Impacts of ammonia on zinc oxide nanoparticle toxicity to *Nitrosomonas europaea*

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Abstract. A Although the toxicity effects of engineering nanoparticles (NPs) in biological wastewater nitrogen removal (BNR) system have been extensively attracted, the impacts of co-existing contaminants from wastewater on NP toxicity have been less addressed. In this study, the effects of ammonia on ZnO NP toxicity to typical ammonia oxidizing bacteria-*Nitrosomonas europaea* were investigated, as indicated by the cell density, membrane integrity, ammonia oxidation rate, and AMO activity. After 6-h’s exposure to 10 mg/L ZnO NPs, the cell density, membrane integrity, ammonia oxidation rate, and AMO activity was dramatically suppressed despite of the increasing ammonia loading. Ammonia at varying concentrations did not obviously affect ZnO NPs toxicity to cell density. The presence of ammonia at 100 or 200 mg/L significantly alleviated the antibacterial effects of ZnO NPs on cells. The reduction of the concentration of released Zn²⁺ might be responsible for the compromised ZnO NPs toxicity. However, the presence of extremely dosed ammonia at 200 mg/L imposed restrictions on further alleviation of ZnO NPs toxicities probably due to the production of free ammonia and acclamation of nitrite. All these findings would provide new insights for risk assessment of the combined effects of NPs with other co-existing contaminants in the BNR system.

1. Introduction
With the rapid development of nanotechnology, diverse nanomaterials featuring at least one dimension with less than 100 nm in size have been increasingly employed in various fields due to their unique physicochemical properties, such as surface effect, interface effect, quantum size effect, and antibacterial performance [1, 2]. Among them, Zinc oxide nanoparticles (ZnO NPs) with some special properties (e.g. catalytic activity, electrical conductivity, dissolved ability and antibacterial effects) have been increasingly utilized in chemical industry and commercial products [3]. As listed as one of the most used engineering NPs in the world, the number of ZnO NPs products have increased to more than 38 according to Consumer Products Inventory (CPI) [4]. The introduction of massive ZnO NPs have raised remarkable concerns on their ecotoxicity and biosafety in the surroundings [5, 6].

Wastewater treatment plants (WWTPs) have been considered as an essential intermediary for NPs transport into ecosystems [7]. By modeling prediction, 1.7 ~ 2.1 µg/L ZnO NPs passed through WWTPs, and 17 ~110 mg/Kg ZnO NPs were retained in the activated sludge [8]. The presence of
these emerging contaminants in the biological treatment process would pose a great threat to metabolic activities of the critical functional bacteria. It has been extensively reported that ZnO NPs exerted negative effects on the ammonia removal and total nitrogen removal efficiency, the related ammonia oxidizing or nitrifying activity, and the abundance of related ammonia oxidizing bacteria (AOB) or nitrifying bacteria in biological nitrogen removal (BNR) system [9, 10, 11]. In particular, ammonoxidation, which is acknowledged as the primary and the rate limiting step for nitrification, are highly sensitive to ZnO NPs stress [10, 12].

Currently, the toxicity effects of NPs on ammonoxidation have been widely addressed, which is considered to be induced by physical interruption, reactive oxygen species (ROS) generation and dissolved zinc release [13, 14]. However, most of these studies excluded the combined effects of diverse existing contaminants in wastewater. Ammonia, as one of the main contaminants in wastewater, could theoretically interact with released zinc ions, affect the physicochemical properties of ZnO NPs, and therefore their antibacterial effects. Kostigen et al found that the presence of ammonia affected the silver NPs dissolution and toxicity to Nitrosomonas europaea [15]. Furthermore, the concentration of ammonia in the influent of WWTPs varies from circumstance to circumstance. During the ammonoxidation, the variation of concentration of oxidation product-nitrite will in turn affect the metabolic activity of AOB [16, 17].

The object of this study is to investigate the effects of ammonia on the physiological responses of the typical AOB-N. europaea to ZnO NPs and the potential mechanisms. N. europaea is a model chemolithotrophic ammonia oxidizer, widely present in biological nitrogen removal process and highly sensitive to toxicants stress. In this study, the cell density, ammonia oxidizing rate, cell membrane integrity and ammonia oxidase (AMO) activity was determined to indicate the bacterial physiological signal variations.

2. Materials and methods

2.1. Bacterial cultivation

N. europaea (ATCC 19718) was constantly cultured in a chemostat bioreactor (working volume = 3 L, hydraulic retention time = 2.3 d) in the dark at 28 °C. The dissolved oxygen (DO) concentration was kept at (0.50 ± 0.02) mg/L by constant aeration. Compressed air was pumped into the culture after filtered through a 0.22 µm filter (Merck Millipore, Billerica, MA, USA). The feeding culture medium containing 280 mg-N/L was designed according to our previous publications [9]. The pH in the bioreactor was maintained (7.50 ± 0.10) by a pH controller (Meller, Taiwang, China). The buffer of 80 g/L NaHCO₃ was automatically added into the culture while pH decreased.

2.2. Nanoparticle characterization

ZnO NPs were bought from Sigma-Aldrich (St. Louis, MO, USA). The size distribution and morphology of ZnO NPs were characterized using a scanning electron microscope (SEM, Japan Electronics Co., Ltd, Japan). The average size of ZnO NPs was (96 ± 25) nm in length. The particle size distribution (PSD) and ζ potential were determined by dynamic light scattering (DLS) using a Malvern nano ZS90 zetasizer. The ZnO NPs suspensions at 10 g/L was ultrasonicated for dispersion (17 W, 40 kHz, 20 min) before exposure experiment.

2.3. Nanoparticle exposure experiment setup

300 mL of cultures were withdrew from the bioreactor into 500 mL flask for bath assays. A series of flasks containing 300 mL cultures were added with 15 g/L NH₄⁺-N to reach the final concentrations at 25, 50, 100, and 200 mg/L, respectively, and acted as controls. Another two series of 4 flasks containing both 300 mL cultures and 25, 50, 100, or 200 mg/L NH₄⁺-N was loaded 10 mg/L ZnO NPs and 3.4 mg/L Zn²⁺, respectively. All the three series were incubated in the dark at 25 °C for 6-h. The designed concentration of added Zn²⁺ at 3.4 mg/L was obtained according to our previous study [18]. After 6-h exposure, cultures were sampled to determine the physiological signals of cells.
2.4. Analytical methods
The concentrations of NH$_3$-N and NO$_2$-N of all samples were determined referring to the Standard Methods [19]. The cell density was measured with bacterial count methods under a OLYMPUS-BX41 microscope (OLYMPUS-BX41, Olympus, Japan). The cell membrane integrity was obtained using a LIVE/DEAD® BacLightTW Bacterial Viability Kit (Life Technologies, Waltham, MA, USA) according to the manufacturer’s instructions. The specific activity of AMO enzyme in charge of ammonia oxidation, was examined according to the procedure described previously, and indicated by the nitrite production rate per unit of protein. The dissolved Zn (DZn) concentrations were measured by a AAAnalyst 400 Atomic Absorption Spectrophotometer (PerkinElmer, Norwalk, CT, USA).

2.5. Statistics analysis
All the results were expressed as mean ± standard deviation. A Student’s t-Test method was applied for statistical analysis by Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). The tests were considered to be significant when $P$-value ≤ 0.05.

3. Results and discussions

3.1. Impacts of ammonia on zinc ion release
In our previous study, the concentration of released Zn$^{2+}$ from 10 mg/L ZnO was determined to be (3.4 ± 0.9) mg/L [18], therefore, we set up a series of cell culture exposed to 3.4 mg/L Zn$^{2+}$ to determine the toxicity effects exerted by ZnO NPs dissolution. As presented in Figure 1, the concentration of Zn$^{2+}$ displayed no obvious variations between these two groups. In addition, the presence of ammonia at 25 and 50 mg/L did not significantly affect the concentration of Zn$^{2+}$ released from ZnO NPs, while ammonia dosed at 100 and 200 mg/L remarkably lower the Zn$^{2+}$ concentration by (36.7 ± 6.0) % and (56.3 ± 1.9) % after exposure to 10 mg/L ZnO NPs when compared with the control (Figure 1). NH$_3$ is considered to be able to react with Zn$^{2+}$ to form (Zn (NH$_3$)$_4$)$^{2+}$. This reaction would probably lower the Zn$^{2+}$ concentrations and potentially affect ZnO NPs toxicity.

![Figure 1](image_url)

**Figure 1.** Impacts of ammonia on zinc ion release. # indicates the statistically significant difference for the samples in the presence of ammonia at different concentrations when compared with those loaded by 25 mg/L ammonia.
3.2. Impacts of ammonia on NP toxicity to cell density and membrane integrity
It can be seen from Figure 2A that cell density significantly decreased under both Zn\(^{2+}\) and ZnO NPs stress despite of the increasing ammonia loading. The ammonia did not obviously impact ZnO NPs inhibition on cell density. However, 10 mg/L ZnO NPs exerted more negative effects on cell density than 3.4 mg/L Zn\(^{2+}\), which confirmed our previous findings that the size-effect of NP it-self could still be mainly responsible for Zn NPs toxicity although the released Zn\(^{2+}\) contributed part of the NP toxicity effects [18].

The interruption of membrane structure and function have been considered as main toxicity mechanisms of NPs [9]. In this study, the cell membrane integrity was inhibited by (7.8 ± 0.1) %, (7.4 ± 0.1) %, (5.4 ± 0.1) %, and (5.2 ± 0.1) % respectively after 6-h’s exposure to ZnO NPs with ammonia concentration increasing from 25 to 200 mg/L (Figure 2B), which suggested the cell membrane damage caused by ZnO NPs or generated ROS [14]. However, the damage of membrane integrity was significantly alleviated when ammonia was dosed up to 100 or 200 mg/L. This might be explained by the reduced Zn\(^{2+}\) concentration [20]. It is noteworthy that there is no significant differences on the membrane integrity in the presence of ammonia at 100 and 200 mg/L, which implied that the presence of extremely high concentration of ammonia did not always decrease the NP toxicity.

3.3. Impacts of ammonia on NP toxicity to ammonia oxidation rate
*N. europaea* derives energy only from ammonia oxidation to assimilate inorganic carbon for substance synthesis [21]. Therefore, the concentration of ammonia plays a role in ammonia oxidation rate. In is study, the ammonia oxidation rate of *N. europaea* dramatically decreased by (42.9 ± 0.4) %, (35.0 ± 0.3) %, (13.4 ± 0.2) %, and (21.2 ± 0.2) % respectively under ZnO NPs stress when compared with the control (Figure 3A). As ammonia loading concentration rose from 25 to 100 mg/L, the inhibition of ZnO NPs on ammonia oxidation rate was increasingly compromised (Figure 3A). Since the cell was withdrew from the chemostat with high and stable activity, the “starving” cells in the presence of high dose of ammonia would accelerate the ammonia oxidation [22], which resulted in the obvious lightened NP suppression. However, when ammonia dosed at 200 mg/L, the inhibition of ammonia oxidation rate increased to (21.2 ± 0.2) % although the ammonia oxidation rate was enhanced without adding NPs or Zn\(^{2+}\). This might be related to more production of (NH\(_4\))\(^+\), as well as the accumulation of nitrite. It has been found that the presence of higher concentration of free ammonia and nitrite would suppress the ammonia oxidation and metabolic activity of AOB [10, 12].
3.4. Impacts of ammonia on NP toxicity to AMO activity

As a membrane protein in charge of ammonia oxidation [23], AMO activity was seriously inhibited by (56.5 ± 2.1) %, (51.6 ± 1.2) %, (47.0 ± 0.6) %, and (50.7 ± 1.0) %, respectively compared to the control with the concentration of ammonia ranging from 25 to 200 mg/L (Figure 3B). Ammonia loaded at 25 or 50 mg/L did not induce significant variations of cellular AMO activity, while the addition of ammonia at 100 or 200 mg/L remarkably increased the AMO activities. In accordance with the impacts of ammonia on NP toxicity to ammonia oxidation rate, the inhibition of NP on AMO activity was lightened as ammonia concentration increased from 25 to 100 mg/L. Nevertheless, the addition of ammonia at 200 mg/L aggravated the NP toxicity to the AMO activity, which further demonstrated that the extremely high concentration of ammonia would make AOB more sensitive to ZnO NPs stress.

![Graph A](image1.png)

![Graph B](image2.png)

Figure 3. Impacts of ammonia loading on ZnO NPs toxicity to ammonia oxidation rate (A) and AMO activity (B). * represents the statistically significant difference for samples when compared with control. # indicates the statistically significant difference for the samples in the presence of ammonia at different concentrations when compared with those loaded by 25 mg/L ammonia.

4. Conclusion

In this study, the impacts of ammonia on the NP toxicity to typical AOB N. europaea were investigated, as indicated by the cell density, the membrane integrity, the ammonia oxidation rate, and the AMO activity. After 6-h’s exposure to 10 mg/L ZnO NPs, the cell density, the membrane integrity, the ammonia oxidation rate, and the AMO activity was significantly inhibited despite of the increasing ammonia loading. Ammonia at varying concentrations did not obviously influence ZnO NPs toxicity to cell density. The presence of ammonia at 100 or 200 mg/L significantly alleviated the antibacterial effects of ZnO NPs on cells. The reduction of the concentration of released Zn$^{2+}$ might contribute to the compromised ZnO NPs toxicity. However, the presence of extremely dosed ammonia at 200 mg/L restricted further alleviation of ZnO NPs toxicities probably owing to the production of free ammonia and acclamation of nitrite. Our findings would provide deep understandings of the influences of wastewater constituents on the NP toxicity effects in BNR system.

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