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# Diversity, Abundance, and Distribution of *nirS*-Harboring Denitrifiers in Intertidal Sediments of the Yangtze Estuary

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**Abstract** Denitrification plays a critical role in nitrogen removal in estuarine and coastal ecosystems. In this study, the community composition, diversity, abundance, and distribution of cytochrome *cd1*-type nitrite reductase gene (*nirS*)-harboring denitrifiers in intertidal sediments of the Yangtze Estuary were analyzed using polymerase chain reaction (PCR)-based clone libraries and quantitative PCR techniques. Clone library analysis showed that the *nirS*-encoding bacterial biodiversity was significantly higher at the lower salinity sites than at the higher salinity sites. However, there was no significant seasonal difference in the *nirS* gene diversity between summer and winter. Phylogenetic analysis revealed that the *nirS*-harboring denitrifier communities at the study area had distinctive spatial heterogeneity along the estuary. At the lower salinity sites, the *nirS*-harboring bacterial community was co-dominated by clusters III and VII; while at the higher salinity sites, it was dominated by cluster I. Canonical correspondence analysis indicated that the community compositions of *nirS*-type denitrifiers were significantly correlated with salinity, ammonium, and nitrate. Quantitative PCR results showed that the *nirS* gene abundance was in the range of $1.01\times10^6$ to $9.00\times10^7$ copies per gram dry sediment, without significant seasonal variation. Among all the environmental factors, the *nirS* gene abundance was only significantly related to the change of salinity. These results can extend our current knowledge about the composition and dynamics of denitrification microbial community in the estuarine ecosystem.

**Keywords** Denitrification · Nitrite reductase · *nirS* · Intertidal sediments · Yangtze Estuary

## Introduction

Global nitrogen (N) overload has been identified as a main emerging environmental issue in this century, due mainly to the excessive anthropogenic N input [1, 2]. Much of the anthropogenic N is transported into estuarine and coastal regions, which has already exerted a serious threat to the environmental quality of estuarine and coastal ecosystems [2]. However, microorganism-mediated denitrification is capable of removing significant quantities of the excessive N load (>50 % of which is nitrate) from these N-enriched environments, providing a sink for N, and thereby, playing an important role in decreasing the degree of eutrophication [3–6]. Denitrification is the dissimilatory reduction of nitrate and nitrite to gaseous products (NO, $N_2O$, and $N_2$) under suboxic conditions. It is a major biological loss term for fixed N from terrestrial and aquatic ecosystems to the atmosphere, especially in estuaries, where it was estimated to contribute up to 93.4 % to the total nitrogen loss while the anaerobic oxidation of ammonium to nitrogen gas (anammox) was much less quantitatively significant [7, 8]. Considering the importance of denitrification for nitrogen removal, it is critical to understand the community dynamics and distribution of the underlying denitrifiers in estuarine ecosystems.

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Denitrification proceeds by a diverse assemblage of microorganisms, encoding different types of metabolic enzymes, such as nitrate reductases, nitrite reductases (Nir), nitric oxide reductases, and nitrous oxide reductases (Nos) [9]. Nitrite reduction to nitric oxide, catalyzed by either cytochrome *cd-1* NirS nitrite reductase or copper-containing NirK enzymes, is the rate-limiting step in denitrification [9]. Besides, Nir distinguishes the true denitrifiers (gas producing) from nitrate-respiring microbes (including those performing dissimilatory nitrate or nitrite reduction to ammonium) [9], and thus, cytochrome *cd1*-type nitrite reductase (*nirS*) and *nirK* (functionally equivalent but structurally different) genes have been most frequently targeted for molecular diversity studies of denitrifiers [6]. However, *nirS* gene is much more commonly used than *nirK* gene in estuarine systems as it is reported that cytochrome *cd-1* NirS nitrite reductase is far more abundant than NirK in estuarine environments [6, 9–11].

In the present study, we selected the Yangtze Estuary as our study area to explore the diversity, abundance, and distribution of denitrifiers in estuarine ecosystems based on *nirS* gene. The Yangtze Estuary located in the subtropical monsoon climate zone is China's largest estuary, covering an area of about 8,500 $km^2$. The Yangtze River delivers more than $7.5\times10^{10}$ moles of N nutrients per year to the East China Sea through the estuarine regions [12]. Especially in recent decades, the Yangtze Estuary has been receiving an increasing load of anthropogenic nitrogen from fish farming, agricultural activities, and both industrial and domestic wastewater discharge, which has resulted in severely eutrophic status in the estuarine and adjacent coastal area [13, 14]. Therefore, the microbial nitrogen removal is of major concern in the Yangtze Estuary. To date, however, the molecular dynamics and distribution of nitrite reductase genes in intertidal sediments along the Yangtze Estuary still remains unexplored. Previously, we have examined the anammox bacterial communities [8] and ammonia-oxidizing prokaryotic communities [15] in these sediments of the Yangtze Estuary, showing distinctive spatial heterogeneities along the estuary which were correlated significantly with salinity. While a similar pattern might be expected for the distribution of *nirS*-harboring denitrifiers along the estuary as the latter were tightly linked with anammox bacterial and ammonia-oxidizing communities, the diversity and abundance of denitrifiers might also be related to key environmental factors such as reactive nitrogen and organic carbon which most denitrifiers were dependent on [9].

The objectives of this study were (a) to investigate the diversity, community composition, and abundance of denitrifiers in the Yangtze Estuary based on the *nirS* gene, (b) to elucidate the distribution pattern of *nirS*-harboring denitrifiers in the estuarine ecosystem, and (c) to explore potential links of estuarine environmental variables with the dynamics of *nirS*-harboring denitrifiers.

## Materials and Methods

### Field Sampling

In this study, surface sediment samples were collected from seven representative sites from the intertidal flats along the Yangtze Estuary (Fig. 1), including Xupu (XP), Liuhekou (LHK), Wusongkou (WSK), Bailonggang (BLG), Daxingang (DXG), Yinyang (YY), and Luchao (LC). Field surveys were conducted in January (winter) and August (summer) 2011, respectively. At each site, triplicate surface sediments (0–5 cm) were collected with stainless steel tubes and shovels. The sediment samples were then stored in sterile plastic bags, sealed and transported to the laboratory on ice within 4 h. Upon return to the laboratory, triplicate surface sediments from each site were homogenized immediately under a nitrogen atmosphere as one composite sample. Subsequently, one part of the sample from each site was preserved at −80 °C for deoxyribonucleic acid (DNA) extraction and subsequent molecular analysis. The other part was stored at 4 °C for denitrification rate measurements and sediment physiochemical analyses.

The denitrification rates and environmental parameters in association with these sediment samples from the Yangtze Estuary were analyzed and have been shown in our previous publication (Supplementary Fig. S1 and Table S1) [8].

### DNA Extraction, Gene Amplification, Cloning, and Phylogenetic Analysis

Total genomic DNA was extracted from duplicate composite sediment samples (~1.0 g) using Ultraclean™ soil DNA Isolation Kits (MOBIO, USA) following the manufacturer's instructions. The *nirS* gene fragments (~840–890 bp) were amplified from pooled sediment DNA extracts using the polymerase chain reaction (PCR) primers *nirS*-1F (5′-CCTAYTGGCCGCCRCART-3′) and *nirS*-6R (5′-CGTTGAACTTRCCGGT-3′) [16]. PCR was performed in a total volume of 50 μL containing 1 μL of *Taq* DNA Polymerase (5 U $\mu L^{-1}$, Sangon), 5 μL of 10×PCR buffer (without $MgCl_2$, Sangon, China), 4 μL of $MgCl_2$ (25 mM, Sangon), 1 μL of dNTP (each 10 mM, Sangon), 1 μL of forward primer (10 μM, Sangon), 1 μL of reverse primer (10 μM, Sangon), and 1 μL of genomic DNA. PCR reactions were carried out using a thermal program of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 56 °C for 40 s, and 72 °C for 40 s, and a final extension cycle at 72 °C for 5 min.

Appropriately sized *nirS* gene fragments were confirmed and separated by electrophoresis in 1 % agarose gel and purified using Gel Advance-Gel Extraction system (Viogene, China). The purified products were cloned using the TOPO-TA cloning kit (Invitrogen, USA) in accordance with the manufacturer's instructions. Clones were randomly selected for

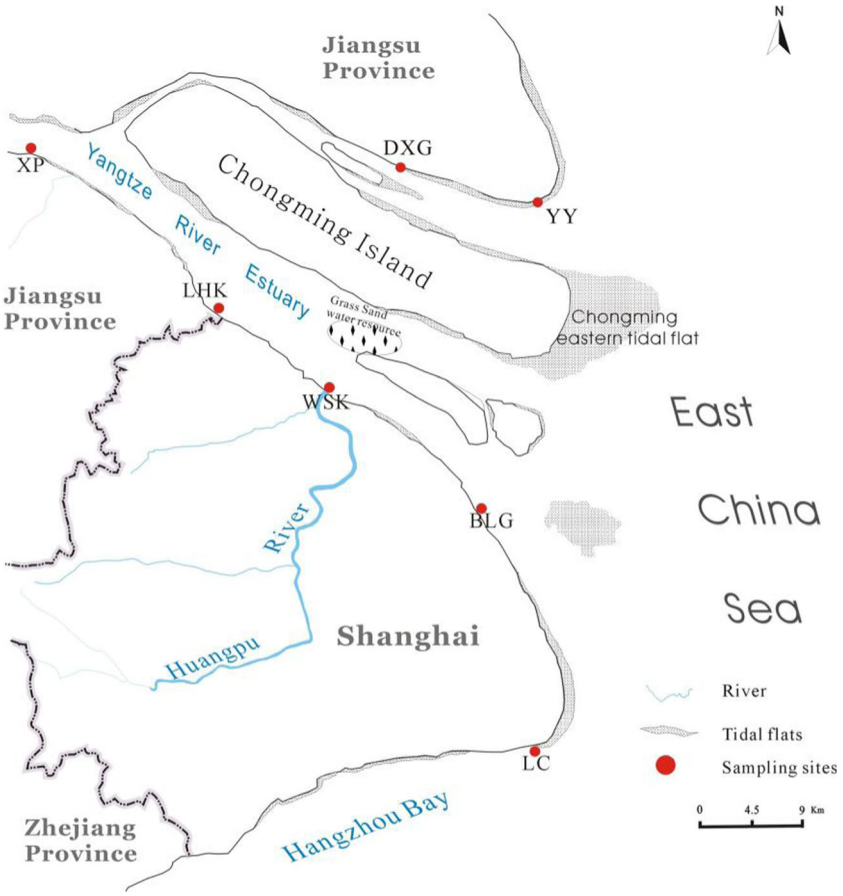


**Fig. 1** The study area showing the sampling sites

sequencing with an ABI Prism 3730 Genetic Analyzer (Applied BioSystems, USA) using Sanger method. The obtained *nirS* sequences were edited using DNAstar software package (DNASTAR, USA), and possible chimeras were checked using the CHECK CHIMERA program of the Ribosomal Database Project [17]. The unique *nirS* gene sequences obtained in this study are available in GenBank under accession numbers KM891747 to KM892364. The *nirS* gene sequences were analyzed initially using the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST/) to aid the selection of the closest matches. All the sequences and their closest matches obtained from the NCBI were aligned by ClustalX program (version 2.1) [18]. The sequences with more than 95 % identity were grouped into one operational taxonomic unit (OTU) using Mothur (http://www.mothur.org/wiki/Main_Page) by the furthest neighbor approach [19]. Neighbor-joining phylogenetic tree was constructed using MEGA software (version 5.03) [20], and the relative confidence of the tree topologies was evaluated by performing 1,000 bootstrap replicates [21].

## Real-Time Quantitative PCR

Plasmids carrying a *nirS* gene fragment were extracted from *Escherichia coli* hosts using a Plasmid Mini Preparation Kit (Tiangen, China) for standard curve construction. Plasmid DNA concentrations were measured using a Nanodrop-2000 Spectrophotometer (Thermo, USA). Triplicate samples and standard reactions were performed with an ABI 7500 Sequence Detection System (Applied Biosystems, Canada). The primer set composed of cd3aF (5′-GTSAACGTSAAGGARACSGG-3′) and R3cd (5′-GASTTCGGRTGSGTCTTGA-3′) was used to quantify the *nirS* gene (The size of the PCR products is 425 bp.) [22]. The quantitative PCR (qPCR) was performed in a total volume of 25 μL containing 12.5 μL of Maxima SYBR Green/Rox qPCR Master Mix (Fermentas, Lithuania), 1 μL of template DNA, and 1 μL of each primer (10 μM). The specificity of the qPCR amplification was determined by the melting curve and gel electrophoresis. The qPCR protocol was as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 95 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min. Negative controls containing no template DNA were subjected to detect and exclude any possible contamination. The *nirS* gene abundance was calculated based on the constructed standard curve, and then converted into copies per gram of sediment, assuming the DNA extraction efficiency was 100 %.

## Statistical Analysis

The Mothur program was used to generate rarefaction curves for the observed OTUs and to determine the species richness Chao1

estimator and diversity indices (Shannon-Weiner and Simpson indices) [19]. The coverage of *nirS* gene clone libraries was calculated by dividing the observed number of OTUs by Chao1 estimator [23]. Correlations between *nirS*-encoding bacterial assemblages and environmental parameters were explored with the canonical correspondence analysis (CCA; the maximum gradient length was 4SD based on the detrended correspondence analysis, showing that the responses of OTUs to environmental variables were unimodal) using the software Canoco (version 4.5) [24]. Community classification of the sediment *nirS*-harboring denitrifiers was performed via principal coordinates analysis (PCoA) using the UniFrac program (http://bmf.colorado.edu/unifrac/index.psp) [25, 26]. Also, UniFrac was used to form the distance matrix of the constructed clone libraries, and all *P* values have been corrected for multiple comparisons by multiplying them by the number of comparisons that were made (the Bonferroni correction) [25, 26].

## Results

### Diversity of *nirS* Clone Libraries

The *nirS* gene fragments were successfully amplified from all the sampling sites of the Yangtze Estuary in both summer and winter (Supplementary Fig. S2). A total of 14 nitrite-reducing bacterial *nirS* gene clone libraries were generated, and overall 1,840 sequences were obtained for further analysis (Table 1). When 5 % divergence in nucleotides was used as the cut-off value, 15 to 42 OTUs were obtained within each individual clone library. Rarefaction analysis as well as the Shannon-Weiner and Simpson indices showed that the greatest *nirS* gene biodiversity was observed at the lower salinity site XP in summer, while the lowest value was occurred at the higher salinity site DXG in winter (Fig. 2 and Table 1). Generally, the diversity of *nirS* gene was significantly higher at the lower salinity sites than at the higher salinity sites in the intertidal sediments of the Yangtze Estuary (Student's *t* test, $P=0.01$). However, no obvious seasonal difference in the *nirS* gene diversity between summer and winter was observed (Student's *t* test, $P>0.05$) (Supplementary Table S2). The estimated coverage values of the clone libraries were between 88.2 and 96.6 %, indicating that the majority of the *nirS* sequence types were captured, and this was further confirmed by the gradually flattening rarefaction curves (Table 1 and Fig. 2).

### Phylogenetic Analysis of *nirS* Sequences

In addition to comparing the relative richness and biodiversity of the *nirS* gene, we examined the phylogenetic relationships of these sequences. For the purposes of discussion, we have grouped the sequences into 10 broadly defined clusters (I–X) based on evolutionary distance (Fig. 3). Within these clusters, sequences were closely affiliated with other environmental *nirS* clones retrieved from the sediments of Jiaozhou Bay (EU048441) [27], Bohai Gulf (JN257765), Bahía del Tóbari Estuary (KC614304) [28], Changjiang Estuary (EU235754;

**Table 1** Diversity characteristics of *nirS* gene clone libraries

| Season | Sample | No. of clones | OTUs [a] | Chao1[b] | Shannon[c] | 1/Simpson[d] | Coverage (%)[e] |
|---|---|---|---|---|---|---|---|
| Summer | XP | 142 | 42 | 43.5 | 3.62 | 42.78 | 96.6 |
| | LHK | 139 | 30 | 31.5 | 3.12 | 20.23 | 95.2 |
| | WSK | 142 | 30 | 31.5 | 3.08 | 17.66 | 95.2 |
| | BLG | 124 | 33 | 34.5 | 3.32 | 28.24 | 95.7 |
| | DXG | 122 | 20 | 22 | 2.41 | 7.53 | 90.9 |
| | YY | 115 | 25 | 26.5 | 2.82 | 11.21 | 94.3 |
| | LC | 143 | 20 | 22 | 2.25 | 5.61 | 90.9 |
| Winter | XP | 136 | 33 | 34.5 | 3.25 | 23.01 | 95.7 |
| | LHK | 134 | 40 | 42.5 | 3.52 | 34.14 | 94.1 |
| | WSK | 136 | 34 | 35.5 | 3.33 | 27.32 | 95.8 |
| | BLG | 119 | 25 | 27 | 2.88 | 14.57 | 92.6 |
| | DXG | 122 | 15 | 17 | 2.10 | 5.78 | 88.2 |
| | YY | 127 | 28 | 29.5 | 3.01 | 16.99 | 94.9 |
| | LC | 139 | 23 | 24.5 | 2.51 | 7.37 | 93.9 |

[a] OTUs are defined at 5 % nucleotide acid divergence

[b] Nonparametric statistical predictions of total richess of OTUs based on distribution of singletons and doubletons

[c] Shannon diversity index. A higher number represents more diversity

[d] Reciprocal of Simpson's diversity index. A higher number represents more diversity

[e] Percentage of coverage: percentage of observed number of OTUs divided by Chao1 estimate

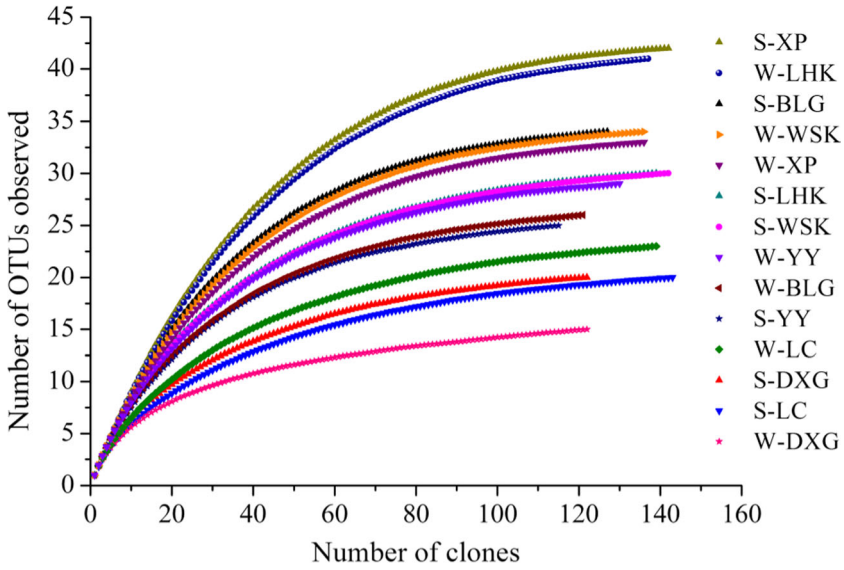


**Fig. 2** Rarefaction analysis of *nirS*-harboring denitrifier communities in the marsh sediments of the Yangtze Estuary. OTUs are defined by <5 % divergence in nucleotide sequence. *S* and *W* before the hyphen character (−) represent summer and winter, respectively

EU235973), Chesapeake Bay Estuary (DQ675714; DQ676127; DQ676123) [6], East Lake (HQ427998), Pearl River Estuary (HM773302), coastal and deep sea regions (DQ159648; GU348416) [29], as well as clones obtained from activated sludge (GU564887) [30] and the cultivated denitrifier *Dechloromonas sp.* (AM230913) [31].

In the sediment of the Yangtze Estuary, the *nirS* sequences in Cluster I were the dominant type at the higher salinity (10 to 20 ppt: YY, LC, and winter DXG) habitats, accounting for 41.8 to 79.1 % of the *nirS*-type denitrifier communities. In the lower salinity (0 to 1.5 ppt: XP, LHK, WSK, and BLG) sites, however, the *nirS*-type denitrifier communities were co-dominated by cluster III and VII (accounting for 46.3 to 78.2 %; Figs. 3 and 4). In addition, the *nirS* sequences in Clusters VI were exclusively obtained from the lower salinity stations, and apparently correspond to "low-salinity" groups of estuarine denitrifiers (Fig. 3 and Supplementary Table S3). However, clusterV harbored the most omnipresent *nirS*-type denitrifiers of the Yangtze Estuary which occurred in all the 14 constructed clone libraries (accounting for 1.3 to 16.1 %; Supplementary Table S3). In this study, no apparent season-specific cluster (except cluster IX) was found throughout the whole phylogenetic tree (Fig. 3).

## Distribution of *nirS*-Harboring Denitrifiers

Spatiotemporal distribution of *nirS*-harboring denitrifiers was investigated using weighed UniFrac PCoA analysis (Fig. 5). The first two PCoA principal coordinates (P1 and P2) explained 61.48 % of the community changes of *nirS*-harboring denitrifiers among all the sampling sites. The denitrifier assemblages at the study area were divided into two distinctive groups. The *nirS*-encoding bacterial communities in group I were recovered in the lower salinity sediments (XP, LHK, WSK, BLG, and summer DXG) whereas those in group II were retrieved in the higher salinity sediments (YY, LC, and winter DXG). Furthermore, the observed variations in the community composition were statistically compared using the UniFrac distance matrix (Fig. 6). Results showed that the *nirS* clone library obtained from the lowest salinity site (winter LHK) was significantly different from the clone libraries obtained from the highest salinity sites (summer YY and winter YY) ($P<0.05$). Also, the predominant groups between the lower and higher salinity sites were different based on the phylogenetic analysis (Figs. 3 and 4). However, based on the distance matrix, as well as the UniFrac PCoA analysis, *nirS* gene libraries at all 7 sampling sites were statistically indistinguishable between summer and winter ($P>0.05$; Figs. 5 and 6). This lack of statistically significant differences between summer and winter libraries suggests that *nirS*-harboring denitrifier community compositions were relatively stable in the sediments of the Yangtze Estuary.

## Quantification Analysis of *nirS* Gene

In this study, the standard curve spanned a range from $1.37\times10^4$ to $1.37\times10^9$ copies per microliter. Real-time qPCR consistency was confirmed by the strong linear inverse relationship between the threshold cycle ($C_T$) and the log10 value of *nirS* copy number of the standard curve ($R^2=0.9959$), with an amplification efficiency of 97.1 %. Melting curve analyses showed only one observable peak at 88.47 °C, while no detectable peaks associated with primer–dimer artifacts or other non-specific PCR amplification products were observed, confirming that fluorescent signals were derived from specific PCR products.

qPCR results showed great heterogeneous distribution of the *nirS* gene abundance among the sampling sites (Fig. 7). The highest number of *nirS* gene copies ($9.00\times10^7$ copies per gram dry sediment) was observed at site XP in winter,

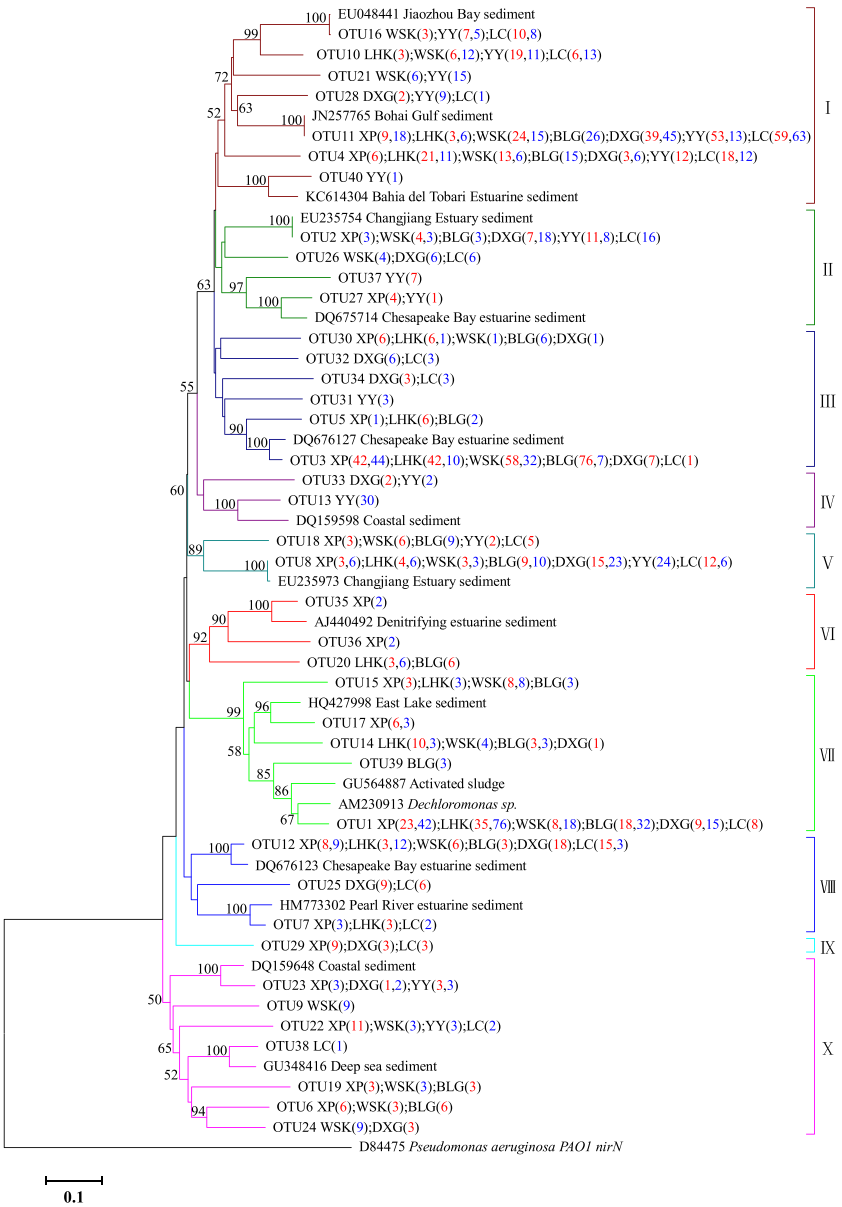


**Fig. 3** Neighbor-joining phylogenetic tree of *nirS* sequences derived from the intertidal marshes of the Yangtze Estuary, with the *nirN* gene from *Pseudomonas aeruginosa PAO1* (accession no. D84475) used as an outgroup. OTUs here were defined at 25 % nucleotide acid divergence for the phylogenetic tree construction. Bootstrap values greater than 50 % of 1,000 resamplings are shown near nodes. The *scale* indicates the number of nucleotide substitutions per site. GenBank accession numbers are shown for sequences from other studies. *Numbers in parentheses* following each site name indicate the number of sequences recovered from each sampling site in summer (*red*) and winter (*blue*)

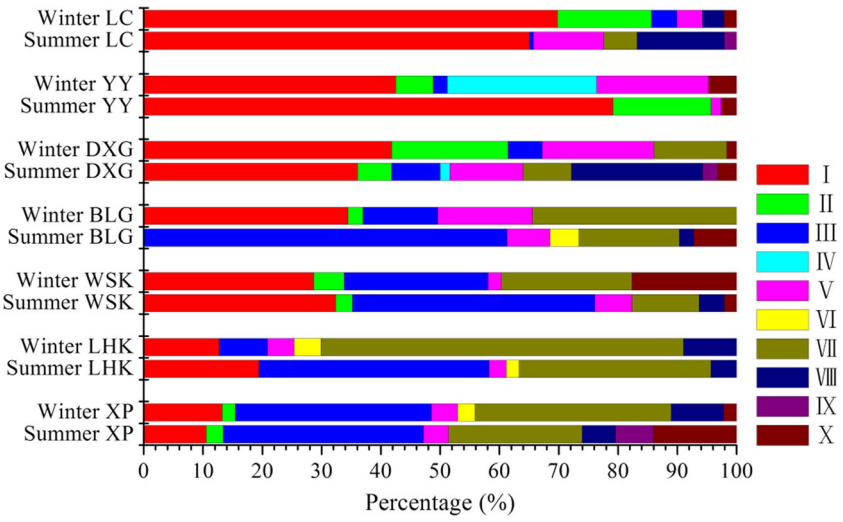


**Fig. 4** The community compositions and distributions of *nirS*-harboring denitrifiers in intertidal sediments of the Yangtze Estuary. Higher salinity sites (10 to 20 ppt): YY, LC, and winter DXG. Lower salinity sites (0 to 2.3 ppt): XP, LHK, WSK, BLG, and summer DXG

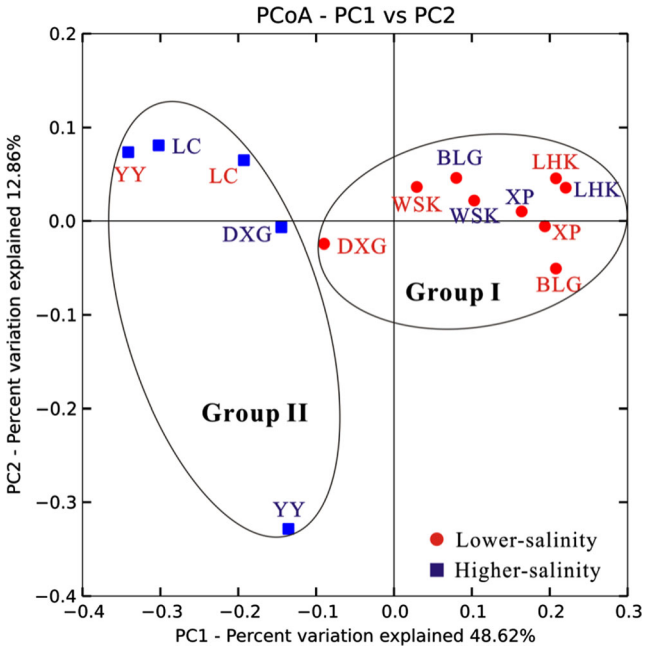


**Fig. 5** The UniFrac weighed PCoA analysis of the *nirS*-harboring denitrifier communities. The first two principal coordinate axes (*PC1* and *PC2*) are shown. *Red circle* and *blue square* symbols represent samples from the lower salinity and higher salinity sites, respectively. The summer samples are in *red font* while the winter samples are in *blue*. Higher salinity sites (10 to 20 ppt): YY, LC, and winter DXG. Lower salinity sites (0 to 2.3 ppt): XP, LHK, WSK, BLG, and summer DXG

whereas the lowest gene copy number ($1.01\times10^6$ copies per gram dry sediment) was recorded at site LC in summer. The abundance of *nirS*-harboring denitrifiers was significantly higher at the lower salinity sites ($6.37\times10^6$ to $9.00\times10^7$ copies per gram dry sediment) than at the higher salinity sites ($1.01\times10^6$ to $7.50\times10^6$ copies per gram dry sediment; $P<0.05$). Overall, no significant seasonal variation of *nirS* gene abundance was observed in intertidal sediments of the Yangtze Estuary (Student's *t* test, $P>0.05$; averaged $3.09\times10^7$ and $2.49\times10^7$ copies per gram dry sediment in summer and winter respectively), with the only exception of site BLG where the *nirS* gene abundance was 4.5 times higher in summer than in winter.

## Relationships of *nirS*-harboring denitrifier communities and abundance with environmental variables

The potential relationships between the *nirS*-harboring denitrifier communities and environmental factors in the estuarine marshes were examined by the weighted CCA analysis (Fig. 8). The first two CCA axes (CCA1 and CCA2) explained 27.0 % of the total variance in the *nirS*-harboring denitrifier composition and 40.6 % of the cumulative variance of the genotype-environment relationship. The results indicated that the *nirS*-harboring bacterial community structures in the sediments of the Yangtze Estuary correlated significantly with salinity ($P=0.002$, $F=2.37$, 499 Monte Carlo permutations), ammonium-N ($P=0.004$, $F=1.65$, 499 Monte Carlo permutations), and nitrate-N ($P=0.049$, $F=1.48$, 499 Monte Carlo permutations). These factors had significant correlation with the composition and distribution of the communities, and provided 32.0 % of the total CCA explanatory power. Although the contribution of all other measured environmental factors (temperature, grain size, nitrite-N, organic carbon, and total phosphorus) was not statistically significant ($P>0.05$, 499 Monte Carlo permutations), the combination of these variables provided additionally 34.5 % of the total CCA explanatory power. Of all the environmental factors investigated, only salinity showed a significant correlation with the *nirS* gene diversity ($r=-0.549$, $P=0.042$, $n=14$). We also investigated the correlations of *nirS* gene abundance with environmental variables. Pearson correlation analyses revealed that the *nirS* gene abundance was significantly related to the

**Fig. 6** The UniFrac Distance Matrix of *nirS* gene clone libraries. The summer samples are in *red font* while the winter samples are in *blue*. The values show the UniFrac distances between each pair of clone libraries. *Boldface blue data* indicate that the libraries are drawn from significantly different communities ($P<0.05$). All *P* values have been corrected for multiple comparisons by multiplying the calculated *P* value by the number of comparisons made (Bonferroni correction)

| | XP | XP | LHK | LHK | WSK | WSK | BLG | BLG | DXG | DXG | YY | YY | LC | LC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| XP | | 0.26 | 0.28 | 0.35 | 0.31 | 0.23 | 0.35 | 0.35 | 0.33 | 0.43 | 0.58 | 0.51 | 0.47 | 0.55 |
| XP | 0.26 | | 0.21 | 0.25 | 0.27 | 0.25 | 0.26 | 0.26 | 0.33 | 0.39 | 0.54 | 0.47 | 0.42 | 0.50 |
| LHK | 0.28 | 0.21 | | 0.27 | 0.33 | 0.28 | 0.28 | 0.29 | 0.42 | 0.44 | 0.58 | 0.52 | 0.45 | 0.54 |
| LHK | 0.35 | 0.25 | 0.27 | | 0.43 | 0.37 | 0.38 | 0.30 | 0.43 | 0.46 | **0.63** | **0.55** | 0.49 | 0.58 |
| WSK | 0.31 | 0.27 | 0.33 | 0.43 | | 0.26 | 0.29 | 0.32 | 0.33 | 0.37 | 0.44 | 0.46 | 0.33 | 0.40 |
| WSK | 0.23 | 0.25 | 0.28 | 0.37 | 0.26 | | 0.27 | 0.33 | 0.37 | 0.38 | 0.49 | 0.47 | 0.41 | 0.45 |
| BLG | 0.35 | 0.26 | 0.28 | 0.38 | 0.29 | 0.27 | | 0.38 | 0.39 | 0.44 | 0.61 | 0.50 | 0.48 | 0.57 |
| BLG | 0.35 | 0.26 | 0.29 | 0.30 | 0.32 | 0.33 | 0.38 | | 0.31 | 0.34 | 0.51 | 0.48 | 0.37 | 0.47 |
| DXG | 0.33 | 0.33 | 0.42 | 0.43 | 0.33 | 0.37 | 0.39 | 0.31 | | 0.25 | 0.40 | 0.39 | 0.21 | 0.34 |
| DXG | 0.43 | 0.39 | 0.44 | 0.46 | 0.37 | 0.38 | 0.44 | 0.34 | 0.25 | | 0.35 | 0.39 | 0.28 | 0.27 |
| YY | 0.58 | 0.54 | 0.58 | **0.63** | 0.44 | 0.49 | 0.61 | 0.51 | 0.40 | 0.35 | | 0.47 | 0.29 | 0.18 |
| YY | 0.51 | 0.47 | 0.52 | **0.55** | 0.46 | 0.47 | 0.50 | 0.48 | 0.39 | 0.39 | 0.47 | | 0.43 | 0.46 |
| LC | 0.47 | 0.42 | 0.45 | 0.49 | 0.33 | 0.41 | 0.48 | 0.37 | 0.21 | 0.28 | 0.29 | 0.43 | | 0.25 |
| LC | 0.55 | 0.50 | 0.54 | 0.58 | 0.40 | 0.45 | 0.57 | 0.47 | 0.34 | 0.27 | 0.18 | 0.46 | 0.25 | |

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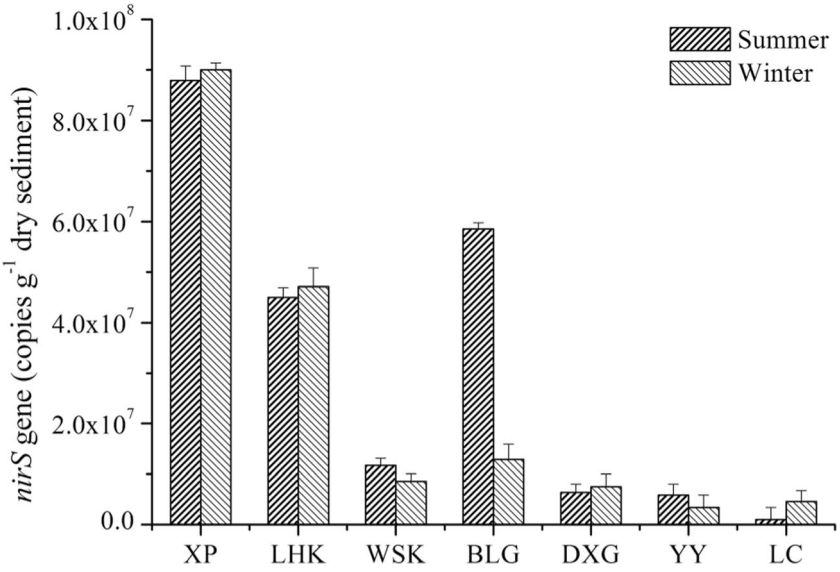


**Fig. 7** The spatiotemporal variations of *nirS* gene abundance in the marsh sediments of the Yangtze Estuary. *Vertical bars* indicate standard error ($n$=3)

change of salinity ($r$=−0.589, $P$=0.027, $n$=14), as compared with other environmental factors.

## Discussion

In the present study, spatial and seasonal variations of *nirS*-harboring bacterial community structure, biodiversity, and abundance were measured in the intertidal sediments of the Yangtze Estuary to provide deeper insights into the microbial mechanisms driving denitrification in the estuarine environments. Diversity estimators of the *nirS* gene libraries in this study were within the same range previously reported at other environmental ecosystems [6, 27, 29, 32–35]. However, the *nirS* gene diversity at the study area was significantly higher than that of the bacterial and archaeal *amoA* genes, and anammox bacterial 16S rRNA gene (Student's $t$ test, $P$= 0.000) [8, 15], showing that a greater variety of microorganisms were involved in the denitrification process in the estuarine ecosystems [36]. Nevertheless, the dominant *nirS* genotypes observed here are not obviously affiliated with known denitrifying strains, which implies that we know very little about the group of organisms that are numerically dominant and ubiquitous in the estuarine systems.

The community of *nirS*-harboring denitrifiers at the study area showed distinctive spatial heterogeneity along the estuary (Figs. 4 and 5). At the lower salinity sites, the *nirS*-harboring bacterial community was co-dominated by cluster III and VII which on average occupied 62.8 % of the total detected

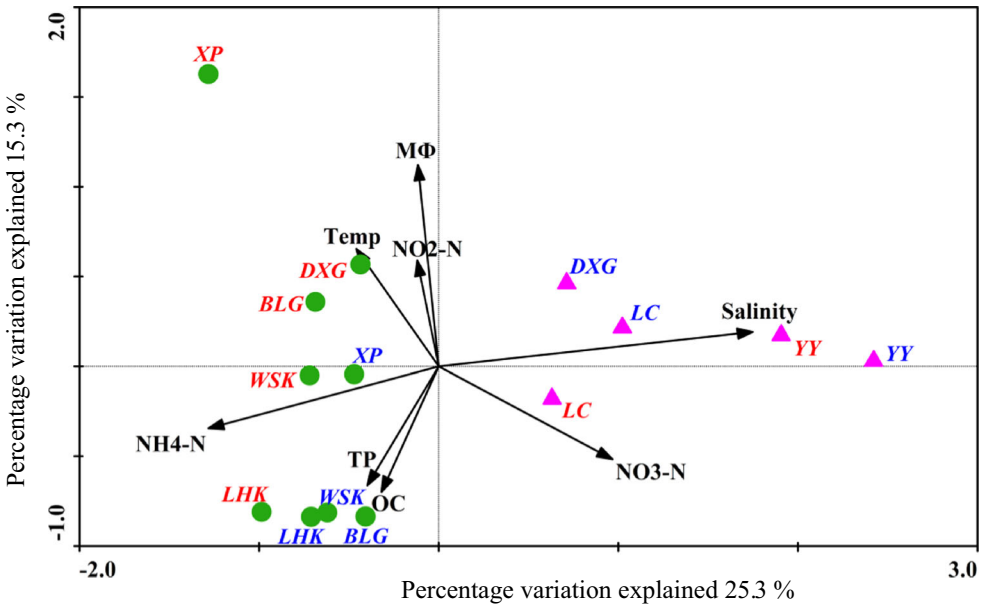


**Fig. 8** CCA ordination plots for the first two principal dimensions of the relationship between *nirS*-harboring denitrifier communities with the environmental parameters. The summer samples are in *red font* and the winter samples are in *blue. Green circle* and *pink triangle* symbols represent samples from the lower salinity and higher salinity sites, respectively. *Temp*, *NH4-N*, *NO3-N*, *NO2-N*, *TP*, *OC*, and *MΦ* represent temperature, ammonium, nitrate, nitrite, total phosphorus, organic carbon, and sediment mean size, respectively

sequences. At the higher salinity sites, however, it was dominated by cluster I which on average accounted for 59.7 % of the sequences. These results indicated that salinity was a key environmental factor regulating the biogeographical distribution of the *nirS*-type denitrifer community structure in the Yangtze Estuary. This trend was consistent with the previous results in other estuarine sediments [6] and was also consistent with a study reporting that *nirS* diversity was inversely correlated with salinity in a wastewater treatment plant [37]. Furthermore, this distribution pattern was supported by the CCA analysis in this study (Fig. 8). Interestingly, according to our previous study, the same distribution pattern was also observed for the anammox bacterial communities [8] and ammonia-oxidizing prokaryotic communities [15] in the sediments of the Yangtze Estuary, implying that salinity might play a critical role in the whole N cycle in the estuarine environments.

While salinity has a direct, although imperfectly understood, effect on the observed distribution patterns of *nirS*-harboring bacterial assemblages, it is worth noting that a variety of physical/chemical environmental parameters might also be important in structuring estuarine denitrifiers with complex interactions [6, 27]. In this study, the communities and spatial distributions of *nirS*-harboring denitrifiers also had significant correlations with ammonium and nitrate concentrations. Consistent with our results, it has been reported that ammonium contributed significantly to the denitrifier–environment relationship in the sediments of Jiaozhou Bay [27]. Nitrate supply is known to have a strong impact on denitrification rates, and might also have great impact on defining denitrifier community structures [6, 38]. Also, the impact of nitrate on the distribution of *nirS*-containing denitrifiers has been proposed in sediments within an oxygen-deficient zone off the Pacific coast [39]. Grain size of sediments might also have significant influence on *nirS*-containing bacterial communities, because it might control many physicochemical characteristics of sediment as it was related to in situ hydrological conditions, such as river runoff, tides, water mixing, and the intensity and dynamics of these activities [40]. However, no significant correlation between grain size and *nirS*-type denitrifier communities was observed in this study. Additionally, organic carbon, the primary electron donor for denitrifying bacteria, was previously reported to contribute significantly to the denitrifying bacteria–environment relationship, which was not confirmed in this study [27, 34]. However, the *nirS*-encoding denitrifier communities at the sampling sites did not show apparent seasonal shifts between summer and winter, suggesting that their population distribution in the Yangtze Estuary reflects an adaptation to site-specific characteristics.

The numbers of *nirS*-containing bacteria quantified in the marsh sediments of the Yangtze Estuary were comparable to those measured in the sediments of Bahía del Tóbari estuary ($2.72\times10^6$ to $8.82\times10^7$ copies per gram sediment) [28] and San Francisco Bay estuary ($5.4\times10^5$ to $5.4\times10^7$ copies per gram sediment) [41], and were relatively higher than those measured in deep-sea subsurface sediments of the South China Sea ($4.29\times10^5$ to $2.06\times10^6$ copies per gram sediment) [34] and paddy field soil ($6.0\times10^5$ to $1.2\times10^6$ copies per gram soil) [42]. Dong et al. observed a decline in the abundance of *nirS*-type denitrifiers in sediments as nitrate concentration decreased along the hyper-eutrophic Colne estuary (United Kingdom) [43]. However, we did not detect any significant correlations between *nirS* gene abundance and the environmental factors (temperature, ammonium, nitrate, nitrite, total phosphorus, organic carbon, and sediment mean size) analyzed in this study, with the only exception of salinity. In the present study, we observed that *nirS* gene abundance was significantly affected by salinity, and this result further confirmed that salinity might play a vital role in the N cycle of the estuarine ecosystems. Overall, the abundance of *nirS*-encoding denitrifiers did not show significant seasonal variations in this study, indicating that the number of the *nirS* type denitrifiers was not sensitive to the change of temperature. This relationship might also suggest that their population distribution in the Yangtze Estuary tend to show an adaptation to site-specific features.

In the intertidal sediments of the Yangtze Estuary, *nirS*-encoding nitrite-reducing bacteria had significantly higher abundance than anammox bacteria (anammox bacterial 16S rRNA gene abundance: $2.63\times10^6$ to $1.56\times10^7$ copies per gram sediment) [8] (Student's $t$ test, $P=0.023$), indicating the *nirS*-encoding bacteria might play a more important role on nitrite reduction than anammox bacteria. Actually, it was estimated that denitrification contributed 87.1 % to 93.4 % to the total nitrogen loss from the intertidal sediments of the Yangtze Estuary based on the $^{15}N$ tracing experiments [8]. In addition, the abundance of *nirS* gene was significantly higher than that of *amoA* genes (bacterial *amoA* gene abundance: $7.36\times10^4$ to $5.20\times10^6$ copies per gram sediment; archaeal *amoA* gene abundance: $5.70\times10^4$ to $6.84\times10^5$ copies per gram sediment) in the Yangtze Estuary [15], which supported the hypothesis described by Corredor et al. [44]: at the estuarine level, ammonia oxidizers are less abundant than denitrifiers. The abundance of denitrifiers may be reflected by the denitrification rates. However, in this study we did not observe any significant correlations between *nirS* gene abundance and denitrification rates ($P>0.05$). We have to note that this lack of correlation might be explained by the fact that we only targeted *nirS*-encoding type of the denitrifying abundance.

In summary, we explored the diversity, abundance, and distribution of cytochrome *cd1*-type nitrite reductase (*nirS*) sequences in intertidal sediments of the Yangtze Estuary. Generally, *nirS*-encoding bacterial biodiversity was significantly higher at the lower salinity sites than at the higher salinity sites, while no obvious seasonal variations were observed.

The composition of *nirS*-harboring denitrifier communities at the study area showed distinctive spatial heterogeneity along the estuary, which were significantly correlated with salinity, ammonium, and nitrate. The *nirS* gene abundance varied between $1.01\times10^6$ and $9.0\times10^7$ copies per gram dry sediment, which were significantly related to salinity. However, no significant seasonal variation in the *nirS* gene abundance was observed at the study area. The present study showed the dynamics of *nirS*-harboring denitrifiers, and thereby, provided an opportunity to further understand the microbial mechanisms of the denitrification process.

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