

Supporting Information

Strategies for robust and accurate experimental approaches to quantify nanomaterial bioaccumulation across a broad range of organisms

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Statistical considerations related to ENM bioaccumulation measurements

Statistically representing the differences among means of compounded data may require propagating the error associated with each measurement into the final assessment of error. For example, bioaccumulation, biomagnification, or trophic transfer factors are calculated from the division of two concentrations. Researchers would have two choices: either calculate individual factors for each replicate experiment and then calculate the mean factor with its standard error, or calculate the mean concentrations and associated standard errors for each the denominator and the numerator, then calculate the factor as the ratio of the means; for the latter, the error for each mean would be propagated into the overall error, using standard methods.¹ When applicable, a best practice would be to calculate the final factor standard error using both approaches, since this would also ensure careful examination of the original data with its variances.

Preparation and characterization of ENM test suspensions in water

There are two basic approaches to prepare ENM test suspensions for exposure in water: either by adding the ENM powder directly or by use of an ENM stock suspension that is added to the test medium.² In either case, the initial ENM powder and/or stock suspension should be fully characterized before addition to the test medium, including ENM morphology, size, zeta potential, elemental composition, etc. The protocol used to prepare the ENM stock suspension needs to be fully described, such as the use of sonication (probe vs. bath; intensity and duration), shaking, or stirring for mixing and if a dispersing agent is used. It is important to test the pH of the test medium both before and also after adding ENMs, and if necessary to adjust the pH if ENM addition has changed the pH to a level unsuitable for organism growth. The actual ENM concentration in the beginning of the exposure should be confirmed in accordance with good practice for toxicity testing.^{3,4} The stability of ENMs in the exposure medium at different concentrations could be investigated separately before exposure.³ If ENMs tend to dissolve or agglomerate, relevant controls (e.g. dissolved ions and/or bulk counterparts) need to be included in the test, with the aim of determining if ENM bioaccumulation is a nanoscale-specific process.^{3,5,6}

Spiking of ENMs to soils

There are two main approaches to prepare an ENM-soil mixture: by adding either ENM powder^{7,8} or a stock suspension^{6,9} into the matrix. It is important to specify which method was used,⁴ as different approaches will influence ENM bioavailability and resulting effects in the soil matrix.¹⁰ In the published literature, standard protocols exist for performing exposure and hazard studies, with adaptations for spiking ENMs.¹¹ These protocols to some extent alleviate the issue of variation as they align the spiking methods, but even with such approaches there may be heterogeneity which may be especially important when exposing smaller invertebrates compared to larger invertebrates.^{4,12} After adding ENMs, it is important to ensure homogeneous mixing of ENMs into the soil. This is often achieved by using a rolling mill or food mixer; importantly, the mixing techniques should be reported, including the procedures used to avoid contamination across treatments.^{7,13} After mixing, the storage conditions and duration of time prior to testing should be reported; as noted above, storage may cause aging or weathering of ENMs which can modify behavior and bioavailability. In addition, plants were exposed to ENMs by irrigating plants using

ENM solutions.¹⁴ Obviously, the actual ENM concentrations need to be confirmed analytically at the beginning of the exposure, and preferably, also at the end of the exposure period.

Impact of body size on bivalve ENM measurements

In aquatic animals that take up chemicals via the gills, including bivalves that can filter large volumes of water, the exposure dose to the gill (or equivalent tissue) is controlled by ventilation volume and respiration rate. Thus, the respiratory physiology of the animal can influence bioaccumulation. Respiratory function is also dependent on body size. One study showed that volume specific respiration rates are higher for small mussels compared to large ones, and consequently, the Zn ENM accumulation rate in the small mussels was approximately seven times greater than for the large animals.¹⁵ This highlights the importance of detailing animal body length, water temperature, and body mass in bioaccumulation studies with ENMs. The scaling of body organs also changes with body size, and so the mass of the organ as a percentage of total body mass will also change with age. It may therefore be more revealing to report tissue concentrations as a fraction of the whole-body dose or correct for mass specific metabolic rate; but such reports remain rare for ENMs. In addition, bivalves have the potential to limit their intake of water for periods by closing their shell which could cause additional variability in ENM bioaccumulation measurements especially for shorter (several h) periods.

Relationship between volume of ENM suspension and *D. magna* ENM bioaccumulation

Depending on the uptake phase duration and ENM exposure concentration, the exposure volume should be carefully chosen as daphnids are filter feeders known to filter large volumes of water. An older study¹⁶ derived equations to estimate the volume filtered by several *Daphnia* species and is used to calculate the filtration rate for *Daphnia* with lengths of 1 mm or 4 mm. Considering an average body length of 4 mm for an adult *D. magna*, the filtration rate can be calculated for a 20 °C exposure to be around 10 mL/animal/h. In the OECD standardized protocol for the immobilization test that lasts for 48 h¹⁷, a volume of at least 2 mL of test solution per organism is specified. This amount was chosen based upon the lower filtration rates (1 mm size \approx 0.208 mL/animal/hour) for < 24 h juveniles which are tested in this method. In the case of bioaccumulation tests, the variability in terms of exposure volume is reported, but volumes smaller than 2 mL/organisms are typically not used. In addition, two main procedures are used regarding exposure and sampling. In the majority of cases, “destructive” replicates are used, i.e. at each sampling time some replicate beakers are removed from the experiment, in order to collect organisms for chemical analysis (e.g., e.g. ¹⁸). Conversely, in the case study of Ribeiro et al.¹⁹, replicates were 2 L jars with 120 daphnids in each, and 15 organisms were sampled per replicate per time point. In both cases, and depending on the media change frequency, organisms will potentially be exposed to a gradually lower concentration over longer exposure periods. Thus, the choice for the exposure volume used should be a compromise between exposure time, the age of the organisms (related to size) and the ENM concentration used.

Characterization of ENMs in soils, sediments, and organisms

Characterization and quantification of different forms of NMs in soils, sediments and organisms beyond measures of total metal forms, including speciation, are often restricted to dried

tissue samples (using, for example, micro X-ray absorption near-edge micro-spectroscopy (μ XANES) spectra^{20, 21} or electron microscopy (EM)^{22, 23}), soil pore water (e.g., by dynamic light scattering (DLS) or EM²⁴) or in the spiking matrix (organic soil extract by asymmetrical flow field-flow fractionation inductively coupled plasma mass spectrometry (AF4-ICP-MS) and EM²⁵ or DLS²⁶). Such analyses each represent one part of the characterization (e.g. oxidation state, atomic structure or simply total elemental concentration), which is why a combination of techniques is often preferred. Also, aspects relating to the complex nature of soil and tissues, including the presence of background metals, or colloidal materials that may interfere with analysis demands for targeted analytical strategies, using the strengths of different techniques. Furthermore, it should be noted that transformations of ENMs in the soil environment may change their speciation or form, potentially resulting in changes such as non-spherical particles or secondary particles formed in the soil.

Different forms of EM have been applied to detect and characterize ENMs in exposure media, including soil or soil extracts²⁷ and tissues of soil invertebrates (e.g., worms^{25, 27} and isopods²⁸). Transmission EM (TEM) has the advantage of precise and exact assessment of the dimensions of ENMs, although this technique can only be applied to ENMs extracted from the soil with potential inclusion of artefacts. Scanning EM (SEM) has been applied to detect ENMs *in situ* in exposure medium²⁹ and in worms, including the use of energy dispersive X-ray spectroscopy (EDX) to assess the chemical composition.²⁵ In a study on isopods exposed to tungsten oxide nanofibers, SEM-EDX was used to detail the interactions between the ENMs and cells in the organisms' hepatopancreas.^{28, 30} Although for SEM, ENMs do not need to be extracted from the matrix in which they occur, sample preparation includes, for example, drying of the materials which may also introduce artefacts such as agglomeration of the ENMs. These examples show that EM techniques can be used to specifically characterize ENMs in media (including soil) and tissues of soil organisms, although artefacts may be related to sample preparation and quantification of the ENMs in the samples is very laborious.

Confocal laser scanning microscopy (CLSM) can image ENMs based on autofluorescence, fluorochrome tagging or staining, or reflectance from objects that have higher scatter potential including metal particles.³¹ Stacking of separate 2-D images can be used to generate three-dimensional reconstructions that can show internal localisation of assimilated ENMs³². Studies of fluorescein-stained CeO₂ uptake in maize using confocal techniques have shown ENM aggregates in the cell walls of epidermis and cortex tissues.³³ In soil species, confocal microscopy has been used to image routes of uptake of silica ENMs in *C. elegans*, pointing to the pharynx and the vulva.³⁴ Confocal microscopy was also used for ENM detection in a study in which colloidal pH sensitive silica ENMs were used to assess pH in the gut of *C. elegans*.³⁵ However, apart from these studies on *C. elegans*, no studies are known to the authors that use this technique in other soil species. Uptake and localisation of Ag in isopods has also been imaged using synchrotron X-ray fluorescence (XRF).³⁶ These XRF observations were collected at the micron scale and showed the cell specific occurrence of Ag in the hepatopancreas of the organisms, co-distributed with Cu and S. XRF and μ -XANES were used sequentially in order to localise and characterise Cu in

earthworm tissues.²⁰ XRF was used at the micron scale to localise Cu, while μ -XANES was applied to those localized spots in order to characterise the speciation of the Cu. Speciation analysis of Ag in soil can be performed with extended X-ray absorption fine structure (EXAFS), a method that can be used to identify the oxidation state of Ag *in situ*³⁷. Other imaging techniques, like coherent anti-Stokes Raman scattering microscopy (CARS), use the inelastic scattering of monochromatic laser light to characterise the vibrational transitions of molecules. In the marine polychaete *Arenicola marina*, CARS was used to investigate the 3D internal distribution of small agglomerates of TiO₂ ENMs,³⁸ directly illustrating that the agglomerates did not pass the epithelium. In *C. elegans*, Raman imaging was used to monitor the biodistribution of rod, bipyramidal and quasi spherical TiO₂ ENMs.³⁹ Images showed the presence of agglomerates of TiO₂ NMs in phalangeal and vulva tissues, notably for rod-shaped ENMs which were also shown to show the highest impairment of pharyngeal function, reproduction and larval growth.³⁹

Secondary ion mass spectrometry (SIMS) uses a focused ion beam to release secondary ions whose mass/charge ratios can be measured to determine the sample elemental, isotopic, or molecular composition. Application of SIMS in soil ecology is developed jointly with the use of TEM to address the issue related to soil carbon dynamics at the micron-scale.⁴⁰ Although this potentially useful imaging technique has shown to be effective in imaging ENMs in tissues of aquatic organisms, to date no studies are known for soil species, with EM and X-ray spectroscopy methods largely being used instead.⁴¹

One approach to obtain information on the size-distribution in samples is by AF4 methods to separate different sizes of ENMs in samples, prior to their detection, similar to LC in LC-MS. In a studies on the effects of Ag²⁵ or Au⁴² ENMs on earthworms, AF4 in combination with UV-detection was used to characterize ENMs in extracts of the exposure soil. However, it may prove challenging to optimize the conditions of AF4 analyses for different types of relevant samples.

A good example of measuring ENM bioaccumulation in plants under soil exposure condition is a systematic investigation of CeO₂ and ZnO ENM bioaccumulation in soybean plants grown to bean production in an agricultural soil.^{8, 43, 44} At harvest, plants were dissected into different tissue types (stem, leaves, pods, roots, and nodules), with roots and nodules rinsed three times using deionized water.⁸ Subsamples of each tissue type were dried and analyzed for total ENM metal concentration by ICP optical emission spectroscopy (ICP-OES),⁸ metal distribution within plant tissues using EDS and X-ray microscopy,⁸ and synchrotron μ -XRF and micro-X-ray absorption near-edge structure (μ -XANES) to determine Ce and Zn speciation and map their distribution inside plant tissues.⁴⁴ Control plants without ENM exposure, the control soil, and the irrigation water were sampled and measured for background ENM metal concentration using ICP-OES.^{8, 43} ZnO ENMs were taken up by soybean and translocated into aboveground tissues.⁸ ZnO ENMs were not detected inside plants, but Zn-citrate was detected in the seeds.⁴⁴ On the other hand, CeO₂ NMs mostly accumulated belowground, in roots and root nodules.⁸ Although less was translocated to aboveground, most of the Ce in soybean pods was as ENMs, which indicates the potential of CeO₂ NMs introduction into the food chain.⁴⁴

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