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Ecological Distribution and Diversity of Key Functional Genes for Denitrification in Surface Sediments of the Northern South China Sea: Implications for Potential N_2O Emissions

Hua Xiang[1,2,3], *Yiguo Hong*[1]*, *Jiapeng Wu*[1] and *Aimin Long*[2,3]*

[1] Key Laboratory for Water Quality and Conservation of the Pearl River Delta, Institute of Environmental Research at Greater Bay Area, Ministry of Education, Guangzhou University, Guangzhou, China, [2] State Key Laboratory of Tropical Oceanography (LTO), South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China, [3] University of Chinese Academy of Sciences, Beijing, China

Denitrification is an important pathway for nitrogen sink and N_2O emissions, but little is known about the ecological distribution of key functional genes of denitrification and their potential N_2O emissions in marine sediments. In this study, we analyzed the abundance, ecological distribution, and diversity of key functional genes (*nir* and *nos*Z) for denitrification in the northern South China Sea (SCS) surface sediments. Our results showed that the gene abundances varied from 10^5 to 10^8 and from 10^6 to 10^7 copies·g^{-1} for the *nir*S and *nir*K, respectively. The *nos*Z II/*nos*Z I gene abundance ratios were 1.28–9.88 in shallow-sea and deep-sea sediments, suggesting that the *nos*Z II gene should play a dominant role in N_2O reduction in the northern SCS sediments. Moreover, the significantly higher abundance ratios of *nir*/*nos*Z in deep-sea surface sediments implied that there might be stronger N_2O emissions potential in deep-sea sediments than in shallow-sea sediments. The ecological distribution profiles of the *nir*S, *nos*Z I, and *nos*Z II gene communities varied with water depth, and denitrification genes in shallow-sea and deep-sea sediments differed in their sensitivity to environmental factors. Water temperature was the major factor affecting both the abundance and the community distribution of the *nir*S gene in deep-sea sediments. Nitrate was the major factor shaping the community of *nos*Z I and *nos*Z II genes in shallow-sea sediments. Our study provides a pattern of ecological distribution and diversity for the *nir* and *nos*Z genes and emphasizes the role of these key functional genes in potential N_2O emissions of the northern SCS surface sediments.

Keywords: denitrification, functional genes, ecological distribution, potential N_2O emissions, South China Sea

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East China Normal University, China

***Correspondence:**
Yiguo Hong
yghong@gzhu.edu.cn
Aimin Long
longam@scsio.ac.cn

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INTRODUCTION

Nitrogen is essential for all creatures and is a major nutrient supporting marine primary production (Wang et al., 2019a). With the rapid development of industry, increased amounts of reactive nitrogen generated by anthropic activities have flooded into estuaries, coastal bays, and oceans (Seitzinger et al., 2010). Fortunately, 40–50% of the external sources of dissolved inorganic nitrogen (DIN) input can be removed by denitrification and released into the ambient environment as N_2 to maintain the nitrogen cycle balance in marine sediments (Ward, 2013). Meanwhile, N_2O, a powerful and long-lived greenhouse gas with 300 times higher global warming potential than CO_2, may also form during denitrification (Makowski, 2019).

The denitrification process includes four stepwise reduction steps: NO_3^- reduction, NO_2^- reduction, NO reduction, and N_2O reduction (Knowles, 1982). Among them, NO_2^- reduction and N_2O reduction are two key steps. Two enzymes with different structures but similar functions catalyze NO_2^- reduction; the *nir*S gene encodes the copper-containing NO_2^- reductases and the *nir*K gene encodes the cytochrome cd1-containing NO_2^- reductases (Zumft, 1997). In most cases, the *nir*S gene or *nir*K gene occurs in bacteria as a single copy (Jones et al., 2008; Graf et al., 2014); the two genes cannot co-exist in microorganisms (Coyne et al., 1989). However, many research on NO_2^- reductase has only focused on the *nir*S gene (Smith et al., 2007; Christopher et al., 2013). The *nir*K gene was detected in only 30% of denitrifying microorganisms but with a broader range of taxa (Coyne et al., 1989). The *nor*B gene encodes the NO reductase (responsible for the reduction of NO to N_2O), but as compared with the *nir*S or *nir*K genes, research on the potential of the *nor*B gene as a molecular marker started relatively late (Guo et al., 2011). N_2O reductase (Nos), which catalyzes N_2O reduction, is encoded by the *nos*Z gene. The *nos*Z gene generally occurs as a single copy, except in *Pseudomonas raps* and *Thiomonas denitrificans* with the form of a double copy (Sanford et al., 2012). A significant deviation between the predicted N_2O emissions based on the gene abundance and expression of the *nos*Z and the actual N_2O emissions detected (Henderson et al., 2010; Morales et al., 2010), indicating that a class of previously undiscovered functional N_2O reductase genes might exist. Sanford et al. (2012) compared the amino acid sequences of *nos*Z genes in public databases and found that, in addition to the known typical *nos*Z I gene, there was also an emerging atypical *nos*Z II gene. Emerging studies about *nos*Z II have focused on the soil as a significant source of N_2O, such as arable soil (Graf et al., 2016), grassland soil (Di et al., 2014), and salt marsh ecosystems (Dini-Andreote et al., 2016). The *nos*Z II gene has been found to have a particularly significant effect on N_2O reduction in soils (Hallin et al., 2018). However, there is little research on the *nos*Z II gene in oceans whose surfaces cover over 70% of the Earth. The abundance and community diversity of the *nos*Z II gene and its relative importance on N_2O reduction in oceans are poorly understood, as well as how these four genes (*nir*S, *nir*K, *nos*Z I, and *nos*Z II) collectively affect N_2O emissions by denitrification in oceans.

The SCS is a largest marginal sea in China and an ideal region for research on the nitrogen cycle (Su, 2004; Yang et al., 2021). The SCS was reported to be a contributor of atmospheric N_2O, with about 132% N_2O saturation in its waters (Tseng et al., 2016; Ren et al., 2017). The atmospheric contribution of N_2O from the SCS has been estimated to be 0.15-0.24 $Tg{\cdot}a^{-1}$, which was much larger than that of the open ocean (Zheng et al., 2009; Ren et al., 2017). Moreover, the N_2O concentration in coastal waters of northern SCS increased gradually from the surface to the bottom (Xu et al., 2006; Han et al., 2013), suggesting that a source of N_2O might exist in deep waters or sediments. The present research on N_2O in the SCS mainly focuses on the distribution of N_2O in waters and its correlation with apparent oxygen utilization (AOU) (Xu et al., 2006; Zheng et al., 2009; Han et al., 2013; Tseng et al., 2016; Ren et al., 2017). As a hot spot for denitrification, sediment is generally overlooked for N_2O emissions in the SCS. The balance between *nir* (*nir*S and *nir*K) and *nos*Z (*nos*Z I and *nos*Z II) genes should determine N_2O emissions to some extent (Domeignoz-Horta et al., 2015). Combining the abundances and communities of denitrification-related functional genes will provide valuable information to explain potential N_2O emissions driven by the denitrification process.

In this study, we analyzed the distribution and relative importance of key functional genes (*nir* and *nos*Z) in N_2O emissions during denitrification by using qPCR and high-throughput sequencing analysis. We aimed to provide valuable insight into the implications of key functional genes on N_2O emissions by denitrification in marine sediments.

MATERIALS AND METHODS

Sample Collection and Environmental Parameters Measurement

The South China Sea (SCS) lies in the Northwest Pacific Ocean, expanding from the equator to latitude 23°N and from 99°E to 121°E (Su, 2005). It covers an area of about 3.50×10^6 km^2, combined with an average water depth of 1000 m (Chen et al., 2001). A total of 17 surface sediments (0–5 cm) were obtained during 3 cruises of R/V Shiyan 3 (in May 2014, October 2015, and October 2016). The sampling map is shown in **Figure 1**. Site description, sampling details, and methods of analysis are provided by Wu et al. (2019). Water depth and water temperature was continuously recorded, and the salinity and DIN (including NO_3^-, NO_2^-, and NH_4^+) of porewater was examined as described by Wu et al. (2016) and Guan et al. (2017).

DNA Extraction and PCR Amplification

Details of DNA extraction and quality determination processes were described by Wu et al. (2019). The primer pairs cd3aF/R3cd (Throback et al., 2004; Yergeau et al., 2007), *nos*Z 2F/*nos*Z 2R (Henry et al., 2006), *nos*Z IIF/*nos*Z IIR (Jones et al., 2013) were used to amplify the genes of the *nir*S, *nos*Z I, and *nos*Z II, and the products' lengths were 426, 267, 749 bp, respectively. Detailed protocols about PCR amplification are listed in **Supplementary Table 1**. PCR products recovered from gel electrophoresis were

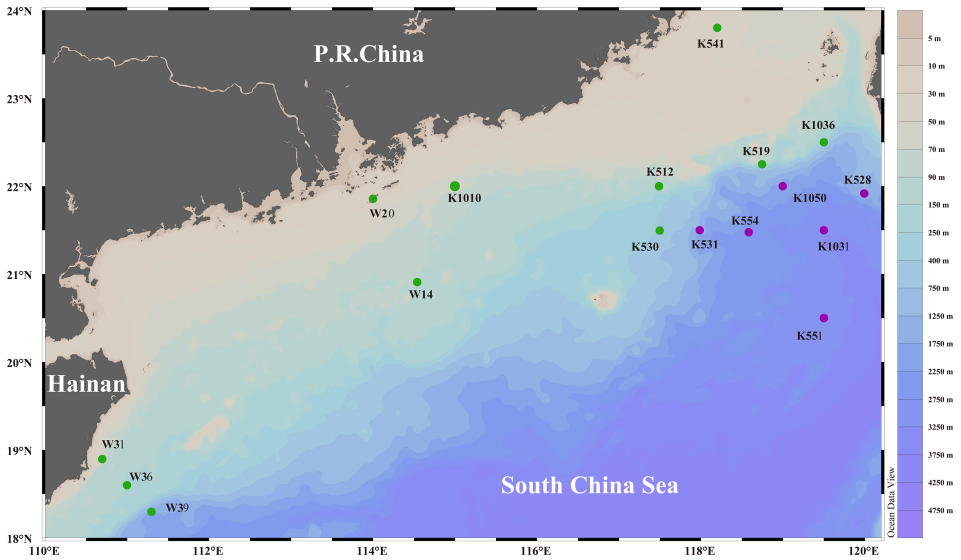

FIGURE 1 | The map of sampling sites. The dots denoted sampling stations (green dots represent shallow-sea stations and purple dots represent deep-sea stations), and the scale bar denoted water depth.

purified with an agarose gel DNA extraction kit (TaKaRa Bio, Japan).

High-Throughput Sequencing Analysis

The purified products were sequenced on an Illumina HiSeq platform (Genewiz Corporation, Suzhou, China) and then analyzed using Mothur (version 1.39.5), according to high-throughput sequencing standard procedures reported by Schloss et al. (2009). The *nos*Z I and *nos*Z II gene sequences were downloaded in Fungene (http://fungene.cme.msu.edu/) as reference sequences, and the reference sequences of *nir*S gene were derived from Fungene and NCBI (https://www.ncbi.nlm.nih.gov/) as described by Liu et al. (2020).

Briefly, valid raw data were derived after the removal of corresponding primers. The trimmed sequences were generated after the processes of binning, denoising, and trimming, and then they were aligned with prepared reference databases. To ensure the quality of the sequences, screen.seqs and chimera.uchime commands were employed to filter out poor-quality reads and to remove chimeric sequences. Finally, the classifying.seqs command was employed and the level of confidence was set at 85% for the *nir*S and *nos*Z genes (Wu et al., 2021a). A 97% sequence identity was used to define the representative sequence of each operational taxon unit (OTU) for these genes for further analysis (Wu et al., 2021a; Wu et al., 2021b). Rarefaction curves, Shannon curves, ranking abundance, and the alpha-diversity index were analyzed by Mothur based on observed OTUs (Schloss et al., 2009) and visualized by Origin 2019b. Origin 2019b was employed to describe the composition of microbial community. The neighbor-joining phylogenetic tree was constructed and visualized using MEGA7.0 (https://www.megasoftware.net/) and EvolView (He et al., 2016). A heat map was plotted by Microsoft Excel (Microsoft Excel 2016).

qPCR Analysis of Key Functional Genes for Denitrification

The abundances of *nir* and *nos*Z genes were detected using a LightCycler ® R480 II Real-Time PCR (Roche, Switzerland), and the primers and detailed protocols are listed in **Supplementary Table 1**. In addition to the primer pairs mentioned above, the *nir*K gene was amplified and quantified by primer sets F1aCu/R3Cu (Hallin and Lindgern, 1999). Each sample was quantified in triplicate, and negative controls were performed simultaneously. A standard curve was constructed with a plasmid DNA of the target gene with known concentration and copy number. The specificity of PCR products was examined by both the melting curve and gel electrophoresis. High amplification efficiencies (90–110%) and correlation coefficients ($R^2 > 0.97$) were shown for the qPCR results.

Statistical Analysis

The map of the sampling area was drawn using Ocean Data View (http://odv.awi.de). Both the bacterial communities and their correlations with environmental variables were explored by canonical correspondence analysis (CCA), redundancy analysis (RDA) and principal coordinates analysis (PCoA) with Canoco 5 (ter Braak and Šmilauer, 2012). The Pearson correlation analysis of abundance and diversity with environmental variables was constructed and visualized using IBM SPSS statistics 19 and Microsoft Excel (Microsoft Excel 2016). Non-parametric test statistics (Mann-Whitney test) was used to examine the differences of gene abundance ratios in shallow-sea and deep-sea sediments as the data did not conform to a normal distribution.

Nucleotide Sequence Accession Numbers

Raw gene sequences of *nir*S, *nos*Z I, and *nos*Z II were deposited in NCBI under the submission numbers PRJNA792035, PRJNA792063, and PRJNA792068, respectively.

RESULTS

Environmental Characteristics of Surface Sediments

The environmental characteristics of surface sediments were described in (Wu et al., 2019). The water depth at the sediments can reach 2,980 m, with water temperatures varying from 1.58 to 25.36°C (**Supplementary Figure 1**). Moreover, the water temperature showed a general drop with water depth (**Supplementary Table 2**), and the average water temperature at depths over 2000 m water was as low as 1.72 ± 0.12°C. Little change was observed in the salinity and pH of porewater, with an average of 35.55 ± 1.65 and 8.31 ± 0.23, respectively. DIN concentrations varied at sampling sites, and mainly existed in the form of NO_3^-, with NO_3^- concentration ranging from 1.8 to 46.43 μM. The concentration of NO_2^- remained at a low level (<1.11 μM) in most samples. The NH_4^+ concentration of the W31 sample was as high as 10.61 μM but consistently low (<1.11 μM) in other samples. In addition, DIN concentrations were positively correlated with the NO_3^- concentrations (r = 0.983 and $p < 0.01$) (**Supplementary Table 2**).

Abundance and Ratios of Key Functional Genes and Their Relationships With Environmental Factors

The *nir* and *nos*Z genes were all detected in sediments (**Figure 2** and **Supplementary Table 3**). An obvious variation was observed in the gene abundance of the *nir*S, ranging from $(6.04 \pm 2.78) \times 10^5$ to $(1.52 \pm 0.05) \times 10^8$ copies·g^{-1}; maximum and minimum values of *nirS* gene abundance were reached in the W20 and K554 samples, respectively. The *nir*S gene abundance inversely correlated with water depth (r = -0.486 and $p < 0.05$) and correlated favorably with water temperature (r = 0.488 and $p < 0.05$) (**Supplementary Table 2**). Moreover, the *nir*S gene abundance showed a favorable correlation with the *nos*Z I gene (r = 0.768 and $p < 0.01$) and the *nos*Z II gene (r = 0.933 and $p < 0.01$) (**Supplementary Table 2**). The *nir*K gene abundance fluctuated between $(1.23 \pm 0.22) \times 10^6$ and $(6.25 \pm 1.60) \times 10^7$ copies·g^{-1}, and it was strongly correlated with the concentration of NH_4^+ (r = 0.752 and $p < 0.01$) (**Supplementary Table 2**). The *nir*K/*nir*S ratios were <1 in shallow-sea surface sediments (except for the W31 and W14 samples) and >1 in deep-sea surface sediments (**Figure 2B** and **Supplementary Table 3**).

The gene abundances of the *nos*Z I and *nos*Z II varied from $(5.51 \pm 0.39) \times 10^5$ to $(7.18 \pm 0.09) \times 10^7$ copies·g^{-1} and from $(3.60 \pm 0.17) \times 10^6$ to $(2.92 \pm 0.41) \times 10^8$ copies·g^{-1}, respectively (**Figure 2A** and **Supplementary Table 2**). The *nos*Z I gene abundance positively correlated with water temperature (r = 0.520 and $p < 0.05$) (**Supplementary Table 2**). Furthermore, the gene abundance of the *nos*Z II was found to be higher than the *nos*Z I, with the *nos*Z II/*nos*Z I ratio >1 in each sample (**Figure 2C**). Gene abundances of the *nir* was calculated as the total gene abundances of the *nir*S and *nir*K, and gene abundances of the *nos*Z as the total gene abundances of the *nos*Z I and *nos*Z II. The gene abundances of the *nir* and *nos*Z varied from 10^6 to 10^8 copies·g^{-1}. The *nir*/*nos*Z gene abundance ratios were >1 in deep-sea surface sediments (except for the K528 sample) and <1 in most shallow-sea surface sediments (**Figure 2D**). Besides, there were significant differences in *nir*K/*nir*S ratios ($p < 0.01$) and *nir*/*nos*Z ratios ($p < 0.05$) between shallow-sea and deep-sea sediments (**Figures 2B, D**).

Community Composition and Diversity of the *nir*S Gene

After executing a series of quality control procedures, an average of 8400 ± 380 high-quality reads of *nir*S gene sequences per sample were screened (**Supplementary Table 4**). Rarefaction curves (**Supplementary Figure 2A**), Shannon index (**Supplementary Figure 2B**), rank abundance (**Supplementary Figure 2C**) and high coverage (**Supplementary Table 4**) all suggested that the sequencing results provided adequate bioinformation to study the *nir*S gene community composition and diversity. Moreover, the community diversity of the *nir*S gene declined with increasing water depth (OTUs, r = -0.722 and $p < 0.01$; Ace, r = -0.753 and $p < 0.01$; Chao1, r = -0.760 and $p < 0.01$; Shannon, r = -0.535 and $p < 0.05$), decreasing water temperature (OTUs, r = 0.55 and $p < 0.05$; Ace r = 0.617 and $p < 0.01$; Chao1, r = 0.608 and $p < 0.05$), and decreasing salinity (Shannon, r = 0.496 and $p < 0.05$; Simpson, r = -0.505 and $p < 0.05$; Evenness, r = -0.250 and $p < 0.05$) (**Supplementary Table 5**).

The dominant OTUs (top 50 OTUs, covering 49.24% of the sequences after removing rare OTUs) of the *nir*S gene were phylogenetically associated with 10 different clusters (**Figures 3A, B** and **Supplementary Figures 3 and 4**). Moreover, only C6 belonged to Alpha-proteobacteria, whereas the other 9 clusters were affiliated to Gamma-proteobacteria (**Figure 3B** and **Supplementary Figure 4**). C6 contained only OTU45 and existed exclusively in the K530 sample, closely affiliated to *Azospirillum* (**Figure 3B** and **Supplementary Figure 4**). C1 (29.59% of the total sequences) and C5 (17.15% of the total sequences) showed some similarity with the uncultured bacteria in Changjiang Estuary and the East China Sea (Dang et al., 2008), San Francisco Bay (Lee and Francis, 2017), respectively. Moreover, C1 and C5 were more abundant in deep-sea surface sediments than those sampled in shallow-sea surface sediments (**Figure 3A** and **Supplementary Figure 3**). C2 (belonging to *Chromohalobacter*), C8 (belonging to *Pseudomonas*), and C9 (belonging to *Thermomonas*) were found almost exclusively in deep-sea surface sediments (**Figures 3A, B**). PCA revealed the *nir*S gene community in deep-sea surface sediments was separated from the shallow-sea surface sediments (accounted for 82.58% of the variance, **Supplementary Figure 5**). For shallow-sea surface sediments, the environmental variables detected in this study explained 47.49% of the total variance, and nitrate was the major factor shaping the spatial distribution of the *nir*S gene community (pseudo-F = 1.5 and $p > 0.05$) (**Figure 3C**). But for deep-sea surface sediments, the environmental variables detected in this study explained 65.66% of the total variance, and water temperature turned to be the major factor affecting the spatial distribution of the *nir*S gene community (pseudo-F = 2.1 and $p < 0.01$) (**Figure 3D**).

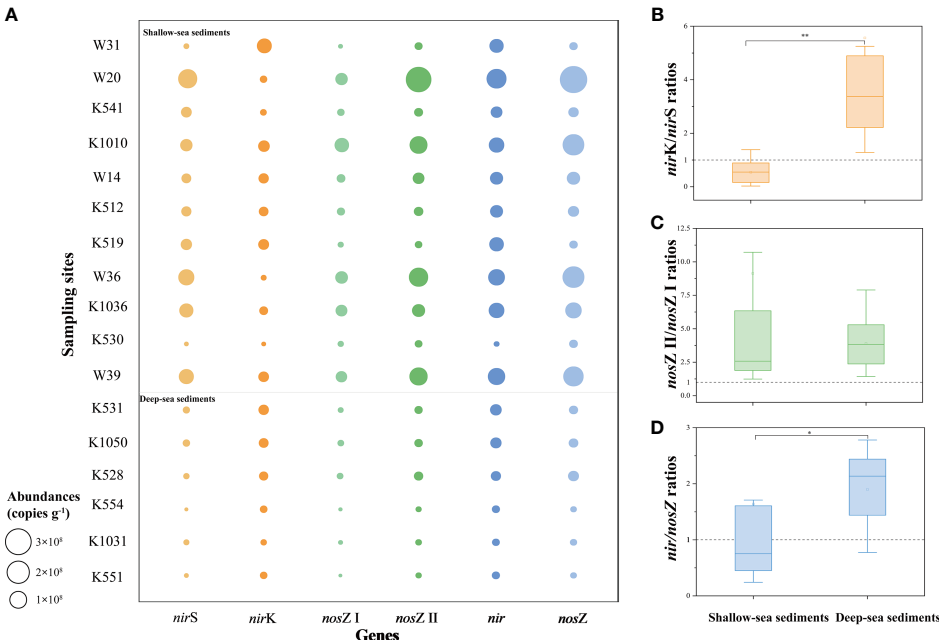

FIGURE 2 | Abundances and ratios of key functional genes for denitrification in surface sediments of SCS. The gene abundances include the *nir* (*nir*S and *nir*K) and *nosZ* (*nosZ* I and *nosZ* II) genes **(A)**, gene ratios include the *nirK*/*nirS* gene abundance ratios **(B)**, the *nosZ* II/*nosZ* I gene abundance ratios **(C)** and the *nir*/*nosZ* gene abundance ratios **(D)**. The asterisks denoted statistically differences by Mann-Whitney test, two asterisks (**) denotes $p < 0.01$ and one asterisk (*) denotes $p < 0.05$.

Community Composition and Diversity of the *nosZ* Gene

A sum of 0.17 million *nosZ* I raw sequences for 17 samples and 0.10 million *nosZ* II raw sequences for 10 samples were detected. Rarefaction curves (**Supplementary Figures 6A, 7A**), Shannon index (**Supplementary Figures 6B, 7B**), and rank abundance (**Supplementary Figures 6C, 7C**) suggested that the sequencing results were sufficient enough to reveal the diversities and community compositions of the *nosZ* gene. The basic alpha diversities of the *nosZ* gene are presented in **Supplementary Tables 6, 7**. Moreover, the coverage of the *nosZ* gene was above 0.99, indicating that the library OTUs of the *nosZ* gene had been adequately captured (**Supplementary Tables 6, 7**). The community diversity of the *nosZ* I gene was impacted by water depth (OUT, r = -0.605 and $p < 0.05$), followed by pH (Shannon, r = 0.499 and $p < 0.05$) (**Supplementary Table 8**). However, for the *nosZ* II gene, its diversity was only affected by water depth (Shannon, r = -0.755 and $p < 0.05$; Simpson, r = 0.694 and $p < 0.05$) (**Supplementary Table 9**).

The dominant OTUs of the *nosZ* I gene were divided into 12 clusters, all of which belonged to *Proteobacteria* (**Figure 4B** and **Supplementary Figure 8**). Extensive sequences of the 50 OTUs, including C1, C4, C6, and C8, matched with soil or sediment samples. Many cultured pure bacteria have also been detected, such as *Aestuarium* (C3), *Azospirillum* (C7), *Ruegeria* (C9), *Polymorphum* (C10), *Rhodobaca* (C11), and *Pseudomonas* (C12). Among the 12 clusters, only *Pseudomonas* (C12) belonged to the Gamma-proteobacteria, whereas the other 11 clusters belonged to Alpha-proteobacteria (**Figure 4B** and **Supplementary Figure 8**). Moreover, *Pseudomonas* (C12) was only observed in deep-sea surface sediments. *Aestuarium* (C3), uncultured pure bacteria (C6), and *Ruegeria* (C9) were detected to be more abundant in surface sediments of deep sea as compared with those in shallow sea. However, the *nosZ* I gene in shallow-sea surface sediments was richer in uncultured *Proteobacteria* (C5) and *Azospirillum* (C7) than those in deep-sea surface sediments (**Figures 4A, B**; **Supplementary Figure 9**).

Dominant OTUs (top 50 OTUs) of *nosZ* II gene sequences were divided into 10 clusters, including *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* (**Figures 5 A, B** and **Supplementary Figure 10**). C1–C5, containing 69.50% of the total *nosZ* II gene sequences, belonged to *Caldilinea aerophila*. C6 (4.37% of the total sequence) was closely related to *Magnetospirillum gryphiswaldense*. C1, C2, C4, and C6 were richer in shallow-sea surface sediments than deep-sea surface sediments. On the contrary, C3 was only found in deep-sea surface sediments. C7 (9.75% of the total sequence), belonging to *Lutibacter profundi*, was more abundant in deep-sea surface sediments than shallow-sea surface sediments. C8 (*Rhodothermaceae bacterium*), C9 (*Myxococcales* bacterium), and C10 (uncultured bacterium in petrochemical soil and Fyrisan Fen) were just observed in some samples.

PCoA discovered that the gene communities of the *nosZ* I and *nosZ* II in the deep-sea group were separated from the shallow-sea group (explained by 74.22% and 76.49% of the variance for *nosZ* I and *nosZ* II gene, respectively) (**Supplementary Figures 12, 13**). For shallow-sea surface sediments, nitrate was the major factor shaping the community of *nosZ* I gene (pseudo-

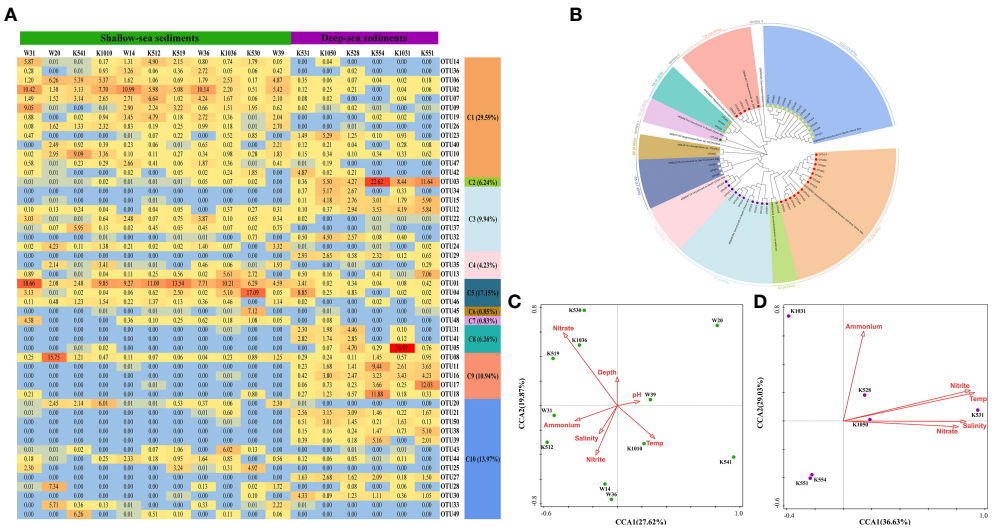

FIGURE 3 | Microbial characteristics of the *nir*S gene in surface sediments of the northern SCS. **(A)** The heat map of dominant OTUs for the *nir*S gene (Top 50 OTUs). **(B)** Neighbor-joining phylogenetic tree of dominant OTUs for the *nir*S gene (Top 50 OTUs) and the reference sequences from Genbank (bootstrap 1000). **(C)** The canonical correspondence analysis (CCA) for the *nir*S gene derived from shallow-sea surface sediments. **(D)** The canonical correspondence analysis (CCA) for the *nir*S gene derived from deep-sea surface sediments.

F = 1.9 and $p < 0.05$) (**Figure 4C**) and *nos*Z II gene (pseudo-F = 1.5 and $p < 0.05$) (**Figure 5C**). And for deep-sea surface sediments, ammonium and water temperature became the most important factor affecting the community of *nos*Z I (pseudo-F = 2.6 and $p > 0.05$) (**Figure 4D**) and *nos*Z II gene (pseudo-F = 5.3 and $p > 0.05$), respectively (**Figure 5D**).

DISCUSSION

Ecological Distribution and Diversity of the *nir* Gene

In this study, the gene abundances of the *nir*S and the *nir*K was focused on in the northern SCS surface sediments, with *nir*S gene abundance ranging from 10^5 to 10^8 copies·g^{-1} and *nir*K gene abundance ranging from 10^6 to 10^7 copies·g^{-1} (**Figure 2** and **Supplementary Table 3**). The gene abundances of the *nir*S and *nir*K were similar to the values shown in Fitzroy Estuary (Abell et al., 2009) and Elkhorn Slough (Smith et al., 2014). The abundances of the *nir*K or *nir*S genes might have been higher than examined, owning to the primer coverage limitations, since the abundances of the *nir*K or *nir*S genes could not be detected with any single universal detection method (Helen et al., 2016). The abundances of *nir* gene indicate the potential of N_2O production in surface sediments of the SCS, as it catalyzes NO_2^- reduction to NO (an essential precursor of N_2O). The *nos*Z gene co-occurred with the *nir*S gene (*nos*Z I, r = 0.768 and $p < 0.01$; *nos*Z II, r = 0.933 and $p < 0.01$) at a relatively higher rate than with the *nir*K gene (*nos*Z I, r = -0.007 and $p > 0.05$; *nos*Z II, r = -0.127 and > 0.05), indicating that the *nir*S gene might share a common regulatory mechanism that inhibits the loss of the *nos*Z gene (**Supplementary Table 2**). However, this mode of genomic linkage was not observed in the *nir*K gene (**Supplementary Table 2**). This result was in accordance with a finding that only 10-30% of *nir*K-harboring bacteria contained the *nos*Z gene (Clark et al., 2012). Meanwhile, there was also a positive correlation between the gene abundance of the *nir*K and N_2O emissions (Clark et al., 2012). Additionally, Cuhel et al. (2010) observed a significant negative correlation between *nir*S gene abundance and the N_2O/N_2 ratio. These results suggested that the *nir*K gene was more prone to be incomplete denitrifiers, therefore, contributed more to N_2O emissions. To some extent, the ratio of *nir*K/*nir*S can be seen as an indicator of the percentage of incomplete denitrification, i.e., potential N_2O emissions. In this study, we found *nir*K/*nir*S >1 in deep-sea surface sediments and *nir*K/*nir*S <1 in shallow-sea surface sediments (except for the W31 and W14 samples) (**Figure 2B**; **Supplementary Table 3**). Moreover, the ratio of *nir*K/*nir*S was around 5.28 in most deep-sea surface sediments (**Figure 2B** and **Supplementary Table 3**). These results suggest that the *nir*K gene cannot be ignored during denitrification, especially in the northern SCS deep-sea surface sediments. More importantly, our study indicated that the northern SCS deep-sea surface sediments had a relatively higher but not strong potential N_2O emissions as compared with the northern SCS shallow-water surface sediments (**Figure 2** and **Supplementary Table 3**).

In addition, the abundance of the *nir*S gene decreased with increasing depth (r = -0.486 and $p < 0.05$) and decreasing water temperature (r = 0.488 and $p < 0.05$) (**Supplementary Table 2**). CCA also showed that water temperature was the major factor affecting the community of *nir*S gene in deep-sea sediments (**Figure 3D**). The results might indicate warm environment is

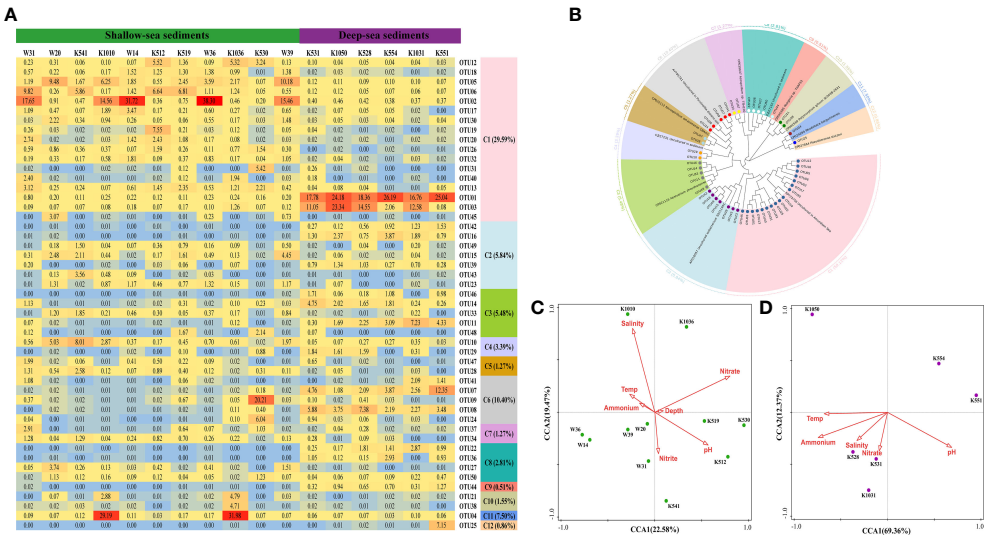

FIGURE 4 | Microbial characteristics of the *nosZ* I gene in surface sediments of the northern SCS. **(A)** The heat map of dominant OTUs for the *nosZ* I gene (Top 50 OTUs). **(B)** Neighbor-joining phylogenetic tree of dominant OTUs (Top 50 OTUs) and the reference sequences from Genbank (bootstrap 1000). **(C)** The canonical correspondence analysis (CCA) for the *nosZ* I gene from shallow-sea surface sediments. **(D)** The canonical correspondence analysis (CCA) for the *nosZ* I gene from deep-sea surface sediments.

more suitable for *nir*S gene to survive, which was consistent with a previous finding that warm incubation enhanced the growth of *nir*S-harboring bacteria (Warneke et al., 2011). The lower abundance of the *nir*S gene in deep-sea sediments also resulted in the significantly higher *nir*K/*nir*S ratios (**Figure 2B**). Some exclusive *nir*S genes were observed in deep-sea surface sediments, such as those in *Chromohalobacter* (C2) and *Pseudomonas aeruginosa* (C8), on average occupying 14.00 ± 9.86% and 17.06 ± 20.30% in the northern SCS deep-sea surface sediments, respectively (**Figures 3A, B** and **Supplementary Figure 3**). Similar to the anammox detected in the SCS, the community shift between shallow-sea and deep-sea sediments of the *nir*S gene might be an adaptation to the low-temperature environment in the deep sea (Oshiki et al., 2016; Wu et al., 2019). Research on surface sediments of San Francisco Bay showed that the environment with higher nitrate and lower water temperature might be more preferred for *nir*S and *nir*K genes, respectively (Lee and Francis, 2017). Our study observed that the *nir*S gene community diversity decreased with increasing salinity (**Supplementary Table 5**, Shannon, r = 0.496 and $p < 0.05$; Simpson, r = -0.505 and $p < 0.05$; Evenness, r = -0.250 and $p < 0.05$). In addition, NH_4^+ concentration was associated with the community composition of the *nir*K gene (Lee and Francis, 2017). In this study, a positive and stronger correlation between *nir*K gene abundance and NH_4^+ concentration was found (r = 0.752 and $p < 0.01$) (**Supplementary Table S2**). Further investigation is needed for a comprehensive understanding between complex environmental characteristics and bacterial community.

Ecological Distribution and Diversity of the *nosZ* Gene

There are biotic processes (denitrification, nitrification, and the reduction of dissimilatory nitrate to ammonium, i.e., DNRA) and an abiotic process (chemodenitrification) that can be the sources of N_2O (Stremińska et al., 2012). However, the only known N_2O sink is the N_2O reduction to N_2 by the N_2O reductase encoded by the genes of *nosZ* I or *nosZ* II (Sanford et al., 2012; Jones et al., 2013). Although the *nosZ* II gene has been found to be a different type of N_2O reductase gene (Sanford et al., 2012; Yoon et al., 2016), many research on the *nosZ* gene still ignored the differences between the two distinctive clades, especially in estuaries and oceans (Wang et al., 2019b; Aamer et al., 2020). In this study, the *nosZ* I and *nosZ* II genes were both widely distributed in the northern SCS surface sediments, with gene abundances ranging from 10^5 to 10^7 copies·g^{-1} and from 10^6 to 10^8 copies·g^{-1}, respectively (**Figure 2** and **Supplementary Table 3**). The gene abundances of the *nosZ* I were higher than the level reported at Gloucester Point (Wu et al., 2021a) but lower than that measured in the rhizosphere (Zhao et al., 2019). The gene abundances of the *nosZ* II were similar to the values detected at Gloucester Point (Wu et al., 2021b) and in the riparian zone (Zhao et al., 2018). The gene abundances of the *nosZ* I were correlated to the *nir*S gene (r = 0.768 and $p < 0.01$) rather than the *nir*K gene (r = -0.007 and $p > 0.05$), and the gene abundances of the *nosZ* II were closely associated with the *nir*S gene (r = 0.933 and $p < 0.01$) rather than the *nir*K gene (r = -0.127 and $p > 0.05$), indicating that the *nosZ* gene might be closely coupled to the *nir*S gene rather than the *nir*K gene during

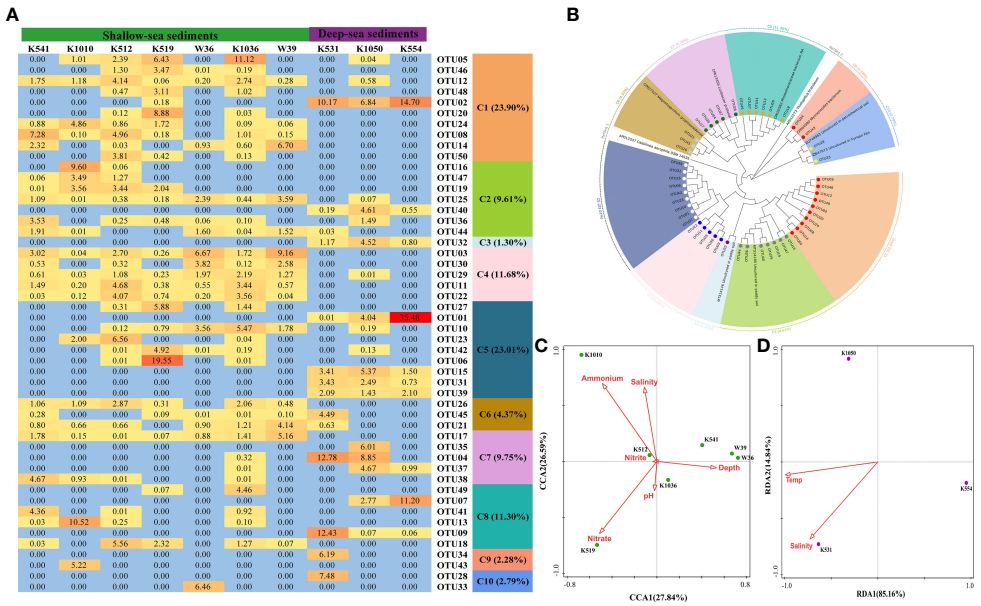

FIGURE 5 | Microbial characteristics of the *nosZ* II gene in surface sediments of the northern SCS. **(A)** The heat map of dominant OTUs (Top 50 OTUs). **(B)** Neighbor-joining phylogenetic tree of dominant OTUs (Top 50 OTUs) and the reference sequences from Genbank (bootstrap 1000). **(C)** The canonical correspondence analysis (CCA) for the *nosZ* II gene from shallow-sea surface sediments. **(D)** The redundancy analysis (RDA) for the *nosZ* II gene from deep-sea surface sediments.

evolution. These results were also observed in Tibetan alpine meadows (Malviya et al., 2016; Samad et al., 2016) and were consistent with a former understanding that *nir*S-carrying bacteria were more potentially complete denitrifiers (Graf et al., 2014).

Although the higher gene abundances of the *nosZ* II over the *nosZ* I at 1.5–10 has been commonly reported in a variety of environments such as soil, sewage treatment plants, wetlands, and rice fields (Tsiknia et al., 2015; Graf et al., 2016), little research has been conducted on the *nosZ* II gene in marine ecosystems. At Gloucester Point, the gene abundance of the *nosZ* II was two orders of magnitude larger than the *nosZ* I based on PCR quantification (Wu et al., 2021a). Another two studies based on metagenomic analysis also supported the dominance of the *nosZ* II gene in the marine oxygen minimum zone (Bertagnolli et al., 2020) and in salt marshes (Graves et al., 2016). In this study, the *nosZ* II/*nosZ* I ratios in the SCS surface sediments were 1.28–9.88, showing that the *nosZ* II gene was dominant in both shallow- and deep-sea surface sediments (**Figure 2C** and **Supplementary Table 3**). The genome of microorganisms can contain different combinations of denitrification genes, and organisms solely equipped with the *nosZ* gene coding Nos are non-denitrifying N_2O microorganisms (Roco et al., 2016; Lycus et al., 2017). Non-denitrifying N_2O microorganisms are more likely to be true N_2O sinks than denitrifying N_2O microorganisms, as they only complete N_2O reduction without contributing to N_2O production (Hallin et al., 2018). Intergenomic comparisons have shown that 51% of microorganisms with the *nosZ* II gene, which lacked the *nir* gene, were non-denitrifying N_2O reducers (Graf et al., 2014); only 17% of microorganisms with the *nosZ* I gene which lacked the *nir* gene were non-denitrifying N_2O reducers (Graf et al., 2014). Pioneering research has also highlighted the role of the *nosZ* II gene instead of the *nosZ* I gene acting as an N_2O sink in soil (Jones et al., 2014).

Coherent with past research (Jones et al., 2014; Wittorf et al., 2016), the community diversity of the *nosZ* II gene was higher than the *nosZ* I gene in the northern SCS surface sediments. At the phylum level, the *nosZ* II gene was detected in *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* (**Figure 5B** and **Supplementary Figure 10**), and the *nosZ* I gene was only found in *Proteobacteria* (**Figure 4B** and **Supplementary Figure 8**). A competitive advantage might be provided by higher community diversity of the *nosZ* II gene in adjusting to different ecology environments. This hypothesis was supported by the Pearson analysis, i.e., no environmental factors except depth were found to be correlated with the community diversity of the *nosZ* II gene (Shannon, r = -0.755 and $p < 0.05$; Simpson, r = 0.694 and $p < 0.05$; Evenness, r = -0.742 and $p < 0.05$) (**Supplementary Table 9**). For the *nosZ* II gene community, Alphaproteobacteria were more abundant in shallow-sea surface sediments than deep-sea sediments, but the opposite was true for *Bacteroides* (**Figure 5B** and **Supplementary Figure 11**). The high abundance and community diversity of the *nosZ* II gene indicated that strong N_2O reduction potential might exist in the northern SCS surface sediments. For shallow-sea sediments, nitrate was the major factor affecting the community of both *nosZ* I and *nosZ* II gene (**Figures 4C** and **5C**), suggesting nitrate may be in short supply in shallow-sea sediments relative to the demand for denitrifying bacteria.

Implications of Key Functional Genes for Potential N_2O Emissions by Denitrification

N_2O can be produced through nitrification, denitrification, and DNRA; however, N_2O exists as an intermediate product in denitrification and as an end product in nitrification and DNRA processes (Zumft, 1997; Sanford et al., 2012; Harris et al., 2021). These microbial processes involving N_2O can happen simultaneously in the marine ecosystems, an N_2O pool might be formed before it is reduced to N_2 through denitrification. Under such a circumstance, denitrification plays an important role in the conversion of N_2O (Wang et al., 2014; Zhao et al., 2021). Different abundances of denitrifying genes were associated with varying denitrification enzyme activities (Holtan-Hartwig et al., 2000; Rich et al., 2003) and indicated potential N_2O production or consumption (Bergaust et al., 2010; Morales et al., 2010). The abundance analysis of denitrifying genes might offer a closer connection for potential N_2O emissions (Cuhel et al., 2010). The *nir* and *nos*Z genes are vital denitrification genes and closely related to N_2O emissions. The enzyme encoded by *nir* gene produce a precursor for N_2O synthesis; the enzymes encoded by *nos*Z gene reduces N_2O to N_2 (Butterbach-Bahl et al., 2014). The gene abundance ratio of *nir*/*nos*Z can be regarded as an indicator of potential N_2O emissions. Previous research has shown the balance between *nir* and *nos*Z genes can partially determine the level of N_2O emissions in soils and lakes (Domeignoz-Horta et al., 2015; Saarenheimo et al., 2015). In this study, the *nir*/*nos*Z ratios were calculated in the northern SCS sediments. In the shallow-sea surface sediments, the *nir*/*nos*Z ratios ranged from 0.27 ± 0.03 to 5.72 ± 0.51, which was in accordance with the results found in rhizosphere soils (Zhao et al., 2019). Most of the *nir*/*nos*Z ratios in the shallow-sea surface sediments were < 1, implying that the N_2O produced might be reduced in shallow-sea surface sediments. This implication was supported by the relatively low value of N_2O concentration (around 12.5 $nmol{\cdot}L^{-1}$) in shallow-sea water (Tseng et al., 2016). However, in deep-sea surface sediments, the *nir*/*nos*Z ratios were > 1, with the highest value of 2.55 ± 0.33 (except for the K528 sample). These results suggested that potential N_2O production was slightly higher than that of reduction during denitrification, thus, resulting in a small amount of N_2O emissions. This implication was supported by a relatively higher value of N_2O concentration (around 25 $nmol{\cdot}L^{-1}$) in deep-sea waters (Tseng et al., 2016). In this study, the *nir*/*nos*Z ratios suggested that deep-sea surface sediments might have stronger potential N_2O emissions than shallow-sea sediments as the significantly higher *nir*/*nos*Z ratios in deep-sea sediments (**Figure 2D**). Another study also showed N_2O concentrations in bottom waters were about 2.5 times than that of surface water in the northern SCS, indicating that N_2O was abundantly accumulated in deep sea (Zheng et al., 2009; Ren et al., 2017). Although most research based on significant correlations among N_2O, AOU, and NO_3^- has provided circumstantial evidence that N_2O was mainly derived from nitrification (Xu et al., 2006; Tseng et al., 2016; Ren et al., 2017). Marchant et al. (2016) reported N_2O was mainly produced by denitrification in sediments of the Wadden Sea. Denitrification process cannot be excluded in an oxygen-depleted deep sea of the SCS (Xu et al., 2006; Zheng et al., 2009; Tseng et al., 2016; Ren et al., 2017). Denitrification is more likely to occur under low oxygen conditions, and N_2O emissions can be accelerated due to the inhibitory effect of oxygen on the activity of Nos (Körner and Zumft, 1989; Codispoti, 2010). Tseng et al. (2016) reported N_2O maxima peaked at depths where dissolve oxygen was minimal at SCS and the West Philippines Sea. Besides, mineralization of organic matter and the product of nitrate from nitrification in the upper water column may provide substrate for denitrification in deep-sea sediments (Gao et al., 2018), and sediments generally contain minerals which can preserve organic substrates (Kleber et al., 2021). Therefore, the substrate can support the abundance of *nir*K and *nos*Z genes in the deep-sea sediments. Additional direct evidence based on SP values measured for N_2O has verified that both nitrification process and denitrification process can produce N_2O in the SCS waters (Zhang et al., 2019). These results all confirmed our speculation based on *nir*/*nos*Z ratios. The gene analysis may provide new insight and act as an indicator of N_2O emissions, especially when there are difficulties obtaining *in situ* N_2O measurement.

CONCLUSION

In this study, we investigated the ecological distribution and diversity of key functional genes (*nir* and *nos*Z) for denitrification in the northern SCS shallow-sea and deep-sea surface sediments. The results showed that both *nir* (*nir*S and *nir*K) and *nos*Z (*nos*Z I and *nos*Z II) genes were widely distributed in the northern SCS surface sediments. Denitrification genes in shallow-sea and deep-sea sediments differed in their sensitivity to environmental factors. The community distribution and diversity of these genes were affected by water depth, nitrate and water temperature. Furthermore, the significantly higher abundance ratios of *nir*/*nos*Z in deep-sea as compared with shallow-sea sediments suggested that deep-sea sediments might have strong potential N_2O emissions as compared with shallow-sea sediments. In summary, this study provides an ecological distribution pattern of functional genes of denitrification and the implication on N_2O emissions by denitrification in marine sediments.

DATA AVAILABILITY STATEMENT

Raw gene sequences of *nir*S, *nos*Z I, and *nos*Z II were deposited in NCBI under the submission numbers PRJNA792035, PRJNA792063, and PRJNA792068, respectively.

AUTHOR CONTRIBUTIONS

HX conducted the experimental operation and wrote the original draft. YH was responsible for the conceptualization. HX, YH, and AL reviewed and edited the manuscript. JW was responsible

for field sampling. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.912402/full#supplementary-material

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