



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

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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<sub>2</sub>). 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<sub>2</sub> fixation, possibly by SRB, plays a role in N cycling within the seasonally hypoxic sediments from the Eckernförde Bay, Baltic Sea. Monthly samplings were performed over the course of one year to measure nitrogenase activity (NA) 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, the active N-fixing community was examined via molecular tools. Integrated rates of N<sub>2</sub> fixation (approximated from NA) and sulfate reduction showed a similar seasonality pattern, with highest rates occurring in August (approx. 22 and 880 nmol cm<sup>-3</sup> d<sup>-1</sup> of N and SO<sub>4</sub><sup>2-</sup>, respectively) and October (approx. 22 and 1300 nmol cm<sup>-3</sup> d<sup>-1</sup> of N and SO<sub>4</sub><sup>2-</sup>, respectively), and lowest rates occurring in February (approx. 8 and 32 nmol cm<sup>-3</sup> d<sup>-1</sup> of N and SO<sub>4</sub><sup>2-</sup>, respectively). These rate changes were positively correlated with bottom water temperatures and previous reported plank-

ton 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<sub>2</sub> fixing SRB, namely *Desulfovibrio vulgaris* and *Desulfonema limicola*, supporting the hypothesis that some of the nitrogenase activity detected may be attributed to SRB. 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  $10^6$  km<sup>2</sup> (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; Midelburg 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 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 Farías (2009) have shown that significant rates of nitrification can also be observed, even at oxygen concentrations  $\leq 5$   $\mu$ M. However, all of these processes may lead to a loss of oceanic fixed N, mostly through the eventual production of molecular nitrogen (N<sub>2</sub>) 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  $\sim 200$  Tg N yr<sup>-1</sup> (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 an incorrect estimate of sources and sinks (Capone and Knapp, 2007). Because biological N<sub>2</sub> 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<sub>2</sub> fixation has traditionally received much attention. While the majority of studies concerning marine N<sub>2</sub> 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). However, the majority of benthic studies have focused on N<sub>2</sub> fixation linked to phototrophic activity, and not on dark heterotrophic-driven N<sub>2</sub> fixation. For both pelagic and benthic N<sub>2</sub> fixation research, much of the current work is focusing on methodological refinements (e.g. underestimation of N<sub>2</sub> fixation rates when using the bubble technique; Mohr et al., 2010; Großkopf et al., 2012) 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.

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 N<sub>2</sub> 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., 1996b; 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 N<sub>2</sub> fixation (based on nitrogenase activity) and sulfate reduction, along with pore-water geochemistry, were determined monthly. Additionally, at several selected time points, molecular analysis of key functional genes was performed for determination of which microorganisms were present with the genetic potential of performing N<sub>2</sub> fixation. The overall goal was to determine whether N<sub>2</sub> fixation, especially dark heterotrophic N<sub>2</sub> fixation, is occurring in Eckernförde Bay sediments, and if so, whether this activity (1) shows seasonal variability, and (2) is coupled to sulfate reduction.

## 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 mean water depth of  $\sim 28$  m, located in the southwestern Baltic Sea. Because of the shallow water depth, there is a strong benthic-pelagic coupling that results in organic-rich sediments (Orsi et al., 1996b) 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 Orsi et al. (1996b) and Treude et al. (2005).

## 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 R/V *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, and oxygen concentrations (based on optode measurements). Profiles were saved and viewed on a PC using the OceanLab 3 software (Hydro-Bios). Following the cast, a miniature multiple corer (MUC; K. U. M., Kiel, Germany) equipped with four cores liners (length  $\sim 60$  cm, inner diameter 10 cm) was used to obtain altogether 6–12 sediment cores of  $\sim 35$  cm length. Cores were then brought back to GEOMAR and taken to a 10 °C cold room where the cores were processed within a few hours of collection.

## 2.3 Porewater geochemistry and sediment properties

Porewater samples were collected each month from two MUC cores. One core was sampled using anaerobic (i.e. flushed with N<sub>2</sub> gas) 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  $\mu$ m 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 sulfate concentrations. Ammonium measurements were performed on a Hitachi UV/VIS spectrophotometer following standard photometric procedures with a precision of 5.5 % (Grasshoff et al., 1999). Because high sulfide concentrations may interfere with ammonium measurements, these sub-samples were acidified with 20  $\mu$ L of HCl and bubbled with argon to strip any hydrogen sulfide. Sulfate concentrations were measured using ion chromatography with the IAPSO seawater standard for calibration (precision of 1.5 %). 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 collected for determination of porosity. Porosity was calculated from the water content (weighing fresh and freeze-dried sediment samples) assuming a dry solid density of 2.65 g cm<sup>-3</sup>.

Additionally, as an indication of seasonal benthic bioirrigation 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. (2013). In short, Br<sup>-</sup> 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 Br<sup>-</sup> was determined in the squeezed porewater by ion chromatography using a Metrohm ion chromatograph with a conventional anion exchange column with IAPSO seawater standard for calibration (relative precision of < 2 % for natural seawater samples). The determination of the depth distribution of Br<sup>-</sup> allowed for the estimation of irrigation rates by modeling the transient infiltration of Br<sup>-</sup> into the sediment. For more details on this method and for further explanation concerning the calculation of the porewater-derived bioirrigation coefficient  $\alpha$ , see Dale et al. (2013).

## 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-cm<sup>3</sup> sediment samples from each depth horizon were placed in 60 mL serum vials flushed with N<sub>2</sub>. Each vial was crimp sealed, injected with 5 mL of C<sub>2</sub>H<sub>2</sub> to saturate the nitrogenase, if present, and the increase in C<sub>2</sub>H<sub>4</sub> 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. N<sub>2</sub> fixation rates were calculated from the linear portion of the NA results using a conversion factor of 3 C<sub>2</sub>H<sub>4</sub> : 1 N<sub>2</sub>, which is based on previous direct <sup>15</sup>N<sub>2</sub> 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-cm<sup>3</sup> samples from each depth interval were placed into 15 mL serum vials flushed with N<sub>2</sub> and used as control samples. To one vial set, no C<sub>2</sub>H<sub>2</sub> was added to determine if natural C<sub>2</sub>H<sub>4</sub> production was occurring and to the other set, 1 mL of 37.5 % Formalin was added along with 1 mL of C<sub>2</sub>H<sub>2</sub> to serve as a kill control. No increase in C<sub>2</sub>H<sub>4</sub> was detected in these control samples.

## 2.5 Benthic sulfate reduction rates

During each sampling month, 2 MUC cores were subsampled 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  $\mu\text{L}$ , 200 kBq, specific activity 37 TBq  $\text{mmol}^{-1}$ ) 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 Amplification, cloning and sequencing of benthic *nifH*

DNA was extracted from  $\sim 100$  mg of sediment, collected in September 2010, using the FastDNA<sup>®</sup> SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA, USA). The *nifH* gene (encoding for one subunit of nitrogenase, the key enzyme of  $\text{N}_2$  fixation) was PCR amplified using primers and PCR conditions according to Zehr et al. (1998). Cloning of PCR amplicons was performed using the TopoTA Cloning<sup>®</sup> Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Sanger sequencing (74 *nifH* 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*, sequence differences were set on a minimum of 5 %.

## 2.7 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 processing within a few hours of collection. All cores were subsampled 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-cm<sup>3</sup> sediment samples from each depth horizon were placed in

120 mL serum vials flushed with  $\text{N}_2$  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

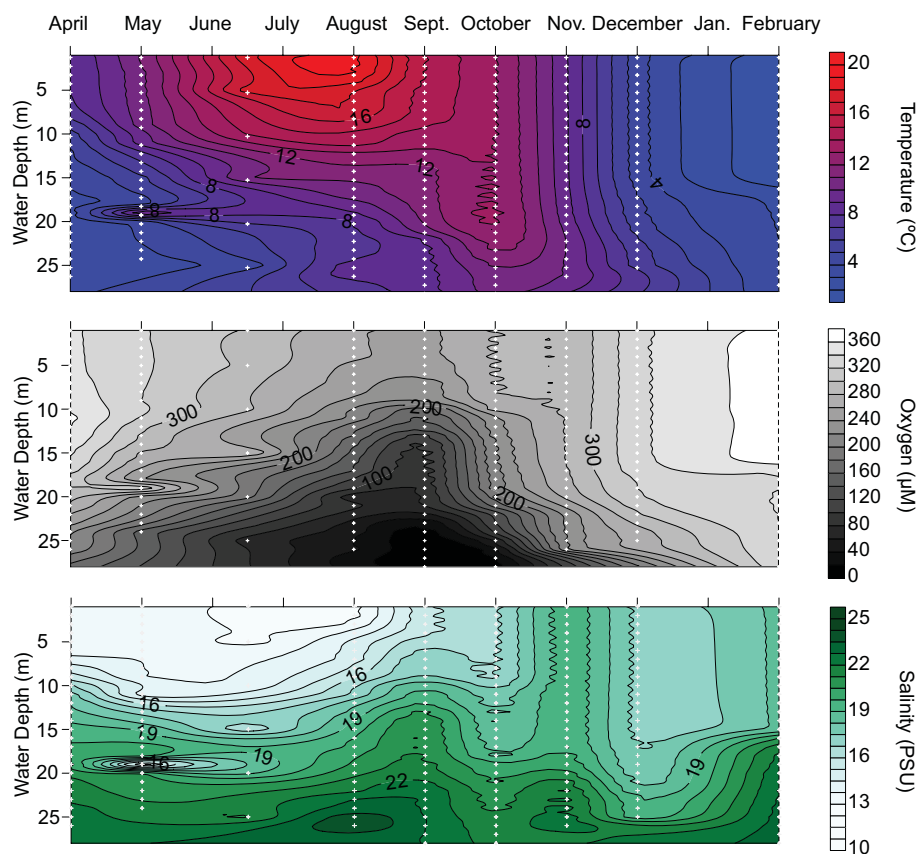
### 3.1 Water column parameters

Over the course of the year, water column temperature, oxygen, and salinity 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, reaching lowest values (1–3 °C) in February. In contrast, water column oxygen concentrations were highest ( $> 360 \mu\text{M}$ ) in February. Hypoxic waters ( $< 63 \mu\text{M}$ ) formed in the deeper water depths of August and persisted through October, reaching lowest values ( $\sim 5 \mu\text{M}$ ) in September. Bottom water salinity ranged from 19–24 ‰, while surficial water salinity ranged from 12–19 ‰. The 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 ‰), supporting the idea of summer stratification, which was also seen with water column temperatures and oxygen concentrations (Fig. 1).

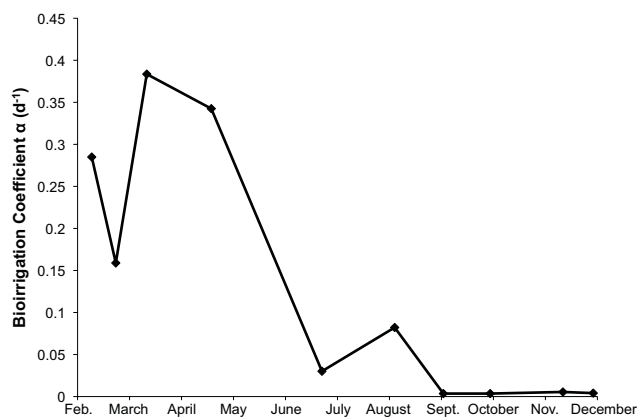
### 3.2 Sediment parameters

Sediment porosity remained relatively stable over the course of the year. Typically, surface sediments had a porosity of  $\sim 0.92$ . Porosity then gradually decreased with sediment depth, reaching a porosity of  $\sim 0.80$  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., 2013). 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 their lowest values ( $< 5 \mu\text{M}$ , Fig. 1).



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



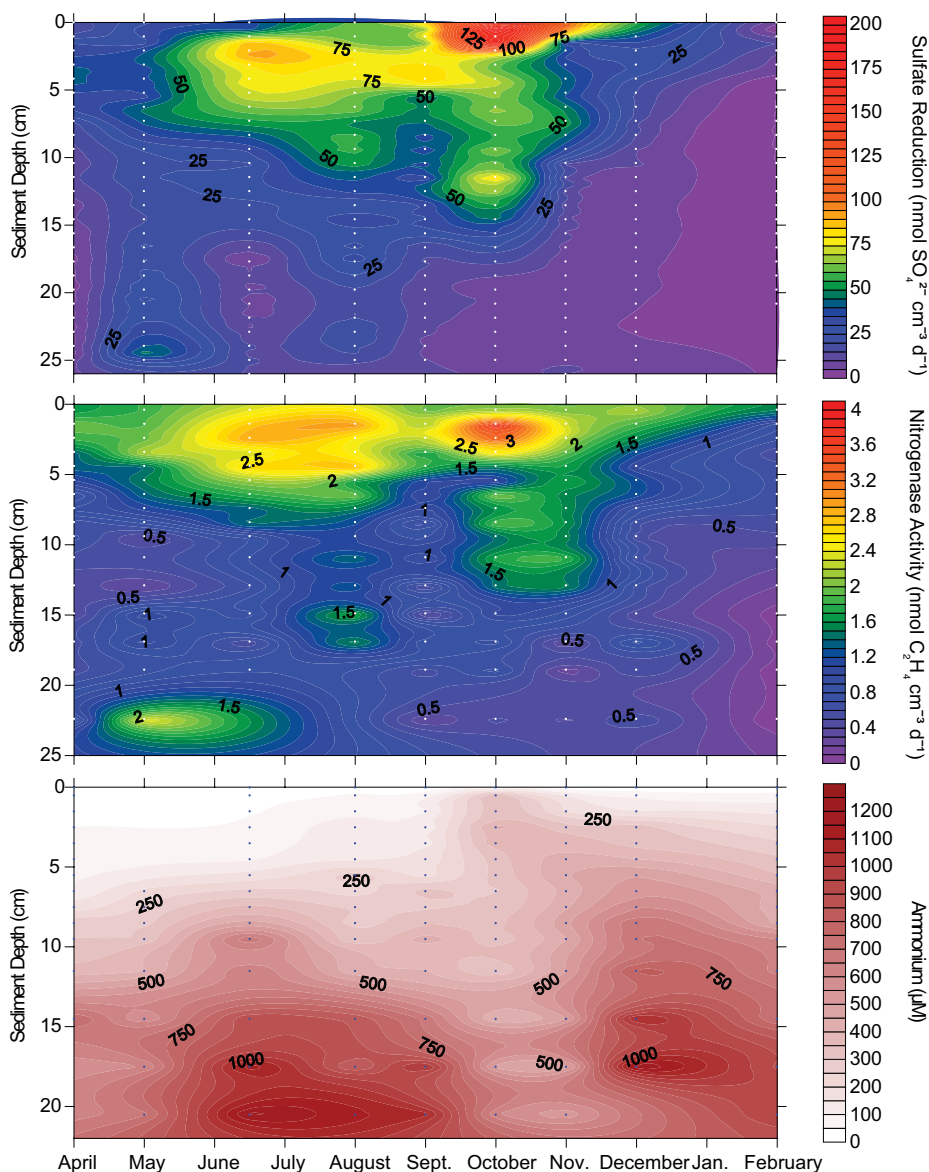
**Fig. 2.** Bioirrigation coefficients ( $\alpha$ ) for sediment cores collected from February through December 2010, as determined by Dale et al. (2013).

### 3.3 Benthic biogeochemical rates and geochemistry

Benthic nitrogenase activity (NA) closely mirrored sulfate reduction 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  $2.4 \pm 1.6 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ . Integrating these microbial activities each month down to a sediment depth of 18 cm (Fig. 7), sulfate reduction rates ranged from  $32.4 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$  (February) to  $1301.2 \pm 146.3 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$  (October) and NA ranged from  $7.6 \pm 0.4 \text{ nmol N}_2 \text{ cm}^{-2} \text{ d}^{-1}$  (February) to  $22.1 \pm 0.5 \text{ nmol N}_2 \text{ cm}^{-2} \text{ d}^{-1}$  (August).

In November and February, there were two replicates for sulfate reduction measurements that displayed unusually high rates, over 1000 and 5000  $\text{nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ ,

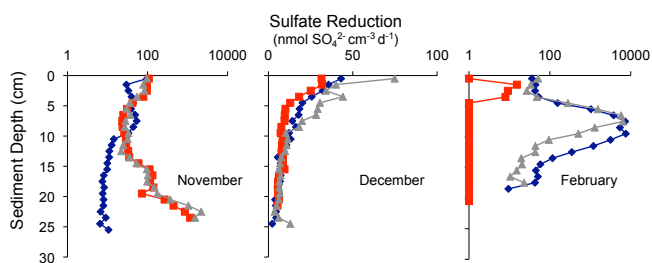


**Fig. 3.** Contour plots of benthic sulfate reduction rates (upper), nitrogenase activity (middle), and porewater ammonium concentrations (lower graph) over the course of the study, from April 2010 to February 2011. The white and blue dots represent data collection points. Note that the isoline scale 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.

respectively, although all replicates in December displayed typical rates with depth (Fig. 4). Because these high rates appear to be rather localized, these were not used in formation of the sulfate reduction contour plot and integrated rates, 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 concentra-

tions ( $416 \mu\text{M}$ ) 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 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.



**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 grey) represents a replicate sub-core used for the rate measurements. Please note the logarithmic scale for November and February.

### 3.4 *nifH* 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. 5). 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).

### 3.5 Temperature experiment

In general, sulfate reduction rates and NA were higher in October 2011 samples incubated at 13 °C versus 3 °C (Fig. 6). 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

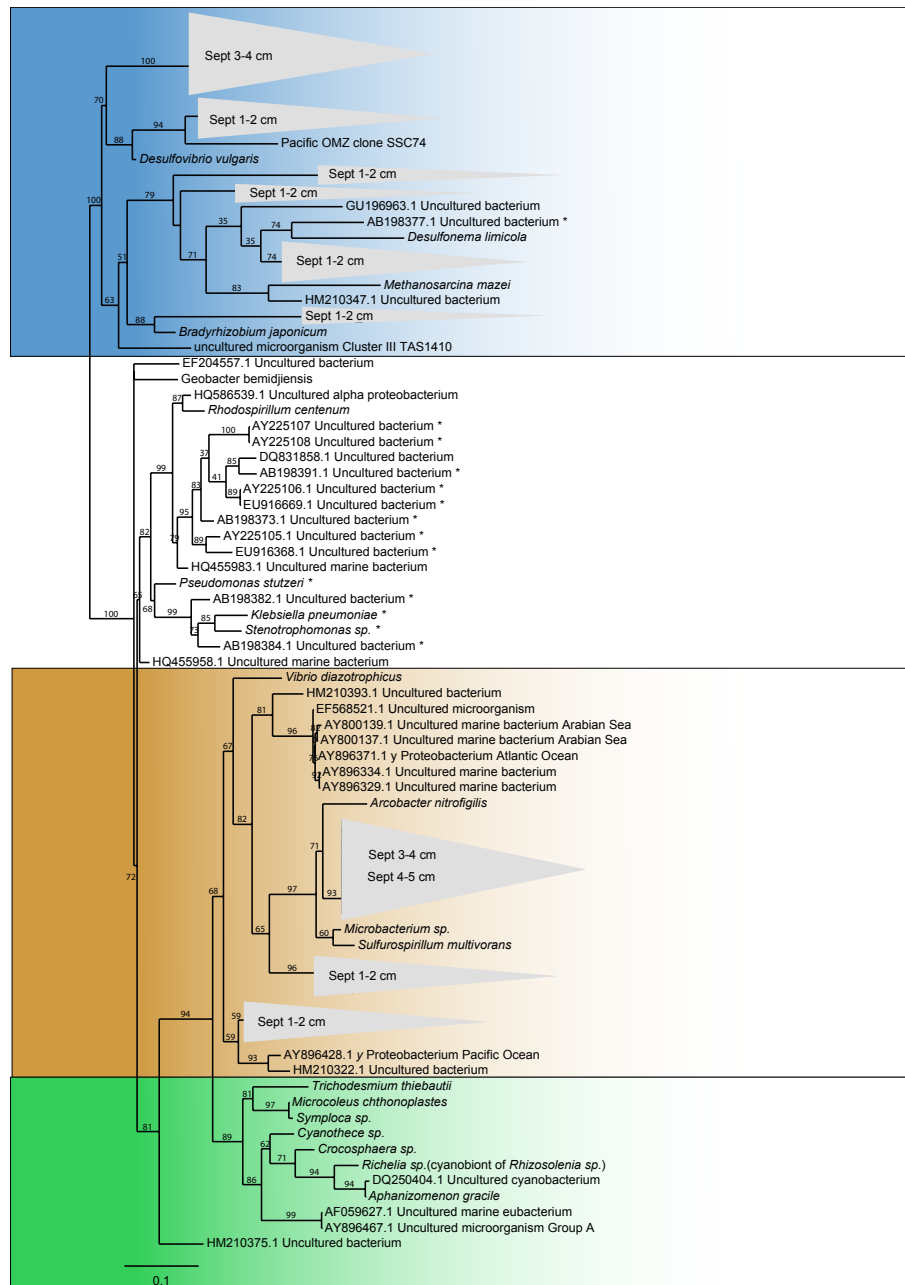
### 4.1 Benthic nitrogen fixation coupled to sulfate reduction

Sulfate reduction is one of the most dominant biogeochemical processes in marine sediments, especially in coastal re-

gions 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  $\text{N}_2$  (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., 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).

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. 7), both activities showed 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  $\text{N}_2$  fixation and sulfate reduction changes dramatically over the course of the year, signifying that different environmental factors may be impacting these rates. These factors will be discussed in more detail in the next section. However, the overall trend similarities again suggest that  $\text{N}_2$  fixation may be coupled to, or at least respond to, some of the same environmental factors as sulfate reduction. This idea was further supported by the results from the *nifH* gene analysis (Fig. 5), in which the majority of benthic Eckernförde Bay *nifH* sequences obtained in this study were related to two known  $\text{N}_2$  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.

Interestingly, Eckernförde Bay sediments are not generally thought to be deficient in reduced N, having porewater ammonium concentrations as high as  $1200 \mu\text{M}$  (Fig. 3). Because ammonium is a known inhibitor of  $\text{N}_2$  fixation (Postgate 1982; Dixon, 1984) and is a source of fixed N to the microbial community, it is unclear why SRB would perform  $\text{N}_2$  fixation in these sediments. However,  $\text{N}_2$  fixation has been shown to remain apparently unaffected by substantial (>  $100 \mu\text{M}$ ) ammonium concentrations in many other benthic environments (for review of these studies see Knapp, 2012), suggesting that ammonium inhibition in sediments may be more complex than originally thought. These complexities are even more apparent when looking at the high rates of  $\text{N}_2$  fixation in dark, ammonium-rich sediments (e.g. Bertics et al., 2010). One possible explanation for  $\text{N}_2$  fixation in the



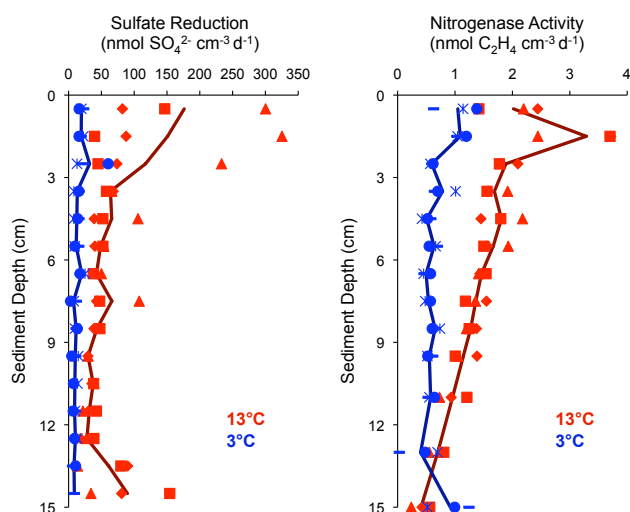
**Fig. 5.** Phylogenetic tree based on the analysis of 74 *nifH* gene sequences retrieved in this study. Novel identified clusters are indicated by grey triangles. Cyanobacterial reference sequences are highlighted in green, proteobacterial sequences are in brown, and Cluster III sequences as defined 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.

presence of high ammonium concentrations is that benthic  $N_2$  fixation may serve as an excess electron sink, especially if a viable Calvin–Benson–Bassham pathway is not present (Joshi and Tabita, 1996; Tichi and Tabita, 2000).

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 could suggest that perhaps there is some de-

gree of N deficiency, possibly on the micro-scale level, which could be overlooked or lost when using traditional porewater extraction techniques that combine all porewater collected over a given depth interval. However, it should be noted that some of the ammonium variations seen between the different sampling months could be due to spatial variability, as only one core from each month





**Fig. 6.** 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.

was used for ammonium measurements. It is surprising to find that NA was still detectable when ammonium concentrations were  $> 1000 \mu\text{M}$ , ranging from  $0.42 \pm 0.14$  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  $\text{N}_2$  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  $\text{N}_2$  fixation.

## 4.2 Environmental controls on nitrogen fixation and Sulfate reduction in Eckernförde Bay Sediments

### 4.2.1 Temperature

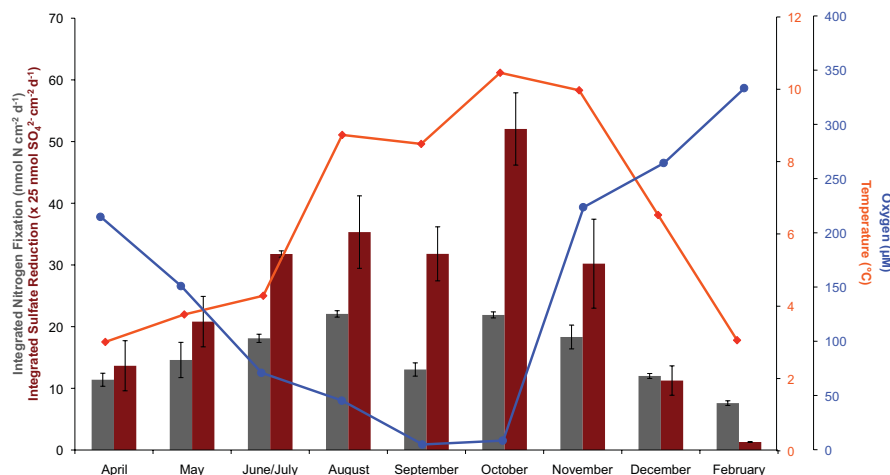
During spring and summer months, typically between March and September, the Eckernförde Bay water column becomes highly stratified (Graf et al., 1983; Orsi et al., 1996b; Bange et al., 2011), causing warmer waters at the surface and colder waters at depth (Fig. 1). In autumn months, when strong winds increase the water column mixing depth, warmer waters begin reaching the sediment surface (Graf et al., 1983; Orsi et al., 1996b). 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. 7). To test whether temperature directly affected micro-

bial 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 versus 3 °C (Fig. 6). However, NA appeared to be less affected by temperature changes, having a  $Q_{10}$  of 2, versus a  $Q_{10}$  of 5.2 for sulfate reduction rates, which is higher than the previous reported  $Q_{10}$  of  $3 \pm 0.5$  for sulfate reduction in Aarhus Bay sediments (Thamdrup et al., 1998). This same temperature pattern was seen during the monthly sampling (Fig. 7), where NA varied less drastically with changing temperature than sulfate reduction rates.

The effect of temperature on Eckernförde Bay NA and sulfate reduction was not surprising based on a previous study in Eckernförde Bay (Treude et al., 2005), which found that another microbial process, the 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 activity change along with temperature for the microbial processes 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., 2013). This reasoning provides a 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 likely caused by hot spots of labile organic matter rather than methane transport.

### 4.2.2 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  $\text{N}_2$  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



**Fig. 7.** Monthly integrated rates of sulfate reduction (red bars) and nitrogen fixation (grey 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.

being deposited on the sediment surface (Treude et al., 2005). Large phytoplanktonic blooms are known to occur in Eckernförde 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 N<sub>2</sub> fixation rates, in general, were higher in summer and autumn months than in winter months (Fig. 7). Additionally, integrated sulfate reduction and N<sub>2</sub> fixation rates slightly decreased in September ( $794.9 \pm 109.5$  nmol SO<sub>4</sub><sup>2-</sup> cm<sup>-2</sup> d<sup>-1</sup> and  $13.1 \pm 1.1$  nmol N cm<sup>-2</sup> d<sup>-1</sup>, respectively) when compared to August ( $883.2 \pm 146.8$  nmol SO<sub>4</sub><sup>2-</sup> cm<sup>-2</sup> d<sup>-1</sup> and  $22.1 \pm 0.5$  nmol N cm<sup>-2</sup> d<sup>-1</sup>, respectively), and then increased again in October ( $1301.2 \pm 146.3$  nmol SO<sub>4</sub><sup>2-</sup> cm<sup>-2</sup> d<sup>-1</sup> and  $21.9 \pm 0.5$  nmol N cm<sup>-2</sup> d<sup>-1</sup>, 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, one hypothesis for the high sulfate reduction rates seen in Eckernförde Bay sediments during this time (Fig. 4) is that microbial “hot spots” of activity may form around localized patches of increased organic matter. It has been speculated that winter storms may lead to macrophyte erosion and cause macrophyte debris to reach the sediment (Meyer-Reil, 1983). Along with this debris, storms

may deposit large quantities of sand and mud, sometimes several centimeters thick, on the sediment surface (Khandriche et al., 1986; Milkert, 1994; Orsi et al., 1996a), which could assist in the rapid burial of macrophyte and other surficial organic material. Treude et al. (2005), using chlorophyll *a* concentrations and chlorin indexes, found that in early September, fresh organic material was located mostly at the sediment surface while in the winter, fresh organic matter appeared to be buried deeper into the sediment, producing irregular profiles in the top 10 cm of the sediment. The burial of macrophyte debris and patches of other surficial organic material could provide a highly localized concentration of organic matter that supports increased benthic microbial processes, forming a microbial “hot spot” of activity, such as was seen with sulfate reduction in November and February (Fig. 4). This type of microenvironment activity has been well documented in other coastal benthic habitats (e.g. Brandes and Devol, 1995; Bertics and Ziebis, 2010). Other sources of localized organic matter may include dead fauna (e.g. worms, fish, etc.), macrofaunal fecal pellets, and for February, the onset of bioturbation, which will be discussed more in Sect. 4.2.4. 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 subsampling and the MUC cores from December, did not hit these patches of increased organic matter and thus did not show elevated microbial rates.

#### 4.2.3 Oxygen concentrations

During times of stratification in Eckernförde Bay, organic matter from 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., 1996b). 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 7). 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), it is possible for these microbial activities to increase with decreasing oxygen concentrations. In general, integrated sulfate reduction and  $N_2$  fixation rates in Eckernförde Bay were higher when bottom water oxygen concentrations were lower (Fig. 7). However, relatively high integrated rates were still seen in November, despite the presence of  $\sim 220 \mu\text{M}$  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) and was previously shown to penetrate only 2 mm into Eckernförde Bay sediments (Preisler et al., 2007), 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 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  $N_2$  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.

#### 4.2.4 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 con-

tent 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) and  $N_2$  fixation rates (Bertics et al., 2012) as well as increased rates of  $N_2$  fixation coupled to sulfate reduction (Bertics et al., 2010).

Bioirrigation results (Fig. 2; Dale et al., 2013) indicated that bioirrigation was present in early spring, and continued through the summer. As bottom water oxygen concentrations began to decrease (Fig. 7), bioirrigation decreased and 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  $N_2$  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., 2013). 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 NA 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 (Roden and Tuttle, 1992; 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., 2013). Those months that indicated the strongest presence of bubble irrigation were May, September and October (Dale et al., 2013). During these same months, porewater ammonium concentrations did not show the typical increase with sediment depth, but instead

**Table 1.** Integrated N<sub>2</sub> fixation rates in Eckernförde Bay sediments compared to rates from other benthic environments. Only the highest and lowest integrated values from Eckernförde Bay are shown. AR indicates acetylene reduction.

Environment	N <sub>2</sub> fixation (mmol N m <sup>-2</sup> d <sup>-1</sup> )	Sediment Depth (cm)	Method	Reference
Eckernförde Bay				
February 2011 (lowest)	0.08 ± 0.004	0–18	AR	this study
August 2010 (highest)	0.22 ± 0.005	0–18	AR	this study
Recent Studies				
Eutrophic estuary	0–18.5	0–20	Net N <sub>2</sub> fluxes in benthic flux chambers and MIMS	Rao and Charette (2012)
Bioturbated coastal lagoon	0.8–8.05	0–10	AR	Bertics et al. (2010)
Lagoon microbial mat	1.17	mat	AR	Charpy et al. (2007)
Mangrove sediments	0–1.21	0–1	AR	Lee and Joye (2006)
Intertidal microbial mat	1.63 ± 1.15	mat	AR	Steppe and Paerl (2005)
Traditional Averages by Benthic Environment				
Lake sediment				
Heterotrophic	0.02 ± 0.03	–	–	Howarth et al. (1988)
Phototrophic	0.03 ± 0.02	–	–	Howarth et al. (1988)
Atlantic Ocean (2800 m)	0.00008	–	–	Howarth et al. (1988)
< 200 m sediments	0.02 ± 0.01	–	–	Capone (1983)
Bare estuarine sediments	0.08 ± 0.03	–	–	Capone (1983)
<i>Zostera</i> estuarine sediments	0.39	–	–	Capone (1983)
Coral reef sediments	6.09 ± 5.62	–	–	Capone (1983)
Mangrove rhizosphere	0.56	–	–	Capone (1983)
Mangrove mats	1.66	–	–	Capone (1983)
Salt marsh rhizosphere	5.27 ± 3.64	–	–	Capone (1983)
Salt marsh surface sediment	0.38 ± 0.41	–	–	Capone (1983)

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 NA seen at deeper sediment depths during these times (Fig. 3).

#### 4.3 Impact on benthic N cycling

Comparing integrated rates of N<sub>2</sub> fixation in Eckernförde Bay with rates from other studies/environments (Table 1), Eckernförde Bay rates most closely resemble those from bare estuarine sediments, salt marsh surface sediments, or those sediments present at water depths < 200 m (Capone, 1983). Most likely because there was cyanobacterial mat present at the surface of the Eckernförde Bay sediment cores, extremely high rates of N<sub>2</sub> fixation were not measured. However, a more suitable question would concern how rates of N<sub>2</sub> fixation rates compare with rates of N loss within this system. Previous modeling of Eckernförde Bay N-cycling estimated that sediments from this location have a fixed N loss rate of 0.08 mmol N m<sup>-2</sup> d<sup>-1</sup> during the winter, i.e. in February (Dale et al., 2011). Comparing this rate estimate with the integrated rate of N<sub>2</sub> fixation from February during this study (Fig. 10, Table 1), it appears as though N losses and N gains would be roughly equal, having a ra-

tio of ~1.1. However, it is uncertain how rates of fixed N loss vary over the course of the year, especially considering the high seasonal variability of environmental conditions and other microbial processes at this location. Instead, it is most likely that there exists 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). To date, there are no published studies directly measuring N turnover rates in Eckernförde Bay sediments, as also noted by Dale et al. (2011), making it impossible to produce an N budget for this system. Additionally, rates of N losses from around the Baltic Sea are highly variable and typically impacted by fluctuating hypoxia and macrofaunal presence (Karlson et al., 2005; Hietanen and Lukkari, 2007; Conley et al., 2009), and so a possibly inaccurate view of Eckernförde Bay benthic N cycling could be formed if comparing N<sub>2</sub> fixation rates from this study with N losses from other studies around the Baltic Sea. In an attempt to provide some level of context, a study by Deutsch et al. (2010) found benthic denitrification rates from the southern and central Baltic Sea to range between 0.01 to 0.7 mmol N m<sup>-2</sup> d<sup>-1</sup>, which would indicate that the N<sub>2</sub> fixation rates presented here could account for somewhere between 10–100 % of the N lost via denitrification, depending on which numbers are used for comparison. Again, direct measurements of N losses in Eckernförde Bay

sediments are necessary and would prove highly valuable for determination of N budgets within this system.

## 5 Conclusions

This study examined the possibility of benthic N<sub>2</sub> 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. NA 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 N<sub>2</sub> fixing SRB, *Desulfovibrio vulgaris* and *Desulfonema limicola*, further supporting the hypothesis that some of the observed NA may be coupled to sulfate reduction. Overall, these benthic processes appeared to be greatly impacted by many seasonally varying environmental conditions, such as temperature, planktonic blooms, 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 concentrations, stressing the importance of time series measurements in these coastal environments.

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