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In situ nitrogen removal processes in intertidal wetlands of the Yangtze Estuary

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ABSTRACT

Estuarine and intertidal wetlands are important sites for nitrogen transformation and elimination. However, the factors controlling nitrogen removal processes remain largely uncertain in the highly dynamic environments. In this study, continuous-flow experiment combined with ^{15}N isotope pairing technique was used to investigate *in situ* rates of denitrification and anaerobic ammonium oxidation (anammox) and their coupling with nitrification in intertidal wetlands of the Yangtze Estuary. The measured rates varied from below the detection limit to 152.39 µmol N/(m^2·hr) for denitrification and from below the detection limit to 43.06 µmol N/(m^2·hr) for anammox. The coupling links of nitrogen removal processes with nitrification were mainly dependent on nitrate, organic carbon, sulfide, dissolved oxygen and ferric iron in the estuarine and intertidal wetlands. Additionally, it was estimated that the actual nitrogen removal processes annually removed approximately 5% of the terrigenous inorganic nitrogen discharged into the Yangtze Estuary. This study gives new insights into nitrogen transformation and fate in the estuarine and intertidal wetlands.

Introduction

Nitrogen (N) is a limiting nutrient for eutrophication in coastal marine ecosystems (Damashek and Francs, 2017; Howarth and Marino, 2006). With increased human activities (*e.g.*, excessive application of nitrogen fertilizers and fossil fuel burning), a large amount of reactive nitrogen has been released into aquatic ecosystems through riverine runoff, atmospheric deposition and sewage discharge during the past several decades (Finlay et al., 2013; Galloway et al., 1996; Galloway et al., 2003). Since the era of industrial revolution, the input of reactive nitrogen has been nearly doubled into estuarine and coastal regions (Galloway et al., 2004; Howarth et al., 2011). Excess N loading has thus led to many aquatic ecological issues, such as eutrophication, water quality deterioration and hypoxia (Yabe et al., 2009). Therefore, understanding nitrogen removal processes and their controlling factors is essential for the elimination of nitrogen pollution in estuarine and coastal environments.

Dissimilatory nitrate reduction processes, denitrification and anaerobic ammonium oxidation (anammox), can remove reactive nitrogen from aquatic ecosystems permanently through converting oxidized nitrogen (NO_x^-) to dinitrogen gas (N_2) (Yin et al., 2015). Therefore, these two processes play a vital role in counteracting the adverse effects caused by nitrogen overload. Depending on the source of NO_x^-, nitrogen removal processes can generally be identified as uncoupled denitrification/anammox (D_w/A_w) which remove the NO_x^- from the overlying water, and coupled nitrification-denitrification/anammox (D_n/A_n) which eliminate the NO_x^- from the nitrification process (Jetten et al., 2001; Mulder et al., 1995; Nielsen, 1992; Risgaard-Petersen et al., 2003). The activity of uncoupled nitrogen removal processes

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(NR_w) and coupled nitrification-nitrogen removal processes (NR_n) are closely related to environmental factors. For instance, Seitzinger et al. (2006) indicated that high NO_x^- concentration in overlying water favors the uncoupled denitrification while low NO_x^- concentration in overlying water is beneficial to the coupled nitrification-denitrification. Previous studies suggested that under hypoxic condition, NR_n is inhibited, mainly due to the adverse effect of low dissolved oxygen (DO) on aerobic nitrification and its coupling with nitrogen removal processes (Kuparinen and Tuominen, 2001; Li et al., 2019; Rasmussen, 1992). However, low DO may be in favor of NR_n in some cases (Hansen and Blackburn, 1991; Rysgaard-Petersen et al., 1994). Under anoxic condition, the final form of nitrogen released from the sediment is altered, and ammonium generally dominates the efflux of nitrogen. With the increase of ammonium concentration, nitrification may be enhanced and thus stimulate NR_n (Abbasi and Adams, 2000; Enrich-Prast et al., 2016). Moreover, salinity variation can change the link between nitrification and nitrogen removal processes (Hu et al., 2019; Liu et al., 2019; She et al., 2016). However, the factors controlling NR_w and NR_n remain largely uncertain especially in a specific aquatic ecosystem.

Under the tidal fluctuation, the sediment in estuarine and intertidal wetlands experiences periodic exposure and inundation, which causes frequent redox changes (Robinson et al., 2006). Due to high sensitivity of nitrogen transformation to redox state, the intertidal area is thus a hotspot of nitrogen cycle (Damashek and Francis, 2017). During the ebb tide, oxic condition can enhance the activity of nitrification, and then during the flood tide, anoxic condition may support higher rates of NR_n (Jiao et al., 2018; Senga et al., 2019). Therefore, NR_n may be the main nitrogen removal pathway in intertidal wetlands (Peng et al., 2016). However, the intertidal ecosystems have been subjected to excessive loading of artificial contaminants (*e.g.*, reactive nitrogen and organic matter) in recent decades, which may alter the relative importance of NR_n and NR_w in intertidal wetlands (Galloway et al., 2004; Howarth et al., 2011; Liu et al., 2019; Peng et al., 2016). Therefore, it is of environmental and ecological significance to understanding the interaction of nitrogen removal processes with environmental factors and the relative importance of NR_n and NR_w for the intertidal wetlands.

Our previous research determined the potential rates of denitrification and anammox in the Yangtze estuarine and intertidal wetlands, using sediment-slurry incubation experiments (Hou et al., 2013). However, slurry incubation greatly changes nitrate availability and biogeochemical gradients, due to the mixing of the sediment. Therefore, the measured rates through sediment-slurry experiments cannot reflect the actual nitrogen removal from the sediment (Behrendt et al., 2013). In contrast, continuous-flow experiments could preserve the structural integrity of the sediments and simulate water flow, to some extent reflecting the *in situ* environment (Hou et al., 2012; Aoki and Mcglathery, 2017). In the present study, continuous-flow experiments combined with ^{15}N isotope pairing technique were thus conducted (1) to determine the actual rates of nitrogen removal processes and discriminate the uncoupled and coupled nitrogen removal processes, (2) to illustrate the main factors controlling the actual nitrogen removal rates and determining the relative importance of both NR_n and NR_w, and (3) to estimate environmental implications of nitrogen removal processes in intertidal wetlands of the Yangtze Estuary. This study helps us improve the understanding of nitrogen cycling in the estuarine and intertidal wetlands.

1. Materials and methods

1.1. *Study area and sample collection*

The Yangtze Estuary is the largest estuary in China, connecting the Yangtze River and the East China Sea. It is located in a typical subtropical monsoon region. Under the interaction between riverine runoff and tidal current, a marked salinity gradient occurs along the estuary. Extensive intertidal wetlands have developed along the estuarine and coastal zone, mainly due to the deposition of tremendous discharge of suspended sediment from the catchment. In this study, six sampling sites were selected along the intertidal wetlands of the Yangtze Estuary (Fig. 1), including Luchaogang (LCG), Donghai (DH), Bailonggang (BLG), Shidongkou (SDK), Liuhekou (LHK) and Xupu (XP). Sites BLG, SDK, LHK and XP are located in the freshwater area, while sites LCG and DH are located in the brackish area. Field surveys were conducted in April (spring), July (summer), October (autumn) 2017, and January (winter) 2018. At each sampling site, six intact cores (7.0 cm in diameter and 15 cm deep) were collected during the ebb period. Three of these cores were used to carry out the continuous-flow experiments. Surface sediment (0–5 cm deep) was also taken from the remaining cores for sediment characteristics measurements and slurry incubation experiments. Meanwhile, 30 L of overlying tidal water was collected from each site for continuous-flow experiments.

1.2. *Continuous-flow experiments*

Three replicate intact sediment cores from each site were installed respectively into a gas-tight continuous-flow system (Yin et al., 2016; Liu et al., 2019). The system was mainly composed of a gas-tight water bag (Tedlar), an acetol plunger with a Viton o-ring and two Peek tubings, an intact sediment core, and a multi-channel peristaltic pump (Appendix A Fig. S1). The acetol plunger was placed about 5 cm above the water-sediment interface. One end of inlet Peek tubing was near the sediment surface, and the other end was connected through a peristaltic pump with a gas-tight bag containing overlying tidal water enriched with $^{15}N\text{-}NO_3^-$ (90–99% $^{15}N\text{-}KNO_3$) to final concentrations of 100 μmol/L $^{15}N\text{-}NO_3^-$ (depending on *in situ* nitrate concentrations). One end of outlet Peek tubing was near the interface between the acetol plunger and overlying tidal water, and the other end was used for collecting incubation water. The water in the incubation system was replaced at a rate of 1.5 mL/min (Gardner and Mccarthy, 2009). The continuous-flow experiments were conducted at near *in situ* temperature and dissolved oxygen. After an overnight pre-incubation, the continuous-flow system reached a steady state (Mctigue et al., 2016). Within the next 24 hr, inlet and outlet water samples were collected with gas-tight vials (12 mL Exetainer, Labco, UK) every 6 hr. After collection, 200 μL saturated $ZnCl_2$ solution was injected into each vial to eliminate microbial activity. The concentrations of dissolved nitrogen gasses (including $^{29}N_2$ and $^{30}N_2$) in water samples were measured by membrane inlet mass spectrometry (HPR-40, Hiden Analytical, UK), with a detection limit of 0.1 μmol N_2/L (Yin et al., 2014). The actual denitrification (D_{14}) and anammox (A_{14}) rates, as well as the uncoupled (D_w and A_w) and coupled (D_n and A_n) denitrification/anammox rates were estimated according to the concentration changes of dissolved nitrogen gasses during the continuous-flow incubation (Liu et al., 2019). A detailed calculation method is provided in Appendix A.

1.3. *Analysis of environmental factors*

The *in situ* temperature, salinity, pH and DO in overlying tidal water were measured using a multi-parameter water quality meter (AZ86031, AZ Instrument, China). Concentrations of dissolved inorganic nitrogen (including NH_4^+, NO_3^- and NO_2^-) in overlying tidal water were analyzed by a continuous-flow nutrient analyzer (SAN Plus, Skalar Analytical B.V., the Netherlands), with detection limits of 0.5 μmol/L for NH_4^+ and 0.1 μmol/L for NO_3^- and NO_2^- (Hou et al., 2015). Water content of sediment (WC) was calculated from weight loss of fresh sediment by freeze-drying. Grain size of sediment was determined using a LS 13 320 Laser Grain Sizer (Beckman Coulter, USA). Sediment organic carbon (OC) was analyzed by the $K_2Cr_2O_7\text{-}FeSO_4$ method (Wang et al., 2007). Sediment sulfide concentration was determined using Sure-Flow combination silver-sulfide electrode (Orion, USA), with a detection limit of 0.1 μmol/L (Hou et al., 2012). Total extractable ferrous iron and ferric iron in sediments were analyzed using the ferrozine method described by Lovley et al. (1987). Sediment inorganic nitrogen was extracted using a 2 mol/L KCl solution and then analyzed using a continuous-flow nutrient analyzer (Liu et al., 2019). The data on these measured environmental factors are provided in Appendix A Table S1 and Table S2.

1.4. *Data analysis*

Analysis of variance (ANOVA) was used to examine the spatial and temporal changes in nitrogen removal processes (including D_{14}, A_{14}, D_w, A_w, D_n, and A_n). Pearson's correlation was performed to analyze the links of nitrogen removal processes with environmental variables. Redundancy analysis (RDA) was conducted to illustrate the effects of environmental factors on the uncoupled (D_w and A_w) and coupled (D_n and A_n) nitrogen removal processes, using CANOCO 5.

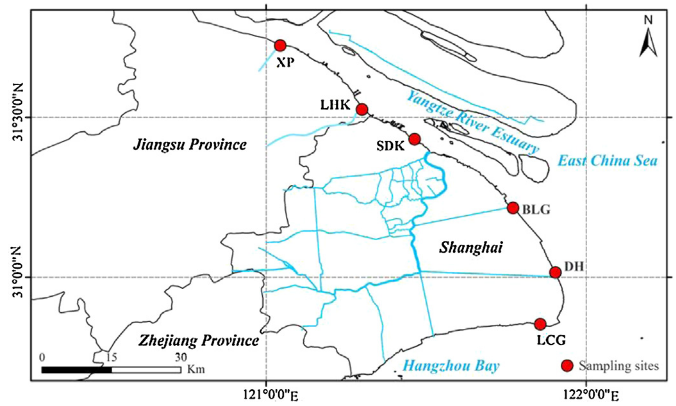

Fig. 1 – Sampling sites along the intertidal wetlands of the Yangtze Estuary. LCG: Luchaogang; DH: Donghai; BLG: Bailonggang; SDK: Shidongkou; LHK: Liuhekou; XP: Xupu.

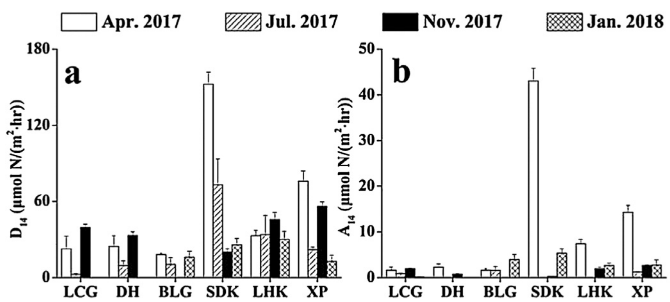

Fig. 2 – Actual rates of (a) denitrification (D_{14}) and (b) anammox (A_{14}) in the intertidal wetlands of the Yangtze Estuary.

2. Results

2.1. Actual rates of denitrification and anammox

The rates of D_{14} and A_{14} varied from below the detection limit to 152.39 μmol N/(m^2·hr) and from below the detection limit to 43.06 μmol N/(m^2·hr), respectively (Fig. 2). In the study area, denitrification was the dominant nitrogen removal process, contributing to about 89% of the total nitrogen removal (sum of denitrification and anammox). A significant spatial difference was found in the D_{14} rates (two-factor ANOVA, $F = 218.813$, $df = 5$, $P < 0.001$) and A_{14} rates (two-factor ANOVA, $F = 643.867$, $df = 5$, $P < 0.001$). In general, these two nitrogen removal rates were higher at the freshwater sites than at the brackish sites. There were no identical seasonal changes for D_{14} and A_{14} rates among all sampling sites. According to annual data of nitrogen removal rates and environmental factors, we conducted Pearson's correlation analysis (Fig. 3). The results showed that both D_{14} and A_{14} rates were not only positively correlated with nitrite ($r = 0.58$, $P < 0.01$ and $r = 0.65$, $P < 0.01$, respectively) in overlying tidal water but also positively correlated with OC ($r = 0.60$, $P < 0.01$ and $r = 0.53$, $P < 0.01$, respectively) and nitrite ($r = 0.85$, $P < 0.01$ and $r = 0.51$, $P < 0.05$, respectively) in sediment. In addition, the D_{14} rates were also positively correlated with sulfide ($r = 0.51$, $P < 0.05$) in sediment.

2.2. Uncoupled/coupled nitrogen removal process

NR_w includes D_w and A_w, and NR_n includes D_n and A_n. In this study, the rates of D_w and A_w ranged from below the detection limit to 152.39 μmol N/(m^2·hr) and from below the detection limit to 43.06 μmol N/(m^2·hr), respectively, while the rates of D_n and A_n varied from below the detection limit to 20.87 μmol N/(m^2·hr) and from below the detection limit to 2.04 μmol N/(m^2·hr), respectively (Fig. 4). The average rate of NR_w (31.12 μmol N/(m^2·hr)) was much higher than that of NR_n (4.48 μmol N/(m^2·hr)), indicating that NR_w was the main nitrogen removal pathway in intertidal wetlands of the Yangtze Estuary. The rates of NR_w were generally higher at the freshwater sites than at the brackish sites, while the rates of NR_n increased with the increasing salinity.

2.3. Effects of environmental factors on nitrogen removal pathways

RDA was used to investigate the effects of environmental factors on uncoupled and coupled nitrogen removal processes. According to the annual data of different nitrogen removal rates and environmental factors, we performed the RDA analysis (Fig. 5). The first two RDA axes (principal components) explained 66.9% of the cumulative percentage variance. The first and second axes accounted for 48.5% and 18.4% of the total variations of D_w, D_n, A_w, and A_n, respectively. NR_w rates were positively correlated with contents of inorganic nitrogen, OC and sulfide. NR_n rates were positively correlated with contents of WC and ferric iron. It is shown that the environmental factors played an important role in regulating the rates of different nitrogen removal pathways.

3. Discussion

Due to human activities, the nitrogen load imported into aquatic ecosystems has greatly increased, and led to severe nitrogen pollution. At present, denitrification and anammox are considered to be the two main pathways of N removal, which can effectively relieve the adverse effects caused by nitrogen overload (Yin et al., 2015). Although estuarine wetlands occupy only a small area of the earth's land surface, they play an important role in global nitrogen removal (Mctigue et al., 2016; Seldomridge and Prestegaard, 2011; Zheng et al., 2019). Our previous study determined the potential rates of denitrification and anammox in intertidal wetlands of the Yangtze Estuary through sediment slurry incubations (Hou et al., 2013), which generally overestimated the ability of sediment nitrogen removal. In order to obtain the actual rates of denitrification and anammox in the study area, continuous-flow experiments were conducted in combination with ^{15}N isotope pairing technique. According to our results, the rates of D_{14} and A_{14} ranged from below the detection limit to 152.39 μmol N/(m^2·hr) and from below the detection limit to 43.06 μmol N/(m^2·hr), respectively. Both of D_{14} rates and A_{14} rates were generally higher in the freshwater area than in the brackish area. Among the 6 sampling sites, the highest D_{14} and A_{14} rates appeared at site SDK, which might be related to sewage discharge near the site. Sewage containing a lot of nitrogen was conducive

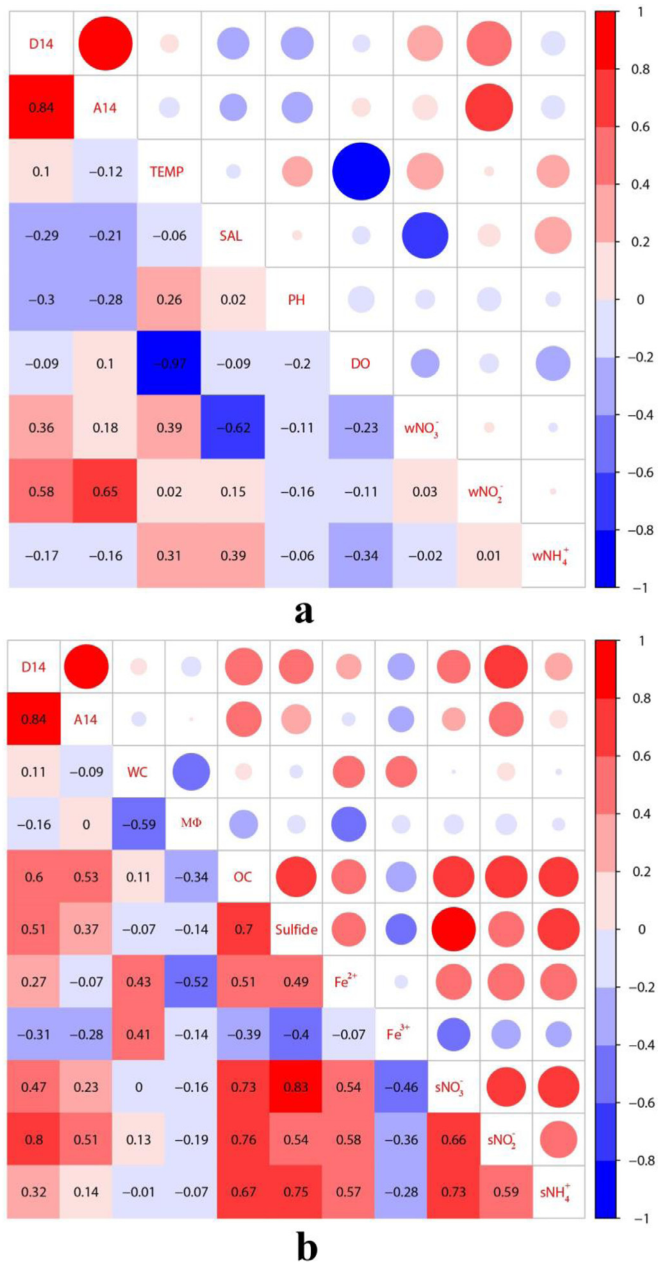

Fig. 3 – Correlations of actual nitrogen removal rates with physicochemical characteristics of (a) overlying tidal water and (b) sediment. TEMP: temperature; SAL: salinity; DO: dissolved oxygen; wNO_3^-: NO_3^- concentration in overlying water; wNO_2^-: NO_2^- concentration in overlying water; wNH_4^+: NH_4^+ concentration in overlying water; WC: water content; MΦ: median grain size; OC: organic carbon; Fe^{2+}: ferrous iron; Fe^{3+}: ferric iron; sNO_3^-: NO_3^- concentration in sediment; sNO_2^-: NO_2^- concentration in sediment; sNH_4^+: NH_4^+ concentration in sediment.

to producing more organic matter and sulfide and forming anoxia condition, which was in favor of nitrogen removal processes (Senga et al., 2019). However, although BLG site was also near a sewage outlet, the D_{14} and A_{14} rates at this site were lower, which was likely attributed to sediment grain size. The grain size of sediment at BLG was larger than that of other sampling sites (Appendix A Table S2). In general, coarse sediment with small specific surface area is not favorable to the attachment of nitrogen removal microorganisms and thus decreases the rate of nitrogen removal process (Xia et al., 2017). The actual nitrogen removal rates in intertidal wetlands of the Yangtze Estuary were several times higher than those examined in the adjacent sea of the Yangtze Estuary (Liu et al., 2019). There may be two reasons for this difference. Firstly, compared with the adjacent coastal sea, the intertidal wetland has higher nitrogen load in overlying water, which is beneficial to nitrogen removal process. Secondly, during the ebb tide, air enters the intertidal wetland, and the substrate is replenished with oxygen, subsequently promoting nitrification to produce more nitrate/nitrite for nitrogen removal processes (Li et al., 2015a).

Environmental factors play an important role in controlling nitrogen removal. However, for a specific environment, the environmental factors controlling nitrogen removal processes remain largely uncertain (Enrich-Prast et al., 2016). In the present study, the D_{14} rates were positively related to nitrite ($r = 0.58$, $P < 0.01$) in the overlying tidal water and nitrite ($r = 0.80$, $P < 0.01$) and nitrate ($r = 0.47$, $P < 0.05$) in sediment, indicating that the importance of NO_x^- as the substrate in controlling denitrification. This result was in consistence with other studies (Koop-Jakobsen and Giblin, 2010; Liu et al., 2018). Canonical denitrification belongs to heterotrophic dissimilatory nitrate reduction, in which organic matter is required to provide energy and electrons (Gao et al., 2017). Therefore, more organic matter is conducive to denitrification, which was confirmed by the positive relationship between the D_{14} rates and OC in our study (Fig. 3). Sulfide is a toxic substance, and high concentration of sulfide can threaten aquatic ecosystems. Some

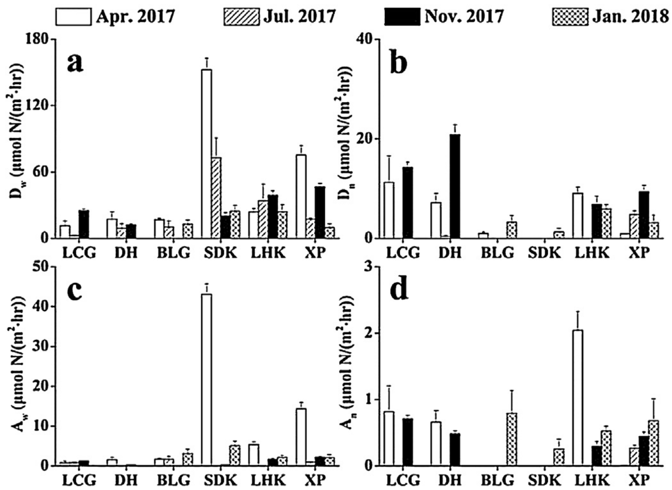

Fig. 4 – Rates of (a) uncoupled denitrification (D_w), (b) coupled nitrification-denitrification (D_n), (c) uncoupled anammox (A_w), and (d) coupled nitrification-anammox (A_n) in the intertidal wetlands of the Yangtze Estuary.

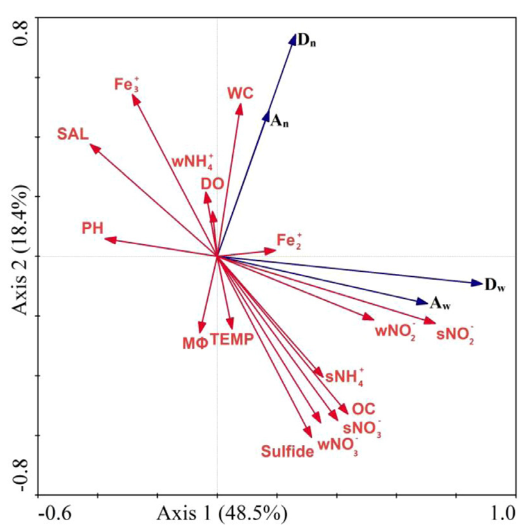

Fig. 5 – RDA analyses of uncoupled and coupled nitrification nitrogen removal processes with environment factors.

studies reported that the excessive sulfide in sediment can reduce the denitrification rate by inhibiting the metabolism of denitrifiers (Aelion and Warttinger, 2010; Brunet and Garcia-Gil, 1996). However, it was also found that sulfide as a strong reducing substance can promote denitrification by providing electrons (Deng et al., 2015; Yang et al., 2015). This contradiction might depend on the form of sulfide. H_2S can inhibit denitrification, while FeS and elemental sulfur may be in favor of denitrification (Burgin and Hamilton, 2007). In the present study, there was a significant positive correlation between sulfide and ferrous iron (Fig. 3), suggesting that FeS may be the main sulfide form in the Yangtze estuarine and intertidal wetlands. It explained why there was a positive correlation between the D_{14} rates and sulfide.

Ammonium and nitrite are the substrates of anammox reaction, so anammox activity may depend on the levels of ammonium and nitrite. In this study, it was observed that the A_{14} rates were not correlated to ammonium but to nitrite in the overlying water ($r = 0.65$, $P < 0.01$) and sediment ($r = 0.51$, $P < 0.05$) (Fig. 3). These relationships indicated that nitrite is the limiting substrate for anammox activity in intertidal wetlands of the Yangtze Estuary. It's worth noting that the autotrophic anammox process was positively correlated with organic carbon ($r = 0.60$, $P < 0.01$). Previous studies have reported that nitrite produced by denitrification was a significant source for anammox (Gao et al., 2017; Shan et al., 2016), which was supported by the significant correlation between the A_{14} and D_{14} rates in our study ($r = 0.84$, $P < 0.01$). Therefore, organic carbon might indirectly promote the rate of A_{14} through stimulating denitrification activity.

According to the sources of NO_x^-, nitrogen removal processes can be divided into NR_w in which NO_x^- is derived from the overlying water and NR_n in which NO_x^- is obtained from nitrification (Fernandes et al., 2016; Nielsen, 1992; Risgaard-Petersen et al., 2003). Previous studies show that the rates of NR_w were high under aquatic ecosystems with high nitrate concentrations, organic matter contents, or reducing substances (*e.g.*, sulfide and ferrous iron), while the rates of NR_n were high under aquatic ecosystems with high concentrations of ammonium or DO (Abbasi and Adams, 2000; Burgin and Hamilton, 2007; Liu et al., 2019; Mctigue et al., 2016; Neubacher et al., 2011; Seitzinger et al., 2006;). So, the changes of NR_w and NR_n rates strongly depend on the environmental factors of aquatic ecosystems. Due to human activity, NO_x^- concentration in tidal water of the Yangtze Estuary has greatly increased in recent decades. In the 1960s, the concentration of NO_x^- in tidal water was below 10 μmol/L, but now it increased to about 120 μmol/L (Yang et al., 2019). During the flood tide, the intertidal sediment is submerged by tidal water with high concentration of nitrate. High concentration of nitrate is beneficial to the diffusion of nitrate into the sediment (Chen et al., 2015), thus enhancing the availability of nitrate to dissimilatory nitrate reducers in the sediment (Yin et al., 2015). In this case, the rates of NR_w were greatly promoted in intertidal wetlands of the Yangtze Estuary (Fig. 5), which was consistent with previous studies (Koop-Jakobsen and Giblin, 2010; Liu et al., 2019; Piehler and Smyth, 2011; Seitzinger et al., 2006). On the other hand, high nitrate concentration might also be in favor of the production of organic matter and sulfide (Senga et al., 2019). This assumption was supported by the positive correlations of inorganic nitrogen with organic carbon and sulfide in sediment (Fig. 3). Because organic matter and sulfide can directly provide energy and electrons or indirectly supply more NO_2^- for NR_w, the activity of NR_w was enhanced with the increase of organic carbon and sulfide (Fig. 5). However, there was no correlation between NR_n and organic carbon (or sulfide). The main pathway of NR_n includes two steps: ammonia oxidation (AO) and nitrite reduction (NIR), and AO is the first step of nitrification which is an autotrophic process and a rate-limiting step of NR_n (Miao et al., 2018; Liu et al., 2019). Therefore, the organic carbon and sulfide had a little effect on AO and NR_n. Compared with summer, the remaining seasons had high rates of NR_n (Fig. 4b and d). This was likely because the DO concentrations were higher in other seasons than in summer (Appendix A Table S1), which was in favor of nitrification. An interesting result in this study was that with the increase of ferric iron concentrations, the rates of NR_n were promoted (Fig. 5). According to Li et al. (2015b), ferric iron could promote the rates of anaerobic ammonium oxidation coupled with Fe(III) reduction (Feammox), which produces more NO_x^- and in turn favors NR_n (Appendix A Fig. S2).

Previous studies demonstrated that NR_n was an important nitrogen removal pathway in estuarine and intertidal wetlands (Jenkins and Kemp, 1984; Peng et al., 2016; Seitzinger et al., 2006; Welsh et al., 2000; White and Howes, 1994). This was partly because tide provided

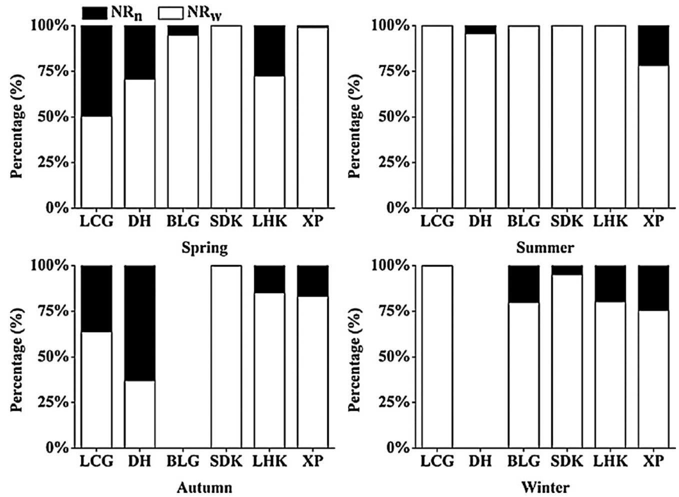

Fig. 6 – Percentage of uncoupled nitrogen removal processes (NR_w) and coupled nitrification-nitrogen removal processes (NR_n) in the intertidal wetlands of the Yangtze Estuary.

sufficient oxygen for nitrification to produce a large amount of NO_x^- and subsequently the nitrification-derived NO_x^- fueled nitrogen removal processes (An and Joye, 2001; Senga et al., 2019). However, this study found that compared with NR_n, NR_w was the dominant nitrogen removal pathway, contributing to about 89% of total nitrogen removal in intertidal wetlands of the Yangtze Estuary. The Yangtze Estuary had high NO_x^- concentrations in tidal water due to intensive human activities, which may alter the relative importance of both NR_n and NR_w. In the intertidal wetlands with low NO_x^- concentrations, nitrogen removal microorganisms can primarily rely on NO_x^- derived from the product of nitrification and oxygen is the dominant factor indirectly controlling nitrogen removal. In contrast, in the intertidal wetlands with high NO_x^- concentrations, nitrogen removal microorganisms could obtain sufficient NO_x^- from overlying water, and NR_w was thus a primary nitrogen removal pathway (Piehler and Smyth, 2011; Seitzinger et al., 2006). Although NR_n was not dominated, the relative importance of NR_n also showed the significant temporal and spatial variations in intertidal wetlands of the Yangtze Estuary (Fig. 6). The relative importance of NR_n in summer was generally lower than that in other seasons, which was likely attributed to low DO in summer. Low DO is adverse to aerobic nitrification and NR_n, but is beneficial to anaerobic NR_w (Rasmussen, 1992). The relative importance of NR_n was generally higher at the brackish sites than at the freshwater sampling sites. There are two reasons for this spatial difference. Firstly, due to the dilution of sea water, the concentration of nitrate in the brackish area was lower than that in the freshwater area (Appendix A Table S1), which thus enhanced the relative importance of NR_n. Secondly, salinity might play an important role in controlling the relative importance of different nitrogen removal pathways. Some studies suggested that as the salinity increases, ammonia oxidation is promoted, while nitrite oxidation is inhibited, which leads to the accumulation of NO_2^- (She et al., 2016). A large amount of NO_2^- accelerates nitrite reduction, and the relative importance of NR_n might thus be enhanced with the increase of salinity.

Nitrogen removal processes of intertidal wetland are critical to reduce nitrogen overload in estuarine and coastal ecosystems. Based on the average of actual nitrogen removal rates in the study area, the annual amount of removed nitrogen was estimated according to the following equation (Lin et al., 2017):

$$F = R \times a \times s \times t$$

where F (t N) denotes the annual amount of nitrogen removal; R (μmol N/(m^2·hr)) denotes the annual average of nitrogen removal rate in the study area; a denotes the unit conversion factor (0.14); s (km^2) denotes the area (approximately 1.2×10^4 km^2) of the tidal flats and adjacent zone along the Yangtze Estuary; and t (year) denotes the time. It is estimated that approximately 5.2×10^4 t N was annually removed from the study area, which accounted for approximately 5% of the total terrigenous inorganic nitrogen discharged into the Yangtze Estuary (Lin et al., 2017). The calculated contribution to nitrogen removal was lower than the estimation (25%) from sediment-slurry incubation (Deng et al., 2015). This comparison partly reflects that the role of sediment in nitrogen removal is largely overestimated by sediment-slurry experiments. Therefore, the actual capacity of nitrogen removal should be taken into account for the control of nitrogen pollution in the estuarine and intertidal wetlands.

4. Conclusions

This study first quantified the *in situ* rates of nitrogen removal processes, denitrification and anammox, and their coupling links with nitrification in the Yangtze estuarine and intertidal wetlands. The *in situ* rates of denitrification and anammox varied from below the detection limit to 152.39 μmol N/(m^2·hr) and from below the detection limit to 43.06 μmol N/(m^2·hr), respectively. Meanwhile, the rates ranged from below the detection limit to 195.45 μmol N/(m^2·hr) for the uncoupled nitrogen removal processes and from below the detection limit to 21.35 μmol N/(m^2·hr) for the coupled nitrogen removal processes. The uncoupled nitrogen removal pathways were generally associated with nitrate, OC and sulfide, while the coupled nitrogen removal pathways were related to DO and ferric iron. It was estimated that *in situ* denitrification and anammox processes annually removed approximately 5.2×10^4 t N from the study area, which was approximate to 5% of the total terrigenous inorganic nitrogen discharged into the Yangtze estuarine and coastal environments.

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Appendix A. Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.03.005.

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