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Effects of graphite nanoparticles on nitrification in an activated sludge system

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HIGHLIGHTS

- This is the first tentative investigation of the mechanism of graphite nanoparticles effect on the nitrification process.
- Nitrification efficiency decreased significantly with graphite nanoparticles exposuring.
- Graphite nanoparticles highly induced bacterial lethal on the nitrifiers.
- The phyla *Gammaproteobacteria*, *Deinocccus*, and *Bacteroidetes* exhibited greater stability than the Nitrifiers.

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ABSTRACT

Graphite nanoparticles (GNPs) might result in unexpected effects during their transportation and transformation in wastewater treatment systems, including strong thermo-catalytic and catalytic effects and microbial cytotoxicity. In particular, the effects of GNPs on the nitrification process in activated sludge systems should be addressed. This study aimed to estimate the influence of GNPs on the nitrification process in a short-term nitrification reactor with exposure to different light sources. The results indicated that GNPs could only improve the efficiency of photothermal transformation slightly in the activated sludge system because of its photothermal effects under the standard illuminant (imitating 1 × sun). However, even with better photothermal effects, the nitrification efficiency still decreased significantly with GNP dosing under the standard illuminant, which might result from stronger cytotoxic effects of GNPs on the nitrifying bacteria. The disappearance of extracellular polymeric substances (EPS) around bacterial cells was observed, and the total quantity of viable bacteria decreased significantly after GNP exposuring. Variation in bacterial groups primarily occurred in nitrifying microbial communities, including *Nitrosomonas* sp., *Nitrosospira* sp., *Comamonas* sp. and *Bradyrhizobiace* sp. Nitrifiers significantly decreased, while the phyla *Gammaproteobacteria*, *Deinocccus*, and *Bacteroidetes* exhibited greater stability during GNP treatment.

1. Introduction

Nanomaterials, including metal oxide nanoparticles (NPs), zero-valent metal NPs, carbon nanotubes and composite nanomaterials (Dongye and Shubo, 2015), have been widely used. Nano-carbon systems, such as carbon nanotubes, grapheme and the products of graphite, also possess specific abilities, such as enhancing energy conversion mechanisms. Carbon nanotubes have been widely studied for its characterization of solar energy harvesting-based photovoltaic chemical energy conversion (Strano, 2015), as well as the nanotube membranes for efficient water desalination (Corry, 2008). Ghasemi et al. (2014) reported a steam generation system based on a double-layer structure (DLS) that consisted of a carbon foam layer and an exfoliated graphite layer. The DLS could concentrate solar power and improve the solar thermal efficiency by up to 85% at 10 KWm^{-2} by keeping the bulk liquid below the reaction layer at low temperatures (Ghasemi et al., 2014). The applications of some nanomaterials have also included the catalytic degradation, adsorption and redox of environmental contaminants in wastewater treatment. With the extensive consumption of nanotech products, concern has grown over the increasing release of various nanoparticles into the environment.

Graphite nanoparticles (GNPs) have been widely used in modern industry due to their excellent photo-thermal performance

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(Wang et al., 2016), suitable properties for application in the lubrication industry (Su et al., 2015), and ideal carrying capacity for drug delivery to cancer cells (Zakrzewska et al., 2015). Graphite nanoparticles were used as additives in a paraffin/water emulsion to improve the thermal conductivity and photothermal conversion in Wang's study (2016). Some modifications were also made based on graphite nanoparticles, and the best output efficiency was found to occur with a 0.5 cm water depth for solar stills (Sharshir et al., 2016). With the development of material GNPs, the accidental and unpredictable release of GNPs during the production and application processes will ultimately result in GNPs entering wastewater treatment plants (WWTPs).

However, unpredictable adverse effects of nanomaterials also accompany their extraordinary physical and chemical properties (Kang et al., 2007; Maynard et al., 2006). The microbial cytotoxicity of carbon-based nanoparticles to eukaryotic cells has been reported as a complex physicochemical property of nanomaterials in previous studies (Kang et al., 2009). Bactericidal activity has also been found in both monocultured model gram-positive and gram-negative bacteria (Lyon et al., 2006; Mohanty et al., 2007). Contingent cytotoxicity was found to occur after direct contact with the cell membrane, and further research on this physicochemical mechanism showed that the toxicity of carbon-based nanomaterials in microbial monocultures was not a suitable predictor of microbial deactivation in biologically and chemically complicated environmental samples (Kang et al., 2009). Ecological composition and microbial structure might affect the environmental implications of cytotoxicity, and the nanoparticles showed different toxicities across each bacterial species (Handy et al., 2008). The effects of nanomaterials, including ZnO, TiO_2, SiO_2 and Ag NPs, on the environment and the process of wastewater treatment have previously been studied (Zhang et al., 2016; Sibag et al., 2015; Tan et al., 2015). Because the nitrifying bacteria grow very slowly and have a relatively smaller population than most of other bacteria in the activated sludge system, the nitrification process is sensitive to inflowing toxic compounds or physical disturbances during wastewater treatment (Jönsson et al., 2000; Zheng et al., 2016). Negative influences on nitrification in the wastewater treatment process have been found in previous studies, including for ZnO, TiO_2, Al_2O_3, and Cu NPs (Zheng et al., 2011a, 2011b; Chen et al., 2012; Clar et al., 2016). Specifically, Ag NPs were found to present cytotoxicity to *Nitrosomonas europaea* in monoculture, and Fe, TiO_2, CeO_2 NPs were found to affect the microbial community distinctly to a certain extent, which suggested potential long-term effects (Arnaout and Gunsch, 2012; Tan et al., 2015; Liu et al., 2016; Ma et al., 2015). However, the potential effects of GNPs on nitrification and the complex microbial community are still unknown; thus, assessing the microbial cytotoxicity of GNPs to the complex activated sludge in the wastewater treatment system may facilitate a deeper understanding of the influence of waste nanomaterials on wastewater treatment. Research on nanomaterial risks and the development of related regulatory policies on risk management are both urgent in modern society.

In this study, the impacts of GNPs on the nitrification of nitrifying bacteria under photothermal effects and the variation of the microbial community in a simulated activated sludge system were first investigated. Scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses were used to determine the impacts on the surface and cell sectional status of the activated sludge. The methods of PMA-qPCR (quantitative polymerase chain reaction) and PMA-Miseq sequencing were also applied to analyze the effects of the GNPs on the viable microbial community during the dosing period.

2. Materials and methods

2.1. Experiment design

Three short-run laboratory-scale nitrification reactors (1 L) were constructed and run until reaching stable operation with a sludge concentration of 0.898 g/L. The reactor was operated with a primary medium of ammonia for one year to dominate the nitrifying bacteria. They were applied under different conditions during the experiment: standard illuminant (1 sun intensity) with GNP treatment (SIN), sunlight with GNP treatment (SLN) and sunlight without GNP treatment (SL). The initial inflowing NH_4^+-N concentration was prepared at 37 mg/L, and the hydraulic retention time was set as 3 h. Graphite nanomaterials were purchased from the XianFeng Material Technology Co., LTD, NanJing, China.

2.2. Sample preparation and PMA treatment

Supernate samples were collected from each reactor to determine the NH_4-N concentration every 20 min. Sludge was collected before and after the operation for microbial analysis. To specifically analyze the viable microbial community, samples were treated with propidium monoazide (PMA) dye (Biotium Inc., USA). First, 2.5 μl of PMA (20 mmol/L) was added to 500 μl of the sludge for a final treatment concentration of 100 μmol/L of PMA dye (Pang et al., 2016). Samples were blended and incubated for 5 min in a lucifugal centrifuge tube with occasional mixing. The centrifuge tube was subsequently placed onto ice and irradiated with a 650 W halogen light at a distance of 20 cm for 4 min. After photo-activation, prepared samples were centrifuged at 1000 g for 5 min for the following DNA extraction procedures. Total DNA was extracted following the manufacturer's instructions (FastDNA Spin Kit for soil, MP, USA).

Samples were also collected for SEM and TEM observations. Concentrated sludge was immobilized with 2.5% glutaraldehyde. Morphological and structural analyses of the activated sludge were performed using a SEM at 15.00 KV (FEI- Quanta200) and TEM (HITACHI, HT7700) operating at 80.00 kV.

2.3. Miseq sequencing and quantitative PCR

DNA was prepared and the universal primers (338F/806R) of the 16S-V3-V4-region were applied in Miseq sequencing on the Illumina Miseq platform (Illumina, USA). In order to minimize the influence of previous PCR errors, three parallel PCR products for each sample were incorporated for constructing amplicon libraries. The amplicons were purified and sequenced on an Illumina MiSeq Platform at Majorbio Bioinformatics Technology Co., Ltd., Shanghai, China. The original sequences were uploaded to the NCBI genebank (https://www.ncbi.nlm.nih.gov/) and the assession numbers are SAMN06099118, SAMN06099133 and SAMN06099134, respectively.

qPCR was performed with 16SrDNA universal primers F341/R534. The original plasmid standard (1.43×10^{11} copies/μl), including the target segments, was prepared with the above primers, and a PCR standard curve was established via 10-fold dilution of the standard solution with the final dilution concentration of plasmid ranging from 1.43×10^{11} to 1.43×10^{0} copies/μl. qPCR was performed on the IQ5 real-time PCR (Bio-Rad, USA). Amplification reactions (25 μl) contained 12.5 μl of SYBR® premix Ex Taq™ (Takara, RR420A), 0.5 μl of each primer (10 μM), 2 μl of the DNA template and 9.5 μl of dd water. The cycling parameters were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s, then 60 °C - 95 °C (0.5/cycle). The quantity of targeted copies was calculated by plotting threshold