Methods, Mechanisms and Typical Bio-Indicators of Engineered Nanoparticle Ecotoxicology: An Overview

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Review

Methods, Mechanisms and Typical Bio-Indicators of Engineered Nanoparticle Ecotoxicology: An Overview

As nanotechnology industries increase production, increased release of engineered nanoparticles (NPs) to aquatic environments suggests a rising need for monitoring and evaluation of their potential toxicity. Based on previous and latest research results in the related field, this paper reviews methods, mechanisms, and typical bio-indicators of nanomaterials ecotoxicological research. It outlines detecting methods of NPs in ecotoxicologial studies, and discusses suspension methods of nanoparticles in this field. Proteomics and genomics technologies are predicted to be indispensable means in ecotoxicological studies of NPs. It also points out that biology of particle-induced oxidative stress is an important mechanistic paradigm, and so are solvent effects and the effects of NPs on other substances. Typical bio-indicators in aquatic systems are claimed to be determined to avoid unnecessary animal testing whenever possible and to reduce unnecessary testing costs. In ecotoxicological research of NPs, fish species are generally considered as preferred species; for nanoparticle ecotoxicity, suspension-feeding invertebrates may be a unique target group, therefore invertebrate testing is also very important; because of sensitivity to pollutants, phytoplankton testing should be strengthened. This paper also summarizes some conflicting results in current experiments, and gives some possible explanations. Finally, future research directions are proposed.

Keywords: Aquatic systems; Bio-indicators; Ecotoxicology; Nanoparticles

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1 Introduction

It is about half a century ago that the physicist and Nobel laureate Richard Feynman challenged the science community to think small in his lecture "There's Plenty of Room at the Bottom" (www.nanotechproject.org/inventories/consumer/). Since then, tens of thousands of researchers have endeavored to obtain nanostructured materials with perhaps every possible morphology, e.g. nanowires, nanofibers, nanobelts, nanotubes, nanodendrites, nanowalls, nanosheets, nanorings, nanomesh, nanocages, nanocorolla, multiarmed nanostar and so on. It has been well proved that nanosized materials exhibit surprising and novel phenomena linked to their sizes and shapes, which make them greatly differ from their bulk counterparts. Nanosized materials become promising candidates for a wide variety of technological applications, e.g. photocatalyst [1], adsorbent [2, 3], degradation agent [4]. Nanotechnology is now maturing rapidly. Taking consumer products for example, it is reported that more than 800 consumer products containing nanoscale components now are on the market as of August 2008 (www.nanotechproject.org/inventories/consumer/). Some reports estimate that the commercial value of products containing some aspect of nanoscale technologies will grow from about $300 billion in 2005 to around $3 trillion in 2014 (www.innovateuk.org/_assets/pdf/Corporate-Publications/NanoscaleTechnologiesStrategy.pdf).

It is about a decade ago that people noticed the potential risks posed by engineered nanoparticles (NPs) to human health and environment. In April 2003, "Science" first put forward that it is necessary to carry out toxicological studies on nanoscale materials [5]. Subsequently, "Nature" published an article in July of the same year proposing that if not made to carry out biological effects research of nanoscale materials and nanotechnology, it would threaten the trust and support of nanotechnology by the government and the public [6]. Ecotoxicology, the science of contaminants and their effects on constituents of the biosphere, has experienced several decades of developments from "the dark ages" in the 1950s and before to "the microbiotesting 1990s" [7]. The clear development tendency of ecotoxicology transfers from traditional ecotoxicology to toxicogenomics [8]. With more and more attention paid to the research on potential environmental risks of NPs, Kahru and Dubourguier [9] named the next era of ecotoxicology as "the (eco) toxicogenomical and nano(eco)toxicological 2010s".

United Kingdom launched a worldwide survey in 2008, and issued a report in March 2009, which is the world's first summary report on

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Abbreviations: **BSO**, buthionine sulfoximine; **CTD**, comparative toxicogenomics database; **LPC-SWNT**, lysophosphatidylcholine-coated single-walled carbon nanotube; **NAC**, *N*-acetylcysteine; **NP**, nanoparticle; **ROS**, reactive oxygen species; **SEM**, scanning electron microscopy; **TEM**, transmission electron microscpy; **THF**, tetrahydrofuran; **ZVI**, zero-valent iron

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the world's research projects and progresses in issues of nanomaterials and nanotechnology to the human environment, health and security. On January 29, 2009, Canada enacted a law requesting domestic companies and research institutes to declare the quantities, uses and known toxicity of NPs for an annual purchase of more than 1000 g. According to "The Nanotechnology Consumer Products Inventory", the most common products of nanomaterial are carbon products (29 kinds) including nanotubes and fullerenes. The second most common nanomaterial is silver (25 kinds), and the followings are silica (14 kinds), titanium dioxide (TiO_2, 8 kinds), zinc oxide (ZnO, 8 kinds) and cerium oxide (CeO_2, 1 kind) [9]. In recent years, with the increased concerns over potential toxicological properties of NPs, the number of research papers on the risk of NPs is increasing dramatically.

Aquatic ecosystems provide endless food and energy resources and mineral resources; the ocean, the most important ecosystem on earth, is also the largest place of natural purification, and the majority of engineered NPs discharged into environments will ultimately run into the ocean. Aquatic sediments are an important sink for NPs, like carbon nanotubes and fullerenes, thus potentially causing adverse effects on the aquatic environment, especially to benthic organisms [10]. Therefore, ecotoxicological research on NPs to aquatic ecosystems is particularly important. After entry of NPs into the aquatic environment the suspended particles will be taken up by planktonic or sediment dwelling invertebrates through different exposure routes such as direct uptake from the water phase and/or through food uptake [11].

Although scholars around the world have already started research in this area, the data available are still very scarce. To date, scholars have discussed the mechanisms of ecotoxicity of NPs, toxicity of different NPs, representative species for nanoparticle ecotoxicity research, and the entering waterways, uptake and bioavailability of NPs. The reactive oxygen species (ROS) is considered to be the most commonly used species in nanoparticle ecotoxicity research; particle size, surface characteristics, solubility, and the combined effects with metal ions are regarded as closely related to the toxicity of NPs. In this paper, methods, mechanisms and typical species of nanoparticles/nanomaterial ecotoxicology are elaborated. We also point out some problems existing in the course of studies, and give possible explanations for them. Finally, future research directions are proposed.

2 Methods of ecotoxicological studies on nanoparticles

2.1 Analytical techniques

One of the main challenges in nano-ecotoxicological investigations is the selection of the most suitable measurement methods and protocols for nanoparticle characterization [12]. Now various parameter measurements of NPs are mainly offline methods, and many modern developed instruments for morphology, structure and interface observation have been used in the analysis of nanoparticles, such as scanning electron microscopy (SEM), transmission electron microscopy, scanning tunneling microscopy, atomic force microscopy, particle electrophoresis, streaming potential, and X-ray absorption spectroscopy [13]. Inductively coupled plasma MS is used to determine the number concentration of metal containing NPs [14]. In recent years, researchers have developed a number of on-line monitoring and control technologies for nanoparticle characterization in ecotoxicological studies, such as electrical low pressure impactor, scanning mobility particle sizer and laser induced incandescence imaging soot particle analyzer. Wezel et al. [15] have developed a sensitive analytical method to quantify nC_{60} in water, using accurate mass screening LC–hybrid linear ion trap Orbitrap MS, making it possible to detect and quantify aqueous concentrations as low as 5 ng/L.

However, these instruments can measure only a single indicator, and there are differences in measurement results [16–18]. Nanodetection technology is expected to exceed existing technology barriers, bringing about new detection principles, methods and technologies, and providing powerful tools for online testing of environmental NPs.

Several characteristics of NPs have been identified as being important, such as specific surface area, suspension, coating, length and so on, because they govern nanotoxicological activities of NPs; some characteristics are better defined than others, and dispersion stability of NPs is one of them [19]. For example, dispersion and solubility of ZnO NPs vary significantly with solution chemistry [20]. There is some ambiguity as to how to measure dispersion stability in the context of ecotoxicological investigations. The work of Tantra et al. [12] has proved that techniques like zeta potential, particle size via DLS, fluorescence and UV–Vis spectroscopy and SEM can provide useful information on dispersion stability. Nanoparticle tracking analysis can provide useful quantitative information on the concentration of NPs in suspension, but is limited by its inability to accurately track the motion of large agglomerates found in the fish medium [12]. Therefore, in-depth studies on suitable conditions and testing methods are needed.

At the single organism level, supplements of quantitative data on toxicological effects of NPs are still needed. Furthermore, in the environment these materials will encounter a variety of chemicals and turbulent waters which may play the role of dispersing agent and/or stabilizers [21, 22]. Although researchers have started to pay attention to these issues, especially in the natural marine environment, knowledge available on the agglomeration/aggregation state of NPs and their toxic effects is still limited [23–25]. Moreover, typical bio-indicators of nanomaterial ecotoxicity are still not determined.

2.2 Promising technologies

Nanoparticle toxicity can be studied through various means, and traditional toxicology methods have been applied in toxic studies of NPs, the discussion of which is omitted in this paper. As new research tools, genomics and proteomics have been widely applied in nanoparticle toxicology. Under microcosmic and moderate conditions, genomics and proteomics methods are more effective than traditional methods. Some scholars have pointed out that the advantage of proteomics approaches is more obvious than traditional biomarkers of oxidative stress in the research of sentinel species [26].

Pradhan et al. [27] investigated the effects of CuO NPs and Ag NPs on leaf litter decomposition by aquatic microbes, and the results were compared with the impacts of their ionic precursors. In this study, DNA fingerprints were used to reflect the shifts in the structure of fungal and bacterial communities accompanied by these toxicological effects. DNA fingerprinting based on denaturing gradient gel electrophoresis showed that the number of fungal or bacterial operational taxonomic units decreased with increasing concentrations of nano- or ionic metals, confirming that fungal and bacterial communities are affected by nano- and ionic metals [27].

DNA ladder bands are an indicator of acute and chronic chemical stress, loss of cellular function and structure; in addition, DNA cleavage is an indicator of irreversible completion of apoptosis occurring in organisms exposed to toxic nanoparticles. Oberholster et al. [28] assessed the effects of different NPs on sediment-dwelling invertebrate *Chironomus tentans* larvae. In this study, DNA laddering was used to do genotoxic stress assessment. The DNA findings were closely correlated with those obtained for the survival end-point, providing a more intuitive and efficient method for nanoparticle toxicity studies.

In digestive gland, gold NPs may cause modest oxidative stress (such as decrease of protein thiol groups and depletion of GSH/GSSG ratio). However, under these conditions traditional biomarkers show no effect. Human diseases are affected by environmental chemicals. In order to elucidate molecular mechanisms of these environmental chemicals, a Database called comparative toxicogenomics database (CTD; http://ctd.mdibl.org/) has been created [29]. CTD presents scientifically reviewed and curated information on many aspects, such as chemicals, relevant genes and proteins, it also reveals their interactions in vertebrates (human, mice, rat, fish) and invertebrates (daphnids, *Drosophila*, nematode *Caenorhabditis elegans*) [28, 30]. However, it is difficult to use CTD in aquatic toxicology, since sequence information of non-model species is very inadequate [9]. However, with the accumulation of research data, there will be more and more sequence information available, proteomics and genomics technologies will become indispensable means for ecotoxicological studies on NPs.

3 Ecotoxicology mechanisms of nanoparticles

There are a lot of natural NPs, and perhaps organisms have biologically adapted to them. However, synthetic NPs are different from naturally occurring ones, and researchers should pay great attention to their toxicological effects. A large number of studies on mechanisms of nanoparticle toxicology have been carried out. Unusual physicochemical properties of engineered NPs can be attributed to their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings, etc.), solubility, shape, and aggregation [31]. There is a consensus on some general principles of toxicity of NPs: (i) toxicity of larger NPs is less than smaller ones; (ii) in eukaryotes, NPs can cross and damage biomembranes; (iii) oxidative stress is often caused by NPs; (iv) chemical compositions of NPs are closely related to their toxicity; (v) in prokaryotes, NPs often remain outside the cell; while in eukaryotes, they can enter cells or even organelles [26].

3.1 Particle-induced oxidative stress

Studies show that a variety of NPs can adversely affect aquatic organisms. Their toxic effects may be due to oxidative stress or free radical generation, resulting in lipid peroxidation and causing cell membrane damages. Then normal cell functions are lost, causing cell death or apoptosis.

Several NPs characteristics can culminate in ROS generation, which is currently the best-developed paradigm for nanoparticle toxicity [32, 33]. Researchers pointed out the two main traits of nanoparticle cytotoxicity, one is cell signaling pathways, the other is oxidative stress-related changes in gene expression [34–39]. Experimental data show that properties of NPs do not closely depend on chemical properties of macroscopic materials, and different NPs exert similar effects. For example, both C_{60} fullerene and TiO_2 NPs are redox active and cause oxidative stress, even though they have different physical and chemistry structures [40, 41].

Studies have shown that NPs, which entered into the brain can cause oxidative stress and consequent neurotoxicity, therefore microglia can phagocytize NPs, and then ROS occurs [42]. Oxidative stress can be caused by TiO_2 in various fish tissues including brain [40, 43, 44]. Other forms of cell damage can be caused by citrate-capped gold NPs entering cultured human cells when lipid peroxidation occurs (e.g. nuclear localization (2 nm-Au); intracellular membrane damage (10 nm-Au)) [45].

A study showed that lysophosphatidylcholine-coated single-walled carbon nanotubes (LPC-SWNTs) can be fed by *Daphnia magna* through normal feeding behavior, and low concentrations of LPC-SWNTs do not cause the death of *D. magna*, while 10 and 20 mg/L of LPC-SWNTs can lead to 20 and 100% mortality [46]. Templeton et al. [47] found that SWNTs purified by electrophoresis have no significant impact on mortality, growth, and reproduction of *Amphiascus tenuiremis*, but high concentration (10 mg/L) without purification of mixture of SWNTs can significantly increase mortality, and reduce fertility and molting success rates; fluorescent nano-carbon components, by-products of purification process, can also significantly increase mortality and reduce molting success rate in high concentration (10 mg/L). Zhu et al. [48] found that high concentrations of carbon nanotubes can inhibit the growth of *Stylonychia mytilus* cells, and this may be due to damage caused by carbon nanotubes to normal physiological function of large nuclear and membrane of *S. mytilus*.

3.2 Photocatalytic activity

Many metal NPs have photocatalytic activity, which is one of the factors of toxicological effects that cannot be ignored [49]. For example, nC_{60} can be transformed by oxidation, reduction, and photochemical reaction [15]. Studies suggest that photosensitivity of NPs and their ROS production under high-intensity light with specific wavelength may relate to their NP toxicity. Researchers have found that with the near-UV irradiation, cell wall of *Escherichia coli* cultured using TiO_2 thin films degrade first, followed by membrane damage and permeability damage, then the outflow of cell contents, leading to cell death [50]. Ma evaluated phototoxicity of a manufactured nanoparticulate ZnO to *C. elegans* under natural sunlight illumination and compared it to toxicity under ambient artificial laboratory light [51]. It is found that phototoxicity of ZnO NPs to the nematodes under natural sunlight illumination is greater than toxicity under artificial laboratory light illumination; 24 h exposure under ambient laboratory light cause less lethality than 2 h exposure under natural sunlight illumination. The potential ecotoxicity of nanosized TiO_2, SiO_2, and ZnO water suspensions was investigated using Gram-positive *Bacillus subtilis* and Gram-negative *E. coli* as test organisms [52]. The study shows that the presence of light is a significant factor under most conditions tested, presumably due to its role in promoting generation of ROS.

However, it is also observed that bacterial growth inhibition occurs under dark conditions. Ag-modified TiO_2 nanotube arrays showed the antimicrobial activity on *E. coli* even in the absence of light [53]. Another study also shows that there is a difference in toxicity of illuminated and non-illuminated products [54]. These phenomena indicate that undetermined mechanisms together with photocatalytic ROS production are responsible for toxicity.

3.3 Bacterial attachment

Some metal NPs have adsorption characteristics, and they can adsorb on the surface of cells or microorganisms, rather than enter them. The attachment of oxide NPs on bacterial surfaces has several possible mechanisms: electrostatic, van der Waals forces, receptor–ligand and hydrophobic interactions [55, 56]. In general, surfaces of NPs carry charges, e.g. zero potential point of TiO_2 NPs is 5.2, while pH of natural water is generally higher than this value, so the surface of TiO_2 NPs in natural waters carries negative charges [57]. In addition, with a large surface area, NPs can absorb various types of molecules in water. Adsorption of zero-valent iron NPs to Ba^{2+} (10^{-6}–10^{-3} mol/L) meets the Freundlich and Dubinin-Radushkevich adsorption isotherm, and this process is exothermic [58].

Zhang et al. [59] found that adsorption of TiO_2 NPs to Cd is in line with Freundlich isotherm; TiO_2 NPs can significantly speed up absorption of Cd in carps. In bacterial toxicity comparison, ZnO NPs is the most toxic one among nanoscaled titanium, silicon, aluminum and zinc oxides, to bacteria (*Pseudomonas fluorescens*, *B. subtilis*, and *E. coli*) [60]. Transmission electron microscopy images illustrated that NPs attached on bacteria, which suggested that bacterial attachment caused the toxic effects. In eukaryotic organisms, NPs could enter the cell cytoplasm while the cell membrane disrupts. This situation was supposedly unlikely to occur in bacterial cell [61].

So far, mechanism of interaction between oxide NPs and bacterial cell wall is still unclear. There is also no unified opinion on possible permeation of NPs into the cells of bacterium. Its mechanism is inconclusive.

3.4 Solubility, surface characteristics and forms of metal oxides

Toxicity of dissociated metal ions from metal NPs sometimes is greater than NPs themselves. Ecotoxicity of CuO and ZnO NPs in natural water has been studied [62]. Under standard test conditions, toxic effects of CuO and ZnO NPs in natural waters are mainly due to dissolved metal ions. Dissolved metal ions from oxides could also induce toxicity of metal oxide. In the studies of Brunner et al. [63], the tested NPs were divided into soluble and insoluble NPs. The results showed that, before or after NPs entering cells, soluble NPs released soluble metal ions, which produce toxic effects.

However, there are different situations. An investigation on ecotoxicological effects of ZnO NPs towards different soil organisms shows that for some organisms, ZnO NPs exert a higher toxic effect on its insoluble forms compared to that of the same amount of ionic zinc. This view is unlike most of the results reported in literature, which attributed the ecotoxic actions to soluble fraction of the ZnO NPs (i.e. the Zn^{2+} ion). Thus, NPs toxic action can be linked to a chemical effect and/or stress or stimuli caused by peculiar physical characteristics of the nanostate [30]. In a study to compare chronic toxicity of nanoparticulate and ionic zinc to the earthworm, *Eisenia veneta* was exposed to uncoated ZnO NPs and $ZnCl_2$ dosed to soil and at 250 and 750 mg Zn/kg for 21 days [64]. ZnO NPs generally had less impact than $ZnCl_2$ on measured traits. Reproduction declined by 50% at 750 mg Zn/kg; when exposed to ZnO NPs, it was almost completely inhibited by $ZnCl_2$. SEM analysis showed the presence of ZnO particles, suggesting that NPs can be taken up in particulate forms.

Surface characteristics are also important factors in ecotoxicology of NPs. In a research on influence of alumina coating on characteristics and effects of SiO_2 NPs in algal growth inhibition, alumina coated SiO_2 NPs showed lower toxicity than bare SiO_2 NPs at concentrations $\geq$46 mg/L. The author pointed out that the alumina coating completely altered NP interactions. Due to the difference in surface composition, bare SiO_2 NPs had the smallest surface area and were more toxic to the alga than the alumina coated SiO_2 NPs [65].

Thus, to understand mechanisms of ecotoxicological action of metal oxides NPs and their ecological consequences, solubility and speciation are the crucial aspects contrarily [66]; for example, to manufactured carbon NPs, size and aggregation seem to be the key factors.

3.5 Solvent effects and interactions of nanoparticles with other environmental contaminants

As toxicity testing environmental conditions of NPs vary from one to another, and there is no uniform requirement for solvent type and use, toxicity research of NPs does not follow the same principles. In the assessment of ecotoxicology of NPs, toxicity of solvents needs to be considered, and it needs to know whether other substances will produce toxicity under the influence of NPs.

Results of evaluating the effects on plants and microorganisms of model NPs indicate that the effect of NP-solvents was sometimes more significant than that of NPs themselves, a point that is of special interest for future nanotoxicological studies [67]. A study on toxicity and bioaccumulation of xenobiotic organic compounds provided underline that not only the inherent toxicity of NPs, but also interactions with other compounds and characterization of NPs in aqueous suspension are of importance for risk assessment of NPs [68].

Henry et al. [69] investigated changes in survival and gene expression in larval zebrafish *Danio rerio* after exposure to aggregates of C_{60} NPs prepared by two methods: (a) stirring and sonication of C_{60} in water; and (b) suspension of C_{60} in tetrahydrofuran (THF) followed by rotovaping, resuspension in water, and sparging with nitrogen gas. Survival of larval zebrafish was reduced in THF–C_{60} and THF–water but not in C_{60}–water. The greatest differences in gene expression were observed in fish exposed to THF–C_{60} and most in fish exposed to THF–water. THF was not detected but THF oxidation products γ-butyrolactone and tetrahydro-2-furanol were found. It is indicated that a THF degradation product produced toxic effects but not C_{60}, which can also explain toxicity attributed to C_{60} in other studies.

Studies have shown that C_{60} fullerene toxicity on the sensitivity of zebrafish embryos can be effectively modified by *N*-acetylcysteine (NAC) or buthionine sulfoximine (BSO) exposure regimes. Co-exposure to C_{60} with NAC decreased mortality while co-exposure with BSO increased mortality, although no indication showed that NAC could reduce the incidence of fin malformation [70]. A study about the effects of ZnO and TiO_2 NPs on swimming activities of larval zebrafish showed that when co-exposed to BSO, the larvae significantly altered their activities but those co-exposed to NAC did not [71]; in this study, the behavioral effects of TiO_2 NPs were not modified when co-exposed to NAC or BSO. These results indicated that damages to the gills and physical irritation might be also involved besides oxidative stress. For elucidating behavioral effect mechanisms of NPs, further research is still needed.

NPs effects as the basis of toxicity are shown in Tab. 1, which is based on a summary of Nel et al. [31], and some supplements are added.

3.6 Conflict research findings in ecotoxicology of nanoparticles

We find an interesting fact that for the same kind of NPs, some studies have shown that they are non-toxic or slightly toxic, while other studies have shown that they are of high toxicity.

There are only a few reports about ecotoxicological studies on aquatic organisms with TiO_2 NPs. It has been reported that mortality was directly proportional to concentrations of TiO_2 NPs in *D. magna* [72, 73]. However, TiO_2 NPs caused no effects on mortality of carp (*Cyprinus carpio*) or rainbow trout [40, 43]. One study shows that NPs are almost non-toxic to bacteria [59], but other studies have shown that NPs have significant sub-acute toxicity to carps [43]. In a study the earthworm *Lumbricus terrestris* was exposed to TiO_2 NPs for seven days in water or 2–8 wk in soil with NPs at concentrations ranging from 0 to 100 mg/kg [74]. Results showed no mortality, but an enhanced apoptotic frequency, which was higher in the cuticle, intestinal epithelium and chloragogenous tissue than in the longitudinal, and circular musculature was observed. TiO_2 NPs did not seem to enter the coelomic liquid, or to reach the muscular layers. No bioaccumulation of TiO_2 NPs was observed.

A study indicated an increase of burst velocity while a decrease of average velocity when zebrafish larvae were exposed to low levels of nano-TiO_2 (0.1, 0.5, and 1 mg/L); but in higher concentration groups the effects are not significant (5 and 10 mg/L) [72]. The authors speculate that it might be owing to induced antioxidant defense systems in the fish.

A study showed that the addition of 100 mg/L zero-valent iron (ZVI) NPs was not toxic to the indigenous bacterial community in aerobic river water [75]; however, other studies reported that ZVI NPs at lower concentrations (<100 mg/L) showed cytotoxic nature [76–78].

Li et al. [20] examined ZnO NPs solubility/dispersion and toxicity to the earthworm *Eisenia fetida* under the influence of salts and dissolved organic matter. When *E. fetida* was exposed in agar, a dose-related increase in mortality was observed. After exposing to the highest concentration (1.0 g ZnO NPs/kg agar), the mortality was almost 100%. However, on filter paper, the highest mortality was observed at the lowest exposure concentration (0.05 g ZnO NPs/L). Moreover, increasing exposure levels seemed to decrease the mortality. SEM images showed that the aggregation of ZnO NPs was enhanced by the added salts, and then the dissolution behavior and biological availability were affected. Another TEM image showed that the added humic acids dramatically changed solubility and morphology of ZnO NPs.

Therefore, environmental conditions significantly affect the impact of NPs on environmental organism. All the factors need to be considered before the full understanding of toxicity of NPs. The reasons for this result are inconclusive. However, the reasons may include the following: (i) although structures of NPs are the same, particle sizes in experiments are different; (ii) during the experiment, NPs are different in levels of aggregation due to different pretreatment methods; (iii) different solvents are used; (iv) different experimental environments, including temperature, pH, light intensity and other factors; (v) the impact of NPs on other environmental substances; (vi) the use of different species, or the same species with different growth times, or different ways of drugs entering the organisms, and parts of accumulation are different [9]; (vii) other unknown factors.

4 Which are more suitable as typical bio-indicators for ecotoxicological studies of NPs in aquatic systems?

4.1 Fish are generally considered as preferred species

In ecotoxicology, fish is the main vertebrate test organism. It is widely agreed that fish should be the first choice when evaluating the potential acute aquatic toxicity of a substance to avoid the use of inappropriate animals and to save research funds [9].

Most of ecotoxicological studies published are acute studies for fish in different life stages (e.g. embryos, juveniles, and adults), and carbon-based NPs are generally less toxic to fish than metal and metal-oxide NPs, by causing sub-lethal effects such as oxidative stress in liver and gills, as well as pathological liver effects [79]. Studies on zebrafish, rainbow trout, carp etc. have been carried out more. Study shows that exposure to Cu NPs causes gill injury and acute lethality in zebrafish (*D. rerio*) [80]. The results demonstrate that Cu NPs is acutely toxic to zebrafish, with a 48 h LC_{50} concentration of 1.5 mg/L. Histological and biochemical analysis reveal that the gill is the primary target organ for Cu NPs. Ag NPs can also accumulate in gills and liver tissues affecting the ability of fish to cope with low oxygen levels and inducing oxidative stress [81, 82]. Recently, the first report of circadian rhythm gene deregulation by NPs in aquatic animals (zebrafish) is published, indicating the potential for broad physiological and behavioral effects controlled by the circadian system [83].

Higher organisms basically develop from a single cell (fertilized egg), and their early development of embryos is very similar, so toxic studies on early embryos of fish are important. Toxicity of different nanometal oxides to early developmental stages of zebrafish has been compared [41]. It is found that ZnO has the most toxic effects on zebrafish embryos and larvae (exposure of 96 h); suspension of both ZnO NPs and conventional ZnO micro-particles inhibit the zebrafish embryos and larvae, and there is a dose-effect relationship. Al_2O_3 NPs and TiO_2 NPs have no obvious effects on zebrafish embryos and larvae. Another study shows that nano-C_{60}, C_{70} are of similar toxicity to

Table 1. NPs effects as the basis for toxicity

Ecotoxicology mechanisms	Experimental NPs effects
ROS generation	Oxidative stress-related changes in gene expression and cell signaling pathways; lipid peroxidation; cell membrane damages; normal cell functions loss; cell death or apoptosis
Photocatalytic activity	Promoting generation of ROS; DNA damage; protein denaturation; degradation; loss of enzyme activity; auto-antigenicity
Solubility, surface characteristics and forms of metal oxides	The cell membrane penetrating; uptake in neuronal tissues; brain and peripheral nervous system injury
Bacterial attachment	Speeding up the absorption of NPs; inconclusive mechanism
Interactions of NPs with other environmental contaminants	Changing physical and chemical properties; increasing toxicity of NPs; damaging the cell membrane; promoting generation of ROS

zebrafish embryos, 200 μg/L of nano-C_{60}, C_{70} cause some deformities in zebrafish embryo, lower survival rate; however, even an order of magnitude higher concentration of nano-C_{60} $(OH)_{24}$ will not produce significant toxicological effects on zebrafish embryos [70]. This may be owing to the fact that surface features of nano-C_{60} $(OH)_{24}$ change relative to nano-C_{60}; NPs may influence the early development of embryos by disturbing the exchange of information between cells.

Some scholars have recently pointed out that behavioral endpoints are more sensitive in detecting toxicity of NPs on developing fish when comparing with other aspects. Negative impacts could be caused by aberrant locomotion behavior, and the severity depends on the level of activity. These negative impacts related to migration, dispersal, predator-prey interactions, and reproductive behavior, thus fitness of fish is decreased [84, 85]. In toxicity test of TiO_2 NPs on developing zebrafish, behavioral endpoints are more effective compared with other aspects such as hatchability and survival [71]. In aquatic ecosystem, fish is the most important biological community with large biomass. Confirming typical bio-indicators not only saves time for future experiments, but also saves a lot of human and financial resources. Fish species in current studies are few, and the lack of available data makes it not enough to determine whether they are suitable as typical bio-indicators.

4.2 Further aquatic invertebrate testing cannot be neglected in ecotoxicological research of NPs

It is stressed that in ecotoxicological research of NPs, further aquatic invertebrate testing (particularly studies of bioaccumulation and chronic endpoints with long-term low exposure) will be of great significance [11]. Behavioral and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to Cu NPs have been studied [86]. Behavioral impairments were observed in *S. plana* exposed to CuO NPs or soluble Cu whereas in *H. diversicolor*, and only the exposure to soluble Cu led to a burrowing decrease. No obvious neurotoxicity effects were revealed since in both species, no changes in cholinesterasic activity occurred in response to both forms of Cu exposure.

It is supposed that the major mechanism of entry into cells is endocytotic routes, thus potentially causing various types of cell damages in many tissues, particularly in tissues with cells of high phagocytic capacity, such as digestive gland and gills. This phenomenon is particularly common in suspension-feeding invertebrates [11, 87]. Bivalve molluscs represent an ideal group for investigating the effects of manufactured NPs, since they are abundant in a variety of aquatic environments, from freshwater to marine. Hemocytes of the marine mussel *Mytilus* have been proved to be a sensitive target for many environmental contaminants. Different conditions of exposure and different kinds of compound can cause different immunotoxic or inflammatory effects [88–91].

4.3 Because of sensitivity to pollutants, phytoplankton testing is also important

Phytoplankton is an important producer in water environments, occupying an important place in aquatic ecosystems. Toxic effects of NPs to phytoplankton and savings of NPs by phytoplankton can directly or indirectly affect the entire aquatic ecosystems.

For NPs, algae were the most sensitive aquatic organisms [79]. Among metal and metal oxide NPs, ZnO NPs was the most toxic, with substantial growth reduction (EC_{50}) at 42 μg/L for *Pseudokirchneriella subcapitata* in freshwater [92], while Wong et al. [93] observed an EC_{50} of 4.6 mg/L for *Thalassiosa pseudonana* in seawater. Inhibition of nano-C_{60} to *P. subcapitata* growth rate is about 30% when concentration is 90 mg/L. The speed of enrichment of other pollutants within the algal will be accelerated when the algae and nano-C_{60} particle aggregates contact with each other [85]. Gong et al. [94] observed that the NiO nanoparticles had severe impacts on the algae (*Chlorella vulgaris*), with 72 h EC50 values of 32.28 mg NiO/L. Under the stress of NiO NPs, *C. vulgaris* cells showed plasmolysis, cytomembrane breakage and thylakoids disorder. Potentially, NPs associated with phytoplankton could be transferred to higher trophic levels via herbivores grazing the algae.

Advantages and representative species as typical bio-indicators are shown in Tab. 2.

5 Concluding remarks

Ecotoxicological tests are irreplaceable tools for hazard evaluation of NPs because they integrate both harmful and mitigating effects and show the net influence of tested compounds in given experimental conditions. The behavior of NPs in natural waters cannot be predicted by experimental phenomena, especially in coastal waters and estuarine. Such areas are rich in species and are subjected to concentrations of organic substances and fluctuations in salinity. But for all these considerations, potential effects of different NPs to organisms in aquatic systems can be indirectly reflected by the general results of these studies.

The harmfulness of NPs to the environment has been confirmed by a large number of studies, but so far, behaviors of NPs in the environment are still not entirely clear. After entering the environments, forms of transmission and transformation of NPs among various elements of the environments remain an issue needing a comprehensive and in-depth study.

Table 2. Advantages and representative species of fish, marine invertebrates and phytoplankton as typical bio-indicators

Category	Advantages	Representative species
Fish	Main vertebrate test organism in aquatic ecosystems; behavioral endpoints being sensitive to toxicity of detected NPs; the most important biological community with large biomass; showing a dose-effect relationship	Zebrafish; Japanese medaka; fathead minnow; rainbow trout; common carp; three-spined stickleback
Invertebrates	Suitable for long-term low exposure with chronic endpoints and bioaccumulation studies; abundant in different aquatic environments; representing a sensitive target for a number of environmental contaminants	Bivalve molluscs; *Daphnia magna*; *Thamnocephalus platyurus*
Phytoplankton	Important producers in water environments; close contact with NMs and the entire aquatic ecosystem; the transference of NPs associated with phytoplankton to higher trophic levels	*Thalassiosa pseudonana*; *Pseudokirchneriella subcapitat*a; *Chlorella vulgaris*

There is a remarkable lack of information on some key aspects, which prevents a better understanding and assessment of toxicity and ecotoxicity of NPs to ecosystem organisms. The followings are important future research directions: (i) continuing in-depth study on basic mechanisms of environmental toxicity of NPs; (ii) determining typical bio-indicators so as to avoid unnecessary animal using whenever possible and to prevent the waste of funds; (iii) standardizing testing protocol, for example, unifying solvents for suspending NPs, and ways of dealing with suspensions; (iv) in-depth exploring the application of genomics and proteomics methods in ecotoxicological studies of NPs, and completing the relevant online database; (v) carrying out systematic aquatic ecotoxicology studies of NPs. Compound toxicity of NPs with other environmental pollutants on organisms should also be paid close attention to. Some of the knowledge gaps identified above may be filled by database based on the well-established toxicity tests in the near future.

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