Responses of soil nitrogen fixation to *Spartina alterniflora* invasion and nitrogen addition in a Chinese salt marsh

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Biological nitrogen fixation (BNF) is the major natural process of nitrogen (N) input to ecosystems. To understand how plant invasion and N enrichment affect BNF, we compared soil N-fixation rates and N-fixing microbes (NFM) of an invasive *Spartina alterniflora* community and a native *Phragmites australis* community in the Yangtze River estuary, with and without N addition. Our results indicated that plant invasion relative to N enrichment had a greater influence on BNF. At each N level, the *S. alterniflora* community had a higher soil N-fixation rate but a lower diversity of the *nifH* gene in comparison with the native community. The *S. alterniflora* community with N addition had the highest soil N-fixation rate and the *nifH* gene abundance across all treatments. Our results suggest that *S. alterniflora* invasion can increase soil N fixation in the high N-loading estuarine ecosystem, and thus may further mediate soil N availability.

Biological nitrogen fixation (BNF) is a major part of N cycling. The total terrestrial N-fixation rate is about 58 Tg N yr⁻¹, accounting for more than 97% of new N input to unmanaged terrestrial ecosystems²,³. BNF rates vary among ecosystems⁴. Many biotic and abiotic factors such as N-fixing microbe (NFM) diversity, soil C:N ratio, and soil P level can affect BNF⁵,⁶. Recently, some studies have found that plant invasion and N enrichment can considerably affect BNF. For example, *Ageratina adenophora* invasion significantly increases the diversity and abundance of N-fixing bacteria in a forest ecosystem⁷. The amount of fixed N by invasive *Myrica faya* (18 kg N ha⁻¹ yr⁻¹) is far greater than by a native plant (0.2 kg N ha⁻¹ yr⁻¹)⁸. In addition, N enrichment is shown to change N-fixing bacterial community and abundance of functional N-fixation genes⁹,¹⁰. Although these studies suggest that plant invasion and N enrichment can influence BNF, their combined effects remain unclear.

Plant invasions are major threats to native ecosystems. Invasive plants can destroy native habitats, replace native plants, and change nutrient cycling and soil properties¹¹,¹². In the eastern coast of China, exotic grass *Spartina alterniflora* was introduced in 1979¹³. After its initial appearance on the Chongming Island located in the Yangtze River estuary in mid-1990’s, it has replaced native *Phragmites australis* and *Scirpus mariqueter* and becomes a dominant plant species there¹⁴. *S. alterniflora* invasion changes soil microorganism community¹⁴,¹⁵ and stimulates greenhouse gas fluxes¹⁶. *S. alterniflora* invasion also increases soil C and N pool sizes¹⁷. Our previous study suggests that N concentration of *S. alterniflora* litter increases as it decomposes in the air and soil, whereas N concentration of *P. australis* litter decreases¹⁸.

In addition, the Yangtze River estuary has long suffered from high N loading due to human activities. It is estimated that approximately 1.339 × 10⁶ t of dissolved inorganic N (DIN) has been discharged to the Yangtze River estuary in 2007¹⁹. N enrichment in the Yangtze River has increased from 13.6 Tg in 1990 to 19.8 Tg in 2000, and the proportion of anthropogenic N increased from 48% in 1980 to 68% in 2000²⁰. DIN concentrations are predicted to be 2.2–3.0 mg L⁻¹ between 2020 and 2050²¹. N enrichment can lead to altered plant composition²¹.
promote biological invasion22,23, and alter C/N cycling24,25 and microbial activity26. In the Yangtze River estuary, for example, N enrichment stimulates CH4 emission in the S. alterniflora community but does not affect the emission in the native plant communities27. In addition, N enrichment stimulates N2O emissions in both invasive S. alterniflora and native P. australis communities28.

Here we conducted an experimental study in the Yangtze River estuary. Because of the positive relationship between NFM and plant C input to soil29, we hypothesized that invasive S. alterniflora relative to native P. australis has a higher simulative effect on soil N fixation. Previous studies suggest that N enrichment can affect soil N-fixation2. Therefore, we further hypothesized that N enrichment can regulate soil N fixation after plant invasion.

Materials and Methods

Experimental design. We conducted our study in the Dongtan National Nature Reserve, located in the Yangtze River estuary of China (31°25′–31°38′ N, 121°50′–122°05′ E) (Fig. 1). The area has an elevation of 2–6 m, and receives sediment and nutrients from the tide30. The area has a subtropical monsoon climate and receives most of its rainfall between May and September. The growing season begins in April and ends in October31. We established 4 plots which had approximately 500 meters far from each other (Fig. 1). All plots was at the same elevation with similar sediment textures and received the same tidal timing. Each plot had a factorial combination of two plant communities and two N levels (UPC: unfertilized Phragmites australis community; USC: unfertilized Spartina alterniflora community; FPC: fertilized Phragmites australis community; FSC: fertilized Spartina alterniflora community). All plant communities grew naturally in this study. The size of each plot was 0.8 × 0.8 m. In each plot, the same type of plant communities were approximately 2 meters apart. The distance between communities of different plant types was no more than 10 meters. To simulate N enrichment, urea (100 g N m−2 y−1) was added to the fertilized communities during growing season (April to August) in 2012 and 2013. To eliminate possible litter effects, litter was removed thoroughly at the beginning of this study.

At the end of the experiment, aboveground biomass was harvested, oven dried and weighted. Five soil cores were collected from the top 15 cm soil of each plot by using a 5-cm-diameter auger. Collected rhizosphere soils were within the densely rooted portion of the soil profile. After being mixed thoroughly, soil samples were hand-sifted to remove residual roots/rocks and separated into two parts. One part was stored at −80 °C for analyzing N-fixing microbes, and the other part was stored at 4 °C for measuring N-fixation rate and soil properties.

Measurements of N-fixation rate and soil properties. N-fixation rate was determined by a 15N2-based method as described by Hsu and Buckley (2009). Briefly, duplicate subsamples (5 g each) from each soil sample were weighted into two tubes. The headspace of one tube was replaced with 20% oxygen and 80% 15N2 mixed gas, while the headspace of another tube was replaced with 20% oxygen and 80% 14N2 mixed gas. Then, these tubes were incubated at 25 °C for 9 days in the dark. The quantities of 15N and 14N in the incubated soils were determined by an isotope ratio mass spectrometer (EA Flash2000-Delta Advantage, Thermo Electron Corporation, USA). The potential N-fixation rate was calculated by subtracting the quantity of 15N in labeling tube from the quantity of 15N in the control tube32.

Soil pH was determined in situ using an IQ150 pH Meter (Hach, USA). Because urea can naturally change to ammonia within a few days33, we did not measure urea for calculating soil N availability. NH4+ and NO3− were extracted from fresh soil samples using 2 M KCl (1:4, w/v) and assayed colorimetrically with a discrete auto-analyzer (SmartChem200, WestCo, America). A soil suspension was prepared using 3 g of air-dried soil and 40 ml deionized water, and then was analyzed with a Laser particle size analyzer (OMEC LS-CWM(3), China) to determine soil particle size. Soil salinity (1:5, w/v) was determined using a conductivity meter (Metler SevenEasy,
NFM community. Total soil DNA (0.5 g soil) was extracted using a FastDNA® SPIN Kit for Soil (MP Biomedicals, US) following the manufacturer’s instructions. The number of NFM was quantified by a SYBR Green 1-based qPCR method. The nifH gene primers (PolF: TGC GAY CCS AAR GGB GAC TC; PolR: AYT GGC CAT YTC GCC GGA) were used in this study34. Each reaction tube contained 0.5 μl of template DNA (5–10 ng), 10 μl of SYBR® Premix Ex Taq™ (Takara, China), 0.5 μl (10 μM) of each primer, 0.5 μl of BSA (1 μg μl−1), 0.4 μl of ROX reference dye (50X), and 7.8 μl of sterile distilled water. A known copy number of plasmid DNA carrying the nifH gene was used as a template to create a standard curve. Quantitative PCR was carried out using an ABI 7900 (ABI, USA). Thermal cycling was conducted using the following steps: 95 °C for 3 min, followed by 4 cycles of touchdown PCR (95 °C for 15 s, 63 °C for 25 s, and 72 °C for 45 s, with a temperature decrease of 2 degrees every two cycles), then 40 cycles of 95 °C for 15 s, 55 °C for 25 s, and 72 °C for 45 s. The results were analyzed using ABI SDS software (version 2.4, ABI, USA).

The template DNAs isolated from each subsample of each plot were pooled so that each subsample was equally represented. The nifH gene diversity and NFM community were characterized by 454-pyrosequencing. The nifH gene primers IGK3 (GCI WTH TAY GGI AAR GGI GGI ATH GGI AA) and DVV (ATT GCR AAC CRI CCR CAI ACI ACR TC) were used. Before PCR, the primers were barcode labeled. PCR amplification was performed with an initial denaturation at 94 °C for 10 min, then 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min35. The PCR products were purified and then used for 454-pyrosequencing. Approximately 10,000 DNA sequences were obtained from each sample. Next, the nifH gene DNA sequences were filtered using quality files, and the remaining sequences were trimmed with barcodes and forward primers. These sequences were converted to amino acid sequences using the Framebot tool in the RDP function gene pipeline36,37.

The sequences that did not correspond to the nifH gene or that had a termination codon in the middle were removed. The sequences that were too long, too short, or had ambiguous bases were also removed. The remaining quality sequences were aligned with the nifH gene database38, and poorly aligned sequences and chimeras were removed. Then, the remaining clean sequences were used for OTU-based analysis. OTU was defined using 90% and 80% sequence-identity cutoffs. Richness (ACE and Chao1) and diversity analyses were performed after the sequence number in each sample had been normalized. All steps of the quality sequence analysis and diversity analysis were performed using MOTHUR software, version 1.8.5. Normalized nifH OTUs at a sequence similarity cutoff of 80% were also used for Jackknife clustering analysis which was carried out using the online free UniFrac software (http://unifrac.colorado.edu). The unifrac metric through a weighted Jackknife clusters method was determined by relative sequence abundances and genetic distances of the four treatments39. The representing sequences of the OTUs (>1% in the total sequences) in combination with the reference sequences from other two related studies39,40 were used to construct a phylogenetic tree. The accession numbers are shown in the phylogenetic tree. All of the clean sequences were identified using Blastx (http://blast.ncbi.nlm.nih.gov/) to determine functional compositions.

Data analyses. Visual maps for the location of the Chongming Island was created using ArcGIS version 10.2 (ESRI Inc., Redlands, CA, USA). Copy number of the nifH gene per gram of dry soil was log converted [log (the copy number of nifH g−1 dried soil)]. We used two-way ANOVA to analyze the effects of plant type and N enrichment on N-fixation rate and soil properties. Canonical correspondence analysis (CCA) was used to examine the relationships between OTU compositions and environmental factors41. All statistical analyses were performed with R software 3.1.2 (R Core Team, 2014).

Nucleotide Sequence Accession Numbers. The partial nifH sequences recovered in this study were available in the NCBI GenBank Short Read Archive (SRA) under Accession No. SRP066209.

Results. S. alterniflora biomass was significantly higher than P. australis biomass at the same N level (Fig. 2a). N enrichment significantly stimulated biomass of both native and exotic plants (Fig. 2a). Plant type had a significant effect on the copy number of nifH gene (P < 0.01). The highest copy number of nifH gene was found in FSC (Fig. 2b). Plant type also significantly affected N-fixation rate (P < 0.01). N-fixation rate was significantly higher in the soils of S. alterniflora communities than in those of P. australis communities (Fig. 2c). In addition, the copy number of the nifH gene was positively correlated with N-fixation rate (R2 = 0.95, P = 0.03).

The OTU number and richness of the nifH gene were higher in the soils of P. australis communities compared with the S. alterniflora communities (Table 1). N enrichment increased the chao1 and ACE indices in each type of plant community (Table 1). Soil nifH gene diversity was generally higher in the P. australis communities than S. alterniflora communities (Table 1). Compared with the P. australis communities, the soil of S. alterniflora communities had higher proportions of OTU013, OTU002, and OTU036, and lower proportions of OTU005, OTU009, OTU008, OTU003, and OTU014 while N enrichment had little effect on the composition of the nifH gene (Fig. 3a). All dominant OTUs in this study matched the known N-fixation microbial orders (Fig. 4). Generally, the most dominant OTUs accounting for 41.1% of the total sequences were associated with the class Deltaproteobacteria. OTU013, OTU020, and OTU002 were similar to the sequences discovered in a previous study39,40 (Fig. 4). Based on the CCA soil pH had the strongest effect on soil NFM compositions (Fig. 5). OTU013 positively correlated with pH, NH4+, and salinity (Fig. 5). But OTU002, OTU020, OTU016, OTU033, OTU004 negatively correlated with these environmental factors (Fig. 5).
The soils of *S. alterniflora* communities had lower proportions of Deltaproteobacteria, Alphaproteobacteria and Gammaproteobacteria but higher proportions of Cytophagia, Spirochaetales, and Methanomicrobia in comparison with the soils of *P. australis* communities (Fig. 3b). Jackknife cluster analysis suggests that *S. alterniflora* communities had similar *nifH* composition for two N treatments as clustered into one clade. However, the *nifH* compositions in the *P. australis* communities were clustered into different clades for two N treatments (Fig. 6).

There were no significant differences in the salinity, NO$_3^-$/NH$_4^+$ concentrations, and soil particle size across all treatments. However, the soils from the *S. alterniflora* communities had higher C/N concentrations but lower P concentration in comparison with the soils from the *P. australis* communities, leading to higher ratios of soil C:P and N:P in the *S. alterniflora* communities (Table 2).

**Discussion**

It is well known that soil microbial diversity and activity are mediated by environmental properties. Plant invasions are shown to change belowground diversity and processes$^{42,43}$. Our findings add to a growing body of evidence that plant invasions can increase soil N-fixation rate$^{8,12,44}$, possibly because invasive plants has higher stimulative effects on their rhizosphere bacterial activity in comparison with co-occurring native plants. Many studies suggest that soil microbial activity is highly correlated with plant biomass production and C input to soil. In a saltmarsh meadow, for example, soil N fixation rate increases with increasing plant primary productivity$^{29}$. In the ecosystem we studied, the *S. alterniflora* community had much higher aboveground biomass (Fig. 2a), litter production, soil organic matter, microbial biomass, and soil respiration rate than the native *P. australis* community$^{16,45}$. Therefore, increased soil C availability due to *S. alterniflora* invasion might be responsible for the high NFM number and N fixation rate.
Figure 3. Proportions of nifH gene types at 80% similar cutoff (a) and N-fixation bacterial types (b). UPC: unfertilized Phragmites australis community; USC: unfertilized Spartina alterniflora community; FPC: fertilized Phragmites australis community; FSC: fertilized Spartina alterniflora community.

Figure 4. Phylogenetic tree based on the representative sequences (>1% abundance in the total sequences) of the nifH gene. The percentages in parentheses indicate those in the total number of sequences. The sequences which are named with Gamble and Lovell are the sequences from Gamble's and Lovell's studies, respectively. Bootstrap values (%) are only shown when they are greater than 50%.
On the other hand, soil nutrient availability has positive effects on NFM community and N-fixation rate. *S. alterniflora* invasion increases soil N pool size and availability of the Yangtze River estuary because of the
invasion-induced increase in soil N intercept through sedimentation\(^{18,30}\). In addition, litter of the invasive plant can decompose more rapidly than that of the native plant, leading to a faster nutrient recycling rate of the invasive plant\(^{34,36}\). As a result, a high availability of soil nutrient after \textit{S. alterniflora} invasion may lead to a stimulation of \textit{BNF}.

We found that \textit{S. alterniflora} invasion changed soil N:P ratios (Table 2). These changes in soil stoichiometric properties might also affect NFM community. In P-limited (relative to N) ecosystems, for example, N fixation rate is negatively correlated with N:P ratio in grassland and tropical forest soils\(^{46,47}\). However, the soils of \textit{S. alterniflora} communities relative to \textit{P. australis} communities had higher N:P ratios as well as higher N fixation rates (Fig. 2b, Table 2). These results suggest that soil nutrient factors affecting N fixation rate are different between ecosystems\(^2\). Although soil N concentration is relatively higher in the Yangtze River estuary compared with other nutrient-limited ecosystems\(^{46,47}\), N enrichment largely stimulated plant growth (Fig. 2a), possibly suggesting that N relative to P may play a more important role in plant growth in Yangtze River estuary.

Our results are consistent with other studies conducted in salt marshes\(^{39,40}\), showing that the most dominant NFMNs belong to \textit{Deltaproteobacteria} (Figs 4 and 5). Our results indicated that \textit{S. alterniflora} invasion had larger effects than N enrichment on the N-fixation rate and NFM community (Fig. 2c). Soil N fixation can be directly stimulated by increased plant production. However, the main way that soil N fixation is affected by N enrichment is believed to be indirect plant stimulation\(^{49}\). In addition, we found that two-year N enrichment had little effect on soil concentrations of TN, NH\(_4\)\(^+\), and NO\(_3\)\(^-\) (Table 2), possibly because litter was removed in our study, it would reduce litter N retention and N input. In addition, perhaps because the ecosystems in the Yangtze river estuary are so eutrophic that N addition is buffered. This may also lead to a greater influence of plant invasion on N-fixation rate and NFM community than that of N enrichment.

Overall, our research demonstrated that \textit{S. alterniflora} invasion changed the functional microbial community for fixing N and increased N-fixation rate in an invaded estuarine ecosystem. However, N enrichment had little effect on this functional process compared with plant invasion. Our study would improve our understanding of ecosystem consequences caused by plant invasion in the highly disturbed estuarine ecosystem. Our results also suggest that there could be a positive feedback between \textit{S. alterniflora} invasion and eutrophication. The \textit{S. alterniflora} invasion plus N addition may increase N fixation and N input, further accelerating the invasion and N pollution. Thus, the spread of \textit{S. alterniflora} may aggravate the eutrophication in China’s coastal zones. Our results are potentially useful for the modeling, prediction, and management of plant invasion under eutrophication in coastal wetland ecosystems.

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Author Contributions

J.-X.H. and B.L. conceived the study. J.-X.H., M.W., X.X. and S.-Y.Q. performed the experiments. J.-X.H., M.N., Z.-X.Q., M.X. and B.L. interpreted the results and wrote the manuscript.

Additional Information

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