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Anammox, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment

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Abstract. Benthic nitrogen transformation pathways were investigated in the sediment of the East China Sea (ECS) in June of 2010 using the ^{15}N isotope pairing technique. Slurry incubations indicated that denitrification, anammox and dissimilatory nitrate reduction to ammonium (DNRA) as well as intracellular nitrate release occurred in the ECS sediments. These four processes did not exist independently, nitrate release therefore diluted the ^{15}N labeling fraction of NO_3^-, and a part of the $^{15}NH_4^+$ derived from DNRA also formed $^{30}N_2$ via anammox. Therefore, current methods of rate calculations led to over and underestimations of anammox and denitrification respectively. Following the procedure outlined in Thamdrup and Dalsgaard (2002), denitrification rates were slightly underestimated by an average 6 % without regard to the effect of nitrate release, while this underestimation could be counteracted by the presence of DNRA. On the contrary, anammox rates calculated from $^{15}NO_3^-$ experiment were significantly overestimated by 42 % without considering nitrate release. In our study, this overestimation could only be compensated 14 % by taking DNRA into consideration. In a parallel experiment amended with $^{15}NH_4^+ + ^{14}NO_3^-$, anammox rates were not significantly influenced by DNRA due to the high background of $^{15}NH_4^+$ addition. The significant correlation between potential denitrification rate and sediment organic matter content ($r = 0.68$, $p < 0.001$, Pearson) indicated that denitrification was regulated by organic matter, while, no such correlations were found for anammox and DNRA. The relative contribution of anammox to the total N-loss increased from 13 % at the shallowest site near the Changjiang estuary to 50 % at the deepest site on the outer shelf, implying the significant role of anammox in benthic nitrogen cycling in the ECS sediments, especially on the outer shelf. N-loss as N_2 was the main pathway, while DNRA was also an important pathway accounting for 20–31 % of benthic nitrate reduction in the ECS. Our study demonstrates the complicated interactions among different benthic nitrogen transformations and the importance of considering denitrification, DNRA, anammox and nitrate release together when designing and interpreting future studies.

1 Introduction

The East China Sea (ECS) is one of the most expansive continental shelf seas, bounded on the west by mainland China and on the east by the western Pacific Ocean island chain (Fig. 1). On the west coast, there is a large freshwater input to the ECS from the Changjiang (Yangtze River) (Beardsley et al., 1985), while on the east outer shelf; the ECS interacts tightly with the Kuroshio, a warm and salty west boundary current. Due to the strong influence of the river input and western boundary current, the ECS exhibits a complex current system, leading to unique nutrient dynamics (Zhang et al., 2007). Nutrient enriched water is restricted to the west inner shelf, where it is influenced by the Changjiang Diluted Water (CDW), while the outer shelf is dominated by the oligotrophic Kuroshio Surface Water (KSW). Anthropogenic activities have exponentially increased the fixed nitrogen concentrations in the Changjiang estuary by a factor of 3–5 from the 1960s to the end of the 1990s (Wang, 2006; Zhou et al., 2008). In response to increased nutrients, the phytoplankton standing stock has also increased, as has the occurrence and

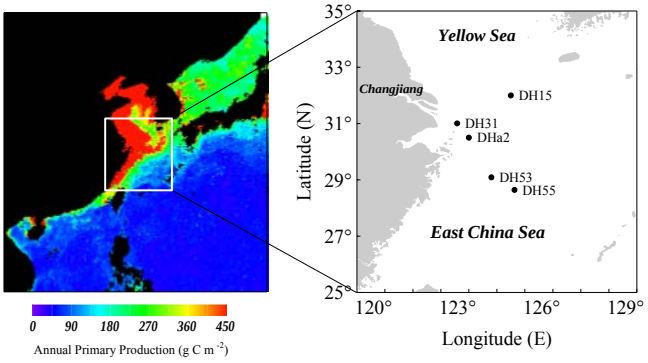

Fig. 1. Sampling locations in the East China Sea. The primary production map is from: http://marine.rutgers.edu./opp/swf/Production/gif_files/PP_Month_9806B.gif.

scale of harmful algal blooms (Zhou et al., 2008). Consequently, eutrophication has become a severe problem in the Changjiang estuary (Zhang et al., 2007), and hypoxic events in the bottom water off the Changjiang estuary have been reported extensively during the past decade (Zhu et al., 2011). N-loss from sediments via denitrification and anammox is the major N sink on continental shelves (Christensen et al., 1987; Trimmer and Nicholls, 2009); however most studies within the ECS have focused only on benthic nutrient fluxes and nitrous oxide (Aller et al., 1985; Zhang et al., 2010), while benthic N-loss has only been investigated at a tidal flat (Wang, et al., 2006).

Denitrification, anammox and dissimilatory nitrate reduction to ammonium (DNRA) are microbially mediated nitrate reduction pathways. Denitrification, in which nitrate is sequentially reduced to N_2 under anaerobic conditions has been found in numerous anaerobic sediments. Anammox, which represents the reduction of nitrite coupled to ammonia oxidation (Mulder et al., 1995), is generally considered to be less important than denitrification but can also account for up to 60–80 % of N-loss in some benthic sediments (Engström et al., 2005; Thamdrup and Dalsgaard, 2002). DNRA is an alternative pathway, by which nitrate is reduced to bioavailable ammonium, thus, no fixed N-loss occurs (An and Gardner, 2002; Koike and Hattori, 1978). Progressively over the last few decades, the importance of DNRA in sediment has been recognised (Dong et al., 2011; Gardner et al., 2006; Koike and Hattori, 1978). For example, DNRA was demonstrated to be the dominant pathway of the benthic nitrate reduction in the tropical estuarine sediment (Dong et al., 2011), it has also been demonstrated that DNRA can be performed by fermentative bacteria (Tiedje, 1988). Meanwhile, it has also been shown that both nitrate storing bacteria (Preisler et al., 2007) and diatoms can perform DNRA (Kamp et al., 2011). Therefore it is now recognised that DNRA, and nitrate storage by organisms performing it, are important parts of the benthic nitrogen cycle (Lomstein et al., 1990; Risgaard-Petersen et al., 2006). Denitrification and anammox represent pathways which remove fixed N from marine systems, therefore they can play a role in reducing eutrophication, and meanwhile DNRA recycles fixed nitrogen which can then be assimilated by primary producers.

Although there have been several reports of the coexistence of anammox, denitrification and DNRA (Bohlen et al., 2011; Dong et al., 2009; Prokopenko et al., 2006; Trimmer and Nicholls, 2009), the contribution of each process in benthic nitrate reduction were not fully studied. The co-occurrence of these processes represented a problem when using the traditional ^{15}N isotope pairing technique (Kartal et al., 2007; Sokoll et al., 2012), as the presence of DNRA may have influenced the apparent isotope distribution of anammox and denitrification. Briefly, $^{15}NH_4^+$ derived from DNRA could have combined with $^{15}NO_2^-$ (derived from $^{15}NO_3^-$ dissimilatory reduction) via anammox to form $^{30}N_2$, the same product as denitrification (Jensen et al., 2011; Kartal et al., 2007). In the study by Jensen et al. (2011), anammox and DNRA was shown to produce $^{30}N_2$ in incubations with $^{15}NO_2^-$, however their calculation approach only works in the absence of denitrification. Spott and Stange (2007) developed an approach to calculate anammox and denitrification rates precisely to avoid the influence of DNRA in open steady-state incubation systems. However, our study, as well as the majority of experimental studies of nutrient cycling in marine sediments is carried out in a closed incubation system. Moreover, if nitrate was stored intracellularly by nitrate storing organisms, use of the isotope pairing technique would be further complicated, as this would represent an excess source of $^{14}NO_3^-$ (Glud et al., 2009; Sokoll et al., 2012). While aerobic denitrification combined with aerobic ammonium oxidation are further processes which could complicate benthic N-cycling in the permeable sediments (Gao et al., 2010), all of our experiments were carried out under anaerobic conditions so these were not in the scope of this study.

In this study, we investigated the nitrate reduction and N-loss pathways within sediments of the ECS continental shelf in slurry incubations using the ^{15}N isotope pairing technique. The influence of nitrate release and DNRA on anammox and denitrification calculations was examined quantitatively.

2 Materials and methods

2.1 Sample collection and preparation

Sediment was collected at five sites from the Changjiang estuary to the outer shelf of the ECS during a cruise on the R/V *Kexue No. 3* from 8 to 22 June, 2010 (Fig. 1 and Table 1). All the sediment samples were collected using a Soutar-type box corer on board; only samples with an undisturbed sediment surface and clear overlying water were used for the subsequent experiments. The bottom water used in the slurry incubations ($\sim$ 2 m above the seafloor) was sampled using Niskin bottles equipped with conductivity,

Table 1. Sampling locations and some general characteristics of bottom water and sediment. The porosity and organic matter content (expressed as LOI %) are the average of the top 8 cm, and data in parentheses represent the variation range.

Station	Latitude (N)	Longitude (E)	Depth (m)	Bottom water Temp. (°C)	Bottom water Salinity (psu)	Bottom water NO_3^- (μM)	Sediment type	Porosity	LOI (%)	Incubation temperature (°C)
DH31	30°57.389′	122°33.922′	19.0	19.40	26.40	27.16	Clayey silt	0.78 (0.65–0.88)	6.2 (4.7–7.2)	23.5
DHa2	30°30.077′	122°59.915′	57.8	18.68	34.08	24.51	Silt-clay-sand	0.71 (0.60–0.87)	5.6 (5.0–6.3)	24.0
DH53	29°05.338′	123°48.202′	78.0	19.58	34.36	4.86	Silt-clay-sand	0.74 (0.61–0.87)	5.3 (4.2–6.8)	24.0
DH55	28°38.571′	124°37.672′	86.4	18.72	34.43	2.23	Fine sand	0.54 (0.50–0.61)	3.6 (3.3–3.8)	23.0
DH15	32°00.010′	124°29.794′	41.4	14.70	31.04	13.12	Silty sand	0.62 (0.52-0.75)	3.8 (3.2-4.1)	18.0

temperature and pressure sensors and stored in 10 L clean polyethylene bottles placed in a seawater bath in dim light. Sediment cores for bulk organic chemical parameters and pore water extraction were collected with large Plexiglas liners (i.d. = 9.5 cm, height = 60 cm). Sediment cores for the slurry incubation were collected with small Plexiglas liners (i.d. = 5 cm, height = 30 cm). Sediment cores for chemical and physical parameters were sectioned at 1-cm intervals, frozen for future analysis and subsequently freeze-dried. Water content of sediments was calculated by weight difference before and after drying.

The overlying water above the sediment surface was collected and filtered through 0.45 μm syringe filters, and then poisoned by the addition of saturated $HgCl_2$ for nutrient analysis with a final Hg^{2+} concentration of $\sim 100\,mg\,L^{-1}$. Sediment cores for pore water extraction were sectioned immediately after collection at 0.5-cm intervals in the upper 5 cm and at 1-cm intervals for the following 15 cm, and at 2-cm intervals for the remainder of the cores. Pore water was extracted using Rhizon Soil Moisture Samplers (19.21.23F Rhizon CSS, Netherlands) (Liu et al., 2011; Seeberg-Elverfeldt et al., 2005). The pore water samples were preserved as described previously.

2.2 ^{15}N slurry incubations

^{15}N tracer sediment slurry incubations, which allow determination of potential rates, have commonly been used to investigate benthic nitrogen transformations (Dähnke et al., 2012; Risgaard-Petersen et al., 2004; Thamdrup and Dalsgaard, 2002). In this study, slurry incubations were conducted in gastight bags as described by Thamdrup and Dalsgaard (2002) to evaluate the presence of anammox, denitrification and DNRA. Briefly, the cores (i.d. = 5 cm) were sectioned into 2-cm slices from the sediment surface down to 8 cm depth, each slice was then mixed with 270 mL He pre-degassed bottom seawater in a gastight plastic bag. The slurries were degassed, and pre-incubated in the dark at room temperature for 24–36 h.

After pre-incubation, $^{15}NH_4^+$, $^{15}NH_4^+ + ^{14}NO_3^-$ and $^{15}NO_3^-$ were added to the incubation bags (^{15}N atom %, 99.3 %, Campro Scientific, Berlin). The experiment amended with $^{15}NH_4^+$ was used as a control experiment (denoted as E_Ctrl), the experiment amended with $^{15}NH_4^+ + ^{14}NO_3^-$ was conducted to confirm the presence of anammox (denoted as E_Amox) and the experiment amended with $^{15}NO_3^-$ was conducted to quantify each process' contribution to nitrate reduction (denoted as E_Denit) (Table 2). In all experiments, the tracers were amended to a final concentration of 100 μM. After each tracer injection and mixing, subsamples were immediately filled into 6 mL Exetainer vials (Labco Ltd, High Wycombe, UK) with 0.1 mL pre-added saturated $HgCl_2$. The temperature of the incubations was between 18–24 °C at different sites (Table 1). In the following 8–12 h, bags were periodically shaken to ensure that the labeled N compounds were homogenously distributed and 5 subsamples were withdrawn in each experiment. Exetainer vials containing the subsamples were sealed and stored at room temperature upside down until subsequent N_2 isotope ratio analysis.

2.3 Chemical analysis

The concentrations of NH_4^+, NO_3^- and NO_2^- in pore water and slurry incubation samples were determined on a segmented flow auto-analyzer (SAN plus, SKALAR) with the standard spectrophotometric methods. The limit of detection for NH_4^+, NO_3^- and NO_2^- was 0.5 μM, 0.06 μM, 0.01 μM, respectively, with a precision of ~ 5 %. The sediment organic matter content was expressed as the percent of weight loss on ignition (LOI %), determined by combustion at 550 °C for 4 h.

Table 2. Slurry incubation approaches performed in this study.

Experiment name	Tracer added	Tracer concentration (μM)	Isotopes measured	Process targeted
E_Ctrl	$^{15}NH_4^+$	100	$^{29}N_2$, $^{30}N_2$	Control experiment
E_Amox	$^{15}NH_4^+ + ^{14}NO_3^-$	100+100	$^{29}N_2$, $^{30}N_2$	Anammox
E_Denit	$^{15}NO_3^-$	100	$^{29}N_2$, $^{30}N_2$, $^{15}NH_4^+$	Denitrification and DNRA

Before N_2 analysis, a 1 mL headspace of helium was introduced to the sample and equilibrated after shaking, isotopic compositions of the N_2 gas were determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS; VG Optima, Manchester, UK) at Max Planck Institute for Marine Microbiology, Bremen and the concentrations of $^{29}N_2$ and $^{30}N_2$ were calculated following Holtappels et al. (2011).

After the measurement of N_2 isotope ratios in the sample from E_Denit and E_Amox, 2 mL of remaining sample was filtered into a new 6 mL Exetainer and treated with hypobromite, converting $^{15}NH_4^+$ to $^{29}N_2$ and $^{30}N_2$ (Preisler et al., 2007; Warembourg, 1993), after which the N_2 isotope ratios were measured using the GC-IRMS as described above.

2.4 Rate calculations

The potential rates of anammox, denitrification and DNRA were calculated from the production of $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ in the slurry incubation using two methods. The first used the quantification technique of Thamdrup and Dalsgaard (2002).

$$D_{(\text{E\_Denit})} = P_{30}/F_{\text{N}}^2 \quad (1)$$

$$A_{(\text{E\_Denit})} = [P_{29} - 2 \times (1/F_{\text{N}} - 1) \times P_{30}]/F_{\text{N}} \quad (2)$$

where, $D_{(\text{E\_Denit})}$ and $\text{A}_{(\text{E\_Denit})}$ denoted the potential rates of denitrification and anammox in E_Denit, respectively. P_{29} and P_{30} were the production rate of $^{29}N_2$ and $^{30}N_2$ in E_Denit. F_{N} represented the $^{15}NO_3^-$ fraction in E_Denit, which was determined from the difference in NO_3^- before and after $^{15}NO_3^-$ addition.

For the experiment of E_Amox, the potential anammox rate was,

$$A_{(\text{E\_Amox})} = P_{29(\text{E\_Amox})}/F_{\text{A(E\_Amox)}} \quad (3)$$

where, $A_{(\text{E\_Amox})}$, $P_{29(\text{E\_Amox})}$ and $F_{\text{A(E\_Amox)}}$ represented the total N_2 production by anammox, production of $^{29}N_2$ and $^{15}NH_4^+$ labeling fraction in E_Amox.

Significant nitrate release and DNRA occurred in our samples (see Sects. 3 and 4), violating the assumptions on which the procedure of Thamdrup and Dalsgaard (2002) was based. Nitrate release from nitrate storing organisms diluted the $^{15}NO_3^-$ fraction in E_Denit, and $^{15}NH_4^+$ production via DNRA would combine with $^{15}NO_3^-$ to produce $^{30}N_2$ through anammox. Therefore, we adapted the previous calculation to take this into account.

First, only the effects of nitrate release by nitrate storing organisms were considered. As proposed by Sokoll et al. (2012), we assumed the anammox rate in E_Denit equaled that from E_Amox, then the derived $^{15}NO_3^-$ fraction (F_{N}^*) could be calculated through Eq. (2) where $A_{(\text{E\_Denit})}$ was substituted by $A_{(\text{E\_Amox})}$.

$$F_{\text{N}}^* = \frac{(P_{29} + 2 \times P_{30}) - \sqrt{(P_{29} + 2 \times P_{30})^2 - 8 \times \text{A}_{(\text{E\_Amox})} \times P_{30}}}{2 \times A_{(\text{E\_Amox})}} \quad (4)$$

If F_{N}^* is significantly less than F_{N}, nitrate release will occur (Sokoll et al., 2012). In this situation, the following calculation would use F_{N}^* instead of F_{N}, $D_{(\text{E\_Denit})}$ and $\text{A}_{(\text{E\_Denit})}$ in Eqs. (1) and (2) would be recalculated and denoted as $D^*_{(\text{E\_Denit})}$ and $A^*_{(\text{E\_Denit})}$. The excess $^{14}NO_3^-$ contributed by nitrate release would be calculated according to Sokoll et al. (2012),

$$\text{Excess } ^{14}NO_3^- = {}^{15}NO_3^- \times (1/F_{\text{N}}^* - 1/F_{\text{N}}) \quad (5)$$

Secondly, the effects of DNRA on the calculation of anammox and denitrification rates were considered. According to the principle of isotope pairing, in E_Denit, for anammox,

$$A_{29} = A^*_{(\text{E\_Denit})} \times [F_{\text{N}}^* \times (1 - F_{\text{A}}) + F_{\text{A}} \times (1 - F_{\text{N}}^*)] \quad (6)$$

$$A_{30} = A^*_{(\text{E\_Denit})} \times F_{\text{N}}^* \times F_{\text{A}} \quad (7)$$

For denitrification,

$$D_{29} = D^*_{(\text{E\_Denit})} \times 2 \times F_{\text{N}}^* \times (1 - F_{\text{N}}^*) \quad (8)$$

$$D_{30} = D^*_{(\text{E\_Denit})} \times (F_{\text{N}}^*)^2 \quad (9)$$

and,

$$P_{29} = A_{29} + D_{29}, P_{30} = A_{30} + D_{30} \quad (10)$$

where, A_{29} and A_{30} denoted the production of $^{29}N_2$ and $^{30}N_2$ by anammox. D_{29} and D_{30} represented the production of $^{29}N_2$ and $^{30}N_2$ through denitrification. F_A represented the fraction of $^{15}NH_4^+$ in E_Denit during the incubation. At each timepoint, F_A could be calculated by the $^{15}NH_4^+$ concentration and total NH_4^+ concentration.

Here, A_{30} was the key parameter linking anammox, denitrification and DNRA. By combining Eqs. (6) to (10), we got,

$$A_{30} = \frac{F_A \times [P_{29} \times F_N^* - 2 \times (1 - F_N^*) \times P_{30}]}{F_N^* \times (1 - F_A) - F_A \times (1 - F_N^*)} \quad (11)$$

Then, the revised anammox and denitrification rates could be calculated by Eqs. (7) and (12),

$$D^*_{(E\_Denit)} = [P_{30} - A_{30}]/(F_N^*)^2 \quad (12)$$

Usually, F_N^* in the anoxic slurry incubations would be constant assuming nitrate release happened only at the beginning as a result of mixing the slurries before subsampling (Sokoll et al., 2012). However, if DNRA occurred, F_A and A_{30} would successively increase over time. We have developed a step-by-step method to quantify the nonlinear $^{30}N_2$ production via anammox (Song et al., 2013). However, from the data in present study, we found that F_A was a semi-linear increase with time, therefore we applied an average F_A during the incubation instead of the actual F_A at each time point to calculate anammox rate.

The DNRA rate could be derived from the accumulation rate of $^{15}NH_4^+$ in E_Denit, however, as mentioned above, a part of $^{15}NH_4^+$ would form $^{30}N_2$ via anammox. Thus,

$$\text{DNRA} = (P_{^{15}NH_4^+} + A_{30})/F_N^* \quad (13)$$

where, $P_{^{15}NH_4^+}$ was the linear slope of apparent $^{15}NH_4^+$ production with time.

DNRA would also influence the anammox rate calculation in E_Amox as $^{14}NH_4^+$ produced by DNRA and remineralisation also diluted the $^{15}NH_4^+$ fraction. We found that the fraction of $^{15}NH_4^+$ in E_Amox decreased linearly with time, thus, $F_{A(E\_Amox)}$ could be replaced by the average value in Eq. 3.

If we assumed that the denitrification rate in E_Amox and E_Denit was equal, then the relative contribution of anammox to the total N-loss in E_Amox would be,

$$\text{ra} = \frac{A_{(E\_Amox)}}{A_{(E\_Amox)} + D^*_{(E\_Denit)}} \quad (14)$$

The production rates of $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ for corrected anammox, denitrification and DNRA rates were calculated from the slope of concentrations versus time. The normality of dependent variable was tested by the Kolmogorov-Smirnov method and standard deviation of the linear rate was derived from the slope standard deviation given by the regression statistic; Pearson correlation was applied to discuss the correlation analysis. The significance level was 0.05. All the statistics were carried out in statistical software (SigmaStat 3.5). To eliminate the discrepancies between the in situ bottom water temperature and the incubation temperature, all the rates were corrected to the in situ temperature using the Arrhenius equation assuming average apparent activation energy of 61 KJ mol^{-1} for all species (Aller et al., 1985). The average temperature correction factor was 0.7.

3 Results

3.1 Water column and sediment characteristics

Bottom seawater and sediment characteristics were investigated at five stations (Table 1). The bottom water NO_3^- decreased sharply from the estuary ($\sim 27\,\mu M$) to the outer shelf ($\sim 2\,\mu M$).

Sediment pore water profiles of NO_3^- and NO_2^- varied from site to site (Fig. 2), as these samples were extracted by Rhizon sampler, any stored nitrate products were excluded in the pore water data. At sites DH31 and DHa2 the nitrate concentration sharply decreased to $< 0.5\,\mu M$ within the upper 1 cm. Nitrate peaked in the layer from 3 to 5 cm at DHa2 with an average NO_3^- concentration of 13 µM, indicating active nitrification or advection of nitrate rich water into the sediment at this depth. At sites DH53 and DH15, there was a nitrate peak in the upper 2 cm, below this, nitrate concentration sharply declined and was $< 0.5\,\mu M$ below 4 cm. At site DH55, NO_3^- mirrored the bottom water concentration then increased to 67 µM at 2 cm, after which it decreased sharply to $\sim 2\,\mu M$ at 5 cm. A second nitrate peak of $\sim 10\,\mu M$ was found at 6–7 cm and then decreased to $< 1\,\mu M$ from 7 to 10 cm. The nitrite profile in the pore water was similar to the nitrate, but generally one order of magnitude lower in concentration. Pore water NH_4^+ concentrations generally increased with depth (Fig. 2). Several µM of ammonium in the upper 0–1 cm of the sediment at most stations indicate that there was a flux of ammonium towards the bottom waters.

3.2 ^{15}N slurry incubations

After 24–36 h pre-incubation in E_Ctrl, NO_3^- was still present in some sediment layers ($5 \sim 15\,\mu M$), especially in the surface layer (0–2 cm) (Table 3). In those layers with residual NO_3^-, significant $^{29}N_2$ accumulation was observed (Fig. 3a and Supplement S1). Although some $^{30}N_2$ was observed in a few layers, it was generally one to two magnitudes lower than $^{29}N_2$ and not quantitatively important ($< 1\,\%$). Therefore, we did not take it into account in later calculations. In the surface layers of DH53 and DH15, NO_3^- was not detectable ($< 1\,\mu M$) after pre-incubation (Table 3), however there was neither measurable $^{29}N_2$ nor

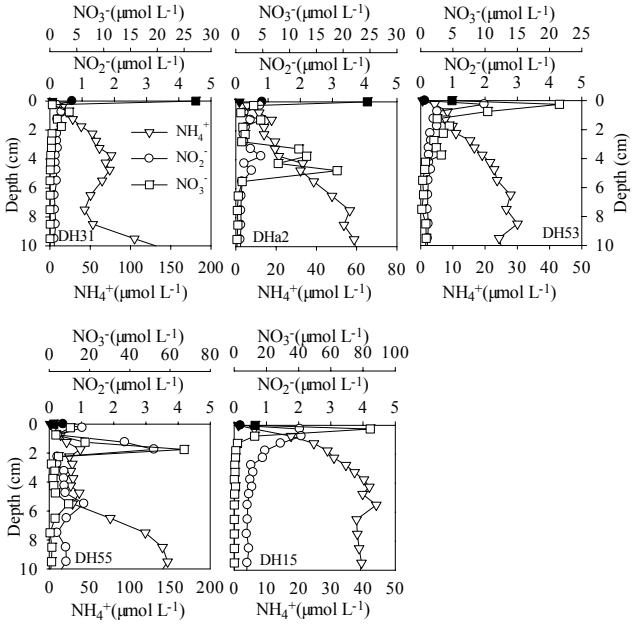

Fig. 2. Pore water profiles of NO_2^-, NO_3^- and NH_4^+ in the East China Sea sediment. The black symbols represent overlying water data.

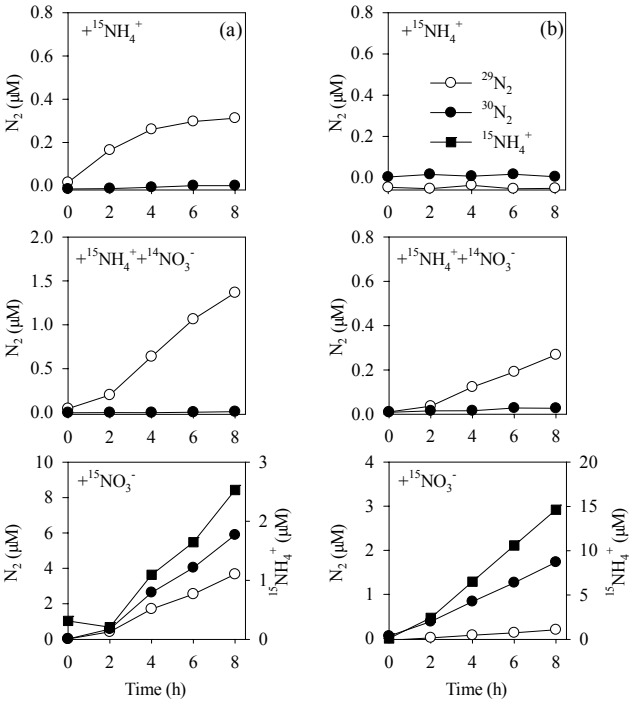

Fig. 3. Production of $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ against time in slurry incubation. **(a)** DH31 0–2 cm sediment. The residual nitrate was not exhausted after the 24 h pre-incubation, thus we observed a slight $^{29}N_2$ production before all the nitrate disappeared; **(b)** DH15 6–8 cm sediment. The nitrate was less than 1 μM after pre-incubation, hence we did not detect significant $^{29}N_2$ production.

$^{30}N_2$ production ($p > 0.05$) during the incubation (Fig. 3b and Supplement S1). Therefore, no coupled nitrification-denitrification in the slurry was observed, and other pathways of anaerobic ammonium oxidation that were of any significance could almost be excluded, e.g. MnO_2 (Luther et al., 1997) and $Fe(OH)_3$ (Yang et al., 2012). This ensured that all the $^{29}N_2$ production in E_Amox was from anammox (see below).

Anammox was observed in E_Amox incubations: $^{29}N_2$ accumulated at all sites over time, while there was no measurable production of $^{30}N_2$ (Fig. 3 and Supplement S1).

In E_Denit, $^{29}N_2$ and $^{30}N_2$ were produced along with $^{15}NH_4^+$ (Fig. 3 and Supplement S1). This showed that dissimilatory nitrate reduction to ammonium (DNRA) occurred concurrently with anammox and denitrification. Nitrate was not a limiting factor in E_Amox and E_Denit. Both $^{15}NH_4^+$ and F_A accumulated linearly over time in all sediment layers ($r^2 > 0.9$, $p < 0.05$) (Figs. 3 and 4). Thus, we used the average F_A to calculate the $^{30}N_2$ production via anammox.

3.3 Nitrate storage and release in the sediment

NO_3^- release occurred at all the sites in the 0–2 cm layer except DH55, at this site NO_3^- release was detected in 2–4 cm and 6–8 cm layers. Generally, the decline of F_N caused by nitrate release ranged from 0.3–10 % with an average of 5.1 %. The calculated excess $^{14}NO_3^-$ ranged from 3 to 83 nmol cm^{-3} with an average of 42 nmol cm^{-3} (Table 3).

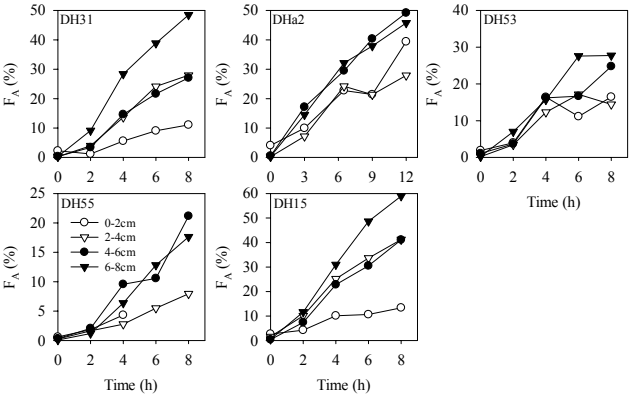

Fig. 4. Time course of $^{15}NH_4^+$ fraction (F_A) in E_Denit.

3.4 N-loss and nitrate reduction in slurry incubation

Denitrification rates calculated from the method of Thamdrup and Dalsgaard (2002) ranged from 0.6 to 20 nmol N cm^{-3} h^{-1} and the average denitrification rate showed a decrease from 14 nmol N cm^{-3} h^{-1} at site DH31, close to the coast, to 2.0 nmol N cm^{-3} h^{-1} at site DH55 furthest from the coast (Fig. 5).

Table 3. Nitrate concentration after pre-incubation, $^{15}NO_3^-$ labeling fraction (F_N) and excess nitrate stored by nitrate storing organisms in each layer. n.d.: not detectable.

Station	Layer (cm)	NO_3^- concentration after pre-incubation (μM)	F_N (%)	Excess nitrate (nmol cm^{-3})
DH31	0–2	15.1	90.7	64
	2–4	9.4	94.2	2.6
	4–6	7.0	95.8	12
	6–8	7.4	95.4	n.d.
DHa2	0–2	9.3	93.9	63
	2–4	8.0	94.2	37
	4–6	6.1	95.8	21
	6–8	9.1	93.4	n.d.
DH53	0–2	0.2	99.2	83
	2–4	0.2	99.2	n.d.
	4–6	0.3	99.1	n.d.
	6–8	0.2	99.2	n.d.
DH55	0–2	10.7	93.2	n.d.
	2–4	1.8	98.8	58
	4–6	1.8	98.8	n.d.
	6–8	2.0	98.8	57
DH15	0–2	0.2	99.2	16
	2–4	0.2	99.1	42
	4–6	0.2	99.1	50
	6–8	0.5	98.9	n.d.

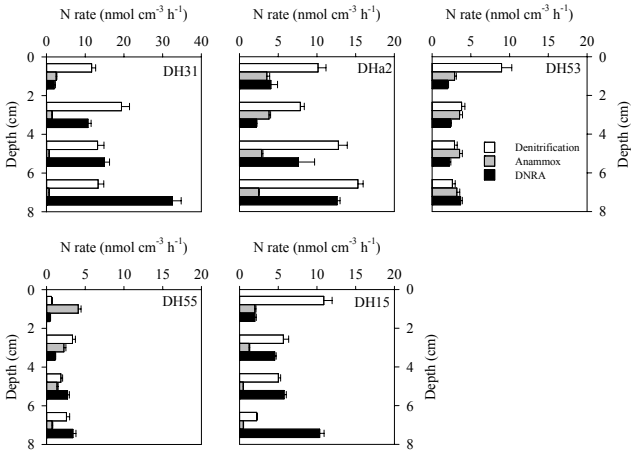

Fig. 5. Vertical distributions of potential denitrification (white bar), anammox (grey bar) and DNRA (black bar) rates in sediment of the ECS from the slurry incubation. The error bar (±1 SE) was calculated from the linear slope standard deviation given by the regression statistic.

Anammox rates from E_Denit, as calculated using the method of Thamdrup and Dalsgaard (2002) ranged from 0.3 to 4.6 nmol N cm^{-3} h^{-1} with an average of 2.0 nmol N cm^{-3} h^{-1}, after correction for nitrate release and DNRA, the anammox rate in E_Denit ranged from 0.3 to 3.5 nmol N cm^{-3} h^{-1} with an average of 1.6 nmol N cm^{-3} h^{-1}. Anammox rates were also calculated from the E_Amox incubation, before the correction by nitrate release and DNRA, anammox ranged from 0.4 to 4.0 nmol N cm^{-3} h^{-1} with an average of 2.1 nmol N cm^{-3} h^{-1}. After correction by DNRA and remineralisation, there was a slight increase (∼ 4 %). The highest anammox rate was found in the surface layer of site DH55 which was located at the outer shelf of the ECS (Fig. 5).

DNRA rates varied from 0.4 to 33 nmol N cm^{-3} h^{-1} with an average of 6.4 nmol N cm^{-3} h^{-1}. The average DNRA rate decreased from 15 nmol N cm^{-3} h^{-1} at site DH31 to 1.9 nmol N cm^{-3} h^{-1} at site DH55 (Fig. 5).

Denitrification rates decreased with increasing sediment depth at sites DH53 and DH15, while at other sites denitrification rates generally showed a slight increase with increasing sediment depth (Fig. 5). Anammox rates generally decreased with increasing sediment depth at all sites except DH53 (Fig. 5). DNRA rate at all sites generally increased with increasing sediment depth (Fig. 5).

Integrated anammox, denitrification and DNRA potential rates were calculated down to the NO_x^- penetration depth where the nitrate+nitrite concentration no longer decreased significantly with sediment depth. The penetration depth of NO_x^- at each site was constrained to 3 cm for DH31, 7 cm for DHa2, 5 cm for DH15, 5 cm for DH53 and 8 cm for DH55. All the integrated denitrification, anammox and DNRA rates showed highest values at site DHa2. At all sites except DHa2, integrated denitrification rates generally decreased with the increasing water depth (Fig. 6a). Opposite to this, the depth integrated anammox rates increased by a factor of 2.7 with increasing water depth. Hence, the relative contribution of anammox to the total N-loss exhibited a significant increasing trend with water depth ($r = 0.93$, $p = 0.02$, Pearson), from 13 % at site closest to the coast DH31 to 50 % at the furthest from the coast, DH55. The contribution of the integrated DNRA rate to the integrated total nitrate reduction rate (sum of DNRA, anammox and denitrification) varied from 23 to 31 % with an average of 26 % (Fig. 6b).

4 Discussion

4.1 The influence of nitrate release and DNRA on anammox and denitrification rates calculation with isotope pairing method

Intracellular nitrate storage has been observed in diverse environments (An and Gardner, 2002; Risgaard-Petersen et al., 2006; Thamdrup, 2012), where it was carried out by a diverse range of benthic organisms, such as sulfur oxidizing bacteria (Fossing et al., 1995; Schulz et al., 1999; Sweerts et al., 1990), benthic foraminifera (Glud et al., 2009; Risgaard-Petersen et al., 2006) and diatoms (Lomas and Glibert, 2000;

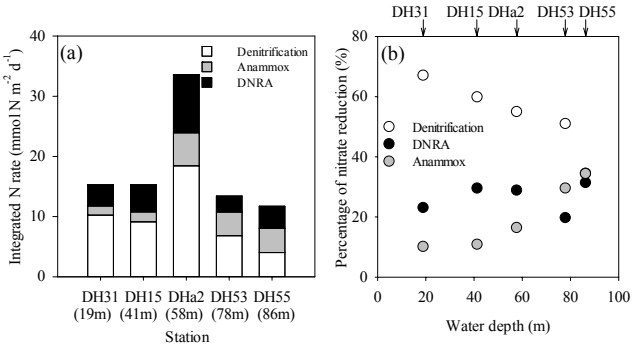

Fig. 6. Total nitrate reduction from denitrification, anammox and DNRA **(a)** and relative contribution of denitrification, anammox and DNRA as a function of water depth **(b)**.

Lomstein et al., 1990). Many of these micro-organisms reduce their intracellular nitrate stores to ammonium (DNRA, Kamp et al., 2011; Otte et al., 1999; Preisler et al., 2007). Therefore, the combined effect of intracellular nitrate storage and DNRA on the isotope pairing method calculations needs to be considered.

The influence of nitrate release on benthic N-loss rate calculation was discussed by Sokoll et al. (2012); therefore we will only briefly discuss this here. The equilibrium and exchange of added labeled $^{15}NO_3^-$ with an intracellularly stored $^{14}NO_3^-$ pool may be related to nitrate release after addition of $^{15}NO_3^-$ and lead to a decrease of F_N (Dähnke et al., 2012; Sokoll et al., 2012). As a result, our denitrification rates were underestimated according to Eq. (1), while anammox rates were overestimated according to Eq. (2). As F_N decreased by 0.3–10 %, the underestimation of denitrification rates in this study ranged from 0 to 19 % with an average of 6 % (Fig. 7a). However, the overestimation of anammox rates varied from 10 to 128 % with an average of 42 % in E_Denit (Fig. 7d). Excluding four samples for which we could not calculate F_N^* (for more detailed information see Supplement S2), all other anammox rates were consistent from the two experiments (Fig. 8a). Consequently we used Eq. (14) to evaluate the relative contribution of anammox to the total N-loss. So far nitrate storage has not been fully considered in most published benthic anammox rates, thus, the relative contribution of anammox to total N-loss in these studies might be overestimated to some extent when determined from $^{15}NO_x^-$ experiments.

Potentially the presence of DNRA would affect the anammox and denitrification rate calculations used in previous isotope pairing technique calculation methods (Holtappels et al., 2011; Nielsen, 1992; Risgaard-Petersen et al., 2003; Thamdrup and Dalsgaard, 2002). Denitrification rates would be overestimated and anammox would be underestimated following the procedure of Thamdrup and Dalsgaard (2002), thus counteracting the influence of nitrate release to some extent. In our study denitrification rates were only slightly overestimated (∼ 1 %) if we did not take the measured DNRA into account (Fig. 7b). Combined with the effect of nitrate release, the actual denitrification rate was only underestimated by 2.5 % in this study (Fig. 7c), below the coefficient of variation for the experiments (∼ 10 %). Contrary to the denitrification rates, anammox rates were underestimated by 16 % in E_Denit without considering DNRA (Fig. 7e). The net effect was that anammox rates would be overestimated by 10 % if we followed the calculation procedure of Thamdrup and Dalsgaard (2002) in this study (Fig. 7f). In E_Amox, anammox rates increased by 4 % after DNRA correction (Fig. 8b) since F_A did not decline significantly due to the high background of $^{15}NH_4^+$ (∼ 100 µM). Previous studies had shown the effect of DNRA on denitrification and anammox (Nicholls and Trimmer, 2009; Trimmer et al., 2003), however, they did not quantify the effect. Our correction calculation in this study allows quantification of the extent of the effects of DNRA on denitrification and anammox rates calculation when ^{15}N isotope pairing method was applied.

4.2 Distribution and regulation of anammox, denitrification and DNRA in ECS sediments

The vertical distribution of potential rates for denitrification, anammox and DNRA may reflect environmental controlling factors in the sediment. In the sediment, nitrate availability in the pore water has usually been considered as a key factor controlling denitrification rates (Seitzinger, 1990). The decrease of potential denitrification rates with sediment depth at DH53 and DH15 reflected the regulation of denitrification rates by pore water nitrate concentrations (Figs. 2 and 5). This pattern was consistent with the result of Laverman et al. (2007) and Sokoll et al. (2012). Oxygen is often considered as an inhibitor for denitrification and included in denitrification models (Laverman et al., 2007; Middelburg et al., 1996), but this would only effect the uppermost mm in cohesive sediments (Glud, 2008; Lohse et al., 1996). Moreover, denitrification and anammox seem to be less effected by oxygen in non-cohesive sediments and waters with regular intrusions of oxygen (Gao et al., 2010; Kalvelage et al., 2011). As we did not measure oxygen penetration in our incubation sediments, we can only speculate if, i.e. the lower denitrification rate in 0–2 cm layer at sandy site DH55 (Fig. 5) could be a reflection of oxygen inhibition on denitrification or organic matter limitation due to oxic respiration.

The availability of organic carbon was also a key factor regulating heterotrophic denitrification. A positive correlation between potential volumetric denitrification rate and organic matter (LOI %) ($r = 0.68$, $p < 0.001$, Pearson), indicated that organic matter content was an important environmental regulator of denitrification. Meanwhile, the integrated denitrification rate also decreased from the shallow coastal site to the outer shelf site except DHa2 (Fig. 6a), agreeing well with the distribution of primary production and/or organic carbon export in the ECS (Gong et al., 2003).

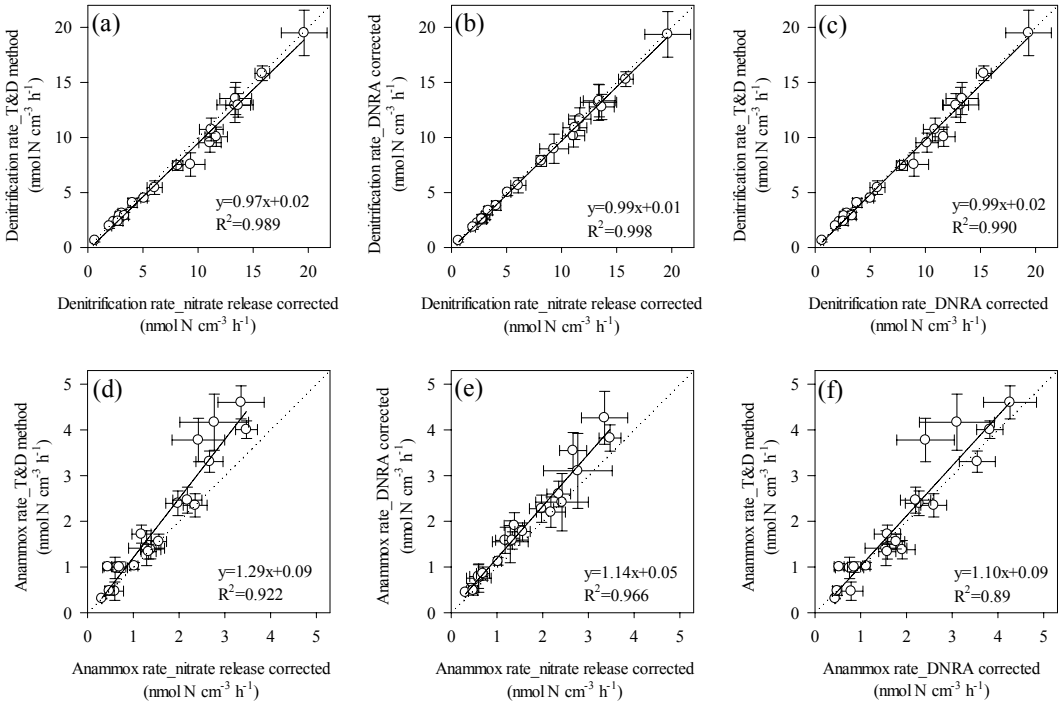

Fig. 7. The influence of nitrate release and DNRA on anammox and denitrification rates in the experiment amended with $^{15}NO_3^-$. T&D method: Thamdrup and Dalsgaard (2002). Nitrate release corrected: the rate calculation corrected by nitrate release. DNRA corrected: after nitrate release correction, the rate calculation was corrected by DNRA. See Sect. 2.4 Rate calculations for more detailed information. The dotted line represents the 1 : 1 line. The linear regression is shown for the interpretation of different processes effects on the anammox and denitrification rates.

Ammonium concentrations in the pore waters were not limiting for anammox activity at our study (Fig. 2). The decreasing pattern of potential anammox rates at four sites in this study may imply that pore water nitrate and/or nitrite regulated anammox rates (Figs. 2 and 5). Availability of nitrate and/or nitrite as electron acceptors is considered an important factor controlling anammox (Dalsgaard et al., 2005). Nitrite could be derived from nitrification or nitrate reduction as the first step of denitrification and DNRA in the sediment. Besides, it has been demonstrated that anammox bacteria could also perform dissimilatory nitrate reduction alone (Kartal et al., 2007). Here, relatively high anammox rates corresponded well with the elevated NO_3^- and/or NO_2^- concentrations in the surface 2 cm layer; this was very consistent with results from the Arabian Sea sediments off Pakistan (Sokoll et al., 2012). However, similar to denitrification, previous studies of anammox rates from slurry incubation also exhibited a large vertical variation at different sites (Gihring et al., 2010; Neubacher et al., 2013; Thamdrup and Dalsgaard, 2002). There was no correlation between volumetric anammox rate and sediment organic matter content ($r = 0.32$, $p = 0.17$, Pearson), implying that the anammox activity was not directly limited by the availability of organic matter in the ECS sediments.

No correlation between DNRA rate and sediment organic matter content was found ($r = 0.06$, $p = 0.81$, Pearson). The increase of DNRA rate with sediment depth (Fig. 5) suggested the deeper sediment layer was more favourable for DNRA, consistent with previous studies (Stief et al., 2010). This could be due to the lower ratio between electron acceptor and donor (Tiedje, 1988). It has also been reported that DNRA showed significantly higher rates in oxic conditions compared to hypoxic conditions (Roberts et al., 2012). Furthermore, it has been argued that potential DNRA rates might result from a stimulation of DNRA bacteria by high concentrations of added nitrate since they rarely obtain nitrate in normal conditions (Binnerup et al., 1992). Considering that the DNRA rates systematically increased with depth in our study and were highest at depths without in situ nitrate (Figs. 2 and 5) the DNRA rates might be more overestimated than the anammox rates which were highest at depths with measurable in situ NO_x^- concentrations (Figs. 2 and 5).

4.3 Biogeochemical significance of anammox, denitrification and DNRA in the ECS sediments

Pore water NO_x^- penetrated in the sediment down to 8 cm (Fig. 2). As our slurry incubations were performed with a resolution of 2 cm, we integrated the potential rates down to the nitrate penetration depth obtained from the pore water

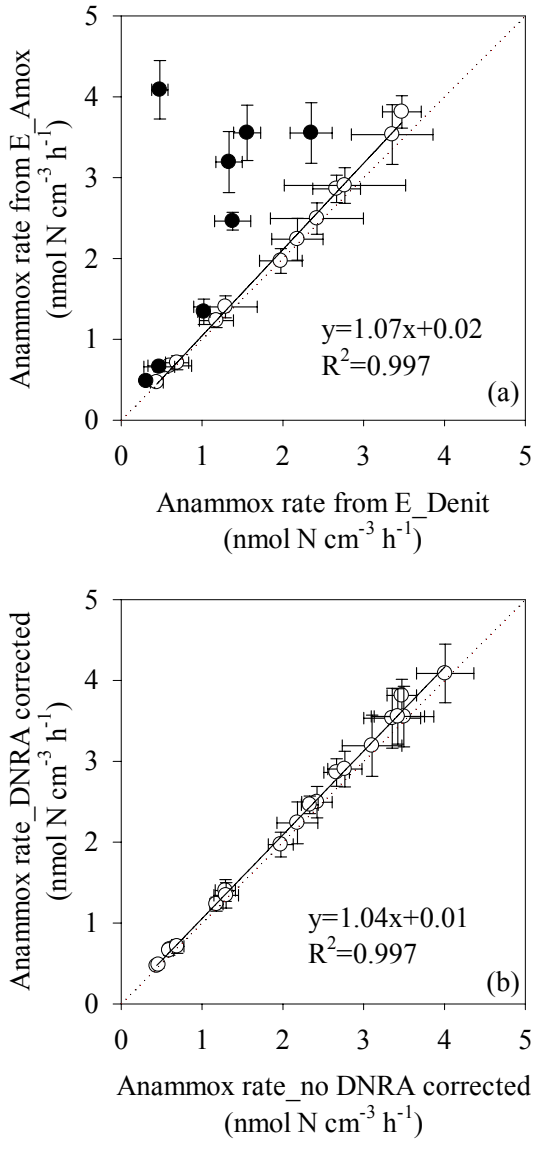

Fig. 8. Comparison of anammox rates from E_Denit and E_Amox **(a)** and DNRA effects on the anammox rate in E_Amox **(b)**. The black dot represents the anammox rate calculated from E_Denit derived from the original F_N since we can not calculate F_N^* or $F_N^* > F_N$ according Eq. (4). The anammox from E_Denit is the rate only after nitrate release correction.

profiles to obtain a full understanding of each process in the sediment. However, it should be noted that the low vertical resolution of nitrate profile might cause some over- or underestimation of nitrate penetration depth, and consequently the relative contribution of each process in total nitrate reduction.

The average relative contribution of anammox to total N-loss was 28 % in our study, which demonstrated that anammox was an important pathway for fixed nitrogen removal in the ECS sediments. This value was in the range of literature values reported for continental shelves sediments and was also similar to the global average value of 23 % in a recent compilation of Trimmer and Engström (2011). Meanwhile, the increase of relative contribution of anammox to total N-loss with water depth also agreed well with the general pattern observed from continental shelves (Thamdrup 2012; Trimmer and Engström, 2011). It was suggested that the increase of the relative contribution of anammox with depth was mainly due to a more significant decrease of denitrification rate than anammox (Trimmer and Engström, 2011). In our study, except at site DHa2, integrated denitrification rates decreased from the shallow estuarine site to the deep outer shelf site, while anammox rates increased and DNRA showed small spatial variation (Fig. 6a). As a result, the percentage of anammox in total nitrate reduction increased with distance from the coast and water depth (Fig. 6b). From this we can infer that in the shallow coastal area, denitrification was the predominant pathway for N-loss. While anammox contributed up to $\sim$ 50 % of the N-loss on the deeper part of the shelf (Fig. 6). This pattern was also a reflection of organic matter availability controlling denitrification. Indeed, organic matter content declined with water depth and distance from the coast in the ECS (Kao et al., 2003).

DNRA has been widely reported in marine sediments but varies in extent (Table 4). Our integrated DNRA rates were in the same range as reported for Colne estuary (Dong et al., 2009); however, they were significantly higher than those from the Atlantic continental shelf (Trimmer and Nicholls, 2009) and Baltic Sea (Jäntti and Hietanen, 2012). Unlike denitrification and anammox, there is no N-loss through DNRA. As a result, fixed nitrogen was still preserved in the system and could then be further used to sustain primary production (Gardner et al., 2006). Thus, competition between N-loss and DNRA determined the fate of benthic nitrate. Benthic N-loss via anammox and denitrification was the principal fate of nitrate, which accounted for $\sim$ 75 % in nitrate reduction in the ECS sediments significantly decreasing the dissolved inorganic nitrogen concentrations and alleviating eutrophication risks. However, the DNRA contribution of 23-31 % of the nitrate reduction is significant for retaining nutrient nitrogen in the system. Combined with the decomposition of settling organic matter, benthic DNRA would lead to enhanced oxygen consumption, contributing to the development of hypoxia in the Changjiang estuary.

5 Conclusions

We have shown the coexistence of anammox, denitrification and DNRA using a modification of the ^{15}N isotope pairing method in the ECS sediments also taking nitrate release by nitrate storing organisms into account. Our calculation demonstrated that nitrate release and DNRA had opposite effects on the denitrification rate calculation, but were of minor importance in most of our experiments due to high label additions ($\sim$ 100 µM). On the contrary, calculated anammox rates

Table 4. DNRA rates reported from other marine sediment. The data in parentheses represent the range.

Location	Depth (m)	DNRA (mmol N m^{-2} d^{-1})	DNRA %[a]	Reference
Tama estuary and		13 (3.6–20)[b]	55 (43–73)	Nishio et al. (1982, 1983)
Gullmar fjord	30	0.03[b]		Enoksson and Samuelsson (1987)
Mokbaai, the Netherlands	0	16[c]	33	Goeyens et al. (1987)
Randers Fjord	< 2	1.5 (0.62–3.0)	85 (79–93)	Bonin (1996)
Two French lagoons	0	0.16 (0.07–0.31)	66 (23–99)	Rysgaard et al. (1996)
Thau lagoon, France	8.5	0.4–161[c]	98	Gilbert et al. (1997)
Gulf of Fos, Marseilles	5.5 (2–10)	1.8 (0.3–6.8)[c]	55 (0–93)	Bonin et al. (1998)
Horsens Fjord trout cage	3–9	∼ 2.2 (0.01–6.8)	53 (25–85)	Christensen et al. (2000)
Fringing marsh–aquifer ecotone		66 (21–147)[c]	37 (5–77)	Tobias et al. (2001)
Texas six estuaries	0–3	0.37 (0–2.4)	24 (0.4–68)	An and Gardner (2002), Gardner et al. (2006)
Kanholmsfjärden	∼ 95	0.8[b]	97	Karlson et al. (2005)
Colne estuary		2.6 (0.1–7.7)	43 (11–60)	Dong et al. (2009)
North Atlantic continental shelf	75 (50–100)	0.02–0.1[d]	< 0.2 %	Trimmer and Nicholls (2009)
Plum Island Sound estuary		2.1 (0.1–7.4)	43 (29–51)	Koop-Jakobsen and Giblin (2010)
Peruvian Oxygen Minimum Zone	470 (78–1005)	1.2 (0–2.9)	33 (0–72)	Bohlen et al. (2011)
Three tropical estuaries		2.0 (0–27)	81 (69–91)	Dong et al. (2011)
Baltic Sea	69 (58–83)	0.3 (0.01–1.1)	52 (17–92)	Jäntti and Hietanen (2012)
Norsminde Fjord estuary	0.5		0–20 %	Binnerup et al. (1992), Rysgaard et al. (1993)
Skagerrak	695		< 2 %	Dalsgaard and Thamdrup (2002)
Washington margin	2000–3000	< 0.02	< 10 %	Engström et al. (2009)
East China Sea shelf	55 (19–86)	4.8 (2.6–9.7)	26 (20–31)	This study

[a] Percentage of DNRA in total nitrate reduction (denitrification + anammox + DNRA).
[b] The rate is calculated by combination of $^{15}NO_3^-$ method and mass balance, others were obtained by direct $^{15}NO_3^-$ method.
[c] The rate was measured by slurry incubation.
[d] The unit is μmol N m^{-2} d^{-1}.

were more sensitive to nitrate release with an average overestimation of 42 % in experiments with $^{15}NO_3^-$ (E_Denit). Within experiments amended with $^{15}NH_4^+ + ^{14}NO_3^-$, where nitrate release had no effect on F_N, anammox rate was not significantly influenced by DNRA (4 %). Thus, we recommended that anammox rates should be calculated from the experiment amended with $^{15}NH_4^+ + ^{14}NO_3^-$ and not $^{15}NO_3^-$.

The denitrification rates decreased with increasing water depth, increasing distance to the coast and decreasing organic matter content. In contrast the integrated anammox rates increased with water depth, leading to an enhanced importance of anammox to benthic N-loss from ∼ 13 % in the coastal area to ∼ 50 % on the outer shelf.

DNRA was also an important pathway accounting for up to 30 % of benthic nitrate reduction in the ECS sediments. The transformation from nitrate to ammonium via DNRA could prolong the residence time of fixed nitrogen. Consequently, eutrophication and seasonal hypoxia in the bottom water off the Changjiang estuary could potentially be enhanced by DNRA.

Supplementary material related to this article is available online at http://www.biogeosciences.net/10/6851/2013/bg-10-6851-2013-supplement.pdf.

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