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Key Points:
- Nitrogen fixation in the intertidal sediments is associated tightly with the abundance of *nifH* gene
- Nitrogen fixation is greatly enhanced by high availability of organic matter, but inhibited by high ammonium concentrations
- Nitrogen fixation may be coupled to activity of sulfate-reducing bacteria

Supporting Information:
- Supporting Information S1

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Nitrogen Fixation in the Intertidal Sediments of the Yangtze Estuary: Occurrence and Environmental Implications

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Abstract Nitrogen fixation is a microbial-mediated process converting atmospheric dinitrogen gas to biologically available ammonia or other molecules, and it plays an important role in regulating nitrogen budgets in coastal marine ecosystems. In this study, nitrogen fixation in the intertidal sediments of the Yangtze Estuary was investigated using nitrogen isotope tracing technique. The abundance of nitrogen fixation functional gene (*nifH*) was also quantified. The measured rates of sediment nitrogen fixation ranged from 0.37 to 7.91 nmol N g^{-1} hr^{-1}, while the abundance of *nifH* gene varied from 2.28×10^6 to 1.28×10^8 copies g^{-1} in the study area. The benthic nitrogen fixation was correlated closely to the abundance of *nifH* gene and was affected significantly by salinity, pH, and availability of sediment organic carbon and ammonium. It is estimated that sediment nitrogen fixation contributed approximately 9.3% of the total terrigenous inorganic nitrogen transported annually into the Yangtze estuarine and coastal environment. This result implies that the occurrence of benthic nitrogen fixation acts as an important internal source of reactive nitrogen and to some extent exacerbates nitrogen pollution in this aquatic ecosystem.

1. Introduction

Reactive nitrogen input to estuarine and coastal areas has increased tremendously in the past few decades, consequently causing eutrophication, hypoxia, and other environmental issues in these aquatic environments (Conley et al., 2009; Deegan et al., 2012; Yin et al., 2014). Anthropogenic input is generally considered a primary source for overloaded reactive nitrogen in estuarine and coastal environments, but nitrogen fixation may also be an important, internal source of reactive nitrogen (Capone et al., 2005; Fulweiler et al., 2013, 2015; Gardner et al., 2006). Nitrogen fixation is a microbial-mediated process that converts atmospheric dinitrogen gas to ammonia or other molecules available to living organisms (Seitzinger & Garber, 1987). This conversion is catalyzed by nitrogenase complex, based on the following stoichiometric equation (LaRoche & Breitbarth, 2005):

$$N_2 + 8H^+ + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16Pi$$

Diazotrophs have the ability to increase reactive nitrogen via fixing atmospheric nitrogen. The nitrogen fixed by diazotrophs may to some extent stimulate primary productivity and contribute to nitrogen pollution in estuarine and coastal ecosystems (Howarth, Marino, Cole, 1988; McGlathery et al., 1998; Montoya et al., 2002).

To date, nitrogen fixation in water columns has been extensively studied in coastal marine environments (Gier et al., 2016, and references therein). In contrast, benthic nitrogen fixation has not received more attention, even though its importance in controlling the nitrogen budgets has been recently identified (Gardner et al., 2006; McCarthy et al., 2015; Mortazavi et al., 2012; Vieillard & Fulweiler, 2012). Benthic nitrogen fixation is performed by several taxa of diazotrophic prokaryotes (Andersson et al., 2014), but it is often associated with the metabolism of sulfate-reduction bacteria in the sediment (Gier et al., 2016, and references therein). For instance, Bertics et al. (2013) and Fulweiler et al. (2013) documented that the benthic nitrogen fixation is coupled to the heterotrophic reduction process of sulfate in the organic matter-enriched sediments of Eckernförde Bay (Baltic Sea) and the subtidal sediments of Narragansett Bay (Rhode Island, USA), respectively. Additionally, it has been found that the nitrogen fixation in the sediment can be affected by various environmental factors, such as salinity (Severin et al., 2012), organic matter (Fulweiler et al., 2008, 2013; Howarth, Marino, Lane, et al., 1988), temperature (Breitbarth et al., 2007), and dissolved inorganic nitrogen

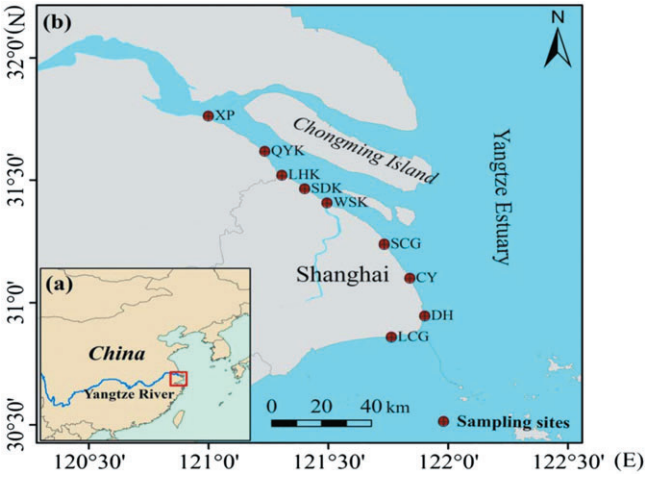

Figure 1. The location of the Yangtze Estuary and the sampling sites.

(Andersson et al., 2014; Bertics et al., 2013; Capone et al., 2005; Knapp, 2012; Moseman et al., 2016). However, the associations of benthic nitrogen fixation with environmental factors remain largely unknown for a specific aquatic environment (Newell, Pritchard, et al., 2016).

The Yangtze Estuary is one of hypereutrophic estuaries around the world (Hou et al., 2013, and references therein). Due to effects of human activities, a huge amount of reactive nitrogen is annually transported into the estuarine and its adjacent coastal area (Hou et al., 2006), consequently leading to severe eutrophication (Hou et al., 2013, 2014). So far, there have been many studies focusing on nitrogen removal processes (including denitrification and anaerobic ammonium oxidation) in the sediments of the Yangtze Estuary (Deng et al., 2015; Hou et al., 2013), but few studies have examined sediment nitrogen fixation in the nitrogen-enriched ecosystem. To the best of our knowledge, only one study documented the effects of *Spartina alterniflora* invasion and nitrogen addition on sediment nitrogen fixation in this estuarine and coastal zone (Huang et al., 2016). The objectives of this work are (1) to measure the spatial and seasonal changes in potential rates of benthic nitrogen fixation and abundance of nitrogen fixation functional gene (*nifH*) in the intertidal flats of the Yangtze Estuary, (2) to examine the links between benthic nitrogen fixation and environmental factors in the hypereutrophic estuary, and (3) to estimate the contribution of benthic nitrogen fixation to the nitrogen budget in the study area. This study on benthic nitrogen fixation improves our understanding of the nitrogen transformation dynamics and budget in the Yangtze estuarine and coastal ecosystem.

2. Materials and Methods

2.1. Study Area

The Yangtze Estuary is the first largest estuary in China, which covers an area of about 8,500 km^2 (Figure 1). It annually receives several hundreds of millions of tons of suspended sediment from the basin (Chen et al., 2001). More than half of the suspended sediment is deposited in the estuarine and coastal area, consequently leading to the development of an extensive intertidal flat. The intertidal flat can be seaward divided into high, medium, and low tidal zones. The intertidal sediment is composed predominantly of clay (<4 μm) and silt (4–63 μm), which both account for approximately 70–90% of the total sediment (Hou et al., 2013). In addition, a large quantity (about 1.1×10^6 t) of inorganic nitrogen is discharged annually into the Yangtze Estuary (Hou et al., 2013), consequently causing various eco-environmental problems (e.g., eutrophication, harmful algal blooms, and extensive hypoxia). Under the effects of both river runoff and tidal current, a distinct salinity gradient occurs in this study area. In general, the intertidal flat between Xupu to Sanchagang is the freshwater-controlling area, while the zone from Chaoyang to Luchaogang (LCG) is the brackish water-controlling area with the salinity of 3 to 15 ‰ (Hou et al., 2003).

2.2. Sample Collection

Along the intertidal flat of the Yangtze Estuary, surface (0–5 cm deep) sediment samples were collected from nine sites in August 2015 and January 2016, respectively (Figure 1). At each site, six replicates of sediment cores were taken and stored in sterilized polyethylene bags. Meanwhile, overlying water samples were also collected with 5 L carboys. After collection, all samples were stored on ice and transported to the laboratory within 4 hr. Upon return to the laboratory, sediment of each core was immediately mixed thoroughly to produce one composite sample under the anoxic condition in an argon-filled glove bag. One part of the sample was promptly incubated to determine potential rates of nitrogen fixation, and the other fraction was used for analyses of sediment physicochemical characteristics and molecular microbiology.

2.3. Analysis of Physicochemical Characteristics

Salinity of overlying water at each site was measured in situ using YSI Model 30 salinity meter. Sediment water content was quantified according to the weight loss of a known amount of wet sediment that was dried at 60 °C to a constant value (Li et al., 2015). Sediment pH was measured with a Mettler-Toledo pH meter, after

the sediment was mixed with CO_2-free deionized water at a sediment/water volume ratio of 1:2.5 (Hou et al., 2013). Sediment organic carbon and nitrogen was determined by a CHN elementary analyzer (VVarioELIII, Elementary, Germany) after carbonate in the sediment was removed by leaching with 0.1 mol L^{-1} HCl (Hou et al., 2013). Inorganic nitrogen (including NH_4^+ and NO_3^- plus NO_2^-) in the sediment was extracted with 2 mol L^{-1} KCl and then measured using continuous-flow nutrient analyzer (SAN plus, *SkalarAnalytical* B.V., the Netherlands) with detection limits of 0.5 μmol L^{-1} for NH_4^+ and 0.1 μmol L^{-1} for NO_3^- (Hou et al., 2015). Sulfide in the sediment was determined using a hydrogen sulfide sensor (H_2S-100, Unisense, Denmark), with a detection limit of 20 nmol L^{-1}. All physicochemical parameters were analyzed in triplicate.

2.4. Measurement of Nitrogen Fixation Rate

Potential rate of nitrogen fixation was measured by sediment-slurry incubations in combination with nitrogen-isotope tracing technique, which was modified on the basis of $^{15}N_2$ gas bubble method (Montoya et al., 1996). In short, slurries were made with fresh sediment and overlying water from each site at a ratio (sediment/water) of 1:7, and purged with argon for 30 min to eliminate background N_2 gas (Li et al., 2015). Then, 40 mL of the purged slurries were transferred into 60 mL serum vials sealed with septum caps under the argon atmosphere. Subsequently, one half of the replicates, designated as initial samples, were immediately preserved with 1 mL of saturated $HgCl_2$ solution (Deng et al., 2015). Meanwhile, the rest of slurries were injected with 2 mL of $^{15}N_2$ (99 atom% ^{15}N; Campro Scientific, Germany), respectively, after an identical volume of gas was removed with an argon-purged gas-tight syringe (Zhang et al., 2012). These injected slurries were shaken gently and incubated in dark for about 24 hr at near in situ temperature (30 °C for August incubation and 5 °C for January incubation). The incubations of these sediment-slurries, designated as final samples, were stopped by addition of $HgCl_2$ as described for the initial samples. The concentrations of ^{15}N-labeled products (including ammonia and organic nitrogen fractions) (Seitzinger & Garber, 1987) generated during the incubations were determined with a membrane inlet mass spectrometer after the samples were oxidized by hypobromite iodine solution (Yin et al., 2014). Potential rates of nitrogen fixation were estimated according to the following equation:

$$R_{\text{N-fixed}} = (^{15}N_{\text{Final}} - {}^{15}N_{\text{Initial}}) \times V \times W^{-1} \times T^{-1}$$

where $R_{\text{N-fixed}}$ (nmol N g^{-1} dry sediment hr^{-1}) denotes the rates of nitrogen fixation; $^{15}N_{\text{Final}}$ and $^{15}N_{\text{Initial}}$ (nmol N mL^{-1}) denote the concentrations of ^{15}N-labeled products in the terminated and initial samples, respectively; V (mL) denotes the volume of the incubation slurries; W(g) denotes the dry weight of sediment; and T (hr) denotes the incubation time.

2.5. Molecular Microbial Analysis

Total genomic DNA was extracted from 0.25 g of sediment using Powersoil™ DNA Isolation Kits (MOBIO, USA). Quantitative polymerase chain reaction (qPCR) was carried out to measure the abundance of *nifH* gene. The extracted *nifH* gene fragments (encoding nitrogen reductase, about 342 bp) of diazotrophs were amplified using polF (5′-TGC GAY CCS AAR GCB GAC TC-3′) and polR (5′-ATS GCC ATC ATY TCR CCG GA-3′) primers (Poly et al., 2001) in triplicate. The abundance of *nifH* gene was conducted using an ABI7500 Sequence Detection System (Applied Biosystems, Canada) and the SYBR green qPCR method (Hou et al., 2013; Zheng et al., 2017). The N_2-fixer PCR cycling was initiated by 50 °C 2 min, then 95 °C 30 s, followed by 30 s at 95 °C, 30 s 59 °C, and 30 s 72 °C for 45 cycles, and finally 5 min at 72 °C. qPCR was performed in a total volume of 25 μL containing 1 μL template DNA, 12.5 μL Maxima SYBR Green/Rox qPCR Master Mix (Ferments, Lithuania), and 1 μL of each forward and reverse primer (10 μmol L^{-1}). The plasmids, which contain the target fragment, were diluted into six gradients as standard curves (from 2.86×10^4 to 2.86×10^9 copies per microliter). The concentration of original plasmid was determined with a Nanodrop-2000 Spectrophotometer (Thermo, USA). Quantification standard curves showed a strong linear relationship between the threshold cycle (C_T) and the log10 values of *nifH* gene copy numbers (R^2 = 0.9949), with an amplification efficiency of 96%.

2.6. Statistical Analysis

Pearson's correlation analysis was conducted using Statistical Package for Social Sciences program (version-19.0) to reveal the relationships among nitrogen fixation rates, *nifH* gene abundance, and environmental factors. Student's *t* test was performed to compare the spatial and seasonal variations in the data obtained from this study.

Table 1
Physicochemical Characteristics of Sampling Sites

	Salinity	pH	NH_4^+ (μg g^{-1})	NO_3^- (μg g^{-1})	H_2S (μmol L^{-1})	OC (%)	C/N
				Summer			
XP	0.2 ± 0.1	7.37 ± 0.03	54.1 ± 4.4	2.9 ± 0.2	21.3 ± 2.5	8.7 ± 0.3	6.3 ± 0.6
QYK	0.2 ± 0.1	7.42 ± 0.02	54.3 ± 0.6	3.1 ± 0.1	22.4 ± 1.4	9.4 ± 1.5	7.2 ± 0.8
LHK	0.2 ± 0.1	7.47 ± 0.05	46.4 ± 1.6	3.3 ± 0.4	28.4 ± 0.3	12.8 ± 0.7	7.5 ± 0.9
SDK	0.2 ± 0.1	7.42 ± 0.01	103.3 ± 2.3	2.7 ± 0.4	21.7 ± 0.3	8.3 ± 1.2	6.8 ± 0.6
WSK	0.2 ± 0.1	7.38 ± 0.07	106.3 ± 6.0	3.1 ± 0.1	24.9 ± 2.5	8.5 ± 1.9	6.2 ± 0.9
BLG	0.3 ± 0.1	7.53 ± 0.13	49.4 ± 11.3	2.6 ± 0.7	24.1 ± 0.4	6.0 ± 0.9	5.9 ± 0.2
CY	2.0 ± 0.3	7.60 ± 0.01	68.7 ± 10.5	4.2 ± 0.3	20.8 ± 3.4	7.8 ± 0.5	7.0 ± 0.7
DH	5.5 ± 0.6	7.44 ± 0.05	44.9 ± 5.7	4.0 ± 0.3	27.2 ± 0.7	11.2 ± 0.6	7.6 ± 0.4
LCG	9.8 ± 0.8	7.81 ± 0.04	44.6 ± 2.3	4.2 ± 0.2	21.5 ± 1.4	5.4 ± 0.76	7.2 ± 0.9
				Winter			
XP	0.1 ± 0.1	7.48 ± 0.01	50.8 ± 5.3	4.4 ± 0.1	8.5 ± 0.2	9.8 ± 0.1	6.1 ± 0.7
QYK	0.2 ± 0.1	7.87 ± 0.02	57.5 ± 9.1	4.5 ± 0.3	7.9 ± 0.2	12.3 ± 0.1	7.7 ± 0.8
LHK	0.3 ± 0.1	7.57 ± 0.07	35.7 ± 3.4	3.9 ± 0.1	8.5 ± 2.5	17.7 ± 0.5	8.7 ± 0.9
SDK	0.2 ± 0.1	7.83 ± 0.03	47.3 ± 13.6	3.7 ± 0.1	7.6 ± 2.1	11.4 ± 0.5	7.4 ± 0.7
WSK	0.2 ± 0.1	7.58 ± 0.01	44.1 ± 0.2	2.7 ± 0.1	9.3 ± 0.2	10.5 ± 2.5	7.5 ± 0.5
BLG	0.3 ± 0.1	8.04 ± 0.06	40.8 ± 6.6	3.2 ± 0.1	5.9 ± 0.1	5.9 ± 0.3	7.9 ± 0.9
CY	2.5 ± 0.5	7.97 ± 0.03	34.1 ± 6.6	4.3 ± 0.2	6.1 ± 1.3	8.0 ± 0.5	8.3 ± 0.4
DH	7.8 ± 0.9	7.50 ± 0.04	49.7 ± 3.8	5.5 ± 1.2	6.3 ± 1.2	9.9 ± 0.2	7.5 ± 0.7
LCG	14.1 ± 1.1	7.71 ± 0.03	45.4 ± 6.1	4.2 ± 0.2	5.1 ± 0.4	4.9 ± 0.1	6.4 ± 0.5

Note. Mean values are shown with standard deviations (n = 3). OC: organic carbon.

3. Results

3.1. Site Characteristics

Physicochemical characteristics of the sampling sites are shown in Table 1. A distinct salinity gradient was observed in the study area, with a low level (0.1–2.5 ‰) in the upper estuary and a high level (5.5 to 14.1 ‰) in the lower estuary. Sediment pH of the study area varied from 7.37 to 7.81 in summer and from 7.48 to 8.04 in winter. Inorganic nitrogen concentrations in the sediments showed significant spatial and temporal variations (Student's t test, $p < 0.05$). Higher concentrations of NH_4^+ (44.6 to 106.3 μg g^{-1} dry sediment) were detected in summer, and lower values (34.1 to 57.5 μg g^{-1} dry sediment) were found in winter. Compared with NH_4^+, the concentrations of NO_3^- were relatively low, with a range of 2.6 to 4.2 μg g^{-1} dry sediment in summer and 2.7 to 5.5 μg g^{-1} dry sediment in winter. Sulfide concentrations in the sediment pore water ranged from 20.8 to 28.4 μmol L^{-1} in summer. In contrast, significantly low concentrations of sulfide were recorded in winter, with values of 5.1 to 9.3 μmol L^{-1} (Student's t test, $p < 0.05$). The contents of sediment organic carbon were in the range of 4.66 to 18.21 mg g^{-1} dry sediment, with relatively higher concentrations at the low-salinity sites than at the high-salinity sites (Student's t test, $p < 0.05$). The C/N molar ratios of sediment varied from 5.9 to 7.6 in summer and from 6.1 to 8.7 in winter.

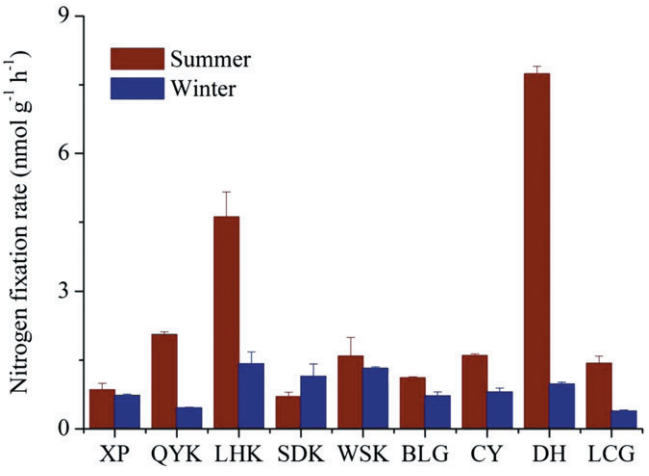

Figure 2. Nitrogen fixation rates in the intertidal sediments of the Yangtze Estuary. The vertical bar denotes standard deviation of triplicate samples.

3.2. Rates of Nitrogen Fixation

Measured rates of nitrogen fixation in the intertidal sediments of the Yangtze Estuary ranged from 0.71 to 7.74 nmol N g^{-1} dry sediment hr^{-1} in summer and from 0.39 to 1.42 nmol N g^{-1} dry sediment hr^{-1} in winter, respectively (Figure 2 and Table S1). Significant spatiotemporal heterogeneity of nitrogen fixation rates was observed in the study area (Student's t test, $p < 0.05$). The highest rate of nitrogen fixation (7.74 nmol N g^{-1} dry sediment hr^{-1}) was observed at site DH in summer, while the minimum rate (0.39 nmol N g^{-1} dry sediment hr^{-1}) occurred at site LCG in winter. Pearson's correlation analyses indicate that the nitrogen fixation rates were correlated significantly and positively with sediment organic

Table 2
Pearson's Correlations of Nitrogen Fixation Rates With Environmental Factors and nifH Gene Abundance (n = 27)

	Salinity	pH	OC (%)	C/N	NH_4^+ (μg g^{-1})	NO_3^- (μg g^{-1})	H_2S (μmol L^{-1})	*nifH* gene (copies g^{-1})
					August			
Coefficients	0.272	−0.126	**0.665**	**0.466**	**−0.442**	0.349	**0.698**	**0.779**
p values	0.169	0.531	**0.0002**	**0.014**	**0.021**	0.074	**<0.0001**	**<0.0001**
					January			
Coefficients	**−0.429**	**−0.383**	**0.610**	**0.388**	**−0.392**	0.289	**0.592**	**0.644**
p values	**0.025**	**0.048**	**0.0007**	**0.045**	**0.043**	0.144	**0.001**	**0.0003**

Note. The coefficients with significant *p* values are shown in bold.

carbon contents ($r = 0.665$, $p = 0.0002$ in summer; $r = 0.610$, $p = 0.0007$ in winter), C/N ratios ($r = 0.466$, $p = 0.014$ in summer; $r = 0.388$, $p = 0.045$ in winter), and sulfide concentrations ($r = 0.698$, $p < 0.0001$ in summer; $r = 0.592$, $p = 0.001$ in winter) (Table 2). In contrast, the nitrogen fixation rates were related significantly and negatively to ammonium contents in the sediments ($r = -0.442$, $p = 0.021$ in summer; $r = -0.392$, $p = 0.043$ in winter). In addition, winter nitrogen fixation rates were related significantly and negatively to the salinity of overlying water ($r = -0.429$, $p = 0.025$) and sediment pH ($r = -0.383$, $p = 0.048$).

3.3. Abundance of *nifH* Gene

Abundance of *nifH* gene in the intertidal sediments of the Yangtze Estuary is shown in Figure 3 and Table S1. Significant spatiotemporal heterogeneity of the *nifH* gene abundance in the sediments was observed in the study area (Student's *t* test, $p < 0.05$). The abundance of *nifH* gene was highest at site DH in summer with a value of 1.20×10^8 copies g^{-1} dry sediment, while the lowest abundance of *nifH* gene was detected at site LCG in winter with a value of 3.64×10^6 copies g^{-1} dry sediment. Pearson's correlation analyses showed that the *nifH* gene abundance was correlated significantly and positively with the nitrogen fixation rates in both summer ($r = 0.779$, $p < 0.0001$) and winter ($r = 0.644$, $p = 0.0003$) (Table 2).

4. Discussion

Due to the excessive input of terrigenous nitrogen mainly caused by human activities, the Yangtze Estuary has been one of highly nitrogen-enriched estuarine and coastal regions around the world (Hou et al., 2013). The nitrogen removal processes (i.e., denitrification and anaerobic ammonium oxidation) and their contribution to nitrogen fate have thus received more attention in the estuarine ecosystem, whereas nitrogen fixation has been neglected for a long time (Deng et al., 2015; Hou et al., 2013). In this work, the occurrence and spatiotemporal distributions of nitrogen fixation rates were examined in the intertidal sediments of the Yangtze Estuary. Also, we evaluated the associated relationships of benthic nitrogen fixation with environmental factors and functional gene (*nifH*) abundance. This study may improve our understanding of nitrogen dynamics in the estuarine and coastal environment.

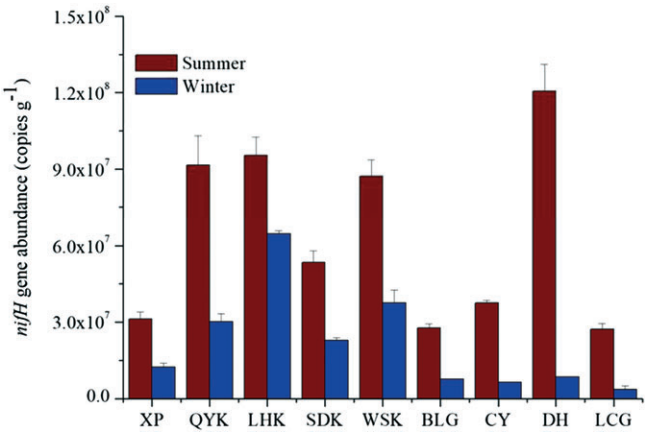

Figure 3. Abundance of the *nifH* gene in the intertidal sediment of the Yangtze Estuary sediments. The vertical bar denotes standard deviation of triplicate samples.

Distinct spatiotemporal changes of the nitrogen fixation rates were observed in the study area. The spatial and temporal variations of nitrogen fixation rates are generally associated with the dynamics of diazotrophic communities (Severin & Stal, 2010). This conclusion is supported by the significant and positive correlations between the *nifH* gene abundance and nitrogen fixation rates ($r = 0.779$, $p < 0.0001$ in summer; $r = 0.644$, $p = 0.0003$ in winter). Meanwhile, Pearson's correlation analyses show that the changes of nitrogen fixation rates in the sediments were influenced significantly by environmental factors. Salinity is generally considered an important factor controlling the nitrogen fixation, because salinity can shape the structure and diversity of diazotrophic bacteria (Herbert, 1975; Severin et al., 2012) and affect the metabolism of diazotrophic bacteria (Tel-or, 1980). In this study, a generally decreasing trend of nitrogen fixation rates with increasing

salinity was observed in the intertidal sediments of the Yangtze Estuary in winter ($r = -0.429$, $p = 0.025$), whereas summer nitrogen fixation rates were not associated significantly with salinity ($r = 0.272$, $p = 0.169$). These relationships suggested that diazotrophic bacteria in summer and winter sediments might have a different degree of sensitivity to salinity. It has been reported that the genus of nitrogen-fixing bacteria *Azotobacter* would not fix nitrogen in the presence of salt or seawater, while the genus *Desulfovibrio* has an obligate salt requirement (Herbert, 1975). In contrast, the genus *Clostridium* is euryhaline, and its nitrogen fixation is not affected significantly by the changes in salinity (Herbert, 1975). Werner et al. (1974) also found that the nitrogen-fixing capability of *Klebsiella pneumonia* isolated from the Oregon coastal sediments was not affected by salinities of up to 23 ‰, whereas the salt-sensitive *Enterobacter aerogenes* from the same area continued to conduct nitrogen fixation at a lower rate. In addition, alkalinity is also an important environmental factor influencing nitrogen fixation. Previous studies reported that nitrogen fixation generally occurs best near neutrality (Belay et al., 1984; Burris, 1994). In this study, winter nitrogen fixation rates had a significantly negative relationship with sediment pH ($r = -0.383$, $p = 0.048$), although it was not significant for summer rates ($r = -0.126$, $p = 0.531$). This result implied that pH may affect sediment nitrogen fixation via modulating the metabolism of diazotrophic prokaryotes (Burris, 1994). However, future work is still required to examine the underlying mechanism of salinity and pH effects on nitrogen fixation in the Yangtze Estuary via analyzing the compositions and diversity of diazotrophic bacteria and their metabolic pathways.

The availability of organic matter is an important driver for microbial metabolic activities such as nitrogen fixation and sulfate reduction by heterotrophic bacteria (Gier et al., 2016). In this study, the nitrogen fixation rates were observed to correlate significantly and positively with the concentrations of organic carbon in the sediments ($r = 0.665$, $p = 0.0002$ in summer; $r = 0.610$, $p = 0.0007$ in winter). Similar associations were also reported in other studies (Capone & Budin, 1982; Moseman et al., 2016). An et al. (2001) documented the increase of nitrogen fixation after glucose addition during sediment-core incubations. In addition, the nitrogen fixation rates was associated closely with C/N ratios in the intertidal sediments of the Yangtze Estuary ($r = 0.466$, $p = 0.014$ in summer; $r = 0.388$, $p = 0.045$ in winter). This relationship shows that the quality of organic matter may play an important role in controlling the nitrogen fixation (Newell, McCarth, et al., 2016). Overall, the observed associations of benthic nitrogen fixation rates with organic matter imply that the nitrogen fixation is mainly heterotrophic in the intertidal sediments of the Yangtze Estuary.

Many studies have reported that ammonium and NO_x (including NO_3^- and NO_2^-) may inhibit nitrogen fixation rates (Andersson et al., 2014; Bertics et al., 2013; Capone et al., 2005; Knapp, 2012; Moseman et al., 2016). In this study, we found that high concentrations of ammonium in the sediments had a negative effect on potential nitrogen fixation rates ($r = -0.442$, $p = 0.021$ in summer; $r = -0.392$, $p = 0.043$ in winter). This inhibition might be attributed to the additional energy cost associated with the conversion of N_2 gas to ammonium by diazotrophic bacteria, compared with direct assimilation of ambient ammonium (Bertics et al., 2010; Knapp, 2012). However, some studies have indicated that no inhibition on benthic nitrogen fixation occurs at high ammonium concentrations (Farnelid et al., 2013; Newell, McCarth, et al., 2016). This comparison demonstrated that diazotrophic bacteria might keep their function of fixing nitrogen regardless of the ambient nitrogen concentrations, and considerable nitrogen fixation occurs in the nitrogen-enriched environments.

The seasonal fluctuation of nitrogen fixation rates may be influenced by temperature changes (Table S1). Although comparable nitrogen fixation rates between lower temperature (2–4 °C) in winter and higher temperature (22–24 °C) in summer were observed in Erie Lake (Howard et al., 1970), many studies have reported that high temperature increases nitrogen fixation rates (Bertics et al., 2013; Fulweiler et al., 2007; Shiozaki et al., 2015). In line with these studies, the nitrogen fixation rates had a remarkable seasonal variation in our study area (Student's t test, $p < 0.05$). The marked seasonal variability in nitrogen fixation rates implied that temperature was a significant factor regulating the nitrogen fixation process in the intertidal sediments of the Yangtze Estuary.

Although the compositions and diversity of diazotrophic bacteria were not detected in the present study, numerous studies reveal that sulfate reduction bacteria are involved in the benthic nitrogen fixation (Bertics et al., 2010; Fukui et al., 1999; Fulweiler et al., 2013; Gier et al., 2016). For instance, Fulweiler et al. (2013) found that the *nifH* gene recovered from the sediments of the Narrangaset Bay was associated with sulfate reducing bacteria, such as *Desulfonema* spp., *Desulfobacter* spp., and *Desulfovibrio* spp., suggesting

Table 3
Comparison of Sediment Incubation Studies on Nitrogen Fixation (Nitrogen Input) and Denitrification (Nitrogen Loss) in the Yangtze Estuary and Other Estuarine and Coastal Ecosystems

Nitrogen fixation rate (μmol N m^{-2} hr^{-1})	Denitrification rate (μmol N m^{-2} hr^{-1})	Method	Location	References
1–253[a]	NA	Acetylene reduction technique	Florida Lake	Keirn and Brezonik (1971)
0–97	0–90	Nitrogen-isotope tracing technique	Texas estuaries	Gardner et al. (2006)
0–650 ± 200	0–530	N_2/Ar ratio technique	Narragansett Bay, RI	Fulweiler et al. (2007)
0–426 ± 35	54 ± 13–615 ± 229	Nitrogen-isotope tracing technique	Lake Waco Wetland	Scott et al. (2008)
8–125	5–72	Acetylene reduction technique	Weeks Bay, Alabama	Mortazavi et al. (2012)
0–147 ± 39	18 ± 0.5–562 ± 70	Nitrogen-isotope tracing technique	Gulf of Mexico hypoxic zone	McCarthy et al. (2015)
23 ± 1–464 ± 10[a]	722–4,028	Nitrogen-isotope tracing technique	The Yangtze Estuary	This study; Hou et al. (2013)

[a]The unit of the data is converted from μmol N g^{-1} hr^{-1} to μmol N m^{-2} hr^{-1}, assuming that the bulk density of dry sediment is 1.19 g cm^{-3}. NA means no data available.

the coupling of sulfate reduction with nitrogen fixation. In this study, the rates of sulfate reduction in the sediments were not measured, but we found that there were significant and positive correlations between the nitrogen fixation rates and sulfide concentrations ($r = 0.698$, $p < 0.0001$ in summer; $r = 0.592$, $p = 0.001$ in winter). These relationships might to some extent reflect that the nitrogen fixation was favored during dissimilatory sulfate reduction to sulfide by sulfate-reducing bacteria in the intertidal sediments of the Yangtze Estuary. However, further work is still needed to verify this hypothesis.

Nitrogen fixation, denitrification, and anaerobic ammonium oxidation (anammox) are the opposite nitrogen transformation processes affecting the fate and budget of nitrogen. The measured rates (0.39 to 7.74 nmol N g^{-1} dry sediment hr^{-1}) of nitrogen fixation in this study are comparable to the values reported from other estuarine and coastal sediments (Table 3), and the rates are much lower than the denitrification rates (12.04 to 67.13 nmol N g^{-1} dry sediment hr^{-1}) while approximately equivalent to the anammox rates (0.94 to 6.61 nmol N g^{-1} dry sediment hr^{-1}) in the study area (Hou et al., 2013). If the annual mean rate (1.65 nmol N g^{-1} dry sediment hr^{-1}) of sediment nitrogen fixation obtained in this study is extrapolated to the entire Yangtze Estuary, it is roughly estimated that approximately 1.02×10^5 t of nitrogen was fixed annually into the estuarine and coastal environment, given that the bulk density of dry sediment is about 1.19 g cm^{-3} (Lin et al., 2016). This calculated amount accounts for about 9.3% of the terrigenous inorganic nitrogen (1.1×10^6 t) transported annually into the Yangtze Estuary (Hou et al., 2013). In contrast, more recently reported rates from other estuarine and coastal sediments showed that the contribution of sediment nitrogen fixation may be at the same level as that of nitrogen-loss processes (Table 3). For example, a nine-year study in Narragansett Bay suggested that about 30% of the nitrogen is added via nitrogen fixation, while approximately 25% of the input nitrogen is removed by denitrification (Fulweiler et al., 2013). In the northern Gulf of Mexico hypoxic zone, nitrogen fixation can reach approximately 23.5% of total nitrogen load, while the nitrogen removed by denitrification is doubled compared with the fixed nitrogen (McCarthy et al., 2015). When it comes to net anthropogenic nitrogen inputs (NANI, about 6.95×10^4 tons yr^{-1}) into the Yangtze Estuary (Chen et al., 2016), the import of nitrogen by sediment biological fixation was nearly 2 orders of magnitude higher than NANI. These results suggested that the benthic nitrogen fixation, as an internal nitrogen source, may play an important role in controlling the nitrogen budgets of the estuarine and coastal ecosystems.

5. Conclusions

This study investigated the occurrence and spatiotemporal distributions of nitrogen fixation in the intertidal sediments of the Yangtze Estuary. Potential rates of nitrogen fixation in the sediments varied from 0.37 to 7.91 nmol N g^{-1} dry sediment hr^{-1}, with significant spatial and seasonal heterogeneity. The benthic nitrogen fixation was associated tightly with the abundance of *nifH* gene in the study area. It is found that high availability of organic matter in the sediments increased the rates of nitrogen fixation, whereas the nitrogen fixation could be inhibited by high ammonium concentrations. The correlations between sulfide concentrations and nitrogen fixation rates suggested that the benthic nitrogen fixation might be coupled with the metabolic

activity of sulfate-reducing bacteria. In addition, the nitrogen fixation in the sediments contributed about 9.3% of the terrigenous inorganic nitrogen transported annually into the Yangtze Estuary. This estimation highlighted the importance of benthic nitrogen fixation, as an internal source of reactive nitrogen, in controlling the budget of nitrogen in the estuarine and coastal environment.

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