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Impacts of different nanoparticles on functional bacterial community in activated sludge ☆

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HIGHLIGHTS

- Silver and ZnO nanoparticles exert different impacts on functional bacterial community.
- Denitrifying bacteria were inhibited by high dosage of silver and ZnO nanoparticles.
- Sludge bulking, heavy metal resistant and biosorption bacteria enriched by both nanoparticles.
- Increase of bulking related bacteria may help to maintain functional redundancy of the community.

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ABSTRACT

Rapidly developing industry raises concerns about the environmental impacts of nanoparticles, but the effects of inorganic nanoparticles on functional bacterial community in wastewater treatment remain unclear. The discriminated effects of silver nanoparticles (Ag-NP) and zinc oxide nanoparticles (ZnO-NP) in a simulated sequencing batch reactor (SBR) system were therefore evaluated by the RNA-based terminal restricted fragment length polymorphism (T-RFLP), 16S rcDNA gene clone library and real-time reverse transcription-PCR (RT-PCR) analyses. Although the COD and NH_4-N removal efficiencies were not or slightly reduced by the addition of ZnO-NP and Ag-NP, the functional bacterial community changed remarkably. The denitrification related species were inhibited by high dosage of ZnO-NP and Ag-NP, including *Diaphorobacter* species, *Thauera* species and those in the *Sphaerotilus-Leptothrix* group. However, the bacteria related to sludge bulking, heavy metal resistant and biosorption were increased, especially by ZnO-NPs treatment, including those closely related to *Haliscomenobacter hydrossis*, *Zoogloea ramigera* and *Methyloversatilis universalis*. In addition, Ag-NP and ZnO-NP treatments influenced the functional bacterial community differently. Increasing of bulking related bacteria may help to compensate the COD removal efficiency and to maintain functional redundancy, but could lead to operation failure of activated sludge system when expose to ZnO-NPs.

1. Introduction

Nanotechnology provides more solutions to solar energy conversion, catalysis, medicine, and water treatment (Sharma et al., 2009), which leads to a great deal of nanoparticle (NP) discharging into sewage. Wastewater treatment plant (WWTP) is therefore one of the final defenses for preventing NPs from discharging into water environments. It is well known that silver nanoparticle (Ag-NP) can conduct bactericidal properties to many Gram-positive and Gram-negative bacteria including multi-resistant strains (Panacek et al., 2006) by a variety of antimicrobial mechanisms (Sharma et al., 2009). In addition, Zinc oxide nanoparticles (ZnO-NPs) and titanium dioxide nanoparticles (TiO_2-NPs) were also proved to have antibacterial abilities (Adams et al., 2006; Zhang et al., 2007). Concern can be therefore raised. Whether the NPs in wastewater treatment plant shall negatively impact the activated sludge microbial community, which eventually hampers the function of WWTP in removing pollutants in wastewater, such as COD, nitrogen, phosphorus.

Recently, studies started to address this issue. Zheng et al. (2011) reported that NH_3-N removal was not significantly influenced by ZnO-NP, but total nitrogen removal efficiency was decreased from 81.5% to 75.6% in the presence of 10 mg L^{-1}

☆ *Note:* Sequences of 16S rRNA gene in this study have been submitted to GenBank database under accession numbers JN609296 to JN609381.

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ZnO-NP. Choi et al. (2008) reported the respiration of autotrophic nitrifying bacteria (ammonia oxidation bacteria or nitrite oxidation bacteria) was inhibited by 86 ± 3% at 1 mg L^{-1} Ag-NP in a continuously stirred tank reactor (CSTR). In addition, both the TiO_2-NP and ZnO-NP nanoparticles caused microbial biomass reduction, bacterial community shifts and diversity decline in soil bacterial communities (Ge et al., 2011). Our previous studies suggested that most of the ZnO-NPs and Ag-NPs could be removed by activated sludge process. In the simulated sequencing batch reactor (SBR) processes, continuous input of ZnO-NPs and Ag-NPs into the wastewater did not significantly reduce chemical oxygen demand (COD) removal but reduced NH_4-N removal by inhibiting the respiration of nitrifying microorganisms (Hou et al., 2012, 2013). However, it is still not clear whether different nanoparticles exert different impacts on functional bacteria in wastewater treatment activated sludge.

Therefore, we investigated the impacts of Ag-NP and ZnO-NP with different doses on the functional bacterial community with the methods based on RNA analyses, including quantitative reverse transcription PCR (RT-PCR), terminal restricted fragment length polymorphism (T-RFLP) and 16S rRNA gene clone library based on cDNA. Results revealed that although the COD and nitrogen removal functions of the activated sludge community did not change remarkably, the functional community structure did changed significantly.

2. Material and methods

2.1. Experiment set-up and sampling

Silver nanoparticles were prepared as described previously (Hou et al., 2012). Zinc oxide nanoparticles were commercial products purchased from Emperor Nanomaterials Co., Ltd., (Nanjing, China) with the properties described previously (Hou et al., 2013). Lab-scale sequenced batch reactors (SBR) (with the volume of 1 L) with the returned sludge from the Xiaojiahe plant as inoculum (with the final biomass concentration of 2.4 g L^{-1}) and their operating realm were described previously (Hou et al., 2012, 2013). Briefly, the SBR cycle involved 10 h of aeration using a mini-aerator (Model AK-8, Risheng Electric Products Inc., Guangdong, China), followed by 2 h of settling. The wastewater used were collected from the effluent from the primary clarifier at the Xiaojiahe plant. In each cycle, after discarding that supernatant treated wastewater, nanoparticles with different dosage were added into the 15 experimental reactors with the fresh wastewater (Table 1). All the treatments were repeated in triplicates with the reactors without NP addition as controls.

In each cycle, 35 mL of completely mixed suspensions were sampled from all the reactors (at the beginning of aeration soon after nanoparticles addition). The samples were numbered with sampling date-reactor number. The samples were immediately centrifuged with 7000 rpm for 15 min at 4 °C. The supernatants of the samples were collected for measuring the concentrations of COD and NH_4-N (Hou et al., 2013); the sludge pellets were collected and used to analyze the sludge concentrations, to extract the DNA and RNA which were subjected to bacterial community analyses by using clone library, T-RFLP and RT-PCR approaches.

Table 1
Reactors and treatments.

Reactors	Treatments	Reactors	Treatments
4#, 5#, 6#	Control (No NPs addition)	13#, 14#, 15#	Addition with 1 mg L^{-1} ZnO-NP
7#, 8#, 9#	Addition with 0.1 mg L^{-1} Ag-NP	16#, 17#, 18#	Addition with 5 mg L^{-1} ZnO-NP
10#, 11#, 12#	Addition with 0.5 mg L^{-1}Ag-NP		

2.2. RNA and DNA extraction and application

The total RNA was extracted from the centrifuged sludge pellets of each sample with the modified method described previously (Griffiths et al., 2000). Briefly, in a 2 mL RNase-free screw-caped tube, about 0.2 g of activated sludge pellets, 0.5 g of RNase-free 0.10–0.11 mm diameter glass beads (Sartorius AG, Goettingen, Germany), 0.5 mL of CTAB buffer, and 0.5 mL of phenol–chloroform–isoamyl alcohol mixture (25:24:1) were homogenized for 30 s twice at 2500 rpm in the Mikro-Dismenbrator S with 1 min on ice between shakings. After centrifugation, 400 μL of aqueous phase was transferred to a new RNase-free tube. The phenol was removed by mixing with equal volume of chloroform–isoamyl alcohol (24:1) followed by centrifugation. Total nucleic acids were precipitated from 300 μL of aqueous layer by 2 volumes of 30% PEG 6000. The pellets were washed in ice-chilled 70% ethanol and re-suspended in 50 μL of DEPC treated water. To obtain pure RNA, 15 μL aliquot was digested by 15U DNase I (TaKaRa, Dalian, China) for 1.5 h, prior to phenol–chloroform–isoamyl alcohol purification. The integrity of RNA was evaluated by electrophoresis, while the amount and purity were estimated spectrophotometrically by measuring the optical density at 260, 230 and 280 nm.

Reverse transcription PCR was conducted for obtaining the cDNA with the specific primer 1492R (5′-GGTT ACCT TGTT ACGA CTT-3′) and random forward primer, which was performed from 500 ng RNA using First Strand cDNA Synthesis kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. In addition, parallel reactions without reverse transcriptase were also made to confirm that no DNA contamination was present in the RNA samples.

From the cDNA amplified, the bacterial 16S rRNA gene fragments were amplified using the bacterial universal primers, 8F (5′-AGAGT TTGAT CCTGG CTCAG-3′) (for clone library construction) or 8F fluorescently labeled with 6-FAM (for T-RFLP analysis) and 1492R (5′-GGTTA CCTTG TTACG ACTT-3′) in a 50 μL of PCR reaction mixture containing 5 μL 10 × PCR buffer, 4 μL dNTP (2.5 mM each dNTP), 0.5 μL Taq™ DNA polymerase (5 units μL^{-1}) (Takara, Dalian, China), 1 μL each primer (10 pmol μL^{-1}) and 1 μL cDNA. The PCR was performed with initial denaturation at 95 °C for 5 min; followed by 14 cycles of 1 min at 94 °C, 45 s at 55 °C and 90 s at 72 °C; and a final extension at 72 °C for 10 min. The PCR amplicons were purified using PCR Purification Kit (BioTeKe, Beijing, China), and subjected to clone library and T-RFLP analyses.

The extraction of the activated sludge DNA, amplification of the bacterial 16S rRNA genes (16S rDNA) for T-RFLP analysis were conducted with the protocols as described previously (Yu et al., 2010; Tang et al., 2012).

2.3. T-RFLP analysis

About 5–8 μL fluorescently labeled PCR products were digested by restricted enzyme *Rsa* I (Fermentas, China), desalted, mixed with formamide and a DNA fragment length internal standard, denatured and subjected to electrophoresis in 3130 Genetic Analyzer (Applied Biosystems) with the protocol described previously (Yu et al., 2010; Tang et al., 2012). The restricted fragments' sizes and peak areas of terminal restriction fragments (T-RFs) were automatically calculated by ABI supplied GeneMapper software (Version 4.0). T-RFs that differed by ±1 bp in different profiles were considered as identical. The relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak area of the respective T-RF divided by the total peak area of all detected T-RFs. Calculation of the pair-wise similarities of T-RFLP profiles was based on Pearson's correlation coefficients (Andoh et al., 2007; Smalla et al., 2007). The T-RFLP profiles of different samples were compared statistically by principal component analysis (PCA)