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Anaerobic ammonium oxidation, 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 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 nitrate release by nitrate storing organisms occurred in the East China Sea sediments. These four processes did not exist independently, the nitrate release therefore diluted the ^{15}N labeling fraction of NO_3^-, 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 Thampdrup and Dalsgaard (2002), denitrification rates were slightly underestimated by on 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. Excluding measurements in which bioirrigation was present, integrated denitrification rates decreased from 10 to 4 mmol N m^{-2} d^{-1} with water depth, while integrated anammox rates increased from 1.5 to 4.0 mmol N m^{-2} d^{-1}. Consequently, 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. This study represents the first time in which anammox has been demonstrated to play a significant role in benthic nitrogen cycling in the East China Sea sediment, 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 nitrogen transformation in the East China Sea. Our study demonstrates the complicated interactions among different benthic nitrogen

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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 East China Sea from the Changjiang (Yangtze River) (Beardsley et al., 1985), while on the east outer shelf, the East China Sea 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 East China Sea 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 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 and anammox both

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represent N-loss 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 reduced to bio-available 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 recognized (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 recognized 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).

The significance and occurrence of each pathway differs in sediments, presently there are only a few reports of the coexistence of anammox, denitrification and DNRA, which are based only on flux calculation (Bohlen et al., 2011). However it is necessary to quantify each process in the benthic nitrogen cycle simultaneously; denitrification and anammox represent pathways which remove fixed N from marine systems, therefore they can play a role in reducing eutrophication, meanwhile DNRA recycles fixed nitrogen which can then be assimilated by primary producers.

The co-occurrence of these processes represents a problem when using the traditional ^{15}N isotope pairing technique (Kartal et al., 2007; Sokoll et al., 2012), as the presence of DNRA influences the apparent isotope distribution of anammox and denitrification. Briefly, $^{15}NH_4^+$ derived from DNRA combines with $^{15}NO_3^-$ via anammox to form $^{30}N_2$, the same product as denitrification (Jensen et al., 2011; Kartal et al., 2007).

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However, there is currently no method to assess the influence of DNRA on anammox and denitrification. Moreover, if nitrate is stored intracellularly by nitrate storing organisms, use of the isotope pairing technique is further complicated, as this would represent an excess source of $^{14}NO_3^-$ (Sokoll et al., 2012). While aerobic denitrification combined with aerobic ammonium oxidation are further processes which may 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 pathways and N-loss 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. To our knowledge, this is the first investigation into the simultaneous occurrence of varied N-cycling pathways in the East China Sea sediment.

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 (~ 2 m above the seafloor) was sampled using Niskin bottles equipped with conductivity, 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

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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 addition of saturated $HgCl_2$ for nutrient analysis. 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 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 predegassed 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, three parallel experiments were conducted with different N amendments (^{15}N atom%, 99.3 %, Campro Scientific, Berlin) (Table 2). In all experiments, the tracers were amended to a final concentration of 100 µM. After each tracer injection and well mixing, subsamples were immediately removed and transferred into 6 mL Exetainer vials (Labco Ltd, High Wycombe, UK) and preserved with 100 µL saturated $HgCl_2$. In the following 8–12 h, bags were periodically shaken to ensure that the labeled N compounds were homogenously distributed and 4 subsamples were withdrawn. Exetainer vials containing the subsamples were sealed and stored at room temperature upside down until subsequent N_2 isotope ratio analysis.

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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 autoanalyzer (SAN plus, SKALAR) with the standard spectrophotometric methods. The detected limitation of NH_4^+, NO_3^- and NO_2^- was 0.5 µM, 0.06 µM, 0.01 µM, respectively (Liu et al., 2011), 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° for 4 h.

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 transferred 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).

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

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

Where, $D_{(E\_Denit)}$ and $A_{(E\_Denit)}$ denote the potential rates of denitrification and anammox in E_Denit, respectively. P_{29} and P_{30} are the production rate of $^{29}N_2$ and $^{30}N_2$ in E_Denit,

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which is obtained by the linear regression of the N_2 isotope concentration against time. F_N represents the $^{15}NO_3^-$ fraction in E_Denit, which is determined from the difference in NO_3^- before and after $^{15}NO_3^-$ addition.

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

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

Where, $A_{(E\_Amox)}$, $P_{29(E\_Amox)}$ and $F_{A(E\_Amox)}$ represent 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) is based. Nitrate release from nitrate storing organisms dilutes the $^{15}NO_3^-$ fraction in E_Denit, and $^{15}NH_4^+$ production via DNRA will 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 are 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 $A_{(E\_Denit)}$ was substituted by $A_{(E\_Amox)}$.

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

As in Sokoll et al. (2012), if $F_N^* < F_N$, we can conclude that nitrate release derived from the nitrate storing organisms occurred. In this situation, the following calculation will use F_N^* instead of F_N, $D_{(E\_Denit)}$ and $A_{(E\_Denit)}$ in Eqs. (1) and (2) will be recalculated and denoted as $D^*_{(E\_Denit)}$ and $A^*_{(E\_Denit)}$. The excess $^{14}NO_3^-$ contributed by nitrate release will be calculated according to Sokoll et al. (2012),

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

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

$$A_{29} = A^*_{(E\_Denit)} \times [F^*_N \times (1 - F_A) + F_A \times (1 - F^*_N)] \quad (6)$$

$$A_{30} = A^*_{(E\_Denit)} \times F^*_N \times F_A \quad (7)$$

For denitrification,

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

$$D_{30} = D^*_{(E\_Denit)} \times (F^*_N)^2 \quad (9)$$

And,

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

Where, A_{29} and A_{30} denote the production of $^{29}N_2$ and $^{30}N_2$ by anammox. D_{29} and D_{30} represent the production of $^{29}N_2$ and $^{30}N_2$ through denitrification. F_A represents the fraction of $^{15}NH_4^+$ in E_Denit during the incubation. At each timepoint, F_A can be calculated by the $^{15}NH_4^+$ concentration and total NH_4^+ concentration.

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

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

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

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

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Usually, F_N^* in the anoxic slurry incubations will be constant assuming nitrate release happens only at the beginning as a result of mixing the slurries before subsampling (Sokoll et al., 2012). However, if DNRA occurs, F_A and A_{30} will successively increase over time. We have developed a step by step method to quantify the non-linear $^{30}N_2$ production via anammox (Song et al., in prep.). However, from the data in present study, we find that F_A is 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 can be derived from the accumulation rate of $^{15}NH_4^+$ in E_Denit, however, as mentioned above, a part of $^{15}NH_4^+$ will form $^{30}N_2$ via anammox. Thus,

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

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

DNRA will also influence the anammox rate calculation in E_Amox as $^{14}NH_4^+$ produced by DNRA and remineralization will also dilute the $^{15}NH_4^+$ fraction. We find that the fraction of $^{15}NH_4^+$ in E_Amox decreases linearly with time, thus in Eq. (3), $F_{A(E\_Amox)}$ can be replaced by the average value.

If we assume that the denitrification rate in E_Amox and E_Denit is equal, then the relative contribution of anammox to the total N_2 loss in E_Amox will be,

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

To eliminate the discrepancies between in situ bottom water temperature and the incubation temperature, all the rates are corrected to the in situ temperature using the Arrhenius equation assuming an average apparent activation energy of 61 KJ mol^{-1} for all species (Aller et al., 1985). The average temperature correction factor is 0.7.

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3 Results

3.1 Water column and sediment characteristics

Near-bottom seawater and sediment characteristics were investigated at five stations (Table 1). The near-bottom water NO_3^- decreased sharply from the estuary (~ 27 μM) to the outer shelf (~ 2 μM).

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 μM 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, the nitrate concentration sharply declined sharply and was < 0.5 μM 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 ~ 2 μM at 5 cm. A second nitrate peak of ~ 10 μM was found at 6–7 cm and then decreased to < 1 μM 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.

3.2 ^{15}N slurry incubations

After 24–36 h pre-incubation in E_Ctrl slurry incubations, NO_3^- was still present in some sediment layers (5 ~ 15 μM), especially in the surface layer (0–2 cm). In those layers with residual NO_3^-, significant $^{29}N_2$ accumulation was observed (Fig. 3a). 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 layer of site DH31 and DH55, NO_3^- was not detectable (< 1 μM) after pre-incubation, however there was neither measurable $^{29}N_2$ nor $^{30}N_2$ production ($p > 0.05$) during the incubation (Fig. 3a). Therefore no coupled

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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).

In E_Denit, $^{29}N_2$ and $^{30}N_2$ were produced along with $^{15}NH_4^+$ (Fig. 3). This shows that dissimilatory nitrate reduction to ammonium (DNRA) occured concurrently with anammox and denitrification. Nitrate was never limiting 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 use the average F_A to calculate the $^{30}N_2$ production via anammox.

3.3 Nitrate storage and release in the sediment

There were some indications of nitrate release by nitrate storing organisms during the slurry incubation: (1) nitrate was not depleted after 24–36 h pre-incubation in some layers (5–15 µM) which was inconsistent with our expected result. (2) The derived fraction of $^{15}NO_3^-$ (F_N^*) calculated by Eq. (4) indicated that at all sampling sites nitrate was released, but not in all sediment layers. Specifically, NO_3^- release was confirmed in all the sites in the 0–2 cm layer except DH55, at this site the 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).

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

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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 (Table 3). After nitrate release and DNRA correction, the denitrification rate showed a slight increase (~ 2.5 %) and there was a good correlation between denitrification rates calculated with the method of Thamdrup and Dalsgaard (2002) and our method (r = 0.997, p < 0.0001, Pearson).

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 remineralization, 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 East China Sea (Table 3).

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 (Table 3).

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 sites DH31, DH15 and DH55, while there was no significant variation with sediment depth at sites DHa2 and DH53 (Fig. 5). DNRA rate at all sites generally increased with increasing sediment depth (Fig. 5).

Integrated anammox, denitrification and DNRA rates were calculated down to the NO_x^- penetration 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 (Table 4, Fig. 2). All the integrated denitrification, anammox and DNRA rates showed highest values at site DHa2. At all sites except DHa2, integrated denitrification rates

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generally decreased with the increasing water depth (Fig. 6). Opposite to this, the depth integrated anammox rates increased by a factor of 2.7 with increasing water depth. Hence, the average relative contribution of anammox to the total N_2 loss exhibited an increasing trend with water depth, from 13 % at site closest to the coast DH31 to 50 % at the furthest from the coast, DH55. Apart from site DHa2, integrated DNRA rates showed only small variations (between 2.6 to 4.5 mmol N m^{-2} d^{-1} with an average of 3.6 mmol N m^{-2} d^{-1}). 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 %.

4 Discussion

The isotope pairing method is the most common tool used to track the different pathways of benthic nitrogen transformation, however high intracellular $^{14}NO_3^-$ concentrations and DNRA activity can influence ^{15}N isotope distribution leading to erroneous interpretation of the results. To date, only one study has considered the potential effect of nitrate storage on anammox rate calculations (Sokoll et al., 2012), and there are no studies of the effect of DNRA on anammox and denitrification calculations.

Benthic DNRA and nitrate storage continue to be observed commonly in diverse environments (An and Gardner, 2002; Risgaard-Petersen et al., 2006; Thamdrup, 2012), and are 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; Lomstein et al., 1990). Many of which can even use their intracellular nitrate stores to carry out DNRA (Kamp et al., 2011; Otte et al., 1999; Preisler et al., 2007). Therefore it is now time to consider the combined effect of these processes on the Isotope Pairing Method calculations.

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4.1 The influence of nitrate release on anammox and denitrification

There is strong evidence that during our incubations nitrate was released from the intracellular NO_x^- pool. This is suggested by the occurrence of high initial t_0 nitrate concentrations and $r29$ values as mentioned in Sokoll et al. (2012), as well as the residual nitrate pool after pre-incubation. Sokoll et al. (2012) suggest that the nitrate is released by the mixing of sediment slurries. Furthermore, the equilibrium and exchange of added labeled $^{15}NO_3^-$ with the intracellularly stored $^{14}NO_3^-$ pool may also cause apparent nitrate release and lead to a decrease of the labeled $^{15}NO_3^-$ in the dissolved pool (Dähnke et al., 2012).

The consequence of this release is a dilution of $^{15}NO_3^-$ by $^{14}NO_3^-$, which decreases the $^{29}N_2$ production by anammox and increases the $^{29}N_2$ production by denitrification, as a result denitrification rates according to Eq. (1) are underestimated. The underestimation of denitrification rates in this study ranged from 0 to 19 % with an average of 6 % (Fig. 7a). Anammox rates calculated from Eq. (2) are also underestimated in E_Denit. This is demonstrated by the first order derivative of Eq. (2), i.e.

$$\frac{dA_{(E\_Denit)}}{dF_N} = \frac{4 \times P_{30} - (P_{29} + 2P_{30}) \times F_N}{F_N^3} = \frac{4 - (r29 + 2) \times F_N}{P_{30} \times F_N^3}$$

Where, $r29 = P_{29}/P_{30}$. Because $0 < F_N < 1$, if $0 < F_N < 4/(r29 + 2)$, $\frac{dA_{(E\_Denit)}}{dF_N}$ will be a positive value and Eq. (2) will be an increasing function. In our study, all the measured $r29 < 2$, thus $4/(r29 + 2) > 1$, therefore when $0 < F_N < 1$, the anammox rate in E_Denit would also decrease with the decreasing of F_N. Indeed, 0.3 % to 10 % nitrate release caused an overestimation of 10 % to 128 % with an average of 42 % of anammox respectively in E_Denit (Fig. 7d).

Furthermore if nitrate release leads to an F_N less than $2/(2 + r29)$, F_N^* becomes a negative value, meaning that Eq. (4) cannot be solved successfully. This was the case for 4 samples in our study, in these cases we recommend the anammox rates

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from E_Amox as the actual rates, as here nitrate release would have no effect. All other anammox rates from the two experiments were consistent (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 considered in most published benthic anammox rates, thus, the relative contribution of anammox to total N-loss in these studies may be overestimated when determined from $^{15}NO_x$ experiments.

Foraminifera and diatoms are the most likely organisms to be responsible for nitrate release, as no large sulfur oxidizing bacteria, and no detectable sulfide concentrations have been observed in Eact China Sea sediments (Lin et al., 2002). Living foraminifera are present in the ESC shelf sediment from the Changjiang estuary to the outer shelf (Wang et al., 1985) with population densities similar areas where nitrate storing foraminifera have been reported (Glud et al., 2009; Risgaard-Petersen et al., 2006). Dormant diatoms are also common in the ECS sediments (Ishikawa and Furuya, 2004) especially during the period after the spring bloom, when this study took place. Excess nitrate concentrations were calculated using conservatively, however they were close to that reported in the surface sediment (0–1 cm, 65 nmol cm^{-3}) of Gullmar Fjord, Sweden (Risgaard-Petersen et al., 2006), and less than that reported in the Arabian Sea off Pakistan (0–6 cm, 135 nmol cm^{-3}) (Sokoll et al., 2012).

Nitrate release occurred in experiments to which $^{15}NO_3^-$ was added, but not in E_Ctrl after $^{15}NH_4^+$ addition. Therefore it seems that cell disruption during slurry mixing did not occur, and that nitrate release was instead an adaptation of the nitrate storing organisms to the external environment. The increase of pore water nitrate by addition of NO_3^- may have stimulated nitrate release or exchange by organisms adapted to store considerable amounts of intracellular nitrate in response to low nitrate concentrations in sediment pore water.

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4.2 The influence of DNRA on denitrification and anammox

Potentially the presence of DNRA would affect the anammox and denitrification rate calculations used all Isotope Pairing Technique calculation methods (Thamdrup and Dalsgaard, 2002; Holtappels et al., 2011; Risgaard-Petersen et al., 2003; Nielsen, 1992). From the calculation equations in Sect. 2.4, DNRA will increase anammox and decrease denitrification rates, which is contrast to the effects of nitrate release, thus the influence by nitrate release will be counteracted 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 1 % 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 (Fig. 7e), when taking into account DNRA (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 have shown the effect of DNRA on denitrification and anammox (Nicholls and Trimmer, 2009; Trimmer et al., 2003), however, they did not quantify this effect. Our correction calculation in this study allows quantification of the extent of DNRA on denitrification and anammox.

So far, this is the first report of DNRA in East China Sea sediment. DNRA has been widely reported in marine sediments but varies in extent (Table 5). Our integrated DNRA rates (2.6–9.7 mmol N m^{-2} d^{-1}) were in the same range as reported for Colne estuary (Dong et al., 2009), however, it was significantly higher than those from the Atlantic continental shelf (Trimmer and Nicholls, 2009) and Baltic sea (Jäntti and Hietanen, 2012). Unlike denitrification or anammox, there is no N-loss through DNRA. As a result, the fixed nitrogen is still preserved in the system and could then be further used to sustain primary production (Gardner et al., 2006). Thus, the competition between N-loss and DNRA determines the fate of benthic nitrate. If benthic N-loss

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exceeds DNRA, then the decrease in dissolved inorganic nitrogen concentration could alleviate eutrophication. Furthermore the decomposition of settling organic matter produced by primary production and re-oxidation of ammonium produced by DNRA would lead to oxygen consumption and contribute to the development of hypoxia in the bottom waters of the Changjiang estuary. Overall the contribution of DNRA to the benthic nitrate reduction accounted for 23–31 % of the total nitrate reduction (sum of DNRA, anammox and denitrification) implying that DNRA plays an important role in the benthic nitrogen transformations in the East China Sea.

4.3 N-loss in the ECS via denitrification and anammox

So far, benthic N-loss rates have never been directly measured in East China Sea sediments using the isotope pairing method. The average integrated total N-loss over the nitrate penetration depth was 13.1 mmol N m^{-2} d^{-1}. This value is more than 1 order of magnitude higher than the value obtained from the modeled value (Christensen et al., 1987) and the denitrification rate at the tidal flat of the Changjiang estuary using the diffusion based on acetylene inhibition method (Wang et al., 2006). The latter will underestimate denitrification rates as coupled nitrification-denitrification is also inhibited by the acetylene and it does not take anammox into account. The increase in NO_3^- concentrations by label addition may have increased the the potential N-loss rates in our slurry incubations, leading to an overestimation of the actual N-loss, however this is unlikely as reported half saturation constants for denitrification are below the initial pore water concentrations measured (Evrard et al., 2012). It seems therefore that the potential total N-loss reported in this study is a reasonable estimation of benthic N-loss on the East China Sea shelf.

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

Benthic N-loss via anammox and denitrification was the main fate of nitrate in the East China Sea sediment contributing ~ 75 % of the nitrate reduction (Fig. 6a). Of these two, denitrification dominated, accounting for on average 72 % of the overall N-loss. This is consistent with previous studies on other continental shelves (Gihring et al., 2010; Neubacher et al., 2011; Trimmer and Nicholls, 2009). The contribution of anammox to N-loss was in the range of the literature values reported for continental shelf sediments (Engström et al., 2005; Trimmer and Nicholls, 2009), but considerably higher than previous studies in shallow estuaries (Meyer et al., 2005; Trimmer et al., 2003).

The availability of organic carbon maybe a key factor regulating hetrotrophic denitrification. A positive correlation between potential volumetric denitrification rate and organic matter (LOI %) ($r = 0.68$, $p < 0.001$, Pearson), reveals that organic matter content is an important environmental regulator of denitrification. Meanwhile, the integrated denitrification rate also decreases from the shallow coastal site to outer shelf site, consistent with the distribution of primary production and/or organic carbon export in the East China Sea (Gong et al., 2003). There is no correlation with volumetric anammox rate ($r = 0.32$, $p = 0.17$, Pearson), implying that the anammox activity is not directly limited by the availability of organic matter in coastal sediments. Organic matter content declines with water depth and distance from the coast in the East China Sea (Kao et al., 2003). Integrated denitrification rates decreased from the shallow estuarine site to the deep outer shelf site, while the relative contribution of anammox increased, except site DHa2 which could be significantly affected by bio-irrigation leading to NO_x^- availability down to 8 cm sediment depth (Figs. 2 and 6a). A similar pattern was observed with water depth (Fig. 6b). From the negative correlation between the relative contribution of denitrification and anammox ($r = -0.92$, $P < 0.05$, Pearson), we can infer that in the shallow coastal area, denitrification was the predominant pathway for nitrate reduction, while anammox mainly controlled the N-loss on the deep outer shelf. This pattern is

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a reflection of organic matter availability controlling the denitrification rate. Bioirrigation enhanced the availability of nitrate for subsequent nitrate reduction, but it did not change the relative partitioning in nitrate reduction (Fig. 6).

The availability of nitrate and/or nitrite as electron acceptors is considered as an important factor controlling anammox (Dalsgaard et al., 2005). The availability of NO_3^- and/or NO_2^- can be derived from extent of nitrification, denitrification and the DNRA in the sediment. Here, relatively high anammox rates corresponded well with the high NO_3^- and/or NO_2^- concentration in the surface 2 cm layer. At sites where bioirrigation was present (DHa2 and DH55) local anammox activity was stimulated. This may be a result of enhanced local nitrification activity and therefore elevated NO_3^- and/or NO_2^- (Gilbert et al., 1998).

There is no correlation between DNRA rate and sediment organic matter content ($r = 0.06$, $P = 0.81$, Pearson). The increase of DNRA rate with sediment depth (Fig. 5) suggested the deeper sediment layer was more favorable to DNRA, which is consistent with previous studies (Stief et al., 2010). This could be due to the lower ratio between electron acceptor and donor (Tiedje, 1988).

5 Conclusions

We have shown the coexistence of anammox, denitrification and DNRA, as well as nitrate release by nitrate storing organisms in the East China Sea sediments using a modification of the ^{15}N isotope pairing method. Our improved calculation demonstrates 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 (~ 100 µM). On the contrary, the calculated anammox rates were more sensitive to nitrate release where an average 6 % decline in the fraction of $^{15}NO_3^-$ would cause a 42 % overestimation of anammox rate when calculated from the E_Denit. Within experiments amended with $^{15}NH_4^+ + {}^{14}NO_3^-$, where no significant nitrate release influence, the anammox rate was not significantly influenced by DNRA (4 %). Thus, we

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recommend that anammox rates should be calculated from experiments amended with $^{15}NH_4^+ + {}^{14}NO_3^-$.

The decrease in integrated denitrification rates with increasing water depth and distance from the coast and the significant correlation between volume specific denitrification rates and organic matter ($r = 0.68$, $p < 0.001$, Pearson) revealed that organic matter was a key parameter regulating denitrification. While integrated anammox rates showed an increasing trend by a factor of 2.7 with water depth; leading to an elevated importance of anammox in the contribution to total N-loss from 13 % on the coastal area to 50 % on the outer shelf.

DNRA was also an important pathway accounting for 20–30 % of benthic nitrate reduction in the East China Sea 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.

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Table 1. Sampling locations and some general parameters characteristics of corresponding bottom water and sediment. The porosity and organic matter content (expressed as LOI%) are the average value 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 (%)
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)
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)
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)
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)
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)

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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

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Table 3. Rates of anammox, denitrification and DNRA in the slurry incubation. Excess nitrate stored by nitrate storing organisms was also shown. T&D (2002): rate calculation method from Thamdrup and Dalsgaard (2002). Our method: rate calculation after nitrate release and DNRA correction. n.d.: not detectable.

Station	Layer (cm)	Denitrification rate (E_Denit) ($nmol\,N\,cm^{-3}\,h^{-1}$)		Anammox rate (E_Denit) ($nmol\,N\,cm^{-3}\,h^{-1}$)		Anammox rate (E_Amox) ($nmol\,N\,cm^{-3}\,h^{-1}$)		DNRA ($nmol\,N\,cm^{-3}\,h^{-1}$)	Excess nitrate ($nmol\,cm^{-3}$)
		T&D (2002)	Our method	T&D (2002)	Our method	before DNRA corr.	after DNRA corr.		
DH31	0–2	10 ± 1	12 ± 1	3.8 ± 0.5	2.4 ± 0.6	2.4 ± 0.2	2.5 ± 0.2	2.0 ± 0.3	64
	2–4	20 ± 2	19 ± 2	1.4 ± 0.4	1.6 ± 0.5	1.3 ± 0.1	1.4 ± 0.1	11 ± 1	2.6
	4–6	13 ± 2	13 ± 2	1.0 ± 0.2	0.76 ± 0.31	0.60 ± 0.04	0.67 ± 0.04	15 ± 1	12
	6–8	14 ± 1	13 ± 1	0.47 ± 0.20	0.79 ± 0.26	0.59 ± 0.05	0.66 ± 0.06	33 ± 2	n.d.
DHa2	0–2	9.5 ± 0.9	10 ± 1	4.6 ± 0.4	4.3 ± 0.6	3.4 ± 0.4	3.5 ± 0.4	4.0 ± 0.5	63
	2–4	7.4 ± 0.4	7.9 ± 0.5	4.0 ± 0.2	3.8 ± 0.3	3.5 ± 0.2	3.8 ± 0.2	2.2 ± 0.04	37
	4–6	13 ± 1	13 ± 1	3.3 ± 0.2	3.5 ± 0.4	2.7 ± 0.2	2.9 ± 0.2	7.6 ± 1.2	21
	6–8	16 ± 1	15 ± 1	1.4 ± 0.2	1.9 ± 0.3	2.3 ± 0.1	2.5 ± 0.1	13 ± 0.2	n.d.
DH15	0–2	11 ± 1	11 ± 1	2.4 ± 0.3	2.3 ± 0.3	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	16
	2–4	5.4 ± 0.6	5.7 ± 0.7	1.7 ± 0.2	1.6 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	4.5 ± 0.2	42
	4–6	4.5 ± 0.3	5.0 ± 0.3	1.0 ± 0.1	0.53 ± 0.11	0.44 ± 0.03	0.47 ± 0.03	5.8 ± 0.3	50
	6–8	2.3 ± 0.1	2.2 ± 0.1	0.31 ± 0.01	0.44 ± 0.01	0.46 ± 0.04	0.48 ± 0.04	10 ± 1	n.d.
DH53	0–2	7.5 ± 1.1	9.0 ± 1.3	4.2 ± 0.6	3.1 ± 0.8	2.8 ± 0.2	2.9 ± 0.2	2.0 ± 0.1	83
	2–4	4.1 ± 0.4	3.8 ± 0.4	2.4 ± 0.3	2.6 ± 0.3	3.5 ± 0.4	3.6 ± 0.4	2.4 ± 0.1	n.d.
	4–6	3.1 ± 0.4	2.9 ± 0.4	1.6 ± 0.2	1.8 ± 0.2	3.4 ± 0.3	3.6 ± 0.3	2.2 ± 0.2	n.d.
	6–8	2.9 ± 0.4	2.6 ± 0.4	1.3 ± 0.2	1.6 ± 0.2	3.1 ± 0.4	3.2 ± 0.4	3.7 ± 0.2	n.d.
DH55	0–2	0.64 ± 0.13	0.63 ± 0.13	0.48 ± 0.10	0.49 ± 0.10	4.0 ± 0.4	4.1 ± 0.4	0.44 ± 0.07	n.d.
	2–4	2.9 ± 0.3	3.3 ± 0.4	2.5 ± 0.3	2.2 ± 0.3	2.2 ± 0.3	2.2 ± 0.3	1.1 ± 0.05	58
	4–6	2.0 ± 0.2	1.9 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	2.7 ± 0.1	n.d.
	6–8	2.4 ± 0.4	2.6 ± 0.4	1.0 ± 0.1	0.85 ± 0.14	0.69 ± 0.08	0.71 ± 0.09	3.4 ± 0.2	57

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Table 4. Integrated rates of anammox, denitrification and DNRA from nitrate penetration depth in slurry incubation. ra%: the percentage of anammox in total N-loss; DNRA%: the percentage of DNRA in total nitrate reduction (sum of anammox, denitrification and DNRA).

Station	Nitrate penetration (cm)	Integrated rates over the nitrate available layer ($mmol\,N\,m^{-2}\,d^{-1}$)			ra%	DNRA%
		Anammox	Denitrification	DNRA		
DH31	3	1.5 ± 0.1	10 ± 1	3.5 ± 0.2	13	23
DHa2	7	5.5 ± 0.2	18 ± 1	9.7 ± 1.1	23	29
DH15	5	1.6 ± 0.1	9.1 ± 0.6	4.5 ± 0.1	15	29
DH53	5	4.0 ± 0.2	6.8 ± 0.7	2.6 ± 0.1	37	20
DH55	8	4.0 ± 0.2	4.0 ± 0.3	3.7 ± 0.2	50	31

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Table 5. 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, 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)
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 uint is $\mu mol\,N\,m^{-2}\,d^{-1}$.

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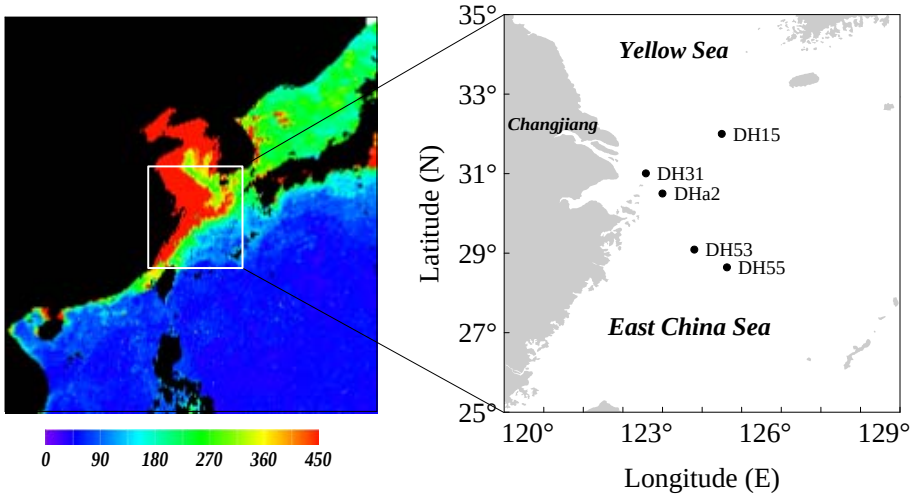

Fig. 1. Sampling locations in the East China Sea. The primary production map is from: http://marine.rutgers.edu./opp/swf/Production/gif_files/PP_Month_9806B.gif.

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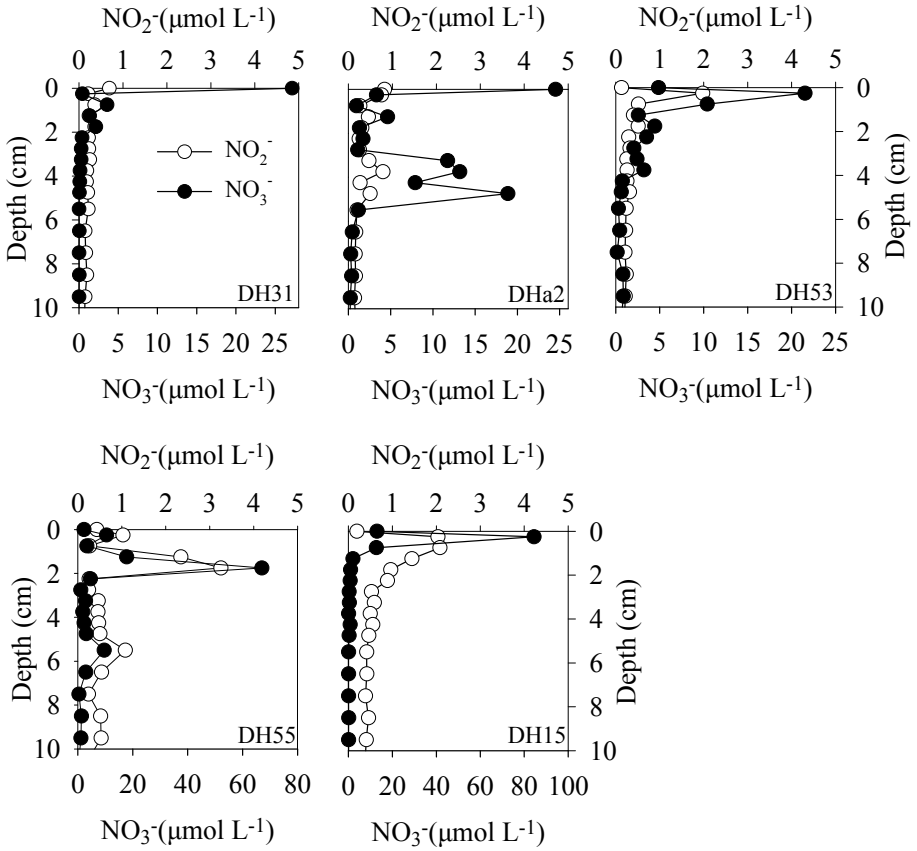

Fig. 2. Pore water profiles of NO_2^- and NO_3^- in the East China Sea sediment.

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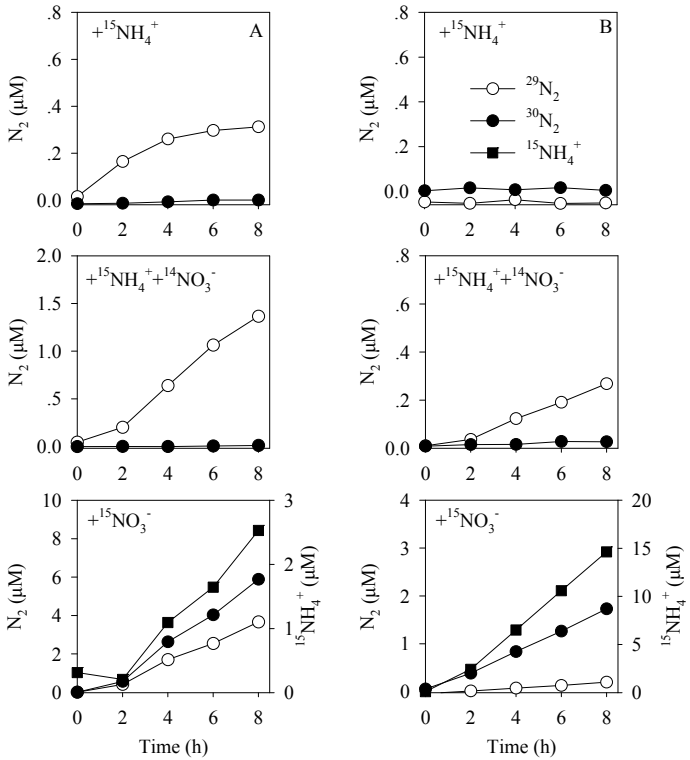

Fig. 3. Production of $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ against time in slurry incubation. **(a)** DH31 0–2 cm sediment. The residual nitrate was not exhausted after the 24 h pre-incubation, thus we observed a slight $^{29}N_2$ production before all the nitrate disappeared; **(b)** DH15 6–8 cm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

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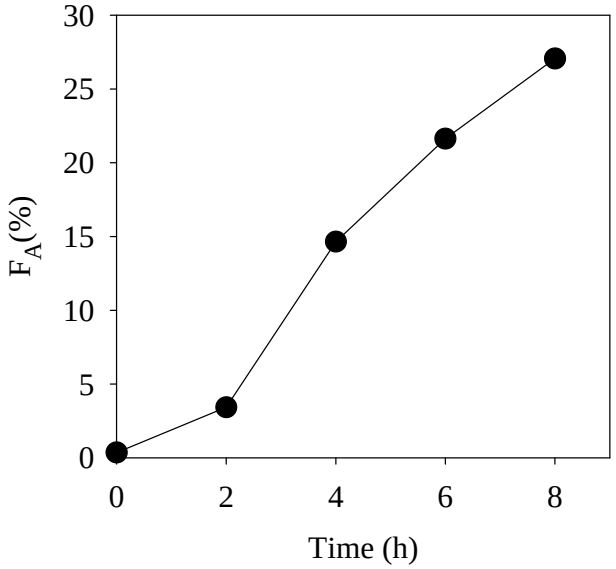

Fig. 4. Time course of fraction of $^{15}NH_4^+$ (F_A) in E_Denit. This example is from DH31 4–6 cm sediment.

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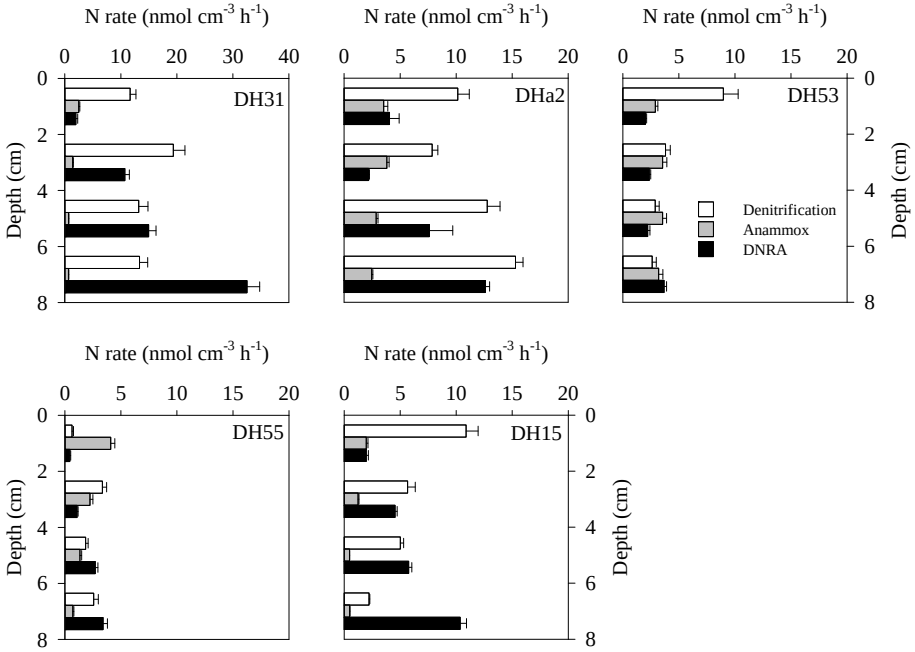

Fig. 5. Vertical distribution of potential denitrification (white bar), anammox (shadow bar) and DNRA (black bar) rates in sediment of the ECS from the slurry incubation. The error bar (± 1 S. E) was calculated from the linear slope standard deviation given by the regression statistic automatically.

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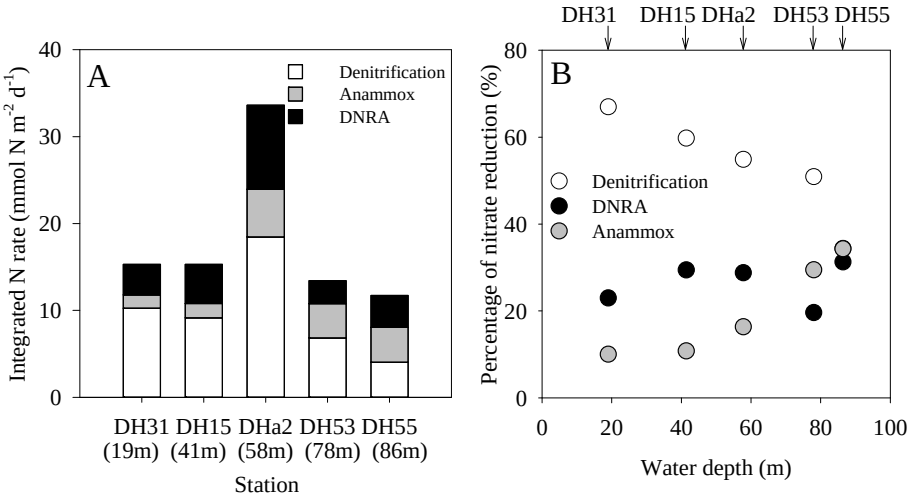

Fig. 6. Total nitrate reduction from denitrification, anammox and DNRA **(a)** and relative contribution of denitrification, anammox and DNRA as a function of water depth **(b)**.

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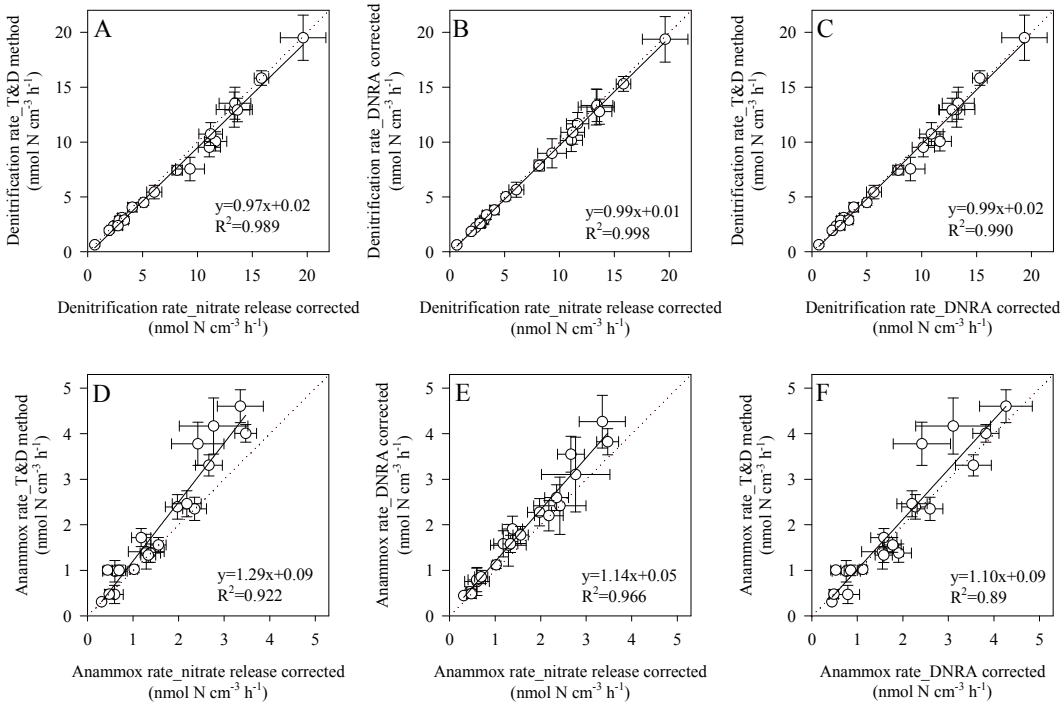

Fig. 7. The influence of nitrate release and DNRA on anammox and denitrification rates in the experiment amended with $^{15}NO_3^-$. T&D method: Thamdrup and Dalsgaard (2002). Nitrate release corrected: the rate calculation corrected for nitrate release. DNRA corrected: after nitrate release correction, the rate calculation was corrected for DNRA. See Sect. 2.4 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.

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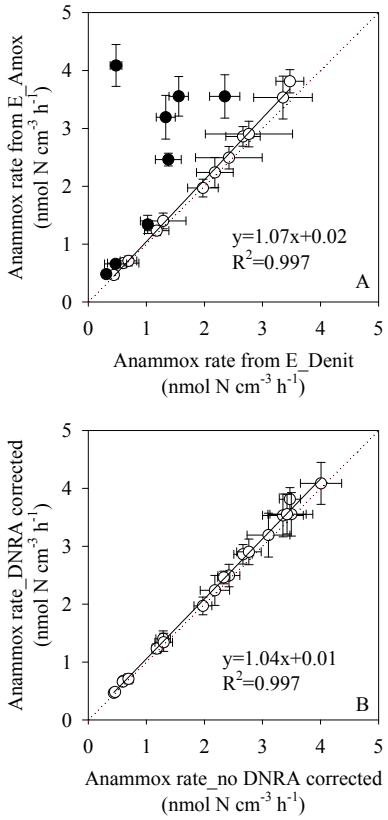

Fig. 8. Comparison of anammox rates from E_Denit and E_Amox **(a)** and DNRA effects on the anammox rate in E_Amox **(b)**. The black dot represents the anammox rate calculated from E_Denit derived from the original F_N since we can calculated F_N^* according Eq. (4). The anammox from E_Denit is the rate only after nitrate release correction (see the text).

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