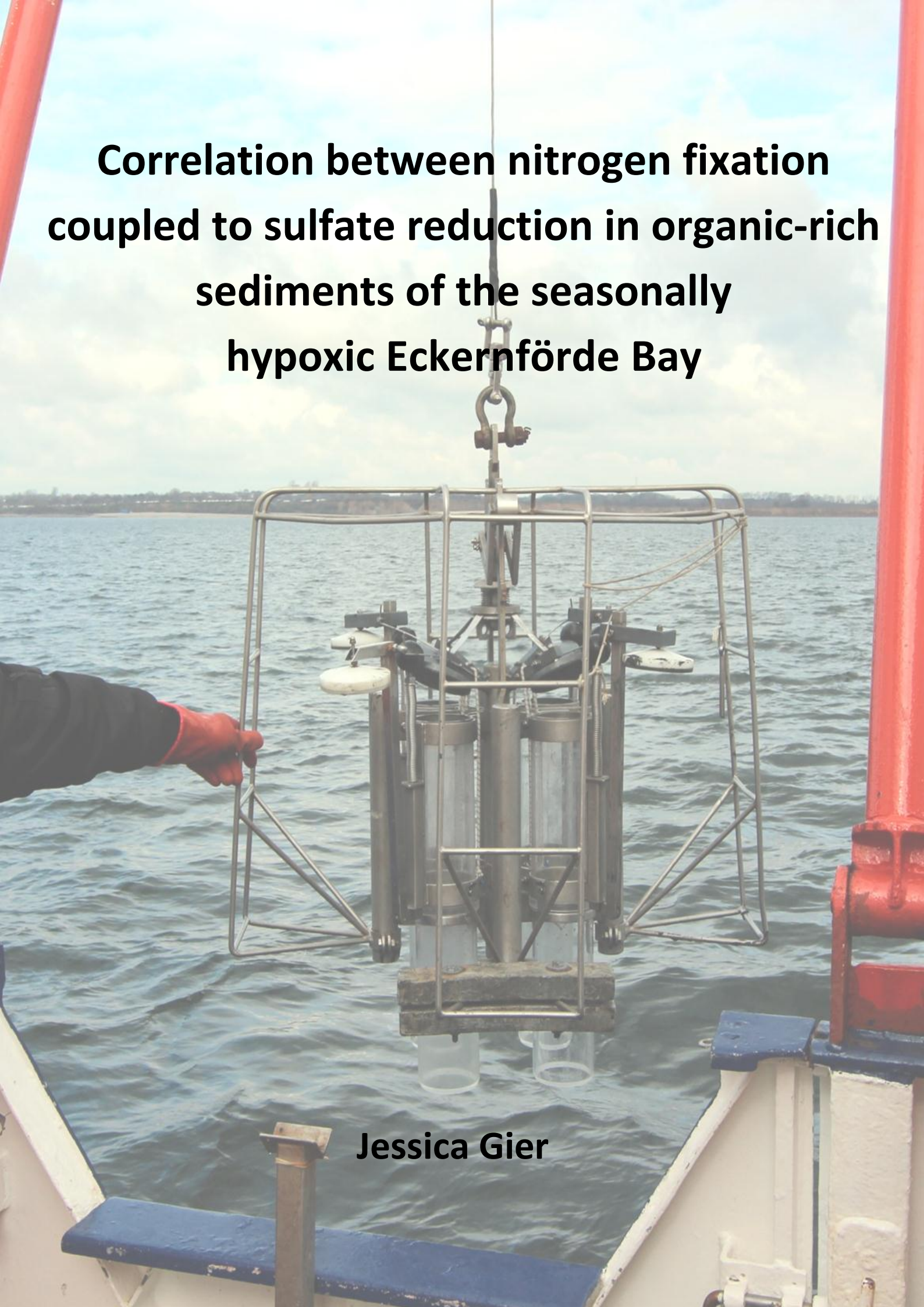


**Correlation between nitrogen fixation
coupled to sulfate reduction in organic-rich
sediments of the seasonally
hypoxic Eckernförde Bay**



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Abbreviation of names, formulas and none SI units

A	Adenine
C	Cytosine
CARD-FISH	Catalyzed Reporter Deposition - Fluorescence in Situ Hybridization
C ₂ H ₂	Acetylene
C ₂ H ₄	Ethylene
CH ₂ O	Formaldehyde
cm	Centi- (10 ⁻²) meter
cm ³	Cubic centi- (10 ⁻⁶) meter
CTD	Conductivity, temperature, depth (instrument)
DAPI	4'.6-diamidino-2-phenylindole
DIN	Dissolved inorganic nitrogen
DNRA	Dissimilatory nitrate reduction to ammonia
DSV 698	<i>Desulfovibrio</i> probe
<i>D. vulgaris</i>	<i>Desulfovibrio vulgaris</i>
EFB	Eckernförde Bay
EDTA	Ethylenediaminetetraacetic acid
et al.	And others (et alii)
EtOH	Ethanol
Fe	Iron
G	Guanine
h	Hour
HCl	Hydrochloric acid
HCO ₃ ⁻	Bicarbonate
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HRP	horseradish peroxidase
H ₂ S	Hydrogen sulfide
kBq	Kilo- (10 ³) becquerel
kJ	Kilo- (10 ³) joule
km	Kilo- (10 ³) meter
L	Liter
min	Minute
µg	Micro- (10 ⁻⁶) gram
µL	Micro- (10 ⁻⁶) liter

μmol	Micro- (10^{-6}) mol
mbar	Millibar
mg	Milli- (10^{-3}) gram
Milli-Q	Ultrapure water of Type 1
Mini-Muc	Mini-Multicorer
ml	Milli- (10^{-3}) liter
mm	Milli- (10^{-3}) meter
mmol	Milli- (10^{-3}) mol
Mn^{2+}	Manganese (II)
MnO_2	Manganese (IV) oxide
N_2	Nitrogen gas
NaCl	Sodium (natrium) chloride
NA	Nitrogenase activity
N_2 Fixation	Nitrogen fixation
nmol	Nano- (10^{-9}) mol
NH_3	Ammonia
NH_3OH	Ammonium hydroxide
NH_4^+	Ammonium
<i>nifH</i> gene	Gene encoding enzymes involved in N_2 fixation
NO	Nitric oxide
NO_2^-	Nitrite
N_2O	Nitrous oxide
NO_3^-	Nitrate
OLAND	Oxygen-limited autotrophic nitrification denitrification
OMZ	Oxygen minimum zone
PBS	Phosphate buffered saline
Ppm	Parts per million
rRNA	Ribosomal ribonucleic acid
SO_4^{2-}	Sulfate
$^{35}\text{SO}_4^{2-}$	Radioactive sulfate
SR	Sulfate reduction/Sulfate-reducing
T	Thymine
T g N y^{-1}	Trillion grams nitrogen per year
TRIS	Tris(Hydroxymethyl)aminomethane
ZnAc	Zinc Acetate

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Abstract

Sulfate reduction (SR) is a fundamental process in the global sulfur cycle. SR bacteria use sulfate as terminal electron acceptor and can contribute to more than 50% to the organic matter degradation in marine benthic environments. Microbial nitrogen (N₂) fixation is introducing bioavailable nitrogen, in the form of ammonia and ammonium, back into the environment. Because bioavailable nitrogen often limits marine productivity, N₂ fixation plays an important role in the N cycle and in global biogeochemical cycles. SR bacteria, such as *Desulfovibrio vulgaris*, were shown to have the genetic capability to fix nitrogen. However, the effect of temperature, as well as organic matter sources, on N₂ fixation coupled to SR, by SR bacteria, are still poorly understood. A previous study conducted with Eckernförde Bay (EFB) sediments, revealed a possible direct correlation between N₂ fixation, SR rates, as well as temperature. To investigate the effect of temperature and organic matter on N₂ fixation coupled to SR, sediment samples were taken in EFB and the microbial processes of N₂ fixation and SR examined, as well as the abundance of *D. vulgaris*.

Two different experiments were conducted in this master thesis. Experiment 1 was focusing on temperature effects on N₂ fixation and SR. Sediment from EFB was incubated under two different temperature conditions (3 °C and 13 °C) and SR rates were determined via radiotracer experiments with ³⁵SO₄²⁻. Nitrogenase activity as a measure of N₂ fixation was examined by the acetylene reduction assay. The catalyzed reported deposition fluorescence in situ hybridization (CARD-FISH) was applied to determine the abundance of *D. vulgaris* in the sediment. In Experiment 2, the effect of organic carbon in respective processes was investigated. Sediment slurries were prepared, adding two different organic carbon species (*phytoplankton* and *macrofauna* material) at two different energy levels. These slurries were incubated at 13 °C for 15 days and were then examined for SR and N₂ fixation rates. The same methods as in Experiment 1 were applied.

Results from Experiment 1 indicated that SR rates were higher at 13 °C than at 3 °C. Integrating SR rates over 0 - 15 cm sediment depth, the 13 °C treatment was five times higher (1052 nmol SO₄²⁻ cm⁻² d⁻¹), than the 3 °C sample (203 nmol SO₄²⁻ cm⁻² d⁻¹). The number of total cells per ml sediment, as well as the number of *D. vulgaris*, was decreasing with increasing sediment depth. Maximum total cell counts were observed in the first cm of

sediment ($1.97 \times 10^9 \text{ ml}^{-1}$), whereas the same applied for *D. vulgaris* counts ($1.02 \times 10^8 \text{ ml}^{-1}$). In Experiment 2, SR rates and nitrogenase activity showed a positive correlation with the energy value of added organic carbon. Additionally, *phytoplankton* samples showed higher rates than *macrofauna* samples.

In conclusion, N_2 fixation and SR in EFB sediments were positively correlated to temperature and might be performed by the organism *D. vulgaris*. Type and energy value of supplied organic matter stimulated N_2 fixation as well as SR rates. Results indicated that microbial processes in EFB are highly influenced by seasonal variations, what was shown in a previous study for AOM rates. An increase in temperature could lead to higher N_2 fixation and SR rates in EFB sediments. However, it is uncertain if increased organic matter in combination with higher temperature would lead to even higher rates because highest rates could be reached for N_2 fixation coupled to SR because bacteria have reached the maximum rate (K_{max}). But as CARD-FISH examines only the natural abundance, it is important for future studies to further focus on the question how much N_2 fixation one cell can accomplish and conduct nanometer-scale secondary ion mass spectrometry or quantitative real time polymerase chain reaction.

1. Introduction

Sulfate reduction (SR) and nitrogen (N₂) fixation are fundamental processes in two globally important biogeochemical cycles, namely the sulfur and the nitrogen cycles. SR is done by sulfate-reducing bacteria, typically in anoxic habitats, where sulfate is used as the terminal electron acceptor for the microbial degradation of organic matter, resulting in sulfide as the final product (Postgate 1965; Jørgensen 1977; Muyzer & Stams 2008). Microbial N₂ fixation is defined as the reduction of dinitrogen gas (N₂) to ammonia- (Postgate 1982; Brandes et al. 2007) and may allow for the introduction of bio-available nitrogen into the surrounding environment. These two processes, SR and N₂ fixation, can occur in both, terrestrial and marine environments, including some marine benthic habitats (Capone 1988; Steppe & Paerl 2002). Various sulfate-reducing bacteria, such as *Desulfovibrio vulgaris*, have been shown to fix nitrogen (Sisler & ZoBell 1951; Riederer-Henderson & Wilson 1970). Additionally, many more sulfate-reducing bacteria have been shown to have the genetic capability to fix nitrogen (Zehr et al. 1995). Combining the ability to fix nitrogen and the high abundance in marine sediments, N₂ fixation by *D. vulgaris* could be an important process, introducing new nitrogen back into marine sediments.

1.1. Microbial degradation process - Sulfate Reduction

Sulfate reduction (SR) is one of the most important anaerobic microbial degradation processes in marine sediments. It is performed for the most part by sulfate-reducing bacteria (SR bacteria), which take up sulfate as a nutrient and reduce it to sulfide (Jørgensen 1977; Muyzer & Stams 2008). Additionally, there exists one genus of hyperthermophilic archaea (*Archaeoglobus*) that carries out SR (Stetter et al., 1993). Two types of SR can be distinguished: (1) assimilatory SR, which is used for building organic sulfur compounds and (2) dissimilatory SR, which is required by microbes to gain energy (Kasten & Jørgensen 2000). The following research will be focusing on the dissimilatory SR. The overall reaction of the bacterial dissimilatory SR is (Eq. 1):



Rates can vary eight orders of magnitude (Goldhaber & Kaplan, 1975; Westrich, 1983), ranging from $2.8 \times 10^{-1} \text{ mol l}^{-1} \text{ y}^{-1}$ measured in laboratory jar experiments for interstitial water sediments (Martens & Berner 1974), to $3.7 \times 10^{-2} \text{ mol l}^{-1} \text{ y}^{-1}$ in coastal sediments (Berner 1972), but reaching as low as $7.3 \times 10^{-8} \text{ mol l}^{-1} \text{ y}^{-1}$ when modeled for the deep

biosphere (Tsou et al. 1973). SR rates vary depending on location, typically with very high rates at continental margins and decreased rates in the basins (Canfield, 1991).

The microbial degradation of organic matter in marine sediments is stratified vertically (Fig. 1), such that electron acceptors and corresponding products disappear and appear, respectively. This stratification is based on the decreasing energy yield per mol organic carbon of the respective oxidant, this process is called 'redox cascade' (Jørgensen 1983; Iversen & Jørgensen 1985). The redox cascade implies that the major electron acceptor in oxic conditions is oxygen, which yields the most energy in aerobic respiration. After all oxygen is depleted, nitrate and reactive metals (MnO_2 , Fe_2O_3) are reduced next, followed by SR (Martens & Berner 1974).

In most cases, sulfate concentrations in porewater samples result in a concave-down profile (SO_4^{2-} in Fig. 1). Concentrations decrease with increasing sediment depth and once the sulfate pool is depleted, methane (CH_4) accumulates as the end product of anaerobic diagenesis via methanogenesis (Martens & Berner 1974; Jørgensen 1983; Martens et al. 1999).

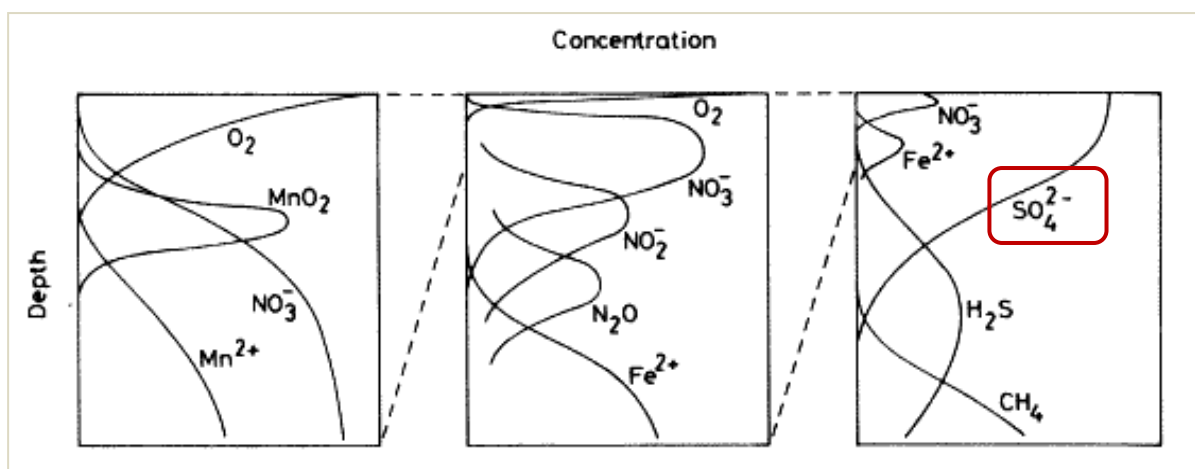


Fig. 1: Graphs of oxidants and corresponding products in porewater of marine sediments. The graphs are nested in each other and the relative depth is shown by the dashed lines. Sulfate reduction occurs as the penultimate degradation process, and methanogenesis as last process (Jørgensen, 1983).

Principally, SR bacteria use sulfate, which is regarded as the most oxidized form of sulfur, as the terminal electron acceptor for the degradation of organic carbon species (Muyzer & Stams, 2008). SR bacteria are typically regarded as anaerobic microorganisms and are abundant in high numbers in a variety of the worlds' ecosystems. These bacteria play an essential role in the biogeochemical cycling of sulfate in marine sediments (Jørgensen 1977;

Plugge et al. 2011). In some benthic environments, SR bacteria can contribute to $\geq 50\%$ of the organic carbon degradation (Jørgensen 1982). Because of the low oxygen concentrations in the water column (areas with $< 22 \mu\text{mol O}_2 \text{ l}^{-1}$ (Wyrteki 1962; Kamykowski & Zentara 1990)), due to the aerobic degradation of organic matter and because of the high organic carbon content reaching the sediment, upwelling regions, such as off Peru or Namibia, typically display high benthic SR rates (Canfield, 1991). SR bacteria have successfully adapted to most marine environments, including very extreme environments, such as deep-sea hydrothermal vent systems (Jeanthon et al., 2002), deep-sea whale falls (Treude et al. 2009) or hydrocarbon seeps, where bacteria are eventually involved in degrading hydrocarbons or petroleum byproducts (Barton & Fauque 2009; Orcutt et al. 2010).

In general, SR bacteria perform two different lifestyles, depending on environmental conditions. Either SR bacteria outcompete methanogens for substrates and perform SR, known as a “sulfidogenic lifestyle”, or the bacteria grow in syntrophy with methanogenic archaea, using a syntrophic metabolism (e.g. producing hydrogen, which a methanogen uses) (Bryant et al. 1977; Muyzer & Stams, 2008).

Studies also indicate that some SR bacteria have the genetic ability to fix molecular nitrogen (Zehr & Paerl 2008). It appeared that these bacteria carry the *nifH* gene, the gene encoding for the nitrogenase enzyme and needed for N_2 fixation. This gene was detected in the genera *Desulfobulbus*, *Desulfobacter*, *Desulfotomaculum* and *Desulfovibrio*, with cultures of *Desulfovibrio gigas* and *Desulfovibrio vulgaris* used in early examinations of N_2 fixation by SR bacteria (Riederer-Henderson & Wilson, 1970).

1.2. Nitrogen Fixation

Nitrogen is a fundamental element in many biogeochemical cycles in marine environments and is essential for all living things (Ward & Bronk, 2001). Only 2 % of nitrogen in the seawater is available for organisms in the form of Nitrate (NO_3^-), whereas much of the remaining nitrogen exists as N_2 . Consequently, a significant limitation of bioavailable nitrogen (NO_3^- and NH_4^+) exists in the oceans (Brandes & Devol, 2002; Gruber, 2008). This limitation makes N_2 fixation to the dominant source of bioavailable nitrogen in marine environments (Falkowski et al. 1998; Strous et al. 1999; Brandes & Devol 2002).

Biological N_2 fixation is defined as the reduction of dinitrogen gas to ammonia and has been established as (Eq. 2) (Simpson & Burris 1984; Kim & Rees 1994):



In general, oceanic nitrogen exists in five forms: (i) Nitrogen gas (N_2), which first has to be converted into ammonium by nitrogen fixing bacteria or archaea, before other organisms can incorporate it into their biomass, (ii) Nitrate (NO_3^-), the most oxidized form and the dominant form in oxic environments, (iii) Ammonium (NH_4^+), the reduced form and the dominant form in anoxic systems, (iv) Particulate nitrogen, in the form of organic nitrogen, mainly found in sediments or in deep-sea waters, (v) Dissolved organic nitrogen (DON), which represents a mixture of compounds (Brandes et al., 2007; McCarthy et al. 1998; Ward & Bronk, 2001).

In a simplified way, the marine nitrogen cycle, including major flux terms, can be summarized in six processes: N_2 fixation, pelagic- and benthic denitrification (converting fixed nitrogen back to N_2), river- and atmospheric input, and lastly sediment burial of organic matter (Brandes et al., 2007; Brandes & Devol, 2002; Galloway et al., 2004).

Over the recent years, a complex global nitrogen cycle has emerged, involving N_2 fixation, denitrification, anaerobic ammonium oxidation, oxygen-limited autotrophic nitrification–denitrification (OLAND), chemodenitrification and dissimilatory nitrate reduction to ammonium (DNRA) (see Fig. 2) (Brandes et al., 2007).

New processes, particularly in sediments, were discovered, such as the anaerobic ammonium oxidation in 1999 (Strous et al. 1999). Because this process includes the conversion of ammonium and nitrite to N_2 , this was revealed to be a second sink, besides denitrification, for bioavailable nitrogen (Brandes et al. 2007). Denitrification in marine sediments and in the water column is occurring in a depth of 200 – 700 m and mainly in oxygen minimum zones (OMZ = areas with $< 22 \mu\text{mol O}_2 \text{ l}^{-1}$ (Wyrski 1962; Kamykowski & Zentara 1990)). These zones are located in the Arabian Sea, the north and south eastern Pacific and at the west coast of South Africa (Brandes & Devol, 2002; Capone & Knapp, 2007).

Highest rates for N_2 fixation were not, as previously thought, in the Atlantic with its iron-rich dust input, but downstream the OMZ in the Pacific Ocean. The Pacific Ocean is depleted in iron but high in water column denitrification. Therefore, N_2 fixation is closely related to nitrogen depleted areas, more precisely to denitrification zones. This finding would lead to the conclusion that the nitrogen cycle stabilizes the oceanic nitrogen budgets over time.

However, the links between the different compounds, as well as the feedbacks, budgets and implications of these pathways, still remain unclear.

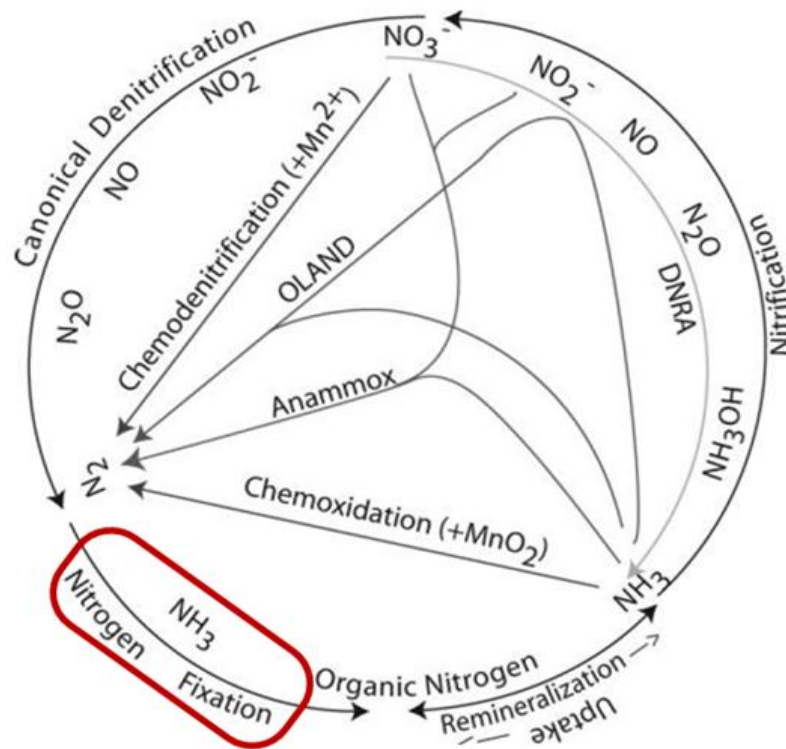


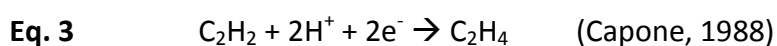
Fig. 2: Today's global nitrogen cycle including all processes and compounds of each step. OLAND: oxygen-limited autotrophic nitrification denitrification; DNRA: dissimilatory nitrate reduction to ammonium. Arrows indicate the direction of process. (Brandes et al., 2007)

Estimates of sources and sinks of global nitrogen budgets are still a topic of much debate and several estimates exist in the literature. One estimate of sources for global marine N_2 fixation is in a range of $120 \pm 50 \text{ T g N y}^{-1}$ (Gruber, 2004), whereas one estimate for total global nitrogen sources is at 294 T g N y^{-1} (Codispoti, 1995). One estimate for global nitrogen sinks is 482 T g N y^{-1} (Codispoti, 1995). In summary, estimated budgets of sinks and sources for nitrogen seem to be unbalanced, which means that sources (N_2 fixation) do not equal sinks (denitrification), and Brandes et al. (2007) come up with a net budget of up to $-200 \text{ T g N y}^{-1}$. Consequently, there must be an under- or overestimation of budgets (Brandes 2002; Capone & Knapp 2007). However, Gruber (2004) estimated sources to be $265 \pm 50 \text{ T g N y}^{-1}$ and sinks to be $275 \pm 50 \text{ T g N y}^{-1}$, which would result in roughly a balanced budget. Nevertheless, there are several estimates present and this estimation is still a topic of much debate.

However, all estimated nitrogen budgets agree that the marine nitrogen cycle is extremely dynamic, with the residence time of fixed nitrogen estimated to be only 3,000 years (Codispoti, 1995). In comparison, the residence time of nitrogen gas in the ocean is $\sim 54,000$

years (Gruber, 2008). Due to the short turnover time of fixed nitrogen, slight changes in sources and sinks could affect the global productivity and also CO₂ levels in the atmosphere (Ganeshram et al. 2000).

Microorganisms capable of N₂ fixation are termed diazotrophs. These microbes are categorized either as free-living diazotrophs, which include e.g. *Desulfovibrio* spp and *Trichodesmium* spp (Capone et al. 1997) or as symbiotic diazotrophs that live in symbiosis with other organisms, such as the cyanobacterium *Anabaena azollae*, which is the endosymbiont of the aquatic fern *Azolla* (Wagner, 1997). Diazotrophs have an extremely oxygen sensitive molybdenum-iron-, iron- (Kim & Rees, 1994) or vanadium- (Robsen et al., 1986) enzyme complex, referred to as nitrogenase. Nitrogenase breaks the triple bonds in nitrogen gas to form bioavailable nitrogen (Capone 1988; Brandes et al. 2007). Three genes (*nifH*, *nifD* and *nifK*) encode for nitrogenase, with *nifH* often used for phylogenetic studies among the diazotrophs. Amazingly, nitrogenase enzymes from various organisms show a remarkable homology (Ruvkun & Ausubel 1980; Zehr & Paerl 2008). Nitrogenase is able to reduce a broad range of analogous molecules besides nitrogen gas (Lockshin & Burris 1965), such as acetylene (Dilworth, 1966). This ability can be used to determine nitrogenase activity (NA) via the acetylene reduction assay (Capone, 1988). The acetylene reduction assay is an *in situ* method to detect NA in a sample, based on the reduction of acetylene (C₂H₂) to ethylene (C₂H₄) by the nitrogen-fixing complex. The temporal increase of ethylene in samples can be measured by flame ionization gas chromatography (Hardy et al. 1968; Stewart et al. 1967). Thereby, the amount of acetylene reduced to ethylene serves as an indication for N₂ fixation rates. The application of acetylene reduction assay is sensitive, cost effective and a simple method to determine NA (Capone, 1993). Therefore, this method has several advantages compared to other methods, such as the δ¹⁵N₂, what was revealed to underestimate N₂ fixation rates when δ¹⁵N₂ was injected as gas bubble (Mohr et al. 2010). The reaction for C₂H₂ reduction is (Eq. 3):



To convert from C₂H₄ to N₂, a ratio of 3:1 is appropriate for benthic samples, whereas a 4:1 ratio gave good agreements with δ¹⁵N₂ results for planktonic samples (Capone, 1993).

However, still a lot of unanswered questions remain, concerning the benthic and pelagic marine N₂ fixation, including trace elements, nutrients, temperature, organic carbon availability and also anthropogenic effects (Sohm et al., 2011).

1.3. Marine benthic nitrogen fixation by sulfate-reducing bacteria

Certain species of SR bacteria have shown evidence of the ability to fix molecular nitrogen. This possibility was discovered more than 60 years ago, when a survey nitrogen/argon ratios examined (Sisler & ZoBell, 1951). More recent work has shown that many additional SR bacteria have the genetic ability to fix molecular nitrogen (Zehr et al. 1995). However, the role of SR bacteria in the global nitrogen cycle is not yet fully understood.

Most of the previous research has focused on pelagic N₂ fixation, but benthic N₂ fixation can also occur. This occurrence has been investigated in recent years, with many scientists concentrating on microbial mats and plant rhizospheres (Herbert 1975; Steppe & Paerl 2002). Productive coastal sea-grass rhizosphere sediments show very high rates of SR and N₂ fixation due to the high input of organic matter from the sea-grass roots. The high input of organics results in high microbial activities in the surrounding sediment and, consequently, to high rates of SR and N₂ fixation (Herbert, 1975). Calculations show that the amount of bacterial N₂ fixation in benthic rhizosphere systems accounts for 10 – 50% of the total nitrogen requirement for photosynthetic sea-grass growth, making N₂ fixation by SR bacteria a fundamental process in marine rhizosphere-systems (Capone 1988).

One important remaining question is whether SR bacteria also considerably contribute to nitrogen cycling in other marine benthic systems.

In coastal waters, burrowing ghost shrimps were found that create a three dimensional zonation pattern in the sediment. This zonation pattern was created via the processes of bioturbation and bioirrigation, that supported increased rates of SR and N₂ fixation (Bertics et al. 2010b). Bioturbation and bioirrigation introduced organic matter deeper into the sediment, which led to N₂ fixation by SR bacteria that resulted in new bioavailable nitrogen in marine sediments.

In conclusion, N₂ fixation by SR bacteria could be an important process, introducing new nitrogen back into marine sediments. Still, there are many debates about N₂ fixation mediated by SR bacteria, or benthic bacteria in general, and their contribution to the nitrogen cycle. However, the global volume of bioturbated sediment was suggested to be > 20 700 km³ (Teal et al. 2008), given this number, benthic N₂ fixation by SR bacteria may play an important role in the global marine nitrogen cycle. Up to now, not much research has been devoted to this question.

1.4. The sulfate-reducing bacterium *Desulfovibrio vulgaris*

The sulfate-reducing bacterium *Desulfovibrio vulgaris* is found in marine benthic environments (Mussmann et al. 2005; Muyzer & Stams 2008). *D. vulgaris* carries the *nifH* gene, which encodes for the nitrogenase enzyme, needed to fix N₂ and previous work has shown that it is capable of fixing molecular N₂ when in culture (Riederer-Henderson & Wilson, 1970). Given these features, the current study will be focussing on *D. vulgaris*.

D. vulgaris is a nonsporulating, gram-negative bacteria with single polar flagella. Mainly found in soils, aquatic environments and animal intestines. It is curved rod-shaped and can use acetate, pyruvate, formate and some primary alcohols as a carbon source (Postgate 1965; Postgate & Campbell 1966).

The genus *Desulfovibrio* belongs to the Phylum Proteobacteria. The associated order Desulfovibrionales is known to use SR as the respiratory dissimilatory process and typically grows anaerobically (Fig. 3 and Fig. 4).

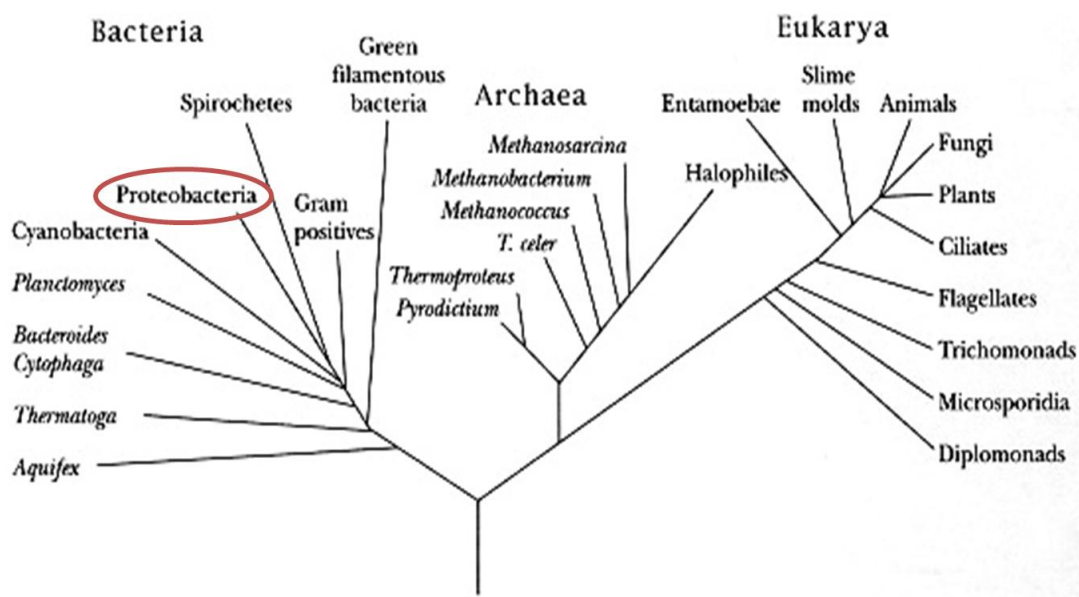


Fig. 3: Phylogenetic tree of Bacteria, Archaea and Eukarya. The genus *Desulfovibrio* belongs to the phylum of the Proteobacteria that are highlighted. Source: www.textbookofbacteriology.net/themicrobialworld/tree.jpeg

It has been demonstrated that *D. vulgaris* can live in syntrophy with the methanogen *Methanobacterium formicicum*. In the absence of sulfate and the presence of the respective methanogen, *D. vulgaris* produces hydrogen that the methanogen uses for growth. Because *D. vulgaris* can live in association with the methanogen, it is possible for the bacterium to be

metabolically active in environments where sulfate is depleted (Bryant et al., 1977). *D. vulgaris*, is even capable of respiring oxygen (Cypionka, 2000).

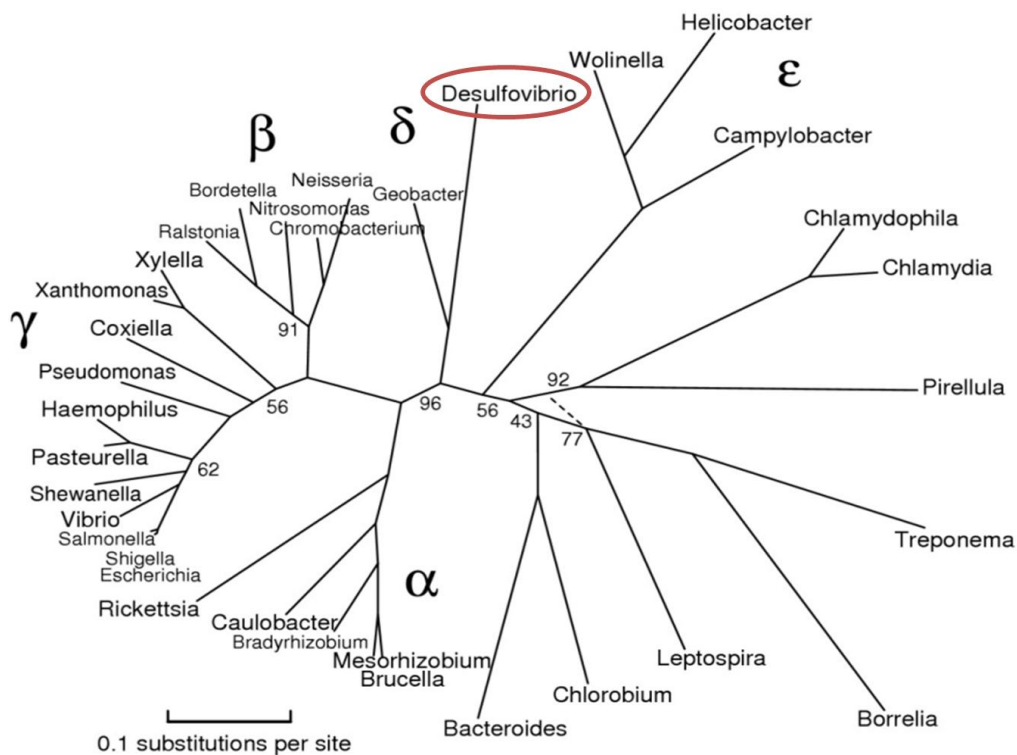


Fig. 4: Phylogenetic tree of Proteobacteria. The genus *Desulfovibrio* belongs to the delta-domain and is highlighted. Source: www.biomedcentral.com/content/figures/1471-2148-5-34-2-1.jpg

Desulfovibrio species are able to use a various number of electron acceptors, such as sulfate, thiosulfate, nitrate and nitrite for respiration and growth (Postgate 1979). In addition to the above electron acceptors, these bacteria have been shown to enzymatically reduce several forms of metals, for instance manganese (IV), iron (III) (Coleman et al. 1993) and chromium (VI) (Lovley, 1993). In particular, some strains of *D. vulgaris* are capable of reducing uranium (VI) (Lovley & Phillips, 1992). Reducing the soluble oxidized form of uranium (VI) to insoluble uranium (IV) might have an important influence on the geochemistry of contaminated environments. Therefore, one could use *D. vulgaris*, to remove uranium from contaminated waters, or in combination with an extraction technique, *D. vulgaris* could be used to remove uranium from soils. Previous studies show that the enzyme that is responsible for this reaction is the cytochrome c_3 reductase and the electron donor is either hydrogen (Lovley et al. 1993) or lactate, with pyruvate as the electron source (Payne et al. 2002).

In summary, the genus *Desulfovibrio* lives in different environmental conditions and is capable of different types of metabolism, making these bacteria extremely fascinating when studying activity rates and metabolism potentials.

1.5. Study site - Characteristics Eckernförde Bay

Eckernförde Bay (EFB) is located in the western Baltic Sea (54° 30' N, 10° 02' E), which is one of the largest brackish water systems on earth. EFB is a semi-enclosed bay with a maximum water depth of ~ 28 m and has a total length of 19 km and a width of 9 km (Fig. 5) (Bange 2010). The eastern area is divided into two troughs by the moraine remnant “Mittelgrund”, where the water depth is only 10 m (Schlüter et al. 2004).

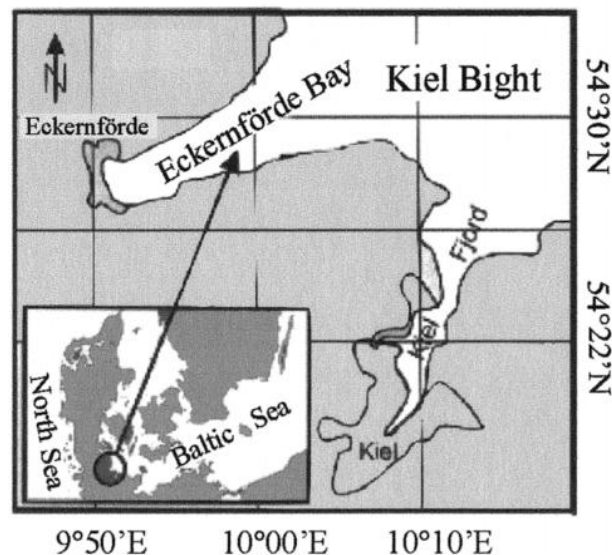


Fig. 5: Study area of Eckernförde Bay (54° 30' N, 10° 02' E) in the western Baltic Sea. (Schlüter et al., 2004)

Because the inflow of North Sea water into the Baltic Sea solely occurs through the Kattegat (depth ca. 20 m) and the Great Belt, EFB as a semi-enclosed area, can be considered as a representative for the southwest Baltic Sea.

Pelagic N₂ fixation rates in the Baltic Sea indicate high seasonal variability. Values range from 20 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ during winter, whereas extensive heterocystous cyanobacterial blooms during summer result in values up to 2500 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Bianchi et al., 2000; Sohm et al., 2011; Wasmund et al. 2001).

Due to the high freshwater inflow, the salinity of the Baltic Sea is, on average, lower than the worlds' oceans (which averages around a salinity of 35) and varies in value depending on

location site. At Kattegat, the salinity is 21 in the surface layer and 31.6 in the deep layer, whereas the average Baltic Sea salinity was determined to be 7.2 in the surface layer and 12.3 at 200 m water depth (Winsor, Rodhe, & Omstedt, 2001). Due to the inflow of more saline water from the North Sea, that leads to stratification of the water column, a pycnocline at about 15 m water depth forms during March to September. This stratification results in slow inert water exchange that prevents oxygen renewal in bottom waters. Consequentially, a temporal oxygen minimum zone (OMZ) develops in some parts of the Baltic Sea (Orsi et al. 1996; Bange 2010). In general, shallow coastal waters are heavily influenced by hypoxia ($O_2 < 63 \mu\text{mol L}^{-1}$) and anoxia ($O_2 = 0 \mu\text{mol L}^{-1}$). Previous studies suggested that OMZs are increasing all over the world (Diaz, 2001; Diaz & Rosenberg, 1995; Stramma et al. 2008). This observation is also true for EFB, where anoxic events have been increasing over the last 25 years (Bange et al. 2011). The time series station at Boknis Eck in EFB has been monitoring oxygen levels over the past 55 years and recorded just one anoxic event from 1957 - 1983, whereas from 1986 – 2011, ten anoxic events occurred (Bange et al., 2011). Hypoxic and anoxic conditions occur seasonally in EFB, influencing the ecological and biogeochemical cycles year round (Bange, 2010; Orsi et al., 1996). Because of high organic matter degradation in the water column, which is using all the oxygen, in combination with phytoplankton blooms, anoxic events mostly occur in summer bottom water (Graf et al. 1983; Meyer-Reil, 1983).

The composition of benthic macrofauna in EFB sediments is linked to seasonal oxygen availability in bottom waters. Hypoxic and anoxic conditions result in regular disturbances of the benthic community, which control the complexity of benthic organisms. Therefore, the benthic community in EFB is composed of low-level organisms. The benthic fauna is dominated by surface deposition feeders, such as polychaetes (*Polydora ciliata*), whereas oligochaetes (*Tubificid* spp) are primarily responsible for mixing the top 0.5 – 1 cm of the sediment in a rapid time scale of < 14 days (D'Andrea et al. 1996).

Phytoplankton blooms in EFB occur mainly in autumn (Sep. – Nov.) and in spring (Feb. - March) (Bange et al. 2011). A third smaller bloom can be observed during summer, depending on environmental conditions, such as temperature and water column mixing. The daily carbon flow to the seafloor was calculated to be $0.2 \text{ g C m}^{-2} \text{ d}^{-1}$ for winter, representing the lowest value, and $1.4 \text{ g C m}^{-2} \text{ d}^{-1}$ for spring and autumn, representing the highest value (Fig. 6) (Graf et al. 1983).

The surface sediments of EFB (upper 2 m) are characterized by a high organic carbon content (4 – 5 wt%) (Orsi et al., 1996), and consequentially has a high oxygen demand (Whiticar, 2002).

	Sediment depth cm	Daily carbon flow gC m ⁻² d ⁻¹
Summer stagnation 9. 9.–1. 10. 81	5	0.8
Breakup 2.10.–27. 10. 81	5	1.4
Autumn input 28.10.–10. 12. 81	2	0.3
	5	0.9
Winter input 11.12.–6. 3. 82	2	0.2
Spring input 7.3.–26. 4. 82	4	1.4

Fig. 6: Daily carbon flow (g C m⁻² d⁻¹) during different periods of the year and in diverse sediment depths with the highest value of 1.4 g C m⁻² d⁻¹ during spring/breakup and the lowest rate of be 0.2 g C m⁻² d⁻¹ for the winter input (modified from Graf et al. 1983).

A trend towards warmer sea surface temperatures was observed in EFB over the last decades (Bange et al. 2011). This increase is supported by the fact that cold events, with a temperature of 0 °C in the water surface, did not occur in the last 14 years.

The amount of total dissolved inorganic nitrogen (tDIN = NH₄⁺ + NO₂⁻ + NO₃⁻) in the EFB water column started decreasing from a value of about 12 µmol L⁻¹ in 1980 to a level of 3 µmol L⁻¹ in 2010 (Bange et al. 2011). A rate for nitrogen loss processes, such as denitrification and anaerobic ammonium oxidation is estimated to be 0.08 mmol m⁻² d⁻¹ N₂ for EFB (Dale et al., 2011), which is low compared to rates between 0.15 – 0.65 mmol m⁻² d⁻¹ N₂ calculated for the Baltic Sea (Tuominen et al. 1998). Nevertheless, due to bioirrigation in the sediment, EFB seems to be a net source of DIN during winter (Dale et al., 2011).

Methane (CH₄) flux to the atmosphere shows strong seasonal variation, with highest numbers in winter between 200 – 400 µmol m⁻² d⁻¹ and lowest numbers of about 1 µmol m⁻² d⁻¹ during spring (Bussmann & Suess, 1998). Additionally, rates for microbial SR and anaerobic oxidation of methane (AOM) in EFB vary seasonally (Treude et al. 2005).

Sediments of EFB are known to recycle inorganic nutrients efficiently (Fig. 7, Bange et al., 2011; Dale et al., 2011). Because EFB is regarded as a muddy coastal sediment rich in organic matter that is degraded in the upper sediment surface, oxygen typically only penetrates a few mm into the sediment (Revsbech et al., 1980; Gundersen & Jørgensen, 1990; Bange et al., 2011). The only processes that are known to transport oxygen deeper into the sediment is either through bioturbation/bioirrigation or by winter storm mixing (Orsi et al., 1996; Bange et al., 2011; Dale et al., 2011).

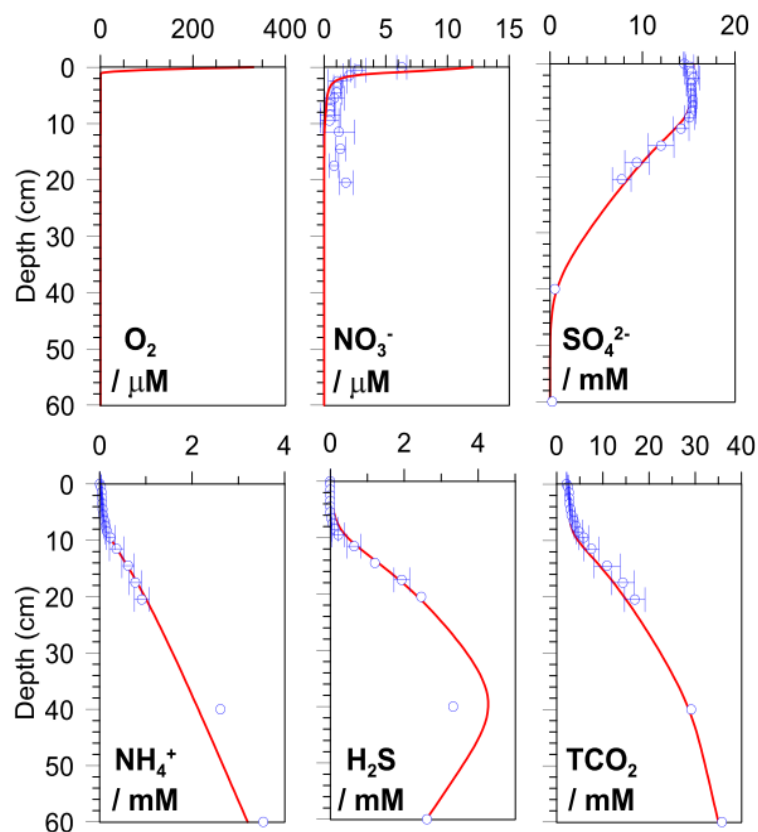


Fig. 7: Porewater concentrations of Boknis Eck sediments during winter 2010. Electron acceptors O_2 , NO_3^- and SO_4^{2-} plus reduced products NH_4^+ , H_2S and TCO_2 (total carbon dioxide) over a depth of 60 cm. Lines = modelled response, points = measured values (Bange et al. 2011; Dale et al. 2011).

Porewater concentrations of EFB sediments (Fig. 7) show that electron acceptors NO_3^- and SO_4^{2-} decrease with sediment depth and SO_4^{2-} shows a concave-down profile due to SR bacteria oxidizing organic matter in the sediment. This pattern implies that sediments of EFB are a sink for these compounds via aerobic and anaerobic microbial respiration. Simultaneously, the concentration of reduced compounds, such as NH_4^+ , H_2S , and total carbon dioxide (TCO_2), is increasing with increasing sediment depth. Therefore, these

compounds must be either diffusing upwards to the surface and released into the water column, or are consumed by microbes in the sediment (Bange et al. 2011).

In summary, the above mentioned features make EFB an ideal place to study microbial degradation and element cycling in sediments below a seasonally hypoxic water column.

1.6. Aim of this thesis

Our knowledge surrounding SR bacteria, including budgets and implications, has improved during the last decades (Jørgensen 2008; Plugge et al. 2011). Nevertheless, many questions remain that are worth investigating. The ability of SR bacteria to fix N_2 make these microbes even more interesting in terms of estimating global sinks and sources of nitrogen. Furthermore, there is little data available on the effect of environmental factors, such as temperature and organic matter availability, on N_2 fixation mediated by SR bacteria.

A time series, which was conducted over almost one year, revealed that there seems to be a direct correlation of peaks between N_2 fixation, SR rates and temperature (Fig. 8) (Bertics et al. 2012). The trend in decreasing nitrogen and SR rates along with decreasing temperature is obvious between October and February. This decrease could be coupled either directly to temperature, or indirectly to the input of organic matter from the water column, e.g. via phytoplankton blooms. In this scenario, the particulate organic matter sinks onto the seafloor, is buried by transport mechanisms, such as mixing or bioturbation, and can then be degraded by microbes. The degradation of organic matter eventually would result in higher N_2 fixation and SR rates.

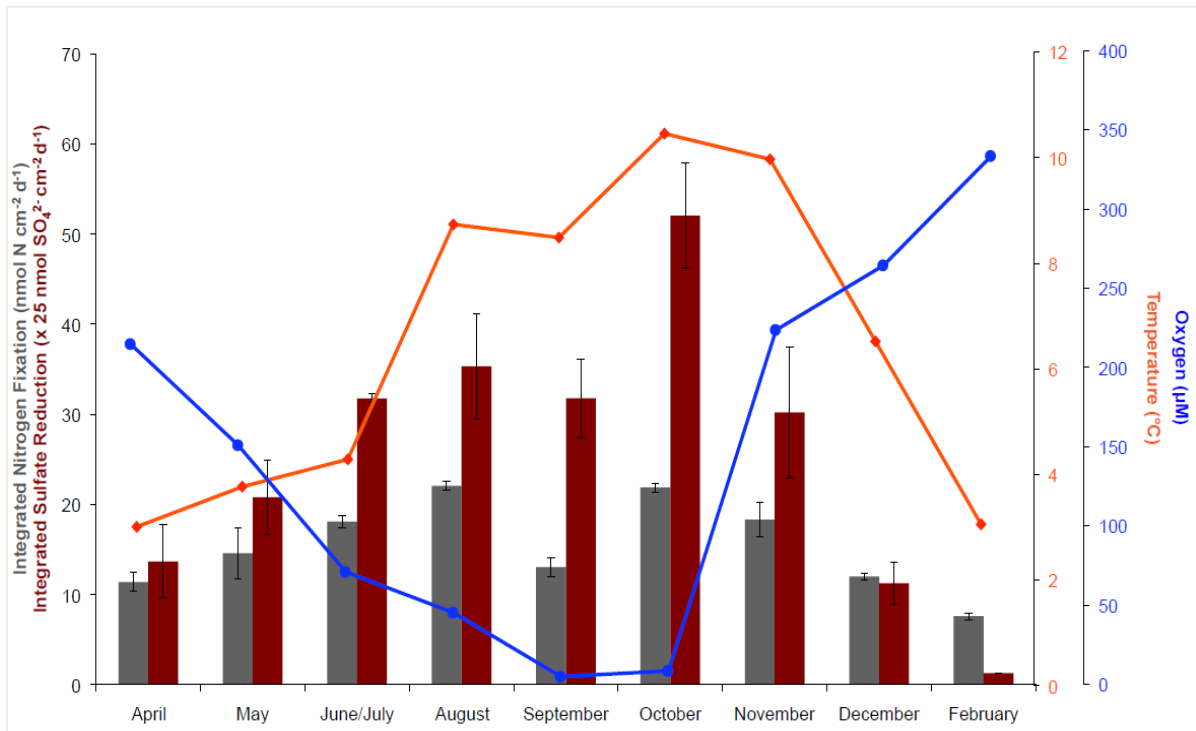


Fig. 8: Time series of integrated sulfate reduction ($\times 25 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$, red bars) and nitrogen fixation ($\text{nmol N cm}^{-2} \text{ d}^{-1}$, grey bars) in EFB sediment from April 2010 to February 2011, including oxygen (μM , blue curve) and temperature ($^\circ\text{C}$, red curve) in bottom water. Error bars indicate standard deviation (Bertics et al. 2012).

The present study is part of a larger project within the research group of T. Treude, where V. Bertics was analyzing N_2 fixation rates coupled to SR in EFB sediments over the course of one year.

One major aim of my study was to investigate if there is a direct correlation between SR, N_2 fixation rates and temperature in EFB sediment. Therefore, my work concentrated on the comparison of SR rates and N_2 fixation (the latter parameter was determined by V. Bertics) at two different temperatures, i.e. the highest and lowest bottom water temperature detected in EFB over the course of year, which occurred in February 2011 (3°C) and October 2010 (13°C), respectively.

It appeared that the SR bacterium *D. vulgaris*, which carries the *nifH* gene, the gene encoding for the nitrogenase enzyme needed for N_2 fixation, is abundant in EFB (Bertics et al. 2012). Therefore, this species was selected as the study organism and quantified, together with the total number of cells, within the sediment via Catalyzed Reporter Deposition Fluorescence in Situ Hybridization (CARD-FISH).

My hypotheses were:

- SR and N₂ fixation activity in sediments of EFB is correlated to temperature.
- The abundance of *D. vulgaris*, a SR bacterium capable of mediating N₂ fixation in the sediment, is correlated with the activity of SR and N₂ fixation.

The second major aim of my study was to investigate the correlation between organic matter availability and N₂ fixation, as well as SR. In this experiment, EFB sediment was mixed with two types of organic carbon (phytoplankton and macrofauna carbon source) at two different energy levels (0.5194 kJ and 3.6361 kJ).

My hypotheses were:

- SR and N₂ fixation rates are positively correlated to the energy value of added organic carbon material.
- Addition of phytoplankton organic matter results in higher SR and N₂ fixation rates, compared to the addition of macrofaunal organic matter.

2. Materials & Methods

Two different experiments were performed for this master thesis. Experiment 1 was focusing on temperature effects, where microbes were incubated under two different temperature conditions (3° C and 13 °C). To get an insight into the sulfate reduction (SR) rates, radiotracer experiments with $^{35}\text{SO}_4^{2-}$ were carried out, using the whole-core-injection method (Jørgensen 1978; Fossing & Jørgensen 1989). Moreover, Catalyzed Reported Deposition Fluorescence in Situ Hybridization (CARD-FISH) was performed (Pernthaler et al. 2002; Ishii et al. 2004), to determine microbial abundance of the SR bacterium *Desulfovibrio vulgaris* in sediment layers. This experiment is described in the first section of the Materials and Methods (2.1.).

In Experiment 2, organic carbon was investigated as a changing variable. Sediment slurries were prepared using two different organic carbon species (phytoplankton and macrofauna material) at two different energy levels. Beforehand, the energy values for both specimens were determined by bomb calorimetry. The slurries were incubated under the same temperature condition (13 °C) and were examined for SR and nitrogen (N_2) fixation rates. The aim of this experiment was to determine how the organic carbon source drives N_2 fixation and coupled SR. Experiment 2 is described in the second section of the Materials and Methods (2.2.).

2.1. Impact of Temperature (Experiment 1):

2.1.1. Sampling

Samples were taken during a one-day cruise with the GEOMAR RC “*Littorina*” in Eckernförde Bay (Boknis Eck Station, 54°31.823 N, 10°02.764 E) on the 4th of October 2011. The sampling site had a water depth of approximately 28 m and a bottom water temperature of 12.7 °C.

A CTD (Hydro-Bios, Kiel, Germany) with an attached oxygen sensor was used to measure conductivity, temperature and density. Based on these parameters, salinity was calculated (OceanLab 3 software). Sediment samples were taken with a miniaturized Multicorer (Mini-Muc) (Fig. 9a), which takes four parallel sediment push cores simultaneously (Barnett et al. 1986). These cores were 60 cm in length with an inner diameter of 10 cm. Sub-sampling of sediment push cores was done onboard with small core liners of 20 cm length and 26 mm inner diameter. The 60 cm cores were placed on an extruder and pushed down until the top area of the sediment reached the upper edge of the core liner (Fig. 9b). Then, the small cores

were pushed into the sediment and closed with rubber stoppers on both sides. Cores and subsamples were stored at in situ temperature before further processing in the laboratory.

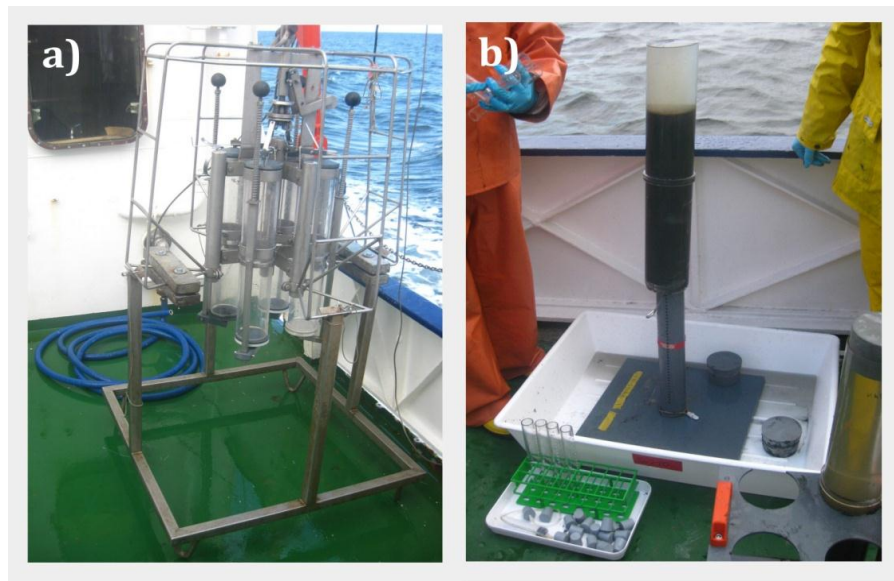


Fig. 9: Sampling on board. a): Mini-Muc, which samples four sediment corer tubes simultaneously, on the rack. b) Sediment core (60 cm) on an extruder prepared for subsampling with small push cores.

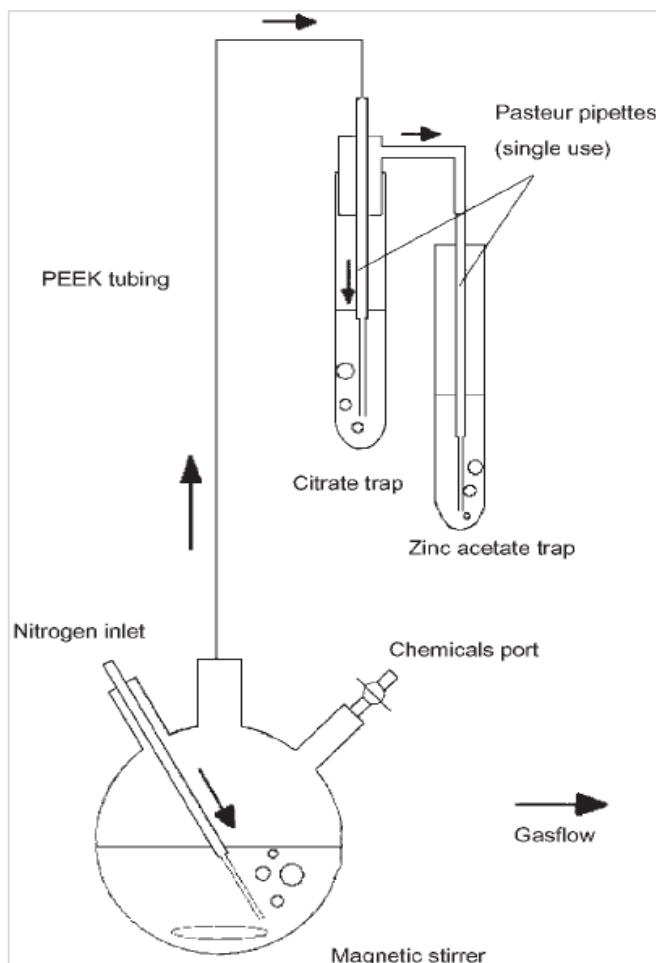


Fig. 10: Cold distillation apparatus to determine the sulfate reduction rates (Kallmeyer et al., 2004).

2.1.2. Whole-core injection method

SR rates were determined using the whole-core injection method with six replicate- and one control push core (length 30 cm, inner diameter 26 mm) (Jørgensen 1978; Fossing & Jørgensen 1989). In the laboratory, carrier-free radioactive sulfate $^{35}\text{SO}_4^{2-}$ (injection volume $6\mu\text{l}$, 200kBq , and specific activity 37 TBq mmol^{-1}) was injected in 1 cm intervals into the small push corers.

Three replicates of radio-labeled cores were incubated at 3°C and three other replicates at 13°C , all for 24 hours in the dark. After incubation, cores were sectioned in 1 cm increments and transferred into 50 ml plastic centrifuge

vials filled with zinc acetate (20%). One control core served as a blank value, with no injection of radiotracer.

SR analyses were done, using the cold single-step chromium distillation method (Fig. 10) (Kallmeyer et al. 2004). This method can detect very small amounts of reduced sulfur species and needs less radiotracer, than the hot single-step chromium reduction method of Fossing and Jørgensen (1989). Aerosols, including $^{35}\text{SO}_4^{2-}$, were filtered into a citrate trap and released sulfide was filtered into a zinc acetate trap, producing zinc sulfide. Finally, SR rates were determined by scintillation counting and calculated according to Kallmeyer et al. (2004) (Eq. 4). For porosity, existing values were used (Bertics et al. 2012).

Eq. 4:

$$SR \text{ rates } (nmol \text{ cm}^{-3} d^{-1}) = [SO_4] * P_{SED} * \frac{a_{TRIS}}{a_{TOT}} * \frac{1}{t} * 1.06 * 1000$$

SO_4 = Porewater sulfate concentration ($mmol \text{ L}^{-1}$)

P_{SED} = porosity of sediment ($mL \text{ porewater cm}^{-3} \text{ sediment}$)

a_{TRIS} = Radioactivity of TRIS (counts per minute)

a_{TOT} = total radioactivity used (counts per minute)

t = incubation time (d)

1.06 = Correction factor (Jørgensen & Fenchel, 1974)

2.1.3. Identification and quantification of microorganisms by Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH)

For identification and quantification of microorganisms in environmental samples, the CARD-FISH method (Fig. 11) was applied. In FISH, a rRNA-targeted probe, which is combined with a fluorescently-labeled oligonucleotide, allows the identification of specific microbes through epifluorescent microscope techniques (Amann et al. 1990). Signals of FISH in aquatic samples were often below the detection limit but can be enhanced by catalyzed reporter deposition (CARD). The CARD part is a further development of FISH, particularly suitable for aquatic habitats, with small, slowly growing, starving bacteria with low rRNA content, based on the tyramide signal amplification (Bobrow et al. 1989). The CARD-FISH protocol applied in the present study, represents a modification of two protocols (Pernthaler et al., 2002; Ishii et al., 2004). Therefore, every step will be described below (for additional details and recipes of reagents - see appendix (1)).

One sediment core (length 60 cm, inner diameter 10 cm) from Eckernförde Bay was sectioned in 1 cm intervals in the uppermost layers (0 – 10 cm) and in 2 cm intervals below (10 – 16 cm). Subsamples of 0.5 ml from each sediment depth were fixed with 1.5 ml 4% formalin/phosphate buffered saline (PBS) solution, for 2 – 4 h at 4 °C. After three washing steps with 1xPBS, each followed by centrifugation (1400 g, 10 min, 4 °C), sediment was finally stored in 1.5 ml 1xPBS/EtOH (50:50) at -20 °C in 2ml cryovials.

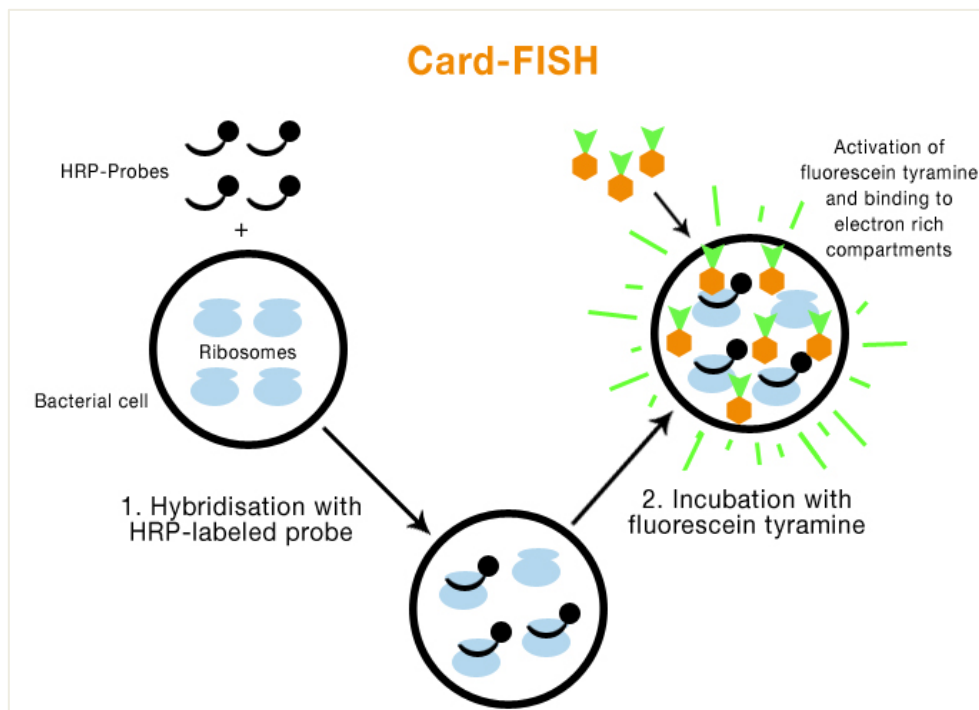


Fig. 11: Principle of CARD-FISH (Source: <http://www.arb-silva.de/fish-probes/fish-protocols/>).

A 1:40 working dilution of the sediment was prepared using cell saver tips that have an extra-large tip orifice to ensure the transfer of cells and bigger sediment particles. Sediment dilutions were sonicated (Type Bandelin Sonoplus GM 200, 2 cycles: 20 sec, 20% intensity, cycle 20) in an ice bath (Fig. 12a and b). Sonication was applied to separate bacteria from sediment particles.

Further sediment dilutions were applied to the respective samples because microbial abundance in sediments may vary depending on sampling site and depth. The optimum cell density was < 30 cells per microscopic view. A pilot study with sediment from the same area was carried out beforehand (testing dilutions between 1:500 and 1:4000), resulting in an optimal final dilution of 1:3000 for sediment between 0 and 10 cm depth, and 1:2000 for sediment between 10 and 16 cm depth.

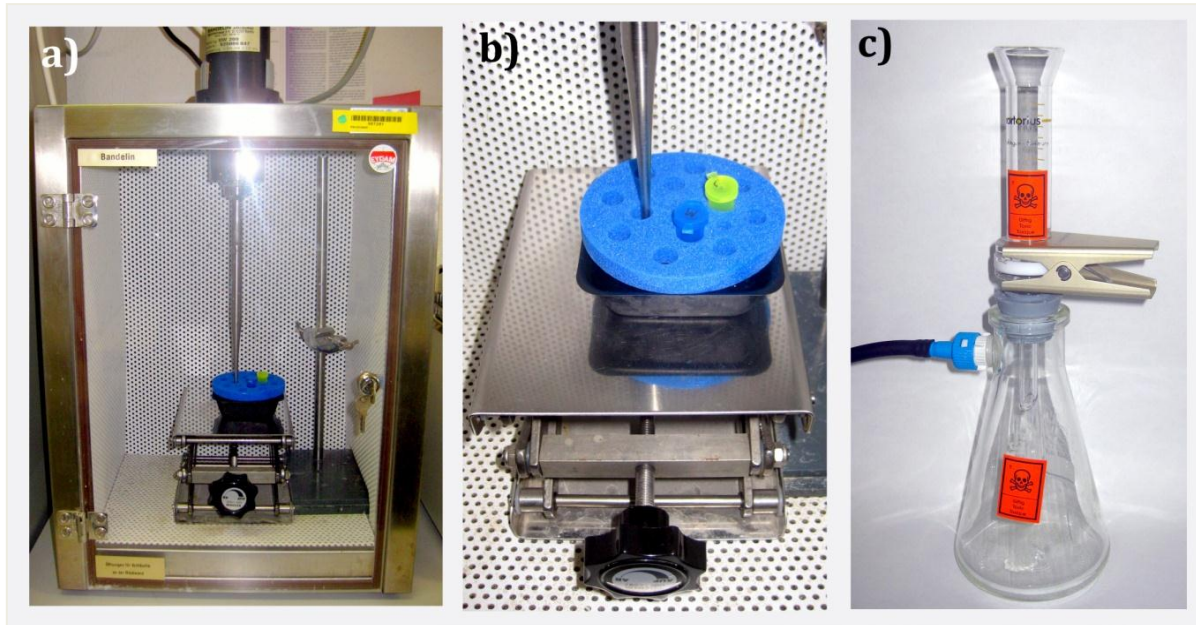


Fig. 12: Principle of sonication and filtration for CARD-FISH. a) Sonicator including a box of iced water with a soft rack on top. b) Close-up image from the inner part of the sonicator. Samples are positioned inside the soft rack and the sonicator tip is inside the iced water. c) Filtration apparatus including filtration tower, filter, claw, drain and flask plus vacuum system.

For filtration (Fig. 12c), a Whatman Glass Microfibre Filter (GF/A 25 mm) was placed on the filtration device, with a Whatman Nuclepore Track-Etch Membrane Filter (0.2 μm , 25 mm ID) “shiny side up”, placed on top. Afterwards, 10 ml of 1xPBS were pipetted onto the filter, 1 ml vortexed sample was added, and the entire liquid was evacuated with -500 mbar. The filtration tower was removed and the filter dried on Whatman Chromatography paper.

For the inactivation of endogenous peroxidases, only a small piece of filter was required. Therefore, the filter was sectioned with a sterile knife into four parts and further analyses were performed on individual sections. Each section was labeled with a pencil and the rest of the filter was stored in the freezer. From here on, the filter was always placed shiny side down into petri dishes. The filter was incubated in 0.1 M TRIS HCl for 60 sec, washed shortly in 1xPBS and incubated in 3% H_2O_2 for 10 min. Afterwards, the filter was shortly washed in sterile Milli-Q, then shortly in 96 % ethanol, and finally dried on Whatman paper.

For the permeabilization of target cells, the filter was incubated in fresh Lysozyme solution (50 mg Lysozyme + 4 ml sterile Milli-Q + 500 μl 0.5 M EDTA + 500 μl 1 M TRIS HCl) under rotation (10 rpm) at a temperature of 36 $^\circ\text{C}$ for 1 h. Pre-hybridization was done in 35 % hybridization buffer under rotation at 46 $^\circ\text{C}$ for 1 h after shortly washing the filter first in Milli-Q and then in ethanol.

Hybridization was done with the DSV 698 probe (Probe sequence: GTT CCT CCA GAT ATC TAC GG, Formamide concentration: 35% (Manz et al., 1998)), which labels the target organisms of this study (*Desulfovibrio spp*). The sample was stored in a reaction vial with a 1:150 dilution of hybridization buffer and DSV 698 probe, and incubated at 46 °C overnight.

Afterwards, the filter was washed in 2x saline-sodium citrate (SSC) and stored in 100 µl blocking reagent (Invitrogen TSA Kit) for 30 min. After storage in the blocking reagent, the filter was incubated for 30 min in a Streptavidin - Horse Radish Peroxidase (HRP) mixture (1:100, 3 µl HRP + 297 µl blocking reagent). HRP is a marker enzyme that attaches to the probe and facilitates the coupling of tyramide and microbes. Finally, the filter was washed in 1xPBS for 10 min.

Due to the dye's sensitivity to light, the following steps were conducted under low-light conditions. During the CARD-part of the protocol, fluorescent-labeled tyramide (either Alexa 488 (stains green) or Alexa 546 (stains red), based solely on availability, results were the same with either) was used to stain *Desulfovibrio vulgaris* specimens. For this step, 300 µl of amplification buffer were mixed with 1 µl of 30% H₂O₂ and 3 µl of the fluorescent-labeled tyramide. The filter was incubated in this mixture for 15 min at 46 °C and following, was washed in 1xPBS for 10 min.

The filter was stored in 0.01 M TRIS HCl for 10 min, to inactivate the HRP and avoid unspecific binding. Afterwards, three short washing steps followed- 1xPBS, Milli-Q, and 96% Ethanol – and the filter was dried on Whatman paper.

Every filter was counter-stained with “4',6-diamidino-2-phenylindole” (DAPI) to cross-check whether CARD-FISH stained objects were related to actual cells and to quantify total cell numbers of the sample. For staining, 50 µl of DAPI (1mg/l) were pipetted on a petri dish and each filter was stored upside down on the drop for 10 min, followed by washing in Milli-Q for 5 min, in 96% ethanol for 1 min and drying on Whatman paper for 30 sec. For microscopy, one drop of Citifluor (Citifluor Ltd., Glycerol/PBS solution) was added on a glass slide. The filter was placed on the drop (“shiny side up”) and another drop of Citifluor was added on top of the filter. A cover slide was placed on top, and then the slide was labeled and placed in the freezer (-20 °C) until further investigation.

The complete above procedure was repeated with a plain filter (i.e. free of sediment), which served as a control against false positives of signals.

Epi-fluorescence microscopy (Leitz Aristoplan) counts of DAPI and CARD-FISH stained cells were done by switching between appropriate filter cubes, depending on dye (A for DAPI, L5 for Alexa 488, Y3 for Alexa 546; details in Tab. 1). Counts were done either until a cell number of 500 or 70 randomly chosen views were reached. Counting was done using a 100x (oil objective) –magnification. The area counted per grid was 0.0121 mm².

Tab. 1: Filter cubes of the microscope for epi-fluorescent microscopy, including details of excitation range, excitation – and suppression filter, dichromatic mirror and corresponding dye for cell detection. BP = bandpass filter. (Source: Leica brochure - Leica Microsystems Fluorescence Microscopy)

Filter	Excitation range [nm]	Excitation filter [nm]	Dichromatic mirror	Suppression filter [nm]	Dye
A	UV	BP 340-380	400	LP 425	DAPI
L5	Blue	BP 480/40	505	BP 527/30	Alexa 488
Y3	CY3 green	BP 535/50	565	BP 610/75	Alexa 546

Mean values of total cells and hybridized cells were calculated according to the following equation (Eq. 5):

$$\text{Eq. 5} \quad \text{Bacterial number/ml sediment} = ((\text{CC} - \text{BC}) * \text{MF} * \text{DF})$$

CC: Average cell number/view

BC: Blank filter

GF: Size of counting grid (mm²)

*FF: Filter area (in mm² by measuring the inner diameter of the filtration tower $\pi * r^2$)*

MF: Microscope factor $MF = FF/GF$

DF: Dilution factor

2.1.4. Porewater analysis and sediment properties

After returning from field sampling, samples for pore-water analysis of ammonium, sulfate and sulfide concentrations were collected, using anaerobic Rhizon soil moisture samplers (Rhizosphere Research Comp., Wageningen, The Netherlands) (Seeberg-Elverfeldt et al., 2005). Cores were sampled in 1 cm intervals to a depth of 10 cm and in 3 cm intervals up to a maximum depth of 30 cm. Samples were sterile filtered with a 0.2 µm filter into plastic vials and analyzed with a Hitachi UV/VIS spectrophotometer following standard photometric

procedures (Grasshoff & Kremlingl, 1999). Sulfate was analyzed using ion chromatography (Metrohm ion-chromatograph with anion-exchange column, using carbonate-bicarbonate solution as eluent) with the IAPSO seawater standard for calibration (for results of porewater analysis see Appendix Tab. 4).

For determination of the porosity, a known volume of wet sediment was dried at 60 °C for 48 h. After drying, the sample was weighted again and from the difference of these two weights and the known volume, porosity was calculated.

2.2. Impact of Organic Carbon (Experiment 2):

2.2.1. Sampling

Samples for Experiment 2 were taken during a one-day cruise with the GEOMAR RC “*Littorina*” on the 13th of April 2012, at the same position as in Experiment 1. Sediment samples were taken with a Mini-Muc (see chapter 2.1.1.). All sediment cores were stored in the 10°C cold room for later laboratory experiments.

2.2.2. Preparation of organic matter samples

Two types of organic matter were used for this experiment: (1) algal material (DT’s Blend Premium alive Phytoplankton), which included the species *Nannochloropsis oculata*, *Phaedactylum tricornutum* and *Chlorella*, and (2) macrofaunal material from the sludge worm *Tubifex tubifex* (JBL Novo Fex Tubifex cubes), an oligochaete, belonging to the family Tubificidae.

In order to determine the energy value of the two organic carbon sources, measurements with a bomb calorimeter were performed (Linde, 2007; Pinhack, 2002).

Because it was not possible to combust the phytoplankton in the bomb calorimeter in the liquid stage, it needed to be freeze-dried before. This procedure freezes material and then reduces the pressure in order to let the frozen water sublimate. Freeze-drying was done at the “Institut für Polarökologie” in the laboratory of M. Bölter’s research group. An undefined amount of Phytoplankton was filled into 10 ml glass vials, which were incubated in the freeze-dryer (Christ ALPHA) for 24 h at -60 °C. *Tubifex* material was purchased in the freeze-dried.

2.2.3. Bomb calorimetry

Bomb calorimetry (IKA Kalorimetersystem 200) was done in the “Institut für Tierernährung und Stoffwechselfysiologie” at the Christian-Albrechts-University Kiel in the laboratory of S. Wolfram. Bomb calorimetry determines the heat created by a sample when burning under an oxygen-rich atmosphere (~99.95%). From this generated heat, the sample’s energy value can be calculated. The *phytoplankton* sample had seven replicates- and *macrofauna* three replicates. The different amount of replicates is due to the fact that *macrofauna* replicates resulted in a high standard deviation following three measurements and therefore, a higher number of replicates was measured for statistical reasons. Each replicate was weighted and inserted into the combustible crucible with an underlying cotton thread (Fig. 13a, b, c). The decomposition vessel was closed and an oxygen rich atmosphere was created by flushing the vessel for 30 sec with pure oxygen (99.95%) (Fig. 13d). Afterwards the tank in the bomb calorimeter was filled with water, the vessel was placed inside, and the bomb calorimeter was closed (Fig. 13e). Finally, the cotton thread started the combustion of the sample inside the crucible.

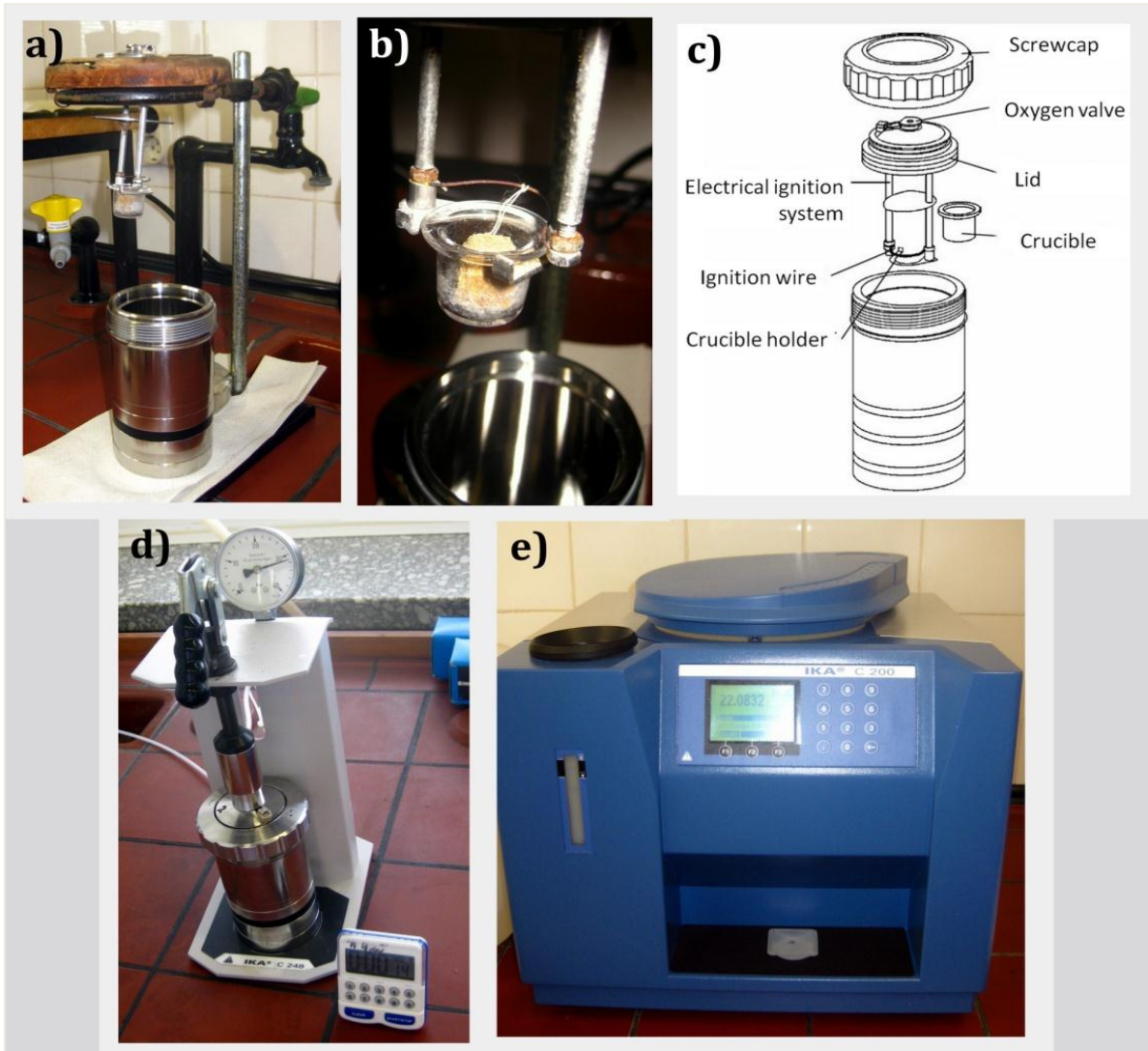


Fig. 13: Measurements with the bomb calorimeter. a) Combustible crucible including a *Tubifex* sample and hanging below the composition vessel. b) Close-up of the combustive crucible with sample and cotton thread. c) Scheme of combustive crucible and composition vessel © by IKA C 200 bomb calorimeter. d) Oxygen station to create an oxygen rich atmosphere. e) Bomb calorimeter “IKA C 200”.

After 10 min the first burning took place and after another 10 min the second one. The values, which the instrument displayed at the end, were the temperature differences (in K) in the water tank, before and after burning. From the difference of the two temperatures and the initial weight of each replicate, the energy value was calculated according to the formula in the “IKA C 200” protocol. Afterwards, mean values were calculated.

The only differences between *phytoplankton* and *macrofauna* were (1) *phytoplankton* were freeze-dried beforehand (see chapter 2.2.2.) and (2) the *phytoplankton* sample was filled into an Acetobutyrate capsule for better burning, because after freeze-drying, it was a fine powder that was not combustible. The value of this capsule was subtracted in the formula.

2.2.4. Artificial seawater medium for sulfate-reducing bacteria (modified by Widdel & Bak 1992)

Artificial seawater medium for SR bacteria was prepared. The artificial seawater had a salinity of 23 and therefore, it was suitable for bacteria, living in the Baltic Sea. This preparation was done according to the protocol (Widdel & Bak, 1992) and used to create the 1:1 medium:sediment slurry used in Experiment 2 (see 2.2.5.) (composition of all reagents see appendix (2) and Tab. 5). After all salts were dissolved in a 1 L measuring cylinder, that included a magnetic stir bar, 1 ml Resazurin was added, which turns pink color when in contact with oxygen. The solution was transferred into a glass bottle equipped with a septum stopper and autoclaved for 35 min at 121°C. After the autoclave cooled down to 85 – 90 °C, the bottle was removed and stored in iced water. After purging with N₂ (5 min, 0.2 bar) and N₂/CO₂ (15 min, 0.2 bar, 80:20), 1 ml/l of the following solutions were added through a 0.2 µm PES filter under constant stirring (for details see Appendix (2)):

- 6 Vitamin solution
- Thiamine solution
- Cyanocobalamine solution
- Riboflavin solution
- Selenite/Tungstate solution
- Trace elements solution
- Na₂S solution (0.5 M)

Flushing with N₂/CO₂ continued until the medium became clear again, indicating the medium was anoxic. The pH was adjusted (pH meter: Schott Instruments, Lab 850) with NaOH (0.1 M and 10 M) or HCl (6.5%) to 7.5.

2.2.5. Experimental design

The upper 0 – 5 cm of two sediment cores (Fig. 14) were sliced and transferred into a 1 L glass vial so that the bottle was filled with roughly 500 ml sediment followed by the addition of 500 ml of medium. The slurry (1:1 mixture sediment:medium) was flushed with N₂, sealed with a rubber stopper and screw cap and incubated at 13 °C overnight.

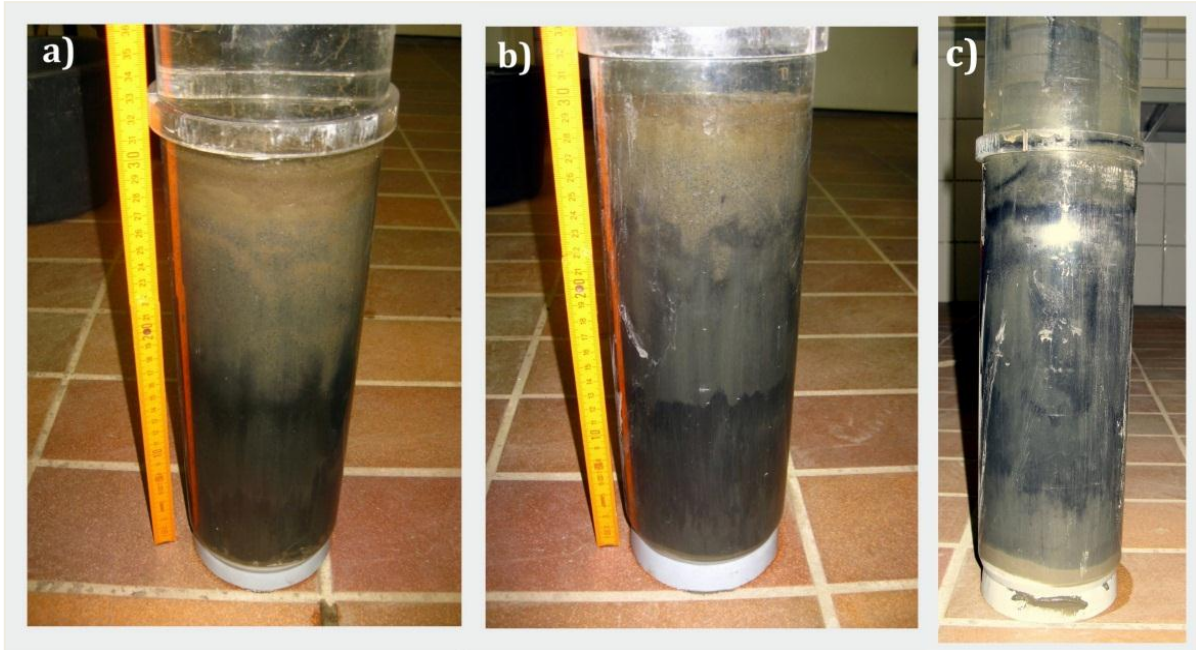


Fig. 14: Sediment cores for the organic matter experiment. a) and b) Cores that were sliced (0 – 5 cm) for the slurry. c) Core that served as control and was sampled 15 days after core a and b. All three cores had a core length of approximately 30 cm.

The next day, the 200 ml slurries for the organic matter experiment were prepared (dilution 1:1 with artificial seawater) (see Fig. 15).

In order to add reasonable amounts of carbon to the sediments, reflecting the natural organic matter input to the sediments used in this study, values reported by Graf et al. (1983) served as orientation. Graf et al. (1983) estimated that the daily carbon flow to the sediments in the Western Baltic Sea was $0.2 \text{ g C m}^{-2} \text{ d}^{-1}$ during winter, representing the lowest value and $1.4 \text{ g C m}^{-2} \text{ d}^{-1}$ in spring, representing the highest value.

These minimum and maximum values were extrapolated to a time interval of 3 weeks and reduced to the surface area of the sediment core, resulting in the amount of $0.042 \text{ g phytoplankton}$ in the low concentration scenario and $0.294 \text{ g phytoplankton}$ in the high-concentration scenario (Tab. 2). Based on these amounts, the corresponding energy value for the two *phytoplankton* concentrations was calculated to be 0.5194 kJ and 3.6361 kJ for the low- and high concentration scenarios, respectively. Because the same energy should be added for *macrofauna* as was added for *phytoplankton*, the values were converted by defining the corresponding concentration of macrofauna in gram to the above mentioned energy values. The amount of added *macrofauna* resulted in 0.0079 g for the low- and 0.055 g for the high concentration scenarios.

Tab. 2: Overview of the type and amount (g) of organic matter added to each 200 ml 1:1 sediment:media slurry. Shown is also the corresponding energy value (kJ) for each carbon species in the high and low amount based on amounts calculated for the Western Baltic Sea, extrapolated to three weeks and the sediment core area.

Organic matter type	Concentration (g)	Energy value (kJ)
<i>Phytoplankton</i> (low)	0.042	0.5194
<i>Phytoplankton</i> (high)	0.294	3.6361
<i>Macrofauna</i> (low)	0.0079	0.5194
<i>Macrofauna</i> (high)	0.055	3.6361

Before mixing with the sediment slurries, the organic carbon material (freeze-dried *phytoplankton* and *macrofauna*) was grinded, weighted and sonicated (Bandelin Sonoplus GM 200) in order to disrupt the material, in Eppendorf caps on a low frequency (2 cycles: 10 sec, 20% intensity, cycle 10). Finally, the proper amount of each carbon material (Tab. 2) was transferred into a 250 ml glass vial that was then sealed with a rubber stopper and screw cap. All bottles were then flushed with N₂ for 5 min to remove oxygen and finally 200 ml of the slurry was added. Bottles were shaken well and incubated horizontally for 15 d at 13 °C. Every second to third day the slurries were shaken gently and on day 14 of the incubation, bottles were arranged upright to have a clear supernatant for sulfide measurements on the following day. For a detailed experimental setup, see Fig. 15.

After 15 days of incubation at 13 °C, serum vials for SR rates and for nitrogenase activity determination, according to the experimental setup (Fig. 15 – “serum vials”), were prepared. For investigation of SR rates, 20 ml of each 200 ml slurries were extracted and injected into 40 ml serum vials (see “SRR serum vials” in Fig. 15). Because more headspace was needed for measurements with the gas chromatograph, the vials for nitrogenase activity had a volume of 60 ml, and included 20 ml sample, as well (see “NA serum vials” in Fig. 15). Serum vials for nitrogenase activity were acid washed and combusted at 450 °C for 24 h in the combustion oven (Nabertherm B 150) prior to use, to remove all metals and organic particles, respectively.

Three samples with three replicates each, were extracted from the control slurry without organic material (20 ml for (i) SRR, (ii) NA (nitrogenase activity), (iii) killed NA with 4 ml Formalin) and transferred into 40 ml (SR) and 60 ml (NA) serum vials.

A third sediment core (Fig. 14c and Fig. 15 “Control core”) was sampled on the same day that the incubations were terminated. This sampling was done in order to have control samples, without organic carbon material. The top 5 cm of the core were sampled, transferred into a beaker, and mixed to create a homogenous mixture.

For SR rates, the control core was sampled with cut-off syringes and about ~ 20 ml sediment was transferred into 40 ml serum vials (see control core “SRR serum vials” in Fig. 15). The controls for SR rates were (i) no media and no organic carbon, (ii) no media, no organic carbon, including ZnAc (40 ml), whereas the latter represented the killed control.

For nitrogenase activity determination, the control core was sampled in the same way as for SR rates, but sediment was transferred into 60 ml serum vials (see control core “NA serum vials” in Fig. 15). The controls for nitrogenase activity were (i) no media and no organic carbon, (ii) no media, no organic carbon and no acetylene injection, (iii) a killed control including Formalin (2 ml) with no media and no organic carbon.

Finally, control core- and slurry serum vials were sealed with a rubber stopper and a crimp seal. To create an anoxic atmosphere, the vials were flushed with N₂. Afterwards, SR rates were determined using the radiotracer technique (2.2.7.) and nitrogenase activity was measured with the acetylene reduction assay (2.2.8.).

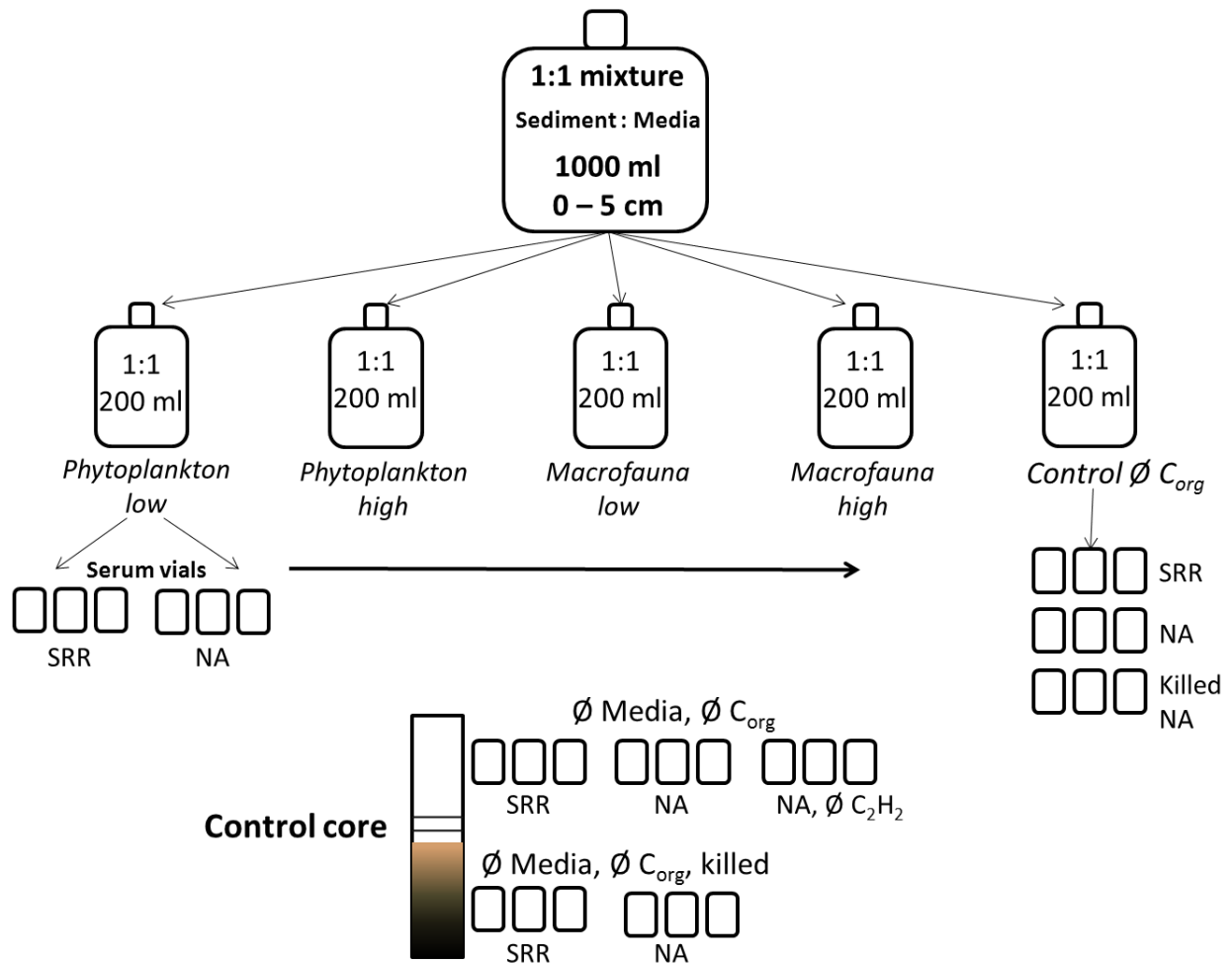


Fig. 15: Setup of the organic matter experiment (Experiment 2) with four different slurry setups (dilution of sediment 1:1 with artificial seawater): phytoplankton low, phytoplankton high, macrofauna low and macrofauna high (for amounts added see Tab. 2). One slurry without (\emptyset) organic matter served as control ($\emptyset C_{org}$), whereas one “control core” served as control without organic carbon and without media ($\emptyset C_{org}$ \emptyset Media). After 15 days of incubation, “serum vials” were prepared for determination of sulfate reduction (SR) rates and nitrogenase activity (NA).

2.2.6. Radiotracer injection to determine sulfate reduction rates

SR rates were determined by injecting the radioactive tracer $^{35}\text{SO}_4^{2-}$ (6 μl , 200kBq, incubation time 19.5 h) into samples (Jørgensen, 1978) and by the cold single-step chromium distillation procedure (Kallmeyer et al. 2004). For details refer back to chapter 2.1.2.

The total number of samples, including controls, was seven- with three replicates each (for sample details see Fig. 15 “SRR”).

2.2.7. Measuring nitrogenase activity by the acetylene reduction assay

To quantify nitrogenase activity, the acetylene reduction assay was applied (Capone, 1993). This method is based on the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) by the enzyme nitrogenase, which can be measured by gas chromatography (see also section 1.2.).

The total number of samples was nine, with three replicates each. Five ml of C_2H_2 were injected into serum vials via a syringe to saturate the nitrogenase enzyme, whereas one sample from the control core (without organic carbon and without medium) served as control without any injection of C_2H_2 (for sample details see Fig. 15 “NA”).

Before and after every measurement, an ethylene standard (Matheson Tri-Gas Micro Mat 14, 100 ppm) was measured twice on a gas chromatograph (Carrier gas: Helium, Air: 20 % O_2 in N_2 , Temperature column: 75 °C, Temperature detector: 160 °C) with a flame ionization detector. Injections were done with a 100 μ l gas tight syringe.

One hour after injection of C_2H_2 , the first measurement (t_0) was done. From each serum vial, a 100 μ l sample from the headspace was extracted and injected into the gas chromatograph. Three samples were injected consecutively (0, 0.4 and 0.8 min), with no overlap in peaks. The first peak of each injection was the nitrogen peak, the second was methane, the third one the ethylene peak and the last one the acetylene peak (Fig. 16).

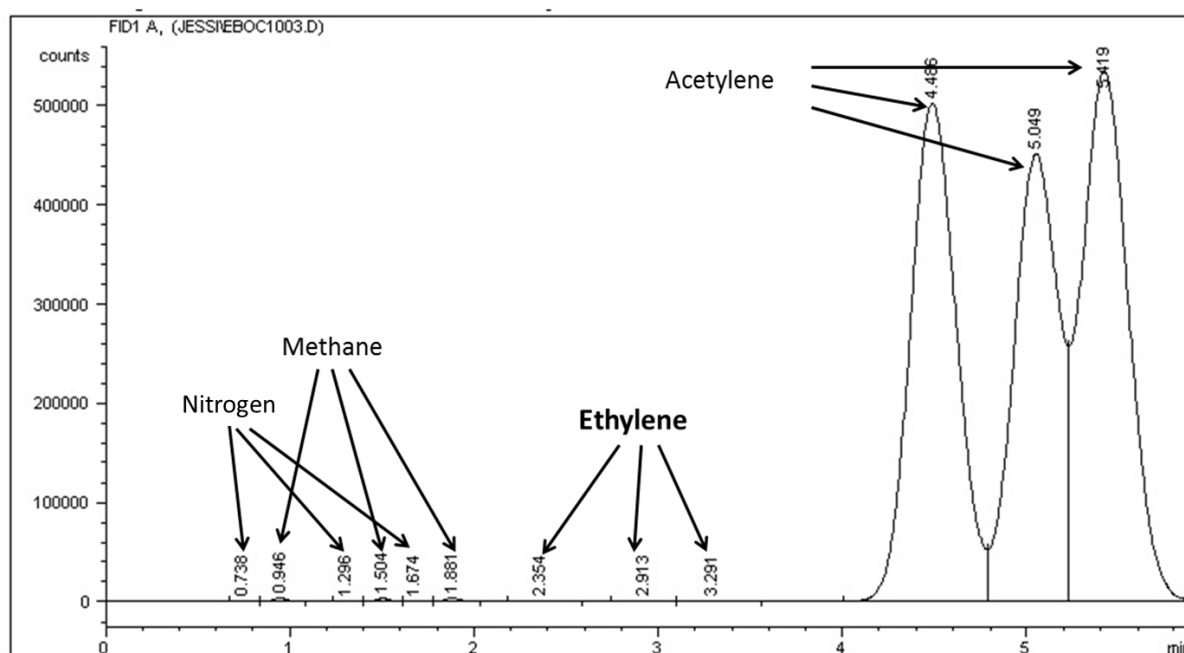


Fig. 16: Chromatogram in the program HP ChemStations. X-axis illustrates time (min) and y-axis displays the counts. For each sample: 1st peak = Nitrogen, 2nd = Methane, 3rd = Ethylene and 4th = Acetylene.

After each measurement, the gas chromatograph-program (HP ChemStations) displayed the peak area and the time of injection. Measurements were done at a total of six time points (t_0 – t_5) including time zero. During and between each measurement, all serum vials were stored in the dark and at *in situ* temperature (13 °C).

C_2H_4 production was calculated by the following equation (Capone, 1988):

$$\text{Eq. 6} \quad \text{nmol C}_2\text{H}_4 = \frac{\text{pk area, unk}}{\text{pk area, std}} * [\text{std}] * (\text{GPV}) * (\text{SC})$$

(pk area, unk) = Peak area response for C₂H₄ for 100 µl of sample

(pk area, std) = Peak area response for C₂H₄ for 100 µl standard

[std] = concentration C₂H₄ standard in nmol ml⁻¹

(GPV) = Gas phase volume

(SC) = Solubility correction for C₂H₄ in aqueous phase

The solubility correction (SC) takes C₂H₄ into account, formed in the aqueous phase of the sample and was calculated according to the equation (Flett et al. 1976):

$$\text{Eq. 7} \quad \text{SC} = 1 + \left(\alpha * \frac{A}{B} \right)$$

α = Bunsen coefficient for C₂H₄ at appropriate temperature and salinity

A = Volume of aqueous phase

B = Volume of gas phase (GPV)

The Bunsen coefficient was defined to be 0.133 according to 12°C and a salinity of 23 (Breitbarth et al. 2004).

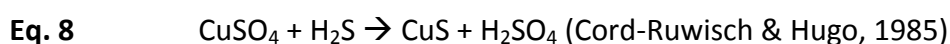
Afterwards, values for C₂H₄ production versus incubation time were graphed in a scatter plot. Rates were determined by linear regression analysis and were averaged per sample. To get an estimate of how much nitrogen was fixed, the rate of C₂H₄ production was converted to a rate of N₂ fixation based on a proper ratio, which is defined to be 3 C₂H₄ : 1 N₂ (O'Donohue et al. 1991; Orcutt et al. 2001).

2.2.8. Sulfide measurement according to Cord-Ruwisch

After 15 d of incubation, sulfide concentrations were measured in the clear supernatant of the 200 ml slurries using a photometer (Shimadzu spectrophotometer, UV mini-1240), following the method according to Cord-Ruwisch (1985). Previously, a sulfide concentration of 16 mmol/l was found to slow down and even inhibit the growth of pure cultured SR bacteria (Reis et al. 1992).

In short, 100 µl of supernatant was injected into a glass vial containing 4 ml copper sulfate solution (Recipe see Appendix (3)) and then transferred into a 4.5 ml quartz cuvette. Absorption of the formed copper sulfide precipitate was measured at a wavelength of 480 nm

The corresponding reaction is (Eq. 8):



Final sulfide concentrations were determined using a calibration line from 0.5 – 20 mM sulfide. The starting sulfide concentration for the slurries was at 0.415 mmol/l and reached 1.998 mmol/l, which is still in the lower range of values compared to 16 mmol/l that could inhibit SR (results see appendix Tab. 6).

2.2.9. Sediment properties

To determine the initial sulfate concentration in each 200 ml slurry prior to 15 d incubation, 3 ml of each sample supernatant were extracted and filtered through a 0.2 µm PES filter into cryovials. These samples were stored in the fridge for later analysis via ion chromatography (Metrohm ion-chromatograph with anion-exchange column, using carbonate-bicarbonate solution as eluent) with the IAPSO seawater standard for calibration.

2.791 g sediment from the control core was extracted and stored in a plastic vial in the fridge for later CNS analyses.

Porosity for the 1:1 sediment:media slurries was calculated based on the average sediment porosity in the upper 0 – 5 cm depth (0.89) and the proportion of media. Then, one had volume of water divided by total volume.

2.3. Statistical analysis

Statistical analysis, such as determination of normal distribution by the Shapiro-Wilk normality test, produce Box-Whisker-Plots and test the significance between samples, was performed by using the software program “R” (version 2.15.0., The R Foundation for Statistical Computing). One-factorial analysis of variance (1-way ANOVA) with subsequent Post-Hoc-Test (TukeyHSD-Test) was used to analyze differences between three or more samples. Differences between two samples were tested using the Student t-test. The level of significance was 5% (p-value < 0.05).

3. Results

The focus of this thesis was to study the effect of temperature and organic matter on nitrogen (N₂) fixation coupled with sulfate reduction (SR) by the SR bacterium *Desulfovibrio vulgaris*. Not much research has been done concerning these effects and to get an insight, SR and N₂ fixation rates were examined. In the first experiment, the variable parameter was temperature (chapter 3.2.). In the second investigation, the variable parameter was the type and the energy level of added organic matter (chapter 3.3.).

3.1. Sediment parameters

Sediment porosity for the surface was in the range of ~ 0.93 for each experiment. It continuously decreased with increasing sediment depth and reached the lowest value of ~ 0.85 within 15 cm depth (data not shown). The 1:1 sediment slurries with artificial seawater had a porosity of 0.95 that was calculated based on the average porosity in the upper 0 – 5 cm depth (0.89) and the water percentage. Porosity was used for formula calculation of SR rates and N₂ fixation.

3.2. Impact of Temperature – Experiment 1

3.2.1. Sulfate reduction rates

Results indicated that SR rates were greater at higher temperature (13°C) than at lower temperature (3 °C) (Fig. 17). SR rates for the 3 °C incubation showed highest values in the upper parts of the sediment (0 – 5 cm) and decreased with increasing sediment depth. The maximum SR rate was 60 nmol SO₄²⁻ cm⁻³ d⁻¹ at 2 – 3 cm sediment depths, whereas the lowest SR rate was located at 14 – 15 cm (8 nmol SO₄²⁻ cm⁻³ d⁻¹). The blue trend line in Fig. 17 illustrates the average rates that stayed constant at ~ 20 nmol SO₄²⁻ cm⁻³ d⁻¹ from top until 7 cm sediment depth. After this depth, rates were decreasing and remained constant at ~ 10 nmol SO₄²⁻ cm⁻³ d⁻¹ until the bottom of the core (15 cm).

The 13 °C sample had much higher rates of SR, compared to the 3°C sample. In the surface sediment (0 – 4 cm), rates were decreasing with increasing sediment depth, but after 12 – 13 cm depth, rates were increasing again. The maximum rate was located at 0 – 2 cm (325 nmol SO₄²⁻ cm⁻³ d⁻¹), which was more than 5 times higher compared to the upper sediment (0 – 5 cm) of the 3 °C incubation. The lowest value (13.5 nmol SO₄²⁻ cm⁻³ d⁻¹) was found at 12 – 13 cm, below which the rates increased again to levels more than one order of magnitude

higher ($154 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$ at 14 – 15 cm). The red trend line in Fig. 17 illustrates the average rates.

Integrating SR rates over 0 - 15 cm sediment depth, rates in the 3 °C ($203 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$) treatment were five times higher than rates in the 13 °C treatment ($1052 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$).

The Student t-test revealed a significant difference between integrated rates of the two treatments ($p = 0.0002$).

The comparison between SR rates and the results of the corresponding nitrogenase activity, that V. Bertics analyzed, will be reviewed in the discussion.

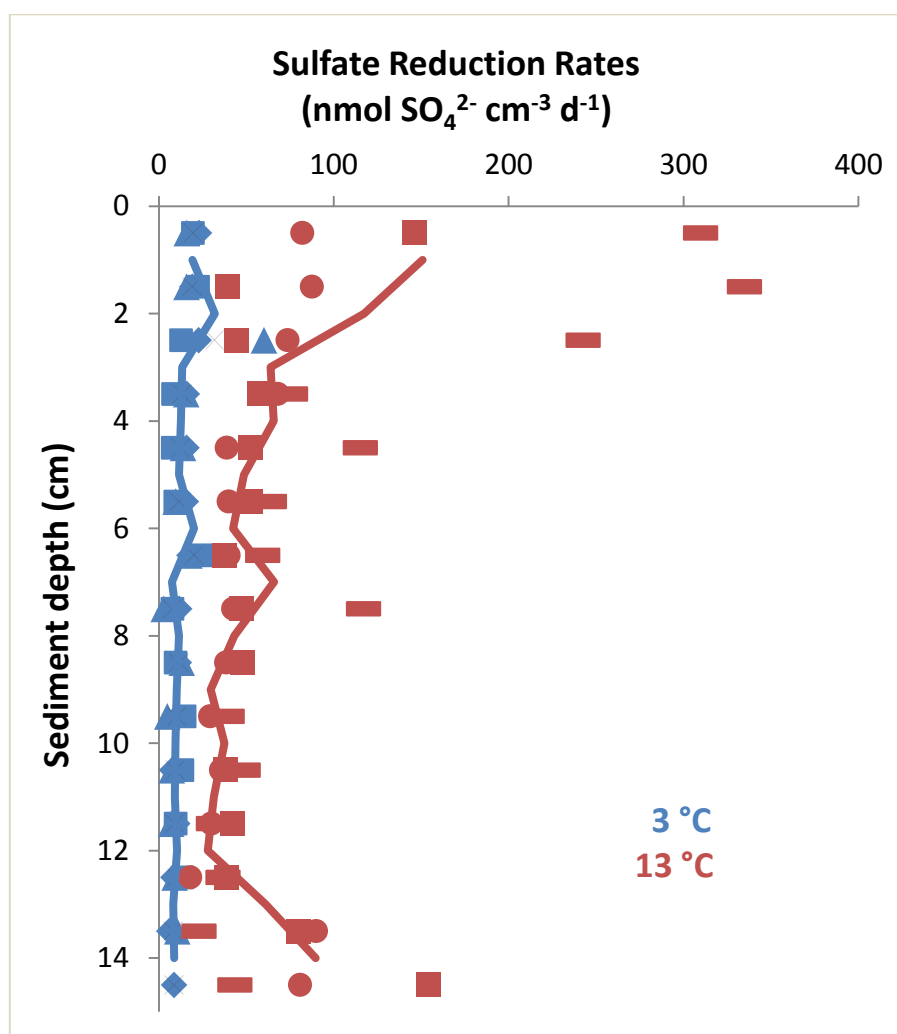


Fig. 17: Sulfate reduction rates of the temperature experiment, determined by the whole core injection method. Displayed on the x-axis are the SR rates ($\text{nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$) and on the y-axis the sediment depth (cm). Blue symbols illustrate the 3 °C incubation while the red symbols signify the 13 °C incubation (three replicates each). Lines represent the averages of respective replicates.

3.2.2. Catalyzed Reported Deposition Fluorescence In Situ Hybridization (CARD-FISH)

The catalyzed reported deposition fluorescence in situ hybridization (CARD-FISH) method was applied to determine total cell numbers in Eckernförde Bay (EFB) sediment and in particular to examine the number of the SR bacteria *D. vulgaris* (For detailed counts see Appendix

Tab. 3). One sediment core was sectioned into 1 cm (0 – 10 cm) and 2 cm (10 - 16 cm) intervals, according to the sectioning for the N₂ fixation method. The ideal dilutions of EFB sediments are 1:300 for sediment in 0 – 10 cm depth interval and 1:200 between 10 and 16 cm, resulting in not more than 30 cells per microscopic view.

Cells were double stained with 4',6-diamidino-2-phenylindole (DAPI) to see the total number of cells per ml EFB sediment (Fig. 18) and a fluorescent-labeled dye (Alexa 488 or Alexa 546 and a DSV 698 probe) was used to stain *Desulfovibrio* cells (Fig. 19 B and D).

Especially in the upper sections, DAPI stained cells were sometimes accumulating in patches within sediment grains and therefore, several microscopic planes had to be examined to count all the cells (Fig. 18).

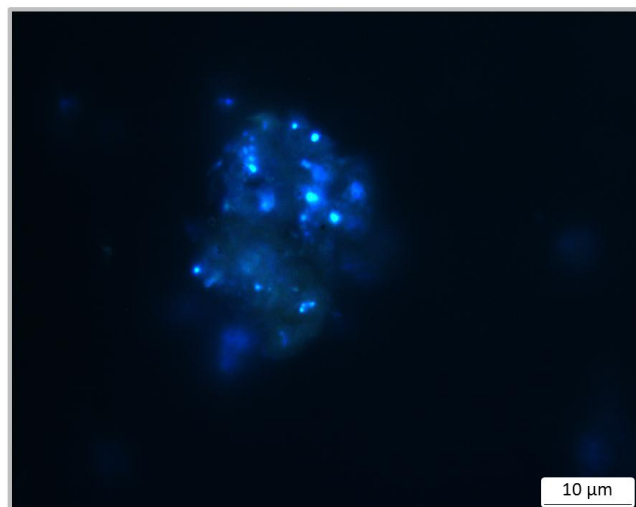


Fig. 18: Patch of bacteria in a sediment grain. Total bacteria stained with 4',6-diamidino-2-phenylindole (DAPI); microscopic filter A (see tab. 1); magnification 100x; sample: 0 – 1 cm, dilution 1:3000.

CARD-FISH stained *D. vulgaris* cells were counted per microscopic view and the DAPI staining served as a control to confirm that cells stained with the fluorescent dye (Alexa 488 or Alexa 546) represented actual cells. Only those cells that were stained by both techniques, were regarded as the target bacteria *D. vulgaris* (As in Fig. 19).

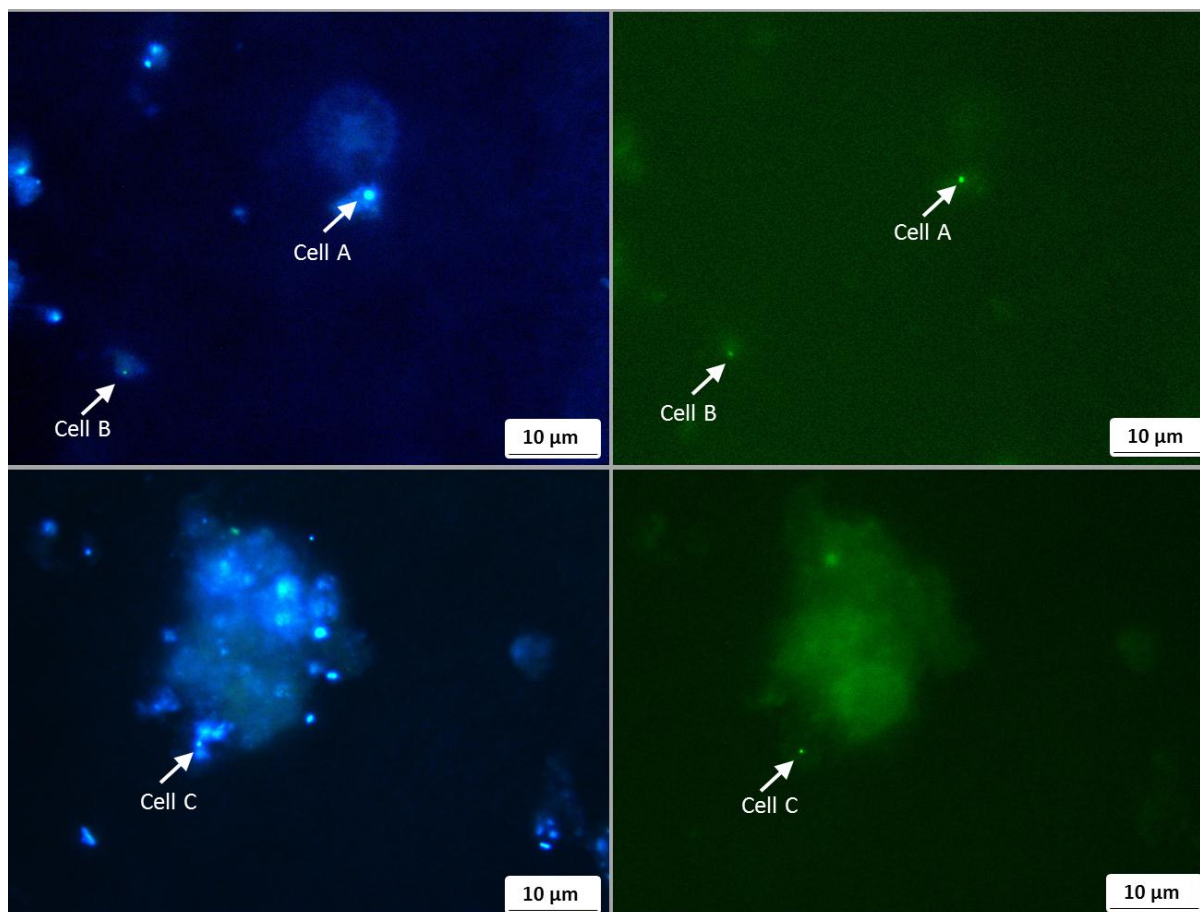


Fig. 19: *Desulfovibrio vulgaris* cells stained with DAPI (A and C) and with Alexa 488 + probe DSV 698 (B and D). Stained bacteria are marked with an arrow; A and C: microscopic filter A; B and D: microscopic filter L5 (microscopic filters see tab. 1); magnification 100x; sample: 0 – 1 cm, dilution 1:3000.

CARD-FISH results indicated that the number of total cells per ml sediment was decreasing with increasing sediment depth (Fig. 20). Maximum numbers were observed in the first cm of sediment ($1.97 \times 10^9 \text{ ml}^{-1}$), below which numbers decreased, reaching the lowest value in 14 – 16 cm ($5.69 \times 10^8 \text{ ml}^{-1}$), which also represented the lowermost section of the sediment core.

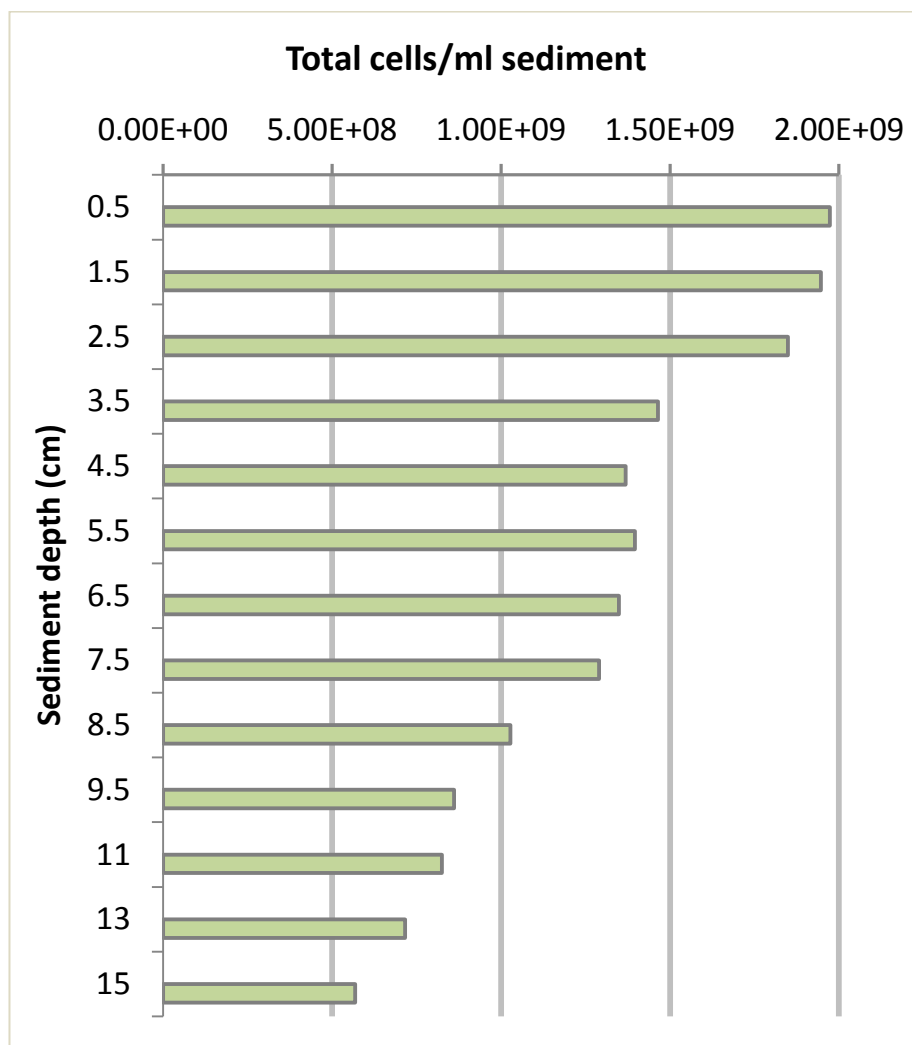


Fig. 20: Total numbers of DAPI stained cells in the Eckernförde Bay sediment. The x-axis displays the number of cells per ml sediment, whereas the y-axis illustrates the sediment depth (0 - 16 cm).

Similar to the total number of bacteria, the number of *D. vulgaris* was also decreasing with increasing sediment depth (Fig. 21). A starting value of 1.02×10^8 target cells per ml sediment was observed in 0 – 1 cm sediment depth, and represented the highest number in all layers. The lowest value for *D. vulgaris* represented $9.79 \times 10^6 \text{ ml}^{-1}$, which was calculated for the deepest layer in 14 – 16 cm depth.

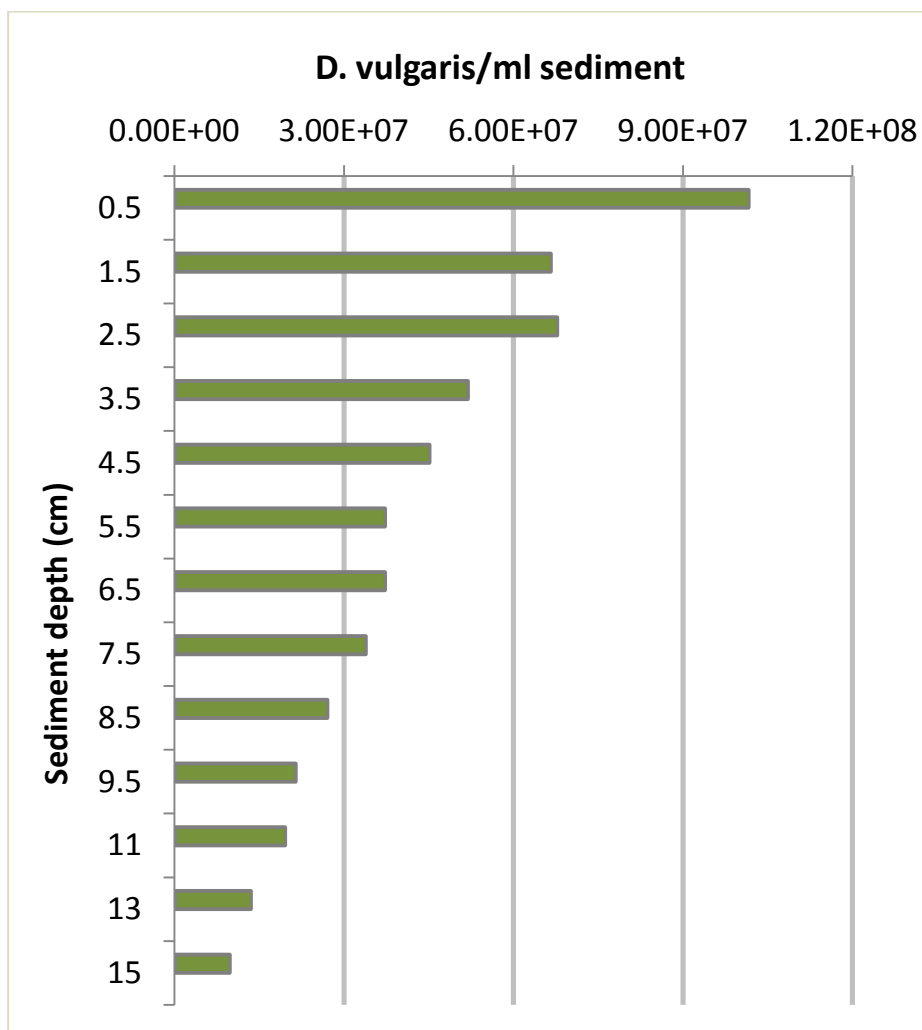


Fig. 21: Number of *Desulfovibrio vulgaris* cells determined by CARD-FISH in the Eckernförde Bay sediment (fluorescent-labeled tyramide Alexa 488/Alexa 546 and a DSV 698 probe). On the x-axis is the number of *D. vulgaris* per ml sediment and on the y-axis the sediment depth (0 - 16 cm).

The relative abundance of *D. vulgaris* cells was decreasing with increasing sediment depth (Fig. 22). In the upper sediment section (0 – 1 cm), *D. vulgaris* reached 5.15% of the total cells and their relative abundance continuously decreased to 1.72% in the deepest section (14 – 16 cm).

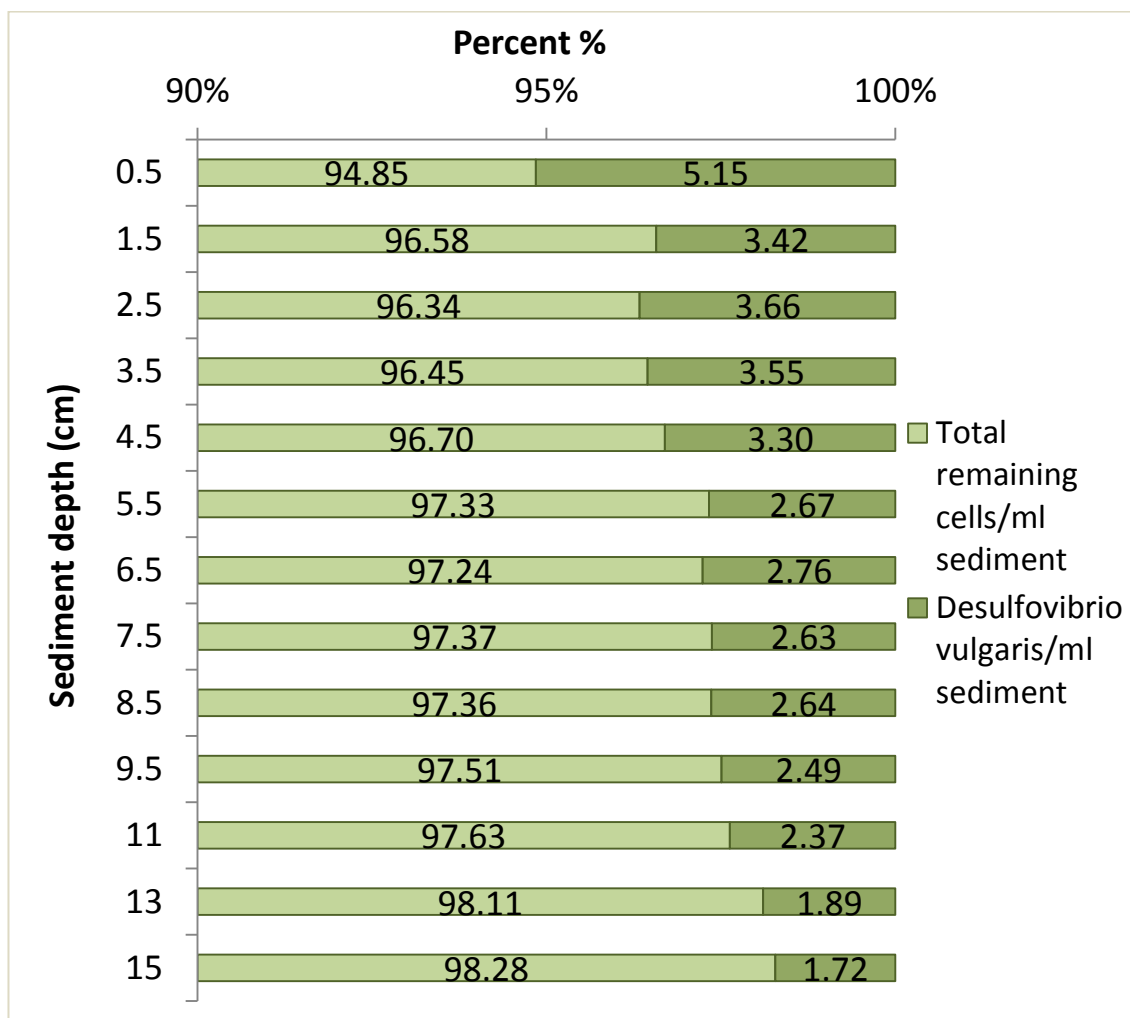


Fig. 22: Abundance of *Desulfovibrio vulgaris* (fluorescent-labeled tyramide Alexa 488/Alexa 546 and DSV 698 probe; x-axis), relative to total cells (100%) (DAPI stained) in each sediment depth (y-axis in cm).

3.3. Impact of Organic Matter – Experiment 2

3.3.1. Sulfate reduction rates

SR rates for the organic matter experiment indicated a correlation between the type and the energy value of the added organic carbon (Fig. 23). In summary, the *phytoplankton* samples showed higher rates than the *macrofauna* samples and a positive correlation between energy value and measured rates was observed (For details in experimental setup see Fig. 15 , chapter 2.2.5.).

Unfortunately, one replicate of the *macrofauna high* sample and one of *macrofauna low* was not available. Consequentially, no averages are presented in these cases, but instead each single replicate is shown (Fig. 23).

The *phytoplankton low* replicates had a high variability and SR values ranged between 7.4 - 65.3 nmol SO₄²⁻ cm⁻³ d⁻¹. The second phytoplankton sample, *phytoplankton high*, had higher rates compared to its analogue, ranging between 94.2 - 111.8 nmol SO₄²⁻ cm⁻³ d⁻¹.

Both *macrofauna* samples had a wide range of SR rates with very low and high values. Rates for *macrofauna low* were between 4.4 and 41.3 nmol SO₄²⁻ cm⁻³ d⁻¹, and *macrofauna high* values ranged from 24.6 – 89.5 nmol SO₄²⁻ cm⁻³ d⁻¹. Interestingly, two of three replicates of *macrofauna high* sample showed values in a similar range as two replicates of the control sample $\emptyset C_{org} + Media$ (\emptyset = without, + = including).

The control containing $\emptyset C_{org} + Media$, resulted in relatively lower rates, which were on average at 23.3 ± 11.9 nmol SO₄²⁻ cm⁻³ d⁻¹.

The control sample without organic carbon and without media ($\emptyset C_{org} \emptyset Media$), displayed in contradiction to all other samples with C_{org} addition, extremely high rates, with an average of 284.2 ± 44.9 nmol SO₄²⁻ cm⁻³ d⁻¹.

The reason for such high rates could be due to natural variability, as the sediment came from a different core than sediment used for the C_{org} samples. Furthermore, the core was sampled at a different time, i.e. two weeks after sediment was taken for the C_{org} samples. It is possible that Polychaetes and other macrofauna, that were present in the cores on the day of recovery, died during the two weeks due to oxygen deficiency and hence provided an

additional source of C_{org} to the microbial community. In conclusion, it was decided that this sample could not serve as a control in this experiment, and was therefore excluded from the discussion. In a future investigation, the control core $\emptyset C_{org} \emptyset Media$, should be sliced on the same day as the cores for the other samples were sliced.

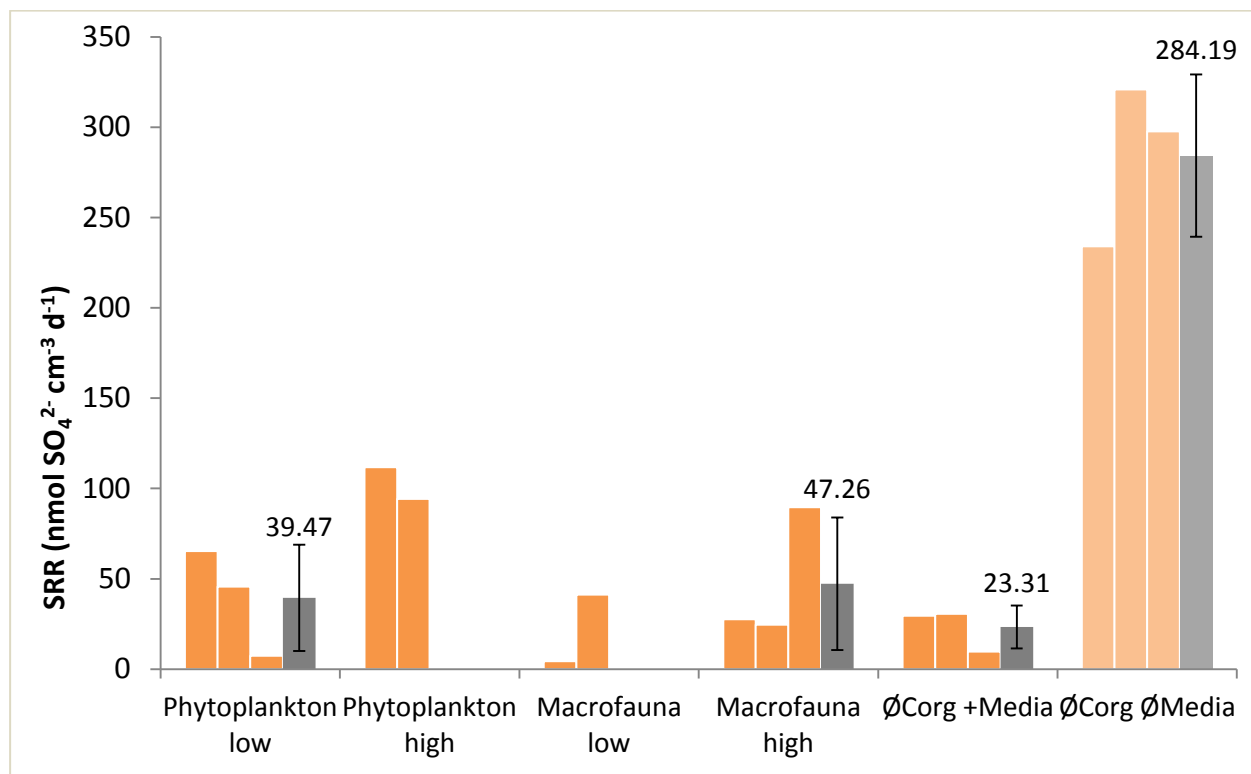


Fig. 23: Sulfate reduction rates (nmol SO₄²⁻ cm⁻³ d⁻¹ on the y-axis) per sample: phytoplankton low, phytoplankton high, macrofauna low, macrofauna high, controls $\emptyset C_{org} +Media$ and control $\emptyset C_{org} \emptyset Media$, with 2 or 3 replicates each. Energy value of C_{org} added: low = 0.5194 kJ, high = 3.6361 kJ. Grey bars represent the averages for samples with three replicates, error bars indicate standard deviation.

One-way ANOVA was applied to examine significant differences between samples. Only samples with three replicates were allowed to be tested, i.e., macrofauna *high*, macrofauna *low* and $\emptyset C_{org} +Media$. Significant differences were not found (p-value = 0.593) and therefore, the null hypothesis, that all samples are equal, was accepted.

3.3.2. Nitrogen fixation rates

In general, nitrogenase activity, as a measure for N₂ fixation determined by the acetylene (C₂H₂) reduction assay, displayed trends in the C_{org} samples similar to SR (Fig. 25).

Both *phytoplankton* samples resulted in comparatively high nitrogenase activity rates with small standard deviations. The average nitrogenase activity rate for *phytoplankton high* was at $4.101 \pm 0.171 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ($\text{C}_2\text{H}_4 = \text{Ethylene}$) and for *macrofauna high*, it was at $4.070 \pm 0.281 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$.

In Box-and-Whisker-plots (Fig. 24), the box diagrams do not look normally distributed; however, after applying a Shapiro-Wilk-test for normality, the p-value was above critical ($p < 0.05$). Therefore, the null hypothesis, that samples are not normally distributed, was rejected. Consequentially, this result allowed performing a 1-way ANOVA.

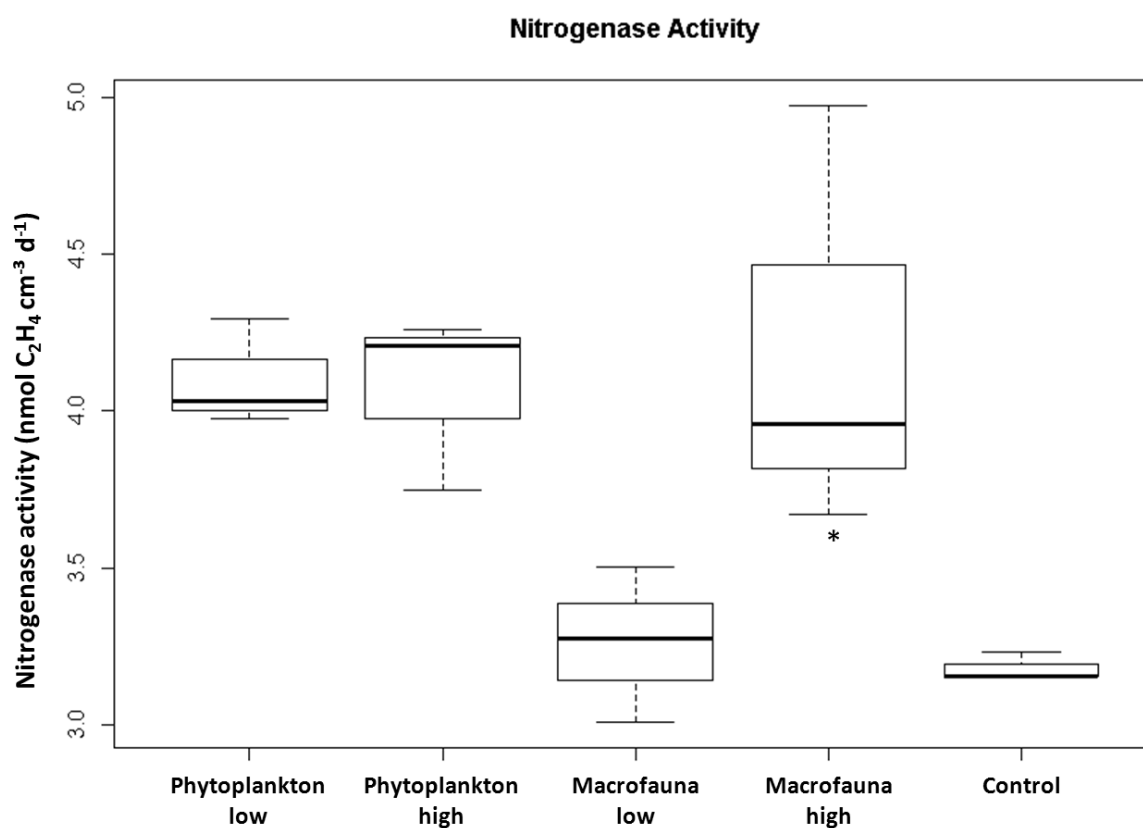


Fig. 24: Box-and-Whisker-Plot of all nitrogenase activity samples and error bars as standard deviation produced by the statistic software program “R”. Thick lines represent the median, top and bottom of a box represent the upper and lower quartile, which means 25% of data are greater or less than this value, respectively. Top whiskers indicate maximum - and bottom whiskers minimum values. The x-axis shows the respective sample and the y-axis the nitrogenase activity ($\text{nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$). Asterisks indicate significant difference from the control $\emptyset\text{C}_{\text{org}} + \text{Media}$ ($p < 0.05$).

By comparing, it was observed that the *macrofauna low* sample had a lower nitrogenase activity rate ($3.262 \pm 0.248 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$), than the *macrofauna high* sample ($4.202 \pm 0.684 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$). The latter also represented the highest rate detected in all

nitrogenase activity samples. Both *macrofauna* samples were not significantly different from each other, when running a 1-way ANOVA ($p < 0.05$). However, 1-way ANOVA, which tested significant differences between samples, resulted in a significance ($p = 0.0111$) and the Tukey HSD post-hoc-test revealed a significant difference ($p = 0.036$) between sample *macrofauna high* and the control $\emptyset C_{org} + Media$. Nevertheless, for all remaining samples, the null hypothesis had to be accepted ($p < 0.05$).

Similar to the SR rates, the control $\emptyset C_{org} \emptyset Media + C_2H_2 \emptyset Formalin$, which was taken from a different core (see 3.1.1), also resulted in extremely high rates ($6.612 \pm 0.189 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$). Rates were twice as high as in the control $\emptyset C_{org} + Media$. For reasons identical to the SR experiment, these control samples will not be further used or discussed.

Three other types of samples served as control in the acetylene reduction assay. Control $\emptyset C_{org} \emptyset Media \emptyset Acetylene \emptyset Formalin$, where no acetylene had been injected, and which checked for natural ethylene production. Results showed that there was no signal of ethylene over the course of time. The same was true for the other two controls, firstly, $\emptyset C_{org} \emptyset Media + C_2H_2 + Formalin$ and secondly, $\emptyset C_{org} + Media + C_2H_2 + Formalin$ ($\emptyset = \text{without}$, $+ = \text{including}$), which were killed controls. The purpose was to examine if there was C_2H_4 production without bacteria. The results for both controls show that there was no C_2H_4 produced over the course of time.

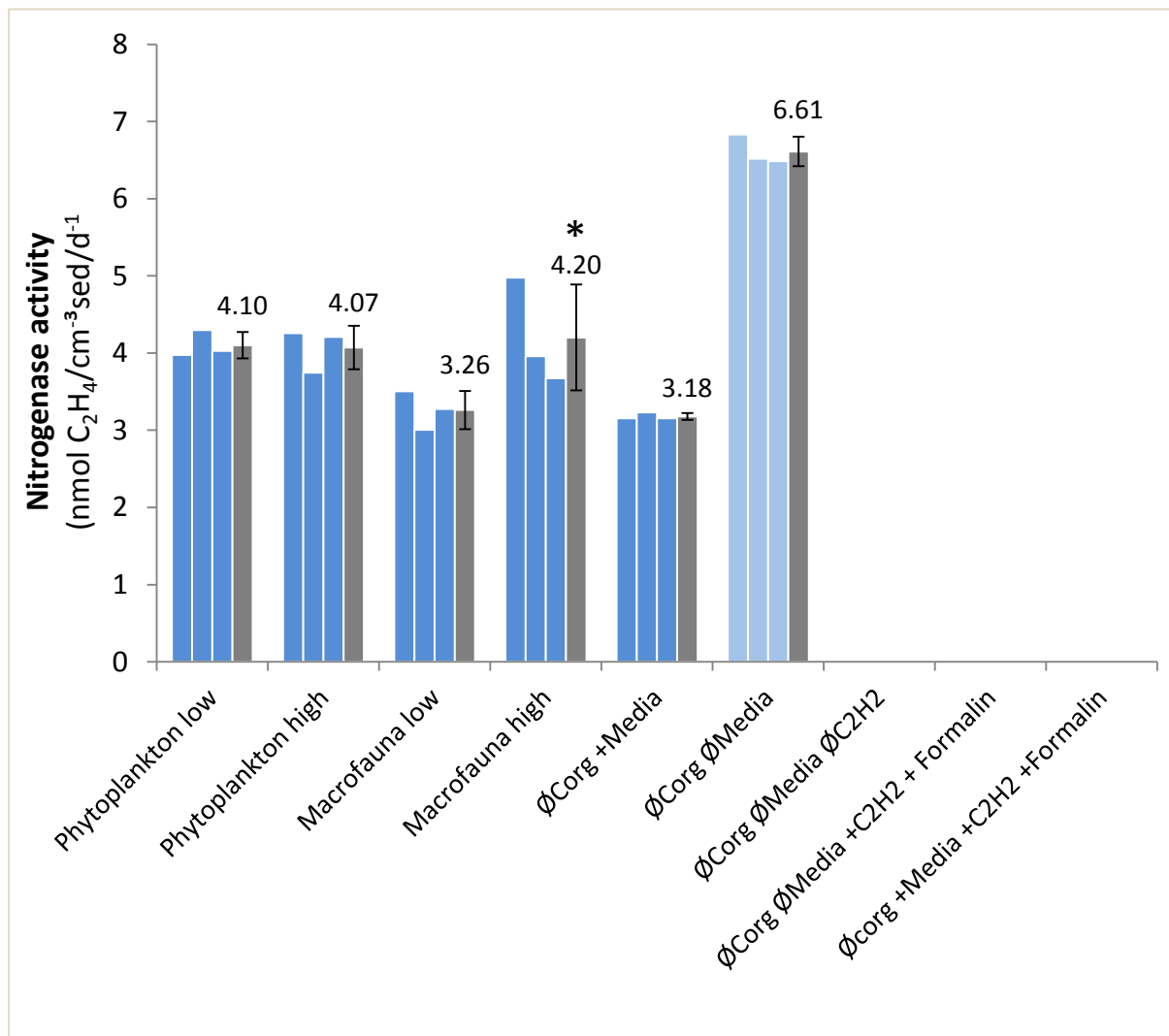


Fig. 25: Nitrogenase activity on the y-axis (nmol C₂H₄ cm⁻³ d⁻¹) for each sample (*phytoplankton low*, *phytoplankton high*, *macrofauna low*, *macrofauna high*, ØC_{org} +Media, ØC_{org} ØMedia, ØC_{org} ØMedia – C₂H₂, ØC_{org} ØMedia +C₂H₂ +Formalin, ØC_{org} +Media +C₂H₂ +Formalin) with three replicates, on the x-axis. Grey bars represent averages, brighter bars control sample that was excluded. Error bars are included and represent standard deviation; asterisks indicate the significant difference between respective samples and control ØC_{org} +Media.

To conclude, the comparison between SR rates and nitrogenase activity indicated a tendency, concerning the energy value, as well as the type of added organic matter (Fig. 26). However, these trends were more pronounced for SR than for nitrogenase activity. This observation could be due to the fact that N₂ fixation is a very energy consuming process for organisms and it may take some time until higher rates are pronounced (see stoichiometry Eq. 2 p. 3).

Phytoplankton low and *high energy* samples indicated higher rates for SR and N₂ fixation, than *macrofauna low* and *high energy* samples (Fig. 26). Interestingly, highest rates for N₂

fixation were not observed in the *phytoplankton high* sample. However, the standard deviation of the *macrofauna high* sample, which displayed the highest nitrogenase rate, was the greatest among all N₂ fixation samples. Eventually, more replicates in the *phytoplankton high* sample might have resulted also in higher nitrogenase activity in the respective sample.

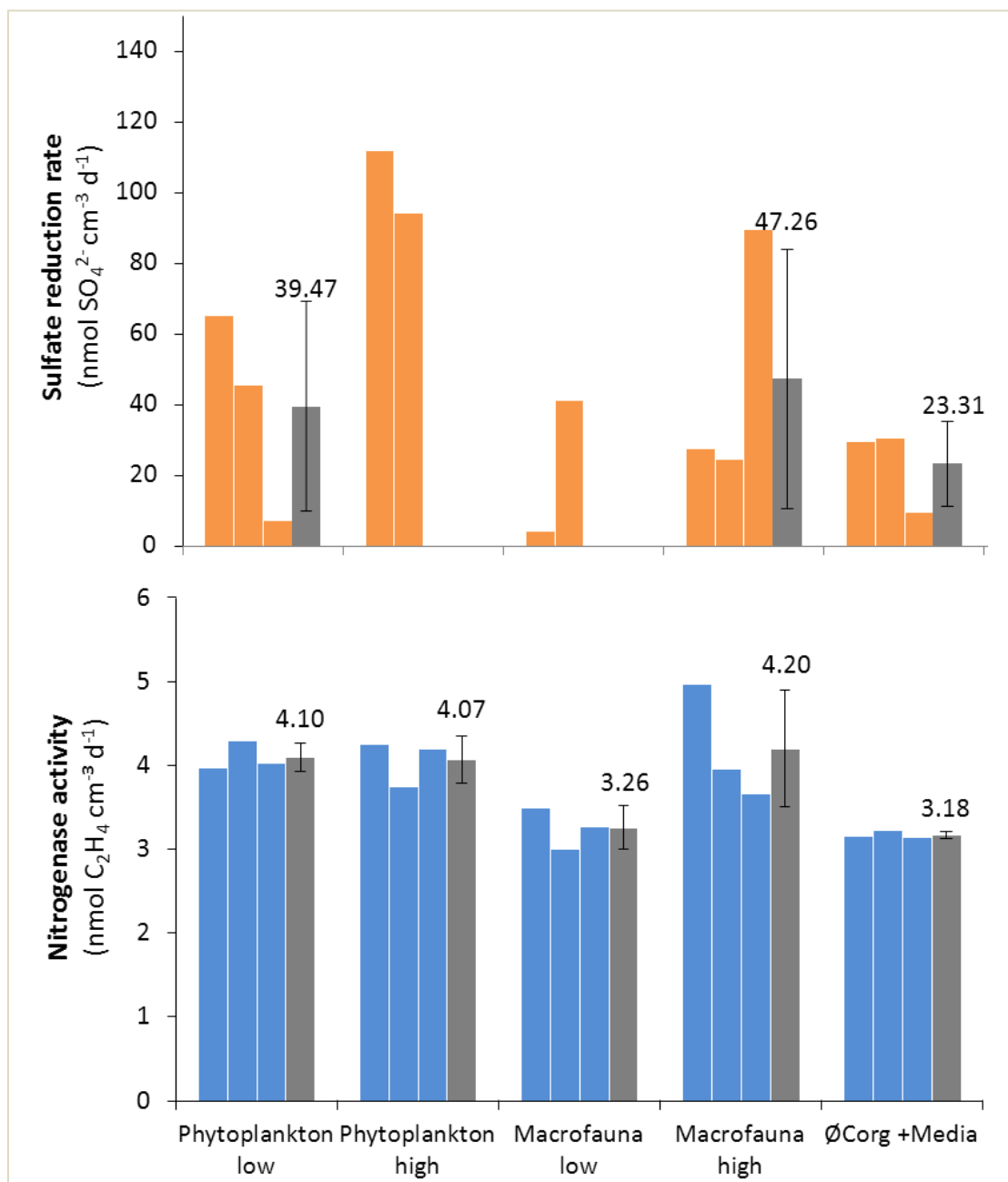


Fig. 26: Comparison of sulfate reduction rate (upper graph, y-axis: nmol SO₄²⁻ cm⁻³ d⁻¹) **and nitrogenase activity** (bottom graph, y-axis: nmol C₂H₄ cm⁻³ d⁻¹) **of organic matter experiment**. Grey bars represent averages and error bars indicate standard deviations. Up to three replicates are shown, when available. On the x-axis respective samples are shown: phytoplankton low, phytoplankton high, macrofauna low, macrofauna high, controls ØC_{org} +Media (*low energy value*: 0.5194, *high energy value*: 3.6361, Ø: without, +: including).

4. Discussion

Microbial nitrogen (N_2) fixation in the ocean is a fundamental part of the marine nitrogen cycle, as it creates and introduces bioavailable nitrogen, in the form of ammonia and ammonium, into the environment (Brandes et al., 2007; Ward & Bronk, 2001). Sulfate reduction (SR) is a major process in the global sulfur cycle, as SR by sulfate-reducing bacteria can contribute to more than 50% of the organic matter mineralization in marine sediments (Jørgensen 1982).

Both processes were found in marine benthic habitats (Capone, 1993; Dale et al., 2011; Steppe & Paerl, 2002), where some organisms, such as the SR bacterium *Desulfovibrio vulgaris*, possess the *nifH* gene, which encodes for the nitrogenase enzyme. Therefore, *D. vulgaris* has the genetic ability to fix nitrogen (Sisler & ZoBell 1951; Riederer-Henderson & Wilson 1970; Zehr et al. 1995).

Little is known about the temperature effects on SR bacteria, mediating N_2 fixation. Often, only two of these processes were addressed (Nielsen et al., 2001; Sohm et al., 2011). Nevertheless, a previous study (Bertics et al. 2012) revealed a possible direct correlation between N_2 fixation, SR rates, as well as temperature, in marine sediments (Fig. 8). However, to our knowledge, a correlation between organic matter sources and how these sources drive N_2 fixation and coupled SR, has not been investigated, yet.

In this study, two main questions concerning N_2 fixation coupled to SR in marine sediments of Eckernförde Bay (EFB) were addressed. The effect of temperature was investigated in Experiment 1. Here, the hypothesis was investigated that N_2 fixation activity and SR rates in sediments of EFB are correlated with temperature.

The abundance of the SR bacterium *D. vulgaris* in sediment layers was determined using catalyzed reported deposition fluorescence in situ hybridization (CARD-FISH) (Pernthaler et al. 2002; Ishii et al. 2004). The hypothesis was that the abundance of *D. vulgaris* would correlate with the activity of SR and N_2 fixation in EFB sediments. Results of Experiment 1 will be discussed in chapter 4.1.

In Experiment 2, the study focused on the correlation of organic carbon availability with SR and N_2 fixation activity. Sediment slurries were prepared, mixed with two organic carbon species (phytoplankton and macrofauna) in two different energy levels and incubated under the same temperature condition (13 °C). Afterwards, SR and N_2 fixation rates were examined and one attempt was to answer the question if N_2 fixation and SR are positively correlated to

the energy value of added organic matter. Secondly, the hypothesis was tested that the addition of algal- compared to macrofaunal organic matter, result in higher SR and N₂ fixation rates. Results of Experiment 2 will be discussed in chapter 4.2. Possible future research is discussed in the last chapters 4.3.

4.1.1. Impact of Temperature – Experiment 1

Results of Experiment 1 indicated a direct correlation of N₂ fixation and SR, with temperature (Fig. 27). The correlation was evident from high SR and N₂ fixation rates in the 13 °C incubated cores, but low rates in the 3 °C incubated cores. Highest SR rates in the 3 °C incubation were in the upper sediments (0 - 5 cm) at 60 nmol SO₄²⁻ cm⁻³ d⁻¹, whereas the 13 °C incubation reached maximum SR rates of 325 nmol SO₄²⁻ cm⁻³ d⁻¹ in surface sediments. In addition, the nitrogenase activity for the 3 °C incubation was at about 1 C₂H₄ cm⁻³ d⁻¹, and the 13 °C sample reached a maximum rates of almost 4 C₂H₄ cm⁻³ d⁻¹. Therefore, the hypothesis that N₂ fixation and SR are positively impacted by increases in temperature in EFB sediments was suggested to be correct.

The rates for SR in the 13°C incubation resulted in a concave down profile, which was observed for EFB sediments in a previous study (Dale et al., 2011). This profile was the result of the relation between downward diffusing sulfate, the uptake of sulfate by microbes and the upward diffusion by advection (Jørgensen, 1983). Therefore, it was suggested that more microbial degradation by SR bacteria was accomplished in the 13 °C samples, than in the 3 °C samples.

The Student t-test revealed a significant difference between integrated rates of SR in temperature incubations ($p = 0.0002$). Therefore, the hypothesis that SR rates in temperature incubated samples are similar was rejected. Integrated rates for nitrogenase activity resulted in a significant difference ($p = 0.0011$). Therefore, the hypothesis that nitrogenase activities between integrated rates are similar was rejected.

Surface sediment in the present study resulted in high SR rates (Fig. 28) (on average 176 nmol SO₄²⁻ cm⁻³ d⁻¹), which were decreasing with increasing sediment depth (27 nmol SO₄²⁻ cm⁻³ d⁻¹). Peaks in SR activity in the 13 °C incubation were in good agreement with nitrogenase activity measured in parallel (Bertics et al., 2012). Nitrogenase activity was high in the surface sediments (~ 3.5 nmol C₂H₄ cm⁻³ d⁻¹) (Fig. 27) and low (~0.5 nmol C₂H₄ cm⁻³ d⁻¹) at 15 cm. In conclusion, the data suggested that both N₂ fixation and SR in EFB sediments,

are positively correlated with temperature. Therefore, it was proposed that N_2 fixation and SR are coupled to temperature and might be performed by the same organism.

One conceivable reason for low SR and nitrogenase activity in the lowermost sediment depth, could be a lower amount of organic matter available in deeper layers, compared to the surface sediment (Rullkötter, 2006).

These results are in good agreement with another experiment carried out with EFB sediment. Anaerobic oxidation of methane (AOM) rates were found to be directly correlated to temperature and potential AOM rates doubled when increasing temperature from 4 °C to 12 °C (Treude et al. 2005). The highest values of AOM were found in March and the lowest in September. This pattern was seen in the present study as well, where lowest rates were observed in the 3 °C incubation (winter/spring scenario) and highest in the 13 °C incubation (summer/autumn scenario). Therefore, this results were not surprising since seasonal variations in microbial processes were found in EFB, before.

Jørgensen (1977) proposed that SR rates in marine sediments increased by a factor of three for every increase in temperature by 10 °C. This temperature effect was tested in short-term radiotracer incubation experiments with sediments from Limfjorden (Denmark). In the present study, integrated rates for SR do not agree with this suggestion. SR rates for the 3 °C sample were at $203 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$, whereas the 13 °C sample was integrated to $1052 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$. This resulted in an increase in SR rates by a factor of five, which is higher than what Jørgensen proposed. Integrating nitrogenase activity rates for the 3 °C ($22 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$) and 13 °C ($11 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$) incubations resulted in a factor of two. The reason why N_2 fixation did not increase the same why as SR, could be either due to the fact that N_2 fixation is a very energy consuming process for organisms, requiring large amounts of ATP (16MgATP) (see stoichiometry Eq. 2 p. 3). Another reason could be that enough bioavailable nitrogen was abundant in sediments and organisms were able to obtain their required nitrogen by taking up Nitrate and Ammonium (Postgate, 1982).

Replicate cores of the 13 °C incubation resulted in high standard deviations, which are at $136 \text{ nmol SO}_4^{2-} \text{ cm}^{-2} \text{ d}^{-1}$ for the upper sediment layer. The same was true for nitrogenase activity, where 0 – 2 cm resulted in high variability (Fig. 27). However, the high standard deviations were probably due to the high heterogeneity of EFB sediments. The majority (60 – 80%) of global coastal regions were found to exhibit heterogeneity in sediments (Holland & Elmore, 2008). This heterogeneity found in sediments, could be a result of the patchy distribution of

macrofauna in EFB sediments. Processes, such as winter storm mixing could have resulted in sediment heterogeneity. But also bioturbation and bioirrigation processes, which were performed by macrofaunal organisms, could result in sediment heterogeneity (Dale et al., 2011; Orsi et al., 1996). The taxonomic composition of EFB sediments showed that, among macrofauna, Polychaetes (e.g. *Nephtys sp*, *Polydora ciliate* and *Scoloplos armiger*) were the abundant group. Depth of particle bioturbation in EFB was found to be limited to the top 0.5 – 1.0 cm sediment depth (D'Andrea et al., 1996). This finding is not totally in accordance with the present study, where high heterogeneity was found up to 3 cm sediment depth. In conclusion, there must have been other organisms, such as clams or crabs, that mixed the sediment up to 3 cm or a storm event occurred before sampling that resulted in sediment heterogeneity.

In the present study, the summer (13 °C) and winter (3 °C) situation resulted in SR rates that fluctuated by about one order of magnitude between temperatures. This result is in agreement with the finding that SR rates vary between one order of magnitude depending on summer or winter temperatures (Westrich & Berner 1988). That study was undertaken with sediments from the coastal area near Long Island Sound, south of Connecticut, in a water depth between 9 and 17 m and a porosity between 0.65 and 0.91. Because of an organic carbon content of up to 3.5 wt% in this previous study, that research is comparable with the present study, where EFB sediments resulted in an organic carbon content between 4 and 5 wt% (Whiticar, 2002). In conclusion, SR rates are in the range than previously observed for other organic rich sediments.

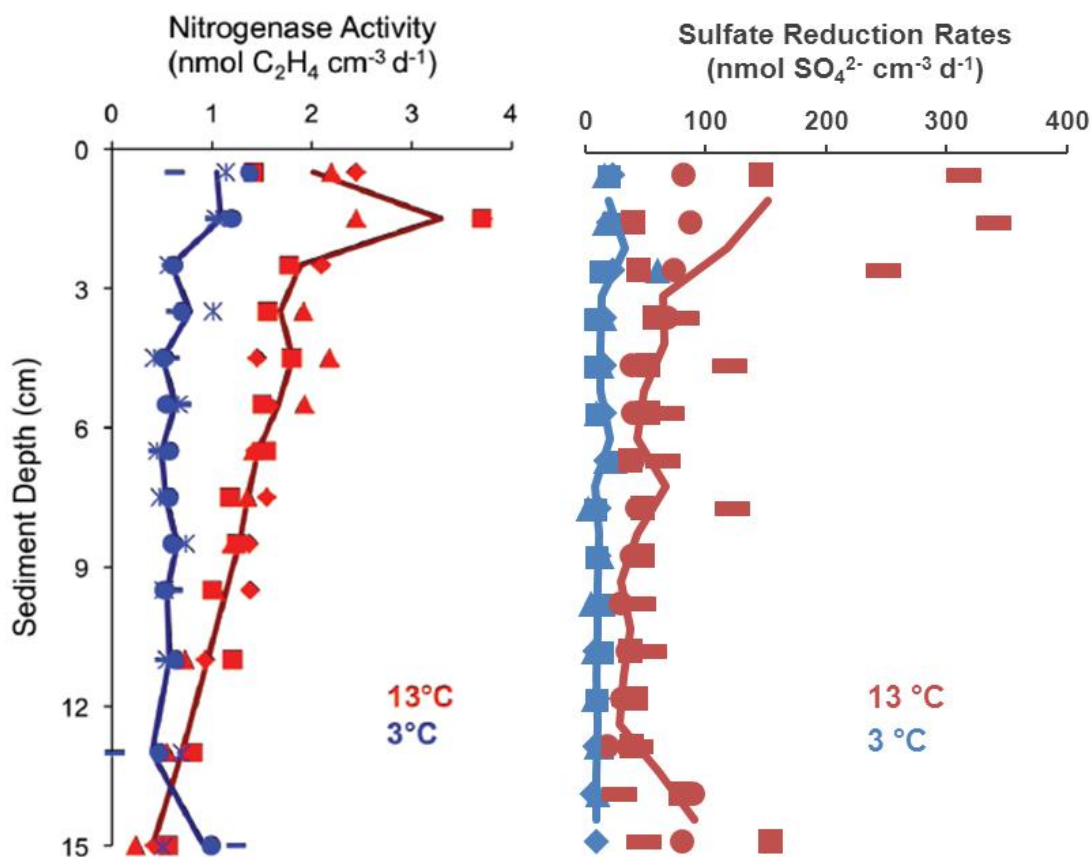


Fig. 27: Nitrogenase activity (x -axis, $\text{nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$) **and sulfate reduction rates** ($\text{nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$) **by SR bacteria over sediment depth** (y -axis cm) determined by the acetylene reduction assay (from Bertics et al. 2012) and whole core injection, respectively. Three replicates (symbols) and the average (solid line) are presented per temperature (3 °C and 13 °C).

The comparison between results of N_2 fixation and SR rates measured directly at the respective month where temperatures occurred (Bertics et al. 2012), and incubated (3 °C and 13 °C) samples in the present study, indicated for the 13 °C measurements, a similar pattern (Fig. 17 and Fig. 28). Some of the cores, which were measured in February directly after sampling at in situ temperature (3 °C), resulted in some extremely high rates (Fig. 28, 3 °C) (with more than $5000 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$). These high rates were very localized between 5 and 15 cm depth, what indicated patches of high organic matter input (Bertics et al. 2012). These so-called “hot spots” were observed in EFB for winter months and may be the result of macrophyte erosion due to winter storms. This organic carbon material would sink to the bottom, brought deeper into the sediment by bioturbation or bioirrigation and provide a high organic matter input into the sediment, which increased microbial activity (Meyer-Reil, 1983).

The results measured directly in October 2010 (Bertics et al. 2012) were in good agreement with the rates measured in the 13 °C incubation of the present study. Both samples indicated high SR rates in surface sediments that decreased when reaching deeper sediment layers. One replicate core measured directly in October 2010 resulted in a similarly high rate, of about 300 nmol SO₄²⁻ cm⁻³ d⁻¹, as the 13 °C incubation core from the current research. These results indicated that sediments of EFB can be variable, depending on location and season, and that there are various factors, such as organic matter input and mixing that may have a high influence on the microbial metabolic activity (Canfield, 1991). However, the results of the present study indicated a trend that was correlating with rates measured directly in the respective month. Eventually the other cores for N₂ fixation and SR rate measurements did not hit one of these hot spots and therefore did not result in such high microbial rates.

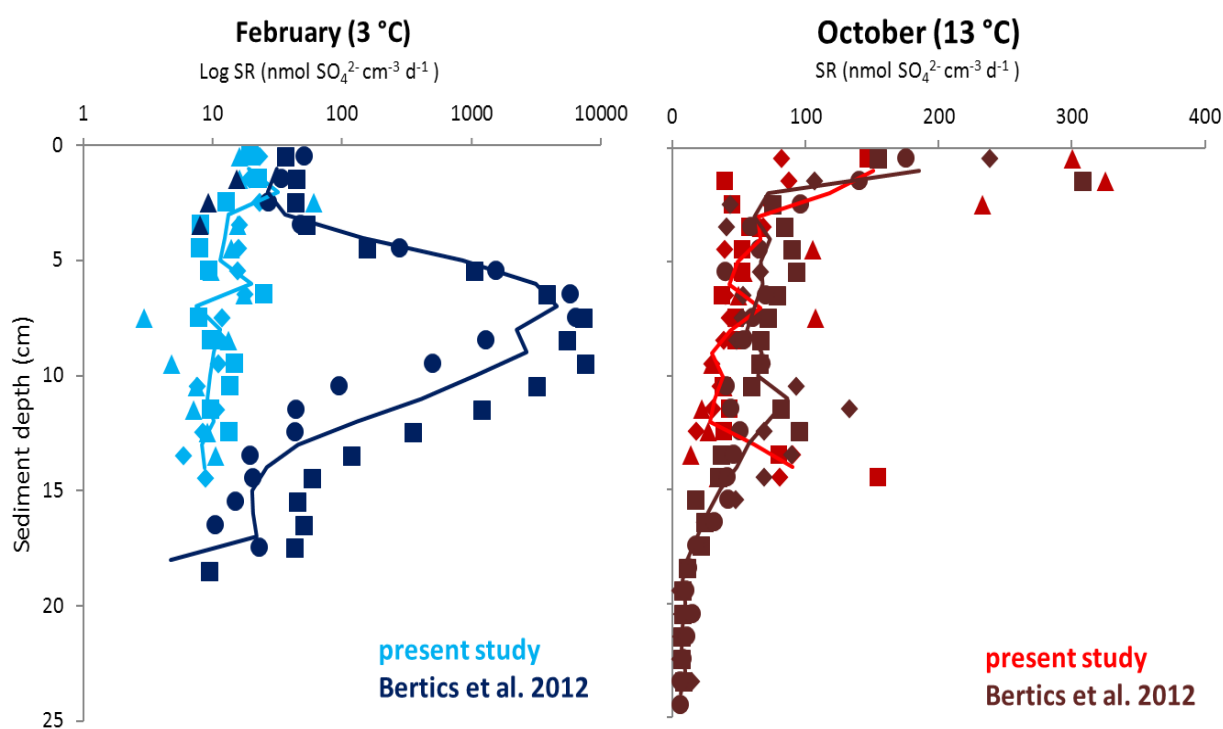


Fig. 28: Sulfate reduction depth profiles from sediment cores taken and measured directly in February (3 °C) and October (13 °C) (Modified after Bertics et al., 2012) and rates of the current temperature incubation experiment (3 °C and 13 °C). Displayed on the x-axis are the sulfate reduction rates (nmol SO₄²⁻ cm⁻³ d⁻¹) and on the y-axis the sediment depth (cm). Three replicates (symbols) and the average (solid line) are presented per temperature (3 °C and 13 °C). Note the logarithmic scale for February. One replicate core (triangles, previous study) of February samples, resulted in 0 nmol SO₄²⁻ cm⁻³ d⁻¹, except for three depth intervals and because of the log scale, values are not plotted.

4.1.2. Abundance of *Desulfovibrio vulgaris* in Eckernförde Bay sediments

To examine whether nitrogenase activity and SR rates are correlated with an increase in numbers of SR bacteria, total cell counts and *Desulfovibrio vulgaris* counts were accomplished. *D. vulgaris* was found to be abundant in EFB (Bertics et al. 2012) and carries the *nifH* gene, the gene encoding nitrogenase and needed to fix N₂ (Riederer-Henderson & Wilson, 1970). Because of the above mentioned features, *D. vulgaris* was selected as the study organism in the present research. The determination of cells and the target bacteria was done by staining total cells with DAPI and performing CARD-FISH (Ishii et al. 2004). This procedure included a fluorescent labeled dye that labels the target organisms (*in this case Desulfovibrio spp*) (Manz et al., 1998).

Highest amounts of total, as well as *D. vulgaris* cell counts, were detected in EFB surface sediments (0 – 3 cm) (Fig. 20, Fig. 21). The relative proportion of *D. vulgaris*, compared to total cells, was highest in the surface layer (0 – 1 cm) with about 5.15% and decreased with increasing sediment depth (1.72 % in 14 – 15 cm) (Fig. 23).

The fact that there was a high amount of *D. vulgaris* in the upper sediments, which was similar to SR rates and nitrogenase activity, supports the hypothesis that the abundance of *D. vulgaris* in EFB sediments was correlated with the activity of SR and N₂ fixation. Because *D. vulgaris* was found to carry the *nifH* gene (Riederer-Henderson & Wilson, 1970), it was suggested that some of the observed nitrogenase activity in the current research may have been carried out by the target bacterium.

Overall, total cell numbers in the present study ranged between 1.37 and 1.97 x 10⁹ per cm³ in the upper 5 cm sediment. These numbers are high compared to another study undertaken in EFB (Vardeh, Diploma thesis, 2011), which examined cells by the same method that was used in the current study (CARD-FISH). This previous study came up with 6.5 x 10⁸ total cells per cm³, however this discrepancy could be due to sampling in a different season. Sediments for the Vardeh (2011) study were sampled in April, whereas sediment for CARD-FISH analysis in the present study was sampled in October. The different cell numbers might be a result of different organic matter availability during that period of the year.

Total cell numbers are in agreement with previous counts in EFB (Treude et al. 2005). In this study, the effect of seasonal changes on anaerobic oxidation of methane (AOM) and the abundance of AOM mediating bacteria was examined. DAPI results indicated total cells of 1.4 x 10⁸ between 24 and 26 cm sediment depth, whereas the current research found 5.69 x 10⁸ for the deepest section (14 – 16 cm). Although the deepest sediment layer in the

previous study was 10 cm below the deepest layer in the present study, results are in the same order of magnitude. In the same previous study, the abundance of the SR bacteria *Desulfococcus* and *Desulfosarcina* in EFB sediments was examined. Results indicated about 3.4×10^6 bacteria of the branches *Desulfococcus* and *Desulfosarcina* in 24 – 26 cm sediment depth, whereas the present study resulted in 9.79×10^6 *D. vulgaris* cells in the lowermost section (14 – 16 cm). These numbers are relatively similar, although there were differences between the previous and the present study. In the Treude et al. (2005) study, SR was linked to AOM, and moreover, the SR species and the sediment depth were different.

In conclusion, total cell counts and *D. vulgaris* counts seemed to be reasonable and the hypothesis that the abundance of *D. vulgaris* was correlated with the activity of SR and N_2 fixation, was strengthened. In order to clarify these results and prove that N_2 fixation is really done by *D. vulgaris*, one could perform either qPCR or nanometer-scale secondary ion mass spectrometry (NanoSIMS) (Polerecky et al., 2012), which will be discussed in the outlook section (4.4.)

4.2. Impact of organic matter – Experiment 2

In Experiment 2, the impact of the correlation between organic matter availability, SR and N_2 fixation was investigated. Here, EFB sediment was homogenized and made into a slurry, after which two types of organic matter were added (phytoplankton and macrofauna material), at two different energy values, based on low- and high carbon concentration scenarios (Graf et al., 1983) (0.5194 kJ and 3.6361 kJ). SR rates were determined by the whole core injection method (Jørgensen 1978; Fossing & Jørgensen 1989) and using the cold single-step chromium distillation method (Kallmeyer et al. 2004). Nitrogenase activity, as an indication of N_2 fixation, was measured via the acetylene reduction assay (Kallmeyer et al., 2004).

The results of Experiment 2 supported the hypothesis, that rates are positively correlated with energy value (Fig. 26). This result was statistically confirmed only in the nitrogenase activity with the *macrofauna high* sample (1-way ANOVA, $p = 0.036$). However, a trend that support this hypothesis can be observed from the data, suggesting that more replicate cores might have been needed for statistical confirmation. Unfortunately, for SR rates one replicate of the *macrofauna high* sample and one of *macrofauna low* was not available.

Consequentially, no statistical test was done to check for significances because at least three replicates were required.

Nevertheless, a trend that the energy content of added organic carbon material has a direct influence on SR and N₂ fixation activity can be observed (Fig. 26). This correlation was found for SR rates. Higher SR rates were observed, when a larger amount of organic carbon material, i.e. more energy, was added. There was one exception where this correlation could not be confirmed and that was with the nitrogenase activity of the *phytoplankton* sample. Here, *high* and *low* energy values resulted in similar nitrogenase activity with small standard deviations. This pattern is most likely not due to sediment heterogeneity, as was discussed already in section 4.1. (Holland & Elmore, 2008) because for this experiment homogenized sediment slurries from the same core were used. This exception could be an indication that a *low phytoplankton* amount was enough, to enhance nitrogenase activities in the same way as a *high phytoplankton* amount.

The trend that the overall rates for SR and nitrogenase activity were higher with *phytoplankton* than with *macrofauna*, supported the hypothesis that algal organic matter resulted in higher N₂ fixation rates and SR when compared to macrofaunal organic matter. These observations indicated that *phytoplankton* material was more easily degraded than *macrofauna* material, at least for bacteria in the present study. Present results agreed with the previous finding that the composition of organic matter was an important factor, influencing microbial degradation rates (Boetius & Lochte 1996). In this past study, sediment slurries of the upper 4.5 cm of each sediment core were enriched directly after sampling, with equal quantities of different substrates and were then incubated for two months. The results of the Boetius and Lochte (1996) study showed that substrate supply, in terms of lipids, starch, cellulose, chitin and albumin, strongly influenced microbial degradation processes. Moreover, the different substrates resulted in variations in bacterial growth rates. This was not tested in the present study but would be worth investigating to and see if this pattern is also true for *D. vulgaris*.

The results from a different incubation (10 days) study showed that the addition of organic carbon material to sediment slurries from Long Island Sound (1 m depth) resulted in enhanced bacterial SR rates (Westrich & Berner, 1984). This previous study suggested that

SR rates and the amount of added organic carbon material were linearly proportional. This correlation was found to be a direct evidence of the validity of the simple G model (Berner, 1964). This model suggested a correlation between SR and the concentration of organic carbon. The previous results of both studies were confirmed in the present study, where a positive correlation between amount of added organic carbon and SR rates was observed.

Nevertheless, the Berner (1964) and Westrich and Berner (1984) studies only investigated the amount, and not the type, of added organic matter. The investigation of microbial degradation of a whale fall, in which anoxic EFB sediment was enriched with whale biomass, to study the activity of SR bacteria, revealed that SR connected to the degradation of animal carbon was delayed up to 35 days (Verdeh, 2011, Diploma Thesis). The reason for this time lag was hypothesized to be the fact that SR bacteria are not typically capable of degrading large organic molecules. Usually, there are aerobic processes that degrade molecules beforehand, and because these processes were missing in the previous and in the present study, it could be possible that SR bacteria were not able to degrade the animal material directly. These results showed that it could take bacteria longer to degrade animal material, compared to phytoplankton material. This idea is in agreement with the current research, where phytoplankton material resulted in high SR rates and nitrogenase activity, compared to macrofauna material. With sufficient time allowed, the macrofauna samples might have resulted in higher SR rates in the present study as well.

However, not just the benthic aerobic degradation of molecules is important for SR bacteria; also, the degradation in the water column is essential. Organic matter, expected that with a benthic origin, is altered in the water column by grazing, microbial degradation or chemical processes while it is sinking on the seafloor (Karl et al. 1988). This alteration step was missing in the present study and Karl et al. (1988) suggested that bacteria, in general, need this aerobic degradation by other organisms, in the water column, to be able to consume macrofauna material. Another important step that was missing in the current study, was the aerobic degradation of organics on the sediment surface. In an oxic environment, organic matter is degraded by aerobic bacteria and by macrofaunal species first, and so both of these organisms contribute to the mineralization of organic matter (Lee, 1992; Middelburg et al. 1993). Thus, it might be difficult for SR bacteria in the present study to break up the

large molecules and degrade the macrofaunal material without aerobic organisms breaking up the larger molecules beforehand.

Bioirrigation by benthic organisms, such as polychaetes, was investigated recently in EFB sediments (Dale et al. 2012). In this recent study, the bioirrigation coefficient for EFB sediments was estimated and resulted in highest values ($\alpha = 0.38 \text{ d}^{-1}$) in March (Fig. 29). The lack of bioirrigation from September to December, and consequently of organisms performing this process, was probably due to decreased bottom water oxygen concentrations. These anoxic conditions in EFB have increased in frequency over the last 25 years (Orsi et al. 1996; Bange et al. 2011). Because bioirrigating organisms need oxygen to survive, the absence of oxygen resulted in many dead animals, specifically at the sediment surface (V. Bertics, pers. observ.) and therefore, added an additional source of organic matter to the sediments (Meyer-Reil, 1983). On the other hand, bioturbation can introduce organic matter deeper into the sediment, where it enhances microbial degradation processes (Bertics et al., 2010). Organisms increase organic matter availability via burrow systems in the sediment either by bioturbation (Aller & Aller, 1986) or by bioirrigation (Bertics et al., 2010). The amount of organic matter originating from phytoplankton blooms that reaches the seafloor of EFB is varying with season and is dependent on temperature, water column mixing and phytoplankton blooms (Graf et al., 1983; Meyer-Reil, 1983). For the present study, this seasonality of organic matter availability and bioirrigation would imply that high SR and N_2 fixation rates are expected in spring and low SR and N_2 fixation rates in winter.

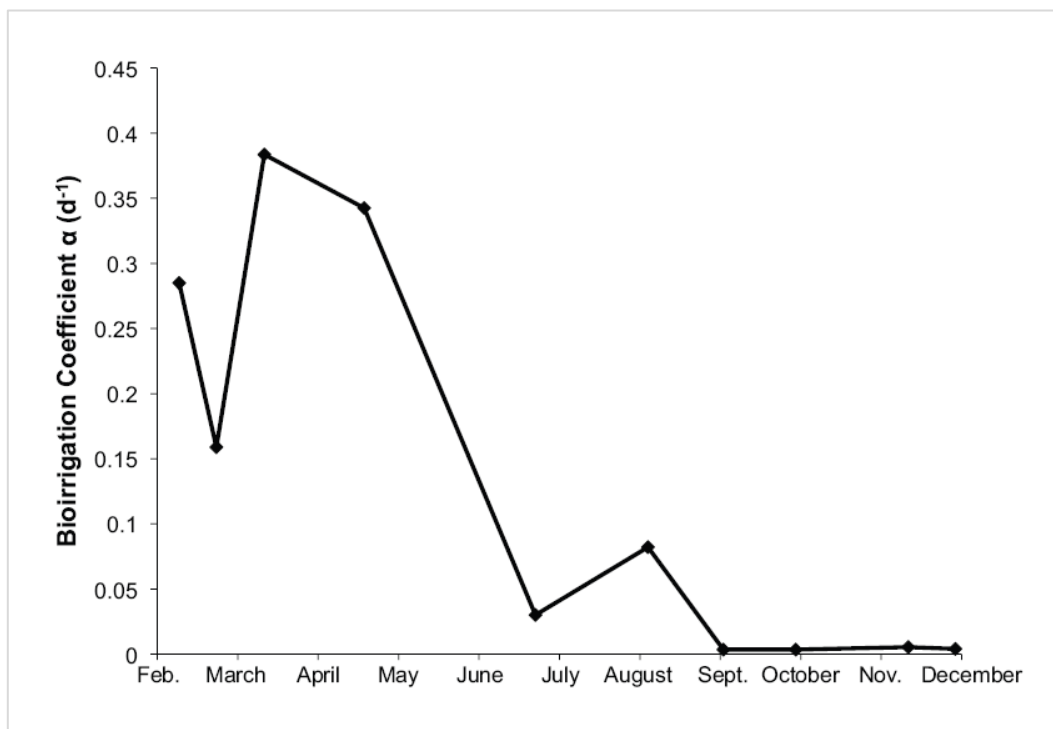


Fig. 29: Bioirrigation coefficient (x-axis: α (d^{-1})) for EFB sediments with time series starting from February 2010 to December 2010 on the y-axis (Bertics et al. 2012; Dale et al. 2012).

In an in situ experiment, the response of a benthic community to a phytodetritus pulse was examined (Witte et al., 2003). During this experiment, the response of an abyssal benthic community (4800 m) was quantified over a period of 2.5 to 23 days. A benthic chamber was used for measurements and for the introduction of freeze-dried ^{13}C labeled algae suspension (*Thalassiosira rotula*). Macrofauna were determined to be very important for initial carbon degradation, whereas the bacterial response was delayed. These results are in agreement with our study. We showed that in the absence of macrofaunal organisms, large organic molecules could not be degraded as well as phytoplankton material. Moreover, Witte et al. (2003) found that the microbial degradation of particulate organic carbon was restricted to the upper sediment layers (0 – 2 cm). Because the present study revealed the occurrence of high SR and N_2 fixation rates at sediment depths deeper than 2 cm, the previous result is not in agreement with the current research. A reason for different results could be due to the fact that Witte et al. (2003) examined deep sea sediment with a low organic carbon content (0.4 wt%), whereas EFB sediment is rich in organics (4 – 5 wt%) (Whiticar, 2002). This organic matter was brought deeper into the sediment by processes, such as bioirrigation or bioturbation, and therefore, enhanced microbial degradation processes, when compared to deep-sea sediments.

4.3. Conclusion

Experiment 1 indicated a positive correlation between nitrogen (N_2) fixation coupled to sulfate reduction (SR) and temperature in Eckernförde Bay (EFB) sediments. The abundance of the target bacterium *D. vulgaris*, was correlated with respective rates in sediments, suggesting that these bacteria were performing some of the observed SR and N_2 fixation.

Results of Experiment 2 suggested a trend between the amount of energy added as organic material and SR, as well as N_2 fixation rates. Therefore, higher energy values resulted in higher SR and N_2 fixation rates. Moreover, *phytoplankton* samples showed higher SR and N_2 fixation rates than *macrofauna* samples. It was suggested that *macrofaunal* carbon material was more difficult to digest for SR bacteria, than *phytoplankton* carbon material. This difficulty could be due to the absence of macrofaunal organisms that would perform aerobic degradation beforehand causing anaerobic microbial degradation processes in the sediments to possibly slow down.

In conclusion, the current research revealed that temperature and type of organic matter, as well as energy concentration, drove N_2 fixation and SR rates in EFB sediments.

These correlations could be also relevant for organic rich sediments in other marine environments. SR bacteria could influence N_2 fixation rates in oxygen minimum zones of the coast of Peru, which have a high organic carbon content and low oxygen concentrations. Rates of N_2 fixation coupled to SR rates could be enhanced either following a phytoplankton bloom or with higher temperatures.

Other organisms that carry *nif* genes, the genes that encode the nitrogenase enzyme, and that were found in EFB sediments (Bertics et al. 2012), are SR bacteria of the species *Desulfonema limicola* (Fukui et al. 1999). This organism is also coupled to SR, like *Desulfovibrio vulgaris*, and capable of N_2 fixation. This bacterium was identified by polymerase chain reaction (PCR) amplification of EFB sediments (Bertics et al. 2012). Another organism that was found in EFB by Bertics et al (2012), and that carry the *nifH* gene, is *Acrobacter nitrofigilis* (McClung et al. 1983). However, this bacterium performs SR and fixes N_2 under aerobic conditions (facultative anaerobic).

In conclusion, the correlations found in the present research could be also relevant for other marine environments and organisms, making N_2 fixation by SR bacteria in marine sediments an important but underestimated process.

4.4. Outlook

Although some studies imply that SR bacteria can contribute to N₂ fixation in marine sediments of EFB (Bertics et al. 2012), this topic still needs future investigation. Results of the present study suggested a correlation between N₂ fixation, SR rates and temperature in benthic environments of EFB. In addition, results indicated that these processes could be performed by SR bacteria, possibly by *D. vulgaris*, but also this needs to be confirmed by future studies. Present results showed that organic matter in the form of *phytoplankton* was more easily and faster degraded than organic matter in the form of *macrofauna*, which was confirmed by higher SR and N₂ fixation rates. Additionally, the effect of climate change, i.e. higher temperatures, and if the type of organic matter present in oxygen minimum zones result in higher N₂ fixation, coupled to SR, should be investigated. The metabolic capabilities of SR bacteria deserve future attention, especially the question concerning why SR bacteria fix N₂ in sediments when reduced nitrogen species are abundant. Another question that remains, is how other processes within the nitrogen cycle are affected by N₂ fixation rates by SR bacteria. In conclusion, there are several gaps in our knowledge concerning SR, N₂ fixation and temperature, as well as organic matter. Here, some of these remaining questions are addressed in more detail.

Effect of metabolic activities. Catalyzed Reporter Deposition Fluorescence in Situ Hybridization (CARD-FISH) has the possibility to examine the natural abundance of a specific target organism, in this case *D. vulgaris*, in marine sediments. It would be interesting to know how much N₂ fixation one cell can accomplish. Therefore, metabolic activities with the analysis of nanometer-scale secondary ion mass spectrometry (NanoSIMS) (Polerecky et al., 2012) could be performed. This method facilitates the determination of elemental and isotopic compositions of cells and the quantification of metabolic activities. Moreover, the quantitative real time polymerase chain reaction (qPCR) could be used for investigation. Only by these methods, N₂ fixation by the SR bacteria *D. vulgaris* could finally be confirmed.

Effect of Climate Change. Future studies, dealing with sulfate reduction (SR) and nitrogen (N₂) fixation should also focus on the effects of climate change (IPCC, 2007). Higher water temperatures could lead to more stratification and an increase in oxygen minimum zones (OMZ) (Diaz, 2001; Stramma et al.2010). Therefore, anaerobic processes, i.e. SR and N₂ fixation, should be examined in OMZs, especially in terms of temperature correlation. A high

oxygen concentration would inhibit SR and N₂ fixation, but anoxic conditions, such as those found in OMZs, could increase SR and N₂ fixation rates. In addition, the type of organic matter would be interesting to examine in OMZs because these areas are upwelling regions high in nutrients. High organic matter concentrations could enhance SR and N₂ fixation, as SR bacteria need organics for microbial degradation. Another question is, if there will be more SR and N₂ fixation due to higher temperatures in marine sediments, in general.

Effect of Ammonium. Why SR bacteria would fix N₂, when bioavailable nitrogen species are abundant, is still a topic of much debate. Although EFB sediment was shown to be rich in ammonium (up to 1200 μM) (Bertics et al. 2012) and although ammonium was suggested to be an inhibitor of N₂ fixation (Postgate, 1982), nitrogenase activity was not inhibited in EFB sediment (Bertics et al. 2012). However, it is unclear at which ammonium concentrations N₂ fixation by SR bacteria would be inhibited in EFB sediments. Therefore, it would be interesting to conduct an experiment changing ammonium concentrations in sediment slurries of EFB and examine respective N₂ fixation rates.

Effect of Phytoplankton Blooms. EFB sediments are exposed to heavy phytoplankton blooms during spring and autumn (Bange et al. 2011). In the current research, the changing parameters were in Experiment 1, temperature and in Experiment 2, organic carbon. Now it would be interesting to conduct an experiment focusing on the combined effects of temperature and organic carbon material. Higher temperatures in combination with *phytoplankton* organic material could result in even higher rates of SR and N₂ fixation than found in the present study. However, eventually highest rates, could be reached for N₂ fixation coupled to SR because bacteria have reached the maximum rate (K_{max}) and are saturated with substrate.

Effect on the Nitrogen Cycle. High N₂ fixation rates in sediments should have an influence on the other processes within the nitrogen cycle. Processes that would be influenced include, for example denitrification, a process that uses nitrate as an electron acceptor and converts it to N₂. Another sink for bioavailable nitrogen, besides denitrification, was shown to be anaerobic ammonium oxidation (anammox) (Strous et al., 1999), which converts nitrite and ammonium back to N₂. Because these processes are a source for N₂, there could be a

correlation between denitrification and N_2 fixation, as well as anammox and N_2 fixation. These correlations could be investigated in an experiment similar to the present study, but then nitrate, nitrite and ammonium should be measured and examined in respect to N_2 fixation rates.

References

- Aller, J. Y., & Aller, R. C. (1986). Evidence for localized enhancement of biological activity associated with tube and burrow structures in deep-sea sediments at the HEBBLE site, western North Atlantic. *Deep Sea Research*, 33(6), 755-790.
- Amann, R. I., Krumholz, L., & Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.*, 172(2), 762-770.
- Bange, H. W. (2010). LOICZ-Affiliated Activities - Boknis Eck Time Series Station. *Meeresforschung - Reports On Marine Research*, (June), 12-14.
- Bange, H. W., Dale, A., Hansen, H. P., Karstensen, J., Malien, F., Petereit, C., Laß, K., et al. (2011). LOICZ-Affiliated Activities Boknis Eck Time Series Station (SW Baltic Sea): Measurements from 1957 to 2010. *INPRINT 2011/1*, 16-22.
- Barnett, P. R. O., Watson, J., & Connelly, D. (1986). A multiple corer for taking virtually undisturbed samples from shelf, bathyal and abyssal sediments. *Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences*, 88, 304-305.
- Barton, L. L., & Fauque, G. D. (2009). *Advances in Applied Microbiology, Volume 68 Chapter 2 - Biochemistry, Physiology and Biotechnology of Sulfate-Reducing Bacteria* (pp. 43-63).
- Berner, R. A. (1964). An idealized model of dissolved sulfate distribution in recent sediments. *Geochimica et Cosmochimica Acta*, 28, 1497-1503.
- Berner, R. A. (1972). Sulfate reduction, pyrite formation, and the oceanic sulfur budget. In D. Dyrssen & D. Jagner (Eds.), *THE CHANGING CHEMISTRY OF THE OCEANS; PROCEEDINGS OF THE 20TH NOBEL SYMPOSIUM* (pp. 347-361).
- Bertics, V. J., Löscher, C. R., Salonen, I., Dale, A. W., Schmitz, R. A., & Treude, T. (2012). Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea. *Biogeosciences Discussions*, 9, 6489-6533. doi:10.5194/bgd-9-6489-2012
- Bertics, V. J., Sohm, J. A., Treude, T., Chow, C., Capone, D., Fuhrman, J. A., & Ziebis, W. (2010). Burrowing deeper into benthic nitrogen cycling: the impact of bioturbation on nitrogen fixation coupled to sulfate reduction. *Marine Ecology Progress Series*, 409, 1-15. doi:10.3354/meps08639
- Bianchi, T. S., Engelhaupt, E., Westman, P., Andren T., Rolff C., & Elmgren R. (2000). Cyanobacterial blooms in the Baltic Sea: natural or human- induced? *Limnol. Oceanogr.*, 45, 716-726.
- Bobrow, M. N., Harris, T. D., Shaughnessy, K. J., & Litt, G. J. (1989). Catalyzed reporter deposition, a novel method of signal amplification application to immunoassays. *Journal of Immunological Methods*, 125(1-2), 279-285. doi:10.1016/0022-1759(89)90104-X

- Boetius, A., & Lochte, K. (1996). Effect of organic enrichments on hydrolytic potentials and growth of bacteria in deep-sea sediments. *Marine Ecology Progress Series*, *140*, 239-250.
- Brandes, A., Devol, A. H., & Deutsch, C. (2007). New developments in the marine nitrogen cycle. *Chemical reviews*, *107*(2), 577-89. doi:10.1021/cr050377t
- Brandes, J. A., & Devol, A. H. (2002). A global marine-fixed nitrogen isotopic budget: Implications for Holocene nitrogen cycling. *Global Biogeochemical Cycles*, *16*(4). doi:10.1029/2001GB001856
- Breitbarth, E., Mills, M. M., Friedrichs, G., & LaRoche, J. (2004). The Bunsen gas solubility coefficient of ethylene as a function of temperature and salinity and its importance for nitrogen. *LIMNOLOGY and OCEANOGRAPHY: METHODS*, *0*(2), 282-288.
- Bryant, M. P., Leon Campbell, L., Reddy, C. A., & Crabill, M. R. (1977). Growth of *Desulfovibrio* in Lactate or Ethanol Media Low in Sulfate in Association with H₂-Utilizing Methanogenic Bacteria. *Applied and environmental microbiology*, *33*(4), 1162-1169.
- Bussmann, I., & Suess, E. (1998). Groundwater seepage in Eckernförde Bay (Western Baltic Sea): Effect on methane and salinity distribution of the water column. *Continental Shelf Research*, *18*, 1795-1806.
- Canfield, D. E. (1991). Sulfate reduction in deep-sea Sediments. *American Journal of Science*, *291*, 177-188.
- Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B., & Carpenter, E. J. (1997). Trichodesmium, a Globally Significant Marine Cyanobacterium. *Science*, *276*, 1221-1229.
- Capone, D. G. (1993). Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In P. F. Kemp, B. F. Sherr, E. B. Sherr, & J. J. Coles (Eds.), *Handbook of methods in aquatic microbial ecology* (pp. 621-631). Boca Raton: CRC Press LLC.
- Capone, D. G. (1988). Benthic Nitrogen Fixation. *Nitrogen cycling in coastal marine environments* (pp. 85-123).
- Capone, D. G., & Knapp, A. N. (2007). A marine nitrogen cycle fix? *Nature*, *16*(445), 159-160. doi:10.1029/2001GB001856
- Codispoti, L. A. (1995). Is the ocean losing nitrate? *Nature*, *376*, 724. doi:10.1038/376724a0
- Coleman, M. L., Hedrick, D. B., Lovley, D. R., White, D. C., & Pye, K. (1993). Reduction of Fe(III) in sediments by sulfate-reducing bacteria. *Nature Publishing Group*, *361*, 436-438.
- Cord-Ruwisch, R., & Hugo, P. V. (1985). A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *Journal of Microbiological Methods*, *4*, 33-36.

- Cypionka, H. (2000). Oxygen respiration by desulfovibrio species. *Annual review of microbiology*, 54, 827-848.
- Dale, A. W., Sommer, S., Bohlen, L., Treude, T., Bertics, V. J., Bange, H. W., Pfannkuche, O., et al. (2011). Rates and regulation of nitrogen cycling in seasonally hypoxic sediments during winter (Boknis Eck, SW Baltic Sea): Sensitivity to environmental variables. *Estuarine, Coastal and Shelf Science*, 95(1), 14-28. doi:10.1016/j.ecss.2011.05.016
- Dale, A. W., Bertics, V. J., Treude, T., Sommer, S., and Wallmann, K. (2012) Modeling benthicpelagic nutrient exchange processes and porewater distributions in a seasonally-hypoxic sediment: evidence for massive phosphate release by *Beggiatoa*?, *Biogeosciences Discussions*.
- Diaz, R. J. (2001). Overview of hypoxia around the world. *Journal of environmental quality*, 30(2), 275-81.
- Diaz, R. J., & Rosenberg, R. (1995). Marine benthic hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna. *OCEANOGRAPHY AND MARINE BIOLOGY ANNUAL REVIEW*, 33, 245-303.
- Dilworth, M. J. (1966). ACETYLENE REDUCTION BY NITROGEN-FIXING PREPARATIONS FROM CLOSTRIDIUM PASTEURIANUM. *Biochimica et biophysica acta*, 127, 285-294.
- D'Andrea, A. F., Craig, N. I., & Lopez, G. R. (1996). Benthic macrofauna and depth of bioturbation in Eckernförde Bay, southwestern Baltic Sea. *Geo-Marine Letters*, 155-159.
- Falkowski, P. G., Barber, R. T., & Smetacek, V. (1998). Biogeochemical Controls and Feedbacks on Ocean Primary Production. *Science*, 281(5374), 200-7.
- Flett, R. J., Hamilton, R. D., & Campbell, N. E. R. (1976). Aquatic acetylene-reduction techniques: solutions to several problems. *Canadian Journal of Microbiology*, 22(1), 43-51. NRC Research Press Ottawa, Canada. doi:10.1139/m76-006
- Fossing, H., & Jørgensen, B. (1989). Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. *Biogeochemistry*, 8(3), 205-222. Springer Netherlands. doi:10.1007/BF00002889
- Fukui, M., Teske, A., Assmus, B., Muyzer, G., & Widdel, F. (1999). Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (genus *desulfonema*). *Archives of microbiology*, 172(4), 193-203.
- Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., et al. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry*, 70, 153-226.
- Ganeshram, R. S., Pedersen, T. F., Calvert, S. E., McNeill, G. W., & Fontugne, M. R. (2000). Glacial-interglacial variability in denitrification in the World's Oceans: Causes and consequences. *Paleoceanography*, 15(4), 361. American Geophysical Union. doi:10.1029/1999PA000422

- Goldhaber, M. B., & Kaplan, I. R. (1975). Controls and consequences of sulfate reduction rates in recent marine sediments. *Soil Science*, 119(1), 42-55.
- Graf, G., Schulz, R., Peinert, R., & Meyer-Reil, L.-A. (1983). Benthic response to sedimentation events during autumn to spring at a shallow-water station in the Western Kiel Bight I. Analysis of processes on a community level. *Marine Biology*, 77, 235-246.
- Grasshoff, K., & Kremling, K. (1999). *Methods of Seawater Analysis* (3rd ed.). Verlag Chemie.
- Gruber, N. (2004). The dynamics of the marine nitrogen cycle and its influence on atmospheric CO₂ variations. In T. Oguz & M. Follows (Eds.), *Carbon Climate interactions* (pp. 97-148).
- Gruber, N. (2008). The Marine Nitrogen Cycle : Overview and Challenges. In D.G. Capone, D. A. Bronk, M. R. Mulholland, & E. J. Carpenter (Eds.), *Nitrogen in the Marine Environment* (2nd ed., pp. 1-50).
- Gundersen, J. K., & Jørgensen, B. B. (1990). Microstructure of diffusive boundary layers and the oxygen uptake of the sea floor. *Letters to nature*, 345, 604-607.
- Hardy, R. W., Holsten, R. D., Jackson, E. K., & Burns, R. C. (1968). The acetylene-ethylene assay for N₂ fixation: laboratory and field evaluation. *Plant physiology*, 43(8), 1185-207.
- Herbert, R. A. (1975). Heterotrophic nitrogen fixation in shallow estuarine sediments. *Journal of Experimental Marine Biology and Ecology*, 18(3), 215-225.
- Holland, K. T., & Elmore, P. a. (2008). A review of heterogeneous sediments in coastal environments. *Earth-Science Reviews*, 89(3-4), 116-134.
doi:10.1016/j.earscirev.2008.03.003
- IPCC. (2007). *Climate Change 2007 : An Assessment of the Intergovernmental Panel on Climate Change*.
- Ishii, K., Mussmann, M., MacGregor, B. J., & Amann, R. (2004). An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. *FEMS microbiology ecology*, 50(3), 203-13.
doi:10.1016/j.femsec.2004.06.015
- Iversen, N., & Jørgensen, B. B. (1985). Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark), 1983(5), 944-955.
- Jeanthon, C., Haridon, L. S., Cuffe, V., Banta, A., Reysenbach, A.-L., & Prieur, D. (2002). *Thermodesulfobacterium hydrogenophilum* sp. nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent at Guaymas Basin, and emendation of the genus *Thermodesulfobacterium*. *International Journal of Systematic and Evolutionary Microbiology*, 52, 765-772. doi:10.1099/ijs.0.02025-0.The

- Jørgensen, B B. (1977a). The sulfur cycle of a coastal marine sediment (Limfjorden, Denmark). *Limnology and Oceanography*, 22(September), 814-832.
- Jørgensen, B B. (1977b). The Sulfur Cycle of a Coastal Marine Sediment (Limfjorden , Denmark). *Limnology and Oceanography*, 22(5), 814-832.
- Jørgensen, B B. (1978). A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. *Geomicrobiology Journal*, 1, 11-27.
- Jørgensen, B B. (1982). Mineralization of organic matter in the sea bed-the role of sulfate reduction. *Nature*, 296, 643-645.
- Jørgensen, B B. (1983). SCOPE 21 -The Major Biogeochemical Cycles and Their Interactions. Processes at the Sediment-Water Interface.
- Jørgensen, B. B., & Fenchel, T. (1974). The sulfur cycle of a marine sediment model system. *Marine Biology*, 24(3), 189-201. doi:10.1007/BF00391893
- Kallmeyer, J., Ferdelman, T. G., Weber, A., Fossing, H., & Jørgensen, B. B. (2004). OCEANOGRAPHY : METHODS A cold chromium distillation procedure for radiolabeled sulfide applied to sulfate reduction measurements. *Limnology and Oceanography: Methods*, 2, 171-180.
- Kamykowski, D., & Zentara, S.-J. (1990). Hypoxia in the world ocean as recorded in the historical data set. *Deep Sea Research Part A. Oceanographic Research Papers*, 37(12), 1861-1874. doi:10.1016/0198-0149(90)90082-7
- Karl, D. M., Knauer, G. A., & Martin, J. H. (1988). Downward flux of particulate organic matter in the ocean: a particle decomposition paradox. *Nature*, 332, 438-441.
- Kasten, S., & Jørgensen, B. (2000). Sulfate reduction in marine sediments. In H. D. Schulz & M. Zabel (Eds.), *Marine Geochemistry* (pp. 263-282). Berlin.
- Kim, J., & Rees, D. C. (1994). Nitrogenase and biological nitrogen fixation. *Biochemistry*, 33(2), 389-97.
- Lee, C. (1992). Controls on organic carbon preservation : The use of stratified water bodies to compare intrinsic rates of decomposition in oxic and anoxic systems. *Geochimica et Cosmochimica Acta*, 56, 3323-3335.
- Linde, K.-O. (2007). Measuring calories in food: the bomb calorimeter.
- Lockshin, A., & Burris, R. H. (1965). Inhibitors of nitrogen fixation in extracts from *Clostridium pasteurianum*. *Biochimica et biophysica acta*, 111(1), 1-10.
- Lovley, D. R. (1993). Dissimilatory metal reduction: from early life to bioremediation. *Annual review of microbiology*, 47, 263-90. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA. doi:10.1146/annurev.mi.47.100193.001403

- Lovley, D. R., & Phillips, E. J. (1992). Reduction of uranium by *Desulfovibrio desulfuricans*. *Applied and environmental microbiology*, 58(3), 850-6.
- Lovley, D. R., Widman, P. K., Woodward, J. C., & Phillips, E. J. (1993). Reduction of uranium by cytochrome c3 of *Desulfovibrio vulgaris*. *Applied and environmental microbiology*, 59(11), 3572-6.
- Manz, W., Eisenbrecher, M., Neu, T. R., & Szewzyk, U. (1998). Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. *Microbiology Ecology*, 25, 43-61.
- Martens, C. S., Albert, D. B., & Alperin, M. J. (1999). STABLE ISOTOPE TRACING OF ANAEROBIC METHANE OXIDATION IN THE GASSY SEDIMENTS OF ECKERNFOERDE BAY, GERMAN BALTIC SEA. *American Journal of Science*, 299, 589-610.
- Martens, C. S., & Berner, R. A. (1974). Methane Production in the Interstitial Waters of Sulfate-Depleted Marine Sediments. *Science*, Vol. 185(no. 4157), 20-22.
doi:10.1126/science.185.4157.1167
- McCarthy, M. D., Hedges, J. I., & Benner, R. (1998). Major Bacterial Contribution to Marine Dissolved Organic Nitrogen. *Science*, 281(5374), 231-234.
doi:10.1126/science.281.5374.231
- McClung, C. R., Patriquin, D. G., & Davis, R. E. (1983). *Campylobacter nitrofigilis* sp. nov., a Nitrogen-Fixing Bacterium Associated with Roots of *Spartina alterniflora* Loisel. *International Journal of Systematic Bacteriology*, 33(3), 605-612.
- Meyer-Reil, L.-A. (1983). Benthic response to sedimentation events during autumn to spring at a shallow water station in the Western Kiel Bight II. Analysis of benthic bacterial populations. *Marine Biology*, 77(247-256).
- Middelburg, J. J., Vlug, T., Jaco, F., & van der Nat, W. A. (1993). Organic matter mineralization in marine systems. *Global and Planetary Change*, 8(1-2), 47-58.
doi:10.1016/0921-8181(93)90062-S
- Mohr, W., Grosskopf, T., Wallace, D. W. R., & LaRoche, J. (2010). Methodological underestimation of oceanic nitrogen fixation rates. *PloS one*, 5(9), e12583.
doi:10.1371/journal.pone.0012583
- Mussmann, M., Ishii, K., Rabus, R., & Amann, R. (2005). Diversity and vertical distribution of cultured and uncultured Deltaproteobacteria in an intertidal mud flat of the Wadden Sea. *Environmental microbiology*, 7(3), 405-18. doi:10.1111/j.1462-2920.2005.00708.x
- Muyzer, G., & Stams, A. J. M. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature reviews. Microbiology*, 6, 441-54. Nature Publishing Group.
doi:10.1038/nrmicro1892

- Nielsen, L. B., Finster, K., Welsh, D. T., Donnelly, A., Herbert, R. A., de Wit, R., & Lomstein, B. A. (2001). Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from *Zostera noltii* and *Spartina maritima* meadows. *Environmental Microbiology*, 3(1), 63-71. doi:10.1046/j.1462-2920.2001.00160.x
- Orcutt, B. N., Joye, S. B., Kleindienst, S., Knittel, K., Ramette, A., Reitz, A., Samarkin, V., et al. (2010). Impact of natural oil and higher hydrocarbons on microbial diversity, distribution, and activity in Gulf of Mexico cold-seep sediments. *Deep Sea Research Part II: Topical Studies in Oceanography*, 57(21-23), 2008-2021. doi:10.1016/j.dsr2.2010.05.014
- Orcutt, K. M., Lipschultz, F., Gundersen, K., Arimoto, R., Michaels, A. F., Knap, A. H., & Gallon, J. R. (2001). A seasonal study of the significance of N₂ fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site. *Deep Sea Research Part II: Topical Studies in Oceanography*, 48(8-9), 1583-1608.
- Orsi, T. H., Werner, F., Milkert, D., Anderson, A. L., & Bryant, W. R. (1996). Environmental overview of Eckernförde Bay , northern Germany. *Geo-Marine Letters*, 16, 140-147.
- O'Donohue, M. J., Moriarty, D. J. W., & Rae, I. C. M. (1991). Nitrogen Fixation in Sediments and the Rhizosphere of the Seagrass *Zostera capricorni*. *Microbiology Ecology*, 22, 53-64.
- Payne, R. B., Gentry, D. M., Rapp-giles, B. J., Casalot, L., & Wall, J. D. (2002). Uranium Reduction by *Desulfovibrio desulfuricans* Strain G20 and a Cytochrome c 3 Mutant. *Applied Environment Microbiology*, 68(6), 3129-3132. doi:10.1128/AEM.68.6.3129
- Pernthaler, A., Pernthaler, J., & Amann, R. (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and environmental microbiology*, 68(6), 3094-101.
- Pinhack, H. (2002). Counting calories. *World coal*.
- Plugge, C. M., Zhang, W., Scholten, J. C. M., & Stams, A. J. M. (2011). Metabolic flexibility of sulfate-reducing bacteria. *Frontiers in microbiology*, 2, Article(May), 8. doi:10.3389/fmicb.2011.00081
- Polerecky, L., Adam, B., Milucka, J., Musat, N., Vagner, T., & Kuypers, M. M. M. (2012). Look@NanoSIMS - a tool for the analysis of nanoSIMS data in environmental microbiology. *Environmental microbiology*, 1-15. doi:10.1111/j.1462-2920.2011.02681.x
- Porter, K. G. and Feig, Y. S.: The Use of DAPI for Identifying and Counting Aquatic Microflora, *Limnology and Oceanography*, Vol. 25, No. 5 (Sep., 1980), pp. 943-948
Published by: American Society of Limnology and Oceanography
- Postgate, J. R. (1965). Recent advances in the study of the sulfate-reducing bacteria. *Bacteriological reviews*, 29(4), 425-41.
- Postgate, J. R. (1979). *The Sulphate-Reducing Bacteria*. Cambridge University Press.

- Postgate, J. R. (1982). *The Fundamentals of Nitrogen Fixation*. Cambridge University Press.
- Postgate, J. R., & Campbell, L. L. (1966). Classification of Desulfovibrio Species, the Nonsporulating Sulfate-reducing Bacteria. *BACTERIOLOGICAL REVIEWS*, 30(732-737).
- Reis, M. A., Almeida, J. S., Lemos, P. C., & Carrondo, M. J. (1992). Effect of hydrogen sulfide on growth of sulfate reducing bacteria. *Biotechnology and bioengineering*, 40(5), 593-600. doi:10.1002/bit.260400506
- Revsbech, N. P., Jorgensen, B. B., & Blackburn, T. H. (1980). Oxygen in the Sea Bottom Measured with a Microelectrode. *Science*, 207, 1355-1356. doi:10.1126/science.207.4437.1355
- Riederer-Henderson, M.-A., & Wilson, P. W. (1970). Nitrogen Fixation by Sulphate-reducing Bacteria. *Journal of General Microbiology*, 61, 27-31.
- Robsen, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M., & Postgate, J. R. (1986). The alternative nitrogenase of Azotobacter chroococcum is a vanadium enzyme. *Nature*, 322, 388-390.
- Rullkötter, J. (2006). Marine Geochemistry. In H. D. Schulz & M. Zabel (Eds.), *Marine Geochemistry* (pp. 129-172). Berlin/Heidelberg: Springer-Verlag. doi:10.1007/3-540-32144-6
- Ruvkun, G. B., & Ausubel, F. M. (1980). Interspecies homology of nitrogenase genes. *Proceedings of the National Academy of Sciences - USA*, 77(1), 191-195.
- Schlüter, M., Sauter, E. J., Andersen, C. E., Dahlgaard, H., & Dando, P. R. (2004). Spatial distribution and budget for submarine groundwater discharge in Eckernförde Bay (Western Baltic Sea). *Limnol. Oceanography*, 49(1), 157-167.
- Seeberg-Elverfeldt, J., Schlüter, M., Feseker, T., & Kölling, M. (2005). Rhizon sampling of porewaters near the sediment-water interface of aquatic systems. *LIMNOLOGY and OCEANOGRAPHY: METHODS*, 3, 361-371.
- Simpson, B. F., & Burris, H. R. (1984). A Nitrogen Pressure of 50 Atmospheres Does Not Prevent Evolution of Hydrogen by Nitrogenase. *Science*, 224(June), 1095-1097.
- Sisler, F. D., & ZoBell, C. E. (1951). Nitrogen Fixation by Sulfate-reducing Bacteria Indicated by Nitrogen/Argon Ratios. *Science*, 113, 511-512.
- Sohm, J. A., Webb, E. A., & Capone, D. G. (2011). Emerging patterns of marine nitrogen fixation. *Nature reviews. Microbiology*, 9(7), 499-508. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. doi:10.1038/nrmicro2594
- Steppe, T. F., & Paerl, H. W. (2002). Potential N₂ fixation by sulfate-reducing bacteria in a marine intertidal microbial mat. *Aquatic Microbial Ecology*, 28, 1-12. doi:10.3354/ame028001

- Stetter, K. O., Huber, R., Blöchl, E., Kurr, M., Eden, R. D., Fielder, M., Cash, H., et al. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature*, *365*, 743-745.
- Stewart, W. D. P., Fitzgerald, G. P., & Burris, R. H. (1967). IN SITU STUDIES ON N₂ FIXATION USING THE ACETYLENE REDUCTION TECHNIQUE. *Proceedings of the National Academy of Sciences of the United States of America*, *58*, 2071-2078.
- Stramma, L., Johnson, G. C., Sprintall, J., & Mohrholz, V. (2008). Expanding oxygen-minimum zones in the tropical oceans. *Science (New York, N.Y.)*, *320*(5876), 655-8. doi:10.1126/science.1153847
- Stramma, L., Schmidtko, S., Levin, L. A., & Johnson, G. C. (2010). Ocean oxygen minima expansions and their biological impacts. *Deep Sea Research Part I: Oceanographic Research Papers*, *57*(4), 587-595. Elsevier. doi:10.1016/j.dsr.2010.01.005
- Strous, M., Kuenen, J. G., & Jetten, M. S. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and environmental microbiology*, *65*(7), 3248-50.
- Teal, L. R., Bulling, M. T., Parker, E. R., & Solan, M. (2008). Global patterns of bioturbation intensity and mixed depth of marine soft sediments. *Aquatic Biology*, *2*, 207-218. doi:10.3354/ab00051
- Treude, T., Krüger, M., Boetius, A., & Jørgensen, B. B. (2005). Environmental control on anaerobic oxidation of methane in the gassy sediments of Eckernförde Bay (German Baltic). *Limnology and Oceanography*, *50*(6), 1771-1786.
- Treude, T., Schüßler, G., & Bertics, V. (2012). CARD-FISH for Sediment Samples Sampling at Sea.
- Treude, T., Smith, C., Wenzhöfer, F., Carney, E., Bernardino, A., Hannides, A., Krüger, M., et al. (2009). Biogeochemistry of a deep-sea whale fall: sulfate reduction, sulfide efflux and methanogenesis. *Marine Ecology Progress Series*, *382*, 1-21. doi:10.3354/meps07972
- Tsou, J.-L., Hammond, D., & Horowitz, R. (1973). *Interstitial Water Studies, Leg 15, Study of CO₂ Released from Stored Deep-Sea Sediments*. doi:doi:10.2973/dsdp.proc.20.212.1973
- Tuominen, L., Heinänen, A., Kuparinen, J., & Nielsen, L. P. (1998). Spatial and temporal variability of denitrification in the sediments of the northern Baltic Proper. *Marine Ecology Progress Series*, *172*, 13-24.
- Wagner, G. M. (1997). Azolla: A review of its biology and utilization. *The Botanical Review*, *63*(1), 1-26.
- Ward, B. B., & Bronk, D. A. (2001). Net nitrogen uptake and DON release in surface waters: importance of trophic interactions implied from size fractionation experiments. *Marine Ecology Progress Series*, *219*, 11-24. doi:10.3354/meps219011

- Wasmund, N., Voss, M., & Lochte, K. (2001). Evidence of nitrogen fixation by non-heterocystous cyanobacteria in the Baltic Sea and re-calculation of a budget of nitrogen fixation. *Marine ecology. Progress series*, 214, 1-14. Inter-Research.
- Westrich, J. T. (1983). Consequences and controls of bacterial sulfate reduction in marine sediments. *PhD Thesis, Yale University*.
- Westrich, J. T., & Berner, R. A. (1984). The role of sedimentary organic matter in bacterial sulfate reduction : The G model tested '. *Limnol. Oceanography*, 29(2), 236-249.
- Westrich, J. T., & Berner, R. A. (1988). The effect of temperature on rates of sulfate reduction in marine sediments. *Geomicrobiology Journal*, 6(2), 99-117.
- Whiticar, M. J. (2002). Diagenetic relationships of methanogenesis , nutrients , acoustic turbidity , pockmarks and freshwater seepages in Eckernförde Bay. *Marine Geology*, 182, 29-53.
- Widdel, F., & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. *The Prokaryotes* (Vol. IV, pp. 3352-3378). Springer-Verlag.
- Winsor, P., Rodhe, J., & Omstedt, A. (2001). Baltic Sea ocean climate : an analysis of 100 yr of hydrographic data with focus on the freshwater budget. *Climate research*, 18, 5-15.
- Witte, U., Wenzhöfer, F., Sommer, S., Boetius, A., Heinz, P., Aberle, N., Sand, M., et al. (2003). In situ experimental evidence of the fate of a phytodetritus pulse at the abyssal sea floor. *Nature*, 424(6950), 763-765. doi:10.1038/nature01799
- www.arb-silva.de/fish-probes/fish-protocols/
- www.biomedcentral.com/content/figures/1471-2148-5-34-2-l.jpg
- www.textbookofbacteriology.net/themicrobialworld/tree.jpeg
- Wyrski, K. (1962). The oxygen minima in relation to ocean circulation. *Deep Sea Research and Oceanographic Abstracts*, 9(1-2), 11-23. doi:10.1016/0011-7471(62)90243-7
- Zehr, J P, Mellon, M., Braun, S., Litaker, W., Steppe, T., & Paerl, H. W. (1995). Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. *Applied and environmental microbiology*, 61(7), 2527-32.
- Zehr, J. P., & Paerl, H. W. (2008). Microbial Ecology of the Oceans Chapter 13. Molecular Ecological Aspects of Nitrogen Fixation in the Marine Environment. In D. L. Kirchman (Ed.), (2nd ed., pp. 481-525). Hoboken, NJ, USA: John Wiley & Sons, Inc. doi:10.1002/9780470281840

Appendix

1. Impact of Temperature – Experiment 1

(1) Recipe for CARD-FISH reagents and buffers

10xPBS

137mM NaCl (MW 58,44) 80g
 2,7mM KCl (MW 74,55) 2g
 10mM Na₂HPO₄ (MW 141,96) 14,1g
 (17,8g of Na₂HPO₄*2H₂O (MW 178,01))
 2mM KH₂PO₄ (MW 136,09) 2,7g
 Solve in 800ml MQ, adjust Ph to 7,3, adjust to 1 L
 Autoclave

1xPBS

100ml 10xPBS
 900ml sterile MQ
 Sterilize again by filtration (Puradisc Filter 0,2µm) before use

1xPBS/EtOH (1:1)

25ml 1xPBS
 25ml 96% EtOH

4% Formalin in 1xPBS

108,1 ml 37% Formalin
 891,9 ml 1xPBS
 Store at 4°C – **toxic!** Use beaker only for Formalin!

5M NaCl

5M NaCl (MW 58,44)
 Solve 146,1 g NaCl in 500 ml MQ
 Autoclave

H₂O₂ (store at 4°C)

General stock solution is 30% H₂O₂
 3% H₂O₂ – 30µl 30% H₂O₂ +270 µl X

10% SDS (sodium dodecyl sulfate)

10 g SDS
 Adjust volume to 100ml with autoclaved MQ
 Fill it into a autoclaved bottle
 Filtrate with 0,2µm

1M Tris – HCl pH 7,3

1M Tris (MW 121,14) 121,14g

Solve in 800 ml MQ, adjust pH to 7,3 with NaOH pellets, adjust to 1L
 Autoclave
 Filtrate with 0,2µm

0,5M EDTA pH 7,3

73,06g EDTA (MW 292,24)
 Solve in 500 ml MQ, adjust pH to 7,3 with NaOH pellets
 Autoclave
 Filtrate with 0,2µm

Sterile MQ

Fill milliQ water into 500ml bottles
 Autoclave

Lysozyme (5mg/ml)– always prepare freshly

Prepare 10mg (20.000U/µl) in caps and store at -20°C
 For use add 2ml 0,05M EDTA/0,1M Tris-HCl
 (1ml 0,5M EDTA, 1 ml 1M Tris-HCl, 8ml MQ)

Achromopeptidase buffer

Solve 3g Achromopeptidase (1000U/mg) in 1ml MQ

Proteinase K

15 µg/ml in 0,05M EDTA/0,1M Tris-HCl

Hybridizationbuffer

5 M NaCl	3,6ml
1M Tris-HCL	0,4ml
Blocking Reagent	2,0ml
10% SDS	20µl
Formamide	x ml
MQ	x ml

35% 7ml Formamide + 7ml MQ

Add 2g dextran sulfate (Dextran sulfate disodium salt from Leuconostoc spp., Sigma), vortex and dissolve dextran at 40-60°C

2x Saline-Sodium Citrate (SSC)

300mM NaCl (MW 58,44) 17,5g
 34mM Na-Citrat*2H₂O (MW 294,1) 10,03g
 Dissolve in 800ml MQ, adjust pH to 7.0 and fill to 1L

Blocking Reagent in 1xPBS

Dissolve 100 mg Blocking Reagent (from kit) in 10 ml 1xPBS,

Streptavidin-HRP in Blocking Reagent in 1xPBS

Dissolve Streptavidin-HRP 1:100 in Blocking Reagent in 1xPBS (3µl+297µl), prepare fresh.

Tyramide-Solution

Add 300 µl Amplification buffer (Kit) , 1µl 0,5% H₂O₂ (f. 0,0015%) and 3µl fluorescently labeled Tyramide (like Alexa488 or Alexa 546), prepare fresh

Tab. 3: Results of the DAPI and CARD-FISH stained counts of total bacteria and *Desulfovibrio vulgaris*. Shown is the average cell number per counting grid and the calculated number of bacteria/*D.vulgaris* in each sediment depth (cm).

Depth (cm)	DAPI (total bacteria)		CARD (<i>D. vulgaris</i>)	
	Average cell number/field	Bacterial Number/ml sediment	Average cell number/field	Bacterial Number/ml sediment
0-1	24.9524	1.97E+09	1.2857	1.02E+08
1-2	24.6190	1.95E+09	0.8429	6.66E+07
2-3	23.3913	1.85E+09	0.8571	6.78E+07
3-4	18.5185	1.46E+09	0.6571	5.20E+07
4-5	17.3103	1.37E+09	0.5714	4.52E+07
5-6	17.6552	1.40E+09	0.4714	3.73E+07
6-7	17.0667	1.35E+09	0.4714	3.73E+07
7-8	16.3226	1.29E+09	0.4286	3.39E+07
8-9	13.0000	1.03E+09	0.3429	2.71E+07
9-10	10.8913	8.61E+08	0.2714	2.15E+07
10-12	15.6563	8.25E+08	0.3714	1.96E+07
12-14	13.5946	7.17E+08	0.2571	1.36E+07
14-16	10.7872	5.69E+08	0.1857	9.79E+06

Tab. 4: Porewater measurements for samples taken in October 2011, including Chloride (mmol), Brom (µmol) and Sulfate (mmol) per sediment depth (cm)

Depth (cm)	Cl mmol	Br µmol	SO ₄ mmol
0.00	377.06	578.77	19.211
0.50	389.61	598.75	19.389
1.50	338.63	518.85	16.91
2.50	373.64	584.96	16.94
3.50	371.32	600.66	15.309
4.50	364.75	562.51	14.327
5.50	360.98	547.44	13.625
6.50	355.02	572.58	13.306
7.50	355.39	565.26	12.856
8.50	355.28	562.42	12.415
9.50	350.05	542.43	12.313
10.50	353.70	546.072	12.738
11.50	353.66	549.19	12.355
12.50	353.66	568.84	11.833

13.50	354.57	526.47	11.486
14.50	349.84	546.57	12.14
15.50	353.51	500.45	11.316
16.50	353.41	540.5	10.55
17.50	324.31	553.6	11.833
18.50	358.04	545.49	10.261
19.50	346.72	500.46	9.636
20.50	334.42	530.5	9.286
21.50	345.27	515.14	8.793
22.50	346.22	543.21	8.21
23.50	347.7	524.99	7.467
25.50	347.85	558.35	7.23
26.50	343.98	506.41	7.02
27.50	347.97	522.61	6.585
28.50	347.355	514.65	5.806
29.50	344.64	514.83	5.723

2. Impact of Organic Matter – Experiment 2

(2) Solution and ingredients and salt recipe for sulfate medium for SR bacteria in the Baltic Sea (PSU 23).

- 6 – vitamin – solution (filtered sterile): 20 mM NaP-buffer (0.356 g Na₂HPO₄, 0.27 g NaH₂PO₄; pH 7, 100 ml); 4-aminobenzoate (4 mg); D-(+) biotine (1 mg); nicotinic acid (10 mg); calcium-d-(+)-panthotheadate (5 mg); pyridoxaminedihydrochloride (15 mg); lipoic acid (1.5 mg)
- Thiamine (B1) (filtered sterile): 10 mg of thiamine in 100 ml of 25 mM NaP-buffer (pH 3.4 – 3.7, 0.345 g NaH₂PO₄), filter (sterile) into Eppendorf tubes, store 1 mL aliquots at -20 °C
- Cyanocobalamine (B12) (filtered sterile): 5 mg of cyanocobalamine dissolved in 100 ml of ultra-clean water, filter (sterile) into Eppendorf tubes, store 1 mL aliquots at -20 °C
- Riboflavine (B2) (filtered sterile): 5 mg of riboflavine dissolved in 100 ml of acetic acid (20 mM), filter (sterile) into Eppendorf tubes, store 1 mL aliquots at -20 °C
- Selenite / wolframate (autoclaved): In 1000 ml of ultra clean water: 400 mg NaOH, 8 mg Na₂WO₂, 6 mg Na₂SeO₃, filter sterile in glass bottle, autoclave
- Trace elements (autoclaved): Add 12.5 ml HCl (37 %, fuming), 2100 mg FeSO₄ * 7 H₂O, 30 mg H₃BO₃, 100 mg I₂*4H₂O, 190 mg CoCl₂ * 6 H₂O, 24 mg NiCl₂*6H₂O, 2 mg CuCl₂*2H₂O, 144 mg ZnSO₄, and 36 mg Na₂MoO₂ * 2 H₂O into a large cylinder and fill it up to 1000 ml with ultra-pure water, filter sterile into autoclaved glass bottle

- Bicarbonate solution (autoclaved): Milli-Q water (1000 mL) + NaHCO₃ (84 g, MW: g). Solution with a concentration of 30 mM of NaHCO₃, flush with CO₂ (5 min), autoclave, store 30 mL aliquots at RT
- Nitrogen / phosphate – solution (autoclaved): Milli-Q water (1000 mL) + NH₄Cl (5 g, MW: g) + KH₂PO₄ (4 g, MW: 136.09 g) Solution with a concentration of 4.67 mM of NH₄Cl and 1.47 mM of KH₂PO₄
- 6.5 % HCl solution (autoclaved): 25 % HCl solution (26 mL) + Milli-Q water (74 mL), into glass bottle and autoclave
- 1 M NaOH – solution (autoclaved)
- 1 M sulfide solution: Milli-Q water (100 mL) + Na₂S*9H₂O (24 g, MW: g), in autoclaved glass bottle, flush with N₂ (5 min), store at 4 °C
- Resazurin (autoclaved): Milli-Q water (100 mL, MW: g) + Resazurin (100 mg, MW: 251.18 g); Fill in glass bottle, autoclave, store at RT

Tab. 5: Salts and their concentrations for 1 l sulfate reducing bacteria medium for the Baltic Sea.

Salts (molecular weight):	Concentrations [mM]; g for 1 l medium:	
KBr (119.01 g)	0.4968 mM;	0.05912 g
KCl (74.56 g)	5.29 mM;	0.3944 g
CaCl ₂ 2H ₂ O (147.02 g)	6.5714 mM;	0.9661 g
MgCl ₂ 6H ₂ O	18.3277 mM;	3.7260 g
MgSO ₂ 7H ₂ O	18.1371 mM;	4.4689 g
NaCl	296.3714 mM;	17.3199 g

(3) Copper Sulfate Solution (CuSO₄) (according to Cord-Ruwisch 1985)

- 1.25 g CuSO₄ x 5 H₂O was dissolved in ultrapure H₂O.
- 6.51 mL HCl (26 %) was added and the solution filled up to a total volume of 1 L with ultrapure water.

Tab. 6: Sulfide (S²⁻) measurements (by photometer) of the sediment:medium slurries. Each sample and the according sulphide concentration (mM L⁻¹) was measured three times and an average was calculated.

Measure-ment	Phyto high	S ²⁻ (mM L ⁻¹)	Phyto low	S ²⁻ (mM L ⁻¹)	Macrofa una high	S ²⁻ (mM L ⁻¹)	Macrofa una low	S ²⁻ (mM L ⁻¹)	Control	S ²⁻ (mM L ⁻¹)
1	0.008	0.384	0.041	1.943	0.017	0.815	0.009	0.445	0.015	0.701
2	0.011	0.517	0.039	1.848	0.022	1.043	0.012	0.569	0.018	0.853
3	0.007	0.346	0.047	2.204	0.020	0.948	0.010	0.474	0.011	0.521
Mean	0.009	0.415	0.042	1.998	0.020	0.935	0.010	0.496	0.015	0.692

Tab. 7: Porewater measurements for each sample including Chloride (mmol) and Sulfate (mmol) plus average and standard deviation. CNS (%) measurements consist of total carbon (TC), N (Nitrogen), S (Sulfur) and organic carbon (C_{org}).

Treatmet	Chlorid mmol		Sulfat mmol		Sulfat average	Chlorid average	SD Sulfat mmol	SD Chlorid mmol	CNS	
Phytoplankton high	403	402.3	18.54	18.58	18.56	402.65	0.034	0.495	N	0,623 %
Phytoplankton low	356.86	367.8	16.38	16.79	16.59	362.33	0.291	7.736	TC	5,507
Macrofauna low	365.5	368	16.65	16.77	16.71	366.75	0.088	1.768	S	1,01 %
Macrofauna high	369.29	368	13.82	13.97	13.90	368.64	0.101	0.912	C_{org}	5,461 %
$\emptyset C_{org}$ +Media	370.05	370	17.33	17.26	17.30	370.02	0.049	0.035		
$\emptyset C_{org}$ \emptyset Media	313.69	311.62	16.06	15.88	15.97	312.65	0.124	1.464		

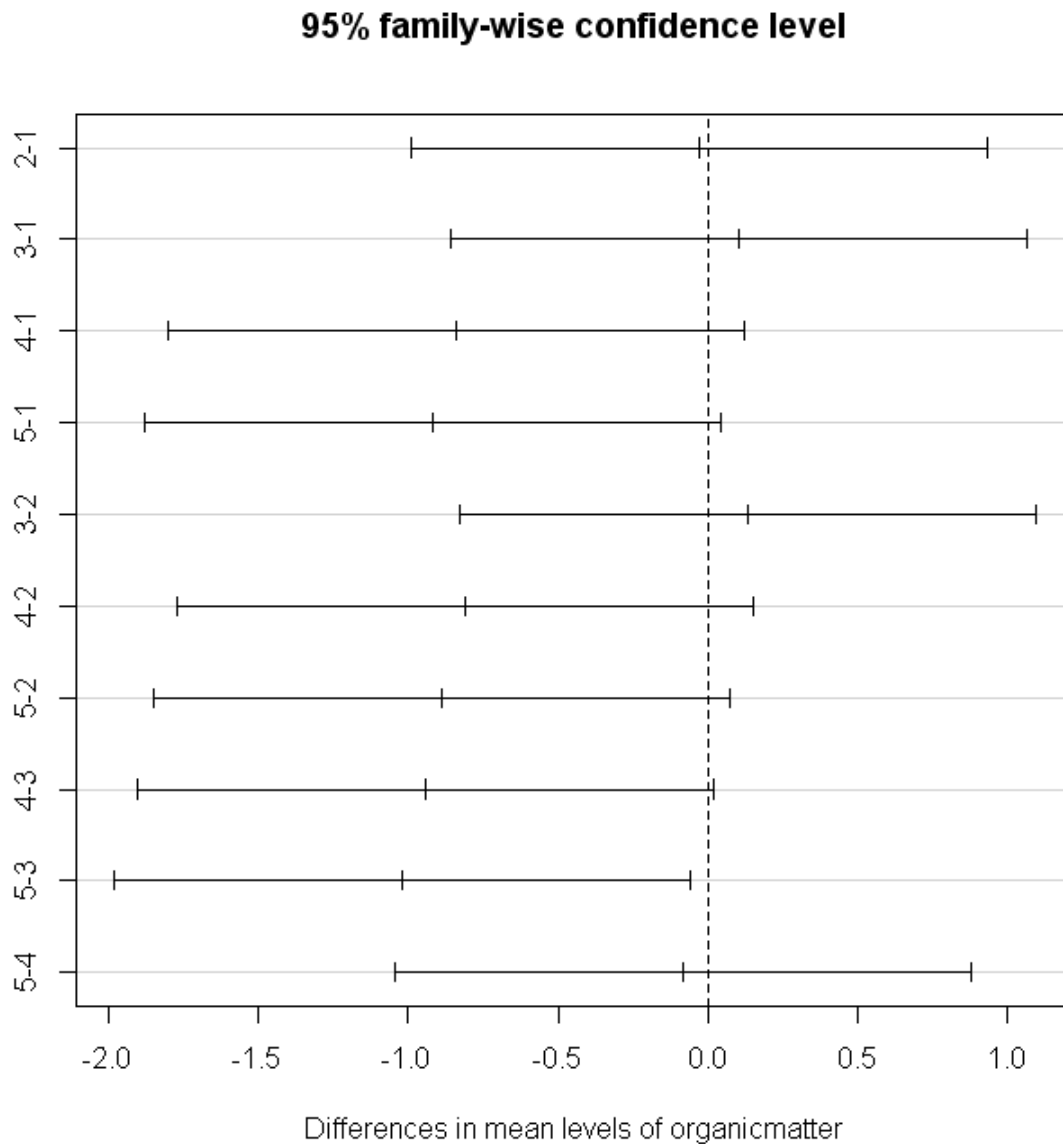


Fig. 30: Plot of the TukeyHSD-test to check for significant differences between samples of nitrogenase activity from the organic matter experiment. The x-axis displays the differences in mean levels of samples and the y-axis the samples which are tested against one another. Sample 1: phytoplankton low, 2: phytoplankton high, 3: macrofauna high, 4: macrofauna low, 5: Control (\emptyset C_{org}, +Media)

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Declaration of Authorship

I certify that the presented thesis

Correlation between nitrogen fixation coupled to sulfate reduction in organic-rich sediments of the seasonally hypoxic Eckernförde Bay

is, to the best of my knowledge and belief, original and the result of my own investigations and that I exclusively used the indicated literature and resources.

I assure that this thesis has not been submitted otherwise in order to obtain academic title.

I agree to include this thesis in the library of the GEOMAR | Helmholtz-Zentrum für Ozeanforschung Kiel and the Christian-Albrechts-Universität, Kiel.

I declare that the master thesis I presented in the written exemplars are identical with that I presented in the electronic version.

Erklärung

Hiermit erkläre ich, die vorliegende Masterarbeit

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selbstständig und mit keinen anderen als den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

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Ich bestätige, dass die gebundene Arbeit mit der elektronischen Version übereinstimmt.

Kiel, den 12. Juli 2012

Jessica Gier