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Enhanced benthic nitrous oxide and ammonium production after natural oxygenation of long-term anoxic sediments

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Abstract
Coastal and shelf sediments are central in the global nitrogen (N) cycle as important sites for the removal of fixed N. However, this ecosystem service can be hampered by ongoing deoxygenation in many coastal areas. Natural reoxygenation could reindate anoxic sediments as sites where fixed N is removed efficiently. To investigate this further, we studied benthic N cycling in previously long-term anoxic sediments, following a large intrusion of oxygenated water to the Baltic Sea. During three campaigns in 2016–2018, we measured in situ sediment-water fluxes of ammonium (NH$_4^+$), nitrate (NO$_3^-$), oxygen (O$_2$), dissolved inorganic carbon, and NO$_3^-$ reduction processes using benthic chamber landers. Sediment microprofiles of O$_2$, nitrous oxide (N$_2$O), and hydrogen sulfide were measured in sediment cores. At a permanently oxic station, denitrification to N$_2$ was the main NO$_3^-$ reduction process. Benthic N$_2$O production appeared to be linked to nitrification, although net N$_2$O fluxes from the sediment were detected. At newly oxygenated sites, dissimilatory NO$_3^-$ reduction to NH$_4^+$ comprised almost half of the total NO$_3^-$ reduction. At these stations, the removal of fixed N was inefficient due to high effluxes of NH$_4^+$. Sedimentary N$_2$O production was associated with incomplete denitrification, accounting for 41–88% of the total denitrification rate. Microprofiling revealed algae aggregates as potential hotspots of seafloor N$_2$O production. Our results show that transient oxygenation of euxinic systems initiates benthic NO$_3^-$ reduction, but may not lead to efficient sedimentary removal of fixed N. Instead, recycling of N compounds is promoted, which may accelerate the return to anoxia.

The doubling of fixed nitrogen (N) inputs to the ocean via rivers and the atmosphere since pre-industrial times (Seitzinger et al. 2010; Beusen et al. 2016; Jickells et al. 2017) has resulted in a strong research focus on the processes affecting the fate of fixed N in the marine environment (Seitzinger et al. 2006; Galloway et al. 2008; Voss et al. 2013). In this respect, coastal sediments play an important role as they receive large amounts of N from land and are sites of intense N cycling. It is estimated that at least 25% of the global marine fixed N loss takes place in coastal and shelf sediments (Voss et al. 2013), even though they comprise only ~8% of the global seafloor area (Sarmiento and Gruber 2006). However, coastal zones globally are under pressure from the spread of oxygen (O$_2$) depletion (Diaz and Rosenberg 2008; Altieri and Gedan 2015; Breitburg et al. 2018). Oxygen exerts a strong control on the N cycle by activating or suppressing many of the microbial processes involved in N transformation (Canfield et al. 2005; Thamdrup and Dalsgaard 2008; Kuypers et al. 2018). A key question is thus how changing O$_2$ conditions in coastal areas will affect the rates of and partitioning between processes in the N cycle, and ultimately the fate of fixed N in the ocean.

Under oxic conditions, ammonium (NH$_4^+$) produced during degradation of organic matter is oxidized to nitrite (NO$_2^-$) and nitrate (NO$_3^-$) by nitrifying bacteria. Any NO$_2^-$ and NO$_3^-$ (here collectively called NO$_x^-$) entering an anoxic environment can...
be used as an electron acceptor in one of three NO\textsubscript{3} reduction processes: denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonia (DNRA). Of these respiratory processes, denitrification and anammox constitute sinks for fixed N by reducing NO\textsubscript{3} to gaseous N\textsubscript{2}, while during DNRA, NO\textsubscript{3} is instead converted back to readily bioavailable NH\textsubscript{4}, thereby keeping fixed N in the system (Canfield et al. 2005; Giblin et al. 2013).

Although these NO\textsubscript{3} reduction processes take place in anoxic environments, they are indirectly dependent on O\textsubscript{2} for the supply of NO\textsubscript{3}. In long-term anoxic systems where NO\textsubscript{3} is depleted, oxygenation events can promote removal of fixed N by activating nitrification which supplies NO\textsubscript{3} for denitrification and anammox (De Brabandere et al. 2015). Denitrification is commonly the major N removal process in coastal sediments due to high availability of organic matter in coastal systems, favoring heterotrophic denitrification over autotrophic anammox as N\textsubscript{2} production process (Devol 2015). However, DNRA appears to be important in highly reduced sediment, especially where the NO\textsubscript{3} concentration is low relative to the electron donor (Kraft et al. 2014; Hardison et al. 2015; Kessler et al. 2018). Furthermore, hydrogen sulfide (H\textsubscript{2}S), which is often prevalent in sediments of previously long-term anoxic coastal systems, may inhibit denitrification and anammox, thus slowing down these processes or leading to accumulation of intermediates such as the strong greenhouse gas nitrous oxide (N\textsubscript{2}O) in the case of denitrification (Sørensen et al. 1980; Senga et al. 2006; Jensen et al. 2008). Some organisms that carry out DNRA can couple the process to H\textsubscript{2}S oxidation (Fossing et al. 1995) or instead reduce sulfate (Dalsgaard and Bak 1994), however, and so are more tolerant to (or even dependent upon) H\textsubscript{2}S.

Recently, benthic NO\textsubscript{3} reduction processes were studied during forced oxygenation of the long-term anoxic By Fjord in Sweden (De Brabandere et al. 2015). DNRA was initially the dominant NO\textsubscript{3} reduction process, but denitrification became increasingly important with time and eventually became the main NO\textsubscript{3} reduction process. Although insightful, the study does not necessarily describe natural conditions since the system was kept artificially oxygenated continually for 2 yr, substantially longer than natural oxygenation events last in similar systems (by fjord, De Brabandere et al. 2015; Baltic Sea, Meier et al. 2018; Saanich Inlet, Michiels et al. 2019). However, a natural oxygenation event in the long-term anoxic Baltic Sea recently offered a unique chance to study the onset of NO\textsubscript{3} reduction processes. After 10 yr of O\textsubscript{2} depletion, a so-called Major Baltic Inflow (MBI) brought oxygenated water to the long-term anoxic bottom waters of the central Baltic Sea. Three months after the MBI reached the Eastern Gotland Basin in the central Baltic Sea, Hall et al. (2017) observed an increase in the bottom water NO\textsubscript{3} concentration from <0.5 to ~10 \mu M. During these initial samplings, measurements of NO\textsubscript{3} reduction process rates showed a dominance of DNRA over denitrification.

In this study, we describe the long-term impact of this natural oxygenation event on the fate of fixed N in the sediment. Our goals were to investigate (1) whether the newly oxygenated sediments were sites of efficient removal of fixed N through denitrification and anammox, (2) which environmental factors affected the division between NO\textsubscript{3} reduction processes, and (3) whether NO\textsubscript{3} from the water column or sedimentary nitrification was fuelling NO\textsubscript{3} reduction processes in the sediment. To this end, we conducted three yearly samplings in 2016, 2017, and 2018 where we performed in situ measurements of NO\textsubscript{3} reduction process rates and sediment–water fluxes of nutrients, O\textsubscript{2}, and dissolved inorganic carbon (DIC). We additionally collected sediment samples and measured sediment microprofiles of O\textsubscript{2}, N\textsubscript{2}O, and H\textsubscript{2}S to gain further insights into the spatial distribution of the processes in surface sediments.

**Material and methods**

**Study site**

The Baltic Sea is a semi-enclosed brackish water sea in Northern Europe (Fig. 1a). The central and southern parts of the Baltic Sea are naturally prone to hypoxia due to slow water exchange and strong water column stratification (Liblik et al. 2018). The situation has been worsened by decades of anthropogenic nutrient release from land, causing eutrophication which in turn has led to extensive O\textsubscript{2} depletion (Carstensen et al. 2014). Every few years, the deep water in the Baltic Sea is ventilated by large inflows, MBIs. After a stagnation period of 10 yr, the third largest MBI in 60 yr took place in December 2014 and brought O\textsubscript{2} to long-term anoxic parts of the Baltic Sea (Liblik et al. 2018).

The MBI reached the Eastern Gotland Basin in the central Baltic Sea in March 2015 (Fig. 1b; Liblik et al. 2018). The water column in the Eastern Gotland Basin is normally O\textsubscript{2} depleted below the halocline at 60–80 m depth, but the inflow created a layer of oxygenated water between 140 m depth and the seafloor (maximum depth 249 m; Hall et al. 2017). This O\textsubscript{2} was consumed within 6 months, but another inflow in January 2016 oxygenated the area again until May (Liblik et al. 2018). In 2014–2017, several small inflows also brought O\textsubscript{2} to the layer between the halocline and 170 m depth (Fig. 1b; SMHI 2021).

We conducted yearly samplings at four stations in the Eastern Gotland Basin in April 2016–2018, along a depth transect from the island of Gotland toward the deepest part of the basin (Fig. 1a). Sta. A is situated at 60 m depth and is permanentlyoxic. Stas. D, E, and F were oxygenated at different times during the sampling campaign (Fig. 1b, Table 1). Stas. E (170 m) and F (210 m) were oxygenated by the MBI and following inflows. These stations became anoxic again in 2018 and 2017, respectively. Sta. D (130 m) was anoxic during our sampling in 2016, but had likely been oxygenated briefly prior to our visit (Sommerset al. 2017; SMHI 2021). The site was reached by anoxic inflow in 2017, but was anoxic again in 2018.
Sediment properties and microsensor measurements

Sediment cores for analysis of solid-phase carbon (C) and N were collected with a multiple corer (inner Ø 9.9 cm). The cores were vertically sectioned in 0.5, 1, or 2 cm intervals down to a sediment depth of 20 cm, with highest resolution at the top. The samples were freeze dried and homogenized using mortar and pestle prior to analysis. Due to technical issues, there are no solid phase samples from 2018. Sediment cores for pore-water sampling were processed under anoxic conditions in an N₂-filled glove bag. Sediment cores were sectioned in the same intervals as described above, before extraction of pore water using Rhizon samplers (Rhizon SMS, Rhizosphere). Samples were stored dark and cold until analysis.

Sediment depth microprofiles of O₂, H₂S, and N₂O (only 2017 and 2018) were measured in cores (inner Ø 5 cm) collected from a modified box corer (Blomqvist et al. 2015) and transferred to a 10 L aquarium where they were kept at in situ temperature and O₂ concentration. Within a few hours of sediment retrieval, microprofiles were measured as described by Marzocchi et al. (2018a), using microsensors (Revsbech 1989; Kühl et al. 1998; Andersen et al. 2001) constructed at Aarhus University. At each station, 3–5 microprofiles were measured in each replicate core (n = 2–3 for Stas. A, D, and F, and n = 1 for Sta. E). Due to sensor breakage, no N₂O profiles were measured at Stas. F and A in 2017 or Sta. D in 2018. In 2017, O₂ and N₂O were also measured in three aggregates of algal material deposited on the sediment surface of cores from Sta. E.

Lander incubations

Benthic solute fluxes were measured in situ using the Gothenburg benthic chamber landers. Deployment of the landers

Table 1. Station information, bottom water properties and solid phase data. NOₓ red. meas.: C = cores, S = slurry, L = benthic chamber lander. Core and slurry incubations were only used to correct rates measured with the benthic chamber lander. Values of sedimentary OC and N are averages for the top 2 cm; there are no samples from 2018.

| Stations | Year | NOₓ red. meas. | Salinity | Temp (°C) | O₂ (µM) | NOₓ (µM) | OC (%) | N (%) | OC : N (mol:mol) |
|----------|------|----------------|----------|----------|---------|----------|--------|-------|-----------------|
| A        | 2016 | 7.3–7.4       | 3.6–3.9  | 330–350  | 2.9–3.3 | 0.9      | 0.1    | 10.5             |
|          | 2017 | C, L, S       | 7.7–8.9  | 4.0–5.0  | 100–300 | 3.3–8.6  | 1.2    | 0.2   | 7.0             |
|          | 2018 | L             | 7.4–7.5  | 2.6–3.0  | 352–355 | 2.9–3.3  |        |       |                 |
| D        | 2016 | C, L          | 12.5–12.7| 6.5–6.6  | < 0.5   | 0.2–0.8  | 7.9    | 0.9   | 10.2            |
|          | 2017 | L, S          | 12.7     | 6.7–6.8  | 8–13    | 7.3–8.0  | 10.5   | 1.3   | 9.4             |
|          | 2018 | L             | 12.4–12.6| 6.6–6.9  | < 0.5   | 2.6–2.7  |        |       |                 |
| E        | 2016 | C, L          | 12.9–13.1| 6.8–6.9  | 7–20    | 8.3–11.9 | 12.1   | 1.3   | 10.9            |
|          | 2017 | L             | 13.1     | 6.9–7.2  | 3–13    | 2.0–6.3  | 10.1   | 1.2   | 9.8             |
|          | 2018 |              | 13.1     | 6.8–7.1  | < 0.5   | 0.03–0.19|        |       |                 |
| F        | 2016 | C, L          | 13.8–13.9| 7.3–7.4  | 22–30   | 9.7–11.0 | 12.1   | 1.3   | 10.9            |
|          | 2017 |               | 13.5     | 7.2–7.4  | < 0.5   | 0.03–0.07| 11.7   | 1.4   | 9.7             |
|          | 2018 |               | 13.1–13.3| 6.9–7.15 | < 0.5   | 0.09–0.18|        |       |                 |
has been described in detail elsewhere (Kononets et al. 2021). Briefly, the big and small landers were equipped with four and two incubation chamber modules, respectively. Sediment and bottom waters were incubated for 36 h at Sta. A and 14 h at Stas. D–F. The conditions inside and outside the chambers were monitored with O2 optodes (Model 3830; Aanderaa Data Instruments) and conductivity and temperature sensors (Model 3919; Aanderaa Data Instruments). Chambers that initially contained O2 did not become anoxic during the incubations. Nine water samples per chamber were collected with syringes at preset times; water from the syringes was processed immediately after lander recovery. Nutrient samples were filtered through pre-cleaned syringe filters (cellulose acetate, 0.45 μm pore size), and were thereafter kept refrigerated until analysis immediately after the cruises. Samples for DIC were analyzed onboard.

In half of the chambers, denitrification and DNRA were measured following the protocol of De Brabandere et al. (2015) with minor modifications. A sample was withdrawn 10 min after the start of the incubation. After 10 more minutes, a solution of Na15NO3 (99.4 atom%6, Sigma-Aldrich) in Milli-Q water was injected into the chambers, increasing the in situ concentration of NO3 by ~70 μM. The tracer was mixed with the chamber water for 10 min, after which a second water sample was withdrawn for the calculation of the 15NO3 enrichment. The remaining seven water samples were withdrawn at regular intervals. Samples for analysis of the isotopic compositions of N2 and N2O were collected in 12 mL Exetainer (Labco Limited) to which 200 μL of 7 M ZnCl2 was added. Another water sample for nutrient and 15NH4 analysis was filtered (cellulose acetate, 0.45 μm pore size) and frozen. The water and upper 7 cm sediment were then homogenized with a glass rod. A sample for analysis of the isotopic composition of N2 and N2O was transferred from the homogenized core to 12 mL Exetainer to which 200 μL of 7 M ZnCl2 were added. One more sample was extracted with Rhizon samplers (Rhizon SMS, Rhizosphere) and immediately frozen for nutrient and 15NH4 analysis.

To estimate the relative contribution of anammox to the N2 production, slurry incubations (Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2003) were conducted at Stas. A and D in 2017. Anammox was assumed to not be active at Stas. E and F, since it was not detected at these sites by Hall et al. (2017) in 2015. Sediment for the slurry incubations was collected with a multiple corer (inner Ø 9.9 cm). The top 1.5 cm from two sediment cores were homogenized and 100 mL of sediment were mixed with 900 mL of filtered (cellulose acetate, 0.45 μm pore size), anoxic bottom water in a glass bottle. The slurry was subsequently bubbled with N2 for 10 min to remove any O2 contamination. The slurry was transferred to 12 mL Exetainers (n = 35), each containing a 4-mm glass bead. The samples were left to preincubate on a rotating wheel for about 10 h to remove any residual O2 and NO3. The slurries were then treated with 150 μL 9 mM deoxygenated solutions of either 15NO3 (n = 15) or 15NH4 + 14NO3 (n = 15), injected through the vial septa, or were left without additions to serve as controls (n = 5). The slurries were incubated on the rotating wheel for 8 h. Directly after addition of the tracers and every 2 h during the incubation, 200 μL of 7 M ZnCl2 were injected to three Exetainers from each treatment and one control to stop the incubation. All operations were conducted at in situ temperature.

Onboard core and slurry incubations

Onboard sediment core incubations were conducted to measure the partitioning of denitrification and DNRA products between water and sediment (De Brabandere et al. 2015). Sediment cores (inner Ø 4.6 cm, n = 15) were collected as described for the microsensor measurements. The sediment cores were transferred to a cold room kept at in situ temperature and were placed uncapped in a 25-liter incubation tank filled with bottom water. The water in the tank was purged with a mixture of N2 and air to maintain in situ O2 concentrations. Externally driven magnetic stirrers were positioned halfway down the water column of each core, gently stirring the water.

Triplicate water samples from the tank were filtered through pre-cleaned syringe filters (cellulose acetate, 0.45 μm pore size) and frozen for later analysis of nutrients (NH4 and NO3). A 200 mM 15NO3 solution was added to the water to increase the in situ concentration of NO3 by ~70 μM, after which three more water samples were collected for nutrient analysis. The cores were left in the tank for 1.5–3.5 h to allow for the added 15NO3 to mix with the endogenous NO3 and to establish a linear production of 15N–N2 (Robertson et al. 2019). After this preincubation, the cores were capped with rubber stoppers and were incubated for 12–16 h. The O2 concentration was measured in each core with an O2 sensor spot (OXSP5, Pyro Science) just before capping and after uncapping to ensure that the concentration did not decrease below 30% of the initial value (Dalsgaard et al. 2000). At each time point (directly after capping the cores and every 3–4 h during the incubation) samples were taken from three cores. From each core, a water sample for analysis of the isotopic composition of N2 and N2O was transferred to 12 mL Exetainer (Labco Limited) to which 100 μL of 7 M ZnCl2 was added. Another water sample for nutrient and 15NH4 analysis was filtered (cellulose acetate, 0.45 μm pore size) and frozen.

Chemical analyses

Solid phase C and N samples were analyzed on an elemental analyzer (Carlo ERBA N1500g, precision ±1%). Samples for organic C (OC) were treated with acid fumes (HCl, 37%) to remove carbonate solids before analysis.

Concentrations of NH4 and NO3 from the lander chambers were determined by segmented flow colorimetric analysis (Alpkem Flow Solution IV, OI Analytical), using standard
procedures based on those described by Koroleff (1983). The analytical uncertainties were 0.036 μmol N L⁻¹ for low (0.036–1.43 μmol N L⁻¹) and 2.4% for high (>1.43 μmol N L⁻¹) concentrations of NH₄⁻ and 0.021 μmol N L⁻¹ for low (0.014–1.43 μmol N L⁻¹) and 2.5% for high (>1.43 μmol N L⁻¹) concentrations of NO₃⁻. DIC concentrations were determined on the ship by infrared detection of CO₂ (LI-6262 CO₂ analyzer, LI-COR) after acidification with phosphoric acid as described by Nilsson et al. (2021). The analytical uncertainty for DIC was 0.2%.

The isotopic compositions of N₂, N₂O, and NH₄⁺ were analyzed by coupled gas chromatography–isotope ratio mass spectrometry (GC-IRMS) following the procedure described in De Brabandere et al. (2015). A 2 mL helium (He) headspace was created in the Exetainers, which were then left upside down for 1 day in order for the gases to equilibrate between water and headspace. Subsamples of the headspace were injected to a custom-made GC extraction line coupled to an IRMS (Thermo Delta V Plus). Both N₂ and N₂O were analyzed in the same sample injection whereby N₂ and N₂O were separated in the GC column and any N₂O was reduced to N₂ in a combustion oven (600°C), thus resulting in two temporally separated N₂ peaks. Samples for measurement of ¹⁵NH₄⁺ were treated with alkaline hypobromite to oxidize NH₄⁺ to N₂ (Warembourg 1993) and were then analyzed as described above.

Calculations and statistics
Sediment–water fluxes were calculated as the change in concentration per unit sediment surface area and time in each chamber following Hylén et al. (2021). Fluxes of O₂ were calculated from linear fits of the chamber O₂ concentration over time. The range of O₂ data used in the calculations was selected as the number of data points during a minimum of 30 min in the initial portion of an incubation that gave the highest r² value (typically better than 0.99). For calculation of fluxes from discrete water samples, the concentrations were first corrected for the small dilution of chamber water by ambient hypobromite to oxidize NH₄⁺ to N₂ (Warembourg 1993) and were then analyzed as described above.

Production rates of ¹⁵N-labeled N₂, N₂O, and NH₄⁺ were calculated from the slopes of linear regression models fitted to data of excess ²⁹,³⁰N₂, ⁴⁵,⁴⁶N₂O, and ¹⁵NH₄⁺ vs. time. The denitrification rate (N₂ and N₂O production; D_tot), and denitrification driven by NO₃⁻ from the overlying water (D_w) or sedimentary nitrification (D_n), were calculated using the isotope pairing technique (IPT) (Nielsen 1992). If anammox is contributing to the N₂ production, rates must be calculated using the revised IPT (Risgaard-Petersen et al. 2003). However, no anammox was found (see Results section), so denitrification rates were calculated using the classical IPT. DNRA rates (DNRA/n) and DNRA driven by NO₃⁻ from the overlying water (DNRA/w) or sedimentary nitrification (DNRA/n) were calculated according to Risgaard-Petersen and Rysgaard (1995). The fractions of denitrification and DNRA that were driven by NO₃⁻ from sedimentary nitrification were equally large. Hence, both fractions are presented as nitrification – NO₃⁻ reduction (% NO₃⁻ redn), calculated as:

\[ \% \text{NO₃⁻ redn} = \frac{D_n}{D_{tot}} \times 100 = \frac{\text{DNRA}_n}{\text{DNRA}_{tot}} \times 100. \]

As the chambers of the lander only captures the N₂, N₂O, and NH₄⁺ that diffuse to the water column (De Brabandere et al. 2015), total denitrification and DNRA rates in the sediment incubated by the chambers of the lander were calculated as follows:

\[ R_{tot} = R_{W-\text{l-lan}} \times (R_C/R_W), \]

where \( R_{W-\text{l-lan}} \) is the rate in the water column of the lander chamber, while \( R_C \) and \( R_W \) are the rates in the homogenized sediment core phase (i.e., sediment and water) and water phase alone, respectively. It was assumed that the partitioning of the added ¹⁵NO₃⁻ between water and sediment was the same in the lander chambers and sediment cores.

Data from the in situ incubations were used to calculate correlations between environmental parameters (bottom water O₂ and NO₃⁻, sedimentary O₂ uptake and DIC release) and NO₃⁻ reduction rates. Calculations were done in R (v. 4.0.2; R Core Team 2020). Since Shapiro–Wilks tests (shapiro.test, “stats” v. 4.0.2) showed that some data were not normally distributed, Spearman’s rank correlation coefficient was used (corr. test, “psych” v. 2.0.7; Revelle 2020). Correlations with \( p \) values <0.05 were considered significant. Due to the low number (≤7) of complete data pairs per station and year, correlations were not calculated for sediment parameters (e.g., OC), bottom water salinity and bottom water temperature.

Results
Bottom water conditions and sediment properties
The bottom water at Sta. A was fully oxygenated (100–355 μM O₂) with low NO₃⁻ concentrations (2.9–8.6 μM; Table 1). In 2017, a storm between two lander deployments caused the halocline to move downward which in turn
increased the O$_2$ concentration substantially (Table 1; van de Velde et al. 2020), whereas the NO$_x$ concentrations did not change. The sediment at Sta. A was generally characterized by three distinct layers: a rust-colored layer down to 2 cm depth, a light-brown layer between 2 and 4 cm depth, followed by post-glacial clay. The OC and N contents in the top 2 cm of sediment were low (0–5% and 0–1%, respectively) but showed some variability between samplings (Table 1; Supporting Information Fig. S1), suggesting spatial heterogeneity. The heterogeneity was also reflected in the OC : N ratios, which varied between 7.0 and 10.5. During the 2018 sampling, we occasionally retrieved sediment of a different type to that which is generally found at the station, finer sediment with darker layers and a deeper post-glacial clay horizon. Animals (polychaetes, isopods and bivalves) were found in the sediment during all samplings.

At Stas. D–F, the sediment was clearly laminated and there were no signs of animal activity. Bottom water concentrations of O$_2$ and NO$_x$ were highest immediately after the inflow, after which they decreased rapidly (Table 1). The OC and N contents in the top 2 cm of sediment were relatively high (7.9–12.1% and 0.9–1.4%, respectively) and the OC : N ratios varied between 9.4 and 10.9. Mats of large filamentous sulfur bacteria were visible on the sediment surface at Sta. D in 2018 and Stas. E and F in 2017 and 2018.

At Sta. A, the O$_2$ penetration depth (OPD) was approximately 5 mm and H$_2$S was below the detection limit of the sensor (1 μM) in the top 10 mm, with the exception of one profile in 2015 (Fig. 2). In 2018, we measured decreasing N$_2$O concentrations from the sediment surface (0.02 μM) to 5 mm depth, where the concentration stabilized at zero. The N$_2$O concentration at Sta. A was an order of magnitude lower than

Fig. 2. Sediment microprofiles. Note different scales of the y-axes on Sta. A compared to Stas. D–F, and the N$_2$O/H$_2$S axes for Sta. A in different years and compared to D–F. Profiles of O$_2$ and H$_2$S in 2016 and 2017 are from Marzocchi et al. (2018), all profiles in 2018 are from Broman et al. (2020). Error bars show standard errors.
production of NO where post-glacial clay started to occur. At Stas. D and F, H2S appeared between the surface and 1 mm depth, indicative of increased organic matter contents and denitrification rates (> 80%) of NOx for denitrification and DNRA at Sta. A (Fig. 5d). Values of $R_c/R_W$ at Sta. A were 1.2 for denitrification and 2.9 for DNRA. In situ incubations at Sta. D in 2016 (before oxygenation) did not show any $^{29}$N2 production (data not shown), indicating that a potential denitrification rate ($D_{13}$) could be calculated. However, production of $^{30}$N2 and $^{15}$NH4 demonstrated that there was a potential for NOx reduction at the station.
Following oxygenation of Stas. D–F, denitrification (37–112 μmol m$^{-2}$ d$^{-1}$) and DNRA (33–71 μmol m$^{-2}$ d$^{-1}$) were detected, with the latter process contributing 31–56% to the total NO$_x$ reduction (% DNRA; Fig. 5a,c). The fraction of denitrification that resulted in N$_2$O production (% N$_2$O) was 41–88% of the total denitrification and did not change substantially between samplings (Fig. 5a,d). Compared to Sta. A, a much smaller fraction (0–63%) of the NO$_x$ reduction was driven by nitriification (Fig. 5d). Values of $R_{C}/R_W$ at Stas. D–F were 1.5 for denitrification and 1.4 for DNRA.

The denitrification rates correlated negatively with the DIC fluxes and DNRA rates did not correlate with any environmental parameters (Table 2; Supporting Information Fig. S3). However, both % DNRA and % N$_2$O correlated positively with DIC fluxes and bottom water concentrations of NO$_x$, and negatively with bottom water O$_2$ concentrations and sedimentary O$_2$ uptake. The % NO$_x$ red$_n$ correlated positively with both bottom water O$_2$ concentrations and sedimentary O$_2$ uptake, whereas there was a negative correlation with bottom water concentrations of NO$_x$. The extent of the oxygenation is also likely to have an effect on the partitioning between NO$_x$ reduction processes. There was a decrease in % DNRA with increasing O$_2$ exposure time during artificial oxygenation of the By Fjord on the west coast of Sweden (from ~65% to ~45% in 2 yr; De Brabandere et al. 2015). In our study, however, no clear change in either rates or % DNRA with time was observed over the few consecutive samplings (Fig. 5a,c). This discrepancy in response can likely be explained by different degrees of oxygenation between the two studies. The bottom water O$_2$ concentration in the By Fjord
was continuously 40–140 μM following oxygenation and the OPD increased with time, suggesting that the sediment was gradually becoming less reduced (De Brabandere et al. 2015). In the Eastern Gotland Basin, the natural oxygenation of the bottom water was likely too weak and not constant enough (<50 μM O₂, Table 1; Fig. 1; Hall et al. 2017) to change the sediment geochemistry sufficiently for a shift in the balance between denitrification and DNRA to occur.

The bottom water O₂ concentration also correlated negatively with % N₂O (Table 2). This correlation, however, as well as those with bottom water NOₓ reduction rates and O₂ uptake, was driven by the large differences between Stas. A

| Table 2. | Spearman’s rank correlation coefficient for correlations between NOₓ⁻ reduction rates and environmental parameters. Correlations marked in bold were statistically significant. |
|----------|----------------------------------------------------------|
|          | BW O₂ | BW NOₓ⁻ | Sed. O₂ uptake | Sed. DIC release |
| Denitr.  | 0.31  | −0.35   | 0.33           | −0.46*           |
| DNRA     | 0.13  | 0.09    | 0.13           | −0.05            |
| % NOₓ⁻ redₙ | 0.71*** | −0.52** | 0.71***        | −0.25            |
| % N₂O    | −0.49* | 0.65*** | −0.48*         | 0.69***          |
| % DNRA   | −0.51** | 0.61*** | −0.52**        | 0.47*            |

*p < 0.05; **p < 0.01; ***p < 0.001.
and D–F rather than showing a trend over all stations (Supporting Information Fig. S3). Bottom water O$_2$ concentrations were thus unlikely to have been a major driver behind % N$_2$O patterns at the newly oxygenated stations. Instead, the last step in the denitrification process was likely inhibited by H$_2$S (Sørensen et al. 1980; Senga et al. 2006) at Stas. D–F, which reached the sedimentary NO$_3^-$ reduction zone at these newly oxygenated stations (Fig. 2). No net production of N$_2$O through denitrification was detected at Sta. A as determined from $^{15}$N isotope incubations (Fig. 5), in agreement with previous studies of permanently oxic sites in the Baltic Sea (Hellemann et al. 2017, 2020; Bartl et al. 2019). A small N$_2$O peak was visible in the oxic part of the sediment in 2018 (Fig. 2), suggesting production through nitrification. The microprofiles suggest that little to no of this N$_2$O was released from the sediment at Sta. A, however.

Interestingly, microprofiling of individual aggregates deposited on the seafloor at Sta. E showed that they were sites of production of N$_2$O (Fig. 3b). The N$_2$O production peaks were located in the anoxic parts of the aggregates, suggesting that denitrification was the responsible process. Aggregates can form as phytoplankton sink from the surface (Thornton 2002), and also as fresh and reactive organic matter is resuspended and transported from shallow to deeper areas in the Eastern Gotland Basin (Nilsson et al. 2021). Aggregates are locations of high degradation of organic matter and thereby form anoxic micro-niches with intense N cycling, both while sinking and once settled on the seafloor (Klawonn et al. 2015; Bianchi et al. 2018; Marzocchi et al. 2018b). The positive correlation observed between % N$_2$O and the DIC fluxes further suggests a connection between N$_2$O production and hot spots of organic matter degradation. These hot spots could occur in the sediment due to local heterogeneities, but also in aggregates consisting of fresh organic matter.

**Strongly reduced sediments favor DNRA**

The contribution from DNRA to the total NO$_3^-$ reduction rates is generally found to be higher in active, more reduced systems with high concentrations of organic matter (Giblin et al. 2013; Kessler et al. 2018). This trend is often attributed to the ratio between NO$_3^-$ and electron donors (e.g., OC and H$_2$S). Although denitri-fication yields more energy per mole of electron donor, more electrons are transferred per mole of NO$_3^-$ during DNRA (Tiedje et al. 1983). As such, DNRA tends to be favored in environments with high availability of electron donors relative to NO$_3^-$ (Kraft et al. 2014; Hardison et al. 2015; van den Berg et al. 2015).

The positive correlation between % DNRA and the bottom water NO$_3^-$ concentrations observed in this study (Table 2; Supporting Information Fig. S3) seems to contradict the theory that DNRA dominates under lower NO$_3^-$ conditions. However, the ratio between NO$_3^-$ and electron donors differed substantially between the permanently oxic Sta. A and the newly oxygenated Stas. D–F. Although the bottom water NO$_3^-$ concentrations generally were lower at Sta. A than at Stas. D–F during years of oxygenation, the OC content in the sediment was one order of magnitude higher at Stas. D–F than at Sta. A (Table 1) and H$_2$S was present <2 mm from the surface at the former sites (Fig. 2; Marzocchi et al. 2018a). The ratios between bottom water NO$_3^-$ and these potential electron donors were thus substantially higher at Sta. A than at Stas. D–F, which likely contributed to the observed partitioning between processes. The positive correlation between DIC fluxes and % DNRA, and negative correlation between DIC fluxes and denitrification rates, further suggested that DNRA was favored at sites with high organic matter degradation. It is also possible that % DNRA changed over the year. The sampling for this study was conducted right before the spring bloom each year (Hylén et al. 2021). The importance of DNRA could therefore have grown in the months after the samplings, as the spring bloom was deposited on the sediment and increased the supply of reactive organic matter, for example, in the form of aggregates.

Whereas sediment denitrification generally is heterotrophic (Seitzinger et al. 2006), we cannot conclusively say which electron donor (OC or H$_2$S) that was used in DNRA. Nevertheless, detection of sulfur bacteria (cable bacteria and Beggiatoa spp.) able to mediate DNRA in the sediment from the Eastern Gotland Basin (Yücel et al. 2017; Marzocchi et al. 2018a, 2021) suggests that a fraction of DNRA was coupled to H$_2$S oxidation. Furthermore, it has recently become clear that Fe$^{2+}$ also can be an important electron donor for DNRA (Robertson et al. 2016; Kessler et al. 2018). Low concentrations (<10 μM) of Fe$^{2+}$ appeared in the pore water at Stas. D–F after the MBI, as iron oxides that had formed in the water column sank and were reductively dissolved in the sediment (van de Velde et al. 2020). However, the concentrations of Fe$^{2+}$ were one order of magnitude lower than at sites where it has been shown to enhance DNRA (Robertson et al. 2016) and the Fe$^{2+}$ concentrations in the sediment at Stas. D–F were considerably lower than those of OC and H$_2$S. We thus consider it unlikely that Fe$^{2+}$ was an important electron donor for DNRA in this system or played a significant role in the partitioning between denitrification and DNRA.

While both denitrification and DNRA were important at Stas. D–F, denitrification dominated at Sta. A. The NO$_3^-$ reduction rates at Sta. A varied substantially between years but were comparable to previous measurements from well-oxygenated sites with low sedimentary OC content in the Baltic Sea (Jänthi and Hietanen 2012; Bonaglia et al. 2017; Hellemann et al. 2020). The large difference in rates between years was likely caused by spatial heterogeneity at Sta. A, as explained above, resulting in incubation of more reactive sediment in lander chambers in 2018. The layered and darker appearance of the sediment in 2018 compared to 2016–2017, in combination with high DIC and O$_2$ fluxes, suggest that the location was richer in OC and that the mineralization rates were higher. Since the bottom water at Sta. A was well-oxygenated...
with low NO\textsubscript{x}\textsuperscript{-} concentrations, a higher sedimentary OC content would likely have stimulated denitrification, as reported from other sites in the Baltic Sea (Jantti et al. 2011; Bartl et al. 2019).

In contrast to some relatively organic matter-poor (2.5–5.1% OC) sites in the Baltic Sea where anammox constituted 5–32% of the NO\textsubscript{x}\textsuperscript{-} reduction (Bonaglia et al. 2014, 2017; van Helmond et al. 2020), the process was insignificant at Sta. A. Although the sedimentary OC content is low at the site, the material is readily degraded compared to other areas of the Baltic Sea (Nilsson et al. 2021). Compared to sites where anammox has been shown to significantly contribute to NO\textsubscript{x}\textsuperscript{-} reduction, it is possible that the available OC at Sta. A was reactive enough that heterotrophic denitrification would out-compete anammox (Devol 2015).

**NO\textsubscript{x}\textsuperscript{-} limited denitrification and DNRA**

The potential NO\textsubscript{x}\textsuperscript{-} reduction rates \( (D_{15}) \), Supporting Information Table S1) measured at Sta. D in 2016, before introduction of O\textsubscript{2} and NO\textsubscript{x}\textsuperscript{-}, suggest that microorganisms capable of NO\textsubscript{x}\textsuperscript{-} reduction were present in the sediment at the time of the MBI. Sta. D experiences minor inflows more regularly than Stas. E and F (Supporting Information Fig. S5; SMHI 2021) and is more likely to maintain active NO\textsubscript{x}\textsuperscript{-} reduction metabolisms. Potential for NO\textsubscript{x}\textsuperscript{-} reduction has been previously measured in long-term anoxic sediments (De Brabandere et al. 2015). Thus, as have been seen at other sites, NO\textsubscript{x}\textsuperscript{-} reducing activity was rapidly resumed in the sediments of Stas. D–F as NO\textsubscript{x}\textsuperscript{-} became available after the MBI, even after a prolonged period of NO\textsubscript{x}\textsuperscript{-}-free bottom water.

The denitrification and DNRA rates at Stas. D–F were in the lower range of rates measured in the Baltic Sea at sites with low bottom water O\textsubscript{2} concentrations and high sedimentary OC content (Bonaglia et al. 2014; Hellemann et al. 2020). A likely reason for the low rates is NO\textsubscript{x}\textsuperscript{-} limitation. Nitrification was not measured in this study, but substantial sedimentary NH\textsubscript{4}\textsuperscript{+} effluxes (Fig. 4) and relatively low bottom water NO\textsubscript{x}\textsuperscript{-} concentrations (Table 1) suggest that the process was inhibited at Stas. D–F. This could be the result of restrictions caused by the very narrow horizon of oxygenated sediment where nitrification could occur, potentially in combination with inhibition of nitrification by H\textsubscript{2}S (Joye and Hollibaugh 1995). Denitrification and DNRA rates were indeed slightly higher directly after the inflow in 2015 (50–160 and 160–530 \( \mu \)mol m\textsuperscript{-2} d\textsuperscript{-1}, respectively), when bottom water O\textsubscript{2} concentrations were higher (Hall et al. 2017).

Sedimentary nitrification contributed half of the NO\textsubscript{x}\textsuperscript{-} being reduced at Stas. D–F (Fig. 5d), despite shallow OPDs (1–2.2 mm, Fig. 2). The only exception was Sta. D in 2018, where anoxia had returned and the water column was the only source of NO\textsubscript{x} for reduction. The highest % NO\textsubscript{x}\textsuperscript{-} red\textsubscript{fl} (> 75%) was measured at the permanentlyoxic Sta. A, where the OPD was consistently deeper (4–7 mm) than at Stas. D–F and pore-water NO\textsubscript{x}\textsuperscript{-} profiles (Supporting Information Fig. S2) suggest that bioturbation enabled nitrification further down in the sediment. Higher bottom water O\textsubscript{2} concentrations tend to increase the importance of NO\textsubscript{x}\textsuperscript{-} red\textsuperscript{fl}, since deeper O\textsubscript{2} penetration pushes the zone of nitrification further down into the sediment, which decreases the diffusional loss of NO\textsubscript{x} to the water column (Rysgaard et al. 1994). The positive correlation between % NO\textsubscript{x}\textsuperscript{-} red\textsubscript{fl} and bottom water O\textsubscript{2} concentration (Table 2) suggest that this was indeed occurring at our sites. On the other hand, high bottom water concentrations of NO\textsubscript{x} can decrease the relative importance of NO\textsubscript{x}\textsuperscript{-} red\textsubscript{fl}, as diffusion of water column NO\textsubscript{x} into the sediment increases (Rysgaard et al. 1994; Seitzinger et al. 2006; De Brabandere et al. 2015). In agreement with this theory, we also saw a negative correlation between % NO\textsubscript{x}\textsuperscript{-} red\textsuperscript{fl} and bottom water NO\textsubscript{x}\textsuperscript{-} concentrations (Table 2).

Interestingly, the sedimentary uptake of NO\textsubscript{x} was ~5–7 times higher than the NO\textsubscript{x}\textsuperscript{-} reduction rates at Stas. D–F. A plausible explanation for this excess uptake is intracellular NO\textsubscript{x} storage by organisms such as large sulfur bacteria, dia- toms, and foraminifera (McHatton et al. 1996; Kamp et al. 2015). Mats of the large sulfur bacteria Beggiatoua spp. were visible on the sediment surface in 2017 and 2018. Members of the Beggiatoaceae family use O\textsubscript{2} and NO\textsubscript{x} to oxidize H\textsubscript{2}S, and are capable of storing NO\textsubscript{x} at concentrations over 100 \( \mu \)M in their vacuoles (McHatton et al. 1996). These bacteri- a are known to perform both denitrification and DNRA, but DNRA is believed to be the dominant pathway in sulfide-rich sediments (Schutte et al. 2018).

The presence of organisms with intracellular NO\textsubscript{x} storage can cause two main issues that may compromise the IPT unless additional experiments are carried out (Robertson et al. 2019). First, since the added \( ^{15} \text{NO}_3 \) is unlikely to equili- brate with the intracellular pools of NO\textsubscript{x}, the reduction of \( ^{14} \text{NO}_3 \) from intracellular pools would not be captured by the IPT and the total NO\textsubscript{x} reduction rate would be under- estimated. Second, the combination of added \( ^{15} \text{NO}_3 \) and \( ^{14} \text{NO}_3 \) from intracellular pools during denitrification would give an increased production of \( ^{29} \text{N}_2 \), which would be mis- taken as NO\textsubscript{x}\textsuperscript{-} red\textsuperscript{fl}. The NO\textsubscript{x}\textsuperscript{-} reduction rates presented here should therefore be seen as a lower limit as the true amount of NO\textsubscript{x} reduction from intracellular NO\textsubscript{x} and water column-derived NO\textsubscript{x} may be underestimated in our calculations.

**Conclusions and environmental implications**

Sedimentary NO\textsubscript{x}\textsuperscript{-} reduction was stimulated in the Eastern Gotland Basin after the MBI, however, we demonstrate that these processes had only a small impact on the capacity to remove N as N\textsubscript{2} through denitrification at the newly oxygenated sites. The weak and inconsistent oxygenation combined with high sedimentary production of reduced species (e.g., H\textsubscript{2}S) likely limited nitrification, and thus nitrification-denitrification. Due to the high NH\textsubscript{4} release and low denitrification rates, the denitrification efficiency (N\textsubscript{2}N\textsubscript{2}O-N/[N\textsubscript{2}N\textsubscript{2}O-N]
+ fluxes of NH$_4^+$ and NO$_3$) $\times$ 100%; Eyre and Ferguson 2009) was only 0.7–2.4% at Stas. D–F, compared to 9.1–72.5% at Sta. A. Denitrification rates at the newly oxygenated stations were in the lower range of measurements at similar sites in the Baltic Sea (Bonaglia et al. 2014; Hellemann et al. 2020) and also in the lower range of denitrification rates at the oxic–anoxic interface in the water column of the Eastern Gotland Basin (60–211 $\mu$mol N m$^{-2}$ d$^{-1}$; Dalsgaard et al. 2013). As the MBI and following inflows created a second oxic–anoxic interface in the water column, the pelagic N removal is likely to have increased considerably, substantially exceeding the sedimentary N removal (Hall et al. 2017). In conclusion, the sediment at the newly oxygenated stations was not sites of efficient removal of fixed N. The natural oxygenation event stimulated production of N$_2$O and recycling of fixed N through DNRA, while increased organic matter degradation lead to elevated release of fixed N as NH$_4^+$ (Hylén et al. 2021). Consequently, the main outcome from this temporary oxygenation was that the sediments continued to contribute to accumulation, rather than removal, of fixed N in the system.

Our results showing dominance of denitrification at the site with less organic matter (A) and a substantial contribution from DNRA at the hypoxic sites with organic rich and reduced sediments (D–F) follow patterns observed in the Baltic Sea (Jäntti and Hietanen 2012; Bonaglia et al. 2017; van Helmond et al. 2020) and elsewhere (e.g., estuaries in Victoria, Australia, Kessler et al. 2018; the By Fjord, Sweden, De Brabandere et al. 2015; sediments below the Peruvian O$_2$ minimum zone, Bohlen et al. 2011). The high proportion of N$_2$O production from incomplete denitrification, however, was unexpected. While N$_2$O released from the sediment at Stas. D–F was likely oxidized in the water column (Myllykangas et al. 2017; Broman et al. 2020), a similar release in shallower areas with hypoxic bottom waters could potentially reach the atmosphere. As O$_2$-depleted zones are expanding in coastal areas (Altieri and Gedan 2015), the exact mechanisms behind increased N$_2$O production observed under fluctuating oxygen conditions (this study; Myllykangas et al. 2017; Broman et al. 2020) deserve attention in future studies. Shallowing of O$_2$-depleted zones could have an even stronger effect on the sedimentary N$_2$O release if aggregates are important sites of N$_2$O production, as suggested by our measurements. In shallow areas, a larger fraction of sinking aggregates escapes degradation in the water column. A higher deposition of relatively more labile aggregates may stimulate N$_2$O production at shallow sites where the severity and persistence of low-O$_2$ bottom waters is increasing. The shorter transport distance from sediments to surface waters may thus result in an increased N$_2$O release to the atmosphere in O$_2$-depleted coastal zones, thereby intensifying global warming.

The spreading of oxygen-depleted zones in coastal areas around the world has led to a growing interest in the response of the benthic N cycle to hypoxia (De Brabandere et al. 2015; Song et al. 2020). Studies have shown varying results depending on local environmental conditions, but there is a tendency toward decreased removal of fixed N under severe hypoxia and anoxia (Jäntti and Hietanen 2012; Caffrey et al. 2019; Song et al. 2020). These studies generally focus on seasonally hypoxic areas, however. Here, we expand the studied areas to include long-term anoxic (euxinic) sediments that have been affected by eutrophication for decades (Carstensen et al. 2014). Our observations suggest that transient oxygenation of these strongly reduced systems may not lead to efficient sedimentary removal of fixed N, and instead the sedimentary release of reduced N compounds can accelerate the return to the original anoxic state.

Data availability statement
Upon publication, all data included in this manuscript will be available to download from the Swedish National Data Service.

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Conflict of interest

None declared.