Chapter 3
Evaluating Microplastic Experimental Design and Exposure Studies in Aquatic Organisms

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Abstract Environmental microplastic particles (MPs) represent a potential threat to many aquatic animals, and experimental exposure studies, when done well, offer a quantitative approach to assess this stress systematically and reliably. While the scientific literature on MP studies in aquatic environments is rapidly growing, there is still much to learn, and this chapter presents a brief overview of some of the successful methods and pitfalls in experimental MP exposure studies. A short overview of some experimental design types and recommendations are also presented. A proper experimental exposure study will yield useful information on MP-organism impacts and must include the following: a comprehensive MP characterization (e.g., density, buoyancy, type, nature, size, shape, concentration, color, degree of weathering/biofilm formation, an assessment of co-contaminant/surfactant toxicity and behavior, an understanding exposure modes, dose and duration, and the type and life stage of the target species). Finally, more conventional experimental

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considerations, such as time, costs, and access to clean water, specialized instrumentation, and use of appropriate controls, replicate, and robust statistical analyses are also vital. This short review is intended as a necessary first step towards standardization of experimental MP exposure protocols so one can more reliably assess the transport and fate of MP in the aquatic environment as well as their potential impacts on aquatic organisms.

3.1 Introduction

Environmental plastic pollution is a ubiquitous phenomenon, affecting even the most remote environments on Earth, such as the Himalayas, the Arctic, and even the deepest marine trenches (Bergmann et al. 2017; Chiba et al. 2018). In addition to visible, macro-sized plastic litter that adversely may affect megafauna, there is another component of aquatic plastic pollution that remains harder to constrain, the microplastic particles (MP) (GESAMP 2015). MP has been conventionally defined as plastic particles less than 5 mm in size (Hidalgo-Ruz et al. 2012) and is either manufactured (primary MP) or the result of fragmentation and weathering of larger plastics (secondary MP). Some of the principal sources of MP in the aquatic environment are from rivers, wastewater treatment plants, atmospheric deposition (e.g., *municipal dust*), and some marine activities such as fishing and shipping (Cole et al. 2011).

It has been reported that more than 200 marine animal species have already been exposed to MP during some phase of their life cycles (Gall and Thompson 2015), either through direct ingestion or by trophic transfer of plastic-laden food (Lusher et al. 2017; Rochman 2015; Au et al. 2017; Auta et al. 2017; Paul-Pont et al. 2018; Botterell et al. 2019; Nelms et al. 2019). While the ubiquitous nature of MP pollution is an obvious potential threat to many aquatic organisms, we still lack a fundamental understanding of its impacts on biological systems (de Sá et al. 2018; Burns and Boxall 2018; Connors et al. 2017; Bucci et al. 2020). Carefully designed experimental exposure studies will enhance our understanding of the effects and underlying mechanisms of MP toxicity towards aquatic organisms. Such information can then guide policy decisions to strengthen and protect coastal and marine ecosystems.

3.2 MP Parameters

To design and conduct a meaningful MP exposure experiment using aquatic animals, the following parameters must be considered: MP type, chemical form, degree of weathering (or not), size, shape, concentration, color, density, presence of additives, sorbed chemical co-contaminants, exposure pathway and duration, target organism, and life stage (Fig. 3.1).

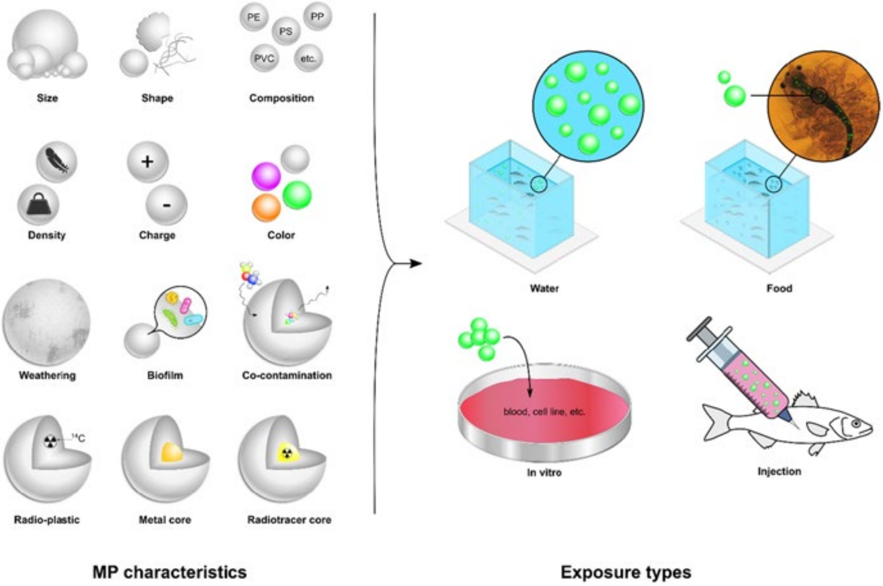

Fig. 3.1 An overview of the characterization of microplastic particles and their potential experimental exposure pathways (food and water) to aquatic organisms

3.2.1 Chemical and Physical Character of MP

In natural aquatic environments, MP are found as complex mixtures with different buoyancies, surface charge, color, composition (e.g., polymer type, presence of adsorbed contaminants and/or chemical additives, presence of biofilm and microorganisms), densities, shapes, and sizes. While some MP characteristics are quite easy to define and control, most require specific considerations. The following section discusses MP characteristics.

There are six plastic polymers that are most widely produced and thus observed in nature: polypropylene (PP), polyethylene (PE) that can occur both as high- and low-density polyethylene (HDPE, LDPE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET), and polystyrene (PS) (Browne et al. 2010; Karapanagioti et al. 2011; Vianello et al. 2013; Isobe et al. 2014; Enders et al. 2015; Frère et al. 2017). Among them, PE, PP, PS, and PET have been found to be the most abundant MP in the marine environment, followed by PVC (Rezania et al. 2018). PS is usually easiest to obtain and thus most widely used in laboratory exposure experiments. For MP fish exposure studies, PE is most utilized, followed by PS and PVC (Phuong et al. 2016; Botterell et al. 2019; Jacob et al. 2020).

MP can also exist in many shapes, such as spheres/beads, pellets, granules, fibers, films, fragments, and foams (Free et al. 2014; Karami 2017). While spheres are most often indicative of a primary MP, fragmentation and weathering will produce secondary MP that can irregularly shape spheres and fibers, films, fragments, and foams (Thompson 2015; Napper and Thompson 2016). Frydkjaer et al. (2017) found that irregular MP fragments were egested at a slower rate than spherical beads in experimental studies using *Daphnia magna*. The shape of a MP is thus an important factor in determining its effects in aquatic organisms (Bucci et al. 2020).

A wide range of MP size classes have been used in experimental exposure studies (Mattsson et al. 2015; Galloway et al. 2017; Ter Halle et al. 2017). According to some studies, the bioavailability and toxicity of MP can be highly size-dependent (Koelmans et al. 2020), with smaller particles generally exhibiting higher toxicity (Betts 2008, Jeong et al. 2016; Wright et al. 2013b; Bucci et al. 2020; Riberio et al. 2019; Wang et al. 2019) due to an increase in bioavailability and potential for translocation across the cell membrane (Browne et al. 2008). Physical blockage in the digestive tract has also been observed with certain MP size classes (Anbumani and Kakkar 2018). Currently, the selection of MP size for exposure experiments is often based on what is commercially available.

MP color can also vary widely, ranging from brightly colored to opaque and clear particles (Shaw and Day 1994; Su et al. 2016; Peters et al. 2017; Wang et al. 2017; Rezania et al. 2018; Zhang et al. 2018). Weathering will fade the original color into a secondary, usually less bright color (Chen et al. 2019). Importantly, the color of some MP may resemble natural food such as phytoplankton, which can affect ingestion rates and/or biological impacts to higher-trophic aquatic organisms (Wright et al. 2013).

The particle surface charge of MP is also an important characteristic that is affected by the ionic strength of natural waters. The shift from freshwater to seawater can dramatically change the aggregation properties and surface charge of particles, including MP. Generally, the physicochemical characterization of MP and its weathering will determine the efficiency of interactions with other particles and/or associated contaminants. The role of the MP surface charge on the toxicity for aquatic organisms is still not well understood (Paul-Pont et al. 2018). However, it has been suggested that the MP charge can play an important role in the transport, fate, and environmental effect of MP in the marine environment (Leslie 2012). The charge and surface properties of MP can play an important role in determining their effects to organisms, primarily due to their interaction with biological membranes (Cole et al. 2013; Rossi et al. 2013).

Polymer density will affect buoyancy and therefore bioavailability to target organisms. For example, high-density particles such as PET quickly sink, increasing bioavailability to benthic dwelling organisms, while pelagic filter/suspension feeders and planktonic feeders will be more readily exposed to low-density MP, such as PE (Wright et al. 2013). Continuous interaction of MP with other marine particles (i.e., ingestion/egestion, adsorption/desorption, aggregation/disaggregation, and biofouling) can also play a role in particle density (Cole et al. 2011, 2016; Kooi et al. 2017; Botterell et al. 2019).

3.2.2 Primary vs. Weathered MP

Primary MP consists of various off-the-shelf polymers such as PP, PE, PVC, PUR, PET, and PS, which are most often not directly released into the aquatic environment. Once natural weathering processes occur (e.g., biofouling, organic coatings, or aggregation of MP with other marine particles), a change in the chemical and physical properties will alter the bioavailability and toxicity (White 2006; Cole et al. 2011, 2016; Kooi et al. 2017; Lambert et al. 2017; Botterell et al. 2019; Chen et al. 2019). MP introduced to natural waters for any length of time will develop an organic biofilm that will drastically impact the fate and behavior of MP and associated co-contaminants. The use of weathered MP in exposure studies more closely reflects the natural environment; thus, it is important to account for these weathering changes during an exposure experiment. It is worth noting that most studies to date typically use primary MP for their exposure experiments (Bråte et al. 2018; Paul-Pont et al. 2018; Botterell et al. 2019; Jacob et al. 2020) or have used experimentally weathered MP (e.g., by immersing plastic particles in water for a few weeks or introducing microorganisms to the MP).

3.2.3 Microplastic Co-contaminants

Microplastics are complex pollutants consisting of polymer blends, residual monomers, plastic additives, and diverse co-contaminants (Rochman 2015). A large number of chemicals and some persistent organic pollutants (POPs) are added to MP during manufacturing to increase polymerization properties and durability, and these can contribute up to 60% (e.g., PVC: Net et al. 2015) of the plastic polymer mass. The additives most commonly used in the manufacturing process are plasticizers, thermal stabilizers, pigments, lubricants, flame retardants, and acid scavengers. It has been reported that chemicals leached from primary MP pellets may cause more deleterious effects than the ingestion of the MP itself (Botterell et al. 2019). However, studies quantifying the effects of plastic additives on organisms are still rare (Browne et al. 2013; Rochman et al. 2013), and desorption processes of plastic-associated chemicals and their effects on aquatic biota including human health remain poorly understood. Expectedly, organisms with longer gut retention times (i.e., some fish) have the potential for increased exposure and therefore for increased toxicity of MP co-contaminants.

Due to their large surface-to-volume ratio and charged hydrophobic surfaces, MP provide an excellent sorption site to scavenge some particle-reactive, dissolved contaminants (e.g., PBTs, PBDEs, DDT, PAHs, and pharmaceuticals), trace metals (e.g., copper, zinc, lead), and other plastic additives (Teuten et al. 2007, 2009; Beckingham and Ghosh 2017; Ribeiro et al. 2019). Consequently, MP can also become a potential, albeit diffuse source for diverse co-contaminants (Koelmans et al. 2013, 2016; Avio et al. 2015; Brennecke et al. 2016; Nakashima et al. 2016;

Alimi et al. 2018). It has been reported that the transport of HOCs (hydrophobic organic compounds) via MP is insignificant compared to their transport via natural particles (Burns and Boxall 2018; Riberio et al. 2019). Frydkjaer et al. (2017) found that C^{14}-labeled phenanthrene (a three-ring PAH used as a tracer molecule) sorbed more to planktonic organisms than to PE MP in laboratory experiments. Moreover, little is known about the effects of these co-contaminants in the smaller size fractions of microplastics (Velzeboer et al. 2014).

As MP exist as a complex mixture of weathered polymers, additives, organic contaminants, and trace metals, it is very difficult to perform laboratory exposure experiments and differentiate the effects of each component (Galloway et al. 2017; Paul-Pont et al. 2018). Thus, there is a need to carefully characterize the sorbed chemicals and plastic additives when exposing organisms to these MP. As many studies are struggling to accurately characterize the MP itself (Costa et al. 2019), proper quantification of plastic-sorbed chemicals prior to and after an experimental exposure study is even more challenging. Analytically it is often difficult to differentiate the toxicological effects of co-contaminants vs. MP, especially at lower, environmentally relevant exposure concentrations.

3.2.4 Application of Labelled Microplastics in Experimental Exposure Studies

Some exposure experiments incorporate labeled MP with either fluorescent or embedded radioisotopes to obtain unique information on transport processes and bioaccumulation kinetics (Cole 2016; Lanctôt et al. 2018). Using fluorescence-labeled MP (i.e., Nile red dye) may enhance imaging (Cole et al. 2016), but one needs to be mindful as MP may also contain an inherent fluorescence which may compromise interpretation. Similarly, stable isotope-labeled MP tracers, using, for example, ^{13}C-labelled MP (Berto et al. 2017), can yield important information on processes such as translocation, cycling, and biological impacts. Gamma- or beta-ray spectrometers are highly sensitive and not readily affected by typical interferences; thus, radiolabeled MP can be accurately quantified, even at trace levels, in complex environmental/biological samples and importantly, even in real time on live target organisms (Lanctôt et al. 2018). Radiolabeled MP can also be used to assess uptake and excretion routes, sorption/desorption kinetics, gut retention time, bioaccumulation, and trophic transfer.

3.3 How to Design a Meaningful Experimental Exposure Study?

Anyone who has worked with MP in controlled exposure studies can attest to the abundant difficulties and challenges. MP introduced to an experimental aquarium will tend to accumulate at the water/air interface and will attach indiscriminately to any surface, including pumps, filters, the exterior of test organisms, and aquaria walls. Thus, MP contact with the target organism must often be facilitated. Experimentalists will almost always have to add a complexing agent/surfactant to the MP to better control the distribution of the MP. The synergistic toxicity of this organic surface-active agent should be carefully evaluated in the context of realistic exposure studies.

An ideal exposure experiment should thus be designed with careful consideration of the physical and chemical properties of MP, the sorbed co-contaminants and additives, as well as the MP concentration, the life cycle of the target organism, and mode and duration of exposure. Environmental parameters such as temperature, salinity, and the pH of ambient aquaria water should be carefully maintained and monitored as these too may have an important effect on the intrinsic chemical properties of the MP. Quantification of MP exposure and retention time, bioaccumulation rates, as well as the concentration of MP are critical for toxicokinetic studies to determine how and where MP is transported in an organism.

3.3.1 Mode of Exposure

Of the four conventional contaminant vectors (food, sediment, water, and parent-to-offspring transfer) commonly traced in experimental exposure studies on aquatic organisms, the two primary pathways of exposure for MP are water and food. For the water pathway, a known concentration of well-characterized MP can be directly introduced into the water column of a controlled aquarium; target organisms can be selected to match the nature of the introduced MP (i.e., bottom- vs. water column-dwelling, life cycle). For the food pathway, target organisms can also be fed prey organisms contaminated with MP so that the target organism ingests the MP with the food (Figs. 3.2 and 3.3). This is a well-proven method to overcome some of the challenges of introducing a toxicant such as MP to living organisms.

3.3.2 Concentration of MP for Exposure Studies

The use of environmentally realistic concentrations of MP in exposure experiments is essential to obtain meaningful information for ecological risk assessments and resource protection (Huvet et al. 2016; Burton 2017; Karami 2017; Nyangoma de

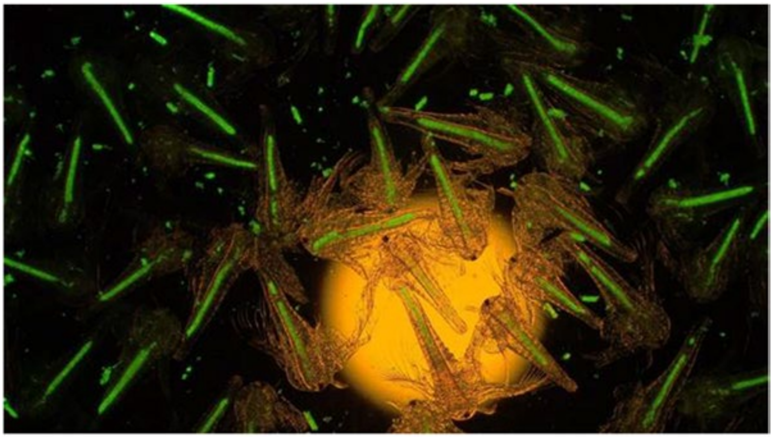

Fig. 3.2 Fluorescent microplastic particles line the stomach of artemia which are used as a microplastic-laden food for experimental exposure studies. (Photo credit: F. Oberhaensli, IAEA, Monaco)

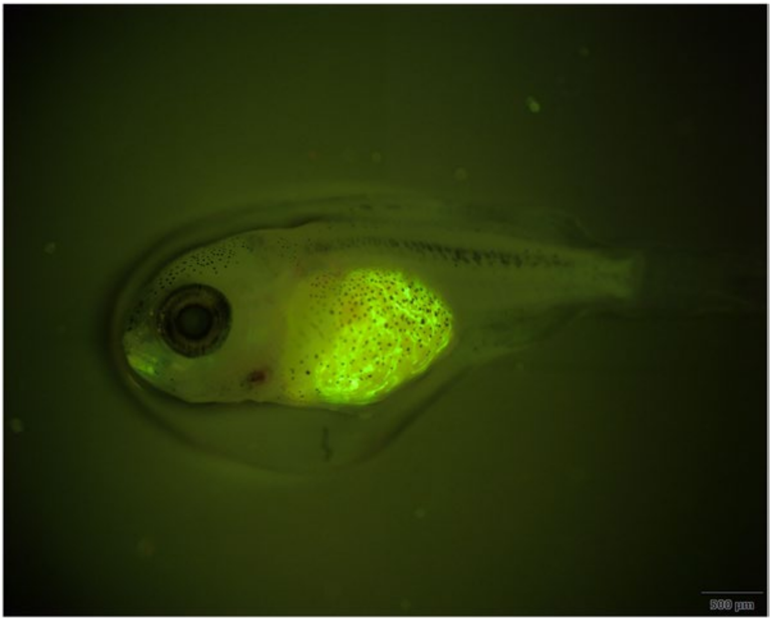

Fig. 3.3 Fluorescent microplastic particles line the stomach of a spiny chromis (*Acanthochromis polyacanthus*) fish. (Photo credit: M Besson, IAEA, Monaco)

Ruijter et al. 2020; Koelmans et al. 2020). Currently, MP concentrations used in laboratory experiments are still often unrealistically elevated (Lenz et al. 2016; Rochman 2016), although we still have a lot to learn about MP abundance in nature (Brandon et al. 2019). Moreover, the deliberate use of elevated concentrations of MP in experiments can be a powerful approach to identify underlying mechanisms and processes that define MP transport and toxicity. The selection of environmentally realistic concentrations of MP for exposure studies is limited mainly by our analytical capabilities (Filella 2015; Lenz et al. 2016; Rochman 2016).

3.3.3 Surfactants

Natural and anthropogenic surfactants are ubiquitous in the aquatic environment, and their inherent toxicity to organisms is generally well-known. Due to the amphiphilic nature of surfactants, the surface tension of the water molecules is decreased which in turn increases the solubility of the HOCs. Surfactants are commonly used in MP exposure experiments to disperse the MP and increase bioavailability. The presence of a surfactant generally increases the formation of homo-agglomerates and promotes adhesion. Indeed, the added presence of a surfactant (MP + surfactant) may increase the toxicity of MP using a surfactant such as Triton X-100 or Tween 20 (Renzi et al. 2019), resulting in higher rates of immobilization. Smaller-sized MP dispersed throughout the water column by surfactants can produce mechanical damage such as impairment of filtration, affecting organism gut residence time, and translocation from the gut into tissues (Cole et al. 2013; Ma et al. 2016; Rehse et al. 2016). Using *Daphnia magna* as a test organism, Renzi et al. (2019) observed the formation of homo-agglomerates of MP, which can adhere to the surfaces of organisms, thereby reducing their motility and increasing energy consumption.

3.3.4 Duration of Exposure

Exposure duration of MP to a target organism is one of the most important parameters that can be easily controlled and one that will directly influence the outcome of an experiment. For example, the residence time and/or retention time of MP within an organism will play a major role in defining its toxicity and will also impact where the MP will eventually reside. The ingestion of MP also depends on the duration of exposure and frequency of feeding which contributes to tissue/organ accumulation and incorporation. Water changes in experimental aquaria must be completed carefully to not remove particles which would change the exposure concentration for the target organisms. Depending upon the duration of the exposure, MP and associated co-contaminants can be leached into the surrounding water column over time with possible additional consequences for aquatic organisms. For example, Pittura et al. (2018) suggested that it might take up to 28 days for a gradual shift in the toxicity of these MP from being mechanical to chemical in nature. Modeling time-series data of chemical toxicity in target organisms can help define acute vs. chronic effects. One of the advantages of using radiolabeled-MP with gamma-emitting radiotracers to study the fate and transport of MP in organisms is that experimental results can be obtained in real time using live target organisms at environmentally relevant concentrations. This permits a real-time assessment of experiment duration to reach an “equilibrium state,” and subsequent experimental adjustments can be made to yield the desired outcome. There are few aquarium-based studies that expose test organisms with various concentrations of MP for both short- and long-term in order to determine both the acute and chronic effects of MP simultaneously (Critchell and Hoogenboom 2018; Wang et al. 2019).

3.4 Recommendations

Based on a literature overview (Table 3.1), there exists a need to better standardize MP exposure experiments to be able to provide meaningful and reproducible results. Working with MP in experimental aquaria is challenging, and one needs to keep track of many physicochemical parameters that will affect the experimental outcome, including the chemical form, shape, size and nature (primary vs. weathered, secondary), and the presence of a biofilm and/or co-contaminants (Burns and Boxall 2018; Bucci et al. 2020). Basic experimental exposure study considerations include the following: (i) at which MP concentrations should the experiment be designed, (ii) what are the reporting units, and (iii) what are the QA/QC parameters? An experiment designed with MP concentrations that are close to environmental levels will yield different information than if the experiments are conducted with elevated MP concentrations. Microplastic concentrations are typically expressed in milligram per liter for most toxicity studies although it may be more accurate to report as the number of particles per liter, since different MP types will have variable size ranges. It is therefore important to count the number of particles using a flow cytometer or other suitable counting methods. Surface charge and density considerations are also essential if the MP is to make proper contact with the selected target species (benthic vs. water column species).

Carefully designed experiments can provide useful insight to better understand MP impacts from cellular to organ, organism, and ecosystem levels. Exposure experiments should incorporate a carefully developed approach that includes physical, chemical, and biological factors that have a strong influence on both the target organism. Furthermore, conducting complementary field and/or laboratory-based studies could better define the scientific lacunae in representative sentinel species in single and combined exposure studies. Such complementary field data may provide useful information to better interpret laboratory-based studies to develop realistic assessments of organismal stress to MP (Anbumani and Kakkar 2018; Wright et al. 2019).

Table 3.1 An overview of priorities and recommendations for experimental exposure studies of microplastics on aquatic organisms

Priorities and recommendations	
Microplastics	Use MP with varying physical and chemical properties
	Evaluate the ecotoxicological effects of MP and associated co-contaminants
	Assess the bioaccumulation pathways of MP and co-contaminants through aquatic food webs.
	Use of primary and/or weathered MP to assess the specific organismal impacts
Target organisms	Use multispecies approach with emphasis on early life stages
	Investigate the impacts of MP on less-studied organisms (e.g., echinoderms, cnidarians, and sponges)
	Examine the link between MP, primary producers, and carbon flow
	Assess biological effects on the community, population, and ecosystems
	Investigate the transfer of MP to higher trophic species
Exposures	Use high concentrations to study MP modes of action, kinetics, and processes
	Assess the scavenging potential of natural particles versus MP
	Investigate potential dose rate or threshold responses by using gradient MP concentrations and experiment durations
	Use environmentally relevant MP concentrations to assess potential ecological impacts
	Study MP ingestion and trophic transfer in fish and compare the use of artificial feed or live food
Methods	Develop specific biomonitoring indicators that can track organismal stress including inflammation, intestinal dysbiosis, neurotoxicity and behavioral change, and metabolic alterations
	Develop and use a best practice guide for MP research
	Assess the impacts of MP on various biological functions, e.g., enzymatic, genetic, histological, reproductive, developmental and physiological functions, as well as immune and stress-related responses, cell signaling, energy homeostasis
	Avoid external contamination with MP of experiments to determine accurate impact by a regular monitoring of experimental conditions
	Study the effects of MP at different levels of biological organization (atomic, molecular, cellular, tissue/organ, individual, community, trans-generational)

Previous studies have generally focused on MP effects on target organisms by treating MP as a single pollutant as opposed to a more realistic mixture of pollutants. There is thus the need to conduct experiments on MP and associated co-contaminant mixtures (Burns and Boxall 2018). Because we still have a lot to learn on proper characterization techniques for MP, special emphasis should be placed on the development and standardization of optimized analytical methods. The application of radiolabeled MP exposure experiments can provide better detection limits even at environmental or trace concentrations and can be an excellent method for elucidating the trophic transfer and movement of MP in live organisms.

The ideal experimental setup should be simple in design and should yield reproducible results using realistic MP concentrations, exposure routes, times, and target organisms. Depending on the specific research question, experimental MP exposure studies may first incorporate a simplified experimental design where one indicator species is exposed to a single type of MP. Subsequent studies may then build on these results and more complex experimental designs will yield more precise information on the organismal effects of MP. While the best laboratory exposure experiments currently address the effects of MP on target organisms under a set of environmental conditions, the next generation studies could address synergistic effects of mixed MP and associated co-contaminants on multiple species. This would be a logical extension of current state-of-the-art exposure experiments and would provide information that more closely resembles a natural aquatic ecosystem.

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