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Review Article

Using a holistic approach to assess the impact of engineered nanomaterials inducing toxicity in aquatic systems

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ABSTRACT

In this report, we critically reviewed selected intrinsic physicochemical properties of engineered nanomaterials (ENMs) and their role in the interaction of the ENMs with the immediate surroundings in representative aquatic environments. The behavior of ENMs with respect to dynamic microenvironments at the nano–bio–eco interface level, and the resulting impact on their toxicity, fate, and exposure potential are elaborated. Based on this literature review, we conclude that a holistic approach is urgently needed to fulfill our knowledge gap regarding the safety of discharged ENMs. This comparative approach affords the capability to recognize and understand the potential hazards of ENMs and their toxicity mechanisms, and ultimately to establish a quantitative and reliable system to predict such outcomes.

1. Introduction

Along with the rapid growth of nanotechnology, there arises the need for a definition of engineered nanomaterials (ENMs) for regulatory purposes. Definitions help to avoid misunderstandings and to ensure efficient communication. For regulatory purposes, a definition should be as clear and simple as possible, but at the same time unambiguous and comprehensive [1]. The European Commission's Joint Research Center Reference Reports of 2010 recommended that a definition for regulatory purposes should only concern particulate nanomaterials, be broadly applicable in European Union (EU) legislation and in line with other approaches worldwide, and use size as the only defining property [1]. The definition of the term nanoscale as recently given by International Organization for Standardization encompasses the size range from approximately 1 nm to 100 nm, a range which has been adopted in a number of other definitions as well [1]. It is noteworthy that the van der Waals diameter of a C_{60} molecule is approximately 1.1 nm. The nucleus to nucleus diameter of a C_{60} molecule is approximately 0.71 nm. Most nanoparticles (NPs) used for drug delivery are in range 200–400 nm.

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The ENMs offer great promise in many industrial and biomedical applications. However, their production processes may potentially result in human exposure by inhalation, dermal, or ingestion routes [2]. Aside from unintentional exposure during manufacture, ENMs might also be delivered to the public in our foods and medicines. Among a number of studies devoted to such issues, the work of Weir and co-workers [3] who investigated the titanium content of some common foods should be mentioned. The titanium content of the products was as high as 100 mg/serving for powdered donuts, and many products with the highest titanium contents could be characterized as sweets or candies. In addition, tremendous progress has been achieved in applying ENMs for drug delivery and tissue engineering, which also may impose risks to the patient, and even surpass their benefits [4]. Inevitably, the discharge of those anthropogenic nanomaterials along with the ones used for environmental remediation may enter water supply sources [5,6]. The quantity and form of the nanomaterials released into aquatic environments should be determined to assess the environmental risks of nanotechnology.

Clearly, the impact of these novel materials warrants strenuous and ongoing toxicological research. The fact that the physicochemical properties of the ENMs *per se*, as well as their interactions with immediate surrounding environments, determine their potential hazards, acute or chronic, direct or indirect, is extremely important [7–11]. Lack of knowledge about the transport, fate, and behavior of ENMs in the aquatic environment largely constrains our ability to assess their potential risks in a quantitative and predictable way. Furthermore, their adverse impacts on biota also vary depending on the tested species and their trophic levels. Thus, it is essential to elucidate their hazards under conditions that resemble natural environments in which they would be encountered. Consequently, two critical issues arise—first, the need to establish a reliable and standard system to clarify the real-time states of targeted ENMs in natural aquatic environments; second, the need for a nanotoxicological study with a holistic approach. This approach will provide a database on vulnerable species in multiple trophic or structural levels in an aquatic ecosystem for assessing the potential hazard of ENMs in a comprehensive way. This database may also afford an opportunity to compare results with other studies to support the formulation of more reliable conclusions.

Much of the existing research on nanotoxicity has concentrated on empirical evaluation of the toxicity of various ENMs with less attention being given to the relationship between nanomaterial physicochemical properties (including chemical composition, shape, size and size distribution, dispersion, aggregation state, surface area, surface chemistry, surface charge, and porosity) and toxicity [12]. This approach gives limited information and should not be considered adequate for predicting the toxicity of seemingly similar ENMs. Instead, if condition permits, we should adopt a systematic approach for studying the toxicity of ENMs by focusing on a group of test nanomaterials of similar physicochemical properties with variation in one particular parameter [13]. It is also noteworthy that characterization of supplied nanomaterial may not actually represent physicochemical properties of the material during or following administration. Therefore, wherever possible, independent characterization of test nanomaterials should be conducted prior to and after administration [12].

In this report, we reviewed the progress of research from many scientific entities from the standpoint of the nature and behavior of the nanomaterials themselves, to the response of organisms in aquatic ecosystems when exposed to them, and also the different approaches to nanotoxicological assessment. It is the purpose of this review to use existing databases on ENMs based on our proposed holistic approach and bridge the gap between ENMs and aquatic ecosystems.

2. Fundamental knowledge needed to understand nanotoxicology

In 1985, Harold Kroto and colleagues [14] discovered C_{60}, and shortly thereafter came to discover the fullerenes. This discovery led to much research into the behavior of these unique particles and eventually to the advent of engineered or anthropogenically produced nanomaterials, or materials on the nanoscale. Today, there are numerous ENMs that are providing the impetus to significant advances in science, engineering, industry, and commercial enterprise. The manufacture, processing, and application of nanomaterials provide opportunity for them to come into contact with humans and other organisms and plants that make up the planet's ecosystems. Along with the technology is the need for nanotoxicology, because the extent of any risk posed by these products is not known completely. Nanotoxicology can be defined as a safety evaluation of engineered nanostructures and nanodevices. Collectively, some emerging concepts of nanotoxicology can be identified from the results of studies already completed.

The ways and means of nanotoxicology discussed in this review are a summarization of the progress in discovering the mechanisms of toxicity of these nanomaterials. It is prudent to investigate not only the response from organisms exposed to them but also the fate of the ENMs themselves as they enter the natural environments. This necessitates a careful examination of the physicochemical characteristics of the nanomaterials and the use of surrogate models ranging from microorganisms, to invertebrates, to vertebrates, particularly, those of aquatic species. Building the experimental database of the relationships between the physicochemical properties of ENMs and their biological end-point response is required for the development of predictive toxicology. Relative to experimental evaluation of the safety of ENMs, computational methods are less expensive and time saving. They may also be efficient alternatives for predicting the potential toxicity and environmental impact of new nanomaterials prior to when they are mass produced. Therefore, the quantitative structure—activity relationship (QSAR) study commonly used to predict the physicochemical properties of chemical compounds is quickly emerging in pharmaceutical and nanotoxicological research.

Among many physicochemical characteristics, crystal structure of NPs is critical in determining their toxicity. The specific spatial arrangement of atoms is closely relevant to their potential oxidative reactivity. NPs demonstrate different

chemical reactivity with respect to various crystal faces [15,16]. It has been shown that the chemical activity of NPs can be tailored by controlling the exposed crystal facets of particles, although the underlying mechanism is not well understood yet. The toxicity of the studied nanomaterials depends on their crystalline form accordingly [17−19]. Crystal morphology was found to be closely related to crystal structure [16,20,21]. The external crystal morphologies can be rationalized in terms of the intermolecular interactions between the growth units. Thus, the morphology factor is also of great importance. A NP's morphology can strongly affect its chemical activity [15,16,22,23], which ultimately affects total cell uptake [24] and toxicity [25].

Within a given crystal structure and morphology, the dimension of NPs becomes a primary property in determining the behavior and fate of NPs in aquatic ecosystems. Although some reports claim that the chemical activity or toxicity is not dependent on particle size [15,26], others agree that it continues to play a critical role in engineering green NPs [27−32]. Most of those studies carrying criticisms used only a few different sizes in range, which may lead to an incomplete conclusion. Furthermore, other properties, such as, morphology and crystal structure, need to be carefully evaluated and confined when size is studied. Noticeably, crystallites are known to be highly sensitive to phase transformation as the size changes, especially in the case of TiO$_2$ NPs. This phenomenon was suggested to be involved in both chemical activity and toxicity toward cellular organisms [33,34]. Other than primary size, aggregation of particles, especially in the case of metal-based NPs, could play a critical role as well [35,36]. Overall, the level of aggregation was found to be concentration, rather than size dependent [29]. Furthermore, surface chemistry along with the environmental factors might also contribute to their propensity to form aggregates in aquatic systems [29]. For example, in natural aquatic ecosystems, the presence of ions may increase the rate of sedimentation, decreasing potential risks to pelagic organisms while increasing the risk to benthic organisms [36]. Thus, rapid aggregation in hard groundwater would be expected for most NPs [10]. These interactions affect the aggregation of the NPs in solution, as well as their attachment to environmental surfaces, which ultimately would influence the results of the *in vivo* or *in vitro* nanotoxicity studies.

Surface modification is another powerful process to tune the chemical properties of ENMs, alter their behavior and fate in biological systems, and consequently change their toxicity. Through such a modification, particle size can be changed, stability can be improved, and surface reactivity can be enhanced [9,37−39]. In addition, interaction with the environment (biological and physical) can be altered and controlled by proper surface modification. These interactions ultimately affect the drug delivery efficiency [37,38], photocatalytic activity [40,41], and may yield increased or decreased toxicity toward organisms [42].

Although these aforementioned correlations are known, no QSAR has been developed upon them. There are only limited physicochemical properties of ENMs that have been developed into predictive paradigms for nanotoxicity. Recently published were some reports concerning the prediction of toxic nanomaterials through a band-gap database

[43,44]. Burello and Worth [44] proposed a theoretical model that predicts the oxidative stress potential of oxide NPs by evaluating the ability of these materials to perturb the intracellular redox state. They claimed that NPs with band energy values that are comparable with the cellular redox potential could trigger and accelerate electron transfers resulting in oxidative stress and a cytotoxic response *in vitro*. For example, NPs with an energy gap (in electron volts) between −4.2 and −4.8 (e.g., Cr$_2$O$_3$, CoO, Co$_3$O$_4$, Ni$_2$O$_3$, and Mn$_2$O$_3$) are deemed to be toxic materials, which is in good agreement with the band-gap predictions [43]. These reports suggested that it is possible to predict the toxicity of NPs by structure−activity relationships based on band-gap energy levels.

As discussed by Puzyn et al [45], cation charge is closely related to ΔH_{Me+}, which refers to the enthalpy of formation of a gaseous cation having the same oxidation state as that in the metal oxide structure. They developed a QSAR upon ΔH_{Me+} of metal oxide NPs for their toxicity prediction. Positive values of ΔH_{Me+} increase with increasing charge, which in turn mediates the cytotoxicity of the metal oxide NPs (Fig. 1). Consequently, the release of Me^{n+} cations having smaller charges is more energetically favorable than the release of cations with larger cation charges. This explains why the toxicity of the studied oxides decreases in the following order: Me^{2+} > Me^{3+} > Me^{4+}. For example, it is commonly recognized that the toxicity from arsenic exposure varies in the order As^{3+} > As^{5+} [46,47]. Hu and co-workers [48] also reported that the cytotoxicity of seven metal oxide NPs was highly correlated with their cation charges. A similar conclusion, that is, the higher the cation charge, the lower the cytotoxicity of the metal oxide NPs, was made.
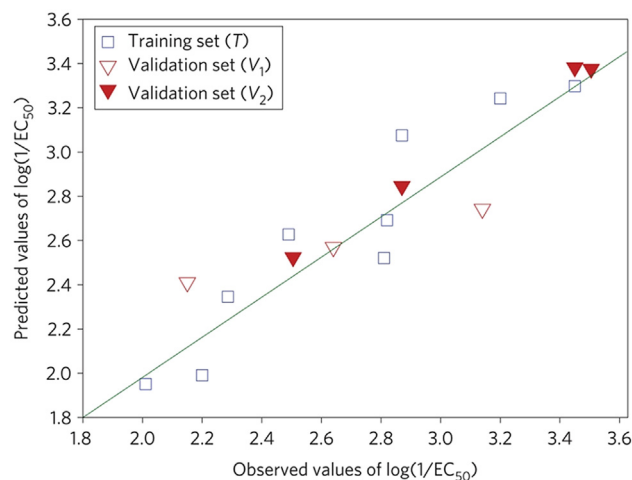

Fig. 1 − Plot of experimentally determined (observed) versus predicted log values of 1/half maximal effective concentration (EC$_{50}$). The straight line represents perfect agreement between experimental and calculated values. The distance of each symbol from the line corresponds to its deviation from the related experimental value. *Note.* From "Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles," T. Puzyn, B. Rasulev, A. Gajewicz, et al, 2011, *Nat Nanotechnol*, 6, p. 175−8. Copyright 2011, *Nature Publishing Group*. Reprinted with permission.

3.　Common mechanisms of nanoecotoxicity

3.1.　Reactive oxygen species

It is well-known that reactive oxygen species (ROS) plays an important role in eliciting the adverse health effects of NPs, leading to a series of biochemical reactions that can ultimately result in cell damage [e.g., depletion of reduced glutathione (GSH), lipid peroxidation (LPO), and DNA damage] and even cell death [49]. Normally, cellular redox homeostasis is well self-regulated by its own antioxidant defense complex. However, excessive ROS generation could cause consequent oxidative stress, weakening antioxidant defense. Starting with NP uptake across the cell membrane, generation of ROS and the activation of redox-sensitive signaling cascades are then initiated [50]. Thus, it leads to the damage of cellular proteins, lipids, and DNA [51]. Li et al [52] comprehensively reviewed the role of oxidative stress in the toxicity of ENMs. It is clear that certain NPs are capable of producing ROS under abiotic and biotic conditions. For example, Pathakoti and co-workers [53] recently reported that TiO_2 NPs were involved in the generation of abiotic ROS and intracellular ROS. The observed increase in abiotic and intracellular ROS levels was correlated with a corresponding increase in cytotoxicity. Thus, once the NPs entered the cell, their interaction with subcellular organelles (e.g., mitochondrion) and biological systems can cause further ROS formation. Consequently, oxidative stress is directly related to NP-induced toxicity.

Semiconductor nanomaterials could elicit an excited energy state that leads to the generation of $O_2^{•-}$ [54]. This radical is capable of damaging cellular macromolecules or mediating cellular signaling pathways to cause cellular dysfunction. In addition, further radical reactions (by dismutation or Fenton chemistry) of superoxide anions can lead to the generation of additional ROS, including singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($•OH$). For example, the excited CdSe quantum dots (QDs) are capable of injecting electrons into TiO_2 [55]. In addition, $O_2^{•-}$ could also be transformed by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) [56]. Moreover, electron hole pairs formed through photoactivation of TiO_2 could also generate $O_2^{•-}$ and $•OH$ radicals [57]. Noticeably, the metal-ion dissolution of certain NPs (e.g., ZnO NP) could be involved in both the generation and mitigation of ROS. For example, Xia et al [58] reported that ZnO dissolution increases the concentration of intracellular Zn^{2+} and is correlated with high levels of ROS production. Thus, ROS generation by nanomaterials could lead to possible adverse biological effects through an oxidant injury mechanism.

Although some inert nanomaterials are not capable of generating ROS directly under abiotic conditions, it is still possible that they may catalyze the formation of ROS indirectly, by reacting with intracellular systems. Xia et al [59] reported that NH_2–polystyrene (PS) nanospheres are incapable of spontaneous ROS formation, yet are strong inducers of H_2O_2 and $O_2^{•-}$ production at the cellular level. For example, cationic PS nanospheres are capable of leading to mitochondrial damage and adenosine triphosphate (ATP) depletion, which in turn increases the generation of intracellular ROS [60].

In addition, generation of ROS is closely correlated to unique physical (size [61,62], shape [62], crystallinity [19,35], and surface charge [63]) and chemical (surface coating [64,65] and elemental composition [62]) attributes. These physico-chemical properties of nanomaterials can facilitate creation of chemical conditions to induce a pro-oxidant environment in the cells, causing an imbalanced cellular energy system dependent on redox potential and thereby leading to adverse biological consequences, ranging from the initiation of inflammatory pathways to cell death.

Detailed discussion on the mechanisms of ROS production can be seen in the work by Fu et al in this issue.

3.1.1.　GSH depletion
GSH, one of the most abundant soluble antioxidants, consists of cysteine, glycine, and glutamate. The GSH/glutathione disulfide (GSSG) complex serves to regulate intracellular redox balance and protect cells from ROS damage. A decrease in the cellular GSH/GSSG could directly lead to severe intracellular oxidative stress. Thus, depletion of GSH could significantly affect cellular metabolism [66], cause mitochondrial dysfunction [67], and result in ATP depletion [68]. The GSH depletion is also associated with cellular predisposition to apoptosis [69].

The depletion of GSH, associated with reduced mitochondrial membrane potential and increased ROS levels, has been found to be closely related to a presence of ENMs [70]. It has been suggested that the loss of GSH resulting from nanotoxicity may weaken cellular antioxidant defenses and result in the accumulation of ROSs that are generated as by-products of normal cellular function [71]. For example, NP-induced decline in GSH could cause mitochondrial transmembrane potential ($\Delta\Psi m$) depolarization [72,73], increase mitochondrial H_2O_2 production [72], lead to Bax translocation [72], result in cytochrome c release [72], initiate downstream caspase 3 activation [72,74], and trigger apoptosis [72,74].

3.1.2.　LPO
LPO is the process by which ROS oxidizes membrane lipids in biological systems, initiating a free radical chain reaction. Once the reaction chain is triggered, severe damages to the cell membrane may occur, as the reaction continues. Moreover, end products of the reaction chain may be mutagenic and carcinogenic. Among them, malondialdehyde has been considered as the most commonly used end product for the quantification of LPO. Furthermore, the LPO levels increase with the elevation of ROS formation. Thus, LPO has been recognized as one of the most critical indicators for cellular oxidative stress.

Correlation between LPO and nanotoxicity has been widely studied and reported. Numerous data suggest that NP-induced oxidative stress consistently produces LPO. Pathakoti et al [53] reported that the LPO levels were elevated with the increased production of ROS after treatment of *Escherichia coli* with the four studied TiO_2 NPs. Similarly, LPO and resultant membrane damage have been proposed to be mainly due to the cytotoxicity of nano-sized C_{60} [75], QDs [76], Fe_2O_3 [77], SiO_2 [78], Ag [79], CuO [80], Au [81], ZnO [82], and Ni [74]. Moreover, LPO associated with NPs may further lead to adverse effects in the brains and gills of mammals and fish, resulting in irreversible severe damage [83,84].

3.1.3. Mitochondrial damage

Mitochondria are involved in a range of cellular processes such as signaling, supplying energy (i.e., ATP), cellular differentiation, cell death (apoptosis), and cell growth. It has been recognized that the mitochondrion is one of the most critical indicators for the toxicity of many exogenous compounds. In addition, it is well-known that mitochondrial damage is closely correlated with the generation of intracellular ROS. The disorder of intracellular ROS imbalance could lead to structural disruption of the mitochondrion. Excessive generation of ROS can drastically alter the permeability of the mitochondrial membrane, disrupt the respiratory chain, and lead to DNA damage, affecting cell cycle progression [85]. Furthermore, the mitochondrion is also a key factor in the process of cell apoptosis. The Bcl-2 protein family and CytC are both involved in the regulation of mitochondria-mediated apoptosis, especially the changes of Bcl-2/Bax ratio, and the formation of apoptosome and the caspase-9 activation, respectively [86].

It has been revealed that ENMs could specifically deposit in mitochondria and lead to oxidative stress-mediated mitochondrial damage [59,86–89]. Mitochondria associated with the cell membrane and cell nucleus have been considered as major cell compartments relevant in possible NPs-induced toxicity [50]. For example, Ag NPs could induce a mitochondria-dependent apoptotic pathway by ROS generation [88]. Upon Ag NP exposure, Bax and Bcl-2 expressions were altered to further lead to the loss of mitochondrial integrity, resulting in the release of cytochrome c and the disruption of $\Delta\Psi$m. Cytochrome c release was followed by caspase 9 and caspase 3 activation. In addition, the loss of $\Delta\Psi$m was a result of the downregulation of Bcl-2, and upregulation of Bax. Thus, it is clear that ROS generation could lead to mitochondrial damage by cellular regulation.

3.1.4. Membrane leakage of lactate dehydrogenase

In addition to the aforementioned cell apoptosis, lactate dehydrogenase (LDH) release is an indicator of necrosis due to cell membrane damage [90]. The LDH leakage assay is most commonly used for membrane leakage testing, which is based on the measurement of extracellular LDH activity. In addition, it is well-known that there is a close correlation between ROS formation and LDH activity [91–93]. Thus, the loss of intracellular LDH and its release into the extracellular medium is an indicator of ROS generation, resulting in irreversible cell membrane damage and further leading to cell death.

The cytotoxicity of nanomaterials may be mainly associated with membrane damage and high LDH leakage [71,94]. Disruption of membranes resulting from nanomaterials has been revealed to be caused by the toxicity induced by a wide range of nanomaterials, including carbon nanotubes [95], semiconductor nanomaterials [96], semimetal-based nanomaterials [73,91], and other metal and metal oxide nanomaterials [71,93,94,97]. It has been demonstrated that monitoring of LDH activity can be a useful tool for evaluating the toxic effects of NPs [98]. For example, Lin and co-workers [91] reported that there was a linear correlation between LDH activity and cell viability, which was also highly correlated with the levels of ROS generated by silica NPs. Furthermore, the steep elevation of LDH associated with cell membrane disruption could act as an indicator for cell necrosis [73,93].

3.1.5. DNA damage

A large number of NPs have been shown to be genotoxic, in addition to being cytotoxic, resulting in DNA damage, chromosome aberrations, and cell death above a certain threshold concentration. Although the exact mechanism behind nanotoxicity is yet to be elucidated, many studies have suggested oxidative stress playing an important role in NP-elicited DNA damage and subsequent cell death [99–101]. The ROS family could react with DNA, leading to DNA mutation and impaired/defective repair [102]. Among the ROS, the highly reactive •OH reacts with DNA by adding to double bonds of DNA bases (i.e., purine and pyrimidine bases) and the deoxyribose backbone as well [103]. For example, the generation of •OH by TiO$_2$ NPs could elevate the levels of formamidopyrimidine DNA glycosylase-sensitive sites, indicating the oxidation of purine DNA bases (i.e., guanine), in both the absence and presence of UV-A irradiation [99]. Furthermore, genes could be up/down-regulated by the oxidative stress associated with NPs. Li and colleagues [100] reported that polynucleotide kinase 3′-phosphatase and the cyclooxygenase-2 were significantly upregulated in Au NPs-treated lung fibroblasts. Consequently, it could arrest the cell cycle at a certain stage in response to DNA damage. Duan et al [73] revealed that the silica NPs caused G$_2$/M arrest in human umbilical vein endothelial cells induced by oxidative DNA damage.

3.2. Metal-ion leaching

In addition to oxidation-induced damage, metal ions released from certain nanomaterials could also act as a key factor in causing cell damage. The release of metal ion is always not only associated with, but is also independent from, the generation of ROS for the NPs with a high level of dissolution [33,104]. Normally, metal-ion leaching is a direct consequence of the breakage of chemical bonds in the crystal lattice or redox reactions. In the latter case, the released ions always tend to enhance the toxicity induced by ROS. Moreover, it could also in turn elevate ROS formation, leading to further cellular damage [105,106]. For example, not only does ROS increase Zn^{2+} release from ZnO NPs but also Zn^{2+} could elevate mitochondrial ROS generation [70,107,108]. It was suggested that the released Zn^{2+} may contribute to mitochondrial and extramitochondrial ROS generation, resulting in aging-related neurodegeneration by activation of nicotinamide adenine dinucleotide phosphate-reduced oxidase as well as NO production and generation of peroxynitrite (ONOO$^-$) [109,110]. In addition, the concentration of dissolved Zn^{2+} tends to equilibrate in the complete cell medium [93], cytosol [111], lysosome [112], endosome [111], and mitochondria [111], maintaining a high level of Zn^{2+}. The elevated intracellular Zn^{2+} could then lead to inflammation, [Ca^{2+}]$_i$ flux malfunction, lysosomal damage, mitochondrial dysfunction, and caspase activation [58,111]. Through the interference with the intracellular homeostasis of Zn^{2+}, it could also inhibit cellular respiration by interfering with cytochrome bc1 in Complex III as well as with α-ketoglutarate dehydrogenase in Complex I [58]. Noticeably, dissolved metal ions other than

nanomaterials *per se* may be more readily bioavailable. Ultimately, high levels of Zn^{2+} could result in apoptosis/necrosis in target cells [58,111,113]. Moreover, other dissolvable nanomaterials are also reported to be toxic, owing to their high level of metal-ion release. For example, Ag^+ released from zero-valent silver NPs was reported to cause oxidative stress following the generation of ROS [114,115]. Auffan et al [116] and Keller et al [10] also suggested that the release of Fe^{2+} and Fe^{3+} cations from the metal surface was associated with ROS formation. The toxicity of CuO NPs was also suggested to be attributed to the release of Cu^{2+} [117].

With this knowledge in mind, it is critical to consider the way to stabilize dissolvable metal or metal oxide NPs in determining their fate in the environment and within the organisms. The process of dissolution and subsequent toxicity are known to be related to physicochemical properties, such as the crystallinity, specific surface area, size, and conduction band energy. Thus, it is possible to predict and control the toxicity of nanomaterials through decreasing metal-ion release. Generally, surface doping is the most commonly used technique to stabilize nanomaterials. It was found that physicochemical properties, for example, ζ-potential and aggregation state, of NPs could be modified by surface doping [118]. For example, Zn^{2+} release was reduced by iron doping [107,118,119]. Thus, the stability of doped NPs could be strongly improved, leading to decreased metal-ion leaching. Ultimately, the stabilized nanomaterials become less toxic toward living organisms. For example, Derfus et al [120] reported that CdSe-core QDs can be rendered nontoxic through appropriate surface coating. In addition to slowing metal-ion release, surface doping could also mediate cellular metabolism. George and co-workers [107] reported that Fe doping on ZnO NPs could slow the loss of mitochondrial membrane potential, propidium iodide uptake, and $[Ca^{2+}]_i$ increase in BEAS-2B cells with increased atomic percentage of Fe. It also may interfere with the inhibitory effects of Zn^{2+} on zebrafish hatching, be associated with decreased polymorphonuclear cell counts and interleukin-6 messenger RNA production, and lead to decreased heme oxygenase 1 expression in the murine lung [119]. All considered, surface doping is a possible safe design strategy for preventing the toxicity of dissolvable nanomaterials in living organisms and the environment.

3.3.   Nano−bio−eco interfaces

The physicochemical properties of nanomaterials are highly correlated with the surrounding environments. Here we summarize their intrinsic properties with two scenarios, namely, biochemical and environmental systems. Nano−bio interfaces refer to the interactions between nanomaterials and biomolecules (e.g., proteins and DNA) in biological environments. For biomolecules, on the one hand, these interactions may lead to the formation of protein coronas, denaturation of proteins, nano−bio complexes or ion corona without protein, particle wrapping, and biocatalytic processes that could have adverse impacts [7,9,11]. For nanomaterials, on the other hand, the interactions may cause phase transformations, free energy releases, particle aggregation, restructuring, and dissolution at the nanomaterial surface [7]. Moreover, nano−eco interfaces here refer to the interactions

between nanomaterials and abiotic natural environments. Natural dissolved organic matter (DOM), surfactant, temperature, ionic strength, and pH may lead to changes in physicochemical properties (e.g., size, aggregation state, surface chemistry such as surface charge and surface modification, and dissolution [10,121]). In addition, geological activities (e.g., water cycling) may alter their bioavailability; others, such as sunlight, may initiate photocatalytic activity [8,13,122,123]. Consequently, their toxicity could be altered. Readers are encouraged to retrieve additional relevant information on these topics from the review report by Hwang et al [13].

Characterizing the nano−bio interface is largely dependent on the fair understanding of the immediate surrounding environments and its interaction with nanomaterials. The dynamic microenvironments could result in uncertainty about the fate and behavior of nanomaterials at the cellular level. It is now increasingly acknowledged that the dynamic biomolecules, as a result of cellular metabolism, could modify the surface of nanomaterials, leading to biophysicochemical interface complexes known as coronas [124−127]. The binding of protein with nanomaterials to form coronas can critically affect and determine their intrinsic toxicity toward living organisms [128,129]. Tenzer and co-workers [130] reported that rapid corona formation is found to affect hemolysis, thrombocyte activation, NP uptake, and endothelial cell death at an early exposure time. In addition, the quantitative characterization of such nano−bio interfaces under biological conditions could open a quantitative avenue in predictive nanomedicine development, risk assessment, and safety evaluation of nanomaterials [131,132].

It has been noticed that the first step in conducting nano-ecotoxicity research on the potential risks to ecosystems and human health of nanomaterials is to determine the means of exposure after release into the environment [133]. In natural ecosystems, it may always involve physicochemical interactions at abiotic aqueous−geological−nanomaterials interfaces. Thereby, it has been known that some nanomaterials could be more toxic in natural ecosystems, whereas others may be less toxic under such conditions and more toxic in pure culture experiments [123]. Through their interactions, the transport of both nanomaterials and natural colloids/contaminants could be facilitated [134−136], the deposition of NP could be augmented [136], and the bioaccumulation of certain contaminants could be enhanced [137]. For example, carbon nanotubes suspensions could be stabilized by dispersants [134], such as surfactants and polymers, as well as DOM [135].

Indeed, understanding these various nano−bio−eco interfaces can allow the development of a knowledge base between predicted and actual toxicity that accounts for both the nanomaterial properties and its surrounding environments. This may turn out to be an important paradigm for understanding the *in vitro* and *in vivo* fate, behavior, and subsequent toxicity of nanomaterials.

4.   Calling for a holistic approach to assessing nanotoxicity

In response to these developments in the field of nanotoxicology,   more   complex   forms   of   nanomaterials'

environmental behaviors have evolved and been revealed. These forms of behavior have been related to various species and cells in various ways. They allow us to predict the fate and toxicity of ENMs in realistic environments, to reduce their risks simultaneously, to introduce green nanotechnology into industry, and to bring greater detail to our knowledgebase. Underlying the above is a realization by chemists, biologists, and ecologists that in order to be cost effective and highly reliable, the modern ENMs also need to be eco-friendly and safe to human beings. Indeed, a broad category of researchers have studied the safety of nanomaterials and assessed their risks when released into environments or inhaled by humans. However, large contradictive results in the database have also attracted public attention. Intuitively, the behavior and subsequent adverse impacts of the same NPs can vary drastically among different species, across trophic levels, and in various environments. Nonetheless, more systematic studies should be available for the construction of a knowledge base where the physicochemical properties of the nanomaterials, surrounding environments, and tested species are truly varied and well characterized. Thus, a review of environmentally related literature by the authors led to the conclusion that a holistic, robust, complete, detailed, and sound risk assessment system is needed. It should encompass the various types of test species and their surroundings, and take into account all possible properties of nanomaterials in terms of risks and quantification of risks posed by them.

A number of knowledge bases have been found in the literature reviewed to date and one of the most common approaches is to simulate the cellular environment and carry out organotypic assessment in vitro. It has been noticed that there are three-dimensional (3D) cell culture models that mimic the morphological and functional features of their in vivo living tissues [138−140]. Because there are no essential cellular functions present in tissues of pure cell cultures, it could not be accurate to predict the cellular responses of real organisms. Therefore, establishing 3D cultures as a mainstream approach may, according to Pampaloni and coworkers [141], have a strong impact on toxicity assessment and will also decrease the use of laboratory animals. It thus could be one of the most capable candidates to assess toxicity in vitro, thereby improving the predictive power of in vitro NP toxicology [142].

Although a large number of approaches to nanotoxicological assessment have been developed on the basis of in vitro surveys, mostly 2D cells, the aforementioned efforts in the progress of toxicity assessment suggest that such approaches alone may not be sufficient. It has also been discovered that in vivo assessment is very critical because we do not have a valid and reliable candidate yet [143−145]. The successful development of valid in vivo assays for evaluating the toxicity of nanomaterials is critical for early stage nanomaterial design. In addition, successful development of in vivo toxicity screening systems could provide high accuracy and predictability compared with their in vitro counterparts. The former provides information about whole, living organisms, which is used to estimate the effects of exposure to whole organisms. These observed overall effects of an experiment on a living organism are the main features of the former that are used to validate the latter.

Although it is not possible at this time to develop a detailed, mature predictive paradigm, scientists acknowledge the need to address the development of in vitro and in vivo QSAR to perform the nanotoxicological study. On the basis of our efforts to integrate experiments with computational research for nanomaterial hazard assessment, a specific aim of the National Science Foundation-funded Jackson State University Interdisciplinary Nanotoxicity Centers of Research Excellence in Science and Technology is to study the mechanisms of the toxic action of nanomaterials, as well as provide relevant scientific information for making informed decisions regarding the cost-effective management of nanomaterials based on QSAR [45,146−148]. Contemporarily, the development of rapid throughput or high-throughput screening (HTS) and high-content screening procedures to assess nanotoxicity is also adopted in many current studies, enabling a large quantity of samples to be screened using several different physical, chemical, and biological assays in a short period [108,118,149,150].

The earlier discussion illuminates the need for a holistic approach to conducting nanotoxicity research. Here, we focus mainly on early stage design of experiments with respect to a variety of different species and trophic levels in aquatic ecosystems.

4.1. Microorganisms

Among the in vitro assays, the microorganism paradigm is often favored, owing to its merits of rapidity and cost effectiveness. It is well-known that microorganisms play an important role in food webs and in organic matter decomposition and nutrient cycling. In addition, they also influence the whole aquatic ecosystem metabolism through their decomposing activities, mediating pH, alkalinity, and redox conditions in the aquatic environments. Hence, it is appropriate to evaluate any adverse effects of xenobiotic chemicals on applicable microorganisms when constructing predictive models for determining the use and clean-up of those contaminants [151,152]. The increasing need for the use of nanotechnology for environmental remediation has extensively increased the potential risks to microorganisms. The discharged nanomaterials first enter the food chain through microorganisms and eventually affect the equilibrium and homeostasis of aquatic systems, even the biosphere [153]. Therefore, it is appropriate in the first step to investigate the potential hazards of ENMs using microorganism models in evaluating their cytotoxicity (Table 1).

Existing reports on the adverse effects of nanomaterials on microorganisms have shown that the interaction of nanomaterials with biomolecules directly, or indirectly, could lead to strong antimicrobial activity [154]. Some of the reports indicated that morphological changes were induced as a result of nanotoxicity. This could be associated with disorganized cell walls [155]. The ROS could cause bacterial cell membrane damage, including membrane disorganization, membrane permeability change, and membrane breakage, and lead to subsequent cell death [156,157]. Moreover, intracellular metabolism could be altered as a result of the changes in the redox cycle in the cytosol, intracellular radical accumulation, and dissipation of the proton motive force for ATP

Table 1 – Examples from the literature review, including studied elements of the approaches using aquatic microorganisms.

Models	Tested ENMs	Highlights
Escherichia coli	17 Metal oxide nanoparticles [45,147], Ag [114,115], iron-based [116], TiO_2 [53], ZnO [82,157,168], CeO_2 [169,170], SWNTs [171], MWNTs [171], C_{60} [171], and graphene and graphene oxide nanowalls [172]	Most widely studied prokaryotic model organism. QSAR model has been established [45]
Vibrio fischeri	ZnO [117], CuO [117], TiO_2 [117], and Ag [173]	In symbiotic relationships with marine animals. Reported QSAR model for nonpolar narcotics and polar narcotic phenols [174]
Bacillus subtilis	ZnO [118], CeO_2 [169], SWNTs [171], MWNTs [171], and C_{60} [171]	Widely used as a model organism as the Gram-positive equivalent of *E. coli*
Shewanella oneidensis	CeO_2 [169] and Ag [175]	Reduces poisonous heavy metals, also used for the biosynthesis of metal nanomaterials
Cupriavidus metallidurans	TiO_2 [176], Al_2O_3 [176], and MWCNTs [176]	Ideal organism to study heavy metal disturbance of cellular processes
Pseudomonas putida	ZnO [118]	Widely used for bioremediation
Pseudomonas fluorescens	Ag [177]	Antiphytopathogenic and used for biocontrol
Yeast: *Saccharomyces cerevisiae*	ZnO [178], CuO [178], and TiO_2 [178]	Most intensively studied eukaryotic model organism in molecular and cell biology; also used in aquaria
Protozoan: *Tetrahymena thermophila*	CuO and ZnO [164]	Common in freshwater, widely used as model organisms in biomedical research
Protozoan: *Paramecium caudatum*	Ag [179]	Bioremediation of wastewater

ENM = engineered nanomaterial; MWCNTs = multiwalled carbon nanotubes; MWNTs = multiwalled carbon nanotubes; QSAR = quantitative structure–activity relationship; SWNTs = single-walled carbon nanotubes.

synthesis [158,159]. In addition, it could also disturb the activity of the microbial community, as well as photosynthetic or respiratory processes, through inhibiting their attachment with consequent proliferation and biofilm formation [160,161]. Microorganisms mainly exist in nature as biofilms and perform or contribute to a range of essential environmental processes such as biogeochemical cycling. The disruption of biofilms could lead to the failure of microorganisms to adapt to adverse and dynamic environments, dysfunction of community communication and metabolism, and eventually death.

Although some excellent work on pure bacterial cultures has been carried out for decades, it is also necessary to focus on conducting and promoting toxicological studies on natural communities of microorganisms (e.g., complex microbial communities and natural bacterial assemblages). Once released into the aquatic environments, ENMs tend to precipitate onto the surface of sediment when they reach the threshold of aggregation size [162]. As discussed in the section related to mechanisms, environmental factors, such as DOM [163–165], may affect the chemistry and behavior of released ENMs. Therefore, water samples [164,166] and sediment samples [163,167] should be considered for supplementing nanomaterial databases. Examples of ENMs tested [168–179] in previous studies are shown in Table 1.

4.2. Crustaceans

Among aquatic biota, crustaceans, in addition to microorganisms, as representatives of different food-web levels, are commonly used to assess and predict the potential hazard of chemicals to ecosystems. In natural ecosystems, crustaceans work as shredders, being involved in the degradation of organic material and nutrient recycling, thus acting as pivotal components of the trophic food webs. The EU chemical regulation Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) required that ecotoxicity of xenobiotic chemicals should be tested on crustaceans [180]. In addition, according to the data provided by the Food and Agriculture Organization of the United Nations [181], more than 12 million tons of crustaceans were produced and consumed by humans in the year 2011. Thus, based on a literature review and an overview of toxic effects of ENMs in aquatic invertebrates, it has been suggested that crustaceans should be considered for nanoecotoxicology as sensitive and relevant test organisms [182]. However, there is insufficient research being conducted in this field.

Existing reports on crustaceans for nanoecotoxicity, for example, genotoxicity and ecotoxicity [183], show that the majority of the studies have used *Daphnia magna* as the test organism [184–187]. Other models such as *Tigriopus japonicus* and *Elasmopus rapax* [188], and *Thamnocephalus platyurus* [189] were also studied previously (Table 2). To date, the limited number of studies have indicated acute toxicity of ENMs to aquatic invertebrates in the low milligram/liter range and higher, although some indications of chronic toxicity and behavioral changes have also been described at concentrations in the high microgram/liter range. NPs have also been found to act as contaminant carriers of co-existing contaminants and this interaction has altered the toxicity of specific chemicals toward *D. magna*. Baun and co-workers [182] recommended that crustaceans, as representatives, can be used to advance the level of knowledge in nanoecotoxicology through standardized short-term (lethality) and long-term low-exposure tests. These tests, with chronic end points along with more research in bioaccumulation, can serve as a basis for investigating behavior and bioavailability of ENMs in the aquatic environment.

Table 2 − Examples from the literature review, including studied elements of the approaches using crustaceans.		
Models	Tested ENMs	Highlights
Daphnia magna	Zero-valent iron [10], C_{60} [186], ZnO [117,164], CuO [117,164], TiO_2 [117,183,187], Ag [173], C_{60} [182], CeO_2 [183], and SiO_2 [183]	Has been specified to be used in the OECD Guidelines for the Testing of Chemicals [184,185]
Tigriopus japonicus	ZnO [188]	Extensively used for toxicity assessment and identification as a marine copepod
Elasmopus rapax	ZnO [188]	Extensively used for toxicity assessment and identification as a marine amphipod
Thamnocephalus platyurus	ZnO [117,164], CuO [117,164], TiO_2 [117], and Ag [189]	Extensively used for toxicity tests as freshwater crustacean
ENM = engineered nanomaterial; OECD = Organisation for Economic Co-operation and Development.		

The ultimate goal of studying crustaceans, as with other species from different trophic levels, is to provide a predictive paradigm for the potential ecotoxicity of ENMs. It has been suggested that *D. magna* could be used for predicting the toxicity of ENMs in aquatic ecosystems [117].

4.3. Phytoplanktons

Phytoplanktons are the dominant producers in the aquatic ecosystems as they comprise the base of food webs, as well as a dominant component of biogeochemical cycles. They are also the most probable organisms in aquatic ecosystems that are exposed to the released ENMs, especially in coastal zones and municipal aquatic environments. It has been noticed that algae along with crustaceans were the most sensitive and thus probably the most vulnerable organism groups in aquatic exposure to ENMs [190]. In particular, phytoplanktons are highly dependent on sunlight for photosynthesis, thus making them more vulnerable to phototoxicity induced by ENMs than other trophic levels. Information on the impact of emerging contaminants on phytoplankton, and the potential interaction of ENMs with environmental variables such as irradiance is required for predicting potential impacts on aquatic food webs and the ecosystems that they support.

According to REACH, the basic ecotoxicological assessment is required for the substances manufactured, or imported in quantities of 1−10 tons/year, including short-term or long-term toxicity testing on aquatic plants (recommend using algae) [191]. The alga and cyanobacteria, growth inhibition test [Organisation for Economic Co-operation and Development (OECD) 201 assay] was used due to its regulatory relevance and due to severe shortage of algal toxicity data [192]. The algae *Pseudokirchneriella subcapitata* has been widely recognized as the representative species for phytoplanktons according to existing publications. It is also often used for monitoring of water quality and hazard assessment of wastewaters because they respond in a predictable manner to the presence of most types of pollutants (Table 3).

In addition, the data could be used for comparison with the available toxicity data from the literature and databases as well as with QSAR predictions. Menard et al [193] suggested that physicochemical characteristics of ENMs could be linked to their toxicity with the algae *P. subcapitata*. They found a correlation between toxicity data for algae and specific surface area and particle size in media (72-hour half maximal effective concentration values). Moreover, Aruoja and co-workers [194] exploited the possible QSAR prediction using the algae *P. subcapitata* to test the toxicity of 58 substituted anilines and phenols. Thus, it is also feasible to use *P. subcapitata* as a test organism for predicting nanoecotoxicity at the phytoplankton level. Further models used in previous studies [195−204] are shown in Table 3.

4.4. Fish cell lines

Although research on the aforementioned species could provide a basic understanding of how ENMs impact aquatic ecosystems, the investigations on fish cell lines could allow one to efficiently evaluate their toxicity toward higher tropic levels prior to conducting *in vivo* studies. *In vitro* primary cell lines have shown their growing importance for evaluating ecotoxicology [205,206]. Fish cell lines have been successfully used for assessing the toxicity of xenobiotic chemicals. In addition, they are also a potential candidate for supplementing and eventually replacing *in vivo* animal tests. Cells isolated from their parent fish tissues are closely related to the acute toxicity *in vivo*, allowing one to investigate mechanisms of nanotoxicity at the molecular and cellular levels with a small sample volume. For example, fish gill cell lines have been investigated for nanotoxicological studies [207,208], suggesting a close correlation with its toxicity *in vivo* [209,210]. The correlations between *in vitro* cell lines and *in vivo* fish could also be extended to the genetic level to enable a better understanding of toxicity mechanisms. Furthermore, fish cell lines are also valuable, rapid, and cost-effective tools for the assessment of nanotoxicity and environmental samples. Through such an *in vitro* experimental system, we can establish a reliable, well-explained structure−activity relationship using QSAR approaches [211].

Based on our literature review, fish cell lines used for assessing nanotoxicity are mainly from certain organs, including the liver [53,212,213], gill [207,208], gonad [214], fin tissue [215], and skin tissues [99]. This review summarizes the existing literature on cytotoxicity studies with these fish cells lines and considers the selection of cell lines as one of the holistic approaches in nanotoxicological studies (Table 4). They have great potentials for the detection of genotoxic [99,214,215], neurotoxic [216], hepatotoxic [212,216], nephrotoxic [216], hematotoxic [217], and histopathological [216] effects, and cell lines are now applied for investigations in environmental diagnostics as a bioanalytical tool [206].

Table 3 – Examples from the literature review, including studied elements of the approaches using phytoplanktons.

Models	Tested ENMs	Highlights
Skeletonema costatum (diatom)	ZnO [188]	OECD 201, ISO 10253:1995, EU Directive 92/69/EEC—Method C.3, and OPPTS 850.5400 for the aquatic toxicity tests [195]
Thalassiosia pseudonana (diatom)	ZnO [188,196,197], zero-valent iron [10], and TiO_2 [198]	First eukaryotic marine phytoplankton; for whole-genome sequencing
Thalassiosira weissflogii (diatom)	Ag [199]	Used to feed shrimps and shellfish larvae in hatcheries
Skeletonema marinoi (diatom)	ZnO [197]	Easy to collect, isolate, and maintain in cultures
Isochrysis galbana (microalga)	Zero-valent iron [10] and TiO_2 [198]	Widely used for use in the bivalve aquaculture industry
Pseudokirchneriella subcapitata (microalga)	Zero-valent iron [10], C_{60} [186], CuO, ZnO, and TiO_2 [200]	OECD and US EPA model organism; QSAR model was reported in predicting the toxicity of anilines and phenols [194]
Dunaliella tertiolecta (marine alga)	Zero-valent iron [10] and TiO_2 [198]	Extensively used for toxicity assessment
Desmodesmus subspicatus (freshwater green alga)	TiO_2 [187] and Ag [173]	OECD 201 used for toxicity assessment. Formerly known as *Scenedesmus subspicatus*
Scenedesmus sp. (green algae)	Al_2O_3 [201]	OECD 201 used for toxicity assessment
Chlorella sp. (green algae)	Al_2O_3 [201,202], SiO_2 [202], ZnO [202], and TiO_2 [202]	Used in OCDE/GD(97)19 test [203]
Chlamydomonas reinhardtii (green algae)	Ag [204]	Used as a model organism for research in cell and molecular biology
Synechocystis (cyanobacteria)	CeO_2 [170]	One of the most highly studied types of cyanobacteria as a model microorganism

ENM = engineered nanomaterial; EPA = Environmental Protection Agency; EU = European Union; ISO = International Organization for Standardization; OECD = Organisation for Economic Co-operation and Development; OPPTS = Office of Prevention, Pesticides and Toxic Substances; QSAR = quantitative structure–activity relationship.

4.5. Fish

The present literature review shows that there is an increasing number of reports on toxic effects of ENMs in fish, and the mechanistic basis of both exposure and effect are widely studied (Table 5). Fish are also recommended in regulatory testing of ecotoxicological hazards of pure chemicals (OECD guidelines) [218–220]. *In vivo* fish studies, in addition to *in vitro* fish cell lines, could provide further fundamental findings with respect to the mechanisms of bioavailability, absorption, distribution, metabolism, and excretion of ENMs in the fish body [221]. Furthermore, one can specifically target the fish organs or tissues for conducting toxicological research. It has been evidenced that the gills, gut, liver, and sometimes the brain are the favorable accumulation sites for ENMs, leading to injury [222]. For example, using a rainbow trout (*Oncorhynchus mykiss*) model, Federici et al [209] investigated the toxicity of TiO_2 NPs in the gill of rainbow trout. They found that accumulated NPs could result in significant decreases in Na^+K^+—adenosine triphosphatase activity, as well as statistically significant increases in the total GSH levels.

Moreover, some specific fish models, such as zebrafish (*Danio rerio*) and medaka fish (*Oryzias latipes*), are transparent and can be used for real-time study of transport and effects of

Table 4 – Examples from the literature review, including studied elements of the approaches using fish cell lines.

Tested models	Tested ENMs	Highlights
From the skin of goldfish (*Carassius auratus*)	TiO_2 [99]	Successfully developed to evaluate chemical toxicity
From rainbow trout (*Oncorhynchus mykiss*) gonadal tissues	TiO_2 [214]	
Rainbow trout (*O. mykiss*) gill cell line, RTgill-W1	WC [207] and Ag [208]	Commercially used for *in vitro* toxicology, fish virology, and fish biochemistry
OLHNI2 cells, a medaka (*Oryzias latipes*) cell line established from adult fin tissue	Ag [215]	Used extensively as a model for human health
Primary cell line from rainbow trout (*O. mykiss*) liver cells (hepatocytes)	Ag [213] and Au [213]	Has previously been shown to be capable of detecting toxic end points of single chemicals and environmental samples
Channel catfish (*Ictalurus punctatus*) primary hepatocytes	ZnO [212], TiO_2 [212], CuO [212], and Co_3O_4 [212]	Used extensively as a model for toxicity assessment
Zebrafish (*Danio rerio*) liver cells	TiO_2 [53]	Commercially available for studies of liver cell metabolism and xenobiotic formation. Also used for *in vitro* toxicological studies

ENM = engineered nanomaterial.

Table 5 – Examples from the literature review, including studied elements of the approaches using fish.		
Models	Tested ENMs	Highlights
Zebrafish (*Danio rerio*)	Ag [79,208,225], Au [225], Pt [225], Al$_2$O$_3$ [225], SiO$_2$ [225], ZnO [225], QD [225], and TiO$_2$ [53]	Important vertebrate model organism in toxicological research
Medaka fish (*Oryzias melastigma*)	ZnO [188]	An effective substitute for exotic larvicidal fishes
Medaka fish (*Oryzias latipes*)	SiO$_2$ [224] and C$_{60}$ [227]	Model organism in toxicological research
Rainbow trout (*Oncorhynchus mykiss*)	TiO$_2$ [209]	Important aquaculture species worldwide and also a model research organism
Carp (*Cyprinus carpio*)	TiO$_2$ [137,228], Ag [229], CuO [230]	Extensively used for determining
Fathead minnow (*Pimephales promelas*)	TiO$_2$ [231] and C$_{60}$ [84,227]	toxicity of chemicals to aquatic species
Carp (*Carassius auratus*)	C$_{60}$ [232]	
Largemouth bass (*Micropterus salmoides*)	C$_{60}$ [83]	
Eurasian perch (*Perca fluviatilis*)	Ag [233,234]	
Crucian carp (*Carassius carassius*)	Ag [234] and polystyrene [235]	
Bleak (*Alburnus alburnus*)	Polystyrene [235]	
Rudd (*Scardinius erythrophthalmus*)	Polystyrene [235]	
Tench (*Tinca tinca*)	Polystyrene [235]	
Pike (*Esox esox*)	Polystyrene [235]	
Atlantic salmon (*Salmo salar*)	Polystyrene [235]	
ENM = engineered nanomaterial.		

NPs *in vivo*, allowing direct observation in a phototoxicity trial. Lee et al [223] directly characterized the transport of single Ag NPs into *in vivo* zebrafish embryos and investigated their effects on early embryonic development at single-NP resolution in real time. Using a transparent medaka fish (*O. latipes*), Lin and co-workers also investigated the biodistribution of mesoporous silica NPs within a living organism without tissue dissection [224].

Eventually, a relation between the physicochemical properties of ENMs and their physiological effects *in vivo* could be also modeled within a reliable QSAR approach [193] or HTS toolkit [225]. George and co-workers [225] highlighted that the use of a HTS, *in silico* data handling, and zebrafish testing may constitute a paradigm for rapid and integrated ENM toxicological screening [225]. In addition, it is also noted that *in vivo* fish toxicological studies could be linked to the corresponding *in vitro* level [225,226]. Various other fish models were also used to study the effects of ENMs [227–235] as shown in Table 5. These valuable endeavors could eventually provide us with a predictive paradigm with a lower cost at the cellular level, compared with the more relevant but also more expensive *in vivo* systems. George et al [225] used a z-score-transformed high-volume data set to construct heat maps for *in vitro* hazard ranking as well as for showing the similarity patterns of selected NPs and response parameters through the collection of raw data *in vivo* with zebrafish embryo models.

4.6. Amphibians

Being sensitive to toxic contaminants due to their biphasic life cycle, permeable eggs, skin, and gills, amphibians have been proved to be valuable bioindicators and sensitive models for ecotoxicological studies. The OECD guideline 231 describes an amphibian metamorphosis assay intended to screen substances, which may interfere with the normal functioning of the hypothalamo–pituitary–thyroid axis [236]. In addition, amphibian larvae may form micronuclei by genome mutations, making it possible for screening clastogenic compounds. Despite the growing current information, there is

little known about the effects of ENMs on amphibians. Given the increasing amounts of ENMs released into the environment and the previously published effects of NPs on fish, there is a reason to believe that frogs may be affected by ENMs. It is noteworthy that not only are frogs sentinel species, but they also undergo a postembryonic metamorphosis from a tadpole into a frog in a process that is directly dependent on thyroid hormones. The disruption of thyroid hormones could cause dysfunction of their metamorphosis, which makes them more vulnerable. In addition, this information could provide a note of caution in their use, particularly during sensitive life stages, as they may alter stress pathways and critical hormone-mediated cellular function.

Emerging research has been carried out regarding acute toxicity and genotoxicity of ENMs on amphibian species (Table 6). Micronuclei have been proposed as biomarkers to assess significant adverse biological impacts (changes at the genetic/molecular level) with suborganismal (molecular, biochemical, and physiological) responses preceding those occurring at higher levels of biological organization such as the cellular, tissue, organ, whole-body levels, and ultimately at the population level [237,238]. Various other amphibian models were also used to study the effects of ENMs [239–243]. In this way, the use of the micronucleus test may provide an important tool for the prediction of the potential long-term effects on amphibians in the environment. Nevertheless, more information on amphibians is urgently needed to supplement the nanotoxicological data at this trophic level.

5. Implications

This literature review indicates that conducting nano-ecotoxicity tests is both multidimensional and multifocal. It is multidimensional in that it is not only outward driven but can also be inward driven. Outward-driven approaches consider the holistic setup for supplementing the knowledge base of nanoecotoxicology. These approaches have made an attempt

Table 6 – Examples from the literature review, including studied elements of the approaches using amphibians.		
Models	Tested ENMs	Highlights
Xenopus laevis	Carbon nanotubes [237–239] and nanodiamond [240]	Used for toxicity assessment by OECD [241]
Ambystoma mexicanum	Carbon nanotubes [242]	
Rana catesbeiana	QDs [243], ZnO [243], and Ag [243]	The micronucleus technique used with *R. catesbeiana* tadpoles has been developed [244]
ENM = engineered nanomaterial; OECD = Organisation for Economic Co-operation and Development.		

to connect all aquatic trophic levels together and make a comprehensive hazard assessment. Inward-driven considerations require more efforts on understanding basic physicochemical properties of ENMs in their process of synthesis, application, and disposal. Outward-driven and inward-driven activities do influence each other in a certain way, as we have reviewed. It is multifocal in that nanoecotoxicology does not focus on one form of interactions of ENMs with cells or organisms, but on the form of interactions of ENMs with their surrounding microenvironments. Ultimately, a reliable, reproducible, and predictive paradigm could be achieved through such a strategic approach.

These existing data indicate that the endeavors that previous researchers dedicated to assessing the health effects of a single or a few ENMs at a time could also be applied to establishing a predictive paradigm overall. This would suggest that the alteration of ENM characteristics contributes to the tendency to undergo changes in fate and behaviors *in vitro* and *in vivo*. Environmental factors that interact with ENMs eventually, in fact, reveal a tendency toward their outward toxicity. Similarly, the distinct toxicological profile correlated in previous research with various test species in a predictable way overall. Thus, it could provide valuable suggestions and directions on the design and synthesis of ENMs in a quantitative way, as well as assure the application safety and proper disposal protocols in the later period.

This suggests that the assessment of nanoecotoxicity should be pursued in a holistic way, rather than by a single methodology. This is because it was found that although intrinsic physicochemical properties of ENMs are factors partially responsible for their toxicity, their fate, behavior, and subsequent adverse impacts in a majority of cases were not confined to their own properties. Rather, such phenomena involve a range of interactions that interface with various factors to influence the extent of their ultimate toxicity.

A holistic approach to nanoecotoxicity is better able to mirror the more sophisticated forms of nano–bio–eco interference that determine the threshold of exposure and its consequences. Furthermore, the holistic approach also offers a more comprehensive basis for the elaboration on *in vitro*, *in vivo*, and *in situ* toxicity mechanisms at different trophic levels. There is an increasing number of ENMs being released into aquatic environments on a global basis, driven by emerging needs for such high technology and decreased costs of bringing new products to market that ENMs afford. In the exploitation of this technology for medical applications, environmental remediation, commercial products, and industry, human, and environmental health will suffer as a result of increasing exposure [245].

Ultimately, from the perspective of policy makers, the holistic approach to the health impacts of ENMs has significant implications. This can be illustrated in the case of governmental assistance programs. Government organizations, through programs of nanosafety assessment (e.g., Nanoscale Science, Engineering, and Technology Subcommittee, Nanotechnology Environmental Health Implications working group), endeavor to support Federal activities to protect public health and the environment affected by ENM exposure. In general, this approach lags behind the development of nanotechnology because it requires a systematic cooperation among research institutions with extensive manpower and management. With a collaborative arrangement with government guidance, a majority of toxicological data could be collected and analyzed in a comprehensive, efficient, and publicly beneficial way.

6. Limitations and directions for future research

In conclusion, in this report we highlighted that it is desirable to adopt the systematic approach to study the relationship between physicochemical parameters of ENMs and biological end-point response in a nanotoxicity study. The fate of ENMs with respect to their modification by immediate surrounding conditions and the resulting impact on toxicity was elaborated. We have discussed possible effects of biological and nonliving factors on the outcomes of nanotoxicity assays. Because nanoecotoxicology is a relatively new field, the concepts of dose metrics, exposure assessment, hazard identification, and risk characterization are still far away from the state in which a conclusive picture emerges.

One of the limitations of this comparative approach is the source of the test models used for such a pursuit. In addition, the application of model test data to humans remains to be perfected. In the absence of a comprehensive investigation on proposed levels, more efforts are needed to fulfill this knowledge gap. Furthermore, presumably, the results of actual human exposure medical cases could provide data that may serve to steer further nanotoxicological research to develop the complete picture of the hazard of ENMs.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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