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Responses and recovery assessment of continuously cultured *Nitrosomonas europaea* under chronic ZnO nanoparticle stress: Effects of dissolved oxygen

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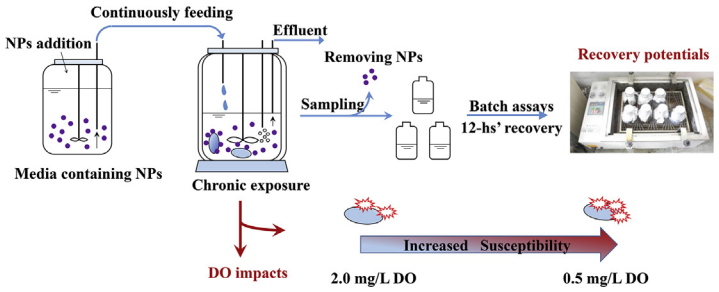
HIGHLIGHTS

- Cells in chemostat were more resistant to NP toxicity than batch cultured ones.
- Low DO caused cells more sensitive to NP stress than under high DO condition.
- NP impaired cells possessed physiological and metabolic recovery potentials.
- NP exposure duration determined cell's damage level and recoverability.
- Membrane and associated metabolic activity preservation were critical for recovery.

GRAPHICAL ABSTRACT



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ABSTRACT

Although the antibacterial performances of emerging nanoparticles (NPs) have been extensively explored in the nitrifying systems, the impacts of dissolved oxygen (DO) levels on their bio-toxicities to the nitrifiers and the impaired cells' recovery potentials have seldom been addressed yet. In this study, the physiological and transcriptional responses of the typical ammonia oxidizers - *Nitrosomonas europaea* in a chemostat to the chronic ZnO NP exposure under different DO conditions were investigated. The results indicated that the cells in steady-growth state in the chemostat were more persevering than batch cultured ones to resist ZnO NP stress despite the dose-dependent NP inhibitory effects were observed. In addition, the occurred striking over-expressions of *amoA* and *hao* genes at the initial NP exposure stage suggested the cells' self-regulation potentials at the transcriptional level. The low DO (0.5 mg/L) cultured cells displayed higher sensitivity to NP stress than the high DO (2.0 mg/L) cultured ones, probably owning to the inefficient oxygen-dependent electron transfer from ammonia oxidation for energy conversion/production. The following 12-h NP-free batch recovery assays revealed that both high and low DO cultured cells possessed the physiological and metabolic activity recovery potentials, which were in negative correlation with the NP exposure time. The duration of NP stress and the resulting NP dissolution were critical for the cells' damage levels and their performance recoverability.

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The membrane preservation processes and the associated metabolism regulations were expected to actively participate in the cells' self-adaption to NP stress and thus be responsible for their metabolic activities recovery.

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1. Introduction

Nitrification, the process of oxidation of ammonia to nitrate via nitrite, has a pivotal role in global nitrogen cycle and nitrogen removal from biological wastewater treatment plants (WWTPs) (Prosser, 1990). As the primary and the rate-limiting step for nitrification, ammoxidation catalyzed by ammonia oxidizing bacteria (AOB) is of particular interest in response to environmental changes or toxicant stress (Prosser, 1990; Kowalchuk and Stephen, 2001). Generally, most of AOB are chemolithoautotrophs, which fix CO_2 for biosynthesis and derive energy solely from ammonia oxidation (Kowalchuk and Stephen, 2001). Ammonia oxidation is achieved by two steps: oxidization of NH_3 to hydroxylamine (NH_2OH) via ammonia monooxygenase (AMO) and further oxidation to NO_2^- via hydroxylamine dehydrogenase (HAO) (Whittaker et al., 2000; Arp et al., 2002). During ammonia oxidation, only two of four electrons are obtained as the sole energy source for the growth of AOB (Prosser, 1990). Therefore, the low level of net obtained energy from ammonia oxidation is widely acknowledged to make AOB extremely sensitive to environmental changes (e.g. dissolved oxygen levels) and toxicant stress (Prosser, 1990; Stein et al., 1997; Park and Ely, 2008, 2009).

Recently, nanomaterials (NM) have been widely incorporated in numerous consumer/industrial products due to their unique physical and chemical properties and served as potential environmental stressors (Vance et al., 2015). Among them, zinc oxide (ZnO) NMs generally display high photoelectric activity, gas sensitiveness, and antimicrobial activity and are extensively used in clothing, medicines, paintings, cosmetics and textiles (Kumar et al., 2017). Due to their wide application, ZnO nanoparticle (NP) could inevitably lead to their release into the environment. According to probabilistic material flow analysis, Gottschalk et al. (2009) and Sun et al. (2014) demonstrated that ZnO NP contents in natural sediment and activated sludge in Europe rose from 0.003 to 0.052 and 13.6–57.0 mg/kg in 2009 to 0.18–1.00 and 17.0–110.0 mg/kg in 2013, respectively. Meanwhile, ZnO NPs have been extensively reported to cause negative effects on biological nitrogen removal performances, and related AMO and nitrite oxidoreductase activities, and microbial diversities in activated sludge of WWTPs and natural nitrifying systems (Puay et al., 2014; Wang et al., 2016; He et al., 2017). Particularly, the relative abundance of ammonia oxidizer and their oxidation activities were expected to be significantly affected under NP stress (Yang et al., 2014; Zhang et al., 2017).

Currently, many researches have explored the antibacterial effects of ZnO NPs and the associated toxicity mechanisms (Zheng et al., 2014; Arakha et al., 2017; Zhang et al., 2017). The ZnO NP's bio-toxicity has been speculated to take effects through three potential interaction pathways: NP physical adsorption or “stab”, released zinc ion stress, and oxidative damage via reactive oxygen species (ROS) production (Lopes et al., 2014; Cervantes-Avilés and Cuevas-Rodríguez, 2017; Kumar et al., 2017; Reshma and Mohanan, 2017). Nevertheless, the majority of these researches mainly concentrated on the acute shock loading or batch test models. Given that the leakage of NPs into the surroundings generally undergo a gradual and perennial instead of temporary process, evaluations of ZnO NPs' long-term effects on nitrifying system and the nitrogen removal related bacteria, especially AOB, are essential and significant. Furthermore, the potential recoverability of NP impaired microorganisms in nitrogen removal system has seldom been addressed yet. Just a few researches ever indicated the potential resistance or metabolic recovery capacities of nitrogen cycling involved bacteria under NP stress. For instance, Alito et al (Alito and Gunsch, 2014). found that ammonia oxidizers were capable of adapting to silver NP stress and retrieved 90% of their ammonia oxidation activities in a sequencing batch reactor (SBR). The denitrifiers-*Bacillus subtilis* were persistent under fullerene NP (nC60) stress via changing membrane composition or associated phase behaviors (Fang et al., 2007). However, more systematic studies are needed on the recovery potentials and the related mechanism of microbial functions for the establishment of efficient emergency regulation strategies in response to NP contaminations.

As the generally accepted NP toxicity inducer, the generation of ROS was reported to be affected by dissolved oxygen (DO) concentration (Yang et al., 2013). The colloidal stability and oxidative dissolution rate of Ag NPs were ever observed to significantly vary at different DO levels (Zou et al., 2017). In addition, previous study indicated that the oxygen involved energy production/conversion pathways in *N. europaea* were actively affected by ZnO NP stress (Wu et al., 2017). Thus, the inevitable variations of DO levels in the environment are expected to significantly impact ZnO NP toxicity to AOB.

In this study, *N. europaea*, a representative chemolithotrophic AOB in WWTP system, was selected as the model AOB (Mobarry et al., 1996) for continuous cultivation and long-term ZnO NP impacts discovery under different DO concentrations. Meanwhile, the recovery potentials of the impaired cells were evaluated at both the physiological and metabolic levels. The transcriptional expressions of functional genes in *N. europaea* cells during chronic NP stress were concurrently quantified by real-time quantitative polymerase chain reaction (qPCR) for the associated mechanism investigation.

2. Materials and methods

2.1. Cell cultivation

N. europaea (ATCC, 19,718) was continuously cultivated in a chemostat in triplicate at 28 °C in the dark. The details of the reactor operations (pH: 7.4–7.5, HRT: 2.2 d, 3 L working volume) and the cultivation medium composition containing 10 mM $(NH_4)_2SO_4$ were provided in our previous publication (Yu et al., 2016b). The DO concentration was maintained at (2.0 ± 0.2) mg/L or (0.5 ± 0.2) mg/L by filter-sterilized air aeration according to the experiment design.

2.2. NP characterization

The tested ZnO NPs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The scanning electron microscope (SEM, Japan Electronics Co., Ltd, Japan) was used to characterize the NP size and surface morphology. ZnO NPs displayed rod-shaped with an average diameter of (94 ± 24) nm (Fig. 1). The average NP

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