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ORIGINAL RESEARCH ARTICLE

Seasonal patterns and environmental drivers of *nir*S- and *nir*K-encoding denitrifiers in sediments of Daya Bay, China

Rongjun Shi [a,1], **Shumin Xu** [a,b,1], **Zhanhui Qi** [a,*], **Honghui Huang** [a,*], **Qingyang Liang** [a]

[a] *Guangdong Provincial Key Laboratory of Fishery Ecology and Environment and Key Laboratory of Open-Sea Fishery Development, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Guangzhou, China*
[b] *College of Marine Science of Shanghai Ocean University, Shanghai, China*

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Summary The seasonal patterns of the denitrifiers (denitrifying bacteria) in the sediment of Daya Bay, southern China, were examined using quantitative PCR and high-throughput MiSeq sequencing methods in spring, summer and winter. The abundance and diversity of *nir*S-encoding denitrifiers were much higher than that of *nir*K-encoding denitrifiers, indicating that the former probably dominated the denitrification processes in sediments of Daya Bay. The average abundance and diversity of *nir*S-encoding denitrifiers were much higher in spring than that in summer and winter, on the other hand, the abundance of *nir*K-encoding denitrifiers showed the opposite pattern. The species composition of *nir*S-encoding denitrifiers community in spring differed significantly from that in summer and winter, whereas, no significant difference existed between summer and winter. The dominant environmental drivers for the diversity of community species were NO_2^-, NO_3^- and DO concentrations. The abundances of dominant genera of *nir*S-encoding denitrifiers, *Accumulibacter* sp. and *Cuprizvidus* sp., were significantly higher in

* Corresponding authors at: South China Sea Fisheries Research Institute, 231 West Xingang Road, Guangzhou 510300, Guangdong Province, China. Tel.: +86 02084457660; fax: +8602084451442
E-mail addresses: qizhanhui@scsfri.ac.cn (Z. Qi), huanghh@scsfri.ac.cn (H. Huang).
[1] Contributed equally to this work.
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summer and winter than that in spring, and were negatively correlated with NO_2^-, NO_3^-, and DO concentrations ($p < 0.05$). In contrast, the abundances of *Azoarcus* sp. and *Halomonas* sp., were highest in spring, and were positively correlated with NO_3^- and NO_2^- content ($p < 0.05$). For *nir*K-encoding denitrifiers, a significant difference in community composition was observed between spring and winter. No obvious correlation was found between community composition of *nir*K-encoding denitrifiers and environmental parameters.

1. Introduction

Agricultural and other anthropogenic activities, e.g., mariculture, result in increasing nitrogen load to freshwater and marine systems, causing eutrophication problems. Therefore, denitrification that removed "excess" N from the water bodies plays an important role in alleviating eutrophication (Abell et al., 2013; Bowen et al., 2014). Based on the consequences of denitrification reactions, the nitrate was consequently catalyzed by four kinds of enzymes, i.e., nitrate reductase which was encoded by *nap*A or *nar*G genes, nitrite reductase encoded by *nir*S or *nir*K genes, nitric oxide reductase encoded by *nor*B or *nor*Z genes, and nitrous oxide reductase encoded by *nos*Z gene (Shrewsbury et al., 2016). Among of them, the nitrite reductase which catalyzing the reaction that leads to the formation of gaseous nitrogen (nitric oxide) through nitrite, is the rate-limiting enzyme for the denitrification processes (Baker et al., 2015; Chen et al., 2013).

Nitrite reductase encoded either by *nir*K or *nir*S are evolutionarily distinct but functionally equivalent (Glockner et al., 1993; Shrewsbury et al., 2016). Therefore, the bacteria which have either *nir*S or *nir*K gene are often considered to be denitrifying bacteria and are found in a variety of environments, including marine, estuarine, wetland and bay (Braker et al., 2000; Gao et al., 2016; Lee and Francis, 2016; Li et al., 2017a). Previous studies reported that the abundance and distribution of denitrifying bacteria in sediments could be affected by various environmental factors, including temperature, dissolved oxygen (DO), dissolved inorganic nitrogen (DIN, e.g., NO_3^-, NO_2^- and NH_4^+) and organic matter content in sediments (Gao et al., 2016; Huang et al., 2011; Li et al., 2017a; Reyna et al., 2010). Reasonably, there were markedly spatial and temporal variations in denitrifiers community profiles, e.g., the species diversity and abundance (Gao et al., 2016; Yang et al., 2015a).

Daya Bay, located in the northwestern part of the South China Sea, is a typical subtropical bay with an area of approximately 600 km^2. Approximately 60% of the water in the Bay is less than 10 m deep, and most of its water originates from the South China Sea and three small rivers (Ni et al., 2015; Wang et al., 2006). The rapid expanding of coastal aquaculture and industries increased nitrogen loading to the bay, which contributed to various environmental issues in Daya Bay, such as eutrophication, annual algal blooms, and hypoxia (Song et al., 2009). In recent years, many studies have been carried out to address the ecological and anthropogenic factors contributing to these effects (Jiang et al., 2016; Ma et al., 2014; Wu et al., 2016, 2017a). Microorganisms associated with nitrogen cycle play important role in the cycling and removal of nitrogen from coastal waters. However, information on the distribution profiles of *nir*K- and *nir*S-encoding denitrifiers, and the major environmental drivers are still very limited.

In this study, quantitative polymerase chain reaction (qPCR) and high-throughput MiSeq sequencing were used to examine the seasonal patterns and environmental drivers of denitrifying bacteria in sediments from Daya Bay. The objectives were: (1) to investigate the seasonal patterns of the *nir*S- and *nir*K-denitrifying bacteria inhabiting the sediment of Daya Bay, and (2) to examine the key environmental factors which determine the spatial-temporal heterogeneities of *nir*S- and *nir*K-denitrifying bacterial community in Daya Bay. We hope to provide useful information to further understand the responses of denitrifying bacteria communities to environmental factors.

2. Material and methods

2.1. Study area and sample collection

Surface sediments were collected using an Ekman-Birge type bottom sampler in winter (December 2015), spring (March 2016), and summer (August 2016) from four stations, i.e., D1, D2, D3 and D4, in Daya Bay, Southern China (Fig. 1). Sediment samples were gently placed on a white enamel tray (30 cm × 60 cm), the top 2 cm sediment was collected using a stainless steel ruler. For each sampling station, triplicate samples of surface sediment were collected, mixed and homogenized. Each sediment sample was further divided into two parts: one was stored at −80°C for DNA extraction and another one was stored at −20°C for sedimentary chemical parameters analyses.

2.2. Physiological and chemical parameters analyses

At each sampling station, temperature, pH, salinity and DO of overlying water were measured with a multiprobe sonde (Yellow Springs Instrument Co., Dayton, OH, USA). Moisture content (MC), total nitrogen (TN), sulfide, total organic carbon (TOC), nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) of sediment were measured in laboratory. Sediment samples (about 2 g) were weighed and then reweighed after

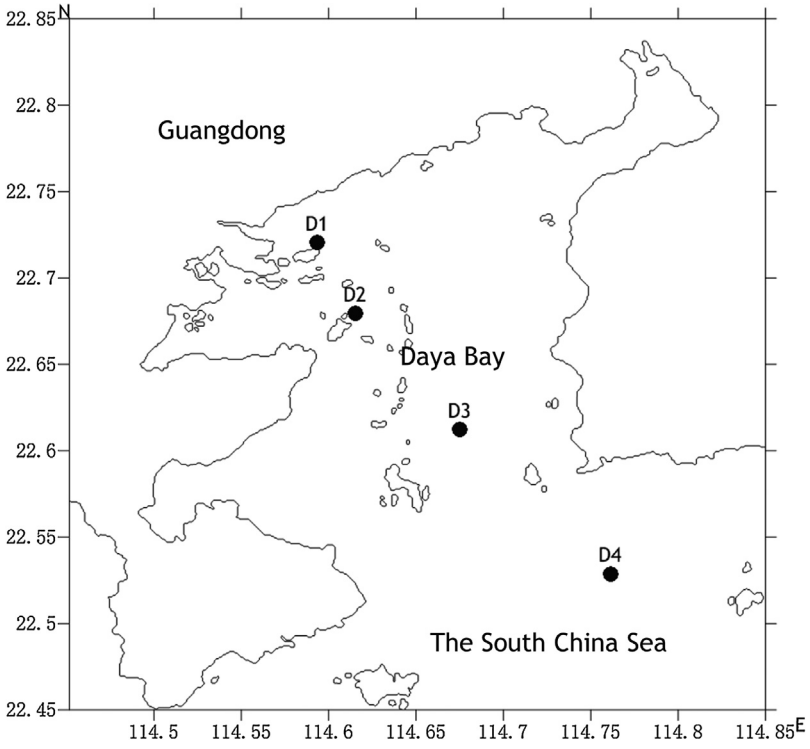

Figure 1 Sediment sampling stations in Daya Bay, Southern China.

dried at 60°C to a constant weight. The differences in weights were defined as moisture content. In addition, TOC were detected according to the method described in Yang et al. (2015b). TN was measured using potassium persulfate oxidation method. Sediment samples (about 0.1 g) that had already been dried at 60°C were dissolved in 25 ml oxidant solutions (NaOH:$K_2S_2O_8$ = 0.15 M:0.15 M) after homogenizing and grounding to fine powder. Aliquots of the samples were then heated for 1 h at 124°C and centrifuged at 3000 × *g* for 10 min. 2 ml of the separated supernatants were diluted with ultrapure water to 25 ml to manually determine TN with a spectrophotometer (Shanghai Precision Science Instrument Co. Ltd., Shanghai, China) according to marine monitoring specifications (GB/T 12763.4-2007, 2007).

Sediment samples (10 g) mixed with KCl solution (2 M, 40 ml) were shaken with an oscillator for 1 h (200–300 rpm) (Keeney and Nelson, 1982; Shrewsbury et al., 2016). Aliquots of the samples were centrifuged at 3000 × *g* for 10 min and then the supernatants obtained were diluted with KCl solution to 75 ml to determine NO_3^-, NO_2^- and NH_4^+ concentrations according to marine monitoring specifications (GB/T 12763.4-2007, 2007).

2.3. DNA extraction and high-throughput MiSeq sequencing

The samples were centrifuged at 1000 × *g* for 30 s to ensure consistency of sampling quantity. The centrifuged samples (approximately 0.25 g) were then used for DNA extraction with the ZymoBiomics DNA mini kit (Zymo Research Co., Irvine, CA, USA) following the manufacturer's instructions. Purity and concentrations of the extracted DNA were examined using a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and were visualized with agarose gel electrophoresis. The resultant DNA was then amplified through PCR (the primers are listed in Table 1) and sequenced using Solexa MiSeq Genome Analyzer (Illumina Inc., San Diego, CA, USA). All the sequences used in this study are publicly available at the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra) under gene accession no. SRP125795.

2.4. Fluorescence quantitative PCR (qPCR)

The primers listed in Table 1 targeting the *nir*S and *nir*K genes were applied to determine the abundance of denitrifying

Table 1 Oligonucleotide sequences of primers used for PCR and qPCR assays.

Target gene	Primer	Sequence [5′→3′]	References
*nir*K	*FlaCu*	ATCATGGTSCTGCCGCG	Hallin and Lindgren (1999), Yi et al. (2015)
	R3Cu	GCCTCGATCAGRTTRTGGTT	
*nir*S	*cd3aF*	GTSAACGTSAAGGARACSGG	Throbäck et al. (2004), Zheng et al. (2015)
	R3cdR	GASTTCGGRTGSGTCTTGA	

bacteria by qPCR according to Yi et al. (2015). Linearized plasmids containing *nir*S and *nir*K were used to generate standard curves. All standard and sample reactions were performed in triplicate, and all the standard curves showed excellent correlations between the DNA template concentration and the crossing point with high coefficients of determination ($R^2 > 0.99$). The results indicated that the qPCR efficiency values for *nir*S and *nir*K were 0.924 and 1.006, respectively. Finally, the abundance of *nir*S and *nir*K genes was calculated based on the constructed standard curve, and then converted into copies per gram of sediment.

2.5. Data analysis

Raw tags were quality-filtered by Trimmomatic (Bolger et al., 2014) and merged by FLASH (Magoc and Salzberg, 2011) to obtain the effective tags. After quality filtration, sequence analysis of effective tags was performed using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/), and sequences with ≥97% similarity were assigned to the same OTUs according to Wu et al. (2017b). A representative sequence of each OTU was aligned with sequences downloaded from the FunGene database (Fish et al., 2013). Statistical analyses were performed using R version 3.3.3 (R Core Team, Vienna, Austria). Alpha diversity indices (Chao1, Pielou's evenness, Shannon–Wiener, and Simpson indices), Correspondence analysis (CA) plots, heat maps and Mantel tests were calculated and generated using R. Besides, Bray–Curtis distance was used for the genera of *nir*S and OTUs of *nir*K distance metric, and euclidian distances were used to describe the underlying structure of our environmental variables in mantel tests (Hollister et al., 2010; Yang et al., 2015b).

Extended error bar plots were created using STAMP (Parks et al., 2014). Phylogenetic tree was created by mega 7 program with the maximum likelihood method and the reliability of the tree topologies was estimated by performing 1000 bootstrapping replicates.

3. Results

3.1. Physiological and chemical characteristics of sediment

The spatial and temporal patterns of salinity, pH, temperature, DO, MC, TOC, NO_2^-, NO_3^-, NH_4^+, and TN at each sampling station are shown in Table 2. Temperature was higher in summer than that in spring and winter. Salinity in D1 was lower than the other stations in all seasons. Water DO and NO_2^- concentrations in all stations were highest in spring, followed by winter and summer. TOC at D1 and D2 stations were higher than that at D3 and D4 stations. No obvious spatial and temporal pattern of the other environmental parameters was observed.

3.2. Abundance of *nir*S-encoding and *nir*K-encoding denitrifiers

The abundance of *nir*S-encoding denitrifiers was 6.48–20.34 × 10^{10} copies g^{-1} sediment in spring, which was about 10 times higher than the abundance in summer and winter (1.44–66.76 × 10^8 copies g^{-1} sediment) ($p < 0.05$) (Fig. 2A, B). However, no significant differences were observed among the three seasons, although the abundances of *nir*K-encoding denitrifiers in summer and winter were higher than that in spring (Fig. 2C, D). In addition, the abundance of *nir*S was higher than that of *nir*K in all the three seasons. In particular, *nir*S abundance in spring was higher than abundance of *nir*K in all seasons ($p < 0.05$). Generally, the abundance of *nir*S-encoding and *nir*K-encoding denitrifiers at stations D2 and D3 was higher than that at stations D1 and D4.

Table 2 Water and sediment selected properties at experimental sites in Daya Bay.

Season	Station	Salinity	pH	T[a]	DO[b]	MC[c]	TOC[d]	NO_2^-[e]	NO_3^-[e]	NH_4^+[e]	TN[f]
				[°C]	[mg L^{-1}]	[%]	[%]	[nmol g^{-1} dry weight]			[μmol g^{-1} dry weight]
Spring	D1	31.05	8.04	19.9	13.08	64.02	1.4	17.42	19.60	0.13	26.99
	D2	31.84	8.14	18.8	11.82	69.49	1.43	19.26	24.77	6.80	9.55
	D3	32.77	8.2	19.1	9.98	49.69	0.85	13.46	13.72	3.09	59.61
	D4	33.22	8.3	17.4	8.27	53.34	0.75	14.31	30.75	7.38	69.1
Summer	D1	30.40	8.44	30.8	5.7	56.01	1.66	7.19	2.18	4.33	28.26
	D2	31.95	8.45	30.0	6.47	63.95	1.42	8.25	9.03	0.01	21.59
	D3	32.01	8.05	30.8	6.68	60.78	1.31	8.22	7.94	0.01	60.21
	D4	31.85	8.16	30.9	7.45	39.71	0.74	3.67	4.21	0.005	32.11
Winter	D1	33.34	8.16	19.34	8.32	72.97	1.98	15.11	25.97	2.19	26.19
	D2	33.59	8.15	19.68	8.31	71.66	1.83	12.58	18.98	34.73	31.02
	D3	34.47	8.13	18.16	8.01	44.63	0.64	6.42	13.61	18.03	6.37

[a] T, water temperature.
[b] DO, water dissolved oxygen.
[c] MC, the moisture content of sediment.
[d] TOC, total organic carbon content of sediment.
[e] NO_2^-, NO_3^- and NH_4^+ concentrations of sediment.
[f] TN, total nitrogen content of sediment.

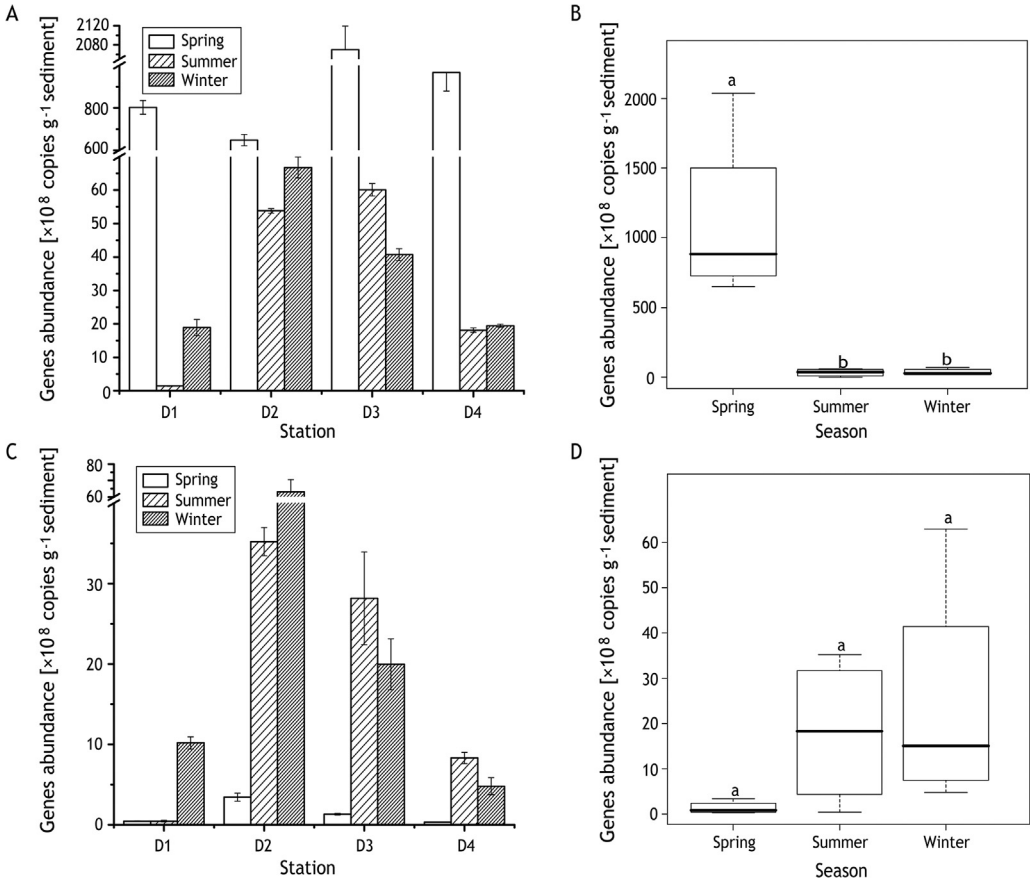

Figure 2 Abundances of *nir*S (A, B) and *nir*K (C, D) genes in the sediment of Daya Bay, China. (A) and (C) error bars represent the standard error of triplicate samples. (B) and (D) different lowercase letters represent statistically significant differences between seasons ($p < 0.05$).

3.3. Community diversity of *nir*S- and *nir*K-encoding denitrifiers

Totally 533,461 effective tags of *nir*S were obtained from sediment samples. These tags were clustered into 4296 OTUs with a similarity cut-off of 97%. The Shannon–Wiener indices of *nir*S-encoding denitrifiers were 0.68–0.71 in spring, which was significantly higher than that in summer and winter (0.53–0.58) ($p < 0.05$) (Table 3). In addition, Pielou's evenness and Simpson (1/*D*) indices exhibited similar patterns as the Shannon–Wiener index. No significant difference in the species richness index (Chao1) was observed among the three seasons ($p > 0.05$).

Similarly, 571,199 *nir*K tags were obtained and clustered into 1123 OTUs with a similarity cut-off of 97%. The Chao1, Shannon–Wiener, and Simpson (1/*D*) indices of *nir*K-encoding denitrifiers were lower than that of *nir*S-encoding denitrifiers, particularly in spring, although no significant difference among the three seasons for any of the indices was observed ($p > 0.05$).

3.4. Spatial and temporal distribution profiles of *nir*S- and *nir*K-encoding bacterial communities

Spatial and temporal distributions of denitrifier communities were compared using CA based on all OTUs (Fig. 3). The distribution of *nir*S-encoding denitrifiers did not differ between summer and winter. However, the distribution was significantly different between spring and the other seasons (Fig. 3A). An obvious difference in *nir*K-encoding denitrifier communities was detected between spring and winter (Fig. 3B). In spring, the community structures of *nir*S- and *nir*K-encoding denitrifiers in station D1 were significantly different from other stations.

3.5. Composition of *nir*S- and *nir*K-encoding denitrifiers

The OTUs of *nir*S-encoding denitrifiers were assigned to 191 genera representing 20 different phyla. The dominant phyla were Proteobacteria (42.43–64.89% of the total OTUs at all stations) and Actinobacteria (0.09–3.75%, Fig. 4A). Further analysis indicated that the identified members of Proteobacteria belonged to four classes, i.e., Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria, of which Betaproteobacteria and Gammaproteobacteria were the dominant classes.

The relative abundance of Betaproteobacteria in spring (14.06–27.64% of the total OTUs) was slightly lower than that in summer and winter (26.95–33.0%, Fig. 4A). However, all genera abundance of Betaproteobacteria (except *Vogesella* sp.) was significantly different between spring and the other

Table 3 Richness and diversity estimates of *nir*S- and *nir*K-encoding bacteria in the Daya Bay sediment.

Gene	Season	Station	OTUs	Chao1	Pielou's evenness index	Shannon–Wiener index [*H*]	Simpson index [1/*D*]
*nir*S	Spring	D1	830	951.63	0.71	4.76	35.90
		D2	868	961.51	0.68	4.60	33.13
		D3	730	872.08	0.71	4.67	42.63
		D4	746	866.23	0.68	4.50	30.58
	Summer	D2	953	953.19	0.53	3.61	12.32
		D3	1108	1118.66	0.57	4.02	16.02
		D4	980	965.81	0.58	3.99	14.56
	Winter	D2	1136	1175.42	0.58	4.12	17.52
		D3	709	722.28	0.54	3.57	12.60
		D4	1168	1211.16	0.56	3.94	15.07
*nir*K	Spring	D1	619	688.00	0.68	4.38	24.05
		D2	631	645.53	0.67	4.33	14.58
		D3	602	653.07	0.63	4.02	8.46
		D4	528	578.83	0.50	3.16	5.05
	Summer	D2	665	724.70	0.57	3.68	8.16
		D3	672	711.28	0.61	4.00	8.38
		D4	616	658.02	0.59	3.80	7.15
	Winter	D2	666	740.45	0.50	3.23	5.70
		D3	613	662.08	0.57	3.68	8.07
		D4	589	641.64	0.64	4.10	16.70

seasons ($p < 0.05$, Fig. 4B). *Azoarcus* sp. and *Polymorphum* sp. were the dominant genera in spring, whereas *Accumulibacter* sp. and *Cuprizvidus* sp. were the dominant genera in summer and winter. The relative abundance of Gammaproteobacteria was significantly higher in spring (24.56–29.23%) than in summer and winter (9.37–12.36%). *Halomonas* sp. and *Pseudomonas* sp. were highest in spring compared to that in summer and winter ($p < 0.05$).

Compared with Betaproteobacteria and Gammaproteobacteria, the abundance of Alphaproteobacteria and Deltaproteobacteria was found to be lower. The dominant genera of the Alphaproteobacteria were *Azospirillum* sp. and *Polymorphum* sp. in spring and *Magnetospirillum* sp. and *Bradyrhizobium* sp. in summer and winter, and the dominant genus of Deltaproteobacteria was *Myxococcus* sp. in summer and winter. The abundance of *Polymorphum* sp., *Bradyrhizobium* sp., and *Myxococcus* sp. differed significantly between spring and the other seasons ($p < 0.05$, Fig. 4B). In addition, the relative abundance of Actinobacteria, whose dominant genus was *Kocuria* sp., was highest in spring ($p < 0.05$). There was no significant difference in the abundance of *nir*S-encoding denitrifiers between summer and winter.

Phylogenetic analysis indicated that a large proportion of unique OTUs matched uncultured environmental *nir*K assemblages. It was also observed that the majority of OTUs closely matched to sequences retrieved from GenBank belonged to samples isolated from estuaries (e.g., San Francisco Bay and Yellow River Estuary), marine habitats (e.g., Arabian Sea and

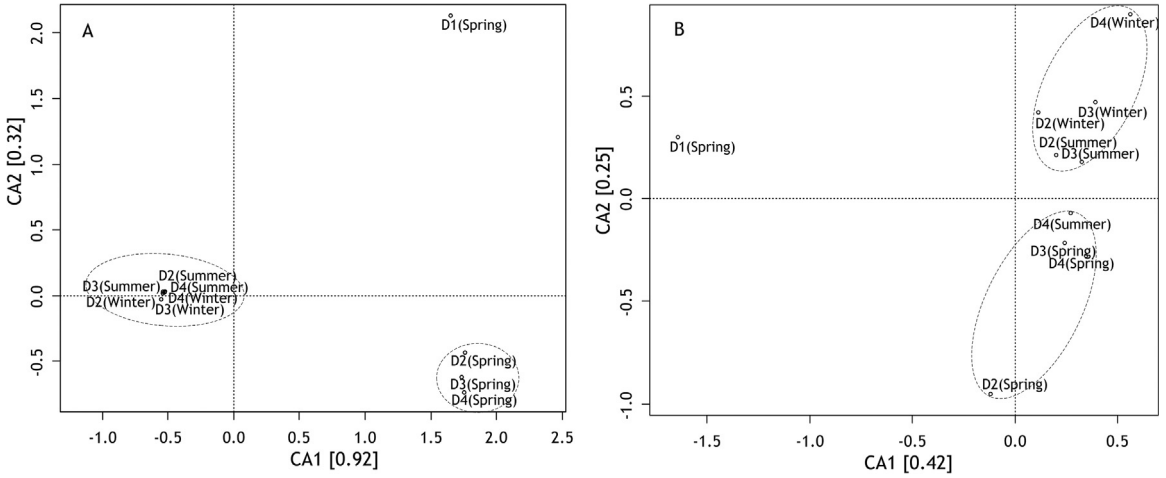

Figure 3 Community structures of *nir*S- (A) and *nir*K-encoding bacteria (B). Ordination plots from correspondence analysis (CA) were generated based on OTUs of denitrifying bacteria in sediment of Daya Bay, China.

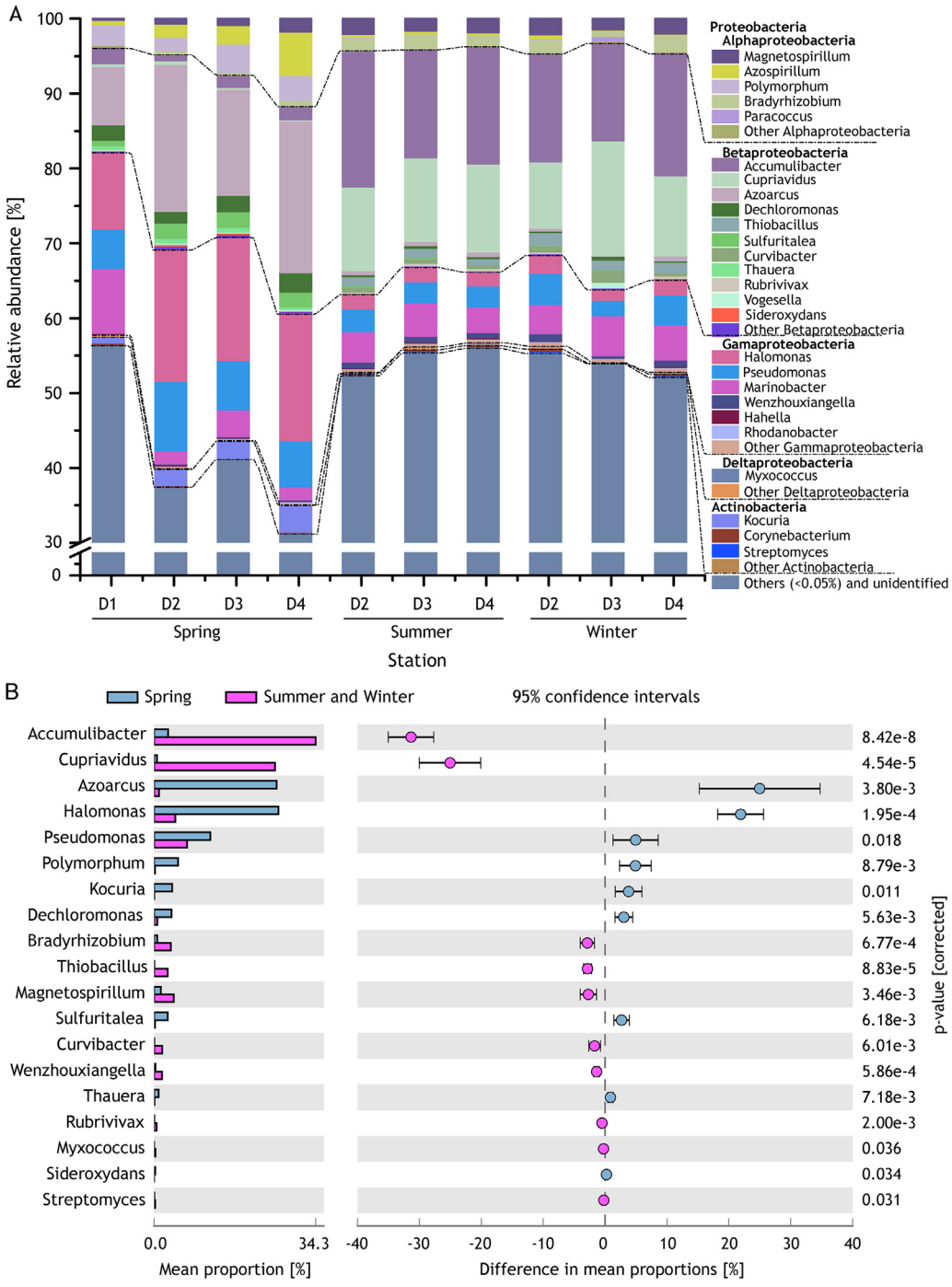

Figure 4 Taxonomic classification (A) and extended error bar plot (B) of *nir*S-encoding denitrifiers. Genera with low proportions (<0.05%) at all stations were grouped with corresponding phyla, classes, or others to increase readability.

East China Sea), wetland restoration sites, and landfill cover soils (Fig. 5). Furthermore, database alignment indicated that only 4.73% of *nir*K OTUs could be assigned to Proteobacteria because of limited available databases. Therefore, further analysis of the *nir*K gene was restricted to the most abundant 50 OTUs, which were assigned to clusters A–D based on phylogenetic analysis (Fig. 5). The dominant cluster at all sampling stations was cluster B (26.66–67.49% of the total OTUs at all stations), followed by cluster A (5.23–44.13%), cluster D (6.44–13.0%), and cluster C (0.53–4.46%, Fig. 6A).

The results indicated that the relative abundance of cluster A, which was phylogenetically similar to Proteobacteria, was highest in winter (25.80–44.13%), followed by summer (10.43–28.76%), and spring (5.23–23.96%, Fig. 6A). A decreasing trend in the relative abundance of cluster A was observed from stations D2, D3 to D4 in all seasons. Whereas, the relative abundance of cluster B was

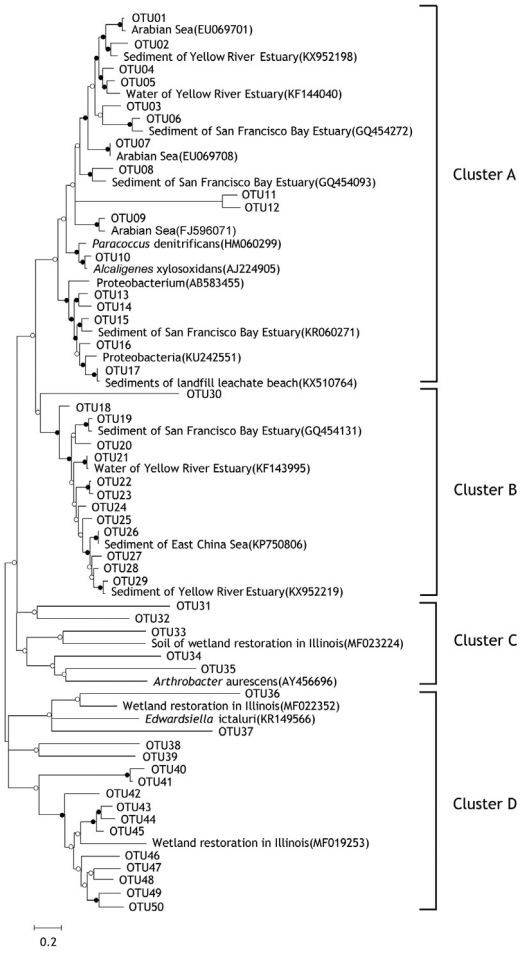

Figure 5 Maximum likelihood phylogenetic tree of *nir*K sequences isolated from Daya Bay, China. Bootstrap values greater than 50% ($n = 1000$) are shown with solid circles and those less than 50% are shown with open circles on the corresponding nodes.

highest in spring (30.15–67.49%), followed by summer (34.35–47.74%), and winter (26.66–36.86%) and an increasing trend was observed from stations D2, D3 to D4 in all seasons. Compared with clusters A and B, the relative abundance of clusters C and D was relatively low and evenly distributed across all stations and seasons. In addition, the abundance of OTU15, OTU23, and OTU37 was significantly higher in summer compared with spring ($p < 0.05$, Fig. 6B). However, the distribution of OTU24 and OTU31 was significantly higher in spring than in winter ($p < 0.05$). The distribution of OTU26, OTU44, and OTU49 was significantly higher in summer than in winter ($p < 0.05$).

3.6. Relationships between denitrifying bacteria and environmental factors

Mantel test indicated that DO, NO_2^- and NO_3^- contents were the most key factors significantly shaping *nir*S-encoding denitrifier communities (Table 4, $p < 0.05$). Thus, *nir*S-encoding denitrifiers, whose proportions were higher than 0.05% in all sampling sites, were divided into three groups based on the correlation between their abundance and environmental factors (Fig. 7A). The representative genera of the first group were *Marinobacter* sp., *Myxococcus* sp., *Bradyrhizobium* sp., *Cuprizvidus* sp., *Magnetospirillum* sp., and *Accumulibacter* sp. Their abundances were negatively correlated with NO_2^-, NO_3^- and DO content, however, positively correlated with temperature. In contrast, for the second group, the representative genera were *Azospirillum* sp., *Azoarcus* sp., *Halomonas* sp., *Polymorphum* sp., and *Kocuria* sp. Their abundances were positively correlated with NO_2, NO_3^- and DO content, however, negatively correlated with temperature. For the third group, the representative genera were *Paracoccus* sp., *Pseudomonas* sp., and *Corynebacterium* sp., abundance was correlated with salinity and TOC.

No significant correlationship between environmental factors and *nir*K-encoding denitrifier communities was observed (Table 4, $p > 0.05$). However, for those *nir*K-encoding denitrifiers whose mean relative abundance of OTUs $> 0.5\%$ still can be divided into four groups (Fig. 7B). The abundance of representative OTU of OTU15 in the first group was significantly negatively correlated with NO_2^-, NO_3^- and DO content ($p < 0.05$), but positively correlated with temperature. For the second group, the abundances of OTU22 and OTU36 were positively correlated with TN content, but negatively correlated with TOC ($p < 0.05$). For the third group, the abundances of OTUs of OTU25, OTU33, OTU39, OTU41 and OTU47 were positively correlated with DO. For the fourth group, the abundances of OTU11 and OTU12 were positively correlated with TOC, MC and NH_4^+.

4. Discussion

In the present study, both *nir*K- and *nir*S-encoding denitrifiers, which had been reported to be widespread in various sediments, were detected in the sediment of Daya Bay, China (Alcántara-Hernández et al., 2014; Chen et al., 2017; Li et al., 2017a; Tang et al., 2016), and the abundance and community diversity of *nir*K-encoding denitrifiers were much lower than *nir*S-encoding denitrifiers. This was probably due to their different ecological strategies or niches, such as the requirement for different enzyme substrates (Huang et al., 2011). Our results were consistent with previous studies which reported that *nir*S-encoding denitrifiers were more widespread than *nir*K-encoding denitrifiers in sediments of the Yellow River Estuary (Li et al., 2017a), San Francisco Bay (Lee and Francis, 2016) and the Elkhorn Slough Estuary (Smith et al., 2015). Since the proportion of *nir* gene determines the potential denitrification rate, the denitrifying potential of *nir*S-encoding bacteria is significantly greater than that of *nir*K-encoding bacteria (Graham et al., 2010). In addition, the higher community diversity of *nir*S-encoding denitrifiers indicated that they had stronger environmental adaptiveness than *nir*K-encoding denitrifiers.

It is reasonable that higher variations in environmental factor, e.g., temperature variation between summer and winter, may lead to significant changes in biological communities. However, in the present study, the abundance and composition of *nir*S-encoding denitrifiers did not differ significantly between summer and winter. A similar finding was reported by Gao et al. (2016), they found that the

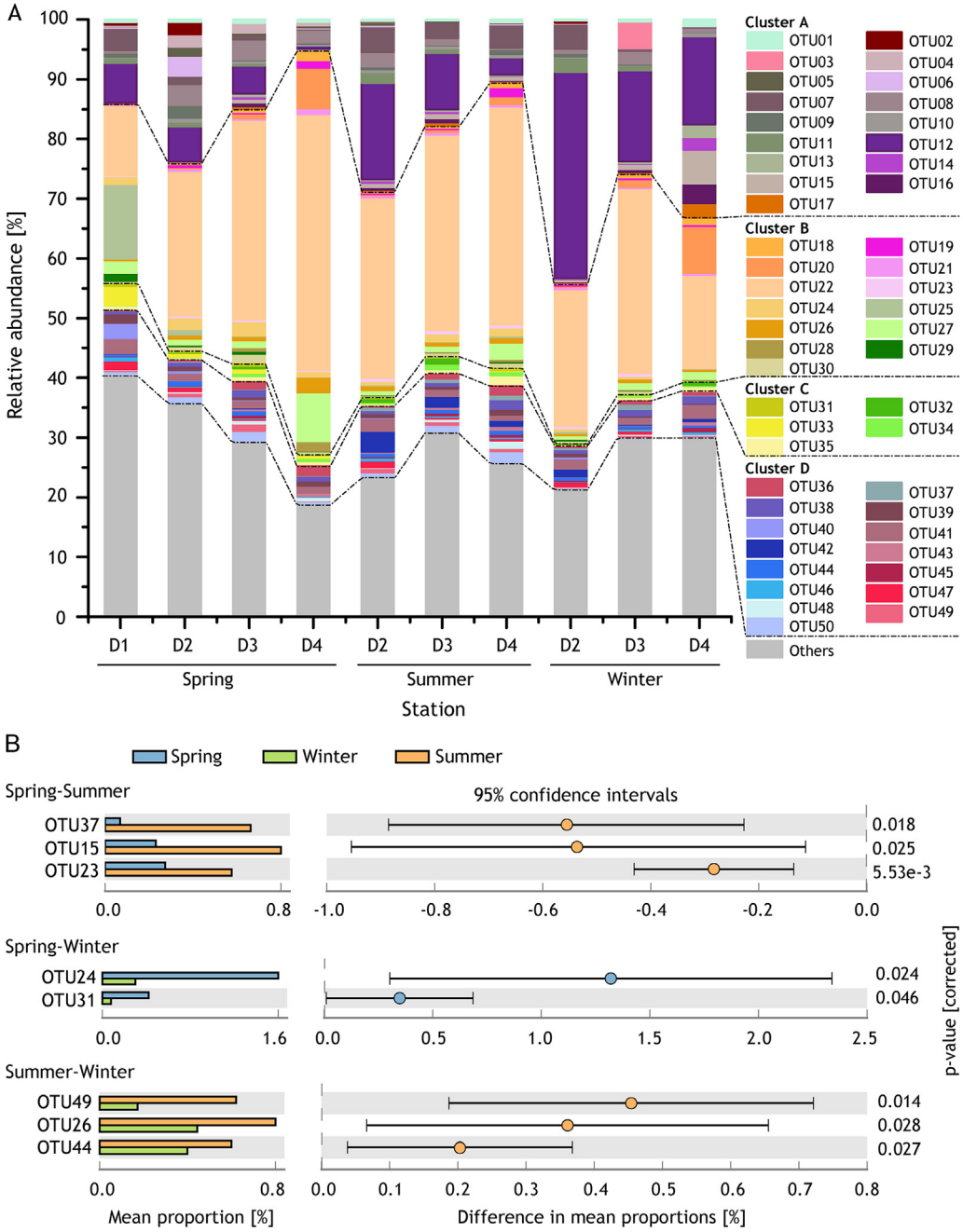

Figure 6 Taxonomic classification (A) and extended error bar plot (B) of *nir*K-encoding denitrifiers. The analysis was based on the most abundant 50 OTUs from different sampling station.

distribution and composition of *nir*S-encoding denitrifiers had a significant latitudinal differentiation, but without a seasonal shift between summer and winter. Nevertheless, greater differences were found between spring and the other seasons. For example, significant differences in abundance and composition of *nir*S-encoding denitrifiers were detected between spring and the other seasons, with NO_2^- and DO concentrations as the dominant environmental drivers (Figs. 7 and 8). Similarly, significant community differences in *nir*K-encoding denitrifiers were also detected between spring and winter. The abundance of *nir*S-encoding denitrifiers was highest in spring ($p < 0.05$), however, the abundance of *nir*K-encoding denitrifiers was lowest in spring.

The significant community differences between spring and the other seasons may be due to the different requirements of bacteria on DO, $NO_2^-$and NO_3^-, which tended to be higher in spring than in summer or winter in Daya Bay, located at a subtropical zone. In spring, the light intensity and water temperature could promote the growth of phytoplankton, which might result in high levels of DO in the overlying water. High levels of DO promote ammonia-oxidizing process at the surface of sediment, providing the substrates (e.g., NO_2^- and NO_3^-) for denitrifying bacteria (Abell et al., 2013; Smith et al., 2015). Consistently, in the present study, the evenness and diversity of *nir*S-encoding denitrifiers were positively correlated with DO, NO_2^- and NO_3^- concentrations

Table 4 Relationships between environmental factors and community composition of *nir*S genera or *nir*K OTUs based on Spearman's rank correlation of Mantel test.

	*nir*S		*nir*K	
	Mantel statistic (*r*)	Significance (*p*)	Mantel statistic (*r*)	Significance (*p*)
Salinity	0.18	0.13	0.29	0.10
pH	−0.10	0.67	−0.05	0.54
Temperature	0.13	0.19	−0.18	0.83
DO[a]	**0.44**	**0.024**	0.29	0.13
MC[b]	−0.13	0.77	−0.003	0.50
TOC[c]	−0.12	0.77	0.16	0.19
NO_2^-	**0.61**	**0.004**	0.16	0.19
NO_3^-	**0.35**	**0.025**	0.23	0.16
NH_4^+	0.03	0.41	0.12	0.29
TN[d]	−0.03	0.52	0.07	0.33

[a] DO, dissolved oxygen; [b] MC, moisture content; [c] TOC, total organic carbon; [d] TN, total nitrogen.
Bold-faced entries indicate significance values in which $p < 0.05$.

(Fig. 8). In addition, the bacterial growth can be promoted by the dissolved organic matters from phytoplankton metabolism (Li et al., 2017b). On the other hand, the competition between *nir*S-encoding and *nir*K-encoding denitrifiers could be a possible reason for the lower abundance of *nir*K-encoding denitrifiers in spring. Furthermore, the abundance and distribution of *nir*S- and *nir*K-encoding denitrifiers at station D1 were significantly different from that at the other stations. This could be due to D1 located near the Dan'ao River estuary in Daya Bay. This river lead to higher nutrients (e.g., nitrogen and phosphate) input to the bay (Ke et al., 2017). This may also influence the amount of phytoplankton and other environmental factors related to the denitrification processes.

In accordance with previous studies (Braker et al., 2000; Kim et al., 2016), the compositions of *nir*S-encoding and *nir*K-encoding denitrifiers were typical of sediment environments, with Proteobacteria being the dominant phylum. This phylum exhibits relatively high phylogenetic and phenotypic diversities, which may explain its ability to colonize a large range of aquatic environments (He and Zhang, 2016; Liu et al., 2015). Within the Proteobacteria community, Betaproteobacteria and Gammaproteobacteria were the two dominant classes of *nir*S-encoding denitrifiers in the Daya Bay. In particular, Betaproteobacteria, which has a strong ability to decompose organic matter (Fahy et al., 2006; Patel et al., 2014), was the most abundant denitrifier in the present study. In addition, a minority of *nir*S-encoding

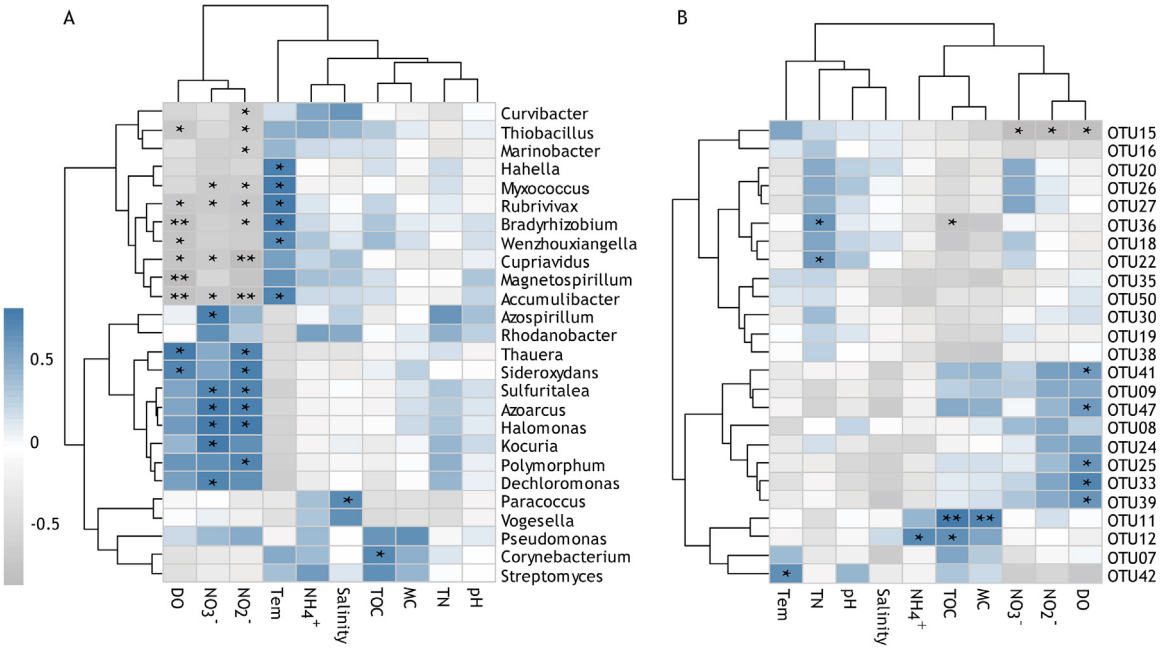

Figure 7 Correlation coefficients between environmental factors and abundance of *nir*S- (A) and *nir*K-encoding denitrifiers (B). Genera (A) and OTUs (B) with low proportions (<0.05% and 0.5%, respectively) at all sites were removed to increase readability. * and ** represent the significant correlation ($p < 0.05$) and extremely significant correlation ($p < 0.01$), respectively.

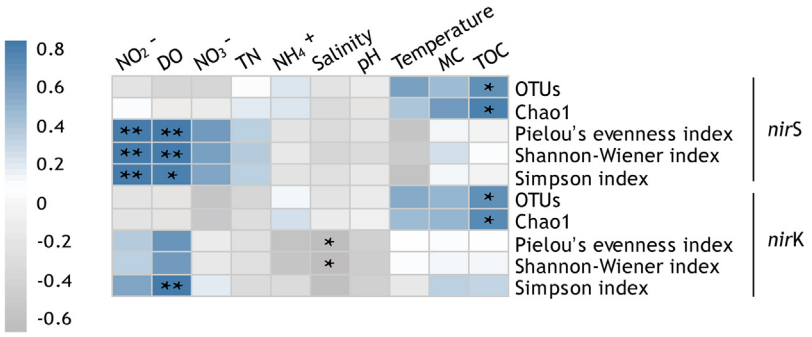

Figure 8 Correlation coefficient between richness and diversity of *nir*S- and *nir*K-encoding denitrifiers and environmental factors. * and ** represent the significant correlation ($p < 0.05$) and extremely significant correlation ($p < 0.01$), respectively.

denitrifier was assigned to the Actinobacteria phylum. Reasonably, the dominant clusters of *nir*K-encoding denitrifiers at all stations were B and A clusters, which were phylogenetically similar to Proteobacteria.

Overlying water quality and sediment characteristics such as salinity, temperature, TOC, MC, NO_2^-, and NH_4^+ concentration have been demonstrated to be associated with sediment denitrification in aquatic ecosystems (Gao et al., 2016; Giles et al., 2017; Liu et al., 2018; Long et al., 2017; Wang et al., 2013). The present study indicated that NO_2^-, NO_3^- and DO were the most important factors influencing *nir*S-encoding denitrifier communities in Daya Bay. It had been reported that denitrification occurred predominantly in suboxic and anoxic environments (Huang et al., 2011; Gao et al., 2016; Yang et al., 2015a), however, serious hypoxic condition could inhibit the growth of denitrifying bacteria due to the lack of NO_2^- and NO_3^-, since they were produced from ammonia-oxidizing reactions which need enough oxygen in the sediment (Testa et al., 2015). The deficiency of reaction substrates resulted in the dominance of *Accumulibacter* sp., *Cuprizvidus* sp., *Bradyrhizobium* sp., *Magnetospirillum* sp., and *Myxococcus* sp. in *nir*S-encoding denitrifiers communities in summer and winter, with the abundance of *Accumulibacter* sp. and *Cuprizvidus* sp. significantly higher than that in spring ($p < 0.05$). The community structure of *nir*S-encoding denitrifiers changed in spring due to the higher levels of NO_3, NO_2^- and DO. Accordingly, the genera *Azoarcus* sp., *Polymorphum* sp., *Halomonas* sp., *Pseudomonas* sp., *Azospirillum* sp., *Polymorphum* sp., and *Kocuria* sp. became the dominant denitrifying bacteria in spring. The abundances of *Azoarcus* sp. and *Halomonas* sp. were highest in spring and were positively correlated with NO_3^-, and NO_2^- concentrations ($p < 0.05$, Fig. 7A). On the other hand, correlations between the OTUs of *nir*K-encoding denitrifiers and environmental factors were relatively weak. The possible reasons for this could be due to *nir*K-encoding denitrifiers were not so sensitive to environmental variation as *nir*S-encoding denitrifiers and/or in this study the correlation coefficients were calculated based on a relatively small taxon of OTUs.

It has been reported that almost all denitrifying bacteria are heterotrophic and the spatial distribution of them is probably impacted by TOC, which is the primary electron donor for respiration by denitrifiers (Burgin and Hamilton, 2007; Huang et al., 2011; Ibekwe et al., 2016; Wu et al., 2017b). In the present study, positive relationships between the sediment TOC content and richness of denitrifying bacteria were detected ($p < 0.05$, Fig. 8), this was in agreement with previous studies (Bruesewitz et al., 2011; Small et al., 2016). In addition to the amount of organic matter, the diversity of *nir*S- and *nir*K-encoding denitrifiers may also be regulated by the quality of organic matter such as organic composition and size spectra (Liu et al., 2018; Stelzer et al., 2014). Further studies are needed to test the influences of seasonal variations in organic matter quality in sediment on denitrifiers community in Daya Bay.

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