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### Authors

Cai, Xianlei  
Yao, Ling  
Wang, Siyu  
et al.

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# Properties of ammonia-oxidising bacteria and archaea in a hypereutrophic urban river network

Xianlei Cai<sup>1,2</sup>  | Ling Yao<sup>1</sup> | Siyu Wang<sup>1</sup> | Yuanyuan Hu<sup>1</sup> | Randy A. Dahlgren<sup>1,3</sup> | Zhenfeng Wang<sup>1,2</sup> | Huanhuan Deng<sup>1,2</sup> | Lizhen Liu<sup>4</sup>

<sup>1</sup>Zhejiang Provincial Key Laboratory of Watershed Science and Health, Wenzhou Medical University, Wenzhou, China

<sup>2</sup>Southern Zhejiang Water Research Institute, Wenzhou, China

<sup>3</sup>Department of Land, Air and Water Resources, University of California, Davis, California, USA

<sup>4</sup>Poyang Lake Research Center, Jiangxi Academy of Sciences, Nanchang, China

## Correspondence

Xianlei Cai, Zhejiang Provincial Key Laboratory of Watershed Science and Health, Wenzhou Medical University, Wenzhou 325035, China.  
Email: caixl@wmu.edu.cn

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## Abstract

1. Ammonia oxidation by ammonia-oxidising archaea (AOA) and bacteria (AOB) is the first and rate-limiting step of nitrification. By providing substrate to the nitrogen-removing denitrification processes, nitrification is an important process for attenuation of allochthonous nitrogen loads in urban rivers, thereby mitigating eutrophication of downstream ecosystems. However, the relative importance of AOA and AOB communities in ammonia oxidation, the distribution and varied patterns of these groups and the underlying mechanisms have received limited study in hypereutrophic urban river networks.
2. To investigate spatial and temporal patterns of particle-attached and free-living ammonia-oxidising microbial communities, we conducted a 2-year study of four different water quality/habitat conditions within a hypereutrophic urban river network of eastern China. The structure and dynamics of ammonia-oxidising microbial communities were determined by high-throughput sequencing based on the AOA and AOB *amoA* gene.
3. We found significant spatial and temporal heterogeneity of ammonia-oxidising microbial communities, but no significant differences between particle-attached and free-living fractions. Analysis revealed that changes in the diversity and composition of ammonia-oxidising microbial communities were mainly related to substrate availability (e.g., ammonia, nitrite, dissolved oxygen). AOB communities were more complex and had a higher species richness and diversity compared with AOA communities.
4. Our findings indicate that high anthropogenic nitrogen loadings appear to promote higher ammonia-oxidising microbial richness and diversity, and AOB are more adaptable to the hypereutrophic conditions, thereby playing a more important role than AOA in nitrogen transformations in hypereutrophic urban river networks.
5. This study highlights the importance of ammonia-oxidising microorganisms in nutrient-rich river systems and provides valuable insights into ammonia transport and transformations in urban river networks.

## KEYWORD

ammonia-oxidising bacteria, free-living, nitrogen, particle-attached, water quality,

## 1 | INTRODUCTION

Reactive nitrogen (refers to all forms of nitrogen except atmospheric  $N_2$ ) is both a necessary resource (e.g., agricultural nutrient) and a potential pollutant (e.g., surface and ground waters, greenhouse gas— $N_2O$ ) of increasing worldwide concern (Gu et al., 2015). As such, reactive nitrogen has a great influence on terrestrial and aquatic ecosystems (e.g., eutrophication/hypoxia, harmful algal blooms) (Galloway et al., 2008; Liu et al., 2013; Peterson et al., 2001). Urban aquatic ecosystems are highly impacted by human activities and are often heavily polluted by excess nitrogen (Lu et al., 2011). This is especially true in rapidly developing countries, such as China, where rapid urbanisation and economic development coupled with lagging waste/storm water infrastructure development result in excessive anthropogenic nitrogen loadings to urban rivers causing severe impairment to aquatic ecosystem health and function (Qiu, 2011; Zhang & Xu, 2011). Urban rivers provide numerous ecosystem services, including a water source for domestic and industrial use, aquaculture, wildlife habitat, recreation, and water transportation (Araya et al., 2003; Vörösmarty et al., 2010). Hence, the control and removal of excess nitrogen in urban rivers is a grand challenge for improvement of urban river ecology and prevention of downstream ecosystem impairment, such as coastal water eutrophication/hypoxia.

Traditional nitrification is a two-step biological process including the oxidation of ammonia to nitrite and the oxidation of nitrite to nitrate (Prosser, 1989). Ammonia oxidation by ammonia-oxidising archaea (AOA) and bacteria (AOB) is the first and rate-limiting step of nitrification (Hempel et al., 2018, 2020). By providing substrate to the nitrogen-removing denitrification processes, nitrification is an important process for attenuation of allochthonous nitrogen loads in urban rivers, thereby mitigating eutrophication of downstream ecosystems (Peterson et al., 2001). Ammonia and dissolved oxygen, as necessary substrates for ammonia oxidation, influence the species composition, community structure, and metabolic activity of AOA and AOB, thus leading to niche specialisation (i.e., adaptation to particular sets of abiotic and biotic characteristics) and differentiation (i.e., different patterns of resource utilisation) between AOA and AOB (Prosser & Nicol, 2012). For example, when ammonia availability increases, the competitive advantage of AOB over AOA is significantly improved (Herrmann et al., 2011). Further, as dissolved oxygen decreases, AOA have purported better adaptability than AOB (Liu et al., 2015). It is common in China for urban rivers to be impaired by high ammonia and low dissolved oxygen concentrations (Cai et al., 2016; Lu et al., 2011; Qiu, 2011; Zhang & Xu, 2011). In such aquatic environments, the relative importance of AOA and AOB communities in ammonia oxidation, and the distribution and varied patterns of these groups have received limited study.

The Wen-Rui Tang River is a typical plain river network that flows through a densely populated urban area (c. 3.0 million) in Wenzhou, eastern China. High riverine nitrogen pollution is a widespread problem in the Wen-Rui Tang River, as in much of China, due

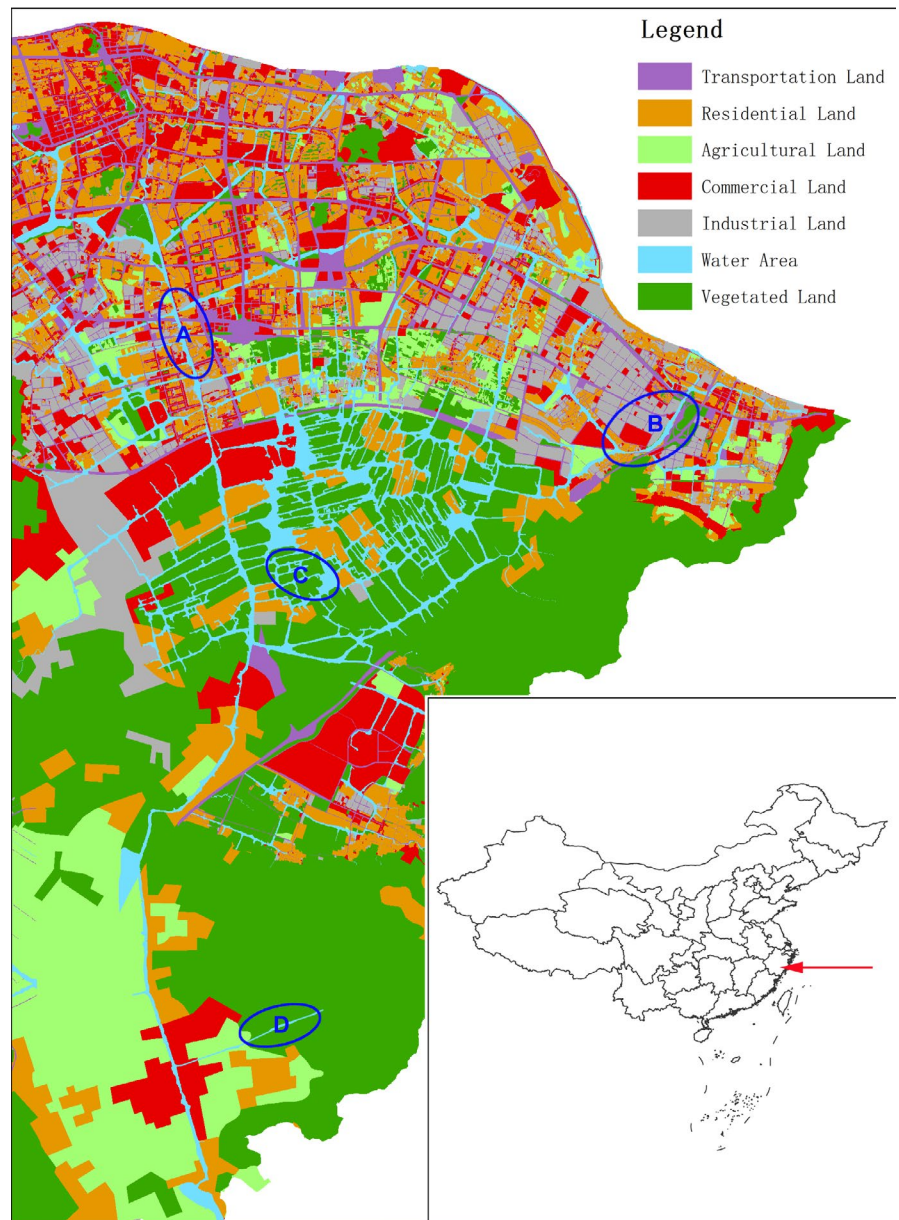
to rapid socio-economic development, urbanisation, and lagging waste/storm water collection and treatment (Lu et al., 2011). Water quality of the Wen-Rui Tang River is characterised by high ammonia (>2 mg N/L), low dissolved oxygen (<4 mg/L), and high turbidity (>20 NTU) (Cai et al., 2018, 2019). Thus, there is a critical need for research examining ammonia transport and transformations in the Wen-Rui Tang River network to provide fundamental knowledge of microbial ecological functions for management and remediation of eutrophic urban river systems. To date, our knowledge of the relationship between AOA and AOB in eutrophic urban river networks is incomplete, and the distribution and varied patterns of these communities are largely unknown. Hence, the Wen-Rui Tang River was selected as a representative research area to investigate AOA and AOB communities at different water quality/habitat conditions within the urban river network. The objectives of this 2-year study were: (1) to examine the diversity and composition of AOA and AOB communities in both particle-attached and free-living assemblages; (2) to investigate the distribution and spatial/temporal patterns of AOA and AOB communities; (3) to identify the main environmental factors that influence AOA and AOB communities; and (4) to determine the relative importance of AOA and AOB in ammonia oxidation in a hypereutrophic urban river.

## 2 | MATERIALS AND METHODS

### 2.1 | Study sites and sampling protocols

Water samples were collected from four distinct water quality/habitat conditions in the Wen-Rui Tang River network: River A (27.97°N, 120.67°E; commercial/residential; water quality classification = exceeding Type V; river depth 2.2–3.0 m; artificial bank with stone and concrete); River B (27.96°N, 120.74°E; industrial/commercial/residential; Type III to exceeding Type V; river depth 1.0–2.8 m; artificial bank with stone and concrete); River C (27.94°N, 120.68°E; vegetated/residential/agricultural; Type III to exceeding Type V; river depth 1.8–2.6 m; natural bank); and River D (27.87°N, 120.68°E; natural areas/vegetated; Type I to Type II; river depth 0.5–1.0 m; natural bank; Figure 1). Water quality was classified by the concentration of ammonia under the guidance of National Water Quality Guidelines for Surface Water (State Environment Protection Bureau of China, 2002). The four sites represent water quality classifications ranging from severely degraded waters (exceeding Type V—the lowest water quality classification that supports aquatic ecosystem health) to minor impairment (Type I—appropriate as a drinking water source). Climate is subtropical monsoon with a mean annual temperature of c. 18°C and annual precipitation c. 1,800 mm. Rainfall mainly occurs in May–September with a typhoon season in July–September. Samples were collected quarterly from July 2017 to March 2019 (8 sampling times) and consisted of three replicate samples at each site separated by a 100-m to 1-km distance. Samples were collected from surface waters (top 50 cm) using a 3-L Schindler

**FIGURE 1** Map of Wen-Rui Tang River, China, showing land use and river locations examined in this study



sampler, placed in sterilised polyethylene bottles, transported to the laboratory in a cooler with ice packs and processed within 12 hr.

After sampling, the microbes in water samples were divided into particle-attached and free-living assemblages in the laboratory. Particle-attached microbes were retained by filtering 100–600 ml of sample through a 5.0- $\mu\text{m}$  polycarbonate membrane (47-mm diameter, Millipore); and free-living microbes were collected by filtering 100–300 ml of the 5.0- $\mu\text{m}$  filtrate through a 0.2- $\mu\text{m}$  polycarbonate membrane (47-mm diameter, Millipore) (Hugoni et al., 2013; Kellogg & Deming, 2009; Tang et al., 2015). After filtration, the membrane was cut into pieces, placed in a 2-mL sterile centrifuge tube, and stored at  $-80^{\circ}\text{C}$  for subsequent DNA extraction. All laboratory equipment and operating procedures followed strict aseptic protocols to prevent post-collection contamination.

## 2.2 | Water quality characterisation

Water temperature (T), pH, and dissolved oxygen (DO) were measured in the field using a multi-parameter water quality sonde (YSI 650MDS/6920, Xylem). Water depth and Secchi depth were measured at each location, and used to calculate the Secchi:water column depth ratio (hereafter referred to as SD). The other physicochemical parameters were done after taking the water samples to the laboratory immediately. Ammonia ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), and orthophosphate ( $\text{PO}_4^{3-}$ ) were determined on filtered subsamples (0.2  $\mu\text{m}$ ), while total organic carbon (TOC), chlorophyll *a* (Chl *a*), and suspended solids (SS) were analysed in non-filtered subsamples using standard methods (Jin & Tu, 1990). We calculated dissolved inorganic nitrogen as  $\text{DIN} = \text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$ . All analytes were quantified at concentrations well above analytical detection limits.

## 2.3 | Molecular analysis of ammonia-oxidising microorganisms

Molecular analyses of AOA and AOB communities were conducted according to previous studies (Cai et al., 2019). The three replicate samples per river from each sampling date were pooled into one sample for DNA extraction. Total DNA for ammonia-oxidising microorganisms was extracted from frozen filters with the FastDNA<sup>®</sup> SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocols. DNA concentration and quality were determined by NanoDrop spectrophotometer (Nano-200; Hangzhou Allsheng Instruments) and agarose gel electrophoresis. Qualified samples were amplified with the forward and reverse primers of Arch-amoA26F/Arch-amoA417R (Park et al., 2008) and amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOA and AOB, respectively. The 25- $\mu$ L reaction mixture contained 50 ng template DNA, 1  $\mu$ M forward and reverse primers, and 12.5  $\mu$ L Phusion<sup>®</sup> Hot Start Flex 2x Master Mix (NEB). PCR amplification followed: denaturation at 98°C for 30s; 40 cycles of denaturation at 98°C for 10 s, annealing at 52.5°C for 30 s, extension at 72°C for 40 s; and a final extension at 72°C for 10 min. PCR amplicons were further analysed by NanoDrop spectrophotometer and agarose gel electrophoresis. The length of sequencing amplicons for AOA and AOB were 491 and 416, respectively.

The purified, barcoded amplicons were sequenced using an Illumina platform at LC-Biotechnology. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (Tanja & Salzberg, 2011). Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the fqtrim (V 0.94; <http://ccb.jhu.edu/software/fqtrim>). Chimeric sequences were filtered using Vsearch software (v2.3.4) (Rognes et al., 2016). Denoised sequences were clustered to operational taxonomic units (OTUs) at a 97% similarity level by Vsearch (v2.3.4). A representative sequence for each OTU was assigned a taxonomic identity using the RDP classifier (version 2.2) (Cole et al., 2014) based on the FGR functional gene database derived from GenBank (<http://fungene.cme.msu.edu>). OTU abundances were normalised using a standardising sequence number corresponding to the sample with the least sequences. OTU data were assessed by Good's coverage, Chao1 richness estimator, and Shannon index; all indices were calculated with QIIME (Version 1.8.0) (Caporaso et al., 2010).

## 2.4 | Statistical analyses

Univariate data are presented as mean  $\pm$  standard error. Differences among water quality parameters and diversity indices for ammonia-oxidising microorganisms were assessed using a repeated measures

ANOVA and three-way ANOVA with no interaction, respectively, followed by Duncan's multiple range test using a  $p < 0.05$  significance level. The assumptions of normality and variance homogeneity of residuals were checked graphically, and if necessary, subjected to square-root, arcsine square-root or log transformation. For the repeated measures ANOVA, the Greenhouse–Geisser adjustment was used if violation of sphericity occurred. Statistical comparisons between means were performed using SPSS 22.0 (SPSS Inc.), and data were plotted using SigmaPlot 12.0 (Systat Software).

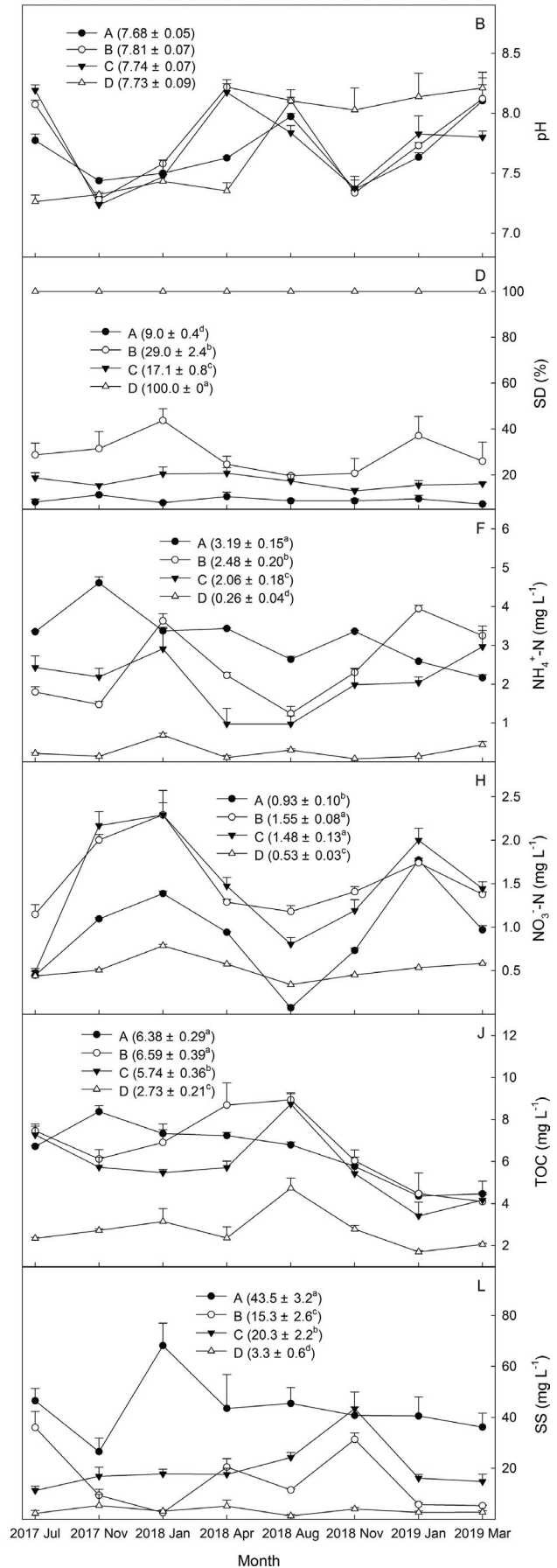
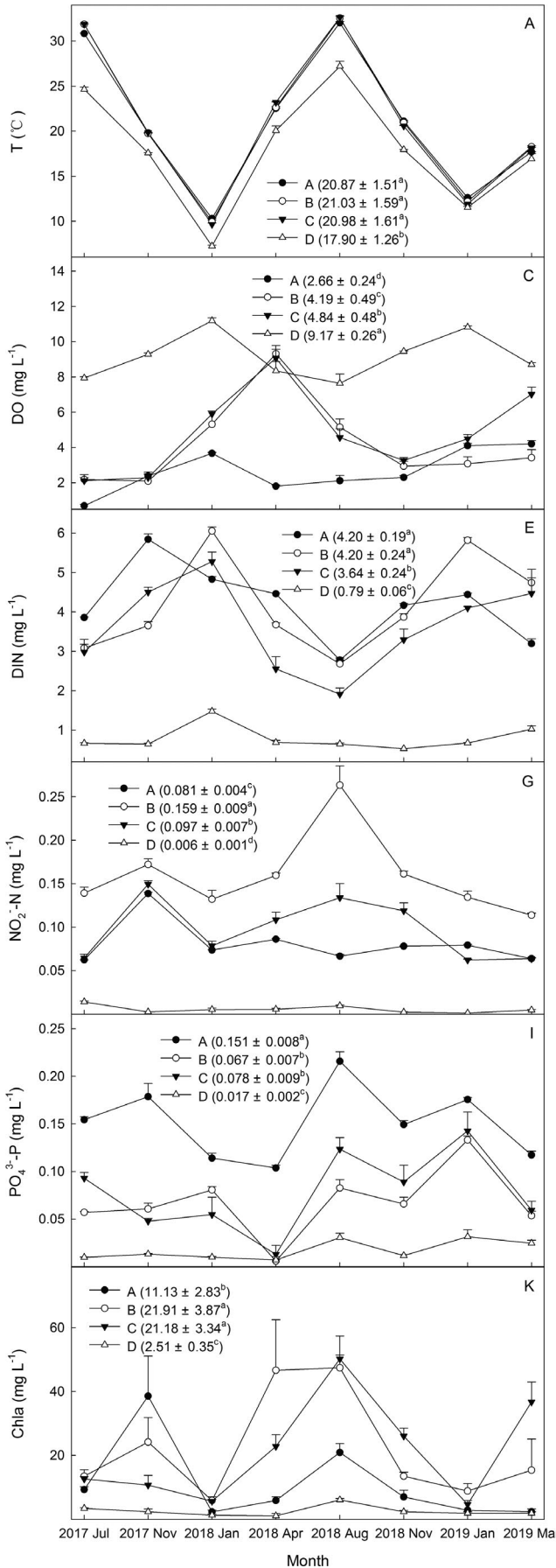
Multivariate analyses of ammonia-oxidising microorganism communities were performed for the relative abundance of OTUs. We used detrended correspondence analysis to assess how community level varied among sampling dates and river sites, and to compare differences between particle-attached and free-living communities. Analysis of similarity (ANOSIM) is a non-parametric test for differences between two or more groups (Clarke, 1993), and was used to test the effects of sampling date, river site and assemblage type (particle-attached vs. free-living) on ammonia-oxidising microorganism communities. The significance of ANOSIM was computed by 10,000 permutations based on Bray–Curtis indices. We used canonical correspondence analysis to examine correlations between ammonia-oxidising microorganism communities and water quality parameters, and assess which parameters were significant in explaining the microbial data. The significance of the canonical correspondence analysis was assessed by Monte Carlo permutation tests with 999 permutations under the reduced model. Multivariate statistical analysis was conducted using Canoco for Windows 5.0 (Ter Braak & Šmilauer, 2012) and PAST (PAleontological STatistics v3.14) (Hammer et al., 2001).

## 3 | RESULTS

### 3.1 | Water quality characterisation

Water quality at the four sites in the Wen-Rui Tang River network showed several significant differences (Figure 2). DO and SD were significantly lower, and concentrations of  $\text{PO}_4^{3-}$ ,  $\text{NH}_4^+$ , and SS were significantly higher in river A compared to the other rivers.  $\text{NH}_4^+$  was the dominant form of DIN with the exception of river D, which had a higher  $\text{NO}_3^-$  concentration. Additionally, most physicochemical water quality characteristics displayed irregular seasonal variations, except for T, DIN,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and Chl *a*. T exhibited a similar seasonal variation among different rivers with a maximum temperature in July–August (33°C) and minimum in January (7°C; Figure 2a). In the more nutrient-rich rivers (A, B, and C vs. D), DIN and  $\text{NO}_3^-$  concentrations revealed significant seasonal changes with a minima in spring–summer and maxima in autumn–winter, whereas  $\text{PO}_4^{3-}$  and Chl *a* concentrations were lower in spring and winter.

**FIGURE 2** Water quality parameters of surface waters at the four rivers in Wen-Rui Tang River network. Numbers in parentheses represent the mean  $\pm$  SE; means followed by different letters were significantly different according to Duncan test ( $p < 0.05$ )



### 3.2 | Richness and diversity of ammonia-oxidising microorganisms

Species richness and diversity indices for AOA and AOB based on *amoA* gene sequences are displayed in Table 1. The lowest sequence amounts for AOA and AOB that were used in the standardisation were 9,593 and 8,006, respectively. Mean Good's coverage for AOA and AOB was 98.9% and 97.7%, respectively, demonstrating that most phylotypes were detected. Across all samples, AOB communities had higher species richness and diversity than AOA communities as assessed from high-throughput sequencing data (Table 1). OTUs, Chao1, and Shannon for AOB communities displayed significant seasonal changes with maxima in autumn–winter and minima in spring–summer (Figure 3a,d,g). In contrast, there was no distinct seasonal change in Shannon values for the AOA communities (Figure 3g). In addition, for AOB communities, the number of OTUs and Chao1 value were significantly lower at river D compared to the other rivers. For AOA communities, the number of OTUs and Chao1 value were significantly lower at rivers B and D compared to rivers A and C (Figure 3b,e). Moreover, there were no significant differences in ammonia-oxidising microbial richness and diversity indices between particle-attached and free-living communities (Figure 3c,f,i).

### 3.3 | Taxonomic identification and community composition of ammonia-oxidising microorganisms

For ammonia-oxidising archaea, unclassified, unclassified *Thaumarchaeota*, *Candidatus Nitrosocosmicus* sp., unclassified *Crenarchaeota*, and *Candidatus Nitrosopumilus* sp. were dominant, accounting for >88% of AOA species across all samples (Figure 4a–c). For ammonia-oxidising bacteria, unclassified, unclassified *Nitrosomonadaceae*, uncultured beta proteobacterium UMTRA 728-D5, unclassified *Nitrosomonas*, and *Nitrosomonas* sp. Nm47 were dominant (Figure 4d–f). Compared with AOA, temporal and spatial changes in the relative abundance of dominant AOB species were more pronounced (Figure 4d,f). Notably, the relative abundance of unclassified was high in many samples, particularly in AOB communities (Figure 4). Additionally, the dominant species were similar between particle-attached and free-living communities, both for AOA and AOB (Figure 4c,f).

### 3.4 | Relationships between ammonia-oxidising microbial communities and environmental factors

Although a few samples (three of 64 for AOA and six of 64 for AOB) failed to adequately sequence, it had little effect on the overall statistical analyses. Detrended correspondence analysis showed ammonia-oxidising microbial communities were largely overlapping for particle-attached and free-living assemblages, regardless of AOA or AOB. Among sites, river D ammonia-oxidising microbial communities were distinct from those at the other river sites, especially for AOB (Figure 5a,b). Differences in ammonia-oxidising microbial composition within seasons were smaller than those between seasons, especially for AOB (Figure 5a,b). Analysis of similarity (ANOSIM) determined significant differences in ammonia-oxidising microbial community structure among both river sites and sampling dates, but there was no significant difference between particle-attached and free-living assemblages, regardless of AOA or AOB (Table 2).

We might expect to find strong regulation of ammonia-oxidising microbial communities by environmental factors associated with water quality conditions. Canonical correspondence analysis indicated that 12 water environmental parameters contributed significantly to the variances in ammonia-oxidising microbial communities (Figure 6a,b, Table 3). The top six explanatory parameters (SD, DIN,  $\text{NH}_4^+$ , DO,  $\text{NO}_2^-$ , and TOC) were the same between AOA and AOB, but some differences existed in their relative order. Most notably, the variation explained by DIN or  $\text{NH}_4^+$  was greater than that explained by DO for AOA; however, the opposite relationship occurred for AOB (Table 3).

## 4 | DISCUSSION

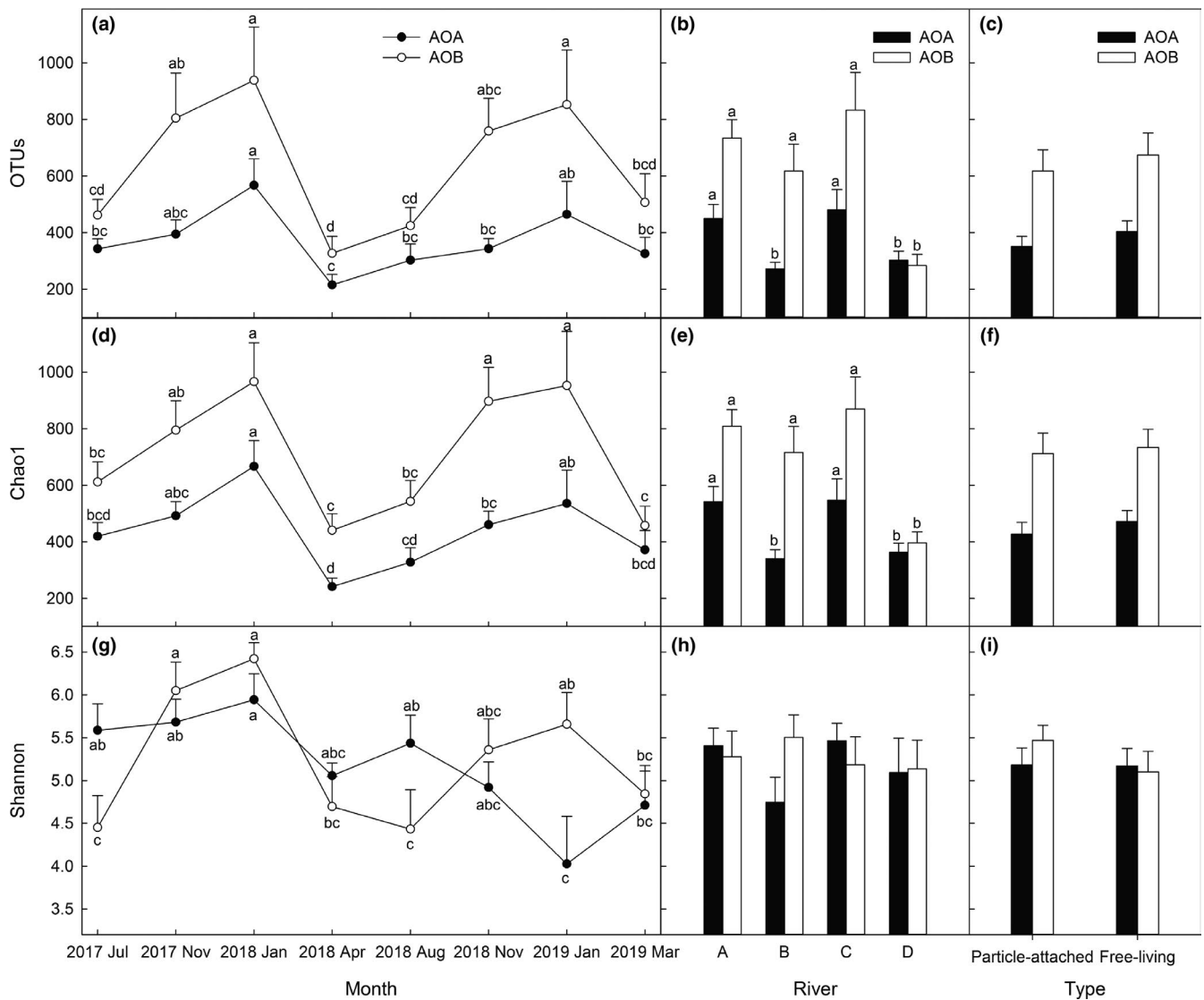
### 4.1 | Particle-attached and free-living ammonia-oxidising microbial communities

An objective of this work was to evaluate whether particle-attached and free-living ammonia-oxidising microorganisms differed in diversity and composition. Thus, the first step vital to microbial community characterisation was to distinguish particle-attached from free-living assemblages with fractional filtration. Although the possibility of population turnover (growth or lysis) in the source water during filtering was deemed unlikely (Padilla et al., 2015), there is the

	Valid tags	Coverage (%)	OTUs	Chao 1	Shannon
AOA	18,277 ± 1,322	98.9 ± 0.08	376 ± 26	449 ± 28	5.17 ± 0.14
AOB	24,514 ± 2,390	97.7 ± 0.18	645 ± 54	723 ± 48	5.28 ± 0.15

TABLE 1 Estimates of richness and diversity for AOA and AOB in Wen-Rui Tang River

Abbreviations: AOA, ammonia-oxidising archaea; AOB, ammonia-oxidising bacteria; OTUs, operational taxonomic units.



**FIGURE 3** Changes of the richness and diversity indices for AOA and AOB for different sampling dates, river sites and life types (particle-attached vs. free-living). Different letters indicate significant difference at  $p < 0.05$  (determined by Duncan test). AOA, ammonia-oxidising archaea; AOB, ammonia-oxidising bacteria

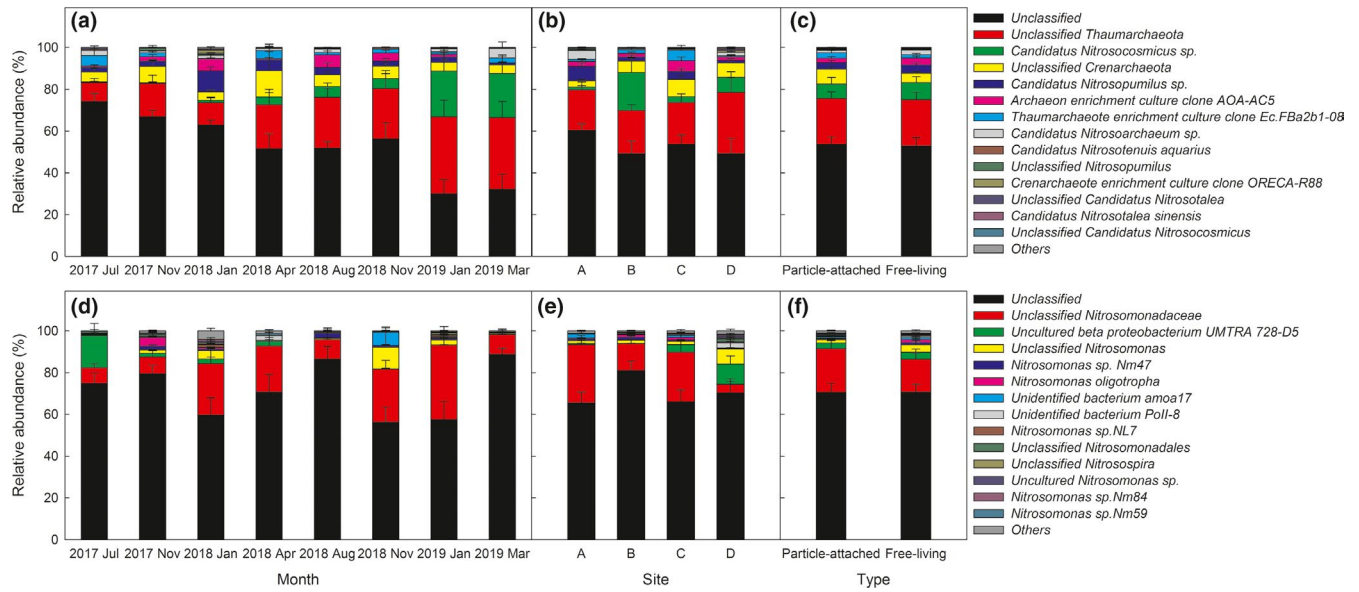
possibility that some free-living microorganisms could be detained on filters due to clogging, or alternatively, particle-attached microorganisms could enter into the free-living fraction due to detachment from particles (Padilla et al., 2015; Xie et al., 2020). To minimise these alterations, we collected particles using 5.0- $\mu\text{m}$  filters and a low vacuum filtration system, which are purported to be an appropriate choice for isolating particle-attached microbial communities under different trophic levels (Xie et al., 2020). Further, we adjusted the volume filtered to minimise clogging, and we completed the collection, isolation, and preservation steps as fast as possible (<12 hr) to minimise microbial attachment/detachment from particles.

In general, distinct differences are often detected for abundances, morphologies and functions between particle-attached and non-attached counterparts (Dang & Lovell, 2016; Grossart, 2010). However, studies such as Tang et al. (2015) report few differences between particle-attached and free-living bacterial communities

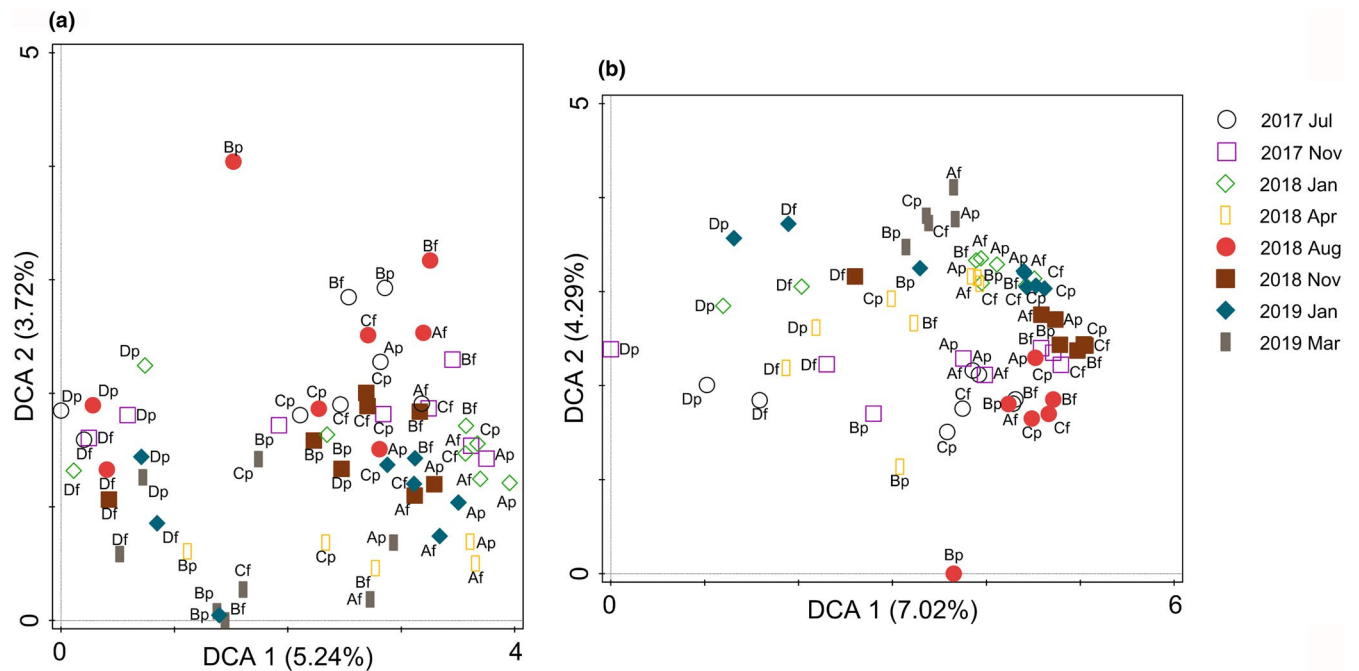
in Meiliang Bay, Lake Taihu (China). Overall, our monitoring of ammonia-oxidising microbial communities (AOA and AOB) over the course of 2 years in a hypereutrophic urban river network revealed little difference in diversity indices and species composition between particle-attached and free-living fractions. Although aquatic microorganisms can be classified into particle-attached and free-living microorganisms according to their different lifestyles (Grossart, 2010), the research from Tang et al. (2017) indicated that some rare aquatic bacterial species are truly particle-attached or truly free-living bacteria. Hence, in aquatic ecosystems, many microorganisms may alternate frequently between particle-attached and free-living stages during their complex life history (Grossart, 2010; Tang et al., 2017). This may be one reason for the similar ammonia-oxidising microbial communities between particle-attached and free-living fractions.

Recently, some researchers suggested that hydrodynamic forcing and the concentration and size of suspended particles play vital roles





**FIGURE 4** Changes in compositions of ammonia-oxidising microorganisms for different sampling dates, river sites and life types (particle-attached vs. free-living) at species level. The sum for the relative abundance of dominant species exceeded 99%. (a–c) Ammonia-oxidising archaea (AOA); (d–f) Ammonia-oxidising bacteria (AOB)



**FIGURE 5** Detrended correspondence analysis (DCA) showing ammonia-oxidising archaea (a) and bacteria (b) assemblages. A: River A, B: River B, C: River C, D: River D; p: particle-attached, f: free-living

in the high overlap between particle-attached and free-living microbial community composition (Tang et al., 2017; Yang et al., 2020). The Wen-Rui Tang River is a network of urban waterways in the Wen-Rui plain. There is continuous ship traffic in this river causing localised water mixing and sediment entrainment. Moreover, this river experiences frequent typhoon impacts in summer. In the present study, we found the average concentration of SS, which is an indicator of sediment resuspension and detritus, was higher than that in Meiliang Bay, Lake

Taihu (Tang et al., 2015), which is a large, shallow, eutrophic subtropical lake experiencing appreciable wind-induced sediment resuspension (Tang et al., 2017). High concentrations of suspended particles driven by hydrodynamic mixing and shear stress may contribute to collision of particles resulting in rapid exchange of microbes between particle-attached and free-living forms (Simon et al., 2002; Tang et al., 2010, 2015). Additionally, under microscope observation, the majority of suspended particles consisted of debris from zooplankton, phytoplankton,

and unidentifiable components, and the size of most particles was  $<100\ \mu\text{m}$ . This suspended particle size is appreciably smaller than that ( $>500\ \mu\text{m}$ ) in pelagic systems experiencing distinct differences between particle-attached and free-living fractions (Simon et al., 2002). Some research suggests that the size of suspended particles may influence the overlap between particle-attached and free-living assemblages, with smaller particles offering fewer niches to support truly particle-attached microbial colonisation (Tang et al., 2017). Thus, there are several possible reasons to explain the lack of differences between particle-attached and free-living assemblages.

## 4.2 | Spatial and temporal patterns of ammonia-oxidising microbial communities

We investigated the spatial and temporal patterns of ammonia-oxidising microbial communities across several water quality/habitat conditions within the urban river network. It is documented that ammonia-oxidising microbial communities are affected by nutrient

**TABLE 2** Analysis of similarity showing differences in ammonia-oxidising microbial assemblages for different sampling dates, river sites and life types (particle attached vs. free-living)

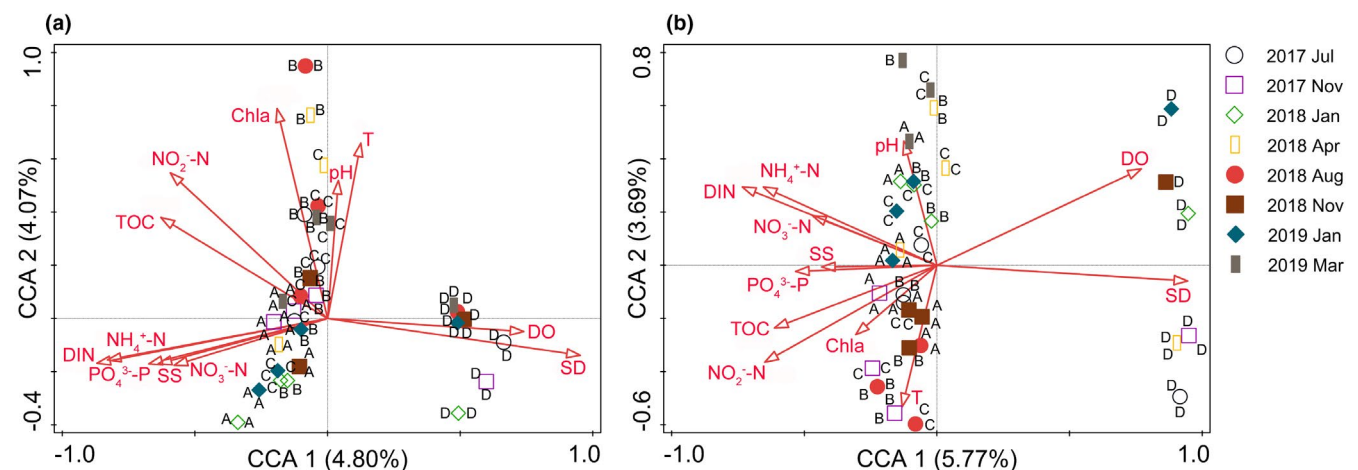
	Comparison	Sample statistic R	p value
AOA	Types	0.053	0.034
	Months	0.269	$<0.001$
	Rivers	0.427	$<0.001$
AOB	Types	0.022	0.130
	Months	0.454	$<0.001$
	Rivers	0.295	$<0.001$

Abbreviations: AOA, ammonia-oxidising archaea; AOB, ammonia-oxidising bacteria.

levels (Herrmann et al., 2011; Lage et al., 2010). When contrasted with river D (lowest  $\text{NH}_4^+$  concentrations; lowest anthropogenic nutrient loadings), there was a pronounced higher richness and diversity in ammonia-oxidising microbial communities at rivers A, B, and C, with the exception of indices for AOA communities at river B. These results suggested that anthropogenic nutrient loadings have a strong impact on ammonia-oxidising microbial community structure and significantly increase their diversity and richness. While the nutrient concentrations at river B appear sufficient to support the richness and diversity of AOA communities, the appreciably lower richness/diversity of AOA was possibly associated with inputs of untreated industrial wastewater (i.e., toxicity potential) from the surrounding industrial area (Subrahmanyam et al., 2014).

We investigated temporal changes of ammonia-oxidising microbial communities to assess their relationship to seasonal variations in water quality conditions. Microbial communities are highly dynamic, experience fast growth rates and are highly sensitive to environmental fluctuations (Araya et al., 2003; Paerl et al., 2003). Hence, seasonal variations in microbial communities are very common and often linked to seasonal changes in environmental conditions. In the present work,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were the two main forms of DIN, and they together contributed to the seasonal variations of DIN. In general, there were similar seasonal changes for the three nitrogen components ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and DIN) with highest concentrations in autumn–winter. The high anthropogenic nitrogen loadings in autumn–winter appear to promote higher ammonia-oxidising microbial richness and diversity compared to the other seasons. Our results indicate that the seasonal variations of ammonia-oxidising microbial communities were closely related to the periodic changes of anthropogenic nitrogen loadings, which in turn are influenced by the seasonal precipitation/river discharge pattern (wet summer–dry winter).

Additionally, seasonal algal blooms may be an important factor that could affect the temporal patterns of ammonia-oxidising microbial communities. Some researchers suggested



**FIGURE 6** Canonical correspondence analysis (CCA) showing the effects of environmental factors on ammonia-oxidising archaea (a) and bacteria (b) communities. Ammonia-oxidising microbial communities were completely overlapping for particle-attached and free-living assemblages, regardless of AOA or AOB. A: River A, B: River B, C: River C, D: River D

**TABLE 3** Percentage of the total variance for ammonia-oxidising archaea (AOA) and bacteria (AOB) communities explained by environmental factors as determined by canonical correspondence analysis

Explanatory variables	Explained variance %	F value	p value
<b>AOA</b>			
SD	4.6	2.8	0.001
DIN	4.1	2.5	0.001
NH <sub>4</sub> <sup>+</sup>	3.9	2.4	0.001
DO	3.8	2.3	0.001
NO <sub>2</sub> <sup>-</sup>	3.7	2.2	0.001
TOC	3.5	2.1	0.001
Chl <i>a</i>	3.3	2.0	0.001
PO <sub>4</sub> <sup>3-</sup>	3.3	2.0	0.001
T	3.3	2.0	0.001
SS	3.2	1.9	0.001
NO <sub>3</sub> <sup>-</sup>	3.1	1.9	0.001
pH	2.5	1.5	0.003
<b>AOB</b>			
SD	5.4	3.2	0.001
DO	4.5	2.6	0.001
DIN	4.2	2.5	0.001
NO <sub>2</sub> <sup>-</sup>	3.9	2.3	0.001
TOC	3.7	2.1	0.001
NH <sub>4</sub> <sup>+</sup>	3.7	2.1	0.001
T	3.3	1.9	0.001
Chl <i>a</i>	3.1	1.8	0.001
NO <sub>3</sub> <sup>-</sup>	3.0	1.7	0.002
PO <sub>4</sub> <sup>3-</sup>	2.9	1.7	0.001
pH	2.9	1.7	0.001
SS	2.5	1.4	0.015

Abbreviations: NO<sub>2</sub><sup>-</sup>, nitrite; NO<sub>3</sub><sup>-</sup>, nitrate; PO<sub>4</sub><sup>3-</sup>, orthophosphate; Chl *a*, chlorophyll *a*; DIN, dissolved inorganic nitrogen; DO, dissolved oxygen; NH<sub>4</sub><sup>+</sup>, ammonia; SD, the ratio of Secchi depth/Water depth; SS, suspended solids; T, water temperature; TOC: total organic carbon.

that ammonia-oxidising microorganisms compete with algae and other primary producers for NH<sub>4</sub><sup>+</sup> (Glibert et al., 2016; Hampel et al., 2018, 2020). In the present study, the results of water quality analysis showed that the increases in the concentrations of Chl *a* in the April–August months were related to the decreases in the concentrations of DIN, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and PO<sub>4</sub><sup>3-</sup> in the relevant months, suggesting that summer algal blooms were possibly responsible for contributing to the decrease of ambient nutrient concentrations. Meanwhile, contrasted with the maxima in autumn–winter, there was a significantly lower richness and diversity in ammonia-oxidising microbial communities in spring–summer, with the exception of the irregular Shannon for the AOA communities. Considering that ammonia-oxidising microorganisms were poor competitors for NH<sub>4</sub><sup>+</sup> during the algal bloom (Hampel et al., 2018), these results

suggested that summer algal blooms might be one reason for the decrease of diversity and richness in ammonia-oxidising microbial community in spring–summer.

### 4.3 | AOA and AOB communities in the hypereutrophic urban river network

An analysis of species composition based on the *amoA* gene found that the dominant AOA and AOB species were somewhat contrary to our previous studies of urban rivers (Cai et al., 2018, 2019). Previously, we found a dominance of the AOA genus *Nitrososphaera* and AOB genus *Nitrosomonas* in attached ammonia-oxidising microbial communities comprising biofilms. These contrasting findings indicate that ammonia-oxidising microbial communities are complex and dynamic in hypereutrophic urban river networks. As research continues, molecular techniques provide more detailed information to challenge some earlier hypotheses, such as AOB dominate ammonia oxidation in high-nutrient or terrestrial-influenced systems (Hampel et al., 2020). These contrasting findings further indicate that ammonia-oxidising microbial communities are complex and vary across time and space. Therefore, the main environmental factors that influence AOA and AOB communities, and the contributions of AOA and AOB communities to ammonia oxidation in different environmental conditions have yet to be fully quantified.

Based on our 2-year data record, six parameters (SD, DIN, NH<sub>4</sub><sup>+</sup>, DO, NO<sub>2</sub><sup>-</sup>, and TOC) were identified as the most important environmental factors influencing variation in microbial communities of AOA and AOB. Clearly, multiple physicochemical parameters interact to influence the ammonia-oxidising microbial communities, and shape the ecological niches of AOA and AOB in this urban river network. The DIN, NH<sub>4</sub><sup>+</sup>, and NO<sub>2</sub><sup>-</sup> parameters all reflect the importance of nitrogen substrates in the ammonia oxidation process (Hatzenpichler, 2012; Kowalchuk & Stephen, 2001).

The SD parameter reflects the proportion of the water column that is within the photic zone (i.e., zone with sufficient light to drive photosynthesis). Interestingly, in hypereutrophic urban rivers, the concentration of Chl *a*, which is used as an indication of algal biomass, not only followed the seasonal variations in water temperature (a measure of incident solar radiation), but also revealed significant spatial differences that were negatively correlated with SS concentration, which decreases the photic zone. In contrast to rivers B and C, the growth and biomass of algae was inhibited significantly in river A due to its high SS concentration and low SD values. Under adequate nutrient conditions, a larger photic zone promotes greater algal photosynthesis and biomass that in turn leads to higher DO production to facilitate the aerobic ammonium oxidation process (Guisasola et al., 2005). Thus, the six most influential water quality parameters are fully consistent with the ammonia oxidation process requiring both NH<sub>4</sub><sup>+</sup> and DO as substrates.

Our results further demonstrated that the relative importance of water quality parameters on the microbial communities of AOA and AOB were somewhat different, indicating contrasting controls on AOA

and AOB community dynamics. Our analyses indicated that AOA communities were more sensitive to  $\text{NH}_4^+$  concentration, whereas the AOB community was more sensitive to dissolved oxygen concentration. In the present study, AOB communities demonstrated greater complexity than AOA communities. We posit that AOB are better adapted to hypereutrophic urban river conditions than AOA, as evidenced by the composition, richness and diversity of ammonia-oxidising microbial communities. Our results agree with previous research indicating that AOB communities are commonly tightly linked in ammonium-rich environments (Aalto et al., 2018; Hampel et al., 2018). These findings are also consistent with our previous study (Cai et al., 2019) and infer that AOB may play a more important role than AOA in this urban river network with high ammonia and low dissolved oxygen concentrations. These results provide new information for understanding the dynamics of ammonia-oxidising microorganisms in nutrient-rich, urban river systems, and provide valuable insights into ammonia transport and transformation processes in hypereutrophic urban river networks.

Considering that urban rivers are hotspots for regional nitrogen pollution (Yang et al., 2021; Zhang et al., 2015), it is of crucial importance to investigate the microorganism-mediated nitrogen cycles in such environments for water quality improvement and human health. Here, we studied the spatial and temporal heterogeneity of particle-attached and free-living ammonia-oxidising microbial communities within a hypereutrophic urban river network of eastern China. We demonstrated that ammonia-oxidising microbial communities were similar between particle-attached and free-living assemblages, and complexity of ammonia-oxidising microbial community composition was present. Furthermore, our results indicate that multiple physicochemical parameters interact to influence ammonia-oxidising microbial communities, with factors affecting substrate availability (e.g.,  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , DO) having the strongest effects in shaping the ecological niches of AOA and AOB. Given the importance of  $\text{NH}_4^+$  as a substrate for the AOA/AOB communities, anthropogenic nitrogen loading has a disproportionate impact on ammonia-oxidising microbial community structure. High anthropogenic nitrogen loadings appear to promote higher ammonia-oxidising microbial richness and diversity in this urban river network. However, additional studies are still needed to further elucidate the underlying mechanisms. Results showed that AOB communities had a more complex and higher species richness and diversity compared to AOA communities. This suggests that AOB are better adapted for survival, and may play a more important role than AOA in hypereutrophic urban river networks.

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## DATA AVAILABILITY STATEMENT

The raw sequence data of bacterial and archaeal *amoA* gene in this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRP288451 and SRP288464. The data supporting the conclusions of this article are included within the article.

## ORCID

Xianlei Cai  <https://orcid.org/0000-0003-2074-2821>

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