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The impact of zinc oxide nanoparticles on nitrification and the bacterial community in activated sludge in an SBR

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The impact of zinc oxide nanoparticles on nitrification and the bacterial community in activated sludge in an SBR

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Zinc oxide(ZnO) nanoparticles (NPs) have been reported to induce adverse effects on organisms. The impacts of ZnO NPs on nitrification and the nitrobacteria community in activated sludge were investigated in a simulated SBR. It revealed that ZnO NPs at low concentrations (5 and 10 mg/L) slightly inhibited nitrification. At these concentrations the activity of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR) as well as the cell membrane integrity of *nitrosomonas europaea* were almost unaffected. Concentrations of 20 mg/L and 50 mg/L ZnO NPs had significantly adverse effects on the activity of AMO and NOR and on the transformation of NH_4^+-N to NO_2^--N and NO_2^--N to NO_3^--N. Analysis by denaturing gradient gel electrophoresis (DGGE) revealed that higher concentrations of ZnO NPs significantly inhibited the growth of the typical ammonia-oxidizing bacteria (AOB) that were mainly responsible for oxidation of ammonia to nitrate. Moderate concentrations of ZnO NPs could accelerate the growth of some types of denitrifying bacteria and promote the growth of some pathogenic bacteria. Moderate and high concentrations of ZnO NPs could obviously destroy the integrity of the cell membrane of *Nitrosomonas europaea*. These findings meaningfully assessed the adverse effects of ZnO NPs on activated sludge in wastewater treatment.

1. Introduction

The development and application of nanotechnology have raised significant concerns about the adverse effects of nanoparticles (NPs) on human health and the environment.[1] NPs can be more toxic than larger particles of the same composition because of their large specific surface area and unique size-effect.[2,3] Many studies were thus conducted to predict their environmental concentrations and investigate the behavior of NPs in the environment.[4,5,6] The increasing utilization of products containing NPs, however, was

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observed to result in the release of NPs into wastewater treatment plants (WWTPs).[7,8,9] Large amounts of zinc oxide nanoparticles (ZnO NPs) have been used in semiconductors, plastic additives, pigments and cosmetics.[10] A recent study confirmed that ZnO NPs were present in sewage sludge and effluents. According to the report issued by the USEPA in 2009, an examination of 84 WWTPs showed that the zinc content in WWTP biosolids was 8.55 g/kg-SS.[11] The investigations in China in 2011 (139 WWTPs in total) and in 2009 (107 WWTPs in total) showed that the average concentration of Zn in biosolids was 1.03 g/kg-SS and that the maximum concentration was 9.14 g/kg-SS.[12,13,14] Concerns can therefore be raised about whether the NPs in WWTPs have negative impacts on the microbial community in activated sludge, which may eventually hamper the function of WWTPs in removing pollutants from wastewater, such as chemical oxygen demand (COD), nitrogen and phosphorous.

Recently, studies have started to address this issue. Zheng et al.[15]reported that the presence of 10 and 50 mg/L of ZnO NPs decreased the total nitrogen removal efficiencies from 81.5% to 75.6% and 70.8%, respectively, compared with the absence of ZnO NPs. Additionally, several other publications indicated that different NPs and exposure times showed different effects on biological nitrogen removal. For instance, Zheng et al. [15] reported that 1 mg/L of SiO_2 NPs caused no adverse acute and chronic effects on sludge viability and wastewater nitrogen removal, while chronic exposure to 50 mg/L SiO_2 NPs depressed the total nitrogen (TN) removal efficiency from 79.6% to 51.6% after a 70-day exposure. Chen et al. [16] found that short-term exposure to 1 and 50 mg/L Al_2O_3 NPs induced only marginal influences on wastewater nitrification and denitrification. Nevertheless, prolonged exposure to 50 mg/L Al_2O_3 NPs was observed to decrease the TN removal efficiency from 80.4% to 62.5%. Ni et al.[17] found that the short-term presence of 50-200 mg/L of NPs decreased the TN removal efficiency resulting from the acute toxicity of a shock load of magnetic NPs, while long-term exposure to 50 mg/L magnetic NPs was observed to significantly improve the TN removal efficiency. Zheng et al. [7] found that concentrations of 1 and 50 mg/L TiO_2 NPs had no acute

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effects on nitrogen removal from wastewater after a short-term exposure (1 day), while 50 mg/L TiO_2 NPs (higher than its environmentally relevant concentration) was observed to significantly decrease the TN removal efficiency from 80.3% to 24.4% after long-term exposure (70 days). Li et al. [18] found that 2-50 mg/L of TiO_2 NPs did not adversely affect nitrogen removal, but when the activated sludge was exposed to 100-200 mg/L of TiO_2 NPs, the effluent TN removal efficiencies were 36.5% and 20.3%, respectively, which were markedly lower than the values observed in the control test (80%). Most of the above studies demonstrated that NPs hampered the function of the WWTP in removing nitrogen from wastewater. However,it is still not well known how ZnO NPs affect nitrification in activated sludge and which step of nitrification is more sensitive to ZnO NPs.

As is well known, the biological removal of nitrogen in wastewater is achieved by complex microbial populations that are responsible for nitrification and denitrification.[7,19,20] Therefore, the diversity of the microbial populations and a stable bacterial community structure both play important roles in achieving a high efficiency of biological nitrogen removal. Previous publications noted that silver NPs could cause a 50% inhibition of the respiration of nitrifying bacteria at a concentration of 0.14 mg/L, [21] whereas Cu NPs showed no inhibitory effect on the respiration of ammonia-oxidizing bacteria at the level of 10 mg/L .[22] Chen et al.[16] indicated that, compared with the control, 50 mg/L Al_2O_3 NPs decreased the abundance of denitrifying bacteria in activated sludge according to quantitative polymerase chain reaction (PCR) assays. Ni et al.[17] reported that a short-term exposure to 50 mg/L magnetic NPs led to the abatement of nitrifying bacteria according to fluorescence in situ hybridization (FISH) assays. According to Zheng et al. [7], denaturing gradient gel electrophoresis (DGGE) profiles showed that 50 mg/L TiO_2 NPs clearly reduced the diversity of the microbial community in activated sludge, and FISH analysis indicated that the abundance of nitrifying bacteria, especially ammonia-oxidizing bacteria, was significantly decreased after long-term exposure to 50 mg/L TiO_2 NPs. Similarly, according to Li et al.,[18] the DGGE profiles showed that 200 mg/L of TiO_2 NPs

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significantly reduced the microbial diversity in the activated sludge. Sheng et al.[23] found that the microbial susceptibility to Ag NPs was different for each microorganism. For instance, *Thiotrichales* is more sensitive to Ag NPs than other biofilm bacteria. These results indicated that different types of NPs showed different influences on bacteria in WWTPs and that the microbial susceptibility to NPs is different. However, to date, how the presence of ZnO NPs affect the nitrifying bacterial community in activated sludge is still not well known.

The objectives of this study are to (a) evaluate how ZnO NPs affect nitrification in an aerobic activated sludge system; (b) determine the toxicity of ZnO NPs to the typical bacteria in activated sludge, which leads to the deterioration of biological nitrogen removal; and (c) explore the effects of ZnO NPs on the typical bacterial diversity in activated sludge.

2. Experimental

2.1 Materials and methods

2.1.1 Nanoparticles suspension

A commercially produced ZnO NP suspension (1.7 g/L, <100-nm particle size) was purchased from Sigma-Aldrich (St Louis, MO, USA). Before use, the ZnO NPs suspension (1700 mg/L) was prepared by dispersing 1 mL of ZnO NPs (1.7 g/L) in 1 L of deionized water, followed by 1 h of ultrasonication (25℃, 250 W, 40 kHz).[24] The primary particle size of ZnO NPs was less than 100 nm, and the average particle size in the stock suspension was measured to be in the range from 30 to 60 nm by dynamic light scattering (DLS) analysis via a Malvern Autosizer (4700, Malvern Instruments, UK). According to transmission electron microscopy (TEM) and scanning electron microscopy (SEM), the ZnO nanoparticles used in this study were spherical..

2.1.2 SBR operation

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The reactor was inoculated with conventional activated sludge taken from the Harbin Taiping municipal wastewater treatment plant, Harbin, China. All of the reactors were fed with synthetic wastewater containing the following components: NH_4^+-N (NH_4Cl), 30-50 mg/L; COD (sucrose), 350-450 mg/L; KH_2PO_4, 30 mg/L; $MgSO_4 \bullet 7H_2O$, 50 mg/L and $CaCl_2 \bullet 2H_2O$, 10 mg/L. The activated sludge was successfully cultured with the synthetic wastewater.

The activated sludge was cultured in the SBR with a working volume of 4 L, which was operated to achieve biological nitrogen removal. The SBR operated at 25-28 ℃ with three cycles each day. Each cycle (8 h) consisted of 5.6 h of aeration, followed by 1 h for settling, 15 min for decanting and 1 h for idling. The influent pH was adjusted to 7.5 by adding NaOH, $NaHCO_3$ and HCl. Air was provided intermittently by using an on/off controller with an online DO detector to maintain DO at an appropriate level. Sludge was wasted to keep the solids retention time (SRT) at approximately 22 days to maintain the ratio of mixed liquor volatile suspended solid (MLVSS) to mixed liquor suspended solids (MLSS), namely, MLVSS/MLSS at 0.75. The reactor was constantly mixed with a stirrer except during the settling, decanting, and idle periods. After approximately 3 months, the stable removal efficiencies of nitrogen (>90.0%) were achieved. The configution of the reactor setup is shown in Fig.1.

2.1.3 ZnO NPs exposure to activated sludge

Four test concentrations (5, 10, 20, and 50 mg/L) of ZnO NPs were examined in the exposure experiments. The environmentally relevant concentration of ZnO NPs in WWTPs was 1 to 5 mg/L according to the literature.[12,13] Because the environmental release of NPs might increase due to large-scale manufacturing, the potential effects of higher concentrations (20 and 50 mg/L) of ZnO NPs were also investigated. Two SBR reactors with a working volume of 4 L each were used to conduct the experiments. One of them contained ZnO NPs by adding a ZnO NP suspension (5, 10, 20 and 50 mg/L), while the other reactor without ZnO NPs was operated as the control. Each SBR reactor was set up in triplicate to ensure reliable results. After the

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addition of ZnO NPs, the trend of the changes in NH_4^+-N, nitrite nitrogen (NO_2^--N), and nitrate nitrogen (NO_3^--N) during each operating cycle and the removal of NH_4^+-N and COD were all measured.

2.2 Analytical methods

2.2.1 Analysis of regular index

The determinations of COD, NH_4^+-N, nitrite-nitrogen (NO_2^--N), and nitrate-nitrogen (NO_3^--N) were conducted in accordance with the Standard Methods.[25]

2.2.2 DNA extraction, PCR-DGGE Analysis and gene sequencing

Twenty millilitres of the activated sludge mixture from the reactor was centrifuged at 12000 g for 10 min at 6-10℃, and then, the precipitate was resuspended to 30 mL and repeated twice. The pretreated activated sludge was dissolved in 15 mL of TE buffer solution. Bulk genomic DNA was extracted using sodium dodecyl sulfate (SDS) hexadecyltrimethyl ammonium bromide (CTAB), and the products were examined by agarose (1% w/v) gel electrophoresis in Tris/borate/EDTA buffer (TBE).

The 16S rDNA variable region of the extracted DNA was amplified with primers 27F, with a GC-clamp (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1492R (5'-GWATTACCGCGGCKG CTG-3'). PCR amplification was carried out in a total volume of 50 μL containing 2 μL of template DNA, 5 μL of Ex Taq reaction buffer, 0.25 μL of Ex Taq polymerase, 4 μL of dNTPs, 1 μL of forward primers and 1 μL of reverse primers (Takara, Japan) using a PE 2700 thermocycler (Biometra T-Gradient). The amplification program consisted of an initial denaturation step at 94℃ for 5 min, denaturation at 94℃ for 45 s, annealing at 55℃ for 45 s and extension at 72℃ for 60 s, followed by a final extension at 72℃ for 10 min. The PCR products were electrophoresed on 6% polyacrylamide gel in 1×TAE buffer with gradients ranging from 30% to 60% denaturant (100% denaturant: 7 M urea and 40% (v/v) deionized formamide) at a constant voltage of 60 V at 60 ℃ for 12 h.

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The gel was stained with EB for 15 min and viewed with a BioRad Gel Documentation system (BioRad). The prominent bands were then excised from the gel, and after cleanup treatment, the recovered DNA was reamplified (initial denaturation at 94℃ for 3 min, denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s and extension at 72℃ for 90 s, followed by a final extension at 72℃ for 6 min), purified, and cloned into the pMD19-T Simple vector (TaKaRa, Japan). The sequences from this study were submitted to the GenBank database. The closest matching sequences were searched using the BLAST program.

2.2.3 Determination of the activity of Ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR)

Aliquots of activated sludge were obtained at the end of the low DO stage and then washed 3 times with 0.01 M phosphate buffer (pH 7.4) to measure the activities of AMO and NOR. The resuspended pellets were then sonicated at 20 kHz and 4°C for 5min to break down the cell structure of the bacteria in activated sludge. The debris was centrifuged at 12000 g and 4°C for 10min, and the crude extracts in the supernatant were obtained for the enzyme activity measurement. All of the key enzymes activities were based on the protein content as determined by the BCA method. Assays for AMO and NOR activity were performed in stoppered serum vials (10 mL) containing 100 µL of crude extract and 1.9 mL of 0.01 M phosphate buffer (containing 2 mM $(NH_4)_2SO_4$, pH 7.4) or 0.01 M phosphate buffer (containing 1 mM $NaNO_2$, pH 7.4). The vials were shaken in a water bath at 30℃ for 30 min and were then centrifuged immediately. This was followed by measuring the increase of nitrite in the AMO activity assay or the decrease of nitrite in the NOR activity assay in the supernatant. The specific AMO and NOR activities were presented as the production of µmol of nitrite/(min·mg protein) and the reduction of µmol of nitrite/(min·mg protein), respectively [7].

2.2.4 Lactate dehydrogenase release analysis

The cell membrane integrity of activated sludge was measured through the lactate dehydrogenase (LDH) release assay. The LDH activity was determined by using a cytotoxicity detection kit (Tiangen, China)

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according to the manufacturer's instructions. After exposure treatments, the mixed sample was centrifuged at 12 000 g for 5 min. Then, the supernatant was seeded on a 96-well plate, followed by the addition of 50 μL of substrate mix (Tiangen). After incubation at room temperature for 30 min in the dark, 50 μL of stop solution (Tiangen) was added to each well and the absorbance was recorded at 490 nm using a microplate reader (BioTek).

2.3 Statistical Analysis

All of the tests were performed in triplicate, and the results were expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used to examine the significance of the results, and $p<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1 Effects of ZnO NPs on biological nitrification

As is well known, biological nitrogen removal depends on the successful oxidation of ammonia and nitrate denitrification to N_2. Fig. 2 presents the NH_4^+-N removal efficiency of the SBR when exposed to ZnO NPs. It can be observed that NH_4^+-N removal was only decreased from 79.8% to 76.3% when it was exposed to 10 mg/L ZnO NPs, which was not much different from the control, suggesting that 10 mg/L of ZnO NPs showed no measurable effect on nitrogen removal. However, the presence of 20 and 50 mg/L of ZnO NPs decreased NH_4^+-N removal efficiency from 82.4% to 20% and from 88.3% to 0%, respectively, after 5 days of exposure, which were remarkably lower values than those of the control. This indicated that a shock load of 20 and 50 mg/L ZnO NPs had caused significant toxicity to activated sludge and finally deteriorated the nitrogen removal performance of activated sludge. It suggests that the nitrogen removal was inhibited by higher concentrations of ZnO NPs. Similarly, Zheng et al.[7] found that a low concentration of ZnO NPs showed no

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measurable effect on nitrogen removal. In contrast, when the concentration of ZnO NPs was 50 mg/L, TN removal was significantly affected.

To probe the effects of ZnO NPs on nitrification, the transformations of NH_4^+-N to NO_2^--N and of NO_2^--N to NO_3^--N were investigated during one cycle when activated sludge was exposed to different concentrations of ZnO NPs.

As seen from Fig. 3, the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were relatively stable in the presence of 10 mg/L ZnO NPs during one cycle, and the variations of NH_4^+-N and NO_2^--N were not significantly different from their respective controls. Nevertheless, in the presence of 20 mg/L ZnO NPs, the level of NO_3^--N significantly differed from that of the control, suggesting that the presence of 20 mg/L ZnO NPs had an adverse effect on the transformation of NO_2^--N to NO_3^--N.

At the concentrations of 20 and 50 mg/L ZnO NPs (Fig.3), the NH_4^+-N removal was inhibited. Moreover, little NH_4^+-N was oxidized into NO_2^--N which appears to immediately be oxidized to nitrate, suggesting that ZnO NPs affected ammonia oxidizing bacteria not nitrite oxidizing bacteria. . On the basis of Fig 3, when the concentration of ZnO NPs reached 50 mg/L, the variations of NH_4^+-N were not significantly different from 20 mg/L ZnO NPs, but the levels of NO_2^--N and NO_3^--N significantly differed from the respective control, indicating that the transformation of NH_4^+-N to NO_2^--N were significantly inhibited.

Further investigation showed that ZnO NPs influenced the activities of enzymes relevant to nitrogen removal. AMO and NOR are two key enzymes in nitrification. As seen in Fig. 4, 5 and 10 mg/L of ZnO NPs showed less inhibition of AMO and NOR activities. However, when the activated sludge was exposed to 20 and 50 mg/L of ZnO NPs for 5 days, compared to the control, the inhibition rate of AMO activity was 22.42% and 39.24%, respectively, and the inhibition rate of NOR activity was 28.03% and 44.84%, respectively. These results indicated that 20 and 50 mg/L of ZnO NPs significantly inhibited these two enzymes, which led to the inhibition of the normal function of nitrifying bacteria. The inhibition was almost

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in a dosing manner. These observations were also consistent with the changes in the transformations of NH_4^+-N to NO_2^--N and of NO_2^--N to NO_3^--N during one cycle of SBR and the lower NH_4^+-N removal efficiencies at ZnO NPs concentrations of 20 and 50 mg/L, as shown in Fig 3.

3.2 Effects of ZnO NPs on the microbial community structure in activated sludge

Although the antimicrobial capabilities of NPs are widely reported, their impacts on ecological microbial communities are not well understood. Nitrification is carried out by a group of bacteria that are capable of using nitrate in place of oxygen as an electron acceptor for respiration. In previous studies[27], NPs were revealed to be toxic to both Gram-negative and Gram-positive bacteria, suggesting that higher concentrations of ZnO NPs in activated sludge might also decrease the abundance of denitrifying bacteria. For this reason, the effects of ZnO NPs on changes in the bacterial diversity of activated sludge were investigated. It can be seen from Fig. 5 that the activated sludge in the control showed high bacterial diversity. According to the detailed information on the bands in the DGGE profiles (Table 1), 50 mg/L of ZnO NPs significantly inhibited the growth of typical ammonia-oxidizing bacteria (AOB) (band b3 and b12, related to *Nitrosococcus sp.* and band 4, related to *Nitrosomonas sp.*). These microorganisms are mainly responsible for the oxidation of ammonia to nitrate. Moreover, the activity of microorganisms, such as *Thioflavicoccus mobilis* (band 10), which is mainly responsible for vulcanization, was also inhibited. Some bacteria associated with denitrification (band b8, related to *Nitratiruptor sp.* and band b14, related to *Pseudomonas sp.*) would be promoted by 20 mg/L of ZnO NPs, but inhibited by 50 mg/L of ZnO NPs. The activity of other pathogenic bacteria, such as *Xanthomonas hortorum pv*. (band b7) and *Stenotrophomonas maltophilia* (band b9), were promoted by ZnO NPs, two types of which could result in death and the rapid aging of activated sludge. Moreover, ZnO NPs had no significant influence on the activity of other bacteria, such as *Thioalkalivibrio sulfidophilus* (band b13).

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Overall, the results indicated that ZnO NPs would inhibit the growth of bacteria associated with nitrification and **sulfofication**. Moderate concentrations of ZnO NPs (20 mg/L) could accelerate the growth of some types of denitrifying bacteria. ZnO NPs could promote the growth of some pathogenic bacteria, which caused activated sludge to rapidly age and die. Moreover, ZnO NPs had no obvious influence on the activity of bacteria, such as *Thioalkalivibrio sulfidophilus*.

3.3 Effects of ZnO NPs on *Nitrosomonas europaea*(ATCC 19718)

3.3.1 Effects of ZnO NPs on activity of *Nitrosomonas europaea*

The pure Nitrosomonas europea (ATCC 19718) were enriched by ATCC medium 2265 before exposure experiment. As is well-known, *Nitrosomonas europaea* plays an important role in the nitrification process, which can transform NH_4^+-N into NO_2^--N. Accordingly, NO_2^--N production can be used as an indicator of the activity of *Nitrosomonas europaea*. To explore the impact of ZnO NPs on the activity of *Nitrosomonas europaea,* NO_2^--N production was determined at every 30 min over 180 min by using NH_4^+-N as a substrate The results are shown in Fig. 6. Fig. 6 (A) shows the trend of the change in the NO_2^--N concentration when *Nitrosomonas europaea* was exposed to ZnO NPs at the uppermost concentration of 50 mg/L in 180 min. It can be seen that the NO_2^--N concentration increased at all exposure concentrations of ZnO NPs in the first 60 min and maintained a stable concentration of 7.5-8.5 mg/L from 60 to 150 min. In contrast, a decrease in the NO_2^--N concentration was observed from 150 to 180 min at the lower exposure concentrations of 5 and 10 mg/L. To some extent, this could be explained from the DGGE analysis of the bacterial community which found 50 mg/L of ZnO NPs significantly inhibited the growth of typical ammonia-oxidizing bacteria (AOB). Specifically, the low and moderate concentrations of ZnO NPs could accelerate the growth of some types of denitrifying bacteria. To further explore the effect of ZnO NPs on

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246 *Nitrosomonas europaea*, the NO_2^--N production rate was measured for the first 60-min exposure. The
247 results were shown in Fig. 6 (B).

248 It can be observed that concentrations of 20 and 50 mg/L ZnO NPs had a significant inhibitory effect on
249 the rate of nitrite formation within 60 min. For the 5 and 10 mg/L ZnO NPs exposure, the NO_2^--N production
250 rate was similar to the control, suggesting that ZnO NPs did not show a significant inhibitory effect on
251 *Nitrosomonas europaea* at low exposure concentrations within 60 min. At high concentrations (20 and 50
252 mg/L), ZnO NPs might inhibit *Nitrosomonas europaea* by destroying the AMO activity damaging the
253 integrity of the cell membrane. Similarly, Yuan et al. [25] investigated the impact of Ag NPs (7±3 and 40±14
254 nm) with different coatings on *Nitrosomonas europae*, and found that Ag-NPs caused damage to the cell
255 wall and cell membrane of *Nitrosomonas europaea* and caused the nucleoids to disintegrate and condense,
256 leading to the inhibition of some important protein functions.

257 **3.3.2 Effects of ZnO NPs on the cell membrane integrity of *Nitrosomonas europaea***

258 Lactate dehydrogenase (LDH) is one type of intracellular enzyme. For undisturbed cells, no or only a small
259 amount of LDH would be secreted outside of cells. Nevertheless, a large amount of LDH would be detected
260 outside of cells when the cell membranes are ruptured. The LDH level in the cell suspension was used to
261 indicate the cell membrane integrity of *Nitrosomonas europaea* after exposure to ZnO NPs. As shown in Fig.
262 7, when *Nitrosomonas europaea* was exposed to 5 and 10 mg/L ZnO NPs after 4 h, LDH was detected, but
263 the concentrations were relatively low (less than 10.0 U/L) compared with the control, suggesting that no
264 significant LDH leakage and damage to cell membrane occurred. However, the LDH activity was increased
265 to 60.0 and 100.0 U/L, respectively, with the ZnO NP concentrations being increased to 20 and 50 mg/L,
266 respectively. These results suggest that 20 and 50 mg/L ZnO NPs caused obvious LDH leakage and damaged

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the cell membrane integrity. On the whole, the cell membrane integrity was affected by ZnO NPs almost in a dose-dependent manner from 5 to 50 mg/L.

To observe the effects of ZnO NPs on the cell membrane integrity of *Nitrosomonas europaea*, TEM was used to characterize the surface morphology of the cells after *Nitrosomonas europaea* was exposed to different concentrations of ZnO NPs (Fig 8).

As seen from Fig. 8, *Nitrosomonas europaea* appeared as a rod shape, which was consistent with the description in Berger's Manual of Determinative Bacteriology (version 8).[26] Compared with the control (Fig. 8 a), adsorbed ZnO NPs can be observed around the *Nitrosomonas europaea* cells in all of the treatments (Fig. 8 (b), (c), (d) and (e)). From Fig. 8 (b) and (c), it can be found that only a small amount of ZnO NPs was adsorbed on the *Nitrosomonas europaea* surface. A lesser change in the morphology of cells as well as less damage in the cell membrane was observed. Moreover, it can clearly be seen that more and more ZnO NPs were adsorbed on the *Nitrosomonas europaea* surface as the concentrations of ZnO NPs increased to 20 and 50 mg/L (Fig. 8 (d) and (e)). As is known, the adsorption of ZnO NPs might hinder the transport of organics. Additionally, damage to the cell membrane can lead to the leakage of intracellular substances, which affects the normal physiological functions of the cell. These observed results were consistent with the results above (from Fig. 2 to Fig. 7). Similarly, Yuan et al. [25] also found that Ag NPs caused damage to the cell wall and cell membrane of *Nitrosomonas europaea* and caused the nucleoids to disintegrate and condense and thus inhibited some protein functions. In short, the impact of ZnO NPs on *Nitrosomonas europaea* included the adsorption of ZnO NPs onto the cell surface, damage to the cell membrane and loss of cytoplasm (intracellular plasmids and inclusions).

4. Conclusions

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By adding different concentrations of ZnO NPs to SBR, we evaluated how ZnO NPs affect nitrification in activated sludge. We investigated the toxicity of ZnO NPs to typical nitrobacteria in activated sludge and explored the effect of ZnO NPs on the bacterial diversity in activated sludge. It is concluded that low concentrations of ZnO NPs slightly inhibited nitrification and that the activities of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR), as well as the integrity of the cell membrane of *Nitrosomonas europaea,* were almost unaffected. Moderate and high concentrations of ZnO NPs had an adverse effect on the activities AMO and NOR and the transformation of both NH_4^+-N to NO_2^--N. Denaturing gradient gel electrophoresis (DGGE) analysis revealed that high concentrations of ZnO NPs have a significant inhibition on the growth of many typical ammonia-oxidizing bacteria (AOB), which are mainly responsible for the oxidation of ammonia to nitrate. Moderate concentrations of ZnO NPs could promote the growth of some pathogenic bacteria, which caused activated sludge to rapidly age and die. A high concentration of ZnO NPs obviously destroyed the integrity of the cell membrane of *Nitrosomonas europaea*. These findings meaningfully assessed the adverse effects of ZnO NPs on activated sludge in wastewater treatment.

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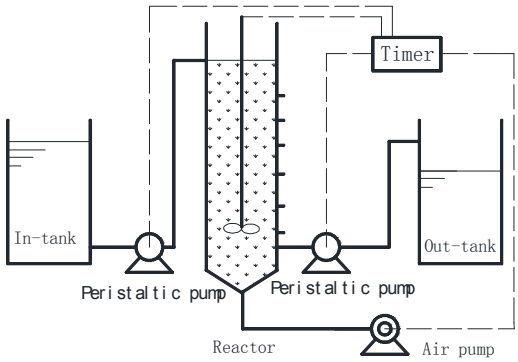

Fig. 1 The setup of sequencing batch reactor (SBR)

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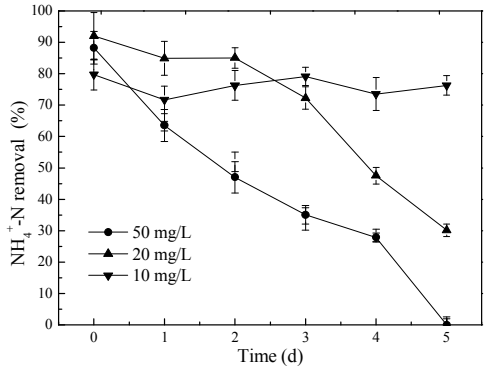

Fig. 2 Impact of ZnO NPs on NH_4^+-N removal

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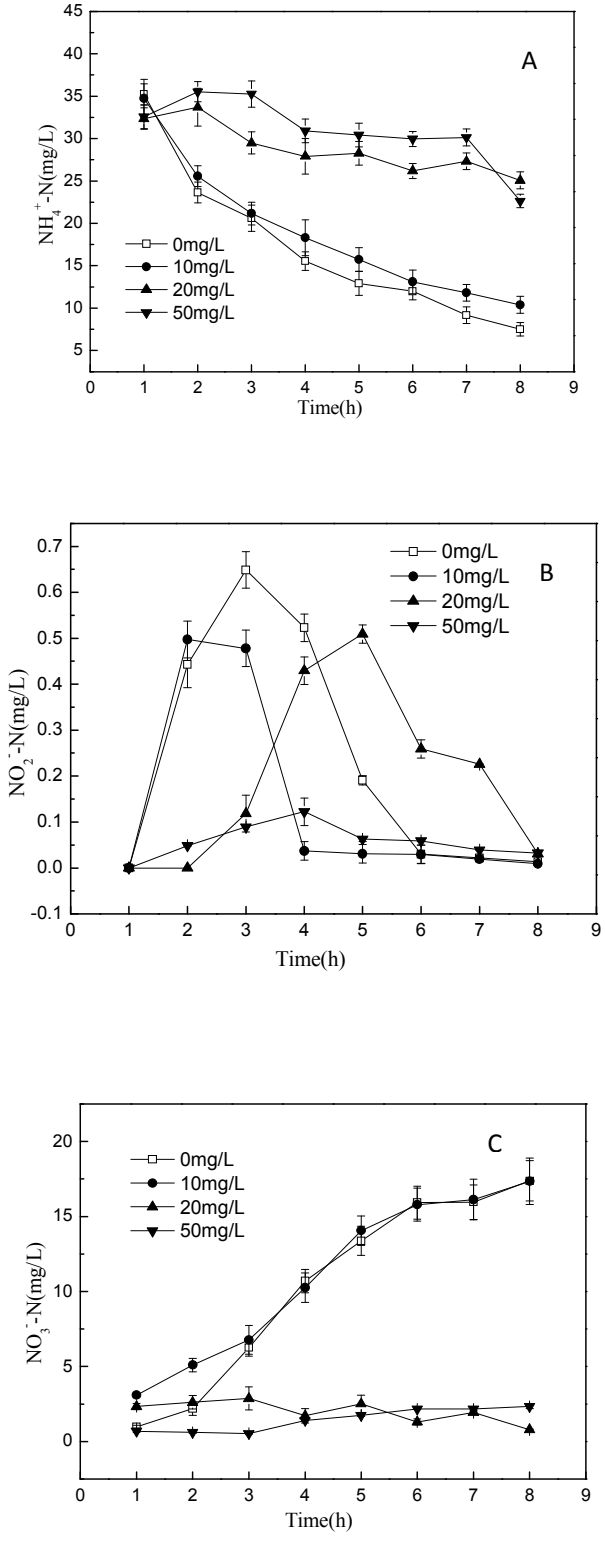

Fig. 3 Effects of ZnO NPs on the transformation of nitrogen to different forms during one cycle of the SBR.

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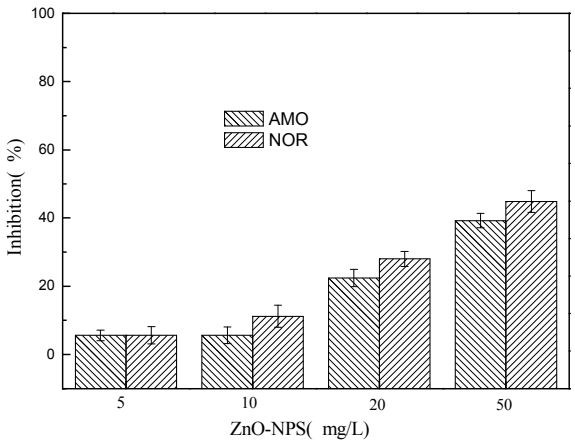

Fig. 4 Effects of ZnO NPs on the activities of AMO and NOR during one cycle of the SBR. Exposure time: 5days

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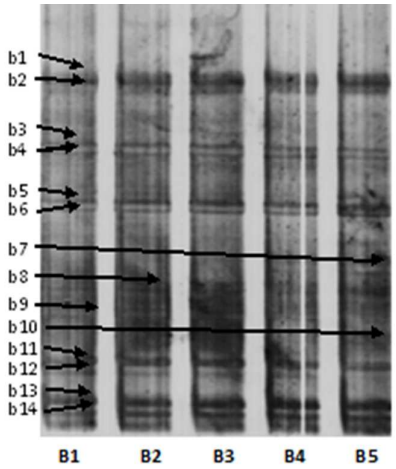

Fig. 5 DGGE profiles of the bacterial communities of activated sludge in SBRs. .B1: without addition of ZnO NPs (Control); B2-B5: with addition of 5, 10, 20 and 50 mg/L ZnO NPs, respectively. Detailed information on the bands (b1-b14) is presented in Table 1.

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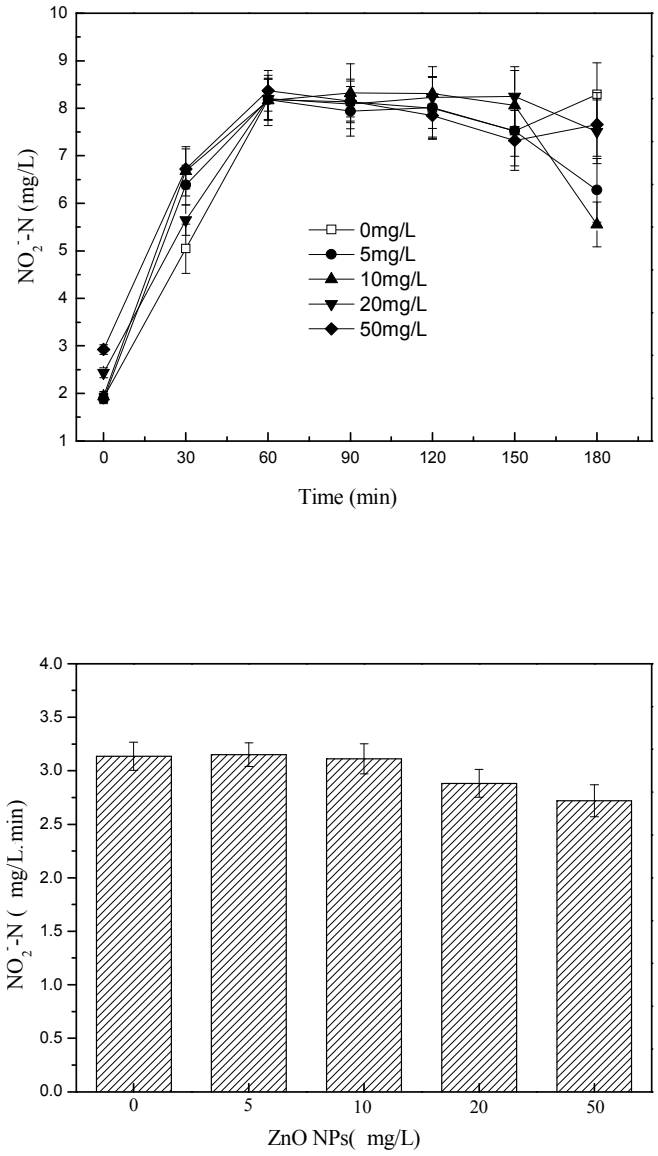

Fig. 6 Effects of ZnO NPs on NO_2^--N production and the production difference. (A): NO_2^--N concentration rate over 180 min; (B): NO_2^--N production rate for the first 60-min exposure.

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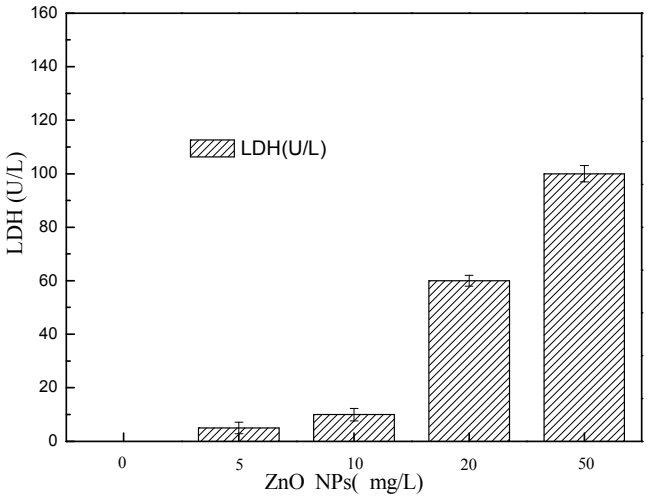

Fig. 7 Effects of ZnO NPs on the integrity of the cell membrane of *Nitrosomonas europaea* Exposure time: 4h.

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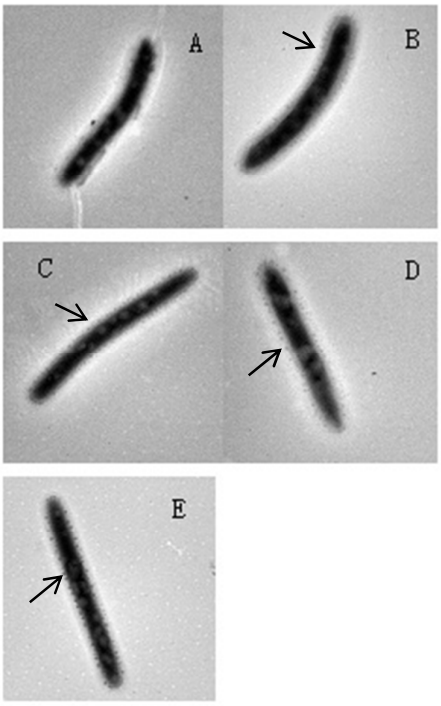

Fig. 8 TEM images of *Nitrosomonas europaea* exposed to different concentrations of ZnO NPs A) control, B) 5 mg/L, C) 10mg/L, D) 20mg/L and E) 50 mg/L of ZnO NPs

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Table 1 DGGE bands and their closely related sequences

Band	Closely related sequences from	Accession no.	Identity (%)
b1	*Pseudoxanthomonassuwonensis*	NC_014924.1	99
b2	*Bifidobacteriumanimalis subsp.*	NC_011835.1	100
b3	*Nitrosococcusoceani*	NC_007484.1	86
b4	*Nitrosomonas sp.*	NC_015222.1	88
b5	*Verrucomicrobiumspinosum*	NZ_ABIZ01000001.1	93
b6	*Arcobacternitrofigilis*	NC_014166.1	83
b7	*Xanthomonashortorumpv.*	NZ_CM002307.1	96
b8	*Nitratiruptor sp.*	NC_009662.1	84
b9	*Stenotrophomonasmaltophilia*	NC_010943.1	96
b10	*Thioflavicoccusmobilis*	NC_019940.1	91
b11	*Pseudomonas denitrificans*	NC_020829.1	94
b12	*Nitrosococcuswatsonii*	NC_014315.1	88
b13	*Thioalkalivibriosulfidophilus*	NC_011901.1	91
b14	*Pseudomonas sp.*	NC_019670.1	95

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