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Dynamics and environmental importance of anaerobic ammonium oxidation (anammox) bacteria in urban river networks☆

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is recognized as an important bioprocess for nitrogen removal, yet little is known about the associated microbial communities in urban river networks which are intensively disturbed by human activity. In the present study, we investigated the community composition and abundance of anammox bacteria in the urban river network of Shanghai, and explored their potential correlations with nitrogen removal activities and the environmental parameters. High biodiversity of anammox bacteria was detected in the sediment of urban river networks, including *Candidatus* Brocadia, Scalindua, Jettenia, and Kuenenia. Anammox bacterial abundance ranged from 3.7×10^6 to 3.9×10^7 copies g^{-1} dry sediment based on 16S rRNA gene, which was strongly correlated to the metabolic activity of anammox bacteria ($P < 0.01$). A strong linkage between anammox bacteria and denitrifiers was detected ($P < 0.05$), implying a potential metabolic interdependence between these two nitrogen-removing microbes was existed in urban river networks. Sediment ammonium (NH_4^+) made a significant contribution to the anammox bacterial community-environment relationship, while anammox bacterial abundance related significantly with sediment total organic carbon (TOC) and silt contents ($P < 0.05$). However, no statistically significant correlation was observed between cell-specific anammox rate and the measured environmental factors ($P > 0.05$). In general, the community composition and abundance of anammox bacteria in different hierarchies of the river network was homogeneous, without significant spatial variations ($P > 0.05$). These results provided an opportunity to further understand the microbial mechanism of nitrogen removal bioprocesses in urban river networks.

1. Introduction

Human activities have more than doubled the reactive nitrogen inputs globally and these excessive anthropogenic nitrogen emissions are consequently becoming one of the most serious global environmental issues, which can potentially cause irreversible ecological problems (Gruber and Galloway, 2008; Rockstrom, 2009; Tan et al., 2019). For example, a series of environmental problems, such as water contamination, algal blooms, acid precipitation, and global warming, were related to anthropogenic nitrogen overload, especially in urbanized estuarine regions (Gu et al., 2012; Pennino et al., 2016; Yu et al., 2013). Currently, more than half the world's population live in cities (Grimm et al., 2008a,b), and urbanization related activities, which bring about extensive nitrogen pollution from industry discharge and domestic emissions, have substantially altered the regional and global biogeochemical cycle of nitrogen (Gu et al., 2012; Zhang et al., 2015). With the ever-growing urbanization, increasing anthropogenic nitrogen loadings have been delivered to the urban river networks, thereby exerting a serious threat to the health of both the aquatic ecosystems and urban residents, as urban river water quality has great significance to residents not only for drinking but also for entertainments (Gu et al., 2012). Thus, it is of crucial importance to investigate the microorganism-mediated nitrogen removal processes in urban river networks for water quality protection and improvement.

Shanghai, one of the world's most developed and urbanized metropolises, located in the Yangtze River Delta and eastern China. The urban river network in Shanghai is dense, with more than 1000 rivers and a total of over 1500 km river length (Yu et al., 2013). The

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water area of the urban river network is about 570 km^2, equivalent to approximately 9% of the total area of Shanghai (6300 km^2) (Yu et al., 2013). Additionally, a huge population of over 24 million made Shanghai under the pressure of high population density (approximately 3800 people per square kilometer) (Dai et al., 2017). With the rapid urbanization of Shanghai, inputs of nitrogen into the surface water of its river network increased approximately 7 times in the past few decades, contributed both by point sources from industrial and domestic wastewater and diffuse sources through agriculture (Gu et al., 2012). This overload of anthropogenic nitrogen has already caused a rapid degradation of water quality in urban rivers of Shanghai and the downstream waterbodies (the Yangtze Estuary), as well as a series of other nitrogen pollution related consequences on regional and even global environment health (Grimm et al., 2008a,b; Hou et al., 2013; Ren et al., 2003). Thus, understandings of the nitrogen loss processes, which are critical in aquatic ecosystems for maintaining homeostasis, and the related microbial mechanisms are of great importance for developing nitrogen managements to protect the river network of Shanghai and the downstream waterbodies (Hale et al., 2014; Naeher et al., 2015; Pennino et al., 2016; Zheng et al., 2016a).

Of the nitrogen removal processes, anaerobic ammonium oxidation (anammox), the bioprocess of oxidizing ammonium (NH_4^+) via reducing nitrite (NO_2^-) under anaerobic conditions, removes reactive nitrogen from aquatic environments permanently and returns it back to the air as nitrogen gas (Kuypers et al., 2003). Anammox was first discovered in a wastewater treatment plant (WWTP) in the Netherlands in 1995 (Mulder et al., 1995). After that, anammox has been detected in various natural ecosystems, such as freshwater sediments, marine sediments, and anaerobic water columns (Jiao et al., 2018; Kuypers et al., 2003; Lam et al., 2009; Naeher et al., 2015; Tan et al., 2019; Yang et al., 2017; Zheng et al., 2016a). Since N_2 rather than N_2O is the direct product, anammox is an environment-friendly nitrogen removal pathway alternative to heterotrophic denitrification (Nie et al., 2018). However, studies about the community structure and importance of anammox bacteria in urban river networks were rarely reported. Due to extensive human modification and distribution, such as impermeable riparian and wastewater outfall (Gu et al., 2012; Grimm et al., 2008a,b; Zhang et al., 2015), environmental conditions of urban rivers might be extremely complex and the anammox bacterial communities and associated activity might also be different from other ecosystems. The objectives of this study were to examine the community biodiversity and abundance of anammox bacteria and their potential correlations with associated nitrogen removal activities and the environmental variables in the urban river network of Shanghai. This work provides useful information for urban river nitrogen control and improves understandings of the microbial nitrogen cycle in urban river networks.

2. Materials and methods

2.1. Study area and sample collection

Huangpu River system (the Huangpu River and its branches including Suzhou Creek, Wenzaobang Creek, Dianpu River, etc.) makes up the backbone of the urban river network of Shanghai. The Huangpu River, a primary shipping channel in Shanghai city, flows northeastwardly into the Yangtze Estuary and originates from the Dianshan Lake. Its annual mean water velocity and discharge are 0.8 $m\,s^{-1}$ and 400 $m^3\,s^{-1}$, respectively (Yu et al., 2013). Since the implement of the coastal development policy in the nineteen-eighties, the land use patterns especially the hydrological infrastructures have changed tremendously, which has brought serious impact on the quality of Shanghai river network (Gu et al., 2012).

In this study, samples of surface sediments and overlying water were collected in January 2015 from 47 representative sampling sites covering urban river network in Shanghai area, considering the river location, river length, and river density (Fig. 1). In this study, based on the river size and their importance, the rivers of Shanghai river network were divided into three hierarchies. 12 sampling sites (F1 to F12) locating along the Huangpu River were the first-order river sites. 16 sites (S1 to S16) locating at the main tributaries of the Huangpu River were the second-order river points. In addition, 19 sites (T1 to T19) locating at the smallest creeks were the third-order river sites. Furthermore, 13 of these sampling sites were located in the center of Shanghai city, while the other 34 sites in the suburbs.

At each sampling station, surface sediments (0–5 cm) were collected in triplicate with plexiglass corers and sealed in airtight bags without air. In addition, samples of overlying water were taken and filtered (0.22 μm, WaterMan) immediately into polyethylene bottles. After collection, all samples were placed on ice and brought back to the laboratory within 4 h. Upon in the lab, sediments from each site were homogenized immediately under helium condition as one composite sample. Subsequently, one part of the homogeneous sediment sample from each site was stored under −80 °C for total DNA extraction and the subsequent molecular analyses, while the remaining part was stored at 4 °C for sediment physiochemical analyses and anammox activity measurement.

Environmental parameters [total organic carbon (TOC), NH_4^+, NO_3^- (plus NO_2^-), Fe(II), Fe(III), sulfide, and grain size of the sediment and pH of the overlying water] and the potential rates of anammox in the sediment from the urban river network of Shanghai were analyzed previously (Cheng et al., 2016) and have been shown in Table S1 and Fig. S1, respectively.

2.2. DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis

Total genomic DNA of the sediment samples was extracted from approximately 0.25 g sediment using DNA Isolation Kits (Powersoil™, MOBIO, USA) according to the instructions from the manufacturer. Subsequently, a nested PCR approach was established for T-RFLP analysis of anammox bacterial 16S rRNA gene sequences, including an initial PCR amplification of *Planctomycetale* 16S rRNA gene with primer pair Pla46f/1390r (Neef et al., 1998; Zheng et al., 1996), which followed by a second PCR targeting anammox bacteria with Amx368f/Amx820r (a final 477 bp PCR product) (Schmid et al., 2000, 2003). During the second PCR, the forward primer Amx368f was fluorescently labeled with 6-carboxyfluorescein (FAM) at the 5′ end (Abdo et al., 2006; Bunbar et al., 2001). The primers, PCR mixtures and conditions can be found in Table S2. PCR products were purified using Gel Advance-Gel Extraction system (Viogene, China). Purified PCR products (50 ng) were digested in duplicate with enzyme *Alu*I at 37 °C overnight (Dale et al., 2009). Subsequently, the digested samples were analyzed using ABI 3130X genetic analyzer (Applied Biosystems, Foster City, CA) and GeneMapper software (version 4.0, Applied Biosystems, Foster City, CA). Only peaks accounting for higher than 4% of the total chromatogram area were taken into account for further analysis (Zheng et al., 2016b).

2.3. Clone library construction, sequencing, and phylogenetic analysis

PCR amplification for anammox bacterial 16S rRNA gene clone library construction was the same as that described above, with

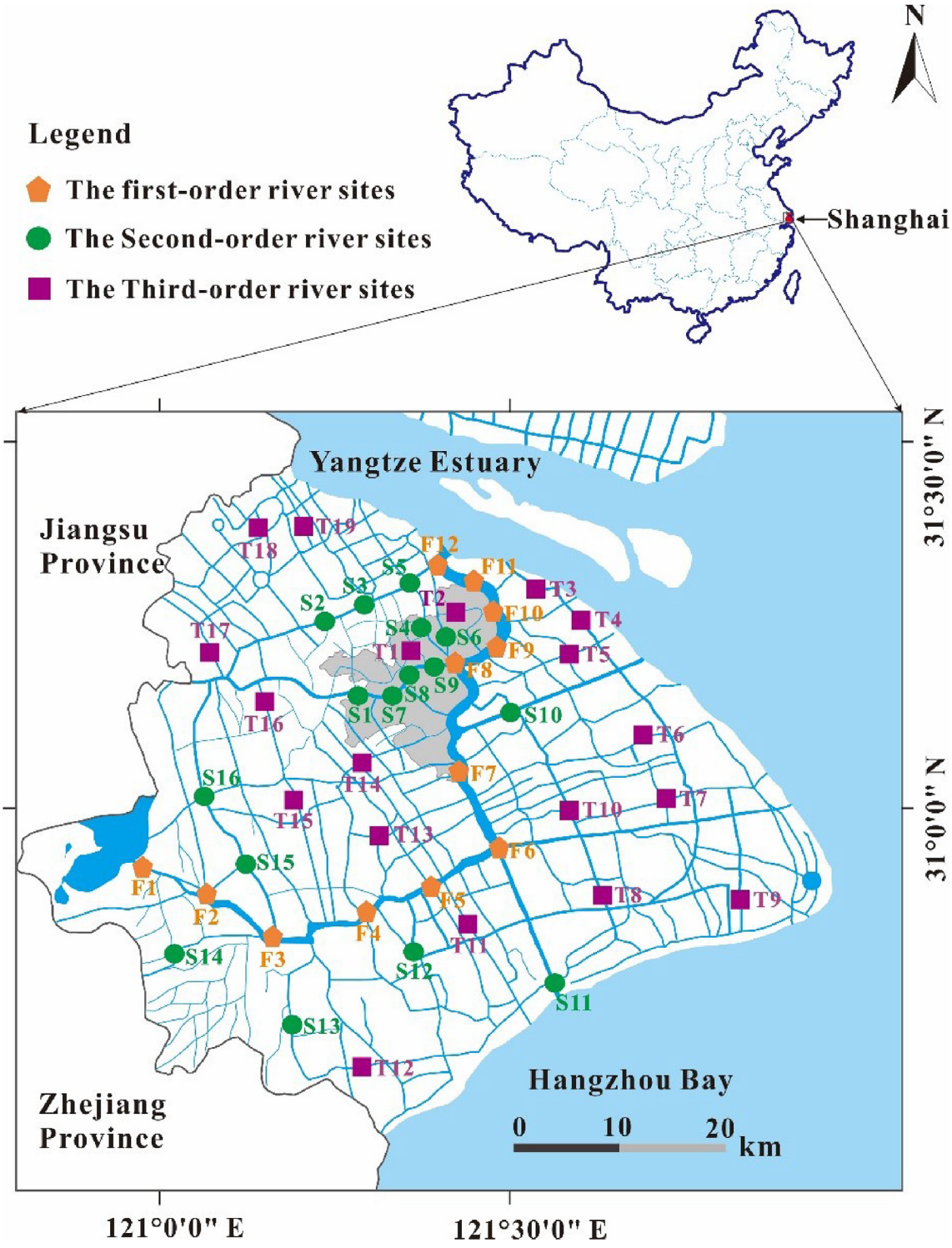

Fig. 1. Study area. The figure shows the location of Shanghai, and the sampling sites in the urban river network of Shanghai.

unlabeled forward primer. Fragments with appropriate size were separated using 1% agarose gel electrophoresis. The purified PCR results were cloned into the TOPO-TA vector (Invitrogen, USA) (Zheng et al., 2016a). Clones were randomly selected and sequenced on an ABI Prism genetic analyzer (Applied Biosystems, Canada). The qualified nucleic acid sequences displaying higher than 97% identity were grouped into one operational taxonomic unit (OTU) using Mothur (version 1.23.0) based on the furthest neighbor approach (Schloss et al., 2009). MEGA software (version 7.0.21) was used to construct a neighbor-joining phylogenetic tree (Tamura et al., 2007). The robustness of the tree topologies was evaluated by carrying out 1000 bootstrap replicates (Tamura et al., 2007). Qualified clones sequenced in this study can be downloaded from GenBank with accession number MK226567-MK226676.

2.4. Real-time quantitative PCR

Anammox bacterial 16S rRNA gene abundance was estimated by real-time quantitative PCR (qPCR). qPCR were conducted in triplicate for each sample on an ABI 7500 Sequence Detection System with SYBR green method (Applied Biosystems, Canada). The primers (Amx-808-F/Amx-1040-R) (Hamersley et al., 2007), qPCR mixtures, and qPCR thermocycling conditions are listed in Table S2. Plasmid Mini Preparation Kit (Tiangen, China) was used to extract plasmids with anammox bacterial 16S rRNA gene inserts from *E. coli* hosts. Standard curve was constructed using a series of tenfold dilutions of the standard plasmids (known copy number) containing anammox bacterial 16S rRNA gene. Then, the constructed standard curve was used to calculate the abundance of anammox bacterial 16S rRNA gene in the samples. Melting curve and gel electrophoresis analysis were conducted for qPCR specificity determination, thus to lower the possibility of overestimation. Moreover, negative controls were always conducted in each qPCR reaction without any DNA template addition. In this study, anammox bacterial abundance was converted into copies of 16S rRNA gene per gram of dry sediment, assuming the efficiency of DNA extraction from the sediment samples was 100%.

2.5. Statistical analysis

The Mothur program (version 1.36.0) was used to construct the rarefaction curve for anammox bacterial clone library and to calculate the species richness Chao1 estimator and biodiversity indicators (Shannon-Wiener and Simpson) (Schloss et al., 2009). Coverage of the clone library was determined by the obtained OTU number divided by Chao1 estimator. The relationship between anammox bacterial community structure and environmental factors were examined with the linear-model-based redundancy analysis (RDA, the maximum gradient length was shorter than 4 standard deviations [SD]) using Canoco 4.5 software (ter Braak and Šmilauer, 2002). The classifications of anammox bacterial communities from different ecosystems were conducted with the principal coordinates analysis (PCoA) with Qiime 1.9.0 package (Caporaso et al., 2010). The spatial variations of anammox bacterial dynamics were determined via one-way analysis of variance (ANOVA) using SPSS (version 16.0). Pearson correlation analyses were performed to explore any underlying relationships. For all analyses, results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Communities and spatial distribution of anammox bacteria

Anammox bacteria in the urban river network of Shanghai were successfully identified in all the sampling sites based on the nested PCR method. T-RFLP analyses showed that anammox communities were dominated by T-RF of 400 bp, which accounted for 11.8–45.9% in all the sampling sites (Fig. 2). However, no significant spatial variations of anammox bacteria in this group was detected among different hierarchies of the river network (One-Way ANOVA, $P > 0.05$). T-RF of 119 bp, accounting for 4.5–38.8%, was also detected in all the sampling sites, without significant spatial variations (One-Way ANOVA, $P > 0.05$). T-RF of 220 bp was detected in 45 of the 47 sampling sites, which was evenly distributed in the first-order (17.3%), second-order (16.2%), and third-order (18.2%) river sites (One-Way ANOVA, $P > 0.05$). However, anammox bacterial group belonging to the T-RF of 282 bp was more dominant in the second-order river sites than in the first- and third-order river sites (One-Way ANOVA, $P < 0.05$), which averagely accounted for 18.7%, 8.8%, and 11.0%, respectively. But, in general, the geographical distribution of anammox bacterial biodiversity in the river network of Shanghai was homogeneous, and the community composition exhibited significant linear relationship in different hierarchies of the river network based on the relative contributions of the major T-RFs to the total integrated peak area in the chromatograms ($P = 0.000$) (Fig. 3).

3.2. Phylogenetic analysis of anammox bacteria

Communities of the anammox bacteria in the urban river network of Shanghai were also analyzed based on clone library construction and sequencing. In this study, 110 qualified sequences were obtained from a composite river network clone library. In total, 75 unique sequences and 21 OTUs were obtained at a 3% nucleotide acid divergence (Table S3). The retrieved anammox bacterial 16S rRNA gene sequences were all anammox-like clones, which was evidenced by Blast search. The coverage of the constructed clone library was quite high (95.5%; Table S3), indicating that the majority of the anammox bacterial diversity were represented in the clone library, which was in consistent with the rarefaction analysis (Fig. S2).

Four known anammox bacterial genera, including *Candidatus* Brocadia, Scalindua, Jettenia, and Kuenenia, and also unknown anammox-like groups were detected in the urban river network of Shanghai based on the phylogenetic analysis (Fig. 4). The study area was dominated by *Candidatus* Brocadia (B. fulgida and B. anammoxidans, with 93–97% sequence similarity), which accounted for 64.5% of the total acquired clones. *Candidatus* Scalindua, Jettenia, and Kuenenia occupied 10.0%, 8.2%, and 2.7% of the total river network sequences, respectively, while the remaining 14.5% of the sequences showed low sequence similarity to the known anammox genera (<92%). These unknown anammox-like sequences had no close relatives among the known organisms. Therefore, it is possible that they are so far uncultivated anammox microbe. The retrieved anammox bacterial 16S rRNA gene clones in the urban river network of Shanghai were also closely affiliated with clones detected in estuarine and marine environments (95–99% sequence identity), including the Yangtze Estuary sediment (Zheng et al., 2016a), Cape Fear River Estuary sediment (Dale et al., 2009), South China Sea sediment (Li et al., 2013), and Golfo Dulce seawater (Schmid et al., 2007) (Fig. 4).

3.3. Abundance of anammox bacteria and associated activity

To quantify anammox bacterial 16S rRNA gene abundance in the urban river network of Shanghai, a standard curve spanning a range of 1.72×10^3 to 1.72×10^9 copies μL^{-1} was constructed in the present study. The qPCR consistency was confirmed by a strong linear inverse correlation between the threshold cycle (C_T) and the $\log_{10}$ value of anammox bacterial 16S rRNA gene copy number of the standard curve ($R^2 = 0.9998$). The amplification efficiency was 94.9%. Only one observable peak at 87.2 °C was detected via melting curve analyses. The qPCR results showed that the highest abundance of anammox bacteria was observed at the second-order river site S13 (3.9×10^7 copies g^{-1} dry sediment), whereas the lowest anammox bacterial abundance was recorded at the third-order river site T7 (3.7×10^6 copies g^{-1} dry sediment) (Fig. 5). Overall, no significant spatial variations of anammox bacterial abundance was observed in different hierarchies of the river network (One-Way ANOVA, $P > 0.05$) (Fig. S3). Also, there was no significant difference in anammox bacterial abundance between the city center and suburban sampling sites (One-Way ANOVA, $P > 0.05$) (Fig. S3).

In the present study, anammox bacterial abundance was found to strongly correlated to the metabolic activity of anammox

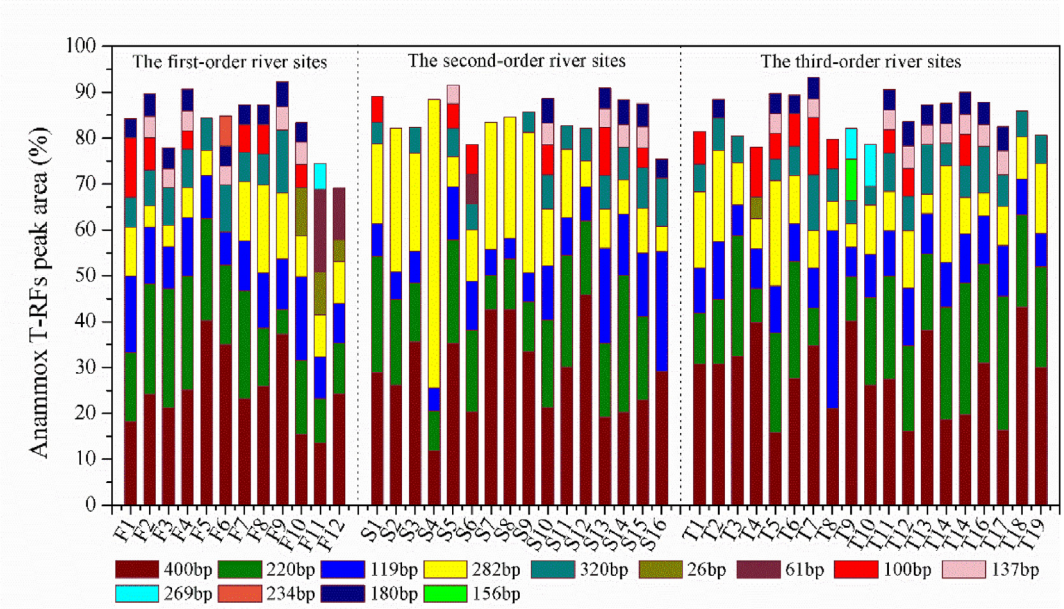

Fig. 2. Community compositions and spatial distributions of anammox bacteria (16S rRNA gene) in the river network of Shanghai based on the relative contributions of major T-RFs (Peak area >4%) to the total integrated area of peaks in the chromatograms.

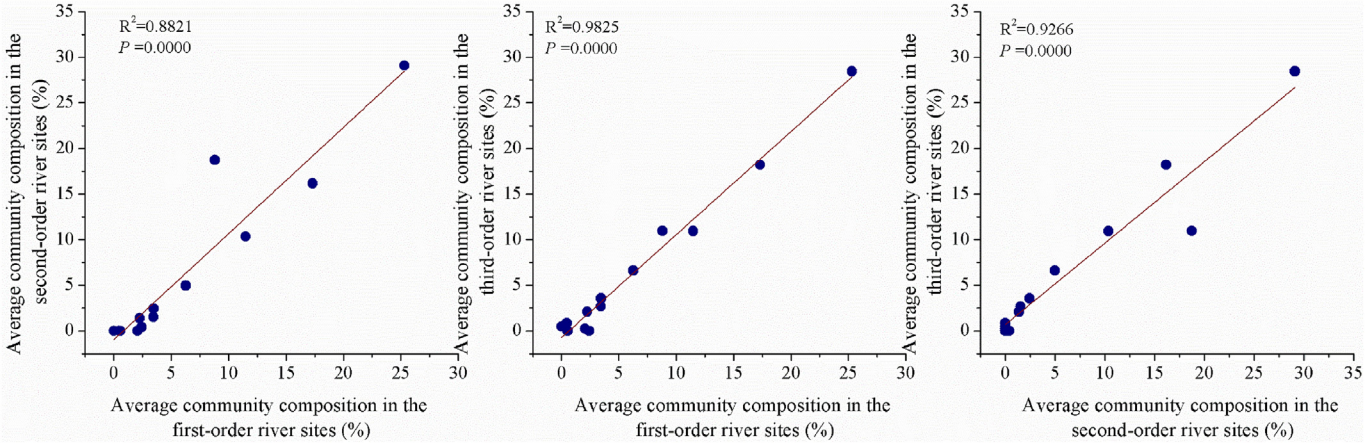

Fig. 3. Correlations of anammox bacterial community composition in different hierarchies of the river network based on the relative contributions of major T-RFs (Peak area >4%) to the total integrated area of peaks in the chromatograms.

bacteria ($R^2 = 0.1285$, $P = 0.0077$) (Fig. 6). Based on ^{15}N tracing technique, the anammox activity was estimated between 0.04 (the second-order river site S14) and 23.7 (the third-order river site T13) nmol N g^{-1} dry sediment h^{-1} in the urban river network of Shanghai (Fig. S1) (Cheng et al., 2016). Supposing each anammox bacterium had equal activity and only contained one anammox bacterial 16S rRNA gene copy, the cell-specific anammox rate was estimated between 0.2 and 59.0 fmol N $cell^{-1}$ d^{-1}, with an average value of 19.1 fmol N $cell^{-1}$ d^{-1}. The average activity of anammox bacteria was higher in the second-order river sites (11.1 nmol N g^{-1} dry sediment h^{-1}) than in the first-order (8.9 nmol N g^{-1} dry sediment h^{-1}) and third-order (10.6 nmol N g^{-1} dry sediment h^{-1}) river sites, though no significant variations were observed in these different hierarchies of the river network (One-Way ANOVA, $P > 0.05$) (Fig. S4). Additionally, no significant spatial heterogeneity of anammox rates was detected between the city center and sub-urban sampling sites (One-Way ANOVA, $P > 0.05$), with an average value of 11.7 and 9.8 nmol N g^{-1} dry sediment h^{-1}, respectively (Cheng et al., 2016).

3.4. Relationships of anammox dynamics with environmental variables

Anammox bacterial dynamics in the urban river networks was influenced by environmental factors (Fig. 7; Table S4). The potential correlations between anammox bacterial community composition and environmental variables were revealed by RDA analysis (Fig. 7). The environmental parameters in the first two RDA dimensions provided 72.3% of the cumulative variance of the anammox genotype-environment relationship. However, only NH_4^+ in the sediment contributed significantly ($P = 0.022$, $F = 2.5$, 499 Monte Carlo permutations) to the anammox-environment relationship, and this factor alone explained 23.8% of the total RDA explanatory power. However, none of the other measured characteristics made significant contributions to the anammox-environment relationship in the urban river network of Shanghai.

Pearson correlation analysis showed that the spatial distribution of the total anammox bacterial abundance in the Shanghai river network was significantly related to sediment TOC and silt contents

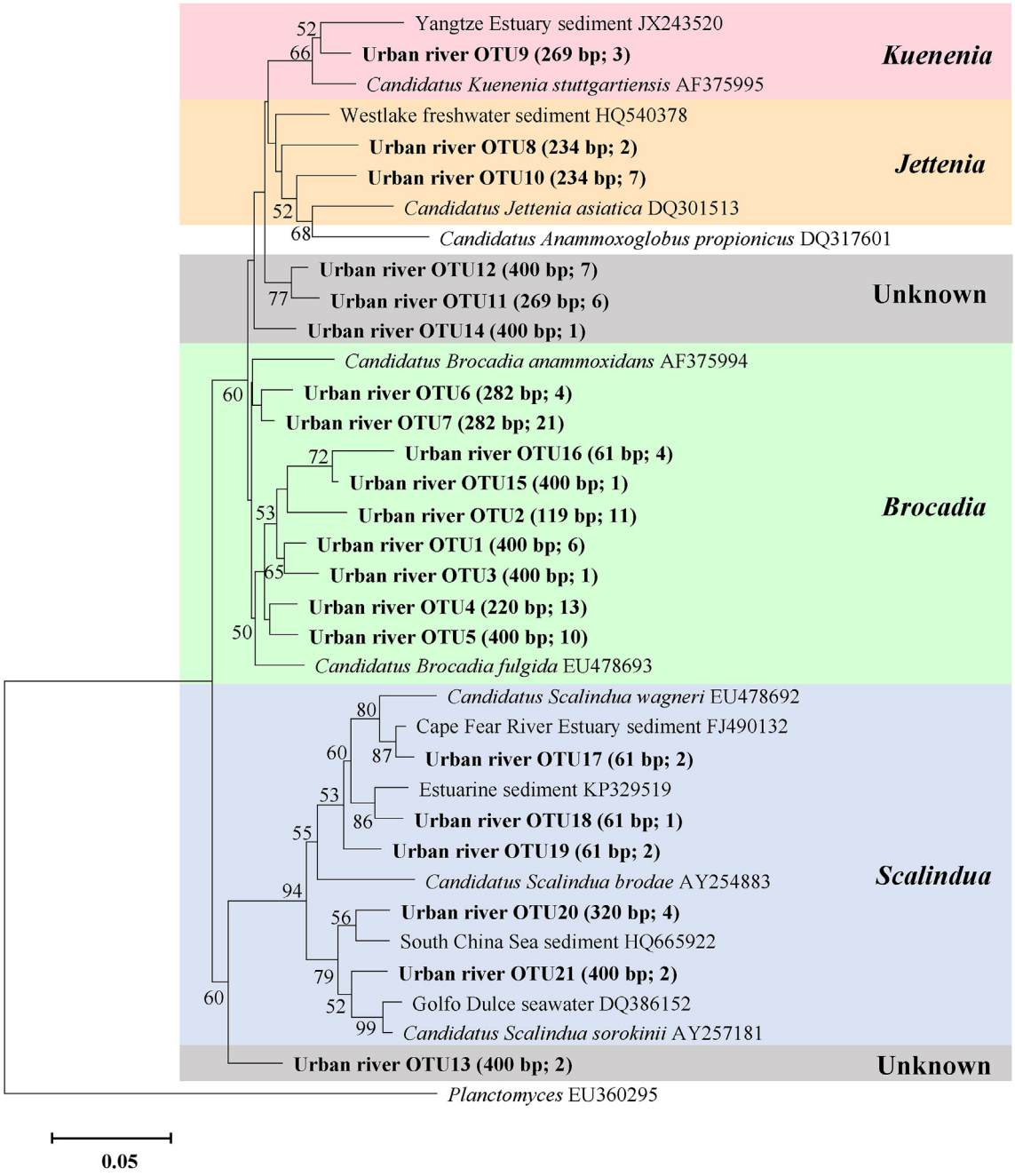

Fig. 4. Neighbor-joining phylogenetic tree of anammox bacterial 16S rRNA gene sequences. Bootstrap values greater than 50% of 1000 resamplings are shown near nodes. The scale indicates the number of nucleotide substitutions per site. GenBank accession numbers are shown for sequences from other studies. Numbers in parentheses followed each OTU indicate the number of sequences. OTUs are defined by <3% divergence in nucleotides.

($P < 0.05$; Table S4). Likewise, abundances of the dominant anammox bacterial groups belonging to T-RFs of 400 bp and 220 bp were also related significantly to sediment TOC and silt contents ($P < 0.05$). However, abundance of 119 bp T-RF showed significant correlation with sediment Fe(III) and TOC contents, while abundance of T-RF of 282 bp was related significantly to sediment TOC and NH_4^+ contents ($P < 0.05$). Additionally, a significant linear relationship between the abundance of anammox bacteria and denitrifier *nirS* gene abundance were observed in the present study ($P < 0.01$) (Fig. 8), which was consistent with the detected linear relationship between anammox activity and denitrification rates ($R^2 = 0.6054$, $P < 0.01$) (Fig. S5). Though significant correlation was demonstrated between the spatial variation of anammox activity and sediment NO_3^-, NH_4^+, Fe (II), sulfide, and TOC contents in the river network of Shanghai ($P < 0.05$), no statistically significant relationship was observed between cell-specific anammox rate and the environmental factors measured in this study ($P > 0.05$; Table S4).

4. Discussion

As an important process of nitrogen cycle, anammox is mediated by chemolithoautotrophic bacteria belonging to *Candidate* Brocadiales of the *Planctomycetes* phylum (Jetten et al., 2010). In the

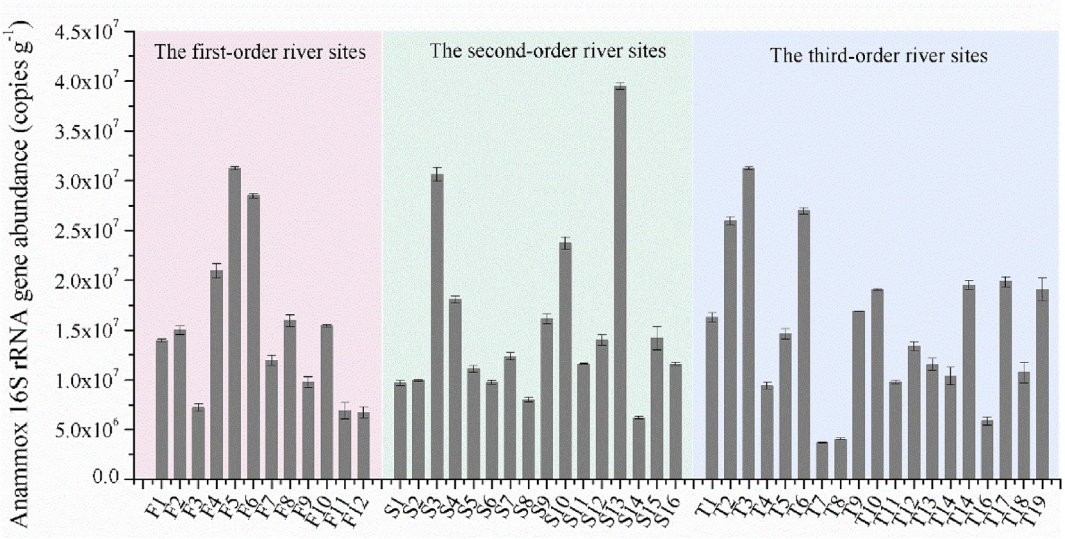

Fig. 5. The spatial variations of anammox bacterial abundance targeting on 16S rRNA gene at each sampling site in the urban river network of Shanghai. Vertical bars indicate standard error (n = 3).

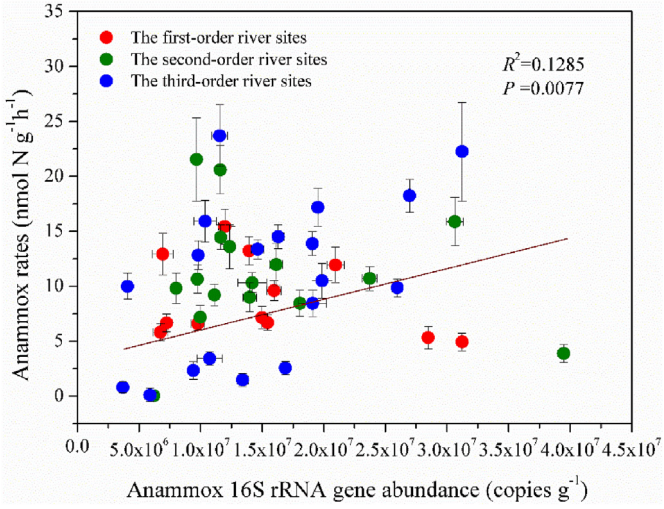

Fig. 6. The correlation between anammox bacterial 16S rRNA gene abundance and anammox rate in the urban river network of Shanghai. Error bars indicate standard error (n = 3).

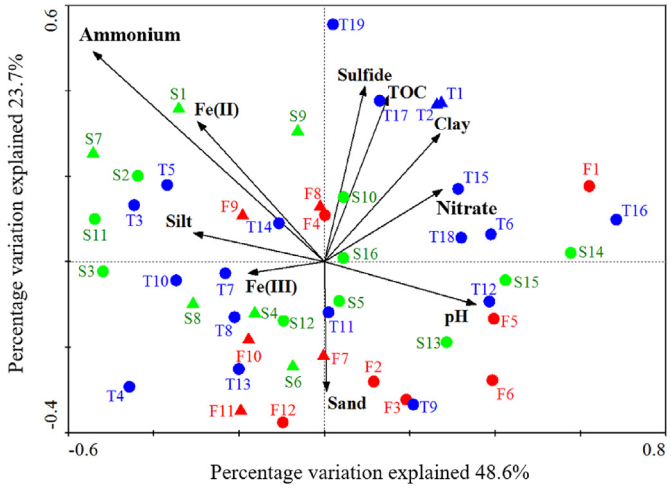

Fig. 7. RDA ordination plots for the first two principal dimensions showing the relationship between environmental factors and the anammox bacterial communities. Correlations between environmental variables and RDA axes are represented by the length and angle of arrows. The red, green, and blue symbols represent the first-, second-, and third-order river sites, respectively. Samples obtained from the city center and the suburban area are shown as up-triangle and circle, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

present study, geographical distributions of anammox bacterial community composition, abundance, and their potential correlations with associated nitrogen removal activities were examined in different hierarchies of the urban river network of Shanghai. Based on molecular techniques, anammox bacteria were ubiquitously present in all the urban river sampling sites (n = 47), as all the retrieved sequences were closely related to anammox bacterial 16S rRNA gene. At present, six genera of anammox bacteria, including *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Scalindua, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia, and *Candidatus* Anammoximicrobium, were identified (Jetten et al., 2010; Khramenkov et al., 2013; Nie et al., 2018). In the urban river network of Shanghai, high biodiversity of anammox bacteria (*Candidatus* Brocadia, Scalindua, Jettenia, Kuenenia, and unknown anammox-like groups) was detected, with *Candidatus* Brocadia being the dominant species. Similarly, high anammox bacterial biodiversity was also reported at the oxic-anoxic interfaces of the estuarine intertidal sediments and the coastal areas (Hou et al., 2013, 2015; Zheng et al., 2016a). Even so, the anammox bacterial communities in the urban river networks were different from those detected both at the freshwater intertidal areas and the high-salinity coastal wetlands (Fig. S6). Furthermore, in marine environments, relatively lower biodiversity of anammox bacteria was reported, which was mainly restricted to *Candidatus* Scalindua genus (Schmid et al., 2007). These differences might be attributed to the fact that different anammox bacterial genera favor different environmental niches, which are influenced by the local hydrodynamic condition, substrate availability, and other environmental factors such as salinity and temperature (Hou et al., 2015; Zhu et al., 2015; Zheng et al., 2016a). For instance, *Candidatus* Scalindua has a high salinity tolerance and adapts better in higher salinity

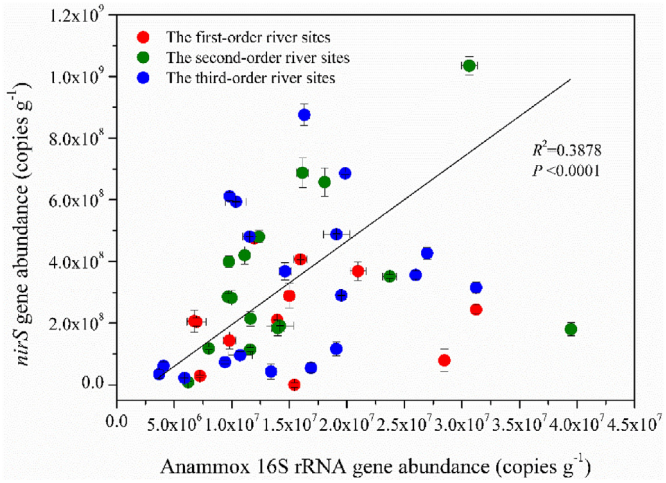

Fig. 8. The correlation between anammox bacterial 16S rRNA gene abundance and denitrifying *nirS* gene abundance in the urban river network of Shanghai. Error bars indicate standard error (n = 3).

conditions whereas the growth of *Candidatus* Brocadia is generally favored in freshwater and (or) brackish conditions (Hu et al., 2012; Jetten et al., 2003; Wang et al., 2012). Therefore, the difference of these anammox bacterial ecophysiology may guide them to different favorable habitats.

In the urban river network of Shanghai, the community composition of anammox bacteria were homogeneously distributed, without significant geospatial variations ($P > 0.05$). This result might be attributed to the fact that all the sampling stations are located at tributaries of the Huangpu River, and they are thus connected by the flowing river water (Gu et al., 2012). The non-significant spatial variation of anammox bacterial biodiversity in the urban river network of Shanghai was mainly defined by sediment NH_4^+ content revealed by RDA analysis, while it was also correlated with environmental parameters such as sediment grain size, salinity, and Fe(II) content in estuarine and coastal ecosystems (Zheng et al., 2016a; Jiao et al., 2018).

Based on the quantitative PCR assays, the abundance of anammox bacterial 16S rRNA gene was estimated between 3.7×10^6 and 3.9×10^7 copies g^{-1} dry sediment (Fig. 4), which was relatively higher than that reported in the Jiaozhou Bay (3.5×10^5 to 5.9×10^6 copies *hzo* gene g^{-1}) (Dang et al., 2010), Cape Fear River Estuary (1.3×10^5 to 8.4×10^6 copies 16S rRNA gene g^{-1}) (Dale et al., 2009), Pearl Estuary (4.22×10^5 to 2.55×10^6 copies 16S rRNA gene g^{-1}) (Fu et al., 2015), and the mashes of the Yangtze Estuary (2.63×10^6 to 1.56×10^7 copies 16S rRNA gene g^{-1}) (Hou et al., 2013). However, high anammox bacterial abundance, ranging from 8×10^6 to 2×10^7 copies hydrazine synthase (*hzsB*) gene g^{-1}, was also reported in the land-freshwater interface, which was identified as a biogeographical hotpot for anammox (Zhu et al., 2013). These results implying that the anammox bacteria might also play an important role in nitrogen transformation in the urban river network of Shanghai. Though the spatial variation of anammox bacterial abundance was not significant in the Shanghai river network ($P > 0.05$), relatively lower anammox bacterial abundance was observed in the upstream of the Huangpu River. However, along with the river flows toward the midstream area, the abundance increased, which was then decreased from the middle to the lower reaches of the Huangpu River (Fig. 5). The same distribution trend was also observed for the abundance of the dominant anammox bacterial group 400 bp T-RF, which might be affected by the sediment TOC and silt contents in the urban river network of Shanghai, as significant correlations were observed ($P < 0.05$; Table S4).

The observed significant correlation between anammox bacterial abundance and sediment TOC content was unexpected, as it has been demonstrated that anammox bacteria are chemolithoautotrophic microorganisms (Jetten et al., 2010). This relationship implies an underlying interaction between anammox bacteria and certain heterotrophic microorganisms (Hou et al., 2015). Actually, a significant linear relationship between anammox bacterial 16S rRNA gene abundance and denitrifier *nirS* gene abundance were observed in the present study ($P < 0.01$) (Fig. 8; Fig. S7), indicating that heterotrophic denitrifiers might stimulate the proliferation of anammox bacteria via providing them with important substrate, such as NO_2^-. Indeed, according to previous studies, NO_2^- was the main limiting factor that affected anammox bacterial population development, and denitrifiers was likely an important source of NO_2^- for anammox bacteria (Thamdrup and Dalsgaard, 2002; Trimmer et al., 2005; Hou et al., 2013). However, previous studies also showed that organic carbon significantly inhibited the anammox activity in bioreactors and marine sediments (Jetten et al., 1999; Jin et al., 2012). Moreover, it was observed that the potential rates of anammox in paddy soil were significantly decreased in the glucose and acetate addition treatments (Shan et al., 2018). Therefore, anammox bacteria might respond differently to organic matters in different environments, and the possibility that the anammox bacteria in urban river networks may perform diverse metabolic pathways can't be excluded. In addition, anammox bacterial abundance in the Shanghai river network was observed to significantly correlate with sediment grain size ($P < 0.05$), which might greatly affect the sediment physicochemical factors as it was related to the *in situ* hydrological conditions, such as water mixing, river runoff, and the dynamics and intensity of those activities (Dang et al., 2010). Anammox bacterial activities in the urban river network of Shanghai (with an average value of 10.3 nmol N g^{-1} dry sediment h^{-1}) (Cheng et al., 2016) were relatively higher than those detected in the estuarine and coastal ecosystems (0–7 nmol N g^{-1} h^{-1}) (Dale et al., 2009; Hou et al., 2013, 2015; Lisa et al., 2014; Naeher et al., 2015; Zheng et al., 2016a). Shanghai is one of the most urbanized and highly developed cities in China. Over the past decades, the total nitrogen delivered into its river network has increased greatly (Gu et al., 2012; Yu et al., 2013), which has caused enormous stress on the health of both the environment and the residents (Gu et al., 2012). Relatively higher, though not significant, anammox rates were observed in the city center than in the suburban areas of the Shanghai river network, implying a higher nitrogen loading in the central region. However, the detected reactive nitrogen concentrations were only slightly higher ($P > 0.05$) in the city center than in the suburban areas (Table S1). In addition, both anammox rates and their relative importance in nitrogen removal were higher, though not significant, in the second-order river sites than those in the first- or third-order river sites (Fig. S4; S8), implying that the living conditions, such as hydrodynamic condition, of the second-order river sites might be suitable for the metabolic activity of anammox bacteria. However, further studies are still needed to reveal the underlying mechanisms.

In this study, anammox bacterial activities were significantly related to the abundance of anammox bacteria ($R^2 = 0.1285$, $P = 0.0077$) but not to their diversity ($P > 0.05$) (Fig. 6; Fig. S9), demonstrating that anammox bacterial abundance may predict anammox activity (Dale et al., 2009), though the existence of anammox bacteria does not mean that they are active *in situ* (Petersen et al., 2012). In addition, the activity of anammox bacteria were significantly and positively correlated with Fe(II) ($P < 0.01$). This result might be due to the reaction of NH_4^+ oxidation coupled

with Fe(III) reduction under anaerobic condition (Li et al., 2015; Guan et al., 2018), which generates Fe(II) and contributes to nitrogen loss. At the same time, NO_x^- is produced and therefore the anammox bacteria are benefited (Li et al., 2015). Furthermore, a significant positive relationship between anammox activity and sulfide was detected ($P < 0.01$), which was unexpected as sulfide might be toxic for anammox bacterial activity (Jensen et al., 2008). However, when partial correlation analysis was conducted to control for Fe(II), which was tightly correlated with sulfide ($P < 0.01$), the correlation between anammox activity and sulfide was not significant ($P > 0.05$). Thus, we postulated that the binding between sulfide and Fe(II) might decrease the bioavailability and toxicity of free sulfide (Deng et al., 2015; Zheng et al., 2016a). The significant correlations between anammox rates and sedimentary NO_x^- and NH_4^+ concentrations ($P < 0.01$) indicated that substrate availability was an important factor affecting the activity of anammox bacteria (Meyer et al., 2005).

Denitrification has long been regarded as the only pathway for aquatic fixed nitrogen loss to the atmosphere. However, in the urban river network of Shanghai, anammox bacteria were estimated to averagely contribute 60% to the total N_2 production (Cheng et al., 2016), showing that the anammox process played a significant role in reactive nitrogen loss from urban river networks and thus relieved the environmental stress. In fact, an annual 5.8×10^4 t of nitrogen removal was estimated to be linked to anammox bacteria in the urban river network of Shanghai, which accounted for approximately 9.9% of the total N delivered annually to the study area (Cheng et al., 2016; Gu et al., 2012; Yu et al., 2013). The contributions of anammox to overall N_2 emissions in the Shanghai river network were relatively higher than those detected in other estuarine and coastal areas (0–54%) (Brin et al., 2014; Tan et al., 2019; Teixeira et al., 2014; Wang et al., 2012). However, in the North Atlantic Basin and the OJ estuary in Zhejiang, anammox was found to contribute as high as 68% and 83% to the sedimentary nitrogen removal, respectively (Trimmer and Nicholls, 2009; Yang et al., 2017). It was also reported that up to 67.6% of N_2 production was contributed by anammox in phreatic aquifer (Wang et al., 2017), and in certain area of the Yangtze Estuary this value was as high as 77% (Zheng et al., 2016a). It has been hypothesized that the contribution of anammox to N_2 production was primarily regulated by organic matter (Dalsgaard et al., 2005), as anammox and denitrifying bacteria possess different metabolic pathways. Heterotrophic denitrifiers obtain both their carbon and energy from the oxidation of organic compounds, while the anammox reaction is catalyzed by the autotrophic anammox bacteria (Kartal et al., 2012). Hence, when organic matter was insufficient, the metabolism of denitrifying bacteria was limited while the anammox bacteria were favored. Significant negative correlation between sediment organic matter content and the relative contribution of anammox process to total nitrogen loss was previously reported (Zheng et al., 2016a). However, in the present study, the role of anammox bacteria in nitrogen removal was not significantly related to organic compounds ($P > 0.05$). In addition, the organic carbon contents (1.0–5.6%) in the urban river network of Shanghai were comparable to those (0.2–12%) in the habitats where the contribution of anammox to N_2 emission was relatively lower (0–46%) (Brin et al., 2014; Hou et al., 2013, 2015; Teixeira et al., 2014; Wang et al., 2012; Zhu et al., 2015). Whereas, the organic carbon contents in phreatic aquifer and the Yangtze Estuary, where the anammox bacteria were reported to make even higher contributions (67.6% and 77%, respectively), were much lower (<0.05%) (Wang et al., 2017; Zheng et al., 2016a). Recently, it was speculated that the most important factor in regulating the relative importance of anammox bacteria and denitrifiers to the total N_2 production might be the sedimentary C/N, as the activity of anammox bacteria and sedimentary C/N was negatively correlated (Tan et al., 2019). However, further studies are still required to verify this statement in urban river networks. Despite the relatively higher nitrogen removal contribution of anammox bacteria, their abundance was approximately one magnitude lower than the observed cytochromecd1-type nitrite reductase gene (*nirS*)-harboring denitrifiers, which showed the possibility that anammox bacteria may achieve metabolic advantage over denitrifying bacteria in this highly dynamic urban river environment, thus reducing the potential nitrous oxide (N_2O) emission risk.

5. Conclusions

This study first investigated the biodiversity, abundance, geographical distribution of anammox bacteria and their importance in the urban river network of Shanghai. Here we demonstrated that high biodiversity of anammox bacteria was present in urban river networks, including *Candidatus* Brocadia, Scalindua, Jettenia, and Kuenenia. In general, the geographical distribution of anammox bacterial abundance, community composition, and metabolic activity in the river network of Shanghai was homogeneous. The present study highlighted that the functional anammox bacteria played an important role in nitrogen removal in urban river networks. These results can extend our current knowledge about the community dynamics and environmental importance of anammox bacteria in the urban river networks.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.112998.

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