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Distribution of typical denitrifying functional genes and diversity of the *nirS*-encoding bacterial community related to environmental characteristics of river sediments

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Abstract

Denitrification in river sediments leads to nitrate removal from the aquatic system; therefore, it is necessary to understand functional diversity of denitrifier communities in the system. Sediment samples (0–25 cm depth) were collected from three typical locations along the Pearl River. The real-time PCR approach was used to measure the abundance of nitrate (*narG*), nitrite (*nirS*, *nirK* and *nrfA*), and nitrous oxide (*nosZ*) reductase genes from the sediment samples. Assemblages of *nirS*, *nirK* and *nosZ* indicated that complete denitrification occurred in sediment cores, with the greatest number of gene copies from 5–15 cm depth. Dissimilatory nitrate reduction appeared to be important below 15 cm depth, based on increasing gene copies of *narG* and *nrfA* with sediment depth. There was a close match (78–94 %) between the *nirS* sequences recovered from Pearl River sediment and those detected in estuarine and marine sediments as well as active sludge, suggesting that domestic sewage inputs and irregular tides. Canonical correspondence analysis indicated that the spatial distribution of denitrifying bacteria was highly correlated with dissolved inorganic N (DIN: NH_4^+, NO_2^- and NO_3^-) concentrations in sediment. We conclude that changes in DIN within the sediment profile influences the distribution of denitrifying genes and the *nirS*-encoding denitrifier community in the river sediment. Our results also reveal a variety of novel denitrifying bacteria in the river sediment.

1 Introduction

Agricultural and other anthropogenic activities result in increasing amounts of nitrogen compounds entering the freshwater and marine systems, causing eutrophication problems. In the microbial process of denitrification, nitrate (NO_3^-) is converted to gaseous forms that are lost from the systems (Canfield et al., 2010). Therefore, the denitrification process should help to minimize the eutrophication problems. Since there is a large amount of denitrifiers and nitrogen compounds in eutrophication sediment, sediment is an important environment for nitrogen removal from an aquatic system through denitrification.

Bacterial denitrifiers are taxonomically diverse due to the fact that each bacterial species may participate in only one step of the denitrification process (Burgin et al., 2007). Denitrification consists of four sequential steps and involves four metalloenzymes. The first step is to reduce NO_3^- to nitrite (NO_2^-) catalyzed by a membrane-bound NO_3^- reductase (*nar*) or periplasmic NO_3^- reductase (*nap*), which are encoded by the *narG* or the *napA* genes, respectively. Denitrifying bacteria contain one or both of the NO_3^- reductase (i.e., *narG* and *napA*), in which *narG* is considered to be more extensive and representative (Deiglmayr et al., 2004; Smith et al., 2007; Reyna et al., 2010). In the second step, NO_2^- is reduced to nitric oxide (NO). This reaction distinguishes denitrifier community from other nitrate-reducing bacteria and is catalyzed by two functionally and physiologically equivalent types of NO_2^- reductases, either a cytochrome cd1 (encoded by *nirS*) or a Cu-containing enzyme (encoded by *nirK*) (Glockner et al., 1993). *Nir* gene sequence diversity in the environment is taken as a measure for diversity of denitrifiers (Prieme et al., 2002; Yan et al., 2003). In the third step, NO is reduced to nitrous oxide (N_2O), catalyzed by NO reductase (encoded by the *norB* gene). N_2O is an obligate intermediate, some of which ultimately escapes to the atmosphere. The reduction of N_2O to nitrogen gas (N_2) is the last step, which is catalyzed by N_2O reductase encoded by the *nosZ* gene present in the periplasm (Throbäck et al., 2004; Chon et al., 2009). These reductase genes are generally used as biological makers of the denitrification process because an approach involving 16S rRNA is not suitable to investigate the diverse community of denitrifying bacteria (Zumft, 1992; Braker et al., 2000).

The distribution of denitrifying genes in sediments are affected by many factors, including concentration gradient of the dissolved inorganic nitrogen (DIN, including NO_3^-, NO_2^- and NH_4^+), organic matter content, dissolved oxygen, and redox potential (Dong et al., 2009; Reyna et al., 2010). As the depth increases, oxygen concentrations decrease rapidly, microorganism changed from facultative aerobic phylotypes to strictly anaerobic phylotypes, and different denitrifiers (base on *nirS* diversity) change with the depth (Tiquia et al., 2006). Concentrations of denitrification substrates, the most important

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being DIN and organic matter, also change with the sediment depth (Dalsgaard et al., 2005; Meyer et al., 2008). More gene copies of *narG* and *nirS* were observed in the river sediment with higher NO_3^- concentrations (Chon et al., 2009). Availability of organic matter and carbon/nitrogen ratio determine bacteria biomass and NO_3^- reduction rates as well as pathways (Burgin et al., 2007; Dodla et al., 2008). Some dissimilatory NO_3^- reducing communities compete for NO_2^- with denitrifying bacteria. These NO_3^- reducing pathways such as anammox (anaerobic ammonium oxidation) and DNRA (dissimilatory nitrate reduction to ammonium), might appear in different sediment depths according to various carbon/nitrogen ratios (Burgin et al., 2007). When sediment conditions favor dissimilatory NO_3^- reducing processes, complete denitrification of NO_3^- to N_2 should be reduced or inhibited. One shortcoming of the previous investigations on this topic is their focus on shallow sediments (0–10 cm depth). Competitions among the different denitrification pathways and communities in the sediment profile are still poorly understood. Therefore, further research is needed to study the denitrifying genes and potentially interacting environmental factors in deeper sediments.

The aims of the current study were twofold: (1) to investigate the spatial distributions of denitrifying functional genes (*narG*, *nirS, nirK*, *nosZ*) as well as *nrfA*, a gene marker for DNRA (Dong et al., 2009) and diversity of denitrifier community along the DIN gradient in the Pearl River sediment, and (2) to determine the relationship between sediment chemistry and denitrifying functional genes in the sediment.

2 Materials and methods

2.1 Sample collection

The Pearl River is located in the subtropical region in the Guangdong Province, Southern China. This river represents one of the most important ecosystems linking the highly developing land area and the South China Sea. In recent years, the Pearl River has experienced high anthropogenic nitrogen loading due to rapid population increase and economic development.

Sediment samples were collected on 6 April 2010 at three locations along the Pearl River near Guangzhou city, China (23°8′ N, 113°17′ E), i.e., from the upstream at Zhujiang Bridge (ZB), midway down the river at Huadi (H), and from the downstream at Ersha (E) (Table 1). The three sample locations were chosen based on the contrasting land use, pollution sources and aquatic conditions. There is a municipal refuse disposal station near location ZB, resulting in a large amount of polycyclic aromatic hydrocarbons deposited in the sediment. Several small factories are around location H, resulting in chemicals such as chlorinated compounds and metals detected in the sediment. Also, a large amount of water lettuces floats on the surface at location H, which causes a hypoxia condition in the water body. Location E is close to residential areas, which input sewage to the river. The water depths at the sample locations were about 3 m and bottom water temperatures were about 23 °C. Salinity was low in this river section, between 1–12‰ depending upon the tide. Three sediment cores were collected at each site, with 2 m between locations selected for core removal. Each sediment core was 8 cm diameter and 30 cm long. Sediment cores were transported on ice and returned to the laboratory immediately. Each sediment core was sectioned into 5 cm slices (0–5 cm, 5–10 cm, 10–15 cm, 15–20 cm, 20–25 cm) and then transferred to sterile containers. About half of the sediment was designated for chemical analysis, which was performed within 24 h of collection on 3 replicates per depth (total = 15 samples). The remaining sediment sub-samples were stored at −80 °C until molecular analysis was performed.

2.2 Analysis of environmental parameters

Sediment pH values were determined using a pH meter (FED20, Mettler, Toledo). Water content and grain sizes of the sediment samples were analyzed using the gravimetric method and pipette method, respectively. By drying a sediment sample overnight at 105 °C to determine the dry weight, the organic matter content was obtained from the subsequent loss of weight after continuously drying the sample at 550 °C at least 2 h. Total carbon content (TOC) was calculated based on the organic matter content and

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the total nitrogen (TN) content was determined using a Foss Kjeltec 2300 Analyzer Unit (Foss Tecator AB, Höganäs, Sweden). Concentrations of NO_3^-, NO_2^-, and NH_4^+ were determined using the methods described by Ryan et al. (2001).

2.3 DNA extraction

Three independent samples of nucleic acids for each sediment slice were extracted from 500 mg sediment samples using the Fast DNA spin kit for soil as described in the manufacturer's instructions (Bio 101, Qbiogene Inc., CA, USA). Extraction of DNA was confirmed by gel electrophoresis. The concentration of the extracted DNA was measured using a Nano-drop spectrophotometer (Nano-Drop Technologies, USA) at 260 nm. A test of replicate extractions was performed to ensure reproducible yields from the DNA extractions.

2.4 Real-time PCR analysis

Table 2 lists the information on the primers selected for amplification of the different genes encoding 16S rRNA, i.e., *narG*, *nirS*, *nirK*, *nosZ,* and *nrfA,*. Each assay contained a standard using a serial dilution of known copies of PCR fragments of the respective functional genes, independent triplicate sediment DNA templates for each sediment slice, and triplicate no template controls (NTC). Experimental Q-PCR triplicates for each DNA sample were then averaged to obtain a single gene copy number. Real-time PCRs were carried out in LightCycler480 with Sequence Detection Software v1.4 (Applied Biosystems, USA). Each PCR mixture (10 µL) was composed of 5 µL of SYBR Premix Ex TaqTM II (2 ×), 0.4 µL 10 nM of each forward and reverse primers, 3.2 µL dH_2O and 1.0 µL of template DNA (TaKaRa Biotechnology, Japan). PCR amplification and detection were performed in LightCycler480 Multiwell (384-well) reaction plates with optical cap (Applied Biosystems, USA). The PCR temperature program was initiated with 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at the specific annealing temperature (Table 2), and 30 s at 70 °C. A melting curve analysis for SYBR

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Green assay was prepared after amplification to distinguish the targeted PCR product from the non-targeted PCR product. The potential presence of PCR inhibitors in sediment DNA extracts was tested by running a real-time PCR assay on serial dilution of sediment DNA extracts. No inhibition was detected in any case. All qPCR tests were carried by triplicate.

2.5 *NirS*-encoding bacterial clone library analyses

The *nirS* gene fragments (∼890 bp) were amplified using the primer pair nirS1F (5′ CCT A(C/T)T GGC CGC C (A/G)C A(A/G)T 3′) and nirS6R (5′ CGT TGA ACT T(A/G)C CCG T 3′) (Braker et al., 1998). PCR amplifications of *nirS* from environmental samples were performed in a total volume of 50 µL, containing 5 µL of 10 × PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris-HCl [pH 8.4]. 0.1 % Triton X-100), 200 µM each deoxyribonucleoside triphosohate, 1.0 U of Taq polymerase (5 U $µL^{-1}$; Appligene Oncor, Illkirch, France), 35 pmol of both primers (5 pmol $µL^{-1}$ each), and DNA (10 ng). After a denaturation step of 5 min at 95 °C, a touchdown PCR was performed (Thermocycler 2400; Perkin-Elmer, Branchburg, N.J.). The touchdown PCR consisted of a denaturation step of 30 s at 95 °C, a primer-annealing step of 40 s, and an extension step of 40 s at 72 °C. After 30 cycles, a final 7 min incubation at 72 °C was performed. During the first 10 cycles, the annealing temperature was decreased by 0.5 °C every cycle, starting at 45 °C until it reached a touchdown at 40 °C. The additional 20 cycles were performed at an annealing temperature of 43 °C. The amplification products were analyzed by electrophoresis on 1 % (wt/vol) agarose gels (Genesnap, SYNGENE) followed by a 15 min stained with Genefinder (0.02 µL mL^{-1}). PCR products were purified by agarose gel extraction and cloned into a PMD19-T vector. Positive recombinant clones were identified by colony PCR and the PCR products were cleaned with ExoSap treatment and sequenced on an automated ABI3730 automatic sequencer (Applied Biosystems, USA).

Six sediment sub-samples were selected and used for an extensive analysis of *nirS* gene diversity as determined through cloning experiments. The six samples were

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obtained from locations ZB and H at three depths (0–5, 5–10, and 10–15 cm), respectively. The *nirS* gene sequences were grouped into operational taxonomic units (OTUs) based on a 5 % sequence distance cutoff calculated using the DOTUR program (Schloss and Handelsman, 2005). Sequences reported in this study were deposited in GenBank database under accession numbers JN016541-JN016591.

2.6 Phylogenetic analysis

Datasets of *nirS* sequences were analyzed using the Bayesian inference (BI) (Huelsenbeck et al., 2001), implemented with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). A best fitting model of nucleotide substitution was identified using the Akaike information criterion (AIC) (Akaike, 1973) as implemented in MrModelTest 2.3 (Nylander, 2004). Bayesian analysis was carried out using GTR+I+G model selected by MrModelTest 2.3, in which model parameters were treated as unknown and estimated through the BI. The following settings were applied: implementing two Markov chain Monte Carlo (MCMC) runs, running four simultaneous Markov chains for 19 million generations, and sampling the Markov chains every 100 generations. Tracer V1.5 (Rambaut and Drummond, 2009) was used to judge convergence of the Bayesian Markov chain Monte Carlo runs. The first 14 000 sampled trees were discarded as burn-in. A consensus tree as show in Fig. 1 was constructed from the remaining sampled trees.

2.7 Statistical analysis

The PCR efficiency was estimated as follows (Bustin et al., 2009)

$$E = 10^{-1/S} - 1 \quad (1)$$

where E is the PCR efficiency and S is the slope of the line of standard curve obtained from tenfold dilution series. Q-PCR data from the different samples were analyzed

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using one way analysis of variance (ANOVA) with the significant level $P < 0.05$. The coverage of each clone library (C) was calculated by (Mullins et al., 1995)

$$C = 100\%[1 - (n/N)] \quad (2)$$

where n is the number of unique OTUs and N the total number of clones in a library. Indices of gene diversity (Shannon-Wiener H and Simpson D) and evenness (E) were calculated using the methods of Schloss and Handelman (2005). Rarefaction analysis and two nonparametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao1 (S_{Chao1}), were calculated using DOTUR (Schloss and Handelman, 2005). Correlations between the genes distributions or *nirS*-encoding clusters assemblage vs. environmental factors were analyzed with the canonical correspondence analysis (CCA) (Dang et al., 2008), in which, the OTUs percentage frequency data in each library were used as the species input. Analyses of Spearman rank, multivariate and stepwise linear regressions of environmental and gene abundance variables were carried out using SPSS v12 on both raw and log-transformed data.

3 Results

3.1 Environment conditions of Pearl River sediments

The sediments were mainly soft silt, changing from gray in the surface layer to black several centimeters below. The black sediment had a light hydrogen sulfide smell. As shown in Table 1, on average, DIN contents were rather high, especially NH_4^+, compared to other river sediments (Richardson et al., 2004; Laverman et al., 2010). DIN concentrations of the sediment surfaces (0–5 cm) increased from locations ZB to E, such that NO_3^-, NO_2^-, and NH_4^+ increased from 8.60, 0.34, 153 mg kg^{-1} to 19.7, 1.28 and 1613 mg kg^{-1}, respectively. DIN concentrations at location E were much higher than those at the other sampling locations, which were attributable to the inputs of domestic wastewater. Tides might be another reason to cause the pollutant accumulation.

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NO_3^- and NH_4^+ increased with the sediment depth at locations ZB and H. Nitrate concentrations at locations ZB and H, respectively, changed from 8.60 and 8.71 mg kg^{-1} in the sediment surface to 12.0 and 17.7 mg kg^{-1} in the bottom layer, while NH_4^+ concentrations changed from 153 and 194 mg kg^{-1} to 1195 and 1218 mg kg^{-1}, respectively. Nitrate and NH_4^+ concentrations at location E were the highest among sample locations, while NO_3^- and NH_4^+ concentrations at location E showed insignificant variations with the sediment depth. In contrast, the vertical change of NO_2^- at location E was significant, and the concentrations were highest at the sediment surface and decreased with depth of 0–15 cm. TOC was abundant in the sediment and the maximum content appeared in E10 (10 cm depth at location E) layer (60.9 g kg^{-1}, Table 1). TOC values increased gradually from locations ZB to E.

3.2 Relative abundances of different denitrifying genes

Standard curves for real-time PCR were produced based on a serial dilution of known copies of PCR fragments of the respective functional genes generated using M13 PCR from clones. The standard curves were used as the references to extrapolate and calculate the concentrations of environmental DNA samples (the indepedent variable was the initial environmental DNA template concentration while the dependent variable was the C_q of the reactions for each primers). All the standard curves showed excellent correlations between the DNA template concentration and C_q with high coefficients of determination ($R^2 > 0.99$). The qPCR efficiency values calculated from Eq. (1) for 16S rRNA, *narG*, *nirS*, *nirK*, *nosZ* and *nrfA* were 0.964, 1.114, 0.863, 0.987, 1.020, and 1.258, respectively. The ratios of denitrifying genes to 16S rRNA gene extracted from total bacteria were determined to evaluate the relative abundances of various denitrifies compared to total bacteria.

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Distributions of the denitrifying genes (*narG*, *nirS*, *nirK, nosZ,* and *nrfA*) varied between the three sampling locations. Although the highest concentrations of DIN and TOC appeared at location E, the relative abundance of denitrifying genes was the lowest at this location. The lowest abundance of 16S rRNA was founded at location H, whereas the relative abundance of denitrifying genes was the highest at this location (Table 3). The relative contributions of *nirS* gene to total bacteria were the highest among the denitrification genes at all locations. Abundances of 16S rRNA decreased with the sediment depth at the three sampling locations. The result showed significant reduction of bacteria abundance with the sediment depth.

The relative abundance of *narG* gradually increased with the sediment depth, except for location ZB with the lowest proportion at 25 cm depth. For *nirS* and *nirK*, the relative abundance was high within the depth of 0–10 cm and decreased rapidly below 10 cm. Since the proportion of *nir* genes can represent the potential denitrification rate (Dong et al., 2009; Graham et al., 2010), the result indicated that nitrite reduction process was mainly carried out above 10 cm in the sediment. For *nosZ*, the highest proportions appeared in the depth of 5–15 cm. Interestingly, different from other denitrifying genes, relative abundance of *nosZ* was higher at location ZB. For *nrfA*, the relative abundance also gradually increased with sediment depth except for location H. It was noted that the total *nrfA* numbers actually decreased with the depth although the relative abundance of *nrfA* increased with the depth.

3.3 Phylogenetic diversity of *nirS*-encoding bacteria

The *nirS* gene libraries from the layers of ZB5, ZB10, ZB15, H5, H10, and H15 resulted in totally 305 *nirS* gene sequences from clones. These *nirS* gene sequences were assigned to 51 OTUs based on a 5 % cutoff. The coverage of these *nirS* clone libraries was 86 %, 79 %, 72 %, 75 %, 71 %, and 65 % in the layers ZB5, ZB10, ZB15, H5, H10, and H15, respectively. The diversity indexes of these libraries suggested a slightly increasing diversity of the denitrifying bacterial community with increasing depth

(Table 4) and the highest diversity was in layer ZB15. The rarefaction curve suggested a similar trend (data not shown).

Nitrite reductase gene sequences from the Pearl River sediment and other typical estuarine, marine environments, sewage treatment system were aligned. A phylogenetic tree indicated that the 51 *nirS* OTUs could be classified into nine clusters (Cluster-A to Cluster-I) (Fig. 1). Cluster-A dominated in all clone libraries with 57 %, 80 %, 52 %, 63 %, 61 %, and 65 % in the layers of ZB5, ZB10, ZB15, H5, H10, and H15, respectively. This cluster belonged to the family *Rhodocyclaceae*which contained mainly aerobic and denitrifying rod-shaped bacteria with very versatile metabolic capabilities (Martins et al., 2010; Wongwilaiwalin et al., 2010). One OTU was closely related to the family *Comamonadaceae*. Both these families were usually found in aquatic sediment habitats, constructed wetland, and activated sludge (Ruiz-Rueda et al., 2007; Spain et al., 2007). Sequences of Cluster-B were similar to species *Ralstonia eutropha strain* (78–90 % indentify), belonging to the family *Burkholderiaceae*. *R. eutropha H16* was previously known as *Alcaligenes eutrophus* and originally isolated from sludge. Cluster-C was closely affiliated with the species *Thiobacillus denitrificans*. This is a chemoautotrophic bacterium using sulfide as electron donor (Beller et al., 2006). The bacterium was only detected in deeper layers at location ZB with 3.3 % and 8.7 % in ZB10 and ZB15. Cluster-D sequences had high sequence similarity (88–94 % identity) with an environmental clone from Chesapeake Bay sediment (Bulow et al., 2008), accounting for 7.1 %, 6.7 %, 13.0 %, 6.3 %, 13.0 %, and 20.0 % in layers of ZB5, ZB10, ZB15, H5, H10, and H15, respectively. Cluster-E was detected in the sediment of location H with a percentage 12.5 %, 17.4 %, and 10 % of the total number of clones in H5, H10, and H15, respectively. Clones in this cluster were related to species *Dechloromonas aromatic*. Cluster-F was related to species *Aromatoleum aromaticum* (80–92 % identity) and mainly appeared in ZB sediment and had the largest percentage (40 %) for layer ZB10. Cluster-G and Cluster-H were only detected in deeper layer at location H, accounting for 4.3 % and 4.4 % in layer H10, 10.0 % and 5.0 % in layer H15, respectively. Sequences of Cluster-G were related

to *Magnetospirillum* sp. (84 % identity), which is a Gram-negative α-proteobacterium. This is a group of facultative anaerobic bacteria related to iron oxidation (Matsunaga et al., 2005). Cluster-H was related with *Herbaspirillum* sp. and belongs to the family *Burkholderiaceae*. These bacteria were usually found in the rhizosphere of soil and sediment environments. They were considered to participate in nitrogen fixation (Jung et al., 2007). Cluster-I belonged to the family *Candidatus Accumulibacter*, which was ecologically significant because it was used to remove phosphorous from waste water (Fukushima et al., 2007). Cluster-I appeared in ZB5, ZB10, ZB15, H5, and H10, accounting for 14.3 %, 13.3 %, 4.4 %, 12.5 %, and 8.7 %, respectively.

3.4 Spatial distributions of denitrifying genes and the *nirS*-encoding bacterial assemblage

Spatial distributions of denitrifying genes and *nirS*-encoding bacterial assemblages might be influenced by nutrient conditions in the Pearl River sediment. The CCA analysis of denitrifying genes abundance and *nirS*-encoding bacterial assemblages in response to environmental variables confirmed the influence. In Fig. 2a, the first two CCA axes (CCA1 and CCA2) explained 73 % of the total variance in the denitrifying gene abundance and 83 % of the cumulative variance of the gene-environment relationship. Dissolved inorganic nitrogen had significant effects on distributions of denitrifying genes. The CCA analysis showed that CCA1 represents the vertical distributions of denitrifying genes. Nitrite reductase genes mainly assembled in 0–10 cm in the sediment and *nosZ* distributed in 5–15 cm, while a large number of *narG* and *nrfA* genes gathered below 15 cm. Distributions of denitrifying genes had obvious stratification in the Pearl River sediment (Fig. 2a). CCA2 represented the nutritional level of the sediment. Results show that TOC was not limited to the distribution of denitrifying genes (Fig. 2a). Concentrations of NH_4^+ were significantly affecting *nrfA* distributions ($P = 0.025$) and *nirS* and *nirK* abundance was correlated with NO_2^- ($P = 0.032$). Distributions of *narG* were affected by the combined effects of DIN. However, *nosZ*

was negatively related to various sediment nutritional factors, which indicated that oligotrophic conditions might promote the activity of *nosZ* (Fig. 2a).

In Fig. 2b, the first two CCA axes (CCA1 and CCA2) explained 71 % of the total variance in the *nirS*-encoding bacteria composition and 81 % of the cumulative variance of the bacteria-environment relationship. CCA1 and CCA2 distinguished the *nirS*-encoding bacterial assemblage of layer ZB15 from those of the other layers. TOC and NO_2^- contributed more to the bacteria-environment relationship than the other environmental factors (Fig. 2b). The distribution of Cluster-D was positively correlated with NH_4^+ and Cluster-E was related to TOC and NO_3^-. However, the distributions of Cluster-F and Cluster-I were negatively correlated with the nutrient-rich conditions. Along the most important CCA1 axis, Cluster-D, -E, -G, and Cluster-H might correspond to nutrient-rich conditions in the sediment, whereas Cluster-F and Cluster-I corresponded to the less eutrophic conditions (Fig. 2b). Denitrifier community diversities were found between different sample locations and between sediment depths.

4 Discussion

The spatial distributions and concentrations of DIN in the sediment clearly showed the urban river characteristics of the Pearl River. Because of a large amount of domestic wastewater inputs, various pollution characteristics appeared at different locations. Concentrations of NH_4^+ in the sediment of this river section were higher than those of other rivers, and the vertical variations of NO_3^- were different from those in other river sediments (Richardson et al., 2004; Tiquia et al., 2006; Laverman et al., 2010). Previous studies showed that denitrifying bacterial diversity was higher at deeper sediment layers, but denitrification rates of deeper sediments could be neglected due to the absence of oxidized NO_3^- and NO_2^- (Tiquia et al., 2006). However, NO_3^- concentrations did not decrease with the sediment depth in the Pearl River and the relative abundance of *narG* was higher at deeper sediment layers (Tables 1 and 3). These results indicated that NO_3^- reduction rates in the deeper sediments could not be neglected in

the river region. Dramatically decrease of abundances of *nirS* and *nirK* below 15 cm did not cause NO_2^- accumulation, indicating that there were other NO_2^- reduction pathways in the deeper sediments. Carbon limited conditions (compared to the high concentrations of DIN) might inhibit DNRA process although relative abundance of *nrfA* increased with the sediment depth. These results suggested that *nrfA* played a key role in the NH_4^+-rich environment while in this environment the community was related to diverse metabolism pathways, such as anammox and sulfate-reducing (Mohan et al., 2004). Anammox, which drives N_2 production in the marine and estuarine environments, might be an important pathway to consume NO_2^- in this river sediment (Kuypers et al., 2003; Dalsgaard et al., 2005). Nevertheless, the N_2 generate efficiency of anammox and its impact on denitrification needs further studies. Our results showed that denitrification processes in different sediment depths were dominated by different NO_3^- and NO_2^- reduction pathways. Therefore, it is essential to study the vertical distribution of denitrification in the river sediment.

In this study, both *nirK* and *nirS* were detected in the Pearl River sediment. However, *nirK* abundance was much lower than *nirS* in the sediment because *nirK* only prevails in conditionally oxygen-exposed environments (Desnues et al., 2007; Knapp et al., 2009). These two nitrite reducing genes have different substrate requirements. Particular environmental conditions might alter the proportion of denitrifiers with *nirK* or *nirS*. Moderate levels of nitrate in the river sediment increase *nirS* diversity (Yan et al., 2003). The *nirS* gene is found to be more widespread in the bacterial communities in various sediments, (Priemé et al., 2002; Liu et al., 2003; Throbäck et al., 2004; Tiquia et al., 2006; Oakley et al., 2007; Dang et al., 2009). Therefore, we used the *nirS* gene to characterize the diversity of denitrifier communities in the Pearl River sediment. Most of the *nirS* sequences in this sediment had a close match with those originally detected in estuarine and marine sediments as well as sludge (Nogales et al., 2002; Tsuneda et al., 2005; Osaka et al., 2006; Ruiz-Rueda et al., 2007; Spain et al., 2007; Bulow et al., 2008), suggesting that this river has both tidal and urban characteristics. When the river is affected by both domestic pollutions and irregularly tides, diversity and distributions of

denitrifying bacteria should become more complicated. Our results strongly supported the hypothesis that the environmental characteristics controlled the composition and distribution of microbial communities, especially the key functional bacterial groups. In comparable environments, similar denitrifying communities may develop despite of different geographical locations (Castro-Gonázlez et al., 2005; Falk et al., 2007). Sediments from location ZB and H were use to study the diversity of denitrifier community according to the high abundance of *nirS* genes in these locations. Some of the *nirS* gene sequences were unique with sampling location, and many *nirS* gene sequences were novel in the Pearl River, as most of them had $< 70\,\%$ nucleic acid identity with those from cultivated strains and $< 90\,\%$ identity with those from environmental clones. Cluster-G and Cluster-H were only detected at location H, corresponding to the serious pollution created by aquatic plants and metal release at this location. Bacteria of Cluster-E and Cluster-F, which utilize different aromatic compounds, appeared only in the sediments of locations H and ZB, respectively, due to different categories of polycyclic aromatic hydrocarbons at the two locations (Chakraborty et al., 2005; Tamang et al., 2009). It is very likely that at least some of these bacteria are the contaminant-degrading species in the *nirS* communities. Lots of novel bacteria exist were also the reason for lower abundance of *narG* compared to *nirS* in this environment (Table 1). Further research is needed to cultivate the novel denitrifying bacteria, to study their ecophysiology, and to understand their roles and mechanisms in N cycling and environmental bioremediation. In addition, more specificity primers should been designed and verified experimentally.

In this study, besides the abundance of denitrifying genes, the composition of denitrifying bacterial communities based on *nirS* gene also showed vertical variations. Denitrifier communities were dominated by a few phylotypes in the surface layers and showed higher diversity in deeper layers. On the other hand, unique denitrifying bacterial phylotypes appeared in different sediment depths (Table 4 and Fig. 1). Denitrifying bacteria changed from facultative aerobic phylotypes to strictly anaerobic phylotypes attributable to the decrease of dissolved oxygen with the sediment depth. Bacteria of

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Cluster-I was facultative denitrifying bacteria, which prefer low dissolved oxygen conditions and usually accumulate in the upper sediments (Fukushima et al., 2007). Nitrification and denitrification processes can simultaneously occur in the surface sediment, thereby increasing the reduction rates of NO_3^- and NO_2^- (Rysgaard et al., 1993; Scott et al., 2008). *R. eutropha H16* of Cluster-B which prefers non-halophilic habitats and does not require oxygen appeared in deeper sediment (Schwartz et al., 2003). With the capability to carry out DNRA processes, bacteria of Cluster-D did not exist in the sediment surface, but the bacteria proportion became remarkable with the sediment depth. All of these results suggested that the vertical distributions of denitrifying genes in this sediment had a similar stratified characteristic, and that transformation, competition, and cooperation of different denitrifier communities were determined by the depth-dependent sediment characteristics. The stratified characteristic showed that the sediments could generally be divided into three layers. At 0–5 cm, synchronic nitrification and denitrification processes dominated in this low oxygen environment. At 5–15 cm, nitrite reductase gene (*nirS* and *nirK*) and *nosZ* were concentrated, potential denitrification ability were high, and the complete denitrification process (with the final product of N_2) dominated in this layer (Graham et al., 2010). Below 15 cm, a strict anoxic condition limited nitrification, thus a large amount of NH_4^+ accumulated and dissimilatory NO_3^- reducing pathways became obvious in this layer. Although the exact depth range of each layer may vary according to sample location conditions, the stratification pattern of denitrification is prevalent in different sediments (Fan et al., 2006; Tiquia et al., 2006).

Among the nutrient characteristics of the Pear River sediment, the TOC and NO_2^- had the most significant impact on the *nirS*-encoding bacterial community structure and spatial distributions (Fig. 2b). Organic carbon was the primary electron donor for the denitrifying bacteria. Although the abundance of *nir* genes was not restricted by the TOC concentrations, the diversity of *nirS* genes was closely related to TOC in the sediment (Fig. 2a, b). As a direct electron acceptor of denitrification, NO_2^- concentrations had great impact on the quantity of *nirS* and *nirK*. However, none of the denitrifier

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clusters was limited by NO_2^- concentrations directly (Fig. 2a, b), suggesting that distributions of denitrifying bacteria in the sediment were determined by combined effects of NO_2^- and other depth-dependent factors. Environmental factors that restrict the *nosZ* distribution also deserve to be studied because *nosZ* is the key functional gene for the complete denitrification. The CCA analysis showed that abundance of *nosZ* was higher at the least nutrient condition in the sediment. This result was contrary to that of Laverman et al. (2010), who considered that higher numbers of *nosZ* appeared in nutrient-rich sediment and clean river might reduce the efficiency of N_2 production. Our result implied that the distribution of *nosZ* was not limited by concentrations of organic carbon or nitrogen but by the ratios of them. Nitrous oxide reductase requires relatively adequate amount of organic carbon as electron donor for NO reduction. The uncertain conclusions about NO reduction and *nosZ* need for further studies.

5 Conclusions

A direct relationship was established between the depth-dependent environmental characteristics and the distributions of various denitrifying genes as well as the structure of *nirS*-encoding denitrifier community in the Pearl River sediment. It was found that the diversity of the denitrifier community was high in the sediment because the river sediment was affected by both domestic sewage inputs and irregular tides. The sediment depth and nutrient conditions were the major factors to control the distributions and diversities of denitrifying genes. Denitrification stratification in the sediment was classified based on the vertical distribution of denitrifying genes and DIN. Many novel *nirS*-encoding denitrifiers were found in this typical sediment environment. The physiology and ecological roles of these novel denitrifiers in the nitrogen transformation and contaminants degradation in the environment should be of great interests in future studies.

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References

Akaike, H.: Information theory as an extension of the maximum likelihood principle, edited by: Petrov, B. N. and Csaki, F., in: Second international symposium of information theory, Akademiai Kiado, Budapest, Hungary, 1973.

Beller, H. R., Chain, P. S. G., Letain, T. E., Chakicherla, A., Richardson, P. M., Coleman, M. A., Wood, A. P., and Kelly, D. P.: The Genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*, J. Bacteriol., 188, 1473–1488, 2006.

Braker, G., Fesefeldt, A., and Witzel, K. P.: Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples, Appl. Environ. Microb., 64, 3769–3775, 1998.

Bulow, S. E., Francis, C. A., Jackson, G. A., and Ward, B. B.: Sediment denitrifier community composition and *nirS* gene expression investigated with functional gene microarrays, Environ. Microb., 10, 3057–3069, 2008.

Burgin, A. J. and Hamilton, S. K.: Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways, Front. Ecol. Environ., 5, 89–96, 2007.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kuista, M., Mueller, R., and Nolan, T.: The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clinical Chemistry, 55, 611–622, 2009.

Canfield, D. E., Glazer, A. N., and Falkowski, P. G.: The evolution and future of earth's nitrogen cycle, Science, 330, 192 pp., 2010.

Castro-Gonázlez, M., Braker, G., Farias, L., and Ulloa, O.: Communities of *nirS*-type denitrifiers in the water column of oxygen minimum zone in the eastern South Pacific, Environ. Microb., 7, 1298–1306, 2005.

Chakraborty, R., O'Connor, S. M., Chan, E., and Coates, J. D.: Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* Strain RCB, Appl. Environ. Microb., 71, 8649–8655, 2005.

Chon, K., Chang, J., Lee, E., Lee, J., Ryu, J., and Cho, J.: Abundance of denitrifying genes coding for nitrate (*narG*), nitrite (*nirS*), and nitrous oxide (*nosZ*) reductases in estuarine versus wastewater effluent-fed constructed wetlands, Ecol. Eng., 37, 64–96, 2009.
Dalsgaard, T., Thamdrup, B, and Canfield, D. E.: Anaerobic ammonium oxidation (anammox) in the marine environment, Res. Microbiol., 156, 457–464, 2005.
Dang, H., Zhang, X., Sun, J., and Li, T.: Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea, Microbiology, 154, 2084–2095, 2008.
Dang, H., Wang, C., Li, J., Li, T., Tian, F., Jin, W., Ding, Y., and Zhang, Z.: Diversity and distribution of sediment nirS-encoding bacterial assemblages in response to environmental gradients in eutrophied Jiaozhou Bay, China, Microb. Ecol., 58, 161–169, 2009.
Deiglmayr, K., Philippot, L., Hartwig, U. A., and Kandeler, E.: Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated atmospheric pCO_2, FEMS Microbiol. Ecol., 49, 445–454, 2004.
Desnues, C., Michotey, V., Wieland, A., Zhizang, C., Fourcans, A., Duran, R., and Bonin, P.: Seasonal and diel distributions of denitrifying and bacterial communities in a hypersaline microbial mat (Camargue, France), Water Res., 41, 3407–3419, 2007.
Dodla, S. K., Wang, J. J., Delaune, R. D., and Cook, R. L.: Denitrification potential and its relation to organic carbon quality in three coastal wetland soils, Sci. Total. Environ., 407, 471–480, 2008.
Dong, L. F., Smith, C. J., Papaspyrou, S., Stott, A., Osborn, A. M., and Nedwell, D. B.: Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Clone Estuary, United Kingdom), Appl. Environ. Microb., 75, 3171–3179, 2009.
Falk, S., Hannig, M., Gliesche, C., Wardenga, R., Köster, M., Jürgens, K., and Braker, G.: *nirS*-containing denitrifier communities in the water column and sediment of the Baltic Sea, Biogeosciences, 4, 255–268, doi:10.5194/bg-4-255-2007, 2007.
Fan, L. F., Shieh, W. Y., Wu, W. F., and Chen, C. P.: Distribution of nitrogenous nutrients and denitrifiers strains in estuarine sediment profiles of the Tanshui River, northern Taiwan, Estuar. Coast. Shelf S., 69, 543–553, 2006.
Fukushima, T., Uda, N., Okamoto, M., Satoh, H., and Mino, T.: Development of the quantitative PCR method for *Candidatus 'Accumulibacter phosphatis'* and its application to activated sludge, J. Water Environ. Technol., 5, 37–43, 2007.

Glockner, A., Jüngst, A., and Zumft, W.: Copper containing nitrite reductases from *Pseudomonas aureofaciens* is functional in mutationally cytochrome cd1-free background ($NirS^-$) of *Pseudomonas stutzeri*, Arch. Microbiol., 160, 2136–2141, 1993.
Graham, D., Trippett, C., Dodds, W., O'Brien, J., Banner, E., Head, I., Smith, M., Yang, R., and Knapp, C.: Correlations between in situ denitrification activity and nir-gene abundances in pristine and impacted prairie streams, Environ. Pollut., 158, 3225–3229, 2010.
Huelsenbeck, J. P., Ronquist, F. R., Nielsen, R., and Bollback, J. P.: Bayesian inference of phylogeny and its impact on evolutionary biology, Science, 294, 2310–2314, 2001.
Jung, S., Lee, M., Oh, T., and Yoon, J.: *Herbaspirillum rhizosphaerae* sp. nov., isolated from rhizosphere soil of *Allium victorialis* var. *platyphyllum*, Int. J. Syst. Evol. Micr., 57, 2284–2288, 2007.
Knapp, C., Dodds, W., Wilson, K., O'Brien, J., and Graham, D.: Spatial heterogeneity of denitrification genes in a highly homogenous urban stream, Environ. Sci. Technol., 43, 4273–4279, 2009.
Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jørgensen, B. B., Kuenen, J. G., Sinninghe Damsté, J. S., Strous, M., and Jetten, M. S.: Anaerobic ammonium oxidation by anammox bacteria in the Black Sea, Nature, 422, 608–611, 2003.
Laverman, A. M., Garnier, J. A., Mounier, E. M., and Roose-Amsaleg, C. L.: Nitrous oxide production kinetics during nitrate reduction in river sediments, Water Res., 44, 1753–1764, 2010.
Liu, X., Tiquia, S. M., Holguin, G., Wu, L., Nold, S.C., Devol, A. H., Luo, K., Palumbo, A. V., Tiedje, J. M., and Zhou, J.: Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off the pacific coast of Mexico, Appl. Environ. Microbiol., 69, 3549–3560, 2003.
Martins, M., Faleiro, M. L., Chaves, S., Tenreiro, R., Santos, E., and Costa, M.: Anaerobic bio-removal of uranium (VI) and chromium (VI): comparison of microbial community structure, J. Hazard Mater., 176, 1062–1072, 2010.
Matsunaga, T., Okamura, Y., Fukuda, Y., Wahyudi, A. T., Murase, Y., and Takeyama, H.: Complete genome sequence of the facultative anaerobic magnetotactic bacterium *Magnetospirillum* sp. strain AMB-1, DNA Res., 12, 157–166, 2005.
Meyer, R. L., Allen, D. E., and Schmidt, S.: Nitrification and denitrification as sources of sediment nitrous oxide production: A microsensor approach, Mar. Chem., 110, 68–76, 2008.

Mohan, S., Schmid, M., Jetten, M. and Cole, J.: Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification, FEMS Microbiol. Ecol., 49, 433–443, 2004.

Mullins, T. D., Britschgi, T. B., Krest, R. L., and Giovannoni, S. J.: Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities, Limnol. Oceanogr., 40, 148–158, 1995.

Nogales, B., Timmis, K. N., Nedwell, D. B., and Osborn, A. M.: Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA, Appl. Environ. Microb., 68, 5017–5025, 2002.

Nylander, J. A. A.: MrModeltest v2. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, 2004.

Oakley, B. B., Francis, C. A., Roberts, K. J., Fuchsman, C.A., Srinivasan, S., and Staley, J. T.: Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone, Environ. Microbiol., 9, 118–130, 2007.

Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N. and Inamori, Y.: Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing, Microbial Ecol., 52, 253–266, 2006.

Priemé, A., Braker, G., Tiedje, J. M.: Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils, Appl. Environ. Microb., 68, 1893–1900, 2002.

Rambaut, A. and Drummond, A. J.: Tracer v1.5, Available at http://beast.bio.ed.ac.uk/Tracer (last access: 5 March 2010), 2009.

Reyna, L., Wunderlin, D. A., and Genti-Raimondi, S.: Identification and quantification of a novel nitrate-reducing community in sediments of Suquía River basin along a nitrate gradient, Environ. Pollut., 158, 1608–1614, 2010.

Richardson, W. B., Strauss, E. A., Bartsch, L. A., Monroe, E. M., Cavanaugh, J. C., Vingum, L., and Soballe, D. M.: Denitrification in the Upper Mississippi River: rates, controls, and contribution to nitrate flux, Can. J. Fish. Aquat. Sci., 61, 1102–1112, 2004.

Ronquist, F. R. and Huelsenbeck, J. P.: MrBayes 3: Bayesian phylogenetic inference under mixed models, Bioinformatics, 19, 1572–1574, 2003.

Ruiz-Rueda, O., Trias, R., Garcia-Gil, L. J., and Bañeras, L.: Diversity of the nitrite reductase gene *nirS* in the sediment of a free-water surface constructed wetland, Int. Microbiol., 10, 253–260, 2007.

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Ryan, J., Estefan, G., and Rashid, A.: Soil and plant analysis laboratory manual. Jointly published by International Center for Agricultural Research in the Dry Areas (ICARDA) and the National Agricultural Research Center (NARC), USA, 2001.

Rysgaard, S., Risgaard-Petersen, N., Nielsen, L. P., and Revsbech, N. P.: Nitrification and denitrificaiton in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing, Appl. Environ. Microb., 59, 2093–2098, 1993.

Scala, D. J. and Kerkhof, L. J.: Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments, FEMS Microbiol. Lett., 162, 61–68, 1998.

Schloss, P. D. and Handelsman, J.: Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness, Appl. Environ. Microb., 71, 1501–1506, 2005.

Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B., and Gottschalk, G.: Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid enconding key enzymes of H2-based lithoautotrophy and anaerobiosis, J. Mol. Biol., 332, 369–383, 2003.

Scott, J. T., McCarthy, M. J., Gardner, W. S., and Doyle, R. D.: Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland, Biogeochemistry, 87, 99–111, 2008.

Smith, C. J., Nedwell, D. B., Dong, L. F., and Osborn, A. M.: Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments, Appl. Environ. Microb., 73, 3612–3622, 2007.

Spain, A. M., Peacock, A. D., Istok, J. D., Elshahed M. S., Najar, F. Z., Roe, B. A., White, D. C., and Krumholz, L. R.: Identification and isolation of a Castellaniella species important during biostimulation of an acidic nitrate- and uranium-contaminated aquifer, Appl. Environ. Microb., 73, 4892–4904, 2007.

Sun, Y., Gustavson, R. L., Ali, N., Weber, K. A., Westphal, L. L., and Coates, J. D.: Behavioral response of dissimilatory perchlorate-reducing bacteria to different electro acceptors, Appl. Microbiol. Biotechnol., 84, 955–963, 2009.

Tamang, D. G., Rabus, R., Barabote, R. D., and Saier Jr., M. H.: Comprehensive analyses of transport proteins encoded within the genome of "*Aromatoleum aromaticum*" strain EbN1, J. Membrane Biol., 229, 53–90, 2009.

Throbäck, I. N., Enwall, K., Jarvis, Å., and Hallin, S.: Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE, FEMS Microbiol. Ecol., 49, 401–417, 2004.

Tiquia, S. M., Masson, S. A., and Devol, A.: Vertical distribution of nitrite reductase genes (*nirS*) in continental margin sediments of the Gulf of Mexico, FEMS Microbiol. Ecol., 58, 464–475, 2006.

Tsuneda, S., Miyauchi, R., Ohno, T., and Hirata, A.: Characterization of denitrifying polyphosphate-accumulating organisms in activated sludge based on nitrite reductase gene, J. Biosci. Bioeng., 99, 403–407, 2005.

Wongwilaiwalin, S., Rattanachomsri, U., Laothanachareon, T., Eurwilaichitr, L., Igarashi, Y., and Champreda, V.: Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system, Enzyme Microb. Tech., 47, 283–290, 2010.

Yan, T., Fields, M., Wu, L., Zu, Y., Tiedje, J., and Zhou, J.: Molecular diversity and characterization of nitrate- and uranium-contaminated groundwater, Environ. Microbiol., 5, 13–24, 2003.

Zumft, W.: Cell biology and molecular basis of denitrification, Microbiol. Mol. Biol. Rev., 61, 533–616, 1997.

Table 1. Characteristics of sample locations in the Pearl River.

Environmental characteristics	Sample location: Zhujiang Bridge (ZB) 0–5 cm	5–10 cm	10–15 cm	20–25 cm	Huadi (H) 0–5 cm	5–10 cm	10–15 cm	20–25 cm	Ersha (E) 0–5 cm	5–10 cm	10–15 cm	20–25 cm
Latitude (N)	23°6′ 50″				23°6′8″				23°7′36″			
Longitude (E)	113°17′2″				113°13′22″				113°13′16″			
Sediment Depth layers	ZB5	ZB10	ZB15	ZB25	H5	H10	H15	H25	E5	E10	E15	E25
TOC($g\,kg^{-1}$)	21.9	32.1	32.3	23.9	39.7	42.5	47.9	49.8	25.7	60.9	33.6	18.9
NO_3^- ($mg\,kg^{-1}$)	8.60	9.52	10.7	12.0	8.71	10.8	22.4	17.7	19.7	27.0	13.7	19.8
NO_2^- ($mg\,kg^{-1}$)	0.34	0.38	0.36	0.37	0.52	0.58	0.47	0.50	1.28	0.60	0.32	0.32
NH_4^+ ($mg\,kg^{-1}$)	153	313	818	1195	194	349	1273	1218	1613	1867	1324	1252

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Table 2. Real time PCR primers used for the amplification of denitrifying genes.

Primer	Target gene	Sequence (5′–3′)	Annealing temp	Reference
narG328F	*narG*	GACAAACTTCGCAGCGG	61	Reyna et al. (2010)
narG497R	*narG*	TCACCCAGGACGCTGTTC	61	Reyna et al. (2010)
nirS3F	*nirS*	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	57	Braker et al. (1998)
nirS5R	*nirS*	GCCGCCGTC(A/G)TG(A/C/G)AGGAA	57	Braker et al. (1998)
NirK1F	*nirK*	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	57	Braker et al. (1998
NirK5R	*nirK*	GCCTCGATCAG(A/G)TT(A/G)TGG	57	Braker et al. (1998
nos1527F	*nosZ*	CGCTGTTCHTCGACAGYCA	57	Scala and Kerkhof (1998)
nos1773R	*nosZ*	ATRTCGATCARCTGBTCGTT	57	Scala and Kerkhof (1998)
nrfA-2F	*nrfA*	CACGACAGCAAGACTGCCG	60	Smith et al. (2007)
nrfA-2R	*nrfA*	CCGGCACTTTCGAGCCC	60	Smith et al. (2007)
1055f	16S rRNA	ATGGCTGTCGTCAGCT	57	Casamayor et al. (2002)
1392r	16S rRNA	ACGGGGCGGTGTGTAC	57	Casamayor et al. (2002)

Table 3. Copy numbers of denitrifying genes and bacterial 16S rRNA genes in the Pearl River sediment. Error bars indicate standard errors of PCR of the three replicate DNA extractions.

Layer	16S rRNA gene g^{-1} sediment($\times 10^{11}$)	Ratios				
		narG/16SrRNA ($\times 10^{-3}$)	*nirS*/16SrRNA ($\times 10^{-2}$)	*nirK*/16SrRNA ($\times 10^{-3}$)	*nosZ*/16SrRNA ($\times 10^{-3}$)	*nrfA*/16SrRNA ($\times 10^{-3}$)
ZB5	4.91 ± 0.18	7.24 ± 0.12	13.1 ± 1.24	6.37 ± 1.22	7.24 ± 2.22	17.5 ± 4.11
ZB10	1.58 ± 0.42	9.69 ± 0.23	11.3 ± 1.03	3.22 ± 0.32	11.6 ± 5.67	21.7 ± 5.17
ZB15	1.75 ± 0.66	6.65 ± 0.11	3.30 ± 0.17	2.10 ± 0.09	9.68 ± 4.32	23.6 ± 3.39
ZB25	0.31 ± 0.14	3.52 ± 0.08	4.88 ± 0.19	2.05 ± 0.17	2.10 ± 0.15	25.9 ± 5.26
H5	1.26 ± 0.33	3.06 ± 0.10	26.8 ± 3.28	7.35 ± 1.03	4.94 ± 0.23	32.4 ± 3.57
H10	0.98 ± 0.64	10.9 ± 0.38	20.7 ± 2.17	4.21 ± 0.54	6.63 ± 0.28	29.9 ± 2.48
H15	0.57 ± 0.14	12.2 ± 0.42	6.70 ± 0.28	2.87 ± 0.12	2.73 ± 0.08	37.1 ± 2.16
H25	0.20 ± 0.05	13.7 ± 0.33	4.38 ± 0.12	2.33 ± 0.13	6.31 ± 0.48	33.9 ± 3.11
E5	3.37 ± 0.29	1.37 ± 0.03	2.94 ± 0.11	5.21 ± 0.23	0.34 ± 0.05	7.98 ± 1.21
E10	2.40 ± 0.09	2.48 ± 0.12	9.45 ± 0.80	2.45 ± 0.09	1.93 ± 0.08	9.29 ± 2.47
E15	1.39 ± 0.07	2.43 ± 0.14	5.60 ± 0.58	2.01 ± 0.11	1.48 ± 0.27	10.6 ± 1.18
E25	1.63 ± 0.32	4.88 ± 0.16	6.55 ± 0.71	1.87 ± 0.07	1.09 ± 0.09	33.5 ± 5.02

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Table 4. Biodiversity and predicted richness of *nirS* sequences recovered from six layers in the Pearl River sediment.

Layer	Coverage (%)	Shannon(H)	Simpson($1/D$)	Evenness(E)	S_{ACE}	S_{Chao1}
ZB5	85.5	2.82	7.72	0.83	66.0	64.3
ZB10	78.5	3.07	8.16	0.82	97.9	119
ZB15	72.4	3.43	9.13	0.78	129	130
H5	74.8	3.25	8.91	0.75	109	168
H10	70.8	4.15	13.6	0.86	146	142
H15	65.3	4.39	15.6	0.84	135	107

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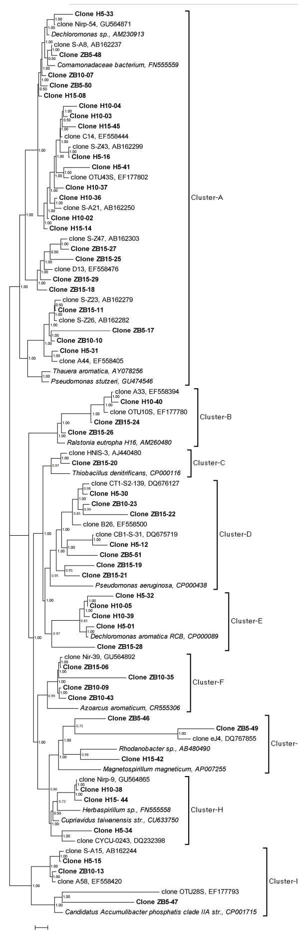

Fig. 1. Phylogenetic tree of PCR-amplified *nirS* gene. The tree was constructed using the Bayesian inference (BI) methods with *nirS* sequences from the Pearl River sediment and from other typical estuarine and marine environments, as well as sewage treatment systems. Sequences determined in this study are in bold. Bootstrap values were based on 1000 replicates each and are shown at the nodes with >50 % bootstrap support. OTUs with bootstrap value either >80 % were utilized to designate the *nirS* phylogenetic clusters. The scale bar represents 10 % sequence divergence.

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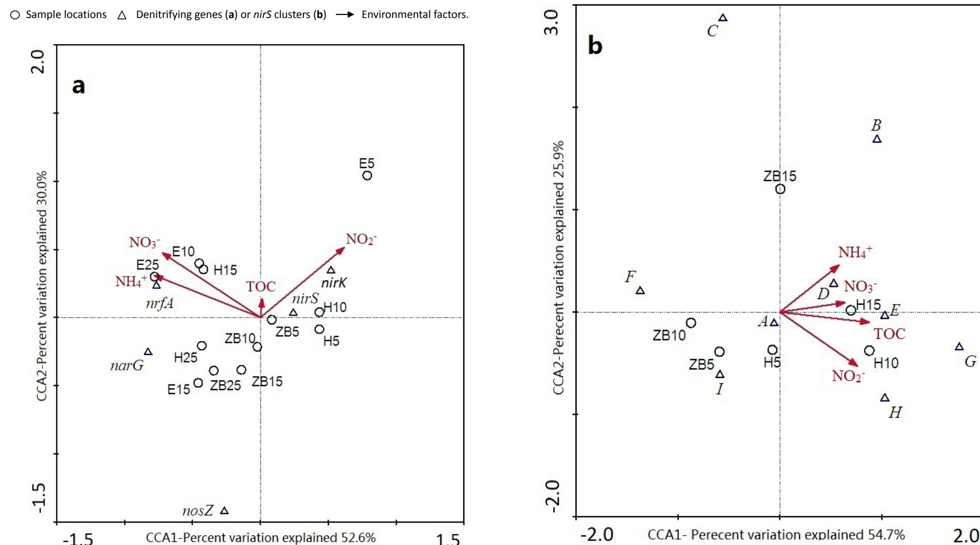

Fig. 2. Ordination plots of the canonical correspondence analysis (CCA) for the first two dimensions of CCA of the relationship between the distributions of **(a)** denitrifying genes and **(b)** *nirS* clusters vs. the nutrients in the Pearl River sediment. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors).

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