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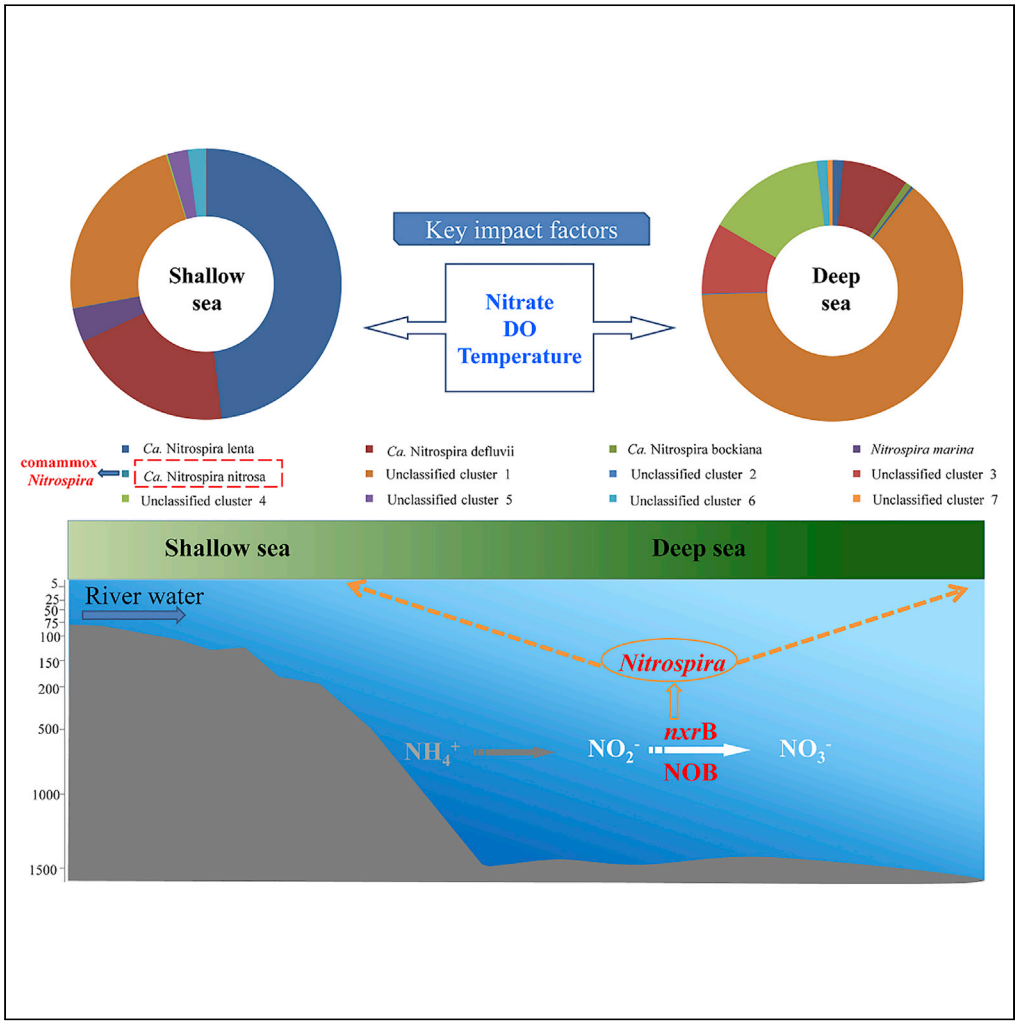


Wei Sun, Lijing Jiao, Jiapeng Wu, Jiaqi Ye, Mingken Wei, Yiguo Hong

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## Highlights

Communities of *Nitrospira* were collected at 25–1500 m depths in the South China Sea

Communities of *Nitrospira* varied spatially between the shallow and deep sea

Comammox *Candidatus* Nitrospira nitrosa was discovered in the marine ecosystem

Nitrate, temperature, and DO shaped the niche differentiation of *Nitrospira* species

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# Existence and distribution of novel phylotypes of *Nitrospira* in water columns of the South China Sea

Wei Sun,[1,2,3] Lijing Jiao,[2,3] Jiapeng Wu,[2,3] Jiaqi Ye,[2,3] Mingken Wei,[1] and Yiguo Hong[2,3,4,*]

## SUMMARY

**In the biological nitrogen cycle, nitrite oxidation is performed by nitrite oxidation bacteria, of which *Nitrospira* is widespread and diverse. Communities of *Nitrospira* were collected at 25–1500 m depths in the South China Sea. Phylogenetic diversity, community composition, and environmental factors were investigated using high-throughput sequencing targeting the *nxr*B gene and statistical analyses. The community composition of *Nitrospira* varied spatially and by depth. Among the 24 OTUs with relatively high abundance, 70% were unclassified and not affiliated with the known *Nitrospira* genus, suggesting a previously unrecognized high diversity of marine *Nitrospira*. Five known *Nitrospira* genera were detected, of which the common marine *Nitrospira marina* was not the dominant species, whereas *Candidatus Nitrospira lenta* and *Candidatus Nitrospira defluvii* dominated in shallow habitats. Comammox *Candidatus* Nitrospira nitrosa was discovered in the marine ecosystem. The niche differentiation of versatile *Nitrospira* species was mainly shaped by nitrate, temperature, and DO.**

## INTRODUCTION

Nitrification, the oxidation of ammonia to nitrite or nitrate, is a crucial aerobic process of the biogeochemical nitrogen cycle. Nitrification, highlighting a principal example of a tight metabolic interaction between free-living microorganisms, was previously considered to be carried out by ammonia-oxidizing microorganisms (AOMs) and nitrite-oxidizing bacteria (NOB) (Koch et al., 2015). The discovery of complete ammonia-oxidizing comammox bacteria performing the two-step process in a single microorganism has made investigators rethink the conventional concept of the nitrification process (Daims et al., 2015). However, comammox bacteria have not been discovered in marine ecosystems (Daims et al., 2015; Mao et al., 2020). Nitrification not only supplies nitrate as the primary source of biologically available nitrogen for assimilation by phytoplankton and microorganisms in surface water (Gruber, 2018; Bayer et al., 2021) but also contributes markedly to carbon fixation in the dark ocean (Pachiadaki et al., 2017; Zhang et al., 2020). It may also be a source of nitrous oxide for the atmosphere (Prosser et al., 2020; Breider et al., 2019). Therefore, nitrification in marine ecosystems has received increasing attention in recent years.

Knowledge about NOB in marine ecosystems is limited compared with that of AOMs. Three of the seven known NOB genera (Hong et al., 2021), *Nitrospina*, *Nitrococcus*, and *Candidatus Nitromaritima*, are restricted to marine ecosystems according to current insights into the ecology of NOB (Watson and Waterbury, 1971; Füssel et al., 2012; Ngugi et al., 2016). Among the known NOB, members belonging to the *Nitrospira* genus are the most widespread in various engineered and natural ecosystems and the most diverse phylogenetically (Koch et al., 2015; Daims et al., 2016). *Nitrospira* consists of at least six phylogenetic sub-lineages that are widely distributed in freshwater habitats (Altmann et al., 2003), seawater (Watson et al., 1986), and even some extreme ecosystems, such as geothermal springs (Lebedeva et al., 2010) and permafrost soil (Alawi et al., 2007). *Nitrospira* adapts well to human-made environments with low nitrite concentrations (Nowka et al., 2015). *Nitrospina* seems to be the dominant genus in the open marine environment composed of an extremely low nitrite concentration (Pachiadaki et al., 2017), whereas *Nitrospira* prefers deep-sea sediments with relatively high nitrite concentrations (Xu et al., 2008). However, *Nitrospira* also surpasses *Nitrospina* in some deep-sea trench environments (Nunoura et al., 2015; Hiraoka et al., 2020). Thus, *Nitrospira* may play a crucial role in nitrite oxidation in marine ecosystems, although the composition and function of the actual marine *Nitrospira* community are still highly unknown.

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Despite the ecological and biotechnological importance of *Nitrospira*, little is known about its eco-physiological characteristics and importance for understanding its eco-niche distributions due to difficulty in its laboratory cultivation (Koch et al., 2015). *Nitrospira* is not strictly chemolithoautotrophic. Some *Nitrospira* species obtained from activated sludge or marine ecosystems are mixotrophic and can make use of simple organic carbon sources, such as formate, glycerol, and pyruvate, in addition to $CO_2$ and nitrite (Daims et al., 2001; Watson et al., 1986; Gruber-Dorninger et al., 2015; Keuter et al., 2011). Genomic analyses of some representative species of *Nitrospira* suggest their versatile metabolism. *Nitrospira defluvii* lacks pivotal genes for defense enzymes that resist oxidative stress (Lücker et al., 2010), whereas other *Nitrospira* species encode enzymes tolerant to oxygen (Koch et al., 2015; Ushiki et al., 2018), indicating that different members of the *Nitrospira* genus may adapt to differentiated environmental niches of oxygen availability. Interestingly, not all members of *Nitrospira* conduct strict nitrite oxidation. *Nitrospira moscoviensis* grows on hydrogen under aerobic conditions (Koch et al., 2014) or uses formate as an electron donor and energy source to reduce nitrate under anoxic conditions (Koch et al., 2015). The newly discovered comammox, belonging to the sublineage II *Nitrospira*, has not been reported in marine ecosystems, possibly due to the high salinity in the marine environment (Zhao et al., 2021). However, comammox *Nitrospira* indeed exists in mangrove and estuary ecosystems with high salinity levels (Liu et al., 2020a; Zhao et al., 2021).

Because *Nitrospira* was primarily studied previously using 16S rRNA sequencing, not all *Nitrospira* sublineages could be amplified (Pester et al., 2014). Nitrite oxidation was conducted by the key enzyme nitrite oxidoreductase (NXR), which was proven to be the best candidate for an NOB-specific functional gene marker (Hou et al., 2018; Wertz et al., 2008). The *nxr*B gene is more suitable than the *16S rRNA* gene for studying *Nitrospira* due to its stronger discernibility (Hong et al., 2021; Vanparys et al., 2007). The *Nitrospira nxr*B gene with high specificity was designed and optimized constantly. A new pair of primers (Nxr-f27/Nxr-r617) was proven to be specific with high amplification efficiency (Hong et al., 2020). A recently expanded taxonomic database was based on *Nitrospira nxr*B gene sequences collected from enriched, pure cultured, and various environmental samples, which were proven to be effective for analyzing the diversity of *Nitrospira* in diverse environments (Hong et al., 2020). However, studies about the composition and distribution of *Nitrospira* in marine ecosystems using the *nxr*B gene are very limited to date (Mao et al., 2020).

The South China Sea (SCS) is the largest marginal sea in the world. With an area of $3.5 \times 10^6$ km$^2$ and an average depth of 1,350 m, it is connected to the North Pacific through the Luzon Strait (Morton and Blackmore, 2002). The SCS belongs to the East Indies Triangle, and its modern marine and terrestrial biodiversity is the highest in the world (Chen et al., 2021). However, the biogeochemical cycling of nitrite in the SCS remains unclear (Chen et al., 2021). In the present study, we explored the composition and distribution of *Nitrospira* from different depths in the SCS along the transect from shallow to deep sea using high-throughput sequencing targeting the *nxr*B gene. We also detected the crucial environmental factors influencing the differentiation of the *Nitrospira* niches in the SCS. By understanding the characteristics of the distribution of *Nitrospira* in the SCS, we aimed to elucidate unexpected ecological niches among the versatile known or unknown *Nitrospira* species in the marine ecosystems and obtain a thorough comprehension of the marine nitrogen cycle.

## RESULTS

### Physiochemical parameters of profile stations in the SCS

The physicochemical parameters of the four stations in the SCS water profile are shown in Figure 2. The sampling was performed in summer, and the SCS is in the low latitude zone (Figure 1); hence, the water temperature of the SCS was comparatively high at the surface (close to 30°C) and decreased with depth ($p < 0.01$), with the lowest temperature of 2.7°C observed at 1,500 m depth. Water pressure reached 1,515 MPa at a depth of 1,500 m. The salinity ranging from 33.51% to 34.82% increased with depth; the highest salinity occurred between 150 and 200 m depth. The water density also increased with depth, especially in the shallow sea close to the continental shelf. The maximum water density was 24.37 in the 100-m water layer at stations 12 and 18. The DO level significantly decreased from the surface to the bottom ($p < 0.01$) and fluctuated in the range of 90.21–210.11 μmol L$^{-1}$.

Dissolved inorganic nitrogen in the water column was shown in Figure 2. The concentrations of nitrite and ammonium always maintained low levels [$c(NO_2^-) < 2$ μmol L$^{-1}$; $c(NH_4^+) < 1.5$ μmol L$^{-1}$], whereas the nitrate concentration gradually increased from the surface to the bottom. In the water layers below 200 m, the nitrate concentration was higher than 10 μmol L$^{-1}$ and maintained high concentrations throughout the deep water. These results suggested that the SCS belongs to an oligotrophic class of sea areas.

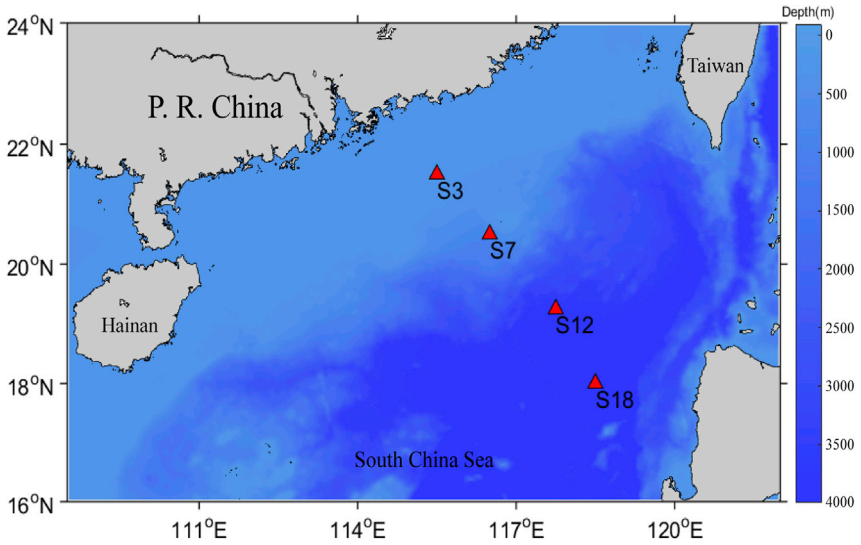


**Figure 1. Sampling stations in the South China Sea**

The water samples were collected at four sites from different depths in the SCS along the transect from the shallow (S3 and S7) to the deep sea (S12, S18).

## Diversity analysis of *Nitrospira* based on high-throughput sequencing of *nxr*B gene

After eliminating a few samples with sequence numbers fewer than 1,000, a total of 16 water samples were input to the diversity analysis, and each sample screened 2,500 sequences. The Shannon index curve tended to be flat with increasing sequencing depth (see Figure S1), suggesting that the sequencing number in the present study was sufficient for reflecting the majority of information on the *Nitrospira* community in the SCS and that subsequent analyses of *Nitrospira* diversity could be conducted.

A total of 32,140 high-quality sequences and 449 OTUs were gained by preprocessing and cluster analyses using the MOTHUR platform. After disposing of rare OTUs, *Nitrospira* species in the SCS were divided into 109 OTUs according to the *nxr*B gene. The alpha diversity indexes in the SCS water body are shown in Table 1. The coverage of *Nitrospira* was higher than 98.00% in almost all samples (except for sample S12_50 with 86.84% coverage), which indicated the confidence of various alpha diversity indexes. The OTU number was the highest at S18_75 (with the number of 69) and lower than 20 in surface water samples at stations 3, 7, and 12 (S3_25, S3_100, S7_75, S7_100, and S12_50), with the lowest OTU number 8 being observed at station 7 (S7_100). Although the OTU number was low at S12_50, the coverage of *Nitrospira* was only 87%, which indicated that some species and genera at this site may not have been collected, resulting in the underestimation of the OTU number. Shannon indexes were comparatively lower than 0.80 at S3_25, S3_100, S7_100, and S18_1000, whereas they were higher than 1.00 at other stations. The Chao1 were lower than 30.00 at S3_25, S3_100, S7_75, S7_100, and S12_50, whereas they were higher than 30.00 at other stations. The Simpson indexes were higher than 0.80 at S3_ 25, S3_100, and S18_1000. In all, the alpha diversity indexes indicated comparatively lower diversity in the surface water of the shallow ocean except for samples at S18_1000.

## Phylogenetic analysis and OTU-level community composition of *Nitrospira* in the SCS

According to the *Nitrospira* sequence analysis, 24 OTUs in the water columns of the SCS were dominant (with the number of sequences >0.1%) and comprised 96% of the total number of sequences. Based on OTU clustering results, the phylogenetic tree was constructed using the representative sequences of dominant OTUs and reference sequences as shown in Figure 3. OTU02 and OTU18 were closely related to *Candidatus Nitrospira lenta* with more than 92.0% similarity. OTU03 and OTU12 were closely related to *Candidatus N. defluvii* with more than 91.0% similarity. OTU11 was closely grouped with *Candidatus Nitrospira bockiana* with 92.1% similarity. OTU07 and OTU22 were similar to *Nitrospira marina*. OTU21 was the most similar to *Candidatus* Nitrospira nitrosa, which belonged to comammox *Nitrospira*, with 94.58% similarity. The residual OTUs (OTU01, OTU04, OTU05, OTU06, OTU08, OTU09, OTU10, OTU13, OTU14, OTU15, OTU16, OTU17, OTU19, OTU23, and OTU24) did not belong to the known species with taxonomic information but still constituted discriminative branches close to *Nitrospira*. OTU01, OTU13, and OTU14 formed unclassified cluster 1 (U1), which clustered

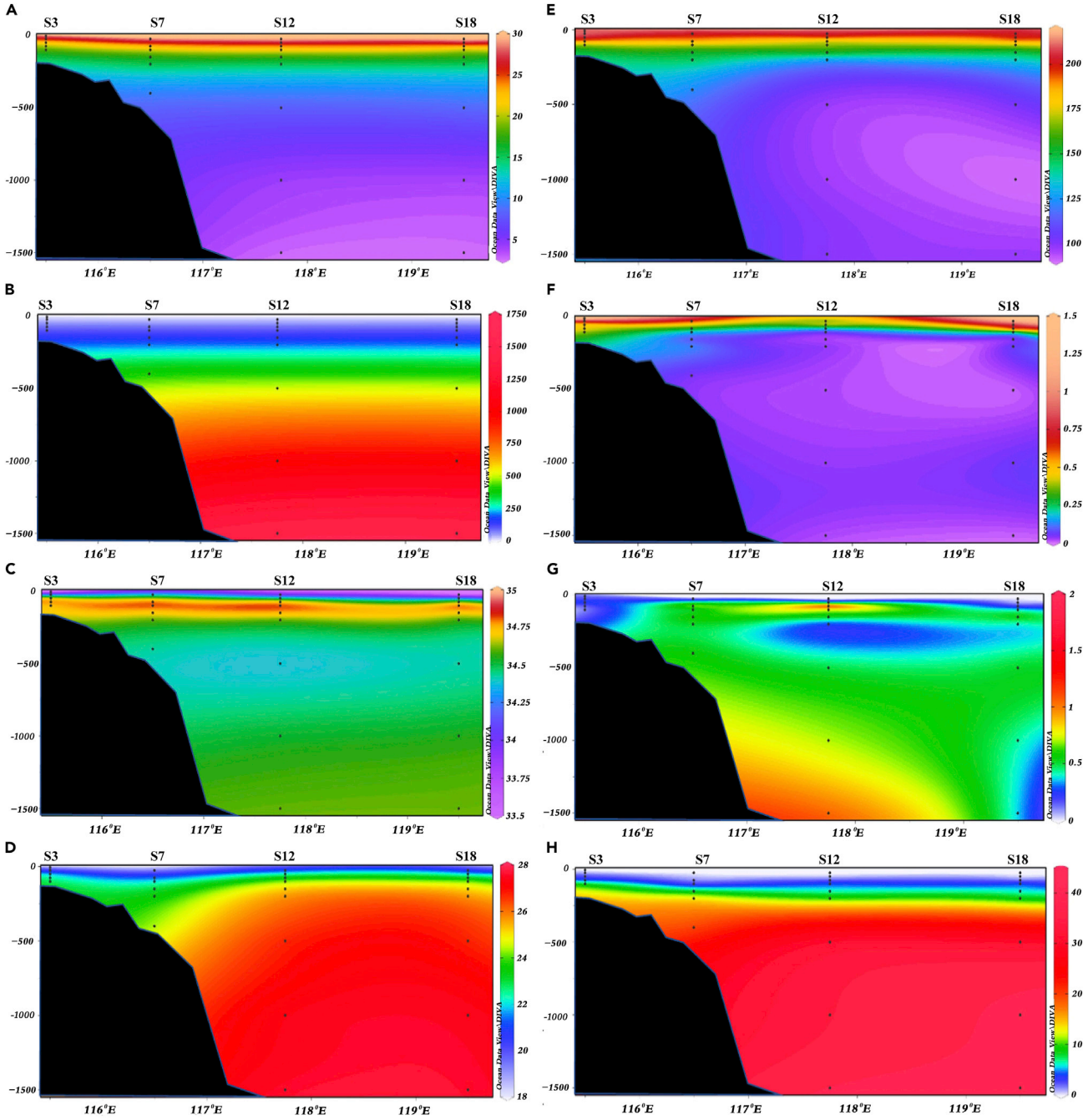


**Figure 2. Physicochemical parameters of the water columns in the South China Sea**

(A) Temperature; (B) water pressure; (C) salinity; (D) water density; (E) DO; (F) $NH_4^+$; (G) $NO_2^-$; (H) $NO_3^-$.

closely between *N. moscoviensis* and *Ca. N. bockiana* with more than 88.0% similarity to these two known genera. OTU24 formed unclassified cluster 2 (U2) closely related to *Ca. N. lenta*. OTU04 formed unclassified cluster 3 (U3), which grouped with *Nitrospira japonica* with 90.4% similarity. OTU05, OTU08, OTU10, OTU16, OTU17, OTU19, and OTU23 formed unclassified cluster 4 (U4), which grouped closely with *N. marina*. OTU09 and OTU06 formed unclassified cluster 5 (U5) and cluster 6 (U6), respectively. OTU15 and OTU20 formed unclassified cluster 7 (U7).

The *Nitrospira* species distribution was drawn according to the relative abundance of the 24 dominant OTUs in the phylogenetic analysis as shown in Figure 4. The known species *Ca. N. lenta* and *Ca. N. defluvii* were widely

**Table 1. Summary of alpha diversity in water samples of the South China Sea**

| Sample | Coverage | OTUs | Chao1 | Shannon | Simpson |
|---|---|---|---|---|---|
| S3_25 | 99.88 | 10.00 | 10.75 | 0.15 | 0.95 |
| S3_100 | 99.72 | 14.00 | 19.25 | 0.38 | 0.82 |
| S7_75 | 99.71 | 17.00 | 27.50 | 1.32 | 0.34 |
| S7_100 | 99.88 | 8.00 | 11.00 | 0.76 | 0.50 |
| S12_50 | 86.84 | 9.00 | 12.33 | 1.30 | 0.44 |
| S12_75 | 98.96 | 64.00 | 83.25 | 1.51 | 0.44 |
| S12_100 | 98.99 | 66.00 | 89.33 | 1.63 | 0.43 |
| S12_150 | 99.01 | 64.00 | 81.50 | 1.55 | 0.45 |
| S12_500 | 99.00 | 67.00 | 76.50 | 1.57 | 0.44 |
| S12_1500 | 98.72 | 67.00 | 110.88 | 1.57 | 0.45 |
| S18_25 | 98.75 | 32.00 | 39.86 | 1.18 | 0.54 |
| S18_75 | 98.97 | 69.00 | 80.88 | 1.65 | 0.44 |
| S18_100 | 99.15 | 66.00 | 77.77 | 1.57 | 0.45 |
| S18_150 | 98.96 | 65.00 | 80.00 | 1.65 | 0.40 |
| S18_200 | 99.06 | 62.00 | 79.00 | 1.61 | 0.43 |
| S18_1000 | 99.56 | 25.00 | 34.17 | 0.31 | 0.91 |

distributed in every water layer of the SCS. Niche differentiation of *Nitrospira* existed in the surface water at station 3, with *Ca. N. lenta* and U1 predominant at S3_25 and S3_100, respectively. Moreover, the relative abundance of U6 content was 8.50% at S3_100, but it was not detected at S3_25. Niche differentiation of *Nitrospira* also existed in the surface water layers at station 7, where the community structure of *Nitrospira* at S7_75 was mainly composed of *Ca. N. lenta, Ca. N. defluvii,* and *N. marina*, whereas *Ca. N. defluvii* and *Ca. N. lenta* were dominant at S7_100. Moreover, the relative abundance of *Ca. N. lenta* was lower than that of *Ca. N. defluvii* at S7_100 and higher than that of *Ca. N. defluvii* at S7_75. There was no obvious niche differentiation among the water layers at S12. U1, as the predominant species, was similar in all water layers at S12, and the relative abundance of *Ca. N. defluvii* ranged from 7.58% to 11.11%. U1 also predominated in the euphotic layers at S18 (from S18_25 to S18_200) with a relative abundance above 65.16%. The relative abundance of *Ca. N. defluvii* ranged from 7.72% to 11.13%, which was similar to the *Nitrospira* distribution at S12. However, *Nitrospira* belonging to the unknown species cluster U3 predominated in the deep sea at S18_1000.

A heatmap was drawn based on the clustering results and the distribution of the relative abundance of dominant OTUs, as shown in Figure 5. OTU01, OTU02, OTU03, and OTU05 were dominant and widely found in all samples. OTU01 accounted for 90.61% of the sequences at S3_100 while being uniformly distributed at S12 and S18 (68.94%–75.58% of the sequences) except at S18_1000. OTU02 was dominant at S3_25, S7_75, and S7_100. OTU03 was dominant at S7_100. OTU05 was distributed uniformly at S12 and S18 (except at S18_1000) with 10.86%–15.05% sequences. Nevertheless, OTU04 accounted for 96.02% of the sequences and only predominated at S18_1000. Overall, the *Nitrospira* community compositions were diverse from the shallow (S3 and S7) to the deep sea (S12 and S18). Moreover, MRPP analysis showed that the *Nitrospira* community compositions at S3 and S7 were significantly different from those at S12 and S18 ($P_{S3/S12}$ = 0.039, $P_{S3/S18}$ = 0.047, $P_{S7/S12}$ = 0.036, $P_{S7/S18}$ = 0.032, see Table S1), which further indicated that the *Nitrospira* species significantly differentiate between the shallow and deep sea (p = 0.001, see Table S1).

The results of PcoA are shown in Figure 6, in which the rates of the first and second axis contributions are 70.43% and 20.74%, respectively. The community structures at S3_25, S7_75, and S7_100 were similar and clustered together, whereas the community structure at S18_1000 was significantly different from that at the other stations. The community structures of *Nitrospira* were similar among the remaining water samples. PcoA indicated that the community structures of *Nitrospira* at S7 exhibited obvious differentiation from the other samples along the physiochemical gradients of the SCS. Moreover, the MRPP results suggested that the *Nitrospira* community composition of S7 was different from those of S12 and S18, based on the OTU levels ($P_{S7/S12}$ = 0.029*, $P_{S7/S18}$ = 0.037*, see Table S2).

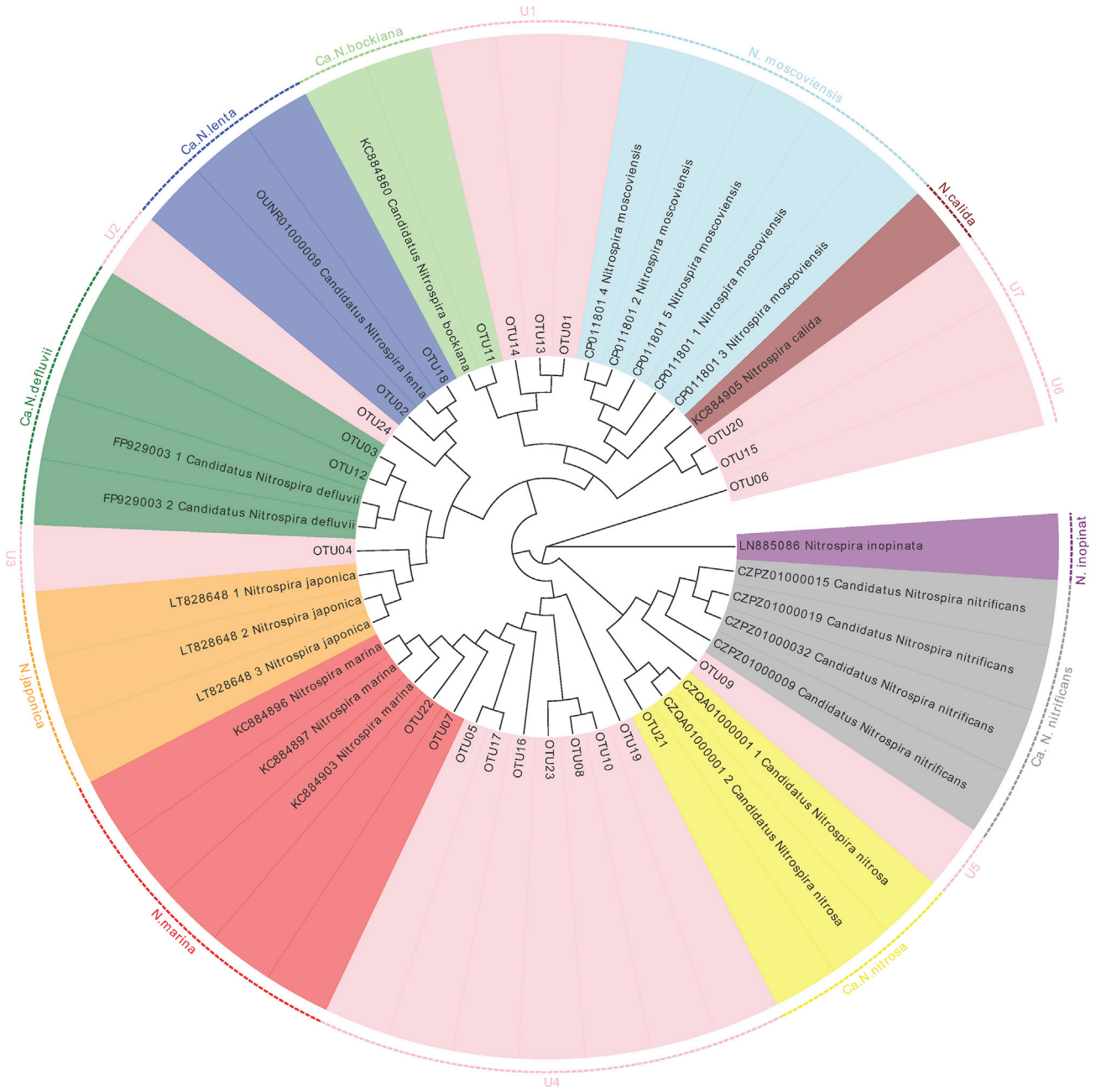


**Figure 3. Neighbor-joining phylogenetic tree of *Nitrospira* in the water columns of the SCS based on dominantly representative OTU sequences and *Nitrospira* species**

Sequences with bootstrap values (1,000 replicates) higher than 50% are grouped together and denoted by the same color. OTUs, which could not be affiliated with any identified *Nitrospira* species, are shadowed in pink.

## Relationship between *Nitrospira* community composition and environmental factors

The correlations between *Nitrospira* community structures based on main OTUs and environmental factors were analyzed using redundancy analysis (RDA). The RDA results showed that the analysis model was invalid (Monte Carlo test $F = 1.8$, $p > 0.05$), which indicated that RDA may not be appropriate to explain the distribution of *Nitrospira* in the present study. The OTU number and dominant OTUs (OTU02, OTU04, and OTU05) showed statistically significant correlations with the environmental factors, as shown in Table S3 (see Table S3). The OTU number in the water body of the SCS was positively related to the

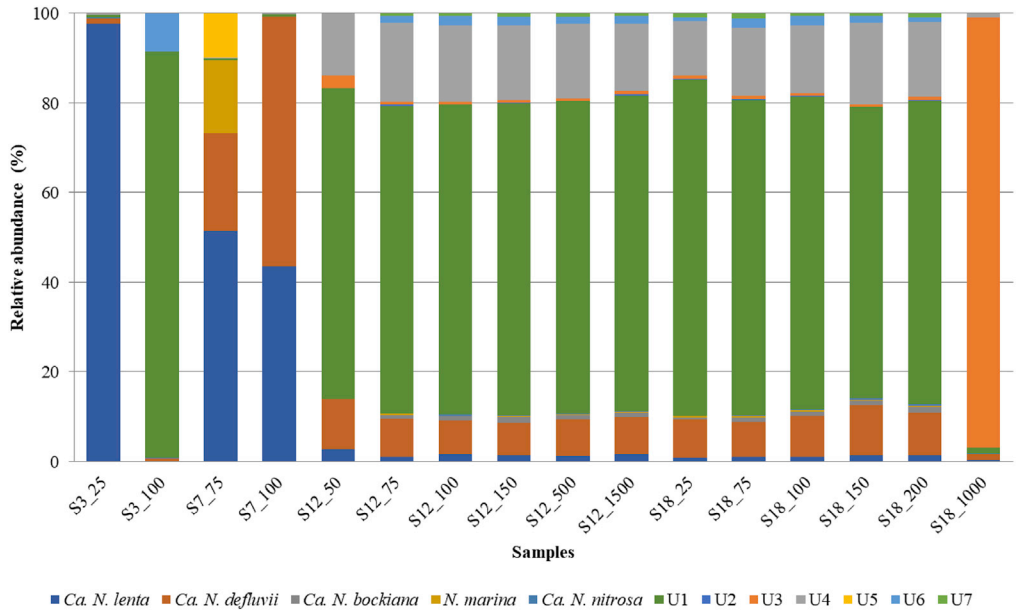


**Figure 4. The relative abundance of *Nitrospira* based on phylogenetic analysis using the dominantly representative OTU sequences distributed at different depths in the South China Sea along the transect from shallow to deep sea.**

seawater density ($r = 0.621$, $p < 0.05$), which implied that the OTU number should be relatively higher in the areas with higher seawater density. In addition, the distribution of OTU05 was positively correlated with the seawater density ($r = 0.542$, $p < 0.05$). The relative abundance distribution of OTU02 was negatively correlated with the water pressure ($r = -0.57$, $p < 0.05$), which indicated that OTU02 was mainly distributed in areas with low water pressure. The relative abundance of OTU04 was positively related to $NO_3^-$ ($r = 0.585$, $p < 0.05$) and was negatively associated with temperature ($r = -0.505$, $p < 0.05$) and DO ($r = -0.504$, $p < 0.05$), which implied that species belonging to OTU04 might adapt to environments with a certain degree of hypothermia and hypoxia.

## DISCUSSION

### Diversity and distribution of *Nitrospira* community in the South China sea

A steep increase in our knowledge of *Nitrospira* in various natural and engineered ecosystems has been witnessed with the rapid development of molecular approaches combined with traditional methods (Watson et al., 1986; Lebedeva et al., 2010; Ushiki et al., 2013; Boris et al., 2015; Daims et al., 2016; Hong et al., 2021; Sun et al., 2022). *Nitrospira* seems to be monophyletic at the phyloevolutionary level, but it consists of six genetic sub-lineages according to the *16S rRNA* gene and is the most diversified genus among the known NOB (Lebedeva et al., 2010; Daims et al., 2016). Interestingly, these sub-lineages do not seem to be uniformly distributed in nature but show pronounced habitat specificity (Daims et al., 2016). Nevertheless, all lineages of *Nitrospira* could not be amplified by the "universal" primers of the *16S rRNA* gene (Hong et al., 2020). The phylogenetic features and diversities of functional communities may be investigated by the functional genes as suitable molecular signatures, which code the pivotal units for enzyme catalytic reactions (Sun et al., 2022).

The *nxr*B functional gene of *Nitrospira* is considered to be an appropriate molecular signature for the exploration of *Nitrospira* ecology (Pester et al., 2014). A new *nxr*B primer set (Nxr-f27/Nxr-r617) and analysis method have been designed and optimized to detect the diversity of *Nitrospira* (Hong et al., 2020). In the present study, the new method was used to explore the diversity and distribution of *Nitrospira* across the physiochemical gradients from shallow to deep areas in the SCS. Seven novel *Nitrospira* clusters (U1–U7), which took up 70% of the total sequences, dominated the water columns of the SCS, which implied a previously unrecognized diversity of marine *Nitrospira*. Phylogenetic analysis of *Nitrospira* in Zhanjiang Bay showed a similar result: 74% of the 58 detected OTUs were not affiliated with any previously described

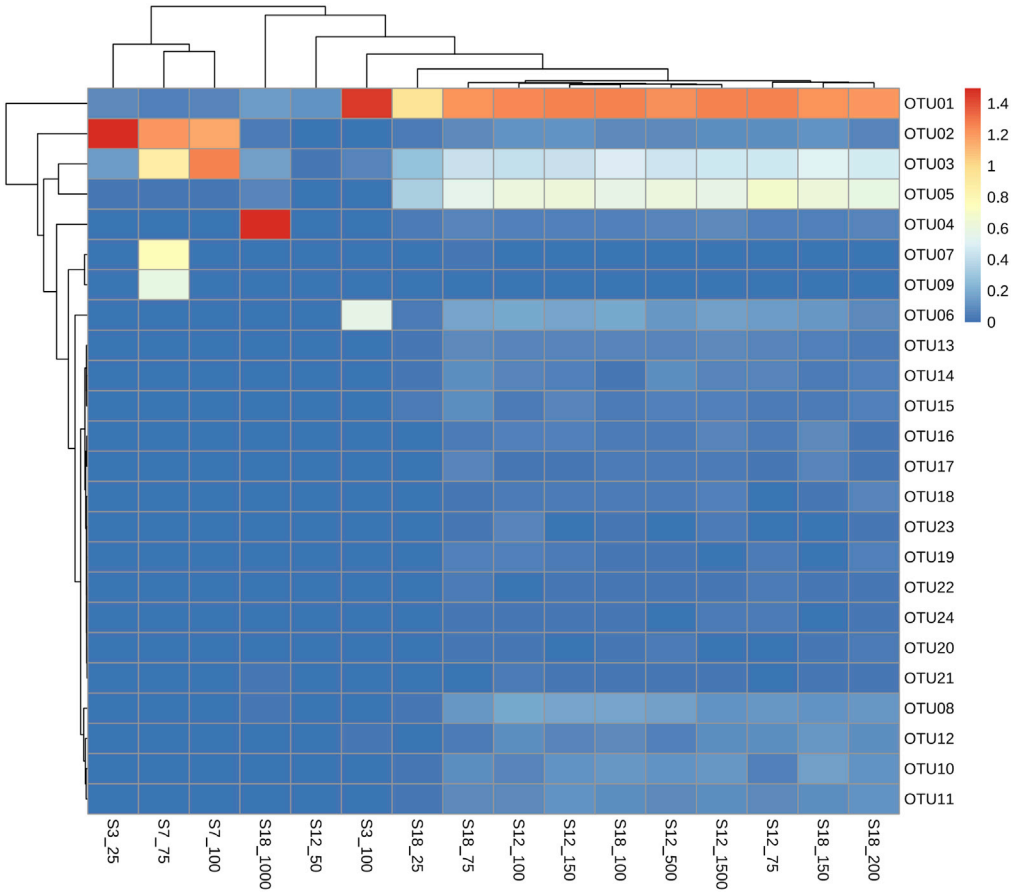


**Figure 5. Heatmap of *Nitrospira* in the water body of the South China Sea based on dominant OTUs (relative abundances >0.01%).**

*Nitrospira* species (Mao et al., 2020). These data further sustain the concept that *Nitrospira* distributes widely in nature as the most diverse NOB genus (Mao et al., 2020). In addition, this investigation of the community structure of *Nitrospira* in the SCS revealed distinct differences (70% sequences unclassified and dominated in the SCS) from those of the known non-marine groups. This discovery is consistent with the distribution of nitrite-dependent anaerobic methane oxidation (n-damo) bacteria in the SCS reported by Chen et al., which suggested that the community compositions of marine n-damo bacteria were distinct from that of the non-marine groups (Chen et al., 2014, 2015). The primers selected in this study were developed from limited *Nitrospira* sequences in enriched, pure cultured, and environmental samples, which might result in underestimating the diversity of *Nitrospira* in marine ecosystems. These results suggest that the unique niche specificity of functional bacteria should be ubiquitous in the marine ecosystem and implies their potential important ecological roles in marine N or C cycling.

The alpha diversity indexes were higher in the deep sea habitat (S12 and S18) than in the shallow ocean habitat (S3 and S7) in the SCS. Among the five known *Nitrospira* genera distributed in the SCS water samples, *Ca. N. lenta* and *Ca. N. defluvii*, belonging to the *Nitrospira* sub-lineages II and I, respectively, dominated in the shallow ocean habitat and were ubiquitous in all samples. Similar results with *Ca. N. lenta* and *Ca. N. defluvii* as the dominant *Nitrospira* species were also found in water columns of the Pearl River Estuary (PRE) (Hong et al., 2021). The SCS shallow habitat is located close to the PRE. Considering the huge flux of the PRE into the SCS, these dominant groups in the SCS water column might derive from the water mixture carried by hydrodynamic force from the PRE.

On the other hand, although *Ca. N. lenta* and *Ca. N. defluvii* were typically found and primarily resided in active sludge of wastewater treatment systems (Boris et al., 2015; Lücker et al., 2010), they were generally

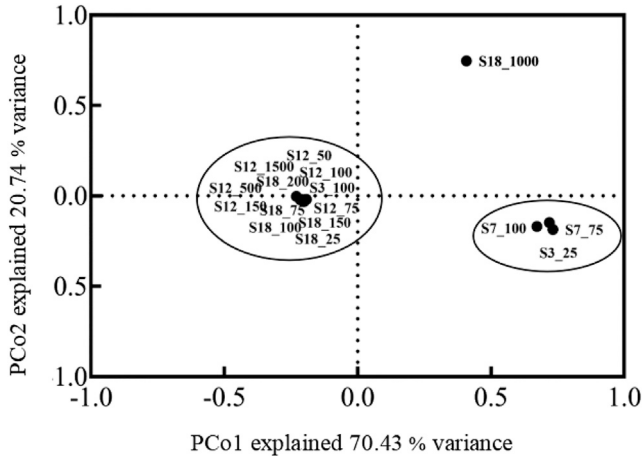


**Figure 6. Principal coordinates analyses *(PCoA)* of *Nitrospira* based on the *nxr*B gene retrieved from the water columns in the South China Sea (SCS)**

Horizontal and vertical axes represent the first two dimensions, PCo1 and PCo2, respectively.

present in many natural environments, including rivers, universal estuaries, subterranean estuaries (STE), and tropical bays (Cébron and Garnier, 2005; Hou et al., 2018; Mao et al., 2020; Hong et al., 2021; Sun et al., 2022). *Ca. N. lenta*, like other members of sublineage II, showed a lower maximum activity (Nowka et al., 2015; Ushiki et al., 2017) and might be outcompeted by sublineage I *Nitrospira* at higher nitrite concentrations (Maixner et al., 2006). If so, the higher relative abundances of *Ca. N. defluvii* at S7 than at S3 could be explained because the nitrite concentration at S7 was five times higher than that at S3. Despite *Nitrospira* species belonging to sub-lineages I and II displaying substantial genomic similarities, most of the *Ca. N. lenta* unique genes lacked any functional prediction (Sakoula et al., 2018). Moreover, according to the first decoded *Nitrospira* genome combined with experimental data, *Ca. N. defluvii* presents obvious discrepancies from other known NOB in many aspects such as NXR, the respiratory chain composition, and the evolution of chemolithoautotrophic nitrite oxidation (Lücker et al., 2010). Thus, *Nitrospira* species affiliated with sub-lineages I and II exhibit ecophysiological differentiation and a clear niche distribution (Lücker et al., 2010; Sakoula et al., 2018).

The OTUs affiliated with unclassified U1 accounted for 49.12% of the total sequences and showed relatively high similarity (88.36%–90.00%) with *N. moscoviensis*. *N. moscoviensis* also represented the same ubiquitous *Nitrospira* sub-lineage II as *Ca. N. lenta*. Surprisingly, the ecophysiology of *N. moscoviensis* suggests a wide distribution of reciprocal feeding interactions between nitrifiers (Koch et al., 2015). The key *Nitrospira* in many ecosystems could convert urea to ammonia and $CO_2$ (Koch et al., 2015). Therefore, urealysis enables *Nitrospira* to provide ammonia to ammonia oxidizers lacking urease and obtain nitrite generated by ammonia oxidation in reward (Koch et al., 2015). The OTUs affiliated to unclassified U3 accounted for 8.08% of the total sequences and showed especially high abundance (96.02%) at S18_1000 and affiliated closely with *N. japonica* with 90.44% similarity. *N. japonica* also belongs to *Nitrospira* sub-lineage II. A comparison of the *N. japonica* 16S rRNA gene sequence showed a higher similarity to *N. moscoviensis* than to *Ca. N. defluvii*, whereas the physiological properties of *N. japonica* were more similar to *Ca. N. defluvii* than to *N. moscoviensis* in some respects, such as the utilization of organic substrate (Ushiki et al., 2013), posing a difficulty in connecting the phylogenetic similarity with physiological traits (Ushiki et al., 2013). Therefore, the unclassified *Nitrospira* species in SCS hint that *Nitrospira* should be unexpectedly diversified and flexible in metabolic activity, which contributes greatly to the N cycle besides nitrite oxidation.

*N. marina* may be one of the most prevalent NOB species in marine environments (Watson et al., 1986). In recent years, *N. marina* was reported to be widely distributed in waters of the PRE, sediments of Daya Bay (STE), and intertidal zones of Zhangjiang Bay with many mangrove plants (Hou et al., 2018; Mao et al., 2020; Hong et al., 2021; Sun et al., 2022). The organic matter in STE and Zhanjiang Bay and strong seawater intrusion in the PRE may properly illuminate the wide or dominant distribution of *N. marina* in these environments (Hou et al., 2018; Mao et al., 2020; Hong et al., 2021; Sun et al., 2022). Nevertheless, *N. marina*-related species accounted for only 1.39% of *nxr*B sequences in the water columns of the SCS in our study. Although

B vitamins have an important role in cellular metabolism, *N. marina* lacks a complete vitamin $B_{12}$ ($VB_{12}$) biosynthesis pathway in contrast to many other bacteria (Shelton et al., 2019; Bayer et al., 2021) and obtains $VB_{12}$ from the salvage of multiple intermediates from the environment (Bayer et al., 2021). Moreover, the salvage acquisition mode is closely correlated with organic ligands ($B_{12}$ precursors or degradation products), which is critical for dissolved cobalt speciation (Saito et al., 2005; Bayer et al., 2021). Interestingly, organic cobalt complexes are rich in the North Atlantic ocean, where *N. marina* was isolated (Noble et al., 2017). However, the concentrations of B vitamins decrease through large tracts of the global ocean (Sañudo-Wilhelmy et al., 2014), and the waters of SCS lack organic matter and nitrogen nutrition to oligotrophic levels. Therefore, we speculated that the lack of $VB_{12}$ and salvage acquisition from organic ligands may be the reasons behind *N. marina* not being the dominant species in the water columns of the SCS.

The discovery of comammox *Nitrospira* with the capability for independently oxidizing ammonia to nitrate (Daims et al., 2015; van Kessel et al., 2015) dramatically challenged our previous perceptions about a two-step process of nitrification. Three comammox species, *Nitrospira inopinata*, *Candidatus Nitrospira nitrificans*, and *Candidatus Nitrospira nitrosa*, formed a branch (named clade A previously) in the phylogenetic tree targeting *nxr*B genes in the present study, which was consistent with the clustering result targeting the ammonia monooxygenase subunit A (*amo*A) gene of comammox (van Kessel et al., 2015; Daims et al., 2015; Xu et al., 2008). Moreover, *Ca. N. nitrosa* was detected in almost all SCS water samples (except S12_50 and S18_25) with low relative abundance, and OTU09 (the U5 cluster) grouped closely within comammox clade A. However, neither *16S rRNA* nor *nxr* genes appropriately distinguish comammox *Nitrospira* (Daims et al., 2016). This discrepancy might be related to the significantly high sequence depth conducted using high-throughput sequencing, high specificity with the optimized *nxr*B primes, and a suitable cutoff value (0.09) for *Nitrospira* species clustering, according to the evolutionary distance of *nxr*B (Hong et al., 2020). *Nxr* genes of comammox *Nitrospira* showed high similarity with the genes of some strictly nitrite-oxidizing *Nitrospira* (Daims et al., 2016). The use of constantly evolving *nxr*B as a genetic marker may open new perspectives on detecting the unknown comammox *Nitrospira* as *amo*A for studying ammonia oxidizers (Pester et al., 2014). Intriguingly, the genomic analysis of all distinguished comammox comprises the *amo*A gene (Xu et al., 2008), so the possibly comprehensive distribution and unrevealed diversity of comammox in the marine ecosystem may be revealed by analyzing the specific *amo*A gene for comammox in future studies. Furthermore, the possible existence of comammox *Nitrospira* may be firstly reported in the SCS water columns, as so far comammox *Nitrospira* seems to be undiscovered or nonexistent in marine systems (Daims et al., 2015; Sun et al., 2021). Many studies indicated that comammox *Nitrospira* may be available for nitrification in aquatic or engineered systems that feature comparatively low ammonia concentrations (Bartelme et al., 2017; Fujitani et al., 2020; Pjevac et al., 2017; Xu et al., 2008; Li et al., 2020). Moreover, the kinetic analysis revealed that comammox *Nitrospira* adapts to hyper-oligotrophic nutrition conditions because of its high affinity to ammonia (Kits et al., 2017). It can be inferred that the existence of comammox *Nitrospira* in oligotrophic sea water is reasonable, but their ecological characteristics in marine ecosystems remain mostly undiscovered, which compels a reevaluation of the various ammonia-oxidizing communities to understand their relative contributions to marine nitrification.

Accordingly, the *Nitrospira* genome provides significant insights for understanding the genomic diversities and physiology of various *Nitrospira* species (Koch et al., 2015; Sakoula et al., 2018; Lücker et al., 2010), which may help understand their broad adaption in various natural habitats and ecological niche differentiation. Moreover, *Nitrospira* has versatile ecological functions that enable it to participate in N-cycle transformation or autotrophic carbon fixation besides nitrification (Koch et al., 2015; Lücker et al., 2010; Li et al., 2020). The important ecological roles within a large group of unclassified and ubiquitous *Nitrospira* in marine ecosystems need to be investigated.

## Environment factors shaping the versatile diversity and distribution of *Nitrospira* in the SCS

The community function is closely related to microbial composition, but environmental changes affect the fluctuation and abundance of the constituent microbes (Sato et al., 2019). Likewise, complex interactions of environmental parameters influence the niche differentiation of NOB and must be evaluated for single species (Wegen et al., 2019). Environmental nitrogen mainly influences the composition of *Nitrospira* (Hong et al., 2021). Comparatively high nitrate concentrations throughout the deep water may be related to the material metabolism and vertical transport process of the euphotic layer. Nitrate, as the dominantly bioavailable nitrogen, could be rapidly assimilated by phytoplankton in surface waters and then accumulate in the deep sea (Gruber, 2018). The presence of nitrate was identified as the one of most important

factors affecting *Nitrospira* distribution in our present analysis. OTU04 was closely grouped with *N. japonica* and correlated positively with nitrate concentration. Compared with ammonia and nitrite, nitrate has been traditionally assumed to be less toxic to cultivated organisms (Camargo et al., 2005). Nitrate toxicity to aquatic organisms rises with growing nitrate contents and exposure duration (Tsai and Chen, 2002; Alonso and Camargo, 2003). On the other hand, nitrate toxicity reduces with water salinity and growing body size (Tsai and Chen, 2002). The shape and arrangement of *Ca. N. defluvii* and *N. japonica* changed with nitrate accumulation and increasing cell density after prolonged incubation (Spieck et al., 2006; Ushiki et al., 2013) and resembled the irregular appearance of *N. moscoviensis* (Ehrich et al., 1995; Spieck et al., 2006). The effects of nitrate on lineage II *Ca. N. lenta* BS10 and lineage I *N. defluvii* were detected at the inhibiting nitrate concentrations of 18 and 25 mM, respectively (Boris et al., 2015). The impacts of nitrate were also evaluated on lineage II *N. moscoviensis* and marine lineage IV *Nitrospira Ecomares* 2.1, which were inhibited by 75 and 80 mM nitrate levels, respectively (Ehrich et al., 1995; Keuter et al., 2011). In contrast, the nitrate concentration in this study increased from the ocean surface to the bottom and ranged from 0.03 to 40 mM, lower than the inhibiting nitrate concentrations reported by Ehrich et al. (1995) and Keuter et al. (2011). This might well explain the strong connection between *Nitrospira* species and nitrates in the SCS water samples. As different *Nitrospira* species showed differentiated adaptations and variations toward nitrate, it cannot be excluded that other new or unclassified *Nitrospira* species may exhibit similar or even higher tolerances to nitrate concentrations in the marine ecosystem.

Besides different adaptations to nitrogen species by *Nitrospira*, the temperature is also identified as the main driver for niche differentiation and community composition of NOB (Wegen et al., 2019; Nowka et al., 2015; Alawi et al., 2009). So far, the optimal growth temperature was reported as 28°C–32°C for most of the *Nitrospira* strains identified from activated sludge (Spieck and Lipski 2011; Ushiki et al., 2013; Fujitani et al., 2014; Nowka et al., 2015; Wegen et al., 2019), except the sublineage II member *N. moscoviensis* at 39°C (Ehrich et al., 1995). However, a few NOB also exist in cold environmental conditions such as in permafrost-affected soils (Wagner et al., 2002; Alawi et al., 2007), and the sublineage II member *Ca. N. lenta* could grow at 10°C together with *Nitrotoga* (Nowka et al., 2015). Moreover, low temperature enhanced the diversity of *Nitrospira* spp. in WWTPs (Siripong and Rittmann, 2007; Kruse et al., 2013). According to experiments in marine systems, the genus *Nitrospira* differed in its temperature preferences with adaption to low temperatures between 10°C and 17°C (Kruse et al., 2013; Alawi et al., 2007). In contrast, the temperature in our study showed a wide range, from 2.8°C to 29.3°C, with a decreasing trend from surface to bottom in the SCS water columns, which might illuminate the ubiquitous spread of versatile *Nitrospira* with adaption to various temperatures of the SCS. Unexpectedly, the relative abundance of OTU04 in our study predominated at S18_1000 with an extremely low temperature of about 4°C, and the relative abundance correlated negatively with temperature, which implied that new unknown *Nitrospira* species tolerant to low temperature might exist in the SCS.

Dissolved oxygen (DO) may play an important role in inhibiting or enhancing *Nitrospira* activity (Mehrani et al., 2020) and thus affect the niche distribution of *Nitrospira* in ecosystems (Hong et al., 2021). *Nitrospira* was divided into low-DO and high-DO species, which were determined by an increased abundance of low-DO *Nitrospira* at an extremely low DO content (0.13 mg/L) and a remarkably increased abundance of high-DO *Nitrospira* at a high DO content (8.7 mg/L) in a bioreactor (Fitzgerald et al., 2015). This suggests that versatile *Nitrospira* adapts to various DO concentrations over a broad range. *Ca. N. lenta* tolerates high DO, whereas *Ca. N. defluvii* might be suppressed by enhanced DO in the PRE water samples (Hong et al., 2021). The abundance of comammox *Nitrospira* was negatively influenced by DO in the Yangtze River (Liu et al., 2020b). Our study showed that DO decreased with temperature and depth in the SCS water column. Various unclassified *Nitrospira* species were widely distributed in different water depths in the deep sea (U1, U2, and U4). Unexpectedly, the relative abundance of OTU4 was highest in the extremely low DO of the deep water at S18 and negatively correlated with DO. The unique genome characteristics of *Nitrospira* explain its good adaption to hypoxic environments and evolution from micro-aerobic or even anaerobic ancestors (Lücker et al., 2010). Therefore, new species of *Nitrospira*, adaptable to aerobic conditions or resistant to micro-aerophilic environments, might exist in the SCS water column. The challenging vertically variable conditions of marine ecosystems require a flexible response from *Nitrospira*, which can variously adapt to decreasing temperature and DO along with increasing water depth. Our results allowed an estimation of the presence and the previously unknown metabolic features of versatile *Nitrospira* ecotypes in marine ecosystems.

In addition to the physicochemical environmental parameters, water pressure and density associated with water dynamics may also be responsible for the niche distribution of the *Nitrospira*. Our results suggested that OTU02 (*Ca. N. lenta*) may be inhibited by increasing water pressure, whereas the OTU numbers and unclassified OTU05 species may be enhanced by increasing water density. However, the effects of water pressure and density on the niche distribution of *Nitrospira* have not been mentioned in previous studies. The complicated bathymetry is crucial not only for water exchange but also for regulating diverse transportation across the main passages in the SCS (Daryabor et al., 2016). The mixed layer depth (MLD) varies in different months but stabilizes throughout the summer (Duan et al., 2012). We collected water samples in summer when the MLD was stable. The rapid increase of water density with depth may be due to temperature variations and the formation of a sharp density interface located between the upper thermocline and the mixed layer (Fan et al., 2015). Therefore, complex hydrodynamic change may be a potential factor explaining the *Nitrospira* niche distributions with a close relationship with water pressure and density in the SCS water column. Exploring the effects associated with hydrodynamic change on the *Nitrospira* species is warranted in a follow-up study.

In conclusion, we explored the diversity, distribution, and community composition of *Nitrospira* using high-throughput sequencing of the *nxr*B gene. We also analyzed their relationship with environmental factors in the SCS water column. The community structure of *Nitrospira* varied markedly from shallow to deep sea and exhibited a remarkably spatial shift. We revealed the previously unrecognized diversity of marine *Nitrospira* and a few novel unclassified *Nitrospira* species that were widely distributed in the SCS water column. Although five known *Nitrospira* genera were detected, *N. marina* was not the dominant species, whereas *Ca. N. lenta* and *Ca. N. defluvii* dominated in the shallow ocean habitat and were ubiquitous in all samples. Moreover, the comammox *Nitrospira*, so far reported to be absent in marine systems, was first detected in the SCS water column in the present study. The physiological characteristics of various *Nitrospira* may allow it to adapt and respond to the physicochemical indexes in the SCS. Statistical analyses revealed that nitrate, temperature, and DO changes with water depth were the key factors controlling the diversity and niche distribution of *Nitrospira* in the SCS. Moreover, the water pressure and density associated with the ocean hydrodynamics should not be ignored, and their effects on the niche differentiation of *Nitrospira* need to be further explored. These findings expand our current knowledge about the niche differentiation of *Nitrospira* in the marine ecosystem and its unexpected physiological characteristics.

### Limitations of the study

In the present study, the largely previously unrecognized *Nitrospira* species imply their potentially important function of nitrite oxidation and physiologic metabolism in the SCS. The results of RT-PCR based on *nxr*B gene showed that the abundance of *Nitrospira nxr*B gene was relatively low in the water columns of the SCS. Thus, the quantitative data are not discussed in this study. The rate and flux of nitrite oxidation for *Nitrospira* are also still unknown. The relative abundance of *Nitrospira* and rates of nitrite oxidation will be conducted in the future research.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104895.

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## AUTHOR CONTRIBUTIONS

Y.H.: Conceptualization; Data curation; Funding acquisition. W.S.: Conceptualization; Writing—Original Draft; Writing—Review and Editing; Funding acquisition. L.J.: Methodology: Formal Analysis; Writing—Original Draft; J.W.: Writing—Review & Editing. J.Y.: Formal Analysis. M.W.: Writing—Review & Editing.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Critical commercial assays | | |
| Power Water DNA Isolation Kit | QIAGEN | Cat.14900-100-NF |
| GoTaq Green Master Mix | Promega | M7122 |
| GoTaq qPCR Master Mix | Promega | A6001 |
| VAHTS Universal Pro DNA Library Prep Kit | VAZYME | ND604 |
| Deposited data | | |
| The raw reads of nitrite oxidoreductase (nxrB)gene sequences of Nitrospira | This paper | Bioproject: CRA005918 |
| Software and algorithms | | |
| Mothur 1.40.5 | Schloss et al. (2009) | https://mothur.org/ |
| MEGA 7.0 | Kumar et al. (2016) | https://www.megasoftware.net/ |
| EvolView | He et al. (2016) | https://www.evolgenius.info/evolview/#/ |
| SPSS Statistics 22.0 | https://www.ibm.com/products/spss-statistics | https://www.ibm.com/products/spss-statistics |
| R 4.1.0 | https://www.r-project.org/ | https://www.r-project.org/ |
| Canoco 5.0 | Jiangshan (2014) | http://www.canoco5.com/ |
| Ocean data view 5.4.0 | https://odv.awi.de | https://odv.awi.de |
| Sigmaplot 12.5 | https://systatsoftware.com/sigmaplot/ | https://systatsoftware.com/sigmaplot/ |
| Other | | |
| Seabird conductivity–temperature–depth sensors | Sea-Bird Electronics | https://www.seabird.com/sbe-911plus-ctd/product?id=60761421595 |
| 0.22-μm pore-size polypropylene filters | Millipore | https://www.sigmaaldrich.com/US/en/substance/fluoroporemembranefilter1234598765 |
| NanoDrop Lite | Thermo Fisher Scientific | https://www.thermofisher.com/order/catalog/product/ND-LITE-PR |
| Bio-Rad iQ5 Real-Time PCR system | Bio-Rad | https://www.bio-rad.com/ |
| Veriti 96-Well PCR Thermal cycler | Applied Biosystems | https://www.thermofisher.com/order/catalog/product/4452300 |
| Illumina sequencing | Illumina | Illumina MiSeq |

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Yiguo Hong (yghong@gzhu.edu.cn).

#### Material availability

This study did not generate new unique reagents.

#### Data and code availability

- The raw reads of *nxr*B gene sequences of *Nitrospira* acquired in the SCS have been submitted to GSA DATA ADMIN and are publicly available as of the date of publication. The accession number is listed in the Key resources table.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## METHOD DETAILS

### Sample collections

A section in the middle and northern SCS was selected for sample collection. A total of 30 seawater samples were collected from different water depths at four stations. The specific sampling locations were S3 and S7 from the shallow sea close to the continental margin and S12 and S18 in the deep sea, as shown in Figure 1. Water samples were collected using the12 5-L Niskin water bottle with Seabird SBE-911 plus conductivity-temperature-depth sensors (CTD). At each site, two 5-L water samples were collected from different water depths. A total of 29 water samples were collected and analyzed for environmental parameters. The surface and bottom water were categorized as depths <200 and >200 cm, respectively. At S3, water samples were collected at every 25-cm depth from 25 to 100 cm and then labeled according to the sampling sites and water depths (S3_25 to S3_100). At S7, water samples were collected at every 25, 50, or 100-cm depth from 25 to 400 cm and then labeled using the same naming policy (S7_25 to S7_400). At S12 and S18, water samples were collected at every 25, 50, or 100-cm depth from 25 to 1500 cm and labeled as previously described.

Microorganisms were collected by filtering 1–2 L water samples through 0.22 μm polycarbonate membranes (47 mm, Millipore, Billerica, MA, USA). The filtered membrane was put into a cryopreservation tube, frozen in liquid nitrogen, and stored at −80°C for total DNA extraction. The filtered seawater was put into PETG plastic bottles (fully washed before use) and stored at −20°C for environmental parameter determination.

### Determination of environmental parameters

The temperature, salinity, pressure, and depth of seawater samples were measured *in situ* by CTD. Water samples for the determination of dissolved oxygen (DO) were collected, and DO was determined using Winkler iodometric titration. Custom-made brown ground glass sampling bottles were used. The sampling tubes were extended into the bottom of the sample bottle and made the water sample overflow more than three times the volume of the sampling bottle. The sampling tubes were pulled out slowly and manganese chloride and alkaline potassium iodide solution were immediately added to the bottles, which were then plugged, sealed, and transferred to the laboratory on board for the determination of DO. The contents of ammonium ($NH_4^+$), nitrite ($NO_2^-$), and nitrate ($NO_3^-$) were detected using a rapid spectrophotometry method optimized at our laboratory (Wu et al., 2016; Guan et al., 2017).

### DNA extraction

At an ultra-clean work table, the filter membranes enriched with water microorganisms were cut up with sterile scissors, and the metagenomic DNA of water microorganisms was extracted according to the operation manual of the water sample DNA Extraction Kit (PowerWater DNA Isolation Kit, MoBio, Carlsbad, CA, USA). The extracted DNAs were detected for quality using a Nanodrop Lite spectrophotometer (Thermo Scientific, Wilmington, ME, USA) and then stored at −20°C until further use.

### PCR amplification and high throughput sequencing

The primer pair Nxr f27/Nxr r617, which was proven to be effective for detecting the community composition of *Nitrospira* (Hong et al., 2020), was selected for amplifying the *Nitrospira nxr*B gene. Because shallow amplification bands of the *Nitrospira nxr*B gene were shown in most of the samples from the SCS, the PCR procedures for the samples were optimized using a step-by-step PCR process. The PCR system consisted of 12.5 μL gotaq green Master Mix (Promega, America), 1.0 μL primer containing barcode in the front primer, 1.0 μL DNA sample, and 10.5 μL nucleic acid-free water. The front primers with different barcodes (8 bp barcode + front primers) were used to distinguish the amplified DNA fragments from different samples. The PCR reaction condition of the first step was conducted as reported in the previous reports (Hong et al., 2020). The PCR system and condition of the second step were the same as the first step, except that the DNA template was replaced by the reaction product of the first step. PCR products from different water samples were purified using the agarose gel DNA kit (Takara, Dalian, China) and then mixed. Finally, the purified PCR products were sequenced on the Illumina HiSeq platform for high-throughput sequencing. The *nxr*B gene of *Nitrospira* could not be amplified by PCR in all water samples, or the effective sequences

in some samples were too low to be analyzed after the high throughput sequencing. Thus, a total of 16 water samples were input to the diversity analysis of *Nitrospira* in the SCS.

### Processing and analysis of high throughput sequencing data

Mothur software (v.1.40.1, https://www.mothur.org/wiki/) was used to analyze *Nitrospira* functional gene *nxr*B sequences using standard operating procedures (Schloss, 2020; Hong et al., 2020). The effective sequences acquired by de-barcoding, de-noising, and trimming were contrasted with the previously constructed reference database (Hong et al., 2020). The operational taxonomic units (OTUs) were separated by the 91% cut-off threshold value (Hong et al., 2020). The alpha diversity-related parameters were obtained from the files generated by Mothur after the removal of rare OTUs (sequence number <0.1‰ of total sequence number). The sampling depth was verified by the Shannon-Wiener curve drawn using OriginPro 2017. Distances between samples were obtained by using the "*dist.shared*" command and used for Principal coordinate analysis (PCoA).

### Phylogenetic analysis

The dominant OTUs were defined according to representative sequences of OTUs covering more than 0.1% of total sequences in each community (24 dominant OTUs, covering 96% of the total sequences). The representative sequences belonging to the dominant OTUs and a seed database were integrated into a FASTA file, and phylogenetic analysis was conducted using bootstrap analysis with 1000 replicates in MEGA7 (Kumar et al., 2016). A heatmap was generated using the software GraphPad Prism 7 (https://www.graphpad.com/scientific-software/prism/) to exhibit the spatial distribution of *Nitrospira* bacteria, based on the relative abundances of dominant OTUs in every sample.

### Accession number of Nucleotide sequences

The *nxr*B gene sequences of *Nitrospira* acquired in the SCS have been submitted to GSA DATA ADMIN, and the accession number CRA005918 was obtained.

### Statistical analysis

The main environmental factors affecting the distribution of *Nitrospira* community structure were analyzed using redundancy analysis (RDA) by importing two excel sheets (with environmental parameters and relative abundance distribution information of dominant OTUs) into Canoco5 (ter Braak and Šmilauer, 2018). The correlations between environmental factors and the distribution of dominant OTUs abundance were analyzed by Pearson correlation analysis using the SPSS statistics software (version 22.0). One-way ANOVA was used to detect significant differences between environmental parameters in different samples by employing SPSS (version 22.0). Multi-response permutation procedure (MRPP) analysis with permutation 999 was used to test differences among the community compositions based on the *Nitrospira nxr*B gene using the R data package Vegan (Zimmerman et al., 1985).