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Protocol

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Exposure protocol for ecotoxicity testing of microplastics and nanoplastics

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Abstract

Despite the increasing concern about the harmful effects of micro- and nanoplastics (MNPs), there are no harmonized guidelines or protocols yet available for MNP ecotoxicity testing. Current ecotoxicity studies often use commercial spherical particles as models for MNPs, but in nature, MNPs occur in variable shapes, sizes and chemical compositions. Moreover, protocols developed for chemicals that dissolve or form stable dispersions are currently used for assessing the ecotoxicity of MNPs. Plastic particles, however, do not dissolve and also show dynamic behavior in the exposure medium, depending on, for example, MNP physicochemical properties and the medium's conditions such as pH and ionic strength. Here we describe an exposure protocol that considers the particle-specific properties of MNPs and their dynamic behavior in exposure systems. Procedure 1 describes the top-down production of more realistic MNPs as representative of MNPs in nature and particle characterization (e.g., using thermal extraction desorption-gas chromatography/mass spectrometry). Then, we describe exposure system development for short- and long-term toxicity tests for soil (Procedure 2) and aquatic (Procedure 3) organisms. Procedures 2 and 3 explain how to modify existing ecotoxicity guidelines for chemicals to target testing MNPs in selected exposure systems. We show some examples that were used to develop the protocol to test, for example, MNP toxicity in marine rotifers, freshwater mussels, daphnids and earthworms. The present protocol takes between 24 h and 2 months, depending on the test of interest and can be applied by students, academics, environmental risk assessors and industries.

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Key points

- In nature, micro- and nanoplastics (MNPs) occur in various shapes, sizes and chemical compositions. Each of these properties can affect both their dynamic behavior and their toxicology and should be considered when performing ecotoxicology experiments to assess their risk.
- Here, MNPs are generated from plastic waste by using either a ball or centrifugal mill. Their physicochemical properties are measured. Ecotoxicology experiments in soil and aquatic systems are described.

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Introduction

Plastic pollution is the subject of environmental and human health concerns at a global scale[1]. The amount of plastic waste is increasing, making it a global threat because of its persistence and poor reversibility[2]. If left unchallenged, this may increase to a level that is beyond a safe operating space for humanity.

Plastic pollution has become an important research topic over recent decades, especially gaining momentum since the discovery of smaller particles, known as microplastics (1 µm < particle size < 5 mm) and nanoplastics (<1-µm particle size) (MNPs)[2,3]. These occur mainly as a result of weathering of larger plastics in the environment through a series of abiotic and biotic processes, leading to fragmentation and the formation of small plastic particles. Moreover, some MNPs are intentionally produced and used in consumer products such as cosmetics and paints[4], leading to direct or indirect environmental emissions and exposure. As a result, MNPs are ubiquitous in the environment[5], and evidence is increasing on the potential adverse effects of these plastic particles. The awareness of this emerging environmental problem has increased rapidly in recent years as reported, for example, by the Science Advice for Policy by European Academies report on Microplastics in Nature and Society in 2019[6].

Plastic particles can cause environmental effects in a range of different ways[7]. MNPs can enter food webs and expose organisms not only to the particles themselves but also to harmful plastic additives or other toxicants associated with the particles[8]. For example, it was reported that microplastics are ingested by all of the fish species that have been studied so far (>150)[9]. MNPs potentially act as stressors affecting energy balances, causing physical damage to the digestive system and causing digestive tract blockage[10]. Importantly, vulnerable fish species may not possess additive genetic variation that would help them to adapt to the increasing MNP pollution[11]. Moreover, microplastics may have indirect effects on the ecosystem; for example, they represent a new surface for microbial biofilm formation for which increased horizontal gene transfer between bacteria has been documented[12]. The consequences of these indirect effects for biodiversity and ecosystem functioning[13], as well as for environmental and human health, remain so far largely unknown.

A need for more environmentally relevant materials and methods

In nature, MNPs occur in different sizes, shapes and chemical compositions. Emerging evidence is showing that size and chemical composition[14] influence the toxicity of MNPs to organisms. However, to date, most ecotoxicological studies have examined commercially available particles with spherical shape as model particles for MNPs. Studies using polystyrene (PS) MNPs are particularly abundant in literature. In these studies, the particles are dispersed in water or spiked to the solid matrices such as soil or food with a certain amount of MNPs for toxicity tests. To advance our knowledge on MNP effects, more environmentally relevant particles and exposure conditions should be studied.

Obtaining MNPs with different physicochemical properties for controlled studies is, however, still challenging, even for commercially available particles. Moreover, using MNPs applied in consumer products to investigate their toxicity is challenging, because of the presence of different chemicals in the matrices of these products. A top-down method based on mechanical treatment has been proposed as an alternative approach to obtain MNPs from different plastic items[15], even plastic waste. These MNPs share similarities with MNPs occuring in nature with respect to their physicochemical properties.

Nanoplastic toxicity is different from microplastic toxicity

Nanoplastics have a size and shape that is comparable to that of large proteins; therefore, their behavior is inherently different from their microplastic counterparts[3], and they may be able to enter cells by crossing biological membranes[16]. In addition, a larger fraction of the molecules in nanoplastics is on the particle surface, which can increase interactions with and reactivity to subcellular components. It is critical to consider the differences between microplastics and nanoplastics when performing toxicity tests using these particles, because nanoplastics

are expected to pose unique risks[3]. Nanoplastics are, however, very challenging to study. This is mainly because of their small size and the limitations in existing analytical methods and instrumentation to track and characterize them in the environment and organisms[17]. Thus, there are particularly large knowledge gaps on the environmental fate and effects of this smallest fraction of plastic particles.

Challenges in comparing results from different studies

Despite the increasing number of studies on the adverse effects of MNPs, comparisons between the results of different studies are challenging. Most of the scientific studies do not follow MNP-specific procedures but rather use protocols and test guidelines developed initially for 'soluble chemicals'. This is largely because of the absence of testing guidelines and protocols specifically developed for toxicity testing of MNPs. Developing a protocol for MNPs involves taking into account the specific properties of each material that distinguish them from soluble chemicals, in particular how to spike MNPs in the exposure matrix to establish reliable and repeatable exposures.

Using inappropriate protocols and guidelines is unlikely to generate accurate, robust and standardized data valid for both scientific and regulatory purposes. This challenges intra-laboratory repeatability and inter- and intra-laboratory reproducibility. To support regulators and policy makers in developing appropriate and informed strategies for risk assessment and management, it is imperative and urgent that we develop an understanding of the fate and any adverse impacts of these emerging pollutants in terrestrial and freshwater ecosystems. A unifying protocol for ecotoxicity testing of MNPs is urgently needed. Here, a protocol is presented, comprising procedures for production of MNPs and developing relevant exposure matrices suitable for standardized and harmonized MNP ecotoxicity tests in different environmental compartments.

Development of the protocol

Many studies have investigated the (eco)toxicity of MNPs to different terrestrial and aquatic organisms at different biological levels (e.g., cellular and organism levels)[18,19]. The current protocol was developed on the basis of the successful results obtained mostly in our laboratories during the last decade and the realization that the exposure matrices, which are used to perform the toxicity testing of MNPs, play a critical role in the accuracy of the test results. The behavior of MNPs in exposure media has been mostly disregarded in toxicological studies. However, it has been increasingly reported that MNPs and nanomaterials have a dynamic behavior in exposure media that can dramatically influence the exposure conditions over time[20]. We applied this information to develop specific exposure matrices for MNPs. The protocol is developed for toxicity testing of MNPs under controlled conditions (e.g., in the laboratory or meso- or macro-cosms) and is not meant to be used for monitoring purposes (e.g., measuring MNPs in field samples or toxicity in the field).

The effect of particle size

Our pioneering study measured ecotoxicity after exposure to 0.05-, 0.5- and 6-µm-sized PS particles[21]. We used Monogonont Rotifer as a model organism, and the association of toxicity to particle size was investigated by measuring several in vivo endpoints and studying the ingestion and egestion by using 0.05-, 0.5- and 6-µm PS MNPs. The results indicated that MNP toxicity depends on the particle size and that nanoplastics are more toxic than microplastics of the same composition. An increase in reactive oxygen species (ROS) as a function of particle size was observed. Exposure to nanoplastics led to a higher cell membrane permeability and molecular damage compared to microplastics. This could be because of the higher number of small particles compared to the number of larger particles the organisms are exposed to when particle mass is used as the dose metric. In another study, we used particle number (3×10^{10} particles/liter) as the dose metric to study the toxicity of 0.2- and 0.6-µm PS particles and co-occurring benzo(a)pyrene effects on daphnids[14]. The data also showed that particle size can influence toxicity to organisms. During the 21 d of exposure to PS of 0.2 µm, the number of broods produced by daphnids was lower compared to PS of 0.6 µm. Size-related toxicity has

been verified, but how other MNP physicochemical properties, such as shape and chemical composition, can drive toxicity needs systematic study using well-defined particles of different properties.

In the EU Horizon 2020 project PlasticsFate (Grant Agreement number 965367), we have developed such an approach to generate MNPs by using a top-down method. The produced particles can be characterized and quantified by using the method described in Steps 2–22 of Procedure 1 and used for different ecotoxicological studies.

Dynamic processes that alter particle concentration

As mentioned earlier, MNPs can undergo dynamic processes that affect their concentration in a medium and their interaction with organisms. These processes include agglomeration, floating, sedimentation, fragmentation and transformation. Because most ecotoxicity tests have been developed for chemicals[9], there is a risk that dynamic particle behavior is overlooked or even disregarded. As a result of this dynamic behavior, the concentrations (and ultimately, the doses) to which organisms are exposed can change substantially during testing.

In previous work, we investigated the dynamic behavior of PS MNPs over time to develop stable exposure in the matrix for toxicity testing of freshwater mussels[22]. Sedimentation and agglomeration are the main processes that influence exposure in laboratory-based experiments. The sedimentation behavior of MNPs was monitored over time[22], and on the basis of these results, the exposure medium was continually mixed by using aeration to keep the particles in dispersion. Despite this mixing of the exposure medium, some of the particles still sedimented.

Sedimentation decreases the particle concentration in the water column while increasing the concentration of particles in the sediments. This makes it difficult to perform long-term toxicity tests, for example, to determine the chronic effects of MNPs and their concentration thresholds. To minimize particle sedimentation, the sonication time, which was used for the dispersion of the particles in the stock, and the exposure duration were optimized. For long-term exposure (>48 h), we advise renewing the exposure medium after investigating the sedimentation and aggregation behavior of the particles in the exposure medium of interest.

Alternatively, the particle behavior can be used to inform which species are most likely to be affected. By using the Organisation for Economic Co-operation and Development (OECD) GD (Guidance Document) 318 developed for nanomaterials to help guide testing and assessment strategies, the test system can be selected to best reflect relevant exposure; for example, a sediment test species may be most relevant to test where most particles are expected to be in the sediments. This will of course depend on the question the study is aiming to answer. In some cases, aquatic testing is needed to address hazards to pelagic species or to reduce interference from other natural particulates in a system, but this guidance can also help ensure that the most relevant test systems are included.

For performing ecotoxicity tests, the existing guidelines and proposed endpoints (e.g., OECD guidelines) can be applied. The OECD (https://www.oecd.org/) has published many guidelines for the testing and assessment of chemicals. The assays most relevant for this work are as follows:

- Test no. 201: freshwater alga and cyanobacteria, growth inhibition test (https://www.oecd-ilibrary.org/environment/test-no-201-alga-growth-inhibition-test_9789264069923-en)
- Test no. 202: *Daphnia* sp. acute immobilisation test (https://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en)
- Test no. 317: bioaccumulation in terrestrial oligochaetes (https://www.oecd-ilibrary.org/environment/test-no-317-bioaccumulation-in-terrestrial-oligochaetes_9789264090934-en)
- Test no. 318: dispersion stability of nanomaterials in simulated environmental media (https://www.oecd.org/env/ehs/testing/test-no-318-dispersion-stability-of-nanomaterials-in-simulated-environmental-media-9789264284142-en.htm)

However, some modifications might be required, because they have not all been designed for nanomaterials. The modifications depend on the behavior of the particles in the exposure media. In a previous study, we exposed unicellular microalgae *Pseudokirchneriella subcapitata* to PS of 0.27 and 0.64 µm in the Woods Hole algal medium prepared according to the OECD

guideline 201 (OECD, 2011)[23]. Accordingly, the particles were first dispersed in the Woods Hole algal medium by sonication of the dispersion for 1 and 5 min for 0.64- and 0.27-µm PS, respectively. To minimize particle agglomeration, the concentration of $CaCl_2$ and $NaNO_3$ in the medium was decreased by 10-fold.

In some cases, microplastics (e.g, polyethylene (PE)) undergo flotation in water. We could not develop a strategy to minimize the flotation of these materials in water. This does not occur for low-density nanoplastics (e.g., PE nanoplastics), because the thermal diffusion (Brownian motion) of the particles at the nanoscale leads to the dispersion of the nanoplastic in water[17].

Overview of the procedures

The protocol consists of three main procedures (Fig. 1): Procedure 1, production of MNPs; Procedure 2, developing defined exposure matrices for soil; and Procedure 3, developing defined exposure matrices for water ecosystems. Procedures 2 and 3 also describe how to perform well-defined toxicity tests with different model organisms.

Procedure 1 is used to produce MNPs from plastic items in a so-called top-down approach. It describes the steps to prepare MNPs of different sizes and shapes. The users can turn different types of plastics into MNPs, even plastics that are collected from the environment. This procedure also more closely resembles the MNPs occurring in the environment in comparison to commercial particles generally used for toxicity tests. The procedure consists of two stages: production of MNPs by using Ball Mill (stage 1) and Centrifugal Mill (stage 2). It is also possible to use commercial MNPs that are supplied by different companies. In that case, researchers may skip this procedure.

Procedures 2 and 3 describe the development of exposure matrices for toxicity tests of MNPs in soil and water, respectively. Given the diversity of polymer types and particle physicochemical properties, developing specific exposure matrices suitable for toxicity testing of different plastic particles of interest is required. Adopting these procedures minimizes experimental errors induced because of the particles' dynamic behavior (agglomeration, sedimentation, etc.) and facilitates inter- and intra-laboratory comparisons. Procedure 3 describes the development of exposure matrices for freshwater, saltwater and brackish water.

Procedures 2 and 3 also explain the implementation of toxicity tests for MNPs by using (micro)organisms from different environmental compartments. This procedure describes performing toxicity tests on some model organisms by using available guidelines. The steps in which modification can be performed are highlighted in each guideline to make the condition suitable for the MNPs of interest. Note that the aim of this procedure is to show the user how the modification in the given ecotoxicity guideline can be carried out to meet the required condition suitable for the MNPs of interest without influencing the conditions suitable for

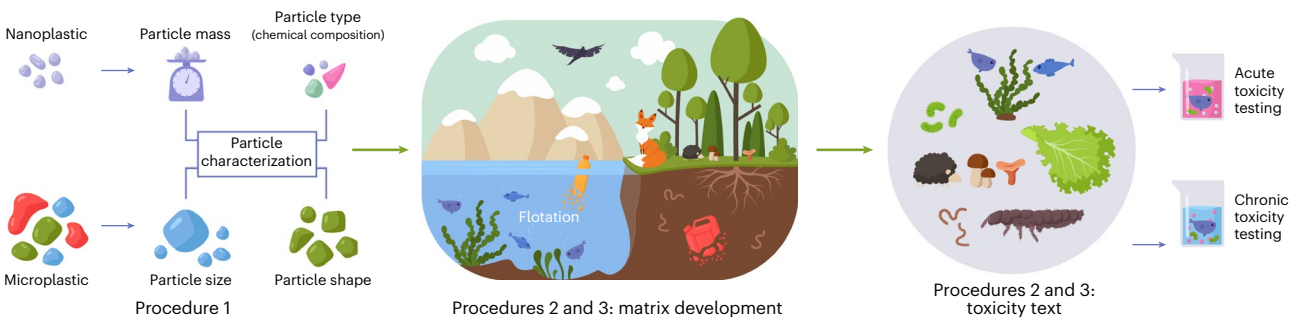

Fig. 1 | An overview of the procedures. Our protocol contains three procedures. Procedure 1 consists of the production of MNPs by using a top-down approach followed by characterization of the MNPs (determining particle mass (the concentration of the MNPs), particle type (the type of polymers forming the MNPs), particle size and shape). Procedures 2 and 3 consist of two sections: (i) developing exposure matrices for soil (Procedure 2) and water (Procedure 3) and (ii) performing the toxicity test with different organisms. For developing the exposure matrices, monitoring particle behavior (e.g., (hetero)aggregation and sedimentation) is required to minimize the loss of the particles. The toxicity tests can be performed by using different organisms and the modified OECD guidelines available for ecotoxicity testing of chemicals. The toxicity assessment might be short term or long term, generating acute and chronic toxicity data. It is important to investigate changes in the properties of the particles over time and to monitor the actual exposure concentration.

the organisms. The procedure demonstrates ecotoxicity tests on algae and daphnids (freshwater models), copepods (saltwater model) and earthworms (soil model).

Applications of the protocol

Our presented protocol covers the development of exposure matrices for MNP ecotoxicity testing for different environmental compartments. This protocol has been developed to be used by students, researchers and commercial laboratories to investigate plastic particle ecotoxicity in a highly standardized and comparable manner. The following purposes can be addressed by our new protocol:

- By ecotoxicologists to assess and understand dose-response relationships after exposure of biota to MNPs, and to determine the influence of physicochemical properties of plastics and of modification of the composition of the test medium on their toxicity to model organisms;
- By the industry sector to design safer plastics and to perform toxicity tests on plastics to fulfill regulatory requirements;
- By regulatory authorities to develop regulatory measures based on potential risks identified.

Comparison with other studies

Unlike most previous studies that used commercial material to simulate plastic particles in the environment, the current protocol describes the production of plastic particles from larger, well-characterized plastic items. This also allows researchers to use aged plastics, including litter collected from the environment, to produce MNPs in their ecotoxicological studies. Furthermore, the behavior of MNPs in the exposure system is systematically investigated and monitored in this protocol. In many previous studies, the behavior of plastic particles in exposure matrices was disregarded[24]. This can lead to highly dynamic exposure systems and consequently different results depending on incubation conditions and approaches.

A number of principles, experiences and recommendations obtained by testing nanomaterials, as described in recent OECD Guidance Documents[25], are in some part transferable to our new protocol for nanoplastic ecotoxicity testing. Especially the OECD GD 317[25] on aquatic and sediment toxicity testing of nanomaterials is relevant for particles with sizes of 1–1,000 nm, with primary or aggregate/agglomerate sizes greater than the range for nanomaterials. However, this GD focuses exclusively on freshwater aquatic and sediment environments, whereas our new protocol focuses on different environmental compartments and includes approaches specific for MNPs.

Limitations

In practice, the production of nanoplastics with a size <50 nm, by using the described procedures, might be challenging. The time and energy input for the grinding mill should be optimized because the generated heat during the production process can lead to particle alteration and degradation. For developing a suitable exposure matrix, sonication is required for some nanoplastics. The sonication process might oxidize the particle surface and thus change the behavior of nanoplastics in the exposure medium and their interaction with (micro) organisms. To partly tackle this limitation, we provide an optimized protocol for the sonication time for some types of plastics to minimize their oxidation and breakage.

The proposed exposure systems are designed for static tests, and while they may be applicable to semi-static tests, they are not applicable for flow-through tests. Another limitation is associated with current analytical challenges in the characterization of MNPs in some exposure media such as sediment and soil[26]. Unlike aquatic systems, the heterogeneous nature of sediment and soil matrices will greatly complicate in situ particle characterization. This necessitates developing new methods for particle isolation from sediment and soil matrices for characterization purposes, which is outside of the scope of this protocol and can be found in a previous publication[26]. Assessment of released additives from plastic is another analytical challenge that needs to be tackled, because many of the chemicals will be present at trace concentrations.

Experimental design

The purpose of this protocol is to develop exposure matrices to support performing short-term and long-term toxicity tests by using MNPs for generating reliable and robust data. The application of the protocol will in certain aspects require high-level expertise. For example, production of MNPs from plastic items requires technical/analytical experts to set up the instrument according to the parameters mentioned in our procedure. Because there are no standard methods for toxicity testing of MNPs, we encourage researchers to modify this protocol where appropriate and according to the intended purposes to ensure the best use of available equipment beyond that listed herein.

MNP production (Procedure 1)

Environmentally relevant model MNPs can be generated by cryomilling of some plastics and subsequent fractionation by sieving[15]. The ideal starting materials for the production of MNP powders are either plastic films or plastic granules whose composition, including additives, is known. From these, undegraded or artificially aged MNP reference materials can be produced.

As a first step, the materials are crushed by grinding. The individual grinding conditions depend on the different polymer types, because the glass transition temperature is crucial. Polymers above the glass transition temperature are flexible, but below it, they become brittle and stiff. To ensure that the polymer stays cold enough to be ground, we recommended cooling by using liquid nitrogen.

Different mills are available, like ball mills and centrifugal mills. In a ball mill (Cryomill), the material to be ground can be continuously cooled with liquid nitrogen, while being crushed with a ball over several cycles. An alternative is a centrifugal mill, in which the plastic items are only pre-cooled and then ground with the help of a ring sieve. Larger quantities of plastic can be ground in a centrifugal mill. Polymers with a low glass transition temperature are easier to grind in a ball mill because of better cooling. Furthermore, the shape of the produced particles is rock-like after use of a centrifugal mill and irregular and frayed with milling by a ball mill.

Any manually changeable equipment (e.g., grinding container, balls and ring sieves) should be used for only one type of polymer, because it is important to avoid contamination, and cleaning alone would not guarantee a contamination-free surrounding. Furthermore, the mills are located in a fume hood.

The ground MNP particles can be further sieved for fractionation into different size classes. Subsequent wet sieving over different sieves leads to the extraction of different size fractions and the removal of particles >1 mm.

MNP characterization (Procedure 1)

MNPs consist mainly of synthetic polymers and some additives. In the past decade, there have been several attempts to develop methods for detection, identification and quantification of MNPs[17,27]. Regardless of how the samples are obtained or prepared, physicochemical characterization of MNPs is required to determine, for example, their size, chemical composition, shape and color[28].

Although developing or prescribing methods for MNP identification and characterization is outside the scope of this protocol, we advise determining their chemical composition if the chemistry of the plastics in a test is unknown. There are a number of spectroscopic and thermoanalytical techniques that can be used to determine the chemical composition of MNPs: for example, Fourier-transform infrared spectroscopy, Raman spectroscopy and pyrolysis gas chromatography-mass spectrometry (GC/MS)[17], which is a technique for chemical analysis in which a sample is heated in an inert atmosphere without oxygen and decomposed into smaller stable compounds through thermal degradation.

In combination, these methods provide information on the polymer mass, type, crystallinity and purity. Microscopic particles are measured with a Raman spectrometer combined with a microscope. The lower measurable limit for particle size is ~1 µm in Raman spectrometry, but smaller particles can be measured if they are agglomerated and the size of the agglomerates is >1 µm[14]. The fully automated thermal extraction desorption (TED)-GC/MS

is suitable for routine measurement of MNPs with minimum absolute masses between 0.06 and 2.2 µg in the crucible[29].

The users of this protocol might, however, apply more suitable techniques (e.g., any newly developed methods in their laboratories) to determine the chemical compositions of MNPs to facilitate developing the exposure matrix of interest.

This protocol is intended for the characterization of clean samples that consist of only one polymer type. There is still no method and technique available for direct characterization of nanoplastics in complex matrices of environmental samples[17].

Exposure matrix development (Section 1 of Procedures 2 and 3)

To minimize variation between studies, some factors need to be considered when developing the exposure matrices for MNPs. In this protocol, the focus is on soil, freshwater, brackish water and saltwater. Ecotoxicity testing procedures for MNPs are similar to those for other chemicals; they have the same test duration and endpoint measurements, and the same biological validation criteria are applied. However, some modifications are required to consider the particle-specific properties of MNPs to make the medium suitable for the exposure test. For microplastics, there are several procedures available for validating spiking concentrations in complex matrices such as soils[30,31]. These are time-consuming procedures that would be difficult to recommend on a routine basis. For example, density-separation, digestion and filtration steps need to be performed over several days to successfully isolate plastic particles from soils[30,31]. For nanoplastics, protocols are not available.

Freshwater. Freshwater ecosystems are present in highly diverse environments including glaciers, lakes, rivers, wetlands and groundwater aquifers. Studying the ecotoxicity of MNPs requires preparation of exposure matrices representative of these distinct aquatic environments. However, freshwaters are so diverse in their composition that they cannot be represented by a single recipe[32]. Few methods have been proposed for the preparation of artificial freshwaters[33], and these mainly focus on the inorganic components of the water matrix. Thus, for freshwater ecotoxicity tests, we propose the application of the media developed by the OECD guidelines after some modifications.

Brackish and saltwater. Artificial brackish water and saltwater can be produced by adding chemicals or commercial sea salts into the water. The salinity of the exposure medium can be set as 10% for brackish water and 35% for saltwater. Different salinities can be used according to the specific requirements of the exposure experiments. In the following section, the main factors that need to be considered for developing exposure matrices are briefly described. Then, a workflow for developing exposure matrices of interest is proposed.

MNP aggregation. Physicochemical properties of plastics influence their behavior in the exposure medium. For example, most MNPs have a hydrophobic surface, and this hydrophobicity influences the interactions of the particles with the surrounding environment. Nanoplastics exhibit a higher free surface energy compared to their microplastic counterparts, which dramatically influences their further interaction in the environment. Nanoplastics are prone to both homo- and hetero-aggregation, which makes spiking the exposure systems (whether soil or water) difficult. Moreover, aggregation (e.g., hetero-aggregation with soil particles or homo-aggregation in water) can lead to a reduction in the particle concentration in the exposure medium. This implies that particle aggregation can decrease the number of plastic particles to which organisms are effectively exposed. Aggregation is influenced by the composition of the exposure medium (e.g., the size of the natural colloids and soil particles[34]) or by the salinity and presence of natural organic matter[17]. To perform a valuable toxicity test, it is necessary to monitor the aggregation behavior of the particles in the respective exposure medium.

MNP floating or sinking. Different types of plastics vary in their density (e.g., PS, ~0.96–1.05 g cm^{-3}; polyvinyl chloride (PVC), ~1.38 g cm^{-3}; and PE, ~0.91 g cm^{-3}). In ecotoxicological studies, organisms

are mostly exposed to a specified mass of MNPs. The lower or higher density of plastics as compared to water makes them float or sediment, respectively, in the exposure medium, and in some cases out of the reach of the exposed organisms. On the other hand, in the case of nanoplastics of the same chemical composition, Brownian diffusion of the particles can facilitate their distribution in the exposure medium.

Maintaining stable dispersions. Considering the issues described in this section, we propose the following workflow (Fig. 2) for developing exposure matrices for MNPs. It is important to understand whether the test is prepared for microplastics or nanoplastics. The powder of the materials should be dispersed by using a suitable dispersant. Note that the amount of dispersants should be limited, if possible, and not induce toxicity to the test organisms of interest. For regulatory acceptance of the test results, it should be noted that <0.1 ml liter^{-1} of dispersant/solvent is allowed in the final exposure medium[35]. The particles in dispersions should be stable against aggregation. This can be achieved by using sonication. In some cases, continuous mixing of the medium can facilitate dispersion of the microplastics. In all cases, we recommend that the behavior of the particles is monitored in the exposure medium during the exposure period.

Exposure considerations for ecotoxicity testing for MNPs (Section 2 of Procedures 2 and 3)

Exposure to and uptake of MNPs mainly occurs inadvertently by adsorption, ingestion and respiration[36], but some organisms may be selective with regard to their interaction with MNPs. For example, visual similarity to prey has been shown to drive microplastic ingestion in several fish species and probably represents an important pathway of ingestion for microplastics[37]. Although fish can recognize microplastics as inedible materials and show rejection behavior, this is not necessarily the case for nanoplastics or for organisms at lower trophic levels[38]. In addition, most organisms can ingest particulate matter under what is considered as the threshold sizes for feeding[39], which is referred to as indiscriminate feeding behavior[40]. These different feeding patterns, as well as the fact that particle exposure is not driven by molecular diffusion, indicate that the steady-state assumption between uptake of a compound in an organism, as assumed for soluble chemicals, and its exposure medium do not hold for MNPs[41].

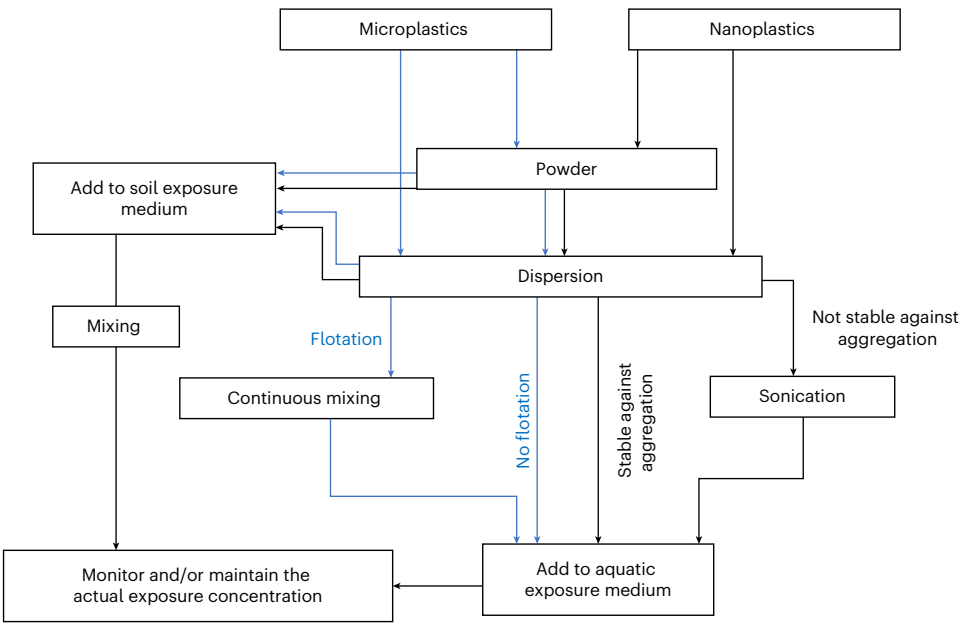

Fig. 2 | A comprehensive workflow. Proposed workflow for developing reliable exposure matrices for microplastics and nanoplastics in soil and aquatic systems.

The exposure route can vary between different soil organisms. For example, exposure may be through the pore water (e.g., for nematodes or springtails) or can be through both pore water and the solid phase of the soils (e.g., earthworms). Relevant routes of exposure should therefore be considered in the selection of organisms and exposures for the question being asked.

Controls and data analysis

Many OECD guidelines that are used for performing ecotoxicity tests might be applied to report the data. We recommend that users apply the available harmonized reporting guidelines for data reporting on MNPs[42]. As with other toxicity tests, it is critical to ensure data assurance and accuracy. See the previous publication for more information[17].

Standards and reference materials

Unfortunately, there are still no standard and reference materials available for MNPs. Reference materials for PS MNPs are available; however, they might not be suitable for PS particles of different shapes. Recently, the application of rare elements such as gadolinium and europium in the structure of nanoplastics has been proposed and tested to provide information on the biological fate of nanoplastics in organisms[17,43]. However, the production of metal-entrapped nanoplastics is extremely challenging; it has been reported only for PS and PVC of certain sizes. It is also important to note that the commercial PS standards expire after a few months because of the limited stability of the dispersion and the potential for bacterial contamination. In addition, these standards often contain surfactants to maintain particle stability in the dispersions, but these surfactants themselves also have a toxicity profile. Thus, if washing of the particles to remove these surfactants is not possible or causes changes to the particles, it will be necessary to include surfactant/carrier control treatments to ensure that toxicity related to these chemicals is accounted for and that toxicity attribution to particles is properly assigned. The applied blank samples could be Milli-Q water. Polymer standards (e.g., fivefold deuterated PS or ^{13}C-labeled PS) can also be used as an internal standard for quality assurance. These can be dissolved with toluene if required and are thus suitable for dosing very small quantities. If these small amounts are added to the samples as an internal standard for each measurement, it is possible to monitor instrumental performance and improve the limit of detection and repeatability.

All the applied chemicals and solutions must be free of polymers and plastic particles. The chemicals should be kept in glass containers rather than plastic ones.

Positive and negative controls

Negative control samples in which organisms are not exposed to MNPs must be applied in toxicity tests. A minimum of three replicates (biological and technical) per concentration must be used to get statistically meaningful results. For the ecotoxicity tests, positive controls with chemicals of well-known toxicity (e.g., potassium dichromate) should be used to document test performance and sensitivity.

Replicate conditions

Because MNPs are particulate materials with dynamic behaviors, it is critical to ensure that the replicate conditions are the same as each other (e.g., by performing the three replicates at the same time or by monitoring the behavior of the particles by using dynamic light scattering (DLS) as described in this protocol[44]). Stock dispersion of interest also needs to be prepared immediately before the experiment or before the spiking of the samples. The spiked samples must be used immediately or kept for at most a few days at 4 °C.

Materials

▲ **CRITICAL** This protocol does not cover monitoring of MNPs in the environment and describes only the preparation of exposure media and how to expose organisms to MNPs in laboratory-based experiments. We have demonstrated the use of this protocol with

model organisms, which are commercially available (e.g., from LGC Standards GmbH) and can be cultured and kept under controlled conditions. The protocol can be used and modified for various other terrestrial and aquatic organisms, including field-collected (micro)organisms. Note that the proposed materials and chemicals are those that we applied in our laboratories. Users of this protocol are not limited to these materials and may apply materials and chemicals from other companies and suppliers.

Biological samples

- Algae (*Raphidocelis subcapitata*). The protocol is described specifically for the algal species *Raphidocelis subcapitata* (previously known as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*) but can also be used for other types of unicellular microalgae such as *Scenedesmus obliquus*.
- Daphnids (*Daphnia magna*), cultured in M7 medium (OECD requirement)
- Copepods (*Paracyclopina nana*)
- Earthworms (*Eisenia fetida*; fully clitelated adults of >300 mg wet weight per individual)

Reagents

- Sodium chloride (NaCl; Sigma-Aldrich, CAS no. 7647-14-5)
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, CAS no. 7786-30-3)
- Magnesium sulfate ($MgSO_4$; Sigma-Aldrich, CAS no. 7487-88-9)
- Sodium carbonate (Na_2CO_3; Sigma-Aldrich, CAS no. 497-19-8)
- Sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich, CAS no. 144-55-8)
- Potassium chloride (KCl; Sigma-Aldrich, CAS no. 7447-40-7)
- Calcium chloride ($CaCl_2$; Sigma-Aldrich, CAS no. 10043-52-4)
- Polystyrene nanoplastic beads (0.05, 0.5 and 6 µm) (Polysciences, cat. nos. 08691, 07307 and 07312, respectively)
- PBS (Life Technologies, cat. no. 70011-044)
- Salts for preparation of algal growth media as outlined in OECD TG-201
- Microplastics (pellets, fragments and foils) (PlasticsEurope)
- Ethanol (absolute; Sigma-Aldrich, CAS no. 64-17-5)
- Toluene (for analysis; Sigma-Aldrich, CAS no. 108-88-3)
- Acetone (for analysis; Sigma-Aldrich, CAS no. 67-64-1)
- Methanol (for analysis; Sigma-Aldrich, CAS no. 67-56-1)
- Internal standard: $^{13}C_6$-labeled PS (Sigma-Aldrich) or fivefold deuterated PS (Polymer Source) dissolved in toluene or others
- Solid phase adsorber (Envea, SorbStar)
- Soil, air-dried and sieved to <2 mm. OECD 222 recommends artificial soil. However, a more natural soil such as Lufa 2.2 (LUFA) has become a standard used routinely in soil ecotoxicological testing.
- Manure to feed: oven-dried, vermicide-free horse manure (needs to be collected; is not supplied)

Equipment

- 20-ml vials (iNexus, cat. no. 27-00018-07)
- 96-well plates (SPL Life Science, cat. no. 30096)
- Duran laboratory bottles, 250 and 500 ml (Sigma-Aldrich, cat. no. 70109091)
- Preserve jar, 1 liter (Kilner, cat. no. 0025.401)
- Thermal desorption tubes (TDU) glass tubes (Sigma-Aldrich, cat. no. CLS9944513)
- Conditioner for TDU glass tubes (Gerstel)
- Tweezers (Sigma-Aldrich, cat. no. T5790)
- 10-µl syringe (Sigma-Aldrich, cat. no. HAM80075)
- Metal spatula (Fisherbrand stainless steel trulla Spatula; Fisher Scientific, cat. no. 11523492)
- Weigh boat (SLS Select polystyrene weighing boat, 100 ml; Scientific Laboratory Supplies, cat. no. ZT1247230S)
- 50–1,000-µl pipette tips (Sigma-Aldrich, cat. no. Z740031-1000EA)

- Pipettes (P1000, P200, P100, P20, and P2; Gilson, cat. nos. F123602, F123601, F123615, F123600 and F144801, respectively)
- Vortex mixer (Sigma-Aldrich, cat. no. HS120214)
- Boxes (1,200-ml natural rectangular tub, 191 × 128 mm; Graham Tyson, cat. no. RB1200M001)
- Analytical balance (SI-234, max 230 g; Denver Instrument, model no. 22006821)
- Centrifuge tubes, 15 and 50 ml (Corning; Sigma-Aldrich, cat. no. 430791)
- Cuvette (Malvern Panalytical Consumables for Zetasizer Series)
- 25-ml glass measuring flasks (e.g., Brand Blaubrand volumetric flask; Merck)
- 20-ml glass scintillation vials with perforated lids (Wheaton liquid scintillation vial with attached foil-lined urea cap; Sigma-Aldrich)
- DLS instrument (Zetasizer Nanodevice, Malvern Panalytical)
- Ultrapure water system (Milli-Q system; Merck Millipore, model no. C85358)
- Temperature incubator (Sanyo, cat. no. MIR-553)
- Warm cabinet for drying
- AlO_x crucibles (150, 600 and 900 µl; size depends on the specific objective and sample material)
- Incubator at 20 °C with a 16:8-h light/dark cycle
- Water bath
- Salinometer (CAS, cat. no. SG-1)
- Shaking table with illumination (e.g., LED vertical illumination table for algal toxicity tests ; see ref. 45)
- Temperature-controlled chamber or room
- Hemocytometer (e.g., Brand counting chambers; Merck) or Coulter counter (e.g., Beckman Multisizer 4e Coulter counter) for cell counting
- Raman microscope (Thermo DXR2xi)
- Glass microscope slides (Fisher Scientific, item no. 11562203)
- A stereomicroscope, 6.3–57× (SZX-ILLK200; Olympus, cat. no. OLY-SZX9-B)
- Milling instrument (e.g., CryoMill (Retsch) including milling vessels and balls made of stainless steel or ZM200, centrifugal mill (Retsch), with cyclone and ring sieves)
- Thermoanalytical method instrument such as TED-gas chromatograph-mass spectrometer (Gerstel)

Software

- The DRC module of R (https://cran.r-project.org/web/packages/drc)
- Software for statistical analysis: SPSS version 23.0
- Software for particle number calculation: Microsoft Excel 2010

Reagent setup

Plastic

Plastics used as starting material should be clean. Using granulate is recommended for pristine polymer MNP, which can be used as delivered. 'Real' plastic products such as plastic beverage bottles, cups or textile fibers should be cleaned for use. In addition, they must be cut in pieces <1 cm^2 before cryomilling.

Brackish and saltwater

1. Pour the intended amount of dechlorinated water into a glass bucket and adjust the water to the desired medium temperature. We used a low-temperature incubator (Sanyo, cat. no. MIR-553) to keep a desired temperature for cultivation.
2. Prepare the chemicals according to Table 1 or directly use commercial sea salt (e.g., ASTM D1141-98) according to the product manufacturer's instructions and dissolve them in 1 liter of water.
3. Adjust pH to 8.2 (7.8–8.4) by adding 0.01 mol $liter^{-1}$ HCl or NaOH.
4. Measure the salinity levels of the water and adjust the saltwater salinity to the required values by adding salt or distilled water.

Table 1 | Amount of chemicals or commercial sea salts for preparation of 1 liter of brackish water (10‰) and saltwater (35‰)

Ingredients	Brackish water (g)	Saltwater (g)
NaCl	8.06	28.22
$MgCl_2$	0.73	2.56
$MgSO_4$	1.03	3.60
Na_2CO_3	0.03	0.13
KCl	0.21	0.74
$CaCl_2$	0.32	1.11
Commercial sea salt	12	42

5. Filter the solution through filter paper and sterilize by autoclaving at 103 kPa and 121 °C for 15 min.

These solutions should be freshly prepared on the day of use. However, the solutions can be stored at 4 °C after cooling for ~3 weeks.

Procedure 1: MNP production and characterization

Production of MNPs

1. Prepare MNPs by using either a ball mill or a centrifugal mill as described in options A or B. Use option A for small mass fractions of a few grams or polymer types with low glass transition temperature that require efficient cooling. If you want to produce hundreds of grams of MNP, use option B.

(A) **Ball mill (e.g., CryoMill, Retsch)**

● **TIMING 1–2 g d^{-1}**

(i) Fill the stainless steel milling container with plastics. One-third of the volume should be plastics, one-third should be the balls and one-third is free volume. Choose the size of the stainless steel balls with a ratio of 1:1,000 according to the size of the plastics to be ground.

▲ **CRITICAL STEP** Before you close the grinding container, wrap the thread with polytetrafluoroethylene tape. This ensures that you have less loss of the ground, fine fraction because of possible minimal loosening of the lid. Note that minimal abrasion of the stainless steel container and balls cannot be excluded.

(ii) Tighten the lid of the grinding container firmly after closing it and put the grinding bowl in the cooling jacket.

◆ **TROUBLESHOOTING**

(iii) Select the program for the milling process. For example, for PS:

- Number of cycles: 3
- Cooling time before the milling: 5 min
- Milling time per cycle: 1 min
- Frequency during milling: 25 Hz
- Cooling time between the cycles: 30 s
- Frequency for the time between the cycles: 5 Hz

▲ **CRITICAL STEP** Other polymers with lower glass transition temperature may need more cycles. This adaptation is easy. The lower the glass transition temperature of the polymer to be ground, the longer the cooling time before the milling and between the cycles. It is better to increase the number of cycles than the cycle duration.

▲ **CRITICAL STEP** More balls lead to more active surface, hence more abrasion and lower particle size of the resulting MNP.

(iv) Check that liquid nitrogen is connected and close the front window. Start the mill by pressing the green 'start' button. The mill stops automatically, after the program is finished.
◆ **TROUBLESHOOTING**

(v) Wait until the grinding chamber is defrosted and loosen the lock nut. Use the dowel pin supplied for this purpose.

(vi) Put the opening aid (right on the device) on the grinding bowl and remove it from the cooling jacket.

(vii) Open the stainless steel milling container and transfer the plastic particles into a beaker by using a clean metal spatula.
▲ **CRITICAL STEP** Open the vessel only when the temperature reaches >0 °C, to minimize water condensation on the sample. If water is incorporated into the product, the sample needs to be dried before proceeding.
■ **PAUSE POINT** The produced particles can be stored at 4 °C for as long as needed before performing exposure experiments (e.g., Procedures 2 or 3).

(B) **Centrifugal mill (ZM 200, Retsch)**
● **TIMING 200 g d^{-1}**

(i) Open the lid of the milling instrument, put the labyrinth disc in the instrument and add the rotor.
◆ **TROUBLESHOOTING**

(ii) Place the collecting vessel and the ring sieve, close the collecting vessel with the cassette cover and the housing cover and press downward.
▲ **CRITICAL STEP** Ring sieves come with different hole sizes. Choose a hole size between 1 mm and 500 µm, if it is not important that many small particles are present. Smaller holes may lead to smaller MNP particles but will reduce the yield per hour. The risk of clogging is increased. The type of plastic has a minor influence on the grinding result, which is dominated by physical processes during grinding.

(iii) Put the plastics in the small dewar and add liquid nitrogen. Pre-cooling for 3 min is recommended. If the milling instrument has a cyclone as equipment, start the hoover.

(iv) Set up the program of the instrument by choosing the velocity (e.g., 16,000 rpm) and start the instrument.
▲ **CRITICAL STEP** Higher velocity leads to smaller MNP particles, but more heat is produced, which can lead to more clogging at the ring sieve.

(v) Fill the pre-cooled plastics from the dewar to the grinding chamber spoon by spoon.
▲ **CRITICAL STEP** Addition of too much plastic at once can clog the ring sieve because of high heat production and melting of the plastics. It is recommended to fill the spoon no more than half full.

(vi) When the grinding process is finished, stop the instrument and the hoover and remove the MNPs from the cyclone's collecting vessel.
■ **PAUSE POINT** The produced particles can be stored at 4 °C for years (if necessary) before performing exposure experiments (e.g., Procedures 2 and 3). Note that aging may occur with very long storage times.

Characterization of MNPs

▲ **CRITICAL** It is important to characterize MNPs to understand their chemical compositions and other physicochemical properties such as size and shape. For the purpose of this protocol, we describe the methodology for determining the chemical composition of MNPs.

Thermodesorption/extraction-gas chromatography/mass spectrometry

● **TIMING 3 h**

▲ **CRITICAL** Cleaning of all equipment and materials used before measurement is mandatory to avoid contamination with plastics. If the use of plastic is unavoidable (e.g., for seals),

use only plastics that are not intended to be measured in the samples. Note that contamination from plastics can have an impact on the toxicity tests. AlO_x crucibles can be reused but must be cleaned carefully before usage by heating in a muffle furnace for ≥8 h at 1,100 °C.

2. Wash the TDU glass tubes with 20 ml of a solution of any concentration of tenside and deionized water. Finally, rinse with acetone and methanol and cover the tubes with paper to avoid contamination. After solvent evaporation overnight, heat the glass tubes at 350 °C under constant nitrogen flow for 30 min. Afterward, put a solid phase adsorber (e.g., SorbStar) in the cooled TDU glass tube and heat both to 250 °C for an additional 30 min.
   ■ **PAUSE POINT** The preheated TDU glass tube with adsorber should be carefully stored free of contamination until usage.
3. Prepare an internal standard solution.
   ▲ **CRITICAL STEP** To make the measurements comparable, to ensure valid quantification and to register any device drifts and carryover, it is recommended that d5-PS or $^{13}C_6$-PS be used as an internal standard. In addition, a blank measurement with internal standard should be performed between each sample measurement.
4. Put a clean and empty AlO_x crucible on an analytical balance and tare it.
5. Add an appropriate sample. (For very high polymer concentrations, microgram intake can be enough. With increasing matrix mass, the overall intake must be raised. Intakes up to 700 mg are possible.) Note the accurate sample intake.
6. Add a volume of internal standard solution to the sample corresponding to an internal standard intake of 4 µg.
   ▲ **CRITICAL STEP** Place the internal standard (stored in the refrigerator) on the laboratory bench at room temperature for a few minutes until it is in thermal equilibrium with the ambient temperature before use.
7. Put the sample-loaded crucible in the thermobalance (TGA) of the TED-GC/MS instrument.
8. Set up the measurement parameters for the GC/MS system as described in Table 2.
9. Use the Maestro software to define the measurement sequence. Start with the TGA measurement.
10. Determine which vial (SorbStar position) should be placed in the TDU coupling of the TGA, followed by determination of the position where the used glass tube is reset and the position for use of the loaded SorbStar for the TDU in the GC/MS.
11. Put the clean TDU glass tube including solid phase adsorber in the tube holder of the GC/MS system.
    ▲ **CRITICAL STEP** Check if the setup parameters match with each other regarding crucible and tube positions. More samples can be measured automatically, if 'autorun' is set.
12. Start the GC/MS run and the Maestro software.
13. Start the TGA to initiate the fully automated measuring process. The device stops automatically, after the program is finished.

Table 2 | Typical measurement setup for TED-GC/MS

Device	TGA 2 (Mettler/Toledo), GC system 7890B with MSD 5977B (both Agilent) and cooled injection system PTV (Gerstel)
Thermal extraction	25–600 °C, 10 K min^{-1}, 30 ml min^{-1} N_2, SorbStar
Thermal desorption	50–200 °C, 40 K min^{-1}, He
Programmed temperature vaporizing injection	−100 to 270 °C, 12 K s^{-1}, splitless, He
GC oven	40–300 °C with 5 °C min^{-1}, He
GC column	HP-5 MS (30 m × 0.25 mm × 0.25 µm)
MS	EI, 70 eV, scan 35–350 amu

amu, atomic mass units; EI, electron ionization; GC, gas chromatography; He, helium; HP5-MS, (5%-phenyl)-methylpolysiloxane phase capillary column; K, Kelvin; MSD, mass selective detector; MS, mass spectrometry; PTV, programmed temperature vaporizing; TGA, thermogravimetric analysis.

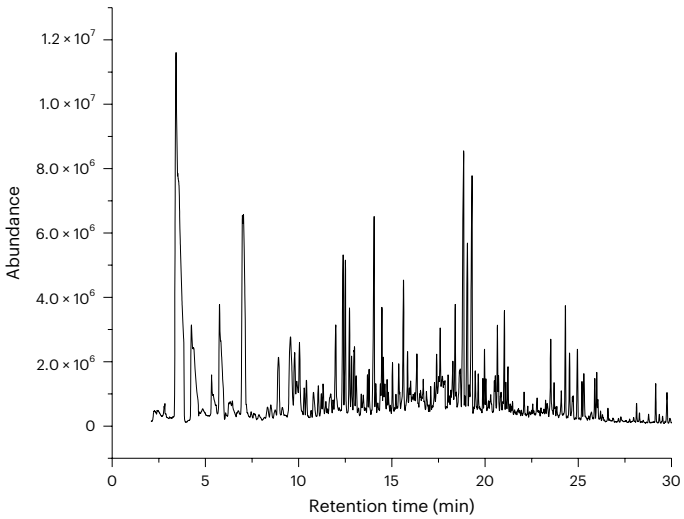

Fig. 3 | Ion chromatogram. Example of a measured total ion chromatogram of a polymer mix containing PE, polypropylene, PS, polyethylene terephthalate, polyamide and styrene-butadiene rubber.

Identification of MNPs

14. Identify plastics by screening the measured total ion chromatogram for individual pyrolysis products of the polymer as the main component.
    ▲ **CRITICAL STEP** The pyrolysis product needs to be specific regarding the polymer type. An example of a chromatogram of a polymer mix is shown in Fig. 3. Table 3 lists recommended pyrolysis products as marker molecules used in the screening protocol to identify and quantify the polymers regarding their polymer type and mass. To identify a polymer correctly, all mass traces for identification named in Table 3 should be visible (see also Supplementary Table 1 for more information).
    ◆ **TROUBLESHOOTING**

Quantification of MNPs

15. Follow the steps in options A and B for quantification of plastics via standard addition or external calibration. Standard addition is more accurate, because it takes into account the effects of the matrix, but it is more time consuming. External calibration is recommended for large datasets.
    (A) **Standard addition**
    (i) In a first measurement, measure a representative subsample as described in the measurement procedure.
    (ii) In a second measurement, prepare a second subsample like the above. Add defined amounts of the pristine polymer types that were found in the first measurement. Measure the sample under identical conditions to the sample of the first measurement.
    ▲ **CAUTION** Add very low amounts of the pristine polymers, not to overload the instrument (Table 3). Note the added mass of the individual pristine polymers (m_{dot}).
    (iii) Determine the individual peak areas of the specific pyrolysis products of the polymers in the first measured chromatogram (A_0).
    (iv) Determine the individual peak areas of the specific pyrolysis products of the polymers in the second measured chromatogram (A_1).
    (v) Determine the peak area of the internal standard ($A_{Int.St.}$) in the first and second measured chromatogram.
    (vi) Normalize A_0 and A_1 to the area of the internal standard ($A_{Int.St.}$) to get the normalized peak area of the added pristine polymer mass (ΔA_{pol}) (equation (1)).
    (vii) Calculate the mass (m_0) in the sample for quantification (equation (2)).

$$\frac{A_1 - A_0}{A_{\text{Int.St.}}} = \Delta A_{\text{pol}} \quad (1)$$

$$\frac{A_0 m_{\text{dot}}}{\Delta A_{\text{pol}}} = m_0 \quad (2)$$

(B) **External calibration**

(i) Prepare seven crucibles as described in the measurement procedure.

(ii) Set one crucible aside (non-spiked sample).

(iii) Add a defined polymer mass to the other crucibles, adjusting the added polymer mass from very low to higher concentrations. Note the amounts.

(iv) Measure all seven crucibles and determine the peak area of the specific pyrolysis products of the polymers.

(v) Normalize the areas (A_1) to the peak area of the internal standard ($A_{\text{Int.St.}}$) (equation (3)).

$$\frac{A_1}{A_{\text{Int.St.}}} = A_{n1} \quad (3)$$

(vi) Subtract the normalized peak areas of the spiked measurements (A_{n1}) from the non-spiked sample (A_{n0}) (equation (4)).

$$A_{n1} - A_{n0} = \Delta A_{n1} \quad (4)$$

(vii) Create a diagram in which the different ΔA_{n1} to ΔA_{n7} (y-values) are plotted as a function of the corresponding polymer weights (x-values).

(viii) Fit a linear regression curve through the data points, where the Pearson value should be close to 1, and determine the equation of the regression curve (equation (5)).

$$f(x) = mx + n \quad (5)$$

with m being the slope of the regression curve and n the point of intersection with the ordinate axis.

▲ **CRITICAL STEP** Mass quantification is allowed only via external calibration within the linear part of the curve.

Table 3 | Pyrolysis products to identify and quantify the polymers with TED-GC/MS

Polymer	Specific pyrolysis products	Mass traces for identification (*m/z*)	Mass traces for quantification (*m/z*)	Limit of detection (µg)	Mass recommended for spiking (µg)
PE	Tetradecadiene	55, 81, 95, 109	81	2.2	100–200
	Pentadecadiene				
	Hexadecadiene				
Polypropylene	Tetrametylundec-10-ene	111, 69, 154, 210	111	0.14	50–150
	Tetrametylundec-10-ene				
	Tetrametylundec-10-ene				
PS	Styrene	51, 78, 104	104	0.08	5–20
	2,4-Diphenyl-1-butene	91, 104, 130, 208	91		
PET	Ethylbenzoate	77, 105, 122, 150	105 and 150	0.24	150–300
	Benzoic acid	51, 77, 105, 122	105		
Polyamide 6	Caprolactam	55, 67, 85, 113	113	0.24	50–150
Styrene-butadiene rubber	Cyclohexenylbenzene	104, 115, 129, 158	104	0.06	5–20
PLA	PLA:3,6-dimethyl-1,4-dioxan-2,5-dione	43, 45, 56, 144	56	0.39	150–300
	PLA*:3,6-dimethyl-1,4-dioxan-2,5-dione				
PBAT	PBAT:adipic acid dibut-3-enyl ester	55, 111, 129, 183	55	0.07	100–200
	PBAT:1.6-dioxacyclododecane-7,12-dione	55, 84, 100, 172	55		
Internal standard (d5-styrene)	d5-Styrene	54, 82, 109	109		4
	2,4-d10-Diphenyl-1-butene	96, 109, 134, 218	96		
Internal standard ($^{13}C_6$-styrene)	($^{13}C_6$)-Styrene	54, 82, 110	110		4
	2,4-Di-($^{13}C_6$)-phenyl-1-butene	97, 110, 136, 220	97		

m/z, mass-to-charge ratio; PBAT, polybutylene adipate terephthalate; PLA, polyactic acid; PLA*, stereoisomer of polylactic acid.

(ix) Measure the real sample. Note the sample intake.

(x) Determine the peak area of the specific pyrolysis products of the polymers (A_{sample}). Normalize it to the peak area of the internal standard (equation (6)).

$$\frac{A_{sample}}{A_{Int.St.}} = A_{n\,sample} \quad (6)$$

(xi) Convert the equation to x and set the value for a sample for the y-value to get the polymer mass (m_0) (equation (7)).

$$m_0 = x = \frac{f(x) - n}{m} \quad (7)$$

Raman spectroscopy

● **TIMING 1 h for measurement and 24 h for sample preparation**

▲ **CRITICAL** The measurement parameters are suitable for this specific instrument model. If other models are used, the parameters may need to be adjusted. A data analysis example is provided for Thermo OMNIC software, but other suitable software can be used.

Sample preparation for nanoplastics

16. Prepare a 500 µg liter^{-1} dispersion of nanoplastics in Milli-Q water.
17. Pipette one droplet of the dispersion on a glass microscope slide, place the slide in a closed Petri dish and let the droplet dry for 24 h in the dark.
    ▲ **CRITICAL STEP** When working with small particles, keep the samples covered to prevent air-borne contamination.

Measurement

18. Place the microscope slide on the Raman microscope stage.
19. In the light microscope image, focus on one larger particle or a dried agglomerate of particles.
20. Measure Raman spectra from the sample surface. Use an objective with 50–100× magnification; laser wavelength of 785 nm and laser power of 30 mW; full-range grating with 400 lines per mm, resulting in a spectral range of 3,300–50 cm^{-1} and spectral resolution of 5 cm^{-1}; a pinhole aperture; exposure time of 0.33 s; and a number of scans of 40.
    ▲ **CRITICAL STEP** The choice of objective/magnification depends on the particle size—the smaller the particles, the higher the magnification needed.
    ▲ **CRITICAL STEP** Suitable laser power, exposure time and number of scans vary between different instruments. The aim is to acquire high-quality spectra with high signal-to-noise ratio (SNR). If the SNR is too low (e.g., if it is less than ~100), increase laser power, exposure time and number of scans.
    ▲ **CRITICAL STEP** High laser power may destroy the sample. Increase the laser power carefully. Focus exactly on the surface of the particles/agglomerates to avoid getting a signal from the underlying glass slide. If the glass signal cannot be prevented, measure one spectrum with the same parameters from the clean surface of the glass slide. The glass spectrum can be subtracted from the sample spectrum.
    ▲ **CRITICAL STEP** The laser may induce fluorescence in the Raman measurement. The fluorescence curve is very wide and overlaps the Raman peaks. If the sample is fluorescent, Raman spectroscopy may not be suitable for identification of polymers.
    ■ **PAUSE POINT** The data can be analyzed at any time before starting the exposure experiments.

Data analysis: spectral library search

▲ **CRITICAL** Compare sample spectra to a spectral library of polymers. The libraries can be either commercial polymer libraries or in-house measured spectra of known plastics.

21. In Thermo OMNIC software, open *Analyze* → *Library Setup* and choose suitable libraries.
22. Click 'Search'. The software calculates correlations between sample and library spectra. In the results window, the library spectra are presented in the order of correlation to the sample spectrum. The rule of thumb is that correlations >70% can be reliable recognitions.

23. If the correlation is <80%, check manually that the peaks are similar in both sample and library spectra. If all peaks have the same Raman shifts (x-axis values) and shapes, and if proportional intensities between peaks are similar, the recognition is reliable. However, if there are extra peaks or missing peaks or peak positions vary, the recognition is not reliable even if the correlation values exceed 70%.

Procedure 2: exposure matrix for soil

▲ **CRITICAL** We describe how to create exposure matrices in soil and use them for studying earthworms.

Developing exposure matrices for a soil system

1. Prepare the soil by air-drying and sieving (<2 mm). It is recommended that each replicate test container contain between 500 and 600 g of dry soil for earthworm testing.
   ▲ **CRITICAL STEP** The use of four replicates is recommended for each test concentration. The mass of soil for all replicates should be spiked as one batch if possible (usually ≤2 kg) to reduce variability within a treatment. The mass of soil needed for each replicate container will depend on the test species. For (earth)worm species used in standardised testing, the following masses are needed: *E. fetida*, 500–600 g of dry weight–equivalent soil (OECD TG 222); *Enchytraeus crypticus*, 20 g of dry weight–equivalent soil (OECD TG 220).
2. Spike the nanoplastics into the soil as either a liquid suspension (option A) or as a solid (option B).
   (A) **Spiking nanoplastics in a liquid suspension**
   ● **TIMING 2 h**
   (i) Prepare a suspension of the MNPs in deionized water with a quantity of particles sufficient for all replicates of one test concentration to be spiked. The suspension can be prepared by adding a known volume of stock suspension to deionized water by using a pipette.
   (ii) Vortex the dispersion for a few seconds to ensure that the suspension is well mixed.
   ▲ **CRITICAL STEP** Although serial dilutions can be performed, separate suspensions prepared for each concentration in the concentration range is better, because errors can be propagated in serial dilutions in which homogeneous suspensions cannot be reliably achieved. This is more likely the case for larger MNPs, whereas for MNPs in the nano range, homogeneous suspensions can be achieved more readily.
   ▲ **CRITICAL STEP** The volume of the suspension should be lower than that needed to reach 40–60% of the water-holding capacity of the soil. Water-holding capacity is measured by using standard procedures (ISO, no. 11268-2)[46].
   ◆ **TROUBLESHOOTING**
   (iii) To the mass of dry soil required, add the prepared suspension for a given concentration to the soil batch.
   (iv) Rinse the tube containing the suspension with a known volume of deionized water and add deionized water to the soil to avoid any loss due to residues remaining in the tube.
   (v) Mix the suspension thoroughly with the soil by using a metal spoon and mix until there is no dry soil and the distribution of moisture in the soil looks homogeneous.
   (B) **Spiking MNPs as a powder**
   ● **TIMING 2 h**
   (i) Weigh the mass of MNPs that is needed to spike the mass of the batch of soil.
   (ii) Weigh the mass of MNPs that is needed to spike the total soil mass for all replicates of one test concentration in a large weighing boat.
   (iii) Add 50 g of dry soil from the weighed-out batch to the MNPs and mix this thoroughly. This helps to reduce static and creates a homogeneous soil-plastic mix before adding to the rest of the soil.
   ◆ **TROUBLESHOOTING**

(iv) Once the small subsample has been mixed thoroughly, add this to the larger soil batch.
(v) Mix this into the soil until mixed thoroughly.

3. Once thoroughly mixed, measure the moisture content of the soil by following the next steps.
4. Add water to 40–60% of the water-holding capacity and mix the soil again.
5. Collect samples from the top, middle and bottom of the spiked batch for validation of spiking.
6. Divide the wetted spiked soil between the four replicate containers, place a lid on each container and record the weight of the box along with the soil and lid.
7. Pierce holes in the lids of the containers.

PAUSE POINT There is no recommended method available for this currently, but samples are stable enough to preserve long term after air-drying. According to OECD guidelines (established for soluble chemicals), the recommendation is to incubate the spiked, wetted soils for 7 d before introducing organisms. In the case of metallic nanomaterials, this duration of incubation was shortened to minimize speciation changes or other potential transformations in the soils before organisms were introduced. In the case of micro- or nanoplastics, it would be assumed that if left dry in the soil, there would be little changes to any materials. As long as the soils are mixed well before wetting, the exposure should be comparable. Thus, it can be possible to have a pause point at which dry particles are added to dry soils.

Ecotoxicity test using earthworms (*E. fetida*)

TIMING 56 d

CRITICAL STEP The test will follow the OECD TG 222 Earthworm Reproduction Test (*E. fetida/Eisenia andrei*). The modifications are highlighted when it is required.

8. Leave the spiked and wetted soils overnight before introducing earthworms. It is also necessary to include a control treatment in replicate, which is soil without MNP amendment.

CRITICAL STEP MNPs without soil also should be used as control to understand the influence of the experimental conditions on the particles of interest (e.g., to ensure that the conditions do not lead to particle degradation or agglomeration). The addition of water to the soil can influence the behavior of the MNPs in the soil or wash the particles to the bottom. Mix the soil carefully after adding any liquid.

9. Perform the earthworm reproduction test as described in OECD TG 222.
10. Rinse the earthworms and dry them carefully with tissue. Record the batch weight of 10 earthworms. This is their initial biomass (B0).
11. To initiate the test, add 10 adult, fully clitellated earthworms (individuals = 300–600 mg in weight) to each replicate container. Add the earthworms to the surface of the soil in the test container.

CRITICAL STEP Some microplastics (e.g., hydrophobic microplastics with size >3 µm) might attach to the surface of the earthworm and lead to abnormal behavior of the organisms in comparison with the control without the particles. This can be considered as part of the experiments, and no action is needed.

12. Feed the earthworms by adding ~5 g of dry weight–equivalent (of, for example, re-wetted horse manure) to the soil surface after the earthworms have burrowed into the soil completely.
13. Place the lids on each container and record the weight of the container.
14. Throughout the exposure, weigh the container every 7 d and add water to maintain the moisture content in the exposure.
15. Incubate the containers in a controled-temperature facility at 20 °C for 14 d.
16. After 14 d, count the number of surviving adults and record their weight.
17. Return the animals to their containers, feed them and incubate for a further 14 d.
18. After 28 d, count and weigh the surviving adults. This is their final biomass (Bt). If reproduction is not the purpose of the toxicity test, the experiment can stop at this point. Otherwise, continue from Step 20.

19. (Optional) If the number of cocoons rather than juvenile numbers are of interest, wet-sieve the soil through a 2-mm mesh to capture cocoons for counting.
20. Return the soil samples, without adults, to the incubator for an additional 28 d to allow juveniles to hatch.
21. At the end of 56 d, count the juveniles. Containers are placed in a water bath at 60 °C, and the heat encourages juveniles to the surface, where they are picked off and counted.
    ▲ **CRITICAL STEP** Adult mortality in the control should not exceed 10% over the 28 d. Juvenile production in the control should exceed 30 juveniles by the end of the test. The coefficient of variation should not exceed 30% for reproduction.

Procedure 3: exposure matrices for water and performing the toxicity test with different aquatic organisms

Preparing and characterizing exposure matrics for aquatic systems

● **TIMING 48 h**

1. Prepare the medium for the toxicity test. Users might apply the following guidelines for their studies.
   - For testing using *D. magna*, use the M7 medium or ISO test water described in OECD guideline 202.
   - For testing using zebrafish, use dechlorinated tap water.
   - For testing using microalgae, use OECD TG 201 medium.
2. After preparing the exposure medium, separate aliquots of the solution. Disperse 1 mg of the MNPs in 100 ml of the prepared medium (i.e., fresh, brackish or salt water), shake the dispersion and leave the samples at room temperature in the dark. This solution will be analyzed to characterize the MNPs in the medium of interest, as well as for doing the toxicity experiments.
3. There are two characterization experiments that should be done. Follow the steps in option A to determine the agglomeration state and option B to determine the sedimentation rate.
   (A) **Determining the agglomeration state of nanoplastics**
   (i) Take 1 ml of the dispersion at time points 0, 1, 6, 12 and 24 h.
   ◆ **TROUBLESHOOTING**
   (ii) Measure hydrodynamic size by using DLS and following a previous protocol[44].
   (iii) Note the polydispersivity index for each measurement to ensure that the DLS operates within the validity range of the instrument used.
   (iv) Plot the hydrodynamic size versus time to obtain the homoaggregation profile of the particles.
   (v) If the polydispersivity index is within the validity range for the instrument and the hydrodynamic size/size distributions did not change significantly over time, the dispersions are stable against nanoplastic agglomeration.
   ▲ **CRITICAL STEP** If the particle size distributions change substantially over time, then the samples should be sonicated by using a bath sonicator (e.g., Elma Schmidbauer) for 10 min before use.
   ▲ **CAUTION** The process of sonication may increase the temperature of the dispersion and lead to degradation of the particles. To avoid this, the sample can be sonicated in an ice bath.
   (B) **Determine the sedimentation rate of MNPs**
   (i) Put the prepared dispersion in Step 2 in 50-ml tubes.
   (ii) Take 500 µl of the dispersion at time points 0, 1, 6, 12 and 24 h from the top 1 cm of the tube.
   ▲ **CAUTION** Leave the samples at room temperature in the dark and do not shake the samples during the experiment.
   (iii) Quantify the concentration of the MNPs as described in Procedure 1, Step 2.

(iv) Plot the graph of concentration versus time. If the concentration was stable over time, no sedimentation took place. If the concentration of MNPs decreased over time, the particles underwent sedimentation.

▲ **CRITICAL STEP** If the particles undergo sedimentation, the experiment could be terminated when the concentration of the MNPs in the dispersion decreases to <80% of the initial concentration (at time 0). If a long-term experiment is required, application of aeration, mixing the exposure medium regularly or replacement of the exposure medium can be applied.

■ **PAUSE POINT** After determining the behavior of the particles in a specific exposure medium, the start of the exposure test can be delayed. However, a new medium should be prepared on the basis of the obtained information immediately before the test.

4. The next step is to prepare fresh solutions as described in Steps 1 and 2 and perform the exotoxicity assays.

▲ **CRITICAL STEP** If the microplastics are prone to agglomeration and/or sedimentation, remember to sonicate and/or follow the advice in Step 3B(iv).

Ecotoxicity test using algae (*R. subcapitata*)

● **TIMING 72 h**

5. Prepare algal growth medium by following OECD 201. Allow for contact with the atmosphere to obtain CO_2 equilibrium (confirmed by a stable pH measurement around 8.0 ± 0.2).
6. Prepare an algal pre-culture 2 or 3 d before testing by adding 10^4 cells ml^{-1} of the desired algal species to the algal medium and placing it at the same conditions (light, temperature and shaking) as for the tests. This is done to ensure that the culture used for testing is in the exponential growth phase.
7. Prepare a stock suspension of your test material in the exposure medium.
8. Measure the pH in the stock suspension, and if this deviates more than 1 unit from the exposure medium, adjust the pH in the stock suspension with NaOH or HCl accordingly.
9. Calculate the volume of pre-culture (inoculum) that you must add to each 25-ml flask (i.e., the volume of test suspension prepared for each tested concentration) to achieve a start concentration of algal cells of 5×10^4 cells ml^{-1}, from the following equation:

$$C_{\text{inocolum}} V_{\text{inocolum}} = C_{\text{test suspension}} V_{\text{test suspension}} \quad (8)$$

where

V_{inoculum} = volume (in milliliters) of the pre-culture (inoculum)
C_{inoculum} = concentration of algal cells in the pre-culture (i.e., 10^6 cells ml^{-1})
$C_{\text{test suspension}}$ = concentration of algal cells in your 25-ml flask (i.e., 5×10^4 cells ml^{-1})
$V_{\text{test suspension}}$= volume of the test suspension in the flask (25 ml).

▲ **CRITICAL STEP** A starting cell density of 5×10^4 cells ml^{-1} is recommended to achieve a high enough SNR for accurate biomass determinations (i.e., to be able to differentiate algal pigment fluorescence from background noise resulting from particulate matter and the color of the test compounds).

10. Decide which concentration series you want to test and select five different concentrations (e.g., 10 µg $liter^{-1}$, 100 µg $liter^{-1}$, 1 mg $liter^{-1}$, 10 mg $liter^{-1}$ and 100 mg $liter^{-1}$). Calculate how much you need of the stock suspension to prepare 25 ml of test solution for each concentration.
11. Mark your 25-ml flasks with the chosen concentrations (including a control).
12. Add to the measuring flasks the amounts of the stock suspension that you need to achieve the different concentrations. Remember to include a control to which no stock suspension is added.
13. Add medium, but do not fill the flasks completely, because space is required for the algal pre-culture.
14. Add the algal pre-culture to the measuring flasks and fill the flasks to 25 ml with medium. Mix the content of the flasks properly.

15. For extraction and quantification of the biomass at the beginning of the experiment, transfer 0.4 ml of each test suspension into glass tubes and add 1.6 ml of acetone (saturated with $MgCO_3$). Use one sample for each concentration. Samples should be stored in the dark.
▲ **CRITICAL STEP** It is important that the lids are tightly closed. Include a blank (medium only) for background correction.
16. Transfer 4 ml of each test solution into 20-ml scintillation glass vials. Use three replicates per concentration and six replicates without added MNPs (negative controls).
17. Put on perforated lids (~1-mm hole) to allow for CO_2 exchange to ensure stable pH and CO_2 levels during testing.
18. Quantify the light absorption in the highest test concentration by using a spectrophotometer to find out whether shading at high particle concentration may be a confounding factor for interpretation of the test results. The issue of shading (i.e., indirect reduction in growth because of particles or sample color) has been extensively described for algal growth inhibition tests with particles (such as engineered nanoparticles)[47,48].
▲ **CRITICAL STEP** To overcome (or minimize) this issue, it is important to ensure homogeneous light distribution throughout the test samples while controling temperature. This can be achieved by using the LED vertical illumination table for algal toxicity tests setup[45].
19. At times 24, 48 and 72 h after exposure, remove 0.4 ml from each sample vial, place it into a glass tube and add 1.6 ml of acetone. Place the algal test vials on a shaking table again (except at the test end at 72 h).
20. Collected acetone-containing samples should be labeled clearly (date, types, experiment, etc.) and stored in the dark. It is important to close the lids tightly to avoid evaporation.
21. At the test end, pool the remaining test suspension from three replicates for each concentration in one vial (this is to have enough liquid for the pH measurement). Measure the pH of all concentrations and the control.
▲ **CAUTION** Discard liquid waste in an appropriate manner, according to applicable health and safety regulations.
22. After an extraction time of 48 h, measure the fluorescence of the acetone-containing samples in the fluorometer.
23. Calculate the algal growth rate (incubation time μ) in each vial as the slope of the growth curve (i.e., the natural logarithm of the flurorescence reading plotted as a function of incubation time).
24. Calclate the inhibition of the growth rate by dividing the growth rate in each vial by the average of the growth rates of the control vials.
25. Construct the concentration-response curves of the experiment by plotting the growth rate inhibition of each vial as a function of the MNP concentration in the respective vials.
26. Estimate the concentration-response curves and the EC_{10} and EC_{50} values by non-linear regression using an appropriate statistical model (e.g., the DRC module in R[49]).

Ecotoxicity test using daphnids (*D. magna*)

● **TIMING 48 h/21 d**

Immobilization

27. Prepare 1, 10, 50 and 100 mg liter^{-1} MNPs of interest in M7 medium or ISO test water.
28. Put 50 ml of the prepared exposure medium (each concentration) in different beakers of 100-ml volume. Use four replicates per concentration and include six replicates without MNP added (negative controls).
29. Select adult *D. magna* (greater than 24 h after hatching) for toxicity testing for 96 h, based on the OECD testing guideline (OECD TG202).
30. Put five individuals in each beaker (concentration).
31. At 48 h after treatment, consider daphnids with no swimming ability (immobilized) for >15 s after gentle agitation as dead.

Reproduction testing

32. Prepare 5 µg liter^{-1} nanoplastics or 10 mg liter^{-1} microplastics of interest in exposure medium.
33. Use 100-ml glass beakers with 50 ml of exposure medium.

34. Put one individual adult in each beaker and monitor 10 replicates for each treatment. Include 10 non-treated replicates as negative controls.
35. Maintain the test organisms at 23 °C and keep them under a 12 h:12 h (light/dark) photoperiod.
36. Renew the exposure medium every 48 h during the entire experiment.
37. For long-term exposure of *D. magna*, feed the organisms every 48 by using *Chlorella vulgaris* (8.92×10^6 cells ml^{-1}) according to the OECD guideline 211. Introduction of algae to the exposure medium can lead to the removal of some of the particles from the dispersion because of the attachment of the particles to the algae cells. Thus, the feeding should be done 6 h before renewing the exposure medium.
38. Observe the beakers daily during the 21-d experiment to determine the number of days to the first brood and the number of offsprings for each organism individually.
39. Determine survival by using Kaplan-Meier survival curves and calculate the cumulative offspring per individual and significant differences between the control and treatment groups.

Ecotoxicity test using copepods (*P. nana*)

● **TIMING 24 d**

Growth test

40. Collect ovigerous females and incubate them in prepared sea water (without particles) for 2 h to examine the retardation in developmental time.
41. Collect newborn nauplii for the experiments. To isolate newborn nauplii, use the proper pore sizes (45, 90 and 150 µm) of mesh (diameter of 75 mm and height of 20 mm); retain adults in 150-µm-pore-size mesh and isolate newborn nauplii with 45- and 90-µm-pore-size mesh. Then, keep them in an incubator during the experiment.
    ◆ **TROUBLESHOOTING**
42. Prepare exposure media with seawater containing the MNPs of interest at concentrations of 0.1, 1, 10 and 20 µg ml^{-1}.
43. Transfer 10 nauplii into a 12-well culture plate (SPL) with 4 ml of each of the exposure media.
44. Observe the developmental stages of nauplii in each of the exposure groups under a stereomicroscope (SZX-ILLK200, Olympus) every 24 h.
45. Finish observation when all nauplii have matured.
46. Continue observations on ovigerous females for 14 d to measure fecundity.

Reproduction test

47. Repeat Steps 40–46 and perform all experiments at 25 °C.
48. Measure fecundity by counting newborn nauplii every 24 h for 9 d.

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Step	Problem	Possible reason	Solution
Procedure 1			
1A(ii)	The lid of the grinding container is not screwed on tightly enough	Screw the lid tightly	Loss of material
1A(iv)	The instrument stops	No liquid nitrogen	Fill up the dewar with liquid nitrogen
1B(i)	Clogging of the ring sieve during milling	The starting material to be ground becomes too warm	Use the spoon to put fewer pellets into the grinding chamber
14	Retention time shift of the decomposition products in the chromatogram	Aging of the GC column	Add pristine polymer to the sample and measure again
	Unexpected polymer peaks in blind values	Carryover of pyrolysis products to next measurements	Clean the TED-GC/MS pyrolysis furnace and the coupling unit manually

Table 4 (continued) | Troubleshooting table

Step	Problem	Possible reason	Solution
Procedure 2			
2A(ii)	Preparing a stable suspension	Because of their density, particles can often float at the surface of the suspension or stick to the walls of the tubes	Adding some surfactant (e.g., SDS) can help produce a better-dispersed suspension
2B(iii)	Validation of test concentrations	Methodologies are not available for routine analysis of concentrations in complex matrices	Validation of spiking solutions would at least give a good measure of the mass added to the soils
Procedure 3			
3A(i)	Plastic particles are attached to the tips used for sampling	Because of hydrophobicity, some plastic particles are attached to the plastic tips	Application of different tips (e.g., glass) could solve the problem
41	Mix with different size of nauplii	Because of their unsynchronized hatching, some nauplii have different sizes	Use of a different size (45 and 90 µm) of pore in mesh

Timing

Procedure 1
Step 1A, ball milling: 1–2 g d^{-1}
Step 1B, centrifugal milling: 200 g d^{-1}
Steps 2–15, thermodesorption/extraction-gas chromatography/mass spectrometry: 3 h
Steps 16–23, Raman spectrometry: 1 h for measurement and 24 h for sample preparation

Procedure 2
Step 2A, spiking of nanoplastics in a liquid suspension: 2 h
Steps 2B–7, spiking of MNPs as a powder: 2 h
Steps 8–21, ecotoxicity test using earthworms (*E. fetida*): 56 d

Procedure 3
Steps 1–4, developing exposure matrices for aquatic systems: 48 h
Steps 5–26, ecotoxicity test using algae (*R. subcapitata*): 72 h
Steps 27–39, ecotoxicity test using daphnids (*D. magna*): 48 h/21 d
Steps 40–48, ecotoxicity test using copepods (*P. nana*): 24 d

Anticipated results

Size-dependent toxicity
In our previous publication, we determined the size-dependent toxicity of PS MNPs (0.05, 0.5 and 6 µm) in the marine rotifer *Brachionus koreanus*[21]. It was shown that small PS MNPs were more toxic in terms of several in vivo endpoints such as growth curve (Fig. 4a), fecundity (Fig. 4b) and life span (Fig. 4c).

Exposure to MNPs directly affected molecular endpoints using reactive oxygen species (ROS) induction (Fig. 5a,b) and MAPK signaling proteins (Fig. 5c,d) including ERK, JNK and P38 under the addition of 0.5 mM *N*-acetylcysteine (NAC) treatment to prove whether PS MNPs induced ROS. The 0.05-µm PS MNPs were the most highly induced ROS in rotifers. In Fig. 5c,d, p-JNK and p-P38, a marker of apoptosis, showed the strongest bands compared to other sizes, indicating that 0.05-µm MNPs were the most toxic among the tested partricles. P-ERK, a marker of proliferation, also showed a positive band, which suggests the dual activation of apoptosis and cell proliferation in 0.05-µm PS MNP–exposed groups.

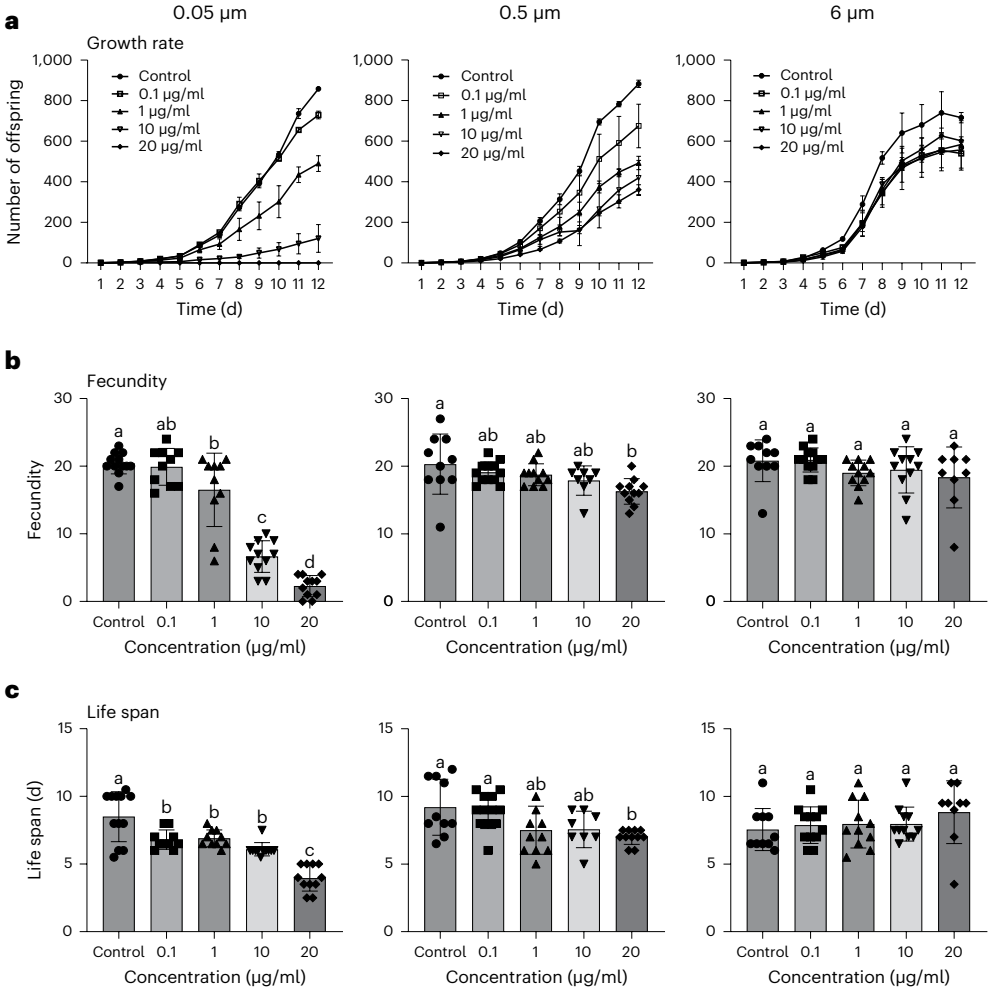

Fig. 4 | Size-dependent toxicity of MNPs. a–c, Effects of exposure to PS MNPs of different diameters (0.05 µm, 0.5 µm and 6 µm) on in vivo endpoints, including growth rate (**a**), fecundity (**b**) and life span (**c**). Different letters above columns indicate significant differences from one-way analysis of variance with post-hoc Tukey test ($P < 0.05$). The data are shown as mean and standard deviation. Figure adapted with permission from ref. 21, ACS.

Testing dynamic behavior of MNPs

In one of the studies, we optimized the exposure medium to minimize particle agglomeration and sedimentation. The ecotoxicity of PS nanoparticles of 270-nm size and their co-occuring benzo[α]pyrene (B[α]P), which was adsorbed from the surrounding water to the surface of the particles, to mussels, *Anodonta anatina*, was tested after 5 d of exposure[22]. The mussels were placed in glass aquaria containing 6 liters of the exposure medium and 2 cm of coarse sand (Beeztees, light aquarium gravel, 3–6 mm). The exposure medium was continually aerated, not only for aeration purposes but also to mix the medium and assist the particles in staying in the dispersion phase for a longer time. The behavior of the particles in the exposure medium was determined. Accordingly, the particles' hydrodynamic size was monitored (as described in Procedure 2, Step 2A(i–v)), and the water parameters were regularly measured to keep the exposure conditions the same over the exposure time. The data showed that the PS nanoparticles could penetrate the organisms' body and accumulate in the gills and digestive gland (Table 5).

When mussels were exposed to the mixture of nanoparticles and B[α]P, the number of particles in the gills and digestive gland was significantly higher (ANOVA, $P < 0.05$) compared

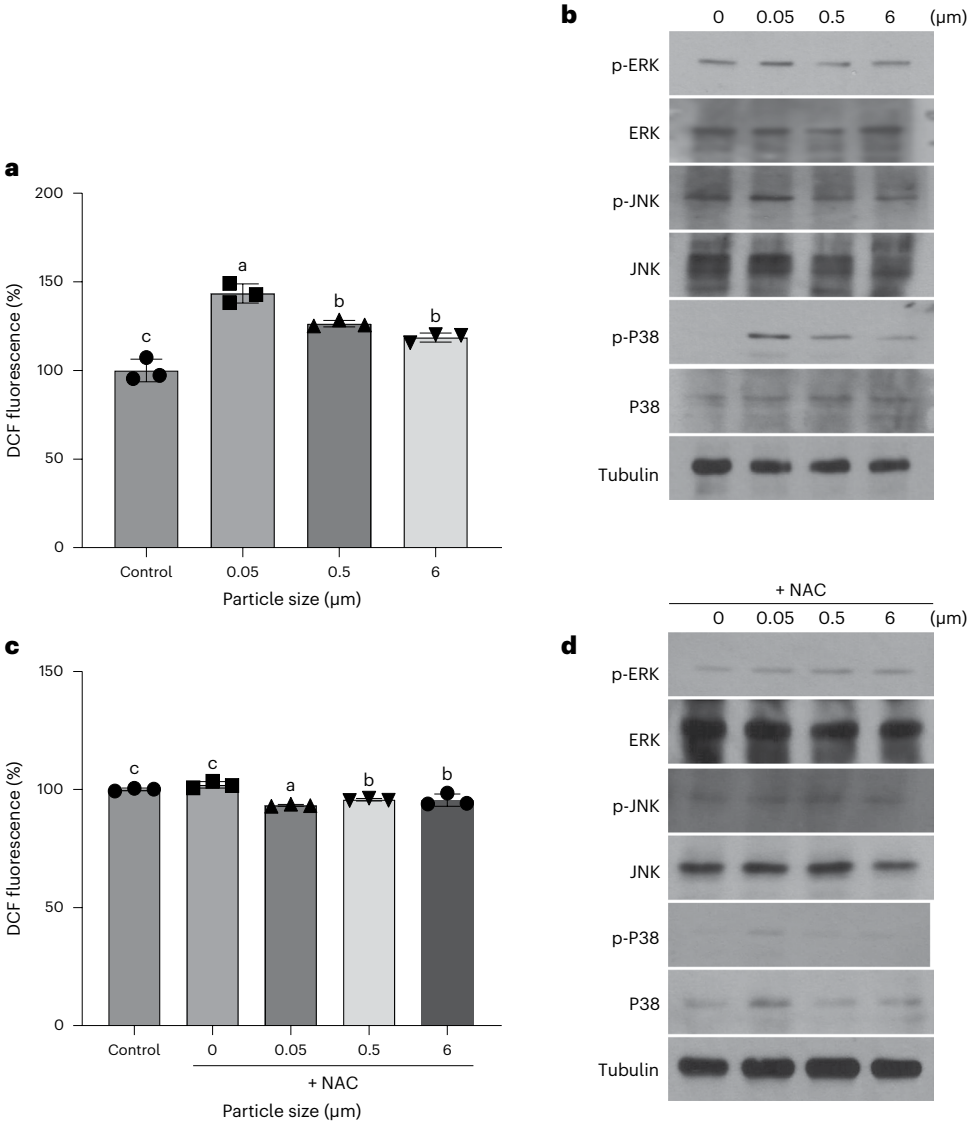

Fig. 5 | Size-dependent toxicity of MNPs on ROS and oxidative signaling. a,**c**, ROS levels without (**a**) and with (**c**) *N*-acetylcysteine (NAC) treatment. **b**,**d**, Phosphorylation of MAPK proteins without (**b**) and with (**d**) 0.5 mM NAC treatment. All PS MNPs were used at 10 μg ml^{-1}. Levels of ROS are represented as percentages of controls. Differences between groups were analyzed for significance by using Tukey's multiple comparison test. Different letters above columns indicate significant differences, defined as $P < 0.05$. Figure adapted with permission from ref. 21, ACS.

Table 5 | Number of PS nanoplastics measured in tissues of the exposed organisms after 72 h

Treatment	Gonad (particles per kg w.w)	Gills (particles per kg w.w)	Foot (particles per kg w.w)	Mantle (particles per kg w.w)	Kidney (particles per kg w.w)	Digestive gland (particles per kg w.w)
Control	ND	ND	ND	ND	ND	ND
PS	ND	$2.7 \times 10^3 \pm 6{,}421^a$	ND	ND	ND	$2 \times 10^4 \pm 2 \times 10^{3a}$
PS and B[α]P	ND	$6 \times 10^3 \pm 385^b$	$3 \times 10^5 \pm 4 \times 10^4$	ND	ND	$2.7 \times 10^4 \pm 2 \times 10^{2b}$

ND, not detected; w.w, wet weight. The results are the average and s.d. of nine replicates. Degrees of freedom = 8. ^{a,b}Significant difference ($P < 0.05$) betwen number of particles in different treatments (mussels exposed to PS MNPs and to the mixture of PS MNPs and B[α]P).

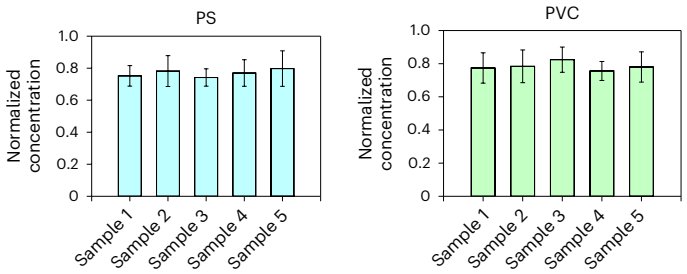

Fig. 6 | Testing homogeneity in MNP distribution in soil. To ensure that the MNPs were homogeneously distributed in the soil, the concentration of MNPs was measured in five samples (Samples 1–5) randomly selected from the spiked soil (mean ± s.d. of three replicates). Figure adapted from ref. 51, under a Creative Commons license CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

to the mussels exposed to PS nanoparticles alone. Moreover, the mixture of PS nanoparticles and B[α]P increased the activity of superoxide dismutase and catalase enzymes in the exposed mussels when compared to the control and to the nanoplastic exposure alone. Our work demonstrated that PS nanoplastics not only accumulate and alter the toxicity of organic chemicals in aquatic organisms, but that the chemicals also can facilitate the uptake of particles by damaging the cells.

Acute and chronic toxicity tests

We recently showed the importance of testing both the acute and chronic toxicity of secondary MNPs to *D. magna*[50]. *D. magna* neonates were exposed to microplastics, nanoplastics and leached plastic additives present in the filtered leachate of weathered plastic debris over a period of 48 h (acute exposure) or 10 d (chronic exposure). Although neonate survival was unaffected in the acute exposure, some sublethal effects were noted as a result of the chronic exposure: a significant increase in body length and reproduction was observed. Moreover, exposure to the plastic leachate led to a significant decrease in the curling of the thoracic appendages.

Developing exposure matrices for toxicity testing in soil

In two studies[51,52], we spiked soil with MNPs of different hydrophobicities (PS, polyethylene terephthalate (PET) and PVC). In the first study[51], troubleshooting was required when the spiked soil was mixed to homogenize the particle distribution. In this step, plastic particles stuck to plastic containers and spoons. To solve the problem, we mixed the soil in glass containers with a stirring bar made of glass. In this particular case, the particles were doped with gadolinum (Gd) to facilitate their detection in the complex matrices of the soil and plant. Note that this step (entrapping metals in nanoplastics) is not part of this protocol, and it is not required for exposure matrix development. The nominal concentration of ~100 mg kg^{-1} for PS and PVC MNPs in soil was selected. We ensured that the MNPs were homogeneously distributed in the soil by measuring the concentration of the particles (using Gd as a proxy) in randomly selected samples of the soil (five samples) (Fig. 6). We also ensured that the Gd did not leach out of the particles in the soil. Finally, we exposed lettuce to MNPs by culturing the plants in the spiked soil for 14 d. The lettuces were harvested, and the particles in the leaves were imaged by using scanning electron microscopy (Fig. 7). The images confirm that the MNPs were taken up by the plants from the soil.

Example for spiking soil

In the second study[52], microplastic fibers and MNPs were added to soil for bioaccumulation assessment. PS MNPs and PET fibers were spiked separately into the soils. In the case of the PS MNPs, the particles were supplied in suspension, and it was decided that serial dilutions would not be suitable to establish different spiking solutions because of potential for losses during

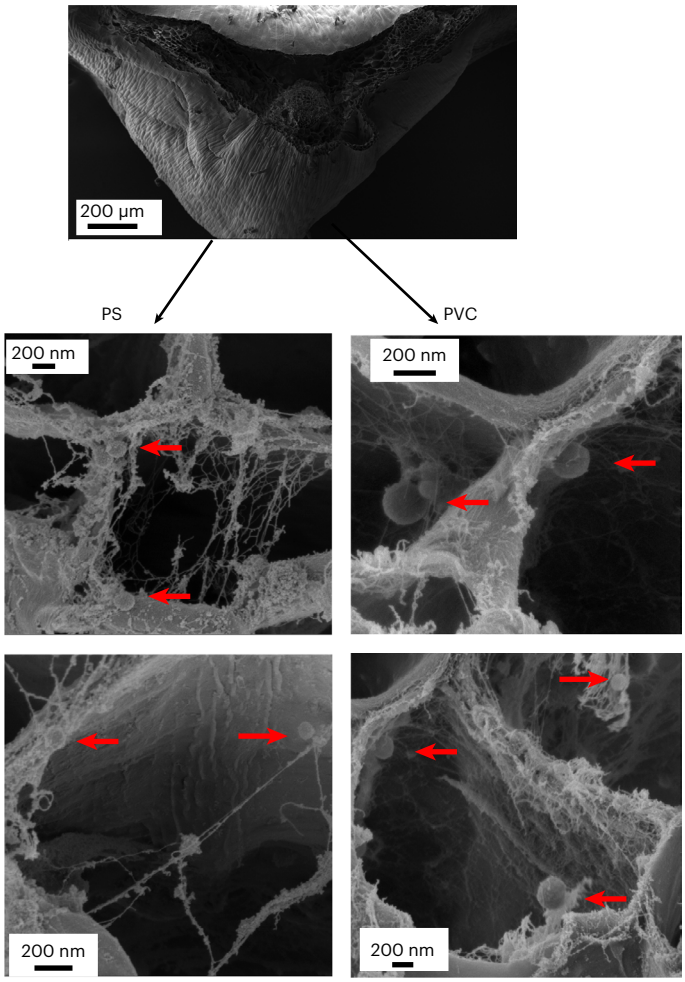

Fig. 7 | Nanoplastic uptake by plants. Scanning electron microscope images showing the presence of PS and PVC MNPs in the leaves of the exposed lettuces. Two images are presented for each particle. Note that the first (top) image of the leaves is just to illustrate from where in the plants the images (bottom) were taken. The red arrows highlight the positions of some of the particles inside the plants, as examples. Figure adapted from ref. 51, under a Creative Commons license CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

dilution; thus, for each concentration, an individual spiking solution needed to be established from the stock solution. In the case of PET fibers, static interference can be an issue in the weighing and mixing of fibers, but the dry spiking procedure was followed (Procedure 2B(i–v)), and this proved to be very reliable in terms of homogeneity of dispersion in the soil matrix achieved and reliability in establishing replicated exposures.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the data that support the plots within this paper have been previously published[21,22,50–52].

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F.A.M. designed the protocol, and conceptualized and supervised the protocol development. All the co-authors contributed to developing, writing, editing and reviewing the protocol.

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