Investigation of Dissimilatory Nitrate Reduction to Ammonium (DNRA) Microbial Communities in Sediments of Urban River Network Along the Huangpu River, China

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Abstract

Dissimilatory nitrate reduction to ammonium (DNRA) is an essential intermediate step in the nitrogen cycle, linking the oxidation and reduction processes of nitrogen compounds. But the detailed research on the environmental nitrogen cycling in urban river networks based on DNRA communities and the functional gene \( nrfA \) is lacking. In this study, the flow line of the Huangpu River in Shanghai was analyzed using isotope tracer, quantitative real-time PCR, and high-throughput sequencing techniques to evaluate the role of DNRA on the stability of the river network and marine. The significant positive correlation between the rate of DNRA and sediment organic carbon was identified. At the genus level, \( Anaeromyxobacter \) is the most dominant. Notably, both heterotrophic and autotrophic DNRA species were discovered. This study added diversity to the scope of urban freshwater river network ecosystem studies by investigating the distribution of DNRA bacteria along the Huangpu River.

1. Introduction

In nature, the nitrogen cycle is a complex process driven by microbes (Wang et al., 2020). Microbial activities such as biodegradation, biochemical transformation, etc., are essential to the ecosystem function of nutrient cycles (Gu, 2016). In recent years, an increasing number of nitrate reduction processes have been examined, from which denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are widely studied. The denitrification process involves reducing of nitrate to \( N_2 \) through a series of intermediate products (NO\(_2^−\), NO, N\(_2\)O) (Burgin and Hamilton, 2007). In the DNRA process, NADH is biologically oxidized to produce NH\(_4^+\) using NO\(_3^−\)/NO\(_2^−\) as electron acceptors (Burgin and Hamilton, 2007). While denitrification permanently removes nitrogen, DNRA effectively protects and recycles nitrogen and reduces nutrient loss during denitrification (Cao et al., 2016; Zhao et al., 2020). Both the denitrification and DNRA processes use nitrate as the reaction substrate, and there is a competitive relationship between the two reactions.

Research on DNRA has usually focused on aquatic ecosystems in the ocean, estuaries, and wetlands (Hardison et al., 2015; Sanford et al., 2012; Shan et al., 2016; Wang et al., 2020). Giblin et al. (2013) measured the nitrate reduction activity at 55 aquatic ecosystem sites and found that DNRA was the dominant reduction pathway at more than one-third of these sites. Thus, DNRA is an essential process in the natural nitrogen cycle and cannot be ignored (Cheng et al., 2016; Davidson, 2009; Galloway et al., 2008; Seitzinger, 2008; Yu et al., 2013). Urban river networks are presumed to have a potentially important role in linking different ecosystems. The urban river network is characterized by high pollution and high reactive nitrogen load (Cheng et al., 2016). Therefore, the urban river network is hotspot for biogeochemical studies of the nitrogen cycle. Cheng et al. (2016) estimated the contributions of anammox, denitrification, and DNRA to nitrate reduction in urban river systems. Denitrification contributed 11.5–99.5% to total nitrate reduction, as compared to 0.343–81.6% for anammox and 0-52.3% for DNRA. However, the \( nrfA \) gene was not analyzed, and the abundance and community composition of DNRA functional bacteria were not further explored. Current research on nitrate reduction
in urban freshwater river network ecosystems is inadequate, and many studies have not explained the abundance and diversity of microbial communities and their interactions with the environment.

The Huangpu River is the primary water source for domestic, industrial, and agricultural uses in Shanghai (Xu et al. 2020a). As an important industrial zone in China, Shanghai produces a high sewage volume due to its high population density and extensive industrial activities. With the continuous progress of urbanization, a large land area along the Huangpu River has been developed for human activities (Xu et al., 2020a); thus, water pollution in the Huangpu River has been in a heavier load condition. Ammonia-based nitrogen pollution is a primary cause of water pollution in the Huangpu River (Zheng et al., 2019). As NH$_4^+$ promotes algal growth and eutrophication at a significantly higher rate than NO$_3^-$, DNRA may be the major contributor to eutrophication in urban freshwater riverine systems (Nogaro and Burgin, 2014; Scott et al., 2008; Wang et al., 2020). However, researchers often overlook DNRA because it is challenging to accurately identify DNRA activity using conventional analytical methods and denitrification is more likely to occur (Zhao et al., 2020). Gu (2021) pointed out that some microorganisms can obtain energy by biochemically transforming pollutants during their metabolic processes. Therefore, it is necessary to explore the microbial degradation processes, microbial species, and their activity changes.

This study selected the Huangpu River in Shanghai, China, as the research site. The obtained samples were analyzed using qPCR, isotope tracing, and high-throughput sequencing techniques to determine the abundance, activity and community structure of DNRA bacteria. The distribution of heterogeneous nitrate reduction in the Huangpu River basin was studied to understand the differences and relevant characteristics of different functional bacteria. The main objectives of the study were; (1) to measure the potential DNRA reaction rates and the abundance of functional genes associated with nitrogen removal, (2) to analyze the dominant communities and function of DNRA along the Huangpu River, and (3) to evaluate the influence of environmental factors on the DNRA process.

2. Materials And Methods

2.1. Description of the research area

The Huangpu River, which flows through the Shanghai region, originates from Taihu Lake. The upstream region of the Huangpu River is predominantly used for agricultural activities, while industrial and residential areas are condensed in the river's downstream region (Xu et al., 2020b). Based on the Huangpu River's natural characteristics and the discharges from each section of the river within Shanghai, six representative sites were selected for sampling (Fig. S1). The six sampling sites were expressed as Chunshentang (CST, 31°06′51″N, 121°26′44″E), Huajinggang (HJG, 31°07′19″N, 121°26′55″E), Suzhouhe (SZH, 31°15′13″N, 121°26′05″E), Shajinggang (SJG, 31°17′24″N, 121°28′35″E), Yunzaobang (YZB, 31°22′12″N, 121°29′20″E), and the Yangtze River Intake (CJ, 31°23′51″N, 121°30′29″E).

2.2. Samples collection and analysis
Sediments from each sampling site were collected in sterile plastic bags and quickly stored at -20 °C for subsequent analytical studies. The six sites were sampled at depths of 0-10 cm. The pH value was measured using a pH meter (PhS-3C, Rex Electrochemistry, China), and the salinity was measured with sediment directed using a conductivity meter (STEC-100, SP, USA). The water content of the sediment samples was determined using the drying method. Nitrogen compounds in fresh sediment were extracted with 2M potassium chloride solution and analyzed using a UV-vis spectrophotometer. The sediment organic carbon (SOC) content was detected using a high-temperature exothermic potassium dichromate oxidation-volume method (Wang et al., 2012).

2.3. \(^{15}\text{N} \) tracer incubations

The potential activity of DNRA in fresh mud samples was determined using mud incubation experiments combined with the \(^{15}\text{N} \) isotope tracer technique (Pang and Ji, 2019). Sediment samples from each station were mixed and divided into four portions of approximately 1 g wet weight and placed in airtight bottles (12 ml capacity). Five milliliters of in-situ water were added to the vial at a ratio of 1:5 and mixed to form a slurry. The vials were purged in argon gas for 15 min and then sealed with a cap. The vials were then placed in a constant temperature (22°C-25°C), a dark anaerobic incubator, for 24 hours of pre-incubation (the purpose of this step was to remove oxygen and nitrate). At the end of pre-incubation, the vial was filled with a syringe of \(\text{Na}^{15}\text{NO}_3 \) (99.3 \(^{15}\text{N} \) atom %) to a final concentration of 100 µmol \(^{15}\text{N} \text{L}^{-1}\). For each set of samples, one vial was injected with 1 ml of ZnCl\(_2\) (50% w/v) to terminate microbial activity; this sample was called the initial sample. The remaining vials not injected with ZnCl\(_2\) were then placed on a thermostatic shaker (180 rpm) and incubated for 8 hours. After incubation, each vial was injected with 1 mL ZnCl\(_2\) (50% w/v) and set as the final sample. After adding hypobromous acid (HBrO) oxidizer to the final water sample, \(^{15}\text{NH}_4^+\) was oxidized to \(^{29}\text{N}_2\) and \(^{30}\text{N}_2\), and the \(^{15}\text{N} \) ratio was determined by isotope mass spectrometry. The potential DNRA rate can be calculated from the change in \(^{15}\text{NH}_4^+\) before and after the reaction using Eq (1):

\[
\text{DNRA} = \left(\left[^{15}\text{NH}_4^+\right]_f - \left[^{15}\text{NH}_4^+\right]_i\right) \times V \times m^{-1} \times t^{-1} \quad (1)
\]

In this equation, \(\left[^{15}\text{NH}_4^+\right]_i\) and \(\left[^{15}\text{NH}_4^+\right]_f\) (mol N L\(^{-1}\)) represent the concentrations of \(^{15}\text{NH}_4^+\)-N before and after culture, respectively. \(V\) (L) represents the volume of the culture system, \(m\) (g) represents the dry weight of the sediment in each vial, and \(t\) (h) represents the shaking time of the culture (Porubsky et al., 2008).

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

According to the manufacturer’s protocol, DNA extraction from natural sediment samples was conducted using a Power Soil DNA Kit (MO BIO Laboratories, USA). The size and integrity of the extracted DNA fragments were determined by 0.8% agarose gel electrophoresis. If the extracted DNA concentration, fragment size, and integrity met the requirements, it was sealed and stored at -20 °C for further analysis.
The abundance distributions of different functional genes were quantitatively compared using a quantitative real-time PCR instrument (Thermo Quant-Studio 5, USA). The target genes tested in this experiment included formic acid-dependent nitrite reductase gene (nrfA), cytochrome cd1 nitrite reductase gene (nirS), copper-type nitrite reductase gene (nirK), and anaerobic ammonia-oxidizing bacteria 16S rRNA gene (Amx). The primers of the genes used in this experiment were nrfAf2aw (CARTGYCAYGTBGARTA) and nrfAR1 (TWNGGCATRTGRCARTC), nirS3cdF (GTSAACGTTAAGGARACGG) and nirS3cdR (GASTTCGGRTGSGTCTTGA), nirK876F (ATYGCGGVCAYGGCGA) and nirK1040R (GCCTCGATCAGRTTRTGGTT), AMX809F (GCCGTAACATGGGCAGT) and AMX1066R (AACGTCTCACGACGAGCTG). The SYBR Green I fluorescent dye qPCR assay was selected and conducted in a 96-well plate for real-time PCR. The reaction mixtures comprised 10 µL of SYBR enzyme, 7.8 µL of ddH₂O (TaKaRa, Japan), 0.4 µL of forward and reverse primers, 0.4 µL of PCR stabilizer, and 1 µL of DNA sample or ddH₂O (as a blank control). The qPCR data were analyzed using Abs Quant/2-order derivatives of Max software (Zhang et al., 2016).

2.5. Biodiversity analysis

In this experiment, the Illumina miseq platform was used for DNA sequencing. The primers nrfAf2aw (CARTGYCAYGTBGARTA) and nrfAR1 (TWNGGCATRTGRCARTC) were used to amplify the partial sequence of nrfA region of bacteria with a length of about 250 bp (Welsh et al., 2014). Electrophoresis of the PCR products was identified and then was purified by gel extraction kit. The gel recovery kit of AXYGEN was used for recycling.

High-quality chimeric sequences were obtained using QIIME (v1.8.0 http://qiime.org/) software with the UCLUST sequence comparison tool (Edgar, 2010). The Operational Taxonomic Units (OTUs) were segmented by 97% sequences identity, and the richest sequence in each OTU was selected as the representative sequence. Indices of microbial community richness from the ACE estimator and Chao 1 estimates were included, and microbial diversity was quantified using the Simpson and Shannon indices. The alpha diversity index of each sample and the distribution of DNRA bacteria in the six samples were determined using the QIIME method. R software was used to construct a heat map of the 50 most enriched genera.

2.6. Statistical analysis

Nonmetric multidimensional scaling (NMDS) analysis based on weighted UniFrac distances was used to evaluate community composition differences at the six sites. Redundancy analysis (RDA) was performed using CANOCO version 4.5 software. The SPSS software package (Standard Version 19.0, SPSS, Inc.) was used for the relevant Pearson correlation analysis. A $P$ value of less than 0.05 was considered a significant correlation in this study.

2.7. Data availability
Data supporting the results of this study are available from the corresponding author upon reasonable request. The sequence of the \textit{nrfA} gene obtained in this study has been submitted to the NCBI Sequence Reading Archive (SRA) with the accession numbers SRP321381:PRJNA732826.

3. Results

3.1. Environmental parameters

The environmental parameters associated with each sediment site are shown in Table S1. The six sample sites' moisture content did not differ significantly, ranging from 0.30% to 0.42%. The pH was weakly alkaline and slightly varied across sites, with the highest pH of 7.71 observed at the HJG site and the lowest pH of 7.35 at the SZH site. The SOC and extractable NH$_4^+$-N concentrations ranged from 9.76 to 15.94 g/kg and 0.34 µmol N/g to 8.01 µmol N/g, respectively. The sediments had the highest extractable NH$_4^+$-N concentration (8.01 µmol N/g) and SOC content (15.94 g/kg) at the YZB site, approximately four times higher than those at the SZH site. On the other hand, the lowest extractable NH$_4^+$-N concentration of 0.34 µmol N/g was observed at the CJ site. The extractable nitrate concentrations ranged from 4.51 to 6.34 µmol N/g, and the CST and HJG sites were associated with slightly higher nitrate concentrations than the downstream HJG, SZH, YZB, and CJ sites.

3.2 DNRA potential rates and abundance of functional gene

The absolute abundances of related genes involved in the nitrate reduction process in the sampled sediments are presented in Fig. 1. The results indicated that the \textit{nrfA} gene, responsible for the metabolic potential of DNRA, was the most predominant among the genes in all sediment samples. The gene abundance of \textit{nrfA} ranged from $4.55 \times 10^{10}$ to $2.85 \times 10^{11}$ copies/g dry weight, with higher values observed at the CJ, CST, and SZH sites ($2.85 \times 10^{11}$, $2.42 \times 10^{11}$, and $1.36 \times 10^{11}$ copies/g dry weight, respectively). The absolute abundances of the denitrification genes \textit{nirS} and \textit{nirK} ranged from $6.24 \times 10^{8}$ to $5.54 \times 10^{9}$ copies/g dry weight and $7.98 \times 10^{9}$ to $2.87 \times 10^{10}$ copies/g dry weight, respectively. The abundance of \textit{Amx} genes representing anammox functions varied from $2.99 \times 10^{9}$ to $1.03 \times 10^{10}$ copies/g dry weight.

The potential rate of DNRA was determined using the $^{15}$N isotope tracer technique. The DNRA rate and the related bacterial functional gene \textit{nrfA} are shown in Table 1. The site with the highest DNRA rate was at the YZB site, with a value of 0.90 nmol N g$^{-1}$ h$^{-1}$, while the lowest value was 0.10 nmol N g$^{-1}$ h$^{-1}$ at the SZH site.

3.3 Diversity of DNRA communities

Sediment samples from the six sites were subjected to \textit{nrfA} pyrophosphate (using the same primers as the qPCR test) sequencing, yielding high-quality sequences ranging from 60550 to 100006. The relationship between the OTUs of these six samples is shown in Table 2. The sediment sample at the CJ
site had the highest number of OTUs, while the SZH sediment sample had the lowest OTUs. To reflect the DNRA bacterial community's alpha diversity, Chao1 and ACE indices characterize community richness, and Shannon and Simpson indices, which represent community diversity, are presented in Table 2. The Chao1 and ACE indices had the highest estimates at the CJ and SJG sites, while low estimates occurred at the SZH and CST sites. This indicated that the community richness occurred at the CJ and SJG sites, followed by the HJG and YZB sites, while the lowest occurred at the SZH and CST sites. Furthermore, the Simpson and Shannon indices were 0.997955 and 10.29, respectively, at the SJG site higher than those at the other sites. This finding further confirmed that the SJG site was rich in diversity. Similarly, the rank abundance curve (Fig. S2) showed the same results.

The NMDS analysis of genus-level community composition was performed using R software to quantify the six sites' differences and associations (Fig. S3). It shows that the SJG, YZB, and HJG sites had relatively similar community structures since the SZH and CJ sites are unique and located relatively far from the other sites.

The DNRA bacterial community structure with relative abundance at the phylum level is shown in Fig. 2 (a). Seven phylum with relatively high abundance (>0.5% abundance at each sample site) were selected. *Proteobacteria* (36.3~68.8%), *Chloroflexi* (4.9~17.4%), *Bacteroidetes* (2.9~15.2%), *Ignavibacteriae* (3.8~15.2%), *Verrucomicrobia* (4.1~8.6%), *Thermus* (1.4~9.2%), and *Firmicutes* (0.8~2.9%) were the dominant bacteria. The phylum *Proteobacteria* dominated the identified bacteria in all sediment samples, with the highest value of 68.8% at the SZH site and the lowest value of 36.3% at the YZB site.

At the genus level, a total of 16 DNRA genera with relatively high abundance (>0.5% abundance at each sample site) were detected. As shown in Fig. 2 (b), *Anaeromyxobacter*, an anaerobic slime bacterium, was the major genus among them and was the most abundant, accounting for 8.5 to 26.4% of the classified sequences. The highest relative abundance of *Anaeromyxobacter* was 26.4% at site CJ. *Pelobacter* and *Geobacter* also had higher abundances of 1.7-24.2% and 1.9-14.1%, respectively. These two genera were more abundant than other genera at the SZH site.

The taxonomic rank tree is shown in Fig. S4, from which the dominant community can be seen. The predominant genera in the Huangpu River sediment samples were *Deltaproteobacteria* (*Proteobacteria*), *Bacteroidia* (*Bacteroidetes*), *Anaerolineace* (*Chloroflexi*), *Ignavibacteria* (*Ignavibacteriae*), and *Opitutae* (*Verrucomicrobia*). *Deltaproteobacteria* was the dominant colony in terms of circle size.

A heat map of 50 bacterial genera in sediment samples from the Huangpu River system's estuary is shown in Fig. 3. Site YZB and site SJG had a more similar cluster composition. Similarly, the community composition was more similar between sites SZH and CJ. The red area in the heat map represents a higher abundance of the specific genus, indicating that the colony structure differed among the sites, and the genus was more abundant at the CST, YZB, and SJG sites than at the other three sites.

### 3.4 Relationship between DNRA rate, gene abundance, diversity, and environmental parameters
The contribution of DNRA in soils or sediments of different ecosystems has been shown in previous studies to be closely related to water content, pH, carbon to nitrogen ratio, and NO$_3^-$-N and NO$_2^-$-N concentrations (Dong et al., 2011; Friedl et al., 2018; Schmidt et al., 2011). RDA was performed to assess the relationship between DNRA abundance, potential rate, and environmental factors (Fig. 4). Pearson analysis was used to determine the correlation between environmental factors on DNRA activity, abundance, and diversity (Table 3). The DNRA rate was positively correlated with SOC and NH$_4^+$ concentrations (Table 3), and it achieved a significant positive correlation with SOC (P-value = 0.002).

4. Discussion

4.1 Potential DNRA rate

The DNRA process is an important contributor of regenerated nitrogen in the ecosystems of the study region. DNRA effectively conserves and recycles nitrogen and reduces nutrient losses due to denitrification (Cao et al., 2016; Zhao et al., 2020). From the qPCR analysis of different genes, the abundance of the DNRA functional gene *nrfA* was significantly higher than the abundances of denitrification and anammox genes. However, this does not necessitate that *nrfA* gene abundance and DNRA potential rate is significantly correlated. It is also not appropriate to judge the corresponding reaction rate based on gene abundance alone since the current qPCR method has some limitations. This is because the *nrfA* gene is not functional in its dormant state (Zhao et al., 2020). Fernandes et al. (2012) found that DNRA rates in Tuvem Mangrove ecosystems ranged from 27.1 to 49.6 nmol N g$^{-1}$ h$^{-1}$. However, DNRA contributed up to 99% nitrate reduction. In exploring the activity characteristics of DNRA in the riparian zone, Kim et al. (2016) found that DNRA rates varied from 9.0 to 53.6 nmol N g$^{-1}$ h$^{-1}$, with a large gap between the slowest and fastest rates. Zhao et al. (2020) also found that the DNRA rates ranged from 0.13 to 0.44 nmol N g$^{-1}$ h$^{-1}$ in wetland ecosystems. The authors found that in the wetland system, the *nrfA* gene concentration was high, but the DNRA rate was low. Therefore, the ability of DNRA to function varies in different aquatic ecosystems. This may be related to various environmental parameters, such as organic matter concentration and the SOC content in the local environment. The physicochemical properties of these sediments influenced the rate of the DNRA process. The rates of DNRA measured in this study ranged from 0.19 to 0.90 nmol N g$^{-1}$ h$^{-1}$, indicating that there are differences in the activity of DNRA bacteria in different parts of the Huangpu River. Among the three different nitrate reduction pathways, the abundance of functional genes associated with the nitrogen cycle was higher at sites CST and CJ. This indicates that the specific environment of these two sites is suitable for the growth of these microbes.

4.2 DNRA dominant genes and their functions

Based on pyrophosphate sequencing of *nrfA* functional genes, six major phyla were identified in the presence of *nrfA* proteins: *Proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, *Planctomycetes*, *Firmicutes*, and *Chloroflexi* (Welsh et al., 2014). Besides, researchers found that *Bacteroidetes* and *Ignavibacteriae* could be employed to detect *nrfA* proteins in the sediments of the Yellow River estuary and wetland,
respectively (Bu et al., 2017; Zhao et al., 2020). *Ignavibacteria* has been found in both natural environments and artificial wastewater treatment systems and is known to be involved in the N cycle and the degradation of aromatic substances (Bu et al., 2017; Han et al., 2020). Moreover, during anaerobic benzene degradation, the presence of *Ignavibacteria* is associated with DNRA (Han et al., 2020).

Among the DNRA bacteria in which the *nrfA* gene has been identified, the amoeboid phylum *Proteobacteria* is the most numerous, with a predominantly *Delta*- and *Gammaproteobacteria* phenotype. Some studies have indicated that *Acidobacteria* is also a type of DNRA bacteria (Decleyre et al., 2015). The highest abundance of *Anaeromyxobacter* was observed at the genus level, and this genus is known to be distributed in rice roots and perform DNRA bacterial functions (Cabezas et al., 2015). The abundance of *Pelobacter* and *Geobacter* was also high. *Pelobacter* and *Geobacter* belong to the Desulfuromonadales order. *Pelobacter* is a fermentative DNRA bacterium, while *Geobacter* can reduce insoluble Fe (III) minerals using organic matter as an electron donor with synergistic nitrate reduction (Kraft et al., 2014; Sanford et al., 2002).

Analyzing the community structure of DNRA in the Huangpu River identified the class *Deltaproteobacteria* (belonging to phylum *Proteobacteria*) as the dominant DNRA group. The *Anaeromyxobacter* genus had the highest abundance at all sites; also, the known DNRA functional bacterium *Geobacter* was more abundant than the other genera at each site.

### 4.3 Coexistence of heterotrophic and autotrophic DNRA

Both heterotrophic DNRA and autotrophic DNRA were identified in our analysis. *Desulfurivibrio* was detected, which is sulfur autotrophic DNRA (S-DNRA) bacteria (Jones et al., 2017). *Desulfurivibrio* can use H₂S as an electron donor for nitrate isomerization reduction and produce sufficient amount of ammonia nitrogen when organic matter is lacking (Jones et al., 2017). *Desulfurivibrio alkaliphilus* of the genus *Desulfurivibrio* can also oxidize sulfide for DNRA processes (Eisenmann et al., 1995). It has been proven that *Desulfurivibrio alkaliphilus* can grow by disproportionation of intermediate sulfur compounds (Pellerin et al., 2019).

The heterotrophic DNRA genera detected with high abundance in this study included *Anaeromyxobacter, Pelobacter, Geobacter,* and *Anaerolinea.* Among them, *Anaeromyxobacter* accounted for 8.5-26.4% of the genera at the six sites. Interestingly, *Anaeromyxobacter* accounted for up to 17.4% of the genera at the HJG site. The abundance of autotrophic DNRA *Desulfurivibrio* was also higher at the HJG site (Fig. 3), indicating the coexistence of autotrophic DNRA and heterotrophic DNRA at high levels. *Pelobacter* accounted for 1.8-24.2% of the genera at all sites. We observed high C/N values at the HJG site (Table S1), which may favor multiple microorganisms' coexistence (Friedl et al., 2018). The SZH site was located at the confluence of the Wusong River and Huangpu River. The environmental parameters at this sampling site were more complex than other sites, which could be a favorable condition for the coexistence of autotrophic and heterotrophic DNRA. The RDA results (Fig. 4) indicated that the SZH site was negatively correlated with the C/N ratio. Therefore, the DNRA bacteria at the SZH site might not make
significant use of C sources but were likely to use sulfur as an electron donor to carry out their life activities. This was likely one of the reasons for the higher abundance of autotrophic DNRA at site SZH. These results suggested that the coexistence of heterotrophic and autotrophic DNRA in the urban river network system was worthy of deeper investigation.

### 4.4 Relationship between the DNRA process and environmental factors

DNRA and denitrification bacteria, which coexist in a variety of natural habitats, compete for reducing nitrate and oxidizing carbon sources. Heterotrophic DNRA and denitrification processes require carbon sources as electron donors and nitrate as electron acceptors in synergistic competition (Sgouridis et al., 2011). The competition mechanism is influenced by the surrounding environmental factors (Davis et al., 2008; Woodward et al., 2009).

In different ecosystems, the contribution of DNRA in soil or sediment is strongly correlated with soil water content, pH value, C/N value, and NO$_3^-$-N and NO$_2^-$-N concentrations (Dong et al., 2011; Friedl et al., 2018; Schmidt et al., 2011). These conclusions were also confirmed in this study (Fig. 4). Combined with RDA and Pearson analysis, the DNRA rate and *nrfA* gene abundance were positively but not significantly correlated with pH and water content. It has been reported that an alkaline environment is more beneficial for DNRA (Yoon et al., 2015). Friedl et al. (2018) pointed out that high moisture content increased unstable carbon in soil and enhanced heterotrophic soil respiration. However, in this study, only moisture was positively but not significantly associated with the *nrfA* gene, and no significant correlation was found with the DNRA rate. The various indices characterizing community richness and the rank abundance curves show that the DNRA community richness was higher at sites SJG and CJ among the six sample sites in this study. Site CJ is close to the river-sea junction, which has more uncultured bacteria and a unique bacterial composition (Bu et al., 2017). This distribution was the reason for the higher community diversity at the CJ site. The high DNRA community diversity at the SJG site was associated with higher concentrations of SOC and lower concentrations of NO$_3^-$. Kraft et al. (2014) reported that a high C/N environment was favored by DNRA bacteria. DNRA rates were positively correlated with SOC and NH$_4^+$ concentrations, and the DNRA rate was significantly positively correlated with SOC ($P_{\text{SOC}}=0.003$). Van den Berg et al. (2016) found that denitrification and DNRA coexisted at a wide range of C/N ratios, and with increasing C/N, the dominant bacteria switched from denitrification to DNRA bacteria. It has been proven that DNRA is carried out mainly by heterotrophic bacteria and tends to occur in places with high organic matter concentrations (Liu et al., 2018; Van den Berg et al., 2015). DNRA processes have also been detected in natural ecosystems like shallow lake sediments and estuarine sediments and mainly involve heterotrophic bacteria (Deng et al., 2015; Pang and Ji, 2019).

### 5. Conclusion

In this study, DNRA rates, *nrfA* gene abundance, microbial community richness, and their interaction with environmental factors in the Huangpu River basin were analyzed. The $^{15}$N isotope tracer technique
showed that the DNRA rates were in the range of 0.1 to 0.9 nmol N g$^{-1}$ h$^{-1}$. The qPCR results showed that the abundance of the nrfA gene was much higher than that of other genes involved in nitrogen removal. The sites with the highest community diversity were SJG and CJ. *Proteobacteria* was the most enriched phylum at all sites based on pyrophosphate sequencing of the nrfA gene. In urban river network systems, heterotrophic DNRA and autotrophic DNRA coexisted. The physicochemical conditions also influenced the composition of the DNRA community in the freshwater river network. This study reveals the critical role of DNRA in urban freshwater river networks and improves the understanding of nitrate reduction processes in urban freshwater networks, particularly the DNRA process.

**Declarations**

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**Authorship contribution statement**

Tong Zhang: Investigation, Writing - original draft. Xuming Zhuang: Methodology. Shakeel Ahmad: Supervision, Methodology. Taeho Lee: Supervision, Methodology. Chengbo Cao: Writing - original draft. Shou-Qing Ni: Investigation, Writing - original draft, Funding acquisition.

**Conflict of interest/Competing interests**

Authors declare no conflict of interest/competing interests.

**Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Consent for publication**

Not applicable.

**Ethics approval and consent participate**

Not applicable.
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Tables

**Table 1.** DNRA rate and nrfA gene abundance at six sampling sites of the Huangpu River Estuary.

| Study site | DNRA rate | nrfA gene |
|------------|-----------|-----------|
| CST        | 0.34      | 2.42×10^{11} ± 5.61×10^{10} |
| HJG        | 0.69      | 8.75×10^{10} ± 2.68×10^{9}   |
| SJG        | 0.63      | 1.04×10^{11} ± 2.21×10^{9}   |
| SZH        | 0.10      | 1.36×10^{11} ± 4.04×10^{10}  |
| YZB        | 0.90      | 4.55×10^{10} ± 5.70×10^{9}   |
| CJ         | 0.19      | 2.85×10^{11} ± 8.77×10^{9}   |

**Table 2.** Number of nrfA sequences, richness and diversity estimators in the Huangpu River Estuary.

| Study site | High quality reads | Simpson | Chao 1 | ACE | Shannon |
|------------|--------------------|---------|--------|-----|---------|
| CST        | 79854              | 0.994060| 2908.72| 2889.62| 9.05    |
| HJG        | 90002              | 0.996805| 3171.23| 3220.37| 9.75    |
| SJG        | 92479              | 0.997955| 3744.38| 3769.41| 10.29   |
| SZH        | 60550              | 0.990977| 2216.00| 2216.00| 9.06    |
| YZB        | 81179              | 0.997223| 3096.04| 3012.67| 9.87    |
| CJ         | 100006             | 0.994438| 3793.80| 3805.11| 10.09   |

**Table 3.** Pearson’s correlation of nrfA gene abundance, DNRA rate, Chao1 and Shannon with the environmental parameters (N = 6)
|                  | pH  | Salinity | NO$_3^-$ N | NO$_2^-$ N | NH$_4^+$ N | SOC  | Moisture content |
|------------------|-----|----------|------------|------------|------------|------|------------------|
| **Coefficients** |     |          |            |            |            |      |                  |
| nrfA gene abundance | -0.129 | 0.412 | -0.048 | -0.355 | -0.810 | -0.575 | 0.621 |
| DNRA rate        | 0.306 | 0.041 | 0.426 | 0.268 | 0.588 | **0.956** | -0.141 |
| Chao1            | 0.309 | 0.460 | 0.197 | 0.149 | -0.453 | 0.375 | 0.454 |
| Shannon          | 0.306 | 0.333 | -0.055 | 0.229 | -0.143 | 0.433 | 0.054 |
| **P values**     |     |          |            |            |            |      |                  |
| nrfA gene abundance | 0.807 | 0.417 | 0.928 | 0.489 | 0.051 | 0.232 | 0.188 |
| DNRA rate        | 0.555 | 0.939 | 0.400 | 0.608 | 0.250 | **0.003** | 0.790 |
| Chao1            | 0.551 | 0.359 | 0.708 | 0.778 | 0.367 | 0.464 | 0.366 |
| Shannon          | 0.555 | 0.519 | 0.918 | 0.662 | 0.787 | 0.391 | 0.919 |

*means highly significant correlation when $P<0.01$.  

**Figures**
**Figure 1**

Abundances of related genes involved in dissimilatory nitrate reduction process at different sites along the Huangpu River.
Figure 2

Distributions of DNRA bacteria in the Huangpu River at the phylum (a) and genus (b) levels.
Figure 3

Richness heat map of 50 most abundant genera of DNRA bacterial communities in the Huangpu River.
Figure 4

Redundancy analysis (RDA) biplot representing the relationship between abundance and activity of DNRA bacteria and environmental factors.

Supplementary Files

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