N₂ production through denitrification and anammox across the continental margin (shelf–slope–rise) of the Ulleung Basin, East Sea

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Abstract

Experimental determinations of nitrogen cycling in deep-sea sediments are strongly underrepresented in the databases. To investigate the total N₂ production rates and relative contribution of denitrification and anaerobic ammonium oxidation (anammox) to benthic fixed-N removal processes, we conducted ¹⁵N isotope-labeling incubation experiments in whole cores and slurries at nine stations across the continental margin from the shelf (< 200 m) and into the deep (> 2000 m) Ulleung Basin (UB) in the East Sea. The total N₂ production rates (anammox plus denitrification) in the center of the UB (8.4 ± 0.2 μmol N m⁻² h⁻¹) were high compared to most other deep-sea sediments at similar water depths. Denitrification rates decreased from the shelf (7.6 ± 0.6 μmol N m⁻² h⁻¹) to the basin (3.2 ± 0.4 μmol N m⁻² h⁻¹), in proportion to benthic oxygen consumption, whereas anammox rates remained relatively constant or even increased slightly (1.3–4.1 μmol N m⁻² h⁻¹). The contribution of anammox to the total N₂ production (ra) increased with increasing water depth from the shelf (ca. 17%) to the basin (ca. 56%). The enhanced ra in the center of the UB was associated with an increased availability of nitrite for anammox, which was likely a result of the competitive suppression of denitrification by manganese reduction under MnO₂-rich conditions. Our results emphasize the importance of anammox as a sink for reactive nitrogen in deep-sea sediments and contribute toward a mechanistic understanding of the factors controlling benthic reactive nitrogen loss in the ocean.

Approximately 50–70% of the removal of fixed nitrogen from the oceans is estimated to occur in organic-rich sediments deposited on the continental margins including shelf, slope, and rise (Codispoti et al. 2001; de Vries et al. 2013). Here, fixed N is transformed into inert dinitrogen gas (N₂) by the two anaerobic microbial processes; denitrification and anammox. Denitrification is a respiratory process whereby nitrate is used as the terminal electron acceptor in the oxidation of either organic matter (5CH₂O + 4NO₃⁻ → 5CO₂ + 2N₂ + 7H₂O) or inorganic compounds including hydrogen, ferrous iron, or reduced sulfur compounds (Straub et al. 1996; Zumft 1997). During anammox (short for anaerobic ammonium oxidation), NO₂⁻ is utilized as an electron acceptor to oxidize NH₄⁺ (NH₄⁺ + NO₂⁻ → N₂ + 2H₂O; Van De Graaf et al. 1995). The average relative contribution of anammox to marine benthic N₂ production is estimated to 28% (Trimmer and Engström 2011). The contribution varies widely, however, with anammox being almost insignificant in many shallow coastal locations while it dominates as N₂ source in some deeper sediments, particularly. This apparent dependence on water depth is mainly driven by decreasing denitrification rates with increasing water depth while anammox rates show lower variability (Dalsgaard et al. 2005; Trimmer and Engström 2011). The decrease in denitrification activity with increasing depth is attributed to a concurrent decrease in the availability of organic carbon (Thamdrup and Dalsgaard 2002; Engström et al. 2005), whereas various factors (i.e., temperature, bottom water NO₃⁻ concentration, and organic carbon content) have been considered to regulate the anammox process (Risgaard-Petersen et al. 2004; Trimmer and Nicholls 2009).
So far, most studies have been restricted to shallow marine sediments (< 1000 m; reviewed by Trimmer and Engström 2011; Thamdrup 2012), while only few studies determined the processes in deep-sea sediment (Engström et al. 2009; Glud et al. 2009; Trimmer and Nicholls 2009). The dearth of experimental determinations of N\textsubscript{2} production in deep-sea sediments seriously limits the accuracy of estimates of the contribution of these sediments as a sink in marine reactive nitrogen budgets.

The East Sea (often referred to as the Japan Sea) is a semi-enclosed marginal sea in the north-western Pacific Ocean, and consists of three deep basins (> 2000 m), the Japan Basin (JB), the Yamato Basin (YB), and the Ulleung Basin (UB) (Fig. 1). Compared to the other deep basins, the UB is characterized by high organic carbon content in the sediment (up to 2.5% dry wt.), which has rarely been observed in deep-sea sediments (Lee et al. 2008). High primary production stimulated by the formation of coastal upwelling and subsequent advection of the highly productive upwelled waters into the center of the UB (Hyun et al. 2009; Yoo and Park 2009; Kwak et al. 2013) were suggested to explain the high organic carbon content accumulated in the sediment of the UB. In association with the high organic carbon content, the central basin is also characterized by remarkably high benthic O\textsubscript{2} utilization rates (6.0–7.1 mmol m\textsuperscript{-2} d\textsuperscript{-1}) (Hyun 2016) and sulfate reduction rates (0.7–1.9 mmol m\textsuperscript{-2} d\textsuperscript{-1}) (Hyun et al. 2010) that are comparable to those reported for highly productive Peruvian and Chilean sites with similar depth range (Fossing 1990; Ferdelman et al. 1997). Thus, the UB is regarded as a biogeochemical hot spot where a substantial amount of carbon and nutrients are recycled (Hyun et al. 2017). An additional intriguing geochemical property of the UB surface sediment is the high content of Mn oxides (> 200 \mu mol cm\textsuperscript{-3}; Cha et al. 2007; Hyun et al. 2010) that supports a large contribution of Mn reduction to C\textsubscript{org} oxidation (45% of total; Hyun et al. 2017). Manganese oxides have previously been suggested to influence benthic
nitrogen transformations either directly through ammonium oxidation (Luther et al. 1996) or by favoring anammox due to competitive inhibition of denitrification by Mn reduction (Thamdrup and Dalsgaard 2002; Trimmer et al. 2013). Yet the role of Mn as a controlling factor for N cycling remains poorly understood.

The objectives of this study were to determine N2 production rates through denitrification and anammox along a transect from the continental shelf to the deep-offshore basin of the UB, and to assess relative significance of the two processes along with varying environmental characteristics, including water depth, organic carbon and manganese oxide content, bottom water NO$_3^-$ concentration, and bottom water temperature. Here, we report that the N2 production rates via denitrification and anammox in the UB were very high even in the deep sediment of the UB compared to other marine sediments at similar water depths, and anammox plays a significant role (up to 51%) in fixed-N removal in the deep-sea sediment of the UB.

Materials and methods

Study sites and sampling

The basin floor of the UB lies at depths of 2000–2300 m, with the boundary between the continental slope and the central basin at approximately 2000 m (Fig. 1). The surface sediments in the basin are defined as fine-grained clay sediments with a mean grain size $<0.004$ mm in diameter (not less than 8 φ) (Cha et al. 2007). In association with the high organic carbon content, the central basin is also characterized by remarkably high Mn oxide ($>200$ μmol cm$^{-3}$) and Fe oxide ($>100$ μmol cm$^{-3}$) content (Hyun et al. 2010).

The study sites (Fig. 1; Table 1) represent a transect from the continental shelf ($<200$ m, Sta. EA4, EA6, and EB1) to the slope ($>1000$ m, Sta. EA5, EB3, and EB7) and the basin ($>2000$ m, Sta. EB6, EB5, and EC1) in the UB, and cover a wide range of bottom water NO$_3^-$ concentrations. Sampling was conducted aboard the R/V $Ounui$ in June 2009, the R/V $Haeyang 2000$ in March 2012, the R/V $Tamyang$ in May 2012, and the R/V $Ieodo$ in July 2012, respectively. The sampling in 2009 occurred in conjunction with a detailed analysis of terminal electron-accepting processes involved in carbon oxidation at the sites EA5 and EB5, (Hyun et al. 2010; sites then denoted M1 and D3, respectively).

At all the sampling locations, a box corer was used to collect sediment samples, and sub-cores were taken for well-preserved surface sediment on deck. The bottom water for dissolved inorganic nitrogen determinations was also sampled at each station using a 20 L Niskin water sampler. The cores and seawater were stored at bottom water temperature in the dark.

Duplicate sub-cores were sliced into depth intervals at 0–2 cm, 2–4 cm, and 4–6 cm for the particulate organic carbon (POC) and nitrogen (PON). The subsamples to determine
POC and PON were fumed with concentrated HCl for 2 h in a desiccator to remove carbonate and were subsequently dried at 60°C. The dried sediments were analyzed using a CHN elemental analyzer (Eurovector 3000 Series, Milan, Italy). Sub-cores for geochemical analysis were collected using polycarbonate barrels (6–9 cm in diameter and 30–40 cm in length) in duplicate or triplicate on board. The sub-cores were immediately sealed with butyl rubber stoppers and placed in a cooler until they were processed in the laboratory on the deck within 6 h. Pore water was extracted using Rhizone soil moisture samplers (Rhizosphere Research Products, Wageningen, The Netherlands) in a N2-filled glove bag. Pore water for moisture samplers (Rhizosphere Research Products, Wageningen, The Netherlands) in a N2-filled glove bag. Pore water for NO₂⁺, NO₃⁻, and NH₄⁺ was stored at 4°C after adding HgCl₂ (125 mM). Pore water for dissolved Mn²⁺ was mixed with HCl (0.1 N) and kept frozen at −20°C. Sediment cores for solid phase analysis were sectioned into 1 cm (down to 10 cm) and 2 cm (10–20 cm) intervals and loaded into polypropylene centrifuge tubes in the N₂ filled glove bag, and the tubes were kept frozen at −25°C until the analysis.

Geochemical constituents

Pore-water NO₃⁻ and NO₂⁻ were measured using an autoanalyzer (Proxima, Alliance). After reducing NO₃⁻ to NO₂⁻ via a cadmium column, the nitrite produced was determined by diazotizing with sulfanilamide and N-(1-naphthyl)-ethylenediamine (Parsons et al. 1984). NO₃⁻ concentration was defined as the difference between the concentrations of NO₃⁻ (NO₃⁻ + NO₂⁻) and NO₂⁻ as determined in non-cadmium-treated samples. Pore water for NH₄⁺ was measured using flow injection analysis (Hall and Aller 1992). Dissolved Mn²⁺ was diluted with HNO₃ (1%) (Thamdrup et al. 1994; Thamdrup and Dalsgaard 2000) and was measured using inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 3300 DV, Perkin Elmer). Total dithionite-citrate-acetic acid extractable Mn (Mn(DCA)) in the sediment was extracted from air-dried sediment in a DCA solution (DCA, pH 4.8) for 4 h (Mehra and Jackson 1960; Lord 1980) and was analyzed using the same ICP-OES.

Oxygen micro-profiles

Vertical profiles of dissolved oxygen were obtained at 50 μm intervals using Clark type microelectrodes (OX25; Unisense, Denmark) while the overlying water was stirred. Microelectrodes were calibrated between 100% air-saturated in situ bottom water and N₂-purged anoxic bottom water. The cores were pre-incubated during 12 h at in situ temperature in order to reestablish in situ O₂ conditions (Glud et al. 1999). Three replicate profiles were measured in dark at in situ temperature. The diffusive boundary layer (DBL) was determined from the intersection of the gradient with the oxygen concentration in the overlying water (Revsbech and Jørgensen 1986). The location of the sediment-water interface was identified from the sudden steepening of the gradient between DBL and the pore-water space.

N₂ production rate determination from intact core incubations

Intact core incubations using ¹⁵N-nitrate followed the isotopic pairing technique of Nielsen (1992) as modified by Rigsøgard-Petersen et al. (2003). Rates of ²⁹N₂ and ³⁰N₂ production from ¹⁵NO₃⁻ are quantified and used to calculate rates of ¹⁴N₂ production (ρ₁₄); when combined with ¹⁵N-tracer slurry incubations (below), rates of anammox, and the relative contributions of anammox and denitrification to p₁₄ can be determined. Fifteen sediment sub-cores (inner diameter = 3.5 cm) were collected from each site to obtain 15 cm of sediment and 15 cm of overlying water. The overlying water was removed from each core and bottom water amended with 50 μmol L⁻¹ of ¹⁵N-nitrate (99 atom% ¹⁵N; Sigma-Aldrich) was added. A small Teflon-coated magnet (0.5 cm × 3 cm) was placed 3–5 cm above the sediment surface in each core and then the core barrels were sealed with rubber stoppers, leaving no headspace. Seawater for nutrient analysis was filtered (0.2 μm) and frozen before and after tracer addition. All incubations were conducted in the dark in bottom water temperature about 0°C. After 6 h of preincubation, three cores from each site were sacrificed at five time points between 0 h and 72 h by adding 1 mL of zinc chloride (50% wt/vol) to the surface sediment and then inverting the cores to thoroughly mix the sediment and water. After settling of the sediment, the water samples for the ²⁹N₂ and ³⁰N₂ determinations were transferred to 12 mL Exetainers pre-filled with 250 μL of 50% (wt/vol) zinc chloride. According to Rigsøgard-Petersen et al. (2003), the N₂ production (ρ₁₄) in intact sediment cores was estimated using the formula,

\[
ρ_{14} = 2ρ_{29}\left[ρ^{29}N_2 + ρ^{30}N_2(1−r_{14})\right]
\]

where \(r_{14}\) is the ratio between \(^{14}NO_3^-\) and \(^{15}NO_3^-\) in the NO₃⁻ reduction zone, and \(ρ^{29}N_2\) and \(ρ^{30}N_2\) are the production of the isotopes \(^{14}N_{2}\) and \(^{15}N_{2}\), respectively. The ratio between \(^{14}NO_3^-\) and \(^{15}NO_3^-\) in the NO₃⁻ reduction zone (\(r_{14}\)) was estimated from

\[
r_{14} = \frac{(1−ra)·R^{29}−ra}{(2−ra)}
\]

where, \(R^{29}\) is the ratio between the \(^{14}N^{15}N\) and \(^{15}N^{15}N\) production, and \(ra\) is the relative contribution of anammox to the N₂ production determined from slurry incubations. The denitrification and anammox rates in the intact cores with added \(^{15}NO_3^-\) were calculated by the following equations:

\[
\text{Denitrification} = ρ_{14} × (1−ra)
\]

\[
\text{Anammox} = ρ_{14} × ra
\]

Slurry incubation for determination of potential rates of denitrification and anammox

Two set of sediment slurry incubation with \(^{15}N\) tracers were performed to determine the relative contribution of
anammox to gross $N_2$ production ($ra$) and the potential for alternative pathways of ammonium oxidation, respectively. Slurry incubations were conducted to determine potential rates (for three depth intervals; 0–2 cm, 2–4 cm, 4–6 cm) of denitrification and anammox by addition of a $^{15}$N-nitrate (Thamdrup and Dalsgaard 2002). Bottom water was sparged with $N_2$ to remove any oxygen and was amended with 50 $\mu$mol L$^{-1}$ of $^{15}$N-nitrate (99 atom%). Subsamples of 2.5 cm$^3$ of the 0–2 cm, 2–4 cm, and 4–6 cm intervals of the sediments were placed in 15 vials (a gas-tight 12 mL Exetainer) for each interval, which were then filled with tracer-amended seawater, leaving no headspace. All the vials were incubated at bottom water temperature and triplicate vials were sacrificed at five time points (0 h, 12 h, 24 h, 48 h, and 72 h). Incubation was stopped by adding 250 $\mu$L of 50% (wt/vol) zinc chloride and the vials were resealed without leaving any headspace. The abundance and concentration of $^{29}$N$_2$ and $^{30}$N$_2$ were determined using a gas chromatograph coupled to a triple-collector isotopic ratio mass spectrometer (Thermo Delta V Plus) as described in Dalsgaard et al. (2012). The potential rates of denitrification and anammox from the slurry incubation were calculated from the measured $N_2$ isotopes ($^{28}$N$_2$ and $^{30}$N$_2$) and the $^{15}$N labeling of the NO$_3^-$ pool according to the following equations (Thamdrup and Dalsgaard 2002):

$$\text{Denitrification} = \rho^{30}N_2 \cdot F_S^2 \quad (5)$$
$$\text{Anammox} = F_S^{-1} \cdot \rho^{29}N_2 + 2 \cdot (1 - F_S^{-1}) \cdot \rho^{30}N_2 \quad (6)$$

where $\rho^{29}N_2$ is the production of $^{14}$N$^{15}$N, $\rho^{30}N_2$ is the production of $^{15}$N$^{16}$N, and $F_S$ is the fraction of $^{15}$N in NO$_3^-$. The potential for alternative pathways of anaerobic ammonium oxidation leading to $N_2$ production, e.g., coupled to Mn oxide reduction (Luther et al. 1996), was explored in experiments with sediment from 0 cm to 2 cm depth collected in 2009 at Sta. EA5 and EB5, using the approach described above with slight modification and with further treatments including $^{15}$NH$_4^+$ and $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$ as well as specific inhibitors. Sediment was slurried in autoclaved bottom water (1 : 2) and ammonium and nitrate were added at final concentrations of $\sim 200$ $\mu$mol L$^{-1}$ and $\sim 50$ $\mu$mol L$^{-1}$, respectively. Additional incubations were made with $^{15}$NH$_4^+$ and either allylthiourea (ATU) as inhibitor of aerobic nitrification (final concentration 0.9 mmol L$^{-1}$) or acetylene as inhibitor of aerobic nitrification, anammox, and $N_2$O reduction (final concentration 0.2 mmol L$^{-1}$; Jensen et al. 2008). Inhibitors and nitrogen compounds were injected after 1 h of preincubation. Duplicate vials were sacrificed at five timepoints from 0 h to 47 h, and stored and analyzed as above.

**Results**

**Geochemical properties**

The water temperature and nitrate concentration in the water near the seafloor showed a pronounced spatial difference between the shelf (EA4, EA6, and EB1), slope (EA5, EB3, and EB7), and basin sites (EB6, EB5, and EC1) (Table 1). The water temperature was close to 0°C at the sites deeper than 500 m, whereas the temperature at the shelf sites varied from 2.3°C to 13.5°C. The nitrate concentrations were considerably higher in the basin (28.7 ± 3.3 $\mu$mol L$^{-1}$) than on the shelf (11.5 ± 5.1 $\mu$mol L$^{-1}$). In contrast to the bottom water, geochemical properties of the sediments were similar both in the shelf and the basin. The mean porosity and density in the upper 6 cm depth of the sediments were 0.8 ± 0.1 and 1.1 ± 0.1 g cm$^{-3}$, respectively. The organic carbon content of the slope and basin sediments ranged from 2.29% to 3.37%, and was highest at Site EA5 located at the lower part of the continental slope.

The depth distributions of O$_2$, NO$_3^-$, NO$_2^-$, NH$_4^+$, and Mn$^{2+}$ in the pore water and Mn$_{(DCA)}$ in the sediment for three representative stations (EB1 for continental shelf; EB3 for continental slope; EB5 for the basin) are presented in Fig. 2. The oxygen penetration depth (OPD) increased with water depth from 0.29 ± 0.00 cm at EB1 to 0.37 ± 0.07 cm at EB3 and 0.44 ± 0.01 cm at EB5. Likewise, NO$_3^-$ was depleted at 2 cm depth at EB1 and EB3 whereas 1–5 $\mu$M persisted to 13 cm at EB5, although there was no consistent gradient below 1 cm. Nitrate was depleted to $<1$ $\mu$M below 1 cm depth at EB1 and EB3, while concentrations >1 $\mu$M persisted to 6 cm depth at EB5. The OPD and nitrate/nitrite profiles thus indicated more oxidized conditions at the center of the basin (EB5). The NH$_4^+$ concentration increased steadily with depth, except for a slight decrease at 0–2 cm at EB3, and was lower at EB5 than at EB1 and EB3. Dissolved Mn$^{2+}$ was completely depleted at EB1. The Mn$^{2+}$ concentration at EB3 increased from 0 cm to 2 cm depth then gradually decreased. At EB5, Mn$^{2+}$ concentration increased down to 6 cm depth, then slightly decreased with depth. Extractable Mn$_{(DCA)}$ was enriched in the surface sediment at EB5 with maximum concentration of 148 $\mu$mol cm$^{-3}$ while a small enrichment of 10 $\mu$mol cm$^{-3}$ was seen at 0–1 cm at EB3 on the slope, and there was no enrichment at EB1 on the shelf.

**Relative contribution of anammox to $N_2$ production**

Potential rates of denitrification and anammox, as determined from the $^{15}$NO$_3^-$-amended anoxic slurry incubations, were typically similar across the three depth intervals analyzed at each site, with a slight tendency to higher rates in the upper 2 cm (Fig. 3). As nitrate distributions indicate that anammox and denitrification are largely restricted to 0–2 cm (Fig. 2), we focus on the rates from this interval. Denitrification and anammox rates in the slurries of the upper 2 cm, ranged from 0.5 nmol N cm$^{-3}$ h$^{-1}$ to 1.0 nmol N cm$^{-3}$ h$^{-1}$ and from 0.1 nmol N cm$^{-3}$ h$^{-1}$ to 0.8 nmol N cm$^{-3}$ h$^{-1}$, respectively (Fig. 4). The denitrification rate decreased with increasing water depth from 0.8 ± 0.1 nmol N cm$^{-3}$ h$^{-1}$ on average at the shelf sites to 0.5 ± 0.1 nmol N cm$^{-3}$ h$^{-1}$ at the basin sites, and hence was inversely correlated with
water depth \((r = -0.80, p < 0.001)\). Conversely, the anammox rates in the sediments increased from \(0.2 \pm 0.0 \text{ nmol N cm}^{-3} \text{ h}^{-1}\) on average in the shelf sites, to \(0.4 \pm 0.0 \text{ nmol N cm}^{-3} \text{ h}^{-1}\) in the slope sites, and \(0.7 \pm 0.1 \text{ nmol N cm}^{-3} \text{ h}^{-1}\) in the basin sites and showed a marked positive correlation \((r = 0.90, p < 0.001)\) with water depth (Fig. 4). The total potential \(\text{N}_2\) production rate, the sum of the rates of denitrification and anammox, ranged from \(1.0 \text{ nmol N cm}^{-3} \text{ h}^{-1}\) to \(1.3 \text{ nmol N cm}^{-3} \text{ h}^{-1}\), and did not show any significant relationship with water depth \((r = 0.13, p = 0.75)\).
The summary of characteristics for the bottom water and sediment from our study sites with various marine sediments are reported in Supporting Information Table S1. A correlation analysis between the relative contribution of anammox (ra) to total N₂ production and some of the measured variables from this study is presented in Table 2. Overall, the relative contribution of anammox (ra) to total N₂ production ranged from 15% to 59% (average 38% ± 18%) across all stations. The ra was positively correlated with water depth, increasing from 17% ± 3% at the shelf sites to 57% ± 5% at the basin sites (r = 0.971, p < 0.001). Similarly, the ra were also relatively good correlation with bottom water NO₂⁻ (r = 0.796, p = 0.010), C/N ratio (r = −0.774, p = 0.014), and total Mn content of sediment (r = 0.783, p = 0.013), but there was no overall simple relationship with water temperature (r = −0.625, p = 0.072) and organic content (r = 0.122, p = 0.754) (Table 2).

![Fig. 3. Potential rates of denitrification (black bar) and anammox (white bar) at three depth intervals determined from the anoxic slurry incubations with homogenized sediments. Number (%) represents the relative contribution of anammox to total N₂ production (ra).](image-url)
Potential for alternative pathways of anaerobic ammonium oxidation

In slurry incubations with $^{15}$NH$_4^+$ but no NO$_3^-$ added, a slight production of $^{15}$N-labeled N$_2$ was detected at rates of $0.005 \pm 0.002$ nmol cm$^{-3}$ d$^{-1}$ and $0.002 \pm 0.001$ nmol cm$^{-3}$ d$^{-1}$ at the slope (EA5) and basin (EB5) station, respectively, which corresponded to 2.1% and 1.5% of $^{15}$N-N$_2$ production measured with $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$ in parallel. No $^{15}$N-N$_2$ production was detected after addition of either ATU as inhibitor of aerobic ammonium oxidation or acetylene as inhibitor of aerobic ammonium oxidation and anammox. This indicates that N$_2$ production without added nitrate occurred through denitrification and/or anammox coupled to aerobic ammonium oxidation, which at the very low rates observed here could be supported by slight leakage of oxygen from the butyl rubber septa of the Exetainer vials (De Brabandere et al. 2012). Thus, there was no evidence of N$_2$ production coupled to, e.g., manganese oxide reduction. In further support of this conclusion, rates of anammox obtained with $^{15}$NH$_4^+$ were similar to those obtained in parallel with $^{14}$NH$_4^+$ + $^{15}$NO$_3^-$ (16% higher and 22% lower than at EA5 and EB5, respectively). We consider this variability to be within the experimental error and therefore there was no indication of the involvement of other pathways than anammox in aerobic ammonium oxidation. This aligns with other studies of $^{15}$NH$_4^+$ transformations in marine sediments (summarized in Thamdrup 2012).

Table 2. Statistical analysis of the relationship between the relative contribution of anammox ($ra$) to total N$_2$ production, and water depth, temperature, nitrate concentration of the bottom water, organic carbon content, C/N ratio, and total Mn oxide content. $R$ and $p$ indicate the correlation coefficient and the significant value, respectively. $n = 9$.

| $ra$ | Depth (m) | Temperature (°C) | NO$_3^-$ (μmol L$^{-1}$) | Organic C (% dry wt.) | C/N (mol : mol) | Total Mn(DCA) (μmol cm$^{-2}$) |
|------|-----------|-----------------|---------------------------|----------------------|----------------|-------------------------------|
| $R$  | 0.971**   | -0.625          | 0.796*                    | 0.122                | -0.774*        | 0.783*                        |
| $p$  | <0.001    | 0.072           | 0.010                     | 0.754                | 0.014          | 0.013                         |

* $p<0.05$; ** $p<0.001$.

**Potential for alternative pathways of anaerobic ammonium oxidation**

In slurry incubations with $^{15}$NH$_4^+$ but no NO$_3^-$ added, a slight production of $^{15}$N-labeled N$_2$ was detected at rates of $0.005 \pm 0.002$ nmol cm$^{-3}$ d$^{-1}$ and $0.002 \pm 0.001$ nmol cm$^{-3}$ d$^{-1}$ at the slope (EA5) and basin (EB5) station, respectively, which corresponded to 2.1% and 1.5% of $^{15}$N-N$_2$ production measured with $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$ in parallel. No $^{15}$N-N$_2$ production was detected after addition of either ATU as inhibitor of aerobic ammonium oxidation or acetylene as inhibitor of aerobic ammonium oxidation and anammox. This indicates that N$_2$ production without added nitrate occurred through denitrification and/or anammox coupled to aerobic ammonium oxidation, which at the very low rates observed here could be supported by slight leakage of oxygen from the butyl rubber septa of the Exetainer vials (De Brabandere et al. 2012). Thus, there was no evidence of N$_2$ production coupled to, e.g., manganese oxide reduction. In further support of this conclusion, rates of anammox obtained with $^{15}$NH$_4^+$ were similar to those obtained in parallel with $^{14}$NH$_4^+$ + $^{15}$NO$_3^-$ (16% higher and 22% lower than at EA5 and EB5, respectively). We consider this variability to be within the experimental error and therefore there was no indication of the involvement of other pathways than anammox in aerobic ammonium oxidation. This aligns with other studies of $^{15}$NH$_4^+$ transformations in marine sediments (summarized in Thamdrup 2012).

**Total N$_2$ production, denitrification, and anammox**

The total N$_2$ production rates in intact sediment cores ranged from 5.5 μmol N m$^{-2}$ h$^{-1}$ to 9.7 μmol N m$^{-2}$ h$^{-1}$ and tended to decrease with increasing water depth, although EB5 and EB6 in the basin had relatively high N$_2$ production rates (8.4 ± 0.1 μmol N m$^{-2}$ h$^{-1}$). The average N$_2$ production rate was 9.2 ± 0.6 μmol N m$^{-2}$ h$^{-1}$ at the shelf sites, 6.9 ± 1.5 μmol N m$^{-2}$ h$^{-1}$ at the slope sites, and 7.4 ± 1.7 μmol N m$^{-2}$ h$^{-1}$ at the basin sites.

The partitioning of N$_2$ production to denitrification and anammox was estimated from the $ra$ values from the slurry incubations of the respective cruise and the total N$_2$ production rate (Supporting Information Table S1). As nitrate distributions indicate that anammox and denitrification are largely restricted to 0–2 cm, $ra$ values for this interval were used (Fig. 4). The denitrification rates varied from 2.7 μmol N m$^{-2}$ h$^{-1}$ to 8.3 μmol N m$^{-2}$ h$^{-1}$ and were higher at the shelf sites (7.7 ± 0.6, $n = 3$) than at the slope (4.3 ± 1.7, S417).
n = 3) and basin (3.2 ± 0.4, n = 3) sites (Supporting Information Table S1). Consequently, denitrification showed a significant negative correlation with depth (r = −0.96, p < 0.001) (Fig. 5B). In contrast to the denitrification, anammox rates increased from the shelf sites (1.6 ± 0.3 μmol N m⁻² h⁻¹) to the basin sites (4.2 ± 1.2 μmol N m⁻² h⁻¹). Sta. EB5 and EB6 in the basin exhibited highest rates of anammox (5.0 ± 0.1 μmol N m⁻² h⁻¹, Fig. 5B) which was responsible for the high total N₂ production rates there (Fig. 5A).

Discussion

Spatial variations of N₂ production along the continental margins

Although there have been several indirect estimations of denitrification in deep-sea sediments (e.g., Middelburg et al. 1996a,b; Hartnett and Devol 2003; Brunnegård et al. 2004; Engström et al. 2009), actual measurements of the N₂ production rate in sediments deeper than 2000 m are scarce (Engström et al. 2009; Trimmer and Nicholls 2009). In the present study, we investigated the total N₂ production rates and relative contribution of anammox and denitrification in sediments from water depths from <100 m to >2300 m, which is, to our knowledge, the first comprehensive report of N₂ production in the deep-sea sediments of the East Asian marginal seas using intact cores to cover such a depth range.

While fluxes were measured in intact cores, thus maintaining the chemical gradients and microbial zonation in the sediment, the separation of denitrification and anammox relied on slurry incubations for determining the relative contribution of anammox to N₂ production (na). An alternative procedure comparing isotope pairing in N₂O and N₂ minimizes the disturbance (Trimmer et al. 2006). A production of N₂O was, however, not detectable with our analytical setup. In a compilation of data from various marine sediments with na values ranging from near zero to ~ 80%, Trimmer et al. (2013) observed a good correlation between na determined in slurries and whole cores, with a slope of 1.06 for na whole core vs. na slurry. Increasing our na values (used in Eq. 2) by this factor would result in an average decrease in the total N₂ production rates of 10% ± 3%, and in relative changes in the anammox rates ranging from 125% at the slope sites (EA4, EA6, EB1) to 25% at the basin sites (EB5, EB6, EC1) (data not shown). Thus, a potential correction of the rates of this magnitude would not affect the overall conclusions of our study substantially.

The total N₂ production rates (anammox plus denitrification) ranged between 5.5 μmol N m⁻² h⁻¹ and 9.7 μmol N m⁻² h⁻¹, and the rates for the shelf (mean 9.2 ± 0.6 μmol N m⁻² h⁻¹) and slope sites (mean 6.9 ± 1.6 μmol N m⁻² h⁻¹) were comparable to those reported at various coastal locations (Supporting Information Table S1), while the N₂ production rates in the center of the UB (mean 8.4 ± 0.2 μmol N m⁻² h⁻¹) were relatively high, supported by an enhanced anammox contribution (Fig. 6). These values are considerably higher than the fixed-N removal measured at sites along a depth transect, from 2000 m to 50 m on the continental slope in the North Atlantic (0.35–6.9 μmol N m⁻² h⁻¹; Trimmer and Nicholls 2009) and comparable to the N₂ production rates at the Washington margin (5.5 ± 1.6 μmol N m⁻² h⁻¹ at a depth range of 2740–3110 m) (Supporting Information Table S1) (Engström et al. 2009; estimated from pore-water profiles). Although different methods were applied in the three studies, the variation in N₂ production rates between them is broadly consistent with differences in

Fig. 5. Rates of total N₂ production (A) and denitrification, and anammox (B) from intact sediment cores as a function of water depth at all stations. Data are mean values for all stations. Error bars represent SE (n = 3).
organic carbon contents of the sediment. The North Atlantic sediments have much lower (0.04% dry wt.) organic carbon contents than the UB (2.5% ± 0.0% dry wt.) and the Washington margin (1.25% dry wt.) (Hartnett and Devol 2003) with these differences expectedly mirroring differences in productivity in the photic zone (Wenzhöfer and Glud 2002). Still, the spatial coverage of the three studies within each region is low, and more measurements are needed for robust inter-region comparisons of benthic N loss. Although some studies have shown a covariation of denitrification and anammox rates (e.g., Brin et al. 2014), in our and other previous studies (e.g., Engström et al. 2005; Trimmer et al. 2013) the two components of N₂ production vary independently, with the former decreasing and the latter increasing slightly with water depth. Thus, a detailed understanding of the controls of benthic N₂ production requires identification of the environmental controls for each of the two processes, and other factors than organic matter availability appear to play a role.

Denitrification rates decreased markedly with depth across the margin; from the shelf (7.6 ± 0.6 μmol N m⁻² h⁻¹), the slope (4.3 ± 1.7 μmol N m⁻² h⁻¹) to the basin (3.2 ± 0.4 μmol N m⁻² h⁻¹). The decrease was proportional to the decrease in total oxygen consumption rates observed during the same cruise from shelf (354 μmol m⁻² h⁻¹ at EB1) to basin (150 μmol m⁻² h⁻¹ at EB5) (S.-H. Kim unpubl.; Supporting Information Table S2), and in general agreement with the increasing OPD (Fig. 2). The proportionality of denitrification to oxygen uptake is consistent with previous observations (Trimmer and Engström 2011) and indicates that denitrification was to a large amount regulated by the availability of organic carbon, as is the case for benthic

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**Fig. 6.** Denitrification rates (A), anammox rates (B), total N₂ production rates (C), and relative contribution of anammox to the N₂ production rate (ra) (D) in sediments plotted as a function of water depth (adopted from Thamdrup 2012). Different types of sediment are marked with different symbols. Indicated are the results by Risgaard-Petersen et al. (2004, open triangle), Dong et al. (2009, pink triangle), Trimmer et al. (2006, sky triangle), Hietanen and Kuparinen (2008, tree diamond), Trimmer et al. (2013, dark green diamond), Risgaard-Petersen et al. (2004, gray diamond), Glud et al. (2009, open diamond), Trimmer and Nicholls (2009, yellow circle), Engström et al. (2009, green circle), this study (blue circle).
oxygen uptake under well-oxygenated bottom waters (Glud 2008). The fact that the potential denitrification rates (Fig. 4) decreased similarly with depth as the whole-core rates, further corroborates that the latter are controlled by electron donor availability. In addition to organic carbon, such donors may include ferrous iron and sulfide as products anaerobic carbon oxidation. Hyun et al. (2010) found that depth-integrated concentrations of soluble Fe(II) were 2.5 times lower at a basin site than at a slope site in the East Sea and that pore-water sulfide was only detectable at the slope site (0.031 mmol m$^{-2}$). In carbon equivalents, denitrification corresponded to only 1–2% of benthic carbon oxidation as estimated from the oxygen uptake (Supporting Information Table S2). The drop in respiration rates of ~60% from the shelf to the basin at >2000 m is small relative to the >90% attenuation of the organic carbon sinking flux between 100 m and 2000 m depth predicted by the Martin curve (Martin et al. 1987). This emphasizes the hotspot-character of UB sediments (Hyun et al. 2010) and suggests that reactive carbon reaches the basin through down-slope transport in addition to vertical sinking, as also indicated for other continental margins (Jahnke et al. 1990; Kim et al. 2017).

In contrast to denitrification, anammox rates remained relatively constant with a slight tendency to increase with depth (1.6–2.8 μmol N m$^{-2}$ h$^{-1}$) and with particularly high rates at the center of the basin (4.9–5.0 μmol N m$^{-2}$ h$^{-1}$) at EB5 and EB6, Fig. 6A,B). Indeed, the latter rates are the some of the highest so far reported from sediments at a similar depth range (Supporting Information Table S1; Fig. 6B). Higher rates were only reported from in Sagami Bay, which was previously recognized as an outlier in a compilation of N$_2$ production rates vs. depth (Trimmer and Engström 2011), likely due to its coastal location and possible anthropogenic influence entering via the adjacent Tokyo Bay.

The factors that regulate anammox in marine sediments are not well understood, but the availability of ammonium and nitrite as substrates, and of hydrogen sulfide as a potential inhibitor have been suggested as important controls (Thamdrup 2012). Ammonium concentrations were relatively high and showed no large differences between the sites (Fig. 2; elevated NH$_4^+$ concentrations in the upper 1–2 cm may indicate release from fauna during centrifugation, e.g., Aller 1998). By contrast, nitrite was present at higher concentrations, >1 μM, at EB5 than EB1 and EB3 (Fig. 2). A half-saturation nitrite concentration of ≤3 μM has been estimated for anammox in marine and estuarine sediments (Dalsgaard and Thamdrup 2002; Trimmer et al. 2003). Our results thus suggest that less intense nitrite limitation contributed to the higher rates in the basin relative to the slope and shelf, thus indicating that nitrite is a controlling factor for anammox in intact sediment cores. The strong depthwise increase in potential anammox rates, measured with addition of both nitrite and ammonium (Fig. 4), which, in relative terms, was even stronger than for the whole-core rates, further indicates that the deeper sites support larger populations of anammox bacteria. This assumes that the potential rates reflect the abundance of enzymes conducting the rate-limiting step, and that the enzymatic inventory per anammox cell does not vary substantially between the sites. An effect of hydrogen sulfide is also possible, as sulfate reduction rates at the shallow sites were always higher than those at the center of the basin in the UB (Lee et al. 2008; Hyun et al. 2010).

**Significance of ra**

In the UB, ra ranged from 13% on the shelf to 55% in the basin, and was significantly correlated with water depth ($r = 0.97$, $p < 0.001$, Table 2, Fig. 4). This trend was in close agreement with the earlier studies reported from various marine sediments (Fig. 6D, reviewed in Thamdrup 2012). The highest contribution of ra (57%) observed at the center of the UB (sites EB5 and EB6) is comparable to that reported in other deep-sea marine sediments, such as Washington margin (40%) and the North Atlantic (65%) (Engström et al. 2009; Trimmer and Nicholls 2009). The results suggest that over the range studied here, the water depth may be a good indicator for the contribution of anammox to the N$_2$ production.

As argued above, the increase in ra with depth was likely influenced by the availability of organic matter (limiting denitrification) and of nitrite (stimulating anammox). Nitrite availability for anammox may, in turn, also be controlled by organic matter through the competition from denitrification, as the nitrate and nitrite reduction steps of this process seem to be more closely coupled in more relative to less organic-rich sediments (Trimmer et al. 2005; Trimmer and Engström 2011), likely due to its coastal location and possible anthropogenic influence entering via the adjacent Tokyo Bay.

The C/N ratio of the organic matter was found to influence ra in oxygen minimum zone waters (Babbin et al. 2014). In the UB, the C/N ratio was more strongly correlated with the ra ($r = -0.77$, $p < 0.05$) than was the organic carbon content ($r = 0.12$, $p = 0.75$) (Table 2). However, the difference in bulk C/N ratio between the basin and shelf (7.2 vs. 7.8) was much too small to explain the difference in ra according to the model of Babbin et al. (2014), and although the C/N signal from the reactive fraction of the organic matter could be swamped by a large unreactive pool, it does not seem likely that the reactive organic matter would have a much lower C/N ratio in the basin than on the shelf, as the C/N ratio of mineralization generally increases with water depth (e.g., Babbin et al. 2014). Furthermore, the observed relationship
may not be a general phenomenon across marine sediments because the C/N ratio in the North Atlantic tended to be high when the contribution of anammox to N₂ production was the highest (Trimmer and Nicholls 2009).

In addition to the availability and quality of organic matter, the balance between denitrification and anammox may be affected by the unusually high Mn oxide content in the center of the UB. Recently, Hyun et al. (2017) reported that the high Mn oxide content in the UB surface sediment results from a combination of highly efficient recycling through reoxidation, with very low permanent burial of authigenic Mn(II) phases, and the UB topography, which may ensure that any Mn²⁺ escaping to the overlying water returns to the basin sediment after reprecipitation. Similar mechanisms likely explain the geochemical focusing of Mn in other basins in, e.g., the Gulf of St. Lawrence and Skagerrak (Sundby and Silverberg 1985; Canfield et al. 1993). As discussed previously for the Skagerrak (Thamdrup and Dalsgaard 2002; Trimmer et al. 2013), competition for organic substrates between denitrifying and manganese-reducing bacteria may limit denitrification in Mn-rich sediments. Indeed, dissimilatory Mn reduction is known to dominate carbon mineralization in the basin (Vandieken et al. 2012; Hyun et al. 2017). Thus, we propose that the elevated nitrite levels in the basin sediment resulted from partial suppression of nitrite reduction, the second step of the denitrification pathway, due to competition from Mn reduction. The accumulation of nitrite due to an uncoupling of nitrate and nitrite reduction is well known from pelagic oxygen minimum zones where ra may approach 1 (e.g., Thamdrup et al. 2006; Lam et al. 2009). The inhibition of nitrite reduction in UB sediment was not strong enough to clearly affect the contribution of denitrification to carbon oxidation (Supporting Information Table S2) and not as strong as observed in the Mn-rich sediment from Skagerrak, where ra reaches ~ 75% and denitrification was suppressed to <1% of carbon oxidation (Trimmer et al. 2013). While the content of Mn oxides in Skagerrak and UB sediments are similar, their susceptibility to microbial reduction might differ as the turn-over time of the oxide pool is an order of magnitude slower in UB compared to Skagerrak (Canfield et al. 2013; Hyun et al. 2017).

In the Mn-rich basin sediment, N₂ could potentially also be produced by the oxidation of ammonium to N₂ coupled to Mn reduction (Luther et al. 1996). We observed slight production of ¹⁵N-N₂ from ¹⁵NH₄⁺ in anoxic slurry incubations without added nitrate, but the production rate was only ~ 2% of the rate obtained with ¹⁵NH₄⁺ in the presence of unlabeled nitrate. Moreover, N₂ production without added nitrate was independent of Mn oxide levels as rates were similar at the Mn-rich basin site and the slope site, with almost 100-fold lower Mn content. Instead, the fact that N₂ production was inhibited by both ATU and acetylene, inhibitors targeting the O₂-dependent ammonium monooxygenase of aerobic ammonium oxidizers (Bédard and Knowles 1989; Gilch et al. 2009), suggests that it could be the result of aerobic nitrification coupled to denitrification, which might result from slight oxygen leakage during the incubations (De Brabandere et al. 2012). We thus conclude that Mn oxide-dependent ammonium oxidation did not contribute substantially to N₂ production in the UB sediments, which is consistent with results from ¹⁵NH₄⁺ incubations at other Mn-rich sites (Thamdrup and Dalsgaard 2000; Engström et al. 2005; Crowe et al. 2012).

In summary, our results provide additional evidence for the significance of anammox as a source of N₂ in the sediment of the deep sea (> 2000 m) of similar importance as denitrification. The results indicate that, in addition to the decrease in denitrification rates caused by lower availability of organic substrates at greater depth, Mn oxide enrichments in the basin sediments may enhance the contribution of anammox to N₂ production by suppressing denitrification and thereby increasing the availability of nitrite for anammox. Mn oxide concentrations were not determined in previous studies of anammox in the deep sea (Engström et al. 2009; Trimmer and Nicholls 2009), and it remains to be determined to which extent this or other factors govern ra in deep-sea sediments on a global scale.

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Conflict of Interest

None declared.

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