water

MDPI

Article

Effect of Mangrove on Nitrogen Removal in the Intertidal Zone of Shenzhen's Deep Bay: From ^{15}N Isotope Tracing to Microbial Analysis

Xinwei Jin [1,2], Jingjing Fu [1], Juan Yang [1], Jing Guo [1], Wenrui Guo [1,3,*] and Yahui Chen [1,4]

[1] PowerChina Huadong Engineering Corporation Limited, Hangzhou 311122, China
[2] College of Oceanography, Hohai University, Nanjing 210098, China
[3] School of Environment, Tsinghua University, Beijing 100084, China
[4] PowerChina Eco-Environmental Group Co., Ltd., Shenzhen 518100, China
* Correspondence: guo_wr@hdec.com; Tel.: +86-(0)-571-56627914

Abstract: This study focuses on the nitrogen removal capability of the mangrove wetland system towards resolving the excessive inorganic nitrogen content in the marine water of Shenzhen's Deep Bay. The nitrogen distribution characteristics, biological nitrogen removal processes, nitrogen removal functional genes, and bacterial community characteristics were investigated in five wetland sites in the intertidal zone of the Deep Bay, viz. the *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites. The results showed that ammonia and nitrate in the marine water were significantly removed in the five wetlands sites, with respective removal efficiencies of 70.9–75.5% and 89.5–94.0%. The concentration of ammonia and nitrate in pore water remained significantly unchanged with depth. Denitrification and anammox were each system's main biological nitrogen removal processes, and their rates were 1.70–3.22 and 0.07–0.36 μmol/(kg·h), respectively. The denitrification rates in the mangroves were higher than in the mud flat site, unlike the anammox rates. The denitrifying functional genes (*nir*S, *nos*Z) and anammox functional gene (*hzs*B) showed an excellent linear relationship with the relevant process rates. *Bacillus* and *Pseudomonas* were the main heterotrophic denitrifying bacteria genera identified. The autotrophic denitrifying bacteria genus *Sulfurovum* was also identified in the systems, while *Candidatus Scalindua* was the only anammox genus identified in this study. The results of this study improve our understanding of the nitrogen removal characteristics of coastal wetlands and the role of mangrove plants in the biological nitrogen removal processes.

Keywords: Deep Bay; mangrove wetland; denitrification; anammox

Citation: Jin, X.; Fu, J.; Yang, J.; Guo, J.; Guo, W.; Chen, Y. Effect of Mangrove on Nitrogen Removal in the Intertidal Zone of Shenzhen's Deep Bay: From ^{15}N Isotope Tracing to Microbial Analysis. *Water* **2022**, *14*, 3507. https://doi.org/10.3390/w14213507

Academic Editor: Christos S. Akratos

Received: 12 October 2022
Accepted: 30 October 2022
Published: 2 November 2022

1. Introduction

The Deep Bay, the inner bay of Shenzhen Bay, is adjacent to the Futian and Mai Po Mangrove Reserves in the east and connected to the central mountain through the Dasha River City Green Corridor in the north. It is an essential ecological source and ecological corridor in the region and a vital link between the land and marine environment. Therefore, the aquatic ecology and environment in the Deep Bay significantly impact the sustainable development of Shenzhen city. However, while the ocean provides opportunities for Shenzhen's rapid development, the city's expansion has also impaired the aquatic ecology and environment of the bay. According to the "2021 Bulletin of Shenzhen's Ecology and Environment Status", the water quality of Deep Bay remained poor. It was consistently classified as grade four, with its inorganic nitrogen far exceeding the grade-four standard while deteriorating yearly. High nitrogen concentrations in seawater directly lead to frequent eutrophication in the bay's water body, threatening the ecosystem and the surrounding environment.

Mangrove wetlands are coastal habitats with critical ecological functions. They can exert ecological functions, maintain biodiversity, provide animal habitats (for fishes, crustaceans, etc.), and have significant water purification effects. As modern sediments are formed from the interactions of rivers and oceans, mangrove wetlands act as sinks, sources, or converters for biogenic elements, such as nitrogen, significantly improving the water quality in bays [1–5]. Leung et al. [6] found that the removal of organic matter and ammonia nitrogen concentrations in mangrove wetland systems was as high as 70.8%–84.9% and 84.8%–99.6%, respectively, while that of total phosphorus could reach 100%. Therefore, the restoration or reconstruction of mangroves in coastal areas is highly significant for improving the bay's water quality.

Research on nitrogen removal characteristics in mangrove wetlands significantly guides mangrove wetland restoration. Some studies on the distribution of nitrogen in mangrove wetlands are documented. In the research of Nie et al. [7], shotgun metagenomic sequencing and quantitative polymerase chain reactions were used to understand the nitrogen cycle in the subtropical mangrove ecosystem in the Beibu Gulf of China. The study clearly illustrates how the mangrove ecosystem mitigates nitrogen pollution through *Desulfobacterales*. Elsewhere, Xiao et al. [8] quantified nitrogen loss in mangrove soils and found that the average nitrogen removal rate was about 2.07 $g/(m^3 \cdot d)$.

Zhang et al. [9] reported the occurrence of anaerobic ammonium oxidation (anammox) in the mangrove wetland of the Zhangjiang Estuary, China. The study verified the coexistence of different genera of anammox microorganisms in mangrove sediments, with *Candidatus Scalindua* and *Candidatus Kuenenia* being the dominant genera. In another study, Baskaran and Prabavathy [10] explored the diversity and distribution of nitrogen fixers and denitrifiers associated with the rhizospheres in the mangroves. The authors found that the mangrove ecosystems are potential sources for identifying unexplored microbial communities contributing to nutrient cycling. Currently, there is insufficient quantitative research on the mechanism of nitrogen removal in mangrove wetland systems, especially under high temperatures in the coastal areas of Guangdong province. What is the contribution of various processes in the system to the total amount of nitrogen removal? What is the effect of mangrove plants on the various nitrogen removal processes and related functional bacterial community structure? These questions require urgent research.

Therefore, selecting the Shenzhen Deep Bay intertidal mangrove wetlands as the research object, we investigated the characteristics of nitrogen distribution, the main biological nitrogen removal processes, the numbers of nitrogen removal functional genes, and the bacterial community structure in the system. The results can help improve our understanding of the nitrogen removal characteristics in coastal wetlands and the effect of mangrove plants on biological nitrogen removal processes.

2. Materials and Methods

2.1. Study Sites

Four mangrove sites on the west and north shores of Shenzhen's Deep Bay (Figure 1) were selected as the study sites: the *Kandelia candel* site (Site KC, 113°57′36″ E, 22°30′27″ N), the *Bruguiera gymnorrhiza* site (Site BG, 113°57′35″ E, 22°30′28″ N), the *Sonneratia apetala* site (Site SA, 113°57′41″ E, 22°30′42″ N), and the *Aegiceras corniculatum* site (Site AC, 113°57′57″ E, 22°31′32″ N). A mud flat site (Site MF, 113°57′38″ E, 22°30′21″ N) was selected as the control group.

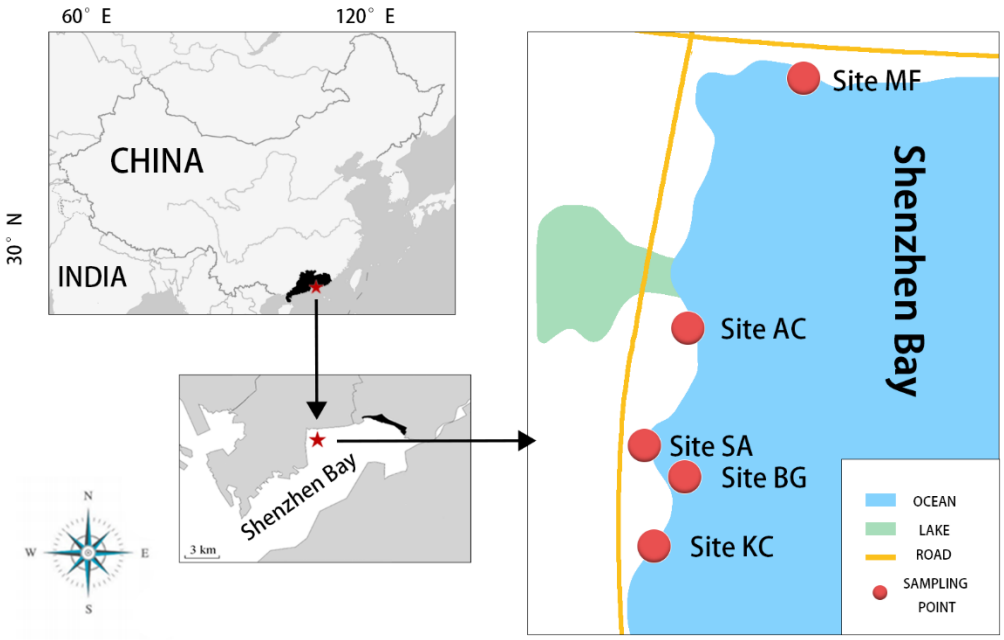

Figure 1. Map showing the research location and sampling sites. KC, BG, SA, AC, and MF are site abbreviations for *Kandelia candel*, *Bruguiear gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flats, respectively.

2.2. Sampling and Analysis

Liquid and solid samples were collected in August 2021. Three sampling points were selected in each of the five sites. The pore water samples were extracted using an AMP-TH DGT device (EasySensor, Nanjing, Jiangsu, China) and HR-Peeper device (Dionex Corporation, Sunnyvale, CA, USA) at 0–150 mm depth. At the same depth, soil samples were simultaneously collected using a columnar sampler.

Nitrate, nitrite, ammonia, and sulfate concentrations were determined using a DX ICS-3000 ion chromatography unit (Dionex Corporation, Sunnyvale, CA, USA). Dissolved oxygen (DO) and chemical oxygen demand (COD) were quantitated using standard methods [11]. The soil organic carbon content was determined via potassium permanganate oxidation, while a total organic carbon analyzer (TOC-VCS/CP, Shimadzu, Japan) quantified the organic carbon content in the liquid. The total sulfur content in sediments was measured using an elemental analyzer (Vario EL III, Elementar, Hanau, Germany).

2.3. Measuring Denitrification, Anammox, and Dissimilatory Nitrate Reduction to Ammonium (DNRA) Rates

Soil slurry incubation experiments measured the potential rates of denitrification and anammox using the ^{15}N isotope-pairing technique and the MIMS (Bay Instruments, Easton, MD, USA) determination of $^{29}N_2$ and $^{30}N_2$ in the soil slurry [12,13]. Briefly, soil slurries were prepared by mixing fresh soils and helium (He)-purged water (soil:water = 1:5) in 12 mL glass vials. The vials were then transferred to a vertical shaker and preincubated for 5–8 days at near in situ temperature (32 °C) to eliminate residual nitrate and oxygen. Subsequently, all the vials were divided into three groups, one spiked with a 100 µL He-purged stock solution of $^{15}NH_4^+$ (99.3% ^{15}N), $^{15}NH_4^+$ plus $^{14}NO_3^-$, or $^{15}NO_3^-$ (99.2% ^{15}N), resulting in a final concentration of 100 µM ^{15}N in each vial. Soil slurry incubations were performed at 32 °C and were stopped by adding 200 µL saturated $HgCl_2$ solution [14] at 0, 1, 3, 5, 7, and 9 h durations. After, the abundance of $^{29}N_2$ and $^{30}N_2$ in the vials was directly determined by MIMS [15]. The potential rate of anammox and denitrification was estimated according to the methods and equations provided in the Supplementary Materials.

Meanwhile, the vials spiked with $^{15}NO_3^-$ were purged with He for 30 min to eliminate $^{29}N_2$ and $^{30}N_2$ produced by denitrification and anammox during incubation. At the same time, 200 µL hypobromite iodine solution was injected to oxidize the DNRA-produced $^{15}NH_4^+$ into ^{15}N gases ($^{29}N_2$ and $^{30}N_2$). The concentration of the ^{15}N gas generated in the initial and final samples was measured by MIMS. The values were used to calculate the potential rate of DNRA according to the methods and equations described in the Supplementary Materials.

2.4. DNA Extraction and Quantitative PCR (q-PCR)

DNA was extracted from 0.5 g of fresh soil using an E.Z.N.A.® Soil DNA Kit (Omega, Norcross, GA, USA). Real-time q-PCR analysis on the extracted DNA determined the abundance of the functional genes of denitrification (*nir*S and *nos*Z), anammox (*hzs*B), and DNRA (*nrf* A). The primers were *nir*SCd3aF/nirSR3cd, *nos*Z-F/*nos*Z-1622R, *hzs*B396F/*hzs*B742R, and *nrf* AF2aw/*nrf* AR1, respectively (Table 1).

Table 1. Characteristics of primer and probe sets for real-time q-PCR.

Primers	Specificity	Sequence (5′ to 3′)	Reference
nirSCd3aF nirSR3cd	*nir*S gene	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	[2]
nosZ-F nosZ-1622R	*nos*Z gene	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	[16]
hzsB396F hzsB742R	*hzs*B gene	WTYGGKTATCARTATGTAG AAABGGYGAATCATARTGGC	[9]
nrfAF2aw nrfAR1	*nrf* A gene	CARTGYCAYGTBGARTA TWNGGCATRTGRCARTC	[3]

2.5. High-Throughput 16S rRNA Gene Sequencing and Analysis

High-throughput 454 GS-FLX pyrosequencing of the 16S rRNA gene was conducted according to standard protocols [11]. The detailed pyrosequencing and analysis methods are described in the Supplementary Materials. BLAST of taxonomic classification down to the phylum, class and genus levels was then achieved using MOTHUR via the SILVA 106 database, with a set confidence threshold of 80%. Abundance of a given phylogenetic group was set as the number of sequences affiliated with that group divided by the total number of sequences per sample.

3. Results and Discussion

3.1. Physical and Chemical Properties of Marine Water

Table 2 illustrates that the inorganic nitrogen concentrations in the five sites ranged from 0.63 to 0.82 mg/L, notably higher than the permissible limit (0.50 mg/L) of the Grade four seawater quality standard in China. Ammonia nitrogen (0.24–0.30 mg/L) and nitrate nitrogen (0.26–0.37 mg/L) were the primary forms of inorganic nitrogen in the seawaters, accounting for 80.0%–91.8% of the total inorganic nitrogen in seawater in each site. These results suggested that the removal of ammonia and nitrate nitrogen should be prioritized. Moreover, the pH, COD concentration, DO concentration, salinity, and sulfate concentration ranges in the sites were 8.3–9.3, 1.8–4.4 mg/L, 2.45–4.20 mg/L, 29,445–37,505 mg/L, and 1215–1535 mg/L, respectively.

Table 2. Physical and chemical parameters of the seawater samples.

Sampling Site	pH	COD (mg/L)	DO (mg/L)	Salinity (mg/L)	Ammonia Nitrogen (mg/L)	Nitrite Nitrogen (mg/L)	Nitrate Nitrogen (mg/L)	Total Inorganic Nitrogen (mg/L)	Sulfate (mg/L)
Kandelia candel site	8.4 ± 0.4	4.4 ± 0.3	2.45 ± 0.26	31655 ± 819	0.277 ± 0.024	0.098 ± 0.007	0.256 ± 0.015	0.631 ± 0.041	1215 ± 77
Bruguiera gymnorrhiza site	9.0 ± 0.6	2.8 ± 0.1	4.20 ± 0.24	33735 ± 983	0.244 ± 0.012	0.149 ± 0.018	0.362 ± 0.020	0.755 ± 0.006	1425 ± 91
Sonneratia apetala site	8.5 ± 0.8	1.8 ± 0.1	2.84 ± 0.11	30160 ± 782	0.269 ± 0.009	0.142 ± 0.014	0.300 ± 0.011	0.711 ± 0.028	1510 ± 87
Aegiceras corniculatum site	8.3 ± 0.3	3.2 ± 0.2	4.22 ± 0.28	29445 ± 721	0.298 ± 0.012	0.149 ± 0.013	0.369 ± 0.014	0.816 ± 0.012	1335 ± 84
Mud flat site	9.3 ± 0.4	2.0 ± 0.2	3.35 ± 0.14	37505 ± 114	0.259 ± 0.010	0.052 ± 0.007	0.322 ± 0.013	0.633 ± 0.022	1535 ± 79

3.2. Vertical Distribution of Soluble Nitrogen Species

As shown in Figure 2, the ammonia nitrogen concentrations in the pore water from each site did not change significantly with the depth ($p > 0.05$). The average ammonia nitrogen concentrations in the pore water from *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites were 0.068 ± 0.026, 0.062 ± 0.014, 0.074 ± 0.025, 0.075 ± 0.024 and 0.076 ± 0.011 mg/L, respectively. Compared with the ammonia nitrogen concentration in the seawater from each site, the reductions were 0.209, 0.182, 0.195, 0.223, and 0.184 mg/L, indicating that each system underwent significant ammonia nitrogen removal, with a respective removal efficiency of 75.5, 72.3, 74.6, 74.8, and 70.9%, respectively. Ammonia nitrogen removal in the mangrove plant sites was higher than in the mud flat site, indicating that mangrove planting promoted removal in the system. Notably, *Kandelia candel* had the most significant promotion effect.

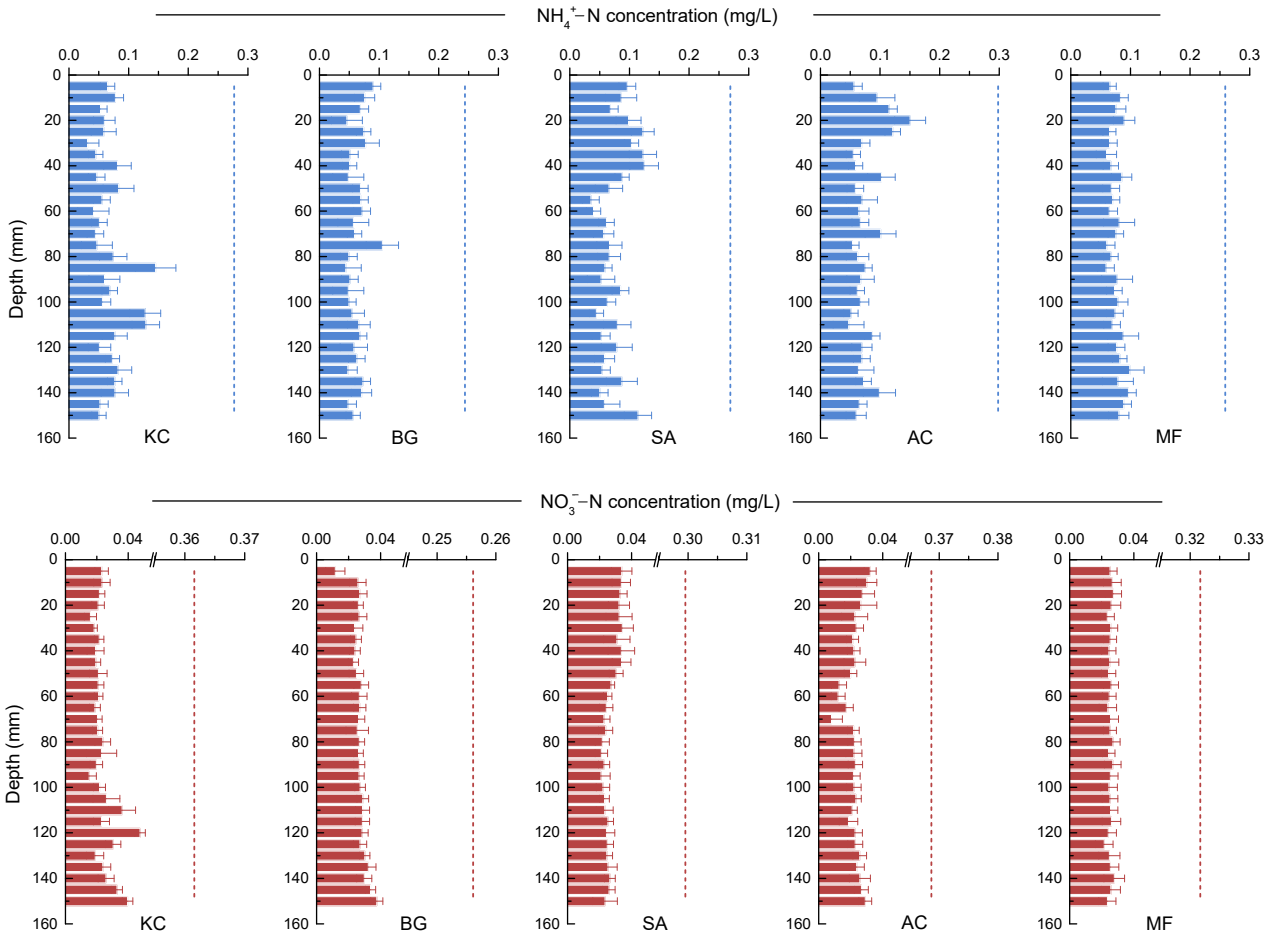

Figure 2. Vertical distribution of NH_4^+−N and NO_3^-−N in the interstitial water samples from the five sites. KC, BG, SA, AC, and MF are site abbreviations for *Kandelia candel*, *Bruguiear gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flats, respectively. Dashed lines indicated the nitrogen concentration in the seawater from each site.

The nitrate nitrogen concentration in the pore water from each site did not change significantly with depth ($p > 0.05$). Its average concentrations in the pore water from *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites were 0.024 ± 0.007, 0.027 ± 0.004, 0.027 ± 0.005, 0.022 ± 0.005, and 0.025 ± 0.001 mg/L, respectively. Compared with its concentration in the seawater samples, a 0.338, 0.230, 0.273, 0.346, and 0.297 mg/L reductions were, respectively observed, indicating that each system exhibited significant nitrate nitrogen removal. The respective removal efficiencies

were 93.4%, 89.5%, 91.0%, 94.0%, and 92.2%. Among them, nitrate removal efficiencies in *Kandelia candel* and *Aegiceras corniculatum* sites were higher than those in the mud flat site. In comparison, nitrate removal efficiencies in the *Bruguiera gymnorrhiza* and *Sonneratia apetala* sites were lower than that in the mud flat site, indicating that *Kandelia candel* and *Aegiceras corniculatum* promoted nitrate removal, and the promotion effect of *Aegiceras corniculatum* was more significant. However, *Bruguiera gymnorrhiza* and *Sonneratia apetala* lowered the nitrate removal efficiency of the system notably.

3.3. Biological Nitrogen Removal

Studies have shown that denitrification, anammox, and DNRA are crucial biological nitrogen removal processes in natural sediments [15,17]. This study determined the potential activities of these three processes in wetland sediments based on soil slurry culture experiments and ^{15}N isotope tracer technology. In incubations with $^{15}NH_4^+$ only, no significant accumulation of $^{29}N_2$ and $^{30}N_2$ was observed in the soil slurries, indicating preincubation consumed soil background nitrate. For soil slurries amended with both $^{15}NH_4^+$ and $^{14}NO_3^-$, the accumulation of $^{29}N_2$ was detected in all soil slurries. In contrast, no accumulation of $^{30}N_2$ was detected, indicating that anammox occurred in the soil samples (data not shown). In incubations with only $^{15}NO_3^-$, significant accumulation of both $^{29}N_2$ and $^{30}N_2$ was observed (Figure S1). The data was used to estimate denitrification and anammox potentials.

As shown in Figure 3, the denitrification rates in *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*e, and mud flat sites were 2.19 ± 0.31, 1.87 ± 0.19, 2.57 ± 0.24, 3.22 ± 0.33, 1.70 ± 0.35 μmol/(kg·h), respectively, while the anammox rates were 0.24 ± 0.10, 0.07 ± 0.03, 0.23 ± 0.09, 0.18 ± 0.11, 0.36 ± 0.15 μmol/(kg·h), respectively. The DNRA rates were extremely low. Hence, they were ignored. The denitrification rates of the systems were about 5–27 times that of anammox, indicating that denitrification was the main biological nitrogen removal process in each system, whose contribution to biological nitrogen removal was as high as 82.5–96.4%. This observation is consistent with the conclusions drawn by Yin et al. [14] and Shan et al. [15] from their respective studies on typical Chinese paddy soils and Chinese coastal sediments.

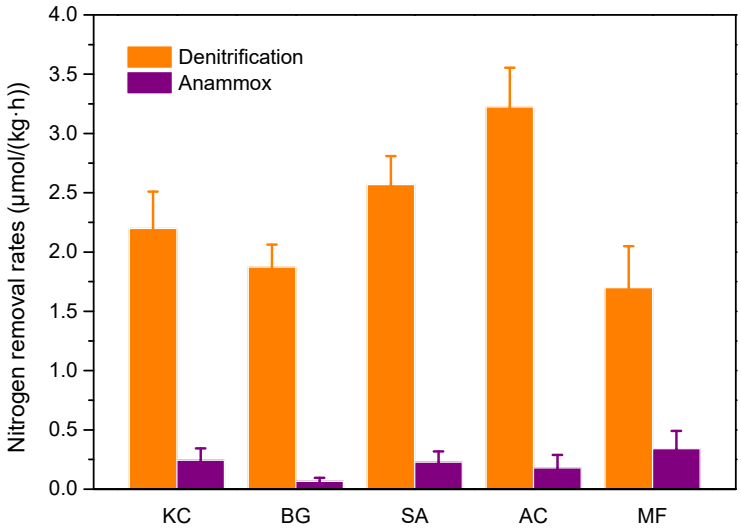

Figure 3. Denitrification and anammox rates at the sampling sites. KC, BG, SA, AC, and MF are site abbreviations for *Kandelia candel*, *Bruguiear gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flats, respectively.

Further analysis showed that the denitrification rates in the plant sites were 1.1–1.9 times that in the mud flat site, indicating that mangrove plants significantly promoted denitrification in the system. Here, the promotion effect of *Aegiceras corniculatum* was the most significant. Research has shown that electron donors affect denitrification in natural

systems. Organic matter and sulfur are essential electron donors for denitrification in natural systems; the former drives heterotrophic denitrification, while the latter drives autotrophic denitrification [18]. As shown in Figure 4, the denitrification rate in each system was significantly and positively correlated with the sediment organic carbon content (R^2 = 0.890). However, it was not significantly associated with the total sulfur content (R^2 = 0.047) in this study. These results indicate that organic carbon significantly influences the denitrification rate, and heterotrophic denitrification was the predominant denitrification mode in each system. Therefore, we opine that the higher level of organic carbon contents (7.63–13.46 g·kg^{-1}) in the sediments of the plant sites than that in the mud flat site (5.99 g·kg^{-1}) was an important reason for the higher denitrification rate in the former sites. The higher organic carbon contents in the sediments of the plant sites were presumed to derive from the secretion of plant roots and the degradation of plant residues.

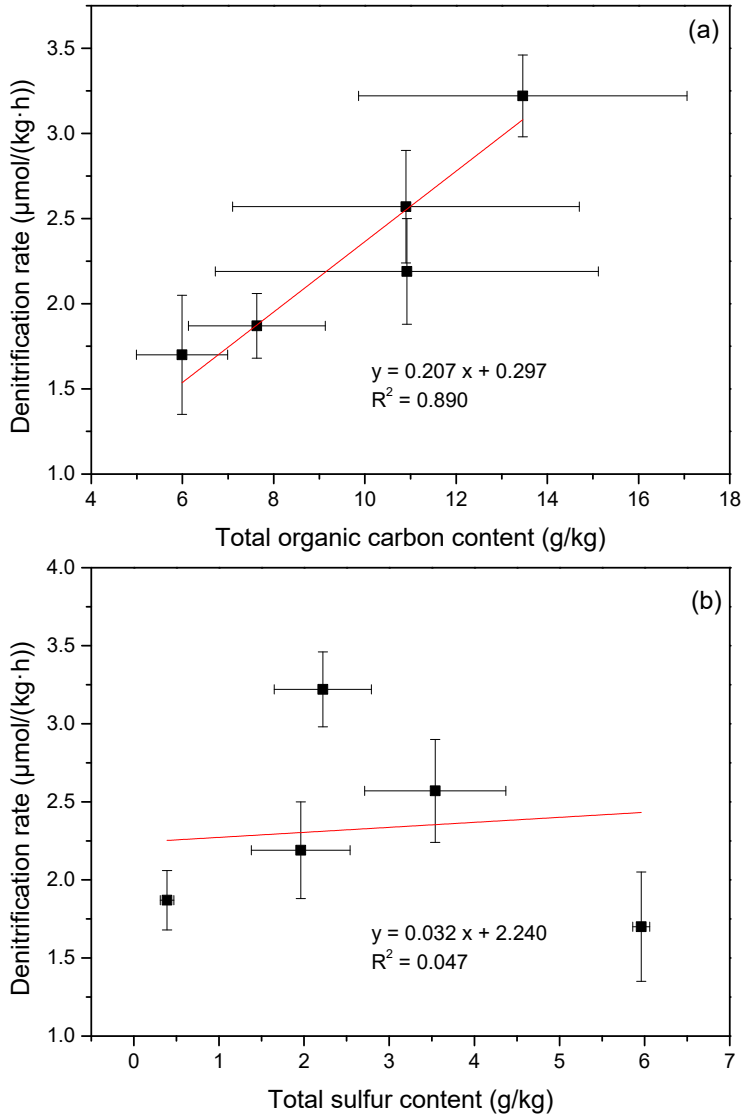

Figure 4. Fitting relationship between the denitrification rate and total organic carbon content (**a**) and total sulfur content (**b**).

Furthermore, the anammox rate in the mud flat site was 1.5–2.0 times that in the plant sites, indicating that the cultivation of mangrove plants restricts anammox. Nitrite nitrogen and ammonia nitrogen are crucial substrates for the anammox reaction, and their amounts significantly impact the anammox rate [19,20]. In anaerobic systems, nitrite is usually produced during denitrification and is an intermediate product of denitrification [21]. In the

current study, a smaller number of denitrification electron donors and weaker denitrification intensity in the mud flat site were more conducive to intermediate nitrite accumulation, resulting in ~0.15 mg/L nitrite concentrations in the mud flat site (i.e., 2.2–10 times the values in the plant sites). In addition, the removal efficiency of ammonia nitrogen in the mud flat site was lower than that in the plant sites (Section 3.2). It demonstrates the ammonia nitrogen concentration in the mud flat site was also slightly higher than that in the plant sites (Figure 2). Higher nitrite and ammonia nitrogen concentrations in the mud flat site were crucial to the higher rate of anammox in the plant sites.

3.4. Nitrogen Removal Functional Genes

Nitrite reductase encoded by *nir*S gene and nitrous oxide reductase encoded by *nos*Z gene, respectively, catalyze the conversion of NO_2 to NO and N_2O to N_2 in denitrification [22]. As depicted in Figure 5a,b, the *nir*S gene copy numbers in the sediments of *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites were 4.53×10^7, 4.21×10^7, 7.31×10^7, 10.06×10^7, and 3.51×10^7 copies/g, respectively. By contrast, the *nos*Z gene copy numbers were 8.65×10^7, 5.12×10^7, 23.4×10^7, 25.1×10^7, and 4.93×10^7 copies/g, respectively. The copy numbers of *nir*S and *nos*Z genes in the plant sites were 1.2–2.9 times and 1.1–5.1 times that in the mud flat site, respectively, indicating that mangrove plants significantly promoted the growth of bacteria containing the aforementioned functional genes in the system. Such an observation could be attributed to organic matter excreted by plant roots and released from plant residues, which provides a sufficient carbon source for the metabolism of denitrifying bacteria (among which *Aegiceras corniculatum* had the most substantial promoting effect on the numbers of *nir*S and *nos*Z). Denitrifying bacteria can compete with anammox bacteria for substrate nitrite [23,24]. Therefore, by promoting the growth of denitrifying bacteria and increasing the number of denitrifying functional genes, mangrove plants have an inhibiting effect on the anammox bacteria growth. The copy numbers of the anammox functional gene *hzs*B in the sediments of *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, and *Aegiceras corniculatum* were 2.50×10^3, 0.17×10^3, 1.92×10^3, and 1.03×10^3 copies/g, respectively (Figure 5c). These values were only 2.6%–38.2% of the *hzs*B gene copy number (6.54×10^3 copies/g) in the sediments of the mud flat site. Further analysis revealed that the denitrification rates of the systems were significantly and positively correlated with the numbers of *nir*S and *nos*Z (R^2 = 0.959 and 0.814, respectively, Figure 6a). Additionally, the anammox rate was positively correlated with the number of *hzs*B (R^2 = 0.806, Figure 6b), indicating that functional genes are crucial to biological nitrogen removal. The numbers of the DNRA functional gene *nrf* A in the sediments of each system were below the detection limit, confirming that the DNRA rates were negligible. The above results revealed the micro-mechanism of the effect of mangrove plants on the biological nitrogen removal processes at the gene level.

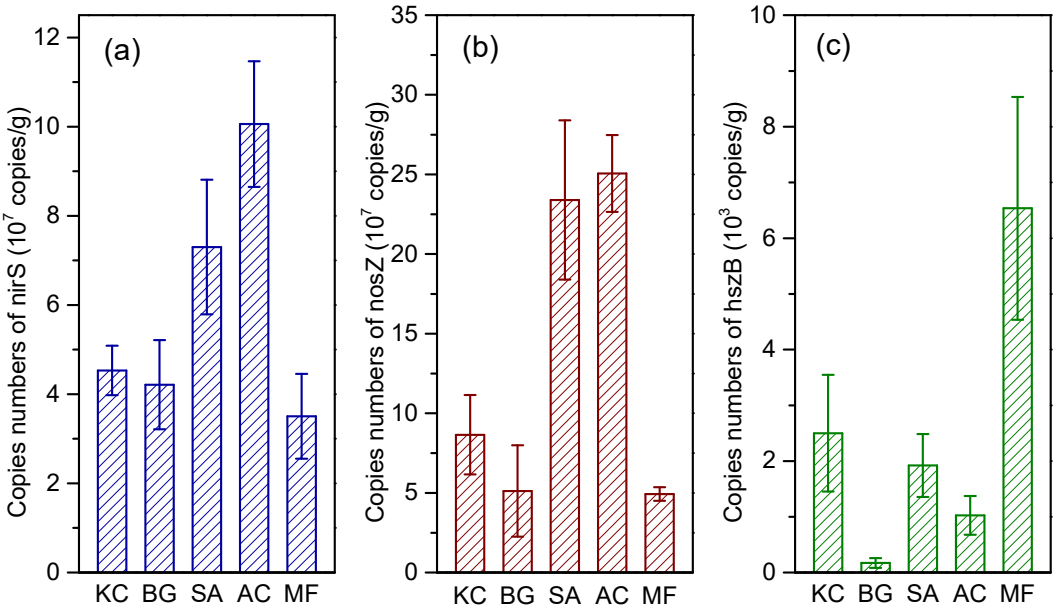

Figure 5. Copy numbers of *nir*S (**a**), *nos*Z (**b**), *hzs*B (**c**) at five sites. KC, BG, SA, AC, and MF are site abbreviations for *Kandelia candel*, *Bruguiear gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flats, respectively.

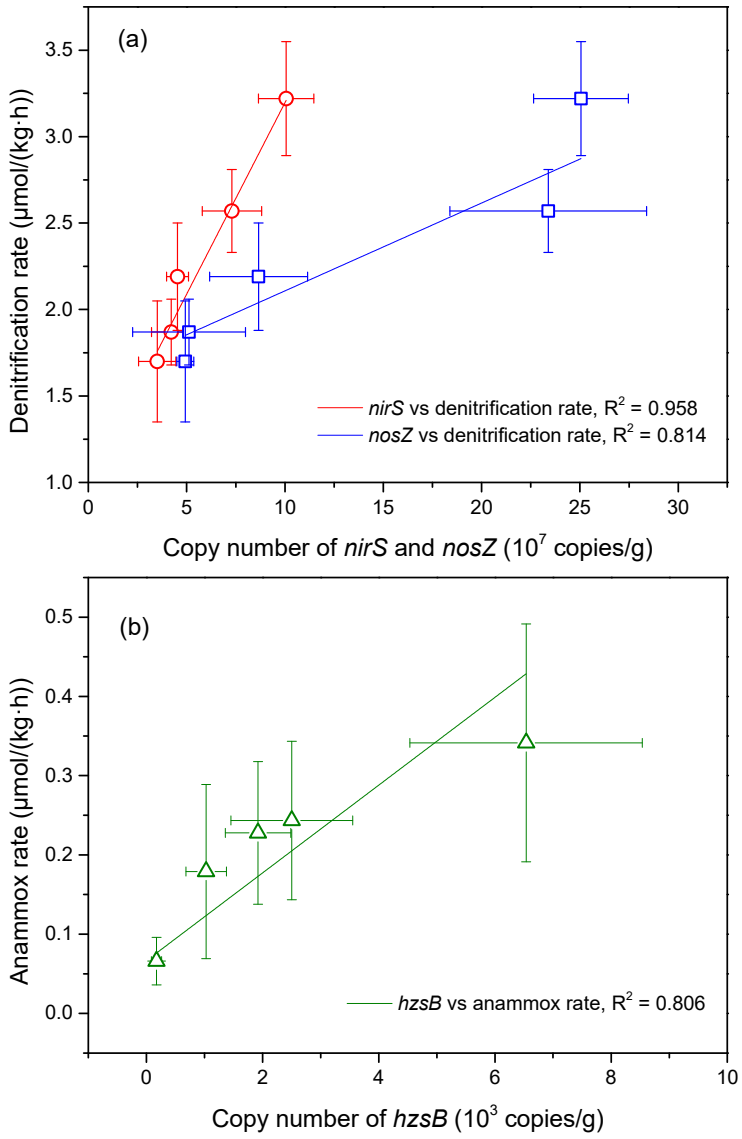

Figure 6. Relationships between (**a**) denitrification rate and copy numbers of denitrification genes (*nir*S and *nos*Z) (**b**) anammox rate and copy number of *hzs*B (**b**).

3.5. Microbial Communities Related to Nitrogen Removal

The Chao index measures species richness, while the Shannon index assesses microbial diversity in a system. As shown in Table 3, the values of these two indices follow the trend: *Kandelia candel* site > *Aegiceras corniculatum* site > *Bruguiera gymnorrhiza* site > *Sonneratia apetala* site > mud flat site. It indicates that the species richness and microbial diversity follow the same trend. Overall, mangrove plants significantly improved the species richness and microbial diversity of the system.

Table 3. Bacterial richness and diversity indices in the sediments.

Sampling site	Chao	Shannon	Coverage
Kandelia candel site	1230	5.98	0.95
Bruguiera gymnorrhiza site	1226	5.71	0.95
Sonneratia apetala site	1344	6.08	0.95
Aegiceras corniculatum site	1069	5.36	0.94
Mud flat site	880	4.91	0.94

In addition, 47, 39, 40, 38, and 46 phyla were identified in Kandelia candel, Bruguiera gymnorrhiza, Sonneratia apetala, Aegiceras corniculatum, and mud flat sites, respectively. Proteobacteria, Firmicutes, Chloroflexi, and Actinobacteriota were the dominant bacterial phyla identified in each system (Figure 7a), with relative abundances ranging from 18.1% to 39.5%, 19.5% to 33.1%, 7.3% to 18.2%, and 2.6% to 12.0%, respectively. At the class level (Figure 7b), Bacilli, Gammaproteobacteria, Alphaproteobacteria, and Anaerolineae were the dominant bacterial classes identified in each system, with relative abundances of 18.6–31.4%, 9.0–23.7%, 8.3–17.0%, 3.2–16.5%, respectively.

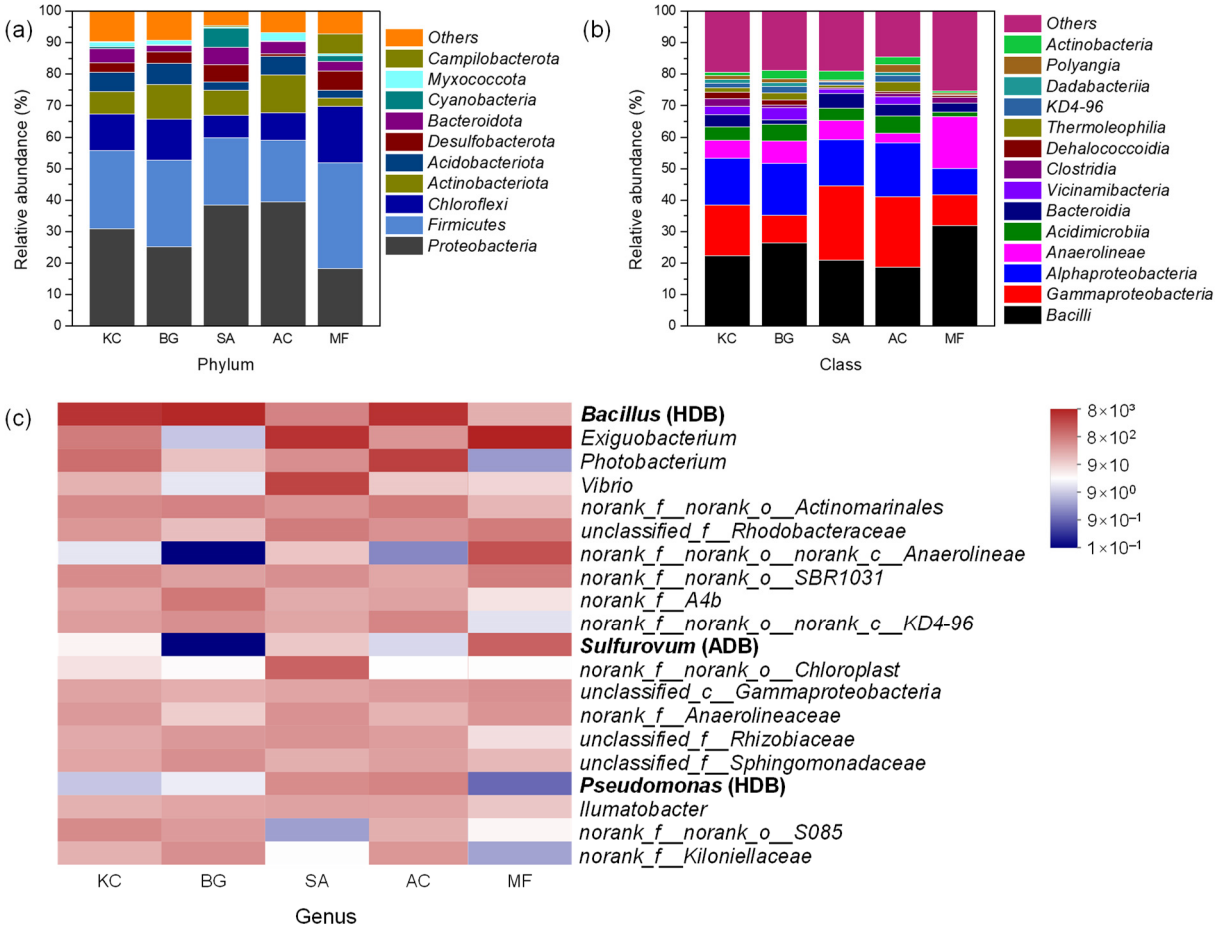

Figure 7. Distribution of phylogenetic taxa at (**a**) phylum level, (**b**) class level, and (**c**) genus level. The genera in bold indicate heterotrophic denitrifying bacteria (HDB) and autotrophic denitrifying bacteria (ADB).

At the genus level (Figure 7c), *Bacillus* and *Pseudomonas* were identified as the main heterotrophic denitrifying bacteria genera. The relative abundances of *Bacillus* in the *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites were 19.3%, 24.8%, 2.4%, 16.8%, and 0.8%, respectively. Additionally, the respective relative abundances of *Pseudomonas* were 0.03%, 0.07%, 1.8%, 2.1%, 0.004%, respectively. The relative abundances of the two genera in the plant sites were significantly higher than those in the mud flat site ($p < 0.05$), indicating that mangrove plants were beneficial to the growth of heterotrophic denitrifying bacteria.

In addition to the heterotrophic denitrifying bacteria genera, an autotrophic denitrifying bacterium genus *Sulfurovum* was also identified in each system. The bacteria in this genus can use reduced sulfur as an electron donor to reduce nitrate [18]. The relative abundances of *Sulfurovum* in each site were 0.15%, 0.0004%, 0.41%, 0.04%, and 6.0%, respectively. The abundances in the plant sites were significantly lower than in the mud flat site,

indicating that mangrove plants were not conducive to their growth. Moreover, *Candidatus Scalindua*, an anammox genus identified in this study (not shown), is frequently detected in marine systems [25]. Here, the relative abundances of this genus in the *Kandelia candel*, *Bruguiera gymnorrhiza*, *Sonneratia apetala*, *Aegiceras corniculatum*, and mud flat sites were 0.06%, 0.22%, 0.01%, 0.04%, and 0.54%, respectively. Their relative abundances in the plant sites were significantly lower than in the mud flat site (p <0.05), indicating that mangrove plants were not conducive for the growth of this genus.

4. Conclusions

In this study, in situ measurement, ^{15}N isotope tracing technique, q-PCR, and 454 high-throughput pyrosequencing were jointly applied to investigate the nitrogen distribution characteristics, biological nitrogen removal processes, nitrogen removal functional genes, and bacterial community characteristics in five coastal wetland sites. The results can improve our understanding of the nitrogen removal characteristics of coastal wetlands and the role of mangrove plants in the biological nitrogen removal processes. The conclusions were:

(1) The ammonia and nitrate in marine water in five wetland systems were significantly removed, with the removal efficiencies ranging from 70.9% to 75.5% and 89.5% to 94.0%, respectively. Among them, *Kandelia candel* and *Aegiceras corniculatum* most significantly promoted ammonia and nitrate removal, respectively.

(2) The denitrification and anammox rates of the five systems ranged from 1.70 to 3.22 μmol/(kg·h) and 0.07 to 0.36 μmol/(kg·h), respectively. The denitrification rates in the plant sites were 1.1–1.9 times that in the mud flat site, while the anammox rate in the mud flat site was 1.5–2.0 times those in the plant sites. These values indicate that mangroves promote denitrification but inhibit anammox.

(3) The numbers of *nir*S, *nos*Z, and *hzs*B in the five systems ranged from 3.51×10^7 to 10.06×10^7, 4.93×10^7 to 25.1×10^7, and 0.17×10^3 to 6.54×10^3copies/g, respectively. The numbers of each gene showed an excellent and positive correlation with the rate of relevant processes.

(4) *Bacillus* and *Pseudomonas* were the predominant heterotrophic denitrifying bacteria genera identified in the systems. The autotrophic denitrifying bacteria genus was *Sulfurovum*, while the only identified anammox genus was *Candidatus Scalindua*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14213507/s1, Section S1. Calculation methods of potential rates of denitrification, anammox, and DNRA; Section S2. Analysis of bacterial community by 454 high-throughput 16S rRNA gene pyrosequencing; Figure S1. Accumulation of $^{29}N_2$ and $^{30}N_2$ in soil slurries spiked with 100 μM $^{15}NO_3^-$ at Sites KC (a), BG (b), SA (c), AC (d), and MF (e).

Author Contributions: Data curation and Formal analysis, W.G.; Investigation and Methodology, J.Y.; Project administration, J.F.; Writing—original draft, X.J. and Y.C.; Writing—review & editing, J.G. and W.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research and the APC were funded by the grant from Postdoctoral Advance Programs of Zhejiang Province (ZJ2021048) and the project of PowerChina Huadong Engineering Corporation Limited (KY2021-GH-02-03).

Data Availability Statement: The data presented in this study are available in insert article or supplementary material here.

Acknowledgments: This work was financially supported by the grant from Postdoctoral Advance Programs of Zhejiang Province (ZJ2021048) and the project of PowerChina Huadong Engineering Corporation Limited (KY2021-GH-02-03).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dai, Y.; Lin, X.; Luo, Y.; Sun, J.; Tian, Y. Molecular analysis of microbial nitrogen transformation and removal potential in mangrove wetlands under anthropogenic nitrogen input. *Sci. Total Environ.* **2021**, *773*, 145632. [CrossRef] [PubMed]
2. Michotey, V.; Méjean, V.; Bonin, P. Comparison of Methods for Quantification of Cytochrome cd1-Denitrifying Bacteria in Environmental Marine Samples. *Appl. Environ. Microb.* **2000**, *66*, 1564–1571. [CrossRef] [PubMed]
3. Yuan, H.Z.; Jia, B.C.; Zeng, Q.F.; Zhou, Y.W.; Wu, J.; Wang, H.X.; Fang, H.; Cai, Y.W.; Li, Q. Dissimilatory nitrate reduction to ammonium (DNRA) potentially facilitates the accumulation of phosphorus in lake water from sediment. *Chemosphere* **2022**, *303*, 134664. [CrossRef] [PubMed]
4. Hao, L.; Huang, J. Effect of Aquaculture Reclamation on Sediment Nitrates Reduction Processes in Mangrove Wetland. *J. Marine Sci. Eng.* **2022**, *10*, 857. [CrossRef]
5. Zhao, L.; Fu, G.P.; Tang, J.; Wu, J.F.; Pang, W.C.; Guo, Z.P. Efficient nitrogen removal of mangrove constructed wetlands: Enhancing heterotrophic nitrification-aerobic denitrification microflora through quorum sensing. *Chem. Eng. J.* **2022**, *430*, 133048. [CrossRef]
6. Leung, J.Y.S.; Cai, Q.H.; Tam, N.F.Y. Comparing subsurface flow constructed wetlands with mangrove plants and freshwater wetland plants for removing nutrients and toxic pollutants. *Ecol. Eng.* **2016**, *95*, 129–137. [CrossRef]
7. Nie, S.Q.; Zhang, Z.F.; Mo, S.M.; Li, J.H.; He, S.; Kashif, M.; Liang, Z.W.; Shen, P.H.; Yan, B.; Jiang, C.J. Desulfobacterales stimulates nitrate reduction in the mangrove ecosystem of a subtropical gulf. *Sci. Total Environ.* **2021**, *769*, 144562. [CrossRef]
8. Xiao, K.; Wu, J.; Li, H.; Hong, Y.; Wilson, A.M.; Jiao, J.J.; Shananan, M. Nitrogen fate in a subtropical mangrove swamp: Potential associationwith seawater-groundwater exchange. *Sci. Total Environ.* **2018**, *635*, 586–597. [CrossRef]
9. Zhang, M.P.; Dai, P.L.; Lin, X.L.; Lin, L.A.; Hetharua, B.; Zhang, Y.M.; Tian, Y. Nitrogen loss by anaerobic ammonium oxidation in a mangrove wetland of the Zhangjiang Estuary, China. *Sci. Total Environ.* **2020**, *698*, 134291. [CrossRef]
10. Baskaran, V.; Prabavathy, V.R. Diverse key nitrogen cycling genes *nifH*, *nirS* and *nosZ* associated with Pichavaram mangrove rhizospheres as revealed by culture-dependent and culture-independent analyses. *Arch. Microbiol.* **2022**, *204*, 109. [CrossRef]
11. Margulies, M.; Egholm, M.; Altman, W.E.; Attiya, S.; Bader, J.S.; Bemben, L.A.; Berka, J.; Braverman, M.S.; Chen, Y.J.; Chen, Z.T.; et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **2005**, *437*, 376–380. [CrossRef] [PubMed]
12. Deng, F.Y.; Hou, L.J.; Liu, M.; Zheng, Y.L.; Yin, G.Y.; Li, X.F.; Lin, X.B.; Chen, F.; Gao, J.; Jiang, X.F. Dissimilatory nitrate reduction processes and associated contribution to nitrogen removal in sediments of the Yangtze Estuary. *J. Geophys. Res. Biogeosci.* **2015**, *120*, 1521–1531. [CrossRef]
13. Risgaard-Petersen, N.; Meyer, R.L.; Schmid, M.; Jetten, M.S.M.; Enrich- Prast, A.; Rysgaard, S.; Revsbech, N.P. Anaerobic ammonium oxidation in an estuarine sediment. *Aquat. Microb. Ecol.* **2004**, *36*, 293–304. [CrossRef]
14. Yin, G.Y.; Hou, L.J.; Liu, M.F.; Liu, Z.F.; Gardner, W.S. A Novel Membrane Inlet Mass Spectrometer Method to Measure $^{15}NH^{4+}$ for Isotope-Enrichment Experiments in Aquatic Ecosystems. *Environ. Sci. Technol.* **2014**, *48*, 9555–9562. [CrossRef] [PubMed]
15. Shan, J.; Zhao, X.; Sheng, R.; Xia, Y.Q.; Ti, C.P.; Quan, X.F.; Wang, S.W.; Wei, W.X.; Yan, X.Y. Dissimilatory nitrate reduction processes in typical Chinese paddy soils: Rates, relative contributions, and influencing factors. *Environ. Sci. Technol.* **2016**, *50*, 9972–9980. [CrossRef] [PubMed]
16. Wang, H.T.; Gilbert, J.A.; Zhu, Y.G.; Yang, X.R. Salinity is a key factor driving the nitrogen cycling in the mangrove sediment. Sci Total Environ. *Sci. Total Environ.* **2018**, *631*, 1342–1349. [CrossRef]
17. Soares, M.I.M. Biological Denitrification of Groundwater. *Water Air Soil Pollut.* **2000**, *123*, 183–193. [CrossRef]
18. Chen, C.; Wang, A.J.; Ren, N.Q.; Kan, H.J.; Lee, D.J. Biological breakdown of denitrifying sulfide removal process in high-rate expanded granular bed reactor. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 765–770. [CrossRef]
19. Jin, R.C.; Yang, G.F.; Yu, J.J.; Zheng, P. The inhibition of the Anammox process: A review. *Chem. Eng. J.* **2012**, *197*, 67–79. [CrossRef]
20. Kuenen, J.G. Anammox bacteria: From discovery to application. *Nat. Rev. Microbiol.* **2008**, *6*, 320–326. [CrossRef]
21. Xi, H.P.; Zhou, X.T.; Arslan, M.; Luo, Z.J.; Wei, J.; Wu, Z.R.; El-Dinb, M.G. Heterotrophic nitrification and aerobic denitrification process: Promising but a long way to go in the wastewater treatment. *Sci. Total Environ.* **2022**, *805*, 150212. [CrossRef] [PubMed]
22. Deng, Y.F.; Wu, D.; Huang, H.; Cui, Y.X.; Van Loosdrecht, M.C.; Chen, G.H. Exploration and verification of the feasibility of sulfide-driven partial denitrification coupled with anammox for wastewater treatment. *Water Res.* **2021**, *193*, 116905. [CrossRef] [PubMed]
23. Ding, S.; Zheng, P.; Lu, H.F.; Chen, J.W.; Mahmood, Q.; Abbas, G. Ecological characteristics of anaerobic ammonia oxidizing bacteria. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 1841–1849. [CrossRef] [PubMed]
24. Li, B.L.; Wang, Y.; Li, J.T.; Yang, L.; Li, X.; Zhou, Z.; Li, Y.; Chen, X.G.; Wu, L. The symbiosis of anaerobic ammonium oxidation bacteria and heterotrophic denitrification bacteria in a size-fractioned single-stage partial nitrification/anammox reactor. *Biochem. Eng. J.* **2019**, *151*, 107353. [CrossRef]
25. Ma, H.R.; Li, J.T.; Dong, H.Y.; Qiang, Z.M. Insights into microbial community variability and functional genes of various Candidatus Scalindua-based anammox processes treating nitrogen-rich saline wastewater. *Sci. Total Environ.* **2021**, *766*, 142544. [CrossRef] [PubMed]