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Coupled relationships among anammox, denitrification, and dissimilatory nitrate reduction to ammonium along salinity gradients in a Chinese estuarine wetland

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ABSTRACT

Salinization in estuarine wetlands significantly alters the balance between their nitrogen (N) removal and retention abilities but these processes have not yet been characterized effectively. In the present study, the potential rates of sediment denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonium (DNRA) were mapped using N isotope tracing methods along salinity gradients across the Yellow River Delta wetland (YRDW) in China. The contribution of anammox to total dissimilatory N transformations in YRDW was merely 6.8%, whereas denitrification and DNRA contributed 52.3% and 40.9%, respectively. The potential rate of denitrification (5.82 μmol/kg/h) decreased significantly along salinity gradients and markedly exceeded DNRA potential rate (2.7 μmol/kg/h) in fresh wetlands, but was lower than that of DNRA in oligohaline wetlands (3.06 and 3.18 μmol/kg/h, respectively). Moreover, a significantly positive relationship between salinity and DNRA/denitrification was obeserved, indicating that increased salinity may favor DNRA over denitrification. Furthermore, total sulfur (TS) content and ratio of total organic carbon to total nitrogen (C/N) increased with the salinity gradient and showed evident positive relationships with the DNRA/denitrification ratio. In this study, we proved that increased salinization resulted in the dominance of DNRA over denitrification, possible through the addition of S and alteration of the C/N in estuarine wetlands, leading to increased N retention in estuarine wetlands during salinization, which would enhance the eutrophication potential within wetlands and in downstream ecosystems.

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Introduction

Estuarine wetlands (EW), which are situated at the interface between land and sea, feature a special dynamic biogeochemical landscape that provides unique ecosystem services. Along with protecting biodiversity and regulating environmental disturbances, EW can purify water that flows adjacent to the sea (Barbier et al., 2008). Given that N is the biologically limiting nutrient along global coastlines, the N removal ability of EW is critical for offshore water quality. Nitrate reduction can be differentiated into pathways of denitrification (from NO_3^- to ultimately N_2) and dissimilatory nitrate reduc-

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tion to ammonium (DNRA) (from NO_3^- to ultimately NH_4^+), in which N can be removed or retained. Anaerobic ammonium oxidation (anammox), that converts NH_4^+ to N_2 using NO_2^- as the electron acceptor is another important N removal pathway (Kuypers et al., 2018).

These three N transformation processes determine the ultimate fate of N in EW. However, their magnitudes for N transformation are inconsistent in different microhabitats. For instance, anammox generated a higher proportion of N_2 in freshwater wetlands than in saltwater wetlands in New England marshes (Koop-Jakobsen and Giblin, 2009). Denitrification attributed to majority of the N removal in freshwater marshes (Giblin et al., 2010; Herbert et al., 2015), while DNRA was equally important as denitrification in coastal systems (Bernard et al., 2015; Dunn et al., 2013). A growing body of research has indicated that the ratio of total organic carbon to total nitrogen (C/N) majorly regulated the partitioning of the relative amount of nitrate consumed by DNRA versus denitrification (Hardison et al., 2015; Hoffman et al., 2019; Jia et al., 2020).

EW have been susceptible to saltwater intrusion mainly due to sea level rise, reductions in riverine freshwater flow, alterations in subsurface freshwater, anthropogenic alteration of coastal geomorphology, and storm surges (Herbert et al., 2015). Salinization has clear implications for the biogeochemical cycling of major elements, such as carbon (C), phosphorous (P), sulfur (S), iron (Fe), and particularly N in wetlands. Generally, salinity has an adverse effect on denitrification in coastal wetlands (Giblin et al., 2010; Neubauer et al., 2019; Rysgaard et al., 1999), but the implications are not always consistent (Fear et al., 2005; Li et al., 2019). The impacts of salinization on C availability and S and Fe cycling may favor DNRA (Herbert et al., 2015). Thus, nitrate reduction could possibly shift from the removal pathway to internal transformation along the salinity gradient in the EW systems. However, this shift is not well characterized in a single EW system and the attendant factors are still undefined.

In this study, we aimed to collect data on variability patterns of denitrification, anammox, and DNRA in EW that experiences saltwater intrusion. We worked in the Yellow River Delta wetland (YRDW), a temperate estuarine wetland in Shangdong province, China. Potential rates of denitrification, anammox, and DNRA as well as other environmental factors, such as salinity, total organic C, total N, and total S were measured using the YRDW sediments. The objectives of this study were to: 1) assess the dominance of dissimilatory N transformation processes in the YRDW, 2) examine the variability patterns of the three processes along the salinity gradient, and 3) illustrate primarily, how salinization affects changes in dissimilatory N transformation processes.

1. Materials and methods

1.1. Site description

The Yellow River merges into the Bohai Sea in Shangdong Province, with its estuary forming an estuarine wetland (the Yellow River Delta wetland, YRDW), that is characterized by weak tides and sandy soil. It is the most comprehensive, extensive, and youngest wetland ecosystem in the warm temperate zone in China. Some areas of the YRDW have experienced a high N load. In addition, due to decreases in precipitation, increase in sea level, high rates of evaporation, high water consumption in human settlements, and low water levels in the estuary, seawater intrusion has occurred often in this wetland (Fan et al., 2012; Ye et al., 2004).

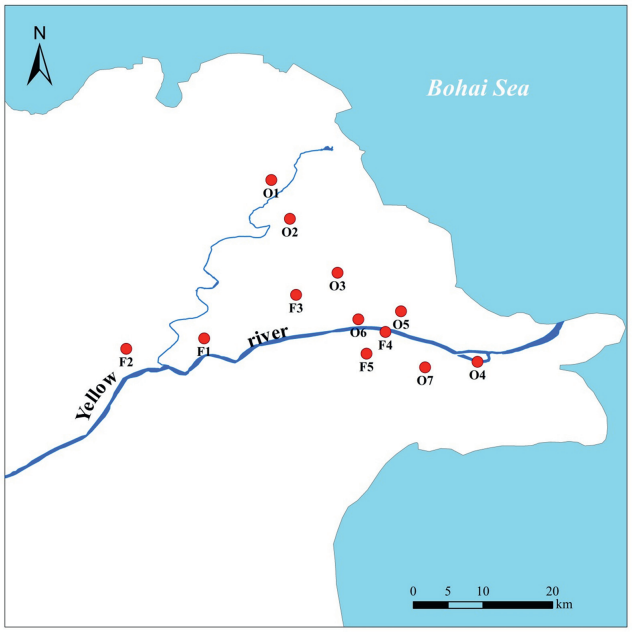

Fig. 1 – Sampling sites in the Yellow River Delta wetland (YRDW). F represents fresh wetlands and O represents oligohaline wetlands.

1.2. Sampling

EW can be classified as fresh (0–0.5 psu), oligohaline (0.5–5 psu), mesohaline (5–18 psu), polyhaline (18–30 psu), and mixoeuhaline (> 30 psu). For this study, 12 sites were examined along the salinity gradient in July 2019; they included five fresh sites (F1–F5) and seven oligohaline sites (O1–O7) (Fig. 1). Three mixed samples of surface sediment (height 0–5 cm) were collected at each site. The samples were stored in a portable fridge at the sampling site and were immediately brought to the laboratory for detection and analysis of chemical characteristics and processes.

1.3. Measurement of sediment properties

Dried aliquots of sediments were ground and homogenized to determine the total N (TN) content using a CHN elemental analyzer (CE-440, EAI, USA). The total organic carbon (TOC) content was determined using a TOC analyzer (Multi N/C 3100, Jena Analytics, Germany). The total S (TS) content was determined using the BaSO4 turbidimetry method (Zhang et al., 2017). For measurements of total P (TP), 0.2 g of finely ground dry soil was combusted at 450 °C in a muffle furnace for 3 hr. After extraction of P with 3.5 mol/L HCl (Ruban et al., 1999), the resulting solutions were initially digested (Ebina et al., 1983)

and then spectrophotometrically analyzed for the presence of orthophosphates (Murphy and Riley, 1986).

1.4. *Potential rates of anammox and denitrification and dissimilatory nitrate reduction to ammonium (DNRA) measurements*

Slurry experiments were conducted to detect the potential rates of anammox, denitrification, and DNRA using N isotope tracing methods, according to the slurry experiment protocols by Engström et al. (2005) and Yin et al. (2014). Briefly, slurries were made with the sampled sediments and artificial seawater at a ratio of 1:7. The slurries were aerated with He for 30 min before being transferred into 12 mL glass vials under a helium (He) atmosphere for 12 hr of pre-incubation at 25 °C, with the aim to eliminate native NO_3^-, NO_2^-, and O. After pre-incubation, these vials were divided into three treatments, which were spiked through the septa of each vial with helium-purged stock solutions of 1) $^{15}NH_4^+$ (100 μmol ^{15}N/L) (Treatment1), 2) $^{15}NH_4^+ + {}^{14}NO_3^-$ (Treatment 2) and 3) $^{15}NO_3^-$ (100 μmol ^{15}N/L) (Treatment 3). After 8 hr incubation, $ZnCl_2$ (50%) was added to inhibit microbial activity. The concentrations of N_2 produced during the incubation period were measured using membrane inlet mass spectrometry (MIMS). In Treatment 1, no significant production of ^{15}N-labeled gasses ($^{29}N_2$ and/or $^{30}N_2$) was detected, indicating that the ambient nitrite and nitrate in slurries were consumed during pre-incubation. In Treatment 2, the production of $^{29}N_2$ was measured whereas no production of $^{30}N_2$ was observed, implying that anammox occurred at our study area. In Treatment 3, both $^{29}N_2$ and $^{30}N_2$ were determined. The potential rates of anammox and denitrification were calculated based on the $^{15}NO_3^-$ incubation (Treatment 3) using the methods developed by Thamdrup and Dalsgaard (2002) and Yin et al. (2014).

The same pre-incubation procedures were conducted before DNRA measurements. After pre-incubation, the slurry vials were spiked with $^{15}NO_3^-$ (final concentration approximately 100 μmol/L; final ^{15}N percentage approximately 90%u−99%, depending on the concentration of background NO_3^-). Furthermore, these vials were divided into two groups having three replicates each. Group 1 represented the initial samples, which were preserved with 300 μL of saturated $ZnCl_2$ solution. The vials in Group 2 were shaken (at 200 r/min) and incubated for approximately 8 hr at a 25 °C. After incubation, the slurries were stirred and purged with He for 30 min to remove any $^{29}N_2$ and/or $^{30}N_2$ generated by denitrification and/or anammox. Later, all vials were injected with 0.2 mL of hypobromite iodine solution to convert NH_4^+ to N_2. The concentrations of $^{29}N_2$ and $^{30}N_2$ produced during the incubation were measured using MIMS. The potential rates of DNRA were calculated according to Yin et al. (2014).

1.5. *Statistical analysis*

Differences between the potential rates and physiochemical parameters in fresh and oligohaline wetlands were analyzed using Analysis of Variance (ANOVA), which was followed by Tukey's test for pairwise comparisons (α=0.05). These analyses were performed using SPSS statistical software (Version21, IBM Corp., Armonk, NY, USA). In addition, Pearson's correlation tests were conducted to identify the relationships between the physiochemical parameters and the potential rates on log(x) transformed data to ensure that the distribution of the data was minimally skewed and to achieve a fit close to a normal distribution. Pearson's correlation tests and graphical representation were done with Sigmaplot 14 software. The level of statistical significance was set at α=0.05 (P value<0.05).

2. Results

2.1. *Physiochemical characteristics of the yellow river delta wetland (YRDW)*

Our sampling sites in the YRDW were classified as fresh and oligohaline wetlands with average salinity values of 0.22 and 1.24 psu, respectively. Variations in the physiochemical characteristics of these wetlands were observed (Table 1). The average TOC contents in sediments from the fresh and oligohaline wetlands were 3610 and 3880 mg/kg, respectively. Furthermore, the TN content in fresh and oligohaline wetlands was 226.7 and 194.4 mg/kg, respectively. The average TS and TP contents were significantly higher in fresh wetland sediments (349.9 and 573.3 mg/kg, respectively) than those in oligohaline wetland sediments (287.2 and 543.3 mg/kg, respectively) (P<0.05). Notably, the TOC/TN ratio (C/N ratio) in oligohaline wetland sediments (21.0) significantly exceeded that in fresh wetland sediments (17.96) (P<0.05).

2.2. *Denitrification, anammox and DNRA activities*

The potential rates of anammox, denitrification, and DNRA were observed in the range of 0.15–1.04, 0.14–9.61, and 0.43–6.33 μmol/kg/hr, respectively. The contribution of anammox to total dissimilatory N transformations in YRDW was merely 6.8%, whereas denitrification and DNRA contributed 52.3% and 40.9%, respectively (Fig. 2a). In addition, the contribution of anammox to total dissimilatory N transformations in fresh and oligohaline wetlands was 6.4% and 7.6%, respectively. The relative contribution of denitrification to the total dissimilatory N transformations in fresh wetland (62.6%) was significantly higher than that in oligohaline wetland (38.8%) while the relative contribution of DNRA was significantly lower in fresh wetland (40.0%) than in oligohaline wetlands (53.5%) (P<0.05) (Fig. 2b).

Moreover, the potential rates of the three dissimilatory N transformations varied significantly between the fresh and oligohaline wetlands; the average values of potential rates of anammox were 0.58 and 0.48 μmol/kg/h, respectively, 5.82 and 2.70 μmol/kg/h, respectively, for denitrification, and 3.06 and 3.18 μmol/kg/h, respectively, for DNRA (Fig. 3). The potential rates of anammox and denitrification demonstrated a significant downward trend from fresh to oligohaline wetlands (P<0.05). In addition, the potential rate of denitrification significantly exceeded anammox and DNRA potential rates in fresh wetlands (P<0.05) while was down from DNRA slightly in oligohaline wetlands (Fig. 3).

Table 1 – Physicochemical characteristics of sediments of different salinities from YRDW.

	Salinity (psu)	TOC (mg/kg)	TN (mg/kg)	C/N	TS (mg/kg)	TP (mg/kg)
fresh	0.22±0.14[a]	3610±600[b]	226.7±90.4[b]	17.96±6.77[a]	287.2±10.9[a]	563.4±39.8[a]
oligohaline	1.24±0.47[b]	3880±880[b]	194.3±53.0[b]	20.89±6.63[b]	349.9±19.8[b]	573.3±38.9[b]

TOC: total organic carbon; TN: total nitrogen; C/N: total organic carbon/ total nitrogen; TS: total sulfur; TP: total phosphorus.
Values are the average-standard deviation of results. Significant difference level of different physicochemical parameters between two types of wetlands are indicated by a and b with $P<0.05$.

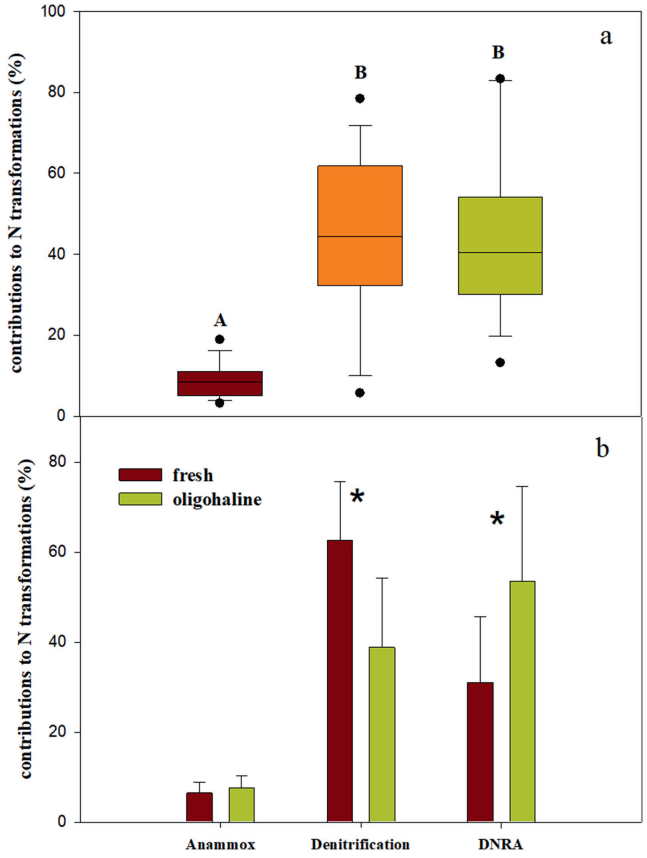

Fig. 2 – (a) Contributions of anammox, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA) to total dissimilatory N transformations from all sites and (b) along salinity gradient in YRDW sediments. Uppercase letters in (a) indicate significant differences among contributions of anammox, denitrification, and DNRA with $P<0.05$. * above the column in (b) indicates there is a significant difference between fresh and oligohaline wetlands ($P<0.05$). Error bars represent standard error of the mean of different sites from YRDW (a) and from fresh and oligohaline wetlands, respectively (b).

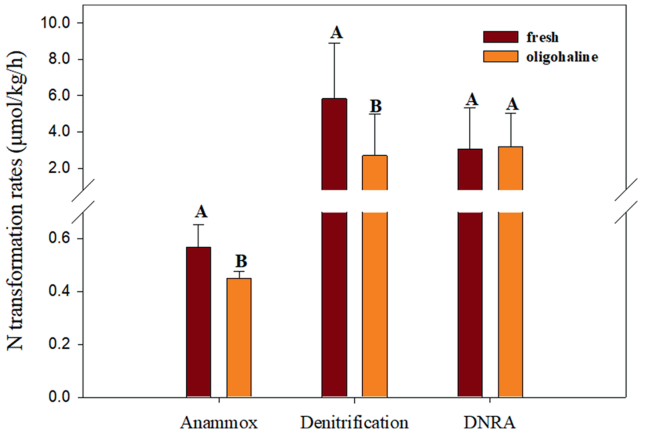

Fig. 3 – Potential rates of dissimilatory N transformations in sediments along a salinity gradient in YRDW. Significant difference level of N transformation rates between types of wetlands were indicated by A and B with $P<0.05$. Error bars represent standard error of the mean of different sites in fresh and oligohaline wetlands, respectively.

2.3. *Relationship between physiochemical characteristics of estuarine wetlands and associated dissimilatory N transformations*

The analyses results of the relationship between the physiochemical characteristics of EW and the potential rates of associated dissimilatory N transformations are shown in Table 2. TOC and TN contents were significantly and positively correlated with the potential rates of anammox, denitrification, and DNRA in the YRDW sediments ($P<0.05$). However, increased salinity reduced the potential rates of anammox and denitrification activities but was scarcely correlated with DNRA; this was also observed during the analysis of the relationship between the C/N ratio and the TS content. Furthermore, the relationships between the ratios of DNRA to denitrification and salinity (Fig. 4) and C/N and TS (Fig. 5) were markedly positive along the fresh and saline gradients ($P<0.05$).

3. Discussion

Salinization in coastal wetlands is a global ecological problem. Salinization in EW can change the dominant biogeochemical processes and eventually alter their ability to provide key ecosystem services (Herbert et al., 2015). One of the most important ecosystem services of EW is their ability to improve water quality by removing N, thereby reducing N loads into the adjacent seas; high N loads are one of the major causes of red tides in the ocean.

Microbes that are susceptible to changes in salinity mediate anammox, denitrification, and DNRA, the magnitudes of which determine the removal or retention of N in the EW system. In our study sites, denitrification and DNRA were signif-

Table 2 – Pearson's correlation between physicochemical characteristics and potential rates of anammox, denitrification, and DNRA.

	lg (Salinity)	lg(TOC)	lg(TN)	lg(C/N)	lg(TS)	lg(TP)
lg(A_{pr})	−0.65*	0.88*	0.85**	−0.57*	−0.56*	0.01
lg(D_{pr})	−0.75**	0.83**	0.79**	−0.55*	−0.72**	0.07
lg($DNRA_{pr}$)	0.4	0.87**	0.60*	0.05	0.21	0.18

A_{pr}, D_{pr} and $DNRA_{pr}$ represents potential rate of anammox, denitrification, and DNRA, respectively.
Significance at **α= 0.01 level; *α= 0.05 level.

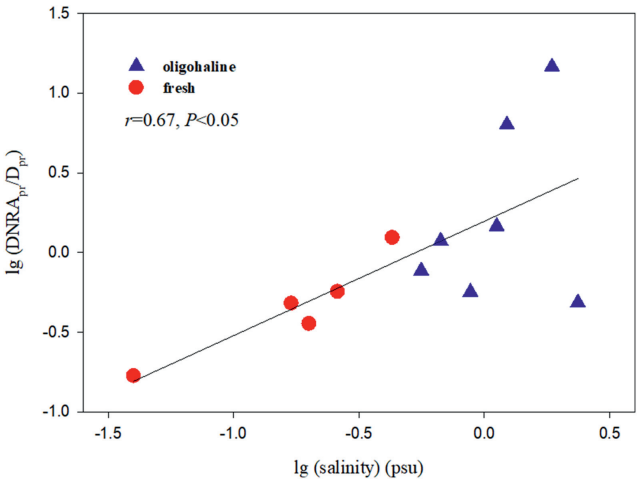

Fig. 4 – Relationship between salinity and ratios of potential rates of DNRA to denitrification in fresh and oligohaline wetlands in YRDW. $DNRA_{pr}$ and D_{pr} represents potential rate of DNRA and denitrification, respectively.

icantly more active than anammox (Fig. 2 and Fig. 3), which merely contributed 6.8% to the total dissimilatory N transformations. Consistent with our study, anammox was shown to decrease along salinity gradients in estuarine environments (Rich et al., 2008; Risgaard-Petersen et al., 2004), thereby indicating that denitrification and DNRA may contribute comparatively great to total dissimilatory N transformations than anammox in EW (Fig. 3). This could be partially attributed to high sediment organic carbon content (2140–5380 mg/kg) since previous studies have established that organic carbon-rich environments generally favored denitrification bacteria over anammox bacteria (Costa et al., 2006; Han et al., 2021). In addition, salinity was critical to determine the distribution of anammox bacteria (Oshiki et al., 2016; Wu et al., 2019). Some freshwater anammox consortium were prevailed in coastal wetland sediments (Li et al., 2020; Han et al., 2021; Zheng et al., 2020), and showed low tolerance to salinity (Dsane et al., 2020; Kartal et al., 2006) and their functional enzyme activities were inhibited significantly under salinization condition (Lin et al., 2021).

Salinization could significantly affect denitrification by altering substrate availability, concentration, and equilibrium (Burgin et al., 2012; Herbert et al., 2015), as well as by mediating the biota of denitrifiers (Han et al., 2021; Neubauer et al., 2019; Wang et al., 2018). Nevertheless, the effects of salinization on denitrification in wetlands were inconsistent in the different types of the latter (Herbert et al., 2015). Denitrification was more active in fresh wetlands than in oligohaline

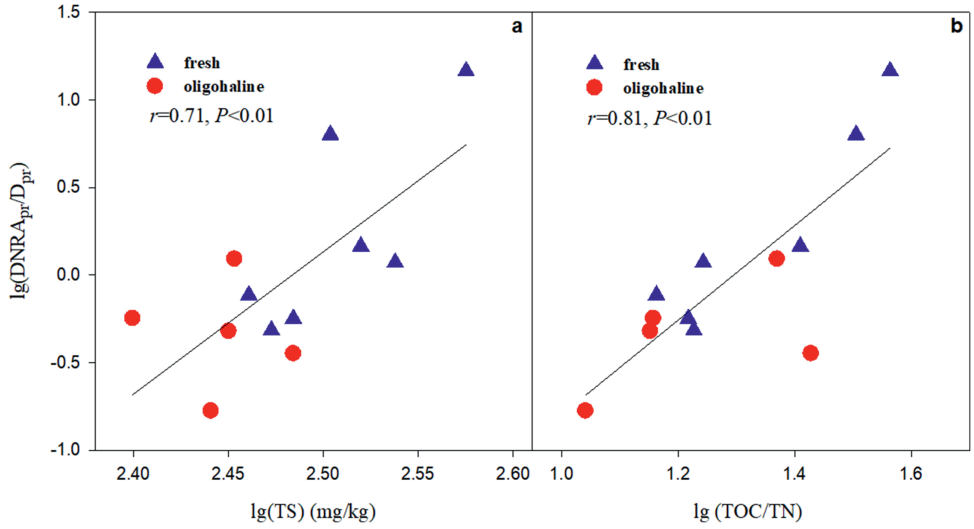

Fig. 5 – Relationships between ratios of DNRA to denitrification and (a) TS content as well as (b) TOC/TN, in fresh and oligohaline wetlands in YRDW. $DNRA_{pr}$ and D_{pr} represents potential rate of DNRA and denitrification, respectively.

wetlands (Fig. 2b and Fig. 3). Furthermore, the potential denitrification rates were significantly and negatively correlated with salinity ($P<0.05$) (Table 2). Additionally, salinity was a major factor inhibiting denitrification activity in the YRDW study area. Similar results were observed in the upper section of the oligohaline Parker River estuary, where denitrification rates were inversely correlated with salinity (Giblin et al., 2010); similar results were observed in the estuarine marshes in Demark (Rysgaard et al., 1999) and South Carolina, USA (Neubauer et al., 2019). DNRA retained N and could contradict denitrification rates in many coastal estuarine environments (Burgin and Hamilton, 2007; Murphy et al., 2020). Denitrification consistently outperformed DNRA in fresh wetlands while it was equally important in oligohaline wetlands in our study (Fig. 3). Furthermore, the ratios of DNRA to denitrification in the fresh and oligohaline wetlands of the YRDW had a significantly positive correlation with salinity ($P<0.05$) (Fig. 4). These results suggested a regime shift from N removal (denitrification) to the internal transformation of N (DNRA), which could be due to increase in salinity in oligohaline EW (Giblin et al., 2010).

DNRA could occur through either a heterotrophic or chemoautotrophic metabolic pathway (Burgin and Hamilton, 2007). Similar to denitrification, organic C sources were important for heterotrophic DNRA; this was indicated by the significant positive relationship between the TOC content and the potential rates of denitrification and DNRA ($P<0.05$) (Table 2). The quality of organic C sources is equally important; the sources are often considered to be the decisive factors for estimating whether NO_3^- reduction occurs via denitrification or fermentative DNRA (Crawshaw et al., 2019; Franklin et al., 2017; Giblin et al., 2013). In our study areas, C/N ratio, which is a function of the quality of organic C, was significantly positively correlated with the ratio of denitrification to DNRA along a fresh to oligohaline gradient ($P<0.05$) (Fig 4). In addition, salinity had positive implications for the C/N ratio, which was higher in oligohaline wetlands than in fresh wetlands ($P<0.05$) (Table 1). This might be due to the suppression of the mineralization of organic C in the higher salinity environment (Setia et al., 2011).

As chemoautotrophic DNRA uses inorganic substrates (for example, S^{2-}) as reductants (Tugtas and Pavlostathis, 2007), higher salinity levels favored DNRA by increasing SO_4^{2-} levels (Burgin and Hamilton, 2008). In addition, sulfide has been shown inhibitory effects on heterotrophic denitrification (Schonharting et al., 1998; Osborne et al., 2015). Moreover, it has been also inhibitor of autotrophic denitrification, despite its potential as an electron donor (Aelion and Warttinger, 2010; Cardoso et al., 2006; Ma et al., 2020; Murphy et al., 2020). High concentration of S^{2-} may inhibit the reductase enzymes that catalyze nitric oxidation and nitrous oxidation in denitrification, and thus the accumulated nitrite can be used by DNRA bacteria (Zhu et al., 2018). The dual influence of S induced by salinity could drive denitrification towards DNRA. This was partially confirmed in our study, in which denitrification activity was lower in oligohaline wetlands ($P<0.05$) (Fig. 3) with higher TS content than in fresh wetlands ($P<0.05$) (Table 1); additionally, the ratio of denitrification to DNRA was also significantly positively related to TS ($P<0.05$) (Fig. 5). Furthermore, due to salting out effect, elevated concentrations of ions induced by increased salinity reduce the solubility of O_2 (Stumm and Morgan 2012), resulting in more negative redox potential in the sediment of salinizing wetlands (Herbert et al., 2015). And more electrons can be transferred per mole NO_3^- through DNRA since it needs three more electrons per mole of reduced NO_3^- than denitrification does (Kraft et al., 2014; Tiedje et al., 1982), DNRA has the advantage over denitrification under more reducing (anoxic) condition (Matheson et al., 2002; Pandey et al., 2019, 2020; Rutting et al., 2011), where electrons donors abound relative to terminal electron acceptors. Under these conditions, an increase in salinity can stimulate reductive conditions to direct NO_3^- reduction towards DNRA.

4. Conclusion

Denitrification and DNRA contributed significantly more than anammox to dissimilatory N transformations in sediments of YRDW. Increase in salinity in the sediments of the wetlands significantly partitioned NO_3^- reduction by altering C, N, and S biogeochemical cycles in YRDW. Firstly, although sediment enrichment with organic C and N could stimulate denitrification and DNRA, higher C/N ratio induced by increase in salinity favored DNRA over denitrification. Secondly, the increase in S contents suppressed denitrification but facilitated DNRA. Thus, the increased wetland salinity eventually shifted the NO_3^- reduction regime from denitrification towards DNRA through the above two possible mechanisms in YRDW.

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