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Original Research Article

Invasive *Spartina alterniflora* accelerates the increase in microbial nitrogen fixation over nitrogen removal in coastal wetlands of China

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ABSTRACT

Salt marsh plants play a vital role in mediating nitrogen (N) biogeochemical cycle in estuarine and coastal ecosystems. However, the effects of invasive *Spartina alterniflora* on N fixation and removal, as well as how these two processes balance to determine the N budget, remain unclear. Here, simultaneous quantifications of N fixation and removal via ^{15}N tracing experiment with native *Phragmites australis*, invasive *S. alterniflora*, and bare flats as well as corresponding functional gene abundance by qPCR were carried out to explore the response of N dynamics to *S. alterniflora* invasion. Our results showed that N fixation and removal rates ranged from 0.77 ± 0.08 to 16.12 ± 1.13 nmol/(g·h) and from 1.42 ± 0.14 to 16.35 ± 1.10 nmol/(g·h), respectively, and invasive *S. alterniflora* generally facilitated the two processes rates. Based on the difference between N removal and fixation rates, net N_2 fluxes were estimated in the range of −0.39 ± 0.14 to 8.24 ± 2.23 nmol/(g·h). Estimated net N_2 fluxes in *S. alterniflora* stands were lower than those in bare flats and *P. australis* stands, indicating that the increase in N removal caused by *S. alterniflora* invasion may be more than offset by N fixation process. Random forest analysis revealed that functional microorganisms were the most important factor associated with the corresponding N transformation process. Overall, our results highlight the importance of N fixation in evaluating N budget of estuarine and coastal wetlands, providing valuable insights into the ecological effect of *S. alterniflora* invasion.

1. Introduction

Intensive industrial and agricultural activities have caused a continuous global increase in anthropogenic reactive nitrogen (N) at a rate of approximately 160 Tg per year [1]. Herein, about 20%–30% of reactive N is transported through hydrological and atmospheric pathways into estuarine and coastal ecosystems [2,3]. Increasingly prominent ecological and environmental issues in estuarine and coastal zones, such as eutrophication, harmful algal blooms, and hypoxia, are closely related to high N levels [4,5]. Alternatively, estuarine and coastal wetlands can regulate N loadings via microbial transformation [6]. Among microbial N transformations, denitrification, anaerobic ammonium oxidation (anammox), and N fixation are the important processes that directly regulate the N budget in aquatic environments [6–8]. Wherein, microbial N fixation, the reduction of atmospheric N_2 to available ammonia, is an internal source of reactive N in ecosystems, while denitrification and anammox can reduce nitrate (NO_3^-)/nitrite (NO_2^-) to gaseous N and result in reactive N removal [9]. For a long time, microbial N fixation has been assumed negligible in nutrient-rich estuarine environments [10,11]. As such, estuarine and coastal wetlands are often recognized as effective N filters, playing an important role in reducing the delivery of reactive N into the ocean through N removal processes [12,13]. However, the

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potential for soil/sediment N fixation may be widespread due to the prevalence of bacteria carrying the *nifH* (a gene associated with N fixation) even in N-rich environments [14,15]. In addition, anaerobic conditions in estuarine and coastal environments also favor the occurrence of N fixation process [15]. Indeed, recent studies show that N fixation occurs strongly in estuarine and coastal systems [15–17], which may largely offset the removal of combined N by denitrification and anammox. Hence, N fixation and removal may occur simultaneously at different microenvironments of estuarine and coastal wetlands [7,17]. Under such circumstances, the ecological function of N purification in estuarine and coastal ecosystems needs to be further evaluated by simultaneously quantifying N fixation and removal.

Exotic plant invasion is one of the most important global ecological problems that threaten the services and function of the ecosystem [18]. In 1979, *Spartina alterniflora*, a perennial C_4 plant, was introduced to China from the eastern coast of North America and the Gulf of Mexico with the aim of providing coastal protection [19,20]. Over the past 40 years, *S. alterniflora* has undergone rapid expansion, and has emerged as a dominant salt marsh plant in China's coastal zone (Fig. 1). *S. alterniflora* invasion not only threatens and replaces some native plants, but also affects ecosystem structure, ecosystem functioning, and element biogeochemical processes [19,21]. To date, many studies have explored the effects of *S. alterniflora* invasion-induced changes on soil/sediment N transformations such as denitrification, anammox, dissimilatory nitrate reduction to ammonium (DNRA), and nitrification [22–24]. In general, invasive *S. alterniflora* can promote denitrification and anammox rates, indicating that this invasive plant has positive effects on N elimination in estuarine and coastal wetlands [23,24]. It has also been noted that *S. alterniflora* invasion increases N fixation rates, which may aggravate N loadings in such ecosystems [14]. However, there have been few studies concerning concurrent quantification of N removal and fixation to evaluate the response of N budgets to *S. alterniflora* invasion in estuarine and coastal wetlands so far.

S. alterniflora is currently distributed in 10 provinces of China, of which Fujian, Zhejiang, Shanghai, and Jiangsu account for 94.13% of the total distribution area (Fig. 1) [19]. To cover the representative zones that have been invaded by *S. alterniflora*, we selected four independent locations from the aforementioned provinces (Fujian, Zhejiang, Shanghai, and Jiangsu) for experimental research. The primary goals of this study were (i) to explore the effects of *S. alterniflora* invasion on microbial N removal (denitrification + anammox) and fixation rates; (ii) to elucidate the key environmental variables influencing soil N removal and fixation rates; (iii) to evaluate how the invasion of *S. alterniflora* affected N loadings in estuarine and coastal ecosystems.

2. Materials and methods

2.1. Study area description and sampling

This study was conducted in Min River Estuary wetland (MR), Yueqing Bay wetland (YQ), Yangtze Estuary wetland (YR), and Yancheng wetland (YC) (Fig. 1). These areas are mainly influenced by subtropical monsoonal climate, with annual mean temperature and precipitation of 13.8–19.3 °C and 1,000–1,390 mm, respectively [25,26]. Following its introduction to China, *S. alterniflora* has dominated as an invasive plant in the coastal wetlands of China. The coasts of Fujian, Zhejiang, Shanghai, and Jiangsu are the most typical area for the invasion of *S. alterniflora* [19]. Thus, we selected an about 800 m long transect spanning *Phragmites australis*, *S. alterniflora* (invaded from 9 to 15 years ago), and bare mudflat in MR, YQ, YR, and YC, respectively. In each habitat, three independent replicate sites were selected, and surface (upper 5 cm) soil samples were collected with stainless steel soil cylinders (inside-diameter 15 cm) in July 2017 and January 2018. A cutting ring (height: 5 cm, diameter: 5 cm) was pressed into the collected soil to determine water content and bulk density [27]. Afterward, the samples were transferred with care into sterile ziplock bags and subsequently stored in a cooler at 4 °C. In the laboratory, the collected soil was homogenized, and visible stones and root residues were removed. Subsequently, the samples were divided into two parts, one frozen at −80 °C for DNA extraction and molecular analysis, and the other stored at 4 °C for physicochemical and N process rate determination.

2.2. Analysis of soil physicochemical parameters

Soil dry bulk density and water content were measured using the cutting-ring and oven-drying methods [28]. Water-filled pore space (WFPS) of soil was further estimated based on the method described by Yang et al. [27]. Soil salinity and pH were measured in fresh soil suspension with deionized water (w:v = 1:2.5) by YSI-30 portable salinity meter and Mettler-Toledo pH meter, respectively [29]. The portable Eh meter (ZKNT-QX6530) was used to determine soil redox potential (Eh). Soil ammonia (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) were extracted using 2 M potassium chloride (KCl), and their concentrations were measured using a continuous-flow nutrient analyzer (Skalar Analytical SAN++, Netherlands) [30]. Soil total organic carbon (TOC) was

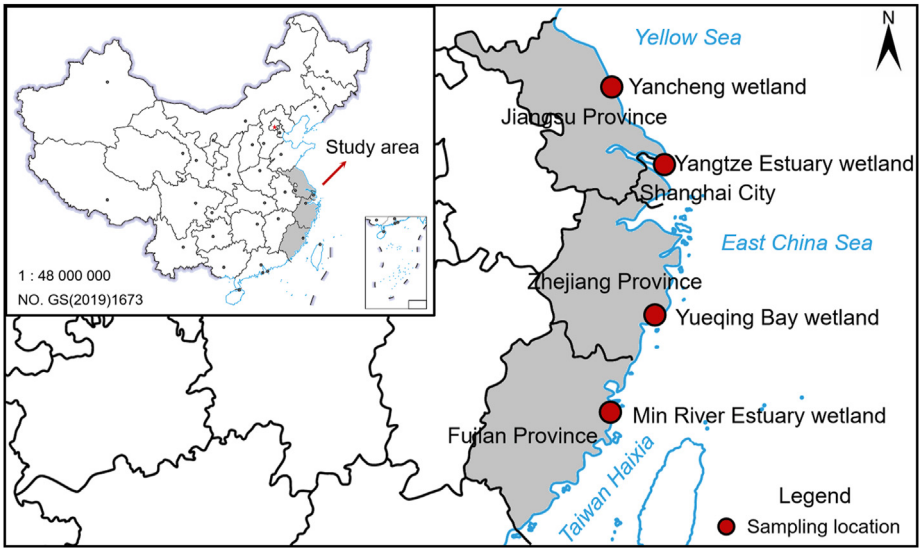

Fig. 1. Study area and sampling locations. Each location has three wetland habitats, including *S. alterniflora*, *P. australis* and bare flat.

analyzed based on the potassium dichromate ($K_2Cr_2O_7$) oxidation method [31]. The Methylene blue spectrophotometric method was used to determine soil sulfide content [32].

2.3. Determination of N transformation rates

Soil-slurry incubations in combination with ^{15}N isotope tracing method were used to measure potential N fixation rates [33]. Briefly, homogenized slurries were prepared by mixing tidal water and fresh soil at a 7:1 ratio (v:w). The mixture was purged with helium for approximately 30 min. Subsequently, the 40 mL prepared slurries were transferred into a 60 mL serum bottle and sealed with septum caps in a helium-filled glove box. Then, all bottles were injected with 2 mL $^{15}N_2$ (99 atom% ^{15}N; Campro Scientific, Germany). Half of the bottles were immediately preserved with 1 mL of saturated mercuric chloride solution and labeled as initial samples. The remaining bottles, labeled as final samples, were incubated at in situ temperature for 24 h and then inhibited by adding 1 mL of saturated mercuric chloride solution at the end of the incubation period. All slurry samples were purged by helium for approximately 30 min to eliminate the residual N_2, and then the slurries were transferred into 12 mL vials (Exetainer, Labco). Subsequently, a 0.1 mL hypobromite iodine solution (oxidizing agent) was injected to oxidize the $^{15}NH_4^+$ and/or organic N fractions into $^{30}N_2$ and/or $^{29}N_2$, and the oxide products in the initial and final vials were measured by membrane inlet mass spectrometry (MIMS) [34,35]. Potential N fixation rates were calculated based on Eq. 1

$$R_{\text{N fixation}} = (^{15}N_{\text{final}} - {}^{15}N_{\text{initial}}) \times V \times W^{-1} \times T^{-1} \quad (1)$$

where $R_{\text{N fixation}}$ [nmol/(g·h)] represents N fixation rates; The $^{15}N_{\text{initial}}$ and $^{15}N_{\text{final}}$ (nmol/mL) denote the contents of ^{15}N-labeled products in initial and final bottles, respectively; T (h) is incubation time; W (g) and V (mL) represent soil dry weight and volume of incubation bottle, respectively [33].

Potential rates of anammox and denitrification were also measured by the slurry experiments combined with N isotope-tracing method [12,23]. The slurries were made as described for the above-mentioned N fixation experiments. Subsequently, soil slurries were transferred into 12-mL vials (Labco Exetainers), which were sealed with butyl rubber stoppers. To consume background NO_x^- (NO_3^- and NO_2^-) and oxygen, these vials were pre-incubated at near field temperature for 48 h in dark conditions. All vials were amended with $^{15}NO_3^-$ (^{15}N at 99%) after pre-incubation, and the concentration of NO_3^- in the vial was approximately 50 μM. Then, one-half of the vials were preserved with saturated mercuric chloride solution (0.1 mL), and these vials were marked as initial samples. The remaining vials were inhibited by injecting saturated mercuric chloride solution (0.1 mL) after 8 h incubation and marked as final samples. Produced $^{30}N_2$ and $^{29}N_2$ concentrations in the vials were determined by MIMS, and denitrification (R_{DNF}) and anammox (R_{ANA}) rates were further estimated based on the production of $^{30}N_2$ and $^{29}N_2$ between the final and initial samples [23,36]. Potential R_{DNF} was determined by ^{15}N tracer techniques based on the assumption of N_2 as the only end product because the ratio of N_2O to N_2 from denitrification in aquatic ecosystems is very small [36]. In this incubation experiment, the preliminary tracer experiment of $^{15}NH_4^+$ was used to confirm the occurrence of anammox [37]. Here, N removal rates were quantified by Eq. 2.

$$R_{\text{N removal}} = R_{DNF} + R_{ANA} \quad (2)$$

where $R_{\text{N removal}}$ denotes N removal rates; R_{DNF} and R_{ANA} represent denitrification and anammox rates, respectively. In addition, soil net N_2 flux ($F_{\text{net } N_2}$) was calculated by Eq. 3.

$$F_{\text{net } N_2} = R_{\text{N removal}} - R_{\text{N fixation}} \quad (3)$$

2.4. Molecular microbial analysis

In the present study, *hzo*, *nirS,* and *nifH* gene abundances, which encode the key enzymes for anammox, denitrification, and N fixation, respectively, were quantified to reveal the potential effects of functional microorganisms on relevant N processes. According to the instructions, the PowersoilTM DNA Isolation Kits (MO BIO, USA) was used to extract soil DNA. Subsequently, *hzo*, *nirS*, and *nifH* gene abundances were analyzed based on the Real-time qPCR using the SYBR green qPCR method and an ABI 7500 Sequence Detection System (Applied Biosystems, Canada). The detailed primer information and q-PCR thermocycling conditions for *nifH*, *nirS,* and *hzo* genes are shown in Table S1 [38–40]. *hzo*, *nirS,* and *nifH* gene abundances were calculated based on the standard curve constructed by a 10-fold dilution series of their respective plasmids DNA standard. The specificity of the qPCR was verified using a single-peak melting curve. The amplification efficiency for these genes was greater than 94%, with a correlation coefficient larger than 0.97.

2.5. Statistical analysis

In this study, the significant differences among soil physicochemical properties, functional gene abundances, and N process rates were tested by One-way analysis of variance (ANOVA) with R (v.4.2.0) *aov* function. The relationships among N process rates, physical-chemical characteristics, and functional gene abundances were evaluated by Spearman's correlation analysis using R (v.4.2.0) *cor* function. The statistical significance was determined at $P < 0.05$. The classification random forest (RF) analysis was used to reveal the potential contributions of abiotic and biotic factors to different N transformation rates [41]. The importance of these environmental variables was evaluated by the percentage increases in mean squared error (MSE) of variables [42]. We also used structural equation modeling (SEM) to reveal the direct and indirect effects of physicochemical factors and functional gene abundance on soil net N_2 fluxes using the *sem* R package.

3. Results

3.1. Soil physicochemical parameters

Soil WFPS ranged from 45.16% ± 5.85% to 64.11% ± 6.10% in summer and from 45.81% ± 2.78% to 58.63% ± 6.42% in winter. Soil WFPS in bare flats was generally higher than in *S. alterniflora* and *P. australis* stands except for YQ wetlands (Fig. S1). Soil bulk density was also higher in bare flats than in *S. alterniflora* and *P. australis* stands. Soil pH in *S. alterniflora* stands was generally lower compared to bare flats, although some differences were not significant. Soil salinity (0.54 ± 0.10 to 2.32 ± 0.12) in YQ and YC wetlands was significantly higher than in MR and YR wetlands. However, no significant difference was observed between different sampling stands within the same wetland (Fig. S1). Soil Eh varied from 135.69 ± 17.44 mV to 445.57 ± 18.11 mV, and the values in bare flats were generally higher than those in *S. alterniflora* and *P. australis* stands. TOC concentrations ranged from 13.00 ± 2.50 g/kg to 27.66 ± 1.82 g/kg, and *S. alterniflora* and *P. australis* stands had relatively higher values compared to bare flats. Soil NH_4^+ and NO_3^- concentrations were in the range of 13.96 ± 2.50 mg N/kg to 26.31 ± 2.34 mg N/kg and 0.72 ± 0.16 mg N/kg to 1.69 ± 0.33 mg N/kg, respectively. There were no significant differences among sampling stands in the same wetland except for YR wetland (Fig. S1). Soil NO_2^- concentrations ranged from 14.14 ± 1.33 μg N/kg to 36.51 ± 6.06 μg N/kg, and the values in bare flats were generally lower than those in *S. alterniflora* stands (Fig. S1). Sulfide contents varied from 1.25 ± 0.26 mg/kg to 6.58 ± 0.98 mg/kg, and higher values generally occurred in *S. alterniflora* stands.

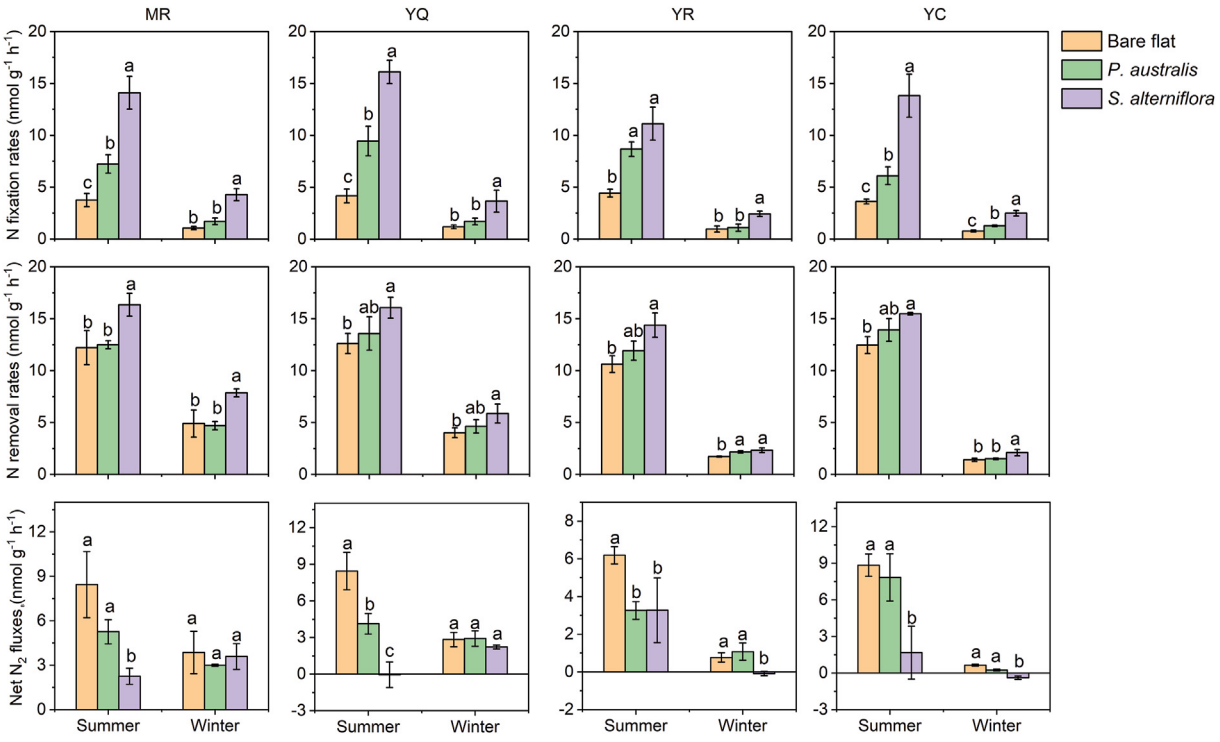

Fig. 2. Soil microbial N fixation and removal rates as well as net N_2 fluxes among different habitats. MR, Min River Estuary wetland; YQ, Yueqing Bay wetland; YR, Yangtze Estuary wetland; YC, Yancheng wetland. Different lowercase letters represent significant differences ($P < 0.05$) among different habitats.

3.2. Microbial N fixation and removal rates

Microbial N fixation rates ranged from 3.62 ± 0.23 nmol/(g·h) to 16.12 ± 1.13 nmol/(g·h) in summer and from 0.77 ± 0.08 nmol/(g·h) to 4.28 ± 0.58 nmol/(g·h) in winter, and the values in winter were significantly lower than those in summer (Fig. 2). In summer, the process rates of N fixation were highest in *S. alterniflora* stands, followed by *P. australis* stands and bare flats. In winter, the highest N fixation rates also occurred in

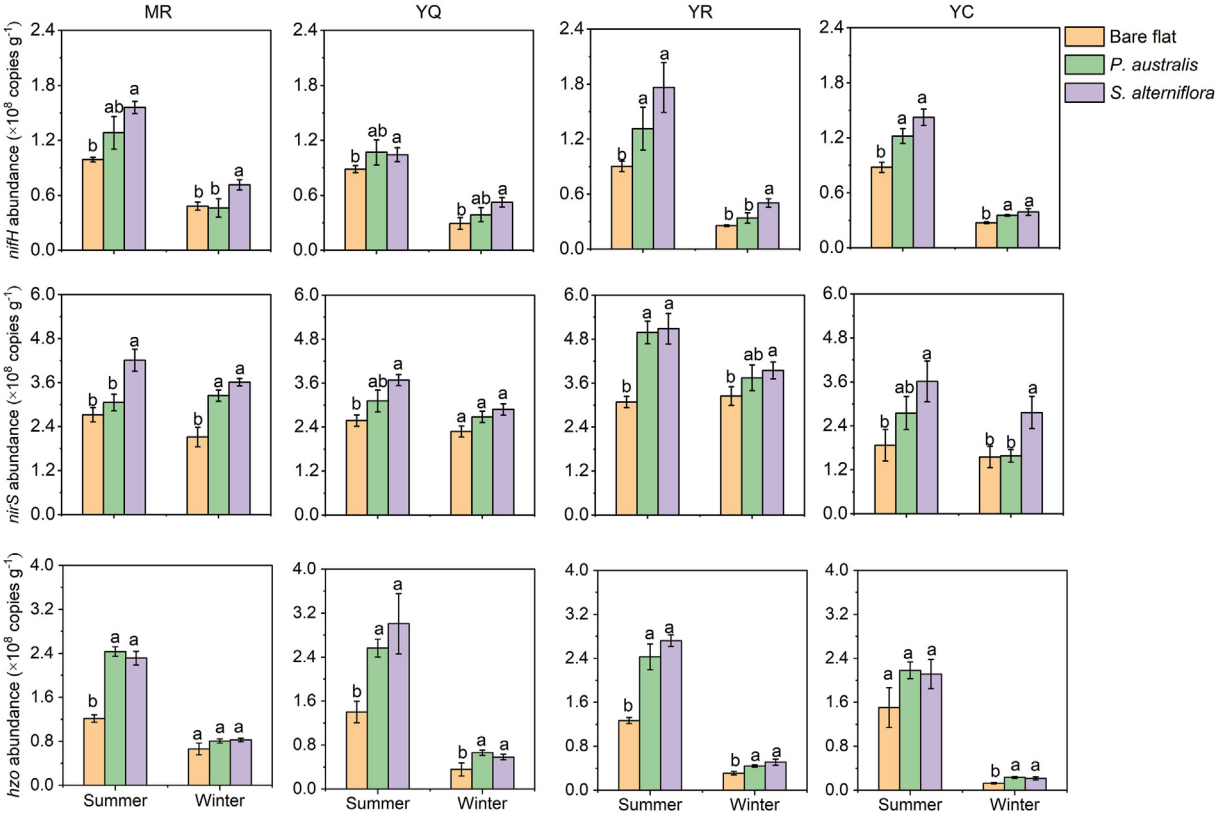

Fig. 3. Soil *nifH*, *nirS* and, *hzo* gene abundance among different habitats.

S. alterniflora stands. However, there were no significant differences between *P. australis* stands and bare flats, except for YC wetland (Fig. 2). Microbial N removal rates in winter [1.42 ± 0.14 nmol/(g·h) to 7.87 ± 0.39 nmol/(g·h)] were also significantly lower than those in summer [10.62 ± 0.81 nmol/(g·h) to 16.35 ± 1.10 nmol/(g·h)]. The N removal rates in *P. australis* stands and bare flats were generally lower than those in *S. alterniflora* stands. However, there was no significant difference in N removal rates between *P. australis* stands and bare flats (Fig. 2). Soil net N_2 fluxes were further estimated based on microbial N removal and fixation rates, with a range of -0.39 ± 0.14 nmol/(g·h) to 8.24 ± 2.23 nmol/(g·h). Net N_2 fluxes were lower in *S. alterniflora* stands than in *P. australis* stands and bare flats, except for MR and YQ wetlands in winter (Fig. 2).

3.3. Relevant gene abundance

Soil *nifH* gene abundance varied from $8.78 \times 10^7 \pm 5.61 \times 10^6$ copies/g to $1.76 \times 10^8 \pm 2.73 \times 10^7$ copies/g in summer and from $2.55 \times 10^7 \pm 7.68 \times 10^5$ copies/g to $7.14 \times 10^7 \pm 5.50 \times 10^6$ copies/g in winter. The abundance of *nifH* gene was significantly lower in bare flats compared to *S. alterniflora* stands, and the *nifH* gene abundance in *P. australis* stands was also generally lower than that of *S. alterniflora* stands, although some differences were not significant (Fig. 3). Soil *nirS* gene abundance ranged from $1.87 \times 10^8 \pm 4.28 \times 10^7$ copies/g to $5.08 \times 10^8 \pm 4.16 \times 10^7$ copies/g in summer and from $1.55 \times 10^8 \pm 2.92 \times 10^7$ copies/g to $3.95 \times 10^8 \pm 2.31 \times 10^7$ copies/g in winter, respectively. The abundances were generally lower in bare flats, followed by *P. australis* and *S. alterniflora* stands (Fig. 3). *hzo* gene abundance in *S. alterniflora* and *P. australis* stands ($2.11 \times 10^8 \pm 2.65 \times 10^7$ copies/g to $3.01 \times 10^8 \pm 5.49 \times 10^7$ copies/g for summer and $2.16 \times 10^7 \pm 2.65 \times 10^6$ copies/g to $8.26 \times 10^7 \pm 3.07 \times 10^6$ copies/g for winter) were generally higher than in bare flats ($1.21 \times 10^8 \pm 6.82 \times 10^6$ copies/g to $1.50 \times 10^8 \pm 3.63 \times 10^7$ copies/g for summer and $1.28 \times 10^7 \pm 1.02 \times 10^6$ copies/g to $6.62 \times 10^7 \pm 1.07 \times 10^7$ copies/g for winter). There was no obvious difference in *hzo* gene abundances between *S. alterniflora* and *P. australis* stands (Fig. 3).

3.4. Environmental variables affecting N process rates

In bare flats, N fixation and removal rates, as well as net N_2 fluxes were significantly correlated to soil Eh, TOC content, *nifH* gene abundance, and *hzo* gene abundance ($P < 0.05$; Fig. 4). In addition, N removal rates and net N_2 fluxes were both significantly correlated to soil bulk density ($P < 0.05$; Fig. 4). We further conducted a random forest (RF) analysis to determine the contribution of environmental variables, both biotic and abiotic, to different N transformation processes. Obviously, *nifH* and *hzo* gene abundance was the most important variable for N fixation and N removal rates as well as net N_2 fluxes (Fig. 4). The best SEM models showed that net N_2 fluxes in the bare flats were mainly explained by *nifH* gene abundance and soil Eh (Fig. 5).

In *P. australis* stands, N fixation and N removal rates, as well as net N_2 fluxes were correlated significantly with soil TOC content, Eh, *nifH* gene abundance, and *hzo* gene abundance ($P < 0.05$; Fig. 4). Besides, N fixation rates and removal were both in relationship with soil sulfide content ($P < 0.05$). The RF analysis revealed that soil Eh, *nifH* gene abundance and *hzo* gene abundance were the most important factors in controlling N fixation, N removal, and net N_2 flux (Fig. 4). *nifH* and *hzo* gene abundance explained mostly the variations of net N_2 fluxes in *P. australis* stands (Fig. 5).

In *S. alterniflora* stands, N fixation and N removal rates as well as net N_2 fluxes were all significantly related to *nifH* gene abundance ($P < 0.05$; Fig. 4). N fixation and N removal rates were both significantly correlated to soil Eh, TOC content, sulfide content and *hzo* gene abundance ($P < 0.05$). Net N_2 fluxes correlated significantly with soil salinity and *nirS* gene abundance ($P < 0.05$; Fig. 4). The RF analysis showed that soil TOC, *nifH* and *hzo* gene abundance were the most important factors for N fixation, N removal and net N_2 flux (Fig. 4). The best SEM models indicated that net N_2 flux in *S. alterniflora* zones was mainly influenced by *nifH* gene abundance and soil salinity (Fig. 5).

4. Discussion

Estuarine and coastal ecosystems are hot spots for N cycling processes [43,44], helpfully mitigating the N pollution via the complete N removal processes of denitrification and anammox [37,45]. Salt marsh plants play a vital role in mediating N biogeochemical cycles in estuarine and coastal ecosystems [46,47]. Exotic *S. alterniflora* invasion can influence soil physicochemical characteristics as well as microbial activities and diversities, further altering N dynamics in estuarine and coastal soils [23, 48]. Generally, soil N removal rates were intimate with redox environments, substrate availabilities, and relevant functional microbial activities [49–51]. In this study, *S. alterniflora* invasion significantly increased soil N removal rates compared to *P. australis* and bare flats (Fig. 2), likely

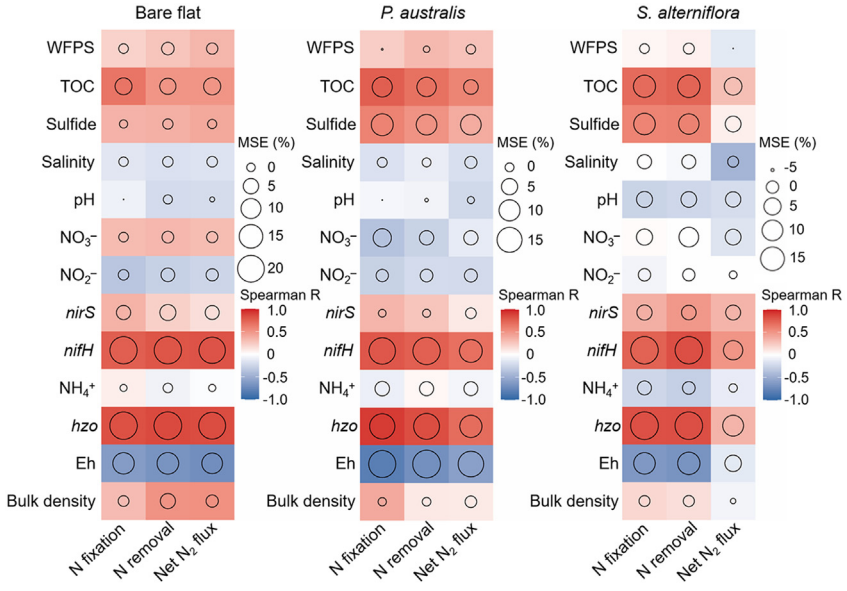

Fig. 4. Potential contributions of abiotic and biotic variables to N transformation rates (N fixation rate, N removal rates and net N_2 fluxes). Circle size denotes the factor importance estimated by percentage of increase of mean square error (MSE%), and higher MSE% values represent more important factor. The shades of colour denote Spearman correlation strength.

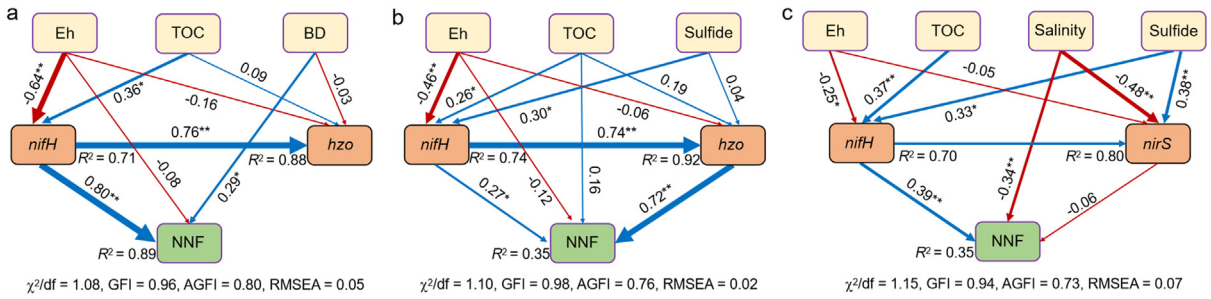

Fig. 5. Maximum variation in net N_2 fluxes (NNF) in bare flat (a), *P. australis* (b) and *S. alterniflora* (c) stands was explained by structural equation modeling (SEM) via edaphic physicochemical factors and function gene (*nifH*, *nirS* and *hzo*) abundance. BD, Bulk density.

due to changes in soil physicochemical properties. TOC can supply electron donors and energy sources for the growth of denitrifiers, and higher TOC contents are generally accompanied by larger denitrification rates [23,50]. Invasive *S. alterniflora* has stronger photosynthesis and more photosynthate carbon will be allotted to soils by rhizodeposition [52]. TOC contents in planted soils were significantly higher than in bare flats in the present study (Fig. S1). Here, the rhizodeposits can facilitate organic matter decomposition and increase the denitrification process rate [8]. Anammox does not require a direct energy source, but the rhizodeposits stimulated the mineralization activity converting organic matter to NH_4^+, and more available NH_4^+ could promote the anammox process rate [33]. The correlation and classification RF analyses supported the importance of TOC in relation to N removal rates (Fig. 4).

In addition, previous studies indicated that higher soil inorganic N (NO_3^-, NO_2^-, and NH_4^+) contents could stimulate N removal processes [53,54]. However, our results showed that the difference in NH_4^+ and NO_3^- concentrations between *S. alterniflora* and the bare flat was mostly not significant (Fig. S1), implying that the change of inorganic N content was not the limiting factor associated with the N removal process in the present study. In general, root exudates of *S. alterniflora* can stimulate microbial activities, and increase soil inorganic N availabilities via special N transformation processes (e.g., N mineralization and nitrification) [55,56]. However, higher N uptake of *S. alterniflora* can weaken this effect [57]. A significant correlation between N removal rates with soil bulk density was observed in bare flats, but there were no obvious relationships in *P. australis* and *S. alterniflora* stands (Fig. 4). It was expected that larger bulk density could decrease the O_2 concentration in soil and thus accelerate anaerobic N removal rates in the bare flat [28]. In contrast, decreased soil bulk density associated with *S. alterniflora* invasion would increase the O_2 concentration in soil, which was adverse to the occurrence of N removal processes. However, this disadvantageous condition for N removal processes could be counteracted by accelerating soil respiration in *S. alterniflora* stands [23,58]. Overall, denitrification and anammox are anaerobic microbial processes that require a lower Eh for optimal conditions [59], which was also supported by the correlation and RF analyses.

It has been noted that N removal processes are mediated by the activity of functional microorganisms, and both of them generally exhibit an intimate relationship [60,61]. Our study showed that *nirS* and *hzo* gene abundance in bare flats was lower than that in *S. alterniflora* stands (Fig. 3). In addition, a significant and positive correlation between N removal rates and *hzo* gene abundance rather than *nirS* gene was observed. This phenomenon suggested that other functional microbes (e.g., *nirK*-denitrifiers), except for *nirS*-denitrifiers, may be the main driver of the N removal process. Nevertheless, functional gene abundance based on DNA level cannot completely reflect the activity of microorganisms [50], so further experiments (metagenomics and/or functional gene expression) should be conducted to determine the role of microbes in soil N transformations in future studies.

Microbial N fixation has been frequently neglected in evaluating N budgets in eutrophic estuaries and coasts in previous studies [6]. However, recent studies, with the development of ^{15}N tracing techniques, have shown that microbial N fixation is a crucial component of N dynamics in estuarine and coastal soils/sediments. [7,15,62], which offered new insight into N biogeochemical cycles in such ecosystems. In the present study, the process rates of microbial N fixation ranged from 0.77 to 16.12 nmol/(g·h) [equal to 0.48–2.69 mmol N/(m^2·d), based on measured soil bulk], which was comparable to those in a bioturbated coastal lagoon [0.8–8.5 mmol N/(m^2·d)], subtidal sediment [0.6–15.6 mmol N/(m^2·d)] and eutrophic estuary [0–18 mmol N/(m^2·d)] [63–65]. We found that invasive *S. alterniflora* generally promoted N fixation rate compared to bare flats and native *P. australis* (Fig. 2). Previous research indicated that the change of N fixation rate was tightly related to the activities of diazotrophic communities [66], which was supported by the significantly positive relationship between *nifH* gene abundance and N fixation rates. In addition, RF analysis indicated that *nifH* gene abundance was the most important controlling factor associated with N fixation in the present study (Fig. 4). Zheng et al. [67] revealed that invasive *S. alterniflora* facilitated the proliferation of sulfate-reducing bacteria characterized by fixing N_2 in Chongming Dongtan wetland of the Yangtze Estuary, which also can explain the increase in N fixation rate in our *S. alterniflora* stands. Spearman's correlation analysis showed that environmental variables also play significant effects on N fixation rates. Organic matter availability is generally considered an important factor controlling N fixation as it provides energy for heterotrophic organisms [68]. In this study, higher soil TOC contents in *P. australis* and *S. alterniflora* stands were observed relative to bare flats and N fixation rates were positively correlated with soil TOC contents (Fig. 4). It can be expected that quantities of photosynthate-carbon to soil continuously increased as the growth of *P. australis* and *S. alterniflora*, and thus stimulated the activities of rhizosphere diazotrophic bacteria due to secretion of rhizosphere organic matter (e.g., organic acids and soluble sugars). Hence, this rhizosphere priming effect (RPE) accelerated N fixation rates in *S. alterniflora* and *P. australis* stands [69]. Jofré et al. [70] reported that salinity can influence the release of root exudates and the acquisition of carbon sources and energy for diazotrophic bacteria, thus mediating microbial N fixation. In this study, there was no significant difference in salinity among *P. australis*, *S. alterniflora*, and bare flat (Fig. S1). In addition, no positive correlation between N fixation rate and salinity was observed in this study (Fig. 4), which implied that the response of diazotrophic bacteria to salinity was not sensitive. Many studies have indicated that sulfide may enhance N fixation rate [68,71]. It can be explained that sulfate was heterotrophically reduced to sulfide, in which sulfate reducers could degrade organic matter and thus provide more energy to microenvironments, which stimulated N fixation [71]. In *S. alterniflora* and *P. australis* stands, N fixation rates were positively related to sulfide concentrations, but this relationship did not occur in the bare flat (Fig. 4). This phenomenon suggested that diazotrophic bacteria in bare flats and salt marsh plants may have various levels of sensitivity to sulfide concentrations. In contrast, some studies found that sulfide could inhibit the N fixation process due to its toxicity to organisms [68,72]. Hence, potential mechanisms of N fixation response to sulfide concentrations need to be explored in the future.

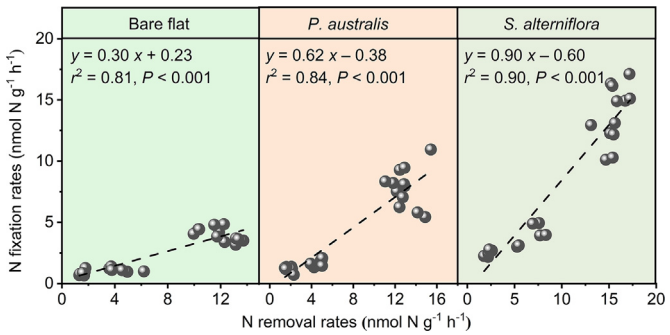

Fig. 6. Correlation between N fixation and N removal rates among the bare flat, *P. australis* and *S. alterniflora* stands.

Generally, invasive *S. alterniflora* significantly increased N removal and fixation rates in estuarine and coastal ecosystems (Fig. 2). However, the potential effects of *S. alterniflora* invasion on N loadings could not be well assessed based on only N removal or fixation rates. The study further evaluated soil net N_2 fluxes with a major focus on the difference between the N removal rate-based sum of denitrification and anammox and N fixation. Our results showed that soil net N_2 fluxes in *P. australis* stands and bare flats were generally higher than those in *S. alterniflora* stands (Fig. 2). The RF analysis and SEM models indicated that the activities of functional microorganisms exerted a key influence on soil net N_2 fluxes (Figs. 4 and 5). The study found a close relationship between N removal and N fixation rates in all stands (Fig. 6), as the increase in TOC content from bare flats to *P. australis* and *S. alterniflora* stands created favorable conditions for both processes [24,73]. The slopes of the linear fitted formulas between N removal and fixation rates in bare flats, *P. australis* and *S. alterniflora* stands were 0.23, 0.62, and 0.90, respectively (Fig. 6). This suggested that N fixation rates were gradually close to N removal rates from bare flats to *S. alterniflora* stands, and the N fixation was nearly sufficient to counteract N removal with *S. alterniflora* invasion. In this context, we deduced that only a fraction of removed N in *S. alterniflora* wetlands might be associated with upstream N enrichment because the increase in N removal caused by *S. alterniflora* invasion may be more than offset by the N fixation process. If *S. alterniflora* further expands, more exogenous reactive N will be exported to offshore zones, thus increasing the N loadings. However, in the present study, N removal processes were merely considered with the sum of denitrification and anammox, other N_2 production processes contributing to soil net N_2 flux, such as Feammox and nitrite/nitrate-dependent anaerobic methane oxidation [74,75], require further study. It should also be noted that *P. australis*, *S. alterniflora,* and bare flats are usually distributed in three different ecological regions, and the N transformations in these three ecological regions may be different due to the variations in hydrological characteristics. Nevertheless, our selected sampling habitats are relatively close to each other in terms of tidal flats scale, so the differences in N transformations between sampling habitats are mainly thought to be caused by vegetation. Here, the N transformations influenced by different hydrological characteristics should be further explored. Overall, our results help improve our understanding of the N budget in estuarine and coastal wetlands, and highlight the important role of considering the N fixation process.

5. Conclusions

In this study, the rates of N fixation and removal were quantified via a ^{15}N tracing experiment, and the functional gene abundance (*nifH*, *nirS,* and *hzo*) was measured by molecular biological techniques. We then dissected the variations in N fixation and removal activity in response to *S. alterniflora* invasion compared to *P. australis* and bare flats in different estuarine and coastal wetlands. The result indicated that *S. alterniflora* invasion generally increased N fixation and removal rates among the different estuarine and coastal wetlands. The functional gene abundance revealed a parallel variation trend to corresponding N transformation rates. In contrast, soil net N_2 fluxes were lower in *S. alterniflora* stands than in *P. australis* stands and bare flats, which suggested that the increase in N removal caused by *S. alterniflora* invasion may be more than offset by N fixation process. The RF analysis and the SEM models revealed that soil Eh, TOC, and functional gene abundance (*nifH* and *hzo*) were the key environmental variables controlling the changes in N removal and fixation rates as well as soil net N_2 fluxes. Overall, our findings shed new light on the ecological role of N purification in estuarine and coastal ecosystems with plant invasion, and emphasize the importance of considering N fixation when evaluating the N budget.

Author contributions

S.T.C.: investigation, formal analysis, writing–original draft, writing–review & editing. D.Z.G.: conceptualization, funding acquisition, methodology, formal analysis, writing–review & editing, supervision. X.F.L. and Y.H.N.: methodology, writing–review & editing. C.L. and D.Y.S.: investigation, writing–review & editing. Y.L.Z., H.P.D., X.L., G.Y.Y. and X.B.L.: writing–review & editing. M.L.: conceptualization, funding acquisition, project administration. L.J.H.: conceptualization, funding acquisition, project administration, writing–review & editing, supervision.

Declaration of competing interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

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