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# Diversity and distribution of planktonic anaerobic ammonium-oxidizing bacteria in the Dongjiang River, China

Wei Sun[a,b,d], Chunyu Xia[a,d], Meiying Xu[a,d,*], Jun Guo[a,d], Aijie Wang[c], Guoping Sun[a,d,**]

[a] Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou 510070, China
[b] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510070, China
[c] Harbin Institute of Technology, Harbin 150090, China
[d] State Key Laboratory of Applied Microbiology Southern China, Guangzhou 510070, China

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ABSTRACT

Anaerobic ammonium-oxidizing (anammox) process has recently been recognized as an important pathway for removing fixed nitrogen (N) from aquatic ecosystems. Anammox organisms are widely distributed in freshwater environments. However, little is known about their presence in the water column of riverine ecosystems. Here, the existence of a diverse anammox community was revealed in the water column of the Dongjiang River by analyzing 16S rRNA and hydrazine oxidation (*hzo*) genes of anammox bacteria. Phylogenetic analyses of *hzo* genes showed that *Candidatus Jettenia* related clades of anammox bacteria were dominant in the river, suggesting the ecological microniche distinction from freshwater/estuary and marine anammox bacteria with *Ca. Brocadia* and *Kuenenia* genera mainly detected in freshwater/estuary ecosystems, and *Ca. Scalindua* genus mainly detected in marine ecosystems. The abundance and diversity of anammox bacteria along the river were both significantly correlated with concentrations of $NH_4^+$-N based on Pearson and partial correlation analyses. Redundancy analyses showed the contents of $NH_4^+$-N, $NO_3^-$-N and the ratio of $NH_4^+$-N to $NO_2^-$-N significantly influenced the spatial distributions of anammox bacteria in the water column of the Dongjiang River. These results expanded our understanding of the distribution and potential roles of anammox bacteria in the water column of the river ecosystem.



## Introduction

In a classical view, the nitrogen (N) cycle contributing to the fixed N removal is simplified using a two-layer model with aerobic ammonium oxidation producing nitrate through nitrification, and then the anaerobic denitrification as the sole pathway to eliminate inorganic N as atmospheric $N_2$ (Moore et al., 2011). The discovery of anaerobic ammonium oxidation (anammox) with ammonium oxidized by nitrite to produce $N_2$ under anoxic conditions revolutionized this view (Mulder et al., 1995; Schubert et al., 2006). This novel N transformation pathway is estimated to be responsible for high $N_2$ removal in marine ecosystems (~50%) (Thamdrup and Dalsgaard, 2002; Brandes et al., 2007), contaminated groundwater (18–36%) (Moore et al., 2011), fertilized paddy soil (4–37%) (Zhu et al., 2011b) and wetland (~33%) (Zhu et al., 2011a). These findings demonstrated that the anammox process represents an important step of the global N cycle in natural environments.

The anammox process is performed by a monophyletic order (*Candidatus Brocadiales*) of bacteria in the *Planctomycetes* phylum, including five described *Candidatus* genera: *Scalindua*, *Brocadia*, *Kuenenia*, *Anammoxoglobus* and *Jettenia* (Dang et al., 2013). Although it is impossible to isolate and culture pure anammox bacteria at present due to their slow growth rates (Schmid et al., 2001), phylogenetic analyses of 16S rRNA and functional genes are the key approaches for the detection of anammox bacterial communities in recent years. However, the diversity of anammox bacteria might be underestimated due to biased coverage and specificity of the 16S rRNA gene PCR primers (Amano et al., 2007; Dale et al., 2009). Functional genes encoding hydrazine synthase (HZS) and hydrazine oxidoreductase (HZO) are essential for the anammox process and proposed as biomarkers for amplifying anammox bacteria from various environments, such as marine and estuarine sediments (Dang et al., 2010, 2013; Hirsch et al., 2011; Wang et al., 2012) and soils (Wang and Gu, 2013), and oilfield (Li et al., 2010a). Both *hzs* and *hzo* could provide physiology information of anam-

* Corresponding author. Tel.: +86 20 87684471; fax: +86 20 87684587.
** Corresponding author. Tel.: +86 20 87681673; fax: +86 20 87684587.
*E-mail addresses:* xumy@gdim.cn (M. Xu), guopingsun@163.com (G. Sun).

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mox bacteria and may be superior to 16S rRNA gene-based analysis (Dang et al., 2013).

Since the identification of anammox process contributed to the N removal in marine ecosystems (Kuypers et al., 2003), gradually increasing proof of wide existence of anammox bacteria and their potential ecological roles was reported in freshwater ecosystem (Penton et al., 2006; Zhang et al., 2007; Moore et al., 2011; Yoshinaga et al., 2011; Zhu et al., 2011a; Hu et al., 2012b; Sun et al., 2014). However, there have been only five known studies characterizing anammox communities in river sediments (Zhang et al., 2007; Hirsch et al., 2011; Hu et al., 2012b; Sonthiphand and Neufeld, 2013; Sun et al., 2014) since the first report of anammox bacteria in the Xinyi River (Zhang et al., 2007). Thus, the information on the characteristics of anammox bacteria in river ecosystems is presently not well-known. Moreover, according to the best of our knowledge, little information is available for the distribution of planktonic anammox bacteria in the water column of river ecosystems. In large rivers, nitrification is significant in the water column due to the comparative small surface to volume (S:V) ratio of the river (Brion et al., 2000), and the coupling N removal process (dinitrification or anammox) may play important roles in the water column of the river. Therefore, it is indispensable to know the characteristics of planktonic anammox bacteria in the river ecosystem.

The Dongjiang River, originating from Jiangxi province and situated mainly in Guangdong province of China, is one of the largest tributaries of the Pearl River (the third largest river in China). The ongoing anthropogenic activity and economic growth are increasing rapidly along the Dongjiang river basin. As a result, the river water quality is imperiled by the pollution from the industrialization, urbanization and agriculture activities. To avoid serious harm to public health and control such pollutions in the river ecosystem, it is indispensable to know the N cycle process associated with the elimination of N compounds and roles of functional microorganisms involved. In previous studies, not only the ammonia-oxidizing archaea and bacteria (AOA and AOB) in the water column of the Dongjiang River, but also the presence of AOA, AOB, and anammox bacteria were confirmed in the sediments of the river (Liu et al., 2011; Sun et al., 2013, 2014). However, anammox organisms could live under low nutrient conditions ($NH_4^+$-N below detection) (Thamdrup et al., 2006; Hamersley et al., 2007) and have a high affinity for nitrite in natural environments (Schmid et al., 2007). Thus, in this study, we hypothesized that anammox bacteria would be favored when low contents of $NO_2^-$-N and $NH_4^+$-N were present in the deep water column of the Dongjiang River with limited oxygen concentrations, and anammox communities might be regulated by the environment factors. We aimed to detect the community composition, diversity and abundance of anammox bacteria using both 16S rRNA and *hzo* genes as targets, and assess key environment factors shaping the planktonic anammox community in the water column along the Dongjiang River. Our results showed that diverse planktonic anammox bacteria existed in the oligotrophic water columns of the Dongjiang River and the diversities and distribution changes of anammox bacterial communities were closely correlated with environmental conditions. This work expands our knowledge about the attributes of anammox bacteria in the water column of river ecosystems and their potential regulatory factors.

## Materials and methods

### *Site description and sample collection*

The Dongjiang River serves as a major source of potable water for 40 million inhabitants in Guangdong province, Shenzhen and Hong Kong. The five sampling sites, with one site Xinfeng (XFW) in the tributary and the others Heyuan (HYW), Guzhu (GZW), Huizhou (HZW) and Qiaotou (QTW) in the mainstream, were selected along the river from upstream to downstream according to nutrient gradients (Fig. S1). The detailed information of the site characteristics was shown in the previous report (Sun et al., 2014). Three water samples from each sampling site were collected by using plexiglass water sampler (WB-PM, Beijing Purity Instrument Co., Ltd., China) from the 1 to 5 m below surface water of the river in the dry season (March) of 2011. All water samples of approximately 1–2 L were filtered through a 0.22-μm nucleopore filter (diameter: 47 mm) within 12 h after water collection, and kept at −80 °C until DNA extraction.

### *Environmental factor analyses*

At each sampling site, pH, temperature, and dissolved oxygen (DO) were measured *in situ* with Universal Pocket Meter Multiline P4 (Universal-Tas-chenmebgerat, Germany). $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N were determined by the methods described by Liu et al. (2011). Total carbon (TC), total organic carbon (TOC), and total nitrogen (TN) were determined on a Total Organic Carbon Analyzer (Elementar, Hanau, Germany).

### *PCR-clone library construction of anammox bacterial 16S rRNA and hzo genes*

DNA was extracted from all water samples as reported previously (Liu et al., 2011, 2012). The amplification of anammox bacterial 16S rRNA genes was conducted using the primer pair Brod541F (5′-GAGCACGTAGGTGGGTTTGT-3′) and Amx 820R (5′-AAAACCCCTCTACTTAGTGCCC-3′) (Li et al., 2010a). The primer set for targeting hydrazine oxidation (*hzo*) genes was Ana-*hzo*1F (5′-TGTGCATGGTCAATTGAAAG-3′) and Ana-*hzo*2R (5′-ACCTCTTCWGCAGGTGCAT-3′) (Quan et al., 2008). The reaction conditions and PCR protocols were detailed in the previous report (Sun et al., 2014). The amplified PCR products from 3 reactions of each sample were firstly examined in 1.0% agarose gels by electrophoresis, pooled to minimize PCR bias and then purified using the Takara Gel Clean-Up Kit (Takara, Japan). Purified DNA fragments were ligated into pMD-19T simple vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5α. Plasmid insert-positive recombinants were selected and verified by PCR amplification with the primer set of RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) and M13R (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′).

### *Sequencing and phylogenetic analysis*

The verified positive clones were sequenced using the ABI 3100 automated sequencer (Applied Biosystems, Foster City, California). DNA sequences were examined and edited using DNASTAR Lasergene SeqMan Program (DNASTAR, Madison, Wisconsin). Chimeras were checked by using Bellerophon (Huber et al., 2004). NCBI BLAST (http://www.ncbi.nih.gov) was used to find the most closely related 16S rRNA gene sequences and HZO protein sequences in the public databases. Phylogenetic analyses were conducted using the MEGA 5.0 program (Tamura et al., 2011). The phylogenetic tree of 16S rRNA gene sequences was constructed using the neighbor-joining topology (Saitou and Nei, 1987) with Kimura's two parameter method (Kimura, 1980). Bootstrap values were obtained from data resampling of 1000 replicates. Phylogenetic analysis of HZO protein sequences translated from *hzo* gene sequences was constructed with the representative phylotypes selected. The phylogenetic tree of HZO protein sequences was constructed using the neighbor-joining