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What is "Environmentally Relevant"? A Framework to Advance Research on the Environmental Fate and Effects of Engineered Nanomaterials

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Environmental Significance Statement

Translating the conclusions elucidated in simplified experimental systems to predict the behavior and effects of engineered nanomaterials (ENMs) in more realistic systems presents a significant challenge. To address this, we propose a framework based on three pillars that collectively define the environmental relevance of a given study, including 1) the properties of the ENMs 2) the experimental conditions, and 3) the exposure scenario and biological endpoints that are assessed. The framework provides an approach for researchers to evaluate and report the environmental realism of their methods. This will assist scientists in placing their work into context with other research and to identify and address research gaps, ultimately helping bridge the translation of knowledge from lab-based to more realistic systems.

Abstract

Environmental nanoscientists and nanotoxicologists have made significant progress towards understanding the various factors and processes that impact the environmental fate and effects of engineered nanomaterials (ENMs); nevertheless, many knowledge gaps remain. This is partly due to a disconnect that occurs when these factors or processes are elucidated in simplified experimental systems and then applied to predict ENM behavior in significantly more complex real-world systems. To aid the translation of findings between these two extremes, we have outlined and demonstrated the use of a Framework for Relevance And Methods Evaluation (FRAME) based on three components or pillars that collectively define the "environmental realism" of a given experimental design. The three pillars include (1) the properties of the ENMs, (2) the experimental conditions, and (3) the exposure scenario and endpoints that are assessed. FRAME provides researchers with an approach for assessing the environmental relevance of alternative experimental designs. It also provides a basis for reporting how an individual study fits within the broader body of scientific knowledge and for identifying areas where additional research is needed. The proposed framework is intended to be used throughout the scientific process, from the initial conception of the experimental design and continuing through to the interpretation of experimental results. Committing to a more complete assessment of environmental realism has the potential to prevent the overgeneralization of results determined in simplified experimental systems and move the field forward more quickly through the identification of critical knowledge gaps.

Introduction

Understanding the processes controlling the environmental fate and effects of engineered nanomaterials (ENMs) is essential for achieving their safe and sustainable use in various applications. Research has shown that the physiochemical properties of ENMs can impact the outcome of these processes.[1-3] These properties, however, are not static but will change throughout an ENM's life cycle in response to physical and chemical transformation processes such as aggregation, dissolution, reduction-oxidation, and the adsorption of organic macromolecules (often referred to as protein- or eco-corona formation, depending on the nature and source of the adsorbing macromolecules).[4-7] These transformation processes can occur simultaneously and are dependent on an array of factors, including the chemical properties of the surrounding media (e.g., pH, ionic strength, temperature, etc.) and its constituents (e.g., bio- and geogenic natural colloids, organic macromolecules, etc.), as well as the physiochemical properties of the ENMs themselves (e.g., size, shape, material chemistry, etc.).

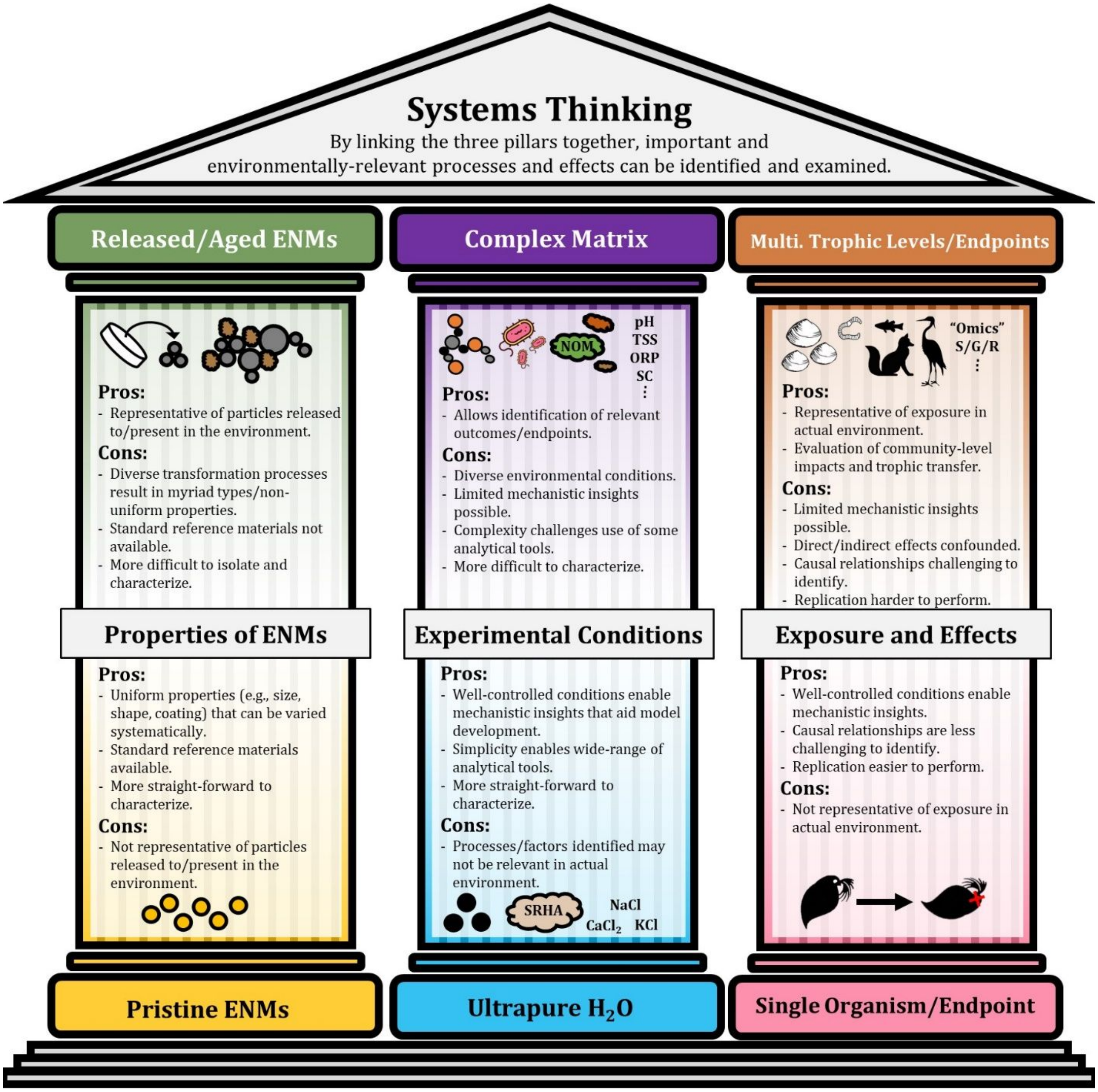
Focusing on aquatic environments, environmental nanoscientists and nanoecotoxicologists have made considerable progress towards understanding the functional fate pathways that are driven by the properties of ENMs and the surrounding media and how those factors dictate the environmental fate and effects of ENMs.[8] To build these connections, researchers have employed a breadth of experimental systems that range from simplified and well-controlled laboratory experiments to more complex and realistic mesocosms.[9] Nonetheless, it remains difficult to predict ENM fate, transport and impact, in part, because of the disconnect that may exist when these processes are elucidated in simplified experimental systems and then applied to significantly more complex real-world systems.

To effectively translate findings between these two extremes, it is critical that research is conducted across the experimental spectrum. An approach is needed that assists researchers in evaluating the environmental relevance of their experimental design while also providing a basis for reporting how their research fits within the broader body of scientific knowledge. Towards this goal, we propose a Framework for Relevance And Methods Evaluation (FRAME) that applies a holistic perspective based on three components or "pillars" that can be used to gauge the environmental realism of a given experimental system. Within each pillar, a spectrum exists that ranges from simplified, lab-based approaches to more realistic and holistic systems that more closely mimic real-world environments. By linking these pillars within a conceptual three-dimensional space, researchers can assess the environmental relevance of their experimental system while also placing it into context with the existing body of literature. In doing so, researchers will not only be able to identify existing knowledge gaps or unexplored exposure scenarios but also use this information to help prioritize future research needs.

Pillars of Environmental Relevance

Our conceptual framework is built on three pillars of environmental relevance that can be used to estimate the realism of an experiment aimed at elucidating the fate and effects of ENMs and ENM-containing products (Figure 1). These pillars, discussed in detail in the following sub-sections, include (1) the properties of the ENMs, (2) the experimental conditions, and (3) the exposure scenario and endpoints that are evaluated when assessing ENM effects. It is important to note that although the three pillars we identify should readily translate between different environmental compartments (e.g., soils, sediments, freshwater, etc.), the following discussion and the details presented in the tables and figures are specific to aquatic environments. Thus, applying FRAME to a different environmental setting would require that the researcher(s) first establish the components/factors that will be used and evaluated.

While each pillar is discussed individually and can be considered as such during experimental design, they are inherently connected (e.g., the physiochemical properties of ENMs are dependent on and influenced by the conditions of their surrounding environment). Thus, assessing the environmental realism of a given experimental design starts by first evaluating each of the three pillars individually and then linking them together to create a conceptual three-dimensional (3-D) space (Figure 2). In this 3-D space, each axis represents a different pillar, where points closer to the origin indicate a simpler and less realistic experimental system and those further from the origin indicate a more realistic (and likely more complex) system. Those points which are furthest from the origin in the 3-D space would be those experiments which most closely mimic the complexity existing in natural environmental systems. Applying a holistic perspective which considers these pillars collectively provides contextual understanding of research aimed at identifying environmentally relevant phenomena.



Systems Thinking

By linking the three pillars together, important and environmentally-relevant processes and effects can be identified and examined.

Released/Aged ENMs

Pros:

- Representative of particles released to/present in the environment.

Cons:

- Diverse transformation processes result in myriad types/non-uniform properties.
- Standard reference materials not available.
- More difficult to isolate and characterize.

Properties of ENMs

Pros:

- Uniform properties (e.g., size, shape, coating) that can be varied systematically.
- Standard reference materials available.
- More straight-forward to characterize.

Cons:

- Not representative of particles released to/present in the environment.

Pristine ENMs

Complex Matrix

Pros:

- Allows identification of relevant outcomes/endpoints.

Cons:

- Diverse environmental conditions.
- Limited mechanistic insights possible.
- Complexity challenges use of some analytical tools.
- More difficult to characterize.

Experimental Conditions

Pros:

- Well-controlled conditions enable mechanistic insights that aid model development.
- Simplicity enables wide-range of analytical tools.
- More straight-forward to characterize.

Cons:

- Processes/factors identified may not be relevant in actual environment.

Ultrapure H_2O

Multi. Trophic Levels/Endpoints

Pros:

- Representative of exposure in actual environment.
- Evaluation of community-level impacts and trophic transfer.

Cons:

- Limited mechanistic insights possible.
- Direct/indirect effects confounded.
- Causal relationships challenging to identify.
- Replication harder to perform.

Exposure and Effects

Pros:

- Well-controlled conditions enable mechanistic insights.
- Causal relationships are less challenging to identify.
- Replication easier to perform.

Cons:

- Not representative of exposure in actual environment.

Single Organism/Endpoint

Figure 1. Framework for Relevance And Methods Evaluation (FRAME). The three components or "pillars" that, in combination, define the environmental realism of a given experimental system. Within each pillar, the experimental approach can range from highly simplified (bottom of each pillar) to more realistic (top of each pillar). Considering the three pillars collectively during experimental design, the evaluation of results, and in connecting one's research to other studies builds holistic knowledge.

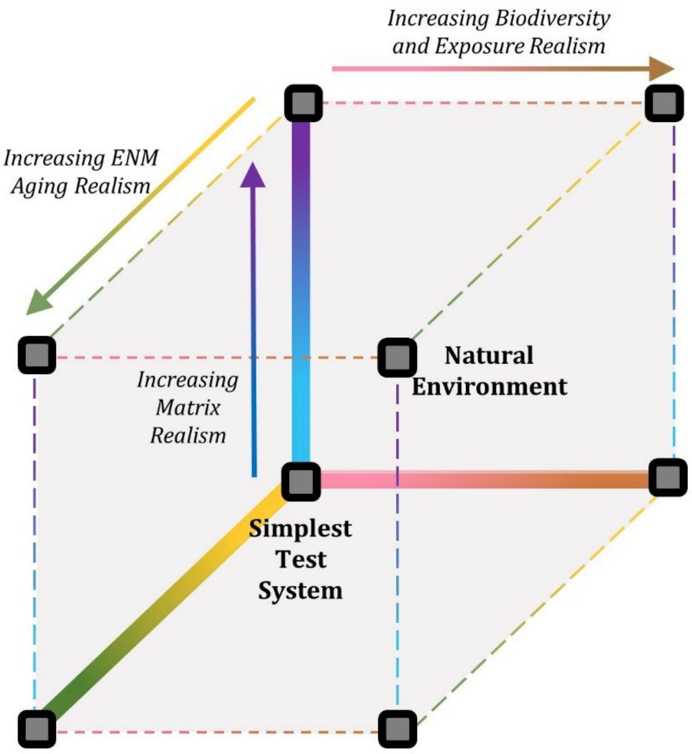


Figure 2. Conceptual 3-D space defined by FRAME. Each axis represents a different pillar shown in Figure 1, where closer to the origin indicates a simplified experimental system and further from the origin indicates a more realistic system. By considering each of these three components, researchers can report and evaluate their study in the context of other research.

Often, individual researchers or research groups may have leading expertise or a particular interest in one or perhaps two pillars. Focusing on a smaller subset of the experimental space can allow for a more detailed analysis of specific mechanisms or may be done out of necessity (e.g., availability of experimental equipment, limited material quantities, or resources available at the home institute). It is important to note that experiments which consider a smaller scope or are conducted in more simplified systems (i.e., nearer the origin of the conceptual 3-D experimental space) are often essential for developing an understanding of the mechanisms and processes which drive ENM transformation, transport and impacts to biota. However, integrating all three pillars together will eventually allow those working in the field of nanomaterial environmental health and safety (nano EHS) to perform more robust assessments of risk and more appropriately suggest factors which could mediate environmental impacts. In recent years, interdisciplinary research teams, often across institutes, have begun leading in this endeavor to cover the breadth of expertise necessary to complete a well-rounded study assessing the environmental fate and effects of ENMs.[8, 10, 11]

Pillar 1: Properties of Engineered Nanomaterials

It has been well documented that the physiochemical properties of ENMs will change over time in response to how the ENMs are incorporated into products, how these products are used, and the various environmental fate pathways that the ENMs may follow.[12-14] Consequently, this first pillar underscores that the properties of ENMs are not fixed and are best understood as existing on a spectrum that ranges from the pristine form of the ENM possessing their as-produced characteristics to a released/aged form having undergone various transformation processes that result in ENMs with significantly different characteristics (Figure 1). A proposed rubric guiding the assessment of environmental realism with respect to ENM properties is proposed in Table 1, with individual elements discussed in greater detail below.

Lowry et al. (2012) provide a broad summary of the types of transformations that can occur when ENMs or ENM-containing products enter the environment, including chemical, physical, and biological transformations as well as interactions of the ENMs with organic macromolecules in engineered systems, the natural environment, or in living systems. In addition, these transformations can occur in combination or simultaneously, resulting in a multitude of ENM forms that are more heterogeneous than the initial ENMs. Some transformations may permanently alter the ENM, such as oxidation and dissolution. Other transformations, such as the aggregation/agglomeration of ENMs or the adsorption of organic macromolecules, may be reversible and change in relation to the environment the ENMs are in. These transformations will ultimately dictate the environmental fate and risk potential of ENMs because their physiochemical properties under realistic environmental conditions, as opposed to more simplified experimental conditions, will drive particle behavior in the environment. As such, assessing the environmental fate and effects of ENMs must contend with the diverse number of ENM forms that exist, such as free versus matrix-embedded particles, monodispersed versus aggregated, or the absence versus presence of an eco- or protein-corona. Complicating matters further is that each of these factors may also vary with the initial ENM size and material chemistry.

Table 1. Example FRAME rubric for evaluating individual studies with respect to each of the three pillars of environmental relevance. Scoring an individual study with respect to these criteria places it within the conceptual 3-D space created by the three pillars (Figure 2).

	Score			
	Less Realistic / More Simplistic		**More Realistic / More Complex**	
Pillar 1 **Properties of ENMs**	**0**	**1**	**2**	**3**
State of Aging	Pristine, commercial or lab-synthesized ENMs	ENMs synthetically aged in simplified / lab-synthesized media (e.g., addition of NOM, cell growth media, etc.)	ENMs synthetically aged in realistic environmental media (e.g., natural waters, WWTP effluent, etc.)	ENMs released from commercially available nano-enabled products or ENMs collected/aged in the environment
Surface Coating	Not considered or reported	Single engineered coating examined	Multiple engineered coatings examined	Matrix embedded, aged, and/or exposed to environmental media for corona formation
Aggregation State	Not considered or reported	Stabilized to limit "natural" aggregation	Characterization of homoaggregates	Characterization of homo- and heteroaggregates
Pillar 2 **Experimental Conditions**	**0**	**1**	**2**	**3**
ENM Concentration	> 10 mg/L	0.01-10 mg/L	0.1-10 μg/L	< 100 ng/L
Ionic Strength / Composition	Ultrapure water	Only simple electrolytes	Synthetic water containing ionic strength/composition representative of a natural water sample	Unadjusted natural water sample
pH	Not considered or reported	Only a single pH value is examined using synthetic waters	A broad range of pH values examined using synthetic waters or adjusted natural water	Unadjusted natural water sample
Organic Matter	Not included	Model compounds utilized (e.g., SRNOM, BSA, etc.)	Natural water sample or OM extracted from a natural or engineered system	Unadjusted natural water sample and realistic NOM:ENM ratio
Natural Colloids	Not included	Simple model particles (e.g., glass beads, monodisperse engineered colloids, etc.)	Natural particles (e.g., clays, silts, sands, algae, microorganisms, etc.) added to synthetic or natural water	Unadjusted natural water sample without particulates removed
Pillar 3 **Exposure and Effects**	**0**	**1**	**2**	**3**
Exposure Scenario	No toxicology or exposure assessed	*In vitro*, single cell type	Model membrane or organ system	*In vivo*, whole organism
Test Organism(s)	No toxicology or exposure assessed	Single cell type	Single whole organism	Multiple trophic levels and/or communities
Endpoint(s)	No toxicology or exposure assessed	Mortality and/or single endpoint (e.g., uptake, etc.)	Additionally, sub-lethal effects (e.g., growth, reproduction, etc.)	Additionally, "-omics" (e.g., proteomics, genomics, etc.)

One starting point for selecting a representative ENM form (or forms) is the identification of the release pathway(s) of the ENM or ENM-containing product into the environment.[13-15] Combining material flow analysis models with fate and transport modeling can provide insights into the sequential transformations that are likely to occur once particles are released into the environment. Those considerations can then be built into a given experimental design by mimicking the physiochemical processes that ENMs may undergo via these release pathways, resulting in aged or weathered ENMs which more closely resemble those particles which are likely to exist in the environment.[16-18] However, creating an environmentally relevant ENM form in the laboratory may be easier said than done. Transformation processes are complex, may take place over a relatively long time, and it is difficult to assess when these dynamic aging processes are "complete". Many researchers have recognized the importance of using well-characterized ENMs and have subsequently improved the baseline characterization of the ENMs used in a given study. Nevertheless, many nanometrology techniques are still far from routine and a detailed understanding of the characteristics of ENMs that have undergone a myriad of transformations can be, at best, challenging and time consuming. Nonetheless, from a nanoecotoxicological standpoint, it is critical that studies determine what transformations are happening during the exposure period to more accurately describe the particle form(s) which an organism is exposed to.

For these reasons, researchers often deliberately choose to evaluate the fate and effects of ENMs using a pristine form, since this affords certain practical advantages. One is the simplicity of obtaining pristine ENMs and the ease of initially characterizing a homogenous starting material. Another advantage is that the physiochemical properties of pristine ENMs can be systematically varied by altering the size, shape, or surface chemistry. A benefit of this approach is that it allows for mechanistic insights to be developed, often leading to the development of mechanistic models describing certain processes. For example, nanoecotoxicologists have produced a significant amount of data studying the effects of pristine ENMs in laboratory systems over the last decade or so of research.[19] Unlike released/aged ENMs, which inherently have non-uniform physiochemical properties, pristine ENMs are more conducive towards the development of standard reference materials (SRMs) and standard methods for assessing the effects of ENMs. Screening methods of similarity concerning fate and hazard have become more important than ever. Through the release of Test Guidelines (TGs) and Guidance Documents (GDs) from the Organization for Economic

Cooperation and Development (OECD) and the availability of ENM SRMs from the National Institute of Standards and Technology (NIST), testing protocols have become increasingly standardized. Aside from these advantages, using pristine ENMs to assess realistic environmental fate and effects is problematic. These materials are not representative of ENMs which are actually used in products or that are released into the environment. Thus, it could be argued that they have limited applicability in nanoEHS research today when the bar for obtaining environmental realism has been raised to a higher degree.

We do not imply, however, that there is one “correct” ENM form to use when investigating the environmental fate and effects of ENMs. Rather, it is critical that research is conducted using ENM forms that exist throughout the spectrum between pristine and released/aged ENMs (Figure 1). The use of pristine ENMs can still support the development of mechanistic models that may then be used to understand the behavior of released/aged ENMs in more realistic environmental systems. Any divergence in the observed versus predicted behavior of the released/aged ENMs can help guide the refinement of mechanistic models. However, to be successful in transferring knowledge between these two extreme cases, it is important that the other pillars are also considered during experimental design.

Pillar 2: Experimental Conditions

The experimental system and its constituents can directly impact ENM environmental fate and thus alter exposure, potential for uptake, and ultimately the dose delivered to an organism. Rather than attempt to provide an exhaustive, all-encompassing discussion describing the range of conditions and environments that ENMs might encounter, the second pillar instead reflects that this range of conditions can be re-created (from an experimental viewpoint) with varying degrees of environmental realism (Figure 1). A proposed rubric for assessing environmental realism with respect to experimental conditions is provided in Table 1. For brevity, the following discussion of experimental conditions centers on aquatic environments. However, this same thought process could also be applied to other environmental compartments and the rubric modified accordingly.

Aquatic environments vary in terms of water chemistry and natural particle loads, which can influence ENM fate. Researchers have assessed how to replicate these important parameters in laboratory conditions to determine how they may influence ENM impacts in the natural environment. The pH and ionic strength of the media are known to impact the aggregation behavior and subsequent sedimentation of aquatic colloids[20-22] and this fundamental knowledge can also be applied to ENMs.[23-25] The pH and concentration of ionic species in the media can strongly drive ENM aggregation behavior, particularly when the pH of the media is close to the ENM's point of zero charge (pH_{PZC}) and polyvalent ions are present.[26-28] Another important factor altering the fate and uptake of ENMs in aquatic systems is the adsorption of organic macromolecules to the ENM surface.[5-7] Natural organic matter (NOM) is ubiquitous in aquatic environments and oftentimes stabilize ENMs against aggregation. Heteroaggregation between ENMs and natural colloids within aquatic systems can drive the aggregation and sedimentation of ENMs in realistic systems.[24, 29-34]

In an effort to control these important parameters, the use of more simplistic experimental systems can be advantageous. Here, the water chemistry can be well-controlled and the constituents in the media can be reduced to a handful of known model surrogates (e.g., Suwannee River NOM, Fulvic Acid, or Humic Acid as a model NOM[35, 36], hematite as a model geogenic colloid[37], etc.). This approach is well-suited for gaining mechanistic insights that can aid the development of models describing the phenomena under investigation, such as the heteroaggregation of ENMs with natural colloids.[38, 39] In addition, the relative simplicity of these systems is amenable to a wide-range of analytical tools, enabling both the detailed characterization of the system and the ENMs introduced into it. A key consideration that underlies this work is the need to ensure that the experimental conditions selected allow for the identification of environmentally relevant phenomena. When seemingly small changes in the experimental conditions result in a cascade of effects that impact the phenomena under investigation, it may be more advantageous to assess ENM fate, transport and ecotoxicity in more complex and realistic systems.

Some of the more environmentally realistic approaches have focused on using mesocosm-scale experimental systems to mimic the diverse and dynamic conditions found in the natural environment.[40-42] Certainly, a distinct advantage of these experimental systems is the ability to mimic a wide range of environmentally-relevant processes though the inclusion of diverse water chemistry parameters and constituents (e.g., biota, bio- and geogenic colloids, organic macromolecules, etc.). In doing so, these systems are well-suited to identify environmentally relevant processes that may be driving the fate and effects of ENMs. However, in striving for environmental realism, these experimental systems inherently come with certain tradeoffs. The first is that the substantial resources and investment that is required to create these experimental systems limits the number of replicate tests that can be conducted and prevents their wide-spread use. Furthermore, these systems can hinder the use of some analytical techniques, thus complicating both the characterization of the systems itself as well as the ENMs introduced into the system. Furthermore, for some ENMs (e.g., TiO_2), background concentrations of certain elements can complicate analytical analyses, as one will need to differentiate between the background versus ENM-specific signal.[43, 44] Lastly, the scale and complexity of these systems can limit the ability for mechanistic insights to be gained, as concomitant processes may simultaneously alter the fate and effects of the ENMs in ways that are not easy to distinguish.

While initial efforts to understand the behavior of ENMs implicitly adopted a research approach based on the assumption that ENM fate and uptake could be predicted from first-principles using appropriate physiochemical properties, recent nanoEHS research has demonstrated that the complexity of real exposures and the multitude of transformations that will occur in the environment have confounded this approach. One approach that is intended to “bridge the gap” between these two distinct types of experimental systems is the concept of functional assays, initially outlined by Hendren et al. (2015).[45] As an alternative evaluation method that has been developed alongside first-principle studies, several semi-empirical functional assays have since been advanced to provide meaningful, system-specific information appropriate for model parameterization and prediction of ENM behavior, including redox potential[46], hydrophobicity[47], attachment efficiency[48], and zeta potential.[49] In addition, diverse groups of researchers have worked to coalesce current knowledge and outline strategies to address the “translation gap” that arises when applying knowledge developed in a variety of simplified, lab-based experimental systems to predict ENM fate and effects in real-world environments.[50-52]

Pillar 3: Exposure and Effects

The rapid development of ENMs and the multi-dimensional nature of their diversity, as has been described above, creates a need to rapidly and cost-effectively assess the risks of numerous ENM types to environmental and human health.[53, 54] The ability to easily assess potential hazards allows engineers to utilize the principles of safer by design when developing new nanomaterials.[10, 55-57] As the body of work relating to the risks of ENMs increases, we develop an increased ability for informatics-based approaches to model and predict nanomaterial hazard *a priori* rather than through additional animal testing. As with Pillars 1 and 2, the research studies that contribute to these models span from simplified test systems that involve a single species in highly controlled laboratory conditions while measuring only a few endpoints (e.g., mortality) to large scale, complex mesocosms comprised of multiple species with multiple, multi-scale measures (e.g., molecular, cellular, organismal, population level effects, etc.; Figure 2). A proposed rubric for assessing environmental realism with respect to exposure and effects is shown in Table 1.

First, consideration must be given to the preparation and handling of the ENMs that can impact nanoecotoxicological test results in both simple and complex experimental systems. For example, ENMs which are well-dispersed into aquatic media can impact subsequent exposure, and thus understanding if one needs to sonicate the stock suspension or not to achieve appropriate and reproducible dosing is essential. A recent meta-analysis of *Daphnia magna* nanoecotoxicity experiments found that inconsistencies in studies could primarily be explained by differences in dispersion protocols, including sonication methodology.[58] Additional factors that could and probably should be considered are the transport, handling, and storage of the ENMs, the temperature (or temperature changes) of the experimental system over time, and the mechanism(s) of delivering the materials to a system (e.g., pipetting, pouring, stirring, etc.). The impacts of such factors are not well studied and are often under-reported in the literature, as they may seem trivial to include. However, repeatability in science is critical and that will only be improved by thorough reporting of methods and techniques that could impact experimental outcomes. For example, ecotoxicological investigations typically do not extensively examine water chemistry parameters as long as they are within the range of what the test organism can tolerate (e.g., temperature, pH, ionic strength, etc.). But given the significant impacts that water chemistry parameters can have on ENM behavior (i.e., oxidation or aggregation state), there is a pressing need to collect and report these measures.

In nanoecotoxicological studies, the simplest but least environmentally relevant studies utilize a single species and measure only one endpoint (e.g., mortality). These studies are still relevant because they can be well-controlled in a laboratory setting and are not confounded by species interaction or secondary effects from other constituents in the system. The most classic, albeit unrefined, measure of toxicity is mortality; however, other single endpoints often analyzed include sublethal impacts such as malformations, altered swimming behavior, physiology, or growth. The analysis of multiple endpoints offers a more refined assessment of the potential impacts on organisms exposed to ENMs and allows for the evaluation of unforeseen responses that may be otherwise overlooked. It should be noted the value gained from a comparative approach in which these simplified, oftentimes rapid, studies can be leveraged to look across wide material classes and provide much needed information on the relative toxicity of those materials. However, the translation of results from those studies to environmental impacts is limited as these exposures are not environmentally realistic. Organisms co-exist with other species that may mitigate or add to the toxicity elicited by an exposure or may alter the amount of ENMs that a particular species is exposed to.

At the other end of the environmental relevance spectrum, the addition or amendment of ENMs to whole lakes or rivers provide direct translation to potential real-world impacts.[59] While these studies have the benefit of direct translation, the drawbacks include the amount of ENMs required for dosing, the sheer volume of waste generated, the potential for large-scale impacts if ENMs are found to be ecotoxicants, and the ability to replicate findings in a paired system. Intermediary to conducting *in situ* exposures, large-scale mesocosm studies can provide realistic information on ecosystem risk and interspecies trophic interactions which may impact exposure and resulting toxicity to specific organisms within the system. The environmental relevance of man-made mesocosms are greater than single species exposures; however, many environmental variables cannot be controlled, such as the weather or entrance of foreign objects or organisms.[60] In addition, the amount of materials required to perform a mesocosm-scale study still limits the ability to test across multiple concentrations and locations. To improve the environmental relevance of single-species laboratory studies and to overcome the limitation for mesocosm studies, small-scale microcosms may offer a means to simulate a naturally occurring ecosystem but in a more controlled environment.[61] Multi-species community toxicological evaluations can provide valuable insight beyond single species toxicity testing, such as toxicity caused by bioaccumulation and biomagnification.[61] They can be rapid, low-cost and efficient, making them amenable for use in inter-laboratory testing strategies to assess potential community impacts from ENM exposure.

Ultimately, the goal of designing nanotoxicological studies should be to determine which factors need to be accounted for to ensure that assessments are robust enough to capture the myriad functional fate pathways that ENMs will take throughout their life cycle. Thus, it is suggested to utilize a cumulative risk framework that would allow for the capture of: multiple stressors (e.g., ENMs and their transformation products), consideration of how those stressors may be synergistic or antagonistic, multiple durations, routes of exposure, and multiple effects from exposure (potentially in different organisms, as would be recommended for a comparative nanotoxicology approach). In consideration of ecotoxicity, guidelines have been established by ASTM International for conducting aquatic microcosm assays.[62] Yet, the approach has not been readily applied to ENMs as they are costly, time-consuming, require large quantities of materials (which are often limited in supply or by cost), and will generate large volumes of waste. Thus, small-scale microcosms that can be used to rapidly assess community level impacts, biopartitioning and species sensitivity would benefit from standardization.[61] Such systems can serve as the critical link along the continuum from simple, laboratory studies to complex, field studies.

Connecting the Pillars to Assess Environmental Relevance

The assessment of realism along the axis of a single pillar is not sufficient to evaluate environmental relevance. Each pillar is connected with the others in significant and complex ways. For example, ENM properties are strongly influenced by the properties of the suspending medium where pH can influence ENM surface charge via the acid/base character of surface oxides or organic acid groups on ENM surfaces; changes in ionic strength or the presence of specific ions can control aggregation behavior; redox conditions can drive transformation processes that control ENM solubility and chemistry; and the type and concentration of organic macromolecules will dictate corona formation. Toxicological effects are also closely linked with both ENM properties and properties of the suspending medium. Changes in ENM aggregation state can impact ENM uptake by organisms; changes in surface chemistry due to corona formation will alter how ENMs interact with cells and tissues; and redox conditions, the presence of light, or the presence of other redox active or facilitating species can control the processes by which ENMs exert toxicity. The clear interdependence of the three pillars highlights the need for ENM researchers to take each of the pillar dimensions into account when designing and reporting their studies.

Researchers are faced with many choices when designing experiments focused on ENM transport, fate and effects and inevitably make compromises to best achieve their experimental aims. Collectively, these choices situate an individual study within the conceptual 3-D experimental space mapped out by the three axes identified above (Figure 2). We postulate that the use of these three pillars as a framework for explicitly and holistically assessing environmental realism will (1) serve as a tool for identifying critical knowledge gaps and (2) aid in the design and justification of experimental protocols to bridge those gaps. Below, we illustrate the use of the proposed framework in these two ways.

It is important to note that when applying FRAME in either manner, care must be taken to first establish the individual factors/components that will be evaluated when placing a study (or studies) within the conceptual 3-D space shown in Figure 2. The rubric shown in Table 1 was collectively developed to define each factor as well as the "scale" that would be used to evaluate each study (or studies). Once defined, the rubric was then applied to evaluate the studies used in our examples. While there is some degree of subjectivity in defining the factors/components that will be evaluated, our expectation is that the FRAME can be applied in an objective manner once the more subjective aspects of the rubric development are addressed . To minimize bias, it is recommended that a diverse range of experts collectively define the features of the pillars before the FRAME is applied, and that inter-rater-reliability be assessed when evaluation/scoring a given study.

Having presented a framework for assessing environmental realism, it is also important to recognize how experiments at differing levels of complexity/realism complement one-another. Often groups of studies that span multiple levels of complexity/realism are necessary to gain a complete understanding of ENM behavior and effects. For example, field-based assessments of ENM occurrence and impacts are highly realistic, but the results are location specific due to site-to-site variability in the many properties and processes that control ENM behavior. Further, such studies are expensive and often yield little mechanistic information. On the other hand, experiments in simple matrices, with well-defined ENMs and single organisms may not represent reality but do allow variables to be controlled independently, yielding mechanistic insights. What follows are selected examples of how individual studies or groups of studies from the literature, when analyzed with the FRAME, illustrate the complexity/realism with respect to one, two, or all three pillars. These examples highlight the necessity of experimentation across multiple levels of complexity/realism.

A large number of studies have examined the aggregation and deposition behavior of pristine ENMs in synthetic and natural waters of varying pH, ionic strength, and organic matter content.[63] These studies reside largely along the axis of Pillar 2, with specific examples that span this dimension illustrated in Figure 3a. Chen and Elimelech examined the aggregation of pristine nC_{60} in synthetic waters of varying ionic strength, ion valence (i.e., 1:1 and 2:1 electrolytes) and in the presence of Suwannee River Humic Acid.[26, 64] By intentionally varying properties of the suspending medium, studies like these established that ENM aggregation and deposition behavior is often well-described by Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory and that organic matter coronas generally stabilize ENMs through electrosteric mechanisms but can also induce aggregation in the presence of elevated divalent cation (e.g., Ca^{2+}) concentrations. Moving away from the origin, Keller *et al.* measured the electrophoretic mobility and aggregation of commercially available TiO_2, CeO_2, and ZnO nanoparticles in filtered waters of varying chemistry (surface water, seawater, groundwater, and wastewater).[65] By using natural waters, these studies confirmed the environmental relevance of mechanisms identified in studies using only synthetic waters. Bridging the gap between the types of studies described above, Ottofuelling *et al.* mapped out TiO_2 ENM zeta potential, aggregation, and settling behavior in synthetic waters of varying chemistry and correlated those results with characteristics and behavior of the same particles in representative natural waters.[66] While effectively spanning much of the vertical axis, all of these studies focus solely on homoaggregation, preventing them from achieving maximum environmental realism along this axis. Further, the fact that these studies use pristine ENMs prevent them from achieving environmental realism along the axis aligned with Pillar 1. The studies shown in Figure 3a were limited in scope for good reason; the simplified systems allowed detailed mechanistic understanding to be developed. However, placing these studies within the conceptual 3-D space makes the limitations of these conditions more transparent and identifies research directions that would extend these findings to more realistic systems. Logical next steps might include repeating the experiments with aged ENMs or in the presence of natural colloids.

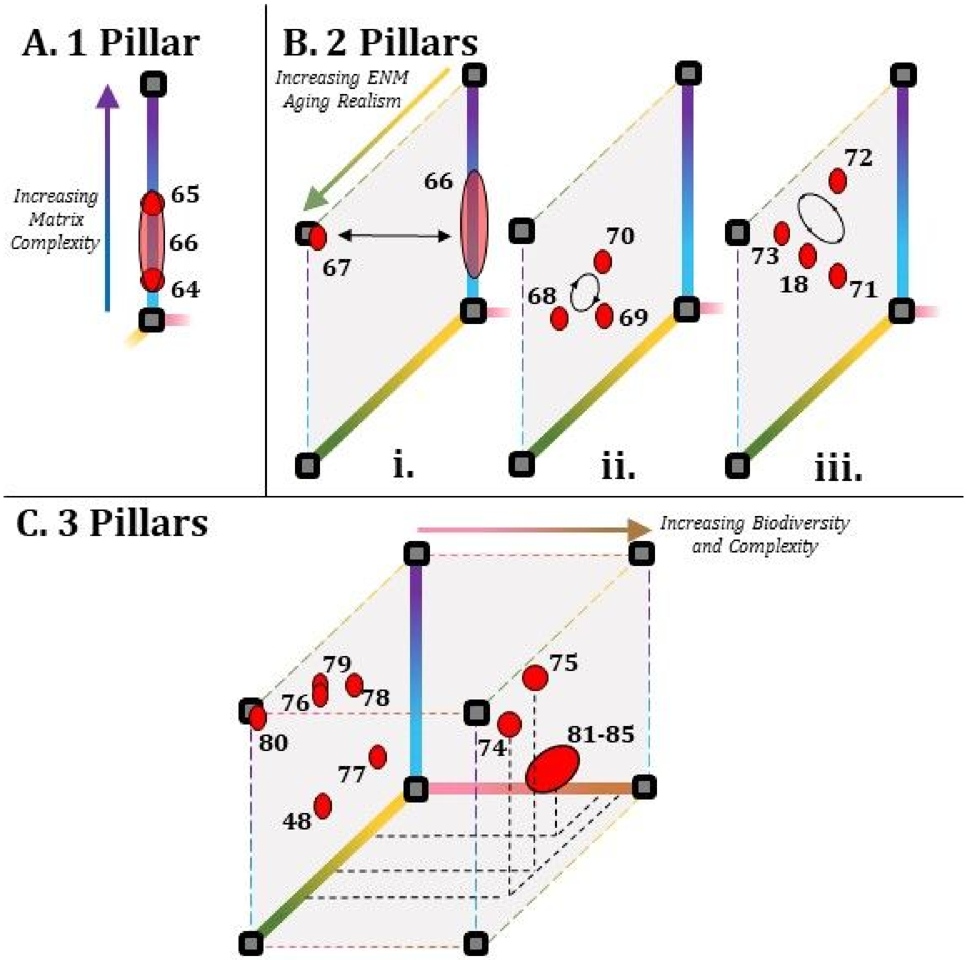


Figure 3. Examples demonstrating how individual studies would be placed within the three-dimensional space that is created when combining the three pillars that define environmental realism: A) studies focused on evaluating thee aggregation and settling of pristine ENMs in synthetic and natural waters; B) studies investigating release of TiO_2 ENMs from sunscreens into a surface water (i), the release of AgNPs from nano-enabled textiles (ii), and aging of AuNPs in wastewater and comparison of behavior with pristine AuNPs (iii); C) Studies examining the fate of CeO_2 NPs in highly realistic wetland mesocosms and supporting work on transport, transformations and toxicity in simpler systems. Numbered labels indicate references for each individual study or group of studies, see reference list. Arrows indicate how individual studies support and complement one another.

Illustrative examples of studies that demonstrate environmental realism along the axes of both Pillars 1 and 2 are shown in Figure 3b. In their study of TiO_2 release from sunscreens at a popular bathing site, Gondikas *et al.* maximized realism with respect to both the properties of the ENM (release from a nano-enabled product) and the experimental conditions (the Danube River).[67] Their findings of low concentrations of TiO_2 ENMs in the water column were strengthened by earlier work focused on aggregation and settling in synthetic and natural waters (Figure 3b-i).[66] Mitrano *et al.* examined the release of Ag^+ and AgNPs from conventional silver and nano-silver textiles during washing, pairing both realistic materials and experimental conditions.[68] Subsequent studies using laboratory prepared nano-enabled textiles, simulated wash water of varying chemistry, and simulating leaching in a landfill allowed deeper mechanistic insight and identification of controlling variables and relevant processes (Figure 3b-ii).[69, 70] Using gold nanoparticles with different engineered coatings as a model system, Surette and Nason first probed aggregation behavior in controlled synthetic matrices with varying ionic strength, pH, and natural organic matter[71]. Subsequent experiments examining the fate of these same ENMs in filtered and unfiltered river water[72] were grounded in the mechanistic insights determined in the simpler system. These same particles were then aged in municipal wastewater to increase realism along Pillar 1[18] and their behavior in river water was compared with the behavior of pristine particles (Figure 3b-iii).[73] Findings at each level of experimental complexity has informed future research while complementing previous work, defining a feedback loop through which a more complete picture of ENM behavior was realized.

Achieving realism and mechanistic understanding in two “dimensions” can often be attained by individual research groups or small collaborations but expanding the scope of inquiry to all three pillars often requires large, interdisciplinary groups. Figure 3c illustrates studies that are highly realistic with respect to all three pillars, along with supporting work of varying realism/complexity focused on the fate and effects of CeO_2. Researchers affiliated with the Center for the Environmental Implications of Nanomaterials (CEINT) performed coordinated and multi-disciplinary work over a decade, culminating in highly realistic mesocosm experiments simulating ponds[74] or wetlands.[75, 76] Wetland systems included multiple environmental compartments, terrestrial and aquatic plants, algae, invertebrates, and fish. Although pristine ENMs are typically used during the initial dosing of the mesocosms, their transformations in realistic aquatic media were followed and characterized. Notably, the mesocosm experiments were supported and complemented by the study of ENM transport[77], transformations[48, 78-80], uptake[81], and toxicity[82-85] in simpler systems. In fact, this group has argued for the use of functional assays that retain key properties of complex/realistic systems, while still allowing mechanistic insight and the determination of parameters needed for modeling efforts.[45, 86]

To illustrate how the FRAME can be used to identify knowledge gaps, we performed an illustrative meta-analysis of studies examining the fate and effects of copper nanoparticles (CuNPs) in aquatic systems. The intent of this illustrative example is to demonstrate how our framework could be applied, as opposed to an exhaustive review that might be used to evaluate the state-of-the-science regarding our selected topic. A literature search was performed to identify a representative group of relevant studies and then those studies were positioned in the conceptual 3-D space shown in Figure 2 using the rubric outlined in Table 1. Details on how the literature search was performed are presented in the Supplementary Information. Briefly, Web of Science was used to identify studies published between 2010 – 2020 that examined the environmental fate, transport, and effects of copper nanoparticles (CuNPs) in freshwater environments. Search results were refined to remove studies that, while meeting the search criteria, were not relevant to the context of the meta-analysis (e.g., impact of nano-TiO_2 on dissolved copper toxicity). This resulted in an initial group of $n = 55$ publications. A representative proportion (targeting ≈50%) were selected using a random number generator that preserved the distribution in publication years of the initial cohort. This resulted in a final cohort of $n = 29$ publications to be evaluated. To position each study within the conceptual 3-D space of our framework, the rubric presented in Table 1 was used to quantify complexity/realism on a scale of 0 (least complex/realistic or not considered/reported) to 3 (most complex/realistic) with respect to each of the three pillars. These values were determined by reviewing each paper to assign a value to the individual factors within each pillar, using the rubric presented in Table 1. Since each factor within a given pillar was equally weighted, the overall value assigned to that pillar is simply the arithmetic average of the values given to each factor. For example, if a hypothetical study evaluated the homoaggregation of pristine, commercially available ENMs that had only a single engineered surface coating, then the value for each factor of Pillar 1 (Properties of ENMs) would be 0 (State of Aging), 1 (Surface Coating), and 2 (Aggregation State), and thus the overall value assigned to Pillar 1 for this study would be 1 (i.e., [0 + 1 + 2]/3).

The results of our illustrative meta-analysis are shown in Figure 4, with the score assigned to each of the three pillars for each study provided in the Supplementary Information (Table S5). Of the 29 studies that were evaluated, the average scores were 0.72, 0.97, and 2.06 for Pillar 1 (Properties of ENMs), Pillar 2 (Experimental Conditions) and Pillar 3 (Exposure and Effects), respectively. None of the 29 studies that were evaluated scored higher than 1.0 for Pillar 1 and only three scored higher than 2.0 for Pillar 2. In contrast, of the 26 studies that evaluated exposure and effects, only a single study scored less than 2.0 for Pillar 3 (there were three additional studies that did not evaluate exposure and effects and thus received a score of zero for Pillar 3).

Through the application of our framework to collectively evaluate and compare these studies, certain insights are possible. For example, the majority of the 29 studies we evaluated typically used less environmentally realistic ENMs and experimental conditions while coupling these with more rigorous and relevant exposure and effects studies. This suggests and is further supported by the "cluster-like" distribution shown in Figure 4, indicating that environmental nanoscientists and nanoecotoxicologists have emphasized using rigorous studies when investigating the effects of CuNPs in freshwater environments but have done so while using less realistic ENMs and experimental conditions. This finding is not necessarily surprising, since exposure and effects studies oftentimes focus on developing mechanistic links between the physiochemical properties of ENMs and their resulting effects of the test organism(s), and these studies are more readily conducted using pristine ENMs and simplified experimental conditions (Figure 1). At the same time, however, this highlights the lack of studies that investigate the fate, transport, and effects of more realistic forms of CuNPs and using more realistic (and presumably more complex) environmental conditions, thus identifying these areas as knowledge gaps that could be addressed through further research.

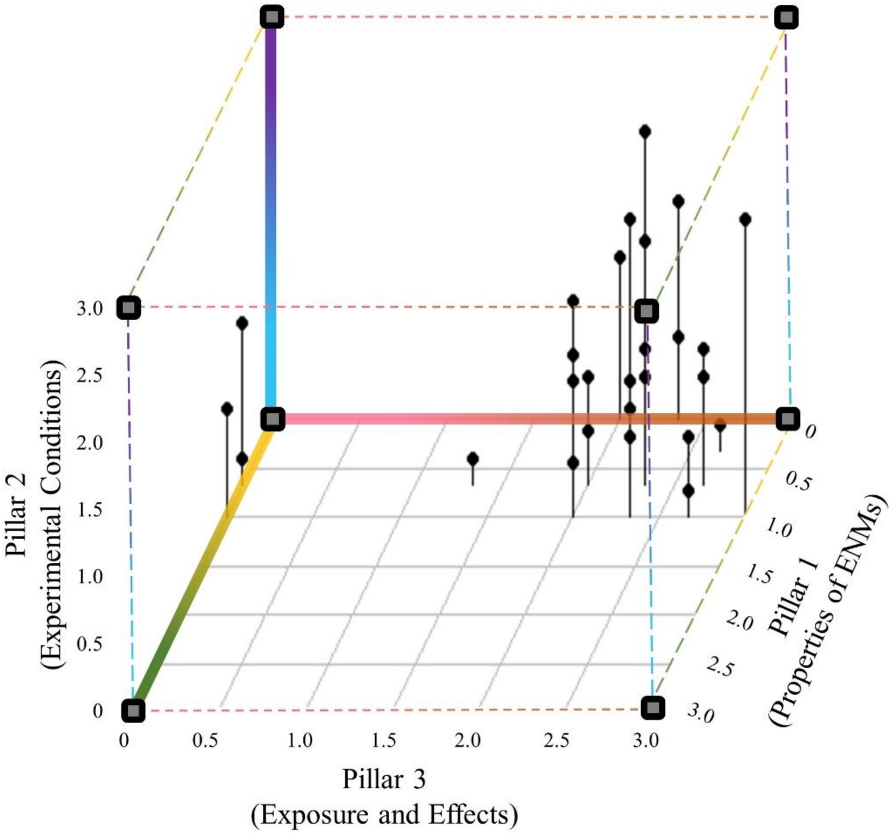


Figure 4. Results of illustrative meta-analysis, showing the positioning of the *n* = 29 studies that were evaluated within the conceptual 3-D space that is created by the three pillars that define environmental realism. Individual scores assigned to each study are provided in the Supplementary Information (Table S5).

While robust conclusions cannot be made, given the relatively small scope our meta-analysis and that its' intent is illustrative rather than exhaustive, our example nonetheless demonstrates the types of insights that can be gained by applying the FRAME concept. However, it is important to note that the factors included in our rubric (Table 1) are specific to the context of our illustrative meta-analysis. In applying our framework to different environmental settings (e.g., soils, groundwater, etc.) and research questions, it is expected that the factors that are evaluated would change.

Likewise, under different contexts in which researchers apply our framework, they may also consider weighting the factors they use. In the examples above, the factors shown in Table 1 were unweighted and thus equally influenced the score that was derived for each pillar. In practice, this approach is recommended due to its relative simplicity and the fact that it eliminates potential concerns associated with applying expert judgement (and thus potential subjectivity) when deciding how to weight the factors comprising a given pillar. In certain circumstances, however, it may be justifiable and more appropriate to emphasize certain factors over others. For example, in the context of ENM fate and transport in aquatic environments, it is generally recognized that ENMs will be transformed (aged) before their release to the environment and, upon their release, will undergo heteroaggregation with suspended particulate matter. Thus, of the three factors comprising Pillar 1 in Table 1 ("Stage of Aging", "Surface Coating", and "Aggregation State"), it may be prudent to weight the first and third factor higher when calculating the value associate with Pillar 1. In doing so, a study that utilizes aged ENMs or fully characterizes their state of aggregation (i.e., accounting for both homo- and heteroaggregation) would be considered "more realistic/more complex" than a study that utilized multiple surface coatings. As discussed previously, the intent of the FRAME is not to suggest that one experimental approach is better than another. However, with the goal of accurately understanding the "state-of-the-science" with regards to environmental realism, weighting certain factors may be prudent and reveal important insights. In any case, weighting schemes and their justification must be explicitly reported.

Finally, we applied a somewhat coarse scale in our rubric (i.e., ranging from 0 to 3). In certain situations, such as when a much larger number of studies are evaluated, it may be more useful to utilize a finer scale to capture small (but potentially important) differences between individual studies. For example, the current four-point scale used in Table 1 for "ENM Concentrations" (Pillar 2) necessitates a broad concentration range per point in order to encompass the wide range of ENM concentrations commonly encountered in the literature (i.e., from low ng/L to high mg/L). Applying a much finer scale, such as a ten-point scale, would enable a more nuanced assessment of individual studies and thus minimize "clumping" when the resulting scores are placed within the conceptual 3-D space shown in Figure 2. While this is relatively straightforward to implement for some factors, it is not as easily applied to others, such as "Organic Matter" or "State of Aging", where there are relatively fewer characteristics that can be defined to distinguish each point on a ten-point scale. Thus, care must be taken when applying the FRAME to balance nuance against practicality and to make explicit and transparent the details of the factors, scales, and weighting factors.

Advancing Fate and Effects Research

Fundamentally, advancing research on the environmental fate and effects of ENMs is dependent on an integrated understanding of various phenomena while also considering the full life cycle of the ENMs or ENM-containing products. Elucidating these functional fate pathways is inherently built on the individual connections that researchers develop between the factors and processes observed in one experimental system and the successful translation of those findings to another system (with a similar or higher level of complexity). The FRAME is intended to help researchers build those connections, with the goal of identifying environmentally relevant phenomena and outcomes.

In practice, we envision that the FRAME can be applied as a guide throughout the scientific process. Considering the FRAME early-on can help researchers keep the "bigger picture" in perspective when they think about the intended outcomes and impact of their work, potentially adjusting their experimental design to better align their approach with their objectives. When evaluating their results, the FRAME can again be used to place their findings into context with other studies. We anticipate that using the FRAME in such a manner would be highly effective with novel or under-investigated ENMs. Another approach that may prove particularly useful is using the FRAME to guide the design of complementary techniques for examining the fate and effects of ENMs, such as utilizing released/aged ENMs and environmentally realistic exposures alongside pristine ENMs and more traditional testing approaches. Alternatively, applying the FRAME in a manner similar to our illustrative meta-analysis will help researchers identify knowledge gaps and define future research directions. To some extent, researchers will often conceptually perform what we have more formally outlined in the FRAME. However, by first defining the evaluation criteria that will be used, the FRAME can serve as a useful diagnostic tool to assist researchers in more objectively evaluating existing research.

In the "Environmental Significance" sections of manuscripts, authors understandably focus on the elements of their work that are expected to translate to relevant environmental systems. Yet, discussions of the ways in which the experimental conditions diverge from or simplify those expected in the environment are less common. As a result, limitations on the application of research results to the “real world” are often neglected. Committing to a more complete assessment of environmental realism has the potential to prevent the overgeneralization of results determined in simplified experimental systems and move the field forward more quickly through the identification of critical knowledge gaps.

It is important to note that the underlying concepts of the FRAME are not limited to investigating the fate and effects of ENMs but could also guide research of other anthropogenic particle contaminants, such as micro- and nanoplastics (as has been suggested by other researchers, e.g., Huffer et al. [2017][87] and Mitrano et al. [2021][88]), or to investigate the transport of natural nanomaterials as part of global biogeochemical cycles (as suggested by Hochella et al. [2019][89]). As in most environmental research, developing a robust understanding of a given phenomenon requires both a broad understanding of diverse factors as well as a strong contextual network to link together what are seemingly disparate factors. Thus, the FRAME could serve as a guide to researchers in other fields aiming to build such a network. Whereas our application of the FRAME utilizes three components to define the environmental realism of a given experiment in order to provide this “contextual network” in nanoEHS, researchers in other fields may identify different features to serve as the guiding aspects that enable them to connect research findings.

Conflicts of Interest

There are no conflicts of interest to declare.

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