

Community assembly of comammox *Nitrospira* in coastal wetlands across southeastern China

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ABSTRACT Complete ammonia oxidizers (comammox *Nitrospira*) are ubiquitous in coastal wetland sediments and play an important role in nitrification. Our study examined the impact of habitat modifications on comammox *Nitrospira* communities in coastal wetland sediments across tropical and subtropical regions of southeastern China. Samples were collected from 21 coastal wetlands in five provinces where native mudflats were invaded by *Spartina alterniflora* and subsequently converted to aquaculture ponds. The results showed that comammox *Nitrospira* abundances were mainly influenced by sediment grain size rather than by habitat modifications. Compared to *S. alterniflora* marshes and native mudflats, aquaculture pond sediments had lower comammox *Nitrospira* diversity, lower clade A.1 abundance, and higher clade A.2 abundance. Sulfate concentration was the most important factor controlling the diversity of comammox *Nitrospira*. The response of comammox *Nitrospira* community to habitat change varied significantly by location, and environmental variables accounted for only 11.2% of the variations in community structure across all sites. In all three habitat types, dispersal limitation largely controlled the comammox *Nitrospira* community assembly process, indicating the stochastic nature of these sediment communities in coastal wetlands.

IMPORTANCE Comammox *Nitrospira* have recently gained attention for their potential role in nitrification and nitrous oxide (N₂O) emissions in soil and sediment. However, their distribution and assembly in impacted coastal wetland are poorly understood, particularly on a large spatial scale. Our study provides novel evidence that the effects of habitat modification on comammox *Nitrospira* communities are dependent on the location of the wetland. We also found that the assembly of comammox *Nitrospira* communities in coastal wetlands was mainly governed by stochastic processes. Nevertheless, sediment grain size and sulfate concentration were identified as key variables affecting comammox *Nitrospira* abundance and diversity in coastal sediments. These findings are significant as they advance our understanding of the environmental adaptation of comammox *Nitrospira* and how future landscape modifications may impact their abundance and diversity in coastal wetlands.

KEYWORDS comammox *Nitrospira*, habitat change, aquaculture reclamation, coastal wetlands, nitrification

Nitrification was traditionally thought to be performed sequentially by two distinct groups of prokaryotes, ammonia oxidizers (AOA or AOB) and nitrite-oxidizing bacteria (NOB). However, this understanding has been revised due to the recent discovery of complete ammonia oxidizers (comammox *Nitrospira*), which can carry out the entire process of nitrification within a single cell (1, 2). Although attempts to isolate and cultivate comammox *Nitrospira* from field samples have had limited success due to their low growth rates and interference from other co-occurring nitrifiers, comammox *Nitrospira* were identified and classified into two monophyletic sister clades A and B (3), with clade A further divided into A.1, A.2, and A.3 using PCR assays of functional genes (4). Comammox *Nitrospira* have been detected in various habitats, including farmlands (5, 6), forests (7), wetlands (8, 9), riparian zones (10), and engineered systems (11, 12). While canonical AOB and AOA produce the greenhouse gas nitrous oxide (N₂O) as a byproduct (13), contributing to climate warming, comammox *Nitrospira* appear to have the necessary functional genes and enzymes to produce N₂O (14), but their overall contribution to N₂O emission remains uncertain (9, 14, 15). Coastal wetlands play a critical role in the global nitrogen cycle (16, 17), and are inhabited by a vast diversity of microorganisms, including comammox *Nitrospira* (18–20). It has been reported that comammox *Nitrospira* thrived in extremely high-salinity sediments (18) and harbored genes that enable them to adapt to low oxygen environment (21). Sun et al. (9) demonstrated that comammox *Nitrospira* played a key role in nitrification and N₂O production in a coastal wetland. However, coastal wetland ecosystems in China are experiencing increasing impact from invasive species and land-use changes, which can modify physicochemical conditions (22, 23) and affect comammox *Nitrospira* in sediment. For example, in the subtropical Shanyutan Wetland, tidal mudflat areas dominated by different native and invasive plant species exhibit distinct comammox *Nitrospira* abundances and diversity (24). Both clades A and B of comammox *Nitrospira* are present in coastal tidal mudflats across the tropical-subtropical gradient in China (19), many of which have been invaded by *Spartina alterniflora* and some of the *S. alterniflora* marshes have been cleared to construct aquaculture ponds (25, 26). These habitat modifications have resulted in significant changes in sediment carbon content (27), carbon mineralization (27), and N₂O production potential (28), but their corresponding effects on comammox *Nitrospira* have not been thoroughly examined. Microbial community assembly can be influenced by deterministic or stochastic processes, and understanding their relative importance is fundamental to comprehending the characteristics and functions of the microbial ecosystem (29, 30). Previous research has investigated community assembly processes in various groups of microorganisms, including protists (31), fungi (32), bacteria (33), archaea (34), and

diazotrophs (35). Comammox *Nitrospira*, which has not yet been reported in marine ecosystems (14), is likely introduced into coastal wetlands from adjacent terrestrial or freshwater habitats through stochastic processes. However, a recent study demonstrated that deterministic processes were more important in influencing comammox *Nitrospira* community assembly in a riverine estuary due to the strong selection by environmental factors (36). Therefore, it remains an open question as to which process plays a more prominent role in the comammox *Nitrospira* community assembly in coastal wetlands, especially those impacted by plant invasion and land-use change. We collected sediment samples from 21 coastal wetland sites across southeastern China, including native mudflats (MFs), adjacent *S. alterniflora* marshes (SAs), and aquaculture ponds (APs), and analyzed their sediment properties and comammox *Nitrospira* communities. Our study aimed to (i) examine the effects of habitat modification on comammox *Nitrospira* communities in coastal wetlands across a large spatial scale; (ii) assess the critical factors that influence the abundance, diversity, and community composition of comammox *Nitrospira* in coastal wetlands; and (iii) explore the assembly mechanism of comammox *Nitrospira* communities in different habitats. We hypothesized that the invasion of mudflats by *S. alterniflora* would increase the abundance, and diversity of comammox *Nitrospira* due to the rhizospheric supply of oxygen and substrates, but the subsequent clearing of *S. alterniflora* marshes to create aquaculture ponds would reverse this trend.

RESULTS

Sediment physicochemical properties in different habitat types

Sediment samples were collected from 21 coastal wetlands in five provinces across subtropical and tropical climate zones (Fig. 1). Habitat modifications have a substantial impact on many sediment physicochemical properties in these coastal wetlands (Table S1). *S. alterniflora* invasion significantly increased soil organic carbon (SOC), $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and microbial biomass nitrogen (MBN) content, while the conversion of *S. alterniflora* marsh into aquaculture ponds significantly increased SO_4^{2-} content but reduced SOC, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and MBN content. The SO_4^{2-} concentration was 8.90 and 9.13 mg L^{-1} in mudflats and *S. alterniflora* marsh, respectively, while aquaculture ponds significantly increased the value to 17.5 mg L^{-1} . Interestingly, both *S. alterniflora* invasion and conversion of *S. alterniflora* marsh into aquaculture ponds did not affect pH, Cl^- , salinity, grain size (proportions of clay, silt and sand), C/N, or microbial biomass carbon (MBC).

Abundances of comammox *Nitrospira* in different habitat types

The abundance of comammox *Nitrospira* clade A was estimated to be 5.71×10^6 copies g^{-1} sediment in the mudflats (Fig. 2A). Neither *S. alterniflora* invasion nor subsequent conversion of *S. alterniflora* marsh into aquaculture ponds significantly altered the abundance of clade A. Random forest analysis revealed that the proportions of clay and silt particles in the sediments were the strongest predictors of clade A abundance across all three habitat types (Fig. 2B). Spearman correlation analysis showed a positive correlation between the abundance of clade A and the proportions of clay and silt particles, while sand particles exhibited a negative correlation (Fig. 2C). Additionally, clade A abundance was positively correlated with sediment water content and organic carbon content but negatively with bulk density (Fig. 2C).

Diversity and community structures of comammox *Nitrospira*

After quality filtering, 3,921,886 sequences were retrieved from 184 sediment samples (five samples were discarded due to negative PCR results), with 10,172–43,868 sequences per sample. The Shannon diversity, Simpson diversity, Chao1 richness, and Observed species of comammox *Nitrospira* were significantly lower in aquaculture ponds, with the average values of 1.32, 0.53, 116, and 62.6, respectively (Fig. 3A, C, E, and G), while no significant difference was found between mudflats and *S. alterniflora* marshes. The Shannon diversity, Simpson diversity, Chao1 richness, and Observed species of comammox *Nitrospira* were most strongly (Fig. 3B, D, F, and H) and negatively (Fig. S1) affected by SO_4^{2-} and salinity. Grain size (proportions of clay, silt, and sand), Cl^- , and C/N were also significant factors. Principal coordinate analysis (PCoA) indicated that the community structures of comammox *Nitrospira* in all three habitat types clustered together (Fig. 4A). However, permutational multivariate analysis of variance (PERMANOVA), analysis of similarities (ANOSIM), and multiple-response permutation procedure (MRPP) analyses showed that comammox *Nitrospira* community structure in aquaculture ponds significantly differed from both mudflats and *Spartina* marshes, whereas the latter two showed no significant differences (Table S1). Notably, sampling sites and their interactions with habitat change explained 33% and 24% of the variations in the community structure of comammox *Nitrospira*, respectively, while habitat change alone explained only 2% (Fig. 4A). Mantel test revealed that sediment water content, bulk density, SO_4^{2-} , Cl^- , salinity, silt and sand fractions, and C/N were critical factors influencing the community structure of comammox *Nitrospira* (Fig. 4; Fig. S2). Canonical correspondence analysis (CCA) showed that all the environmental factors together explained ~11% of the variation in comammox *Nitrospira* community structure among all samples (Fig. S2).

Phylogenetic analysis revealed that all comammox *Nitrospira* sequences belonged to clades A.1, A.2, or B but not clade A.3 (Fig. 5; Table S3). Generally, the relative abundance of clade A.1 was determined to be 81.1% and 78.5% in mudflats and *S. alterniflora* marshes, respectively, with no significant difference, but it was significantly reduced to 65.3% in aquaculture

ponds. In contrast, Clade A.2 OTU1524 and OTU26605 were notably more abundant in aquaculture ponds (Fig. 5). Overall, the invasion of mudflats by *S. alterniflora* did not significantly change the relative abundances of clades A.1, A.2, and B. However, subsequent conversion to aquaculture ponds significantly decreased the relative abundance of clade A.1 and increased the relative abundances of clades A.2 and B (Fig. 6).

Assembly processes of comammox *Nitrospira* community

Our results revealed that dispersal limitation, homogenous selection, and heterogeneous selection were the key processes driving the community assembly of comammox *Nitrospira* across all three habitat types (Fig. 7). Dispersal limitation played a dominant role, accounting for >60% of the relative contributions, while homogenous selection and heterogeneous selection contributed 19%–27% and 11%–13% of the relative contributions, respectively. The bootstrapping test results indicated that habitat modification did not significantly alter the relative importance of these different processes in comammox *Nitrospira* community assembly.

DISCUSSION

Transformation of coastal wetlands due to invasive plants and aquaculture may drastically alter sediment properties and comammox *Nitrospira* communities, but studies in the literature have yielded conflicting results. For example, Lin et al. (24) reported a significant shift in comammox *Nitrospira* community structure between mudflats and *S. alterniflora* marshes in the Min River estuary, whereas Liu et al. (18) found a very similar comammox *Nitrospira* community structure between these two habitats in the Zhang River estuary. These discrepancies suggest that the response of comammox *Nitrospira* communities to habitat change may vary depending on location. In our study, by analyzing samples from three habitat types (mudflats, *S. alterniflora* marshes, and aquaculture ponds) in 21 wetland sites across a broad geographical range, we found that the comammox *Nitrospira* community structure was more influenced by wetland locations and their interactions with landscape modification than by landscape change alone (Fig. 4A).

Our data showed that the invasion of mudflats by *S. alterniflora* increased SOC, ammonium, and nitrate contents of the sediment but had minor effects on comammox *Nitrospira* diversity (Fig. 3B) or community structure (Fig. 4B). These findings are consistent with previous observations in an estuarine wetland (24). In contrast, the conversion of *S. alterniflora* marshes into aquaculture ponds reduced comammox *Nitrospira* diversity, likely due to the elevated SO_4^{2-} concentration in the pond sediments (Table S1). This finding is surprising since microbial diversity was found to be higher in aquaculture ponds than natural wetlands (37) and comammox *Nitrospira* species were firstly enriched and characterized in aquaculture systems (2). It might be possibly due to that the continuous flooding in aquaculture ponds reduced the oxygen penetration into sediments (22), making the conditions more unfavorable for some comammox *Nitrospira* species than other microorganisms. Furthermore, some members of comammox *Nitrospira* are sensitive to sulfate (38), and this was supported by our Random Forest (Fig. 3) and Spearman's correlation (Fig. S1) analyses. Thus, sulfate concentration might be a good indicator to predict the diversity of comammox *Nitrospira* in coastal wetlands during habitat modification in future.

Soil texture plays a critical role in determining soil microbial community structure (39, 40). However, the importance of sediment grain sizes has been often overlooked in previous studies of comammox *Nitrospira* (20, 41, 42). Sessitsch et al. (43) suggested that finer particles were associated with greater bacterial diversity due to higher nutrient availability and better protection from predators. Similarly, Boey et al. (44) reported that the transcripts of AOA and AOB were more abundant in muds than in sands in an estuarine wetland. Consistent with these findings, our data demonstrated a strong and positive correlation between the abundance of comammox *Nitrospira* clade A with the proportions of clay and silt particles, but a negative correlation with sand content (Fig. 2C). Since the sediment grain size distribution did not differ significantly between habitat types (Table S1), the overall abundance of comammox *Nitrospira* clade A did not vary (Fig. 2A). Nevertheless, we did observe some differences when we examined the subdivisions of the clade.

Although there was no significant difference in the relative abundances of clades A.1, A.2, and B between mudflats and *S. alterniflora*, conversion of *S. alterniflora* marshes to aquaculture ponds decreased the relative abundance of clade A.1 and increased that of clades A.2 and B (Fig. 6). Previous studies have shown that clade A.1 is often dominant in coastal wetlands (45, 46), whereas clade A.2 is more abundant in agricultural soils (4, 47). Our findings support these results and suggest that natural substrates may be more favorable for clade A.1, whereas cultivated substrates in farms and aquaculture ponds may be more favorable for clades A.2 and B. Nevertheless, clades A.1 and A.2 were highly diverse containing many different operational taxonomic units (OTUs), indicating that each clade may respond to habitat change differently (Fig. 5). Since clade A.1 was found to be the most active comammox *Nitrospira* communities in nitrification in coastal wetlands (9), the lower relative abundance of clade A.1 and diversity of comammox *Nitrospira* in aquaculture ponds might result in a lower potential activity of comammox *Nitrospira* communities. However, direct evidence was needed to verify this in future study.

Despite the broad range of geographical conditions among the 21 wetland sites that we studied, environmental variables only explained 11.2% of the variations in comammox *Nitrospira* community structure (Fig. S2), and homogeneous and heterogeneous selections together contributed to only about one-third of the comammox *Nitrospira* community assembly process (Fig. 7). These findings seem to contrast with those of Liu et al. (36) who suggested that selection by local environmental factors was the most important in a riverine estuary, and environmental factors explained 60.2% of the comammox *Nitrospira* community variations in their study. Our results, on the other hand, showed that the assembly of the comammox *Nitrospira* community was predominantly shaped by dispersal limitation (Fig. 7). There is no known source of comammox *Nitrospira* from the sea (14). Meanwhile, unlike a river-dominated estuary, the wetlands that we studied had very restricted freshwater flow, which would greatly limit dispersal rate. This was even more evident in the case of the isolated aquaculture ponds, where the importance of dispersal limitation increased to nearly 70% in the community assembly process (Fig. 7). Furthermore, in tidally influenced wetlands, fluctuations in environmental conditions could induce stresses and frequently disrupt the microbial community structure (48, 49). Similarly, the common aquaculture practice of drying out the pond sediments and conditioning them with lime between farming seasons would significantly disrupt the sediment microbial community (50, 51), adding stochasticity to the community assembly process. In addition, we observed that habitat modification did not significantly influence the comammox *Nitrospira* community assembly processes. This is counter-intuitive since there are large ecological differences between the three habitats (52–54). This finding may be due to the fact that sampling sites explained more variance in comammox *Nitrospira* community structure than habitats, and the comammox *Nitrospira* community assembly in coastal wetlands is mainly governed by stochastic processes at a large spatial scale, which weakened the role of habitat modification in influencing community assembly.

In conclusion, our study found that comammox *Nitrospira* was present across the 21 coastal wetlands in the tropical and subtropical zones of China. By comparing samples from different habitat types, we demonstrated that habitat modification affected comammox *Nitrospira* diversity in the sediment but did not impact the abundance of comammox *Nitrospira* clade A. Despite the wide geographical range covered in our study, environmental variables explained only a small fraction of the variations in comammox *Nitrospira* community structure, of which the response to habitat modification appeared to be dependent on wetland location. Additionally, we found that the community assembly process was predominantly influenced by dispersal limitation. The location-dependent response to habitat change and the stochasticity of the community assembly process would make it difficult to predict how comammox *Nitrospira* community and activity change on different spatial and temporal scales without in situ measurements. Nevertheless, our study identified key variables, such as sediment grain size, salinity, and sulfate concentration, that affect comammox *Nitrospira* abundance and diversity, which can be used to make an initial assessment of how future landscape modification may impact comammox *Nitrospira* in coastal sediments.

MATERIALS AND METHODS

Study sites and sampling

Sediment samples were collected from 21 coastal wetland sites in southeastern China (20°42' N to 31°51' N; 109°11' E to 122°11' E) across the subtropical and tropical climate zones (Fig. 1). The latitude and longitude of each site can be found in Table S3. These sites were located in five provinces: two in Shanghai (SH), six in Zhejiang (ZJ), nine in Fujian (FJ), three in Guangdong (GD), and one in Guangxi (GX) (Fig. 1). The annual average temperature ranged from 11.0°C to 23.0°C and the annual average precipitation ranged from 100 to 220 cm across the five provinces. In 2014, the total area of coastal wetlands in these five provinces was approximately 2.58×10^6 ha, accounting for 44.5% of the total coastal wetlands in China (55). Some areas of the mudflats in these coastal wetlands have been converted into marshes by the invasive *S. alterniflora*, and parts of these marshes have been reclaimed and converted into aquaculture ponds. These aquaculture ponds were created about 12–16 years ago by complete removal of original marsh vegetations. The distance between sampled habitats at each site was generally <500 m. In total, *S. alterniflora* marshes cover 3.34×10^5 ha (56) or 61.2% of the total area of *S. alterniflora* marshes in China, whereas coastal aquaculture ponds cover 5.31×10^5 ha (57) or 36.9% of the total area of aquaculture ponds in China.

Surface sediments in the top 20 cm were collected with a steel corer (1.5 m long, 5 cm internal diameter). Sediment samples were collected in triplicates from all three habitat types at each site: native MF, SA, and AP, between December 2019 and January 2020, resulting in a total of 189 sediment samples. All sediment samples were transferred into zip-lock

bags and transported to the laboratory in a constant temperature box containing ice. The soil samples were divided into two parts. One part was stored at 4°C in the dark for no more than 2 weeks to determine soil physicochemical variables, while the other part was stored at -80°C for subsequent DNA extraction.

Physicochemical analysis

Plant residues and stones were removed from the sediment samples before analysis. Grain size was measured using a Master Sizer 2000 Laser Particle Size Analyzer (Malvern Scientific Instruments, Suffolk, UK). Sediment water content and bulk density were determined after drying fresh samples at 105°C for 48 hours (58, 59). Samples were diluted with deionized water in a 1:2.5 ratio to measure pH using an Orion 868 pH meter (USA). Salinity was measured by diluting samples in a 1:5 ratio and using a Eutech Instruments-Salt6 salinity meter (USA). Porewater NO₃-N and NH₄-N were extracted in 2 M KCl solution (60, 61) and measured using a flow injection analyzer (Skalar Analytical SAN++, Netherlands). Concentrations of SO₄²⁻ and Cl⁻ were measured following the methods of Chen and Sun (62) using an ion chromatograph (Dionex 2100, USA). Sediment MBN and MBC contents were determined using the fumigation-extraction method (63).

DNA extraction and quantitative PCR

DNA was extracted from 0.5 g of each freeze-dried sediment sample using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) following the manufacturers' protocols. The quality and quantity of DNA were determined by gel electrophoresis and a spectrophotometer (NanoDrop Technologies, Wilmington, USA). We measured the abundances of comammox *Nitrospira amoA* genes using quantitative PCR (qPCR) on a CFX384 Optical Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to Lin et al. (24).

We used the primer sets CA377f/C576r and CB377f/C576r to amplify the *amoA* genes of comammox *Nitrospira* clades A and B, respectively (64). The qPCR reaction mixture contained 5 µL SYBR qPCR mix, 0.2 µL of optimized concentration of forward and reverse primers, 1 µL of DNA template, and 3.6 µL of sterile distilled water. The thermal conditions were 95°C for the initial 3 minutes, followed by 38 cycles of 95°C for 30 seconds, 55°C for 25 seconds, and 72°C for 20 seconds. A tenfold serial dilution of plasmid DNA containing each target gene was used to generate standard curves. The amplification efficiency was 105.3%, and *R*₂ was 0.997 for clade A. However, clade B amplification was characterized by strong non-specific amplification despite efforts to optimize thermal conditions and change primer pairs. Consequently, data on clade B abundances were excluded from further analysis.

High-throughput amplicon sequencing and bioinformatics analysis

The amplification of comammox *Nitrospira amoA* genes was conducted using a nested PCR approach (65). Primer sets A189Y/C576r and CA209f/C576r were used for the first and second rounds of PCR amplifications, respectively. A unique sample identifying barcode was added to the CA209f primer for the second round. The resultant PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany), and libraries were constructed using a NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, USA). Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq platform.

The raw paired-end reads of each sample were merged using FLASH (Version 1.2.7) and quality controlled by QIIME (66), as previously described (67). Chimeric sequences were subsequently checked and removed using the UCHIME algorithm within the USEARCH package (68). Since there is no established species-level cut-off value for the *amoA* gene of comammox *Nitrospira* (69), high-quality sequences were grouped into OTUs at a 97% similarity level. Singleton and doubleton OTUs were eliminated to reduce the rate of spurious OTUs generated by PCR and sequencing errors (70). Representative sequence of each remaining OTU was subsequently selected and compared to NCBI database, and only those sequences that were affiliated with comammox *Nitrospira* were retained for downstream analysis. A neighbor-joining phylogenetic tree was constructed using representative sequences and reference sequences retrieved from NCBI, using the MEGA-X software (71). The phylogenetic tree was visualized using the Interactive Tree of Life (<http://itol.embl.de>) (72).

Community assembly analysis

A phylogenetic bin-based null model framework (iCAMP) was used to evaluate the relative importance of different community assembly processes (73), using OTU table, phylogenetic tree, treatment file, and taxonomic table. This procedure was conducted in iCAMP (v 1.3.2) with default settings using a pipeline on the Galaxy platform (<http://ieg3.rccc.ou.edu:8080>). The significance of difference in the relative importance of each assembly process among different habitat types was assessed using bootstrapping with 1,000 replications on the Galaxy platform.

Statistical analyses

To investigate the effect of habitat changes on sediment and porewater physicochemical properties, the abundance and diversity of comammox *Nitrospira*, and the abundance of comammox *Nitrospira* clades, one-way ANOVA was performed

with LSD test using the SPSS Statistics 20 (IBM, USA). Alpha diversity indexes including Shannon, Simpson, Chao1 and Observed species were calculated using the *diversity* and *estimateR* functions in the “vegan” package in R. Random forest analysis was conducted to evaluate the importance of environmental factors in influencing the abundance and diversity of comammox *Nitrospira* using the “randomForest” package in R (v 4.2.1). Spearman correlation analysis was used to test for the associations among the abundance and diversity of comammox *Nitrospira*, and the relative abundance of comammox *Nitrospira* clades, and environmental factors, using the “corrplot” and “Hmisc” packages in R. The abundance data of OTU matrices were standardized using the Hellinger transformation in the vegan package in R. Principal coordinate analysis (PCoA) was performed using the *pcoa* function in the “ape” package. Mantel test and CCA were carried out to assess the effects of environmental factors on comammox *Nitrospira* community composition.

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DATA AVAILABILITY

Raw sequences have been submitted to DNA Data Bank of Japan (DDBJ) database with the accession number of DRA015269.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1, Fig. S2, Tables S1 to S3 (AEM00807-23-S0001.pdf). Supporting data.

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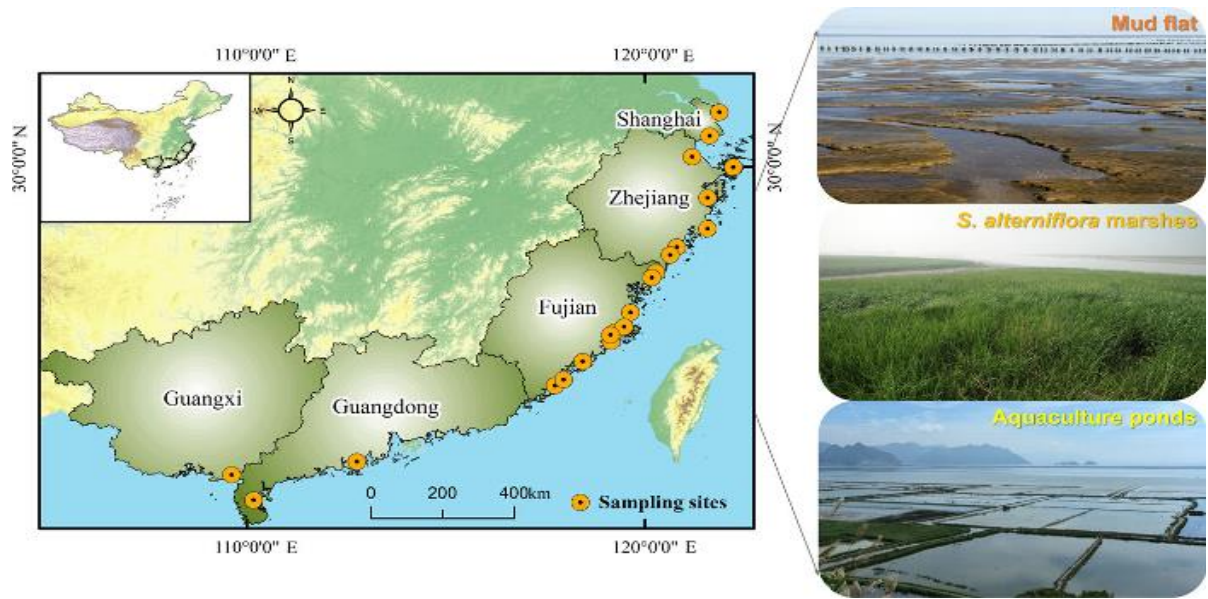


FIG 1 The location of the study area and 21 sampling sites across the coastal regions in southeastern China.

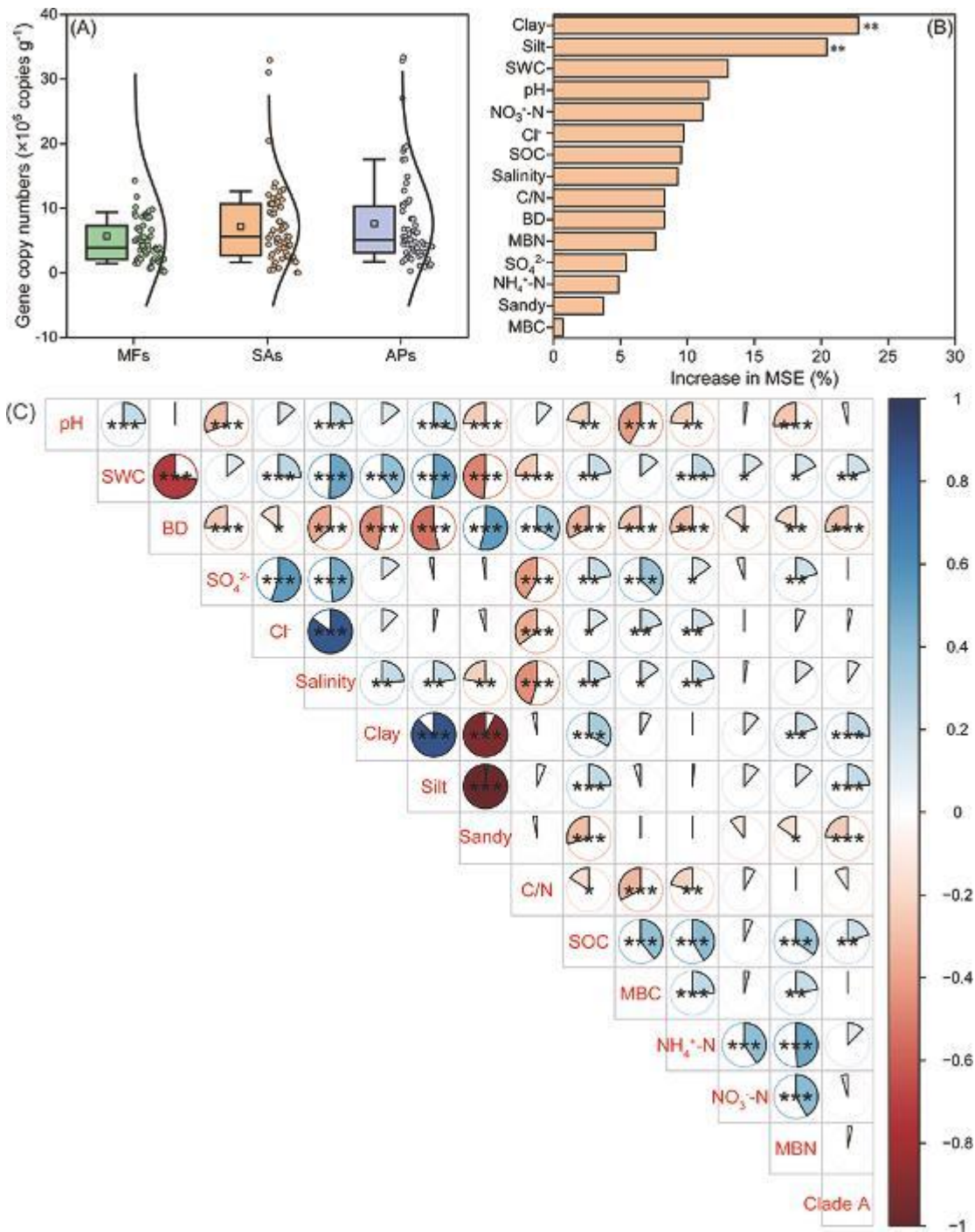


FIG 2 Box-normal plots showing the distribution of the abundance of comammox *Nitrospira* clade A in the three wetland habitat types (A). Random forest analysis showing the importance of environmental factors in influencing the abundance of comammox *Nitrospira* clade A (B). Spearman correlations among environmental variables and the abundance of comammox *Nitrospira* clade A (C). Color of the circle indicates the direction of correlation (blue = positive; red = negative). Size of the circle is proportional to the r value. Asterisks indicate levels of significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). MFs, SAs, and APs represent mud flats, *S. alterniflora* marshes, and aquaculture ponds, respectively. BD, bulk density; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SOC, soil organic carbon; SWC, sediment water content.

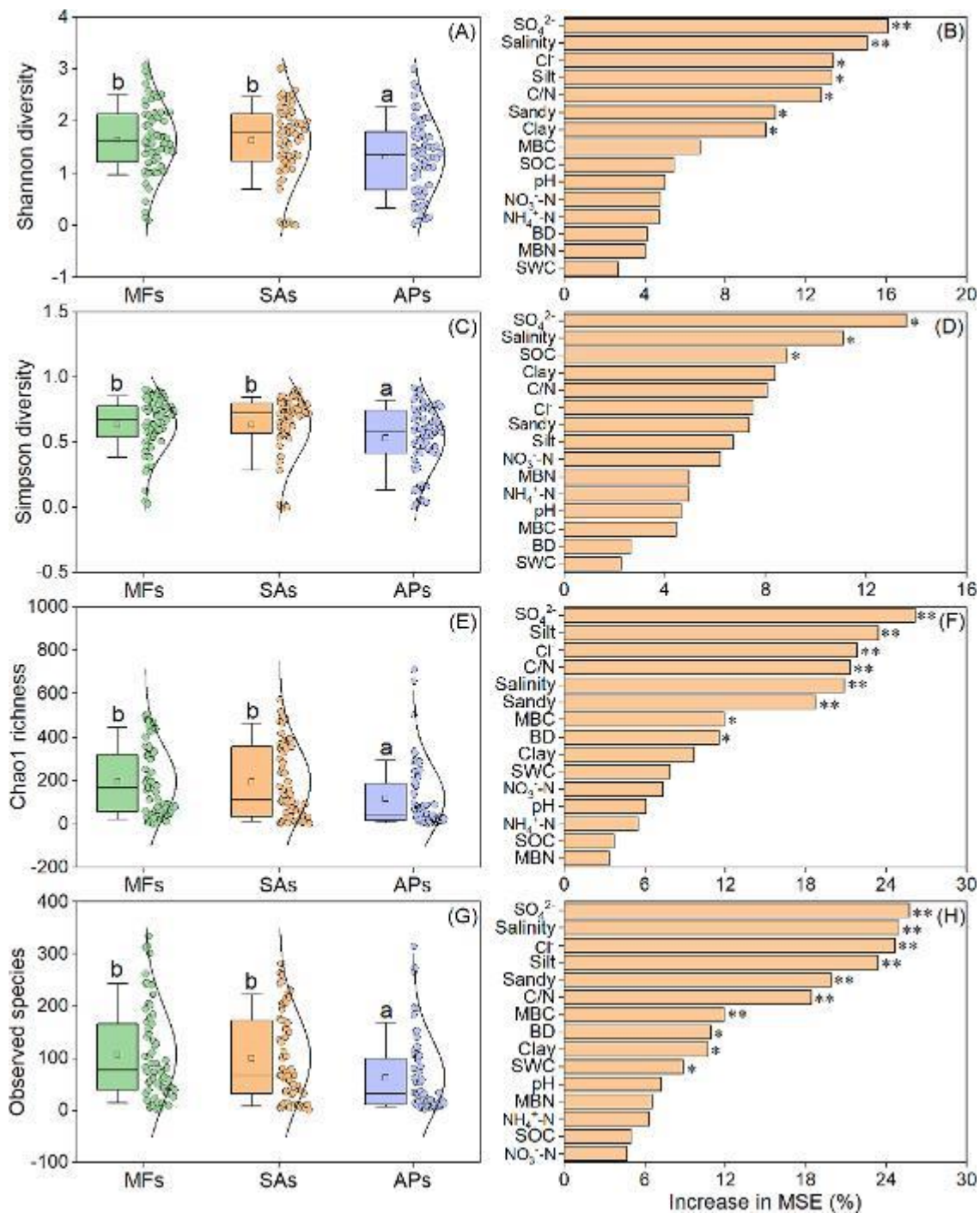


FIG 3 Box-normal plots showing the distribution of the Shannon diversity (A), Simpson diversity (C), Chao1 richness (E), and Observed species (G) of comammox *Nitrospira* in the three wetland habitat types. Random forest analysis showing the importance of environmental factors in influencing the Shannon diversity (B), Simpson diversity (D), Chao1 richness (F), and Observed species (H) of comammox *Nitrospira*. Different lowercase letters above the boxes indicate significant differences between wetland habitat types ($P < 0.05$). Asterisks indicate levels of significance ($*P < 0.05$; $**P < 0.01$). BD, bulk density; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SOC, soil organic carbon; SWC, sediment water content.

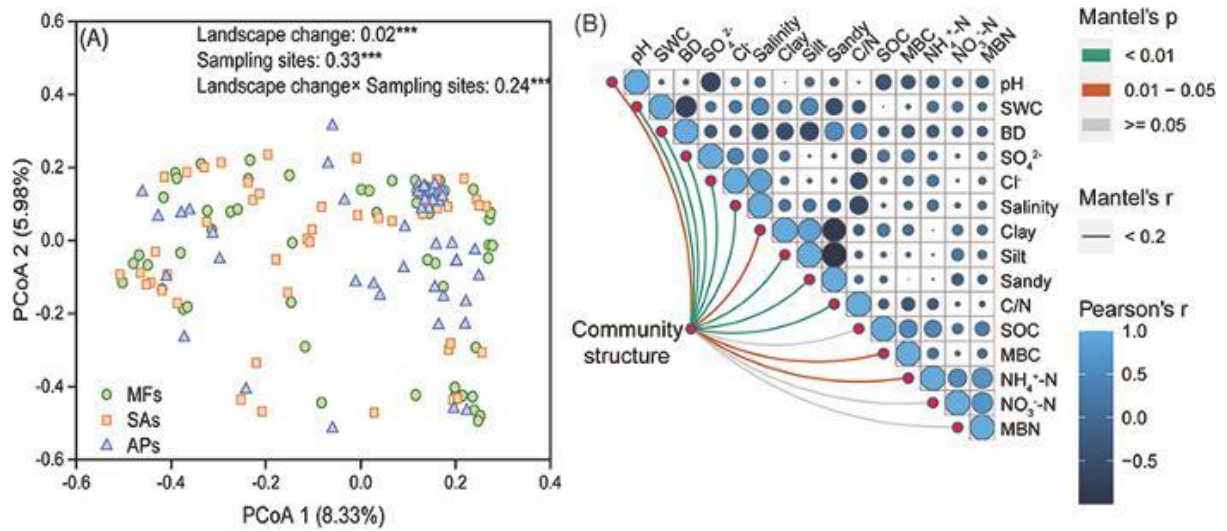


FIG 4 Principal coordinate analysis (PCoA) of comammox *Nitrospira* communities the three wetland habitat types (A). Mantel test showing the relationships between the comammox *Nitrospira* community structure and environmental factors the three wetland habitat types (B). The numbers in PCoA plot indicate the R² values, while asterisks *** represent statistically significant at 0.001 probability level as revealed by PERMANOVA. Edge width of plot B is proportional to Mantel's R value, and the edge colors indicate statistical significance. Pairwise correlation coefficients of environmental factors are shown with color gradients. BD, bulk density; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SOC, soil organic carbon; SWC, sediment water content.

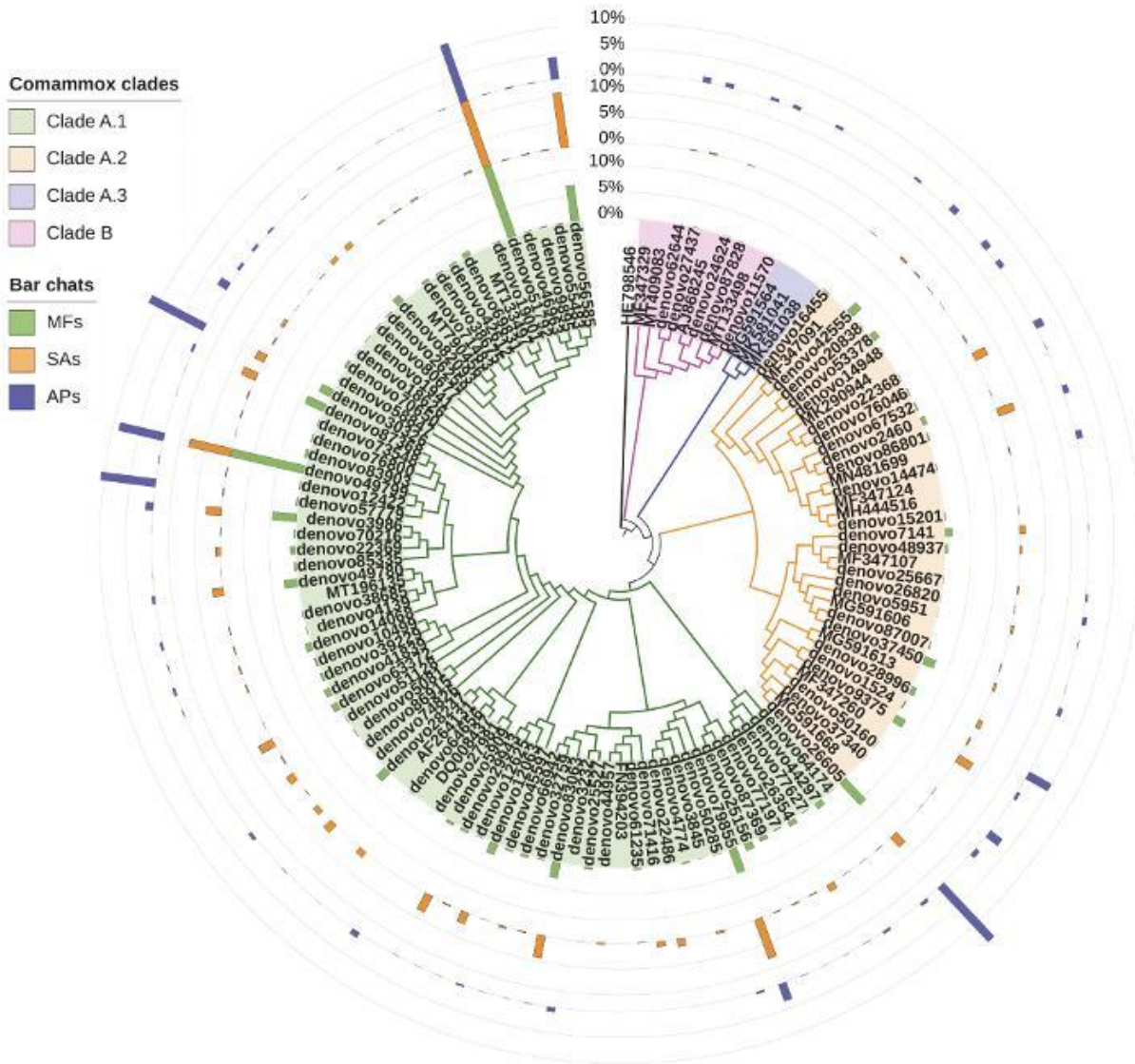


FIG 5 Neighbor-Joining tree showing the relationships among representative sequences in this study (Top 100) and the reference sequences from GenBank. The bar chart showing the relative abundance of each OTU in the three wetland habitat types. The colored ranges indicate different clades of comammox *Nitrospira*. Bootstrap values of >50% based on 1,000 replicates are shown next to the branches. AP, aquaculture pond; MF, mud flat; SA, *S. alterniflora* marsh.

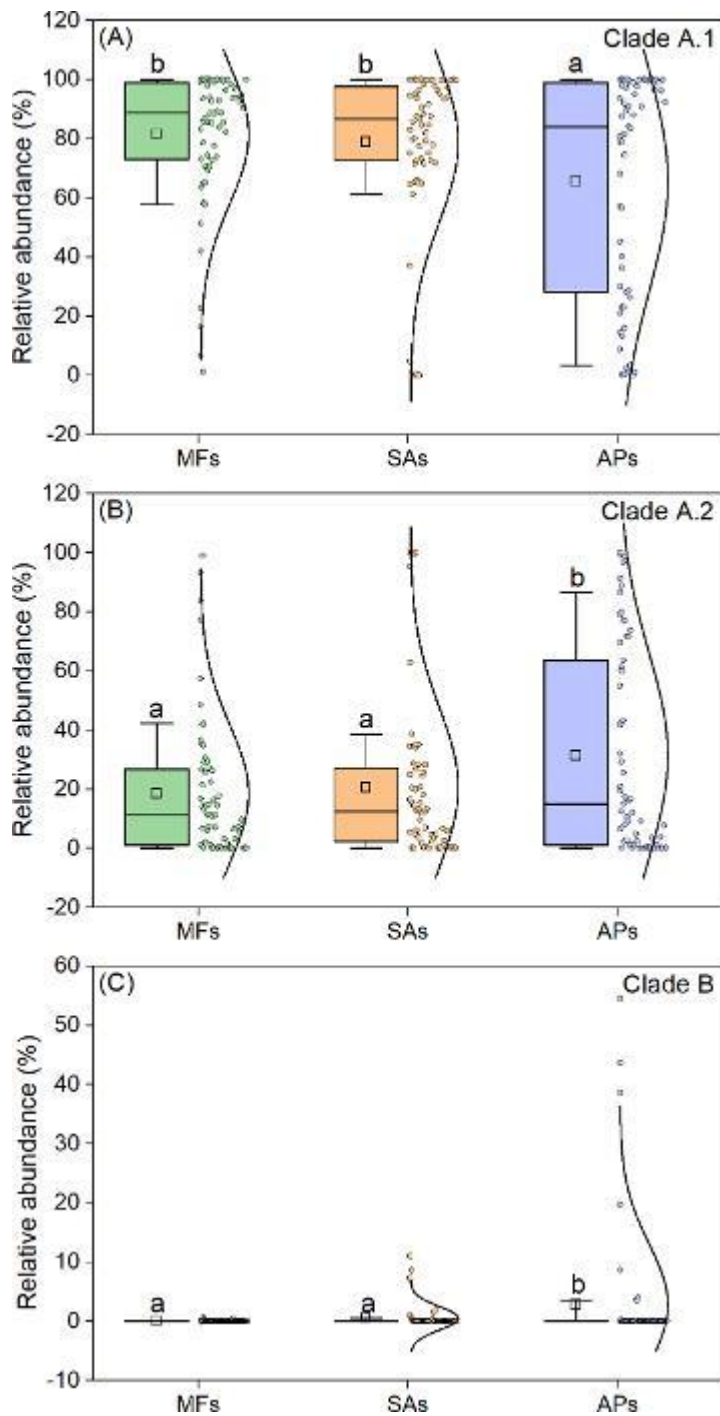


FIG 6 Box-normal plots showing relative abundance of each clade of comammox *Nitrospira* in the three wetland habitat types. Different lowercase letters above the boxes indicate significant differences between wetland habitat types ($P < 0.05$). APs, aquaculture ponds; MFs, mudflats; SA, *S. alterniflora* marshes.

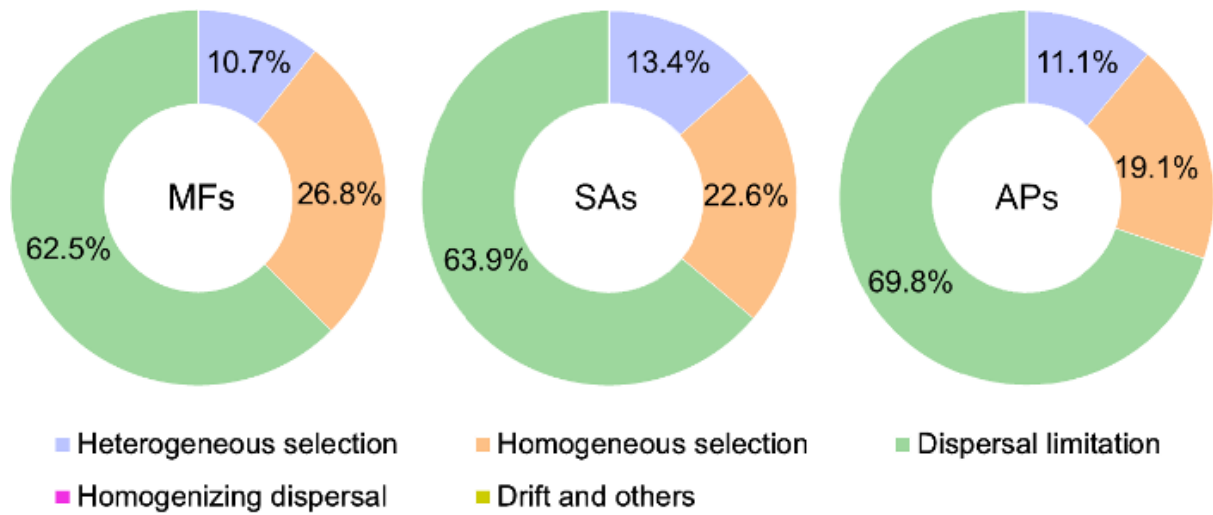


FIG 7 Summary of the relative importance of each process driving the community assembly of comammox *Nitrospira* based on iCAMP analysis. MFs, SAs, and APs represent mudflats, *S. alterniflora* marshes, and aquaculture ponds, respectively.

Supplementary Materials

Community assembly of comammox *Nitrospira* in coastal wetlands across southeastern China

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Table S1 Physico-chemical properties of the sediments and porewater in the three wetland habitat types.

	MFs	SAs	APs
pH	7.99 ± 0.06a	7.95 ± 0.06a	7.82 ± 0.11a
SWC (%)	43.1 ± 1.33a	47.1 ± 1.38ab	47.8 ± 1.70b
BD (g cm ⁻³)	1.29 ± 0.02b	1.26 ± 0.02ab	1.23 ± 0.02a
SO ₄ ²⁻ (mg L ⁻¹)	8.90 ± 0.63a	9.13 ± 0.50a	17.5 ± 1.40b
Cl ⁻ (mg L ⁻¹)	36.8 ± 2.15a	40.9 ± 2.23a	37.8 ± 3.43a
Salinity (‰)	3.96 ± 0.20a	4.54 ± 0.23a	4.21 ± 0.31a
Clay (%)	10.4 ± 0.47a	10.9 ± 0.49a	10.5 ± 0.57a
Silt (%)	54.1 ± 2.29a	52.7 ± 2.41a	50.1 ± 2.56a
Sandy (%)	35.5 ± 2.69a	36.4 ± 2.86a	39.4 ± 3.06a
C/N	14.0 ± 0.60a	13.9 ± 0.62a	12.9 ± 0.83a
SOC (g kg ⁻¹)	6.81 ± 0.22a	10.0 ± 0.54c	8.48 ± 0.49b
MBC (mg kg ⁻¹)	326 ± 23.7a	343 ± 28.6a	385 ± 33.5a
NH ₄ ⁺ -N (mg kg ⁻¹)	13.3 ± 0.82a	25.0 ± 1.40c	16.9 ± 1.03b
NO ₃ ⁻ -N (mg kg ⁻¹)	1.25 ± 0.03a	1.85 ± 0.11b	1.45 ± 0.08a
MBN (mg kg ⁻¹)	12.7 ± 0.65a	26.6 ± 1.99c	16.8 ± 0.82b

SWC: soil water content; BD: bulk density; SOC: soil organic carbon; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen. Different lowercase letters within the same row indicate significant differences at $p < 0.05$. Data are after *Yang et al.* [unpublish] for reference and review only.

Table S2 PERMANOVA, ANOSIM and MRPP analyses of the community structure of comammox *Nitrospira* among three wetland habitat types.

Pairwise comparison	PERMANOVA		ANOSIM		MRPP	
	R^2	P	R	P	R	P
MFs vs. SAs	0.007	0.713	0.000	0.465	-0.001	0.753
MFs vs. APs	0.019	0.002	0.049	<0.001	0.006	<0.001
SAs vs. APs	0.021	<0.001	0.060	<0.001	0.007	<0.001

MFs: mud flats; SAs: *S. alterniflora* marshes; APs: aquaculture ponds.

Table S3 Locations and the relative abundance of each clade of comammox *Nitrospira* in 21 sampling sites.

	Latitude	Longitude	Clade A.1	Clade A.2	B
CMD	31.49 N	121.88 E	56.11 ± 9.37	43.63 ± 9.41	0.25 ± 0.11
FX	30.84 N	121.62 E	23.96 ± 9.37	64.62 ± 8.66	11.43 ± 6.02
HZW	30.21 N	120.79 E	60.59 ± 9.45	39.41 ± 9.45	0
ZS	29.99 N	122.22 E	85.27 ± 4.88	13.93 ± 4.78	0.81 ± 0.81
NB	29.15 N	121.57 E	64.42 ± 13.93	35.58 ± 13.93	0
TZ	28.31 N	121.57 E	79.75 ± 9.89	19.29 ± 8.95	0.96 ± 0.96
LW	27.80 N	120.80 E	75.58 ± 4.02	24.42 ± 4.02	0
AJK	27.59 N	120.63 E	89.97 ± 3.68	10.03 ± 3.68	0
CTG	27.11 N	120.27 E	89.48 ± 3.32	9.69 ± 3.06	0.84 ± 0.55
YCZ	26.97 N	120.17 E	77.66 ± 9.89	16.29 ± 6.42	6.05 ± 6.05
MJR	26.03 N	119.63 E	86.71 ± 3.29	13.08 ± 3.31	0.21 ± 0.17
XHW	25.49 N	119.24 E	40.95 ± 15.46	58.84 ± 15.52	0.21 ± 0.21
FQW	25.63 N	119.48 E	66.75 ± 10.56	33.25 ± 10.56	0
SWY	25.23 N	119.15 E	79.08 ± 5.77	19.69 ± 6.04	1.23 ± 1.23
MLX	25.41 N	119.13 E	98.10 ± 0.62	1.85 ± 0.61	0.05 ± 0.05
JZG	24.02 N	117.74 E	99.96 ± 0.03	0.04 ± 0.03	0
CYG	24.19 N	117.96 E	81.11 ± 6.95	18.89 ± 6.95	0
SJJ	24.68 N	118.44 E	79.53 ± 11.79	19.40 ± 11.81	1.07 ± 1.07
GH	21.94 N	112.77 E	95.40 ± 1.87	4.60 ± 1.87	0
LD	20.89 N	110.17 E	97.78 ± 1.41	2.22 ± 1.41	0
BH	21.59 N	109.62 E	52.91 ± 17.59	47.09 ± 17.59	0

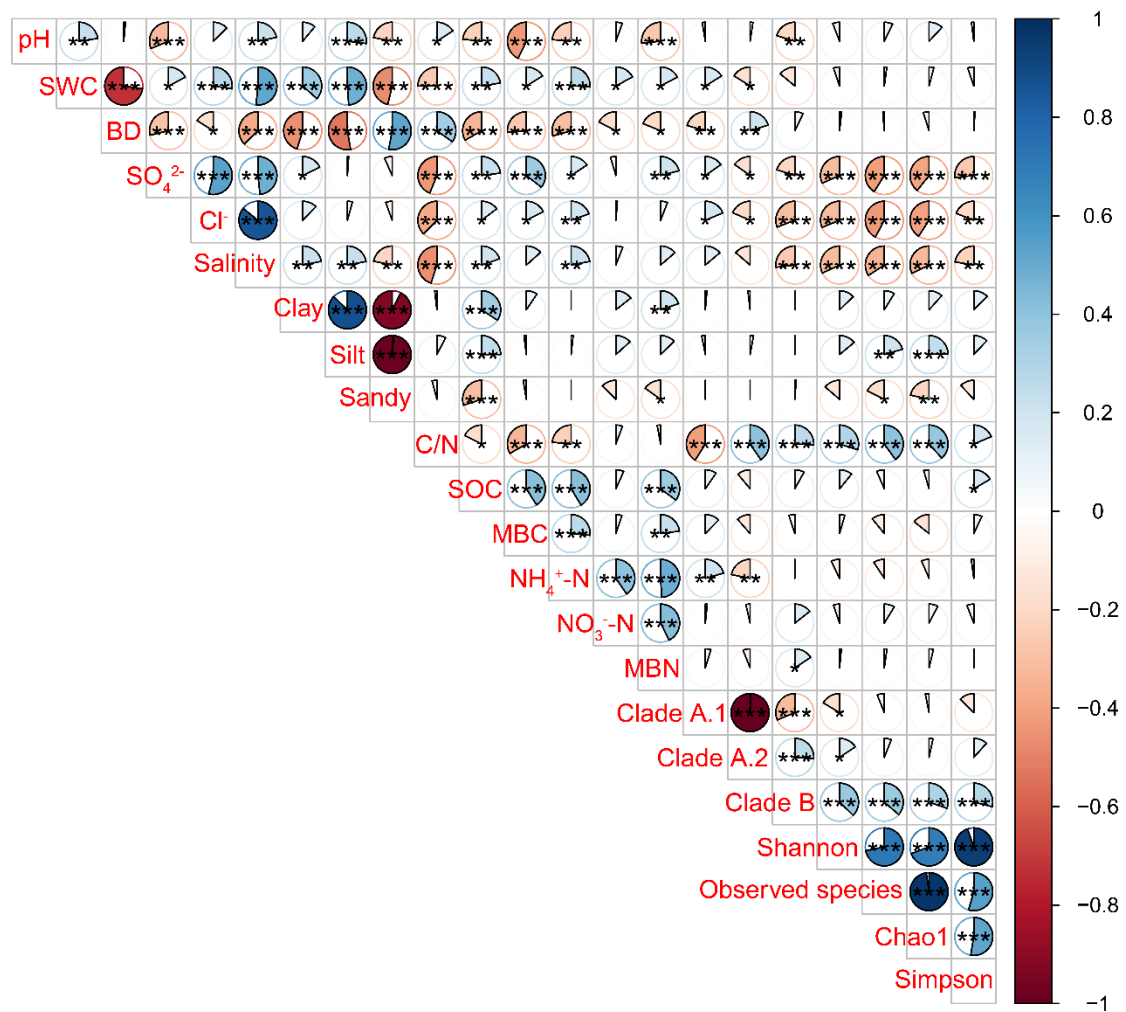


Figure S1 Spearman correlations among environmental variables, the relative abundance of comammox *Nitrospira* clades, and alpha diversity of comammox *Nitrospira*. Color of the circle indicates the direction of correlation (blue = positive; red = negative). Size of the circle is proportional to the r^2 value. Asterisks indicate levels of significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

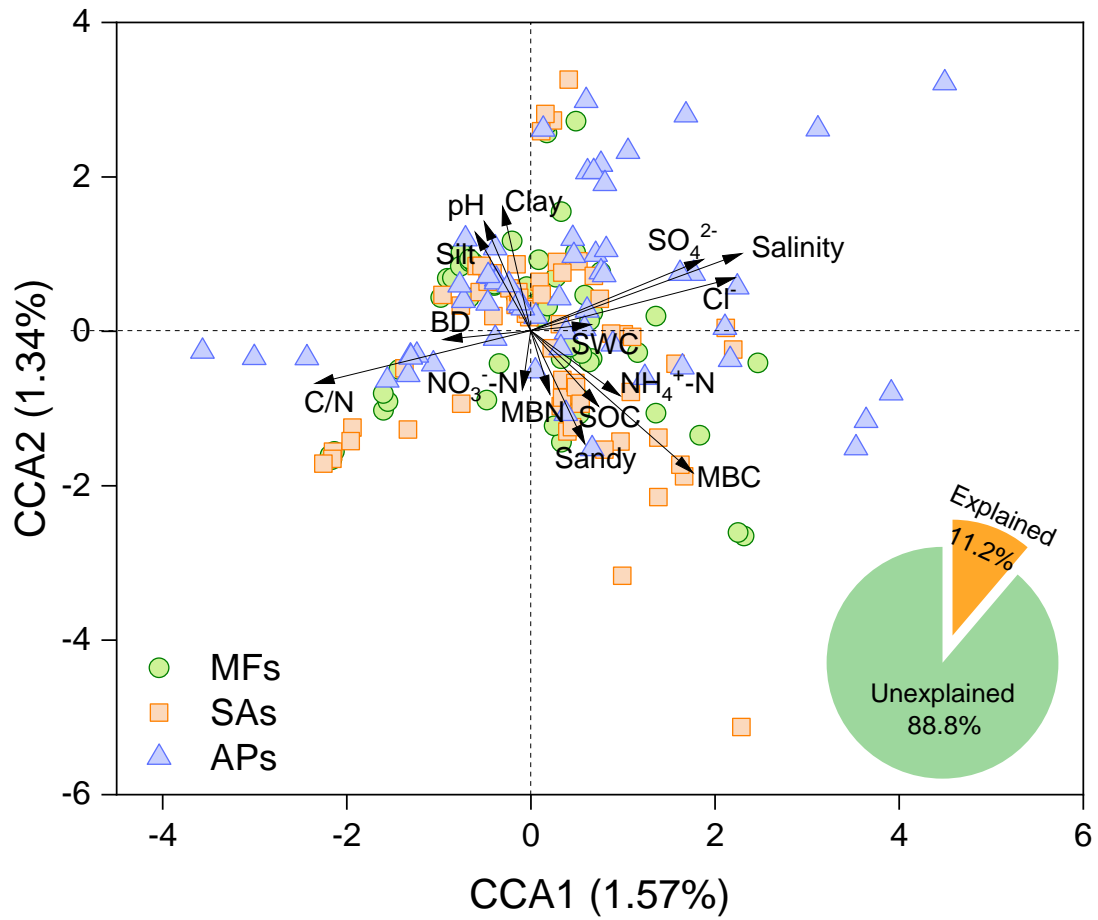


Figure S2 Canonical correspondence analysis (CCA) of the comammox *Nitrospira* community structures. The positions and lengths of the arrows indicate the directions and strengths, respectively, of the effects of soil variables on the comammox *Nitrospira* communities. The pie chart summary the variation that explained by environmental factors.