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RESEARCH PAPER

Particle-attached microorganism oxidation of ammonia in a hypereutrophic urban river

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Abstract

To elucidate the importance and mechanisms of particle-attached microorganisms on ammonia oxidation, we conducted a controlled simulation experiment with samples collected from the Shunao River, an ammonia-rich hypereutrophic urban river in eastern China. The effects of particle concentration, ammonia concentration, organic carbon source and concentration, dissolved oxygen concentration and pH were investigated on ammonia transformation rate (ammonia removal rate and NO_2^-+NO_3^- accumulation rate) and abundance of particle-attached ammonia-oxidizing bacteria (AOB) and archaea (AOA). All these factors significantly influenced ammonia transformation rates. Our results provided direct evidence that microorganisms attached on riverine suspended particles were associated with ammonia oxidation. Sequencing revealed that the AOA genus *Nitrososphaera,* and the AOB genus *Nitrosomonas* were the most dominant in particle-attached ammonia-oxidizing microbial communities. Further analysis showed that AOB communities had higher species richness and diversity compared to AOA communities. Additionally, AOB *amoA* genes were ~10-100 times more abundant than AOA *amoA* genes, and AOB abundance was more strongly correlated with ammonia transformation rates than AOA abundance in most experiments, indicating that particle-attached AOB were more important than AOA in the hypereutrophic urban river. This study adds to our knowledge of particle-attached microorganism oxidation of ammonia.

KEYWORDS

ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), hypereutrophic urban river, suspended particles, water quality

1 INTRODUCTION

Anthropogenic activities have increased bioavailable nitrogen concentrations (NH_4^+-N and NO_3^--N) in many aquatic ecosystems worldwide [1]. Currently, China creates far more reactive nitrogen than any other country [2,3], and about 25% of this reactive nitrogen is exported in rivers [4,5]. Bioavailable nitrogen concentrations are especially high in many urban rivers that receive storm runoff and often appreciable amounts of non-treated waste waters containing a complex mixture of nutrients and organic compounds. Excessive anthropogenic nutrient loading in urban rivers can cause serious eutrophication and decline in ecosystem function, including oxygen-depleted dead zones.

One distinctive feature of several rivers is that enhanced nutrient fluxes are often accompanied by high concentrations of suspended particles [6-8]. Due to the influx of particles from domestic sewage and urban runoff, together with sediment resuspension in shallow coastal plain rivers, it is common to have a large number of suspended organic-rich particles in the water column. These organic-rich particles provide attachment sites for both allochthonous and autochthonous microorganisms in fluvial systems [9-11]. In some shallow aquatic habitats, the abundance of particle-attached bacteria accounted for more than 50% of total bacterial abundance [12]. Increasingly, research is demonstrating that suspended particles serve not only as attachment sites for microorganisms, but also as "hot spots" for microbially catalyzed biogeochemical reactions [13,14]. Particle-associated microorganisms usually possess distinct morphologies (usually larger cells), abundances (often enriched), and functions (often higher per-cell exoenzymatic activities) compared to their non-attached (planktonic) counterparts [13,14]. In eutrophic urban river systems, the microbial community is the primary engine driving important nutrient cycling processes (e.g. aquatic nitrogen cycle) [15,16]. However, the importance of attached versus non-attached microorganisms has not been fully investigated, especially in hypereutrophic urban river systems enriched in ammonia.

Ammonia oxidation is the first step in coupled nitrification-denitrification, which is an important process for removal of ammonia from degraded urban river systems, attenuating potential ammonia toxicity and eutrophication in downstream aquatic ecosystems [17]. For example, coupled nitrification-denitrification was shown to remove a substantial percentage (10~80%) of anthropogenic nitrogen pollution in estuaries [18,19]. Ammonia-oxidizing bacteria (AOB) and archaea (AOA) are considered the most important contributors to catalyze nitrite production during ammonia oxidation. A study from the Chang Jiang River (China) plume showed that nitrifying microorganisms were primarily associated with particles [8]. Similarly, Xia et al. [20] reported that nitrification rates increased with suspended sediment concentration in the Yellow River (China), suggesting the importance of particle-associated microorganisms.

We hypothesize that suspended particles are an important ecological niche for ammonia-oxidizing microorganisms in eutrophic urban river systems. Prosser et al. [21] advocated that future investigations of niche specialization for ammonia oxidizers require a rigorous characterization of a wide range of environmentally representative cultures and an emphasis on experimental studies rather than surveys. Hence, to elucidate the

importance and mechanisms of particle-attached microorganisms on ammonia oxidation, we conducted a controlled simulation experiment in the laboratory with samples collected from an ammonia-rich, hypereutrophic urban river. Additionally, it has also been noted that serious organic and nutrient pollution in the water column and sediments, insufficient water flows and low oxygen reaeration-high oxygen demand are commonly encountered in urban rivers. The aims of this work were to investigate: (1) how the nitrogen transformation rate and *amoA* gene abundance of particle-attached microorganisms were affected by the main experimental factors (particle concentration, ammonia concentration, organic carbon source and concentration, dissolved oxygen concentration and pH), and (2) the relative importance of particle-attached AOA and AOB in ammonia oxidation in a hypereutrophic urban river system. A better understanding of ammonia oxidation in eutrophic river systems may inform bioremediation strategies for nitrogen removal via coupled nitrification-denitrification.

2 MATERIALS AND METHODS

2.1 Sampling site and sampling protocols

The Wen-Rui Tang River (27.772411°-28.064034°N, 120.417398°-120.859893°E) is a typical coastal plain river system located in Wenzhou, eastern China, and has a total catchment area of 740 km^2. Water depth generally ranges from 0.5 to 3.0 m. The river flows through a densely populated region (~3.0 million in urban center and ~9.1 million in region) and is a long-term receptor of industrial/municipal wastes. As a consequence, the Wen-Rui Tang River water quality has degraded dramatically since the 1990s, and is now in a state of serious eutrophication, characterized by high turbidity, high ammonia concentrations and seasonally low dissolved oxygen [22-24].

The sampling site was located on the Shunao River (27.929854° N, 120.705249° E), which is an urban tributary of the Wen-Rui Tang River. The water quality is representative of the impacts resulting from rapid urbanization that has occurred over the past two decades throughout eastern China. This river had high nitrogen, phosphorus, and total organic carbon concentrations. Samples used for collecting suspended particles and 0.22 μm-filtered water were taken from bottom (bottom 30 cm) and surface (top 30 cm) water using a 3 l Schindler sampler in the middle of Shunao River, respectively. Total sampling volume for each experiment exceeded 30 l of water. All samples were transported to the laboratory within 30 min and experimental assays were conducted

within 48 h of sample collection. Samples examining different treatment effects were collected at different time periods in order to facilitate completion of each incubation within a consistent time period. Physicochemical analysis for the river water and suspended particles used in these experiments is summarized in Table 1.

2.2 Laboratory simulation experiment

A simulation experimental system was designed to study the influences of different factors (particle concentration, ammonia concentration, organic carbon source and concentration, dissolved oxygen concentration and pH) on nitrogen transformation rates and *amoA* gene abundance of particle-attached microorganisms. To concentrate the suspended particles, river water was centrifuged (8,000×g) for 10 min [25]. River water was filtered through 0.22 μm pore-size membrane filters to remove all microorganisms [26,27]. The collected suspended particles and filtered water were used as media for the simulation experiments. The experimental system was composed of a series of Erlenmeyer flasks with all experimental treatments having four replicates unless otherwise indicated.

To determine the influence of suspended particle concentration, a 250 ml aliquot of filter-sterilized (0.22 μm pore-size) river water was added to a series of flasks and suspended particles added to achieve concentrations of 0, 10, 50, 100, 500, 1000, 5000 and 10000 mg l^{-1} (fresh weight). A known amount of NH_4^+ (as NH_4Cl) was added to obtain an initial NH_4^+-N concentration of 10 mg l^{-1}.

To investigate the influence of different NH_4^+ concentrations, 500 mg l^{-1} of suspended particles (previously determined as the optimum concentration) was added to a series of flasks containing filter-sterilized (0.22 μm pore-size) river water and NH_4^+-N added to achieve concentrations of 1, 5, 10, 15, 20 and 25 mg l^{-1}.

To investigate the influence of organic carbon source, peptone and glucose were separately added to a series of flasks containing filter-sterilized (0.22 μm pore-size) river water, 500 mg l^{-1} of suspended particles and 10 mg l^{-1} of NH_4^+-N (optimum NH_4^+ concentration). Peptone and glucose was added to obtain final concentrations of 0, 1, 5, 10, 20, 30, 40 and 50 mg l^{-1}.

The influence of pH was investigated by adjusting the pH in the experimental system containing filter-sterilized (0.22 μm pore-size) river water, 500 mg l^{-1} of suspended

particles and 10 mg l^{-1} of NH_4^+-N. The pH in each experimental system was monitored daily using a FiveEasy Plus FE28 pH meter (Mettler-Toledo Instruments, Shanghai Co., LTD). The pH was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using 1M NaOH or 1 M HCl.

The influence of DO was investigated by adjusting the DO concentration in the experimental system containing filter-sterilized (0.22 μm pore-size) river water, 500 mg l^{-1} of suspended particles and 10 mg l^{-1} of NH_4^+-N. DO concentration was monitored daily using a DO electrode (JPSJ-605, Shanghai INESA Scientific Instrument) and was manually adjusted to 0-2, 2-4 or 4-9 mg l^{-1} using filter-sterilized air or N_2. For each treatment at least eight replicates were used.

Samples were incubated in the dark at 25 ^oC. Samples were collected daily and analyzed for NH_4^+ concentration. The incubation was ended when around 80% of the NH_4^+ has been consumed [28]. An aliquot of the sample was collected at the beginning and end of the incubation to determine the suite of nitrogen concentrations (NH_4^+, NO_2^- and NO_3^-). At the conclusion of the incubation, an aliquot was filtered through a 0.22 μm cellulose acetate membrane filter (50 mm diameter) and stored at -20 °C for subsequent molecular analyses.

2.3 Chemical analysis

For determination of ammonium (NH_4^+-N), nitrate (NO_3^--N), and nitrite (NO_2^--N) in water samples, aliquots were first filtered through a 0.22 μm filter. The Nesslerization colorimetric, ultraviolet spectrophotometric, and N-(1-naphthyl)-ethylenediamine colorimetric methods were used for the quantification of NH_4^+-N, NO_3^--N and NO_2^--N, respectively [29]. We used two criteria to assess ammonia transformation rates in this study. Ammonia removal rates were calculated based on changes in NH_4^+-N concentrations with time. Nitrite and nitrate accumulation rates were determined from the cumulative increase in NO_3^--N and NO_2^--N concentrations over time. These two measures of ammonia transformation rates may differ somewhat due to microbial mineralization of N from organic-rich particles and/or microbial assimilation of N during the incubation.

2.4 DNA extraction and quantitative real-time PCR (qPCR) analysis

Total DNA was extracted from frozen filters with the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to manufacturer's protocols. The concentration and quality of extracted DNA was determined with a spectrophotometer (Nano-200, Hangzhou Allsheng Instruments, Hangzhou, China). Real-time quantitative PCR was performed using a StepOne Real-Time PCR System (Applied BioSystems, Foster City, CA, USA) with SYBR Green I fluorescent dye. The AOA and AOB *amoA* genes were amplified using primers Arch-amoAF/Arch-amoAR [30] and amoA-1F/amoA-2R [31], respectively. The 20 μl reaction mixture contained 10 μl 2×TransStart Top Green qPCR SuperMix (TransGen, China), 0.2 μM forward and reverse primers, 0.4 μl Passive Reference Dye (50×), 0.5 μl diluted DNA extract and ultra-pure sterile water. Two different PCR amplification programs were performed as described by Zeng et al. [32]. Briefly, the PCR amplification program used for the AOA *amoA* gene was 3 min at 95 °C; 45 cycles of 30 s at 95 °C, 1 min at 53 °C, 20 s at 72 °C and a final extension of 7 min at 72 °C. The program used for the AOB *amoA* gene was 3 min at 95 °C; 45 cycles of 30 s at 95 °C, 1 min at 55 °C, 20 s at 72 °C and a final extension of 7 min at 72 °C. Copy numbers were determined via standard curve constructed as follows: 10-fold serial dilutions of a known copy number of the plasmid DNA were subjected to quantitative real-time PCR assay in triplicate to generate a standard curve. The PCR product specificity was checked using melting curve analysis and agarose gel electrophoresis. Data were analyzed with StepOne Software Ver. 2.3 (Applied BioSystems). Quantification data were combined with sample volume to calculate the abundance of AOA and AOB *amoA* gene copies ml^{-1}.

2.5 Sequencing and phylogenetic analysis

To investigate the community composition of AOA and AOB in particle-attached microorganisms, DNA samples from selected experimental treatments (treatment 500 mg l^{-1} of particle concentration experiment, marked as P500; treatment 0 mg l^{-1} of organic carbon experiment, marked as CKY; and treatment pH 7.5 of pH experiment, marked as pHD) were analyzed by high-throughput sequencing. PCR amplification of archaeal and bacterial *amoA* genes were performed with the primers Arch-amoA26F /Arch-amoA417R [33] and amoA-1F/amoA-2R [31], respectively. The 25 μl reaction mixture contained 12.5 μl Phusion® Hot Start Flex 2× Master Mix (NEB), 1 μM forward and reverse primers, 50 ng template DNA and ultra-pure sterile water. The PCR amplification

program was 30 s at 98 °C; 40 cycles of 10 s at 98 °C, 30 s at 52.5 °C, 45 s at 72 °C and a final extension of 10 min at 72 °C. Sequencing was performed on the Illumina platform at LC-Biotechnologies (Hangzhou). Denoised sequences were aligned and sorted into operational taxonomic units (OTUs) at the 97% similarity level, and these obtained OTUs were used to calculate Good's coverage, abundance-based coverage estimator (ACE), Chao1 richness estimator, and Shannon index. Taxonomy was assigned using the GenBank NT database with the BLAST software at the National Center for Biotechnology Information (NCBI). All sequence data from this study were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRP159421 and SRP159422.

2.6 Statistical analysis

One-way ANOVA and Duncan tests with a significance level of 0.05 were used to determine significant differences among treatments. Relationships among variables were examined using Pearson's correlation. All statistical analyses were performed using SPSS Ver. 22.0 (IBM-SPSS Statistics, Chicago, IL, USA). Data were plotted using SigmaPlot 12.0 (Systat Software, San Jose, CA, USA).

3 RESULTS

In the present study, we used centrifugation and filtration to collect suspended particles and filtered-sterilized water from an urban river for use in a controlled simulation experiment to investigate the effects of particle concentration, NH_4^+ concentration, organic carbon source and concentration, dissolved oxygen concentration and pH on ammonia transformation rate and abundances of particle-attached AOA and AOB. For the assessment of ammonia transformation rate, we used both ammonia removal and NO_2^- +NO_3^- accumulation rates as indicators of ammonia oxidation. AOA and AOB compositions and abundances were inferred by the *amoA* gene, which encodes subunit A of ammonia monooxygenase that performs the first step in ammonia oxidation by both groups.

3.1 Influence of particle concentration on particle-attached microorganism oxidation of ammonia

Suspended particle concentration had a significant effect on ammonia transformation rate as evidenced by the NH_4^+ removal rate, NO_2^- +NO_3^- accumulation rate, and abundance of

AOA and AOB *amoA* genes (Table 2; Fig. 1A; Fig. 2A). The NH_4^+ removal rate increased with increasing suspended particle concentrations to a maximum at 5000 mg l^{-1} before showing a slight decline at higher concentrations. When suspended particle concentration increased from 100 to 500 mg l^{-1}, the corresponding NH_4^+ removal rate increased from 0.25 to 0.70 mg l^{-1} d^{-1}. The NO_2^- + NO_3^- accumulation rate showed a similar trend as the NH_4^+ removal rate; it increased to a maximum of 0.6 mg l^{-1} d^{-1} at 500 mg l^{-1} suspended particles, followed by a slight decrease at higher suspended particle concentrations.

Abundance of AOA and AOB genes were characterized using real-time quantitative PCR analysis for archaeal and bacterial *amoA* gene copy numbers. The abundance of the bacterial *amoA* gene was significantly higher than the archaeal *amoA* gene for all samples. Archaeal *amoA* gene abundance increased along with suspended particle concentration. A similar trend was found for the abundance of the bacterial *amoA* gene with suspended particle concentrations; increasing abundance was found in the 10 to 500 mg l^{-1} range and then reached a plateau between 500 to 10000 mg l^{-1}. Thus, we chose the 500 mg l^{-1} particle concentration as the optimal condition for subsequent experiments. Additionally, the abundance of the *amoA* gene exhibited a significant positive correlation with the NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate (Table 3).

3.2 Influence of NH_4^+ concentration on particle-attached microorganism oxidation of ammonia

NH_4^+ concentration had a significant effect on ammonia transformations as evidenced in the NH_4^+ removal rate, NO_2^-+NO_3^- accumulation rate, and abundance of AOA and AOB *amoA* genes (Table 2; Fig. 1B; Fig. 2B). When NH_4^+-N concentration increased from 1 to 10 mg l^{-1}, the corresponding NH_4^+ removal rate increased from 0.11 to 0.80 mg l^{-1} d^{-1}. The NO_2^-+NO_3^- accumulation rate showed a similar trend as the NH_4^+ removal rate; it increased to a maximum of 0.71 mg l^{-1} d^{-1} at 10 mg l^{-1} NH_4^+-N concentration, followed by a significant decreased after reaching the maximum, and then remained at a low level between NH_4^+-N concentrations of 15 to 25 mg l^{-1}. The abundance of the bacterial *amoA* gene was significantly higher than the archaeal *amoA* gene. Archaeal *amoA* gene and bacterial *amoA* gene abundance exhibited different trends with NH_4^+ concentration. Archaeal *amoA* gene abundance remained at a relatively stable level between 1 to 10 mg N l^{-1}, while bacterial *amoA* gene abundance increased in the 1 to 10 mg N l^{-1} range, and

then both significantly declined at higher NH_4^+ concentrations. Thus, we chose the 10 mg l^{-1} NH_4^+-N concentration as the optimal condition for subsequent experiments. Additionally, the NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate exhibited a significant positive correlation with the abundance of the bacterial *amoA* gene, but not with the abundance of the Archaeal *amoA* gene (Table 3).

3.3 Influence of organic carbon on particle-attached microorganism oxidation of ammonia

Organic carbon source (peptone vs. glucose) and concentration had significant effects on ammonia transformations and abundance of *amoA* genes (Table 2; Fig. 1C and 1D; Fig. 2C and 2D). The NO_2^-+NO_3^- accumulation rate and NH_4^+ removal rate decreased with increasing concentrations of peptone and glucose. The reduction in the NO_2^-+NO_3^- accumulation rate was greater for glucose than peptone. The abundance of the bacterial *amoA* gene was significantly higher than the archaeal *amoA* gene for both peptone and glucose amendments. Archaeal and bacterial *amoA* gene abundance decreased with increasing peptone concentration. A similar trend was found for the abundance of the bacterial *amoA* gene with glucose concentrations; decreasing abundance was found in the 5 to 50 mg l^{-1} range. In contrast, changes in archaeal *amoA* gene abundance were not significantly different among different glucose concentrations. Additionally, the NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate exhibited a significant positive correlation with the abundance of the bacterial *amoA* gene, but not with Archaeal *amoA* gene abundance following peptone and glucose amendments (Table 3).

3.4 Influence of pH on particle-attached microorganism oxidation of ammonia

Changes of NH_4^+ removal rate, NO_2^-+NO_3^- accumulation rate, and abundance of AOA and AOB *amoA* genes (Table 2; Fig. 1E; Fig. 2E) demonstrated a significant pH effect on ammonia transformations. The NH_4^+ removal rate significantly increased with increasing pH from 6.0 to 7.5 and reached a plateau above pH 7.5. The NO_2^-+NO_3^- accumulation rate showed a contrasting trend demonstrating an increase from pH 7.5 to 9.0.

Archaeal *amoA* gene and bacterial *amoA* gene abundance exhibited different trends with respect to pH. The highest archaeal *amoA* gene abundance was detected in pH 6.0 treatment followed by a decrease to pH 7.5 where it remained at a low level with a further pH increase to 9.0. In contrast, the lowest abundance of the bacterial *amoA* gene was detected in the pH 6.0 treatment. The abundances of the bacterial *amoA* gene

increased with increasing pH and reached a plateau between pH 7.5 and 9.0. In general, the abundance of the bacterial *amoA* gene was significantly higher than the archaeal *amoA* gene at all pH levels. Additionally, the NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate exhibited significant positive correlations with bacterial *amoA* gene abundance, but demonstrated a significant negative correlation with archaeal *amoA* gene abundance in the pH experiments (Table 3).

3.5 Influence of DO on particle-attached microorganism oxidation of ammonia

The NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate showed significant differences among the three DO levels, while archaeal *amoA* gene and bacterial *amoA* gene abundance showed no significant differences (Table 2; Fig. 1F; Fig. 2F). The changes in NH_4^+ removal rate with DO levels were similar to those observed for the NO_2^-+NO_3^- accumulation rate, both showing an increase with increasing DO levels. The bacterial *amoA* gene abundance was significantly higher than the archaeal *amoA* gene abundance at all DO levels. Additionally, the NH_4^+ removal rate and NO_2^-+NO_3^- accumulation rate exhibited a significant positive correlation with bacterial *amoA* gene abundance, but not with archaeal *amoA* gene abundance in the DO experiments (Table 3).

3.6 Diversity and taxonomic identification of particle-attached ammonia-oxidizing microorganism

Species richness and diversity indices of ammonia-oxidizing microbial communities based on bacterial and archaeal *amoA* gene sequences are shown in Table 4. Good's coverage ranged from 99.2 to 99.9%, indicating that the sequencing effort was sufficient to capture the relative complete diversity of the examined samples. Results from high-throughput sequencing showed that AOB communities had relatively higher species richness and diversity compared to AOA communities, as evidenced by OTUs, ACE, Chao1, and Shannon indices (Table 4).

In this study, the similarity analysis of ammonia-oxidizing microbial communities was performed on OTUs (Fig. 3). Venn diagrams give a measure of the similarities among different communities [34]. For ammonia-oxidizing archaea, the selected P500, CKY and pHD samples had 200 OTUs in common; and for ammonia-oxidizing bacteria, these samples had 404 OTUs in common. There were 37 (9.9%) unique OTUs of ammonia-oxidizing archaea for P500, 32 (9.3%) in CKY, and 262 (42.3%) in pHD. Moreover, there were 542 (39.8%) unique OTUs of ammonia-oxidizing bacteria for P500, 184

(18.9%) in CKY, and 128 (16.8%) in sample pHD. In general, the ammonia-oxidizing archaea or bacteria in the three samples shared large number of OTUs in common with low unique OTU numbers.

The relative abundance of dominant genera and species (sum >99%) is shown in Fig. 4 and Fig. 5, respectively. For ammonia-oxidizing archaea, *Nitrososphaera, Candidatus Nitrosocosmicus* and *Archaea noname* were the three predominant genera in all examined samples, especially in P500 and CKY. Compared with P500 and CKY, there was a marked increase in the proportion of *Candidatus Nitrosoarchaeum* and *Candidatus Nitrosotenuis* in pHD (Fig. 4A). For ammonia-oxidizing bacteria, the two predominant genera were *Nitrosomonas* and *Nitrosospira* in all examined samples; the sum of their relative abundance exceeded 99% (Fig. 4B).

At the species resolution, the AOA species *Candidatus Nitrososphaera gargensis* and *Candidatus Nitrosocosmicus franklandus* were dominant in each sample (Fig. 5A). *Nitrosomonas* sp., *Nitrosomonas oligotropha, Nitrosospira multiformis, Nitrosomonas nitrosa* and *Nitrosospira* sp. were the five dominant species in the AOB community of each sample (Fig. 5B). Additionally, it is notable to find that the complete ammonia oxidizing (comammox) bacteria, *Candidatus Nitrospira inopinata* belonging to genus *Nitrospira* [35] was evident in this study, although its relative abundance was low (Fig. 5B).

4 DISCUSSION

It is well documented that aquatic microorganisms can be highly associated with particles in fluvial systems [9-11,36,37]. Studies suggest that particle association can provide microorganisms with benefits such as protection against predators, enhanced environmental resistance and increased nutrient availability [38-41]. Previous research posited that nitrifying microorganisms were attached to particles based on indirect evidence [8,42-44]. As expected, the particle concentration experiment showed that ammonia removal rate, NO_2^-+NO_3^- accumulation rate, and abundance of AOA and AOB *amoA* genes were significantly affected by increasing particle concentration (Table 2; Fig. 1A; Fig. 2A). Additionally, Pearson's correlation analysis indicated a significant positive correlation between abundance of the AOB *amoA* gene and NO_2^-+NO_3^- accumulation rate (R=0.653, p<0.001), as well as for abundance of the AOA *amoA* gene and NO_2^-+NO_3^- accumulation rate (R=0.494, p=0.009) (Table 3). In the present study, we

documented an increase in ammonia oxidizing microorganism abundance with increasing particle concentration that was accompanied by an increase in ammonia removal and NO_2^-+NO_3^- accumulation rates (Fig. 1A and Fig. 2A). Therefore, our results provide evidence in support of our hypothesis that suspended particles are an important ecological niche for ammonia-oxidizing microorganisms in eutrophic urban river systems.

On the other hand, surprisingly, the increase in ammonia removal and NO_2^-+NO_3^- accumulation rates with increasing suspended particle concentrations was not a linear function. Rather, ammonia transformations showed a slow initial increase (0-100 mg l^{-1} suspended particles) followed by a rapid increase (100-500 mg l^{-1} suspended particles) before declining at the higher suspended particle concentrations (Fig. 1A). Because ammonia transformation rates were dependent on microorganism abundance under the same experimental conditions, the slow increase in ammonia removal and NO_2^-+NO_3^- accumulation rates likely reflected the low microorganism abundance at low particle concentrations. At high particle concentrations, ammonia removal and NO_2^-+NO_3^- accumulation rates reached a plateau, possibly due to restriction of microbial population growth in the limited space of the simulation flasks or alternatively due to decreased oxidation efficiency (i.e., competition) associated with high microbial populations. In the present study, there was a slow increase or even saturation in the abundance of the AOA and AOB *amoA* gene at high particle concentrations (Fig. 2A). Based on these results, we chose the 500 mg l^{-1} particle concentration as the optimal condition for subsequent experiments.

Our study was further designed to investigate the effects of the main river water characteristics influencing ammonia transformation rates and abundances of particle-attached AOA and AOB. Results demonstrated that the effects of NH_4^+-N concentration on ammonia transformation rate were significant with the highest rate observed at an NH_4^+-N concentration of 10 mg l^{-1} (Fig. 1B). In general, although the trends in ammonia removal rates among the different treatments in different experiments were similar to those observed for NO_2^-+NO_3^- accumulation rates, there were some marked differences in the absolute values between these two measures, especially for the glucose and peptone amendments (Fig. 1). Some of these differences may result from microbial mineralization of nitrogen from the organic fractions in experimental system, volatilization of ammonia from the water to the atmosphere [45], and/or immobilization

of nitrogen by microbes during C-substrate utilization. It was interesting to find that the NO_2^-+NO_3^- accumulation rates exceeded the ammonia removal rates in pH experiment, as observed in peptone experiment. Obviously, the marked decrease in the ammonia removal rates was linked to the presence of organic nitrogen compounds in pH experimental system. These results implied that ammonia transformation calculated by the cumulative increase in NO_3^--N and NO_2^--N concentrations might be a more suitable assessment of the ammonia oxidation rate in our study. On the basis of the NO_2^-+NO_3^- accumulation rates, we found that increasing organic carbon concentrations resulted in a perceived decrease in ammonia oxidation rates; glucose was more conducive to the decline of ammonia oxidation rates than peptone (Fig. 1C and D). Additionally, factors such as pH and DO significantly influenced ammonia transformation rates with greater rates at higher pH and DO levels (Fig. 1 E and F). Ammonia transformation is well known to be associated with AOA and AOB. Some research has suggested that AOA and AOB often co-exist in the same environment, but the contributions of AOA and AOB to ammonia oxidation has yet to be quantified [46,47]. In the present work, all of the experiments showed a significant positive correlation between AOB *amoA* gene abundance and NO_2^-+NO_3^- accumulation rate, as well as between AOB *amoA* gene abundance and ammonia removal rate (Table 3). The ammonia transformation rate was more strongly positively correlated with the abundance of the AOB *amoA* gene than with abundance of the AOA *amoA* gene. Meanwhile, the ~10-100 time higher abundance of the AOB *amoA* gene relative to the AOA *amoA* gene provides further evidence to support a greater contribution of AOB over AOA to ammonia oxidation in this study.

One of the main reasons for a greater contribution of AOB over AOA to ammonia oxidation results from differences in the optimum conditions for growth between these groups. We found that increasing NH_4^+-N concentrations had different influences on particle-attached AOA versus AOB. Archaeal *amoA* gene abundance remained at a relatively stable level between 1 to 10 mg l^{-1} NH_4^+-N concentrations, while bacterial *amoA* gene abundance increased in the 1 to 10 mg l^{-1} NH_4^+-N concentration range (Fig. 2B). These results were in agreement with French et al. [46] who suggested that AOB had an advantage over AOA at higher NH_4^+ concentrations. Our results also showed that the archaeal and bacterial *amoA* gene abundance, ammonia removal rate and NO_2^-+NO_3^- accumulation rate all significantly declined at higher NH_4^+-N concentrations (≥15 mg l^{-1}; Fig. 2B). Although ammonia is the substrate for ammonia-oxidizing microorganism, high ammonia concentrations in river systems may also reflect a decrease in either abundances

or activities of ammonia-oxidizing microorganism. The results from French et al. [46] not only indicated that strains from AOA cultures are less tolerant to high NH_4^+-N concentration, but also revealed that the members of *Nitrosomonas oligotropha*, which are the dominant species in the AOB community in our study (Fig. 5B), are less tolerant to high NH_4^+-N concentrations.

In addition to high NH_4^+-N concentrations, organic contamination is often another significant characteristic of urban rivers. Hence, we investigated the effects of organic carbon source and concentration on the ammonia transformation rate and abundances of particle-attached AOA and AOB. The NO_2^-+NO_3^- accumulation rate was lower for glucose than peptone. Some researchers suggest that heterotrophic bacteria out-compete nitrifiers for available nitrogen, oxygen and space under a high C/N ratio [48]. Amending incubations with a C-rich substrate is known to immobilize N from the system as microbes take up N from their environment to build body tissue from metabolism of the C-rich substrates. As glucose is more microbially labile than peptone as a C-substrate, glucose would be expected to result in greater N immobilization than peptone resulting in the lower NO_2^-+NO_3^- accumulation rate for the glucose amendment.

Additionally, AOA and AOB responded differently as a function of pH. AOA are found to exist over a wide pH range (3.7 to 8.65) in natural environments [49]. In the present study, a higher abundance of AOA was found under acidic conditions than alkaline or neutral conditions, but a higher abundance of AOB was found under neutral pH values (Table 3; Fig. 2E). These results suggest that AOA from hypereutrophic urban rivers might have a stronger competitive advantage in low pH environment. Moreover, it is generally accepted that AOA are better adapted to low oxygen conditions than AOB [46,49], while AOB are active under more aerobic conditions. However, archaeal *amoA* gene and bacterial *amoA* gene abundance showed no significant differences across the range of DO levels used in this results (Table 2; Fig. 2F).

In the current study, the ammonia-oxidizing microorganisms attached to particles were identified by high-throughput sequencing. Results showed that AOB communities had relatively higher species richness and diversity compared to AOA communities, as evidenced by OTUs, ACE, Chao1, and Shannon indices (Table 4). Sequencing analysis revealed that the AOA genus *Nitrososphaera,* and the AOB genus *Nitrosomonas* were the most dominant in the particles-attached ammonia-oxidizing microbial communities. Further analysis of species composition showed that the AOA species *Candidatus*

Nitrososphaera gargensis and *Candidatus Nitrosocosmicus franklandus*, and the AOB species *Nitrosomonas* sp., *Nitrosomonas oligotropha*, *Nitrosospira multiformis*, *Nitrosomonas nitrosa* and *Nitrosospira* sp. were dominant. Variation in the community compositions of AOA and AOB may affect the ammonia transformation rate. By comparing the results of *amoA* gene sequencing, the dominant genera and species of AOA and AOB communities for the different experimental factors assessed in this study were similar, especially for the AOB communities (Fig. 3, Fig. 4 and Fig. 5). These results of ammonia-oxidizing microbial communities were supported the conclusion that AOB may play a more important role than AOA in the suspended particle fraction of the ammonia-rich hypereutrophic urban river system examined in this study. Moreover, it was notable that the complete ammonia oxidizing (comammox) bacteria *Candidatus Nitrospira inopinata* was presence in our river system, and the relevance of this result should be investigated further.

In conclusion, our results provided direct evidence that microorganisms attached on riverine suspended particles were associated with ammonia oxidation in an ammonia-rich, hypereutrophic urban river. The suspended particles had significant effects on ammonia oxidation in river waters, and the contribution of particle-attached microorganisms to ammonia oxidation depended on suspended particle concentration. Sequencing analysis revealed that the AOA genus *Nitrososphaera*, and the AOB genus *Nitrosomonas* were the most dominant in the particles-attached ammonia-oxidizing microbial communities. Further analysis showed that AOB communities had higher species richness and diversity compared to AOA communities. Moreover, AOB *amoA* genes outnumbered AOA *amoA* genes by a factor of ~10-100 times, and AOB abundances were more strongly correlated with ammonia removal rate and NO_2^-+NO_3^- accumulation rates than those found for AOA abundances in almost all experiments. These results suggest that AOB may play a more important role than AOA in ammonia oxidation in the suspended particle fraction of the ammonia-rich hypereutrophic urban river system examined in this study.

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CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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Figures

FIGURE 1 Effects of particle-attached microorganisms on nitrogen transformation rates for different experimental factors. (A) particle concentrations; (B) ammonia concentrations; (C) peptone concentrations; (D) glucose concentrations; (E) pH; (F) dissolved oxygen (DO) levels. Different lower case letters indicate significant difference at $p<0.05$

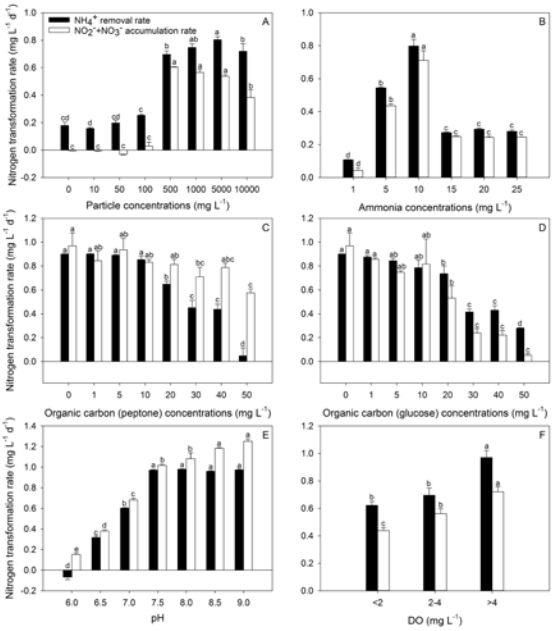

FIGURE 2 Effects of particle-attached microorganisms on *amoA* gene abundance for different experimental factors. (A) particle concentrations; (B) ammonia concentrations; (C) peptone concentrations; (D) glucose concentrations; (E) pH; (F) dissolved oxygen (DO) levels. Different lower case letters indicate significant difference at $p<0.05$

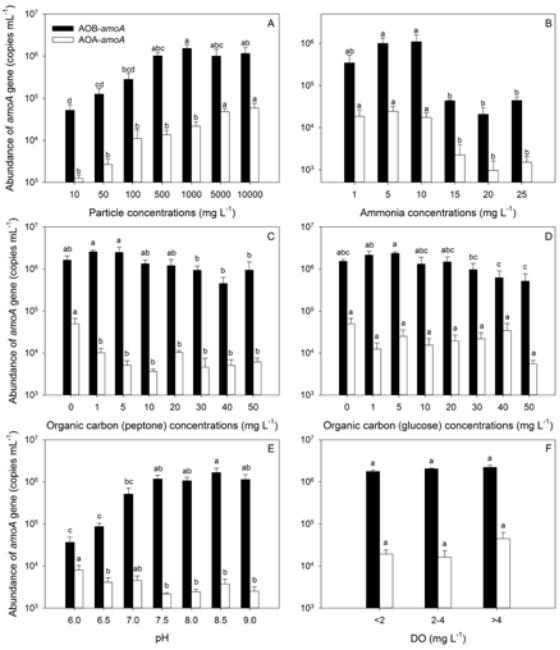

FIGURE 3 Venn diagrams of operational taxonomic units (OTUs) showing the ammonia-oxidizing microbial communities similarity from different experimental samples (P500, CKY and pHD). (A) Ammonia-oxidizing archaea (AOA); (B) Ammonia-oxidizing bacteria (AOB). P500: 500 mg l^{-1} particle concentration treatment; CKY: 0 mg l^{-1} organic carbon treatment; pHD: pH 7.5 treatment

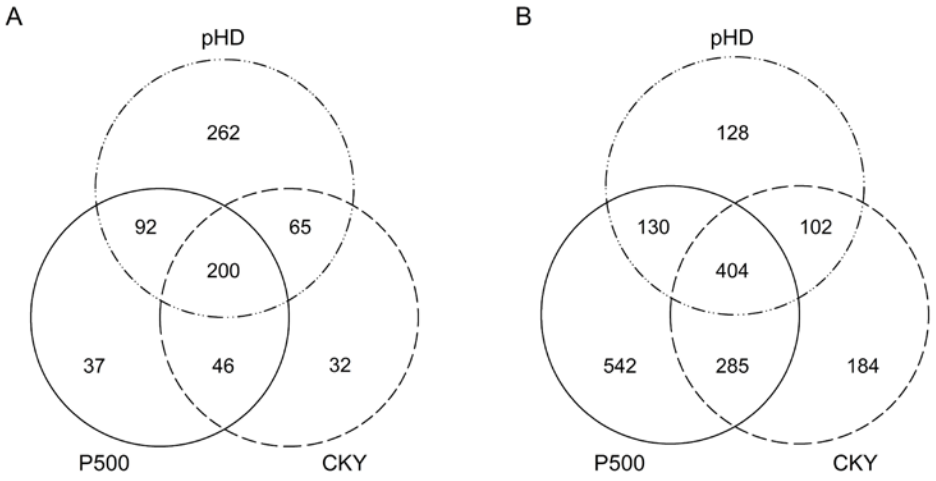

FIGURE 4 Compositions of ammonia-oxidizing microorganisms from different samples at genera level. The sum relative abundance of dominant genera exceeded 99%. P500: 500 mg l^{-1} particle concentration treatment; CKY: 0 mg l^{-1} organic carbon treatment; pHD: pH 7.5 treatment. (A) Ammonia-oxidizing archaea (AOA); (B) Ammonia-oxidizing bacteria (AOB)

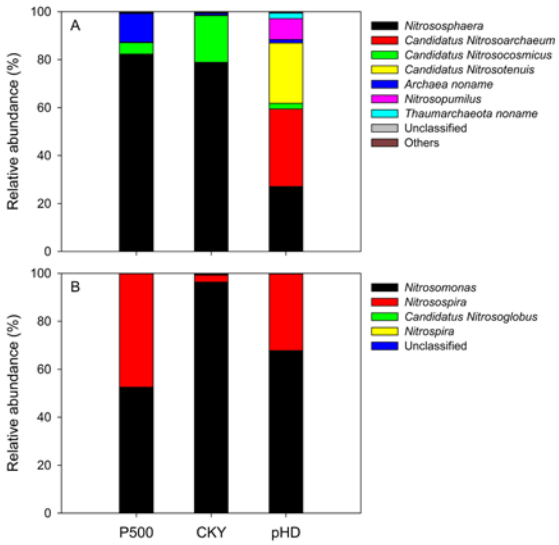

FIGURE 5 Compositions of ammonia-oxidizing microorganisms from different samples at species level. The sum relative abundance of dominant species exceeded 99%. P500: 500 mg l^{-1} particle concentration treatment; CKY: 0 mg l^{-1} organic carbon treatment; pHD: pH 7.5 treatment. (A) Ammonia-oxidizing archaea (AOA); (B) Ammonia-oxidizing bacteria (AOB)

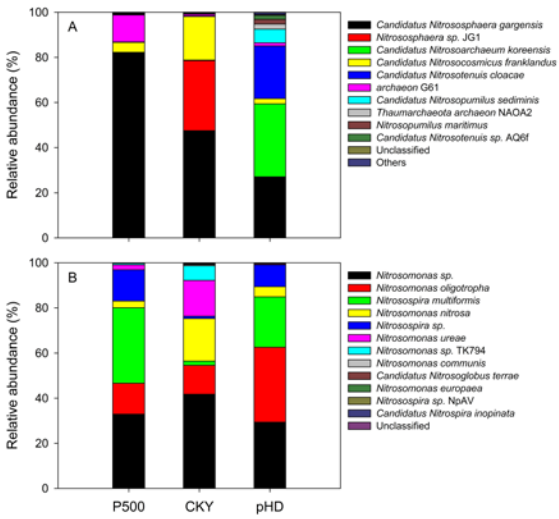

TABLE 1 Physiochemical characteristics of water and suspended particles used in this study

Experiment	Sampling time	Particle water content (%)	Water ($mg \cdot l^{-1}$)			
			NH_4^+-N	NO_3^--N	NO_2^--N	DOC
Particle concentration	21 May, 2017	77.3	0.82	0.70	0.06	-
Ammonia concentration	30 June, 2017	84.9	0.92	2.10	0.15	-
Organic carbon	20 July, 2017	69.5	0.41	0.47	0.04	19.44
pH	26 Nov, 2017	75.8	1.43	1.96	0.06	-
DO	30 Dec, 2017	72.6	1.23	2.79	0.10	-

NH_4^+-N: ammonium; NO_3^--N: nitrate; NO_2^--N: nitrite; DOC: dissolved organic carbon

TABLE 2 Results of one-way ANOVA testing of the effects of different treatments from different experiments on nitrogen transformation rate and *amoA* gene abundance of particle-attached microorganisms

Parameters	F value	P value
Particle concentration		
NH_4^+ removal rate	117.641	<0.001***
NO_2^-+NO_3^- accumulation rate	143.081	<0.001***
Abundance of AOA-*amoA* gene	6.790	<0.001***
Abundance of AOB-*amoA* gene	3.873	0.009**
Ammonia concentration		
NH_4^+ removal rate	209.564	<0.001***
NO_2^-+NO_3^- accumulation rate	78.566	<0.001***
Abundance of AOA-*amoA* gene	4.365	0.009**
Abundance of AOB-*amoA* gene	3.792	0.016*
Organic carbon (peptone)		

NH_4^+ removal rate	67.575	<0.001***
NO_2^-+NO_3^- accumulation rate	3.201	0.015*
Abundance of AOA-*amoA* gene	5.503	0.001**
Abundance of AOB-*amoA* gene	2.741	0.031*
Organic carbon (glucose)		
NH_4^+ removal rate	43.200	<0.001***
NO_2^-+NO_3^- accumulation rate	13.642	<0.001***
Abundance of AOA-*amoA* gene	1.763	0.142
Abundance of AOB-*amoA* gene	3.086	0.018*
pH		
NH_4^+ removal rate	1210.419	<0.001***
NO_2^-+NO_3^- accumulation rate	268.985	<0.001***
Abundance of AOA-*amoA* gene	2.747	0.039*

Abundance of AOB-*amoA* gene	5.359	0.002**
DO		
NH_4^+ removal rate	18.615	<0.001***
NO_2^-+NO_3^- accumulation rate	21.546	<0.001***
Abundance of AOA-*amoA* gene	1.640	0.211
Abundance of AOB-*amoA* gene	1.526	0.234

*: $p<0.05$; **: $p<0.01$; and ***: $p<0.001$; DO: dissolved oxygen

TABLE 3 Correlation analyses for the relationships between nitrogen transformation rate and abundance of *amoA* gene

Experiment	Nitrogen transformation rate (mg l^{-1} d^{-1})	Abundance of *amoA* gene (copies ml^{-1})	
		AOA-*amoA*	AOB-*amoA*
Particle concentration	NH_4^+ removal rate	0.651**	0.638**
	NO_2^-+NO_3^- accumulation rate	0.494**	0.653**
Ammonia concentration	NH_4^+ removal rate	0.316	0.569**

	NO_2^-+NO_3^- accumulation rate	0.222	0.520**
Organic carbon (peptone)	NH_4^+ removal rate	0.273	0.481**
	NO_2^-+NO_3^- accumulation rate	0.154	0.365*
Organic carbon (glucose)	NH_4^+ removal rate	0.260	0.648**
	NO_2^-+NO_3^- accumulation rate	0.187	0.570**
pH	NH_4^+ removal rate	-0.602**	0.698**
	NO_2^-+NO_3^- accumulation rate	-0.537**	0.712**
DO	NH_4^+ removal rate	0.286	0.507**
	NO_2^-+NO_3^- accumulation rate	0.150	0.475**

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level (2-tailed, Pearson's correlation); DO: dissolved oxygen

TABLE 4 Estimates of richness and diversity for ammonia-oxidizing bacteria (AOB) and archaea (AOA)

Type	Sample	Valid Tags	Coverage (%)	OTUs	ACE	Chao1	Shannon
AOA	P500	63978	99.8	375	462	444	4.33
	CKY	59190	99.9	343	396	383	3.69
	pHD	53864	99.9	619	644	632	5.33
AOB	P500	29367	99.2	1361	1521	1461	7.53
	CKY	30191	99.4	975	1090	1067	6.35
	pHD	30840	99.3	764	920	914	5.39

P500: 500 mg l^{-1} particle concentration treatment; CKY: 0 mg l^{-1} organic carbon treatment; pHD: pH 7.5 treatment; OTUs: operational taxonomic units; ACE: abundance-based coverage estimator