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Overall bacterial community composition and abundance of nitrifiers and denitrifiers in a typical macrotidal estuary

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ABSTRACT

Coupled nitrogen cycling processes can alleviate the negative effects of eutrophication caused by excessive nitrogen load in estuarine ecosystems. The abundance and diversity of nitrifiers and denitrifiers across different environmental gradients were examined in the sediment of Hangzhou Bay. Quantitative PCR and Pearson's correlation analyses suggested that the bacterial ammonia-oxidizers (AOB) were the dominant phylotypes capable of ammonia oxidation, while the *nirS*-encoding denitrifiers predominated in the denitrification process. Simultaneously, nitrite and pH were found to be the two major factors influencing *amoA* and *nir* gene abundances, and the distribution of bacterial communities. Moreover, the ratio of *nirS*/AOB *amoA* gene abundance showed negative correlation with nitrite concentration. Fluorescence *in situ* hybridization further demonstrated that AOB and acetate-denitrifying cells were closely connected and formed obvious aggregates in the sediment. Together, all these results provided us a preliminary insight for coupled nitrification-denitrification processes in the sediment of Hangzhou Bay.

1. Introduction

Over the last few decades, coastal eutrophication caused by excessive nitrogen (N) discharge has become a matter of global concern (Howarth et al., 2002). Estuarine eutrophication has resulted in a suite of environmental problems, such as hypoxic events (Rabalais, 2002) and harmful algal blooms (Paerl et al., 2002), and greatly threaten both the economy and human health. Accordingly, bioavailable N from landscapes to coastal waters can be removed by tightly-coupled microbial processes, such as coupled nitrification-denitrification (Seitzinger et al., 2006) or coupled nitrification-anammox (the anaerobic oxidation of ammonium) (Lam et al., 2007). Nitrification, the two-step conversion of ammonium (NH_4^+) to nitrate (NO_3^-) *via* nitrite (NO_2^-), is commonly thought to play a vital role in N cycle (Head et al., 1993). As is known to all, ammonia oxidation is the first and rate-limiting step of nitrification and is catalyzed by ammonia monooxygenase (AMO), which is encoded by the *amoA* gene from both archaea and bacteria. It is generally assumed that ammonia-oxidizing archaea (AOA) not ammonia-oxidizing bacteria (AOB) are the main contributors to ammonia oxidation process in marine environments (Francis et al., 2005; Könneke et al., 2005; Wuchter et al., 2006; Smith et al., 2014), however, the predominant ammonia oxidizers in estuarine ecosystems remain more uncertain. Being an intermediate zone between land and ocean, the estuarine area often experiences salinity and nutrient gradients, which may have important impacts on the temporal and spatial dynamics of ammonia oxidizers (Zheng et al., 2014b). As noted earlier, numerous studies suggest that AOA usually outnumber AOB in estuarine environments and play more important roles in nitrification process, such as the researches in Bahía del Tóbari, Mexico (Beman and Francis, 2006), Monterey Bay, USA (Mincer et al., 2007), the Yangtze Estuary, China (Dang et al., 2008; Zheng et al., 2014b), the Fitzroy Estuary, Australia (Abell et al., 2010), the Plum Island Sound Estuary, USA (Bernhard et al., 2010) and the Pearl River Estuary, China (Jin et al., 2011). Besides, a few studies found that nitrification might be driven by bacteria rather than archaea in estuarine environments, such as the researches in San Francisco Bay, USA (Mosier and Francis, 2008) and the Colne Estuary, UK (Li et al., 2014).

Denitrification, the sequential reduction of NO_3^- to dinitrogen gas (N_2) *via* oxidized intermediates, can remove more than half of the inorganic nitrogen (DIN) inputs from terrestrial ecosystems when coupled

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with nitrification (Seitzinger et al., 2006). Denitrification is considered to be the dominant loss pathway for fixed N in shallow coastal and estuarine systems (Bulow et al., 2008; Mosier and Francis, 2010), although anammox has been recently identified as an alternative microbial pathway of N_2 production (Ward et al., 2009). Two nitrite reductases are key enzymes in the denitrification pathway: copper-containing nitrite reductases and cytochrome cd_1 nitrite reductases (encoded by *nirK* gene and *nirS* gene respectively). Though these two forms of *nir* genes are supposed to be functionally equivalent (Zehr and Ward, 2002), *nirS* gene appears to be more abundant in estuaries according to previous studies, such as the researches in the Fitzroy Estuary, Australia (Abell et al., 2010), San Francisco Bay, USA (Mosier and Francis, 2010) and Laizhou Bay, China (Wang et al., 2014). Crucial factors known to influence the diversity and abundance of denitrifiers include substrate availability (Kemp et al., 1990), oxygen concentration (Smith et al., 2006), salinity, temperature and pH (Salehlakha et al., 2009).

Hangzhou Bay is located in the northern part of Zhejiang Province, China. It is the outer part of the Qiantang River Estuary and adjacent to the East China Sea. Covering an area of approximately 8500 km^2, it is one of the world's largest macrotidal embayments. The tidal amplitude at the mouth is 3–4 m, and it exceeds 4–6 m further upstream. Tidal currents are mainly rectilinear and the maximal flood velocity exceeds 4.0 m/s (Xie et al., 2009). The major rivers discharging directly into Hangzhou Bay are Qiantang River, Cao-e River and Yong River, with average water discharge of 44.4 km^3/a and DIN load of $> 3.8 \times 10^4$ t/a (Zhang et al., 2002). Over the past few decades, excessive anthropogenic N from agricultural production, domestic and industrial wastewater discharge and fish farming has resulted in severe eutrophication of Hangzhou Bay (Huo et al., 2010). Here, the water quality is far worse than Grade IV Sea Water Quality Standard of China (SOA, 2014). Influenced by tidal currents and waves, Hangzhou Bay has a high carrying capacity for suspended particulate matter, in which the net primary production tends to be light-limited (Xie et al., 2009). Thus, N biogeochemistry in such turbid environments, which is almost exclusively reliant on reduction-oxidation reactions, is facilitated primarily by non-phytoplankton microorganisms (Dang and Jiao, 2014). However, to the best of our knowledge, the microbial N cycling processes in this eutrophic macrotidal estuary as yet remain unclear.

This study aims to (I) evaluate the abundance and diversity of nitrifying (AOA *amoA* and AOB *amoA*) and denitrifying (*nirS* and *nirK*) phylotypes with quantitative PCR (qPCR), and examine the major environmental factors controlling the distribution of nitrifiers and denitrifiers in the estuary; (II) characterize the distribution of bacterial communities and its environmental regulation information using Illumina MiSeq sequencing; (III) demonstrate the ecological niche of nitrifiers and denitrifiers by fluorescence *in situ* hybridization (FISH).

2. Materials and methods

2.1. Sampling and environmental parameters

Sediment and overlying water samples were collected from Hangzhou Bay along a salinity gradient in May 2014, when the quantity of phytoplankton was quite low (Cai, 2006) (Fig. 1). Overlying water samples were collected with a 5-L Niskin bottle (Tianjin test center, Tianjin, China). Standard oceanographic properties, including water temperature, salinity, dissolved oxygen (DO) and pH, were measured on board immediately using a Horiba U-52 water quality checker (Horiba, Kyoto, Japan). Then the overlying water from each site was sampled triple from the Niskin bottle, and transferred to acid washed polyethylene bottles. The concentration of chlorophyll a (Chl a) was measured following the standard protocols described previously (Strickland and Parsons, 1972). Sediment samples were taken with a 250-cm^3 Van Veen Grab (Hydro-bios, Kiel, Germany). The upper layer (0–5 cm) sediment of each site was sliced, mixed and homogenized, and then put into a sterile plastic bag and quickly stored in the − 20 °C ice box for further analyses. Inorganic N (NH_4^+, NO_2^- and NO_3^-) were extracted from the sediment using 2 M KCl as previously described (Shen et al., 2013). The concentration of total phosphorus (TP) in sediment was measured colorimetrically by the ascorbic acid-molybdate blue method (Murphy and Riley, 1962).

2.2. DNA extraction

Total genomic DNA of each sediment sample (0.4–0.6 g) was extracted using a FastDNA spin kit for soil (Qbiogene, Carlsbad, CA, USA), following the manufacturer's instructions. Duplicate DNA extractions for each water sample were performed. DNA quality was detected through 1% agarose gel electrophoresis which was stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). The duplicate DNA extractions were then merged together, and stored at − 80 °C for subsequent molecular analysis.

2.3. Quantitative PCR (qPCR)

The abundance of functional marker genes, including AOA *amoA*, AOB *amoA*, *nirK* and *nirS* gene, were quantified by qPCR analysis using a CFX 96C 1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Standard curves were generated using serial tenfold dilutions (10^{-1} to 10^{-5}) of linearized plasmids containing cloned AOA *amoA*, AOB *amoA*, *nirK* and *nirS* genes. The 20 μL reactions contained 0.4 μL of each primer (10 mM), 10 μL of SYBR Premix Ex Taq (Takara, Tokyo, Japan) and 2 μL of template DNA. Primers used in this study are listed in Table S1. The PCR cycle started with 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at the specific annealing temperature and 30 s at 72 °C. The specificity of amplification was checked by the observation of melt curves. The PCR amplification efficiencies were 83–100.7%, and correlation coefficients (R^2) for all assays were > 0.99. All samples and standard reactions were performed in triplicate, and average values were calculated.

2.4. Illumina MiSeq sequencing

Bacterial communities were investigated at the sediment samples of Hangzhou Bay, using high-throughput sequencing according to the protocols described by Caporaso et al. (2011). The V4 regions of the bacterial 16S rRNA gene were amplified from the DNA extracts using the primers 520F (5′-barcode-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) (Klindworth et al., 2015). The barcode is a seven-base sequence unique to each sample. The sequencing was then conducted on the Illumina MiSeq platform (Personalbio, Shanghai, China) and altogether generated 385,806 reads of 16S rRNA gene from nine sediment samples. Raw sequencing data were de-multiplexed and quality-filtered using the default parameters in Qiime version 1.7.0 (Caporaso et al., 2010). Criteria used for the filtering step were recommended by Bokulich et al. (2013). The remaining high quality 16S rRNA gene sequences were then clustered into operational taxonomic units (OTUs; 97% similarity) with uclust in Qiime (Edgar, 2010). A bootstrap cutoff of 50% suggested by the Ribosomal Database Project (RDP) was applied for taxonomic assignment (Wang et al., 2007). Based on OTU numbers, the alpha diversity measures (Chao 1 and Shannon index) were calculated in Mothur version 1.31.2 (Schloss et al., 2009).

All V4 sequence data are available in the NCBI Short Read Archive database (Accession Number: SRP091594).

2.5. FISH analysis

Three 16S rRNA-targeted oligonucleotide probes were used for *in situ* detection of nitrifying and denitrifying bacteria: (1) Cy3-labeled NSO190 probes, specific for ammonia-oxidizing β-subclass *Proteobacteria*; (2) Cy5-labeled DEN67 probes, specific for methanol-