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Fengyu Deng and Lijun Hou contributed equally for this work.

Key Points:
- Dissimilatory nitrate reduction rates were linked closely to site sediment characteristics
- Denitrification contributed 66% to total nitrate reduction in the estuary
- High nitrogen retention contributed substantially to estuarine eutrophication

Supporting Information:
- Supporting Information S1

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Dissimilatory nitrate reduction processes and associated contribution to nitrogen removal in sediments of the Yangtze Estuary

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Abstract Dissimilatory nitrate reduction processes, including denitrification, anaerobic ammonium oxidation (ANAMMOX), and dissimilatory nitrate reduction to ammonium (DNRA), play an important role in controlling the nitrate dynamics and fate in estuarine and coastal environments. We investigated potential rates of denitrification, ANAMMOX, and DNRA in the sediments of the Yangtze Estuary via slurry incubation experiments combined with isotope-tracing techniques to reveal their respective contributions to total nitrate reduction in this hypereutrophic estuarine ecosystem. Measured rates of denitrification, ANAMMOX, and DNRA ranged from 0.06 to 4.51 μmol N kg^{-1} h^{-1}, 0.01 to 0.52 μmol N kg^{-1} h^{-1}, and 0.03 to 0.89 μmol N kg^{-1} h^{-1}, respectively. These potential dissimilatory nitrate reduction process rates correlated significantly with salinity, sulfide, organic carbon, and nitrogen. Denitrification contributed 38–96% total nitrate reduction in the Yangtze Estuary, as compared to 3–45% for DNRA and 1–36% for ANAMMOX. In total, the denitrification and ANAMMOX processes removed approximately 25% of the external inorganic nitrogen transported annually into the estuary. In contrast, most external inorganic nitrogen was retained in the estuary and contributes substantially to the severe eutrophication of the Yangtze Estuary.

1. Introduction

Nitrogen is a key element limiting primary production in estuarine and coastal ecosystems [*Camargo and Alonso*, 2006; *Gardner et al*., 2006; *Crowe et al*., 2012; *Roberts et al*., 2014]. Over the past few decades, reactive nitrogen production has increased by 120%, and global nitrogen overload has become an emerging environmental issue in this century [*Piña-Ochoa and Álvarez-Cobelas*, 2006; *Galloway et al*., 2008; *Virdis et al*., 2010; *Bu et al*., 2011]. Increasing input of nitrogen (mainly in the form of nitrate) is an important driver of ecoenvironmental questions in estuarine and coastal ecosystems [*Seitzinger and Sanders*, 1997; *Vermaat et al*., 2012; *Kennison and Fong*, 2014], by altering food-web structure, decreasing biodiversity, causing widespread anoxia, and increasing frequency of algal blooms [*Carstensen et al*., 2007; *Hautier et al*., 2009; *Howarth et al*., 2011]. An improved understanding of nitrogen transformation is thus required to assess nitrogen fate and control nitrogen pollution in these aquatic environments.

Dissimilatory nitrate reduction processes, including denitrification, anaerobic ammonium oxidation (ANAMMOX), and dissimilatory nitrate reduction to ammonium (DNRA), are important pathways of nitrate transformation in aquatic environments [*Souza et al*., 2012; *Song et al*., 2013]. However, these processes play diverse roles in controlling the fate of nitrate. Both denitrification and ANAMMOX remove nitrate from aquatic ecosystems [*Seitzinger*, 1988; *Mulder et al*., 1995] by conversion to gaseous nitrogen (mainly dinitrogen gas). Denitrification is generally respiratory nitrate reduction to dinitrogen gas in response to oxidation of electron donors such as organic matter [*Seitzinger*, 1988], whereas in ANAMMOX, ammonium is oxidized to dinitrogen gas, with nitrate or nitrite serving as the electron acceptor under anaerobic conditions [*Mulder et al*., 1995]. In contrast to those processes, DNRA does not remove nitrate from aquatic environments in the form of dinitrogen gas but instead reduces it to ammonium [*An and Gardner*, 2002]. Therefore, DNRA still retains the transformed nitrogen in aquatic ecosystems, as a biologically available form. The balance of dissimilatory nitrate reduction processes generally depends on environmental factors, of which the availability of reducing Fe (II), temperature, and sulfide and organic matter concentrations are considered as the most crucial variables [*Bae et al*., 2001; *Weber et al*., 2001; *Campos et al*., 2002; *Weber et al*., 2006; *Jensen et al*., 2008;

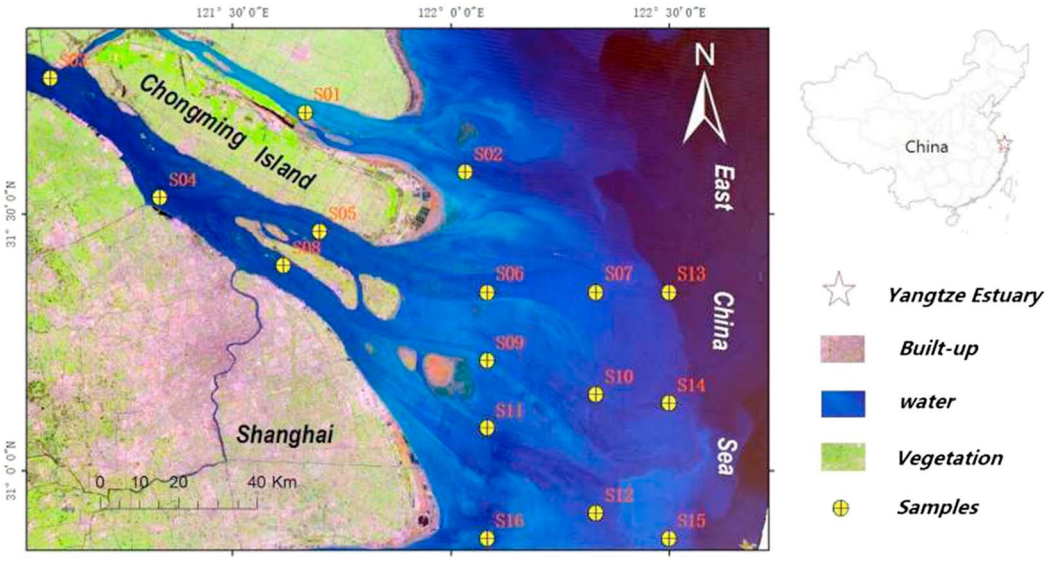

Figure 1. Study area. This figure shows the location of the Yangtze Estuary and the sampling sites during field investigations.

Gameron and Schipper, 2010]. However, particular factors controlling the denitrification, ANAMMOX, and DNRA processes and their respective contributions to the nitrogen removal remain unclear for specific aquatic environments [*Song et al*., 2013; *Yin et al*., 2014a].

The Yangtze River is the largest river in the Euro-Asian continent. It is ranked third in length, fourth in sediment discharge, and fifth in freshwater discharge around the world [*Hou et al*., 2009] and thus plays an important role in global biogeochemical cycles. The Yangtze Estuary is located in one of the most densely populated and industrialized areas of China. The estuarine ecosystem has received large quantities of anthropogenic inorganic nitrogen from the basin of the Yangtze River over the past several decades and caused severe eutrophication and frequent occurrences of harmful algal blooms [*Hou et al*., 2013; *Zheng et al*., 2014]. Therefore, the nitrogen pollution has been identified as the most serious environmental issue in the Yangtze Estuary. The benthic nitrogen fluxes across the sediment-water interface have been well examined [*Hou et al*., 2006; *Chen et al*., 2010; *Deng et al*., 2014], but few studies have addressed rates of dissimilatory nitrate reduction processes in the estuary. In this work, slurry experiments were conducted to quantify the rates of the denitrification, ANAMMOX, and DNRA processes with nitrogen isotope tracing techniques. We also compared the relative contributions of denitrification, ANAMMOX, and DNRA to total nitrate removal at the study area. Furthermore, environmental factors were determined and compared to elucidate their influences on the processes of denitrification, ANAMMOX, and DNRA. This work provides novel insights into the nitrogen dynamics and fate in the Yangtze Estuary.

2. Materials and Methods

2.1. Study Area

The Yangtze Estuary is China's largest estuary, situated on the east coast of China. It is over 250 km long from the tidal current boundary to its mouth and on average about 90 km wide, which covers an area of about 8500 km^2. The tide in the estuary is semidiurnal and irregular, with average tidal amplitudes of 2.4–4.6 m [*Chen*, 1988]. A distinct salinity gradient, ranging from 0 to 30 ‰ in the estuary, is caused by river runoff and tidal current effects [*Li et al*., 2009]. Eutrophication and harmful algal blooms are the most serious environmental issues in this ecosystem due to excessive inputs of inorganic nitrogen. Concentration of inorganic nitrogen in the estuarine and coastal water has increased greatly over the past few decades from about 10 $\mu mol\ N\ L^{-1}$ in the 1960s to over 130 $\mu mol\ N\ L^{-1}$ in the 2000s [*Chai et al*., 2006].

2.2. Sampling and Pretreatment

In this study, 16 representative sampling sites were selected along the salinity gradient in the Yangtze Estuary (Figure 1). Field surveys were conducted in July 2013 and January 2014, respectively. Surface sediments

(0–10 cm) at each site were collected in triplicate by subcoring the box corers with PVC tubes. The sediment samples were sealed in sterile plastic bags and stored at 4°C. After sediment samples were taken to the laboratory, the sediment of each tube was completely mixed under a helium atmosphere to produce one composite sample. One part of the fresh sediment sample was incubated immediately via slurry experiments to measure the rates of the dissimilatory nitrate reduction processes, and the other portion was examined to determine sediment physiochemical characteristics.

2.3. Analysis of Sediment Characteristics

Sediment salinity and pH were measured with YSI Model 30 salinity meter and Mettler-Toledo pH meter, respectively, after the fresh sediments were mixed with deionized water free of CO_2 at a sediment/water volume ratio of 1:2.5 [*Zheng et al*., 2014]. Sediment water content was measured from the weight loss of a known amount of wet sediment dried at 80°C to a constant value [*Zheng et al*., 2014]. Exchangeable ammonium and nitrate (plus nitrite) were measured on a continuous-flow nutrient analyzer (SAN Plus, Skalar Analytical B.V., the Netherlands) after being extracted with 2 M KCl from fresh sediments [*Hou et al*., 2013]. The concentration of Fe (II) was quantified after extraction with 0.5 M HCl from fresh sediments, followed by colorimetric (Ferrozine) analysis [*Roden and Lovley*, 1993]. Sediment sulfide concentration was analyzed using an Orion Sure-flow® combination silver-sulfide electrode [*Hou et al*., 2012]. Organic carbon and nitrogen in sediments were determined with a carbon-hydrogen-nitrogen elementary analyzer (VVarioELIII) after leaching with 0.1 M HCl to remove sedimentary carbonate [*Hou et al*., 2012]. All sediment physiochemical parameters were analyzed in triplicate.

2.4. Denitrification and ANAMMOX Rate Measurements

Potential rates of denitrification and ANAMMOX were determined via slurry experiments combined with a nitrogen isotope tracing method [*Risgaard-Petersen et al*., 2003; *Hou et al*., 2012]. Briefly, slurries were made with site sediment and water at a sediment/water volume ratio of 1:7. The slurries were mixed with a magnetic stirrer and purged by helium for about 30 min. The mixed slurries were transferred into 10 respective 12 mL gas-tight vials (Labco Exetainers) under a helium atmosphere. Subsequently, the vials were placed on a shaker table (150 rpm) and preincubated at near in situ temperature for 24 h to eliminate residual nitrite, nitrate, and oxygen. After preincubation, the vials were spiked with $^{15}NO_3^-$ (final concentration approximately 100 μmol $^{15}N\,L^{-1}$, final % ^{15}N approximately 90–99%, depending on the background nitrate concentration). Subsequently, one half of the replicates were preserved with 100 μL of saturated $HgCl_2$ solution and designated as initial samples. The remaining slurries were shaken (200 rpm) and incubated for about 8 h. At the end of incubations, remaining sample replicates were preserved with $HgCl_2$, as described for the initial samples. The nitrogen gases ($^{29}N_2$ and $^{30}N_2$) produced during the incubations were analyzed with a membrane inlet mass spectrometer (MIMS) [*Kana et al*., 1994; *An et al*., 2001]. Assuming that complete denitrification occurred during the incubations, concentrations of $^{29}N_2O$ and $^{30}N_2O$ were thus not measured in this study [*Crowe et al*., 2012]. Potential rates of denitrification and ANAMMOX were quantified based on the differences in the process-specific ^{15}N-labeled products ($^{29}N_2$ and $^{30}N_2$) between the final and initial experimental samples.

Prior to the slurry experiments, a preliminary $^{15}NH_4^+$ tracer experiment confirmed the occurrence of ANAMMOX at all sampling sites. The denitrification and ANAMMOX rates in the slurry experiments were estimated from the accumulation of $^{29}N_2$ and $^{30}N_2$ during the slurry incubation obtained respectively from denitrification and ANAMMOX [*Thamdrup and Dalsgaard*, 2002; *Trimmer et al*., 2003]. The respective contributions of denitrification and ANAMMOX to $^{29}N_2$ production were quantified by equation (1)

$$P_{29} = D_{29} + A_{29} \tag{1}$$

where P_{29} (μmol N $kg^{-1}\,h^{-1}$) denotes the total, measured $^{29}N_2$ production rates during the slurry experiments and D_{29} and A_{29} (μmol N $kg^{-1}\,h^{-1}$) denote the production rates of $^{29}N_2$ from denitrification and ANAMMOX, respectively. Here D_{29} was obtained by equation (2), assuming random paring of ^{14}N and ^{15}N from $^{14}NO_3^-$ or $^{15}NO_3^-$ [*Nielsen*, 1992; *Risgaard-Petersen et al*., 2003]

$$D_{29} = P_{30} \times 2 \times (1 - F_N) \times F_N^{-1} \tag{2}$$

where P_{30} (μmol N $kg^{-1}\,h^{-1}$) denotes the total, measured production rates of $^{30}N_2$ during the slurry experiments and F_N (%) denotes the fraction of ^{15}N in NO_3^-, which was obtained from the added $^{15}NO_3^-$ and

Table 1. Physiochemical Characteristics of Sampling Sites in the Yangtze Estuary[a]

Sites	Depth (m)		Sulfide (μg S g^{-1})		Fe (mg Fe g^{-1})		Salinity (‰)		pH		OC (mg C g^{-1})		ON (mg N g^{-1})		C/N		NH_4^+ (μmol N g^{-1})		NO_3^- (μmol N g^{-1})	
	July	January	July	January	July	January	July	January	July	January	July	January	July	January	July	January	July	January	July	January
S01	6.0	5.6	13.76	25.6	3.25	5.14	9.36	28.00	8.10	8.53	8.81	5.66	1.06	0.35	7.65	16.17	1.92	2.51	0.10	0.06
S02	10.9	12.5	10.56	21.76	4.63	2.11	24.77	24.83	7.85	8.50	5.59	4.83	0.54	0.30	10.35	16.00	4.36	2.41	0.05	0.05
S03	14.2	12.4	7.68	6.08	1.41	1.21	0.19	0.27	8.05	8.67	0.13	4.15	0.08	0.25	1.63	16.60	1.40	2.39	0.15	0.42
S04	9.0	8.6	43.52	14.72	4.66	2.16	0.20	0.22	8.33	7.76	4.90	7.34	0.24	0.47	20.42	15.62	1.60	3.04	0.02	0.08
S05	15.5	15.9	23.04	16.32	8.05	1.58	0.18	0.87	8.35	8.48	4.58	5.42	0.33	0.41	13.88	13.22	4.34	2.66	0.01	0.11
S06	9.4	9.1	18.24	30.08	1.85	0.86	4.00	18.83	8.10	7.93	3.44	2.56	0.13	0.07	26.46	36.57	1.26	2.43	0.08	0.13
S07	10.6	11.3	12.8	11.2	2.32	0.88	25.50	25.47	7.86	8.06	0.15	4.17	0.10	0.10	1.50	41.70	1.18	2.56	0.09	0.10
S08	11.8	12.5	35.84	7.04	1.88	0.80	0.14	0.83	8.49	8.85	2.24	3.70	0.09	0.10	24.89	37.00	1.79	2.74	0.05	0.13
S09	9.2	9.5	17.28	78.72	4.94	2.81	7.18	7.23	8.11	8.11	3.38	6.49	0.07	0.76	48.29	8.54	1.12	2.37	0.08	0.05
S10	7.1	8.1	12.8	24.0	2.15	0.88	27.00	27.53	7.80	8.58	0.14	2.31	0.21	0.22	0.67	10.50	1.29	2.60	0.14	0.09
S11	7.2	7.8	10.24	17.92	6.50	1.42	13.6	17.83	7.97	8.27	7.19	4.57	0.61	0.23	11.79	19.87	3.86	2.50	0.09	0.12
S12	10.8	10.8	31.68	9.6	5.20	5.41	21.8	24.47	7.95	8.08	4.23	4.97	0.37	0.44	11.43	11.30	4.23	2.37	0.04	0.06
S13	22.6	21.0	111.36	77.76	5.01	1.02	30.23	31.00	8.14	8.11	4.05	4.65	0.42	0.45	9.64	10.33	3.56	2.22	0.08	0.52
S14	16.7	15.7	17.28	25.6	4.72	1.33	31.00	29.00	8.21	8.12	5.33	6.18	0.51	0.71	10.45	8.70	4.59	2.17	0.20	0.06
S15	15.2	16.6	14.75	2.56	8.76	1.65	33.00	28.53	8.67	8.21	4.82	4.44	0.31	0.34	15.55	13.06	3.81	2.25	0.09	0.06
S16	14.2	15.09	24.0	9.28	3.92	2.34	20.00	18.30	8.06	8.31	4.71	4.46	0.50	0.52	9.52	9.06	3.61	2.21	0.10	0.13

[a]These data represent mean values from triplicate analyses.

the measured residual NO_3^- concentrations in the incubation slurries. The potential rates of denitrification and ANAMMOX were quantified by equations (3) and (4)

$$D_t = D_{29} + 2\times D_{30} \quad (3)$$

$$A_{29} = P_{29} - D_{29} \quad (4)$$

where D_t and A_{29} (μmol N kg^{-1} h^{-1}) denote the rates of denitrification and ANAMMOX, respectively.

2.5. DNRA Rate Measurements

Sediment slurries for DNRA measurement were prepared and preincubated, as described for the denitrification and ANAMMOX experiments. After the preincubation, the slurry vials were spiked with $^{15}NO_3^-$ (final concentration approximately 100 μmol ^{15}N L^{-1}, final % ^{15}N approximately 90–99%, depending on the background nitrate concentration). One half of the slurry vials as initial samples were immediately preserved with 100 μL of saturated $HgCl_2$ solution. Incubation of the remaining slurries was stopped by injecting 100 μL of saturated $HgCl_2$ solution after 8 h. The concentrations of $^{15}NH_4^+$ produced over the incubation were measured by OX/MIMS [*Yin et al.*, 2014b]. In brief, the produced $^{15}NH_4^+$ was oxidized into nitrogen gas with hypobromite iodine solution, and the oxidized products ($^{29}N_2$ and $^{30}N_2$) were determined with MIMS. Potential rates of DNRA were estimated from concentration changes in the process-specific ^{15}N-labeled product ($^{15}NH_4^+$) during the incubations.

Potential DNRA rates were quantified in the slurry experiments according to equation (5) [*Porubsky et al.*, 2008]

$$R_{DNRA} = \left(\left[{}^{15}N\ H_4^+\right)_{Final} - \left[{}^{15}N\ H_4^+\right]_{Initial}\right) \times Vol \times W^{-1} \times T^{-1} \quad (5)$$

where R_{DNRA} (μmol N kg^{-1} h^{-1}) denotes the total, measured potential DNRA rates, $\left[{}^{15}N\ H_4^+\right]_{Final}$ and $\left[{}^{15}N\ H_4^+\right]_{Initial}$ (μmol N L^{-1}) denote the concentrations of $^{15}NH_4^+$ in the final and initial samples of the slurry experiments, respectively, Vol (L) denotes the volume of the incubation vial; W (kg) denotes the dry weight of sediment, and T (h) denotes the incubation time.

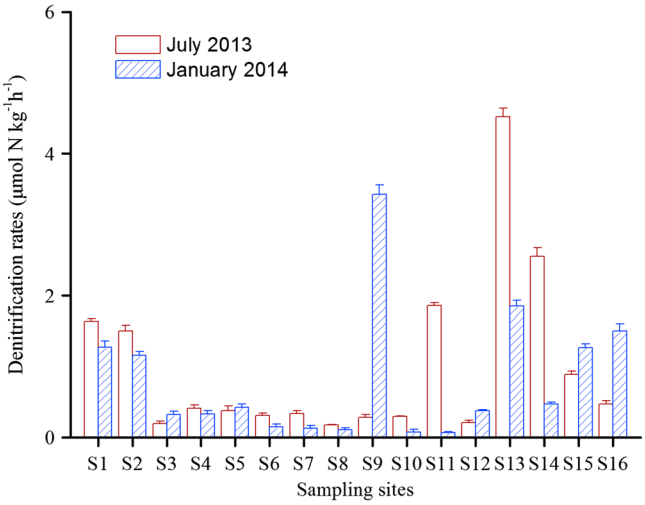

Figure 2. Denitrification rates in the Yangtze Estuary sediments during July 2013 and January 2014, respectively. Vertical bars denote standard error of triplicate samples.

2.6. Statistical Analysis

A one-way analysis of variance (ANOVA) was performed to examine temporal and spatial differences in the collected data. Correlations between variables were analyzed with the Pearson correlation. Statistical analyses were conducted at a 0.05 significance level. All statistical analyses were conducted using Statistical Package of Social Sciences (SPSS, version-19.0).

3. Results

3.1. Sampling Site Characteristics

The physiochemical characteristics of respective sites are shown in Table 1. The water depth of sampling sites varied between 5.6 and 22.6 m. Sediment pH at the study area varied from 7.76 to 8.85 in January and from 7.80 to 8.48 in July, respectively. A marked salinity gradient (salinity range of 0.18–30 ‰) was observed over the study area. The water content of sediments ranged from 22.65% to 44.65% in January and 24.12% to 58.33% in July. The concentrations of sulfide in sediments were in the range of 0.16–3.48 $\mu g\ S\ g^{-1}$, with relatively higher concentrations in July (0.27–3.48 $\mu g\ S\ g^{-1}$) than in January (0.18–2.46 $\mu g\ S\ g^{-1}$). The concentrations of Fe (II) ranged from 0.80 to 8.05 $mg\ Fe\ g^{-1}$ at the study area, with higher Fe (II) concentration in July (1.41–8.05 $mg\ Fe\ g^{-1}$) than in January (0.80–5.41 $mg\ Fe\ g^{-1}$). The contents of organic carbon and nitrogen in sediments of the Yangtze Estuary varied from 0.13 to 8.81 $mg\ C\ g^{-1}$ and 0.07 to 1.06 $mg\ N\ g^{-1}$, respectively. The concentrations of nitrate (plus nitrite) in sediments were relatively higher in January (0.05–0.52 $\mu mol\ N\ g^{-1}$) than in July (0.01–0.20 $\mu mol\ N\ g^{-1}$). In contrast, sedimentary ammonium had relatively higher concentrations in July (1.12–4.59 $\mu mol\ N\ g^{-1}$) than in January (2.17–3.04 $\mu mol\ N\ g^{-1}$).

3.2. Denitrification Rates

Potential denitrification rates varied from 0.09 to 4.52 $\mu mol\ N\ kg^{-1}\ h^{-1}$ and 0.06 to 3.43 $\mu mol\ N\ kg^{-1}\ h^{-1}$ in July and January, respectively (Figure 2). A significant spatial difference in denitrification rates was observed among sampling sites (one-way ANOVA, $n = 16$, $P = 0.04$ for July; one-way ANOVA, $n = 16$, $P = 0.03$ for January). However, no temporal variation for denitrification rates was detected (one-way ANOVA, $n = 32$, $P > 0.05$). In July, the highest denitrification rate appeared at site S13, whereas the lowest denitrification rate was at site S10. In January, the highest rate of denitrification occurred at site S9, and the lowest rate at S11. The rates of denitrification at the study area were significantly related to salinity ($r = 0.355$, $P = 0.046$, $n = 32$), organic carbon ($r = 0.396$, $P = 0.025$, $n = 32$), organic nitrogen ($r = 0.552$, $P = 0.01$, $n = 32$), and sulfide ($r = 0.667$, $P < 0.0001$, $n = 32$) (Table 2). Denitrification contributed 40–96% and 38–88% to total nitrate loss (sum of denitrification, ANAMMOX, and DNRA) in July and January, respectively.

Table 2. Pearson's Correlation Analyses Between Nitrogen Transformation Rates and Physiochemical Characteristics of Sediments ($n = 32$)

Transformation	Fe ($mg\ g^{-1}$)	Salinity (‰)	pH	OC ($mg\ g^{-1}$)	ON ($mg\ g^{-1}$)	NH_4^+ ($\mu mol\ g^{-1}$)	NO_3^- ($\mu mol\ g^{-1}$)	Sulfide ($\mu g\ g^{-1}$)
Coefficients								
DNRA	0.137	−0.089	−0.087	0.622	0.698	0.095	0.298	0.118
ANAMMOX	0.322	0.409	0.335	0.256	0.439	0.251	−0.046	−0.38
Denitrification	0.266	0.355	0.136	0.396	0.552	0.327	0.327	0.667
P values								
DNRA	0.456	0.629	0.673	0.000	0.000	0.605	0.098	0.521
ANAMMOX	0.072	0.020	0.061	0.158	0.012	0.166	0.803	0.032
Denitrification	0.141	0.046	0.458	0.025	0.001	0.068	0.686	0.000

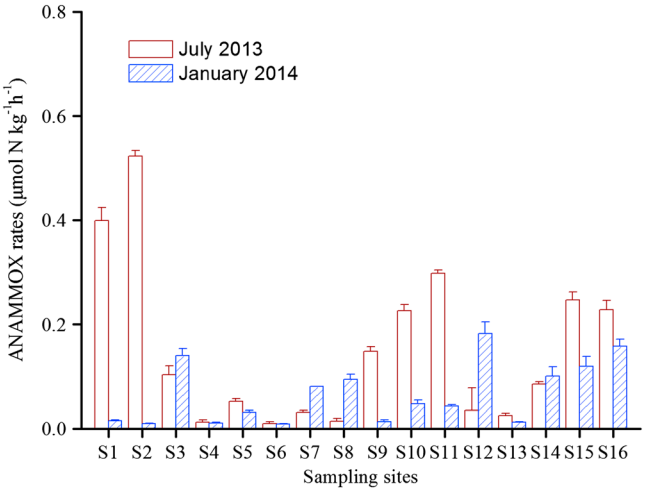

Figure 3. Anaerobic ammonium oxidation (ANAMMOX) rates in the Yangtze Estuary sediments during July 2013 and January 2014, respectively. Vertical bars denote standard error of triplicate samples.

3.3. ANAMMOX Rates

Potential ANAMMOX rates varied between 0.01 and 0.52 μmol N kg^{-1} h^{-1} at the study area (Figure 3). A significant seasonal variation in the ANAMMOX rates was observed (one-way ANOVA, $n = 16$, $P = 0.02$). In general, the ANAMMOX rates at the sampling sites were higher in July (0.01–0.52 μmol N kg^{-1} h^{-1}) than in January (0.01–0.23 μmol N kg^{-1} h^{-1}). Meanwhile, there was a remarkable spatial difference in the ANAMMOX rates among sampling sites (one-way ANOVA, $n = 16$, $P = 0.01$ for July; one-way ANOVA, $n = 16$, $P < 0.0001$ for January). The highest ANAMMOX rates occurred at site S2 in July and at site S12 in January. Of the detected environmental factors, ANAMMOX rates were related significantly to sulfide ($r = -0.38$, $P = 0.032$, $n = 32$). Compared to denitrification, ANAMMOX was of minor importance to nitrate removal, which contributed 1–31% and 1–36% to total nitrogen loss in July and January, respectively.

3.4. DNRA Rates

Rates of DNRA ranged from 0.04 to 0.86 μmol N kg^{-1} h^{-1} and 0.03 to 0.89 μmol N kg^{-1} h^{-1} in July and January, respectively (Figure 4). At the study area, a remarkable spatial difference occurred in DNRA rates (one-way ANOVA, $n = 32$, $P < 0.0001$). No seasonal variation in DNRA rates was observed (one-way ANOVA, $n = 32$, $P > 0.05$). The highest DNRA rates occurred at site S1 and the lowest at site S3 in July. In January, the highest and lowest DNRA rates were detected at sites S14 and S6, respectively. DNRA rates correlated positively with the concentrations of organic carbon ($r = 0.622$, $P < 0.0001$, $n = 32$) and nitrogen ($r = 0.698$, $P < 0.0001$, $n = 32$) in sediments. The contribution of DNRA to total nitrate reduction varied from 3–40% in July and 11–45% in January.

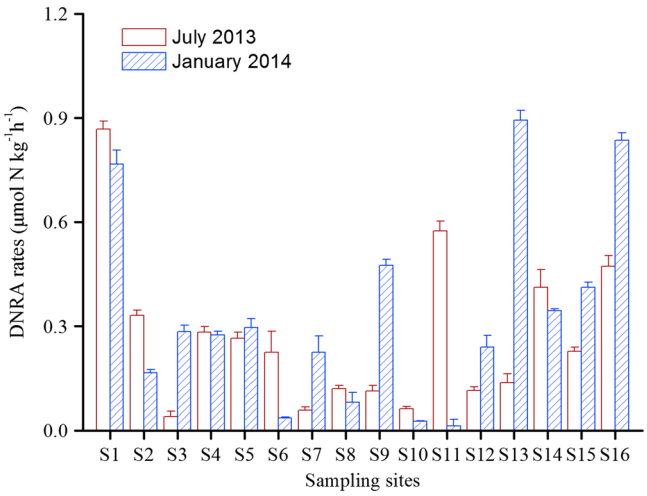

Figure 4. Dissimilatory nitrate reduction to ammonium (DNRA) rates in the Yangtze Estuary sediments during July 2013 and January 2014, respectively. Vertical bars denote standard error of triplicate samples.

4. Discussion

Denitrification, ANAMMOX, and DNRA are the most important processes of dissimilatory nitrate reduction in aquatic environments [*Rysgaard et al.*, 2004; *Dalsgaard et al.*, 2005; *Hou et al.*, 2012; *Song et al.*, 2013]. However, these processes play a diverse role in controlling the fate of nitrate [*Hou et al.*, 2012]. Both denitrification and ANAMMOX processes can remove nitrate "permanently" from aquatic ecosystems, whereas DNRA reduces nitrate to ammonium and thus retains the transformed inorganic nitrogen in ecosystems [*Seitzinger*, 1988; *Hietanen and Kuparinen*, 2008; *Crowe et al.*, 2012]. In the present study, we investigated the distributions of these dissimilatory nitrate reduction processes for the first

time in subtidal areas of the Yangtze Estuary (Figure S1 in the supporting information), thus providing a deep insight into the nitrate dynamics in this estuarine ecosystem.

Denitrification rates varied spatially in Yangtze Estuary sediments. The positive relationship of denitrification rates with salinity suggests that the spatial heterogeneity of denitrification was associated tightly with the salinity gradient at the study area. In general, denitrification rates decrease with increasing salinity [*Seo et al*., 2008], because salinity may cause a physiological stress on denitrification [*Rysgaard and Sloth*, 1999]. However, our study conflicted with that often accepted idea. The positive response of denitrification activity to salinity in this region suggests that high salinity might help halophilic denitrifying bacteria predominate and thereby improve denitrification efficiency [*Yoshie et al*., 2006; *Marton et al*., 2012]. Numerous studies have reported that occurrence of sulfide may decrease denitrification rates [*Schönharting et al*., 1998; *Cardoso et al*., 2006], via inhibiting nitrogen-monoxide and nitrous-oxide reductases which may control reduction of N_2O to N_2 during denitrification [*Sørensen et al*., 1980; *Brunet and Garcia-Gil*, 1996]. However, in this study, denitrification rates related positively to sulfide concentrations in the sediments. We postulate that the observed correlation between denitrification and sulfide may be attributed to involvement of sulfide as an electron donor for nitrate reduction in denitrification [*Sørensen et al*., 1979; *Reyes-Avila et al*., 2004; *Wang et al*., 2009]:

$$1.25S^{2-} + 2NO_3^- + 2H^+ \rightarrow 1.25SO_4^{2-} + N_2 + H_2O \quad (6)$$

Denitrification rates related positively with the contents of organic carbon and nitrogen, as expected from previous studies [*Bachand and Horne*, 1999; *Burgin and Hamilton*, 2007; *Gameron and Schipper*, 2010]. These correlations may relate to the supply of necessary organic substrates for denitrifying bacteria [*Piña-Ochoa and Álvarez-Cobelas*, 2006; *Dodla et al*., 2008; *Yin et al*., 2014a].

Previous studies have reported that the optimal temperature for ANAMMOX is about 30 to 35°C [*Egli et al*., 2001; *Jetten et al*., 2001]. In agreement with these studies, ANAMMOX had a remarkable seasonal variation at our sampling sites. The high ANAMMOX rates in July, compared to January, may relate to the high summer temperatures which promote ANAMMOX bacterial growth and activity [*Strous et al*., 1999; *Rattray et al*., 2010]. Also, spatial variations of ANAMMOX rates were observed at the study area. The spatial fluctuations of ANAMMOX rates were likely attributed to the geochemical characteristics of site sediments. A negative correlation of ANAMMOX rates with sulfide concentrations was observed at the study area. As an important factor regulating ANAMMOX, sulfide has been reported to be an irreversible inhibitor of ANAMMOX [*Jensen et al*., 2008]. This inhibition may be associated with the sulfide toxicity to the ANAMMOX bacteria, and thus low ANAMMOX rates occurred under high sulfide concentrations [*Sears et al*., 2004].

In contrast to ANAMMOX results, our DNRA rates were statistically indistinguishable between July and January. The absence of significantly seasonal variations in DNRA rates suggests that temperature was not an important factor controlling the DNRA activity in the Yangtze Estuary. Previous studies have reported that ammonium production via DNRA is associated with organic carbon content [*Buresh and Patrick*, 1978; *Yin et al*., 2002]. Likewise, the DNRA rates correlated significantly with organic carbon and nitrogen in our study area. The correlation between DNRA rates and organic matter may be attributed to organic matter providing surface structure for DNRA bacteria [*Ståhl and Davidsson*, 2000]. Also, organic matter can be utilized as a substrate to supply electron for the reduction of nitrate during the DNRA reaction [*Burgin and Hamilton*, 2007]. Generally, sulfide serves as a potential electron donor and increases the DNRA rates via inhibiting denitrification in sediments [*Myers*, 1972; *Bonin*, 1996; *Brunet and Garcia-Gil*, 1996; *Sher et al*., 2008; *Lu et al*., 2013]. Furthermore, DNRA may be coupled to Fe (II) oxidation by the following expected stoichiometric equation [*Behrendt et al*., 2013; *Roberts et al*., 2014]:

$$8Fe^{2+} + NO_3^- + 10H^+ \rightarrow 8Fe^{3+} + NH_4^+ + 3H_2O \quad (7)$$

However, we observed no distinct correlations of the DNRA rates with the concentrations of sedimentary sulfide and Fe (II). We postulate that this pattern may be attributed to the binding of Fe (II) with free sulfide, because the formation of iron sulfide precipitates (FeS) decreases the bioavailability of Fe (II) and free sulfide for DNRA activity [*Brunet and Garcia-Gil*, 1996]. Further investigation is still needed to verify the hypothesis.

Due to a diverse role of denitrification, ANAMMOX, and DNRA in dissimilatory nitrate reduction, competition among these processes can determine the fate of nitrate. This study shows that denitrification was the dominant transformation pathway of nitrate in the Yangtze Estuary sediments, on average contributing about 66%

Table 3. Contributions of Denitrification, ANAMMOX, and DNRA to Total Nitrate Loss in the Yangtze Estuary and Other Estuarine and Coastal Ecosystems[a]

Locations	Detrification (%)	ANAMMOX (%)	DNRA (%)	References
Thma Estuary	45(27–57)	NA	55(43–73)	*Nishio et al.* [1982, 1983]
Mokbaai, the Netherlands	67	NA	33	*Goeyens et al.* [1987]
Thau lagoon	<2	NA	98	*Gilbert* [1997]
Gulf of Fos, Marseilles	36(0–43)	NA	64(18–100)	*Bonin et al.* [1998]
Horsens Fjord trout cage	47(7–100)	NA	53(25–85)	*Christensen et al.* [2000]
Skagerrak	33	65	<2	*Dalsgaard and Thamdrup* [2002]
Kanholmsfjärden	2	NA	98	*Karlson et al.* [2005]
Baltic Sea	33(6.8–72.5)	12(10–15)	55(16.9–92.5)	*Hietanen and Kuparinen* [2008]; *Jäntti and Hietanen* [2012]
Colne Estuary	40–89	0–30	11–60	*Dong et al.* [2009]
Mae Klong	22(2–42)	NA	78 (58–98)	*Dong et al.* [2011]
Plum Island Sound	57(49–71)	NA	43(29–51)	*Koop-Jakobsen and Giblin* [2010]
Copnano	98.87(98.03–99.3)	0.67(0.4–1.45)	0.46(0.3–0.52)	*Hou et al.* [2012]
Saltwater Creek	1-15	10-24	75	*Dunn et al.* [2013]
East China Sea	65(17–85)	20(8–66)	15(8–22)	*Song et al.* [2013]
Yangtze Estuary	66.2	8	25.8	This study

[a]The data in parentheses represent the range. NA means no data available.

of total nitrate loss. Compared with denitrification, ANAMMOX played a minor role in total nitrate reduction, accounting for only about 8% of total nitrate loss at the study area. DNRA accounted on average for 26% of total nitrate reduction, implying that DNRA plays an important role in dissimilatory nitrate reduction in sediments of the Yangtze Estuary. The estimated respective contributions of denitrification, ANAMMOX, and DNRA to total nitrate reduction at the study area were in the range of values reported from other estuarine and coastal ecosystems (Table 3). Interestingly, compared with the bottom-water nitrate concentrations in all these estuarine and coastal ecosystems (Table S1 in the supporting information), the contributions of both denitrification and ANAMMOX to total nitrate reduction were observed to enhance logarithmically with increasing nitrate concentrations (Figure S2 in the supporting information). In contrast, the contributions of DNRA to total nitrate reduction showed a logarithmic decrease with increasing nitrate concentrations (Figure S3 in the supporting information). These correlations implied that the level of nitrate may be an important factor modulating the nitrate fate in estuarine and coastal environments.

Extrapolating denitrification and ANAMMOX rates to the entire Yangtze Estuary, we calculated that about 1.3×10^5 t and 1.99×10^4 t of nitrogen was removed annually from the estuarine ecosystem by denitrification and ANAMMOX, respectively, assuming that the bulk density of the sediments from the study area is 2.68 g cm^{-3} [*Hou et al*., 2013]. Denitrification consumed about 22% of the total terrigenous inorganic nitrogen transported annually into the Yangtze Estuary and, as expected, was the dominant pathway for fixed nitrate removal. ANAMMOX accounted for approximately 3% of the total terrigenous inorganic nitrogen and thus also removed significant terrigenous inorganic nitrogen from the Yangtze Estuary. In total, both denitrification and ANAMMOX processes removed about 25% of the total inorganic nitrogen from agriculture activity, fish farming, and industrial wastewater transported into the Yangtze Estuary [*Chai et al*., 2006; *Zheng et al*., 2014]. This result resembles the value estimated in Jinpu Bay [*Yin et al*., 2014a] but is considerably lower than the 50% removed from other estuarine and coastal environments [*Seitzinger*, 1988; *Seitzinger and Kroeze*, 1998]. The relatively low percentage of nitrogen removal from the Yangtze Estuary might be partly attributed to DNRA, because transformation from nitrate to ammonium via DNRA promotes sediment nitrogen retention [*Welsh et al*., 2014]. In addition, total nitrogen exported into the Yangtze Estuary has increased 2-fold over the past two decades [*Wang et al*., 2014], which contributes to the low fraction of inorganic nitrogen removed at the study area. We conclude that most inorganic nitrogen from external sources is retained in this hypereutrophic estuarine ecosystem, despite the high rates of nitrate removal by denitrification and ANAMMOX. The budgets of nitrogen thus suggest that sources and sinks are seriously out of balance, a factor responsible for the severe eutrophication and frequent occurrence of harmful algal blooms at the study area [*Chai et al*., 2006; *Li et al*., 2014; *Zheng et al*., 2015].

5. Conclusions

This study for the first time investigates dissimilatory nitrate reduction processes and associated contribution to nitrogen removal in subtidal sediments of the Yangtze Estuary. The potential rates of dissimilatory nitrate

reduction varied from 0.06 to 4.51 μmol N kg^{-1} h^{-1} for denitrification, 0.01 to 0.52 μmol N kg^{-1} h^{-1} for ANAMMOX, and 0.03 to 0.89 μmol N kg^{-1} h^{-1} for DNRA. The denitrification rates at the study area were related significantly to salinity, sulfide, organic carbon, and nitrogen, whereas ANAMMOX rates were related negatively to sulfide concentrations. DNRA was associated closely with the amount of sediment organic matter. Denitrification, the dominant process removing reactive nitrogen from the Yangtze Estuary, contributed about 66% to total nitrate reduction on average. DNRA was an important pathway of nitrate reduction, accounting for about 26% of total nitrate reduction. Compared with denitrification and DNRA, ANAMMOX contributed only about 8% of total nitrate reduction. Our data suggest that denitrification and ANAMMOX removed about 25% of the total inorganic nitrogen transported into the Yangtze Estuary. Thus, by subtraction, we conclude that the remaining approximately 75% of external inorganic nitrogen is retained in the Yangtze Estuary and may cause severe eutrophication and frequent occurrence of harmful algal blooms. These results should guide future efforts to prevent eutrophication and protect the health of estuarine and coastal ecosystems and water quality in the Yangtze Estuary and other estuaries.

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