in order to preserve OM and kill any organisms present. The sediment has an OM content of 4.21% ± 0.03 (n=5) (de Ruijter et al., 2023), which can be considered as a typical, default standard OM content.

2.5. Test organisms

L. variegatus were cultured at the Aquatic Ecology and Water quality Management Department, Wageningen University and Research (Wageningen, The Netherlands), in Dutch Standard Water (DSW) at 20 ± 1 °C. They were fed twice a week with organic nettle powder. Additionally unbleached kitchen paper was added as a living substrate. DSW was renewed weekly.

2.6. Test design

The ERMP and the ERMS mixtures were each mixed into the sediment in order to obtain six doses: 0, 0.3, 1.0, 2.5, 5.0 and 10.0 % (v/v). Exposure to ERMP and ERMS was standardized by applying doses based on particle volume. This equalizes the number of encounters between organisms and the particles, as well as the bioavailable fractions for particle ingestion and food dilution. However, equalizing by volume does result in a difference in dose based on the weight of the particles. In terms of weight, ERMS doses were 2.68 times higher than those for ERMP. Weight percentages were: 0, 0.26, 0.88, 2.21, 4.43 and 8.91% (wt). Particle number

concentrations were: 0, 3.49 ×10^6, 1.18 ×10^7, 2.97 ×10^7, 5.94 ×10^7 and 1.20 × 10^8 microplastics/kg. Experimental units were prepared in triplicate. For the systems with enriched OM, 1.0 g of organic nettle powder was added to the sediment. Subsequently, the systems were manually homogenized using a stainless steel spoon (Figure S8, 9). Afterwards, DSW was gently added at a 4:1 water-to-sediment ratio. The systems were randomized and left to acclimatize for two weeks before adding the organisms. In each experimental unit, 20 organisms were added. The exposure duration was of 28 days, and the temperature was maintained at a constant 20 ± 1 °C. Dissolved oxygen, pH, temperature, conductivity, salinity and NH_3 concentrations were measured twice a week. DSW was periodically refreshed, and air supply was checked daily. During the exposure period, the test systems were covered with aluminium foil to avoid contamination from the laboratory environment. After 28 days, the organisms were sieved, counted and then dried for 96 hours at 37°C before being weighed per replica. For ERMP treatments, exposure was verified by analysing whole body tissue and egestion samples for microplastic contents using micro-FTIR, which is reported elsewhere (de Ruijter et al., 2023).

Test design, materials, handling of materials, control of background contamination and exposure conditions adhered to the QA/QC criteria as defined and previously described by de Ruijter et al. (2020) and de Ruijter et al. (2023). A summary of how these criteria were met is provided as Supporting information (Table S3).

2.7. Data analysis

All statistical analyses and graphs were performed in R (R Core Team, 2023). Dose response curves and effect concentrations (EC_x) were determined utilizing the drc package in R (Ritz et al., 2015). The continuous data for reproduction and growth responses were fitted using 2 to 4 parameter log-logistic and Weibull models. The best fitting model was selected based on the Akaike Information Criterion (AIC), using the *mselect* function from the *drc* package and visual inspection. Subsequently, model assumptions were checked visually using Q-Q plots and residual plots. To assess the presence of a dose-effect relationship, the dose response model was compared to a linear regression model with a slope of 0, indicating the absence of a dose-response relationship. This was done using the likelihood ratio test (*noEffect function drc*). EC_{10} and EC_{50} values were derived with 95% confidence intervals using the function *ED()* in the *drc* package.

The effects of material type (i.e., ERMP or ERMS), OM content (enriched and average), and exposure concentration on reproduction and growth, were assessed with a three-way ANCOVA. Additionally, the effect of material type (ERMP or ERMS), concentration (0%, 5%, 10%) and day were tested with a repeated three-way ANOVA. Interactions between variables were investigated using ANOVA, AIC, significance of interaction terms and interaction plots. Model assumptions were checked using diagnostic plots (Q-Q and residual plot), as well as statistical tests such as the Shapiro-Wilk normality test and Levene's test. All residuals of reproduction and growth data were normally distributed, and the variances were homogenous. However, residuals of egestion data were not normally distributed, and were log transformed before analysis. Multiple comparisons among different treatments (mixtures, OM content) and concentrations were conducted using Tukey's method.

3. Results & discussion

3.1. Effects of microplastics and mineral particles on reproduction

We exposed worms for 28 days to increasing doses of particles, mixed in the sediment. Water quality measurements temperature, pH and dissolved oxygen were consistent through time and showed no apparent differences between treatments (Table S4). Conductivity 884 ± 68.41 µS/cm was higher than recommended, however no differences between treatments were detected (Table S4) (OECD, 2007). The average number of living worms in the controls increased with a factor of 2.5 ± 0.10, 1.98 ± 0.32, 2.08 ± 0.31, 1.8 ± 0.48 compared to the start of the test for the treatments ERMP with enriched OM content, ERMP with average OM content, ERMS with enriched OM content and ERMS with average OM content, respectively. This indicates that all exposures adhered to OECD guidelines regarding control reproduction, and test conditions were adequate (OECD, 2007).

Our primary objective was to compare the effects of environmentally relevant microplastic (ERMP) with an inert non-polymer material exhibiting a similar level of polydispersity. In a direct comparison of the effects of ERMP on reproduction with those obtained for ERMS, no statistically significant differences were observed (three-way ANCOVA, $p = 0.174$) (Table S6). This implies that neither material is significantly more toxic than the other, even at the highest tested dose of 10%. While this lack of differences does not confirm the existence of specific effect mechanisms such as food dilution (since no effects were observed), it does reduce

the likelihood of material-specific mechanisms. In contrast, a non-specific mechanism like food dilution relies solely on the volume ingested. Thus, it is possible that the ingested volumes for both material types were limited enough to prevent food dilution from occurring. In this context, the results are consistent with a food dilution mechanism. Similarly, previous studies by Silva et al. (2021) and Redondo-Hasselerharm et al. (2018) also did not find any reproductive effects on *L. variegatus* after a 28-day exposure.

Despite these findings, our dose-response model extrapolated a statistically significant effect threshold above the highest tested dose (EC_{50} = 13.68 ± 5.54 v/v%; $1.64*10^8 \pm 6.59*10^7$ microplastics/kg) for ERMP in the 'enriched OM' treatment (Table S5; Figure 1). However, because the EC_{50} value exceeds the highest tested concentration of 10% (v/v), this raises concerns about the reliability of the threshold, which therefore must be interpreted with caution. Nevertheless, this EC_{50} is close to the highest 'hotspot' concentrations reported for Liangfeng River sediments in China with 2.21×10^8 number/kg of dw. For the other treatments, including ERMP with average OM content, ERMS with enriched OM content, and ERMS with average OM content, no significant dose-effect relationships could be established (Table S5; Figure 1).

Significant differences in the reproduction rate were found between the treatments depending on the organic matter content in the sediment (three-way ANCOVA, p = < 0.001)(Table S6). Where the organic matter content was higher, the reproduction rate was also higher with a factor of 1.2 for the treatment ERMP (Tukey HSD, ERMP OM enriched vs ERMP OM average, p = 0.022) and ERMS (Tukey HSD, ERMP OM enriched vs ERMS OM average, p = 0.003)(Figure 1; Table S6). This confirms the important role of food quality and abundance as factors of habitat quality for benthic invertebrates (Amariei et al., 2022; Vroom et al., 2017). Furthermore, this underscores the significance of food conditions when designing microplastics effect studies.

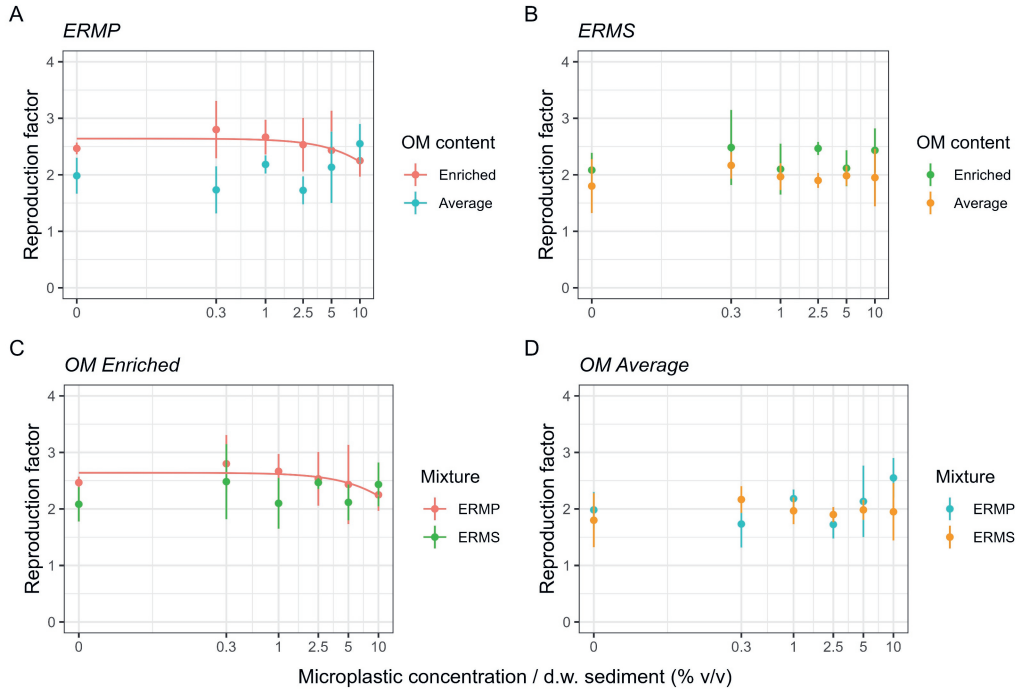


Figure 1. Mean reproduction factor (± s.d.) of *L. variegatus* after chronic exposure to ERMP or ERMS in sediment with enriched versus average organic matter content. Note that concentrations are on a log scale. Additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

3.2. Effects of microplastics and mineral particles on Growth

Chronic exposure to ERMP or ERMS with concentrations of up to 10% (v/v) caused no significant effect on the growth of *L. variegatus,* and no significant dose effect relationships were observed (figure 2; Table S7). This is in accordance with previous studies that exposed L. variegatus to PS and PE, respectively (Redondo-Hasselerharm et al., 2018; Silva et al., 2021). No differences on the growth were detected between the mixtures ERMP and ERMS (three-way ANCOVA, p = 0.592)(Table S8). Hence, also for this endpoint, neither material appeared significantly more toxic than the other, supporting our interpretation of the reproduction data. Interestingly, sediment OM content appeared to be a more important factor explaining the observed growth differences (three-way ANCOVA, p = < 0.001)(Table S8). With enriched OM content, the growth of *L. variegatus* increased on average by a factor of 1.3. These differences were apparent between

the treatments ERMP OM enriched and EMS OM average (Tukey HSD, p = 0.013) and ERMS OM enriched and ERMS OM average (Tukey HSD, p = 0.011)(Table S8).

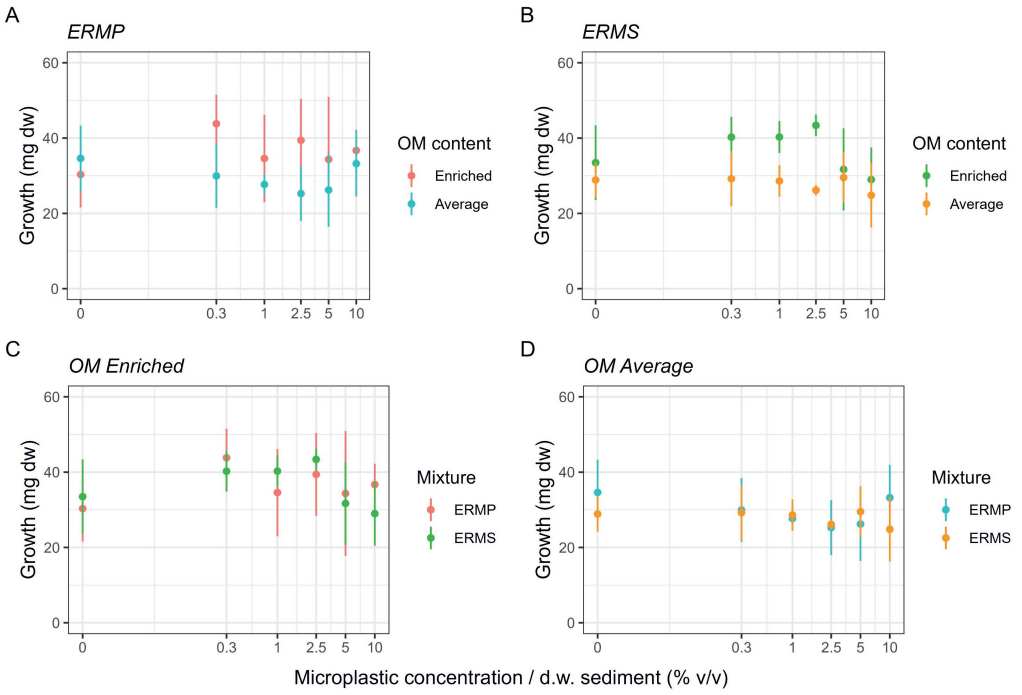


Figure 2. Mean growth mg dw (± s.d.) of *L. variegatus* after chronic exposure to ERMP or ERMS in sediment with enriched versus average organic matter sediment content. Note that concentrations are on a log scale, additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

3.3. Effects of microplastics and mineral particles on egestion

Water quality measurements temperature, pH and dissolved oxygen were consistent over the 14 days of exposure and showed no apparent differences between treatments (Table S9). After 14 days, the controls had an average reproduction factor of 0.91 ± 0.11 and growth of 10.77 ± 3.24 mg dw.

The egestion of faeces by *L. variegatus* was not significantly different between the two mixtures ERMP and ERMS (three-way repeated ANOVA, p = 0.071)(Figure 3; Table S10). Only for one exposure concentration 5 % (v/v),ERMS. *L. variegatus* exposed to ERMP egested more than when exposed to ERMS (three-way repeated ANOVA, Mix x Concentration, p = 0.004) (Tukey HSD, p = 0.010)(Figure 3; Table S10). We can only speculate about the explanation. As egestion is a proxy for ingestion

this indicates that for the same amount of nutrition ingested more energy is spent. Another explanation could be due to the density differences in the particles. As ERMS had a 2.68 times higher density than ERMP, it might take more energy to process the sand and clay particles compared to the microplastics, resulting in less egestion of these particles. However, it remains unclear, because you would also expect this at other doses, which is not observed.

When the dose of ERMP is increased up to 10% (v/v), egestion decreases significantly (Tukey HSD, p = 0.029) and shows no difference compared to the control (Tukey HSD, p = 1.000) (Figure 3; Table S10). We have no conclusive explanation for this observation; we can only speculate how material-specific differences such as particle density or aggregation behaviour might affect the egestion of particles. A possible explanation is that at higher concentrations microplastics were more aggregated or encapsulated in the sediment, rendering them less bioavailable for *L. variegatus*. For the treatment with ERMS the egestion rate is not concentration dependent; no differences are found between the control and the concentrations tested (Tukey HSD, p = 0.310, p = 0.999) (Figure 3; Table S10).

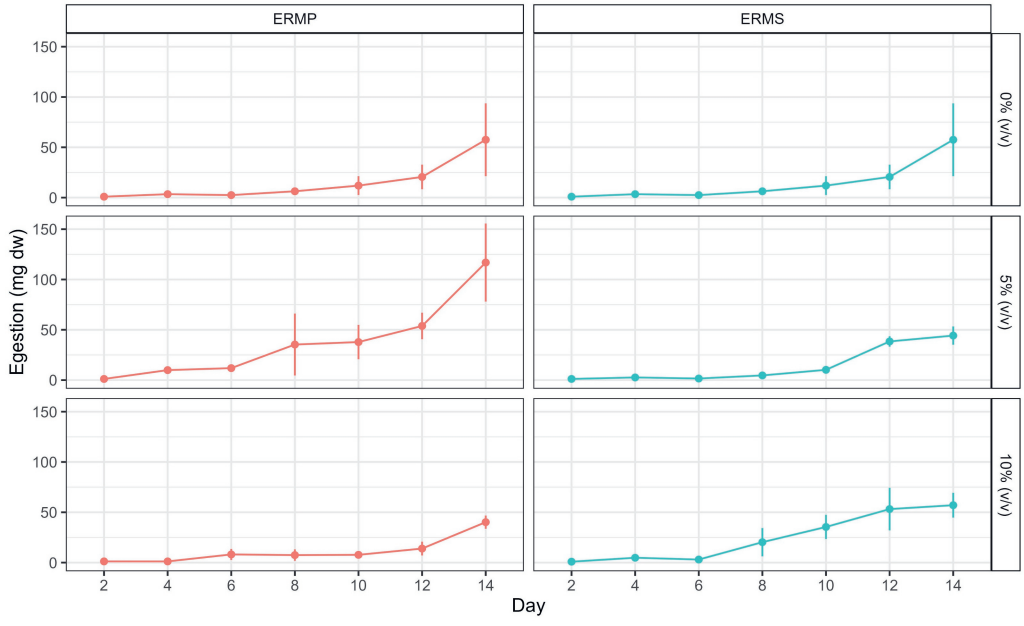


Figure 3. Egestion of faecal pellets (mg dw) after exposure of *L. variegatus* to ERMP or ERMS over 14 days.

4. General discussion and recommendations

In this study, we introduce a novel approach for making environmentally relevant mixtures of particles within the microplastic size range of 1 to 5000 µm. We provide a recipe for making polydisperse mixtures of microplastic and natural sand and clay particles with a focus on achieving size similarity among them. The chosen size range determines if the particles can be ingested, and by expressing exposure using a particle volume-based concentration ratio instead of using mass or particle number concentration, hypothesized effect mechanisms like that of food dilution can be tested.

This study shows that it is possible to conduct effects tests on environmentally relevant, heterogeneous particle mixtures, here ERMP and ERMS, while adhering to strict QA/QC criteria (de Ruijter et al., 2020). There was no difference between the effects of inert ERMP or ERMS particles on the reproduction and growth of *L. variegatus*. This result differs from what has been found in recent meta analyses comparing effects of microplastic to those of natural particles, which generally suggest that microplastics are slightly more toxic than natural particles (Doyle et al., 2022; Ogonowski et al., 2018; Ogonowski et al., 2023; Waldschläger et al., 2022). However, these meta analyses have their limitations. They involve the comparison of data from different studies, each carrying considerable uncertainties, resulting in reduced statistical rigour compared to experiments where all experimental conditions are held constant, as in our study (Waldschläger et al., 2022). Published studies often involve particles that differ significantly in terms of polydispersity, compared to the particles tested in our study. Moreover, the particles used in those studies may contain chemicals, with unknown chemical identities and concentrations that vary between experiments. Consequently, differences in toxicity stemming from chemical contaminants are erroneously attributed to inherent microplastics characteristics. In essence, the toxicity of chemicals primarily pertains to the hazard assessment of those chemicals, and should be considered separately from the assessment of particle effects (Koelmans et al., 2017; Koelmans et al., 2022a; Petersen et al., 2022). Together with the use of inappropriate metrics, these factors complicate comparisons (Ogonowski et al., 2018; Ogonowski et al., 2023).

Only for ERMP with enriched organic matter content we find an adverse effect on the reproduction of *L. variegatus*, higher than the highest dose. However, this is not

an environmentally relevant concentration(Lenaker et al., 2019; Redondo-Hasselerharm et al., 2023; Xia et al., 2021), is not a reliable threshold concentration, and does not alter the fact that the direct comparison between ERMP and ERMS showed no difference. Furthermore, this effect threshold is considerably higher than we found in our previous study (EC_{50} = 2.51 ± 0.44 % d.w). As the experimental set ups were almost identical, this indicates that the repeatability of tests to detect adverse effect induced by microplastic particles may be limited, possibly due to biological variability and the relatively small effect size detected previously.

We did observe a difference in egestion rates between the diverse microplastics and diverse mineral particles tested. Interestingly, when exposed to the same particle volume concentration in the sediment for the two mixtures, *L. variegatus*, albeit only at the 5% (v/v), showed an increase of egestion when exposed to ERMP compared to ERMS. This suggests that in order to acquire the same amount of nutrition, *L. variegatus* is spending more energy. This is in accordance with the findings of Silva et al. (2021), who found that PE-MPs induced depletion of energy reserves (Silva et al., 2021). Notably, this difference in effect diminished at the highest concentrations tested 10% (v/v). Although the sediment was thoroughly homogenized before the start of the experiment, it can be speculated that during the experiments, the bioturbation of the blackworms caused aggregation of microplastics preventing it to become bioavailable. Here the use of a polydisperse mixture increases environmental relevance and gives insight into behaviour of particles as a mixture. One factor explaining the response variables more substantially than the different mixtures tested, is the organic matter content in the sediment. This variable explains significant differences in both the reproduction and weight of *L. variegatus*, highlighting its importance when designing microplastic testing experiments.

Supporting information

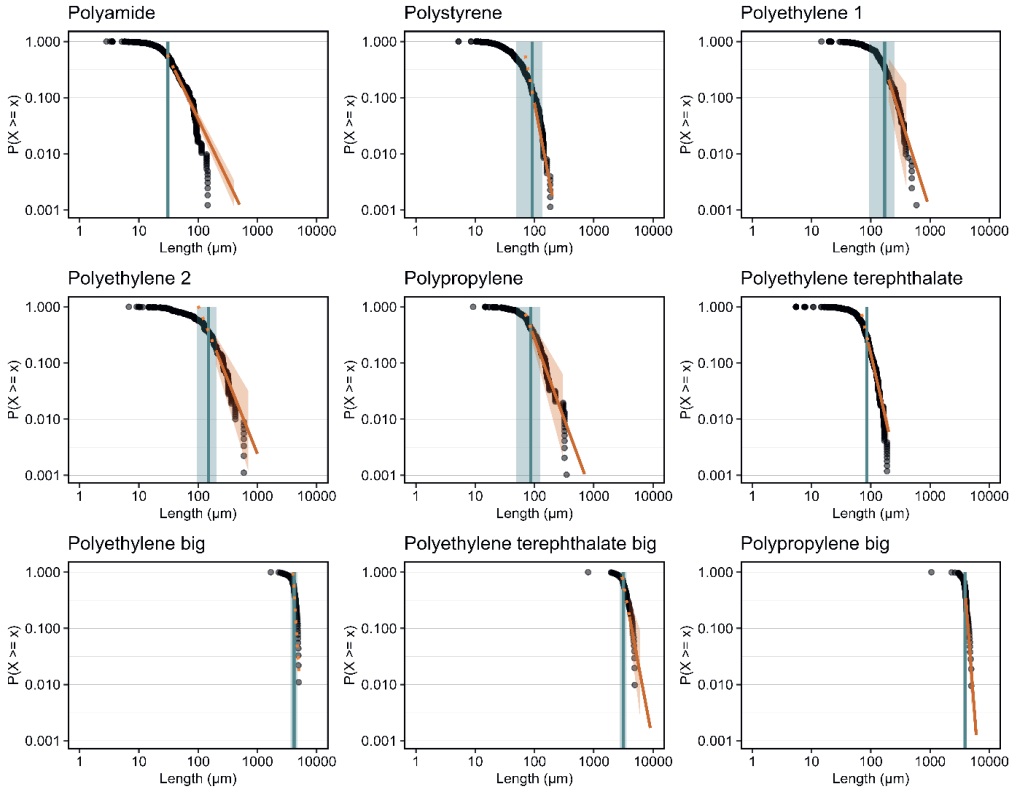


Figure S1. Particle length distributions for the different polymer types. The blue vertical lines indicate the minimum size for which the fitted power law is valid (X_{min}). The orange slopes indicate the fitted power law distributions (α). The mean and standard deviation of both α and X_{min} were determined using a bootstrap method (n = 100) for the different polymer types. α = 3.45 ± 0.23 for PA, 4.59 ± 0.89 for PS, 4.32 ± 0.87 for PE1, 3.83 ± 0.31 for PE2, 3.80 ± 0.29 for PP, 5.26 ± 0.25 for PET, 2.94 ± 0.23 for PE big, 3.86 ± 0.25 for PET big and 3.61 ± 0.23 for PP big.

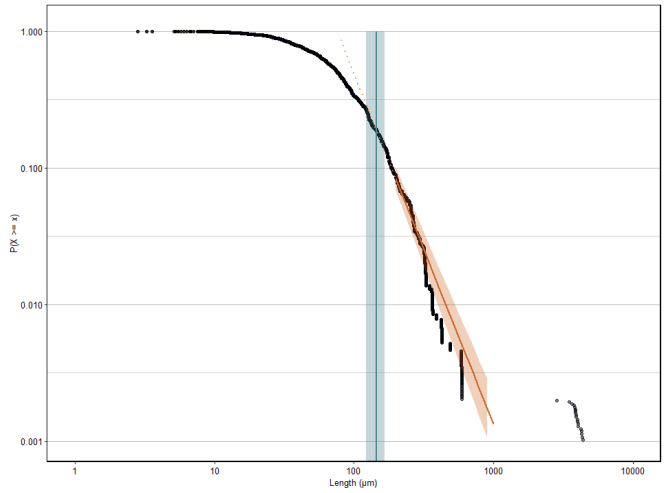


Figure S2. Particle length distributions for the ERMP, mean exponent parameter α = 3.57 ± 0.10. The blue vertical line indicate the minimum size for which the fitted power law is valid. The orange slope indicates the fitted power law distribution.

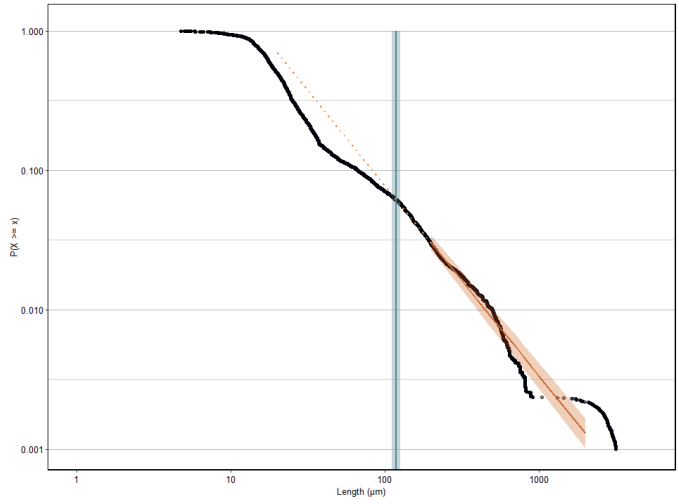


Figure S3. Particle length distributions for the ERMS, mean exponent parameter α = 2.37 ± 0.03 s.d. The blue vertical line indicate the minimum size for which the fitted power law is valid. The orange slopes indicate the fitted power law distributions.

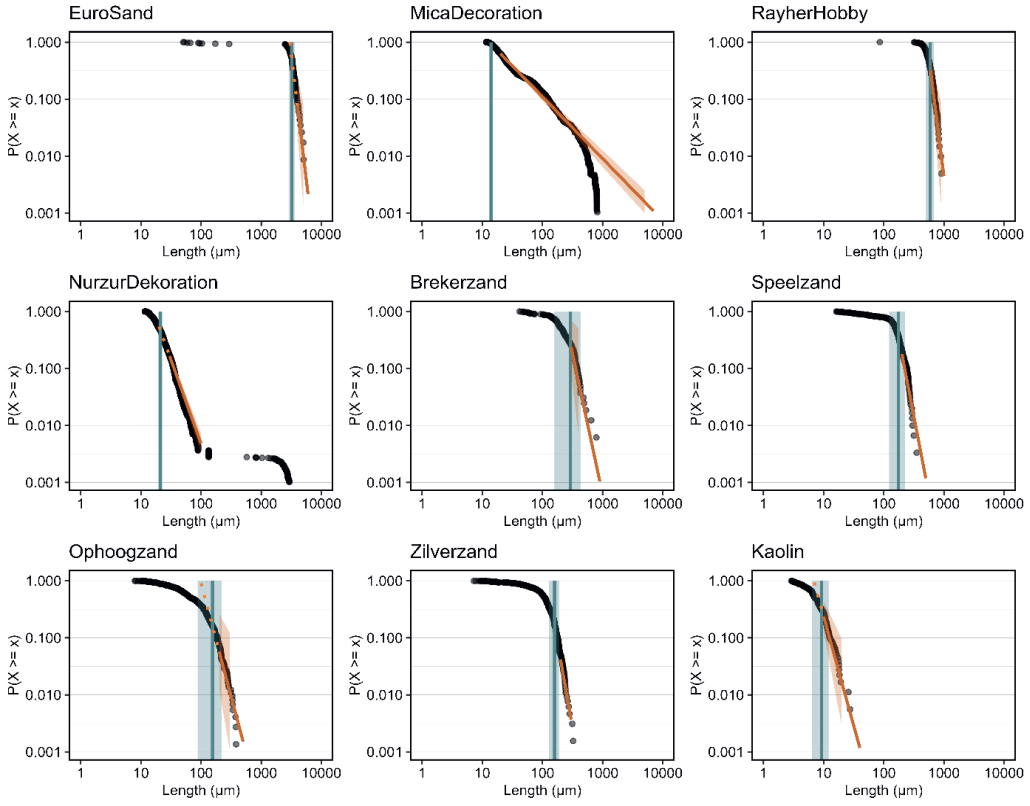


Figure S4. Particle length distributions for the different sand and clay particles. The blue vertical lines indicate the minimum size for which the fitted power law is valid (X_{min}). The orange slopes indicate the fitted power law distributions (α). The mean and standard deviation of both α and X_{min} were determined using a bootstrap method (n = 100) for the different sand and clay particles: α = 9.70 ± 1.72 for Eurosand, 2.08 ± 0.04 for MICADecoration, 9.35 ± 1.90 for RayherHobby, 3.93 ± 0.11 for NurzurDekoration, 5.94 ± 1.86 for Brekerzand, 6.48 ± 2.10 for Speelzand, 4.97 ± 1.05 for Ophoogzand, 7.01 ± 1.03 for Zilverzand and 4.83 ± 0.82 for kaolin.

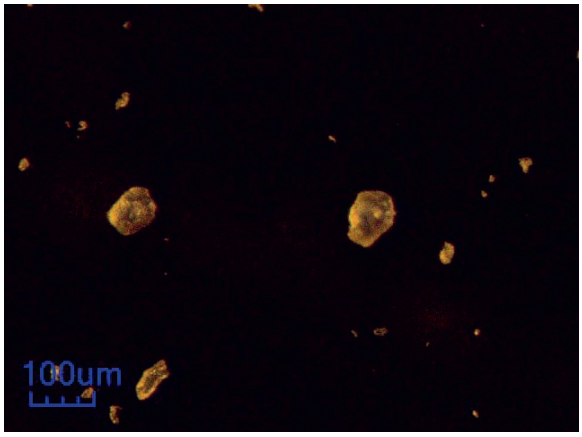


Figure S5.1. Microscope image of Polyamide.

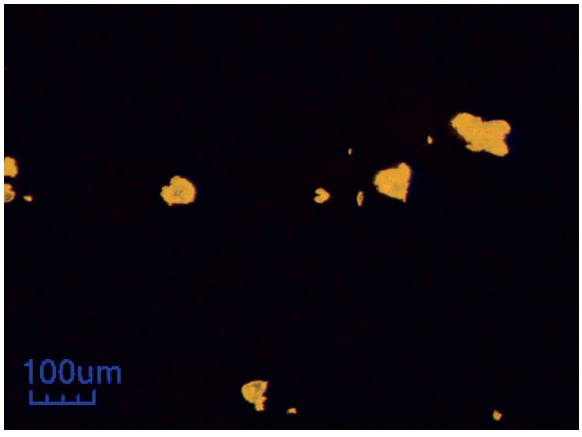


Figure S5.2. Microscope image of Polystyrene.

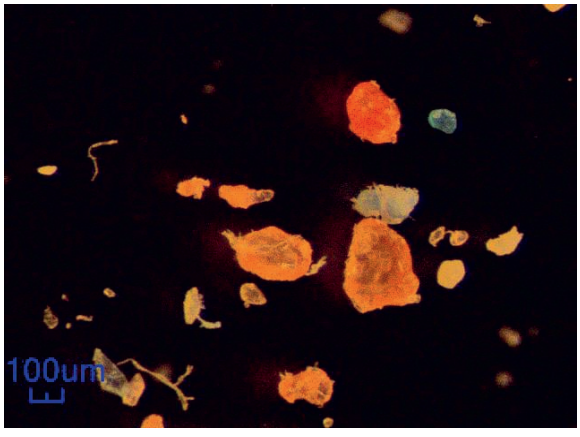


Figure S5.3. Microscope image of Polyethylene.

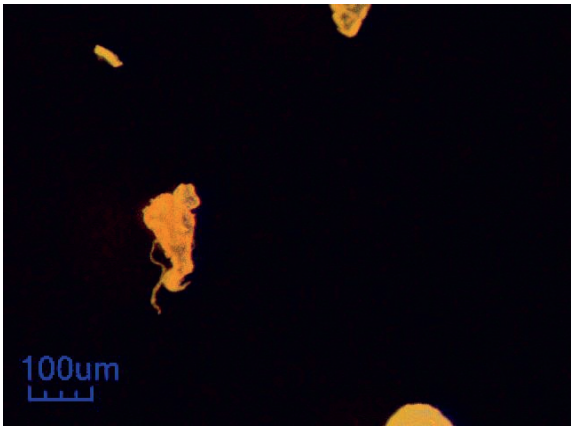


Figure S5.4. Microscope image of Polyethylene.

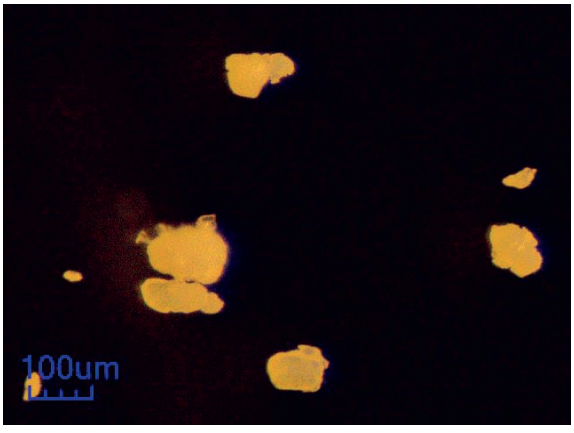


Figure S5.5. Microscope image of Polypropylene

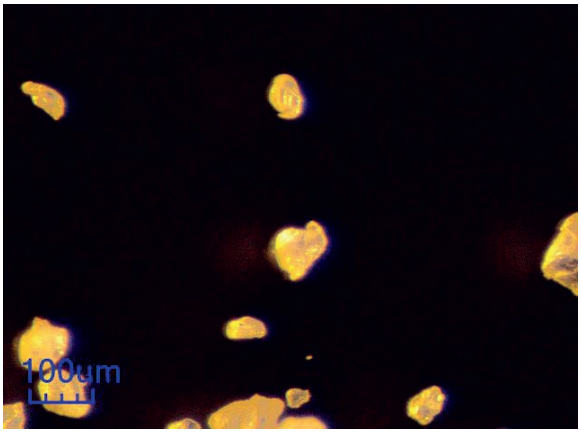


Figure S5.6. Microscope image of Polyethylene terephthalate

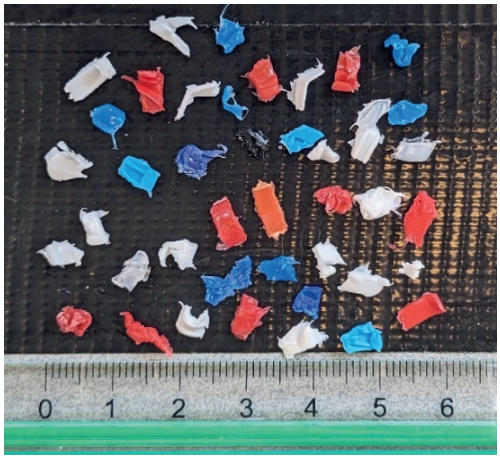


Figure S5.7. Microscope image of Polyethylene big

Figure S5.8. Microscope image of Polyethylene terephthalate big

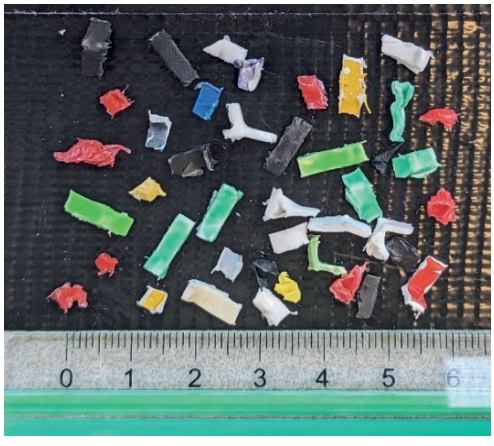


Figure S5.9. Microscope image of Polypropylene big

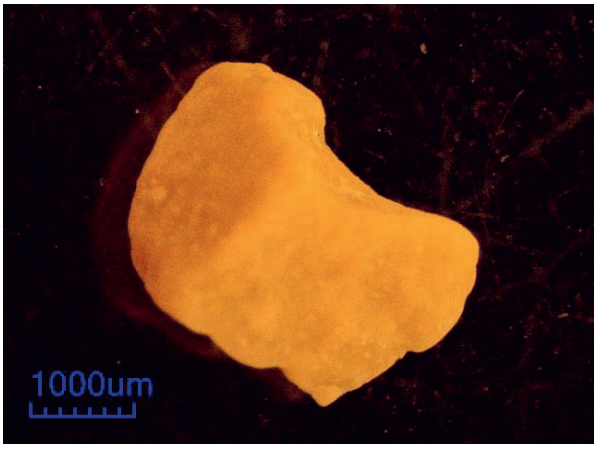


Figure S5.10. Microscope image of Eurosand

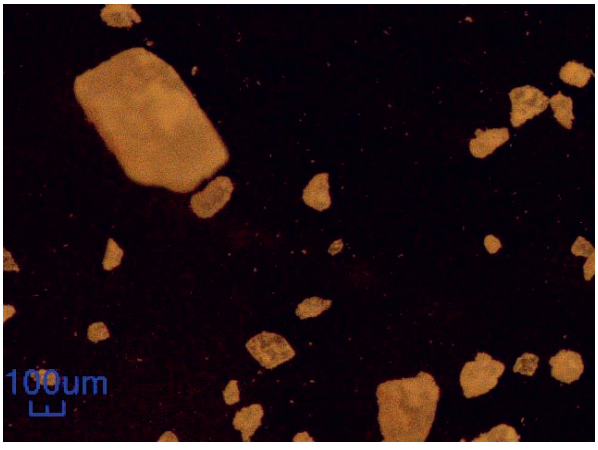


Figure S5.11. Microscope image of MicaDecoration

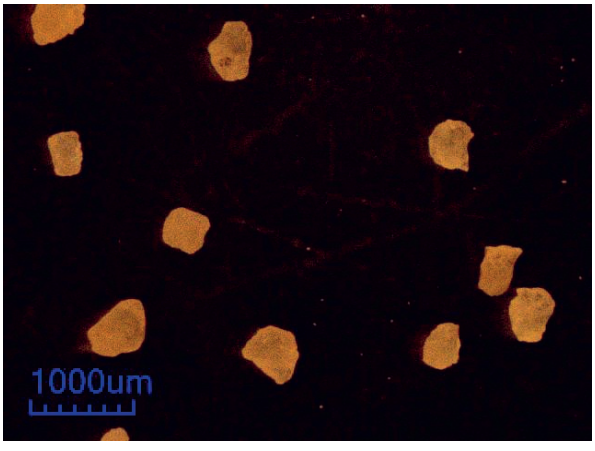


Figure S5.12. Microscope image of RayherHobby

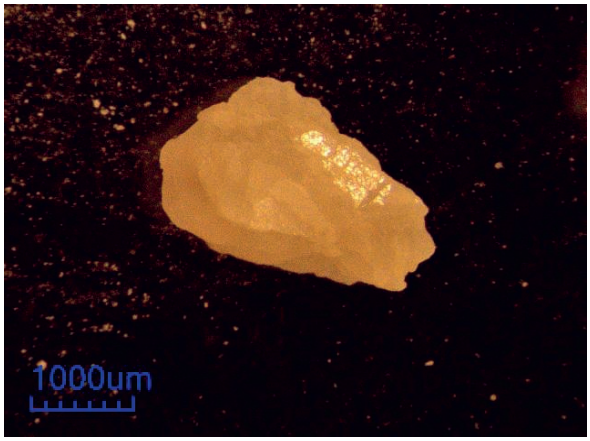


Figure S5.13. Microscope image of NurzurDekoration

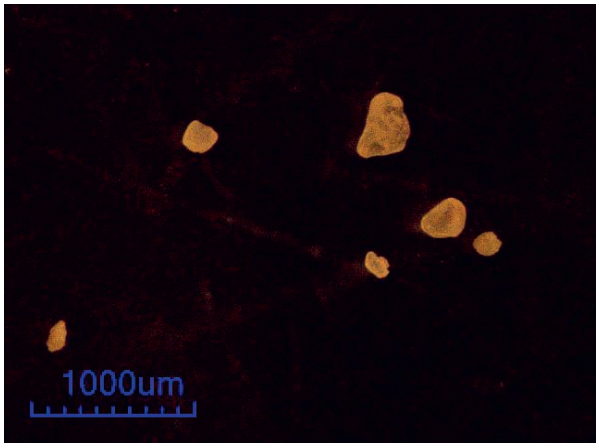


Figure S5.14. Microscope image of Brekerzand

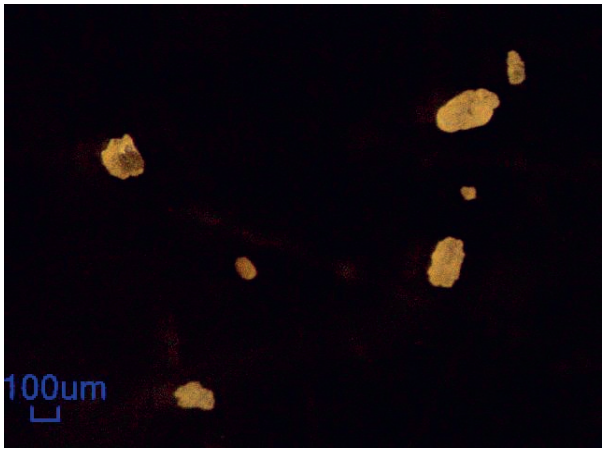


Figure S5.15. Microscope image of Speelzand

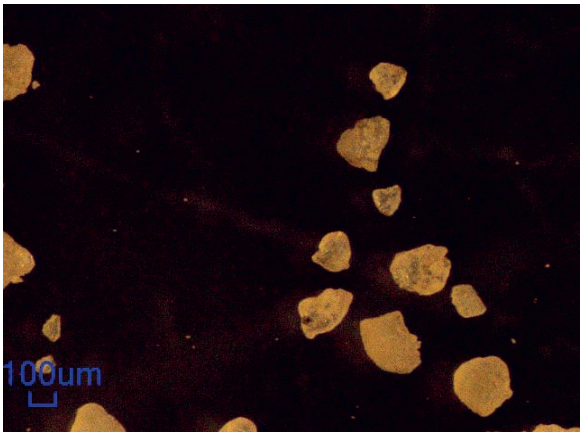


Figure S5.16. Microscope image of Ophoogzand

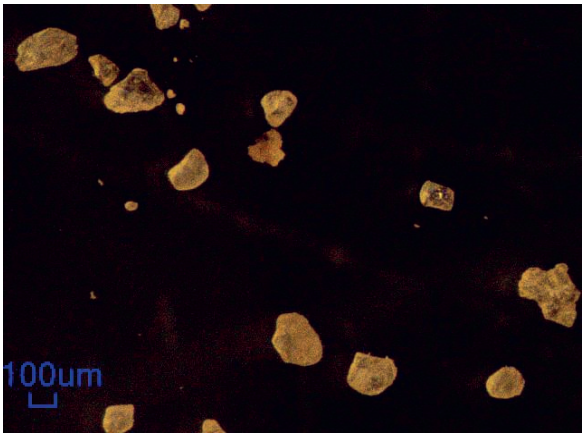


Figure S5.17. Microscope image of Zilverzand

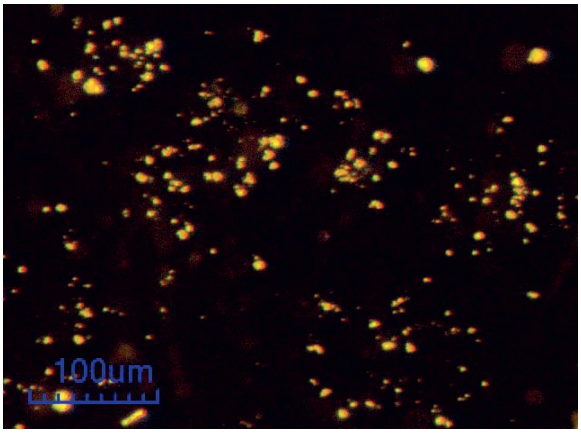


Figure S5.18. Microscope image of Kaolin

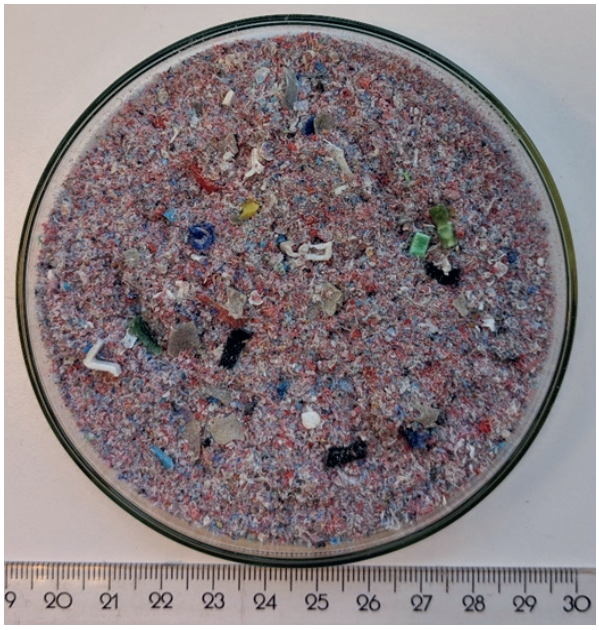


Figure S6. Final mixture of ERMP including all nine size classes.

Figure S7. Final mixture of ERMS including all nine size classes.

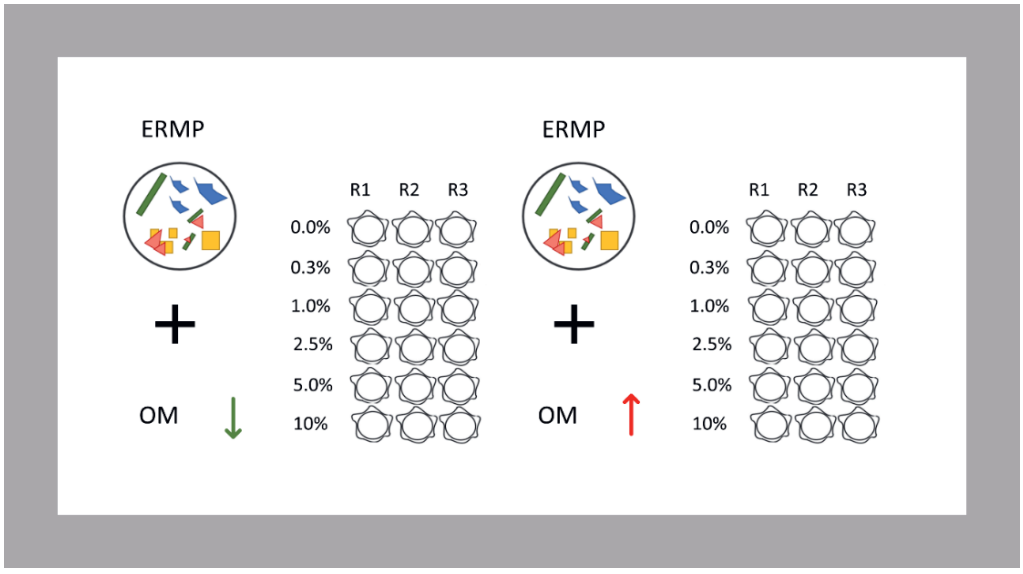


Figure S8. Experimental set up. ERMP with average and enriched organic matter content in sediment. Additionally 6 doses; 0, 0.3, 1.0, 2.5, 5.0 and 10.0 % v/v in triplicate are shown.

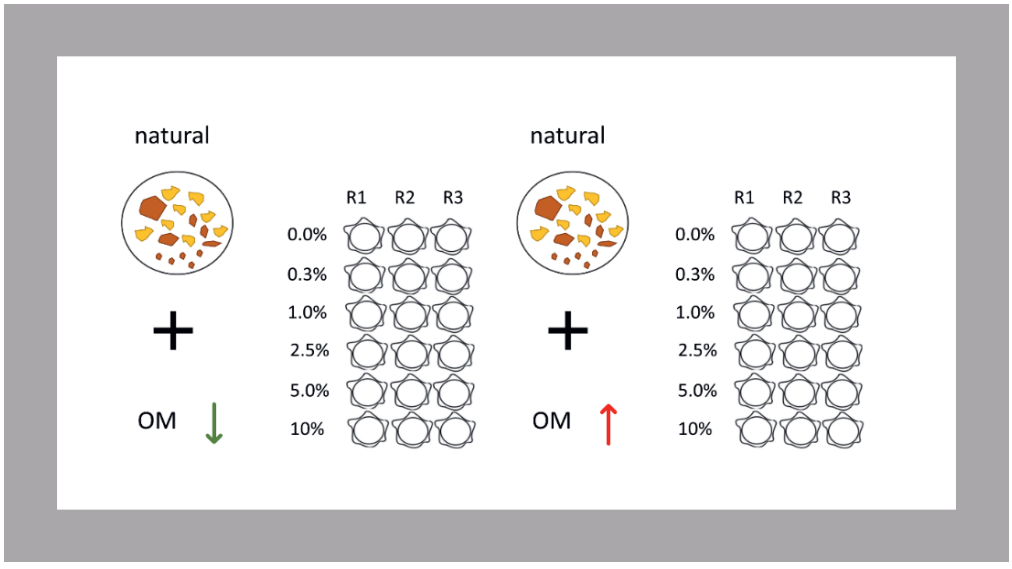


Figure S9. Experimental set up. Natural particle mix with average and enriched organic matter content in sediment. Additionally 6 doses; 0, 0.3, 1.0, 2.5, 5.0 and 10.0 % v/v in triplicate are shown.

Table S1. Summary of different size classes, including polymer type, source, lower and upper limit of size range, mean particle size and density.

Size class	Type	Source	Lower limit (µm)	Upper limit (µm)	Median (µm)	Mean (µm)	Density (g/cm3)	Mass mixture (%)	Particle mixture (%)
1		WeCreate www.korrels.nl					1.16 ± 0.001	1.0	14.4
2	PA	WeCreate www.korrels.nl	3	145	33	39	1.10 ± 0.001	3.0	15.5
3	PS	UniPlastic	5	185	49	56	0.97 ± 0.001	36.7	14.4
4	PE1	UniPlastic	15	589	141	155	0.97 ± 0.002	27.3	15.8
5	PE2	UniPlastic	7	590	123	134	0.93 ± 0.001	3.9	8.6
6	PP	Plastic Recycling Amsterdam	9	348	79	91	1.30 ± 0.015	11.5	29.7
7	PET	Plastic Recycling Amsterdam	5	229	73	74	0.95 ± 0.003	5.6	0.5
8	PE big	Plastic Recycling Amsterdam	3477	16837	8394	8700	1.35 ± 0.002	5.6	0.7
9	PET big	UniPlastic	2858	13409	6456	6583	0.91 ± 0.002	5.6	0.5
	PPbig		4371	18605	8785	8856			

Table S2. Summary of different size classes, including source, lower and upper limit of size range, mean particle size and density.

Size class	Name	Source	EAN	Lower limit	Upper limit	median	mean	Density (g/cm3)	Mass mixture (%)	Particle mixture (%)
1		Dutch QualityProducts	9506864654270					2.69 ± 0.004	53.9	0.39
	ÉuroSand			50	5071	3247	3072			
2		Shoppartners B.v. shoppartners.nl	8711473463286					2.68 ± 0.004	0.3	2.50
	MICADecoration			22	876	104	147			
3		Shoppartners B.v. shoppartners.nl	8718758989967					2.64 ± 0.003	1.2	0.12
	RayherHobby			86	907	557	536			
4		Boetiek Chloë	6090927853821					2.86 ± 0.003	44.3	9.79
	NurzurDekoration			12	4047	137	893			
5		Karwei (constructionshop)	8711297375314					2.64 ± 0.006	0.1	0.58
	Brekerzand			42	787	208	228			
6		Karwei (constructionshop)	8711297134041					2.64 ± 0.003	$4.7*10^{-2}$	5.12
	Speelzand			16	353	157	151			
7		Karwei (constructionshop)	8711297375307					2.64 ± 0.015	0.1	5.12
	Ophoogzand			16	533	98	121			
8		Karwei (constructionshop)	8711297134065					2.64 ± 0.012	0.1	6.91
	Zilverzand			14	413	147	147			
9		Sigma Alderich	1332-58-7 *					2.71 ± 0.055	$7.5*10^{-4}$	69.47
	Kaolin			5	39	11	13			

* CAS number

Table S3. Quality Assurance/Quality control (QA/QC) criteria score for testing effects of MP in aquatic test systems (de Ruijter et al., 2020)

Criteria	Score (0-2)[a)]	Score explanation
Particle Characterization		
1. Particle size	2	Min/max size values, median and average size given and particle size distribution measured and reported.
2. Particle shape	2	High resolution pictures of used microplastics are provided.
3. Polymer type	2	Polymer types (PA, PS, PE, PP and PET) were identified with FTIR.
4. Source of MP	2	Sources of microplastics (Wecreate, UniPlastic, Plastic recycling Amsterdan, the Netherlands) and description how MP was made at industrial grinding company (Netzch Lohnmatecknik GmbH, Bobingen, Germany) reported.
5. Data reporting	2	MP concentrations were reported as volume, mass and particle amount.
Experimental design		
6. Chemical purity	2	To remove additives present, if any, the MP were washed with methanol three times.
7. Laboratory preparation	2	All materials used were washed with Milli-Q water, and non-plastic materials were used whenever possible. Bioassays were covered with aluminium foil to prevent contamination from air.
8. Verification of background contamination	2	Identical lab conditions used as previous study that showed that background contamination was negligible (de Ruijter et al. submitted)
9. Verification of exposure	2	Identical lab conditions used as previous study that measured nominal concentrations (de Ruijter et al. submitted), however no evidence that at least 80% of the nominal concentration throughout the test is maintained.
10. Homogeneity of exposure	2	Method of obtaining homogenous exposure (stirring) was described.
11. Exposure assessment	1	Whole body tissue and egestion samples were quantitatively analysed using FTIR in separate experiment
12. Replication	2	3 replicates were used.
Applicable for Risk assessment		
13. Endpoints	2	Ecologically relevant endpoints (growth and reproduction) for risk assessments at the individual level were used.
14. Presence of natural (food) particles	2	Natural particles and food were added to test systems
15. Reporting of effect thresholds	2	Effect thresholds were reported as EC_{50} and EC_{10} with standard errors reported.
16. Quality of dose-response relationship	2	6 concentrations including control
Ecological relevance		
17. Concentration range tested	2	2 environmentally relevant concentrations were used and motivated from measured environmental concentrations.

18. Aging and biofouling	1	Aged plastic was used. A biofilm was allowed to form during acclimatization, however, the biofilm was not characterized. Consequently only 1 point was given .
19. Diversity of MP tested	2	A variety of MP shapes and sizes was used.
20. Exposure time	2	The exposure time was 28 days.
Total	38 (95%)	

Table S4. Water quality measurements during 28 d chronic exposure, taken every other day and at least one sample of each dose and replicate.

Mixture	OM content	T(°C)	pH	DO(%)	Conductivity (µS/cm)	NH3 (mg/L)
ERMP	Enriched	20.1 ± 0.35	8.37 ± 0.29	85.1 ± 8.1	953 ± 68	0.13 ± 0.12
ERMP	Average	20.5 ± 0.36	8.31 ± 0.28	86.9 ± 7.2	833 ± 82	0.11 ± 0.10
ERMS	Enriched	20.1 ± 0.36	8.38 ± 0.29	85.1 ± 12.1	950 ± 92	0.12 ± 0.12
ERMS	Average	20.1 ± 0.36	8.19 ± 0.30	85.9 ± 6.0	800 ± 79	0.11 ± 0.09

Table S5. Statistical results dose response testing and likelihood ratio test for the endpoint 'reproduction''

Treatment	Endpoint	Type of drc [a] fitted	Log-likelihood ratio test p-value (noEffect)	p-value effect threshold	EC_{10} ± S.E.% (v/v)	EC_{10} # particles/ kg dw sediment	EC_{50} ± S.E.% (v/v)	EC_{50} # particles/ kg dw sediment
ERMP enriched	Reproduction	LL.3	**0.035**	**0.026**	5.43 ± 3.44[b]	6.45 *10^7 ± 4.08 *10^7	13.68 ± 5.54[c]	1.64 *10^8 ± 6.59 *10^8
ERMP average	Reproduction	LL.4	0.073	**0.009**	-	-	-	-
ERMS enriched	Reproduction	W1.3	1.000	0.913	-	-	-	-
ERMS average	Reproduction	LL.4	0.766	NA	-	-	-	-

Significant findings ($p < 0.05$) are highlighted in bold.

[a)] Dose-response curve package in R provides the following models: Weibull type I model (W1.x) and log logistic (LL.x), with 'x' is the number of parameters fitted.

[b)] Lower limit uncertainty range of EC_{10} is zero.

[c)] EC_{50} value higher than the highest concentration of 10% (v/v)

Table S6. Statistical results from three-way ANCOVA for the endpoint 'reproduction'

Response: reproduction	d.f.	Sum sq	Mean sq	*F*	*p*
Mix	1	0.300	0.300	1.886	0.174
OM	1	2.293	2.293	14.404	**< 0.001**

Concentration	1	0.004	0.004	0.022	0.882
Mix:OM	1	0.025	0.025	0.159	0.691
Mix:Concentration	1	0.065	0.065	0.409	0.525
OM:Concentration	1	0.949	0.949	5.961	**0.017**
Mix:OM:Concentration	1	1.451	1.451	9.112	**0.004**
Residuals	64	10.190	0.159		
Tukey's method for multiple comparisons					
ERMP OM Enriched vs ERMP OM average					**0.022**
ERMP OM Enriched vs ERMS OM enriched					0.598
ERMP OM Enriched vs ERMS OM average					**0.003**
ERMP OM average vs ERMS OM Enriched					0.326
ERMP OM average vs ERMS OM average					0.901
ERMS OM Enriched vs ERMS OM average					0.087

Table S7. Statistical results dose response testing and likelihood ratio test for the endpoint 'growth'

Treatment	Endpoint	Type of drc fitted	Estimate effect threshold p-value	Log-likelihood ratio test p-value (noEffect)	EC_{10} ± S.E.%	EC_{10} # particles/ kg dw sediment	EC_{50} ± S.E.%	EC_{50} # particles/ kg dw sediment
ERMP enriched	Growth	LL.3	0.946	1.000	-	-	-	-
ERMP average	Growth	LL.3	0.939	1.000	-	-	-	-
ERMS enriched	Growth	LL.3	0.070	0.107	-	-	-	-
ERMS average	Growth	LL.3	0.209	0.525	-	-	-	-

Table S8. Statistical results three-way ANCOVA for endpoint 'growth'

Response: growth	d.f.	Sum sq	Mean sq	*F*	*p*
Mix	1	18.400	18.400	0.291	0.592
OM	1	1025.300	1025.290	16.199	**< 0.001**
Concentration	1	93.400	93.380	1.475	0.229
Residuals	64	4177.300	63.290		
Tukey's method for multiple comparisons					
ERMP OM enriched vs ERMP OM average					0.084

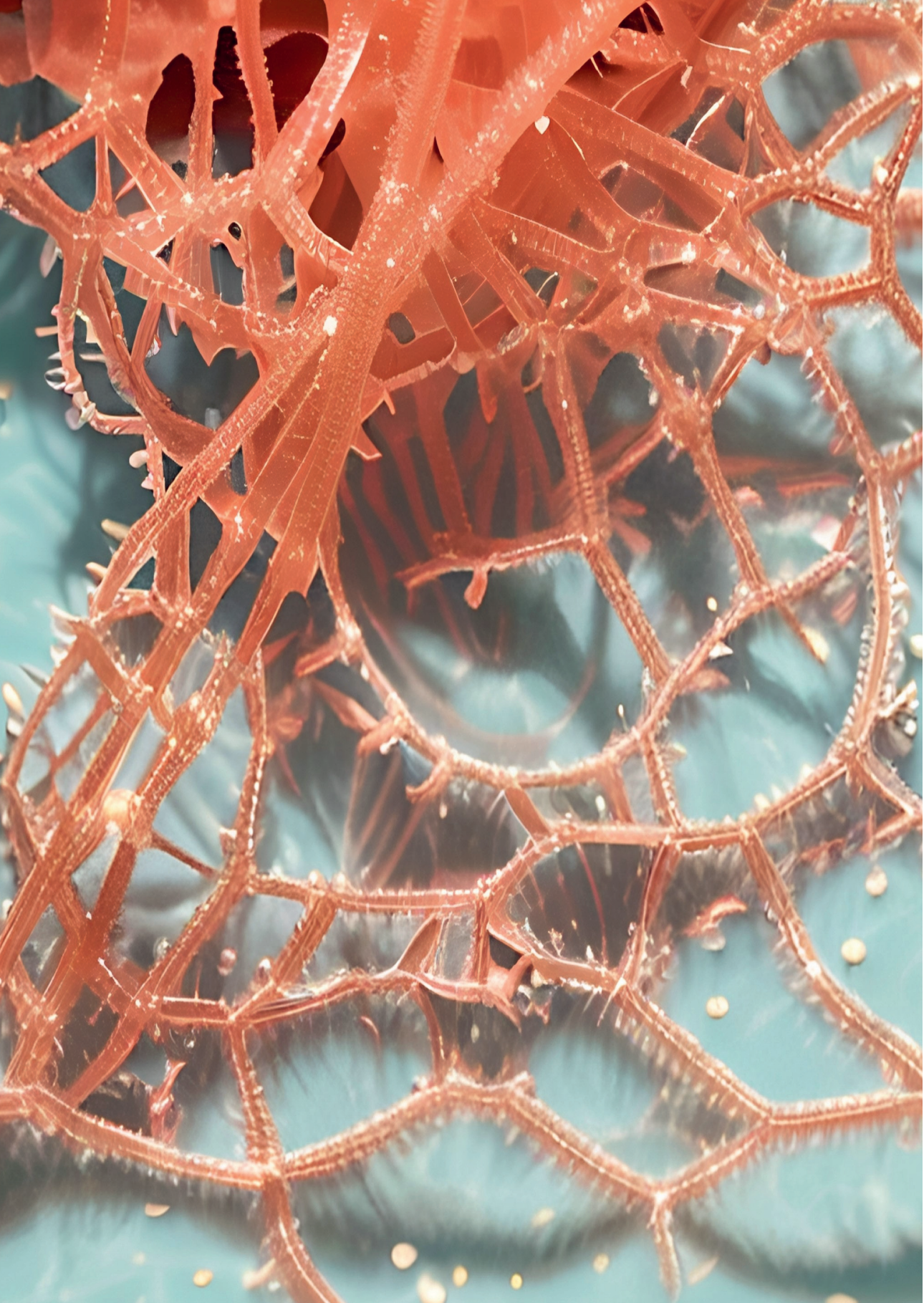
ERMP OM enriched vs ERMS OM enriched	1.000
ERMP OM enriched vs ERMS OM average	**0.013**
ERMP OM average vs ERMS OM enriched	0.080
ERMP OM average vs ERMS OM average	0.898
ERMS OM enriched vs ERMS OM average	**0.011**

Table S9. Water quality measurements during the 14 d egestion study, taken every other two days and at least one sample of each dose and replicate.

Mixture	T(°C)	pH	DO(%)	Conductivity (µS/cm)	NH3 (mg/L)
ERMP	19.7 ± 0.64	8.04 ± 0.36	91.76 ± 4.14	489 ± 59	0.05 ± 0.05
ERMS	19.7 ± 0.70	8.36 ± 0.08	92.36 ± 2.75	467± 58	0.06 ± 0.09

Table S10. Statistical results three-way Repeated ANOVA for egestion.

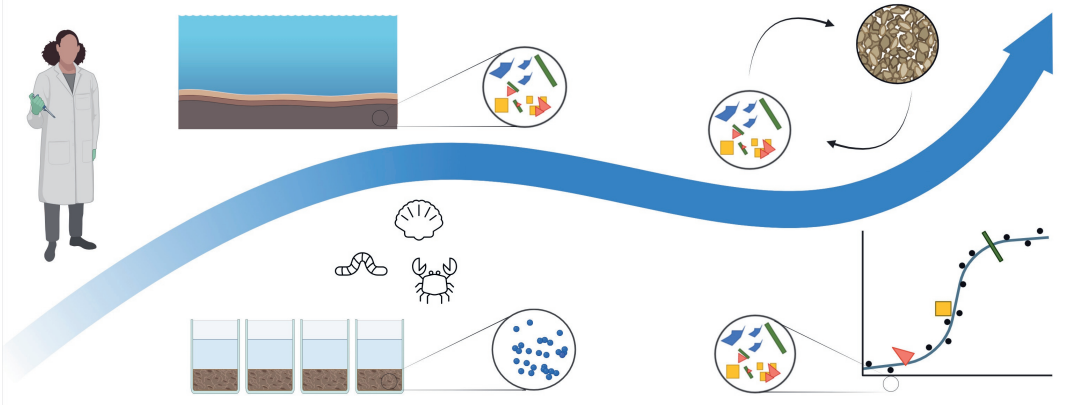
Response: egestion	d.f.	Sum sq	Mean sq	*F*	*p*
Error: Between					
Mix	2	7.789	3.895	3.259	0.071
Concentration	1	2.217	2.217	1.855	0.196
Mix:Concentration	1	14.874	14.874	14.447	**0.004**
Residuals	13	15.535	1.195		
Error: Within					
Day	6	222.65	37.11	102.761	**< 0.001**
Day:Mix	12	10.80	0.90	2.491	**0.008**
Day:Concentration	6	1.11	0.19	0.513	0.797
Day:Mix:Concentration	6	7.12	1.19	3.286	**0.006**
Residuals	78	28.17	0.36		
Tukey's method for multiple comparisons					
Control 0 % – ERMP 10 %					1.000
Control 0 % – ERMP 5 %					**0.010**
Control 0 % – ERMS 10 %					0.310
Control 0 % – ERMS 5 %					0.999
ERMP 10 % – ERMP 5 %					**0.029**
ERMP 10 % – ERMS 10 %					0.480
ERMP 10 % – ERMS 5 %					1.000
ERMP 5 % – ERMS 10 %					0.423
ERMP 5 % – ERMS 5 %					**0.037**
ERMS 10 % – ERMS 5 %					0.562



Chapter 6

General discussion

A brief history of microplastics effect testing: Guidance and prospect



1. Introduction

Over the past few decades, concerns have arisen regarding the potentially adverse effects of microplastic particles (MP) in the environment. Understanding these effects is crucial to quantify them within the context of risk assessments (Koelmans et al., 2017). While the testing of more traditional chemical stressors like pesticides, persistent organic pollutants (POPs), and heavy metals has been well-established, resulting in numerous guidance documents, protocols, and standard operating procedures (SOPs), the MP scientific community has undergone a steep learning curve in the past decade.

MP represent a complex, heterogeneous mixture of particles with varying degrees of aging and weathering Jahnke et al. (2017). They are associated with biofilms and chemicals, including additives and chemicals sorbed from the environment. These characteristics are highly variable in both time and space, posing significant challenges when evaluating the risks associated with this complex contaminant. Despite the challenges causing the research community to take substantial detours and delays in developing valid testing and assessment strategies, some of these key issues are gradually being addressed in response to landmark papers highlighting the limitations of available data (Figure 1).

Our brief history of MP effect testing begins as early as 2016 with the work of Lenz et al. (2016), who stressed the importance of using environmentally realistic MP concentrations. In the same year, Phuong et al. (2016) also argued that laboratory experiments often employed MP concentrations significantly higher than those found in the field. Additionally, they noted that MP exposure conditions typically involve a single type of polymer with a precise size and homogeneous shape, which does not accurately represent the diversity of MPs found in the environment. Connors et al. (2017) also emphasized the need to address the environmental relevance of test concentrations and stressed that studies should provide sufficient detail to convert particle concentrations and characterize particles extensively. Moreover, they highlight the need for relevant controls or reference materials. Karami (2017) underscored the importance of quantifying chemicals associated with MP particles, selecting appropriate test organisms, preventing aggregation, and considering the environmental relevance of particle size. Rist and Hartmann (2018) elaborated on these aspects and emphasized the need of developing clear definitions for plastic particle categorization, including controls for chemical

leaching (e.g., monomers, additives), the development of reference materials for method validation and comparison, and addressing the influence of environmental transformation processes (e.g., 'aging') on MP behaviour and ecotoxicity. De Sá et al. (2018) were among the first to observe the over-representation of certain species in MP effect studies, primarily focusing on fish and small crustaceans. They also highlighted a lack of understanding regarding the mechanisms responsible for MP effects. Burns and Boxall (2018) were the first to provide a systematic comparison of effects across multiple species (Species Sensitivity Distributions) and exposure concentrations, shedding light on low risks in natural settings. However, their analysis still faced challenges due to the incomparability of data, as noted by earlier authors. Advancing further, O'Connor et al. (2020) stressed that testing higher-than-realistic MP concentrations remains essential for assessing dose-dependent effects. Meanwhile, Triebskorn et al. (2019) identified additional imbalances, including an overrepresentation of certain polymers (e.g., polyethylene and polystyrene), species (e.g., fish), and a notable dearth of studies addressing the effects of diverse MP mixtures. Furthermore, Bour et al. (2021) advocated for a more nuanced approach to address the complexity of organism-particle interactions, and Kukkola et al. (2021) reiterated the mismatch between field and laboratory studies regarding plastic types and assessed endpoints. They also emphasized that fibres are more prevalent in field studies, whereas particles dominate laboratory studies. Finally, in 2022, an international group of experts identified data gaps and emphasized the importance of adequate particle characterization and appropriate study design that allow for the derivation of dose-response curves (Thornton Hampton et al., 2022a).

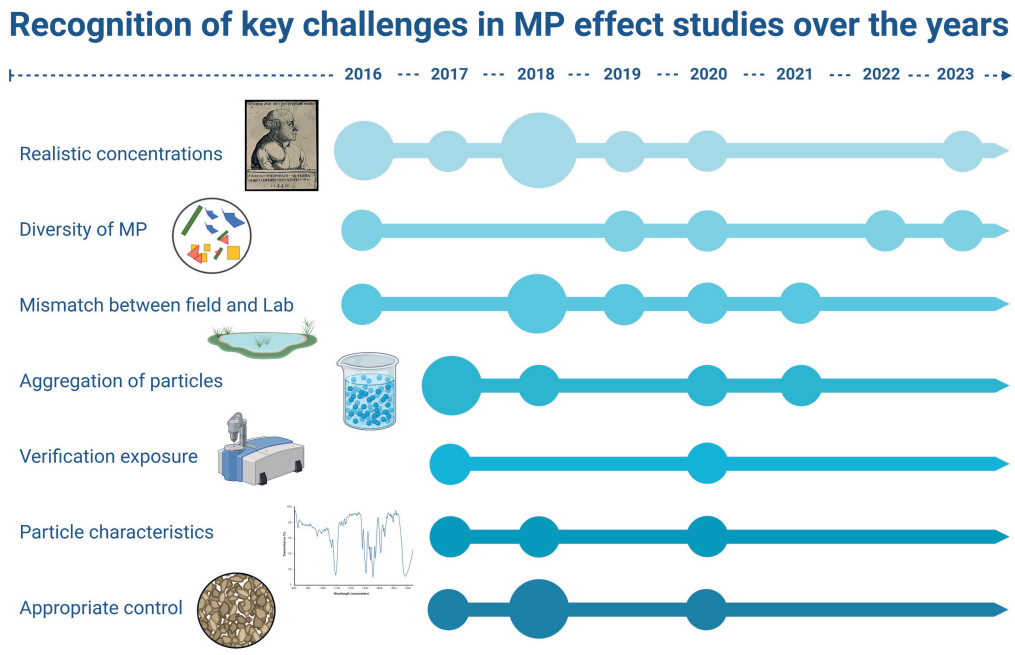


Figure 1. Recognition of key challenges of MP effect studies (n=16) (Bour et al., 2021; Burns and Boxall, 2018; Coffin, 2023; Connors et al., 2017; de Ruijter et al., 2020; De Sá et al., 2018; Karami, 2017; Kukkola et al., 2021; Lenz et al., 2016; O'Connor et al., 2020; Ogonowski et al., 2018; Phuong et al., 2016; Rist and Hartmann, 2018; Thornton Hampton et al., 2022a; Thornton Hampton et al., 2022b; Triebskorn et al., 2019). Already from 2016, researchers have pointed out the same key challenges and proposed recommendations on how to improve MP effect studies. These key challenges include testing of environmentally realistic concentrations, thoroughly characterizing particles, addressing of aggregation of MP particles during exposure studies, verification of exposure concentrations, testing of MP in their diversity, mismatch plastic type field and lab and natural particle as reference material. Bigger circles indicate that more than one study has pointed out a certain key challenge (Table S1)

In 2020, de Ruijter et al. (2020)(**Chapter 2**), synthesized most of these qualitative observations into a quantitative tool for assessing study quality (QA/QC). They retrospectively applied this new tool to all studies that had provided effects data up to that point (n=105 studies), revealing that none of the studies had obtained non-zero scores for all criteria (de Ruijter et al., 2020). This suggests that, if a strict approach were followed, none of the studies would hold relevance for risk assessment in a regulatory context. In the same year, a group of 23 researchers published a set of reporting guidelines aimed at enhancing the comparability and

reproducibility of studies (Cowger et al., 2020). To address disparities in particle testing, Koelmans et al. (2020) proposed a data alignment and rescaling framework, which for the time being would solve the problem of the incomparability of results caused by differences in particles being tested.

In summary, numerous reviews over the years have consistently highlighted the shortcomings of MP effect studies, with many of the same issues identified in 2016 still relevant in 2023 (Figure 1). Solutions have been proposed by de Ruijter et al. (2020), (**Chapter 2**), Cowger et al. (2020), and, to some extent, Koelmans et al. (2020). The question now is to what extent the various guidance documents converge and whether there are still new or unresolved bottlenecks.

The aim of this concluding chapter is to summarize the knowledge accumulated in MP effect testing, compare available guidelines, and provide a comprehensive overview of the knowledge on risk assessment at both the single species and community levels. I will review the preceding chapters, evaluating their contributions to the scientific field and their relevance to our overall objectives. More specifically, I discuss standard test materials, characteristics of MP, and mechanisms explaining their effects. Finally, recommendations for future research directions are provided.

2. Effect testing with relevance for risk assessment: Risk assessment framework

2.1 Assessment of ecological risks using data rescaling and alignment

Similar to more traditional contaminants such as heavy metals and organic micro-pollutants, the evaluation of the risk associated with MP involves comparing exposure to effect thresholds (Koelmans et al., 2017). Exposure is quantified through concentration measurements in environmental samples, while effect thresholds are derived from dose-response relationships measured in the laboratory. One challenge in this regard is that these data are often obtained using different methods, making them incomparable. For instance, exposure to 100 particles/L, ranging in size from 20 to 5000 µm and composed of various polymers, cannot be directly compared to an effect threshold measured in the laboratory for 100 µm-sized MP. These are essentially different stressors, making the comparison like "comparing apples and pears". The only currently available solution is a computational method to correct for these differences. In this approach, all data

are converted to an equivalent value as if they were obtained for a standard MP mixture ranging from 1 to 5000 µm (Koelmans et al., 2020). I will briefly explain the principle here using an example. Suppose the mechanism causing people to become nauseous is consuming too many apples. In this case, the total volume of those consumed apples is a plausible measure of the relevant dose. We can assume that other factors do not matter in the short term. In this example, it can be presumed that the same effect would occur if too many pears were consumed, as long as the total volume of those pears is equal to that of the apples. The effect thresholds of apples and pears can thus be converted into each other based on volume. In this example, volume is the "effect metric," and food dilution is the mechanism. Interestingly, this example is not coincidental; in the literature, this very mechanism has been identified as the most plausible mechanism for the effects of MP on small organisms such as zooplankton, with the ingested particle volume as the relevant exposure metric (de Ruijter et al., 2020; **Chapter 2**; Thornton Hampton et al., 2022b).

2.2 Past Risk Assessments

Several previous risk assessments have been carried out with non-harmonized data (Adam et al., 2019; Besseling et al., 2019; Everaert et al., 2020; Everaert et al., 2018), which will not be covered in the present review. The data rescaling and alignment techniques, as explained in the previous section, have been applied in a limited number of risk assessments. In 2020, the first assessment for MP in surface waters was conducted, providing an indication of risks for 1.5% of the then-known exposure concentrations worldwide (Koelmans et al., 2020). In 2022, the method was employed by an international working group in the context of California state regulations to set risk management thresholds for coastal waters (Mehinto et al., 2022). A risk assessment was performed for MP in San Francisco Bay (Coffin et al., 2022b). Over 75% of the samples exceeded the limit for the most conservative food dilution threshold. Within the Central Bay, 38% of the samples exceeded a higher threshold related to management planning, which was statistically significant within a 95% confidence interval. In 2023, a risk assessment for MP in freshwater sediments worldwide was also conducted (Redondo-Hasselerharm et al., 2023). The exposure concentrations were below or within the uncertainty range of the HC5 values. This implies that the risks of MP for benthic communities at the current freshwater sediment concentrations worldwide could not be ruled out. In 2023, the same methods were used for a risk assessment of MP in the Laurentian Great Lakes

(Koelmans et al., 2023b). Due to uncertainties in the parameters used for corrections and conversions, uncertainties in sample volume, and variability in hydrological conditions, the assessment was performed probabilistically (Koelmans et al., 2023b). The probability of a risk occurring due to food dilution was 24% of pelagic exposure in Lake Ontario, 8.3–10.3% of pelagic exposures in Lake Michigan, Lake Huron, Lake Superior, and Lake Erie, and 13–15% of benthic exposures in Lake Erie and Lake Huron. A recent risk assessment for soils also shows that MP concentrations, for a limited number of soils globally, cause a probability of effects occurrence up to 95% (Redondo-Hasselerharm et al., 2024). All these risk assessments indicate that the highest concentrations observed within an ecosystem or globally, for surface water, sediments, and soils, exceed the effect thresholds measured in the laboratory.

2.3 Assessment of ecological risks using tests with environmentally relevant microplastics

The data alignment methods described above aim to correct the differences between particles in the environment and particles used in laboratory effect tests as accurately as possible. However, a significant drawback is the uncertainty introduced by all these computational corrections. It is preferable to conduct laboratory effect tests using environmentally relevant microplastics (ERMP), a mixture of particles that represents the average MP composition of an environmental compartment as closely as possible (de Ruijter et al., 2023; **Chapter 3**). This mixture should thus have a realistic polymer composition, particle size distribution, and composition of shape categories, e.g., fibres, fragments, pellets, and sheets. In addition, tests with such an ERMP mixture should meet crucial QA/QC criteria (de Ruijter, 2020; Cowger et al, 2020). In de Ruijter et al. (2023)(**Chapter 3)**, we put this into practice by testing 16 invertebrate species while satisfying 20 QA/QC criteria. Consequently, in **Chapter 4** we analyse the bioavailable fraction from the ERMP mixture with FTIR. In **Chapter 5**, a similarly diverse ERMP mixture was used to test one species and compare the effects with a mixture with a nearly identical particle size distribution, but composed of non-polymer particles (de Ruijter et al., 2024). Threshold effects obtained from dose-response relationships for such realistic mixtures could be more directly compared with exposure data to characterize the risk without computational alignments.

3. Quality assurance in single species tests of microplastic particles

In de Ruijter et al. (2020)(**Chapter 2**), 20 QA/QC criteria were proposed for the adaptation of a standardized protocol to test the effects of MP in aquatic test systems. These criteria provide guidance to enhance the quality of effect tests with regard to particle characterization, experimental design, applicability to risk assessment, and ecological relevance. After analysing 105 studies, they showed that no study scored positively on all criteria, highlighting the need for improved quality assurance (de Ruijter et al., 2020; Chapter 2). Specifically, most room for improvement lies in the experimental design, which includes ensuring chemical purity, preventing and measuring contamination in the laboratory, verification of exposure concentrations, and ensuring that tested concentrations are homogeneous and well dispersed within the medium. To enhance applicability to risk assessment, studies should report effect thresholds and include at least 6 doses. Additionally, improving the ecological relevance of studies can be achieved by aging and biofouling MPs rather than testing pristine particles. Furthermore, enhancing the reliability of MP risk assessment can be achieved by diversifying the types of particles tested and extending the exposure time. While acknowledging that some criteria may substantially increase workload, it is essential to recognize that some criteria represent “low-hanging-fruit”, or quick wins that can be easily implemented. Given limited resources, a strategic focus on these achievable criteria holds the potential for substantial improvements in informing risks assessment practices.

In parallel with de Ruijter et al. (2020)(**Chapter 2**), a diverse group of 23 researchers suggested reporting guidelines to increase the reproducibility of MP research, including considerations for toxicology studies (Cowger et al., 2020). Importantly, their recommendations align with those of de Ruijter et al. (2020)(**Chapter 2**) emphasizing the importance of thorough reporting of MP characteristics, such as plastic age, polymer type, size, and shape. Additionally, they underscore the importance of reporting colour, if possibly relevant for organism being tested. Furthermore, they highlight the need for reporting exposure concentrations and their verification, which depend on the exposure media. They also stress the importance of providing detailed descriptions of how test organisms are exposed, and which tissues were analysed.In line with the recommendations of Cowger et al. (2020); de Ruijter et al. (2020)(**Chapter 2**), Alimi et al. (2022) propose reporting guidelines specifically for studies investigating the effects of weathered MPs. This

review once again underlines that the vast majority (90.4%) of MP effect studies only test pristine MP. Alimi et al. (2022) offer highly specific guidance aimed at enhancing comparability and reproducibility when testing weathered MPs. They emphasize the need to clearly report the conditions under which particles have been weathered including exposure time, temperature, humidity, irradiance and also specifying the mimicked weathering pathway.

The body of literature describing the effects of MPs is growing (Granek et al., 2020). Thornton Hampton et al. (2022c) have innovatively designed an open database, complemented by an open source R shiny web application named Toxicity of Microplastics Explorer (ToMEX). This tool enables the compilation and synthesis of existing toxicity data and now includes 160 MP toxicity studies. Moreover, it incorporates the twenty QA/QC criteria proposed by de Ruijter et al. (2020)(**Chapter 2**), filtering out poor-quality data for risk assessment. Interestingly, a subset of 14 criteria, referred to as “red criteria”, was pragmatically selected; otherwise an insufficient amount of data would have passed the QA/QC screening (Mehinto et al., 2022). Although expert groups may make such decisions in the absence of useful data, the consequence is that the resulting assessment did not meet the full set of twenty critical criteria, and thus, it should not be considered reliable. Nevertheless, while some experts still assigned a value of 4 on a scale of 5 for the reliability of the results, others who gave more weight to the reliability of the data assigned only a score of 1 for the quality of the established thresholds (Mehinto et al., 2022). For instance, the “chemical purity” criterion is excluded, despite its importance in distinguishing particle effects from chemical effects arising from MPs, which is a fundamental component of risk assessment. A potential solution involves thorough particle washing with organic solvents to isolate the particle effect; however, this approach may raise concerns about altering particle characteristics (Cowger et al., 2020; de Ruijter et al., 2020; **Chapter 2**). As an alternative, the distribution of plastic-associated chemicals in the exposure system can often be calculated based on chemical distribution or speciation principles. Subsequently, the calculated concentrations for the various exposure media can be compared to threshold effect concentrations for these chemicals to potentially exclude any contribution of the chemicals to the observed effect (e.g. (Besseling et al., 2014; Redondo-Hasselerharm et al., 2020; Redondo-Hasselerharm et al., 2018b; Redondo-Hasselerharm et al., 2021)). Another solution, although laborious, is proposed by Cowger et al. (2020): non-target screening for

the presence for additive chemicals. Although the proposed solutions differ, both research groups agree that it is crucial in toxicity testing to know what is being tested. Additionally, several other crucial criteria, despite their importance, have not yet been incorporated as strict prerequisites in ToMEX. These include preventing and measuring contamination in the laboratory, verifying exposure concentrations, ensuring the homogeneity of these exposure concentrations, assessing exposure in tested organisms, and replication. Hopefully, in the future, studies will be able to incorporate these criteria, thereby reducing uncertainties in risk assessment.

In a study conducted by Coffin (2023), an evaluation applying 20 QA/QC criteria from (de Ruijter et al., 2020; **Chapter 2**), assessed whether the quality of MP toxicity tests (n = 160) improved from 2012 to 2020. While the overall total accumulated score has indeed improved, the rate of progress, at 0.002 points per year, suggests that the research community would require many years to achieve a perfect score. Specifically, advancements have been made in the technical aspects, such as particle characterization and experimental design, of MP toxicity tests. However, critical elements, such as including sufficient doses and incorporating environmentally relevant doses withing the tested range, have not shown improvement. Furthermore, a prevalent trend persists wherein many studies choose to test monodisperse pristine MPs, neglecting the environmentally realistic preference for diverse aged and biofouled particles.

To date, only a few studies (Amariei et al., 2022; Verdú et al., 2022) have specifically aimed to incorporate the QA/QC guidelines proposed by de Ruijter et al (2020)(**Chapter 2**). In a follow-up study de Ruijter et al. (2023)(**Chapter 3**), demonstrated the feasibility of meeting all 20 criteria (i.e. 95% of the maximum score while not having ‘zero scores’) by conducting standardized dose-response tests for 16 benthic invertebrate species. In another study, they successfully met all criteria while also adhering to standards for control mortality. This study compared the effects of MP with the effects of natural particles (de Ruijter et al., 2024; **Chapter 5**).

All 20 criteria remain to be incorporated for ecotoxicology studies focusing on pelagic species. These existing criteria are applicable to both benthic and pelagic tests. However, the criterion “homogeneity of exposure” may be considered more challenging when working with water as the medium, as MPs tend to aggregate and

adhere to glass walls. Nevertheless, solutions such as renewing the media, plankton wheels, or applying gentle aeration during exposure are available (Détrée and Gallardo-Escárate, 2017; Gambardella et al., 2019; Gerdes et al., 2019; Tang et al., 2018). Furthermore, the 'verification of exposure concentrations' is not yet a standard procedure when testing MP in water. However, many studies have successfully done so in the past (Long et al., 2017; Peixoto et al., 2019; Reichert et al., 2019; Sussarellu et al., 2016; Wang, J. et al., 2019; Zimmermann et al., 2020). Some studies have utilized slightly more advanced methods such as a flow cytometer (Long et al., 2017; Sussarellu et al., 2016), coulter counter (Zimmermann et al., 2020) or a fluorescence microscope (Peixoto et al., 2019). Alternatively, it is also possible to filter a subsample of the exposure suspension and simply count the numbers under a stereomicroscope (Reichert et al., 2019; Wang, J. et al., 2019). Interestingly, upon screening the ToMEX database, it is notable that, for each individual criterion, there is always at least one study that manages to incorporate that criterion adequately when testing pelagic species. This indicates that the scientific community possesses the knowledge for conducting high-quality MP effect studies. In conclusion, there is no valid reason for ecotoxicological studies testing pelagic species not to adhere to the 20 criteria proposed in (de Ruijter et al., 2020; **Chapter 2**).

Recently, Jemec Kokalj et al. (2021) put forward new quality criteria for nanomaterial studies using the 20 QA/QC criteria for MP effect studies (de Ruijter et al., 2020; **Chapter 2**) as a starting point and proposed additional nanoplastic specific criteria for nanomaterial studies. Moreover, similar QA/QC evaluation tools have been developed for measuring MP in matrices such as water, sediment, air or biota samples (Hermsen et al., 2018; Koelmans et al., 2019; Redondo-Hasselerharm et al., 2023; Wright et al., 2021). Since the measurement of MP in these matrices is also necessary in the context of effect tests, these QA/QC criteria are relevant to such studies. Although the details are beyond the scope of this review, QA/QC criteria for water and biota samples, for instance, have been implemented by the World Health Organization (World Health Organization, 2019, 2022).

4. Quality assurance in single species tests of microplastic particle-associated chemicals

MP particles in the natural environment always contain chemical substances, either as a result of the production of the polymers from which the MPs originated, or due

to the adsorption of chemicals from the environment. Effects of MPs can, therefore, occur when thresholds for the particles, the chemicals, or both are exceeded, e.g. Tian et al. (2021). When testing these effects in a laboratory test simultaneously, a problem often arises regarding the relevance of exposure to the substances and particles, ideally aiming for exposures that are as environmentally relevant as possible (Koelmans et al., 2016; Koelmans et al., 2022a).

The first issue is that when you test chemically contaminated MPs in an uncontaminated test system, the chemical substances will start to desorb immediately. This needs to be measured to understand the exposure, but this is rarely done (Koelmans et al., 2022a). The second problem is that some researchers accelerate this desorption by actively extracting the chemicals and determine the toxic effects of the substances after concentrating them (Capolupo et al., 2021; Klein et al., 2021; Zimmermann et al., 2021; Zimmermann et al., 2020), while using unrealistically high plastic-to-water ratios up to 80 g/L. They frame testing of methanolic extracts as “worst case scenario” and testing of water extracts as a “realistic scenario”. However, as long as the exposures are not environmentally relevant, this may still be misleading. What is subsequently measured often has little to do with reality. In nature, microplastic-associated chemicals do not desorb as they are in equilibrium with their surroundings, or they desorb very slowly. This means that when chemicals are released, it leaves few effects through dilution. The third problem is that the tests often use relatively clean test organisms, so exposure is caused only by the created thermodynamic concentration gradient. In reality, these gradients are often absent or even reversed, in which case the substances adhere to the plastic, and exposure can actually decrease (Koelmans et al., 2016; Koelmans et al., 2013; Mohamed Nor and Koelmans, 2019; Mohamed Nor et al., 2023). The fact that absorption to plastic is typically discussed in the context of the 'vector effect' concerning hazards and risks but is omitted when it occurs in the context of exposure reduction suggests the existence of biases in the literature. The fourth problem is that tests examining the role of MP in the exposure to plastic-associated chemicals usually do not consider parallel exposure routes (Koelmans et al., 2016; Koelmans et al., 2022a). The flux of chemical substances through the ingestion of MP is relatively small when compared to the flux through water or food (prey). Because plastic, water, and food are part of the same system, they typically contain the same substances. The fact that the flux through water and food is often greater than that through MP is often overlooked in tests, even though it is a crucial

feature of environmentally relevant exposure. Guidance on testing for plastic-associated chemicals is detailed by Koelmans et al. (2022a), so I omit it here. The most important point of attention is that the actual chemical exposure must be determined in the test system, for example with passive samplers, that the exposure must be environmentally relevant, and that the data interpretation explicitly states the degree of environmental relevance.

5. Community level approaches

5.1. Community level approaches as a component of tiered risk assessments

To assess the environmental risks associated with MPs in a cost-effective manner, it is advisable to employ a tiered approach (Koelmans et al., 2017; Posthuma et al., 2008). This framework consists of successive tiers that increase in ecological relevance and complexity, and resource requirements. The lowest tiers encompass the use of databases (Tier 0) and the performance of laboratory single species tests (Tier 1). Toxicity data obtained from these tests can be combined with literature data in Species Sensitivity Distributions (SSDs) (Tier 2). SSDs are cumulative probability distributions that show the sensitivity of a group of species to a stressor. This tool enables the determination of the Hazardous Concentration for 5% of the species (HC5), which is commonly used to estimate the Predicted No Effect Concentration (PNEC) in environmental risk assessments. As previously mentioned, several studies have characterized the risks associated with MPs by constructing SSDs using toxicity data for aquatic organisms (Coffin et al., 2022b; Koelmans et al., 2023b; Redondo-Hasselerharm et al., 2023). Discrepancies among the PNECs reported in the literature were resolved by aligning exposure and effect data, and by selecting data based on quality criteria and other relevant aspects (*e.g.*, included endpoints). When risks are estimated in Tier 2 through the comparison of the PNEC with measured or predicted environmental concentrations, higher-tier experiments become necessary to assess effects at population and community levels. Higher-tier experiments (Tier 3) are conducted in outdoor artificial or semi-artificial systems, such as mesocosms or enclosures, which mimic the natural environment more effectively than single species tests and significantly enhance ecological relevance. One advantage is that interactions between abiotic and biotic factors play a role in higher tiers, enabling the detection of effects under more realistic and complex scenarios. However, these experiments are more expensive and time-consuming, which consequently limits the amount of toxicity data

available for population and communities. Although environmental risks of MPs have been identified for aquatic ecosystems, only a few experiments have been conducted at this level of ecological relevance with marine and freshwater organisms (Foekema et al., 2022; Green, 2016; Marchant et al., 2023; Redondo-Hasselerharm et al., 2020).

5.2. Overview of microplastic community tests

One of these freshwater outdoor mesocosm studies, as conducted by Marchant et al. (2023), assessed the effects of two MP polymer types (polyethylene and biodegradable polylactic acid) under two nutrient conditions (enriched and non-enriched) on pelagic community structure and ecosystem functioning over a 12-weeks period. The study demonstrated that environmentally relevant MP concentrations, even at higher levels, did not have an impact on plankton community composition and taxonomic richness, periphyton productivity, or leaf litter decomposition (Marchant et al., 2023). In another study, a freshwater benthic community in a semi-artificial ditch was exposed to trays containing sediment and either polystyrene NPs or MPs for durations of 3 and 15 months (Redondo-Hasselerharm et al., 2020). While no effects were detected after 3 months of exposure, after 15 months the highest concentration (5% plastic per sediment dry weight) of both NP and MPs led to a reduction in the population of Naididae worms. Furthermore, a freshwater mesocosm study by Yıldız et al. (2022) also showed adverse effects. They reported a significant reduction in the biomass of daphnids, and a significant change in the wing morphology of chironomids following 7 weeks of exposure to MPs through water (polyethylene and polypropylene) and sediments (polystyrene, polyvinyl chloride, polyethylene terephthalate and polyamide) at the highest MP concentration of 20 mg L^{-1} (Yıldız et al., 2022). In a study by Foekema et al. (2022) marine pelagic and benthic communities, including fish, were exposed to 700 µm polystyrene spheres for 2 months using outdoor mesocosms. At the community level, no effects were observed on species richness and diversity. However, reductions in the condition index of fish (*Solea solea*) and the density of barnacles (*Semibalanus balanoides*) were found (Foekema et al., 2022). Lastly, Green (2016) conducted a study that exposed a marine benthic community to MPs. Exposure of polyethylene and biodegradable polylactic acid for 60 days resulted in a significant reduction in species richness and total number of organisms. Furthermore, the abundances of periwinkles (*Littorina* sp.) and isopods

(*Idotea balthica*), as well as the biomass of clams (*Scrobicularia plana*), decreased at the highest tested concentrations of 80 µg L^{-1} (Green, 2016).

5.3. Determining effect thresholds and risks of microplastics based on community test data.

When comparing the effect thresholds of studies for freshwater and marine populations and communities (Table 1), it becomes apparent that establishing safe limits for the occurrence of MPs in aquatic ecosystems is challenging. Some studies report effect thresholds in mass concentration, while others use number concentrations. Moreover, the characteristics of the tested MPs, the chosen exposure pathways and the selected exposure times greatly differ. Here, I provide an example of how environmental risks of MPs could be calculated using high-tier effect thresholds and compare the measured environmental concentrations of MPs in sediments, as reported by Redondo-Hasselerharm et al. (2023), with the PNEC calculated from the $NOEC_{population}$ for Naididae in Redondo-Hasselerharm et al. (2020). One way to account for the uncertainties and variability in the data is to use assessment factors (AF). This is a conservative approach to ensure that the estimated exposure levels are protective. However, currently, there are no widely accepted AFs specifically tailored for MPs. For this reason, I assessed MPs risks in two ways: 1) without the use of an AF (NOEC = PNEC), and 2) using an AF of 5, as recommended for mesocosm studies by the *Technical Guidance for Deriving Environmental Quality Standards* from the European Union for chemicals (European Commission Directorate-General for Health and Food Safety, 2017). The PNEC values were rescaled to encompass the entire MP size range (1 – 5000 µm), while also accounting for the polydispersity of environmental MP and the *bioaccessible* MP fraction (Koelmans et al., 2023b; Koelmans et al., 2020; Mehinto et al., 2022; Redondo-Hasselerharm et al., 2023; Rico et al., 2023). Following earlier studies (Koelmans et al., 2023b; Redondo-Hasselerharm et al., 2023; Rico et al., 2023), the minimum, mean and maximum measured environmental concentrations of MPs in sediments were compared with the PNECs. These comparisons were repeated for two effect mechanisms: food dilution and toxicity triggered upon translocation, which are caused by the volume of the MPs ingested, and the surface area of the MPs, respectively. The *bioaccessible* MP fraction for volume was set at 1 - 130 µm based on the maximum ingestible size of particles reported for Tubifex spp., which belongs to the family Naididae (Juget, 1979; Redondo-Hasselerharm et al., 2023). For surface area, the selected *bioaccesible* MP fraction selected was 1 -

83 µm following previous studies (Koelmans et al., 2023b; Mehinto et al., 2022; Redondo-Hasselerharm et al., 2023; Rico et al., 2023). The rescaled PNECs were 2.68×10^{10} and 7.6×10^{10} particles/kg for volume and surface area when AF = 0, respectively. With AF=5, the rescaled PNECs for volume and surface area were 5.5×10^{9} and 1.5×10^{10} particles/kg. When plotting the rescaled measured environmental concentrations of MPs in sediments against the rescaled PNECs, we observe that no risks are expected, whether using an AF = 0 or an AF = 5 (Figure 2). Therefore, we can conclude that no risks are anticipated for freshwater benthic communities at the current environmentally realistic concentrations of MPs.

Table 1. Microplastic effect thresholds collected from the literature for freshwater and marine populations and communities.

	Taxonomic group	Exposure pathway	Polymer type	Size and shape	Effect threshold	Concentration		Exposure time	Reference
FRESHWATER	**POPULATIONS**								
	Daphnia spp. (Genus)	Surface water	PE, PP	< 500 µm	NOEC	0.07 g/m2		7 weeks	Yıldız *et al.*, 2022 [6]
		Water column	PE	< 500 µm	NOEC	20 g/m3			
		Sediment	PS, PVC, PA, PET	< 500 µm	NOEC	80 g/m2			
	Chironomidae (Family)	Surface water	PE, PP	< 500 µm	NOEC	0.007 g/m2			
		Water column	PE	< 500 µm	NOEC	2 g/m3			
		Sediment	PS, PVC, PA, PET	< 500 µm	NOEC	8 g/m2			
	Naididae (Family)	Sediment	PS	96.3 ± 1.85 nm	NOEC	5 g/kg		15 months	Redondo-Hasselerharm *et al.* (2020) [5]
			PS	20 – 516 µm	NOEC	5 g/kg			
	COMMUNITIES								
	Planktonic communities	Surface water	PE	12.5 – 500 µm	HNOEC	22 × 10^4 particles/L		12 weeks	Marchant *et al.* (2023) [4]
			PLA	12.5 – 500 µm	HNOEC	23 × 10^4 particles/L			
	Benthic communities	Sediment	PS	96.3 ± 1.85 nm	NOEC	5 g/kg		15 months	Redondo-Hasselerharm *et al.* (2020) [5]
			PS	20 – 516 µm	NOEC	5 g/kg			
MARINE	**POPULATIONS**								
	Solea solea (Species)	Surface water	PS	700 µm	NOEC	0.8 g/m2		2 months	Foekema *et al.* 2022 [8]
	Semibalanus balanoides (Species)	Surface water	PS	700 µm	NOEC	0.8 g/m2*			

	Littorina sp. (Genus)	Surface water (mixed with microalgae)	PE	0.6 – 363 µm	NOEC	80 µg/L		60 days	Green *et al.* (2016) [7]
			PLA	0.48 – 316 µm	NOEC	80 µg/L			
	Idotea balthica (Species)		PE	0.6 – 363 µm	NOEC	80 µg/L			
			PLA	0.48 – 316 µm	NOEC	80 µg/L			
	Scrobicularia plana (Species)		PE	0.6 – 363 µm	NOEC	80 µg/L			
			PLA	0.48 – 316 µm	NOEC	80 µg/L			
	COMMUNITIES								
	Pelagic and benthic communities	Surface water	PS	700 µm	HNOEC	80 g/m2		2 months	Foekema *et al.* 2022 [8]
	Benthic communities	Surface water (mixed with microalgae)	PE	0.6 – 363 µm	NOEC	80 µg/L		60 days	Green *et al.* (2016) [7]
			PLA	0.48 – 316 µm	NOEC	80 µg/L			

* Non concentration dependent

Abbreviations: PA: polyamide; PE: polyethylene; PET: polyethylene terephthalate; PLA: polylactic acid; PP: polypropylene; PS: polystyrene; PVC: polyvinyl chloride; NOEC: no observed effect concentration; HNOEC: highest no observed effect concentration.

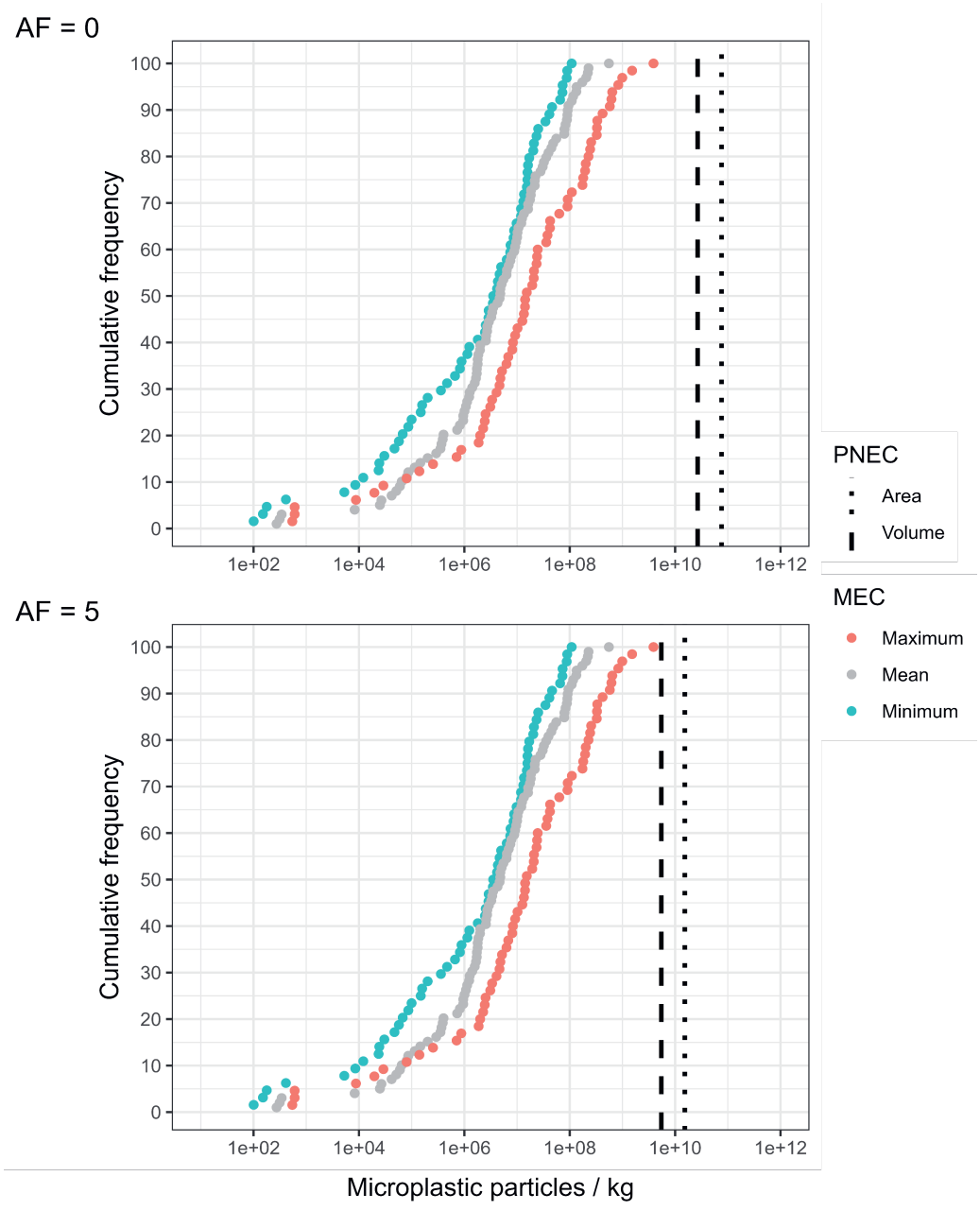


Figure 2. Cumulative frequency distributions of the rescaled minimum, mean and maximum measured environmental concentrations (MECs) of MPs in freshwater sediments compiled by Redondo-Hasselerharm et al. (2023) , plotted together with the predicted no observed effect concentration (PNEC) (dashed lines) derived from $NOEC_{population}$ from Naididae in Redondo-Hasselerharm et al. (2020) for volume (purple) and area (green) as ecologically relevant metrics (ERM) using no assessment factor (upper panel) or an assessment factor

of 5 (lower panel). This higher tier community level risk assessment shows that there are no MEC values that exceed the PNEC values and thus no risks are expected for freshwater benthic communities at the current environmentally realistic concentrations.

6. Standard test materials

To ensure comparability of results across different laboratories, the development of standard reference materials is crucial (ACC TG Environmental Research, 2022; Dehaut et al., 2023; Martínez-Francés et al., 2023; Teague et al., 2021). For instance, standard reference MP particles are essential in calibrating and validating analytical methods, as well as in studies investigating the effects of MPs on organisms. In the latter case, there are valid reasons for using either mono- or polydisperse distributions. While testing monodisperse plastics has provided valuable insights, offering the scientific community a mechanistic understanding of MPs, many researchers have recognized the need to account for the diversity of MPs in study design (Coffin, 2023; de Ruijter et al., 2020; Chapter 2; Koelmans et al., 2023a; Lambert et al., 2017; Rochman et al., 2019; Thornton Hampton et al., 2022a). One of the solutions considered to address this key challenge is proposed by Bucci and Rochman (2022). They advocate the separate testing of various components of MPs, taking into account differences in shape, size and polymer type, to assess their potential hazards. However, although such monodisperse toxicity test results are beneficial for mechanistic research, their environmental relevance may be limited. Translating these results to the natural environment, where MP mixtures are polydisperse, is challenging and conducting separate tests for all possible combinations of characteristics would require extensive resources (Koelmans et al., 2023a). A more relevant and efficient approach is to assess the effects of environmentally realistic polydisperse particles. This approach allows for the evaluation of the causal link between exposure and effects by measuring the bioavailable MPs within the continuum of MPs. By using continuous probability distributions (i.e. probability density functions; PDFs) for shape, size and density from empirical data sets, the multidimensionality of MPs can be characterized across various environmental compartments (Kooi and Koelmans, 2019). This new concept of using polydisperse, environmentally relevant microplastics (ERMP) in effect tests has been implemented in recent studies for a wide range of invertebrate species ((de Ruijter et al., 2023; **Chapter 3**; de Ruijter et al., 2024; **Chapter 5**)). These studies also provide detailed descriptions of the preparation and characterization of the ERMP test mixture (de Ruijter et al., 2024; **Chapter 5**).

Additionally, a step by step protocol for creating such a polydisperse ERMP test material is provided as Supporting information.

7. MP characteristics determining effects and mechanisms explaining effects

The most important MP characteristics that determine effects are concentration, particle size, shape and type (Bucci et al., 2020; Kögel et al., 2020). Furthermore, numerous researchers have emphasized the importance of determining the appropriate units for reporting MP concentration in the context of understanding the effects of MPs (Bucci et al., 2020; Burns and Boxall, 2018; Connors et al., 2017; de Ruijter et al., 2020; Karami, 2017; Kögel et al., 2020). In previous studies, the choice between reporting mass or particle count concentration has complicated the comparison of toxicity studies (Thornton Hampton et al., 2022b). Recently, there has been a positive shift towards adopting these recommendations, with an increasing number of studies reporting both metrics (Thornton Hampton et al., 2022b). Consequently, Thornton Hampton et al. (2022b) assert that reporting mass and number concentration should be the minimum requirement. Additionally, metrics such as particle volume and surface area, which may be informative for certain toxicity mechanisms, should also be reported . Already since 2017, researchers noted that mechanisms behind adverse effects are poorly understood (Connors et al., 2017; De Sá et al.; Ogonowski et al., 2018). de Ruijter et al. (2020)(**Chapter 2**) found that most evidence supports the food dilution mechanism, but there is also evidence for mechanisms like internal physical damage, external physical damage and oxidative stress (de Ruijter et al., 2020; **Chapter 2**). In a study conducted by Thornton Hampton et al. (2022b), researchers were instructed to explore the ToMEx database using their own expertise, knowledge and statistical tools and determine which MP characteristics are most explanatory for understanding MP effects. In line with the food dilution hypothesis, they found that particle volume was a statistically significant predictor of toxicity. Additionally, they found that surface area was an explanatory factor for toxicity of MPs. While MPs need to be relatively small to translocate from the gut to other tissues (Mehinto et al., 2022), once situated within the tissue, it is likely that the surface area correlates with the extent of inflammation and oxidative stress. However, these findings come with uncertainties as the data in the existing toxicity database was not thoroughly screened for quality before data analysis. Moreover, surface area and total plastic volume in the gut are often not comprehensively reported in toxicological studies (Thornton Hampton et al., 2022b).

While all 20 criteria proposed by de Ruijter et al. (2020)(**Chapter 2**) are crucial for testing the effects of MP, three criteria - namely “chemical purity”, ”exposure assessment of organism” and “presence of food” - could enhance the strategic testing of mechanisms. For instance, assessing chemical purity helps distinguish the particle effects from the chemical effects. Moreover, studying how organisms are exposed to MP, preferably quantitatively, provides insight into the mechanisms behind adverse effects. Finally, the criteria focusing on the presence of food are equally important. If no food is provided during exposure to MP, it is remains unclear whether the effect is due to starvation of the organisms or the MPs themselves. Only a relatively small number of MP effect studies have incorporated all of these three criteria to a certain extent (Blarer and Burkhardt-Holm, 2016; Kalčíková et al., 2017; Lu et al., 2023; Murphy and Quinn, 2018; Qiao et al., 2019; Redondo-Hasselerharm et al., 2018b; Sussarellu et al., 2016; Von Moos et al., 2012; Ziajahromi et al., 2017).

Natural particles as controls in MP effect tests

Scientists have often emphasised the importance of including natural particles in the experimental design as a control (Gerdes et al., 2019; Ogonowski et al., 2018; Ogonowski et al., 2023). Some argue that, to inform risks assessment, researchers need to demonstrate that the effects of MPs are indeed different from those of natural particles (Ogonowski et al., 2018). While including natural particles provides insight into the specific causes of toxicity to organisms and places the magnitude of toxicity in a broader context, it is not a prerequisite for determining risk. For instance, if negative effects due to food dilution by MP were to increase with rising plastic pollution, while natural inert particles cause similar negative effects, this would not diminish the adverse effects caused by the MPs. Nevertheless, investigating differences in material type, while keeping particle characteristics as similar as possible, enables the testing of mechanisms such as food dilution, where the volume determines toxicity. Until recently, studies have generally indicated that MPs may be slightly more toxic than natural particles (Gerdes et al., 2019; Ogonowski et al., 2018; Ogonowski et al., 2016; Redondo-Hasselerharm et al., 2018b; Schür et al., 2020; Yap et al., 2020). However a recent meta-analysis by Ogonowski et al. (2023), comparing the toxic effects of MP and naturally occurring suspended solids (SS), indicated that there was no significant difference between the two types of particles. Researchers however, highlighted that the uncertainties, relating to systematic differences in experimental design such as unreported

chemicals in MPs and the predominant use of pristine MPs, were substantial. This underlines the need for studies with a more targeted design (Ogonowski et al., 2023), such as for instance the design provided in **Chapter 5**. Here a similarly diverse ERMP mixture was tested on *Lumbriculus variegatus* and compared to the effects of a mixture with a nearly identical particle size distribution, but composed of mineral particles, —specifically, an a priori designed mixture of ten clay, silt, and sand fractions of different particle sizes (de Ruijter et al., 2024). For both mixtures, no effect was found, which is consistent with the assumption regarding the comparability in potential harmlessness or harmfulness of the particles. Moreover, we observed no differences in growth or reproduction between the two mixtures at particle concentrations of up to 10% (v/v).

8. Recommendations and prospect

We conclude that the quality of microplastic effect studies is gradually improving, and QA/QC practices are increasingly being adopted by MP researchers. However, there are specific aspects that require more attention. The most significant advancements in MP effect studies can be achieved through the improvement in the experimental design. Additional focus should be placed on enhancing the applicability to risk assessment and the ecological relevance of MP effect studies. For instance, diversifying the types of particles tested simultaneously could significantly enhance the reliability of MP risk assessment. While data rescaling and alignments are available, it is recommended to use environmentally relevant microplastic (ERMP) mixtures. In this regard, we have provided a step-by-step protocol for designing polydisperse test materials and a targeted approach to test differences between ERMP mixtures and equally diverse mixtures of natural particles. Given the constraints of limited resources, a focused emphasis on these achievable criteria strategically holds the potential for substantial improvements in enhancing risk assessment practices. Nevertheless, in order to produce reliable data for risk assessment studies should adhere to all QA/QC criteria.

The body of literature providing reliable data on the effects thresholds and mechanisms behind adverse effects is still limited, underscoring the need for more research to advance our understanding and accurately inform risk assessments. Additionally, it could provide insights into the specific organisms that are more sensitive to MPs. To achieve this, well-designed experiments focusing on mechanisms are needed, while adhering to strict QA/QC criteria. For instance, a

comparative study between natural and MP particles with dimensions as closely aligned as possible, could provide insight into the mechanisms of food dilution, where volume determines toxicity.

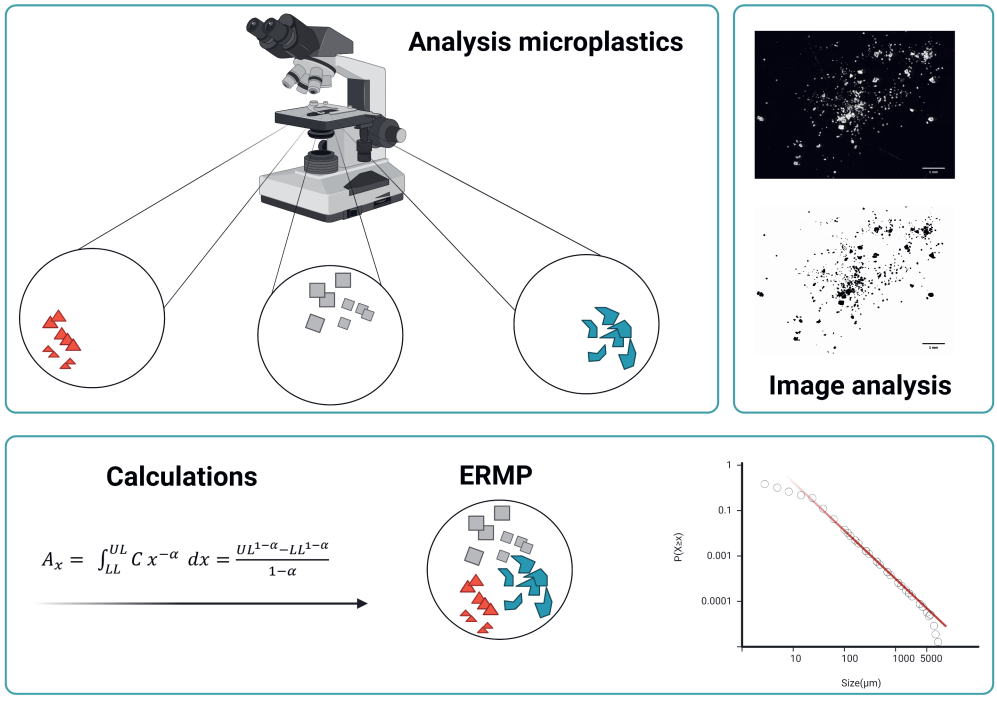
Current risk assessments predominantly indicate that concentrations observed globally and within ecosystems, specifically in surface water, sediments, and soils, exceed the effect thresholds measured for MP toxicity in the laboratory. However, here a higher-tier approach is presented, that reveals no significant risks for freshwater benthic communities at prevailing environmental MP concentrations. Nonetheless, it is important to note that only a limited number of mesocosm studies have been conducted, emphasizing the critical need for broader application of community effect testing and a more comprehensive higher tier risk assessment.

Supporting information

Table S1. Recognition of key challenge of MP effect studies (n=16). These key challenges are 1= testing of environmentally realistic concentrations, 2 = thoroughly characterizing particles, 3 = reporting of concentration metric, 4 = addressing of aggregation of MP particles during exposure studies, 5 = inclusion of sufficient doses, 6 = verification of exposure concentrations, 7 = testing of MP in their diversity, 8 = mismatch plastic type field and lab, 9 = natural particle as reference material and 10 = endpoint selection.

Study	1	2	3	4	5	6	7	8	9	10
Lenz et al. (2016)	x									
Phuong et al. (2016)	x						x	x		
Connors et al. (2017)	x	x	x	x		x			x	
Karami (2017)				x						
Rist and Hartmann (2018)		x		x					x	
De Sá et al. (2018)	x							x		
Burns and Boxall (2018)	x		x					x		
Ogonowski et al. (2018)	x								x	
O'Connor et al. (2020)										
Triebskorn et al. (2019)	x						x	x		
de Ruijter et al. (2020)	x	x	x	x	x	x	x	x	x	x
Bour et al. (2021)										
Kukkola et al. (2021)								x		x
Thornton Hampton et al. (2022a)					x		x			
Thornton Hampton et al. (2022b)			x							
Coffin (2023)	x				x		x			

Protocol for the manufacture of an environmentally relevant microplastics (ERMP) mixture



Selection of materials

- The ERMP mixture consists of several polydisperse microplastic mixtures, each spanning various size ranges
- These mixtures can be obtained through purchase, collection, or grinding, and if necessary, sieving may be required.
- The combined size classes should cover the range of 10 – 5000 µm. The protocol is generic and not dependent on the number of classes used to make the complete mixture. In this explanation, we use four classes (A, B, C, D), to create a polydisperse mixture for a single polymer. For multi-polymer mixtures the approach involves combining mixtures for multiple polymers based on weights.

Characterizing particles

Assuming a mixture of particles from a single polymer with different size categories A (10-100 µm), B (100-500 µm), C (500-1000 µm) and D (1000-5000 µm).

1. Capture high resolution pictures of at least 100 random particles per size category within the overall targeted size range of 10 to 5000 µm. Ensure that a scale is included and particles are as dispersed much as possible. Maintain consistent magnification all the time, otherwise, the data needs to be corrected for '2D subsampling' (for explanation how to correct for this see below). Repeat this process for other size classes B, C and D.
2. Use ImageJ (Schneider et al., 2012) or another image analysis software to assess the exact number of particles ($N_{observed,x}$; x = A, B, C or D) , the minor and major axis of the particles, and the perimeter in the 2D image. In ImageJ a macro can be created to automatically measure the minor and major axis using the "analyze particles" – plugin, this way manual actions do not have to be repeated for each image (Barchiesi et al., 2023). How the image is analysed depends on the image, but prerequisites include setting a scale, enhancing contrast, setting a HSB stack, using an auto threshold to distinguish the particle from the background and if particles are not fully dispersed in the image, the option "watershed" can be applied (Figure S1;S2). Repeat for other size classes B, C and D.
3. For each particle now the volume can be calculated assuming that particles have an ellipsoid shape. For this, the user can follow either the Barchiesi model, the Simon model, or the Tanoiri model (Barchiesi et al., 2023; Simon et al., 2018; Tanoiri et al., 2021). Subsequently, the mass of each particle can be calculated by multiplying the volume with the density of each MP. The densities of the MPs can, after polymer identification with ATR-FTIR or micro-FTIR, be taken from literature (Kooi and Koelmans, 2019). Densities can also be measured with volume displacement or with a gas pycnometer (Barchiesi et al., 2023). Calculate the total mass ($M_{observed,x}$) for the total number of particles ($N_{observed,x}$) of each size category. Repeat for other size classes B, C and D (Table S2).
4. This step is optional, but for each polymer a power law distribution can be fitted for the final mixture using maximum likelihood estimation (Clauset et al., 2009; Newman, 2005). For this use the poweRlaw package in R (Gillespie, 2014).

5. From your ImageJ analysis of microscope pictures determine the lower limit (LL) and the upper limit (UP) for the size class considered (Table S2). Using equation 1, the number of particles (A) can be calculated that is needed given the targeted mean exponent parameter (α) (Kooi et al., 2021), for the known upper limit (UP) and lower limit (LL). This calculations can be made for each - as much as possible complementary - size category and for the total number of all particles in all size classes together, which is set to 100% (10 – 5000 µm), giving the proportions for your mixture (equation 2). Note that these proportions are in particle numbers (table S2; column Particles targeted (%)).

$$A_x = \int_{LL}^{UL} C\, x^{-\alpha}\, dx = \frac{UL^{1-\alpha} - LL^{1-\alpha}}{1-\alpha} \quad (1)$$

$$A_{Total} = A_A + A_B + A_C + A_D \quad (2)$$

Here, A_{total} represents a total number of particles between 10 and 5000 µm, given a power

law slope α, and each A_x represents the number of particles within the LL-UL size fractions

A, B, C, D for the same power law slope.

6. Now consider the number of particles per data set ($N_{observed,X}$) with x is A, B, C or D (Table S2, column) and the calculated total mass per data set calculated in Step 3 (Table S2 "$M_{observed}$,x"). From your observed ImageJ counts ($N_{observed,X}$), you can calculate the observed particle concentration for each of the classes A, B, C and D as a percentage (%). This information is required to determine how many times the number of particles ($N_{observed,X}$) need to be multiplied to match the targeted percentages based on the theoretical design (Eq. 1). Since the targeted particle (%) differs from the observed particle (%) for each size category (Table S2), compute a

multiplication factor (MF = targeted% /observed%). By applying this multiplication factor to the observed weights per polymer fraction, a corrected weight for each polymer fraction can be derived (Table S2, column "$M_{required}$").

7. Determine the desired polymer percentage mass distribution (Table S2, column "$M_{required,x}$"), and translate the required mass to percentages (Table S2, column "$M_{recipe,x}$"), .
8. Duplicate the datasets accordingly to calculated MF (Table S2). Subsequently, a power law distribution can be fitted for the final mixture using maximum likelihood estimation (Clauset et al., 2009; Newman, 2005). For this use the poweRlaw package in R (Gillespie, 2014).

Possible corrections required at Step 1:

If pictures are taken at different magnifications in order to get a focused image of the different particles sizes within a size category, corrections need to be made. Assume pictures are taken, first with a magnification such that a frame (A1) of 1 x 4 mm is covered (blue rectangle). Secondly, images are taken with a higher magnification for smaller particles magnification, leading to a frame (A2) of 0.5 x 2 mm (red rectangle). Compared to the blue rectangle, this should be considered *a subsample in 2D*. Therefore all individual particles analysed from the higher magnification red rectangle should be multiplied with a factor of 4, because that is the ratio of the surface areas of the frames: A1/A2=4.

For example if there would be three measured red areas, then the re-scaling would be by a factor of 4/3 = 1.33. However, the unique datasets with particle measurements cannot be cloned with such a number. In such a case it is possible to randomly select particles from the dataset in the measured read areas. Another solution could be to multiply both datasets with whole numbers in such way that the correct ratio is obtained.

If there would be more than four red areas (the total of the 'high magnification red field of view areas' exceeds the surface of the low magnification blue area, then simply declare that total high magnification area as the sample size, and consider the blue area as subsample.

Table S2. Example table for designing ERMP mixture

Polymer	LL (μm)	UL (μm)	$\alpha_{targeted}$	A_x	Particles targeted (%)	$M_{observed,x}$ (g)	$N_{observed,x}$	Particles observed (%)	$MF_{required}$	$M_{required,x}$ (g)	M_{recipe} (%)
A	10	100	3.25	2.49E-03	99.4	2.53E-04	1763	57.2	1.739	4.40E-04	4.9
B	100	500	3.25	1.37E-05	0.5	1.53E-03	818	26.5	0.021	3.16E-05	0.3
C	500	1000	3.25	2.97E-07	0.0	1.46E-02	338	11.0	0.001	1.58E-05	0.2
D	1000	5000	3.25	7.69E-08	0.0	1.48E+01	164	5.3	0.001	8.56E-03	94.6

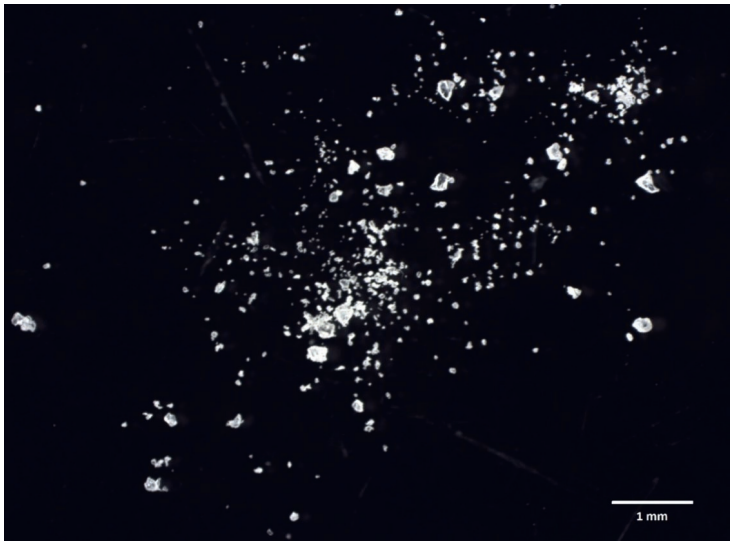


Figure S1. Image microscope polystyrene particles

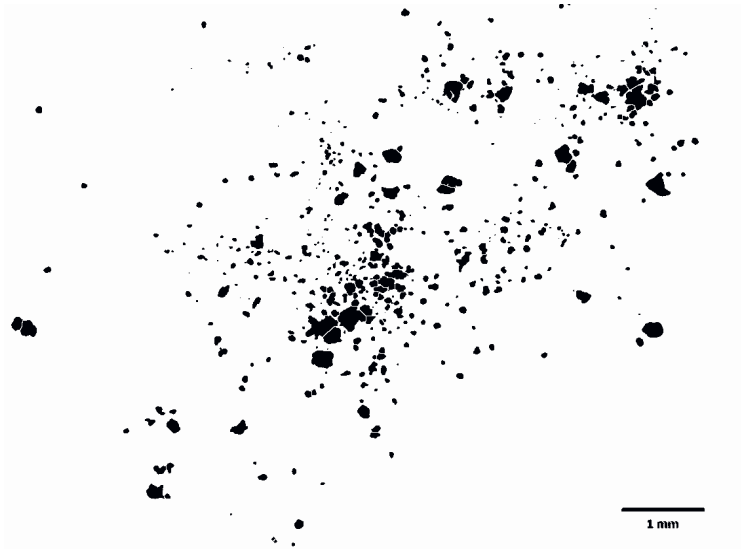
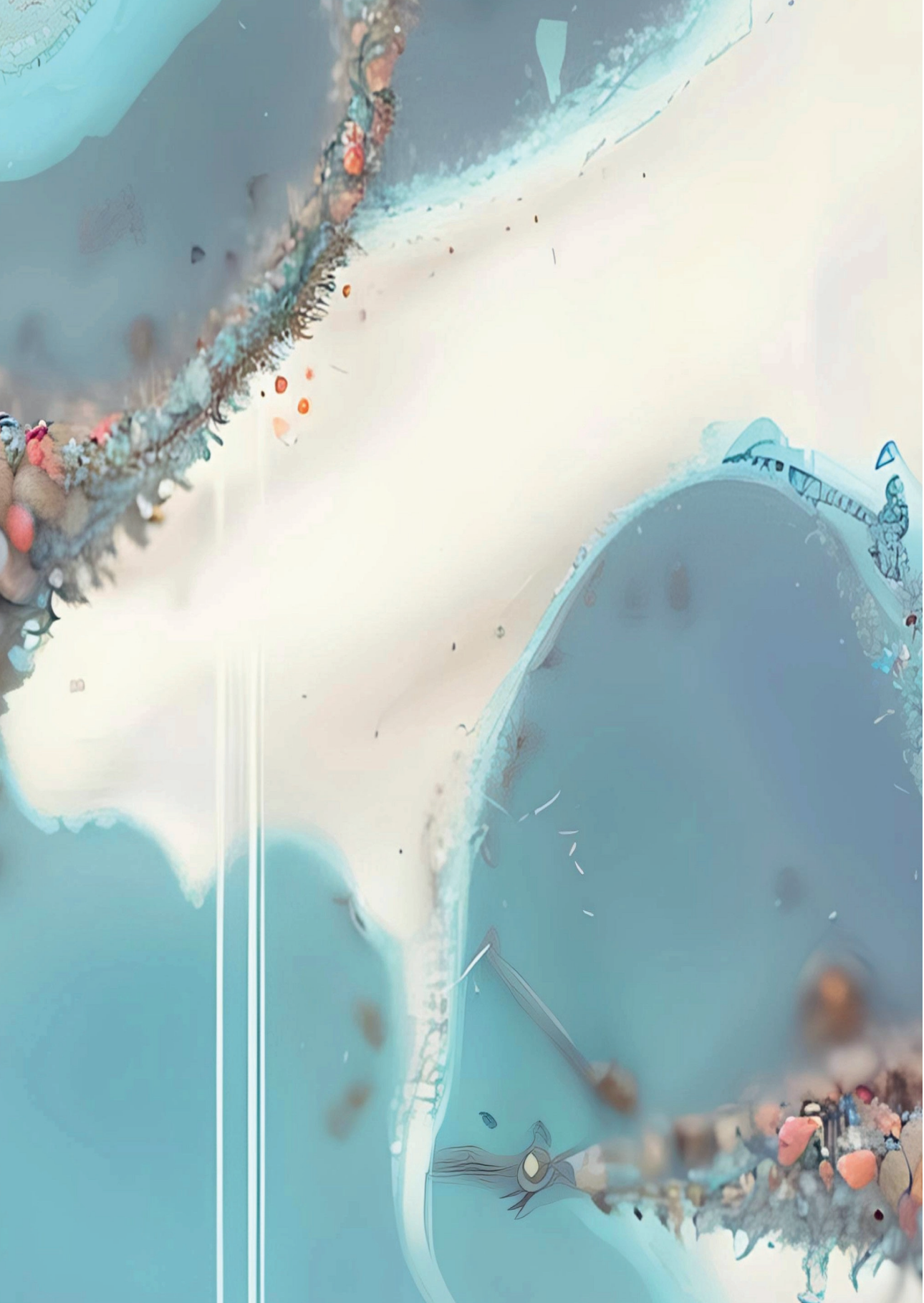


Figure S2. Image polystyrene particles after analysis ImageJ



References

Aberle, N., Hillebrand, H., Grey, J., Wiltshire, K.H., 2005. Selectivity and competitive interactions between two benthic invertebrate grazers (Asellus aquaticus and Potamopyrgus antipodarum): an experimental study using 13C-and 15N-labelled diatoms. Freshwater Biology 50(2), 369-379. https://doi.org/10.1111/j.1365-2427.2004.01325.x.

ACC TG Environmental Research, 2022. Microplastic Reference Materials. American Chemistry Council, TG Environmental Research https://www.americanchemistry.com/chemistry-in-america/research/long-range-research-initiative-lri/catalog-resources/microplastic-reference-materials-invited-expert-workshop.

Adam, V., Yang, T., Nowack, B., 2019. Toward an ecotoxicological risk assessment of microplastics: Comparison of available hazard and exposure data in freshwaters. Environ. Toxicol. Chem. 38(2), 436-447. https://doi.org/10.1002/etc.4323.

Alimi, O.S., Claveau-Mallet, D., Kurusu, R.S., Lapointe, M., Bayen, S., Tufenkji, N., 2022. Weathering pathways and protocols for environmentally relevant microplastics and nanoplastics: What are we missing? J. Hazard. Mater. 423, 126955. https://doi.org/10.1016/j.jhazmat.2021.126955.

Aljaibachi, R., Callaghan, A., 2018. Impact of polystyrene microplastics on Daphnia magna mortality and reproduction in relation to food availability. PeerJ 6, e4601. https://doi.org/10.7717/peerj.4601.

Amariei, G., Rosal, R., Fernandez-Pinas, F., Koelmans, A.A., 2022. Negative food dilution and positive biofilm carrier effects of microplastic ingestion by D. magna cause tipping points at the population level. Environ. Pollut. 294, 118622. https://doi.org/10.1016/j.envpol.2021.118622.

Anbumani, S., Kakkar, P., 2018. Ecotoxicological effects of microplastics on biota: a review. Environmental Science and Pollution Research 25(15), 14373-14396. https://doi.org/10.1007/s11356-018-1999-x.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62(8), 1596-1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.

Andrady, A.L., 2017. The plastic in microplastics: A review. Mar. Pollut. Bull. 119(1), 12-22. https://doi.org/10.1016/j.marpolbul.2017.01.082.

Arthur, C., Baker, J.E., Bamford, H.A., 2009. Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington Tacoma, Tacoma, WA, USA.

Au, S.Y., Bruce, T.F., Bridges, W.C., Klaine, S.J., 2015. Responses of Hyalella azteca to acute and chronic microplastic exposures. Environ. Toxicol. Chem. 34(11), 2564-2572. https://doi.org/10.1002/etc.3093.

Bakir, A., O'Connor, I.A., Rowland, S.J., Hendriks, A.J., Thompson, R.C., 2016. Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. Environ. Pollut. 219, 56-65. https://doi.org/10.1016/j.envpol.2016.09.046.

Barchiesi, M., Kooi, M., Koelmans, A.A., 2023. Adding depth to Microplastics. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.3c03620.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-E ects Models Using lme. J. Stat. Softw. 67, 1--48. https://doi.org/10.18637/jss.v067.i01.

Bellingeri, A., Bergami, E., Grassi, G., Faleri, C., Redondo-Hasselerharm, P.E., Koelmans, A.A., Corsi, I., 2019. Combined effects of nanoplastics and copper on the freshwater alga Raphidocelis subcapitata. Aquatic toxicology 210, 179-187. https://doi.org/10.1016/j.aquatox.2019.02.022.

Benfenati, E., 2011. Quantitative structure-activity relationships (QSAR) for pesticide regulatory purposes. Elsevier.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological) 57(1), 289-300.

Besseling, E., Quik, J.T.K., Sun, M., Koelmans, A.A., 2017. Fate of nano-and microplastic in freshwater systems: A modeling study. Environ. Pollut. 220, 540-548. https://doi.org/10.1016/j.envpol.2016.10.001.

Besseling, E., Redondo-Hasselerharm, P.E., Foekema, E.M., Koelmans, A.A., 2019. Quantifying ecological risks of aquatic micro- and nanoplastic. Critical Reviews in Environmental Science & Technology 49(1), 32-80. https://doi.org/10.1080/10643389.2018.1531688.

Besseling, E., Wang, B., Lurling, M., Koelmans, A.A., 2014. Nanoplastic affects growth of S. obliquus and reproduction of D. magna. Environ. Sci. Technol. 48(20), 12336-12343. https://doi.org/10.1021/es503001d.

Blarer, P., Burkhardt-Holm, P., 2016. Microplastics affect assimilation efficiency in the freshwater amphipod Gammarus fossarum. Environmental Science and Pollution Research 23(23), 23522-23532. https://doi.org/10.1007/s11356-016-7584-2.

Bosker, T., Olthof, G., Vijver, M.G., Baas, J., Barmentlo, S.H., 2019. Significant decline of Daphnia magna population biomass due to microplastic exposure. Environ. Pollut. 250, 669-675. https://doi.org/10.1016/j.envpol.2019.04.067.

Boucher, J., Friot, D., 2017. Primary microplastics in the oceans: a global evaluation of sources. Iucn Gland, Switzerland.

Bour, A., Haarr, A., Keiter, S., Hylland, K., 2018. Environmentally relevant microplastic exposure affects sediment-dwelling bivalves. Environ. Pollut. 236, 652-660. https://doi.org/10.1016/j.envpol.2018.02.006.

Bour, A., Sandgaard, M.H., Syberg, K., Palmqvist, A., Almroth, B.C., 2021. Comprehending the complexity of microplastic organismal exposures and effects, to improve testing frameworks. J. Hazard. Mater. 415, 125652. https://doi.org/10.1016/j.jhazmat.2021.125652.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, Mytilus edulis (L.). Environ. Sci. Technol. 42(13), 5026-5031. https://doi.org/10.1021/es800249a.

Bucci, K., Rochman, C.M., 2022. Microplastics: a multidimensional contaminant requires a multidimensional framework for assessing risk. Microplast. and Nanoplast. 2(1), 7. https://doi.org/10.1186/s43591-022-00028-0.

Bucci, K., Tulio, M., Rochman, C., 2020. What is known and unknown about the effects of plastic pollution: A meta-analysis and systematic review. Ecol. Appl. 30(2), e02044. https://doi.org/10.1002/eap.2044.

Burns, C.W., 1968. The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. Limnology and Oceanography 13(4), 675-678.

Burns, E.E., Boxall, A.B., 2018. Microplastics in the aquatic environment: Evidence for or against adverse impacts and major knowledge gaps. Environ. Toxicol. Chem. 37(11), 2776-2796. https://doi.org/10.1002/etc.4268.

Calfee, R., Piontkowski, D., 1984. Design and analysis of experiments. https://doi.org/10.2307/1271458.

Canniff, P.M., Hoang, T.C., 2018. Microplastic ingestion by Daphnia magna and its enhancement on algal growth. Sci. Total Environ. 633, 500-507. https://doi.org/10.1016/j.scitotenv.2018.03.176.

Capolupo, M., Gunaalan, K., Booth, A.M., Sørensen, L., Valbonesi, P., Fabbri, E., 2021. The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean mussel Mytilus galloprovincialis. Environ. Pollut. 283, 117081. https://doi.org/10.1016/j.envpol.2021.117081.

Catarino, A.I., Macchia, V., Sanderson, W.G., Thompson, R.C., Henry, T.B., 2018. Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal. Environ. Pollut. 237, 675-684. https://doi.org/10.1016/j.envpol.2018.02.069.

Chapron, L., Peru, E., Engler, A., Ghiglione, J.F., Meistertzheim, A.L., Pruski, A.M., Purser, A., Vétion, G., Galand, P.E., Lartaud, F., 2018. Macro-and microplastics affect cold-water corals growth, feeding and behaviour. Sci. Rep. 8(1), 15299. https://doi.org/10.1038/s41598-018-33683-6.

Choi, J.S., Jung, Y.-J., Hong, N.-H., Hong, S.H., Park, J.-W., 2018. Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (Cyprinodon variegatus). Mar. Pollut. Bull. 129(1), 231-240. https://doi.org/10.1016/j.marpolbul.2018.02.039.

Clauset, A., Shalizi, C.R., Newman, M.E., 2009. Power-law distributions in empirical data. SIAM review 51(4), 661-703. https://doi.org/10.1137/070710111.

Coffin, S., 2023. The emergence of microplastics: charting the path from research to regulations. Environmental Science: Advances 2(3), 356-367. https://doi.org/10.1039/d2va00275b.

Coffin, S., Bouwmeester, H., Brander, S., Damdimopoulou, P., Gouin, T., Hermabessiere, L., Khan, E., Koelmans, A.A., Lemieux, C.L., Teerds, K., 2022a. Development and application of a health-based framework for informing regulatory action in relation to exposure of microplastic particles in California drinking water. Microplast. and Nanoplast. 2(1), 12. https://doi.org/10.1186/s43591-022-00030-6.

Coffin, S., Weisberg, S.B., Rochman, C., Kooi, M., Koelmans, A.A., 2022b. Risk Characterization of Microplastics in San Francisco Bay, California. Microplast. and Nanoplast. 2, 19. https://doi.org/10.1186/s43591-022-00037-z.

Cole, M., Coppock, R., Lindeque, P.K., Altin, D., Reed, S., Pond, D.W., Sorensen, L., Galloway, T.S., Booth, A.M., 2019. Effects of Nylon Microplastic on Feeding, Lipid Accumulation, and Moulting in a Coldwater Copepod. Environ. Sci. Technol. 53(12), 7075-7082. https://doi.org/10.1021/acs.est.9b01853.

Cole, M., Galloway, T.S., 2015. Ingestion of Nanoplastics and Microplastics by Pacific Oyster Larvae. Environ. Sci. Technol. 49(24), 14625-14632. https://doi.org/10.1021/acs.est.5b04099.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environ. Sci. Technol. 49(2), 1130-1137. https://doi.org/10.1021/es504525u.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environmental Science Technology 47(12), 6646-6655. https://doi.org/10.1021/es400663f.

Connors, K.A., Dyer, S.D., Belanger, S.E., 2017. Advancing the quality of environmental microplastic research. Environ. Toxicol. Chem. 36(7), 1697-1703. https://doi.org/10.1002/etc.3829.

Cowger, W., Booth, A.M., Hamilton, B.M., Thaysen, C., Primpke, S., Munno, K., Lusher, A.L., Dehaut, A., Vaz, V.P., Liboiron, M., Devriese, L.I., Hermabessiere, L., Rochman, C., Athey, S.N., Lynch, J.M., De Frond, H., Gray, A., Jones, O.A.H., Brander, S., Steele, C., Moore, S., Sanchez, A., Nel, H., 2020. Reporting guidelines to increase the reproducibility and comparability of research on microplastics. Appl. Spectrosc. 74(9), 1066-1077. https://doi.org/10.1364/AS.74.001066.

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proceedings of the National Academy of Sciences 111(28), 10239-10244. https://doi.org/10.1073/pnas.1314705111.

Critchell, K., Hoogenboom, M.O., 2018. Effects of microplastic exposure on the body condition and behaviour of planktivorous reef fish (Acanthochromis polyacanthus). PloS one 13(3), e0193308. https://doi.org/10.1371/journal.pone.0193308.

Dawson, A.L., Santana, M.F., Nelis, J.L., Motti, C.A., 2023. Taking control of microplastics data: A comparison of control and blank data correction methods. J. Hazard. Mater. 443, 130218. https://doi.org/10.1016/j.jhazmat.2022.130218.

de Ruijter, V.N., Hof, M., Kotorou, P., van Leeuwen, J., Van Den Heuvel-Greve, M., Roessink, I., Koelmans, A.A., 2023. Microplastic Effect Tests Should Use a Standard Heterogeneous Mixture: Multifarious Impacts among 16 Benthic Invertebrate Species Detected under Ecologically Relevant Test Conditions. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.3c06829.

de Ruijter, V.N., Redondo-Hasselerharm, P.E., Gouin, T., Koelmans, A.A., 2020. Quality Criteria for Microplastic Effect Studies in the Context of Risk Assessment: A Critical Review. Environ. Sci. Technol. 54(19), 11692-11705. https://doi.org/10.1021/acs.est.0c03057.

de Ruijter, V.N., Xie, X., Koelmans, A.A., 2024. Microplastics versus natural mineral particles. How to create and test them while maintaining environmental relevance.Manuscript submitted for publication.

de Sa, L.C., Luís, L.G., Guilhermino, L., 2015. Effects of microplastics on juveniles of the common goby (Pomatoschistus microps): confusion with prey, reduction of the predatory performance and efficiency, and possible influence of developmental conditions. Environ. Pollut. 196, 359-362. https://doi.org/10.1016/j.envpol.2014.10.026.

De Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029-1039. https://doi.org/10.1016/j.scitotenv.2018.07.207.

Dehaut, A., Himber, C., Colin, M., Duflos, G., 2023. Think positive: Proposal of a simple method to create reference materials in the frame of microplastics research. MethodsX 10, 102030. https://doi.org/10.1016/j.mex.2023.102030.

Détrée, C., Gallardo-Escárate, C., 2017. Polyethylene microbeads induce transcriptional responses with tissue-dependent patterns in the mussel Mytilus galloprovincialis. Journal of Molluscan Studies 83(2), 220-225. https://doi.org/10.1093/mollus/eyx005.

Diepens, N.l.J., Dimitrov, M.R., Koelmans, A.A., Smidt, H., 2015. Molecular assessment of bacterial community dynamics and functional end points during sediment bioaccumulation tests. Environ. Sci. Technol. 49(22), 13586-13595. https://doi.org/10.1021/acs.est.5b02992.

Dowd, C., 2020. A new ECDF two-sample test statistic. arXiv https://doi.org/10.48550/arXiv.2007.01360.

Doyle, D., Sundh, H., Almroth, B.C., 2022. Microplastic exposure in aquatic invertebrates can cause significant negative effects compared to natural particles-A meta-analysis. Environ. Pollut., 120434.

ECETOC, 2018. An Evaluation of the Challenges and Limitations Associated with Aquatic Toxicity and Bioaccumulation Studies for Sparingly Soluble and Manufactured Particulate Substances. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels.

ECHA, 2019. Annex XV Restriction Report Proposal for a Restriction. European Chemicals Agency, Helsinki.

European Commission Directorate-General for Health and Food Safety, 2017. Technical guidance for deriving environmental quality standards, Updated version 2018 ed. European Commission. https://op.europa.eu/en/publication-detail/-/publication/d5b2b9b9-32fb-11e8-b5fe-01aa75ed71a1. https://doi.org/10.2875/018826.

Everaert, G., De Rijcke, M., Lonneville, B., Janssen, C.R., Backhaus, T., Mees, J., van Sebille, E., Koelmans, A.A., Catarino, A.I., Vandegehuchte, M.B., 2020. Risks of floating microplastic in the global ocean. Environ. Pollut. 267, 115499. https://doi.org/10.1016/j.envpol.2020.115499.

Everaert, G., Van Cauwenberghe, L., De Rijcke, M., Koelmans, A.A., Mees, J., Vandegehuchte, M., Janssen, C.R., 2018. Risk assessment of microplastics in the ocean: Modelling approach and first conclusions. Environ. Pollut. 242(Pt B), 1930-1938. https://doi.org/10.1016/j.envpol.2018.07.069.

Feng, Z., Zhang, T., Li, Y., He, X., Wang, R., Xu, J., Gao, G., 2019. The accumulation of microplastics in fish from an important fish farm and mariculture area, Haizhou Bay, China. Sci. Total Environ. 696, 133948. https://doi.org/10.1016/j.scitotenv.2019.133948.

Foekema, E.M., De Gruijter, C., Mergia, M.T., van Franeker, J.A., Murk, A.J., Koelmans, A.A., 2013. Plastic in north sea fish. Environ. Sci. Technol. 47(15), 8818-8824. https://doi.org/10.1021/es400931b.

Foekema, E.M., Keur, M., Van Der Vlies, L., Van Der Weide, B., Bittner, O., Murk, A.J., 2022. Subtle ecosystem effects of microplastic exposure in marine mesocosms including fish. Environ. Pollut. 315, 120429. https://doi.org/10.1016/j.envpol.2022.120429.

Foley, C.J., Feiner, Z.S., Malinich, T.D., Höök, T.O., 2018. A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. Sci. Total Environ. 631-632, 550-559. https://doi.org/10.1016/j.scitotenv.2018.03.046.

Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Environmental Toxicology and Chemistry: An International Journal 25(1), 272-280.

Franzellitti, S., Capolupo, M., Wathsala, R.H.G.R., Valbonesi, P., Fabbri, E., 2019. The Multixenobiotic resistance system as a possible protective response triggered by microplastic ingestion in Mediterranean mussels (Mytilus galloprovincialis): Larvae and adult stages. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 219, 50-58. https://doi.org/10.1016/j.cbpc.2019.02.005.

Frias, J.P., Nash, R., 2019. Microplastics: Finding a consensus on the definition. Mar. Pollut. Bull. 138, 145-147. https://doi.org/10.1016/j.marpolbul.2018.11.022.

Frydkjær, C.K., Iversen, N., Roslev, P., 2017. Ingestion and egestion of microplastics by the cladoceran Daphnia magna: effects of regular and irregular shaped plastic and sorbed phenanthrene. Bulletin of Environmental Contamination and Toxicology 99, 655-661. https://doi.org/10.1007/s00128-017-2186-3.

Galloway, T.S., Dogra, Y., Garrett, N., Rowe, D., Tyler, C.R., Moger, J., Lammer, E., Landsiedel, R., Sauer, U.G., Scherer, G., Wohlleben, W., Wiench, K., 2017. Ecotoxicological assessment of nanoparticle-containing acrylic copolymer dispersions in fairy shrimp and zebrafish embryos. Environmental Science: Nano 4(10), 1981-1997. https://doi.org/10.1039/c7en00385d.

Gambardella, C., Morgana, S., Ferrando, S., Bramini, M., Piazza, V., Costa, E., Garaventa, F., Faimali, M., 2017. Effects of polystyrene microbeads in marine planktonic crustaceans. Ecotoxicol. Environ. Saf. 145, 250-257. https://doi.org/10.1016/j.ecoenv.2017.07.036.

Gambardella, C., Piazza, V., Albentosa, M., Bebianno, M.J., Cardoso, C., Faimali, M., Garaventa, F., Garrido, S., Gonzalez, S., Perez, S., Sendra, M., Beiras, R., 2019. Microplastics do not affect standard ecotoxicological endpoints in marine unicellular organisms. Mar. Pollut. Bull. 143, 140-143. https://doi.org/10.1016/j.marpolbul.2019.04.055.

Gardon, T., Reisser, C.I., Soyez, C., Quillien, V., Le Moullac, G., 2018. Microplastics affect energy balance and gametogenesis in the pearl oyster Pinctada margaritifera. Environ. Sci. Technol. 52(9), 5277-5286. https://doi.org/10.1021/acs.est.8b00168.

Gerdes, Z., Hermann, M., Ogonowski, M., Gorokhova, E., 2019. A novel method for assessing microplastic effect in suspension through mixing test and reference materials. Sci. Rep. 9(1), 1-9. https://doi.org/10.1038/s41598-019-47160-1.

Gillespie, C.S., 2014. Fitting heavy tailed distributions: the poweRlaw package. arXiv preprint arXiv:1407.3492. https://doi.org/10.48550/arXiv.1407.3492.

Goldstein, M.C., Rosenberg, M., Cheng, L., 2012. Increased oceanic microplastic debris enhances oviposition in an endemic pelagic insect. Biol. Lett. 8(5), 817-820. https://doi.org/10.1098/rsbl.2012.0298.

Goncalves, C., Martins, M., Sobral, P., Costa, P.M., Costa, M.H., 2019. An assessment of the ability to ingest and excrete microplastics by filter-feeders: A case study with the Mediterranean mussel. Environ. Pollut. 245, 600-606. https://doi.org/10.1016/j.envpol.2018.11.038.

Gorokhova, E., Könnecke, O., Ogonowski, M., Gerdes, Z., Eriksson Wiklund, A.K., 2018. Alterations in swimming behavior of Daphnia exposed to polymer and mineral particles: towards understanding effects of microplastics on planktonic filtrators. BioRxiv, 406587. https://doi.org/10.1101/406587.

Gouin, T., 2020. Toward an Improved Understanding of the Ingestion and Trophic Transfer of Microplastic Particles: Critical Review and Implications for Future Research. Environ. Toxicol. Chem. 39(6), 1119-1137. https://doi.org/10.1002/etc.4718.

Gouin, T., Becker, R.A., Collot, A.G., Davis, J.W., Howard, B., Inawaka, K., Lampi, M., Ramon, B.S., Shi, J., Hopp, P.W., 2019. Toward the Development and Application of an Environmental Risk Assessment Framework for Microplastic. Environ. Toxicol. Chem. 38(10), 2087-2100. https://doi.org/10.1002/etc.4529.

Granek, E.F., Brander, S., Holland, E., 2020. Microplastics in aquatic organisms: Improving understanding and identifying research directions for the next decade. Limnology and Oceanography Letters 5(1). https://doi.org/10.1002/lol2.10145.

Gray, A.D., Weinstein, J.E., 2017. Size- and shape-dependent effects of microplastic particles on adult daggerblade grass shrimp (Palaemonetes pugio). Environ. Toxicol. Chem. 36(11), 3074-3080. https://doi.org/10.1002/etc.3881.

Green, D.S., 2016. Effects of microplastics on European flat oysters, Ostrea edulis and their associated benthic communities. Environ. Pollut. 216, 95-103. https://doi.org/10.1016/j.envpol.2016.05.043.

Green, D.S., Boots, B., Sigwart, J., Jiang, S., Rocha, C., 2016. Effects of conventional and biodegradable microplastics on a marine ecosystem engineer (Arenicola marina) and sediment nutrient cycling. Environ. Pollut. 208(Pt B), 426-434. https://doi.org/10.1016/j.envpol.2015.10.010.

Green, D.S., Colgan, T.J., Thompson, R.C., Carolan, J.C., 2019. Exposure to microplastics reduces attachment strength and alters the haemolymph proteome of blue mussels (Mytilus edulis). Environ. Pollut. 246, 423-434. https://doi.org/10.1016/j.envpol.2018.12.017.

Green, J.W., Springer, T.A., Staveley, J.P., 2013. The drive to ban the NOEC/LOEC in favor of ECx is misguided and misinformed. Integrated Environmental Assessment and Management 9(1), 12-16. https://doi.org/10.1002/ieam.1367.

Guzzetti, E., Sureda, A., Tejada, S., Faggio, C., 2018. Microplastic in marine organism: Environmental and toxicological effects. Environmental toxicology and pharmacology 64, 164-171. https://doi.org/10.1016/j.etap.2018.10.009.

Hanson, M.L., Wolff, B.A., Green, J.W., Kivi, M., Panter, G.H., Warne, M.S., Agerstrand, M., Sumpter, J.P., 2017. How we can make ecotoxicology more valuable to environmental protection. Sci. Total Environ. 578, 228-235. https://doi.org/10.1016/j.scitotenv.2016.07.160.

Hanvey, J.S., Lewis, P.J., Lavers, J.L., Crosbie, N.D., Pozo, K., Clarke, B.O., 2017. A review of analytical techniques for quantifying microplastics in sediments. Anal. Methods 9(9), 1369-1383. https://doi.org/10.1039/c6ay02707e.

Harris, C.A., Scott, A.P., Johnson, A.C., Panter, G.H., Sheahan, D., Roberts, M., Sumpter, J.P., 2014. Principles of sound ecotoxicology. Environ. Sci. Technol. 48(6), 3100-3111. https://doi.org/10.1021/es4047507.

Hartmann, N.B., Hüffer, T., Thompson, R.C., Hassellöv, M., Verschoor, A., Daugaard, A.E., Rist, S., Karlsson, T., Brennholt, N., Cole, M., Herrling, M.P., Hess, M.C., Ivleva, N.P., Lusher, A.L., Wagner, M., 2019. Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris. Environ. Sci. Technol. 53(3), 1039-1047. https://doi.org/10.1021/acs.est.8b05297.

Hartmann, N.B., Jensen, K.A., Baun, A., Rasmussen, K., Rauscher, H., Tantra, R., Cupi, D., Gilliland, D., Pianella, F., Riego Sintes, J.M., 2015. Techniques and Protocols for Dispersing Nanoparticle Powders in Aqueous Media-Is there a Rationale for Harmonization? Journal of Toxicology and Environmental Health, Part B 18(6), 299-326. https://doi.org/10.1080/10937404.2015.1074969.

Hermsen, E., Mintenig, S.M., Besseling, E., Koelmans, A.A., 2018. Quality Criteria for the Analysis of Microplastic in Biota Samples: A Critical Review. Environ. Sci. Technol. 52(18), 10230-10240. https://doi.org/10.1021/acs.est.8b01611.

Hjorth, R., Skjolding, L.M., Sørensen, S.N., Baun, A., 2017. Regulatory adequacy of aquatic ecotoxicity testing of nanomaterials. NanoImpact 8, 28-37. https://doi.org/10.1016/j.impact.2017.07.003.

Imhof, H.K., Laforsch, C., 2016. Hazardous or not - Are adult and juvenile individuals of Potamopyrgus antipodarum affected by non-buoyant microplastic particles? Environ. Pollut. 218, 383-391. https://doi.org/10.1016/j.envpol.2016.07.017.

Imhof, H.K., Rusek, J., Thiel, M., Wolinska, J., Laforsch, C., 2017. Do microplastic particles affect Daphnia magna at the morphological, life history and molecular level? PLoS One 12(11), e0187590. https://doi.org/10.1371/journal.pone.0187590.

Jabeen, K., Li, B., Chen, Q., Su, L., Wu, C., Hollert, H., Shi, H., 2018. Effects of virgin microplastics on goldfish (Carassius auratus). Chemosphere 213, 323-332. https://doi.org/10.1016/j.chemosphere.2018.09.031.

Jafari, M., Ansari-Pour, N., 2019. Why, when and how to adjust your P values? Cell Journal (Yakhteh) 20(4), 604. https://doi.org/10.22074/cellj.2019.5992.

Jager, T., 2012. Bad habits die hard: the NOEC's persistence reflects poorly on ecotoxicology. Environmental Toxicology Chemistry 31(2), 228-229. https://doi.org/10.1002/etc.746.

Jahnke, A., Arp, H.P.H., Escher, B.I., Gewert, B., Gorokhova, E., Kühnel, D., Ogonowski, M., Potthoff, A., Rummel, C.D., Schmitt-Jansen, M., 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ. Sci. Technol. Lett. 4(3), 85-90. https://doi.org/10.1021/acs.estlett.7b00008.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347(6223), 768-771. https://doi.org/10.1126/science.1260352.

Jâms, I.B., Windsor, F.M., Poudevigne-Durance, T., Ormerod, S.J., Durance, I., 2020. Estimating the size distribution of plastics ingested by animals. Nature Communication 11(1), 1594. https://doi.org/10.1038/s41467-020-15406-6.

Jemec, A., Horvat, P., Kunej, U., Bele, M., Krzan, A., 2016. Uptake and effects of microplastic textile fibers on freshwater crustacean Daphnia magna. Environ. Pollut. 219, 201-209. https://doi.org/10.1016/j.envpol.2016.10.037.

Jemec Kokalj, A., Hartmann, N.B., Drobne, D., Potthoff, A., Kühnel, D., 2021. Quality of nanoplastics and microplastics ecotoxicity studies: refining quality criteria for nanomaterial studies. J. Hazard. Mater. 415, 125751. https://doi.org/10.1016/j.jhazmat.2021.125751.

Jemec Kokalj, A., Kunej, U., Skalar, T., 2018. Screening study of four environmentally relevant microplastic pollutants: Uptake and effects on Daphnia magna and Artemia franciscana. Chemosphere 208, 522-529. https://doi.org/10.1016/j.chemosphere.2018.05.172.

Jeong, C.B., Kang, H.M., Lee, M.C., Kim, D.H., Han, J., Hwang, D.S., Souissi, S., Lee, S.J., Shin, K.H., Park, H.G., Lee, J.S., 2017. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. Sci. Rep. 7, 41323. https://doi.org/10.1038/srep41323.

Jeong, J., Choi, J., 2019. Adverse outcome pathways potentially related to hazard identification of microplastics based on toxicity mechanisms. Chemosphere 231, 249-255. https://doi.org/10.1016/j.chemosphere.2019.05.003.

Jin, Y., Lu, L., Tu, W., Luo, T., Fu, Z., 2019. Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. Sci. Total Environ. 649, 308-317. https://doi.org/10.1016/j.scitotenv.2018.08.353.

Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., Fu, Z., 2018. Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish. Environ. Pollut. 235, 322-329. https://doi.org/10.1016/j.envpol.2017.12.088.

Jovanovic, B., 2017. Ingestion of microplastics by fish and its potential consequences from a physical perspective. Integrated Environmental Assessment and Management 13(3), 510-515. https://doi.org/10.1002/ieam.1913.

Jovanović, B., Gökdağ, K., Güven, O., Emre, Y., Whitley, E.M., Kideys, A.E., 2018. Virgin microplastics are not causing imminent harm to fish after dietary exposure. Mar. Pollut. Bull. 130, 123-131. https://doi.org/10.1016/j.marpolbul.2018.03.016.

Juget, J., 1979. La texture granulometrique des sediments et le regime alimentaire des oligochetes limnicoles. Hydrobiologia 65, 145-154. https://doi.org/10.1007/BF00017420.

Kalčíková, G., Gotvajn, A.Ž., Kladnik, A., Jemec, A., 2017. Impact of polyethylene microbeads on the floating freshwater plant duckweed Lemna minor. Environ. Pollut. 230, 1108-1115. https://doi.org/10.1016/j.envpol.2017.07.050.

Kaposi, K.L., Mos, B., Kelaher, B.P., Dworjanyn, S.A., 2014. Ingestion of microplastic has limited impact on a marine larva. Environ. Sci. Technol. 48(3), 1638-1645. https://doi.org/10.1021/es404295e.

Karami, A., 2017. Gaps in aquatic toxicological studies of microplastics. Chemosphere 184, 841-848. https://doi.org/10.1016/j.chemosphere.2017.06.048.

Karami, A., Groman, D.B., Wilson, S.P., Ismail, P., Neela, V.K., 2017. Biomarker responses in zebrafish (Danio rerio) larvae exposed to pristine low-density polyethylene fragments. Environ. Pollut. 223, 466-475. https://doi.org/10.1016/j.envpol.2017.01.047.

Klein, K., Piana, T., Lauschke, T., Schweyen, P., Dierkes, G., Ternes, T., Schulte-Oehlmann, U., Oehlmann, J., 2021. Chemicals associated with biodegradable microplastic drive the toxicity to the freshwater oligochaete Lumbriculus variegatus. Aquatic Toxicology 231, 105723.

Klimisch, H.-J., Andreae, M., Tillmann, U., 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regulatory toxicology and pharmacology 25(1), 1-5.

Koelmans, A.A., Bakir, A., Burton, G.A., Janssen, C.R., 2016. Microplastic as a Vector for Chemicals in the Aquatic Environment: Critical Review and Model-Supported Reinterpretation of Empirical Studies. Environ. Sci. Technol. 50(7), 3315-3326. https://doi.org/10.1021/acs.est.5b06069.

Koelmans, A.A., Belay, B.M.G., Mintenig, S.M., Mohamed Nor, N., H,, Redondo-Hasselerharm, P.E., de Ruijter, V.N., 2023a. Towards a rational and efficient risk assessment for microplastics. TrAC Trends in Analytical Chemistry, 117142. https://doi.org/10.1016/j.trac.2023.117142.

Koelmans, A.A., Besseling, E., Foekema, E., Kooi, M., Mintenig, S., Ossendorp, B.C., Redondo-Hasselerharm, P.E., Verschoor, A., Van Wezel, A.P., Scheffer, M., 2017. Risks of plastic debris: unravelling fact, opinion, perception, and belief. Environ. Sci. Technol. 51(20), 11513-11519. https://doi.org/10.1021/acs.est.7b02219.

Koelmans, A.A., Besseling, E., Wegner, A., Foekema, E.M., 2013. Plastic as a carrier of POPs to aquatic organisms: a model analysis. Environ. Sci. Technol. 47(14), 7812-7820. https://doi.org/10.1021/es401169n.

Koelmans, A.A., Diepens, N.J., Mohamed Nor, N.H., 2022a. Weight of evidence for the microplastic vector effect in the context of chemical risk assessment. Microplastic in the Environment: Pattern and Process, 155-197.

Koelmans, A.A., Mohamed Nor, N.H., Hermsen, E., Kooi, M., Mintenig, S.M., De France, J., 2019. Microplastics in freshwaters and drinking water: Critical review and assessment of data quality. Water Res. 155, 410-422. https://doi.org/10.1016/j.watres.2019.02.054.

Koelmans, A.A., Redondo-Hasselerharm, P.E., Mohamed Nor, N.H., de Ruijter, V.N., Mintenig, S.M., Kooi, M., 2022b. Risk assessment of microplastic particles. Nat. Rev. Mater. 7(2), 138-152. https://doi.org/10.1038/s41578-021-00411-y.

Koelmans, A.A., Redondo-Hasselerharm, P.E., Mohamed Nor, N.H., Gouin, T., 2023b. On the probability of ecological risks from microplastics in the Laurentian Great lakes. Environ. Pollut. 325, 121445. https://doi.org/10.1016/j.envpol.2023.121445.

Koelmans, A.A., Redondo-Hasselerharm, P.E., Mohamed Nor, N.H., Kooi, M., 2020. Solving the Nonalignment of Methods and Approaches Used in Microplastic Research to Consistently Characterize Risk. Environ. Sci. Technol. 54(19), 12307-12315. https://doi.org/10.1021/acs.est.0c02982.

Kögel, T., Bjorøy, Ø., Toto, B., Bienfait, A.M., Sanden, M., 2020. Micro- and nanoplastic toxicity on aquatic life: Determining factors. Science Total Environment 709, 136050. https://doi.org/10.1016/j.scitotenv.2019.136050.

Kong, X., Koelmans, A.A., 2019. Modeling Decreased Resilience of Shallow Lake Ecosystems toward Eutrophication due to Microplastic Ingestion across the Food Web. Environ. Sci. Technol. 53(23), 13822-13831. https://doi.org/10.1021/acs.est.9b03905.

Kooi, M., Koelmans, A.A., 2019. Simplifying Microplastic via Continuous Probability Distributions for Size, Shape, and Density. Environ. Sci. Technol. Lett. 6(9), 551-557. https://doi.org/10.1021/acs.estlett.9b00379.

Kooi, M., Primpke, S., Mintenig, S.M., Lorenz, C., Gerdts, G., Koelmans, A.A., 2021. Characterizing the multidimensionality of microplastics across environmental compartments. Water Res. 202, 117429. https://doi.org/10.1016/j.watres.2021.117429.

Kooi, M., van Nes, E.H., Scheffer, M., Koelmans, A.A., 2017. Ups and downs in the ocean: effects of biofouling on vertical transport of microplastics. Environ. Sci. Technol. 51(14), 7963-7971. https://doi.org/10.1021/acs.est.6b04702.

Korez, Š., Gutow, L., Saborowski, R., 2019. Feeding and digestion of the marine isopod Idotea emarginata challenged by poor food quality and microplastics. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 226, 108586. https://doi.org/10.1016/j.cbpc.2019.108586.

Kukkola, A., Krause, S., Lynch, I., Smith, G.H.S., Nel, H., 2021. Nano and microplastic interactions with freshwater biota–current knowledge, challenges and future solutions. Environment International 152, 106504. https://doi.org/10.1016/j.envint.2021.106504.

Lambert, S., Scherer, C., Wagner, M., 2017. Ecotoxicity testing of microplastics: Considering the heterogeneity of physicochemical properties. Integrated Environmental Assessment and Management 13(3), 470-475. https://doi.org/10.1002/ieam.1901.

Landis, W.G., Chapman, P.M., 2011. Well past time to stop using NOELs and LOELs. Integrated Environmental Assessment and Management 7(4), vi-viii. https://doi.org/10.1002/ieam.249.

Lee, K.W., Shim, W.J., Kwon, O.Y., Kang, J.H., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod Tigriopus japonicus. Environmental Science Technology 47(19), 11278-11283. https://doi.org/10.1021/es401932b.

Lenaker, P.L., Baldwin, A.K., Corsi, S.R., Mason, S.A., Reneau, P.C., Scott, J.W., 2019. Vertical Distribution of Microplastics in the Water Column and Surficial Sediment from the Milwaukee River Basin to Lake Michigan. Environ. Sci. Technol. 53(21), 12227-12237. https://doi.org/10.1021/acs.est.9b03850.

Lenz, R., Enders, K., Nielsen, T.G., 2016. Microplastic exposure studies should be environmentally realistic. Proceedings of the National Academy of Sciences 113(29), E4121-E4122. https://doi.org/10.1073/pnas.1606615113.

Leung, J., Chan, K.Y.K., 2018. Microplastics reduced posterior segment regeneration rate of the polychaete Perinereis aibuhitensis. Mar. Pollut. Bull. 129(2), 782-786. https://doi.org/10.1016/j.marpolbul.2017.10.072.

Li, J., Yang, D., Li, L., Jabeen, K., Shi, H., 2015. Microplastics in commercial bivalves from China. Environ. Pollut. 207, 190-195. https://doi.org/10.1016/j.envpol.2015.09.018.

Lobelle, D., Cunliffe, M., 2011. Early microbial biofilm formation on marine plastic debris. Mar. Pollut. Bull. 62(1), 197-200. https://doi.org/10.1016/j.marpolbul.2010.10.013.

Löder, M.G., Imhof, H.K., Ladehoff, M., Löschel, L.A., Lorenz, C., Mintenig, S., Piehl, S., Primpke, S., Schrank, I., Laforsch, C., 2017. Enzymatic purification of microplastics in environmental samples. Environ. Sci. Technol. 51(24), 14283-14292. https://doi.org/10.1021/acs.est.7b03055.

Long, M., Paul-Pont, I., Hegaret, H., Moriceau, B., Lambert, C., Huvet, A., Soudant, P., 2017. Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation. Environ. Pollut. 228, 454-463. https://doi.org/10.1016/j.envpol.2017.05.047.

Lu, H.-C., Kumar, A., Melvin, S.D., Ziajahromi, S., Neale, P.A., Leusch, F.D., 2023. Metabolomic responses in freshwater benthic invertebrate, Chironomus tepperi, exposed to polyethylene microplastics: A two-generational investigation. J. Hazard. Mater. 459, 132097. https://doi.org/10.1016/j.jhazmat.2023.132097.

Magni, S., Della Torre, C., Garrone, G., D'Amato, A., Parenti, C.C., Binelli, A., 2019. First evidence of protein modulation by polystyrene microplastics in a freshwater biological model. Environ. Pollut. 250, 407-415. https://doi.org/10.1016/j.envpol.2019.04.088.

Marchant, D.J., Rodríguez, A.M., Francelle, P., Jones, J.I., Kratina, P., 2023. Contrasting the effects of microplastic types, concentrations and nutrient enrichment on freshwater communities and ecosystem functioning. Ecotoxicol. Environ. Saf. 255, 114834. https://doi.org/10.1016/j.ecoenv.2023.114834.

Markic, A., Gaertner, J.-C., Gaertner-Mazouni, N., Koelmans, A.A., 2019. Plastic ingestion by marine fish in the wild. Crit. Rev. Environ. Sci. Technol. 50(7), 657-697. https://doi.org/10.1080/10643389.2019.1631990.

Martínez-Francés, E., van Bavel, B., Hurley, R., Nizzetto, L., Pakhomova, S., Buenaventura, N.T., Singdahl-Larsen, C., Magni, M.-L.T., Johansen, J.E., Lusher, A., 2023. Innovative reference materials for method validation in microplastic analysis including interlaboratory comparison exercises. Analytical and Bioanalytical Chemistry, 1-13. https://doi.org/10.1007/s00216-023-04636-4.

Martínez-Gómez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Marine environmental research 130, 69-76. https://doi.org/10.1016/j.marenvres.2017.06.016.

Mateos-Cárdenas, A., O'Halloran, J., van Pelt, F.N.A.M., Jansen, M.A.K., 2020. Rapid fragmentation of microplastics by the freshwater amphipod Gammarus duebeni (Lillj.). Sci. Rep. 10(1), 12799. https://doi.org/10.1038/s41598-020-69635-2.

Mateos-Cardenas, A., Scott, D.T., Seitmaganbetova, G., Frank, N.A.M.V., John, O., Marcel, A.K.J., 2019. Polyethylene microplastics adhere to Lemna minor (L.), yet have no effects on plant growth or feeding by Gammarus duebeni (Lillj.). Sci. Total Environ. 689, 413-421. https://doi.org/10.1016/j.scitotenv.2019.06.359.

McMahon, R.F., Wilson, J.G., 1981. Seasonal respiratory responses to temperature and hypoxia in relation to burrowing depth in three intertidal bivalves. J. Therm. Biol. 6(4), 267-277. https://doi.org/10.1016/0306-4565(81)90015-2.

Mehinto, A.C., Coffin, S., Koelmans, A.A., Brander, S.M., Wagner, M., Thornton Hampton, L.M., Burton, A.G., Miller, E., Gouin, T., Weisberg, S.B., Rochman, C.M., 2022. Risk-based management framework for microplastics in aquatic ecosystems. Microplast. and Nanoplast. 2(1), 17. https://doi.org/10.1186/s43591-022-00033-3.

Michida, Y., Chavanich, S., Chiba, S., Cordova, M.R., Cozsar Cabanas, A., Glagani, F., Hagmann, P., Hinata, H., Isobe, A., Kershaw, P., 2019. Guidelines for harmonizing ocean surface microplastic monitoring methods. Ministry of the Environment Japan 71.

Moermond, C.T.A., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: Criteria for reporting and evaluating ecotoxicity data. Environ. Toxicol. Chem. 35(5), 1297-1309. https://doi.org/10.1002/etc.3259.

Mohamed Nor, N.H., Koelmans, A.A., 2019. Transfer of PCBs from microplastics under simulated gut fluid conditions is biphasic and reversible. Environ. Sci. Technol. 53(4), 1874-1883. https://doi.org/10.1021/acs.est.8b05143.

Mohamed Nor, N.H., Niu, Z., Hennebelle, M., Koelmans, A.A., 2023. How Digestive Processes Can Affect the Bioavailability of PCBs Associated with Microplastics: A Modeling Study Supported by Empirical Data. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.3c02129.

Monikh, F.A., Praetorius, A., Schmid, A., Kozin, P., Meisterjahn, B., Makarova, E., Hofmann, T., von der Kammer, F., 2018. Scientific rationale for the development of an OECD test guideline on engineered nanomaterial stability. NanoImpact 11, 42-50. https://doi.org/10.1016/j.impact.2018.01.003.

Moore, J., 1975. The role of algae in the diet of Asellus aquaticus L. and Gammarus pulex L. The Journal of animal ecology, 719-730.

Morét-Ferguson, S., Law, K.L., Proskurowski, G., Murphy, E.K., Peacock, E.E., Reddy, C.M., 2010. The size, mass, and composition of plastic debris in the western North Atlantic Ocean. Mar. Pollut. Bull. 60(10), 1873-1878. https://doi.org/10.1016/j.marpolbul.2010.07.020.

Murphy, F., Quinn, B., 2018. The effects of microplastic on freshwater Hydra attenuata feeding, morphology & reproduction. Environ. Pollut. 234, 487-494. https://doi.org/10.1016/j.envpol.2017.11.029.

Newman, M.E., 2005. Power laws, Pareto distributions and Zipf's law. Contemporary Physics 46(5), 323-351. https://doi.org/10.1080/00107510500052444.

O'Connor, J.D., Mahon, A.M., Ramsperger, A.F.R.M., Trotter, B., Redondo-Hasselerharm, P.E., Koelmans, A.A., Lally, H.T., Murphy, S., 2020. Microplastics in freshwater biota: a critical review of isolation, characterization, and assessment methods. Global Challenges 4(6), 1800118. https://doi.org/10.1002/gch2.201800118.

OECD, 2007. Test No. 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment. OECD Publishing, Paris, France. https://www.oecd-ilibrary.org/content/publication/9789264067356-en. https://doi.org/10.1787/9789264067356-en.

OECD, 2014. Test No. 239: Water-Sediment Myriophyllum Spicatum Toxicity Test;. OECD, Paris.

OECD, 2022. Global plastics outlook: economic drivers, environmental impacts and policy options. OECD publishing, Paris. https://doi.org/10.1787/de747aef-en

Ogonowski, M., Gerdes, Z., Gorokhova, E., 2018. What we know and what we think we know about microplastic effects – A critical perspective. Current Opinion in Environmental Science & Health 1, 41-46. https://doi.org/10.1016/j.coesh.2017.09.001.

Ogonowski, M., Schür, C., Jarsén, Å., Gorokhova, E., 2016. The Effects of Natural and Anthropogenic Microparticles on Individual Fitness in Daphnia magna. PLoS One 11(5), e0155063. https://doi.org/10.1371/journal.pone.0155063.

Ogonowski, M., Wagner, M., Rogell, B., Haave, M., Lusher, A., 2023. Microplastics could be marginally more hazardous than natural suspended solids–A meta-analysis. Ecotoxicol. Environ. Saf. 264, 115406. https://doi.org/10.1016/j.ecoenv.2023.115406.

Ogonowski, M., Wenman, V., Barth, A., Hamacher-Barth, E., Danielsson, S., Gorokhova, E., 2019. Microplastic intake, its biotic drivers, and hydrophobic organic contaminant levels in the Baltic herring. Frontiers in Environmental Science, 134. https://doi.org/10.3389/fenvs.2019.00134.

Onink, V., Kaandorp, M.L., van Sebille, E., Laufkötter, C., 2022. Influence of particle size and fragmentation on large-scale microplastic transport in the Mediterranean Sea. Environ. Sci. Technol. 56(22), 15528-15540.

Pan, C.G., Mintenig, S.M., Redondo-Hasselerharm, P.E., Neijenhuis, P.H.M.W., Yu, K.F., Wang, Y.H., Koelmans, A.A., 2021. Automated muFTIR Imaging Demonstrates Taxon-Specific and Selective Uptake of Microplastic by Freshwater Invertebrates. Environ. Sci. Technol. 55(14), 9916-9925. https://doi.org/10.1021/acs.est.1c03119.

Paul-Pont, I., Tallec, K., Gonzalez-Fernandez, C., Lambert, C., Vincent, D., Mazurais, D., Zambonino-Infante, J.-L., Brotons, G., Lagarde, F., Fabioux, C., Soudant, P., Huvet, A., 2018. Constraints and Priorities for Conducting Experimental Exposures of Marine Organisms to Microplastics. Front. mar. sci 5, 252. https://doi.org/10.3389/fmars.2018.00252.

Peixoto, D., Amorim, J., Pinheiro, C., Oliva-Teles, L., Varo, I., de Medeiros Rocha, R., Vieira, M.N., 2019. Uptake and effects of different concentrations of spherical polymer microparticles on Artemia franciscana. Ecotoxicol. Environ. Saf. 176, 211-218. https://doi.org/10.1016/j.ecoenv.2019.03.100.

Petersen, E.J., Barrios, A.C., Henry, T.B., Johnson, M.E., Koelmans, A.A., Montoro Bustos, A.R., Matheson, J., Roesslein, M., Zhao, J., Xing, B., 2022. Potential artifacts and control experiments in toxicity tests of nanoplastic and microplastic particles. Environ. Sci. Technol. 56(22), 15192-15206.

Petersen, E.J., Henry, T.B., Zhao, J., MacCuspie, R.I., Kirschling, T.L., Dobrovolskaia, M.A., Hackley, V., Xing, B., White, J.C., 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. Environ. Sci. Technol. 48(8), 4226-4246. https://doi.org/10.1021/es4052999.

Phuong, N.N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C., Lagarde, F., 2016. Is there any consistency between the microplastics found in the field and those used in laboratory experiments? Environ. Pollut. 211, 111-123. https://doi.org/10.1016/j.envpol.2015.12.035.

Piarulli, S., Vanhove, B., Comandini, P., Scapinello, S., Moens, T., Vrielinck, H., Sciutto, G., Prati, S., Mazzeo, R., Booth, A.M., Van Colen, C., Airoldi, L., 2020. Do different habits affect microplastics contents in organisms? A trait-based analysis on salt marsh species. Mar. Pollut. Bull. 153, 110983. https://doi.org/10.1016/j.marpolbul.2020.110983.

Pikuda, O., Xu, E.G., Berk, D., Tufenkji, N., 2018. Toxicity assessments of micro-and nanoplastics can be confounded by preservatives in commercial formulations. Environ. Sci. Technol. Lett. 6(1), 21-25. https://doi.org/10.1021/acs.estlett.8b00614.

Posthuma, L., Eijsackers, H.J., Koelmans, A.A., Vijver, M.G., 2008. Ecological effects of diffuse mixed pollution are site-specific and require higher-tier risk assessment to improve site management decisions: a discussion paper. Sci. Total Environ. 406(3), 503-517. https://doi.org/10.1016/j.scitotenv.2008.06.065.

Posthuma, L., Suter II, G.W., Traas, T.P., 2001. Species Sensitivity Distributions in Ecotoxicology. CRC press.

Prata, J.C., da Costa, J.P., Duarte, A.C., Rocha-Santos, T., 2019. Methods for sampling and detection of microplastics in water and sediment: A critical review. TrAC Trends in Analytical Chemistry 110, 150-159. https://doi.org/10.1016/j.trac.2018.10.029.

Primpke, S., Cross, R.K., Mintenig, S.M., Simon, M., Vianello, A., Gerdts, G., Vollertsen, J., 2020. Toward the systematic identification of microplastics in the environment: evaluation of a new independent software tool (siMPle) for spectroscopic analysis. Appl. Spectrosc. 74(9), 1127-1138. https://doi.org/10.1177/0003702820917760.

Primpke, S., Dias, P.A., Gerdts, G., 2019. Automated identification and quantification of microfibres and microplastics. Anal. Methods 11(16), 2138-2147.

Primpke, S., Lorenz, C., Rascher-Friesenhausen, R., Gerdts, G., 2017. An automated approach for microplastics analysis using focal plane array (FPA) FTIR microscopy and image analysis. Anal. Methods 9(9), 1499-1511. https://doi.org/10.1039/C6AY02476A

Primpke, S., Wirth, M., Lorenz, C., Gerdts, G., 2018. Reference database design for the automated analysis of microplastic samples based on Fourier transform infrared (FTIR) spectroscopy. Analytical and bioanalytical chemistry 410, 5131-5141.

Prokić, M.D., Radovanović, T.B., Gavrić, J.P., Faggio, C., 2019. Ecotoxicological effects of microplastics: Examination of biomarkers, current state and future perspectives. TrAC Trends in Analytical Chemistry 111, 37-46. https://doi.org/10.1016/j.trac.2018.12.001.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., Lemos, B., 2019. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Sci. Total Environ. 662, 246-253. https://doi.org/10.1016/j.scitotenv.2019.01.245.

Ramsperger, A.F., Stellwag, A.C., Caspari, A., Fery, A., Lueders, T., Kress, H., Löder, M.G., Laforsch, C., 2020. Structural diversity in early-stage biofilm formation on microplastics depends on environmental medium and polymer properties. Water 12(11), 3216. https://doi.org/10.3390/w12113216.

Redondo-Hasselerharm, P., Gort, G., Peeters, E., Koelmans, A., 2020. Nano-and microplastics affect the composition of freshwater benthic communities in the long term. Sci. Adv. 6(5), eaay4054. https://doi.org/10.1126/sciadv.aay4054.

Redondo-Hasselerharm, P.E., de Ruijter, V.N., Mintenig, S.M., Verschoor, A., Koelmans, A.A., 2018a. Ingestion and Chronic Effects of Car Tire Tread Particles on Freshwater Benthic Macroinvertebrates. Environ. Sci. Technol. 52(23), 13986-13994. https://doi.org/10.1021/acs.est.8b05035.

Redondo-Hasselerharm, P.E., Falahudin, D., Peeters, E.T., Koelmans, A.A., 2018b. Microplastic Effect Thresholds for Freshwater Benthic Macroinvertebrates. Environ. Sci. Technol. 52(4), 2278-2286. https://doi.org/10.1021/acs.est.7b05367.

Redondo-Hasselerharm, P.E., Rico, A., Koelmans, A.A., 2023. Risk assessment of microplastics in freshwater sediments guided by strict quality criteria and data alignment methods. J. Hazard. Mater. 441, 129814. https://doi.org/10.1016/j.jhazmat.2022.129814.

Redondo-Hasselerharm, P.E., Rico, A., Lwanga, E.H., van Gestel, C.A., Koelmans, A.A., 2024. Source-Specific Probabilistic Risk Assessment of Microplastics in Soils applying Quality Criteria and Data Alignment Methods. J. Hazard. Mater., 133732. https://doi.org/10.1016/j.jhazmat.2024.133732.

Redondo-Hasselerharm, P.E., Vink, G., Mitrano, D.M., Koelmans, A.A., 2021. Metal-doping of nanoplastics enables accurate assessment of uptake and effects on Gammarus pulex. Environmental Science: Nano 8(6), 1761-1770. https://doi.org/10.1039/D1EN00068C

Rehse, S., Kloas, W., Zarfl, C., 2016. Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of Daphnia magna. Chemosphere 153, 91-99. https://doi.org/10.1016/j.chemosphere.2016.02.133.

Reichert, J., Arnold, A.L., Hoogenboom, M.O., Schubert, P., Wilke, T., 2019. Impacts of microplastics on growth and health of hermatypic corals are species-specific. Environ. Pollut. 254, 113074. https://doi.org/10.1016/j.envpol.2019.113074.

Revel, M., Lagarde, F., Perrein-Ettajani, H., Bruneau, M., Akcha, F., Sussarellu, R., Rouxel, J., Costil, K., Decottignies, P., Cognie, B., 2019. Tissue-specific biomarker responses in the blue mussel Mytilus spp. exposed to a mixture of microplastics at environmentally relevant concentrations. Frontiers in Environmental Science 7, 33. https://doi.org/10.3389/fenvs.2019.00033.

Revel, M., Yakovenko, N., Caley, T., Guillet, C., Châtel, A., Mouneyrac, C., 2020. Accumulation and immunotoxicity of microplastics in the estuarine worm Hediste diversicolor in environmentally relevant conditions of exposure. Environmental Science and Pollution Research 27, 3574-3583. https://doi.org/10.1007/s11356-018-3497-6.

Ribeiro, F., Garcia, A.R., Pereira, B.P., Fonseca, M., Mestre, N.C., Fonseca, T.G., Ilharco, L.M., Bebianno, M.J., 2017. Microplastics effects in Scrobicularia plana. Mar. Pollut. Bull. 122(1-2), 379-391. https://doi.org/10.1016/j.marpolbul.2017.06.078.

Rico, A., Redondo-Hasselerharm, P.E., Vighi, M., Waichman, A.V., de Souza Nunes, G.S., de Oliveira, R., Singdahl-Larsen, C., Hurley, R., Nizzetto, L., Schell, T., 2023. Large-scale monitoring and risk assessment of microplastics in the Amazon River. Water Res. 232, 119707. https://doi.org/10.1016/j.watres.2023.119707.

Rist, S., Baun, A., Hartmann, N.B., 2017. Ingestion of micro- and nanoplastics in Daphnia magna - Quantification of body burdens and assessment of feeding rates and reproduction. Environ. Pollut. 228, 398-407. https://doi.org/10.1016/j.envpol.2017.05.048.

Rist, S., Carney Almroth, B., Hartmann, N.B., Karlsson, T.M., 2018. A critical perspective on early communications concerning human health aspects of microplastics. Sci. Total Environ. 626, 720-726. https://doi.org/10.1016/j.scitotenv.2018.01.092.

Rist, S., Hartmann, N.B., 2018. Aquatic ecotoxicity of microplastics and nanoplastics: lessons learned from engineered nanomaterials, Freshwater Microplastics: Emerging Environmental Contaminants? Springer International Publishing, pp. 25-49. https://doi.org/10.1007/978-3-319-61615-5_2.

Ritz, C., 2010. Toward a unified approach to dose-response modeling in ecotoxicology. Environ. Toxicol. Chem. 29(1), 220-229. https://doi.org/10.1002/etc.7.

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-Response Analysis Using R. PLoS One 10(12), e0146021. https://doi.org/10.1371/journal.pone.0146021.

Rochman, C.M., Brookson, C., Bikker, J., Djuric, N., Earn, A., Bucci, K., Athey, S., Huntington, A., McIlwraith, H., Munno, K., 2019. Rethinking microplastics as a diverse contaminant suite. Environ. Toxicol. Chem. 38(4), 703-711. https://doi.org/10.1002/etc.4371.

Romano, N., Ashikin, M., Teh, J.C., Syukri, F., Karami, A., 2018. Effects of pristine polyvinyl chloride fragments on whole body histology and protease activity in silver barb Barbodes gonionotus fry. Environ. Pollut. 237, 1106-1111. https://doi.org/10.1016/j.envpol.2017.11.040.

Rummel, C.D., Jahnke, A., Gorokhova, E., Kühnel, D., Schmitt-Jansen, M., 2017. Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. Environ. Sci. Technol. Lett. 4(7), 258-267. https://doi.org/10.1021/acs.estlett.7b00164.

SAPEA, 2019. A scientific perspective on microplastics in nature and society. SAPEA, Berlin. https://doi.org/10.26356/microplastics.

Scherer, C., Brennholt, N., Reifferscheid, G., Wagner, M., 2017. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci. Rep. 7(1), 17006. https://doi.org/10.1038/s41598-017-17191-7.

Schmitz, E.H., Scherrey, P.M., 1983. Digestive anatomy of Halella azteca (Crustacea, Amphipoda). Journal of Morphology 175(1), 91-100.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9(7), 671-675. https://doi.org/10.1038/nmeth.2089.

Schrank, I., Trotter, B., Dummert, J., Scholz-Böttcher, B.M., Löder, M.G.J., Laforsch, C., 2019. Effects of microplastic particles and leaching additive on the life history and morphology of Daphnia magna. Environ. Pollut. 255, 113233. https://doi.org/10.1016/j.envpol.2019.113233.

Schür, C., Zipp, S., Thalau, T., Wagner, M., 2020. Microplastics but not natural particles induce multigenerational effects in Daphnia magna. Environ. Pollut. 260, 113904.

Scircle, A., Cizdziel, J.V., Tisinger, L., Anumol, T., Robey, D., 2020. Occurrence of Microplastic Pollution at Oyster Reefs and Other Coastal Sites in the Mississippi Sound, USA: Impacts of Freshwater Inflows from Flooding. Toxics 8(2). https://doi.org/10.3390/toxics8020035.

Seoane, M., Gonzalez-Fernandez, C., Soudant, P., Huvet, A., Esperanza, M., Cid, A., Paul-Pont, I., 2019. Polystyrene microbeads modulate the energy metabolism of the marine diatom Chaetoceros neogracile. Environ. Pollut. 251, 363-371. https://doi.org/10.1016/j.envpol.2019.04.142.

SETAC, 2019. Technical Issue Paper: Recommended Minimum Reporting Information for Environmental Toxicity Studies. Society of Environmental Toxicology and Chemistry, Pensacola (Florida).

Shim, W.J., Hong, S.H., Eo, S.E., 2017. Identification methods in microplastic analysis: a review. Anal. Methods 9(9), 1384-1391. https://doi.org/10.1039/c6ay02558g.

Silva, C.J., Patricio Silva, A.L., Campos, D., Soares, A.M., Pestana, J.L., Gravato, C., 2021. Lumbriculus variegatus (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses. Ecotoxicol. Environ. Saf. 207, 111375. https://doi.org/10.1016/j.ecoenv.2020.111375.

Silva, C.J., Silva, A.L.P., Campos, D., Machado, A.L., Pestana, J.L., Gravato, C., 2021. Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by Chironomus riparius (Diptera) larvae. J. Hazard. Mater. 402, 123775. https://doi.org/10.1016/j.jhazmat.2020.123775.

Silva, C.J.M., Silva, A.L.P., Gravato, C., Pestana, J.L.T., 2019. Ingestion of small-sized and irregularly shaped polyethylene microplastics affect Chironomus riparius life-history traits. Sci. Total Environ. 672, 862-868. https://doi.org/10.1016/j.scitotenv.2019.04.017.

Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying Focal Plane Array (FPA)-based Fourier Transform Infrared (FT-IR) imaging. Water Res. 142, 1-9. https://doi.org/10.1016/j.watres.2018.05.019.

Sjollema, S.B., Redondo-Hasselerharm, P.E., Leslie, H.A., Kraak, M.H.S., Vethaak, A.D., 2016. Do plastic particles affect microalgal photosynthesis and growth? Aquatic toxicology 170, 259-261. https://doi.org/10.1016/j.aquatox.2015.12.002.

Slootmaekers, B., Carteny, C.C., Belpaire, C., Saverwyns, S., Fremout, W., Blust, R., Bervoets, L., 2019. Microplastic contamination in gudgeons (Gobio gobio) from Flemish rivers (Belgium). Environ. Pollut. 244, 675-684. https://doi.org/10.1016/j.envpol.2018.09.136.

Straub, S., Hirsch, P.E., Burkhardt-Holm, P., 2017. Biodegradable and Petroleum-Based Microplastics Do Not Differ in Their Ingestion and Excretion but in Their Biological Effects in a Freshwater Invertebrate Gammarus fossarum. Int. J. Environ. Res. Public Health 14(7). https://doi.org/10.3390/ijerph14070774.

Su, L., Nan, B., Hassell, K.L., Craig, N.J., Pettigrove, V., 2019. Microplastics biomonitoring in Australian urban wetlands using a common noxious fish (Gambusia holbrooki). Chemosphere 228, 65-74. https://doi.org/10.1016/j.chemosphere.2019.04.114.

Su, L., Sharp, S.M., Pettigrove, V.J., Craig, N.J., Nan, B., Du, F., Shi, H., 2020. Superimposed microplastic pollution in a coastal metropolis. Water Res 168, 115140. https://doi.org/10.1016/j.watres.2019.115140.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. Proceedings of the National Academy of Sciences 113(9), 2430-2435. https://doi.org/10.1073/pnas.1519019113.

Tang, J., Ni, X., Zhou, Z., Wang, L., Lin, S., 2018. Acute microplastic exposure raises stress response and suppresses detoxification and immune capacities in the scleractinian coral Pocillopora damicornis. Environ. Pollut. 243, 66-74. https://doi.org/10.1016/j.envpol.2018.08.045.

Tanoiri, H., Nakano, H., Arakawa, H., Hattori, R.S., Yokota, M., 2021. Inclusion of shape parameters increases the accuracy of 3D models for microplastics mass quantification. Mar. Pollut. Bull. 171, 112749. https://doi.org/10.1016/j.marpolbul.2021.112749.

Teague, K., Shaw, K., Corniuk, R., Lynch, J., 2021. Introducing the Polymer Kit 1.0: . https://microplastics.setac.org/spotlights/polymer-kit.

Team, R., 2021. RStudio: Integrated Development Environment for R http://www.rstudio.com/.

Thompson, R.C., Moore, C.J., vom Saal, F.S., Swan, S.H., 2009. Plastics, the environment and human health: current consensus and future trends. Philosophical transactions of the royal society B: biological sciences 364(1526), 2153-2166. https://doi.org/10.1098/rstb.2009.0053.

Thornton Hampton, L.M., Bouwmeester, H., Brander, S.M., Coffin, S., Cole, M., Hermabessiere, L., Mehinto, A.C., Miller, E., Rochman, C.M., Weisberg, S.B., 2022a. Research recommendations to better understand the potential health impacts of microplastics to humans and aquatic ecosystems. Microplast. and Nanoplast. 2(1), 1-13. https://doi.org/10.1186/s43591-022-00038-y.

Thornton Hampton, L.M., Brander, S.M., Coffin, S., Cole, M., Hermabessiere, L., Koelmans, A.A., Rochman, C.M., 2022b. Characterizing microplastic hazards: which concentration metrics and particle characteristics are most informative for understanding toxicity in aquatic organisms? Microplast. and Nanoplast. 2(1), 1-16. https://doi.org/10.1186/s43591-022-00040-4.

Thornton Hampton, L.M., Lowman, H., Coffin, S., Darin, E., De Frond, H., Hermabessiere, L., Miller, E., de Ruijter, V.N., Faltynkova, A., Kotar, S., 2022c. A living tool for the continued exploration of microplastic toxicity. Microplast. and Nanoplast. 2(1), 1-11. https://doi.org/10.1186/s43591-022-00032-4.

Tian, Z., Zhao, H., Peter, K.T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., 2021. A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. Science 371(6525), 185-189. https://doi.org/10.1126/science.abd6951.

Tincani, F.H., Galvan, G.L., Marques, A., Santos, G.S., Pereira, L.S., da Silva, T.A., Silva de Assis, H.C., Barbosa, R.V., Cestari, M.M., 2017. Pseudoreplication and the usage of biomarkers in ecotoxicological bioassays. Environ. Toxicol. Chem. 36(10), 2868-2874. https://doi.org/10.1002/etc.3823.

Triebskorn, R., Braunbeck, T., Grummt, T., Hanslik, L.a., Huppertsberg, S., Jekel, M., Knepper, T.P., Krais, S., Müller, Y.K., Pittroff, M., S. Ruhl, A., Schmieg, H., Schür, C., Strobel, C., Wagner, M., Zumbülte, N., Kohler, H.R., 2019. Relevance of nano-and microplastics for freshwater ecosystems: a critical review. TrAC Trends in Analytical Chemistry 110, 375-392. https://doi.org/10.1016/j.trac.2018.11.023.

Van Cauwenberghe, L., Devriese, L., Galgani, F., Robbens, J., Janssen, C.R., 2015. Microplastics in sediments: A review of techniques, occurrence and effects. Marine Environmental Research 111, 5-17. https://doi.org/10.1016/j.marenvres.2015.06.007.

van Weert, S., Redondo-Hasselerharm, P.E., Diepens, N.J., Koelmans, A.A., 2019. Effects of nanoplastics and microplastics on the growth of sediment-rooted macrophytes. Sci. Total Environ. 654, 1040-1047. https://doi.org/10.1016/j.scitotenv.2018.11.183.

Vandermeersch, G., Van Cauwenberghe, L., Janssen, C.R., Marques, A., Granby, K., Fait, G., Kotterman, M.J.J., Diogène, J., Bekaert, K., Robbens, J., 2015. A critical view on microplastic quantification in aquatic organisms. Environmental Research 143, 46-55. https://doi.org/10.1016/j.envres.2015.07.016.

Vaserstein, L.N., 1969. Markov processes over denumerable products of spaces, describing large systems of automata. Problemy Peredachi Informatsii 5(3), 64-72.

Verdú, I., Amariei, G., Plaza-Bolaños, P., Agüera, A., Leganés, F., Rosal, R., Fernández-Piñas, F., 2022. Polystyrene nanoplastics and wastewater displayed antagonistic toxic effects due to the sorption of wastewater micropollutants. Sci. Total Environ. 819, 153063. https://doi.org/10.1016/j.scitotenv.2022.153063.

Verschoor, A.J., 2015. Towards a definition of microplastics: Considerations for the specification of physico-chemical properties. Rijksinstituut voor Volksgezondheid en Milieu RIVM, Bilthoven, the Netherlands.

Von Moos, N., Burkhardt-Holm, P., Köhler, A., 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel Mytilus edulis L. after an experimental exposure. Environ. Sci. Technol. 46(20), 11327-11335. https://doi.org/10.1021/es302332w.

Vroom, R.J.E., Koelmans, A.A., Besseling, E., Halsband, C., 2017. Aging of microplastics promotes their ingestion by marine zooplankton. Environ. Pollut. 231(Pt 1), 987-996. https://doi.org/10.1016/j.envpol.2017.08.088.

Waldschläger, K., Brückner, M.Z., Almroth, B.C., Hackney, C.R., Adyel, T.M., Alimi, O.S., Belontz, S.L., Cowger, W., Doyle, D., Gray, A., 2022. Learning from natural sediments to tackle microplastics challenges: A multidisciplinary perspective. Earth-Science Reviews 228, 104021.

Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W., Ru, S., 2019. Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (Oryzias melastigma). Environ. Pollut. 254, 113024. https://doi.org/10.1016/j.envpol.2019.113024.

Wang, Y., Zhang, D., Zhang, M., Mu, J., Ding, G., Mao, Z., Cao, Y., Jin, F., Cong, Y., Wang, L., Zhang, W., Wang, J., 2019. Effects of ingested polystyrene microplastics on brine shrimp, Artemia parthenogenetica. Environ. Pollut. 244, 715-722. https://doi.org/10.1016/j.envpol.2018.10.024.

Watts, A.J.R., Urbina, M.A., Goodhead, R., Moger, J., Lewis, C., Galloway, T.S., 2016. Effect of microplastic on the gills of the shore crab Carcinus maenas. Environ. Sci. Technol. 50(10), 5364-5369. https://doi.org/10.1021/acs.est.6b01187.

Weber, A., Scherer, C., Brennholt, N., Reifferscheid, G., Wagner, M., 2018. PET microplastics do not negatively affect the survival, development, metabolism and feeding activity of the freshwater invertebrate Gammarus pulex. Environ. Pollut. 234, 181-189. https://doi.org/10.1016/j.envpol.2017.11.014.

Welden, N.A.C., Cowie, P.R., 2016. Long-term microplastic retention causes reduced body condition in the langoustine, Nephrops norvegicus. Environ. Pollut. 218, 895-900. https://doi.org/10.1016/j.envpol.2016.08.020.

Wesch, C., Bredimus, K., Paulus, M., Klein, R., 2016. Towards the suitable monitoring of ingestion of microplastics by marine biota: A review. Environ. Pollut. 218, 1200-1208. https://doi.org/10.1016/j.envpol.2016.08.076.

WHO, 2019. Microplastics in Drinking-Water. World Health Organization, Geneva.

Woods, M.N., Stack, M.E., Fields, D.M., Shaw, S.D., Matrai, P.A., 2018. Microplastic fiber uptake, ingestion, and egestion rates in the blue mussel (Mytilus edulis). Mar. Pollut. Bull. 137, 638-645. https://doi.org/10.1016/j.marpolbul.2018.10.061.

World Health Organization, 2019. Microplastic in drinking-water, Geneva.
World Health Organization, 2022. Dietary and inhalation exposure to nano-and microplastic particles and potential implications for human health.
Wright, S.L., Gouin, T., Koelmans, A.A., Scheuermann, L., 2021. Development of screening criteria for microplastic particles in air and atmospheric deposition: critical review and applicability towards assessing human exposure. Microplast. and Nanoplast. 1(1), 1-18. https://doi.org/10.1186/s43591-021-00006-y.
Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Current Biology 23(23), R1031-R1033. https://doi.org/10.1016/j.cub.2013.10.068.
Wu, Y., Guo, P., Zhang, X., Zhang, Y., Xie, S., Deng, J., 2019. Effect of microplastics exposure on the photosynthesis system of freshwater algae. J. Hazard. Mater. 374, 219-227. https://doi.org/10.1016/j.jhazmat.2019.04.039.
Xia, F., Yao, Q., Zhang, J., Wang, D., 2021. Effects of seasonal variation and resuspension on microplastics in river sediments. Environ. Pollut. 286, 117403. https://doi.org/10.1016/j.envpol.2021.117403.
Xu, S., Ma, J., Ji, R., Pan, K., Miao, A.J., 2020. Microplastics in aquatic environments: Occurrence, accumulation, and biological effects. Sci. Total Environ. 703, 134699. https://doi.org/10.1016/j.scitotenv.2019.134699.
Yap, V.H., Chase, Z., Wright, J.T., Hurd, C.L., Lavers, J.L., Lenz, M., 2020. A comparison with natural particles reveals a small specific effect of PVC microplastics on mussel performance. Mar. Pollut. Bull. 160, 111703. https://doi.org/10.1016/j.marpolbul.2020.111703.
Yıldız, D., Yalçın, G., Jovanović, B., Boukal, D.S., Vebrová, L., Riha, D., Stanković, J., Savić-Zdraković, D., Metin, M., Akyürek, Y.N., 2022. Effects of a microplastic mixture differ across trophic levels and taxa in a freshwater food web: In situ mesocosm experiment. Sci. Total Environ. 836, 155407. https://doi.org/10.1016/j.scitotenv.2022.155407.
Zeileis, A., Hothorn, T., 2002. Diagnostic Checking in Regression Relationships. R News 2(3), 7--10.
Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the "plastisphere": microbial communities on plastic marine debris. Environ. Sci. Technol. 47(13), 7137-7146. https://doi.org/10.1021/es401288x.
Zhang, C., Chen, X., Wang, J., Tan, L., 2017. Toxic effects of microplastic on marine microalgae Skeletonema costatum: Interactions between microplastic and algae. Environ. Pollut. 220(Pt B), 1282-1288. https://doi.org/10.1016/j.envpol.2016.11.005.
Zhang, C., Jeong, C.B., Lee, J.S., Wang, D., Wang, M., 2019. Transgenerational Proteome Plasticity in Resilience of a Marine Copepod in Response to Environmentally Relevant Concentrations of Microplastics. Environ. Sci. Technol. 53(14), 8426-8436. https://doi.org/10.1021/acs.est.9b02525.
Zhao, T., Tan, L., Huang, W., Wang, J., 2019. The interactions between micro polyvinyl chloride (mPVC) and marine dinoflagellate Karenia mikimotoi: The inhibition of growth, chlorophyll and photosynthetic efficiency. Environ. Pollut. 247, 883-889. https://doi.org/10.1016/j.envpol.2019.01.114.
Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2017. Impact of Microplastic Beads and Fibers on Waterflea (Ceriodaphnia dubia) Survival, Growth, and Reproduction: Implications of Single and Mixture Exposures. Environ. Sci. Technol. 51(22), 13397-13406. https://doi.org/10.1021/acs.est.7b03574.
Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2018. Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. Environ. Pollut. 236, 425-431. https://doi.org/10.1016/j.envpol.2018.01.094.
Zimmermann, L., Bartosova, Z., Braun, K., Oehlmann, J., Völker, C., Wagner, M., 2021. Plastic Products Leach Chemicals That Induce In Vitro Toxicity under Realistic Use Conditions. Environ. Sci. Technol. 55(17), 11814-11823. https://doi.org/10.1021/acs.est.1c01103.
Zimmermann, L., Dierkes, G., Ternes, T.A., Völker, C., Wagner, M., 2019. Benchmarking the in vitro toxicity and chemical composition of plastic consumer products. Environ. Sci. Technol. 53(19), 11467-11477. https://doi.org/10.1021/acs.est.9b02293.
Zimmermann, L., Gottlich, S., Oehlmann, J., Wagner, M., Volker, C., 2020. What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to Daphnia magna. Environ. Pollut. 267, 115392. https://doi.org/10.1016/j.envpol.2020.115392.

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Scientific publications

de Ruijter, V. N., Redondo-Hasselerharm, P. E., Gouin, T., & Koelmans, A. A. (2020). Quality criteria for microplastic effect studies in the context of risk assessment: a critical review. *Environmental Science & Technology*, *54*(19), 11692-11705.

de Ruijter, V. N., Hof, M., Kotorou, P., van Leeuwen, J., van den Heuvel-Greve, M. J., Roessink, I., & Koelmans, A. A. (2023). Microplastic effect tests should use a standard heterogeneous mixture: Multifarious impacts among 16 benthic invertebrate species detected under ecologically relevant test conditions. *Environmental science & technology*, *57*(48), 19430-19441.

de Ruijter, V. N., Xie, X., & Koelmans, A. Microplastics Versus Natural Mineral Particles. How to Create and Test Them While Maintaining Environmental Relevance. *How to Create and Test Them While Maintaining Environmental Relevance*. *Submitted*

de Ruijter, V. N., Milou, A., & Costa, V. (2019). Assessment of microplastics distribution and stratification in the shallow marine sediments of Samos island, Eastern Mediterranean sea, Greece. *Mediterranean Marine Science*, *20*(4), 736-744.

Koelmans, A. A., Redondo-Hasselerharm, P. E., Nor, N. H. M., **de Ruijter**, V. N., Mintenig, S. M., & Kooi, M. (2022). Risk assessment of microplastic particles. *Nature Reviews Materials*, *7*(2), 138-152.

Koelmans, A. A., Belay, B. M. G., Mintenig, S. M., Nor, N. H. M., Redondo-Hasselerharm, P. E., & **de Ruijter**, V. N. (2023). Towards a rational and efficient risk assessment for microplastics. *TrAC Trends in Analytical Chemistry*, 117142.

Thornton Hampton, L. M., Lowman, H., Coffin, S., Darin, E., De Frond, H., Hermabessiere, L., Miller, E., **de Ruijter**, V.N., Faltynkova, A., Kotar, S., Monclús, L., Siddiqui, S., Völker, J., Brander S., Koelmans, A. A., Rochman, Chelsea M., Wagner, M. & Mehinto, A. C. (2022). A living tool for the continued exploration of microplastic toxicity. *Microplastics and Nanoplastics*, *2*(1), 13.

Redondo-Hasselerharm, P. E., **de Ruijter**, V. N., Mintenig, S. M., Verschoor, A., & Koelmans, A. A. (2018). Ingestion and chronic effects of car tire tread particles on freshwater benthic macroinvertebrates. *Environmental science & technology*, *52*(23), 13986-13994.

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born on the 21st of February 1991 in Leiden, The Netherlands

has successfully fulfilled all requirements of the
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Wageningen, 7th of June 2024

SENSE coordinator PhD education

Dr Ir Peter Vermeulen

The SENSE Director

Dr Jampel Dell'Angelo

The SENSE Research School declares that **Vera Nyangoma de Ruijter** as successfully fulfilled all requirements of the educational PhD programme of SENSE with a work load of 39.9 EC, including the following activities:

SENSE PhD Courses

- Environmental research in context (2019)
- Research in context activity: Organising Expert workshop Microplastic effect studies in Seattle (2023)

Other PhD and Advanced MSc Courses

- Design of Experiments – Advanced statistics course, WIAS and PE&RC (2019)
- Searching and Organising Literature, WUR (2019)
- Reviewing scientific manuscript, Wageningen Graduate Schools (2019)
- Tidy data transformation and visualization with R, PE&RC and WIMEK (2020)
- General Toxicology, Postgraduate education in Toxicology (2020)
- Ecotoxicology, Postgraduate education in Toxicology (2021)
- Un-box your PhD process & take charge, Wageningen Graduate Schools (2021)
- Career Perspectives, Wageningen Graduate Schools (2022)

External training at a foreign research institute

- Statistical methods in ecotoxicology using R, SETAC, Dublin, Ireland (2023)
- MP Early career Researcher workshops, online in (2021) and in Athens, Greece (2022)

Management and Didactic Skills Training

- Supervising two BSc students and 6 MSc students with thesis (2019-2023)
- Co-organizing and assisting during practical aquatic ecology and water quality, (2019-2021)
- Supervising two MBO interns and one student assistant (2021-2022)
- Co-organisation of Workshop 'Microplastics in het Milieu. Theorie & Toepassing' (2022)

Selection of Oral Presentations

- *Quality criteria for microplastic effect studies in the context of risk assessment.* MICRO2020 Fates and Impacts of microplastics: Knowledge and responsibilities, 23-27 November 2020, Online
- *Microplastic effect threshold for Aquatic species (METAS).* SETAC North America 42nd Annual meeting, 14-18 November 2021, Online
- *Microplastic Effects Tests Should use a Standard Heterogeneous Mixture: Multifarious Impacts among Sixteen Benthic Invertebrate Species Detected, under Ecologically Relevant Conditions.* SETAC Europe 42nd Annual meeting 2023, 30 April – 4 May 2023, Dublin, Ireland
- *Unveiling the effects of microplastics: QA/QC, effect thresholds and effect mechanisms.* 2nd ICCA Microplastics Advanced Research and Innovation Initiative (MARII) Workshop, 12-14 June 2023, Seattle, USA

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