Long-term effects of engineered nanoparticles on enzyme activity and functional bacteria in wastewater treatment plants

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ABSTRACT

The pervasive use of engineered nanoparticles (NPs) in a wide range of fields raises concerns about their potential environmental impacts. Previous studies confirmed that some NPs had already entered wastewater treatment plants (WWTPs). Wastewater nutrient removal depends on the metabolisms of activated sludge bacteria and their related key enzymes. Therefore, this study compared the possible influences of Al₂O₃, SiO₂, TiO₂, and ZnO NPs on the key enzymes activities and microbial community structures involved in wastewater treatment facilities. It was found that long-term exposure to these NPs significantly affected the microbial communities and changed the relative abundances of key functional bacteria, such as ammonia-oxidizing bacteria. Also, the gene expressions and catalytic activities of essential enzymes, such as ammonia monooxygenase, nitrite oxidoreductase, nitrate reductase, and nitrite reductase, were decreased, which finally resulted in a lower efficiency of biological nitrogen removal.

Key words | inhibition, key enzyme, microbial community, nanomaterial

INTRODUCTION

Engineered nanoparticles (NPs) have been widely used in many industrial and consumer products due to their unique physico-chemical properties (Nel et al. 2006). However, the extensive applications of NPs inevitably lead to their environmental releases. Previous publications have pointed out that many types of NPs have already been found in soils, surface waters, wastewaters, and sewage sludge (Nowack & Bucheli 2007). It can be estimated that the releases of NPs into the environment might be accelerated with their increasing production and utilization. Therefore, the potential environmental impacts of the released NPs attract much attention. Recent studies indicated that the presence of some NPs, such as TiO₂ and ZnO, can affect biological nitrogen and phosphorus removal (Li et al. 2014; Tan et al. 2015). Nevertheless, it remains unknown whether different types of NPs show similar effects on wastewater nutrient removal.

It is well-known that the activated sludge process is a commonly used method for nitrogen and phosphorus removal from wastewater. This process is carried out by large amounts of microbial populations, which are mainly responsible for nitrification, denitrification, and phosphorus removal (Mino et al. 1998; Zeng et al. 2005). Therefore, the microbial community structure plays an important role in wastewater nutrient removal. Meanwhile, biological nitrogen and phosphorus removal depends on many biochemical reactions, which are catalyzed by some key enzymes in activated sludge bacteria. However, to date, the long-term influences of NPs on the abundances of key functional bacteria and expressions of the genes encoding essential enzymes related to wastewater nutrient removal are sparse.

The objectives of this study were: (1) to compare the long-term effects of different types of engineered NPs on biological nitrogen and phosphorus removal; (2) to examine the changes in microbial community structures of activated sludge after long-term exposure; and (3) to determine the influences of these NPs on the gene expressions and catalytic activities of key enzymes closely related to biological nutrient removal. These results can be used to assess the potential risks of nanomaterials to wastewater treatment processes.

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MATERIALS AND METHODS

Preparation of NP suspensions

Engineered NPs used in this study (Al₂O₃, SiO₂, TiO₂, and ZnO NPs) were purchased from Sigma-Aldrich. The NP suspensions (100 mg/L) were prepared by dispersing 100 mg of NPs in 1 L of Milli-Q water followed by ultrasonication for 1 h (25 °C, 250 W, 40 kHz). The primary sizes of NPs in these stock suspensions were determined to be 80–100 nm by dynamic light scattering using a Malvern Autosizer 4700 (Malvern Instruments, UK).

Set-up and operation of sequencing batch reactors

To conduct the experiment, a series of sequencing batch reactors (SBRs) were operated to achieve biological nitrogen and phosphorus removal. The inoculum sludge was obtained from an anaerobic-low dissolved oxygen (0.15 mM MgCl₂) SBR, which had been operated for more than 100 days. After inoculation these SBRs were fed with synthetic wastewater containing different concentrations of NPs (0, 1, and 50 mg/L, respectively) and operated for 70 days. The detailed components of synthetic wastewater were documented in our previous publication (Zheng et al. 2011). All SBRs were maintained at 21 ± 1 °C, and worked with three 8 h cycles per day. Each cycle consisted of 1.5 h anaerobic and 3 h low dissolved oxygen (DO) periods, followed by 1 h settling, 10 min decanting, and 140 min idle periods. The SBRs were constantly mixed with magnetic stirrers except for the settling, decanting, and idle periods. The initial concentrations of chemical oxygen demand (COD), ammonia–nitrogen (NH₄⁺ – N), and soluble ortho-phosphorus (SOP) were approximately 300 mg/L, 25 mg/L, and 10 mg/L, respectively. The solids retention time was controlled at approximately 22 d. The effluent nitrogen and phosphorus concentrations were determined during the long-term exposure period.

PCR-DGGE analysis of bacterial community in activated sludge

After long-term exposure, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis was used to examine the changes in microbial community structures. Briefly, bacterial genomic DNA was extracted and used as the template for PCR amplification according to the literature (Muyzer et al. 1993). The 16S rDNA variable V3 region was amplified with the primers 341f with a GC-clamp (5’-CGCCTACGGGAGGCAGCAG-3’) and 534r (5’-ATTACCGGGGCTGCTGCTG-3’). The PCR amplification was carried out in a total volume of 25 μL containing 10 ng of template DNA, 1× Taq reaction buffer, 1 U Ex Taq polymerase, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 0.5 μM primers (TaKaRa, Japan) using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Germany). The amplification program consisted of an initial denaturation step at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 8% polyacrylamide gel in 1× TAE buffer with gradients ranged from 50 to 60% denaturant (100% denaturant: 7 M urea and 40% (v/v) deionized formamide) at a constant voltage of 80 V for 15 h at 60 °C using a DCode Universal Mutation Detection System (BioRad, USA). The gel was stained with EB for 15 min, and viewed with a BioRad Gel Documentation system (BioRad, USA). Prominent bands were excised, reamplified, purified, cloned to the pMD19-T vector, and sequenced using an ABI PRISM 3730 automated DNA sequencer. The closely related sequences were searched using the BLAST program.

Determination of gene expressions and catalytic activities of key enzymes related to wastewater nutrient removal

Ammonia monoxygenase (AMO), nitrite oxidoreductase (NOR), nitrate reductase (NAR), nitrite reductase (NIR), exopolyphatase (PPX), and polyphosphate kinase (PPK) were regarded as the key enzymes related to biological nitrogen and phosphorus removal. Thus, the gene expressions of these enzymes were determined by the quantification of amoA, nxxA, narG, nirS, ppx, and pph genes via reverse transcriptase quantitative PCR, respectively. Briefly, the total RNA was extracted with TRIzol reagent (Invitrogen, USA), and used to synthesize cDNA at 42 °C. Then, cDNA was purified using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer’s instruction. The qPCR was performed via a StepOne Real-Time PCR System (Applied Biosystems, USA) in a total volume of 20 μL containing 1× SYBR Green PCR Master Mix, 0.5 μM each primer, and 1 μL of cDNA. The primers and amplification conditions were described in the literature (Kim et al. 2007; Gerbl et al. 2014; Zheng et al. 2014). All qPCR assays were performed using three replicates per sample, and contained the control reactions without cDNA.

In addition, the catalytic activities of these key enzymes were determined after long-term exposure to NPs. Briefly, the mixture was withdrawn at 5 min before the end of low DO stage, and washed three times with 0.01 M phosphate.
buffer (pH 7.4) before measuring AMO, NOR, NAR and NIR activities or washed three times with 1.5 M NaCl buffer (containing 0.01 M EDTA and 1 mM NaF, pH 7.4) before determining PPX and PPK activities. Before the assays, the resuspended pellets were sonicated at 20 kHz and 4°C for 5 min to break down cell structure of activated sludge. The debris was centrifuged at 12,000 g and 4°C for 10 min, and the crude extracts in supernatant were obtained for measuring the enzyme activities according to our previous publication (Zheng et al. 2011). All enzyme activities were based on the protein content which was measured using bovine serum albumin as the standard, and the relative enzyme activity was expressed as the ratio of the specific activity in the presence of NPs to that in the absence of NPs.

ANALYTICAL METHODS

The measurements of NH$_4$$^+$-N, NO$_2$$^-$-N, NO$_3$$^-$-N, SOP, mixed liquor suspended solids, and mixed liquor volatile suspended solids were conducted in accordance with Standard Methods (APHA 1998).

Statistical analysis

All tests were performed in triplicate, and an analysis of variance was used to test the significance of results. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Previous studies mainly investigated the potential effects of TiO$_2$ (Li et al. 2014) or ZnO NPs (Tan et al. 2015) on wastewater nutrient removal. This study further compared the influences of different oxide NPs on biological nitrogen and phosphorus removal after long-term exposure, and then explored the mechanisms by analyzing changes in bacterial community structures and gene expressions of key enzymes. It was found that long-term exposure to 50 mg/L TiO$_2$ and

![Figure 1](https://iwaponline.com/wst/article-pdf/72/1/99/177568/wst072010099.pdf)

Figure 1 | Effluent concentrations of NH$_4$$^+$-N (white), NO$_2$$^-$-N (black), NO$_3$$^-$-N (white) and SOP (black) after long-term exposure to 50 mg/L NPs (a) and (b)) or during exposure to released metal ions (c) and (d). Since SiO$_2$ and TiO$_2$ NPs had very low solubility, no silicon and titanium ions were detected in synthetic wastewater. Error bars represent standard deviations of triplicate measurements. Asterisks indicate statistical differences ($p < 0.05$) from the control test.
ZnO NPs showed significant impacts on ammonia oxidation, while the presence of 50 mg/L Al₂O₃ and SiO₂ NPs greatly inhibited the reduction of NO₃⁻ and NO₂⁻ (Figures 1(a) and 1(b)). However, these NPs had no effects on wastewater phosphorus removal. Further studies indicated that among these NPs Al₂O₃ and ZnO NPs released some metal ions into wastewater, and the released zinc ions (1.15 mg/L) had a negative impact on wastewater nitrogen removal during long-term exposure (Figures 1(c) and 1(d)). Clearly, these data suggested that long-term exposure to engineered NPs could lead to an adverse effect on wastewater nitrogen removal, although wastewater phosphorus removal was not affected.

It is well-known that the diversity of bacterial populations and stable microbial community play important roles in achieving successful biological nitrogen and phosphorus removal (Mino et al. 1998). Therefore, the PCR-DGGE analysis was used to examine the changes in microbial community after long-term exposure to these NPs. Figure 2 shows the DGGE profiles of activated sludge after long-term exposure, and Table 1 gives the detailed information on the DGGE bands involved in Figure 2. It can be observed that the presence of these NPs affected the microbial community structures after long-term exposure. Compared with the control (L1), the presence of 50 mg/L TiO₂ and ZnO NPs significantly reduced the microbial diversity of activated sludge. Similar observations were made by previous studies (Ge et al. 2011). It should be noted that long-term exposure to TiO₂ and ZnO NPs greatly decreased the abundance of ammonia-oxidizing bacteria, such as Nitrosomonas sp. (band 3) (Dionisi et al. 2002). Conversely, Candidatus Accumulibacter phosphatis (bands 5 and 8) and Rhodocyclaceae bacterium (band 10) were reported to be typical polyphosphate accumulating organisms (PAOs) (Martin et al. 2006; He et al. 2007), and the presence of Al₂O₃, SiO₂, TiO₂, and ZnO NPs did not affect these PAOs, which was in accordance with no measurable impacts on wastewater phosphorus removal.

It was reported in the literature that TiO₂ and ZnO NPs were able to alter the bacterial composition in soil and reduce microbial populations after 60 days of exposure.
(Ge et al. 2011). It should be noted that *Nitrosomonas* sp. (band 3) was found to be washed out of activated sludge due to the presence of 50 mg/L TiO\(_2\) NPs. In previous study, TiO\(_2\) NPs were reported to be toxic to the pure culture of *N. europaea* (Fang et al. 2010), which might explain the disappearance of *Nitrosomonas* sp. in activated sludge after long-term exposure. Interestingly, *Stenotrophomonas* sp. (band 4) can tolerate high levels of metal contaminants and carry out nitrate denitrification (Alonso et al. 2000; Heylen et al. 2007), which might be the reason for the existence of this micro-organism after exposure to these metal oxide NPs. In addition, *Candidatus Accumulibacter phosphatis* (bands 5 and 8) and *Rhodocyclaceae* (band 10) were found to be the dominant PAOs after long-term exposure to these NPs, which was consistent with marginal effects on biological phosphorus removal.

Moreover, wastewater nitrogen and phosphorus removal are closely linked to some key enzymes, such as AMO, NOR, NAR, NIR, PPX, and PPK. For example, AOB can use AMO to catalyze ammonia oxidation, and the oxidation of NO\(_2^-\)/NO\(_3^-\) is carried out by NOR. Denitrification is mainly catalyzed by NAR and NIR, whereas phosphorus transformation is related to PPX and PPK (Lee et al. 2006). It can be seen from Figure 3 that 50 mg/L TiO\(_2\) and ZnO NPs significantly inhibited the gene expressions of AMO and NOR, while the presence of 50 mg/L Al\(_2\)O\(_3\) and SiO\(_2\) NPs showed negative effects on NAR and NIR. Similar trends were observed in the catalytic activities of these enzymes after long-term exposure to NPs (data not shown). Previous studies showed that engineered NPs could adsorb large amounts of essential micronutrients such as copper and iron ions in synthetic wastewater (Chen et al. 2012), which might be responsible for the decreased activities of NAR and NIR (Granger & Ward 2003). However, it was also found that the gene expressions of PPX and PPK were not affected by the presence of these NPs, which was in accordance with no obvious effects on phosphorus removal after long-term exposure.

**CONCLUSIONS**

In summary, long-term exposure to Al\(_2\)O\(_3\), SiO\(_2\), TiO\(_2\), and ZnO NPs could affect wastewater nutrient removal, and
the different types of NPs showed dissimilar influences. The main reasons for these negative effects were closely related to the changes in microbial community structures and gene expressions and catalytic activities of key enzymes related to wastewater nitrogen and phosphorus removal. In particular, long-term exposure to TiO$_2$ and ZnO NPs greatly decreased the gene expressions and activities of AMO and NOR, which caused the significant inhibition to ammonia removal. The presence of Al$_2$O$_3$ and SiO$_2$ NPs suppressed the gene expressions and activities of NAR and AMO and NOR, which caused the significant decrease in ammonia removal. The presence of Al$_2$O$_3$ and SiO$_2$ NPs also greatly decreased the gene expressions and activities of NAR and AMO and NOR, which caused the significant decrease in ammonia removal. The presence of Al$_2$O$_3$ and SiO$_2$ NPs suppressed the gene expressions and activities of NAR and AMO and NOR, which caused the significant decrease in ammonia removal.

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REFERENCES


Heylen, K., Vanparys, B., Peirsegaele, F., Lebbe, L. & De Vos, P. 2007 *Stenotrophomonas terrae* sp. nov. and *Stenotrophomonas humi* sp. nov., two nitrate-reducing bacteria isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 57, 2056-2061.


