Research Article

Towards a Definition of Harmless Nanoparticles from an Environmental and Safety Perspective

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The rapid development of nanoparticles (NPs), such as silicon nanoparticles (Si NPs) and ferric oxide nanoparticles (Fe_2O_3 NPs), and their use in myriad commercial applications have raised questions of their potential impacts on wastewater treatment systems. In this study, we investigated the consequences of the presence of Si NPs and Fe_2O_3 NPs in the denitrification of anoxic sludge. Fe_2O_3 NPs, at a concentration up to 50 mg/L, had no significant impact on nitrate removal, whereas Si NPs, at concentrations up to 50 mg/L, increased the rate of nitrate removal. We used transmission electron microscopy (TEM) to investigate the effect of Si NPs and Fe_2O_3 NPs. Si NPs exposure enhanced the abundance of *narG*-1 gene, which might promote nitrate removal process directly. Finally, we reviewed and identified the specific properties of a variety of NPs responsible for toxicity and found NPs larger than about 100 nm and without ion release in general possible to energy safety and nontoxic or low toxic to environment. Our results provide useful information to understand the response of anoxic sludge to Si NPs and Fe_2O_3 NPs in complex environmental matrix as well as potent support for wide use of the environmentally friendly NPs.

1. Introduction

With the rapid innovation and commercialization in the field of nanotechnology, nanoparticles (NPs) have been used in an increasing number of consumer and industrial products, as NPs unique size-dependent physicochemical properties [1] present commercial advantages. Many studies have shown that the NPs can be toxic to environmental microbes, plants, animals, and even human cells [2–6].

The increasing utilization of products containing NPs results in the release of NPs into sewage which ultimately enters wastewater treatment plants (WWTP). WWTPs are the last barriers that can prevent the release of NPs into the environment [7, 8]. Previous toxicological studies have shown that Ag, CuO, and ZnO NPs could induce significant growth inhibition of bacteria, mammalian cells, fish, and crustaceans [9]. The evidence for effects on wastewater treatment is

decidedly mixed: one publication demonstrated that ZnO NPs could induce acute effects on wastewater nitrogen and phosphorus removal and impair biological phosphorus removal [10]. Another showed that Ag NPs caused inhibition of biogas production and a slight inhibition in the action of other biomasses [11]. However, TiO_2 NPs were reported to have no obvious impacts on biological nitrogen removal after short-term exposure [12]. Similarly, TiO_2 and Au NPs caused only limited or no inhibition for tested biomasses [11]. Different kinds of NPs have different toxic because of their own characteristics.

NPs with diameter below 30 nm can be readily internalized by cells and will potentially be toxic to the cell. However, little research has explored the combination of nontoxicity of NPs to both eukaryotic (such as mammalian or fish) cells and the impacts on microbial functions. Herein, we explore the impact of silicon and iron oxide nanoparticles, which has

been generally shown to be nontoxic to certain wastewater treatment biofunctions. Si NPs are a new generation of optoelectronic semiconductor materials with a wide gap to semiconductors and also high power laser source materials. Fe_2O_3 NPs are widely utilized as pigments [13] and have attracted considerable attention due to their promising potential in biomedical applications because of their superparamagnetic properties [14] and use in nutritional [15] applications. Under these circumstances, these two kinds of NPs showed low toxicity [16, 17].

In this study we investigated the effects of Si NPs and Fe_2O_3 NPs on nitrate removal in wastewater sludge. Furthermore, we assayed interactions between the NPs and bacterial cells using transmission electron microscopy (TEM) visualization and measurement of reactive oxygen species. These studies identify some distinguishing features of interaction between nontoxic NPs and organisms.

2. Materials and Methods

2.1. Nanoparticles. Si NPs and Fe_2O_3 NPs used in this study were purchased from Chaowei Nanomaterials (Shanghai) and Aladdin, respectively. In this study, the NPs stock suspension (100 mg/L) was prepared by adding 100 mg of NPs to 1 L of Milli-Q water, followed by 30 min of ultrasonication (25°C, 250 W, 40 kHz). The primary particle size of the Si NPs and Fe_2O_3 NPs was characterized with transmission electron microscope (TEM) (JEOL JEM-1230, Japan) operated at 80 kV. The average diameter of the particles and zeta potential in the stock suspension was measured by dynamic light scattering (DLS) and laser Doppler microelectrophoresis using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, UK).

2.2. Operation Conditions of Sequencing Batch Reactors (SBR). Activated sludge was obtained from the Qige Wastewater Treatment Plant (Hangzhou, China) and cultivated in the anoxic parent SBR with a working volume of 12 L. The SBR was operated at 25°C with three cycles each day. The mixed liquor suspended solid (MLSS) was kept at a constant concentration of 3.5 g/L. Synthetic wastewater, consisting of 216.5 mg KNO_3 , 2.08 mg KH_2PO_4 , 1.76 mg K_2HPO_4 , 4 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.96 mg NaCl , 1.12 mg CaCl_2 , and 1.92 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (all per liter), was used to simulate nitrate-contaminated water. In this synthetic wastewater, trace elements with components of 1000 μg EDTA, 300 μg H_3BO_3 , 600 μg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 30 μg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 30 μg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 10 μg $\text{CuCl}_2 \cdot \text{H}_2\text{O}$, 70 μg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 20 μg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ were supplied (again, all per liter). The pH was controlled at 7.0 ± 0.5 ; dissolved oxygen (DO) was maintained below 0.5 mg/L.

To conduct the experiments, 5600 mL of mixture was withdrawn from the parent SBR, centrifuged at 10000 $\times g$ for 5 min, and resuspended in 800 mL of deionized (DI) water. The 0, 5, 20, and 50 mg/L Si NPs were prepared in 4 reactors by adding 0, 35, 140, and 350 mL of Si NPs stock suspension (100 mg/L), respectively. The 0, 5, 25, and 50 mg/L Fe_2O_3 NPs were prepared in 4 reactors by adding 0, 35, 175, and 350 mL of Fe_2O_3 NPs stock suspension (100 mg/L), respectively. Then, 100 mL of resuspended sludge and 50 mL of concentrated

synthetic wastewater were fed into each reactor. DI water was added to make the final volume 700 mL. The initial pH was 7.0 ± 0.5 . All reactors were bubbled with nitrogen gas for 5 min and DO was maintained below 0.5 mg/L. The reactors were fully mixed with magnetic stirrer.

2.3. Determination of Reactive Oxygen Species (ROS) Production Induced by NPs. Intracellular ROS production was determined using a Mouse ROS ELISA Kit (Shanghai Chuanxiang). Sludge was centrifuged at 10000 $\times g$ for 5 min and washed with 0.1 M phosphate buffer (pH 7.4) for 3 times. The sludge particles were resuspended in 0.1 M phosphate and then ultrasonic broken for 3 s on ice, after 5 s static time, and ultrasonic broken for 3 s on ice again and this process will be repeated 100 times. The solution was centrifuged at 10000 $\times g$ for 10 min in 4°C. We added 50 μL of the suspended sample per well in a 96-well plate and then 100 μL of HRP-avidin and then covered the microtiter plate, incubated for 1 h at 37°C. We aspirated each well and washed 5 times with wash buffer (200 μL). After the last wash, remaining wash buffer was removed by aspiration. Then 50 μL of solution A and 50 μL of solution B were added to each well, incubated for 15 min at 37°C. Finally, add 50 μL of stop solution to each well and determine the optical density of each well within 15 min, using a microplate reader ($\lambda = 450 \text{ nm}$).

2.4. Transmission Electron Microscopy. Visual characterization of interior morphology of sludge bearing Si and Fe_2O_3 NPs was conducted using TEM. The sludge sample, randomly withdrawn from each reactor, was washed three times with 0.1 M phosphate buffer (pH 7.4), fixed with 2.5% glutaraldehyde (pH 7.2–7.4) overnight at 4°C, and then washed 3 times with 0.1 M phosphate buffer. Samples were fixed for 2 h by 1% osmic acid before being washed for 3 times with 0.1 M phosphate buffer. Samples were dehydrated by serial passage through increasing ethanol concentrations (30, 50, 70, 90, and 100%, 15 min per step) and then placed in a mixture of Spurr (2) resin and acetone (1:1) for 30 min, followed by 2 h in 2 changes of 100% resin. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70°C for 8–24 h [18]. Ultrathin (70–90 nm) sections were cut with a diamond knife for TEM analysis.

2.5. DNA Extraction and Real-Time PCR. Chromosomal DNA of the activated sludge was extracted according to the instructions from the DNA Isolation Kit purchased from BioTeke Corporation (Beijing, China).

Gene expression of NAR, the key enzyme in denitrification, was monitored by real-time PCR. The bacterial gene NAR design primers were as follows: *narG*-1F (5'-GAC TTC CGC ATG TCR AC-3') and *narG*-1R (5'-TTY TCG TAC CAG GTG GC-3'), *narG*-2F (5'-CTC GAY CTG GTG GTY GA-3'), and *narG*-2R (5'-TTY TCG TAC CAG GTS GC-3') [19]. Real-time PCR was run on the Bio-Rad fluorescence quantitative PCR. The volume of 10 μL reaction system contained 5 ng of DNA template, 5 μL iQ SYBR Green Supermix, and 20 pmol/L forward and reverse primers. Cycle conditions were as follows: initial denaturation for 10 min at 95°C followed by 40 cycles of 95°C for 15 s and

annealing/extension at the temperatures 55°C and 60°C for 1 min. Gene and gene transcript numbers were quantified via comparison to standard curves. Automatic analysis settings were selected to determine the threshold cycle (Ct) values and baseline settings. We used the $2^{-\Delta\Delta C_t}$ method to calculate the relative quantitative gene expression of NAR gene expression to 16s rDNA internal gene expression [20]. 16s rDNA internal gene primers were 338F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3').

2.6. Analytical Methods. All samples were centrifuged and then filtrated with 0.45 μ m filter membrane before analysis. The analysis of nitrate-nitrogen (NO_3^- -N), nitrite-nitrogen (NO_2^- -N), COD, and MLSS, and mixed liquor volatile suspended solid (MLVSS) was conducted in accordance with standard methods [21].

2.7. Statistical Analysis. All tests were performed in triplicate and the results were expressed as mean $\pm$ standard deviation. An analysis of variance (ANOVA) was used to test the significance of results and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characterization of Si NPs and Fe_2O_3 NPs. We initially characterized the NPs using a combination of electron microscopy and WHAT. TEM images of the Si NPs and Fe_2O_3 NPs from the batch used for this study are shown in Figure 1. The average particle sizes of Si NPs and Fe_2O_3 NPs were observed as 50–100 nm and 80–100 nm, respectively. The zeta-potential of Si NPs and Fe_2O_3 NPs was -23.23 – -27.18 and -12.25 – -16.65 mV. All zeta-potentials were negative, indicating relative stability within a suspension. Si NPs had the lower magnitude zeta-potential, and it suggested that their surface morphology was different. The interactions of anoxic sludge with 50 mg/L Si NPs and 50 mg/L Fe_2O_3 NPs were observed in Figure 2, and sludge bulking of the anoxic sludge was caused by 50 mg/L of Si NPs. Si NPs and Fe_2O_3 NPs did not release any ions (data not shown). Si NPs had the lowest magnitude zeta-potential, which explains the greater extent of agglomeration by Si NPs than Fe_2O_3 NPs. Because zeta-potential of all NPs was between -10 mV and -30 mV, the NPs were expected to form microscale particle aggregates [22]. The aggregates would become greater in quantity and larger in size with increasing NPs loading concentrations [23], which should lead to the reduction of NPs' effective surface area and surface reactivity and reduce adverse impacts on bacteria.

3.2. Effects of Si and Fe_2O_3 NPs on Nitrate Removal. To study if Si or Fe_2O_3 NPs could alter biological functions, we investigated nitrate removal efficiencies with different concentrations of NP. Nitrate removal with the addition of Si NPs was nearly 100% at the end of each cycle (Figures 3(a)–3(c)). Compared to the control, with the increase of Si NPs concentrations from 0 to 50 mg/L, the NO_3^- -N removal efficiency decreased from 90.5% to 82.8% at the first hour in the first cycle, but there was no significant difference to the control group ($p > 0.05$). In the third and sixth cycle, the

NO_3^- -N removal efficiencies of Si NPs (5, 20, and 50 mg/L) were higher than that of control group. In the sixth cycle, 50 mg/L Si NPs had positive effect on the NO_3^- -N removal ($p < 0.05$). Thus, Si NPs appeared to promote nitrate removal or at a minimum and had no deleterious impact on nitrate removal.

Similarly we tested the NO_3^- -N removal efficiency in the presence of Fe_2O_3 NPs at concentrations of 5, 25, and 50 mg/L (Figures 3(d)–3(f)). Nitrate removal efficiency was almost 100% with exposure of 5, 25, and 50 mg/L Fe_2O_3 NPs under anoxic conditions, almost the same as those observed in the control test, suggesting that the addition of Fe_2O_3 NPs had no effect on NO_3^- -N removal ($p > 0.05$).

As shown in Figure 2, anoxic sludge exposure to 50 mg/L of Si NPs caused sludge bulking. Under a limited sludge bulking stage, nutrient removal efficiencies were increased [24]. Previously it was reported that the exposure to SiO_2 NPs induced no evident effects on MSTO and 3T3 cells due to its insolubility [25]. SiO_2 NPs and TiO_2 NPs were found to have no significant effects on nitrogen removal after short-term exposure due to its insolubility [12, 26]. Similarly, it is reasonable that no effects of insoluble Si and Fe_2O_3 NPs on nitrogen removal were observed.

3.3. Interaction of Bacterial Cells and NPs. Recently, Herd et al. [27] reported that SiO_2 NPs with various geometries have different orientations at the macrophage cell surface, resulting in distinct NP uptake and toxicity mechanisms. We reasoned that bacteria/NPs interactions might explain why Si and Fe_2O_3 NPs did not negatively influence nitrogen removal efficiency even at high Si and Fe_2O_3 NPs concentrations. TEM was employed to investigate the interaction of sludge and Si, Fe_2O_3 NPs (Figures 4 and 5). We observed that Si NPs did not enter into bacteria (Figures 4(E) and (G)), and there was no obvious damage to the internal structure of bacteria. Si NPs primarily aggregated around the bacteria. In the first 6 h, Si NPs caused plasmolysis of larger bacteria and some death (Figure 4(F)); however, most bacteria appeared normal after 36 h (Figure 4(a)). When anoxic sludge was exposed to Fe_2O_3 NPs, Fe_2O_3 NPs were visibly attached to the cell wall and damaged bacteria at 6 h (Figures 5(b), (D), (E), and (F)). Strikingly, after incubation with Fe_2O_3 NPs (50 mg/L) for 36 h, bacteria were observed surrounded by massive aggregates of Fe_2O_3 NPs (Figure 5(H)). Notably, the majority of bacteria in the sludge system were not affected by NPs [28, 29]. In previous studies anoxic sludge was shown to contain a variety of bacteria, which had strong defenses against NPs [30]. This would explain why both NPs had no effect on nitrate removal (but not why other NPs did affect nitrate removal).

3.4. ROS Production. NPs could cause oxidative stress and thereby induce oxidative damage to cell membrane [31]. ROS generation is considered to be the primary method by which NPs might be toxic to bacterial cells [32]. High ROS production might lead to the damage of cytoplasmic proteins or cell membrane in human cells [33]. Different NPs produced different amount of ROS, which might explain the different toxicities.

FIGURE 1: TEM image of the (a) Si NPs and (b) Fe_2O_3 NPs used in this study.

FIGURE 2: The sludge properties with addition of 50 mg/L Si NPs and 50 mg/L Fe_2O_3 NPs.

We noted that early in the experiment, before 6 h, there was apparent NP toxicity to bacterial populations (Figure 4). We reasoned that this might be due to ROS production and measured intracellular ROS production was measured. ROS generation of sludge bacteria induced by Si NPs was concentration-dependent for the first two hours of the reaction but decreased and leveled off near controls by 6 h (Figure 6). The increase in ROS at these early time points was caused by oxidative stress. The initial oxidative stress upon Si NPs (Figure 6) gradually abated in response to the antioxidant defense system of cells. Therefore they effectively resisted the toxicity of Si NPs and promoted the activity of bacteria inversely. The overall ROS production in the presence of Fe_2O_3 NPs was not different ($p > 0.05$) than the control without Fe_2O_3 NPs.

According to the result of ROS, Si and Fe_2O_3 NPs had no obvious toxic to sludge. The effect of fullerene NPs aggregate size on ROS production and toxicity toward *Vibrio fischeri*

has been reported [34]. However, there is some disagreement regarding the role of ROS in conveying NPs toxicity. The studies reported that Cu NPs were prone to induce the oxidative damage of membrane lipids [35] and proteins [36]. Moreover, it has been reported that toxic effects of fullerene NPs are due to direct oxidative damage by NPs without ROS production [37].

3.5. Gene Expression Analysis. We next asked whether the presence of NPs and the effects on bacteria could have an impact on gene expression. We focused on nitrate reductase (NAR), the key enzyme for denitrification. We measured transcription of the genes *narG-1* and *narG-2* by RT-PCR [19] (Figure 7). We did not detect transcripts from the *narG-2* subgroup in the sludge. Si NPs did not affect the abundance of *narG-1* gene of sludge bacteria in the first and third cycle ($p > 0.05$) (Figure 7(a)). However, 50 mg/L of Si NPs induced the increased abundance of *narG-1* gene ($p < 0.05$) in

FIGURE 3: ((a)–(c)) Nitrate removal efficiency in the reactors with different concentrations of Si NPs. Nitrate removal efficiency of (a) the first cycle, (b) the third cycle, and (c) the sixth cycle. ((d)–(f)) Nitrate removal efficiency in the reactors with different concentrations of Fe_2O_3 NPs. Nitrate removal efficiency of (d) the first cycle, (e) the third cycle, and (f) the sixth cycle. Mean $\pm$ standard.

FIGURE 4: TEM image of sludge without (a) or with (b) the addition of 50 mg/L Si NPs after 6 h. (a) Inset panels (A)–(C) and (D)–(F) show three kinds of bacteria in the sludge. In panel (c), the reaction was allowed to continue for 36 hours in the presence of Si NPs. The insets (G, H) show bacteria at the 36 h time point. Arrows indicate Si NPs.

the sixth cycle, which may partly explain the promotion of nitrate removal. By comparison, Fe_2O_3 NPs did not affect the abundance of *narG*-1 gene in the sludge ($p > 0.05$). The results of RT-PCR were consistent with our previous results on the efficiency of nitrate removal and suggested that NPs can act to result in the increased expression of denitrification enzymes.

4. Discussion

Toxic effects have been observed for many kinds of NPs at a range of concentrations and for a variety of organisms. We collected, analyzed, and summarized the toxicity data from the published literature on NPs. Studies used various measures of toxicity including: minimum inhibitory concentration (MIC), median lethal dose (LD_{50}), half maximal effective concentration (EC_{50}), half maximal inhibitory concentration (IC_{50}), viability, CFU, ROS, and DNA damage. The purpose of reviewing the literature on toxicity was to summarize the physicochemistry characteristics of experimented NPs, so as to distinguish nontoxic NPs (“harmless NPs”) from current widely used NPs.

We grouped the potential risk of NPs, based on toxicity to many kinds of bacteria [38–41], into four categories: (1) toxic at any concentration less than 1 mg/L; (2) toxic at any

concentrations less than 10 mg/L; (3) toxic only at concentrations >10 mg/L; and (4) nontoxic at all tested concentrations (Figure 8). Details for the studies are provided in Supplemental Table 1 (see Supplementary Material available online at <http://dx.doi.org/10.1155/2016/8608567>). We considered the following aspects to determine whether NPs are “harmless NPs.”

4.1. Dissolution and Ion Release. Ag, CuO, ZnO NPs, and CdSe QDs demonstrated high toxicity to microorganisms, and it is noteworthy that metal ions released from metallic NPs played a key role in mediating the toxicity. Radniecki et al. [42] found that 20 nm Ag NPs were more toxic than 80 nm Ag NPs, which was attributed to the higher release rate of Ag^+ from the smaller particles. Similarly, smaller ZnO NPs (8 nm) were found to display greater growth inhibition to *S. aureus* than larger particles ($>1 \mu\text{m}$) [43]. These findings were in concordance with the study from Sadeghi et al. [44] who investigated the influence of different NPs shapes (rods, spherical particles) on bacterial toxicity. It concluded that an increased surface area resulted in increased release of Ag^+ which could explain the increased toxicity of Ag NPs.

4.2. ROS. Toxicity of NPs cannot always be explained by particle dissolution and release of metallic ions [45, 46]. In

FIGURE 5: TEM image of sludge without (a) or with (b) the addition of 50 mg/L Fe_2O_3 NPs after 6 h. (a) Inset panels (A)–(C) and (D)–(F) show three kinds of bacteria in the sludge. In panel (c), the reaction was allowed to continue for 36 hours in the presence of Fe_2O_3 NP. The insets (G)–(I) show bacteria at the 36 h time point. Arrows indicate Fe_2O_3 NPs.

these cases, ROS-mediated toxicity is widely thought to be the mechanism [47–50]. Large amount of ROS could be generated from small amounts of ZnO or CuO NPs [51]. In our study, Si and Fe_2O_3 NPs induced the increase of ROS production at first 2 hours and then dramatically decreased to normal level. We speculate that the increased production of ROS was matched in the case of Si and Fe_2O_3 NPs by the stress response of bacteria and only the continuous high ROS can cause cellular damage [52].

4.3. Type of Organisms. The toxicity values of SiO_2 NPs varied greatly. Jiang et al. [23] found SiO_2 NPs (20 nm) caused a high death rate for *E. coli* (58%) and did not detect any ions released from NPs. They concluded that the observed toxicity of SiO_2 NPs resulted from their nano-size-related properties. In contrast, SiO_2 NPs (10–20 nm, amorphous) doses up to 150 milligram per gram total suspended solids (mg/g-TSS) showed no inhibitory effect on waste activated sludge anaerobic digestion [53]. Hence SiO_2 NPs showed different toxicity to different organisms.

4.4. Photocatalysis. Toxicity of TiO_2 NPs might be attributed to their photocatalytic activity. TiO_2 is a photocatalyst and promotes generation of ROS by interaction with photons in the ultraviolet spectrum. Several studies of toxicity TiO_2 NPs were conducted under ambient laboratory lighting which contains negligible or no UV radiation. It is important to note that energy at wavelength at or below 368 nm accounts for approximately 6% of the sunlight energy reaching the earth's surface (although this varies depending on atmospheric conditions) [54]. As such, other studies have documented that toxicity of TiO_2 NPs was significantly enhanced under natural sunlight [55] as compared to laboratory fluorescent lighting or dark.

4.5. Physicochemistry. Biological effects of NPs are dependent on several factors including NP physicochemistry, dose, contact time, type of organisms, and composition of growth medium [56]. However, the potential toxicity to organisms in the presence of variable environment could be dependent on the physicochemistry of NPs' characteristics [57]. Particle

FIGURE 6: ROS concentrations at 2 h in various reactor cycles in the presence of Si NPs (a, b, and c) or Fe₂O₃ NPs (d, e, and f). (a, d) Cycle 1, (b, e) cycle 3, and (c, f) cycle 6. The error bars represent standard deviations from the mean. Asterisks indicate a statistically significant difference ($p = 0.05$) between the treatment and control groups.

size and shape are known to affect the organisms and NPs interaction [58]. Several studies have demonstrated that NPs always showed more serious toxicity than bulks [59–61] and suggested that particle size was one of the key factors influencing the toxic effect of NPs. 10 nm Ag NPs induced more apoptotic cells than the larger particles (i.e., 50 and 100 nm) [62]. The size of NPs is directly correlated with many essential properties, such as solubility, surface property, chemical reactivity, and nanoparticle-cell interaction that later affect the toxicological behaviors of NPs [63–65]. In

other words, decreasing of NPs size resulted in increasing of NPs specific surface area, which increased the reactivity and enhanced interactions between NPs and organisms [34]. NPs can easily enter into the human cell or bacteria due to their tiny size [66, 67].

Besides size, shape of NPs is another key factor that determined NPs toxicity. For example, the truncated triangular form of Ag NPs was found to have the strongest bactericidal effect on *E. coli*, compared with spherical or rod-shaped form [68]. Rod- and sphere-shape of TiO₂ are more phototoxic

FIGURE 7: Quantitation of *narG-1* transcript levels in reactors. We used RT-PCR to determine the amount of target gene *narG-1* in the reactors with different concentrations of Si NPs (a). Similarly we measured the amount of target gene *narG-1* in the reactors with different concentrations of Fe₂O₃ NPs (b). The error bars represent standard deviations from the mean. Asterisks indicate a statistical difference ($p = 0.05$) between that treatment and the control group.

FIGURE 8: Different toxic levels of different NPs.

than TiO₂ nanotubes and nanosheets [50]. Material morphology influences NPs toxicity by governing how NPs align at the organisms' surface.

We conclude that the essential characteristics of "harmless NPs" are as follows: (1) the size of the nanoparticles should be >100 nm, (2) the nanoparticles should have no photocatalytic activity, (3) the nanoparticles should not generate toxic ions in solution, and (4) the nanoparticles should be highly crystalline.

5. Conclusions

We studied the effects of Si and Fe₂O₃ NPs on denitrification in a wastewater treatment model system. They did not cause inhibitory effects on biological nitrate removal. According

to the results of TEM imaging, ROS, and the abundance of *narG-1* gene, Si NPs and Fe₂O₃ NPs were nontoxic to anoxic sludge. Si NPs and Fe₂O₃ NPs might be considered as "harmless NPs" with regard to denitrification. A definition of "harmless NPs" based on physicochemical properties is needed for future applications in nanotechnology. We have made predictions about the properties of "harmless NPs" based on the literature; however, toxicological experiments should be carried out prior to wide production and application of NPs.

Abbreviations

NP:	Nanoparticle
TEM:	Transmission electron microscopy
WWTP:	Wastewater treatment plants
DLS:	Dynamic light scattering
SBR:	Sequencing batch reactors
MLSS:	Mixed liquor suspended solids
DO:	Dissolved oxygen
DI:	Deionized
ROS:	Reactive oxygen species
MIC:	Minimum inhibitory concentration
LD ₅₀ :	Median lethal dose
EC ₅₀ :	Half maximal effective concentration
IC ₅₀ :	Half maximal inhibitory concentration
TSS:	Total suspended solids.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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