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Nanomaterials in the aquatic environment: An EU-USA perspective on the status of ecotoxicity testing, research priorities and challenges ahead

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Abstract

The US-EU Community of Research (CoR) was established in 2012 to provide a platform for scientists to develop a 'shared repertoire of protocols and methods to overcome nanotechnology environmental health and safety (nanoEHS) research gaps and barriers' (www.us-eu.org/). Based on work within the Ecotoxicology CoR (2012-2015) we provide here an overview of the state-of-the-art of nanomaterials (NMs) in the aquatic environment by addressing different research questions with a focus on ecotoxicological test systems and the challenges faced when assessing nanomaterial (NM) hazards (e.g., uptake routes, bioaccumulation, toxicity, test protocols and model organisms). Our recommendation is to place particular importance on studying the ecological effects of aged/weathered NMs, as-manufactured NMs, as well as NMs released from consumer products in addressing the following overarching research topics: i) NM characterization and quantification in environmental and biological matrices, ii) NM transformation in the environment and consequences for bioavailability and toxicity, iii) alternative methods to assess exposure, iv) influence of exposure scenarios on bioavailability and toxicity, v) development of more environmentally realistic bioassays and vi) uptake, internal distribution, and depuration of NMs. Research addressing these key topics will reduce uncertainty in ecological risk assessment and support the sustainable development of nanotechnology.

Key words: Nano, nanomaterial, ecotoxicology, water, sediment

Introduction

As nanotechnology continues to evolve so do the test methods to assess the potential ecological effects of as-manufactured nanomaterials (NMs) and nanomaterials after their release from products that incorporate them. The widespread use of nanomaterials (NMs) has inevitably resulted in their release into the environment, either as the original (as-manufactured) nanomaterial, or more likely, as degradates of societal nano-enabled goods. Of particular interest is the aquatic environment, including sediments, which tend to be the ultimate sink for particulate contaminants. Once in the aquatic environment, NMs are highly affected by their surroundings and consequently undergo transformations (e.g., agglomeration, aggregation, dissolution, sulfidation). It is now clear that the fate and behaviour of NMs depends both on their physical-chemical properties and on the characteristics of the receiving environment including pH, temperature, concentration of natural organic matter (NOM), ionic strength and salinity, and water hardness (presence of divalent ions such as Ca^{2+} and Mg^{2+}). Aquatic environments can contain substantial amounts of naturally occurring particulates such as organic particles/colloids (e.g., macromolecules of humic acid from degrading leaf litter) and minerals (e.g., iron particles from the weathering of rocks/soil). However, our knowledge is far from adequate in terms of identifying exposure or hazard to enable an environmental risk assessment of NMs that is as robust as those we currently prepare for traditional chemicals. A particular challenge for environmental safety is to understand how the myriad of naturally occurring particles (many at the nanoscale) interacts with engineered NMs. One key concern is modifications to the NM surface by chemical reactions with the environment including the adsorption of organic ligands, metals, and naturally occurring colloids. The formation of the so-called "corona" on the surface of NMs and how it modifies over time is poorly understood. Together, all of these environmental processes may alter the NMs leading to very different physical-chemical properties of aged or released material compared to the original manufactured form. Further, the surface coatings or the development of coronas may alter the bioavailability of

75   NMs [1, 2]. This creates uncertainty when using results of research conducted with as-manufactured

76   NM to predict behaviour and effects in the environment.

77   There are also concerns about which aquatic ecosystems and compartments will be at most risk

78   from NMs. For traditional chemicals, the regulatory testing strategy usually initiates with aquatic

79   tests in the water column [3, 4]. However due to the settling behaviours of particulates, benthic

80   organisms and sediments are more likely to be exposed. Modelled average sediment concentrations

81   of NMs are often several orders of magnitude higher than in the overlying water [5]; for example,

82   the average concentration of CNTs in surface waters ranged from 10^{-3} µg/L to 10^{-5} µg/L while the

83   concentrations in sediments ranged from 1 µg/kg to 1 mg/kg, although these units are not directly

84   comparable. One might argue that benthic organisms especially, and those in the water column,

85   have evolved in the world of natural colloids and other particles. However, the unusual chemistries,

86   reactivities and shapes of engineered NMs may present different hazards. Natural colloids are also

87   critical to many fundamental biological processes (biofilm formation, biocrystallisation, etc.,) and

88   how engineered NMs modify these biological foundations of ecosystem function is poorly

89   understood.

90   Currently, knowledge of biological effects in the aquatic environment is skewed towards studies on

91   as-manufactured NMs in aqueous acute tests using pelagic organisms. This is clearly demonstrated

92   by recent literature searches using the Web of Science (Table 1). While more than 900 000 hits

93   were recorded using 'nano*' as a search criteria, most published literature included the term 'water'

94   with about 31 times fewer papers addressing 'sediments' (Table 1). Clearly, only a small fraction of

95   published research concerns sediments (Table 1). A comparison of hits using 'accumulation' or

96   'effect' together with 'nano' showed that there is a significant bias towards effect studies (20 times

97   more). Furthermore, most published papers seem biased toward pelagic organisms with fewer

98   studies on benthic organisms. Of the benthic studies, the freshwater oligochaete *Lumbriculus*

99   *variegatus* and *Chironomus riparius* and the estuarine polychaetes *Capitella teleta*, and *Nereis*

100   *diversicolor* have been the focus of some sedimentary studies (e.g., [6-14]). Another group that has

been the focus of an increasing number of studies (although still in very low numbers) is the molluscs, with the freshwater snails *Lymnaea stagnalis* and *Potamopyrgus antipodarum* and the marine mussel *Mytilus* spp, being the main focus (e.g., [15-17]).

The number of studies on environmentally modified ('aged') NMs, long-term chronic effects, bioaccumulation, and exposure of benthic (sediment) organisms is substantially fewer. It is recognized, however, that these studies are urgently required to provide a comprehensive understanding of the potential effects of NMs after release into the natural environment. Moreover, the behaviors of NMs (e.g., dissolution, agglomeration) and their potential to cause artifacts in standard aquatic toxicity tests suggest that standard tests will likely need to be modified to test for potential ecological effects of NMs.

The US-EU Community of Research (CoR) was established in 2012 to provide a platform for scientists to develop a 'shared repertoire of protocols and methods to overcome nanoEHS research gaps and barriers' (www.us-eu.org/). The overall goal of the Ecotoxicity Testing and Predictive Modeling CoR (Ecotox CoR) is to encourage the evolution of: i) hazard assessment methods and predictive models built on the foundations of fundamental research characterizing fate (including ageing) of nanomaterials in different environmental compartments and the interactions of nanomaterials with biota and ecosystems: ii) knowledge on state of the art of bioaccumulation, effects and mechanisms and conveying this information to relevant stakeholders: and iii) communication among regulators, experimentalists, and modellers to make data available/presented in a useful format to help modellers, experimentalists and risk assessors (www.us-eu.org/). Based on ongoing work in the Ecotox CoR and three Ecotox CoR workshops (2013-2015) we provide here an overview of the state-of-the-art of NMs in the aquatic environment and discuss the challenges ahead by providing suggestions for future research needs that will enable us to reduce uncertainty in ecological risk assessment and thus improve the quality of NM risk assessment.

This paper builds on our current understanding of as-manufactured NMs in addressing different research questions with a focus on ecotoxicological test systems and the challenges faced when

assessing NM hazards (e.g., uptake routes, bioaccumulation, toxicity, test protocols and model organisms) highlighting the main knowledge gaps, challenges and suggestions on how to focus future research.

Challenges in aquatic toxicity testing of nanomaterials

A key challenge in aquatic toxicology testing of NMs is that exposure is often not constant because particle settling and other transformations typically occur during the tests. In addition, methods to characterize and quantify NMs in experimental media and in environmental samples are time consuming, may require specialized equipment, or may not yet be available for complex matrices (e.g., sediment), thus creating significant uncertainty when trying to relate dose and organism response [18]. There may also be differences in results among laboratories given that the dispersion methods used often vary among laboratories (e.g., probe sonication or stirring in water) and there are many different forms of the same nanomaterial (e.g., graphene, graphene oxide, few layer graphene, etc.) that can be produced by different synthesis methods. Ecotoxicity testing of conventional chemicals, where there is adequate understanding of the contaminant fate and behaviour, can often keep a reasonably constant exposure concentration throughout the bioassay. This is in clear contrast with the testing of particulate contaminants in general and NMs especially. Furthermore, traditional aquatic testing often relies on steady mass concentrations of the test substance over fixed exposure times to deduce the exposure dose (i.e., concentration x exposure time = dose). This simple "two dimensional" approach may be problematic for use with NMs [19]. For example, in a mesocosm test with benthic and pelagic species, settling may result in increasing exposure concentrations for benthic species yet decreasing concentrations for pelagic species. Such problems are not just of scientific concern, but also have practical implications for testing strategies; for example, excessive aggregation might invalidate or limit the use of tests for screening high concentrations of NMs. There has also been much discussion about dose metrics, and whether or not to continue to use mass concentration for NMs or use some other metric such as surface area or

153 particle number concentration. However, there are examples in the literature illustrating both the

154 classic concentration responses and non-monotonic relations with NMs [18, 20]. When possible,

155 depending on sampling and analytical considerations, it may be useful to quantify NM

156 concentration, particle number, and surface area. Further, characterizing these metrics over time

157 during a bioassay would provide insight into the integrated exposure that the organism experiences.

158 This often proves a practical challenge due to lack of available methods.

159

160 *Characterization methods* Regulatory testing requires that the concentration of the test substance is

161 known, that its change during the bioassay is characterized, and that the exposure is confirmed by

162 measuring the test substance in the exposure media and/or the organism. In addition, there is

163 uncertainty about which types of characteristics of the initial material should be measured prior to

164 and during toxicity testing. Standardized methods are available for some but not other NM

165 characteristics and each additional characterization technique raises the cost and increases time

166 required for the ecotoxicity test. One challenge is that there is a lack of characterization methods for

167 detecting and quantifying NMs in complex environmental samples that are accurate, precise and

168 available for use in a standard laboratory (reviews of current methods are available, e.g., [21, 22]).

169 For example, it is possible to detect NMs in tissues using advanced microscopic methods

170 (hyperspectral imaging, confocal microscopy, or near infrared fluorescence) depending on the NM

171 properties. Electron microscopy (EM) can also provide unequivocal identification of intact NMs in

172 tissues, and perhaps even localization/tissue distribution; but these measurements are challenging,

173 time consuming, expensive, and can usually only provide biodistribution information about a

174 limited number of organisms or area of the organism. Furthermore, care should be taken when using

175 EM only to identify NM since artifacts are common [23, 24].

176 There are some emerging approaches that hold significant promise for enabling these

177 measurements, but which are, at this stage, far from being standardized and widely available. One

178 example is single-particle inductively coupled plasma-mass spectrometry (spICP-MS), an approach,

179   which has the advantage of providing a size distribution of the NMs in the tissue of interest [25].

180   However, such methods are limited to metal or metal oxide particles that will survive the chemical

181   digestion processes needed to make a liquid sample for ICP-MS, and the detection of particles <20

182   nm is problematic with this method for some elements. Subcellular fractionation techniques may be

183   used to examine the intracellular compartmentalization of metals administered in different forms

184   (e.g., as metal salt and metal NMs) and can elucidate differences in handling and mechanisms of

185   detoxification of internalized metals. The distribution of the metal among different subcellular

186   compartments can reveal implications for cell and organism health. However, it is important to

187   ensure that the subcellular fractionation procedure (i.e., centrifugation technique) is not altered by

188   the presence of NMs. In addition, for metal NMs, it is often not clear if the particulate form

189   observed within the tissues was taken up as NM, or as a soluble form, which was then precipitated

190   in the tissues in particulate form. Although the latter is less likely, the inclusion of control

191   experiments is important to test for this possibility [24].

192   Having readily available, quantitative methods for NMs in different matrices will provide insight

193   into the potential effects of NMs. For example, linking NM exposure to organism body burden

194   further clarified by quantitative measurements of NM distribution within the organism would likely

195   lead to key mechanistic insights [14, 26]. Further, having reliable and rapid measurements of NM

196   concentrations and transformations in different environmental media could enable more accurate

197   characterization of the exposure dose and provide insight into the benefits of additional

198   concentration metrics such as particle number and surface area. Although this is an important and

199   interesting area, it does rely heavily on the availability of techniques that allow these measurements

200   in aqueous samples. Another key area of research that would be feasible with improved analytical

201   methods is the characterization of NM transformations and concentrations in soils and sediment.

202   This remains a substantial research challenge for many NMs [18]. Finally, an important research

203   area is the study of fate and effects of NMs released from nano-enabled consumer products. Key

204   research topics are summarized in Table 2.

205   *Potential artefacts in nanoecotoxicity testing* One key consideration for testing the ecotoxicological

206   effects of NMs is that they may cause artefacts as a result of their different properties and

207   behaviours compared to stable, water soluble chemicals. These potential artefacts and

208   misinterpretations can occur at all stages of the testing procedure starting from procuring the NMs

209   (their physical-chemical properties sometimes dramatically differ from manufacturer specifications)

210   to assessing their distribution in organisms or cells [3, 4, 24]. Many of these potential artefacts are

211   illustrated in Figure 1. It may also be important to conduct control experiments to differentiate

212   between direct toxicological effects from the NMs on the organisms and indirect effects such as

213   nutrient depletion. Testing for NM artefacts is especially important for photoactive NMs, which

214   may cause damage to biomolecules from light exposure during sample processing after the

215   exposure assay is finished, and for NMs with strong absorbance or fluorescent properties that could

216   impact assay measurements [3, 4, 27, 28]. Including relevant control experiments (described at

217   length in [24] and also in [3, 4]) during nanoecotoxicity testing will enhance the reliability of the

218   data, facilitate standardization, and likely increase agreement among results obtained from different

219   laboratories. Some control experiments include testing the potential effects of ions for NMs that

220   dissolve in water, filtrate only controls to test the potential impact of toxic impurities (e.g., metal

221   catalysts on carbon nanotubes), testing of the same core materials of a larger size, and a coating

222   control to assess if the coating could have a toxic or stimulatory impact.

223

224   *What parameters to measure and report?*

225   One helpful step that will likely increase the reliability of nanoecotoxicology test results is to

226   standardize the supporting measurements and data reporting. Some suggestions along these lines are

227   provided in standard ecotoxicology methods for soluble, stable chemicals. For example, many

228   Organization for Economic Cooperation and Development (OECD) standard aquatic toxicity tests

229   require measurements of the concentration of the chemical compound at the beginning and end of

230   the experiment (e.g., OECD 202 *Daphnia* sp. Acute Immobilisation Test and Reproduction Test).

The specification for many of these tests is that the concentration of the test substance should change by less than 20 % (OECD) or 30 % (ISO and US EPA methods) during the exposure period. Thus, measuring the NM concentration at the beginning and end of an experiment is suggested as a minimum frequency. However, as described above, quantitative measurements of NMs in water may be challenging especially in the presence of natural organic matter or cellular organisms such as algae. In addition, NMs may undergo various changes during the aquatic toxicity test period (dissolution, agglomeration, etc.). While it is well known that NM will be transformed in the environment (e.g., oxidation of carbon NMs), the impact of long-term transformation processes on nanoecotoxicity results has generally been less frequently studied. One exception to this is the sulfidation of silver nano-particles (Ag NPs). This process occurs during transit through wastewater treatment plants, and has been shown to dramatically decrease Ag NP toxicity [29]. Monitoring these changes is even more complex in sediments as a result of analytical difficulties. Environmental modification of NMs may increase their stability in water such as when graphene is oxidized [30]. Alternatively, for metal particles, mineralization or dissolution may also lead to their removal from the water column. Therefore, characterizing changes to the NM, such as agglomeration or dissolution rates in the defined test media, and during the tests when the organisms are present may be critical to understanding the exposure, and thus subsequent toxic effect. Chemical oxidation and other phenomena related to particle stability also raises the issue of what aspects of the test media should be monitored. Often in traditional aquatic toxicity tests, the water measurements are restricted to pH, dissolved oxygen, and the general ionic composition and hardness of the media. However, other measurements may be justifiable for NM tests. For example, would the measurement of redox potential or sulphur compounds give an accurate understanding of what chemical form organisms are being exposed to during a test with Ag NPs? Would such additional measurements be justified in terms of time, cost and resources for a regulatory test?

Quantifying changes to NMs in sediments during ecotoxicity experiments remains especially challenging. Currently, methods for characterizing exposure are limited to measuring total metal

257 concentrations when metal-containing NMs are used. Often the particle size distribution and

258 changes in this due to dissolution or aggregation processes cannot be measured readily in soil or

259 sediment because of the large background of naturally occurring particulates. However, thorough

260 characterization of sediment characteristics (organic matter concentration, particle size, etc.) used in

261 nanoecotoxicity testing is important and considered critical for future modelling efforts. The debate

262 concerning the use of standard artificial sediments (aka OECD protocols) vs natural sediments

263 continues. The latter confer additional reality to the tests and also allows for results to be more

264 widely applicable. The use of standard artificial sediments, however, facilitates laboratory

265 comparability, and this line of thought is not different for NMs when compared to hazard testing of

266 conventional chemicals [3, 4].

267 *Which model organisms to use* Rapid agglomeration and settlings of some NMs suggests that

268 testing pelagic organisms may have less environmentally relevance than benthic organisms. While

269 all pelagic organisms will be exposed to NMs and their transformation products in the water

270 column, the group of filter feeders (e.g., *Daphnia magna*) will be exposed to NMs and their

271 agglomerates in the water column while filtering water for food. For animals that breathe in water,

272 the gills or other respiratory surface are vulnerable to chemicals due the anatomical features that

273 enable respiration to occur, including: a large surface area, small diffusion distances to the internal

274 body fluid (e.g., blood), and high blood flow (perfusion of the respiratory surface). This

275 vulnerability also applies to NMs. Another consideration is mechanical suffocation (non-chemical

276 toxicity) in aquatic organisms; however, measurements to quantify this effect are not currently

277 included in regulatory tests. Benthic species (both epi- and infaunal) will be exposed either via

278 direct body contact with sediment-associated NMs (i.e., bound to sediment particles, from pore

279 water and overlying water while irrigating) or through ingestion of settled NMs associated with the

280 sediment, biofilms, or other food sources. For regulatory testing, these issues are pragmatically

281 framed around the notion of exposure routes (water, food, sediment) for traditional chemicals, and

282 the weighting of evidence in the environmental risk assessment might be more towards the results

283 of (for example) sediment testing where effects on the benthos are a concern. For NMs the overall

284 testing strategy may need adjusting so that more consideration is given to soil/sediment tests

285 compared to the base set of acute aquatic tests (algae, *Daphnia*, fish; [4]). However, such thinking is

286 based on nearly a hundred years of epithelial biology where substances are taken up by ubiquitous

287 active solute transporters, facilitated diffusion, or passive diffusion depending on the membrane

288 biology, water permeability, and anatomy of the biological barrier/organism. This has arguably led

289 to a selection of regulatory test organisms where these features are well-known. However, NMs

290 bring new challenges to epithelial biology. Most materials are too large to use solute transporters or

291 simple diffusional processes, and internalisation via endocytosis and related mechanisms has not

292 been documented. However, with the huge diversity of biological barriers in the animal kingdom

293 alone, there is no guarantee that the traditional test organisms that are used in regulatory

294 ecotoxicology are the "best" or "most representative" organisms to use to account for this mode of

295 uptake. Current legislation is geared towards "protecting most of the organisms most of the time"

296 and without biological barrier or uptake information on NMs across a range of phyla and life stages

297 we may not achieve this with our current test organisms or bioassays. Work on marine species and

298 other organisms currently not used in regulatory ecotoxicology are needed to identify vulnerable

299 anatomical features or groups of organisms.

300

301 *Using the organisms to measure exposure?*

302 The difficulty in measuring NMs in exposure media and complex environmental matrices has

303 already been discussed above; yet, regulatory tests require some confirmation of the exposure. Of

304 course for traditional chemicals, an alternative approach is to define the exposure by measuring the

305 test substance in/on the organism (e.g., apparent bioaccumulation, net uptake), or by quantifying

306 biological responses that are well-known to be associated with the exposure (i.e., biomarkers of

307 exposure). The following sections explore these two approaches, and whether or not they can be

308 applied to NMs.

309

Confirmation of exposure through body-burden assessment?

Bioaccumulation terminology for dissolved chemicals may be misleading for NMs There are several important differences between uptake of NMs and traditional dissolved chemicals that complicate usage of the same terminology. Mainly, the uptake of NMs does not reach a steady-state equilibrium condition and concepts that rely on steady-state concentrations (ratios) between the external compartment and the organism (i.e., bioconcentration factor, biota sediment accumulation factor) are in most cases not appropriate for use with NMs unless caveats are included to clearly distinguish the difference from traditional dissolved chemicals [3, 4, 18]. Instead, terms such as body burden, which do not make assumptions about equilibrium being reached, or the biodistribution in the organism are encouraged. Overall, this is an area where consensus has not yet been reached in the nano-ecotoxicology field. However, a prerequisite for regulatory use would include defining a test or measurement that is analogous to the concept of bioaccumulation for dissolved chemicals. While almost all studies on this topic have demonstrated a lack of NM absorption across epithelial cells, a study with *Drosophila melanogaster* fed with single-wall carbon nanotube spiked food showed that only a small fraction (10^{-8} of the total dose of ingested nanotubes) were translocated to other tissues in the organism [31]. Overall, NMs do not readily pass through the epithelial tissues in the gut tract or the surface skin [32], or may be slower to absorb compared to solutes, so further work on the timescales of such tests will be needed. Wray and Klaine [33] examined the influence of particle characteristics (Au NP surface charge, size and shape) on total body burden in *D. magna* and found no evidence that Au NP were absorbed across epithelial membranes, a result similar to other studies with CNMs [23, 34, 35]. These authors discuss the possibility that a part of the ingested NPs may adsorb to gut structures (e.g., microvilli) and that these have a slower transport out of the gut compared to nanoparticles, which are not in contact with gut structures. In any case, clear terminology should be used so that such measurements for NMs are not confused with those for soluble chemicals with very different

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properties and biokinetic principles. Moreover, NMs may undergo surface transformations in the gut (e.g., coated with a protein corona) with implication for uptake and depuration kinetics in predator organisms. However, only a few studies have been published on trophic transfer [36] so more information is required to address this question.

Body-burden assessment Although bioaccumulation constitutes an important part of risk assessment, there is not much information in the literature on NM bioaccumulation. Of these studies, the majority has reported total body burden after the conclusion of the experiment, while only a limited number have focused on uptake and depuration kinetics and NM transformations in the organisms (examples of recent work in this area are; [8, 9, 14, 16, 17, 26, 35, 37, 38]. Most likely as a result of limitations in availability of analytical methods and instruments, even fewer studies have been published on internal distribution of NMs after exposure [6, 23, 34, 39], or on trophic transfer (examples include [26, 36]). A weight of evidence is needed with different NMs and organisms to confirm the utility of simple body burden measurements for NMs and the theoretical basis (uptake mechanism, rate limiting steps, etc.,) that define the validity or utility of the approach.

Use of reference substances in body burden-related assessments for NMs One approach that has been used to determine the NM component of ecotoxicity for a NM is to compare toxicity results from NM exposure with the toxicity of the ionic form for NMs that dissolve, or of a larger bulk form (e.g., micron scale) of the same chemical substance. This approach provides a means to compare bioavailability and toxicity of NMs with the conventional form of the same chemical substance. Some studies have observed nano-related effects (both including effects on different endpoints and more pronounced effects on the same endpoints) both at the whole-body level and subcellular level, while other studies have shown higher toxicity from the bulk or ionic form (see [40, 41] for examples on metal NPs in sediment systems). For example, in trout the target organs for nano Cu are broadly the same as $CuSO_4$, but the rate of appearance and severity of organ

360   pathologies may be different [42, 43] and toxicity may be at least partly caused by dissolved ions

361   for NPs that dissolve during the test period.

362   In principle, the reference treatment does not need to just relate to the chemical substance (e.g.,

363   dissolved versus particulate), but could be extended to the different forms (crystal structures of the

364   same chemical), size and shapes of NMs. In an aquatic water column test, or cell culture media such

365   reference substances may be less difficult to measure. The matrix of soils or sediments presents a

366   difficult challenge (for the reasons above). However, if we move our thinking away from the test

367   media to the organism itself, measurements may be less problematic (decreased particulate

368   background noise within the organism compared to sediments). A body burden test system with

369   reference chemicals or treatments would require some consistency in the exposure dose. The same

370   concentration of the compound should be included in all treatments. For these types of experimental

371   setups different forms of well-defined test substances (e.g., NM, bulk, ionic metal, different NM

372   sizes and shapes) will be needed so that concentrations are reliably compared. For example, the use

373   of mass concentration (e.g., mg/l) of a metal may require correction for surface coating (oxide

374   formation) or the presence of organic matter that changes the molecular weight of the primary

375   particle. These are not minor considerations when organic surface coating on a 20 nm metal particle

376   might occupy 30 % or more of its mass. Interestingly, gut epithelial cells can distinguish between

377   crystal structures of the same NM, and selectively take up certain crystal forms (e.g., of titania,

378   [44]). How and why this occurs is unclear, but it raises the concern that risk assessments may need

379   to consider crystal structure as well as size when exploring the bioaccumulation potential of NMs.

380

381   *Confirmation of exposure through biological response assessment?*

382   *Internal distribution in organisms and biomarkers of exposure* The alternative to measuring the test

383   substance itself in and on the organism is to determine its presence indirectly from biological

384   responses of the whole organism, or preferably key target organs/cellular compartments. Such ideas

385   are well established for soluble chemicals. For example, the liver is a central compartment for the

metabolism of organic chemicals while chaperone molecules serve to modulate metal concentrations in the blood and inside cells. However, in order to use biomarkers of exposure for NMs, at least two fundamental pieces of information would be needed: (i) where does the NM go inside the organism (choice of target tissue/cells); and, (ii) what does it do when it gets there that provides a unique biological signal of the presence of the material? The former is dogged by the ever-changing corona on the surface of the NM, dissolution and re-precipitation (e.g., in the gut) and how this might influence uptake and biodistribution. For example, in sediment tests it might be expected that the NM corona and speciation will alter in the sediment matrix, leading to measurable differences in bioavailability. Increasing evidence suggests that metal NMs are available for uptake via the dietary route of exposure (diet and sediment) and that sediment-dwelling organisms may accumulate metal NMs. However, the digestive anatomy (chemical environment of the gut) is well known to alter the uptake kinetics of metals and organic chemicals. The effect of the gut lumen chemical environment on corona formation, dissolution and re-precipitation on NMs also needs to be studied. This cannot be done in isolation of the mechanical anatomy of the gut, as some of this biology is specifically designed for sorting food by particle size. For example, polychaetes have a conveyer-belt feeding manner where all particles are transported through the worm and defecated. Mollusks, on the other hand, have an internal sorting mechanism in the gut and digestive diverticula where smaller-sized particles will be retained in the digestive gland and larger-sized particles will be transported in the intestine. The underlying science for understanding the relation between particle size and digestive physiology for accumulation is poorly developed and our ability to predict ecological consequences of different NMs is therefore limited. Similar information is needed for fishes and other vertebrate animals. However, a prerequisite is to understand what corona forms in the exposure media, then in the mucous epithelia of the organism (uptake surface), and then the blood (extracellular fluid) and the tissues (intracellular environment); as well as how this changes over time (degradation/dissolution) within each of these compartments. For fish, NMs

411   might also adsorb to the outside of the gill, and so a measurement of these tissues might provide a

412   more relevant exposure concentration, even if a bioaccumulation parameter cannot be determined.

413   Determining a biological signal that indicates the presence of a NM may be less problematic from

414   the perspective of an analytical biochemistry challenge. Biomarkers are often geared towards the

415   mechanism of toxicity (biomarkers of oxidative stress, ionoregulatory disturbance, etc.), not the

416   physical form and shape of the material. Nonetheless, modifications of existing biomarker screens

417   could include the use of phagocytosis and endocytosis-related assays to confirm the presence of

418   particles [3]. Some information exists suggesting that subcellular endpoints, especially oxidative

419   stress, may be more sensitive for NMs than other more conventional contaminants. For example,

420   Cong et al. [45] reported that sediment-associated Ag-NPs did not impact whole-body endpoints

421   such as mortality and growth in the polychaete, *Nereis diversicolor*, whereas subcellular endpoints

422   were more responsive (e.g., lysosomal damage, DNA damage determined using comet assay). A

423   limiting aspect for biomarkers is crystal structure and particle shape: our understanding of

424   biocrystallisation and how cells sense crystals is far from adequate for toxicological applications.

425

426   *Incorporating increased environmental realism in nanoecotoxicity testing*

427   While most ecotoxicity studies with NMs have examined the impact on individual organisms,

428   alternative approaches such as mesocosm studies can provide a more complex system, which better

429   simulates the environment (e.g., [36, 40]). These studies can provide information regarding the

430   impact of NMs and consumer products containing NMs on the interactions among organisms of

431   different tropic levels or potentially trophic transfer [46]. However, a limitation of mesocosm

432   studies is that it can be challenging to unequivocally interpret the results as a result of the

433   complexity and multiple factors interacting. In addition, it is often challenging to quantify NMs in

434   the complex matrices (e.g., sediment) that are typically present in mesocosm experiments. It is also

435   possible to study food chain transfer in simpler experimental designs, albeit substantially more

complex than single organism testing, by measuring the transfer of NMs along a single food chain (Kalman et al., 2015).

Furthermore, most NM tests to date have been conducted using NM synthesized in house or procured from the manufacturer. For example, Natalio et al. [47] tested the impact of paint with and without vanadium pentoxide (V_2O_5) nanowires (nw) on antifouling on boat hulls (Figure 2). While approaches like this have resulted in significant increases in the scientific understanding of the potential effects of these materials in the aquatic environment, assessing the impact of NM ageing and transformations on their toxicity requires more research as stated above. It is also important to consider the form in which NMs will actually be released into environmental compartments from consumer products. Carbon nanotubes, for example, may be partly encapsulated by polymers if they were released from a polymer nanocomposite [48, 49]. Thus, the form that may reach the environment after usage or disposal of consumer products may differ from that, which is most frequently tested by scientists. However, the exact form of the released particle may differ based on the product application and information about the nanoparticle by itself remains valuable for assessing the potential impact of NM spills. In addition, there have been few measurements of NMs in field samples and it is thus challenging to know exactly what form is present at the highest concentration in the environment. This raises questions concerning mesocosm simulations, for example *i*) what is the realistic test concentration?, *ii*) what is the form we should apply (i.e., aged, with/without corona, size, mono-/poly dispersed), *iii*) should we apply NMs to the water and then follow it to the sediment and eventually to the food chain?, and *iv*) will a freshwater, marine or estuarine system be the most realistic test scenario or do we need all three as they each represents unique chemical-physical parameters as well a biological components? A discussion of the appropriateness of this type of mesocosm setup for NMs is needed, and careful consideration should be placed on these upon designing and performing mesocosm studies. Additional research is needed to test the ecotoxicity of NMs released from consumer products (e.g. Figure 2) [47] and this is now starting to take place [46].

462 *Putting it all together through nanocategorization and modelling*

463 There is a strong desire to find categories that can be used to group NMs [50, 51]. This would

464 enable risk assessment of a NM with unknown toxicity using fate and hazard data determined for

465 other NMs in the same group, a process which could be similar to read-across and grouping

466 strategies for dissolved chemicals. There is still much debate regarding grouping and categorisation

467 of NMs and at this point there is no agreement. Categorization of NMs has recently gained traction

468 for use with human health toxicity [52, 53], but has not yet been developed to the same extent for

469 ecotoxicity, although some inroads have already been made in the environmental area [54]. The

470 progress continuously being made in this area, together with the development in NM quantitative

471 structure activity relationships can support the development of safe products such as through Safe

472 by Design [55].

473 *Where to focus future research to reduce uncertainty in ecological risk assessment?*

474 Validated bioassays, hazard assessment tools, and especially predictive models, remain to be

475 developed and tested for NMs. Even though we have learned much over the last decade, it is still

476 critical that underpinning research continue to be conducted that explores the fundamental

477 principles that define the consequences of the interactions of NMs with biota (e.g., bioavailability,

478 internal deposition, deleterious effects, and bioaccumulation). Due to the complexity of nano-

479 research, efforts should take an interdisciplinary approach to move the research forward and should

480 be founded in current and emerging research needs (e.g., follow technology and production

481 closely).

482 An enhanced understanding of the underpinning science will lead to more environmentally realistic

483 and implementable approaches ensuring the safe use of NMs and thus the potential benefits of

484 products of nanotechnology. Our specific recommendation for future research areas are centered

485 around 6 main topics (Table 2): *i*) NM characterization in environmental and biological matrices, *ii*)

486 NM transformation in the environment and consequences for bioavailability and toxicity, *iii*)

487   alternative methods to assess exposure, *iv*) influence of exposure scenarios on bioavailability and

488   toxicity, *v*) development of more realistic bioassays, and *vi*) uptake, internal distribution and

489   depuration of NMs. Based on our current understanding of fate and effects of as manufactured

490   NMs, we recommend studying the effects of aged and weathered NMs, as manufactured NMs, and

491   NMs released from consumer products when addressing these 6 topics, which are further described

492   in Table 2. While testing the effects of as-manufactured nanomaterials is the most straightforward,

493   albeit still challenging, testing the effects of particles released from consumer products or those

494   altered in the environment are more environmentally realistic. Research addressing these key topics

495   will reduce uncertainty in ecological risk assessment and support the sustainable development of

496   nanotechnology.

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Acknowledgement

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656   **Figure legends**

657   **Figure 1:** Potential artefacts in nanoecotoxicology testing. This schematic is intended to show the

658   ways in which contaminants in the NMs, release of dissolved ions, NM agglomeration, interactions

659   between the organism and NM coating, or interference from the NM with the assay measurement

660   (i.e., absorbance) can potentially cause inaccurate dosing or artefacts in nanoecotoxicology assays.

661   Reprinted with permission from the American Chemical Society [24].

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663   **Figure 2:** Effect of nanoparticles on biofouling in situ [47]. Digital image of stainless steel plates (2

664   cm x 2 cm) covered with a commercially available paint for boat hulls without (-V_2O_5 nw) and with

665   (+V_2O_5 nw) vanadium pentoxide (V_2O_5) nanowires (nw) immediately after fixation (t=0; top row)

666   and after 60 days (t=60; bottom row). The painted stainless-steel plates with no V_2O_5 nw suffered

667   from severe natural biofouling (plate c) whereas biofouling was complete absent on plates with

668   V_2O_5 nw (plate d). Reprinted with permission from Nature Nanotechnology [47].

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670   Fig 1:

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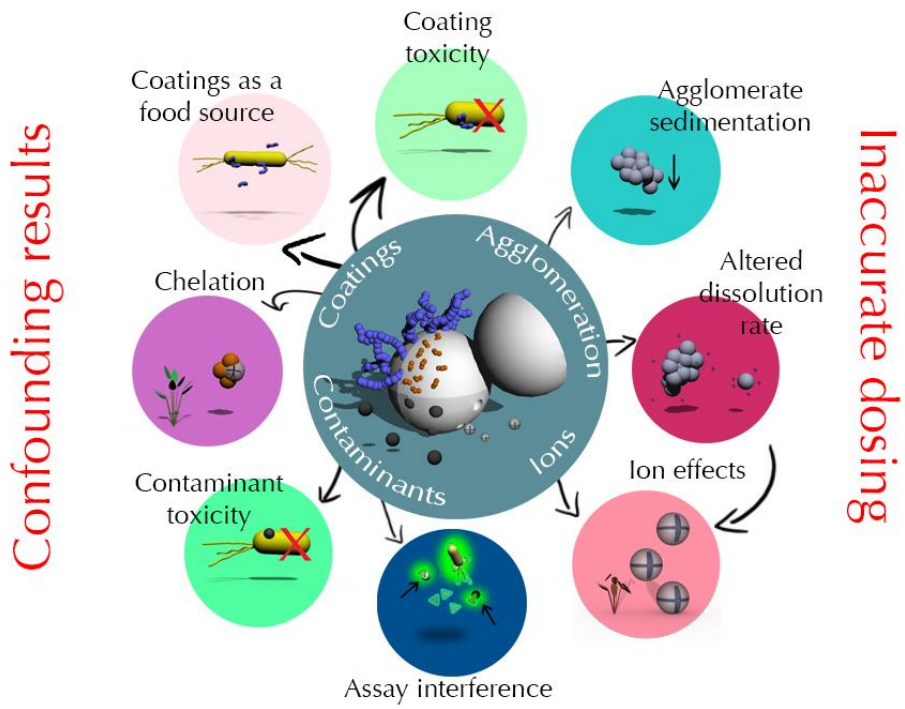
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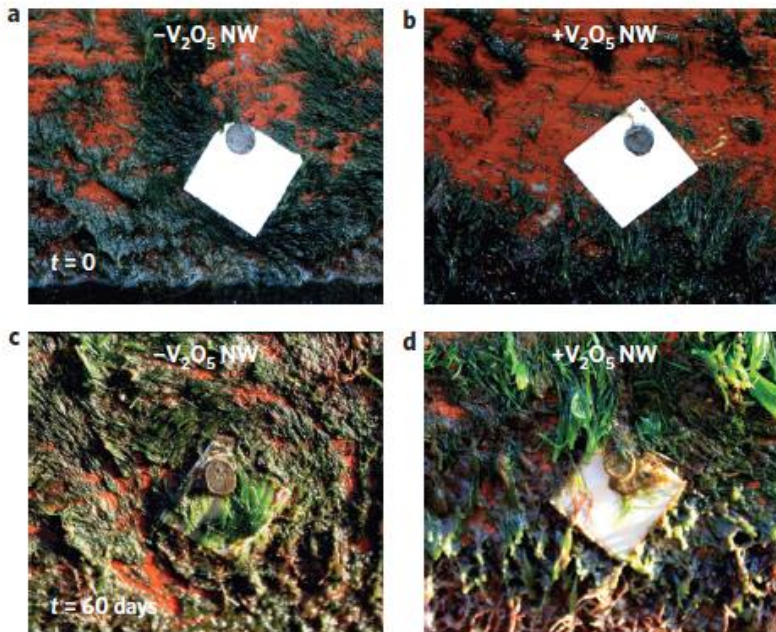
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681 **Text boxes**

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684 TEXT BOX 1

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Recommendations for overarching research topics, which will reduce uncertainty in NM environmental risk assessment

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Emphasis should be placed on studying the ecological effect of aged/weathered NMs, as-manufactured NMs and NMs released from consumer products, addressing:

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- NM characterization and quantification in environmental and biological matrices

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- NM transformation in the environment and consequences for bioavailability and toxicity

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- Alternative methods from conventional to assess exposure

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- The influence of exposure scenarios on bioavailability and toxicity

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- The development of environmentally realistic bioassays

- The uptake, internal distribution and depuration of NMs

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Due to the complexity of nanosafety research, an interdisciplinary approach is key to moving this area forward.

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706 TEXT BOX 2

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Environmental fate of NMs

- NM fate in the aquatic environment depends both on their physical-chemical properties and the characteristic of the receiving environment (pH, temperature, NOM, salinity etc).

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- NMs may interact with naturally occurring particles, which likely modify the NM surface (e.g., creating a corona) thus providing the NM with modified physical-chemical properties which likely alter their fate and bioavailability.

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- Due to the settling behavior of NMs, benthic organisms are likely to be exposed to a higher degree than aquatic organisms

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- There is a need for studies on environmentally modified (aged/weathered) NMs, long-term chronic effects, bioaccumulation and exposure of benthic organisms

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722   TEXT BOX 3

Key challenges in testing and assessing NMs

- Exposure is often not constant.
- NMs are likely to agglomerate/aggregate upon introduction to aqueous media and thus settle out of solution resulting in a reduced aquatic concentration and increased sediment concentration.
- NMs undergo surface modifications (e.g., environmental corona development), which provide them with a new physical-chemical 'identity' thus affecting fate and bioavailability over time.
- Methods to characterize and quantify NMs in experimental media, environmental- and biological samples are time consuming, may require specialized equipment or are not available for complex matrices (e.g., sediment).
- Artifacts may cause inaccurate results and thus careful planning of control experiments is necessary

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739   TEXT BOX 4

Overall considerations and suggestions related to improving NM ecotoxicity testing.

- The overall testing strategy may need adjusting so that more consideration is given to
  - sediment systems compared to the base set of acute aquatic tests (algae, *Daphnia*, fish), although care needs to be taken to compare NM sensitivity between pelagic and sediment-dwelling organisms.
  - more complex ecotoxicity testing such as long-term chronic exposure, increased environmental realism (e.g., mesocosms), and testing with aged/weathered NMs
- Acknowledging the challenges associated with confirming exposure, alternative/complementary approaches could be used to estimate exposure such as
  - by measuring organism NM body burdens
  - by biological response assessment

Both of these approaches require implementation of a reference substance such as the ionic form of NMs that dissolve or a larger /different shape particulate form of the same chemical substance.

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763 **Table 1:** *Literature search on nano-related published literature using Web of Science (June 8th,*

764 *2015). Different search words are listed along with the number of papers (hits) fulfilling the*

765 *specific search criteria. '*' refer to the end of the word being unspecific.*

Search words	Hits	Search words	Hits	Search words	Hits
nano*	952 650	nano* effect*	291 579	nano* accumulat*	13 616
nano* water*	119 143	nano* effect* water	40 624	nano* accumulat* water	1 969
nano* sediment	3 876	nano* effect* sediment	575	nano* accumulat* sediment	222
Organism groups					
nano* alga*	3 266	nano* benth*	323	nano* polychaet*	59
nano* daph*	667	Nano* benthos*	32	nano* oligochaet*	33
nano* fish*	2 314	Nano* invertebrate*	369	nano* mussel*	533
				nano* snail*	190

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Table 2: Key future research topics

Overarching research topic	Future research areas
NM characterization in environmental and biological matrices	Continue developing characterization methods to analyze as-manufactured, 'aged' (although determination consensus has not yet been reached on how to test 'aged' nanoparticles) and weathered NMs in relevant environmental matrices but especially for soils and sediments); however, a consensus has not been reached on how to prepare and test 'aged' or 'weathered' nanoparticles. These methods should be accurate, precise and available for implementation in a standard research laboratory.
NM transformations in the environment	Environmental modification of NMs may affect their stability and fate upon introduction to the natural environment. Differences and fluctuations in natural parameters such as salinity, ionic strength, organic matter, pH, temperature and food availability, which undergo seasonally and yearly fluctuations, will affect e.g., corona development (both environmental and biologically mediated), which may affect their environmental fate (including the distribution between water and sediment compartments) thus affecting which organisms are at most risk for NM exposure. For metal NMs, mineralization or dissolution may lead to their removal from the water column as would sedimentation. We therefore encourage studies characterizing changes to the NM, such as agglomeration, dissolution rates, corona formation and re-precipitation both in laboratory (i.e., in defined test media, and during the tests when the organisms are present) as well as in different aquatic environments (e.g., freshwater, estuarine, marine).

Alternative methods to assess exposure	Due to the challenges associated with quantifying NMs and thus establishing exposure in complex media, it may be possible to instead determine exposure by measuring the test substance in or on the organism (e.g., body burden values), or by quantifying biomarkers of exposure. For body burden values, it is highly recommended to make similar measurements of ionic or bulk particle treatments for comparison, and to use the same exposure concentration (or dose). Measurements of biodistribution of the NMs (and ionic and bulk particles if used for comparison) are highly desirable because NMs may not readily pass through the epithelial tissues in the gut tract or the surface skin, or may be slower to absorb/adsorb compared to dissolved chemicals. A weight of evidence is needed employing different NMs and organisms to confirm the applicability of simple body burden measurements for NMs as a means to assess exposure by examining the theoretical basis (e.g., uptake mechanism, rate limiting steps) that define accumulation. An alternative to measuring the NM in and on the organism is to determine its presence indirectly from biological responses of the whole organism, or key target organs/cellular compartments.
Influence of exposure scenarios on bioavailability and toxicity	While it is well known that NM will be transformed in the environment, the impact of long-term transformation processes on nanoecotoxicity has generally been less frequently studied. Some standardized test methods employ short-term exposures (e.g., 24 h to 48 h), but these methods are not designed to detect delayed and chronic effects. We therefore recommend the assessment of the influence of duration of exposure including ageing and development of environmental corona and thus the

	relation between acute and long-term effects, for fate, bioaccumulation and effects of NMs. Standardized test methods for chronic exposures could potentially be used but modifications for NM testing would be needed.
Development of more realistic bioassays	For regulatory testing, exposure for traditional chemicals has mostly been via water exposure, whereas the weight of evidence in the environmental risk assessment of NMs might suggest sediment testing is most critical when the NMs are not stable in suspension. We therefore recommend rethinking of the overall testing strategy for NMs to place more consideration on sediment tests and organisms that may be more appropriate for this mode of uptake compared to the base set of acute aquatic tests (algae, *Daphnia*, fish), although care should be placed on including water exposure as well when assessing toxicity to determine the most sensitive species.
	Increased realism should be considered through the use of micro/mesocosms and by including nano-enabled products in the mesocosm setup. Despite the challenges that typically are associated with mesocosm experiments: i.e., interpretation of results (i.e., multiple factors interacting, proper controls), these studies can provide information regarding the impact of NMs and nano-enabled products on the interactions among organisms of different trophic levels or potentially trophic transfer. Food chain transfer studies which can be assessed using simpler experimental designs compared to the mesocosm setup, albeit substantially more complex than single organism testing, are encouraged to measure the transfer of NMs along a single food chain.

Uptake, internal distribution and depuration of NMs	The majority of published data have reported total body burden and significantly less has been published on uptake and depuration kinetics and NM transformation and distribution in the organisms. Moreover, the mechanisms of translocation should be documented if uptake occurs. The impact of gut fluids and molecules on transformations and biodistribution of NM should also be studied. More work needs to be done to refine bioaccumulation tests to reflect exposure to particulate material rather than dissolved.

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