

Potential Artifacts and Control Experiments in Toxicity Tests of Nanoplastic and Microplastic Particles

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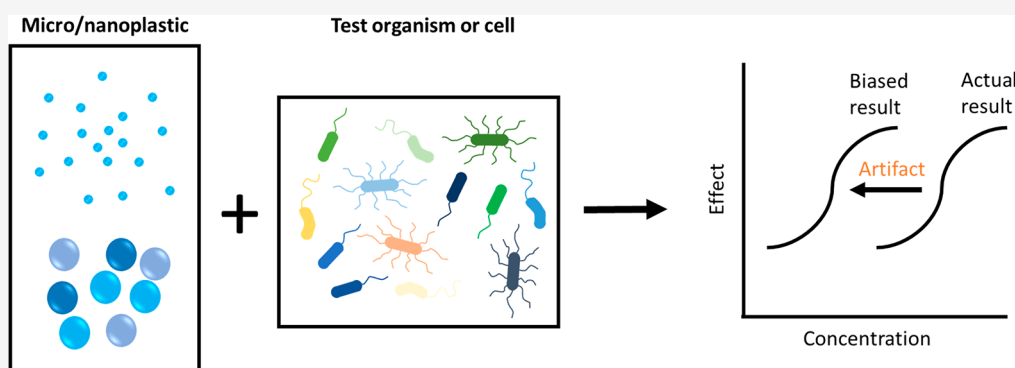


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ABSTRACT: To fully understand the potential ecological and human health risks from nanoplastics and microplastics (NMPs) in the environment, it is critical to make accurate measurements. Similar to past research on the toxicology of engineered nanomaterials, a broad range of measurement artifacts and biases are possible when testing their potential toxicity. For example, antimicrobials and surfactants may be present in commercially available NMP dispersions, and these compounds may account for toxicity observed instead of being caused by exposure to the NMP particles. Therefore, control measurements are needed to assess potential artifacts, and revisions to the protocol may be needed to eliminate or reduce the artifacts. In this paper, we comprehensively review and suggest a next generation of control experiments to identify measurement artifacts and biases that can occur while performing NMP toxicity experiments. This review covers the broad range of potential NMP toxicological experiments, such as *in vitro* studies with a single cell type or complex 3-D tissue constructs, *in vivo* mammalian studies, and ecotoxicity experiments testing pelagic, sediment, and soil organisms. Incorporation of these control experiments can reduce the likelihood of false positive and false negative results and more accurately elucidate the potential ecological and human health risks of NMPs.

KEYWORDS: microplastics, nanoplastics, measurement quality, artifacts, control experiments

INTRODUCTION

There has been increasing research interest in recent years on the potential adverse effects of nanoplastics ($<1\ \mu\text{m}$) and microplastics (between $1\ \mu\text{m}$ and $5\ \text{mm}$) (NMPs) on ecosystems and human health.^{1–13} NMPs are often categorized as primary particles, when a consumer product is designed to contain such particles, or as secondary particles, when the particles are produced by the weathering and degradation of larger pieces of plastic.¹⁴ It is critical to have accurate and reliable measurements to understand potential risks that NMPs pose.^{2,3}

Many standardized toxicity methods are designed to test dissolved substances (e.g., organic chemicals or metals).^{15,16} Guidance on the use of some of these methods has the deliberate aim to remove particles so that the dissolved fraction alone can be tested.^{15,16} As such, modifications to test methods

have been required to evaluate particulate contaminants, such as engineered nanomaterials (ENMs) or NMPs.^{15,17} It is broadly recognized that particulate substances may lead to artifacts in many assays and that control experiments are needed.^{18–22} Moreover, there is also a possibility for misinterpretations if the toxicity is attributed to particulate contaminants, namely NMPs, and control measurements are not performed to determine whether the toxicity is from the particles themselves or substances released from them (i.e.,

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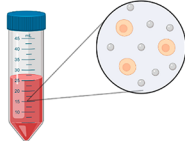


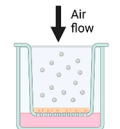
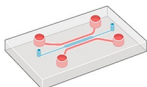

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Table 1. Potential Biases in the Exposure Concentration for Tests with Different Exposure Systems

Category	Graphical example	NMP matrix	Relevant biological test systems	Potential biases	Steps to minimize bias
1		Particles suspended in the test medium	Suspended molecules (e.g., proteins), suspended cells (e.g., algae), or pelagic aquatic organisms (e.g., fish)	The suspended concentration could change based on adsorption of particles to the sidewalls of the container or from agglomeration and sedimentation	Measure the concentration in containers without cells and/or measure the suspended concentration in containers with cells after density gradient centrifugation
2		Particles suspended in the test medium	Submerged, adherent cells at the bottom of microplate wells	Low density polymers may only reach the bottom of the wells at low concentrations according to models that estimated the cell exposure concentration	Adjust the suspended exposure concentration so that a sufficient concentration of particles reaches the cells or perform experiments for longer durations
3		A liquid (e.g., suspension), cream, or solid	Cells or a 3D construct located on an air-liquid interface, <i>in vivo</i> dermal exposure	For liquid exposures, the suspended concentration could change based on adsorption of particles to the sidewalls of the container or from agglomeration	Test the agglomeration and adsorption to the sidewalls using blank inserts without cells; assess the concentration associated with the cells or dermal surface for <i>in vivo</i> exposures
4		Airborne particles from a suspension or a dry powder	Cells or a 3D construct located on an air-liquid interface, <i>in vivo</i> inhalation exposure	The largest particles may not be nebulized/aerosolized or may not get transported throughout the exposure system	Perform control experiments without cells to measure the size distribution and concentration at different steps such as after nebulization/aerosolization, after passage through the exposure system, and onto blank inserts without cells or exposed cells, or into organisms during <i>in vivo</i> exposures
5		Exposure via airborne particles or particles suspended in the test medium	Cells located within an <i>in vitro</i> microphysiological device	Particles may clog the microfluidic channels; particles may adhere to sidewalls of tubing	Perform experiments in the absence of cells and measure the exposure concentration and size distribution that exits the system; perform a mass balance during exposures with cells
6		Particles mixed with soil or sediment	Sediment or terrestrial burrowing organisms	Inhomogeneous mixing of the particles may lead to variable exposure and avoidance of areas with elevated concentrations	If methods are not readily available to quantify the NMP concentration in the test soil or sediment, mixing experiments could be performed with an ENM analogue that is easier to quantify (e.g., gold ENMs of a similar size)

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substances bound to or leached from the NMPs). While these issues have been previously discussed for ENMs,¹⁸ a comprehensive evaluation of the relevant control experiments for NMPs for the broad range of ecological and human health

assays in use is not yet available (although quality criteria have been suggested for ecotoxicity testing²³ and human health related assays²⁴). While these previous reviews motivate screening criteria for the use of data in risk assessment in a

broad sense, this review goes much deeper by focusing solely on the dimension of the control experiments. In addition, many artifacts and biases observed during testing of ENMs have not yet been reported in the NMP literature. Borrowing these lessons learned from previous ENM studies can help raise the quality of future NMP studies.

It is critical to differentiate between operator mistakes, artifacts from testing particles, and uncertainty in experimental measurements. All measurements contain some amount of uncertainty that is often expressed through, for example, standard deviation values or 95% confidence intervals. This is distinct from operator mistakes in the performance of an assay (e.g., pipetting twice the intended volume), and from artifacts that arise from testing particles in assays originally designed to evaluate dissolved substances. For example, if a specific type of NMP has an absorbance signal similar to that being measured in a plate reader to assess a change in cell viability,²⁵ that could be a potential artifact in the assay that, if it is not accounted for, could cause a false positive or a false negative result. In this case, the false positive or false negative result would be an example of a misinterpretation. Misinterpretations can also occur when the mechanism of toxicity is misassigned such as when toxicity caused by impurities present in a mixture is attributed to plastic particles. This could then hinder comparisons among studies using particles that contain different impurities, or could result in misleading perceptions about the risks of plastic toxicity given that different impurities may be present in mixtures tested in laboratory studies versus actual products. Overall, it is critical that protocols include the right control measurements to ensure that operator mistakes and artifacts from the NMPs have been avoided. It should be noted that the magnitude of biases from operator mistakes and artifacts varies among experiments. Some biases (i.e., deviations from the value that would have been obtained in the absence of operator mistakes and artifacts) may be so small that they cannot be separated from typical experimental uncertainty, while others may be so large that they invalidate the measurement and could lead to a misinterpretation.

The aim of this paper is to help raise the quality of NMP research by systematically describing potential artifacts, biases, and misinterpretations that can occur during NMP research, as well as control measurements to identify, and, when possible, strategies to minimize them; no previous reviews have focused specifically on control experiments during NMP testing. While some of these topics are similar to those described for ENMs, there are also many unique issues for NMPs that require different considerations (e.g., the larger size of microplastics can limit ingestion by multicellular organisms). Issues can occur throughout NMP toxicity measurements from sample handling to performing the experiments. One overarching issue that is beyond the scope of this paper is evaluating the environmental relevance of NMPs used in toxicity experiments. There are several recent developments that allow us to better understand the distributions of the properties of environmentally relevant microplastic particles.^{26,27} Aged and weathered particles of various shapes and polymer types can be mixed to approximate these characteristics as much as possible. Nevertheless, this is a complicated topic, especially for nanoplastics, which have rarely been isolated from environmental matrices.²⁸ A consensus has not yet been reached in the field regarding what particles to test, although some suggestions have recently been published for microplastics.²⁹

■ SAMPLE HANDLING—PROCUREMENT, STORAGE, AND DISPERSION PRIOR TO CELL OR ORGANISM EXPOSURE

Depending upon the origin of the NMPs, potentially toxic impurities may be present. For example, antimicrobial compounds may be present for primary NMPs such as polystyrene (PS) spheres.^{30,31} In the absence of antimicrobials, it is possible that biofilms may form on particles especially during long-term storage. This could change the NMPs' surface and their toxicity. The presence of plasticizers is common among a range of plastic particles and may elicit toxicity depending upon the assay and released plasticizer concentration.^{32,33} Heavy metals, such as lead, have also been shown to be released from NMPs and may have been used in the polymerization process.^{34,35} Endotoxins may also become associated with the NMPs during sample preparation, such as the dilution in the test media, or during the production of secondary NMPs.¹⁸ This is especially important when testing for inflammation-related end points.^{18,36,37} It is important to monitor physicochemical changes (e.g., degradation, release of additives) to the NMPs and to the media for those NMPs stored in aqueous media during long-term storage because increased release of toxic compounds could occur.

Most studies conducted on the toxicity of NMPs use PS spheres that may contain surfactants in the formulation or have surface modifications to support their stability. For these particles, dispersion in the test media is relatively straightforward because only dilution is typically required. If sonication is used with these particles or with the secondary NMPs formed from the degradation of larger pieces of plastic, it is important to ensure that this process does not produce unintended changes to the particles (e.g., degradation of the NMPs themselves or of other substances that may be bound to the NMPs or freely available in the media). Extended probe sonication of carbon nanotubes has been shown to substantially degrade them,^{38,39} and a similar effect would likely occur for NMPs. When testing secondary NMPs that contain a heterogeneous mixture of particles, consistently producing dispersions with similar concentrations and size distributions among batches may be challenging. Therefore, additional methodological development may be needed in this area, potentially including the use of natural organic matter to facilitate dispersion.

■ DOSIMETRY ISSUES DURING TOXICITY TESTS

One challenge in summarizing the potential for biases from dosing is the huge variability among model systems, which vary from simple 2-D adherent cell models to microphysiological devices and sediment exposures. Based on the exposure system, there are different potential sources of bias (Table 1). Nevertheless, the potential for adsorption to the sidewalls of test containers,^{40,41} pipettes, or syringes would influence most exposure systems. If this is observed, using other items (e.g., a different kind of test container) can be evaluated to assess if losses decrease. It should be noted that these losses are also often observed in experiments with dissolved substances. In addition, care is required to minimize atmospheric deposition of plastic particles during the experiments, and the appropriate blanks should be used to evaluate this possibility.^{23,42,43}

For exposure systems where exposure occurs to organisms with suspended particles in the test media (e.g., algae, fish), potential sources of bias include adsorption to sidewalls of the

Table 2. Summary of Potential Control Experiments to Identify Assay Artifacts^a

Potential control experiments	Method to perform control experiment	Purpose(s)	Examples of relevant <i>in vitro</i> assays	Test modifications to avoid or minimize artifacts
Bioavailability control ⁵⁸	Perform the assay and then investigate the distribution of the NMPs in the cells	Some cell measurements require that the substance can enter the cells prior to causing toxic effects. If the NMPs do not enter the cells, there may be a false negative result	Ames assay	Change to a different test method
Cell-free control ^{25,64}	Add the NMPs only to the test media and perform the analytical method, or add the NMPs with the assay's reagents and then perform the assay	Assess if NMPs themselves, in the absence of cells, produce or inhibit a signal (e.g., absorbance, fluorescence) or interact with assay reagents in a way that could produce or inhibit the production of a signal similar to the assay measurement. This will identify interferences and potential false positive or false negative results	All absorbance and fluorescence-based assays; DCFDA assay	Change probe (e.g., MTS to MTT) or switch to a similar assay (e.g., LDH release instead of MTS); add control measurements to quantify amount of interference; add steps to remove particles after exposure period
Filtrate only control ^{68,137}	Filter the NMP suspension and then perform assay with the filtrate	Assess potential toxicity of additives, contaminants, and leachates	All assays	Dialyze particles or wash them with alcohol to remove dissolved substances
Heteroagglomeration control ^{30,68}	Incubate the NMP suspension with cells and observe if heteroagglomeration occurs	Evaluate if heteroagglomeration could occur between cells designed as a food source for larger organisms and the NMPs. This could lead to indirect toxicity from starvation	All assays with cells as a food source	When cells are used as a food source, it may be possible to add the cells and particles at different times
Fluorescent dye control ^{104–106}	Quantify if dye has been leached from a fluorescently labeled NMP during a bioaccumulation experiment, and assess the bioaccumulation of the freely available dye in separate experiments	Assess if freely available fluorescent dye has been released from the NMP and could confound bioaccumulation experiments	All bioaccumulation experiments	Dialyze the particles prior to the experiment, change the analytical technique, use a technique that can differentiate between freely available dye versus dye bound to a particle
Nutrient depletion control ^{138,139}	Incubate NMPs with assay medium for the duration of the assay; remove NMPs such as by using filtration, and perform assay with the medium	Assess the extent to which adsorption of media constituents by NMPs could have an indirect toxicity effect on end points	All assays with nutrients in the test media	Increase the nutrient concentration to counteract the loss of nutrients from sorption to the particles
Rinsing control ^{111–113}	Add the NMPs to the test species and then immediately perform the rinsing procedure	Simple rinsing procedures may be insufficient to separate NMPs from small organisms or cells	Bioaccumulation experiments with <i>C. elegans</i> or algae	Perform a density gradient centrifugation
Shading control ^{86–88}	Quantify the amount of decreased light transmission in wells with algae and NMPs, or use a special testing set up that has the light pass through wells with only NMPs and then to the wells with only algae	Assess if the toxicity mechanism is from decreased light transmission due to shading	Algae toxicity experiments	Modifications are not needed since only the interpretation of the toxicity mechanism is changed
Positive spiked control (inhibition/enhancement control) ^{20,22,91}	Perform the assay exposure period with the positive control. Then, add the NMPs to the positive control wells and perform subsequent analysis steps	Assess if the presence of NMPs may inhibit/enhance the signal of cells that would otherwise have a positive response in the assay	Flow cytometry assays, absorbance and fluorescence assays	Perform steps to remove particles after the exposure period
Vector effect control ¹¹⁷	Test the bioaccumulation of a contaminant in the absence of the NMP	By comparing the bioaccumulation of a contaminant in the presence and absence of the NMP, it is possible to assess if there is a vector effect	All co-contaminant bioaccumulation studies	If biomarkers are also evaluated, it may be helpful to include a NMP only control; it may also be important to confirm uptake of the NMP
Zero-hour control ^{64,92,93}	Add the NMPs at a certain step of the assay and then immediately perform the remainder of the assay without modification; this differs from the typical approach in that there is no exposure period after the NMPs are added	Test if NMPs: Cause a toxicological effect (e.g., DNA damage) during processing steps after conclusion of exposure period by evaluating if effects could be observed during the processing steps after the assay is finished Would interact with test reagents or biomolecules and cause a false negative or false positive result May cause a change in the cell stability for suspended cells through heteroagglomeration	Comet assay, DCFDA stress assay	Perform steps to remove particles after the exposure period

This table has been modified and edited with permission from ref 140. ^aAbbreviation: DCFDA, 2',7'-dichlorodihydrofluorescein diacetate.

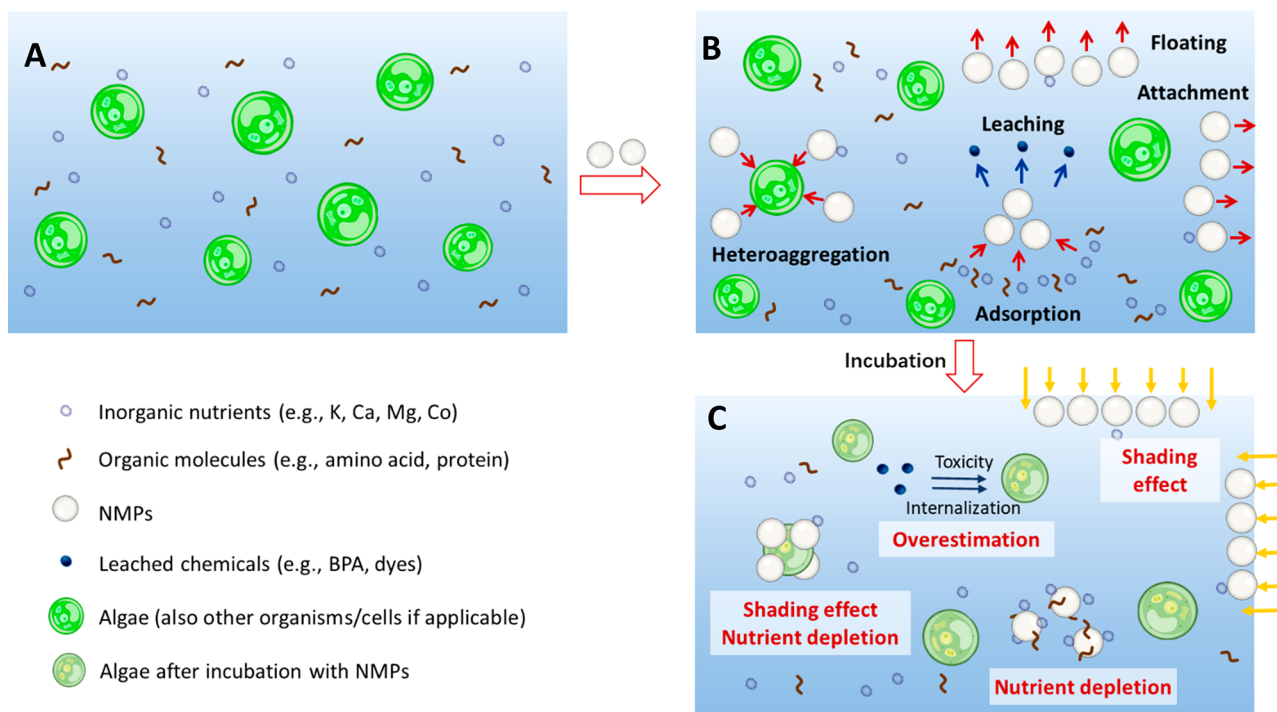


Figure 1. Schematic illustration on indirect interferences/misinterpretations caused by adsorption, leaching/desorption, and attachment processes of NMPs. Initially, all of the particles, cells, and molecules are evenly dispersed (A). Then, processes, such as adsorption, occur that influence the distribution of the different elements (B). This could lead to artifacts and biases (C).

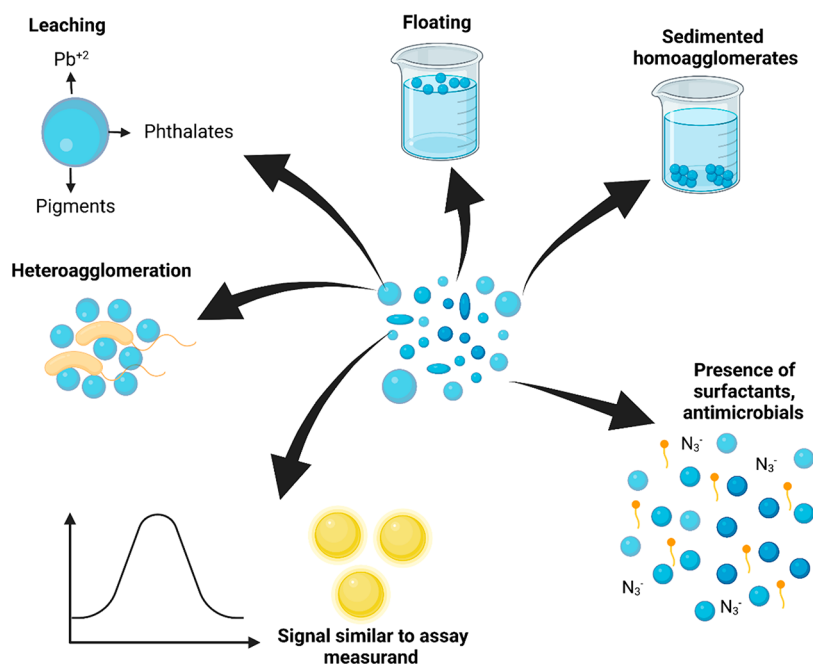


Figure 2. Illustration of different potential artifacts during NMP testing. The NMPs are shown to span a range of sizes and shapes as would be expected in the environment. Created with [Biorender.com](https://biorender.com).

container,⁴⁴ flotation on the air–water interface,^{45,46} and sedimentation of the particles out of the suspension after homo- or heteroagglomeration.^{47,48} Sedimentation can potentially be accelerated in the presence of test organisms by agglomeration during passage through the organism or by attachment to suspended cells (e.g., algae, bacteria).^{40,49,50} For other types of exposure systems, the size of the particles can impact function of the exposure test method. For example,

microplastic particles could potentially clog the channels in microphysiological devices, which often have cross-channels smaller than 1 mm, or flow-through systems with aerosolized particles.⁵¹ In addition, some types of plastic particles (e.g., poly(vinyl alcohol)) can form an amorphous mass after dissolution, a process that can hinder making a uniform concentration in many test systems.⁵² The density of the plastic particles can also impact exposure for some test

systems: for example, for less dense particles or particle agglomerates, only a minimal concentration (6–19% in a study with 60 nm PS spheres)²⁵ may reach 2-D adherent cells at the bottom of the wells in a microplate. This can be estimated using dosimetry models, such as the *in vitro* sedimentation, diffusion, and dosimetry model (ISDD).^{53,54} Some plastic particles have a sufficiently low density that they are buoyant in many test media (e.g., expanded PS floats) and this can impact the ability to generate homogeneous exposure preparations for ecotoxicity testing (to some extent mitigated by extensive stirring). This would impact most exposure systems, except for soil or sediment exposures. One challenge in exposures with soils and sediments is that extraction and quantification methods are still under development.^{55,56} This can hinder evaluating the uniformity of the sample throughout the test container after mixing and ensuring that the test concentration remains constant during the exposure period.

POTENTIAL ARTIFACTS AND CONTROL EXPERIMENTS DURING TOXICITY TESTS

Potential control experiments for NMP toxicity studies are listed in Table 2 including their purpose, their methodology, and strategies to minimize artifacts or biases. It should be noted though that some of the issues being tested in these experiments (e.g., nutrient depletion, shading) could occur at environmental hot spots with elevated plastic concentrations or for specific organisms (e.g., corals). While some of these control experiments are similar to those for ENMs, there are also many important nuances that are specific to NMP toxicity testing. In any aqueous test media, NMPs will undergo adsorption, leaching/desorption, and attachment processes (Figure 1). All these processes could lead to unexpected artifacts and misinterpretations in toxicity assessments if they are not fully understood (Figure 1C). A graphical depiction of selected artifacts and biases is provided in Figure 2. In this section, a set of proposed controls are discussed below to elucidate their importance in toxicity testing to identify potential artifacts.

Bioavailability Control. Substance bioavailability, defined as the extent to which the substance enters a tissue and reacts with biological molecules,⁵⁷ is an important aspect of toxicological tests with NMPs. Some assays require that the toxicants are able to reach a particular location (e.g., the nucleus) to assess certain end points. For example, measuring genotoxicity using the Ames test requires that a particle can travel across the bacterial cell membrane and interact with the nucleus. However, this is not guaranteed to occur and was not observed in studies on ENMs.⁵⁸ In these cases, there is the potential for false negative results. A similar issue is relevant for studies with multicellular organisms. It is important for the NMPs to be distributed to the organ of interest (e.g., the brain)⁵⁹ to cause a toxicological effect there, unless there is a reasonable alternative mechanistic explanation. Given that there is uncertainty in the ability for NMPs to cross epithelial barriers for some organisms as is discussed in more detail in the Potential Artifacts and Control Experiments during Bioaccumulation Tests section, measurements need to be made to confirm that the NMPs can be transported to the tissue of interest. Moreover, the large size of some microplastics (up to 1 mm) limits their ingestion by many species (maximum ingestible size for selected species ranges from 36 to 400 μm).⁶⁰ Currently, it is not possible to advocate for a single analytical method to be used for this purpose, because

the maturity of quantitation methods varies substantially between microplastic and nanoplastic methods, with microplastic methods being substantially more advanced.^{61,62} Nonetheless, NMPs can potentially induce negative effects in tissues independent of whether they are ingested or internalized, for example if the NMPs accumulate on and occlude tissue surfaces and impact photosynthesis.⁶³

Cell-Free Control. This control measurement is relevant for cell-based (e.g., mammalian, bacteria, algae) toxicity experiments. Many cell-based toxicity experiments assess changes to the cells using probes with absorbance or fluorescence at particular wavelengths. However, the NMPs tested may also have a signal at the same wavelengths and therefore could bias the results if they are present when the measurement is performed.^{25,64} Washing steps can be used to remove particles present prior to the measurement,⁶⁵ but the removal process needs to be verified by control measurements that measure the NMP concentration. For some types of measurements (e.g., flow cytometry or Coulter counter), microplastic particles or heteroagglomerates could be misinterpreted as cells, both of which could bias the results.⁴⁰ The potential for a technique to distinguish between particles and cells can also be evaluated by the zero-hour control as described in the Zero-Hour Control section.

Filtrate Control. One control experiment that is relevant across all toxicity tests is the filtrate control (Table 2). Commercially available NMPs (especially nanoplastics) are commonly presuspended in water in suspensions that also contain additional potentially toxic compounds such as preservatives, antimicrobials, or surfactants.^{30–33} For example, sodium azide, added as an antimicrobial preservative in PS NMPs suspensions, was much more toxic to *Daphnia magna* than the particles themselves.³⁰ After dialysis, sodium azide molecules both in the suspension and on NMPs' surface could be removed which resulted in highly reduced toxicity (100% mortality was reduced to nondetectable mortality at 100 mg/L after dialysis).³⁰ Therefore, the toxicity of commercially available NMPs could be overestimated if the contribution of sodium azide was not excluded.

It is also possible for additives and monomers to be released from NMPs during experiments (Figure 1B, C).^{34,35,66,67} For example, both low-density polyethylene (PE) and polycarbonate (PC) NMPs can release bisphenol A (BPA), a commonly used additive. A BPA concentration of 14.68 $\mu\text{g/g}$ leached from PC NMPs after shaking for 3 d in water,⁶⁶ it should be noted that toxic effects from released additives are important for laboratory studies but are less relevant for field conditions where organisms will mainly be exposed to NMPs that have been in the environment for long periods of time. Poly(vinyl chloride) (PVC) and PE NMPs have been observed to release hazardous compounds, such as heavy metal stabilizers and pigments, from the polymeric matrix that contributed to the NMP toxicity to sea urchin (*Paracentrotus lividus*) embryos,³⁵ algal cells (*Microcystis aeruginosa*),⁶⁷ and zebrafish.³⁴

This control functions by testing the potential toxicity of other substances that may be present in a NMP suspension (e.g., additives, leachates, biocides, surfactants), in addition to the particles themselves. The filtrate control should be prepared shortly before the toxicity experiment so that the dissolved substances mirror those in the NMP suspension. If leaching during an experiment is a concern, it would also be possible to obtain and test the filtrate from the NMP

suspension after incubation in test media in the absence of organisms for the duration of the toxicity experiment. Washing NMPs in an organic solvent or dialysis can be used to remove leached impurities.

Heteroagglomeration Control. Similar to ENMs, it is possible for NMPs to heteroagglomerate with suspended cells especially for nanoplastics and smaller microplastics; the situation differs for larger microplastics where cells could adsorb onto them. This could complicate an ecotoxicity assay if heteroagglomeration of NMPs occurs with the food source for another organism. For example, a recent study found that positively charged PS nanoplastics could heteroagglomerate with *Escherichia coli*, the food source for *Caenorhabditis elegans*.^{50,68} This, then, resulted in the formation of particles much larger than 2 μm , the typical size of food consumed by *C. elegans* using pharyngeal pumping. The growth and reproduction inhibition observed was hypothesized to stem from indirect toxicity due to starvation instead of direct toxicity from exposure to the particles. Therefore, it is possible that NMPs under specific conditions may interact with the food source in other assays such as algae feeding of *D. magna* during chronic experiments and limit food intake. This could also likely occur in nature.

To test for potential heteroagglomeration of NMPs and suspended cells intended as a food source, it is possible to perform the experiment without the test organism and microscopically evaluate if larger heteroagglomerates are formed.⁵⁰ Hyperspectral darkfield imaging could be used to probe the heteroagglomerates to assess if they contain cells and NMPs.⁵⁰ If larger heteroagglomerates are observed when NMPs are present, the agglomerate size should be compared to the maximum size that can be ingested by the test organism.

Nutrient Depletion Control. During toxicological tests, essential nutrients are supplemented in the culture media of animal cells, algae, bacteria, protozoa, and plants. These essential nutrients include organic (e.g., proteins, amino acids, vitamins) and inorganic components (e.g., N, P, Ca, Fe) in different culture systems. Engineered nanomaterials have been found to adsorb these nutrients, thus reducing available nutrients for the growth of the tested organisms, such as algae,⁶⁹ plants,⁷⁰ and cells.⁷¹ NMPs (especially, nanoplastics) have comparable surface areas and adsorption capacities with ENMs. However, to date, adsorption-induced nutrient depletion by NMPs has not been directly evaluated in toxicological tests to our knowledge. Conversely, it is also possible for ingestion of microplastics to enhance growth if there are unintentional bacteria associated with microplastic particles that the test organism could use as a food source,⁷² although it is more common to observe food dilution in NMP studies.²³

NMPs have been reported to accumulate nutrients (e.g., N, P) in the sediments from river waters due to adsorption.⁷³ Such nutrient accumulation in sediments may result in nutrient depletion from the decreased concentration of N and P in the aqueous phase if the NMP concentration is sufficiently high (Figure 1C). The presence of PS and polytetrafluoroethylene (PTFE) NMPs at concentrations of 0.25% and 0.5% (mass/mass) reduced the contents of available N and P in the soil for rice growth.⁷⁴ This was attributed to the activity inhibition of soil enzymes (e.g., urease), but this could also be from adsorption-induced depletion. In addition, the potential for NMPs to change the soil environment and thereby impact nutrient availability to onions was hypothesized.⁷⁵ It is worth

noting that NMPs are able to adsorb other nutrients, such as Fe, B, Ca, and amino acids in aqueous phase^{74,76} which could lead to nutrient depletion in some media.

In addition to inorganic nutrients, organic nutrient deficiency could also lead to growth inhibition of test organisms. For example, vitamin B12, an essential nutrient for algal growth,^{77,78} is present in freshwater (e.g., HUT medium) and marine algae (F/2 medium) test media at 0.5 $\mu\text{g/L}$. The strong adsorption capacity of vitamin B12, as high as 1700 mg/g onto poly(vinylidene fluoride) membranes,⁷⁸ suggests that nutrient depletion could occur. Proteins such as fetal bovine serum (FBS) are another important component of some mammalian cell media. Recent studies^{79,80} suggested a strong interaction between proteins and NMPs including a multilayer adsorption pattern. Engineered nanomaterials, such as CeO_2 and TiO_2 , were reported to reduce the viability of human cells (HaCaT and A549 cells) due to the depletion of serum proteins in cell culture media.⁸¹ NMPs, especially nanoplastics, have comparable surface area, stronger hydrophobic interaction, and unique π - π interaction with proteins in comparison with CeO_2 and TiO_2 ENMs. Therefore, protein depletion by NMPs is expected to occur and should be assessed during *in vitro* assays.

To understand the potential for nutrient depletion, adsorption experiments are recommended by adding the same mass concentration of NMPs as in the toxicity experiments to the culture medium and incubating for the duration of the toxicity test.⁶⁹ Then, the NMPs should be removed. A comparison should be made between this supernatant and the regular test media in the ability of each to support the growth of the test organisms (e.g., algae, bacteria, cells, plants). The difference between these two treatments can reveal the degree to which nutrient depletion has occurred. It is important to also note that adsorption of proteins can result in a protein corona that could change the potential toxicological impact of the NMPs.^{82,83}

Heteroagglomeration could also lead to nutrient depletion, for example, by blocking algal pores, thereby inhibiting gas exchange.⁸⁴ Furthermore, heteroagglomeration between NMPs and algae could inhibit biosorption of critical nutrients (e.g., P, N). NMPs-algae heteroagglomeration was reported to accelerate the sedimentation of both NMPs and algae from the water phase to sediment,^{40,49} further enhancing nutrient depletion by constraining algae in a limited space.

In experiments where cells serve as the food source for larger organisms, adsorption of cells onto larger microplastics may not have an impact if the particles can be readily ingested, but could reduce the quantity of the food source if the particles are too large to be ingested. Control experiments with the cells but without test organism can be performed to assess if the suspended cell concentration is significantly reduced by larger microplastics and the size of the particle with adsorbed cells.

Shading Effect Control. Floating NMPs and those attached onto the test container could block the transmission of light, causing shading effects to some test organisms such as algae (Figure 1C). For example, strong attachment of PS NMPs onto the inner wall of flasks was observed while exposing marine algae.⁴⁰ Such attachment could also cause a reduction of the NMP exposure concentration in addition to shading effects (Figure 1C). However, this possibility is rarely evaluated in toxicity mechanism discussions of NMPs.

NMPs can also exert shading effects by directly attaching onto algal cells. This toxicity mechanism has been confirmed

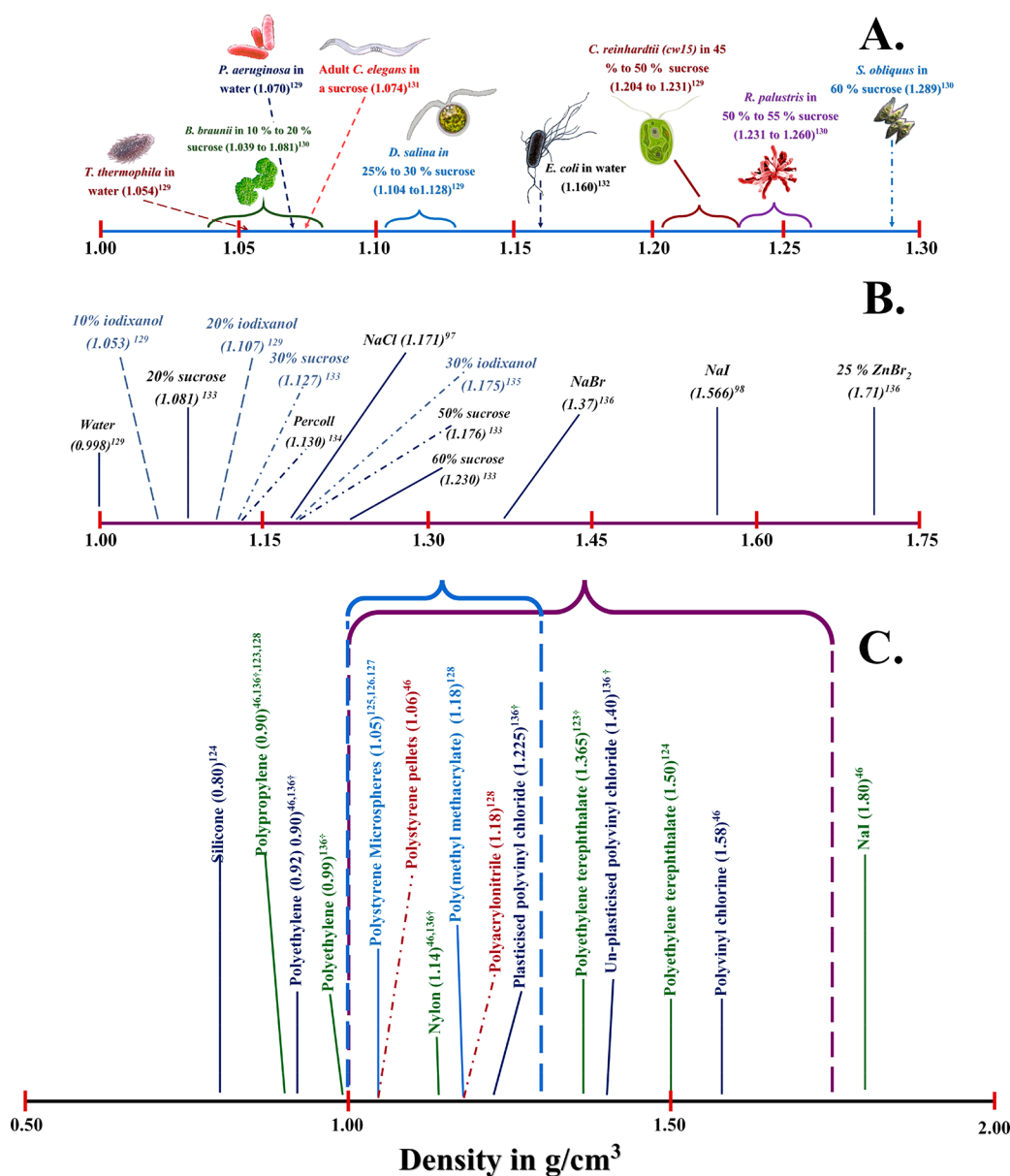


Figure 3. A comparison of the densities among model biological organisms widely used in toxicity studies (A), commonly employed density gradient centrifugation media (B), and microplastic precursors (C). The ‘†’ symbol represents references that provided approximate density ranges for the given density media or NMP materials. Note that the reported density of the biological organisms, density gradient media, and microplastic precursor all fall within the same relative range. Therefore, density gradient separations can be a useful tool. Considerations can be made to select the appropriate media combinations (and centrifugation parameters) that will facilitate successful separation of biological organisms and NMPs in bioaccumulation studies. This figure has been adapted and reprinted with permission from ref 99. Copyright 2019 Royal Society of Chemistry.

for NMPs-induced growth inhibition of algae.⁷⁷ For example, PS NMPs (0.1 to 2 μ m) attached and encapsulated microalgae cells (*Scenedesmus obliquus*), thus blocking light transmission and inhibiting photosynthesis.⁸⁵

Relevant control experiments can be performed to assess the potential for shading effects.^{86–88} The amount of light reduction in particle-only containers can be quantified and then how much this decreased light transmittance would reduce algal growth can be evaluated.^{86,87} Alternatively, it is possible to use a special set up that passes the light first through chambers with the particles but without algae and then to chambers with only algae.^{88,89} These results can be compared to algae directly exposed to particles and to negative control algae without particle exposure.

Positive Spiked Control. The positive spiked control differs from the positive control (Table 2). The positive control tests a substance known to elicit the toxicological effect being tested.⁹⁰ In contrast, the function of the positive spiked control is to evaluate if substances added to positive control samples can inhibit or increase its signal. This control measurement is similar to the zero-hour control measurement (described in the following section). The key difference is that positive control samples (i.e., samples exposed to a positive control thereby exhibiting the toxicological effect under investigation) are used instead of negative control samples (i.e., cells not exposed to any toxicant). This type of control experiment was performed on a study on cell apoptosis and necrosis measurements using flow cytometry with positive control cells spiked with gold and silica ENMs and found that

the presence of ENMs impacted the signal observed.⁹¹ This control could apply to both microplastics and nanoplastics. If, for example, an ELISA study was performed, it is possible that either microplastics or nanoplastics could adsorb excreted cytokines and decrease the effect being studied.

Zero-Hour Control. This control functions similar to the positive spiked control. However, instead of adding substances to positive control samples, they are added to negative control cells and then subsequent processing steps are performed without an exposure period (Table 2).^{64,92,93} The zero-hour control experiments evaluate the extent to which toxicity can be induced after the exposure period concludes. For example, in a genotoxicity assay, particles could interact with cellular DNA thereby changing DNA migration during the gel electrophoresis step of the Comet assay and potentially appearing in the head of the comet.^{18,94} Additionally, particles could induce DNA damage after the exposure period during the subsequent sample processing steps.^{92,93}

It is also possible for NMPs to heteroagglomerate with suspended cells and interfere with cell number measurements (e.g., using flow cytometry or a hemocytometer). This could occur during *in vitro* experiments with suspended cells or ecotoxicity experiments using single-celled organisms, such as algae or bacteria. These artifacts are well-known in the ENM literature and have been observed, for example, for algae after exposure to TiO₂ particles.⁹⁵ While smaller microplastics would be more likely to heteroagglomerate, larger microplastic particles could adsorb cells, which could also significantly interfere with cell number measurements.

■ POTENTIAL ARTIFACTS AND CONTROL EXPERIMENTS DURING BIOACCUMULATION TESTS

One common end point to assess in NMP studies is bioaccumulation,^{96–98} the capacity of organisms to accumulate plastic particles over time. An organism can accumulate particles by their association with external tissue surfaces (e.g., integument and respiratory tissue surfaces), accumulation on external epithelial surfaces within the lumen of the gastrointestinal tract (for the fraction that is ingestible), and also after absorption across epithelial membranes and accumulation within internal tissues/organs.⁹⁹ One key question in these studies is whether the particles have the capacity to travel across epithelial surfaces. This could influence the potential toxicological mechanisms that the particles could cause such as impacts in the organisms' digestive tissue versus those in other tissues.

In an analysis of field studies of microplastics, it was estimated that >99% of the plastic particles were observed to be in the gut tract, suggesting a lack of absorption.^{96,100} However, different results have been observed in laboratory studies with fluorescently labeled plastic particles, suggesting systemic absorption into different tissues.^{101–103} With the fluorescent labeling approach, it is critical to differentiate between the accumulation of fluorescent probes detached from the plastic particles and those that remain attached. If this is not conclusively evaluated, it is possible for artifacts to occur, as has been observed in recent studies with fish and *D. magna*.^{104–106} Control experiments can include dialyzing the particles to remove freely available molecules, testing the stability of the fluorescent particles in various media prior to performing the experiment, and testing the bioaccumulation of the probe molecule by itself. Confidence in bioaccumulation

results can be strengthened when an orthogonal technique (e.g., pyrolysis gas chromatography–mass spectrometry⁵⁵ or metal labeled particles^{10,107}) is used to confirm the results.

An additional consideration for bioaccumulation studies with smaller organisms is to ensure that they can be separated from individual and agglomerated NMPs prior to quantifying the associated NMP concentration.⁹⁹ While simple solvent rinses followed by filtration may be sufficient for removal of particles specifically adsorbed to the surface of organisms,^{108–110} they may not be sufficient to separate the test organism from suspended NMPs. In contrast, density gradient separations have been shown to separate small organisms from particles still suspended in media or even adsorbed to cuticle surfaces (Figure 3).^{111–113} Density separations have not yet been reported for the determination of uptake of NMPs by small organisms, but given the efficiency the technique has shown when employed in ENM uptake studies^{111–113} and the fact that most laboratories may have access to the tools needed to perform the separations, this approach should be evaluated if control measurements (e.g., zero-hour control) indicate an incomplete separation of the test organism and suspended ENMs.

■ ARTIFACTS AND CONTROL EXPERIMENTS DURING CO-CONTAMINANT EXPERIMENTS

Plastic particles are typically associated with chemicals, either from the manufacturer (intentionally added chemicals, i.e., additives) or by absorption of chemicals from the ambient environment. These chemicals often are referred to as plastic-associated chemicals (PACs).^{114,115} The presence of PACs has raised concerns that transport followed by ingestion of microplastics would lead to additional bioaccumulation, exposure, and risks compared to a scenario without any plastic particles present (i.e., “zero microplastic” scenario).^{114–117} Here, the aspect of additional uptake caused by the presence of NMPs, the ‘microplastic vector effect,’ is crucial, because all PACs are ubiquitous in all environmental media at background levels. Whether a “microplastic vector effect” occurs under natural conditions depends on the relative share of exposure via plastic compared to other pathways, and on whether the fugacity gradient for transfer favors desorption from plastic particles.^{114,117–119}

There are several reviews that summarize the weight of the evidence for the vector effect to occur, generally concluding that the evidence base is thin.^{114,117,119,120} This is attributed to misinterpretations typically encountered when results from plastic vector studies are put into an environmental context. First, parallel chemical uptake pathways are not considered, thereby artificially increasing the supposed relevance of the pathway occurring via microplastic ingestion.¹¹⁷

Second, transfer of PAC from microplastics to the organism is studied at the maximum possible concentration gradient, i.e., with clean organisms. Such a situation conflicts with conditions in nature where the gradients for MPs are small or nonexistent.¹¹⁷ In aquatic systems, the time scales of adsorption and desorption of PAC between microplastic particles and water are determined by particle concentration, particle size, the thickness of the stationary boundary layer surrounding the particles, the diffusion rate in that layer, the intrapolymer diffusion coefficient of the PAC, the PAC partition coefficient of plastic to water, the concentration of dissolved organic matter (DOM) and the PAC partition coefficient of DOM to water. The role of these traditional

factors is well understood in environmental chemistry and several studies have shown, based on empirical and validated model simulations, that PAC equilibrium times range from hours to months at most.^{114,121} Given the residence time of NMPs in water systems, this means that equal fugacity (i.e., chemical equilibrium) usually is the standard state for PAC residing in NMP, especially in the oceans. This also applies to noncovalently bound additives that can contain tens of percent of the weight of the plastic particles. Nevertheless, numerous studies explore the artifactual nonequilibrium scenario, whereas the number of studies addressing the opposite case is small, which seems to reflect confirmation bias in the literature.

Third, studies exist that report data of PAC desorption (extraction or migration) from high concentrations of plastics to relatively small volumes of water or cell media (e.g.,^{32,33}). These experimental conditions will easily lead to concentrations that cause responses in, for example, *in vitro* toxicity tests. Often, such studies implicitly suggest that PAC are toxic under environmentally relevant conditions. In reality, however, such migration of PAC would not lead to exceedance of chemical effect threshold concentrations. This is because the concentrations of microplastics in the environment are much lower than those used in these tests and dilution would occur, making such studies confusing.

Fourth and finally, when dealing with the implications of the MP vector effect, the scenario of absorption by NMPs of nonadditives (i.e., reducing the chemical concentration) should be given equal weight to that of leaching of additive chemicals, something which is however usually overlooked. For example, recent reviews postulate biomagnification and gut leaching simultaneously as accumulation pathways,¹²² without considering that biomagnification can increase the fugacity in an organisms' lipids such that gut leaching would not occur, or even could be reversed. Incidentally, the distinction between additives and nonadditives ('sorbed chemicals') is often artificial because many of the same chemicals belong to both categories. Recently, a practical tool has been developed that allows for the accurate simulation of the microplastic vector effect in a food web under all possible hydrophobic organic contaminant exposure scenarios, including "overequilibrium" (e.g., additives) as well as "under-equilibrium" (sorbed chemicals).¹¹⁸ This shows that it is feasible to translate observations from co-contaminant experiments to field conditions by numerically correcting all of the above potential artifacts. In summary, co-contaminant or "vector studies" can be done in many ways, but their results should be put in an environmental context, that is, as long as they are meant to be environmentally relevant.¹¹⁷

Implications. Making robust measurements of the potential toxicity of NMPs and PACs is critical for understanding their risks. While there are a broad range of potential artifacts that can impact toxicity assays evaluating NMPs, the extensive literature on toxicity testing of ENMs provides insights into some shared potential artifacts that are relevant for particle toxicity testing in general. In addition, there are many unique issues for NMP testing that differ from those relevant for ENMs that are also described here in depth (e.g., the potential for some plastic particles to float, and the potential for single-celled organisms to be absorbed onto larger microplastic particles). There is no clear distinction between nanoplastics and smaller microplastics with regards to the potential artifacts and control experiments that are needed. Nevertheless, there are differences with larger microplastics,

which are more likely to have distinct size-related differences such as adsorbing cells rather than forming heteroagglomerates, limited transport through an exposure system, and being too large for ingestion by organisms.

The control experiments described in this paper should be incorporated into future studies to help raise the quality of the published literature, avoid false positive and false negative results, and accurately determine the toxicity mechanism. Given differences among toxicity methods, it is not possible to prescribe *a priori* all of the control measurements that would be relevant for a particular study, but the extensive discussion of relevant control measurements for different types of experiments will facilitate designing a robust experiment. Ultimately, having reproducible results will support future informatics efforts to pull together results among studies, such as with species sensitivity distributions, to identify NMPs that may pose risks, to understand toxicity mechanisms, and to guide the design of safer alternatives.

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Notes

Certain commercial equipment, instruments, and materials are identified in this paper to specify an experimental procedure as completely as possible. In no case does the identification of particular equipment or materials imply a recommendation or endorsement by the National Institute of Standards and Technology or by the Consumer Product Safety Commission (Commission or CPSC), nor does it imply that the materials, instruments, or equipment are necessarily the best available for the purpose. The paper is work of staff and has not been reviewed or approved by and does not necessarily represent the views of the Commission nor does any mention of trade names, commercial products, or organizations imply endorsement by the CPSC.

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