The effects of salinity on coupled nitrification and aerobic denitrification in an estuarine system

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ABSTRACT

Salinity has significant effects on nitrification and denitrification processes, particularly in estuarine systems. A dissolved oxygen-enriched river and its estuary in northern China were selected to investigate the impact of salinity gradients (0.6, 4, 7.6, 11.4 and 14.7‰) obtained from the mixing of river samples and estuarine samples with different proportions on coupled nitrification and aerobic denitrification via incubation experiments (35 and 10 °C). Results indicated that: (a) nitrification and coupled nitrification-aerobic denitrification occurred for all treatments, which resulted in NO_3^- being either accumulated or removed at the end of the incubation; (b) a suitable range of salinity is 4.0–11.4‰ for nitrification and 4.0–7.6‰ for coupled nitrification-aerobic denitrification; and (c) the relatively higher temperature (35 °C) can effectively stimulate N transformation processes compared to the lower temperature (10 °C) in the incubation experiment.

Key words | coupled nitrification-aerobic denitrification, estuary, nitrification, salinity

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INTRODUCTION

Nitrification and denitrification are the central processes of the N cycle. Nitrification is a process whereby ammonia (NH_3) is oxidized to nitrite (NO_2^-) or nitrate (NO_3^-) under aerobic conditions, while denitrification is the process whereby NO_3^- or NO_2^- is reduced to gaseous products such as nitrogen (N_2) or nitrous oxide (N_2O) under anaerobic condition (Ward 1996; Baron *et al.* 2013). Coupled nitrification–denitrification removes NO_3^- provided by nitrification from the aquatic systems, in other words, anaerobic denitrification depends on aerobic nitrification (Seitzinger 1988).

Salinity is a key regulating factor for the biogeochemical cycle of nitrogen (N) in coastal estuaries (Rysgaard *et al.* 1999; Li *et al.* 2009). Kemp & Boynton (1984) reported that nitrification and coupled nitrification–denitrification in sediments of estuaries decreased gradually with the increasing salinity. Furumai *et al.* (1988) found that a salinity range of 0–6‰ did not show a significant effect on NH_4^+ oxidation and NO_2^- oxidation in nitrification, but they were greatly inhibited at a salinity range of 15–30‰. When salinity increased from 0 to 10‰, the rate of denitrification decreased by 50% (Rysgaard *et al.* 1999) but a higher salinity has no obvious effect on it. Wang *et al.* (2002) reported that mineralization is more sensitive to low salinity compared to that of nitrification, and the rate of nitrification is higher than that of mineralization once the micro-organism adapted to the salinity. Li *et al.* (2009) found that salinity, dissolved oxygen (DO) and rate of denitrification showed a significant negative correlation. To our knowledge, denitrification generally occurs at completely anaerobic conditions (Payne 1981; Tiedje *et al.* 1982). However, researchers found that denitrification may occur under aerobic conditions, called aerobic denitrification or coupled nitrification-aerobic denitrification (Capone & Kiene 1988), which is the main NO_3^- removal process in some marine environments, estuaries, rivers and lakes (Seitzinger 1988; Rysgaard *et al.* 1993; An & Joye 2001; Risgaard-Petersen 2003; Sheibley *et al.* 2003; Xu *et al.* 2007; Gao *et al.* 2012). Based on the existing studies, the response of micro-organisms to salinity is uncertain, but estuarine strains in many cases exhibited optimal activity at low or moderate salinity (Rysgaard *et al.* 1999). Since the environmental factors, physico-chemical properties of estuaries and researching methods are different, it is rather difficult to obtain simple or direct conclusions regarding the effects of salinity on the N cycle.

doi: 10.2166/wst.2015.159

In this study, a DO-enriched river (Chaobaixin River) and its estuary located in northern China have been selected to investigate the effects of salinity gradients on coupled nitrification-aerobic denitrification via a combination of dissolved inorganic nitrogen (DIN: NH_4^+ and NO_3^-) and incubation experiments (35 and 10 °C).

MATERIAL AND METHODS

Study sites

The samples were taken from Chaobaixin River and its estuary in November 2012. The Chaobaixin River flows through a rural area and is 81 km in length and has a watershed area of about 1,387 km^2 (Gburek & Sharpley 1998). Animal manure is a potential dominant NO_3^- source as the watershed is an important livestock breeding base for the municipality (Calderon *et al.* 2005; Elmi *et al.* 2005; Shao *et al.* 2010).

Sampling and analyses

Water samples were taken on transects along the Chaobaixin River flow and the corresponding estuary. The filtered riverine and the estuarine samples were mixed well for incubation experiments. Simulating the background temperature (summer at 35 °C, winter at 10 °C) in the study sites, incubation experiments were set up as follows: (1) mixing the river samples and the estuarine samples at different proportions to obtain a salinity gradient of 0.6‰ (100% river sample), 4.0‰ (75% river sample + 25% estuarine sample), 7.6‰ (50% river sample + 50% estuarine sample), 11.4‰ (25% river sample + 75% estuarine sample), and 14.7‰ (100% estuarine sample); and (2) for each salinity treatment, we added 100 mL of the mixing water sample into a 250 mL flask in two replicates and fastened the caps for thermostatic oscillation. A series incubation was carried out for 0, 2, 5, 10, 48, 72 and 120 hours under 35 °C and 10 °C, respectively. At each incubation interval, water samples were taken and immediately measured for DO and DIN concentrations. DO was measured by a portable water quality probe (Thermo Orion, Beverly, MA, USA) in the laboratory. Nitrate and NH_4^+ concentrations were analyzed on a continuous flow analyzer (Auto Analyzer 3, Seal, Norderstedt, Germany). For NO_3^- determination, NO_3^- is reduced to NO_2^- by hydrazine in alkaline solution with a copper catalyst, after which it is reacted with sulphanilamide and NEDD (N-(1-napthyl) ethylenediamine dihydrochloride) to form a pink compound measured at 550 nm. For NH_4^+ determination, the sample is reacted with salicylate and dichloro-isocyanuric acid with a nitroprusside catalyst to produce a blue compound measured at 660 nm. The physico-chemical properties of the salinity treatments at the initial and the end of the incubations are summarized in Table 1. Both NO_3^- and NH_4^+ concentrations will show a time-variation trend in the incubation, since multiple processes such as nitrification, denitrification and mineralization will cause the NO_3^- and NH_4^+ concentrations to increase or decrease. Thus, the mean of variation rate (V_{mean}) between two incubation intervals (t_2, t_1) was calculated as

$$V_{mean} = \frac{C_2 - C_1}{t_2 - t_1} \quad (1)$$

where C_1 and C_2 are the measured DIN concentrations at incubation t_1 and t_2, respectively. A positive V_{mean} represents that substrate is increasing and a negative V_{mean} indicates that substrate is decreasing. An increase in NO_3^- was linked to nitrification as the dominant process, behaving as $V_{mean} > 0$ (defined as gross nitrification rate (GNiR)). In contrast, a decrease in NO_3^- was linked to a coupled nitrification-aerobic denitrification process, behaving as $V_{mean} < 0$ (defined as gross nitrification-aerobic denitrification rate (GNi-ADeR)).

RESULTS AND DISCUSSION

Coupled nitrification-aerobic denitrification in the river samples

The concentrations and mean variation rates of NO_3^-, NH_4^+ and DO varied meaningfully at 35 and 10 °C for the 0.6‰ salinity treatment (100% river) in the incubation experiments (Figure 1). At 35 °C before 2 hours, the concentration of NO_3^- decreased with a relatively higher mean rate of −5.2 μmol L^{-1} h^{-1}, potentially resulting from a NO_3^- removal process. Normally the decreasing NO_3^- concentrations are linked to denitrification, assimilation, or dissimilatory NO_3^- reduction to NH_4^+ (DNRA) processes (Rivera-Monroy *et al.* 2010; Roberts *et al.* 2014). The effects of assimilation might be negated as the filtered water samples were incubated to prevent NO_3^- uptake by photosynthetic organisms. Furthermore, the NH_4^+ concentrations were not accumulated when NO_3^- decreased, indicating that the DNRA process is unlikely to occur. Thus, denitrification is the dominant process for

Table 1 | The physico-chemical properties of the samples for all the treatments

Treatment	Salinity (‰)	DO (mg L^{-1})		NO_3^- (mg L^{-1})			NH_4^+ (mg L^{-1})			
		$C_{ini.}$	C_{End} (35 °C)	$C_{ini.}$	C_{End} (35 °C)	C_{End} (10 °C)	$C_{ini.}$	C_{End} (35 °C)	C_{End} (10 °C)
100% river (mean± SD)	0.6 ± 0.01	9.9 ± 0.1	7.4 ± 0.1	151.2 ± 0.6	155.1 ± 0.1	162.9 ± 0.1	266.9 ± 0.1	219.8 ± 0.8	266.9 ± 0.1
75%river + 25% estuary (mean± SD)	4 ± 0.1	9.5 ± 0.1	6.9 ± 0.1	117.5 ± 0.4	110.8 ± 0.4	118.4 ± 0.3	219.6 ± 0.4	153.7 ± 0.2	185.8 ± 0.1
50% river + 50% estuary (mean± SD)	7.6 ± 0.2	9.7 ± 0.1	6.9 ± 0.1	83.8 ± 0.1	92.1 ± 0.04	97.6 ± 0.1	172.3 ± 0.2	196.9 ± 0.1	96.2 ± 0.04
25% river + 75% estuary (mean± SD)	11.4 ± 0.04	10 ± 0.1	7.7 ± 0.1	50.1 ± 0.1	59.6 ± 0.3	83.0 ± 0.03	125.0 ± 0.1	125.0 ± 0.1	164.5 ± 0.3
100% estuary (mean± SD)	14.7 ± 0.1	10.3 ± 0.2	7.9 ± 0.1	16.4 ± 0.003	18.0 ± 0.1	13.6 ± 0.1	77.7 ± 0.5	107.8 ± 0.1	82.3 ± 0.5

C_{ini} represents initial concentration in the incubation.
C_{End} represents final concentration at the end of the incubation.

NO_3^- removal in the incubation experiments. DO was simultaneously consumed at a mean rate of 1.3 mg L^{-1} h^{-1}. The decrease of DO before 2 hours was potentially related to (1) aerobic nitrification in which DO was consumed to oxidize NH_4^+ to NO_2^- and NO_3^-; and (2) DO balance trend between the liquid and gas phases as the initial DO of 9.9 mg L^{-1} is supersaturated compared to the saturated DO of 7.0 mg L^{-1} at 35 °C. However, the latter DO escaping rate (V_{mean} of −0.1 mg L^{-1} h^{-1} for Milli-Q water as a blank) from liquid to gas phase is lower compared to the overall mean rate of DO consumption (V_{mean} of 1.3 mg L^{-1} h^{-1}) in this study. Nitrification is the dominant DO consumption process. Thus, a coupled nitrification-aerobic denitrification potentially regulated the NO_3^- variations and was considered as the process responsible for removing NO_3^- within the first 2 hours of the incubation. Many researchers confirmed that some pure cultures of bacteria could regulate denitrification under aerobic conditions (Robertson & Kuenen 1984; Ronner & Sorensson 1985; Trevors & Starodub 1987; Robertson *et al.* 1995). Moreover, various heterotrophic and autotrophic nitrifiers have been found responsible for simultaneous nitrification and aerobic denitrification (Castignetti & Hollocher 1984; Robertson *et al.* 1989). In 2–5 hours, the concentrations of NO_3^- increased with a consumption of DO and NH_4^+, indicating that nitrification was the dominant process to accumulate NO_3^-. Although the NO_3^- concentrations demonstrated a shift after 5 hours, the mean rates of NO_3^- tended to be zero after a period of incubation, possibly linked to the dynamic equilibrium of N turnover. Nitrate was increased by 2.6% at the end of the incubation. Note that the concentrations of NH_4^+ showed an accumulation in 48–72 hours, which may result from mineralization as compensation for NH_4^+ consumed in the earlier stage. Thereafter, the added NH_4^+ was consumed for nitrification.

At 10 °C, the nitrification and coupled nitrification-aerobic denitrification were responsible for NO_3^- accumulation and attenuation in the incubation as well. In addition, the variation of NH_4^+ was linked to mineralization and nitrification. Nitrate increased by 7.7% in the incubation. The variation rates of NO_3^- and DO were relatively lower than that at 35 °C as the lower temperature may inhibit microbial activities.

Coupled nitrification-aerobic denitrification in a salinity gradient

To obtain a salinity gradient, the river samples and the estuarine samples were mixed at different proportions as explained

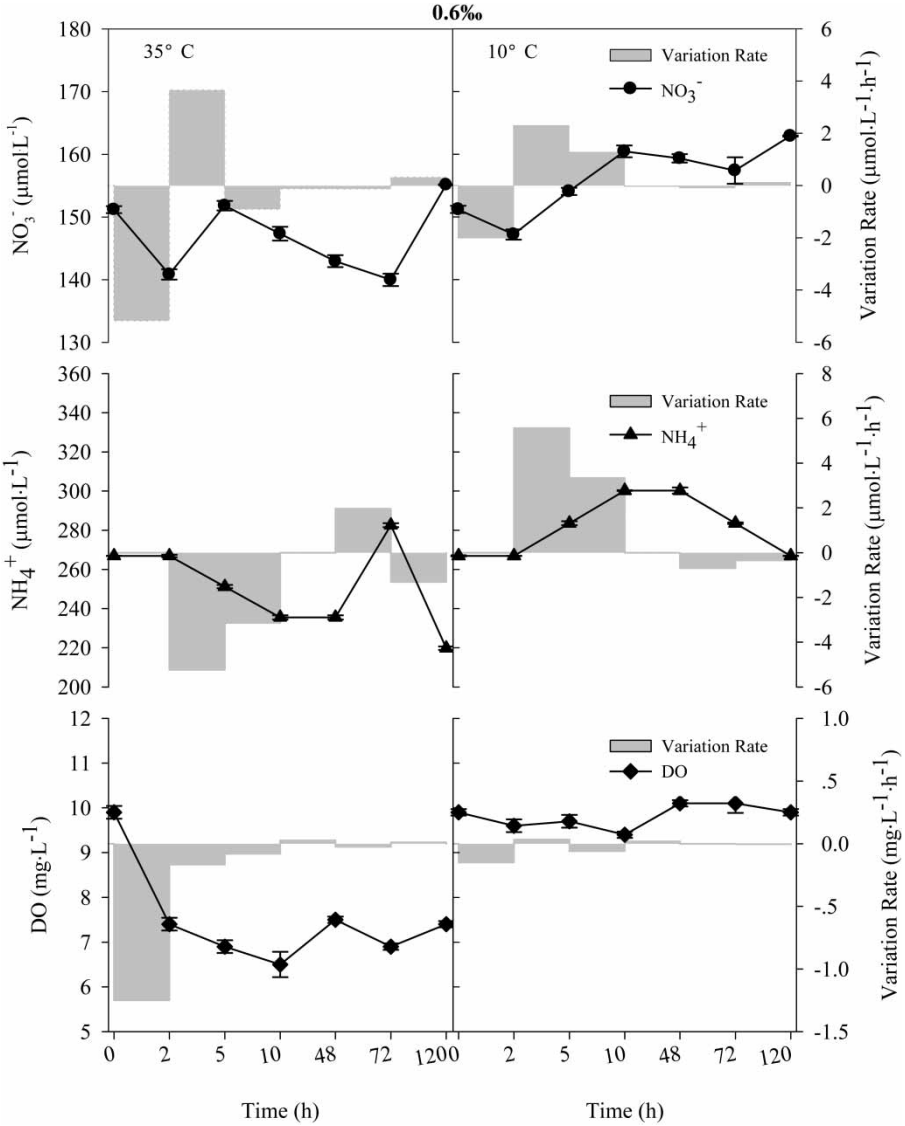

Figure 1 | Variations of NO_3^-, NH_4^+ and DO concentrations and the corresponding mean of the variation rates at 35 and 10 °C for river samples (salinity of 0.6 ‰).

under 'Sampling and analyses'. As shown in Figure 2, the concentrations and mean variation rates of NO_3^-, NH_4^+ and DO changed with the salinity gradient, revealing that salinity has important effects on N transformation in estuaries. In the relatively low salinity (4.0‰) treatment, the concentrations of NO_3^- greatly decreased (maximum V_{mean} is -20.2 μmol L^{-1} h^{-1}) with a consumption of DO due to the coupled nitrification-aerobic denitrification process before 5 hours. Thereafter, the NO_3^- rapidly increased (maximum V_{mean} is 11.8 μmol L^{-1} h^{-1}) as a result of nitrification. In contrast, for the treatments of salinity at 7.6 and 11.4‰, the concentrations of NO_3^- firstly increased with a maximum V_{mean} of 17.0 and 8.2 μmol L^{-1} h^{-1} caused by the nitrification process. Then, a coupled nitrification-aerobic denitrification resulted in NO_3^- attenuation with a maximum V_{mean} of -10.8 and -1.1 μmol L^{-1} h^{-1} for the two treatments, respectively. Ammonium, as a substrate for nitrification, rapidly increased with a maximum V_{mean} of 22.0 and 36.9 μmol L^{-1} h^{-1} for the salinity treatments of 4.0 and 7.6‰, respectively. It seems that the mineralization is more sensitive to the salinity gradient. After 10 hours, the mean variation rates of all the species tended to be zero due to the dynamic equilibrium of N turnover. Nitrate had been decreased by 5.7% for the treatment of salinity at 4.0‰ and increased by 9.9 and 18.9% for the treatments of salinity at 7.6 and 11.4‰ at the end of the incubation.

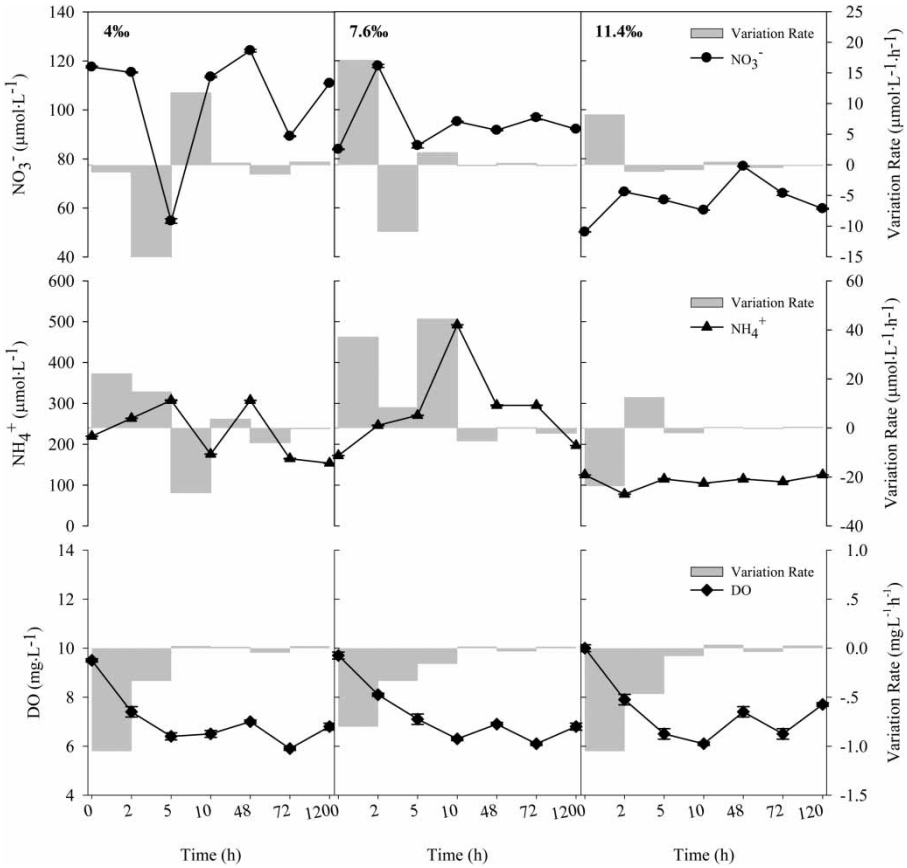

Figure 2 | Variations of NO_3^-, NH_4^+ and DO concentrations and the corresponding mean of the variation rates at 35 °C for a salinity gradient of 4.0, 7.6 and 11.4‰.

At 10 °C, both concentrations and mean variation rates of NO_3^- and NH_4^+ (Figure 3) demonstrated a different pattern compared to that at 35 °C. For the treatments of salinity at 4.0 and 7.6‰, NO_3^- accumulation and attenuation were regulated by the nitrification and coupled nitrification-aerobic denitrification processes. However, for the 14.7‰ salinity treatment, nitrification resulted in NO_3^- continuously accumulating in the entire incubation. At the end, NO_3^- had been increased by 0.8%, 16.5% and 65.8% for the treatments of salinity at 4.0‰, 7.6 ‰ and 11.4‰, respectively. The variation rates of all the species were relatively lower than that at 35 °C.

Coupled nitrification-aerobic denitrification in estuarine samples

As shown in Figure 4, different patterns of NO_3^-, NH_4^+ and DO have been observed in the 14.7‰ salinity treatment (100% estuary). At 35 °C, NO_3^- concentrations were accumulated by nitrification with a V_{mean} of 1.6 μmol L^{-1} h^{-1} before 2 hours, and then were removed by a coupled nitrification-aerobic denitrification with a V_{mean} of −1.7 μmol L^{-1} h^{-1} in 2–5 hours. Ammonium consumed by nitrification was continuously compensated for by mineralization, resulting in NH_4^+ accumulation at the end of the incubation. This could imply that the relatively high salinity is favoring mineralization of dissolved organic N at relatively high temperature. Nitrate was increased by 9.8% in the incubation compared to the early stage.

At 10 °C, the NO_3^- concentrations demonstrated a clear declining trend, linked to the coupled nitrification-aerobic denitrification. Simultaneously, the NH_4^+ concentrations were increased before 10 hours, linked to mineralization. The NO_3^- was decreased by 17.1% in the incubation.

The effects of salinity on coupled nitrification-aerobic denitrification

Nitrification is a process to increase NO_3^- concentrations and has a positive effect on NO_3^- variation rates (defined as GNiR and GNiR >0). In contrast, the coupled nitrification-aerobic denitrification is a process to decrease NO_3^-

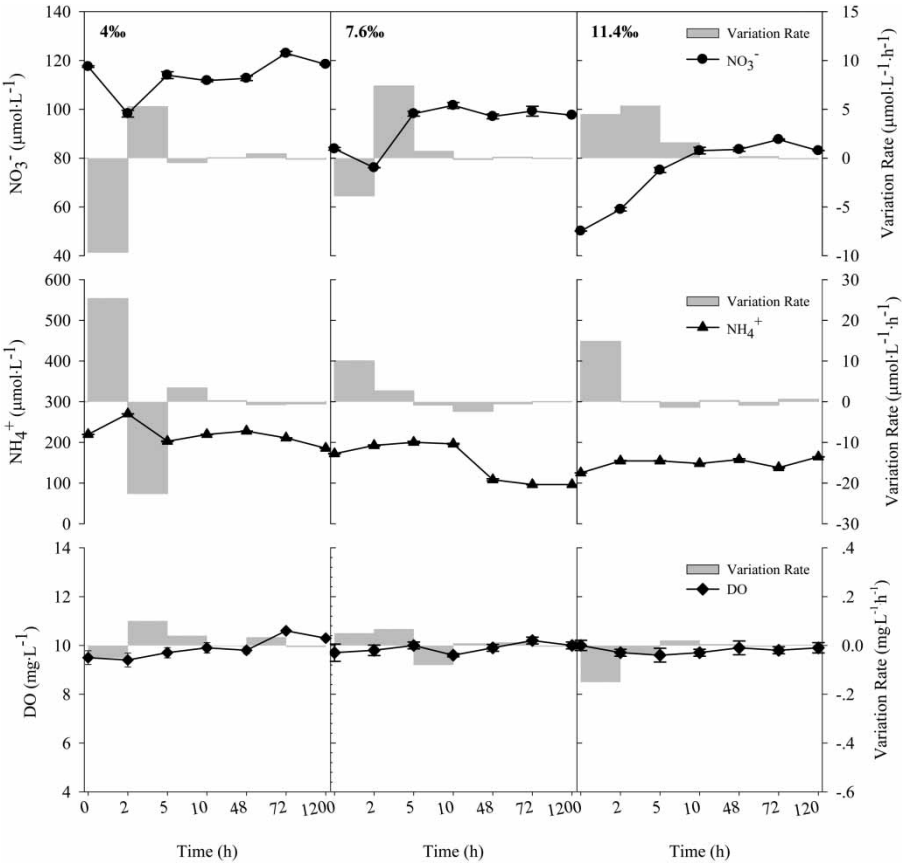

Figure 3 | Variations of NO_3^-, NH_4^+ and DO concentrations and the corresponding mean of the variation rates at 10 °C for a salinity gradient of 4.0, 7.6 and 11.4‰.

concentrations and has a negative effect on NO_3^- variation rates (define as GNi-ADeR and GNi-ADeR <0). The higher the GNiR or GNi-ADeR, the more active is the corresponding process. The maximum mean rates of nitrification and coupled nitrification-aerobic denitrification for different salinity treatment under 35 and 10 °C are shown in Figure 5. It is obvious that the nitrification and coupled nitrification-aerobic denitrification occurred in all the salinity treatments. Both GNiR and GNi-ADeR demonstrated an up–down trend with the salinity increase, potentially illustrating a significant impact of salinity on nitrification and coupled nitrification-aerobic denitrification (Gardner *et al.* 1991; Rysgaard *et al.* 1999). Since these two processes are mediated by micro-organisms, the variation rates of GNiR and GNi-ADeR to some extent may reflect the changes of the activity and function of the micro-organisms in response to the salinity gradient. Furthermore, it is also possible that the rapid salinity variation 'shocks' the microbial population, and changes the initial microbial population pattern in the mixed samples. Nitrification was more active in the salinity range of 4.0–11.4‰, and the peak value (17.0 μmol L^{-1} h^{-1}) of the variation rate occurred in the treatment of salinity at 7.6‰. Coupled nitrification-aerobic denitrification was more active in a relatively narrow salinity range of 4.0–7.6‰ and the peak value (−20.2 μmol L^{-1} h^{-1}) of the variation rate occurred in the treatment of salinity at 4.0‰. The suitable salinity range is different for GNiR and GNi-ADeR, possibly due to the different sensitivity and adaptation ability of the micro-organisms to the salinity gradient (Rysgaard *et al.* 1999). Berounsky & Nixon (1993) reported that the maximum nitrification rate of the Providence River estuary occurred in the salinity range of 20–28‰. However, Furumai *et al.* (1988) provided a suitable salinity range of 0–6‰ for nitrification of estuary sediments, in which nitrification was greatly inhibited in a salinity range of 15–30‰. In a subterranean estuary below a Rhode Island fringing salt marsh, the maximum rate of denitrification was observed in a salinity range of 12–14‰ (Addy *et al.* 2005). Although the effects of salinity on nitrification and coupled nitrification-aerobic denitrification is complex, the

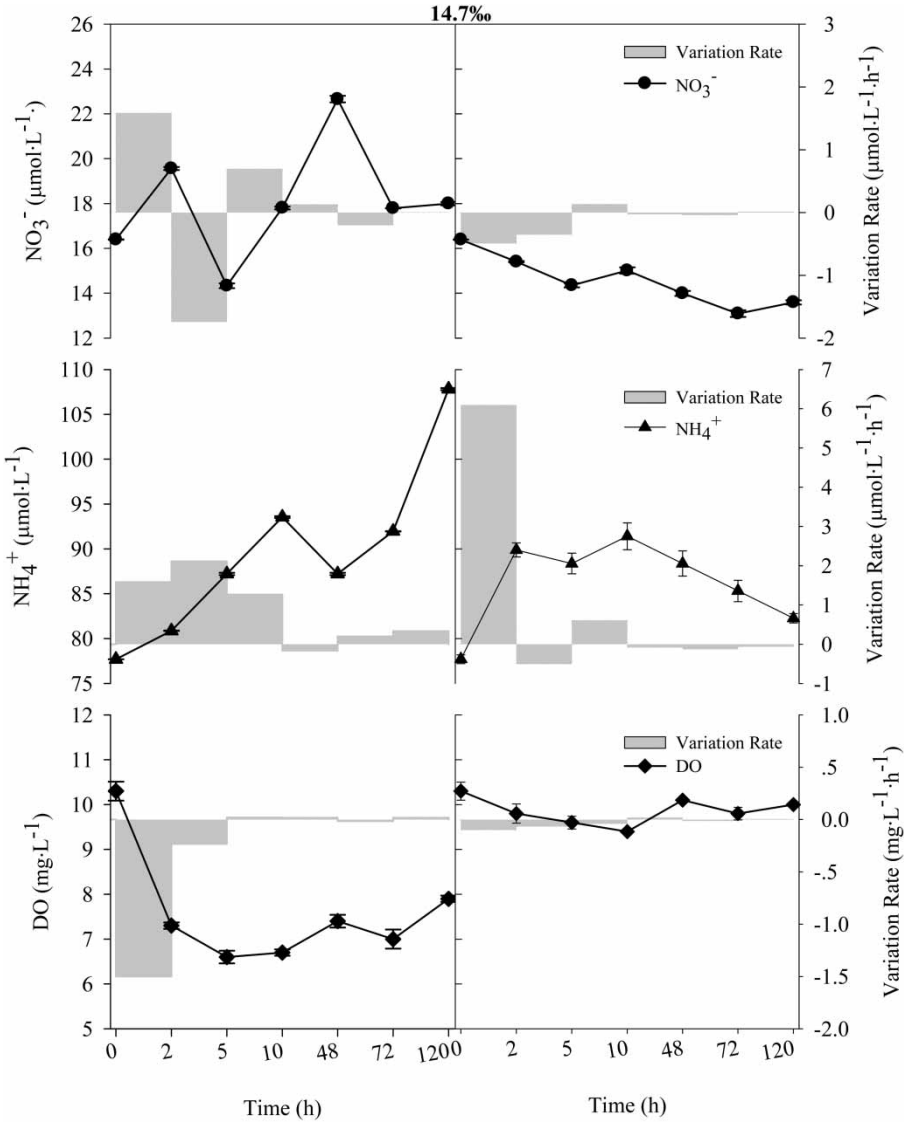

Figure 4 | Variations of NO_3^-, NH_4^+ and DO concentrations and the corresponding mean of the variation rates at 35 °C (left) and 10 °C (right) for estuarine samples (salinity of 14.7 ‰).

literature implies that nitrifying and/or denitrifying organisms could more easily adapt to the low or moderate salinity gradient. This finding was confirmed by our study. In addition, the GNiR and GNi-ADeR for all the salinity treatments were higher at 35 °C, as the activity, proliferation and metabolism of the micro-organisms are easily affected by temperature (Shammas 1986). A significant difference test by one-way analysis of variance (ANOVA) was conducted for all the treatments (Table 2). Results showed that the majority of the salinity treatments show significant difference at the level of $p = 0.05$ at different incubation intervals, except for several pairs of non-significant difference at 35 and 10 °C. Thus, it is obvious that salinity gradients have a significant effect on the nitrification-denitrification processes.

CONCLUSION

In this study, the concentration variations of DIN (NH_4^+ and NO_3^-) and DO for different salinity treatments (0.6‰, 4‰, 7.6‰, 11.4‰ and 14.7‰) indicated that both nitrification and coupled nitrification-aerobic denitrification regulated N transformation processes, resulting in NO_3^- being either accumulated or attenuated at the end of the incubation experiments. Owing to the different sensitivity and

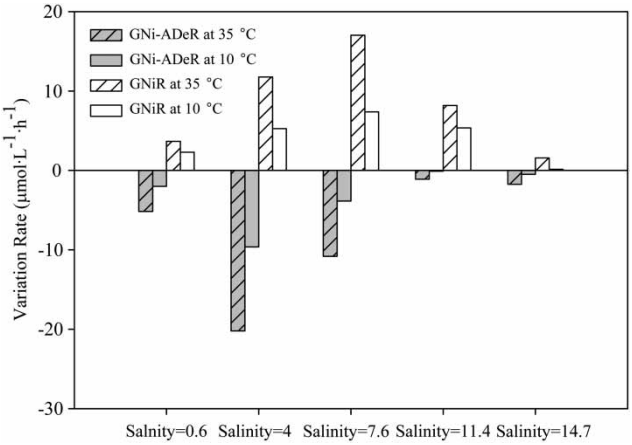

Figure 5 | Maximum GNiR and GNi-ADeR for different salinity treatment under 35 and 10 °C.

Table 2 | The results of significant difference test for all the treatments by one-way ANOVA

Time intervals (h)	Significant difference test by one-way ANOVA ($p < 0.05$)	
	35 °C	10 °C
0–2	All*	All* except T1 vs. T3
2–5	All* except T4 vs. T5	All* except T2 vs. T4
5–10	All* except T1 vs. T4	All* except T1 vs. T4; T2 vs. T5; T3 vs. T5
10–48	All* except T1 vs. T3	T2 vs. T3* ; T3 vs. T4*
48–72	All* except T1 vs. T5	All*
72–120	All* except T3 vs. T4	All* except T2 vs. T4; T3 vs. T5

* represents significant difference at the level of 0.05.
All* represents all the treatments are significant difference at the level of 0.05.
T1 represents the treatment of 100% river (salinity of 0.6‰).
T2 represents the treatment of 75% river + 25% estuary (salinity of 4‰).
T3 represents the treatment of 50% river + 50% estuary (salinity of 7.6‰).
T4 represents the treatment of 25% river + 75% estuary (salinity of 11.4‰).
T5 represents the treatment of 100% estuary (salinity of 14.7‰), respectively.

adaptation ability of the micro-organisms to the salinity gradient, a salinity range between 4.0 and 11.4‰ is more suitable for nitrification, while a salinity range between 4.0 and 7.6‰ is more suitable for coupled nitrification-aerobic denitrification. This study further indicates that nitrifying and/or denitrifying organisms could more easily adapt to the low or moderate salinity gradient. Furthermore, the relatively higher temperature (35 °C) stimulates higher GNiR and GNi-ADeR. However, the mechanism that would drive different nitrification and coupled nitrification-aerobic denitrification at different salinity gradients is not clear. The existing bacterial community should be able to tolerate a wide range of environmental conditions and thus may perform well under different salinities and enriched DO. Further studies should be focused on not only which environmental characteristics determine nitrification and coupled nitrification-aerobic denitrification rates, but also which ones determine the community composition and relative abundance of the micro-organisms. The approach may be applicable in removal of N in aquatic systems, particular for estuaries.

ACKNOWLEDGEMENTS

We gratefully acknowledge Xi Yang and Wenjing Hao for water sampling. This study was financially supported by the National Natural Science Foundation of China (41203001, 41173096), the innovation team training plan of the Tianjin Education Committee (TD12-5037), Tianjin Municipal Science and Technology Foundation (13JCYBJC38200) and Tianjin Normal University Foundation (5RL117).

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First received 7 January 2015; accepted in revised form 20 March 2015. Available online 1 April 2015