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The role of alternative testing strategies in environmental risk assessment of engineered nanomaterials

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Within toxicology there is a pressure to find new test systems and organisms to replace, reduce and refine animal testing. In nanoecotoxicology the need for alternative testing strategies (ATS) is further emphasized as the validity of tests and risk assessment practices developed for dissolved chemicals are challenged. Nonetheless, standardized whole organism animal testing is still considered the gold standard for environmental risk assessment. Advancing risk analysis of engineered nanomaterials (ENMs) through ATS was discussed in September 2014 at an international Society for Risk Analysis (SRA) workshop in Washington, D. C. and serves as the point of departure for this paper. Here we present the main outcomes by describing and defining the use of ATS for ENMs as well as discussing its future role in environmental risk science. We conclude that diversity in testing should be encouraged to avoid "selective ignorance" and that, through an iterative process with low-tier and high-tier testing, data-generation can be validated to ensure relevant endpoints. Furthermore, simplified screening of ENMs could enable early decision-making on material design, while complex multispecies studies should be utilized to skip uncertain environmental extrapolations and give rise to more accurate risk analysis.

Environmental significance

An increasing number of nanoecotoxicological studies focus on non-standardized tests as a way to accelerate data generation, increase environmental relevance and to overcome the limitations of standardized protocols. However, it is currently unclear how these alternative testing strategies (ATS) feed into risk analysis and regulatory decision-making, *e.g.* in the EU and the U.S. In this article we describe the current approaches to ATS in nanoecotoxicology and suggest that, *via* an iterative process, ATS can advance faster and more accurate environmental risk assessment of engineered nanomaterials.

Introduction

Assessing the environmental hazard of new chemicals and materials, such as engineered nanomaterials (ENMs), can be a challenging task as they might have novel properties that could potentially lead to new and unforeseen risks.[1] Over decades standardized tests with the "base set" of test organisms (algae, fish and crustaceans) have been incorporated into regulatory decision making and today have a clear legislative role for assessing environmental impact. Meanwhile, alternative testing strategies (ATS) with new organisms, endpoints and a span of variations in the scale and complexity of the tests have increasingly found usage in the nanotoxicological literature.[2,3]

This is especially the case with high throughput screening (HTS) methods of *in vitro* testing and *in silico* simulation to study the mode of action of ENMs, whereby these methods have grown in popularity as ATS in nanotoxicology. Advocates of using HTS methods point to the staggering number of possible variations of ENMs that can be introduced to the market as well as the possible novel endpoints a test should evaluate.[4] This motivates and drives the need for rapid screening of ENMs with tests inspired from the drug discovery and development process.[5–7] However, the simplicity and lack of environmental complexity in HTS tests have also given rise to so-called "low throughput" studies such as microcosms,

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mesocosms, or field-scale studies to more completely represent factors that can influence fates and effects of ENMs in the environment. The purpose of the different tests performed today in nanoecotoxicology therefore varies considerably, both among alternatives and their departure from standardized regulatory testing approaches. Potential scientific tensions arise, as some tests lack regulatory relevance while other tests lack the exploratory nature needed to properly investigate the impact of ENMs.[8,9]

The use and role of ATS are more clearly defined in human toxicology as they primarily serve as *in vitro* replacements, and as reductions and refinements to the conventional reliance on animal testing. However, in ecotoxicology the pressure to find alternative models is less intense, and the base set of organisms and corresponding *in vivo* tests are therefore still seen as the gold standards for environmental risk assessment (ERA). This raises the question: what is the role of ATS in ERA of ENMs, and how can we facilitate the use of data generated from ATS into risk analysis and decision-making? More broadly, what is the value to risk characterization of question- and hypothesis-based basic research for discovering unidentified ENM interactions with environmental organisms, given that such research typically departs from the constraints of standardized testing protocols? Especially since the very nature of ENM effects on organisms are still mostly undiscovered, research to discover ENM-organism interactions requires applying best scientific practices—and these are unlikely to mirror standard testing protocols.

This was discussed at a Society for Risk Analysis (SRA) workshop entitled "Nano Risk Analysis II" in Washington, D. C. (U.S.) in September 2014, with the overarching theme of how to advance risk analysis of ENMs,[3,10] and such discussion serves as the point of departure for this paper. Here we aim to describe and define the use of ATS for ENMs, and to discuss the future role of ATS in environmental risk science as applied to ENMs.

Environmental risk assessment and standardized ecotoxicity testing

To provide context for what is meant by "alternative" testing, this section gives a brief overview of standardized ecotoxicity testing as well as the development and practice of traditional chemical risk assessment. Importantly, this overview exemplifies that many elements of traditional risk assessment are based less on ongoing scientific research and more on convention.

Although there are regional differences, chemical risk assessment is normally divided into four overall steps: hazard identification, hazard assessment, exposure assessment, and risk characterization. The U.S. National Research Council of the National Academy of Sciences originally proposed this approach for human health-oriented chemical risk assessment in 1983, and the U.S. Environmental Protection Agency (U.S. EPA) adapted the risk assessment framework to ERA during the 1990s. Many of the principles and terminologies for ERA were articulated by the U.S. EPA in 1992 during the publishing of the report "Framework for Ecological Risk Assessment".[11] Although the U.S. EPA was not referenced, the associated principles and terminology were subsequently adopted in the European Union (EU) *via* the publication of the first Technical Guidance Documents for new and old substances in 1993 and 1994.[12,13] The use of standardized testing was initiated in the 1970s and led by the Organization for Economic Cooperation and Development (OECD) as a way to ensure mutual acceptance of data (MAD) for risk assessment.

Ecotoxicological information and ecotoxicity testing using standard test organisms provide the backbone for ERA, as they are used to derive "safe" levels of exposure: the so-called predicted no effect concentration (PNEC). Many of the key procedural elements of how to complete ERAs were decided upon in the late 1980's and early 1990's after discussions between the U.S. and many European countries, *e.g.* The Netherlands, Denmark, and Germany.[14] For instance, in 1989, a scientific advisory committee of the Health Council of the Netherlands was asked to give advice on chemical ERAs.[15] Consequently, it was suggested that acute toxicity data for algae, daphnia and fish from tests performed according to OECD test guidelines should be minimum requirements, and that the lowest EC_{50} should be compared to the (expected or measured) exposure concentration.[16] Similarly, in 1992, participants in an OECD workshop in Arlington, VA (U.S.) recommended three tiers of extrapolation factors or assessment factors, each with a factor of ten, in order to take species-to-species sensitivity, chronicity and laboratory-to-field differences into account. For the purposes of ease and simplicity, all factors were rounded off to the nearest power of ten[17]—an approach that was adopted on a wider scale after the OECD workshop.[14]

The discussion and use of extrapolation factors in ERA stem from a report that the U.S. EPA had published in 1984 called "Estimating concern levels for concentrations of chemical substances in the environment".[13,17] In the report, the U. S. EPA argues that data from three fish species and two crustacean species were largely representative of all relevant species' sensitivity, and thus test requirements could therefore be limited to fish and crustaceans. Algae were subsequently added as a third group.[17] The acute-to-chronic ratio (ACR) factor was set at 10, based on a statistical study of 95 chemicals showing that the median ACR was 8.46 but with large variation as, for example, a reported ACR was 17 551 for the herbicide Propanil.[17,18] Similarly, a laboratory-to-field ratio was derived by comparing experimental acute LC_{50} data with field toxicity data, and was found to span from 12 to 5300.[13]

Ecotoxicity testing of nanomaterials

Ecotoxicity testing of ENMs has only emerged as a new scientific research field within the past decade. Recently, a comprehensive review of the nanoecotoxicological literature published in Thomson Reuters WoS identified more than 200

articles reporting on more than 1500 toxicity values (EC_{50}/LC_{50}/NOEC) across numerous different species for eight different ENMs.[19] It is well established that the ecotoxicity of ENMs is influenced by, and can be related to, the specific particle physico-chemical properties, but it is currently unclear exactly which properties affect ecotoxicity. Overall, Juganson *et al.*[19] reported three major knowledge gaps: i) in most studies the physico-chemical properties of the investigated ENMs are insufficiently described, ii) relatively few studies have been performed with algae and fish, and iii) ecotoxicity tests with standard test organisms were often performed with modified protocols. Whereas the first point underlines the issues with ENM characterization, both ii) and iii) stress that few studies are performed with standard organisms relevant for ERA, and the ones that do tend to deviate from the guidelines. This means that the results of the tests would normally not be considered applicable for risk assessment purposes.[20]

Holden *et al.*[21] recommend "scenario driven" approaches based on expected exposure regimes and magnitudes, encompassing recommendations for enhancing the environmental relevance of hazard assessment. Another recent analysis asserted that near-term attempts to understand the mechanisms of impact as a function of ENM properties (*e.g.* structure activity relationships; SAR) are unlikely to be successful particularly in complex ecosystems, based on the sheer multitude of inextricable influential factors across the materials, environmental compartments, and receptors whose dynamic relationships determine the ultimate effects.[22] The authors propose an alternative approach, utilizing laboratory scale functional assays to measure intermediate processes that are important determinants of material fate and effects that are a function of a complex mixture of material, medium and scenario-based parameters. This approach is part of, and complementary to, the overall scenario-driven tiered approach.[21]

Jurisdictions around the world are applying conventional chemical-based regulatory frameworks to ENMs, consistent with the Council Recommendation by the OECD[23] which states "…to manage the risks of manufactured nanomaterials, apply the existing international and national chemical regulatory frameworks or other management systems, adapted to take into account the specific properties of manufactured nanomaterials". However, these frameworks rely almost entirely on test methods, endpoints, and approaches developed specifically for conventional chemicals. According to the OECD[24] and Brinch *et al.*,[25] there are also a number of great challenges when it comes to ecotoxicity testing of ENMs which can be divided into four areas: 1) material characterization, 2) exposure preparation and delivery of substance to test systems, 3) monitoring of stability and consistency of ENMs during the test and 4) measurements and use of dose metrics. Recently, Skjolding *et al.*[26] also highlighted the difficulty in testing ENMs. Understanding and accounting for these issues is paramount for reliable ecotoxicity testing and therefore also for ATS to provide useful data.[2,27]

Addressing challenges associated with applying conventional chemical-based approaches to ENMs has fostered considerable international cooperation, including the large-scale initiative under OECD's Working Party on Manufactured Nanomaterials (WPMN), which aims at informing on environment and human health safety aspects of ENMs. The WPMN has initiated a variety of projects, including the coordination and generation of high quality research under its Sponsorship Program, evaluation of the appropriateness of OECD Test Guidelines for ENMs, and addressing the development of risk assessment approaches. Under the steering group on Risk Assessment and Regulatory Programs (SG-AP), the WPMN published the report "Important Issues on Risk Assessment of Manufactured Nanomaterials" which identified over 50 issues that need to be addressed to conduct more appropriate risk assessments of ENMs.[24] The SG-AP thus continues to address these constraints by working on pragmatic approaches including understanding the role of ATS for ENMs in risk assessment.

Alternatives to standard ecotoxicity testing of nanomaterials

According to Calow,[28] ecotoxicity testing is performed for two reasons: 1) to anticipate how toxicants are likely to impact ecological systems and 2) to assess what changes are taking place in ecological systems under the influence of released toxic substances. Whereas anticipatory testing is generic in nature, assessment testing is a closer simulation of the environment and is more scenario-specific. In human nanotoxicology, ATS is a move towards anticipatory, generic and predictive hazard assessment without the reliance on assessing ENMs through whole organism animal testing.[3] However, for ATS in nanoecotoxicology, testing is diverging into both more predictive, anticipatory testing and more complex, realistic assessments. This divergence is occurring without clearly delineating the various approaches or overtly comparing across them. It should be noted that the use of alternative hazard assessment approaches is not unique to ENMs, and there has been discourse in conventional chemicals toxicity testing for years concerning what is most relevant to ERA. What is different is that in the course of evaluating ENMs, ATS have matured conceptually to incorporate both particulate as well as chemical behaviors, allowing for the generation of data for human health-related hazard assessment of ENMs.[2] At the same time the validity of ERA for ENMs is challenged.[24,29,30] However there is currently a gap in defining what "alternative" means in the ERA of ENMs.

Defining the current ideas and acknowledging the understanding that such ideas deliver could help to advance the development of ATS towards faster and more accurate risk analysis approaches and strategies. The current range of ecotoxicological approaches as applied to ENMs span from attempting to simulate all environmental complexities in field or mesocosm studies, to laboratory studies attempting

to simplify and remove complexities in order to discover specific mechanisms, or a continuum of studies that are tiered within these extremes.[21] This is also reflected in the order in which they are performed, *i.e.* starting with the most comprehensive environmental simulation ("top-down") and using the results to motivate examining mechanisms, *versus* starting with simplified screening-type studies ("bottom-up") whose results may motivate determining if effects are observable in more complex environmental representations (Fig. 1). The use of these terms is intended to describe a continuum in experimental system complexity and to acknowledge the trade-off along that continuum between environmental realism, at the most complex end of the spectrum, *vs.* control and reproducibility at the most streamlined or simplified end of the spectrum. Further, the authors acknowledge at the outset that the "top" is not truly the maximum level of complexity represented in a real world system; rather, top-down refers to approaches beginning at the highest level of complexity realistically achievable. Similarly, "bottom-up" refers to the most controlled experimental design with fully isolated variables.

Where to start: top-down or bottom-up?

As described above, top-down and bottom-up approaches appear to offer binary alternatives to ecological ENM toxicity testing. However, using complimentary experimental design in both bottom-up and top-down tests that are run in parallel may represent the most effective and efficient path forward. This approach can increase the number of materials that can be screened given limited time and resources, while working to ensure that the selected assays are testing relevant endpoints and are directionally correct in their screening conclusions. It should be clarified that, while top-down and bottom-up approaches are two ends of the ENM ecotoxicity testing continuum, the testing strategies between these end members build biological and environmental complexity along this continuum. This could be demonstrated, for example, by testing cells of a single microbial taxon in a microtiter plate, testing microbial communities in soil where ENM bioavailability limitations could preclude effects, and finally by testing microbes in planted soil mesocosms where plant-microbial interactions can be observed.[21]

Designing ATS with intentional, iterative feedback between top-down and bottom-up approaches can direct the development of higher-throughput methods based on important conclusions unique to the research on complex systems. We expect this to streamline ATS in a number of important ways. As illustrated conceptually in Fig. 1, targeted, iterative communication between top-down and bottom-up approaches can promote well-informed caution and direction. This recognizes the practical need to prioritize when low throughput studies at large scale and over a longer term should be performed while maximizing the possibility that research-based near-term conclusions are available to decision-makers.

Bottom-up approaches

Bottom-up approaches address ENM environmental hazards by following tiers of experiments that begin with simplified systems and continue further experiments along increasingly higher levels of complexity based on the results.

As with HTS for general nanotoxicology[7] hypothesized mechanisms for well-characterized ENMs should guide the

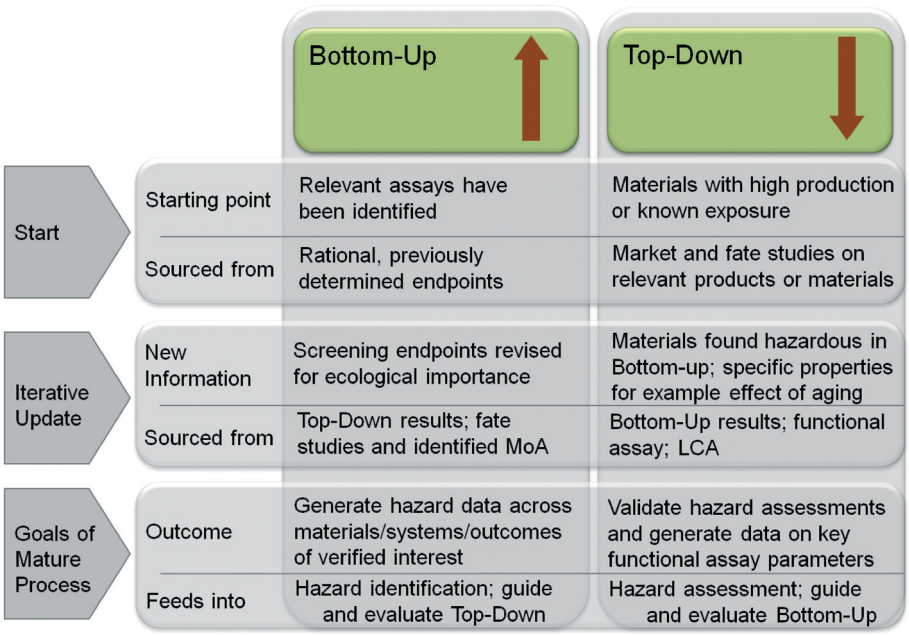

Fig. 1 Detailed differences and feedback loops between two—bottom-up and top-down—parallel nanoecotoxicological approaches, which together constitute a robust ATS scheme. MoA = mode of action, LCA = life cycle assessment.

design of ATS for environmental hazard identification. For example, HTS using environmentally relevant bacteria may focus on assessing ENM effects on population growth, since ecosystem-relevant reactions are often catalyzed *via* growth.[31] HTS approaches are not static; rather, they should improve as the mechanisms of ENM induced effects are discovered and as novel ENMs with novel properties arise. Exposure assessment can also be advanced *via* bottom-up approaches, since ATS can supply basic information for modeling ENM environmental transport and fate processes.[22]

While ecotoxicological and fate testing can move with bottom-up approaches along separate tracks from simple to complex, they can also move in tandem, and can start at intermediate complexities[21] (see Fig. 2). Results would then indicate if next steps should include less or more complexity, for example towards delving into mechanisms, or towards determining ENM bioavailability in complex media such as soil[32] with possible ecosystem-level outcomes,[33] respectively. Selection of the right intermediate tests, potentially including functional assays,[22] to carry out in systems and on endpoints of interest, provides directional insight along a continuum of complexity to guide future higher tier experiments as well as to identify useful lower tier tests.[34] The outcomes of simple first tier testing, whether it begins with subcellular assays[35] or higher, drive testing at higher tiers, and iteratively the testing strategies in the first-tier are influenced by higher tier results.

Bottom-up approaches start with targets that have some importance to environmental processes, and are simple enough to be used in HTS. Microorganisms are suitable, given their sizes and importance in ecosystem function. Bacteria and phytoplankton are environmentally abundant; bacteria are hugely diverse in their genetic makeup[36] and their functions underpin planetary biosphere processes. Phytoplankton fix half of the carbon flowing through the biosphere on Earth.[37] Bacteria catalyze nutrient cycling reactions that recruit N_2 from the atmosphere into mineral forms that feed plants.[38] Bacteria and other microbes decompose tissues, and oxidize reduced forms of C, N, Fe, S and many other elements that consequentially flow through and nourish aquatic and terrestrial plants and animals – the ultimate food for livestock and human consumption. Microbes have a high capacity for sorbing pollutants[39] and could with their predators initiate ENM trophic transfer[40] therefore propagating into food webs. Thus, how ENMs affect microbial processes and how microbes affect ENM fate and transport could conceivably be screened rapidly to determine potential ENM hazard.

The cautions regarding bottom-up approaches include that some rational notion of how ENMs might affect a biological target and some judicious choice of target are necessary. The concept of ENM exposure and effects "scenarios" would drive ATS designs including targets.[21] Other cautions of course are that laboratory-testing configurations, no matter how judiciously targets or ATS are selected, may fail to capture the most important consequences of ENM environmental exposures. For example, HTS using bacteria can be argued for,[31] but would only interrogate one aspect of the complex plant-microbe interactions that drive formation and function of agriculturally relevant root symbioses.[33] In that sense, ATS using environmentally relevant HTS approaches can at best indicate the "potential" for ENMs to inflict harm on biological targets, subject to ENM bioavailability and community or higher level biological interactions. However, whether the potential for impact would be realized is determined by the fate of the ENM, which can either be studied *via* screening assays (Fig. 2) or by scaling tests up to the next tier of complexity and using biological community responses to infer bioavailability.[41] Lastly, as with all ENM environmental hazard assessment approaches, developing mechanistically-based mathematical models of biological effects[42] that could be married to mechanistic exposure models[43] is of high value,[21] especially given the many permutations of ENMs that could be manufactured or that arise from environmental aging. Bottom-up approaches, including using appropriate functional assays, subject to addressing the caveats about careful design and iterative improvements, could be economical vehicles for populating and testing models, and thus could provide for important predictive capabilities in risk assessment.

Top-down approaches

As alternatives to experiments that focus on a single cell-line, species, or strain of organism, top-down approaches use a

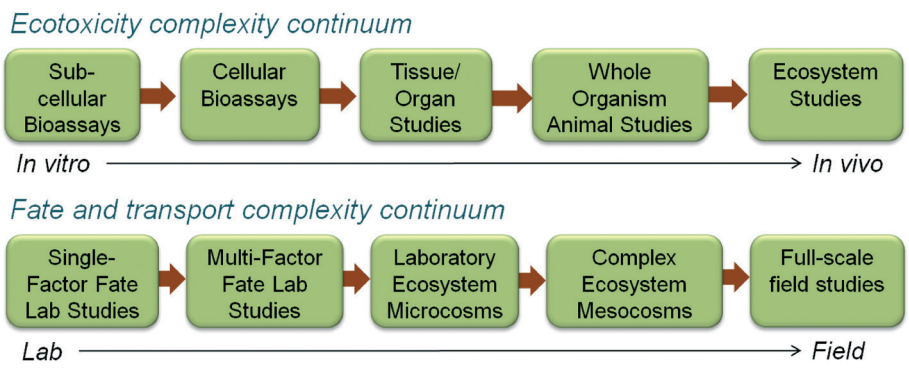

Fig. 2 Bottom-up approaches in nanoecotoxicology in assessing ENM ecotoxicity (top) and fate (bottom). Anticipatory tests are skewed to the left, and assessment tests are skewed to the right.

diverse assemblage of organisms in a representation of their natural physical and chemical environment. In such experiments a contaminant is added and the impacts can be studied across many levels of biological organization including: individual organisms; populations, consisting of organisms within a given species or group; communities, consisting of strategic groupings of many interacting populations; and ecosystems, consisting of communities of organisms and their physical and chemical environment. Any change in chemical regimes can alter the abundance, composition, and function of organisms through a range of mechanisms. The goal of working across these many scales is to determine both the impacts of the contaminants on these different levels of biological organization, but also to determine the impact of the organisms and environment on the fate and transformation of the contaminants.

These types of experiments offer many strengths when evaluating ecological hazards. First, they allow for the examination of the movement and/or accumulation of a contaminant in a food web to place bounds on uptake, trophic transfer, and the potential for biomagnification of a contaminant in an ecosystem.[44] Second, they tend to emphasize environmentally realistic exposure scenarios by testing lower concentrations of contaminants, looking beyond toxicity mechanisms to more ecologically relevant endpoints, and examining the interplay of contaminants and ecosystems over longer time scales.[45] Third and finally, top-down approaches can identify complex indirect effects that would not be observed in single species experiments and thus could be completely missed in identification of potential impacts of a contaminant.[34] The inclusion of a multitude of variables, both controlled and uncontrolled, allows for investigating which variables are driving contaminant fate, transport, and impacts.

However, these strengths are accompanied by challenges inherent to the scale and nature of such experiments. One such challenge is that with so many variables it is not feasible to systematically step through and vary each individual factor to tease out every dependency. Instead, the scenarios tested are more limited and must be as representative as possible of an environmentally relevant potentiality, supporting a contextual search for trends across a variety of metrics. Another challenge is that variability between replicates within a treatment can be sufficient enough to make small treatment effects difficult to observe above the background variability. As noted by Sanderson[46] the interpretability of micro- and mesocosms studies could be enhanced by 1) determine the appropriate experimental design and number of replicates by using power analysis, 2) utilise advanced statistical analysis, such as probabilistic effect distribution and principal response curves, and 3) report, preferably in quantitative terms using power analysis, the risk of type II error. Furthermore, the long time frames and large spatial extent involved limit throughput. Thus the high resource intensity means that fewer materials and scenarios can be tested. Despite the resource-intensive nature of these top-down approaches, they must be part of a viable testing strategy to avoid critical directional errors and false negative conclusions that may arise in the absence of community to ecosystem level investigations. While there may not be universal validation criteria for top-down tests like there are for many bottom-up approaches, general guidelines exist for maximizing their validity (*e.g.*, using relevant endpoints, measuring actual exposure concentrations, and minimizing variability among replicate mesocosms).[21,47] While the degree to which top-down approaches are utilized in a regulatory setting differs, their use always increase the understanding of a compounds or materials ecotoxicological effects.[48,49]

Insights from top-down methods may be used to identify instances where screening methods not only fail to deliver understanding of actual environmental processes, but actually have the potential to generate the wrong conclusion. This could in turn propagate directional errors throughout further research, guidelines and regulation. For example, in a recent experiment, the toxicity of silver nanoparticles (AgNPs) to plants in wetland mesocosms led to a release of labile dissolved organic matter, which in turn led to an increase in microbial respiration.[50] This increased respiration led to decreased O_2 levels, which led to decreased methane consumption. This – when coupled with the abundant C substrate, elevated CO_2, and low O_2 – led to increased methane production from the system. Had a lower complexity, faster experiment been performed examining only plants and AgNPs, methanogens and AgNPs, or methanotrophs and AgNPs, these interactions would not have been linked as clearly and dramatically (forty-fold increase in methane concentration). Had these top down experiments not been conducted, a conclusion would have been that AgNPs have marked impacts on plants and CO_2, while missing the critical impacts on methane production and consumption. Recognition of such interdependent system and material variables has subsequently informed the design of more constrained tests in microcosms to more mechanistically examine the drivers of the observed phenomena.

Another key contribution of top-down approaches may be in helping to identify the appropriate rate-limiting steps or phenomena that will allow meaningfully interpreting data from one ecological endpoint, and applying those reasonably to other endpoints along the biological continuum. However, bridging scales is notoriously difficult in ecosystem ecology. A good analogy of the challenge inherent in attempting to bridge the scale of individual to ecosystem effects from a chemical or material stressor is the challenge of doing so even in the absence of a potential chemical stressor. For example, scaling up a fundamental process in a forest like photosynthesis – up from the chloroplast to the leaf level – is challenging, from the chloroplast to the whole plant is harder still, and from the chloroplast to a stand of trees is likely impossible. Understanding chlorophyll dynamics can help refine estimates of stand level photosynthesis, but not replace other methods of assessing the process.[51] While HTS can inform ecosystem level experiments and *vice versa*, modeling

processes at various scales with linkages between scales would be preferred over inferences in how to take information from one level to another. However, modeling from the scales of individuals to populations is currently feasible,[52] and linking higher scales is more aspirational.

Current regulatory use of ATS

TSCA

There are two main regulatory approaches through which ENMs are evaluated in response to the Toxic Substances Control Act (TSCA) – a major regulatory mechanism to handle new and existing chemicals including ENMs in the U.S. These include premanufacture notifications for new ENMs and an information gathering rule for new or existing ENMs. For the premanufacture notifications, manufacturers of new ENMs must provide information to the U.S. EPA prior to manufacturing or introduction of the ENMs into commerce. After this, the U.S. EPA may decide to take action to control any potential risks to health or the environment (*e.g.* personal protection equipment, engineering controls, limit use, *etc.*). Under TSCA, the U.S. EPA has reviewed more than 170 new chemical notices for ENMs to date including those for carbon nanotubes, quantum dots, and a metal oxide.[53]

Regarding ATS, TSCA specifically mentions the use of screening level techniques to evaluate chemical substances and mixtures.[54] One example used by the U.S. EPA has been through their ToxCast program, whereby HTS approaches were applied to a number of ENMs to provide targeted testing and to identify affected biological pathways (*e.g.* Wang *et al.*[55]). At the same time, however, some authors have noted challenges with applying current testing practices[56] while others have argued that not enough ENMs have been submitted under the premanufacture notice under TSCA to provide for read-across or structure activity relationship (SAR) approaches.[57] These authors have also suggested that the complementary use of animal data with *in vitro* data and *in silico* estimates could support decisions involving ENMs as well as help advance new testing approaches that are potentially also applicable to conventional chemicals.

TSCA has been recently amended with the Frank R. Lautenberg Chemical Safety for the 21st Century Act (June 2016). This new law aims to help improve chemical regulation in the U.S. with a number of important changes including, but not limited to, a mandatory requirement for U.S. EPA to evaluate the safety of existing chemicals on the market in a prioritized manner, evaluate new and existing chemicals using risk-based safety standards, establishing clear and enforceable deadlines to promote timely reviews and actions on identified risks, a greater transparency of chemical information, and help ensuring U.S. EPA has a consistent source of funding to carry out actions related to this new law. It is expected that this amendment will impact the evaluation of new and existing ENMs on the market. The Act explicitly state that a plan to promote the development and implementation of alternatives testing methods shall be developed within two years with reporting to Congress every fifth year on the progress. However, the actual impact of these initiatives is questioned.[58]

REACH

Under the European chemicals legislation on "Registration, Evaluation, Authorisation and Restriction of Chemicals" (REACH) and the Technical Guidance (TG) (r7b) provided by the European Chemical Agency (ECHA), *in vitro* data are listed as a relevant type of information for assessing aquatic toxicity while also noting that there are no EU/OECD guidelines for *in vitro* tests of relevance at the moment. Primary cells from liver and gills are noted to be "particularly suitable for mechanistically oriented studies on cell-specific toxicant fate and action" whereas fish cell lines can be used to measure the cytotoxic effect of chemicals.[20] Information from *in vitro* studies might be considered in a weight of evidence approach provided that they fulfill certain data quality aspects and comply with the Annex XI criteria *e.g.* results are derived from an *in vitro* method whose scientific validity has been established by a validation study and there is adequate and reliable documentation of the applied method.[20] Although the ECHA TG r7b was updated in February of 2016, parts of the TG have not been updated recently and it notes that: "At the present (2006) no *in vitro* tests are available that can substitute for *in vivo* data". At the same time, it also lists development and validation of "…*in vitro* tests and based on this develop guidance how to use *in vitro* tests" as one of the priorities for future research.[20]

The use of data from ATS in a regulatory context faces obstacles in Europe due to the current risk assessment paradigm. In REACH, ecotoxicity studies undergo a quality evaluation to determine how adequately the study can feed into risk assessment based on the relevance and reliability of the produced data. The relevance of a study can change depending on what is being assessed, whereas the reliability is an inherent quality of a study quantified as a fixed score known as the so-called Klimisch score.[59] Furthermore, ECHA has emphasized that "only validated and pre-validated *in vitro* methods can be used under specific conditions for risk assessment".[60]

ATS for ENMs finds limited use in regulatory risk assessment partly due to these issues with reliability scores. Ågerstrand *et al.*[61] evaluated 12 peer-reviewed non-standardized toxicology and ecotoxicology studies and found that information needed for a high reliability score was typically missing in the studies, which they interpreted as an indication of a "general problem with non-transparent reporting in the peer-review literature". Whether the authors behind the studies simply did not obtain the missing information or chose not to report it is unknown, but Ågerstrand *et al.*[61] urge authors to consider what to report in articles (*e.g.* in supplemental materials) and suggest using reporting guidelines (*e.g.* Ågerstrand *et al.*[62]) in order to increase the studies' reliability and regulatory usefulness. This could be an important aspect if ATS for ENMs are to achieve regulatory impact.

The future role of alternative testing strategies

The overview herein of bottom-up and top-down approaches highlights the variety of possible tests within nanoecotoxicology. As noted by Wickson *et al.*,[9] such diversity in testing is important to avoid "selective ignorance", as nanoecotoxicity is still a maturing science. As such, alternative or exploratory testing of ENMs without necessarily assuring regulatory relevance should be encouraged to improve the understanding of a variety of factors (*e.g.* toxic mode(s) of action of ENMs) which indirectly will highlight what should be emphasized for risk assessment. As accounted for herein, a solitary focus on screening-level data generation, even with multiple endpoints and materials, is unlikely to replace standardized testing in ecotoxicology, given the entrenched regulatory frameworks that rest on the latter. Rather, various bottom-up and top-down ATS approaches could comprise informative approaches along the ecotoxicity testing continuum. Each step towards higher complexity in testing increases the environmental relevance of the hazard assessment, and is especially valuable if supported by mechanistic bottom-up studies that verify the investigated endpoint. The behavior and effects of many ENMs are difficult to assess in a beaker or a microtiter plate[63] and some ENMs with high production, exposure or hazard potential, as described in Fig. 1, should be candidates for complex top down testing for accurate ERA with as few extrapolation needs as possible. One such candidate ENM is nanoscale zero valent iron (nZVI), as it is intentionally released into the environment in high quantities.

High-throughput ATS for ENMs have been suggested to play a proactive role in the development of less hazardous ENMs.[64] Early *in vitro* and *in silico* toxicity screening could influence and facilitate decision-making on design parameters, such as material selection, size, shape, surface charge *etc.*, in order to reduce the hazard or exposure potential. Such an approach is also found within chemical alternatives assessment, which could provide the framework for incorporation of ATS generated data into risk analysis and decision-making for ENMs.[65]

Conclusion

In this paper, we propose that alternative testing strategies (ATS) within nanoecotoxicology comprise the testing of engineered nanomaterials (ENMs) hazard and exposure potentials with environmentally relevant organisms or biological levels of complexity, using methods that either accelerate data generation, increase the realism of information, or both, relative to conventional toxicity testing. Importantly, the best use of ATS is likely to be *via* an iterative process where results from bottom-up and top-down approaches feed into each other. Although the regulatory readiness for ATS can be questioned, regulators in both the EU and the U.S. seem aware of the main challenges and efforts to better incorporate ATS data and weight of evidence approaches that have been ongoing. While not a short-term replacement of standardized toxicity testing, ATS could contribute to traditional risk assessment as long as experiments are reported completely and transparently. However, the true strength of ATS lies outside of the current paradigm in environmental risk assessment (ERA). High throughput studies can elucidate mechanistic data and help identify novel and sensitive endpoints as well as predict and guide testing at higher complexity levels. Low throughout studies with high complexity can circumvent the need for extrapolations and assumptions needed in current risk assessment and provide more accurate no-effect levels for environmental risk assessment. For these reasons, ATS for ecotoxicity of ENMs as described here can provide risk assessors with answers to direct environmental concerns and could, in the long-term, be the strategy of choice for ERA.

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