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Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea

V. J. Bertics¹, C. R. Löscher², I. Salonen², A. W. Dale¹, R. A. Schmitz², and T. Treude¹

¹Helmholtz Centre for Ocean Research Kiel (GEOMAR), Wischhofstrasse 1–3, 24148 Kiel, Germany

²Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität Kiel, Am Botanischen Garten 1–9, 24118 Kiel, Germany

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Correspondence to: V. J. Bertics (vbertics@geomar.de), T. Treude (ttreude@geomar.de)

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Abstract

Despite the worldwide occurrence of marine hypoxic regions, benthic nitrogen (N) cycling within these areas is poorly understood and it is generally assumed that these areas represent zones of intense fixed N loss from the marine system. Sulfate reduction can be an important process for organic matter degradation in sediments beneath hypoxic waters and many sulfate-reducing bacteria (SRB) have the genetic potential to fix molecular N (N_2). Therefore, SRB may supply fixed N to these systems, countering some of the N lost via microbial processes such as denitrification and anaerobic ammonium oxidation. The objective of this study was to evaluate if N_2 -fixation, possibly by SRB, plays a role in N cycling within the seasonally hypoxic sediments from Eckernförde Bay, Baltic Sea. Monthly samplings were performed over the course of one year to measure N_2 -fixation and sulfate reduction rates, to determine the seasonal variations in bioturbation (bioirrigation) activity and important benthic geochemical profiles, such as sulfur and N compounds, and to monitor changes in water column temperature and oxygen concentrations. Additionally, at several time points, rates of benthic denitrification were also measured and the active N-fixing community was examined via molecular tools. Integrated rates of N_2 -fixation and sulfate reduction showed a similar seasonality pattern, with highest rates occurring in August (approx. 22 and 880 $\text{nmol cm}^{-3} \text{d}^{-1}$ of N and SO_4^{2-} , respectively) and October (approx. 22 and 1300 $\text{nmol cm}^{-3} \text{d}^{-1}$ of N and SO_4^{2-} , respectively), and lowest rates occurring in February (approx. 8 and 32 $\text{nmol cm}^{-3} \text{d}^{-1}$ of N and SO_4^{2-} , respectively). These rate changes were positively correlated with bottom water temperatures and previous reported plankton bloom activities, and negatively correlated with bottom water oxygen concentrations. Other variables that also appeared to play a role in rate determination were bioturbation, bubble irrigation and winter storm events. Molecular analysis demonstrated the presence of *nifH* sequences related to two known N_2 -fixing SRB, namely *Desulfovibrio vulgaris* and *Desulfonema limicola*, supporting the hypothesis that some of the nitrogenase activity detected may be attributed to SRB. Denitrification appeared

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to follow a similar trend as the other microbial processes and the ratio of denitrification to N₂-fixation ranged from 6.8 in August to 1.1 in February, indicating that in February, the two processes are close to being in balance in terms of N loss and N gain. Overall, our data show that Eckernförde Bay represents a complex ecosystem where numerous environmental variables combine to influence benthic microbial activities involving N and sulfur cycling.

1 Introduction

Dissolved oxygen in the world ocean is a key factor driving marine biogeochemical processes and nutrient turnover, especially in regards to the marine nitrogen (N) and carbon cycles (Helly and Levin, 2004; Bange et al., 2005; Middelburg and Levin, 2009). When substantial organic carbon is available, oxygen demands become higher, and when combined with sluggish or restricted water circulation, oxygen minimum zones (areas with dissolved oxygen <22 μM) can develop in large midwater regions, typically between 200–1000 m water depth (Wyrski, 1962; Kamykowski and Zentara, 1990; Levin, 2003). The same process can lead to hypoxia (dissolved oxygen <63 μM) in silled basins, fjords and enclosed seas (e.g. Black Sea, Cariaco Basin, Koljød Fjord; Anderson and Devol, 1987; Middelburg et al., 1991; Rosenberg et al., 2001). It is estimated that the benthic area of sediments subjected to hypoxia world-wide is over 10⁶ km² (Helly and Levin, 2004). Due to global warming induced changes in the marine environments, as well as human alterations of coastal ecosystems, these oxygen minimum zones and hypoxic regions are expanding and are predicted to continue to do so for the near future (Stramma et al., 2008; Middelburg and Levin, 2009).

N-cycling within oxygen-depleted regions is still poorly understood and therefore has become a topic of much debate. Until recently it was assumed that the dominant process involved in N-loss in these areas was denitrification (Ulloa and Pantoja, 2009), a process that uses nitrate as an electron acceptor for organic matter degradation. In recent years, it has been shown that anaerobic ammonium oxidation (anammox) with

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nitrite, sometimes possibly linked to dissimilatory nitrate reduction (Kartal et al., 2007), can also play an important role (Dalsgaard et al., 2005; Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007). Additionally, despite the fact that oxygen concentrations are so low, Molina and Fariás (2009) have shown that significant rates of nitrification can also be observed, even at oxygen concentrations $\leq 5 \mu\text{M}$. However, all of these processes may lead to a loss of oceanic fixed N, mostly through the eventual production of molecular nitrogen (N_2) and are therefore categorized as a sink for fixed N.

Globally, several estimates of marine fixed N sinks greatly exceed estimates of marine fixed N sources, with some estimates suggesting a deficit as great as $\sim 200 \text{Tg N yr}^{-1}$ (Codispoti et al., 2001; Brandes and Devol, 2002; Hulth et al., 2005; Codispoti, 2007). These results would indicate that either the ocean is decreasing in biologically available N over time, which geochemical data suggest is not the case (Deutsch et al., 2004), or that there is an overestimation or underestimation of outputs and inputs, respectively (Capone and Knapp, 2007). Because biological N_2 -fixation is the primary input of fixed N into the marine biosphere and because biologically available N is often limiting for marine productivity (Thomas, 1966; Carpenter and Capone, 2008; Moisander et al., 2012), the microbial process of N_2 -fixation has traditionally received much attention. While the majority of studies concerning marine N_2 -fixation has focused on the water column, many benthic habitats also have been examined, such as coral reefs, photosynthetic microbial mats, mangrove sediments, and seagrass rhizospheres (Capone, 1983, 1988; Carpenter and Capone, 2008) and more recently, shallow estuarine and bioturbated sediments (e.g. Fulweiler et al., 2007; Bertics et al., 2010). For both pelagic and benthic N_2 -fixation research, much of the current work is focusing on methodological refinements (e.g. underestimation of N_2 -fixation rates when using the bubble technique, Mohr et al., 2010) and exploring locations that were typically thought to be areas of fixed N loss, such as oxygen minimum zones and hypoxic regions, in the hope of resolving the global marine N budget discrepancy.

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As mentioned earlier, oxygen-depleted waters typically form when there is high organic matter loading to the marine system. Much of this organic carbon reaches the sediment surface where it can promote high rates of microbial activity, such as increased sulfate reduction. Because many sulfate-reducing bacteria (SRB) have the genetic potential to fix N (Zehr et al., 1995) and have been shown to do so in a variety of benthic habitats (e.g. Nielsen et al., 2001; Steppe and Paerl, 2002; Bertics et al., 2010) it is possible that these organisms could facilitate the biological fixation of N₂ in sediments beneath hypoxic waters. To test this hypothesis, a region of the German Baltic Sea, Eckernförde Bay, known to undergo seasonal hypoxia in late spring through early autumn caused by strong water stratification (Orsi et al., 1996; Hansen et al., 1999; Bange et al., 2011), and known to support seasonal variations in microbial sulfate reduction (Treude et al., 2005), was selected for an intense year-long study. Over the course of the year, water column oxygen concentrations, salinity, density, and temperature were monitored and benthic microbial rates of N₂-fixation and sulfate reduction, along with porewater geochemistry, were determined monthly. Additionally, at several selected time points, microbial denitrification rates were measured for an indication of benthic fixed N loss and molecular analysis of key functional genes was performed for determination of which microorganisms were present with the genetic potential of performing N₂-fixation or denitrification. The overall goal was to determine whether N₂-fixation is occurring in Eckernförde Bay sediments, and if so, whether this activity (1) shows seasonal variability, (2) is coupled to sulfate reduction, and (3) can at least partially offset fixed N loss from the sediment due to benthic denitrification.

2 Materials and methods

2.1 Study site

Eckernförde Bay, Germany (54°31.15' N, 10°02.18' E) is a semi-enclosed bay with a water depth of ~28 m, located in the southwestern Baltic Sea. Because of the shallow

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water depth, there is a strong benthic–pelagic coupling that results in organic-rich sediments (Orsi et al., 1996) where the bulk of organic matter originates from plankton and macroalgal sources (Balzer et al., 1987). The presence of two water masses (salty North Sea and brackish Baltic Sea water), along with high surface water temperatures, causes strong stratification during the summer months. Consequently, mixing in the water column is reduced, strengthening the formation of a deep halocline (Hansen et al., 1999). During late summer, organic matter degradation coupled to phytoplankton blooms frequently cause oxygen deficiency in the isolated bottom water (Graf et al., 1983; Meyer-Reil, 1983; Hansen et al., 1999). In late autumn and winter, storms and decreased surface water temperatures cause a breakdown in this stratification and an increase in nutrient and oxygen concentrations in deeper waters (Hansen et al., 1999). For more information on Eckernförde Bay, see Bange and Treude (2012).

2.2 Water column and sediment sampling

From April 2010 to February 2011, field sampling was conducted monthly, with the exceptions that June and July sampling was done from 30 June–2 July (this sampling will hereafter be termed June/July) and that January sampling did not occur due to bad weather. All field sampling was conducted onboard the RV Alkor, the RC Littorina, or the RB Polarfuchs. At each sampling time, an initial cast of a 6 bottle rosette with attached CTD (Hydro-Bios, Kiel, Germany) was performed to profile water column temperature, salinity, density, and oxygen concentrations. Profiles were saved and viewed on a PC using the OceanLab 3 software (Hydro-Bios). Following the cast, a miniature multiple corer (MUC) equipped with four cores liners (length ~60 cm, inner diameter 10 cm) was used to obtain altogether 6–12 sediment cores of ~35 cm length. Cores were then brought back to GEOMAR and taken to a 10 °C cold room for immediate processing.

2.3 Porewater geochemistry and sediment properties

Porewater samples were collected each month from two MUC cores. One core was sampled using anaerobic Rhizons (Rhizosphere Research Comp., Wageningen, The Netherlands) and the other core was sampled using a low pressure (1–5 bar argon)

porewater press containing 0.2 μM cellulose acetate Nuclepore[®] filters. Cores were sampled in 1-cm intervals to a depth of 10 cm, below which 3-cm intervals were used. Porewater collected via Rhizons was immediately analyzed for ammonium concentrations and porewater collected via the press was immediately analyzed for sulfide concentrations. Both nutrient analyses were performed on a Hitachi UV/VIS spectrophotometer following standard photometric procedures (Grasshoff et al., 1999). Because high sulfide concentrations may interfere with ammonium measurements, these subsamples were acidified with 20 μl of HCl and bubbled with argon to strip any hydrogen sulfide. Porewater collected via the press also was immediately analyzed for sulfate concentrations using ion chromatography with the IAPSO seawater standard for calibration. For these measurements, a Metrohm ion-chromatograph equipped with a conventional anion-exchange column, using carbonate-bicarbonate solution as an eluent, and a conductivity detector was used. Wet sediment samples were used for determination of porosity. Porosity was determined by drying a known volume of sediment. The wet sediment was weighed, lyophilized, and weighed again. From the difference of the two weights, the proportion of sediment dry mass and the porosity was calculated.

Additionally, as an indication of seasonal benthic bioturbation activity, we present the results from bioirrigation experiments performed on MUC cores obtained, in the same manner as was previously described, on 12 occasions between February and December 2010 by Dale et al. (2012). In short, Br^- was added in large excess to the water overlying the sediment to act as a dissolved conservative tracer. Cores were incubated for a known period of time and determination of the depth distribution of Br^- allowed for the approximation of irrigation rates by modeling the transient infiltration of Br^- into

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the sediment. For more details on this method and for further explanation concerning the calculation of the bioirrigation coefficient α , please see Dale et al. (2012).

2.4 Benthic Nitrogenase Activity (NA)

From each sampling month, one MUC core was sliced using the following sampling scheme: 0–10 cm sediment depth in 1-cm intervals, 10–20 cm sediment depth in 2-cm intervals, and 20–25 cm sediment depth in one 5-cm interval (due to core length, June/July sampling went to 20 cm and August sampling went to 18 cm). All samples were then analyzed for nitrogenase activity (NA) using the acetylene reduction assay (Capone, 1993). In this study, triplicate 10-cm³ sediment samples from each depth horizon were placed in 60 ml serum vials flushed with N₂. Each vial was crimp sealed, injected with 5 ml of C₂H₂ to saturate the nitrogenase, if present, and the increase in C₂H₄ was assayed over a week (total of 5 time points, including a time-zero) on a gas chromatograph (GC) with a flame ionization detector. At each time point, the headspace of each vial was sampled and directly injected into the GC. Incubations were kept in the dark and at in-situ temperature (3–13 °C based on CTD data) based on the bottom water temperature results from the CTD casts. N₂-fixation rates were calculated from the linear portion of the NA results using a conversion factor of 3 C₂H₄:1 N₂, which is based on previous direct ¹⁵N₂ comparisons in seagrass habitats (Patriquin and Knowles, 1972; O'Donohue et al., 1991) and with the marine cyanobacteria *Trichodesmium* (Orcutt et al., 2001; Capone et al., 2005), and additionally has been used in other coastal sediments (e.g. Welsh et al., 1996).

In April and May, 2 sets of triplicate 5-cm³ samples from each depth interval were placed into 15 ml serum vials flushed with N₂ and used as control samples. To one vial set, no C₂H₂ was added to determine if natural C₂H₄ production was occurring and to the other set, 1 ml of 37.5 % Formalin was added along with 1 ml of C₂H₂ to serve as a kill control. No increase in C₂H₄ was detected in these control samples.

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2.5 Benthic sulfate reduction rates

During each sampling month, 2 MUC cores were sub-sampled with smaller core liners (length 30 cm, inner diameter 2.6 cm) for the determination of sulfate reduction rates through whole-core incubation experiments (Jørgensen, 1978) using the radioactive tracer $^{35}\text{SO}_4^{2-}$. In total, there were 4 small cores used for sulfate reduction measurements (3 experimental and 1 control). Each experimental core was injected with $^{35}\text{SO}_4^{2-}$ (6 μl , 200 kBq, specific activity 37 TBq mmol^{-1}) at 1-cm depth intervals throughout the length of the sediment core (length 20–25 cm) and incubated at in-situ bottom-water temperatures (3–13 °C based on CTD data) for 18–24 h in the dark. After incubation, cores were sliced in 1-cm increments and each sub-sample ($\sim 5 \text{ cm}^{-3}$) was transferred to 20 ml zinc acetate (20% w/w). Controls were first transferred to zinc acetate before radiotracer addition. Sulfate reduction rates were determined using the method of Fossing and Jørgensen (1989) modified to a cold chromium distillation procedure after Kallmeyer et al. (2004).

2.6 Potential benthic denitrification rates

In August and September, one MUC core was sliced in the same manner as the NA core (due to core length, August sampling only went to 14 cm) and analyzed for potential denitrification rates. Denitrification was measured using the C_2H_2 inhibition method, in which C_2H_2 blocks the transformation of N_2O to N_2 in the denitrification pathway (Sørensen, 1978), causing an accumulation of N_2O . The accumulation of N_2O was measured using N_2O microsensors (Revsbech et al., 1989; Binnerup et al., 1992). To help alleviate the problem that C_2H_2 may inhibit nitrification, causing a decrease in NO_3^- over time (Hynes and Knowles, 1978; Cornwell et al., 1999), NO_3^- was added to incubations (Joye et al., 1996), thus providing potential rate measurements. In detail, triplicate 5-cm³ samples from each depth were placed into 15 ml serum vials that were flushed with N_2 and contained 500 μl of 360 μM potassium nitrate (KNO_3). This KNO_3 addition

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led to a final concentration of $\sim 20 \mu\text{M NO}_3^-$ in the samples, which was slightly less than the highest NO_3^- concentration ($28 \mu\text{M}$) seen in the June/July porewater sampling. Prior to starting the incubation, initial N_2O concentrations were determined in each vial using an N_2O microsensor that was inserted into the sediment (Unisense[®], Aarhus, Denmark). Microsensor signals were amplified and transformed to millivolt (mV) by a 2-channel picoammeter (PA 2000) (Unisense[®]), and directly recorded on a computer using the software SensorTrace[®] (Unisense[®]). Each vial was then closed with a butyl stopper, crimp sealed and injected with 1 ml of C_2H_2 . Vials were kept in the dark and at in-situ temperature ($\sim 8.5^\circ\text{C}$). After approximately 24 h, analysis of N_2O with microsensors was performed again as an endpoint measurement.

2.7 Amplification, cloning and sequencing of benthic *nifH* and *nirS* genes

DNA was extracted from ~ 100 mg of sediment, collected in September 2010, using the FastDNA[®] SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA, USA). The *nifH* gene (encoding for one subunit of nitrogenase, the key enzyme of N_2 -fixation) was PCR amplified using primers and PCR conditions according to Zehr et al. (1998). Additionally, *nirS* gene fragments (one of the functional marker genes for denitrification) were amplified according to Lam et al. (2007). Cloning of PCR amplicons was performed using the TopoTA Cloning[®] Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Sanger sequencing (74 *nifH* sequences and 88 *nirS* sequences) was carried out by the Institute of Clinical Molecular Biology, Kiel, Germany. Sequences were phylogenetically analyzed using the ARB software package (Ludwig et al., 2004) on a 321 bp fragment for *nifH* and on a 411 bp fragment for *nirS*, sequence differences were set on a minimum of 5%.

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2.8 Temperature experiment

To examine the effects of temperature on NA and sulfate reduction, an additional sampling of Eckernförde Bay was performed on 4 October 2011. As before, an initial CTD cast was used to determine bottom water temperature (13°C). Following the CTD, 5 MUC cores (2 for NA, 2 for sulfate reduction, and 1 for porewater) were collected and brought back to a 10°C cold room at GEOMAR for immediate processing. All cores were sub-sampled in the manner previously described, with the exception that sampling only reached a sediment depth of 16 cm and porewater was collected only via Rhizons.

For NA measurements, 2 sets of triplicate 12-cm³ sediment samples from each depth horizon were placed in 120 ml serum vials flushed with N₂ and analyzed in the manner described above. Incubations were kept in the dark and one set of vials was kept at the lowest in-situ temperature seen in Eckernförde Bay during our study (3°C; February 2011) while the other set was kept at the highest temperature (13°C; October 2011).

For sulfate reduction rate measurements, 2 sets of triplicate sub-cores and one control sub-core were collected and assayed in the same manner as was described earlier. Identical to the NA incubations, one set of sub-cores was kept at 3°C while the other set was kept at 13°C.

3 Results

3.1 Water column parameters

Over the course of the year, water column temperature and oxygen varied greatly between months and with water depth (Fig. 1). Warmer waters (>10°C) developed in the upper water column in May, reaching highest values (>19°C) in August. In October, the entire water column was >10°C, but by November, temperatures began to decrease,

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reaching lowest values (1–3 °C) in February. In contrast, water column oxygen concentrations were highest (>360 μM) in February. Hypoxic waters (<63 μM) formed in the deeper water depths of August and persisted through October, reaching lowest values (~5 μM) in September. Bottom water salinity (data not shown) ranged from 19–24 ‰, while surficial water salinity ranged from 12–19 ‰. Smallest depth variation in salinity was seen in December (surficial 17 ‰ – bottom 19 ‰), indicating water column mixing, most likely due to winter storm events and decreased surface water temperatures. The largest depth variation was seen in August (surficial 12 ‰ – bottom 24 ‰). For August, salinity remained around 12–13 ‰ until approx. 3 m water depth, below which, salinity increased rather linearly until reaching 24 ‰ in the bottom water. This same trend was seen with August density measurements (data not shown), supporting the idea of summer stratification, which was also seen with water column temperatures and oxygen concentrations (Fig. 1).

3.2 Sediment parameters

Sediment porosity remained relatively stable over the course of the year, decreasing only slightly during the period of hypoxia. Typically, surface sediments had a porosity of ~0.92. Porosity then gradually decreased with sediment depth, reaching a porosity of ~0.8 at 25 cm sediment depth.

Bioirrigation was already detectable in cores collected in early February (2010) and reached a maximum coefficient value ($\alpha = 0.38 \text{ d}^{-1}$) in late March (Fig. 2; Dale et al., 2012). Bioirrigation decreased after this time, eventually becoming almost undetectable starting in September ($\alpha < 0.01 \text{ d}^{-1}$) and continuing like this through December. At the same time that bioirrigation became almost undetectable in September, bottom water oxygen concentrations reached lowest values (<5 μM, Fig. 1).

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3.3 Benthic microbial turnover rates and geochemistry

Benthic nitrogenase activity (NA) closely mirrored sulfate reductions rates monthly and with depth in the sediment (Fig. 3). In general, sulfate reduction rates and NA were highest in the top 10 cm of the sediment throughout the year. For both microbial activities, highest rates were seen in the top 5 cm of the sediment in October, reaching a sulfate reduction rate of $190 \pm 44 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ at 0–1 cm sediment depth and a NA of $3.8 \pm 2.5 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ at 1–2 cm sediment depth. In contrast, lowest rates were seen in the bottom 10 cm of sediment in February, dropping to immeasurable sulfate reduction rates for most sediment depths and a NA of $0.02 \pm 0.01 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ in the 20–25 cm sediment depth interval. In May, a co-occurring peak of sulfate reduction and NA appeared in the 20–25 cm sediment depth interval, with a sulfate reduction rate of $49 \pm 7 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ and a NA of $2.4 \pm 1.6 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$. In November and February, there were two replicates for sulfate reduction measurements that displayed unusually high rates, over 1000 and 5000 $\text{nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ respectively, although all replicates in December displayed typical rates with depth (Fig. 4). Because these high rates appear to be rather localized, these rates were not used in formation of the sulfate reduction contour plot, but were instead plotted as individual profiles (Fig. 4). An explanation for these high rates is provided in the Discussion, Sect. 4.2.

Porewater ammonium concentrations (Fig. 3) generally showed an increase with depth in each month, with the exception of October and November, in which concentrations remained rather constant below 4 cm sediment depth. For the top 5 cm of sediment, the highest ammonium concentrations ($416 \mu\text{M}$) were seen in October at a sediment depth of 2–3 cm. For the 5–10 cm sediment depth horizon, the highest ammonium concentrations ($702 \mu\text{M}$) were seen in December at a sediment depth of 9–10 cm. For sediment depths greater than 10 cm, the highest ammonium concentrations ($1214 \mu\text{M}$) were seen in June/July in the very bottom of the core (19–22 cm). Overall, ammonium profiles tended to show the opposite trend when compared to the NA and

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sulfate reduction rate profiles, with months displaying high peaks in microbial activities also showing little variation in, and typically lower concentrations of, ammonium with sediment depth.

Like NA, denitrification rates measured in August were higher than those measured in September (Fig. 5). In August, highest denitrification rates ($>8 \text{ nmol N cm}^{-3} \text{ d}^{-1}$) were seen from 1–10 cm sediment depth. In contrast, September denitrification rates never reached above $4.3 \text{ nmol N cm}^{-3} \text{ d}^{-1}$ (seen in the 7–8 cm depth horizon), with highest rates ($>3 \text{ nmol N cm}^{-3} \text{ d}^{-1}$) occurring from 2–16 cm sediment depth. In August, denitrification rates were, on average, 3–13 times higher than nitrogen fixation rates, in terms of $\text{nmol N cm}^{-3} \text{ d}^{-1}$. Likewise, for September, denitrification rates were, on average, 1.5–13 times higher than N_2 -fixation rates, in terms of $\text{nmol N cm}^{-3} \text{ d}^{-1}$, with the exception that at 18–20 cm, the two rates were close to being balanced (0.5 vs. $0.4 \text{ nmol N cm}^{-3} \text{ d}^{-1}$ for denitrification and nitrogen fixation, respectively). Integrating both denitrification and N_2 -fixation for each month (Table 1), areal denitrification greatly exceeded that of N_2 -fixation in August and September, but the two rates were approximately equal in February.

3.4 *nifH* and *nirS* gene analysis

In general, the *nifH* gene sequences detected in September samples clustered with those from proteobacteria and those from Cluster III, as defined by Zehr and Turner (2001), while there were no cyanobacterial *nifH* gene sequences detected (Fig. 6). Two of the organisms that clustered with September *nifH* sequences were SRB, namely *Desulfovibrio vulgaris* (Postgate, 1984; Heidelberg et al., 2004) and *Desulfonema limicola* (Widdel, 1989; Fukui et al., 1999). Two of the other organisms with which September *nifH* sequences clustered, belonged to genera with members capable of performing sulfur reduction, namely *Acrobacter nitrofigilis* and *Sulfurospirillum multivorans* (Madigan et al., 2003).

The majority of the September *nirS* gene sequences (Fig. 7) clustered with uncultured denitrifying bacterium from the Baltic Sea, as reported by Falk et al. (2007) and

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with uncultured denitrifying bacterium from the Chesapeake Bay, as reported by Francis et al. (2003). Interestingly, one outlier September *nirS* gene sequence from the 5–6 cm sediment depth horizon clustered with an uncultured denitrifying bacterium from the oxygen minimum zone located on the Mexican Pacific coast, as reported by Liu et al. (2003).

3.5 Temperature experiment

In general, sulfate reduction rates and NA were higher in samples incubated at 13 °C vs. 3 °C (Fig. 8). One exception was seen with the NA present in the 14–16 cm sediment depth horizon, where 2 out of 3 replicates incubated at 3 °C displayed higher NA than those incubated at 13 °C. Integrating to 15 cm sediment depth, sulfate reduction rates for incubations at 13 °C ($1052 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$) were 5 times higher ($Q_{10} = 5.2$) than those at 3 °C ($203 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$). For NA, integrating to 16 cm sediment depth, incubations at 13 °C ($22 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-2} \text{ d}^{-1}$) were 2 times higher ($Q_{10} = 2$) than those at 3 °C ($11 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-2} \text{ d}^{-1}$).

4 Discussion

4.1 Benthic nitrogen fixation coupled to sulfate reduction

Sulfate reduction is one of the most dominant biogeochemical processes in marine sediments, especially in coastal regions where sulfate reduction can account for >50 % of organic matter degradation (Jørgensen, 1977a, 1982; Canfield, 1989). In coastal hypoxic sediments, this estimate can be even higher, reaching >75 % of organic matter degradation (Rowe et al., 2002). Many sulfate-reducing bacteria (SRB) have the genetic potential to fix N_2 (Zehr et al., 1995) and have long been shown to do so in both laboratory and environmental settings (e.g. Sisler and Zobell, 1951; Le Gall et al., 1959; Riederer-Henderson and Wilson, 1970; Postgate et al., 1985; Welsh et al.,

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1996; Bertics et al., 2010). Combining this ability with the high abundance of SRB in certain marine sediments, it has been suggested that SRB may play a significant role in supplying fixed N to benthic communities, in particular, those present in N deficient sediments (Herbert, 1975; Nedwell and Aziz, 1980; McGlathery et al., 1998).

5 In this study, sulfate reduction depth profiles correlated with depth profiles of NA for each sampling month, suggesting that these two processes may be linked in Eckernförde Bay sediments. Looking at the contour plots (Fig. 3), areas of increased sulfate reduction activity often displayed co-occurring increases in NA. Integrating these microbial activities down to a sediment depth of 18 cm (Fig. 9), both activities showed
10 a similar seasonality trend, with highest integrated rates occurring during the summer and fall months (June/July–November) and lowest rates during the winter and spring months (December–May). Interestingly, the ratio between N_2 -fixation and sulfate reduction changes dramatically between September and October, signifying that different environmental factors may be impacting these rates. These factors will be discussed
15 in more detail in the next section. However, the overall trend similarities again suggest that N_2 -fixation may be coupled to, or at least respond to the some of the same environmental factors as, sulfate reduction. This idea was further supported by the results from the *nifH* gene analysis (Fig. 6), in which the majority of benthic Eckernförde Bay *nifH* sequences obtained in this study were related to two known N_2 -fixing SRB (*Desulfovibrio vulgaris* and *Desulfonema limicola*) or to organisms in the same genera with other SRB. Therefore, it is likely that much of the NA seen in Eckernförde Bay sediments is carried out by SRB.

25 Interestingly, Eckernförde Bay sediments are not generally thought to be deficient in reduced N, having porewater ammonium concentrations as high as 1200 μM (Fig. 3). Because ammonium is a known inhibitor of N_2 -fixation (Postgate, 1982; Dixon, 1984) and is a source of fixed N to the microbial community, it is unclear why SRB would perform N_2 -fixation in these sediments. Overall, Eckernförde Bay NA was highest ($>2 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$) when ammonium was $<400 \mu\text{M}$. These results indicate that there may be some degree of N deficiency, possibly on the micro-scale level, which

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could be overlooked or lost when using traditional porewater extraction techniques that combine all porewater collected over a given depth interval. However, it is surprising to find that NA was still detectable when ammonium concentrations were $>1000 \mu\text{M}$, ranging from 0.42 ± 0.14 to $0.98 \pm 0.24 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$. Based on several environmental studies it has been suggested that different benthic habitats will display differing levels of ammonium inhibition (Capone, 1988), with one study finding that a 50 mM ammonium concentration was needed to inhibit N_2 -fixation in *Spartina* dominated sediments (Dicker and Smith, 1980). It is therefore uncertain if, and at which point, ammonium concentrations in Eckernförde Bay sediments would be high enough to completely inhibit benthic N_2 -fixation.

4.2 Environmental controls on nitrogen fixation and sulfate reduction in Eckernförde Bay sediments

4.2.1 Organic matter provided through pelagic production

Heterotrophic microbial activity in sediments depends on organic matter availability, which can vary seasonally in most coastal marine environments, leading researchers to question if N_2 -fixation by SRB can vary seasonally as well (e.g. Welsh et al., 1996). Studies in the Kiel Bight (of which Eckernförde Bay is a fjord-like extension) have shown that the amount of organic material (from phytoplankton blooms, resuspension, and macrophyte debris) reaching the sediment varies drastically over the course of the year (Graf et al., 1983; Meyer-Reil, 1983). Increases in organic matter loading to the sediments led to increased microbial abundances and activity, as well as increased diversity and biomass of benthic macrofaunal communities. Focusing on Eckernförde Bay, a study looking at benthic microbial activities found that sulfate reduction rates were higher in early and late September than in early March and that these rates positively correlated with sedimentary chlorophyll *a* concentrations, presumably coming from fresh algal material being deposited on the sediment surface (Treude et al., 2005). Large phytoplanktonic blooms are known to occur in Eckernförde

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Bay in spring and autumn (Bange et al., 2011). From these blooms, >50% (spring) and >75% (autumn) of the material has been estimated to reach the benthic system via sedimentation (Smetacek et al., 1984), where it could then stimulate benthic microbial activity. Summer months (July/August) have been shown to support smaller phytoplanktonic blooms (Bange et al., 2011), of which <25% is estimated to reach the sediment surface (Smetacek et al., 1984). Our results are in good agreement with these previous studies in that integrated sulfate reduction and N₂-fixation rates, in general, were higher in summer and autumn months than in winter months (Fig. 8). Additionally, integrated sulfate reduction and N₂-fixation rates slightly decreased in September (794.9 ± 109.5 nmol SO₄²⁻ cm⁻² d⁻¹ and 13.1 ± 1.1 nmol N cm⁻² d⁻¹, respectively) when compared to August (883.2 ± 146.8 nmol SO₄²⁻ cm⁻² d⁻¹ and 22.1 ± 0.5 nmol N cm⁻² d⁻¹, respectively), and then increased again in October (1301.2 ± 146.3 nmol SO₄²⁻ cm⁻² d⁻¹ and 21.9 ± 0.5 nmol N cm⁻² d⁻¹, respectively). This decrease in activity may be due to less-pronounced phytoplankton blooms in August, which would provide less material to the sediment (settling time of planktonic material to the seafloor during this stratification time is on the order of weeks; Graf et al., 1983) and lead to decreased September benthic activities.

For winter months, it has been speculated that winter storms may lead to macrophyte erosion and cause macrophyte debris to reach the sediment (Meyer-Reil, 1983). This debris would provide a highly localized concentration of organic matter that could support increased benthic microbial processes, forming a microbial “hot spot” of activity, such as was seen with sulfate reduction in November and February (Fig. 4). Because two sulfate reduction sub-cores came from one MUC core, it is understandable that in both cases, only two of the replicates showed co-occurring peaks in activity. It is possible that the other sulfate reduction MUC core, as well as the MUC core used for NA sub-sampling and the MUC cores from December, did not hit these patches of increased organic matter and thus did not show elevated microbial rates.

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4.2.2 Oxygen concentrations

During spring and summer months, typically between March and September, the Eckernförde Bay water column becomes highly stratified (Graf et al., 1983; Orsi et al., 1996; Bange et al., 2011). During this stratification time, organic matter from the phytoplankton blooms reaches the deeper waters and microbial degradation of this material leads to the rapid consumption of oxygen. Eventually, hypoxic, or periodically anoxic, deeper waters can form and have been increasingly doing so in Eckernförde Bay over the last 25 yr (Bange et al., 2011). In autumn, typically in October, strong easterly winds break up this stratification and the oxygen-depleted waters are replaced (Orsi et al., 1996). A similar pattern was clearly seen during this study, in that the formation of hypoxic bottom waters began in June/July and continued through October, after which there was a break up of the stratification and a strong increase in bottom water oxygen concentrations in November (Figs. 1 and 9). Because oxygen is an inhibitor of the nitrogenase protein (Postgate, 1998) and because some sulfate-reducing activities can be suppressed by oxygen (reviewed in Muyzer and Stams, 2008), one would expect these microbial activities to increase with decreasing oxygen concentrations. In general, integrated sulfate reduction and N₂-fixation rates in Eckernförde Bay were higher when bottom water oxygen concentrations were lower (Fig. 9). However, relatively high integrated rates were still seen in November, despite the presence of ~220 μM oxygen concentrations. Two possible explanations for this high activity are: (1) because oxygen is a highly favorable electron acceptor in relatively low concentrations compared to such as sulfate, penetration by diffusion into coastal sediments is only a few millimeters (Revsbech et al., 1980; Gunderson and Jørgensen, 1990), meaning that only the benthic microbial community in the top sediment layer is most likely affected by bottom water oxygen concentrations, and (2) many SRB have developed strategies for overcoming oxygen stress (e.g. Krekeler et al., 1997), with some SRB, of which *Desulfovibrio vulgaris* is one (Cypionka, 2000), even capable of respiring oxygen (e.g. Dilling and Cypionka, 1990). Overall, fluctuations in Eckernförde Bay water column oxygen

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concentrations probably serve as more of an indication of organic matter availability to the benthos (i.e. bacterial degradation of organic matter in the water column leads to consumption of oxygen, with the remaining organic matter settling on the seafloor), rather than directly influencing sulfate reduction and N₂-fixation in the sediment. The exception may be for the surface microbial community (0–1 cm), which did indeed show highest microbial rates when bottom water oxygen concentrations were lowest (Figs. 1 and 3). However, hypoxia may have indirect impacts on the benthic microbial community through its influences on the benthic macrofauna community, which will be discussed in Sect. 4.2.4 of this discussion.

4.2.3 Temperature

Like oxygen concentrations, water column temperatures also varied greatly over the course of the year (Fig. 1), with intense stratification during summer months and warmer waters reaching the sediment surface in autumn months, when strong winds may increase the water column mixing depth (Graf et al., 1983; Orsi et al., 1996). Benthic microbial activities (NA and sulfate reduction) positively correlated with bottom water temperatures over the year, with highest activities and temperatures occurring during the late summer/early autumn months (Fig. 9). To test whether temperature directly affected microbial rates, a temperature experiment was performed and the results indicated that, in general, both sulfate reduction rates and NA increased when sediments were incubated at 13 °C vs. 3 °C (Fig. 8). These results were not surprising based on a previous study in Eckernförde Bay (Treude et al., 2005), which found that microbial rates of anaerobic oxidation of methane (AOM) increased steadily from 4 °C to 20 °C, after which there was a small decline. This same study reported that AOM was higher in early September than in early March, which is in good agreement with the microbial activities observed in the present study. Additionally, it has been suggested that the warm productive autumn season leads to a shallowing of the Eckernförde Bay benthic AOM layer, which in turn leads to a shallowing of the methane-dependent sulfate reduction activity (Treude et al., 2005; Dale et al., 2012). This reasoning provides

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another possible explanation for the high sulfate reduction rates seen at the base of two replicate cores in November (Fig. 4). However, the observed peaks in sulfate reduction rates ($>2000 \text{ nmol cm}^{-3} \text{ d}^{-1}$) greatly exceeded potential AOM rates reported from these depths at Boknis Eck ($\sim 100 \text{ nmol cm}^{-3} \text{ d}^{-1}$, Treude et al., 2005), and we therefore suggest that they were caused by hot spots of labile organic matter rather than methane transport.

4.2.4 Benthic organisms and bioturbation

In late spring, the benthic community, mostly consisting of polychaetes (e.g. *Pectinaria koreni*, *Nephtys ciliata*, *Polydora* sp. and *Capitella capitata*), throughout the Kiel Bight begins to develop (Graf et al., 1982; Meyer-Reil, 1983). These organisms not only add to the organic matter content of the sediment via biomass (Meyer-Reil, 1983), but also via bioturbation (as was recently redefined by Kristensen et al., 2012), i.e. all transport processes carried out by animals that directly or indirectly affect sediment matrices, including both particle reworking and burrow ventilation. Bioturbating organisms can increase benthic organic matter availability, and thereby increase microbial activities, through burrow construction (Aller and Aller, 1986; de Vaugelas and Buscail, 1990), through release of fecal pellets (Jørgensen, 1977b), through irrigation techniques that bring organic particles from the overlying water into the burrow-system (Bertics and Ziebis, 2010), and through rapid subduction of labile organic matter that settles on the sediment surface (Graf, 1989; Jørgensen, 1996; Witte et al., 2003; reviewed in Meysman et al., 2006). The increase in organic matter associated with bioturbation has been shown to lead to the formation of reduced microniches within the sediment that can display increased sulfate reduction rates (Goldhaber et al., 1977; Bertics and Ziebis, 2010), as well as increased rates of N_2 -fixation coupled to sulfate reduction (Bertics et al., 2010).

Bioirrigation results (Fig. 2; Dale et al., 2012) indicated that bioirrigation, or bioturbation, was present in early spring, and continued through the summer. As bottom water oxygen concentrations began to decrease (Fig. 9), bioirrigation decreased and

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eventually reached immeasurable levels in September, presumably because there was not enough oxygen present for the organisms to survive. Between April and August large specimens of errant polychaetes (presumably *Nephtys ciliata*) were frequently detected during core slicing (personal observations). The occurrence of these organisms may have led to the increased rates of sulfate reduction and N₂-fixation seen in the upper half of the sediment cores during this time through the formation of sulfate-reducing microniches (Fig. 3; Bertics et al., 2010). In September, only dead polychaetes were seen on the sediment surface of collected cores (personal observations), which is in agreement with the documentation of a decline in bioturbation activity (Fig. 2; Dale et al., 2012). These dead worms may have provided a substantial organic carbon load to the surface sediments, which could be responsible for the intense sulfate reduction and N₂-fixation activity seen in the top sediment layers in October (Fig. 3).

Aside from macrofaunal bioirrigation, sediments can also be irrigated via bubble movement through the sediment matrix (Haeckel et al., 2007). As bubbles rise through soft sediments, porewaters are mixed, causing solute fluxes, and possibly microbial activities, in the sediment to be enhanced (Haeckel et al., 2007). Porewater profiles in Eckernförde Bay sediments suggest that bubble formation and irrigation may have taken place over the course of this study (Dale et al., 2012). Those months that indicated the strongest presence of bubble irrigation were May, September and October (Dale et al., 2012). During these same months, porewater ammonium concentrations did not show the typical increase with sediment depth, but instead remained relatively uniform (Fig. 3), perhaps due to bubble porewater mixing. It is therefore possible that these decreases in ammonium concentrations could have led to the increased nitrogenase activity seen at deeper sediment depths during these times (Fig. 3). Along with causing the outflux of ammonium from the sediment, this irrigation may also have allowed for the transport of sulfate and organic matter deeper into the sediment, thereby causing the increased sulfate reduction rates seen at depth during these months (Fig. 3).

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4.3 Variability in benthic N cycling

Previous modeling of Eckernförde Bay N-cycling estimated that sediments from this location have a fixed N loss rate of $0.08 \text{ mmol N m}^{-2} \text{ d}^{-1}$ during the winter, i.e. in February (Dale et al., 2011). Yet, it was still uncertain how rates of fixed N loss would vary over the course of the year, especially considering the high seasonal variability of environmental conditions in this location. For this reason, potential denitrification rates were measured in the present study during August and September (Fig. 5), i.e., in times when other microbial rates appeared to be highest. Upon integrating these rates down to the deepest sampling depth (Table 1, Fig. 10) and comparing these rates to the February rate from Dale et al. (2011), denitrification appears to follow the same trend as integrated sulfate reduction and N_2 -fixation (Fig. 9), in that rates are high in August, decrease in September, and reach lowest values in February. The ratio of denitrification to N_2 -fixation is highest in August (~ 6.8) but then decreases in September (~ 3.5). In February, N losses and N gains become roughly equal, having a ratio of ~ 1.1 (Fig. 10). These results indicate that there is high temporal variability in net N cycling in Eckernförde Bay sediments, similar to that seen in other marine sediments (e.g. Joye and Paerl, 1994).

For individual sediment depths from both August and September, average denitrification rates were higher than average N_2 -fixation rates (Fig. 5). Looking at individual replicates, there were several depth horizons in September when N_2 -fixation rates were higher than denitrification rates, notably at depths 1–2, 2–3, 18–20, and 20–25 cm, suggesting the possible formation of fixed N gain microniches during certain months of the year and the possible existence of internal N cycling in deeper sediment layers. Due to the lack of information regarding the spatial distribution of denitrification and N_2 -fixation “hot spots” within a given depth horizon, it is uncertain if these areas overlap and consequently no N budgets should be made. However, these results indicate that there is spatial variability in N cycling related microbial activities within each sediment depth horizon of Eckernförde Bay, as has been seen in other marine sediments (e.g. Gilbert,

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et al., 1995; Bertics et al., 2012). Possible reasons for such spatial variability include limited substrate diffusion to crowded sediment grains (Harms and Zehnder, 1994), organic matter availability (Bertics et al., 2012), and bioturbation (Gilbert, et al., 1995; Bertics et al., 2010, 2012).

5 Conclusions

This study examined the possibility of benthic N₂-fixation, perhaps coupled to sulfate reduction, occurring within the seasonally hypoxic Eckernförde Bay. Presented here is one of only a few studies looking at both of these microbial processes over the course of a year on almost a monthly basis. N₂-fixation and sulfate reduction profiles showed similar patterns both spatially and temporally, suggesting that these two processes might be linked within Eckernförde Bay sediments. Molecular analysis confirmed the presence of *nifH* sequences related to two known N₂-fixing SRB, *Desulfovibrio vulgaris* and *Desulfonema limicola*, further supporting the hypothesis that some of the observed N₂-fixation may be coupled to sulfate reduction. Denitrification also showed a seasonal pattern similar to that of N₂-fixation and sulfate reduction. However, the ratio of denitrification to N₂-fixation changed drastically between summer and winter months. In August and September, denitrification greatly outweighed N₂-fixation, while in February the rates were almost equal. These results indicate that the sediments of Eckernförde Bay may alternate between being a net sink or a net source of fixed N over the course of the year. Overall, all of these benthic processes appeared to be greatly impacted by many seasonally varying environmental conditions, such as planktonic blooms, temperature, oxygen, bioturbation, and storm events.

Areas of coastal marine hypoxia are globally increasing (Middelburg and Levin, 2009) and understanding N cycling in these environments is crucial for predicting how these expanding regions will impact N cycling worldwide. For this reason, areas such as Eckernförde Bay, which are seasonally hypoxic, are valuable study sites for monitoring the response of ecosystems to the changes in available organic matter and oxygen

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concentrations, stressing the importance of time series measurements in these coastal environments.

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Table 1. Integrated Areal Denitrification versus Nitrogen Fixation Rates (\pm SD).

Month	Denitrification ($\text{nmol N cm}^{-2} \text{ d}^{-1}$)	Nitrogen Fixation ($\text{nmol N cm}^{-2} \text{ d}^{-1}$)	Integration Depth (cm)
February	8 ^{a,b}	7.6 \pm 0.4	60 (D), 25 (NF)
August	121.5 \pm 0.8 ^c	18.0 \pm 0.4	14
September	53.1 \pm 1.8 ^c	14.9 \pm 1.0	25

^a Modeled N-turnover rates from Dale et al. (2011).

^b These values represent the total fixed N lost via denitrification and anammox combined.

^c Potential rates.

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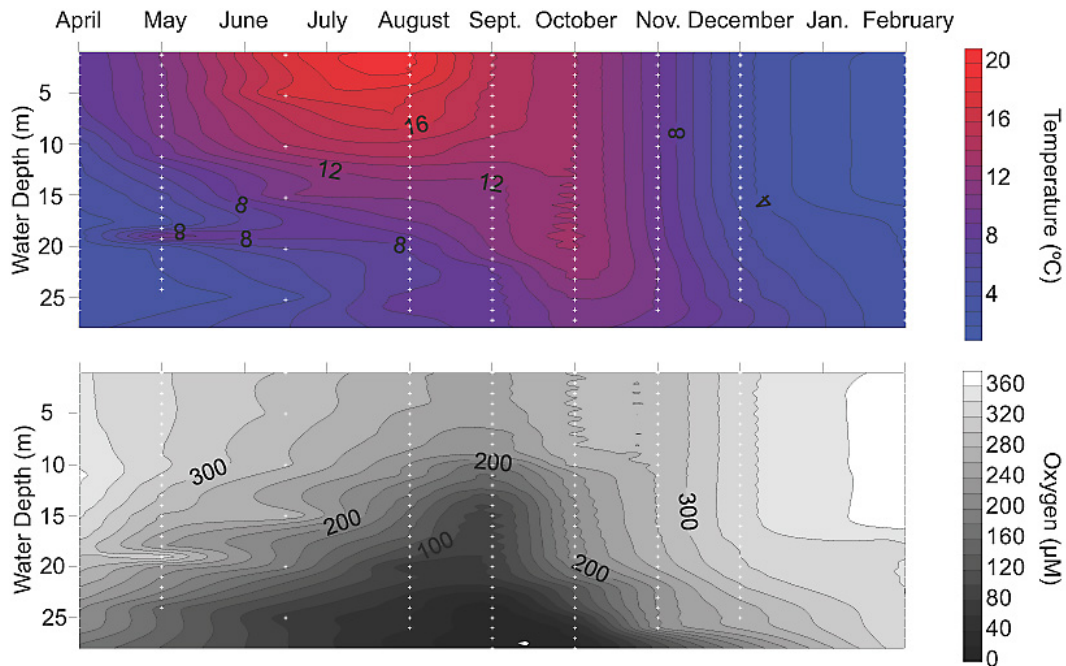


Fig. 1. Contour plots of water column temperature (upper) and oxygen concentrations (lower graph) over the course of the study, from April 2010 to February 2011. White dots represent data collection points.

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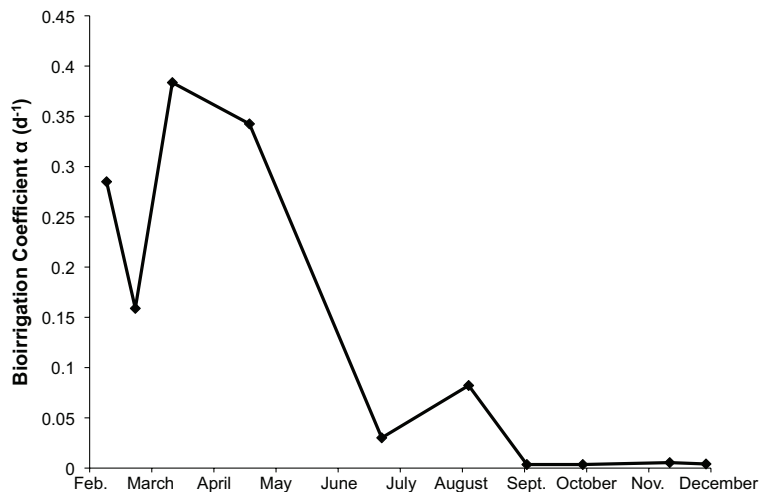


Fig. 2. Bioirrigation coefficients (α) for sediment cores collected from February through December 2010, as determined by Dale et al. (2012).

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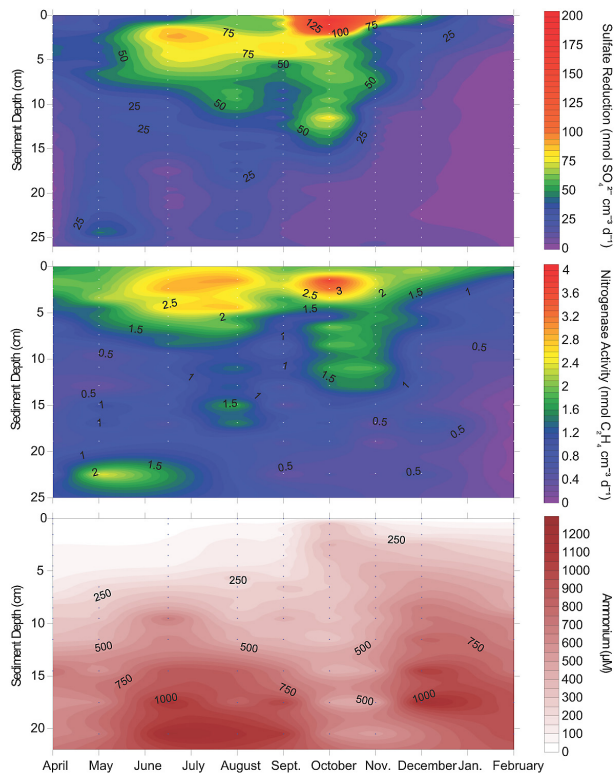


Fig. 3. Contour plots of benthic sulfate reduction rates (upper), nitrogenase activity (middle), and porewater ammonium concentrations (lower graph) over the course of the study, from April 2010 to February 2011. The white and blue dots represent data collection points. Note that the isoline scale on the sulfate reduction graph has been adjusted to allow higher resolution in the 0–100 $\text{nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ range.

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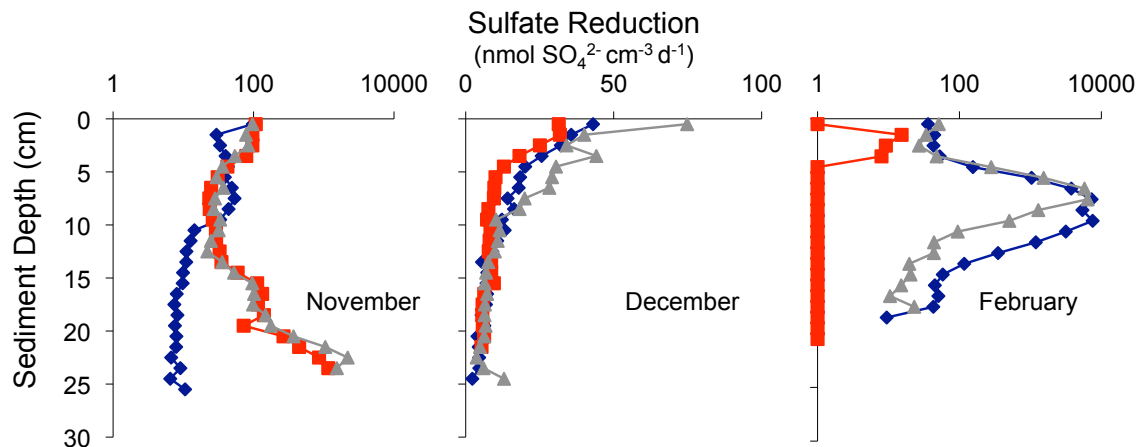


Fig. 4. Sulfate reduction depth profiles of the November 2010 and February 2011 sampling, which revealed exceptionally high rates in some replicates that were not included in contour plots (Fig. 3). Profiles from December 2010 are shown for comparison purposes. Each line (red, blue, and gray) represents a replicate sub-core used for the rate measurements. Please note the logarithmic scale for November and February.

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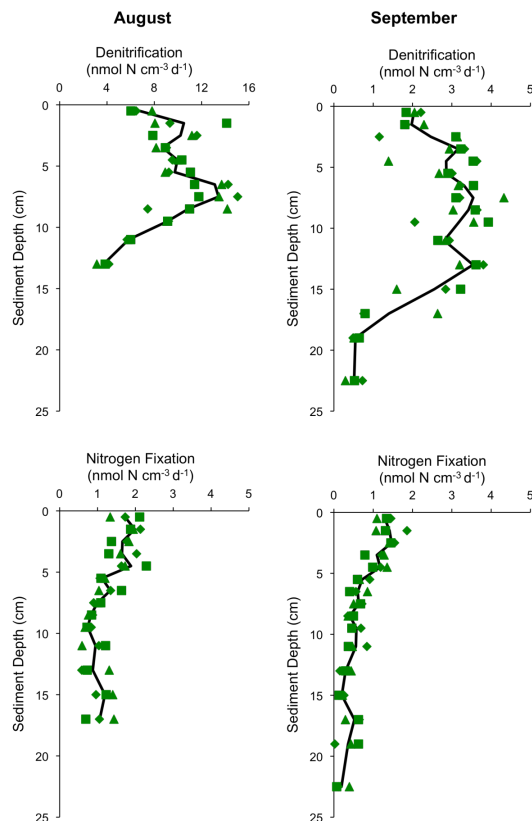


Fig. 5. Denitrification (upper) and nitrogen fixation (lower graph) depth profiles from August and September 2010. Each replicate rate measurement is shown with a symbol and the average depth profile is indicated with the black line. Note the scale difference on the x-axes of the August denitrification profile. Consider that denitrification activity represent potential rates (see methods).

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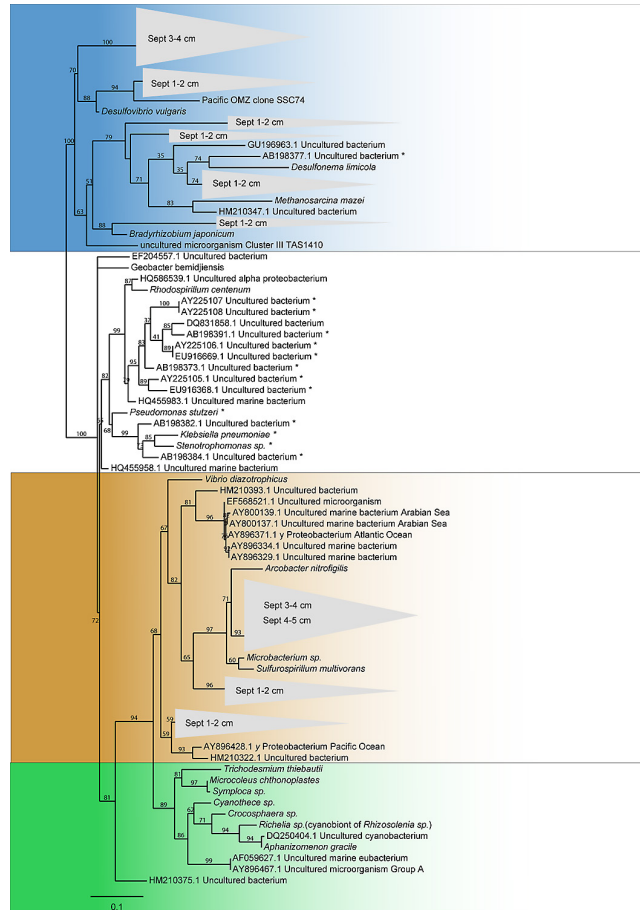


Fig. 6. Phylogenetic tree based on the analysis of 74 *nifH* gene sequences retrieved in this study. Novel identified clusters are indicated by grey triangles. Cyanobacterial reference sequences are highlighted in green, proteobacterial sequences are in brown, and Cluster III sequences as defined by Zehr and Turner (2001) in blue. Bootstrapped values (%) above 50, out of 100, are shown on branches. The scale bar represents 10 % estimated sequence divergence. Sequences marked with an asterisk indicate likely contaminated PCR products previously reported by Turk et al. (2011), the novel clusters are mostly distant from those sequences.

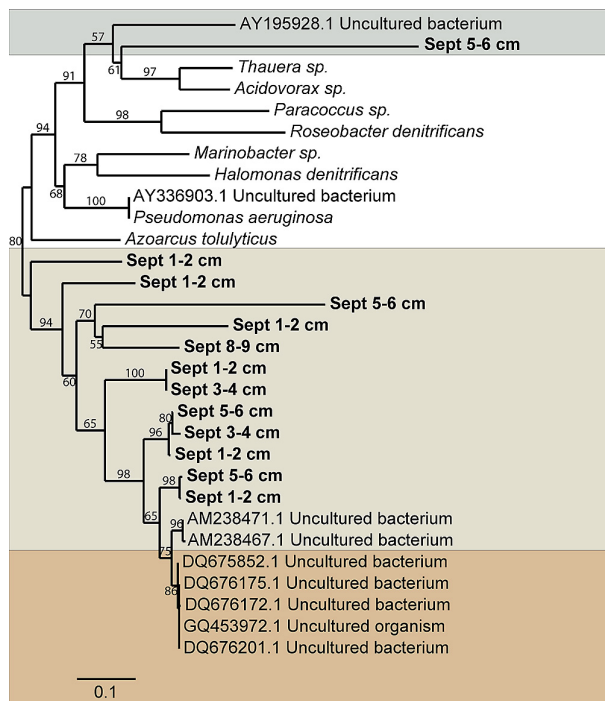


Fig. 7. Phylogenetic tree based on the analysis of 88 *nirS* gene sequences gained during this study. Novel identified clusters are shown in bold. Dark brown reference sequences were derived from Chesapeake Bay sediment samples (Francis et al., 2003), light brown sequences from previous studies of Baltic Sea sediments (Falk et al., 2007) and grey highlighted sequences from sediments underlying OMZ waters off Mexico in the Pacific Ocean (Liu et al., 2003). Bootstrapped values (%) above 50, out of 100, are shown on branches. The scale bar represents 10 % estimated sequence divergence.

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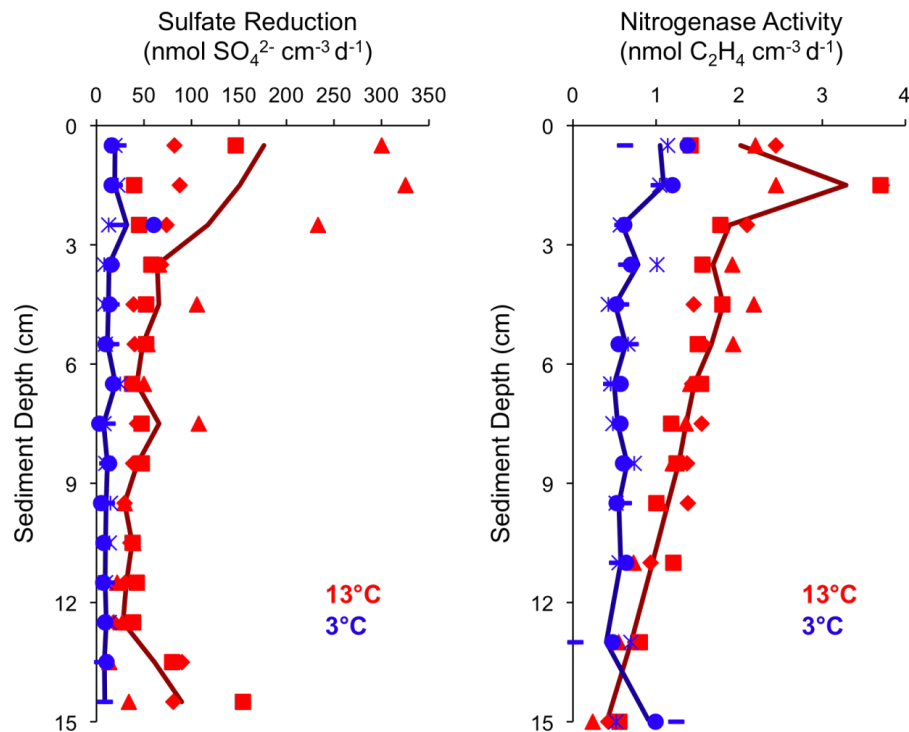


Fig. 8. Sulfate reduction (left) and nitrogenase activity (right) depth profiles obtained during the temperature experiment in October 2011. Results from the 3°C incubation are in blue, while results from the 13°C incubation are in red. Each replicate rate measurement is shown with a symbol and the average depth profile is indicated with a line.

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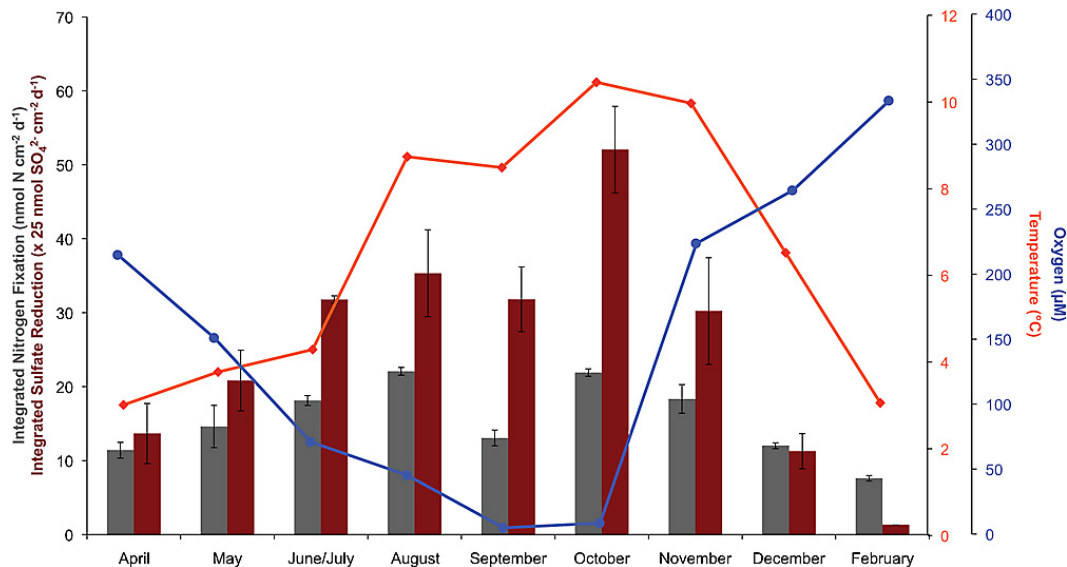


Fig. 9. Monthly integrated rates of sulfate reduction (red bars) and nitrogen fixation (gray bars) down to a sediment depth of 18 cm from April 2010 to February 2011. Overlaying this data are bottom water temperature (orange line) and oxygen concentrations (blue line). Error bars represent standard deviation.

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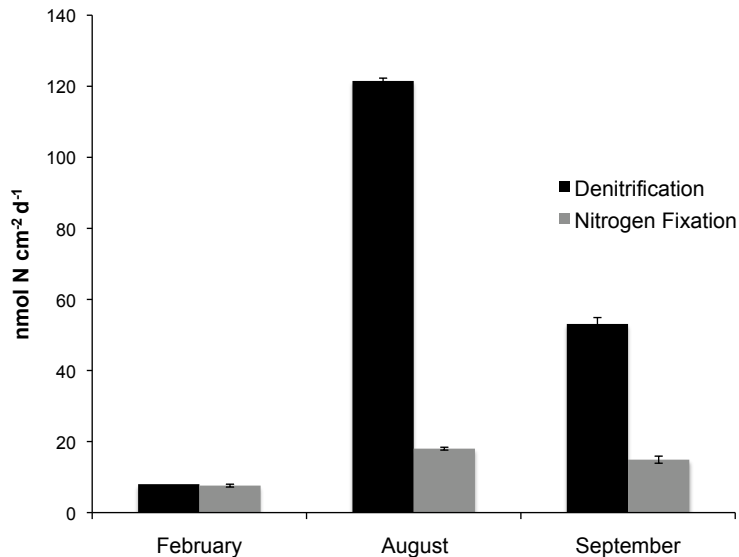


Fig. 10. Areal integrated rates of denitrification and nitrogen fixation over the course of the year. Exact values can be found in Table 1. Error bars represent standard deviation.

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