

Master Thesis

**MICROBIAL SULFATE REDUCTION AND
NITROGEN FIXATION IN OIL-CONTAMINATED
MARINE SEDIMENTS**

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AK-01	<i>Desulfatibacillum alkenivorans</i> strain
ANOVA	Analysis of Variance
ANME	Anaerobic Methanotrophs
ATP	Adenosine Triphosphate
C	Carbon
C ₂ H ₂	Acetylene
C ₂ H ₄	Ethylene
CO ₂	Carbon Dioxide
Conc.	Concentration
cm	Centi-(10 ⁻²) Meter
cm ³	Cubic centi-(10 ⁻⁶) Meter
cm ⁻³	Per Cubic centi-(10 ⁻⁶) Meter Sediment
CuSO ₄	Copper Sulfate
d ⁻¹	Per Day
df	Degrees of Freedom
DNA	Desoxyribonucleic acid
E	East
e ⁻	Electron
EBAY	Eckernförde Bay
Et al.	And others (et alii)
Fe	Iron
FID	Flame Ionization Detector
h	Hour
H ₂	Hydrogen Gas
H ⁺	Proton
HCl	Hydrochloric Acid
HCO ₃ ⁻	Bicarbonate
H ₂ O	Water
H ₂ S	Hydrogen Sulfide
μl	Micro-(10 ⁻⁶) Liter
μmol	Micro-(10 ⁻⁶) Mol
l	Liter
m	Mass

<i>masD</i>	Gene encoding enzymes involved in alkane biodegradation
mg	Milli-(10 ⁻³) Gram
Milli Q	Ultrapure Water
min	Minute
ml	Milli-(10 ⁻³) Liter
mM	Milli-Mol per Liter
mmol	Milli-(10 ⁻³) Mol
Mo	Molybdenum
<i>n</i>	Number
N	North
N ₂	Nitrogen Gas
¹⁵ N ₂	Nitrogen Gas with Radioactive ¹⁵ N Isotope
N ₂ fixation	Nitrogen fixation
NA	Nitrogenase Activity
NAMV	North Alex Mud Volcano
<i>nifH</i>	Gene encoding enzymes involved in N ₂ fixation
nm	Nano-(10 ⁻⁹) Meter
nmol	Nano-(10 ⁻⁹) Mol
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
P _i	Inorganic Phosphate
ppm	Parts per Million
R	Software for Statistical Computing
RV	Research Vessel
RWE	Rheinisch-Westfälische Elektrizitätswerke
SD	Standard Deviation
SO ₄ ²⁻	Sulfate
³⁵ SO ₄ ²⁻	Sulfate with Radioactive ³⁵ S Isotope
SRB	Sulfate-Reducing Bacteria
TS	Total Sulfide
UV/Vis	Ultraviolet-Visible Light
Vol.	Volume

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ABSTRACT

Biodegradation is the most important process in the removal of oil from the environment. However, nitrogen often becomes limiting in oil-contaminated environments. Although the fixation of atmospheric nitrogen (N_2) can overcome nitrogen limitation, there are only a few studies on the impact of oil on N_2 fixation and how this may influence microbial oil biodegradation. Most of the isolated bacteria utilizing oil compounds belong to the sulfate-reducing bacteria (SRB). Interestingly, also many SRB have the genetic potential to fix N_2 . However, the majority of studies dealing with N_2 fixation have focused on N_2 fixation linked to phototrophic activity, and not on dark, heterotrophic N_2 fixation. Additionally, the majority of studies dealing with oil degradation have focused on aerobic environments, and not on anaerobic environments.

The overall goal of the present study was to determine whether dark, heterotrophic N_2 fixation is occurring in marine oil-contaminated sediments from Eckernförde Bay (EBAY), Baltic Sea, and North Alex Mud Volcano (NAMV), Mediterranean Sea. A correlation between sulfate reduction and N_2 fixation had been already suggested for natural EBAY sediment. A correlation between sulfate reduction and oil degradation had been already observed in EBAY and NAMV sediment slurries under laboratory conditions.

In the present study, it was hypothesized that N_2 fixation is coupled to SRB degrading oil compounds in EBAY and NAMV sediment slurries. Furthermore, it was suggested that both sulfate reduction and N_2 fixation are stimulated by oil addition. As a measure of sulfate reduction, total sulfide (TS) production was determined according to Cord-Ruwisch 1985, and as a measure of N_2 fixation, nitrogenase activity (NA) was determined according to Capone 1993. The effect of oil treatment on TS production and NA in *anaerobic* EBAY and NAMV sediment slurries was investigated over 14 days (Experiment 1). The effect of oil treatment on NA in *aerobic* EBAY sediment slurries was investigated over 10 days (Experiment 2).

Oil treatment had a significant *positive* effect on TS production rates in *anaerobic* EBAY and NAMV sediment slurries. However, oil treatment had a significant *negative* effect on NA in anaerobic EBAY sediment slurries. Thus N_2 fixation did not seem to be coupled to SRB degrading oil compounds in anaerobic EBAY sediment slurries. Oil treatment had no significant effect on NA in anaerobic NAMV sediment slurries. Therefore it is uncertain, if N_2 fixation is coupled to SRB degrading oil compounds in anaerobic NAMV sediment slurries.

Oil treatment had no significant effect on NA in *aerobic* EBAY sediment slurries, when incubated with oil for 10 days. However, oil treatment had a *positive* significant effect on NA in aerobic EBAY sediment slurries, when incubated with oil for about three months prior to the start of the experiment, suggesting that oil degradation and N₂ fixation may be coupled or at least that N₂ fixation is not impeded by oil addition in aerobic EBAY sediment slurries.

It could be hypothesized that the co-occurrence of hydrocarbon oxidation and N₂ fixation is rare or physiologically delicate. Further research is needed to understand, whether there is an incompatibility between hydrocarbon degradation and N₂ fixation.

1. INTRODUCTION

Oil fuels the world. The world production of crude oil is more than four billion tons per year, and about half of this is transported by sea (Harayama et al. 1999). It is estimated that about 1.3 million tonnes of oil enter the marine environment each year through natural and anthropogenic sources (McGenity et al. 2012). Massive oil releases from pipelines, wells and tanker accidents usually get most of the public attention, such as the Deepwater Horizon explosion in 2010, where over four million barrels of oil were released during 84 days, making it one of the biggest oil catastrophes to date (Crone & Tolstoy 2010). However, it is estimated that almost 50 % of the oil entering the marine environment actually comes from natural oil seeps at the seafloor, and less than 9 % comes from such catastrophes mentioned above (Prince et al. 2003). Oil released into the sea is degraded either biologically or abiotically (Harayama et al. 1999). Biodegradation is thought to be the most important process in the removal of crude oil from the environment (Head et al. 2006), particularly in the long-term.

The major constituents of oil are hydrocarbons, which can be a source of energy and carbon not only for humans but also for microbes (American Academy of Microbiology 2011). In general, hydrocarbons and their derivatives are ubiquitous in the environment (Yakimov et al. 2007). Thus it is hardly surprising that many microbes have evolved the ability to utilize hydrocarbons (Yakimov et al. 2007). The utilization of hydrocarbons by microbes leads to the degradation of oil released in the environment, called biodegradation. Microbes that use hydrocarbons in the presence of oxygen have been known since the beginning of the 20th century (Widdel & Rabus 2001 and the references therein). However, oxygen is not available in all environments where hydrocarbons are present, for instance in deep sea sediments and oil reservoirs. Nevertheless, it was not until the late 1980s that microbes were definitely shown to degrade hydrocarbons under anoxic conditions. These microbes use nitrate, ferric iron or sulfate as electron acceptors in anaerobic respiration. Among those, sulfate-reducing bacteria (SRB) are thought to play the main role in the utilization of crude oil compounds (Wolicka & Borkowski 2007) due to their significance in anaerobic organic matter degradation in the marine environment. The idea that SRB are major players in oil degradation is further supported by the observation that most isolated bacteria utilizing hydrocarbons so far belong to the SRB (Meckenstock et al. 2000; Müller et al. 2001; Karnachuk et al. 2002; Kniemeyer et al. 2003).

However, the availability of nitrogen and phosphorous can limit the growth and activity of hydrocarbon-degrading microbes, and thus oil biodegradation (Harayama et al. 1999). The

simplest way of stimulating the growth of indigenous hydrocarbon-degrading microbes is therefore thought to be the addition of nitrogen and phosphorus nutrients (Prince 2010). However, this procedure is seen as problematic since the addition of nitrogen and phosphorus nutrients, if not handled carefully, can lead to eutrophication (Nikolopoulou & Kalogerakis 2010).

More recent studies dealing with the biodegradation of oil compounds have therefore shifted towards the search of desired metabolic activities in the indigenous bacterial community (de Lorenzo 2006). For example, the ability to utilize hydrocarbons and to fix molecular nitrogen (N_2) under aerobic (Musat et al. 2006; Taketani et al. 2009; Toccalino et al. 1993; Piehler et al. 1999; John et al. 2011) and anaerobic conditions (Musat et al. 2006) was investigated. The ability to fix N_2 is highly advantageous since it liberates microbes from the dependence on fixed forms of nitrogen, such as ammonium and nitrate (Madigan et al. 2011). Interestingly, many SRB have also the genetic potential to fix N_2 and have been shown to do so in a variety of benthic habitats (Nielsen et al. 2001; Steppe & Paerl 2002; Bertics & Ziebis 2010). It is therefore possible that SRB could facilitate N_2 fixation in oil-contaminated marine sediments.

For a detailed description of the composition of crude oil and different types of crude oil, see Section 1.1. For a description of the fate of crude oil in the ocean, including physical, chemical and biological processes see Section 1.2. For general descriptions of the two processes sulfate reduction N_2 fixation see Section 1.3. For an outline of the overall aim of the present study, as well as the underlying research questions and hypotheses see Section 1.4.

1.1 Composition of Crude Oil

Crude oil is one of the most complex mixture of organic compounds that occurs on Earth with more than 17,000 chemical components identified so far (Head et al. 2006). Four main groups of chemicals can be classified in crude oil: the saturated hydrocarbons and the aromatic hydrocarbons, and the more polar, non-hydrocarbon components the resins and the asphaltenes (Head et al. 2006).

Saturated hydrocarbons contain no double bonds (Harayama et al. 1999). Saturated hydrocarbons are further distinguished into alkanes and cycloalkanes. Alkanes have either

branched or unbranched carbon chain(s) and have the general formula C_nH_{2n+2} . Cycloalkanes have one or more rings of carbon atoms and have the general formula C_nH_{2n} . The majority of cycloalkanes in crude oil have an alkyl substituent(s). Aromatics have one or more aromatic rings with or without an alkyl substituent(s). Alkyl-substituted aromatics generally exceed the non-substituted types in crude oil.

In contrast to the saturated and aromatic hydrocarbons, both the resins and asphaltenes contain non-hydrocarbon polar compounds (Harayama et al. 1999). In addition to carbon and hydrogen, resins and asphaltenes can contain trace amounts of nitrogen, sulfur and oxygen, and can form complexes with heavy metals. Asphaltenes consist of high-molecular weight compounds which are not soluble in a solvent such as *n*-heptane, while resins are *n*-heptane-soluble polar molecules. Resins contain heterocyclic compounds, acids and sulfoxides.

The composition of crude oil can vary substantially. The average composition of 527 crude oil samples investigated is quoted to be 58.2 % saturated hydrocarbons, 28.6 % aromatic hydrocarbons and 14.2 % polar compounds, namely resins and asphaltenes (Tissot & Welte 1984). In general, the major constituents of crude oil are hydrocarbons. Broadly, two different types of crude oil, light and heavy oils, can be distinguished according to the proportion of the four main groups mentioned above (Figure 1). Light oils are usually high in saturated and aromatic hydrocarbons, with a smaller proportion of resins and asphaltenes (Head et al. 2006). Whereas heavy oils have a much lower content of saturated and aromatic hydrocarbons and a higher proportion of resins and asphaltenes. Heavy oils typically result from the biodegradation of crude oil under anoxic conditions *in situ* in petroleum reservoirs.

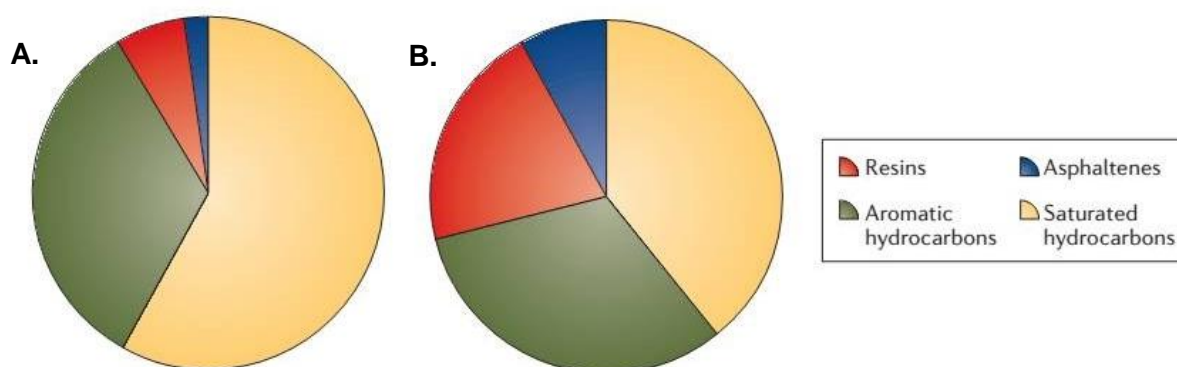


Figure 1 Exemplary compositions of (A.) light crude oil and (B.) heavy oil (modified from Head et al. 2006).

1.2 Fate of Crude Oil in Ocean

When oils enter the oceans from seeps, urban runoff or a spill, they become subject to a variety of processes collectively termed 'weathering' (Prince 2010). Weathering includes the combined effects of physical, chemical and biological modifications. Examples for those weathering processes are evaporation, oxidation, emulsification, dispersion, dissolution and sedimentation, which are described in the following.

Evaporation is usually limited to molecules with less than about 15 carbon atoms (Prince 2010). However, 75 % or more of a diesel spill, and perhaps 20 to 40% of a typical light crude oil can evaporate. However, the composition of the oil and the environmental conditions, such as tidal activity and temperature, play a big role in the fate of oil spilled at sea (American Academy of Microbiology 2011).

Oxidation. Since light cannot penetrate very far into a dark oil slick, photooxidation has little effect on the bulk properties of spilled oil (Prince 2010). However, for instance, aromatic hydrocarbons, particularly the larger and more alkylated ones, can be photochemically oxidized (Garrett et al. 1998), converting them to polar and probably polymerized species.

Dissolution. Aliphatic hydrocarbons are almost insoluble in water, but small aromatic hydrocarbons, particularly benzene, toluene, ethylbenzene and the xylenes, as well as small polar molecules, such as naphthenic acids, dissolve in water and leave the bulk oil behind (Lafargue & Le Thiez 1996; Prince 2010).

Emulsification and Dispersion. When water and oil mix, water may become entrained in the oil to form an emulsion or 'mousse' or oil can disperse into the water column as a suspension of small droplets (Prince 2010). In most oil spills, emulsification happens (Fingas & Fieldhouse 2009), which promotes the persistence of oil at the sea surface. The resulting mounds are thought to be the precursors of tarballs, which can last for decades (Goodman 2003). However, dispersion is likely under storm conditions, as happened during the 1993 Braer spill off the Shetland Islands (Prince 2010). Dispersion facilitates microbial degradation due to the increase in surface area (American Academy of Microbiology 2011).

However, the weathering processes described above only distribute and change the oil in various ways. These processes actually do not remove oil from the environment. Only combustion and biodegradation can remove oil from the environment by converting it to

carbon dioxide and water (Prince 2010). Since the combustion of oil comes with various risks and limitations, biodegradation is the eventual fate of oil spilled at sea.

Biodegradation of Oil. As already mentioned above, microbes that are able to grow on hydrocarbons, the major proportion of oil, are ubiquitous in the environment. However, the degradation of hydrocarbons depends heavily on their chemical structure. The biodegradation of saturated hydrocarbons is quantitatively the most important process in the removal of crude oil from the environment (Head et al. 2006). Saturated hydrocarbons, especially those of smaller molecular weight, are readily biodegraded in marine environments (Harayama et al. 1999). Aromatic hydrocarbons with up to three rings can also be efficiently biodegraded. However, aromatic hydrocarbons with more than four rings are quite resistant to biodegradation. Nevertheless, it should be noted that all hydrocarbons are simultaneously degraded irrespective of their molecular weight (Head et al. 2006). It is simply the lower rates of degradation of the high-molecular weight hydrocarbons that causes the illusion of sequential degradation (Figure 2).

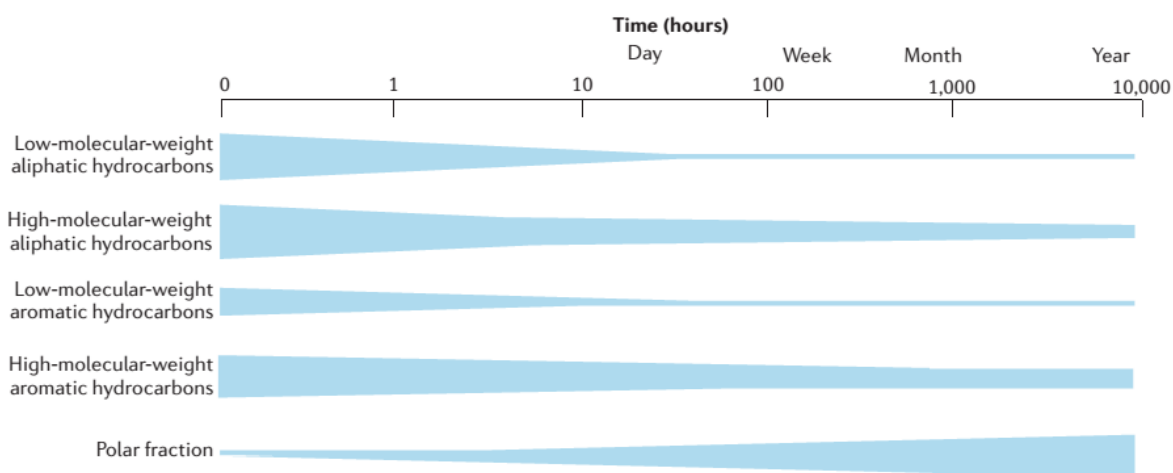


Figure 2 General changes in the composition of spilled oil. Variations in the extent of biodegradation of different crude oils are likely due to the wide range of physical and chemical properties that affect their fate in the environment (modified from Head et al. 2006).

If oil is not rapidly degraded, then it will start to sink into the sediment, where it can remain for decades (McGenity et al. 2012). Undegraded oil below the surface layer of beaches and sediments affected by the Exxon Valdez oil spill in 1989 were detected recently (Boufadel et al. 2010).

Organic Matter Degradation in Marine Sediments. For the degradation of organic matter, bacteria oxidize these reduced organic compounds by the use of an oxidant (terminal electron acceptor), which itself gets reduced during this process (Jørgensen 2000). For microbial degradation of organic matter in marine sediments there is a typical vertical sequence of oxidants, which are used for organic matter degradation in different sediment layers, termed the “redox cascade” (Figure 3). This vertical succession of microbial degradation processes is caused by the decreasing energy yield of the processes and the redox potentials of the oxidants (Jørgensen 2000).

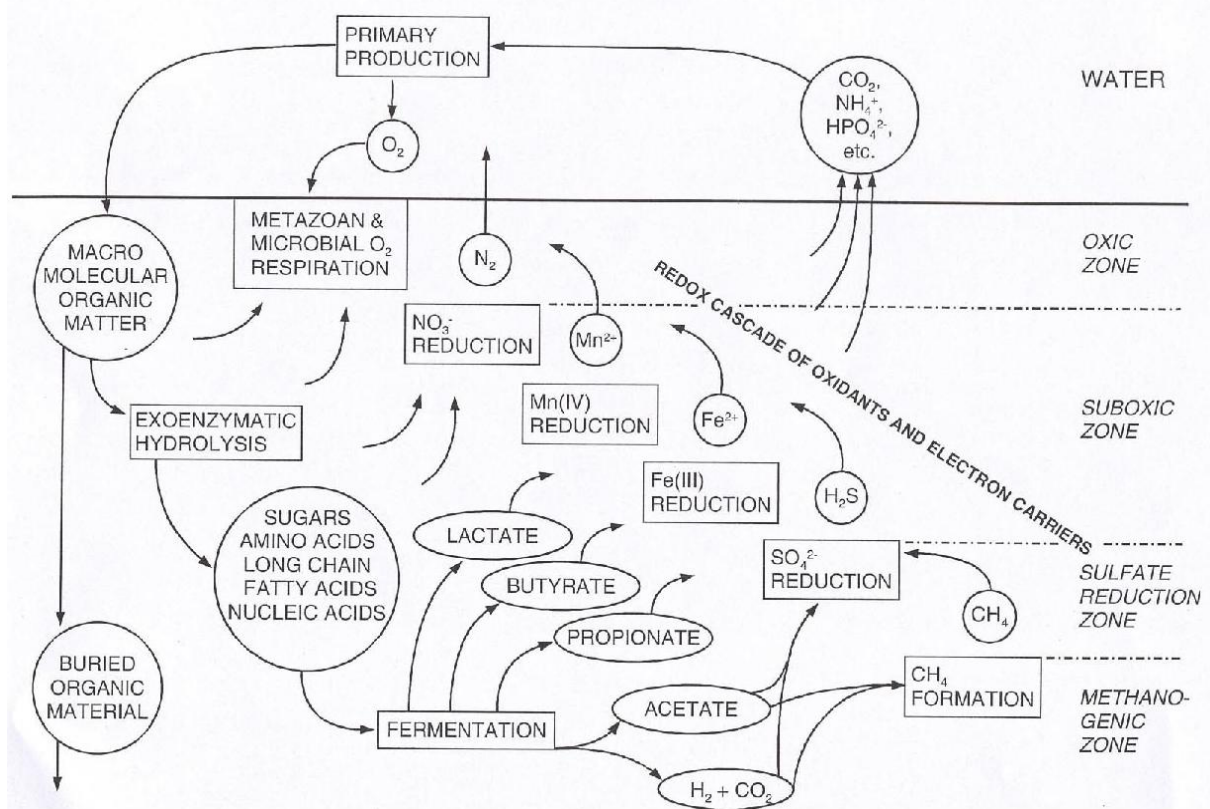


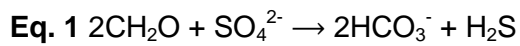
Figure 3 Processes included in the organic matter degradation in marine sediments (Jørgensen 2000)

Biodegradation of Oil in Marine Sediments. Biodegradation of oil compounds occurs under aerobic and anaerobic conditions. As mentioned before, biodegradation of oil components by oxygen-respiring microorganisms has been known for about a hundred years but the utilization of hydrocarbons by bacteria under anoxic conditions has only been discovered in the last ten years (Wolicka & Borkowski 2007). However, the growth of anaerobes is significantly slower than that of aerobic alkane degraders. Nevertheless, anaerobic degradation of alkanes plays an important role in the recycling of hydrocarbons in the environment (Rojo 2009). These microbes use nitrate, ferric iron or sulfate as electron acceptors in anaerobic respiration (Widdel & Rabus 2001).

Effect of Nutrient Addition. It is known that the concentrations of available nitrogen and phosphorous limit the growth and activity of hydrocarbon-degrading microbes in a marine environment (Harayama et al. 1999). To stimulate the growth of indigenous hydrocarbon-degrading microbes and thus stimulating biodegradation, nitrogen and phosphorus can be for instance added in the form of fertilizers. This procedure was first used on a large scale in Alaska, following the Exxon Valdez oil spill in 1989 spill (Prince 2010).

1.3 Sulfate Reduction

Sulfate reduction is the most important anaerobic process for organic matter degradation in marine sediments (Jorgensen 1982) since sulfate is the most abundant electron acceptor in seawater. Electron donors for sulfate reduction include H_2 , organic compounds and even phosphite (Madigan et al. 2011). Sulfate-reducing bacteria (SRB) reduce sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S):



where CH_2O represents organic matter (Kasten & Jørgensen 2010).

It is important to distinguish between the two types of sulfate reduction, the assimilative and dissimilative metabolism. Many organisms use sulfate as a sulfur source for biosynthesis (Madigan et al. 2011). However, the ability to use sulfate as an electron acceptor for energy generating purposes involves the large-scale reduction of SO_4^{2-} and is restricted to SRB (Madigan et al. 2011). Whereas the H_2S formed in assimilative sulfate reduction is immediately converted into organic sulfur, it is excreted in dissimilative sulfate reduction.

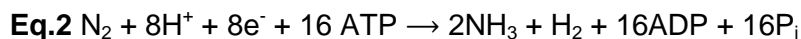
Most known sulfate reducers are among the *Deltaproteobacteria*, although there are some gram-positive SRB in the genus *Desulfotomaculum* in the Firmicutes phylum, and there are several other gram-positive thermophilic SRB (Kirchman 2012a). Sulfate reduction in the archaea is, as far as known, restricted to the genus *Archaeoglobus* (Kirchman 2012a).

SRB are present in most anaerobic environments containing sulfate and organic compounds (Wolicka & Borkowski 2007). However, the most characteristic habitat of SRB are marine sediments. SRB are always present in crude oil and are considered as indicator organisms in the search for new reservoirs. The use of these bacteria in view of their ability to utilize hydrocarbons present in crude oil, in bioremediation of anaerobic areas contaminated with oil-derived products, can also not be ruled out. Bioremediation is a process that uses the

natural ability of microorganisms to biodegrade hazardous substances into less toxic or non-toxic substances. It is estimated that SRB participate in the degradation of over 50 % of the organic matter present in marine sediments along coasts and about 60 % in soils contaminated by petroleum products. SRB play the main role in the utilization of crude oil compounds.

1.4 Nitrogen Fixation

Despite the abundance of molecular nitrogen (N_2) in the atmosphere, N_2 in this form is unavailable for most organisms. However, nitrogen is a major component of many metabolites and structural molecules, such as amino acids, proteins and nucleic acids (Zehr & Paerl 2008). Nitrogen fixation is the conversion of the inert N_2 gas into biologically available compounds (Zehr & Paerl 2008). In nitrogen fixation, N_2 is reduced to ammonia and the ammonia is converted to organic form (Madigan et al. 2011). The stoichiometry of biological N_2 fixation is:



where ATP is adenosine triphosphate, ADP is adenosine diphosphate and P_i represents inorganic phosphates (Zehr & Paerl 2008).

This process is catalyzed by an enzyme complex, called nitrogenase. The two nitrogenase proteins are called component I, the MoFe (molybdenum-iron) protein or dinitrogenase, and component II, the Fe protein or dinitrogenase reductase (Zehr & Paerl 2008). The Fe protein is composed of a pair of identical subunits encoded by the *nifH* gene (Zehr & Paerl 2008). The high degree of similarity of the *nifH* gene among diverse taxa has made it attractive for use in molecular studies, since *nifH* can be amplified with polymerase chain reaction primers (Zehr & Paerl 2008).

Due to the stability of the dinitrogen triple bond, N_2 is extremely inert. The process of nitrogen fixation is highly energy demanding, so that the synthesis and activity of nitrogenase is highly regulated (Madigan et al. 2011). Nitrogen fixation is inhibited by oxygen and by fixed nitrogen, including ammonia, nitrate and certain amino acids. However, the ammonia produced by nitrogenase does not repress the enzyme synthesis because as soon as it is made, it is incorporated into organic form and used in biosynthesis.

Only certain prokaryotes can fix N_2 . The ability to fix N_2 liberates an organism from dependence on fixed forms of nitrogen (Madigan et al. 2011). Since fixed nitrogen is usually

in high demand in microbial ecosystems, the capacity to fix N_2 confers an ecological advantage on those cells capable of the process. Nitrogen fixing microorganisms, also called diazotrophs, can use the large reservoir of N_2 present in the atmosphere, since it readily dissolves in seawater (Zehr & Paerl 2008). However, only relatively few bacteria have been reported to fix nitrogen during growth with oil hydrocarbons (Chen et al. 1993; Prantera et al. 2002; Musat et al. 2006).

1.5 Aim of Thesis

In oil-contaminated environments, chemotrophic N_2 fixation has rarely been detected, despite of the frequent nitrogen limitation (Musat et al. 2006). In cases where N_2 fixation has been observed (Toccalino et al. 1993; Piehler et al. 1999; Eckford et al. 2002), the process could not be directly linked to hydrocarbon-degrading bacteria (Musat et al. 2006).

That is why this study not only focuses on SRB, which are known to play a major role in benthic oil degradation, but also on SRB which have additionally the ability to fix N_2 and can therefore potentially overcome nutrient limitation in the course of oil degradation in marine sediments.

The main research questions to be answered in the present study were:

- How are benthic microbial processes such as sulfate reduction and heterotrophic N_2 fixation affected by crude oil ?
- Is there a correlation between sulfate reduction and heterotrophic N_2 fixation in oil-contaminated marine sediments ?
- Does the addition of oil stimulate SRB to fix more N_2 ?

The corresponding hypotheses were:

- Sulfate reduction is higher in sediment samples incubated with oil than in sediment samples incubated without oil
- Heterotrophic N_2 fixation is higher in sediment samples incubated with oil than in sediment samples incubated without oil
- Sulfate reduction and heterotrophic N_2 fixation are positively correlated in oil-contaminated marine sediments

2. MATERIALS AND METHODS

The overall goal of this study was to determine, whether nitrogen (N_2) fixation is occurring in marine oil-contaminated sediments from two different study sites (see Section 2.1 for Study Site Descriptions). If N_2 fixation is occurring, is it coupled to sulfate-reducing bacteria (SRB) degrading oil compounds? It is hypothesized that both sulfate reduction and N_2 fixation are stimulated by oil addition.

Both processes, sulfate reduction and N_2 fixation, were measured indirectly. As a measure for sulfate reduction, total sulfide (TS) production was determined and as a measure for N_2 fixation, nitrogenase activity (NA) was determined (see Section 2.5.2 for TS Measurement Procedure and Section 2.5.3 for NA Measurement Procedure).

In total, three different experiments were conducted. In the Preliminary Experiment, the effect of dilution on the NA in anaerobic sediment was investigated (see Section 2.2 for how the Preliminary Experiment was conducted). In Experiment 1, the effect of oil treatment on TS production and NA in anaerobic sediment slurries from two different study sites was investigated (see Section 2.1 for Study Site Descriptions and Section 2.3 for how Experiment 1 was conducted). In Experiment 2, the effect of oil treatment on NA in aerobic sediment slurries was investigated (see Section 2.3 for how Experiment 2 was conducted).

For the methods used throughout the present study see Section 2.5 and for the statistical analysis of collected data see Section 2.6.

2.1 Study Sites and Their Characteristics

To study sulfate reduction and N_2 fixation in marine oil-contaminated sediments, sediments from two different sites were chosen. The study sites are Eckernförde Bay (EBAY), southwestern Baltic Sea, and North Alex Mud Volcano (NAMV), eastern Mediterranean Sea (Figure 4). Their characteristics are described in the following.

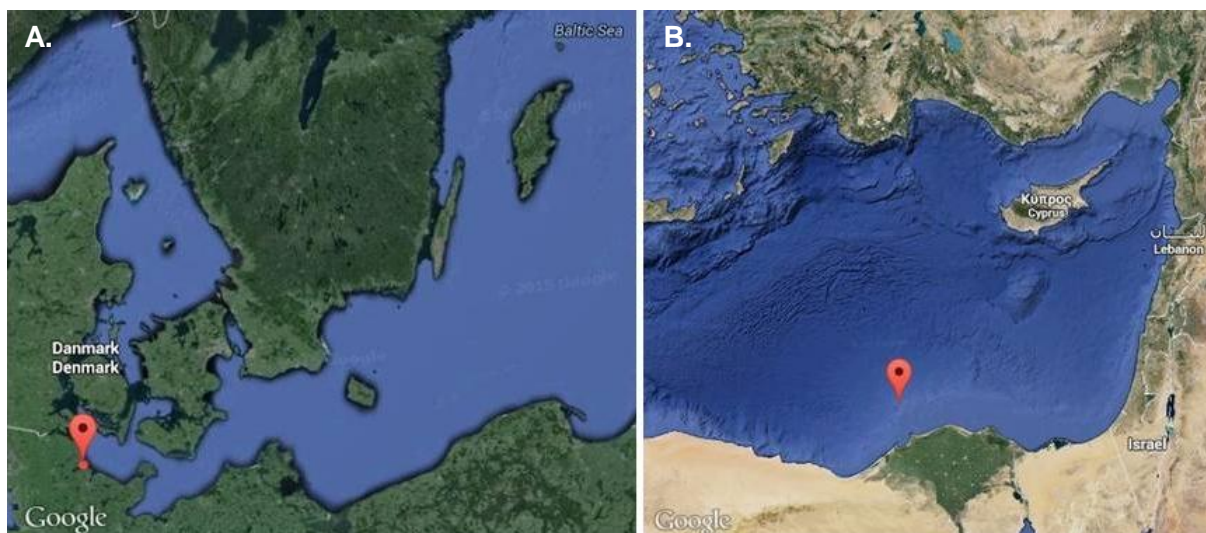


Figure 4 Location of the two study sites (A.) Eckernförde Bay, Baltic Sea and (B.) North Alex Mud Volcano, Mediterranean Sea (Source: www.google.de/maps).

In general, natural sediments from EBAY are assumed to be pristine in terms of oil contamination (Laufer 2012). However, there are EBAY sediment slurries that have been experimentally incubated with oil as only carbon source for about two years until the beginning of the present study (Laufer 2012). It is assumed that the microbial communities in these oil-treated EBAY sediment slurries are therefore adapted to oil. Furthermore, a correlation between dark, heterotrophic N_2 fixation and sulfate reduction has been observed in natural sediments from EBAY (Bertics et al. 2013). However, to the author's knowledge, sulfate reduction and N_2 fixation have not been observed simultaneously in oil-contaminated EBAY sediment.

Microbial communities in natural sediments from NAMV are used to the presence of hydrocarbons due to the nature of the study site (see Section 2.1.2 for further description). In another study, a correlation between oil degradation and sulfate reduction has been observed in NAMV sediment slurries (Sonakshi Mishra, unpublished data). However, to the

authors knowledge, there is no data available about N₂ fixation at this study site, particularly not in correlation with sulfate reduction and oil degradation.

In conclusion, EBAY and NAMV are suitable study sites to test, whether N₂ fixation is occurring in marine oil-contaminated sediments, and if N₂ fixation is coupled to SRB degrading oil compounds.

2.1.1 Eckernförde Bay (EBAY), Baltic Sea

Sediment from Boknis Eck (54°31.2' N, 10°02.5' E) at the entrance of Eckernförde Bay (EBAY) in the southwestern Baltic Sea was used in the present study. Boknis Eck is considered to be one of the best studied and most frequently sampled coastal environments in the Baltic Sea (Whiticar 2002), since a time series with water column characteristics for nearly half a century exists (Bange et al. 2011).

Due to a high freshwater inflow, the Baltic Sea is considered to be one of the largest brackish water systems on Earth. However, the salinity changes horizontally and vertically over a wide range. In the Kattegat, which connects the Baltic Sea with the more saline North Sea, the salinity is around 21 at the surface, increasing up to 31.6 at the bottom (Winsor et al. 2001). The average salinity for the whole Baltic Sea was calculated to be around 7.2 in the surface waters and around 12.3 in the bottom waters (Winsor et al. 2001).

EBAY is a semi-enclosed bay with a water depth of about 28 m and a salinity around 25 (Bange et al. 2011). The water column is characterized by two main phytoplankton blooms in spring and autumn (Bange et al. 2011), followed by a high organic matter degradation, consuming nearly all of the oxygen. The inflow of more saline water from the North Sea through the Kattegat leads to a stratification of the water column usually from March to September (Bange et al. 2011), inhibiting water mixing and exchange of nutrients and oxygen. This results in a low oxygen saturation in the bottom waters during summer, favoring anoxic events (Diaz & Rosenberg 2008; Bange et al. 2011). The muddy surface sediments of EBAY have a high organic carbon content (Orsi et al. 1996), showing strong seasonal variations in N₂ fixation and sulfate reduction rates (Bertics et al. 2013).

EBAY is close to the Schwedeneck oil field (distance about 5 km), where oil was recovered from 1982 to 2000. Consequently, there was the possibility that EBAY sediments were contaminated with oil during oil recovery. However, it is assumed that natural EBAY sediments are pristine in terms of oil-contamination. Firstly, the RWE Dea AG, the operator of the Schwedeneck oil rigs, stated in a press release that oil recovery was performed without contamination after these oil rigs were shut down (Laufer 2012). Secondly, findings

from another study by Laufer 2012 suggested that the microbial community in EBAY sediments was not adapted to oil prior to the laboratory experiments, since relatively long lag-phases of about 125 days were observed following an oil input.

2.1.2 North Alex Mud Volcano (NAMV), Mediterranean Sea

Sediment from the North Alex Mud Volcano (NAMV, 31°58'09.1"N, 30°08'09.7"E) in the eastern Mediterranean Sea was used in the present study. NAMV is a modern deepwater cold seep site located north of Alexandria, off the coast of Egypt, in a water depth of about 500 m (Treude et al. 2011).

Cold seeps are geologically and geochemically active seafloor systems (Omoregie et al. 2009), which have been detected on active ridges and passive continental margins (Omoregie et al. 2009). Cold seeps develop at faults as a result of strong compression in areas with high sedimentation rates such as deep sea fans (Nuzzo et al. 2012). Pore space overpressure is created due to the diagenesis of buried sediment at temperatures usually higher than 60°C accompanied by the release of large amounts of water and gases (Nuzzo et al. 2012). This pore space overpressure in turn leads to seepage of fluids and gases from the deep subsurface transporting electron donors to the surface sediments (Omoregie et al. 2009). Since the Egyptian slope is known for its large subsurface gas and oil reservoirs (Omoregie et al. 2009), these fluid and gas seepages are typically characterized by large quantities of methane and higher hydrocarbons. These seepages lead to the formation of depressions, pockmarks and mud volcanoes at the seafloor (Nuzzo et al. 2012). In a mud volcano, such as NAMV, a mix of hydrocarbon gases, water, petroleum and mud is transported through so called vertical feeder channel to the seafloor (Nuzzo et al. 2012). A steep temperature gradient was observed in the surface sediments of NAMV, ranging between 13°C in the bottom water up and 80°C in 6 m sediment depth.

The eastern Mediterranean basin is considered to be one of the most oligotrophic areas of world's oceans with low particle flux rates, deep oxygen and sulfate penetration into the sediments and consequently low rates of organic matter mineralization and low numbers of microbes involved (Omoregie et al. 2009). Due to the generally low organic matter input, sulfidic environments on the seafloor are usually associated with seepage of fluids and gases from the deep subsurface transporting electron donors to the surface sediments (Omoregie et al. 2009). Microbial processes such as the anaerobic oxidation of methane or sulfate reduction coupled to oil degradation leads to the accumulation of sulfide in the sediments (Treude et al. 2011). It has been suggested that the depth of sulfide environments is inversely proportional to the intensity of upward flux of methane-rich fluid (Nuzzo et al. 2012).

Interestingly, only some of the investigated sediment showed a relatively tight coupling between anaerobic oxidation of methane and sulfate reduction, indicating that at most sites compounds other than methane were fueling sulfate reduction (Omoregie et al. 2009). However, the sulfide is in turn the energy basis for typical chemosynthetic communities including free-living sulfur bacteria, symbiotic clams/mussels and tubeworms (Treude et al. 2011).

2.2 Preliminary Experiment - Anaerobic Incubation (EBAY)

In the Preliminary Experiment, the effect of dilution on the nitrogenase activity (NA) in anaerobic EBAY sediment was investigated via the acetylene (C_2H_2) reduction method over the course of 10 days (see Section 2.2.3 for Experimental Design and Section 2.5.3 for NA Measurement Procedure).

2.2.1 Sediment Sampling and Preparation of Sediment Slurries

During a one-day cruise with the *RB Polarfuchs* to Boknis Eck (EBAY, Baltic Sea) on 13 March 2014, four sediment cores were taken with a Mini-Multicorer to get undisturbed surface sediments (Figure 5A). The core liners had a length of 60 cm and a diameter of 10 cm, containing a sediment core of about 35 cm (Figure 5B). The sediment cores were brought to a cold room (0°C) at GEOMAR, Helmholtz Center for Ocean Research, Kiel within a few hours of sampling. The sediment cores were sliced the following day in a 10°C cold room. For Experiment 2, the top 1 to 2 cm (oxic layer) of each sediment core (Figure 5B and C) were sampled. For the Preliminary Experiment, sediment from 2 to 10 cm (anoxic layer) sediment depth was sampled.

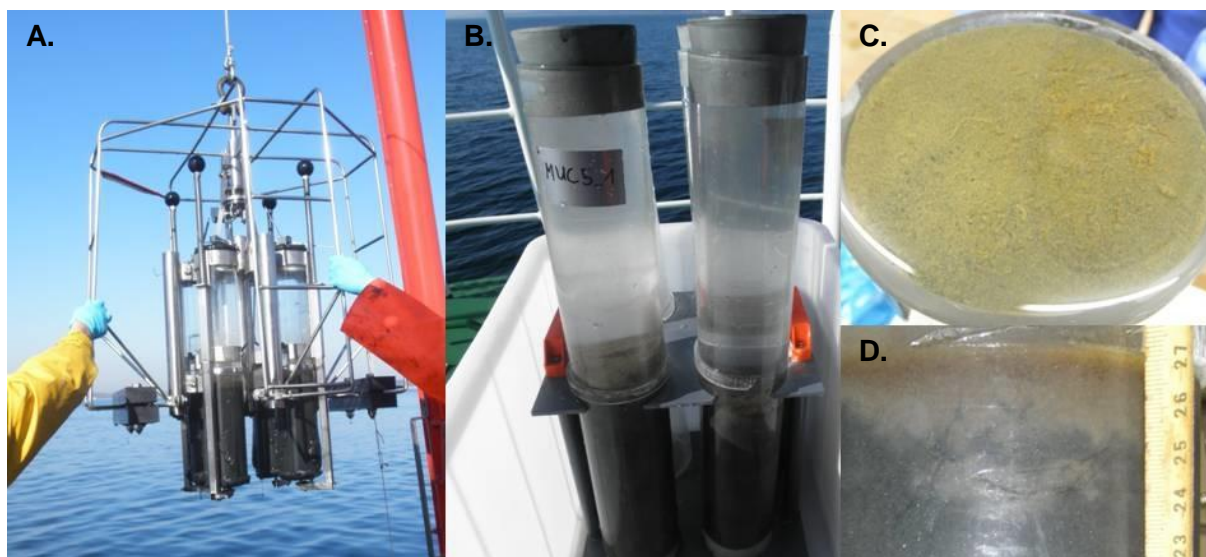


Figure 5 Surface sediments were sampled with a Mini-Multicorer (A), in total four sediment cores from one cast (B) were used in the Preliminary Experiment and Experiment 2. The top 1 to 2 cm of each sediment core represented the oxic layer (C and D) used in Experiment 2.




For the Preliminary Experiment, 60 ml serum vials were used, which were previously acid washed (3% HCl), Milli Q washed and combusted in an oven at 450°C for 24 h to remove all

organic material. Before the addition of sediment, the vials were flushed with N₂ to create anaerobic conditions. Then 10 ml of sediment (see above for sediment sampling) were quickly transferred into each vial with the help of acid washed, cut-off plastic syringes. The vials were immediately closed with autoclaved butyl rubber stoppers and aluminum crimp caps. While the vials were flushed again with N₂ to assure anaerobic conditions, different amounts (10, 20 and 30 ml) of artificial seawater medium for SRB were added with N₂ flushed syringes (see Section 2.5.1 for Preparation of Medium). The sediment slurries were kept anaerobic via the rubber stopper and crimp cap with a N₂ headspace. Incubations were performed in the dark at 13°C.

2.2.2 Experimental Design and Sampling Scheme

Undiluted sediment as well as three differently diluted sediment slurries (dilution factor 2, 3 and 4) were tested for the measurement of NA via the C₂H₂ reduction method (see Section 2.5.3 for NA Measurement Procedure). Each dilution was conducted in triplicate (Table 1). Additionally, one triplicate of undiluted sediment samples was treated with 2 ml of 37.5 % Formaldehyde per vial, serving as a killed control. Another triplicate of undiluted sediment samples was not saturated with C₂H₂ to check for natural ethylene (C₂H₄) production. The headspace of the vials was sampled at day 2, 4, 6, 8 and 10 of the Preliminary Experiment to measure the C₂H₄ production according to the C₂H₂ reduction assay via gas chromatography (see Section 2.5.3 for NA Measurement Procedure).

Table 1 Experimental design of the Preliminary Experiment. To test the effect of dilution on the nitrogenase activity (NA) in anaerobic sediment from Eckernförde Bay, four groups were compared: undiluted sediment (dilution factor 0) and three differently diluted sediment slurries (dilution factor 2, 3 and 4). Sediment samples treated with Formaldehyde served as a killed control. Sediment samples without acetylene served as a control for natural ethylene production.

Dilution Factor	0	2	3	4
NA				
+ Formaldehyde				
- Acetylene				

2.3 Experiment 1 - Anaerobic Incubation (EBAY and NAMV)

In Experiment 1, the effect of oil treatment on total sulfide (TS) production and nitrogenase activity (NA) in anaerobic EBAY and NAMV sediment slurries was investigated over the course of 14 days (see Section 2.3.4 for Experimental Design, Section 2.5.2 for TS Measurement Procedure and Section 2.5.3 for NA Measurement Procedure).

2.3.1 Sediment Sampling and History of Sediment Slurries

Sediment slurries from EBAY and NAMV used in Experiment 1 had undergone experimental work in the laboratory already, which influenced the present study. A history of sediment slurries from both study sites until the start of the present study is therefore described in the following.

Eckernförde Bay (EBAY), Baltic Sea

Sediment samples were collected during a one-day cruise with the *RV Littorina* to Boknis Eck (EBAY, Baltic Sea) on 13 April 2012 (Laufer 2012). Sediment cores were taken with a miniaturized multicorer (Mini-MUC) to get undisturbed surface sediments. The core liners had a length of 60 cm and a diameter of 10 cm, containing a sediment core of about 35 cm. The sediment cores were brought back to a 10°C cold room at GEOMAR, Helmholtz Center for Ocean Research, Kiel within a few hours of sampling and were sliced on 16 April 2012 in the same cold room. The top 0 to 10 cm of each sediment core were sampled. The sediment slurries were prepared on the same day by quickly transferring 20 ml sediment into N₂ flushed 200 ml serum vials and adding 120 ml anaerobic artificial seawater medium for SRB, resulting in a dilution factor of seven (see Section 2.5.1 for Preparation of Medium). The sediment slurries were kept anaerobic with a N₂ headspace by butyl rubber stoppers crimped with aluminum caps.

One triplicate of sediment slurries was contaminated with 0.57 % (crude oil volume/slurry volume) light crude oil. Crude oil was provided by REW Dea AG. Another triplicate of sediment slurries was left without oil to serve as a control. Sediment slurries were incubated horizontally at 13°C in the dark for 181 days. Over the course of the experiment, the TS concentration as well as the change in total alkalinity was measured regularly from the supernatant of the sediment slurries. Methane production was determined by sampling the headspace of the vials and gas chromatography. Afterwards, the sediment slurries were kept in a fridge at 4°C until the revival process in the present study (see Section 2.3.2 for Revival

of Sediment Slurries). The indicator Resazurin, which turns pink in the presence of oxygen, was contained in the medium used in the preparation of sediment slurries. Since the medium did not turn pink during the refrigerated storage, it is assumed that the sediment slurries were kept anaerobic over this period.

North Alex Mud Volcano (NAMV), Mediterranean Sea

Sediment cores from NAMV were collected with *RV Pelagia* on 22 November 2008 with a multicorer. Sediment from 5 to 10 cm of the sediment cores was stored under anaerobic conditions in Duran bottles without headspace. Sediment bottles were stored at 0°C in a cold room until December 2012 for an experiment on crude oil degradation (Sonakshi Mishra, unpublished data). Sediment slurries were prepared in a glove box to maintain anaerobic conditions. Sediment slurries were prepared by transferring 10 ml sediment into N₂ flushed 100 ml serum vials and adding 60 ml artificial seawater medium for SRB (see Section 2.5.1 for Preparation of Medium), resulting in a dilution factor of seven. One triplicate of sediment slurries was treated with 1 % of light crude oil (crude oil volume/slurry volume). Crude oil was provided by RWE Dea AG. Another triplicate of sediment slurries was left without oil to serve as control. Sediment slurries were kept anaerobic under N₂ headspace by butyl rubber stoppers crimped with aluminum caps. Sediment slurries were incubated horizontally at 20°C. TS production in the sediment slurries was measured over the course of 100 days. Afterwards, sediment slurries were stored at 4°C in a fridge until the revival process in the present study (see Section 2.3.2 for Revival of Sediment Slurries). The indicator Resazurin, which turns pink in the presence of oxygen, was contained in the medium used in the preparation of sediment slurries. Since the medium did not turn pink during the refrigerated storage, it is assumed that the sediment slurries were kept anaerobic over this period.

2.3.2 Revival of Sediment Slurries

Sediment slurries from EBAY and NAMV used in Experiment 1 were used in other studies beforehand (see Section 2.3.1 for History of Sediment Slurries). After the end of these studies, sediment slurries were stored refrigerated at 4°C for 1 and 1.5 years (NAMV and EBAY respectively). Besides some oil, which was added to some of the sediment slurries during the previous experiments, the sediment slurries did not receive any other carbon source over this whole period. Thus it was uncertain, if the microbial communities in these sediment slurries were still alive. To check, if SRB were still alive in the sediment slurries from both study sites after the relatively long storage period, sediment slurries had to undergo a revival process, which is described in the following.

The revival process of EBAY and NAMV sediment slurries started 1 April 2014 and lasted for about three months. The revival process consisted of three revival steps, which lasted for about four weeks each. In the beginning of each revival step, as much old medium as possible was removed and the same amount of new medium was added with N₂ flushed syringes (see Section 2.5.1 for Preparation of Medium). These medium exchanges were done while flushing the vials continuously with N₂. Since one triplicate of sediment slurries from each study site has been incubated with oil before, 1 % new oil were added in the beginning of each revival step. Crude oil was provided by RWE Dea AG. Sediment slurries were kept anaerobic with a N₂ headspace via butyl rubber stoppers and aluminum crimp caps. Incubations were performed in the dark at 13°C. The activity of SRB was monitored via TS measurements over the three months of the revival process (see Section 2.5.2 for TS Measurement Procedure). After the activity of SRB in oil-treated sediment slurries was confirmed by TS production during the revival process, Experiment 1 was conducted.

2.3.3 Transfer of Sediment Slurries

In Experiment 1, the effect of oil treatment on TS production and NA in anaerobic EBAY and NAMV sediment slurries was investigated over the course of 14 days. However, TS production and NA could not be measured from the same microcosms. For the NA measurements, sediment slurries with a dilution factor of seven were considered unsuitable. Typically NA measurements are done from undiluted sediment samples or sediment slurries with a dilution factor of two. Just decreasing the dilution factor from sediment slurries in the 100 ml (NAMV) and 200 ml (EBAY) serum vials, that were used throughout the previous studies and the revival process in the present study, did not allow for TS measurements (see Section 2.3.1 for History of Sediment Slurries, Section 2.3.2 for Revival Process and Section 2.5.2 for TS Measurement Procedure). A clear separation between sediment, medium and oil was needed for TS measurements. Thus TS production and NA measurements could not be done from sediment slurries in the same microcosms. For the TS measurements, sediment slurries in 15 ml hungate tubes were used, that allowed a clear separation between sediment, medium and oil despite the relatively low amount of available sediment. For the NA measurements, 60 ml serum vials were used. Accordingly, the sediment slurries from both study sites had to be transferred from the 100 ml (NAMV) and 200 ml (EBAY) serum vials, that were used throughout the previous studies and the revival process in the present study to other microcosms (60 ml serum vials and 15 ml hungate tubes) for Experiment 1.

The 60 ml serum vials and 15 ml hungate tubes were acid washed (3% HCl), Milli Q washed and combusted in an oven at 450°C for 24 h before usage. The transfer of sediment slurries was done in a glove box to maintain anaerobic conditions (Pure Lab, Innovative Technology,

Model# AC-2GB/IL-GP-1). In the glove box, the vials were opened and as much old medium as possible was removed with pipettes. Triplicates of sediment slurries were combined in an autoclaved 250 ml Duran glass bottle and homogenized to achieve equal initial starting points for replicates. The vials were washed with a definite amount of new medium, which was also added to the combined sample to keep the loss of sample material to a minimum (see Section 2.5.1 for Preparation of Medium). The combined sediment samples were thoroughly mixed and carefully transferred into new microcosms (60 ml serum vials and 15 ml hungate tubes) with pipettes. The sediment samples were further diluted with new medium to achieve a dilution factor of two in the vials for the NA measurements and a dilution factor of four in the hungate tubes for the TS measurements (Table 2).

The vials and hungate tubes were closed with autoclaved butyl rubber stoppers and aluminum crimp caps or screw caps respectively. Afterwards, 1 % oil was added with a syringe to five vials and three hungate tubes from each study site (see Section 2.3.4 for Experimental Design). The sediment slurries were kept in the dark at 13°C.

Table 2 Characteristics of sediment slurries from Eckernförde Bay (EBAY) and North Alex Mud Volcano (NAMV) used in Experiment 1. (A.) Characteristics of EBAY and NAMV sediment slurries used in previous studies and (B.) characteristics of EBAY and NAMV sediment slurries used in Experiment 1. Nitrogenase activity (NA) was measured from sediment slurries in 60 ml serum vials, whereas the total sulfide (TS) production was measured from sediment slurries in 15 ml hungate tubes. Note: NA and TS measurements were derived from sediment slurries with different dilution factors.

















































	Microcosm	Microcosm Vol. [ml]	Sediment Vol. [ml]	Medium Vol. [ml]	Dilution Factor
(A.) EBAY	Serum Vial	200	20	120	7
(B.) EBAY	Serum Vial	60	10	10	2
	Hungate Tube	15	3	9	4
(A.) NAMV	Serum Vial	100	10	60	7
(B.) NAMV	Serum Vial	60	5	5	2
	Hungate Tube	15	1.5	4.5	4

2.3.4 Experimental Design and Sampling Scheme

In Experiment 1, sediment samples from two different study sites (NAMV and EBAY) were used (see Section 2.1 for Study Sites Descriptions). Since the two main dependent variables TS and NA could not be measured from the same microcosms (see Section 2.3.3 for further explanations), two sets of microcosms per study site were prepared. For the TS measurements, sediment slurries in 15 ml hungate tubes were used, and for the NA measurements, 60 ml serum vials were used.

For each study site, one triplicate of sediment slurries in 60 ml serum vials was treated with oil and another triplicate was left without oil to serve as a control. Additionally, one triplicate of sediment slurries in 15 ml hungate tubes was treated with oil and another triplicate was left without oil to serve as a control for each study site. Furthermore, one replicate of oil-treated sediment slurry and one replicate of control sediment slurry from each study site was treated with 37.5 % Formaldehyde to serve as a killed control (2 ml for EBAY and 1 ml for NAMV sediment slurries). Another replicate of oil-treated sediment slurry and another replicate of control sediment slurry from each study site was left without acetylene (C_2H_2) to check for natural ethylene (C_2H_4) production (see Table 3 and Figure 6 for Experimental Design of Experiment 1). Unfortunately, it was not possible to do these additional controls in triplicate due to the limited amount of available sample material.

Table 3 Experimental design of Experiment 1. To test the effect of oil treatment on total sulfide (TS) production and nitrogenase activity (NA) in anaerobic sediment slurries from Eckernförde Bay (EBAY) and North Alex Mud Volcano (NAMV), oil-treated and control sediment slurries were compared. Sediment slurries treated with Formaldehyde served as a killed control. Sediment slurries without acetylene served as a control for natural ethylene production. NA was measured from sediment slurries in 60 ml serum vials, whereas TS production was measured from sediment slurries in 15 ml hungate tubes.

	EBAY						NAMV					
	Oil			Control			Oil			Control		
NA												
+ Formaldehyde												
- Acetylene												
TS												

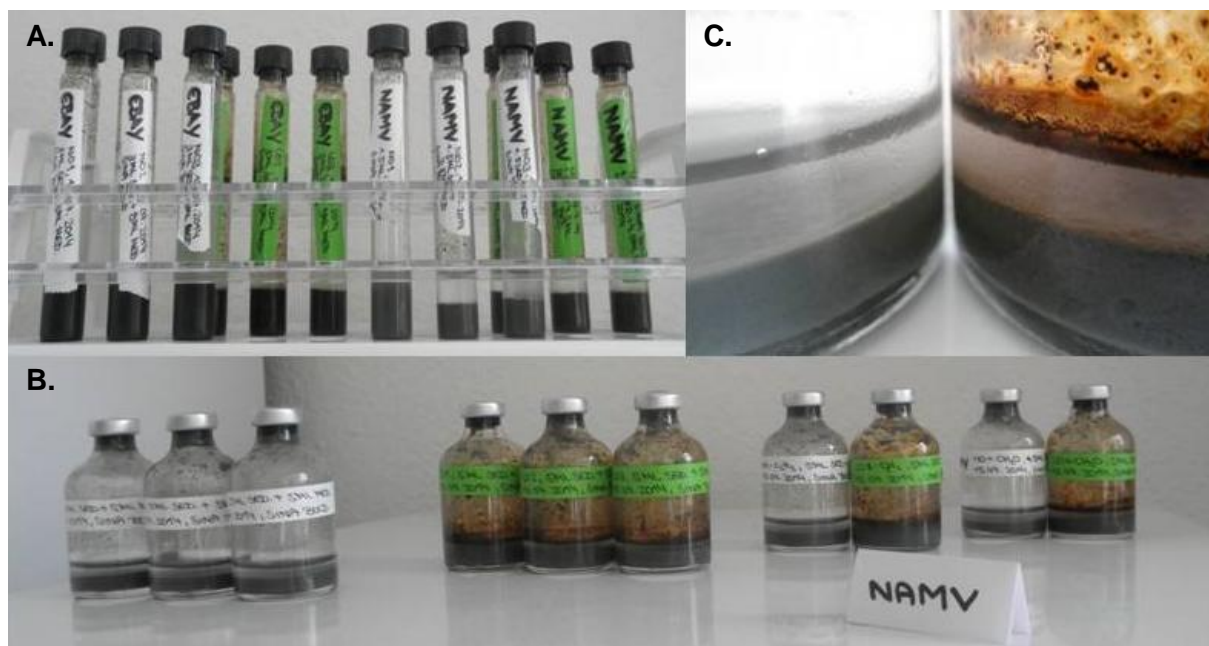


Figure 6 Control and oil treated sediment slurries in Experiment 1. (A.) 15 ml hungate tubes were used to measure the total sulfide concentration, whereas the nitrogenase activity was measured from (B.) 60 ml serum vials. The difference between control (C, left) and oil-treated sediment slurries (C, right) can be seen clearly.

To demonstrate that the microbial community in the controls was still alive, even after years without the addition of a carbon source, acetate was added at day 7 of Experiment 1 to vials and hungate tubes with control sediment slurries from both study sites. A 50 mM acetate stock solution was prepared by dissolving 0.6804 g $\text{CH}_3\text{COONa} \cdot 3 \text{H}_2\text{O}$ in 100 ml Milli Q. This acetate stock solution was then autoclaved and flushed with N_2 to make it sterile and anaerobic. The stock solution was added to control sediment slurries in order to reach an acetate concentration of 1 mM, which resembles natural porewater concentrations of acetate in marine sediments (Widdel 1988). Acetate is the most important organic compound for natural communities of SRB (Kirchman 2012a).

TS production, as a measure of sulfate reduction, was determined via UV/Vis photometry at day 0, 2, 4, 6, 8, 10, 12 and 14 of Experiment 1 (see Section 2.5.2 for TS Measurement Procedure). NA, as a measure of N_2 fixation, was determined via the C_2H_2 reduction assay followed by gas chromatography at day 0, 2, 4, 6, 8, 10, 12 and 14 of Experiment 1 (see Section 2.5.3 for NA Measurement Procedure). For the analysis of ammonium, nitrate and nitrite supernatant was sampled at day 0 and 14 of Experiment 1. Ammonium, nitrate and nitrite samples were stored at -20°C until analysis via UV/Vis photometry (see Section 2.5.4 for Ammonium, Nitrate and Nitrite Measurement Procedure). Sediment samples were taken for DNA extraction and further molecular analysis at day 14 of Experiment 1 (see Section 2.5.5 for DNA Extraction Procedure). Therefore vials were opened in a glove box (Pure Lab,

Innovative Technology, Model# AC-2GB/IL-GP-1) to prevent exposure to oxygen. Triplicates of sediment slurries were combined in an autoclaved 250 ml Duran glass bottle and homogenized. After mixing, 4 ml per former triplicate was transferred into 4 ml cryovials with a pipette and stored at -80°C until DNA Extraction.

2.4 Experiment 2 - Aerobic Incubation (EBAY)

In Experiment 2, the effect of oil treatment on nitrogenase activity (NA) in aerobic EBAY sediment slurries was investigated over the course of 10 days (see Section 2.4.2 for Experimental Design and Section 2.5.3 for NA Measurement Procedure). Whereas one set of sediment slurries has been incubated with oil for about three months prior to the start of Experiment 2 (hereafter called pre-adapted sediment slurries), another set of sediment slurries has been incubated with oil only for the duration of Experiment 2 (hereafter called non-adapted sediment slurries).

2.4.1 Sediment Sampling and Preparation of Sediment Slurries

Sediment cores from EBAY were collected as described in Section 2.2.1. The top 1-2 cm (oxic layer) of four sediment cores were combined in an acid washed and autoclaved 1 l Duran glass bottle and homogenized. About 400 ml sediment was gained this way. The sediment was diluted with 400 ml of natural seawater, which was previously overlying the sediment cores, to get a 'stock slurry' with a dilution factor of two. The 'stock slurry' was kept in the dark at 10°C with an autoclaved cotton plug and continuously shaking to allow oxygen penetration.

For Experiment 2, well mixed 20 ml aliquotes of the 'stock slurry' were transferred with pipettes into 60 ml serum vials, which had been acid washed, Milli Q washed and combusted at 450°C for 24 h previously.

One triplicate of sediment slurries was treated with 1 % oil, whereas another triplicate was left without oil to serve as a control. The sediment slurries were coated with a sterile aluminum foil cap and were continuously shaken to allow oxygen penetration. The sediment slurries were left in the dark at 10°C for about three months to 'pre-adapt' to oil, if present. Before the start of Experiment 2, another set of sediment slurries (non-adapted) was









prepared in the same manner as described above. One triplicate of sediment slurries was treated with 1 % oil, whereas another triplicate was left without oil to serve as a control.

At the start of Experiment 2, the vials had to be closed to allow the measurement of NA via the C₂H₂ method (see Section 2.5.3 for NA Measurement Procedure). Vials were closed with autoclaved butyl rubber stoppers and aluminum crimp caps with an air headspace. The sediment slurries were kept in the dark at 13°C and were manually shook about twice a day.

2.4.2 Experimental Design and Sampling Scheme

For both pre-adapted and non-adapted sediment slurries, one triplicate of sediment slurries was treated with 1 % oil and another triplicate was left without oil to serve as a control. Furthermore, one replicate of oil-treated sediment slurry and one replicate of control sediment slurry was treated with 2 ml of 37.5 % Formaldehyde to serve as a killed control. Another replicate of oil-treated sediment slurry and another replicate of control sediment slurry was left without acetylene (C₂H₂) to check for natural ethylene (C₂H₄) production (Table 4).

Table 4 Experimental design of Experiment 2. To test the effect of oil treatment on nitrogenase activity (NA) in aerobic sediment slurries from Eckernförde Bay (EBAY), one set of sediment slurries was incubated with oil for about three months prior to the start of Experiment 2 (pre-adapted sediment slurries). Another set of sediment slurries was incubated with oil only for the duration of Experiment 2 (non-adapted sediment slurries). Sediment slurries treated with Formaldehyde served as a killed control. Sediment slurries without acetylene served as a control for natural ethylene production.

	Pre-Adapted 3 months		Non-Adapted 10 days	
	Oil	Control	Oil	Control
NA				
+ Formaldehyde				
- Acetylene				

NA, as a measure of N₂ fixation, was determined via the C₂H₂ reduction assay followed by gas chromatography at day 1, 3, 5, 7 and 9 of Experiment 2 (see Section 2.5.3 for NA Measurement Procedure). For the analysis of ammonium, nitrate and nitrite supernatant was sampled at day 0 and 10 of Experiment 2. Ammonium, nitrate and nitrite samples were stored at -20°C until analysis via UV/Vis photometry (see Section 2.5.4 for Ammonium, Nitrate and Nitrite Measurement Procedure). Sediment samples were taken for DNA extraction and further molecular analysis at day 10 of Experiment 2 (see Section 2.5.5 for DNA Extraction Procedure). Triplicates were combined in an autoclaved 250 ml Duran glass bottle to get a homogenized sample. After mixing, 4 ml per sample were transferred into cryovials with a pipette and stored at -80°C until DNA extraction.

2.5 Methods

2.5.1 Preparation of Artificial Seawater Medium (Widdel & Bak 1992)

The artificial seawater medium for SRB was prepared according to Widdel & Bak 1992 with a few modifications. Since sediment samples from two different study sites were used, two different media were prepared to simulate the natural conditions found at each study site (see Section 2.1 for Study Site Descriptions). The two media differed in the salinity. Medium used to prepare EBAY sediment slurries had a salinity of 25 and medium used to prepare NAMV sediment slurries had a salinity of 37. Different salinities were achieved by adding different amounts of salts (Table 5).

Table 5 Amounts of salts for preparation of 1 l of artificial seawater medium for both study sites Eckernförde Bay (EBAY) and North Alex Mud Volcano (NAMV) and the resulting salt concentrations.

Salts	EBAY		NAMV	
	m [g]	conc. [mM]	m [g]	conc. [mM]
KBr	0.059	0.497	0.090	0.756
KCl	0.394	5.290	0.600	8.050
CaCl ₂ ·2H ₂ O	0.966	6.571	1.470	10.000
MgCl ₂ ·6H ₂ O	3.726	18.328	5.670	27.890
MgSO ₄ ·7H ₂ O	4.469	18.137	6.801	27.600
NaCl	17.320	296.371	26.356	451.000

After the salts were dissolved in Milli Q water and thoroughly mixed with a magnetic stirrer within a 1 l measuring cylinder, the solution including the magnetic stirrer was gently transferred into a 1 l Duran glass bottle. Then 0.5 ml of the oxygen indicator Resazurin was added per 1 l medium prepared. The bottles were loosely closed with screw caps and autoclaved for 35 min at 121°C. After cooling down to 85-90°C, the bottles were removed from the autoclave and the screw caps were closed quickly. The medium was further cooled down by placing the bottles in ice water, while flushing the headspace with N₂/CO₂ for 15 min. When the medium was cooled down, 30 ml of sterile bicarbonate solution and 8 ml of sterile NH₄Cl/KH₂PO₄ solution were added with a N₂ flushed syringe through a 0.22 µm filter (GE Healthcare Life Sciences, Whatman Puradisc 25AS, Polyethersulfone membrane) to prevent microbial contamination. Then 1 ml of each of the following vitamin and trace

element solutions were added in the same manner (see Appendix A.1 for Preparation of Solutions):

- 6 Vitamine Solution
- Thiamine Solution
- Cyanocobalamine Solution
- Riboflavin Solution
- Selenite/Tungstate solution
- Trace Elements Solution
- Sulfide Solution

After the addition of each solution, the medium was thoroughly mixed with a magnetic stirrer. In the end, the pH of the medium was adjusted by taking aliquots, measuring them with a pH meter (Schott Instruments Lab 850 with pH electrode Blue Line, SI Analytics), and adding about 1 ml of 10 M NaOH to reach a final pH of about 7.5. Until usage, the medium was stored in a fridge at 4°C.

2.5.2 Total Sulfide Measurement (Cord-Ruwisch 1985)

The total sulfide (TS) production, as a measure of sulfate reduction, was measured photometrically according to Cord-Ruwisch 1985 (see Section 1.3 for General Information on Sulfate Reduction). However, the photometric measurement of TS had to be adjusted to oil-treated samples. Photometric measurements are sensitive to turbidity in the sample, which might have been elevated through sediment particles or oil globules in the supernatant. However, another study confirmed the use of photometric TS measurements according to Cord-Ruwisch for oil-contaminated sediment slurries (Laufer 2012). An increase in absorbance in the TS measurement was accompanied by an increase in total alkalinity.

One day before the measurements, sediment slurries were gently shaken to dissolve sulfide and put upright to get a clear separation between sediment, medium and oil. For the measurement, about 0.3 ml of the supernatant was taken with a N₂ flushed plastic syringe. To remove oil globules suspended in the medium, the supernatant was filtered through a 0.45 µm filter (GE Healthcare Life Sciences, Whatman, PTFE Membrane) or a 0.22 µm Filter (GE Healthcare Life Sciences, Whatman Puradisc 25AS, 0,2µm Polyethersulfone membrane). For the first and second revival step, 0.45 µm filter were used, whereas for the third revival step 0.22 µm filter were used (see Section 2.3.2 for Revival of Sediment Slurries). For Experiment 1, 0.45 µm filter were used. After filtering, 0.1 ml of the supernatant

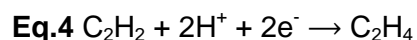
were injected into a glass tube with 4 ml of CuSO₄ solution (2.5 g CuSO₄ in 8.79 ml 37 % HCl, filled up to 2 l with Milli Q). After vortexing for 5 seconds, the mixture was quickly transferred into a 4.5 ml plastic cuvette (VE = 100, Kartell). The following reaction (Eq. 3) leads to the formation of a copper sulfide precipitate, whose absorption was measured at a wavelength of 480 nm at a UV/VIS photometer (Shimadzu Spectrophotometer, UV mini-1240).



TS concentrations were determined via a seven point calibration, consisting of sulfide standards with concentrations ranging from 0 to 20 mM (see Appendix A.2 for Preparation of Standards). For the calculation of TS production rates, TS concentrations derived from the repeated measure of each replicate were plotted against time. TS production rates were calculated with a linear regression analysis for each replicate. TS production rates were corrected for the dilution factor of the sediment slurries and the volume of sediment used to prepare the sediment slurries to allow comparisons between study sites (see Section 2.3.3 for Characteristics of Sediment Slurries in the Revival Process and Experiment 1). TS production rates were averaged per triplicate.

2.5.3 Nitrogenase Activity Measurement (Capone 1993)

The nitrogenase activity (NA), as a measure of nitrogen (N₂) fixation, was measured via the C₂H₂ reduction assay according to Capone 1993 (see Section 1.4 for General Information on N₂ Fixation). The foundation for this method is the reduction of acetylene (C₂H₂) to ethylene (C₂H₄) by the enzyme nitrogenase, which is also responsible for breaking the N₂ triple bond during N₂ fixation (Eq. 4).



Since C₂H₂ usually comes with some level of acetone and phosphine contamination, C₂H₂ was lead through a Milli Q water wash for removal of these contaminants prior to usage (Capone 1993). In the beginning of each experiment, the nitrogenase was saturated by adding 5 ml C₂H₂ per vial with a syringe. Over the course of the experiments, C₂H₄ was quantified in the headspace via flame ionization gas chromatography (GC-2014, Shimadzu Gas Chromatograph, Carrier Gas: Helium, Gas Flow: 22 ml/min, Column Type: HayeSepT, Column Temperature: 70°C, Detector Temperature: 300°C). Therefore 50 µl of headspace were sampled with a 100 µl gas tight syringe (VICI Precision Sampling Inc, Pressure Lok, Precision Analytical Syringe). Three samples were injected consecutively in one run (Figure

7). After each run with oily samples, five C₂H₄ standards were measured (100 ppm C₂H₄ N30 in He N46, Air Liquide).

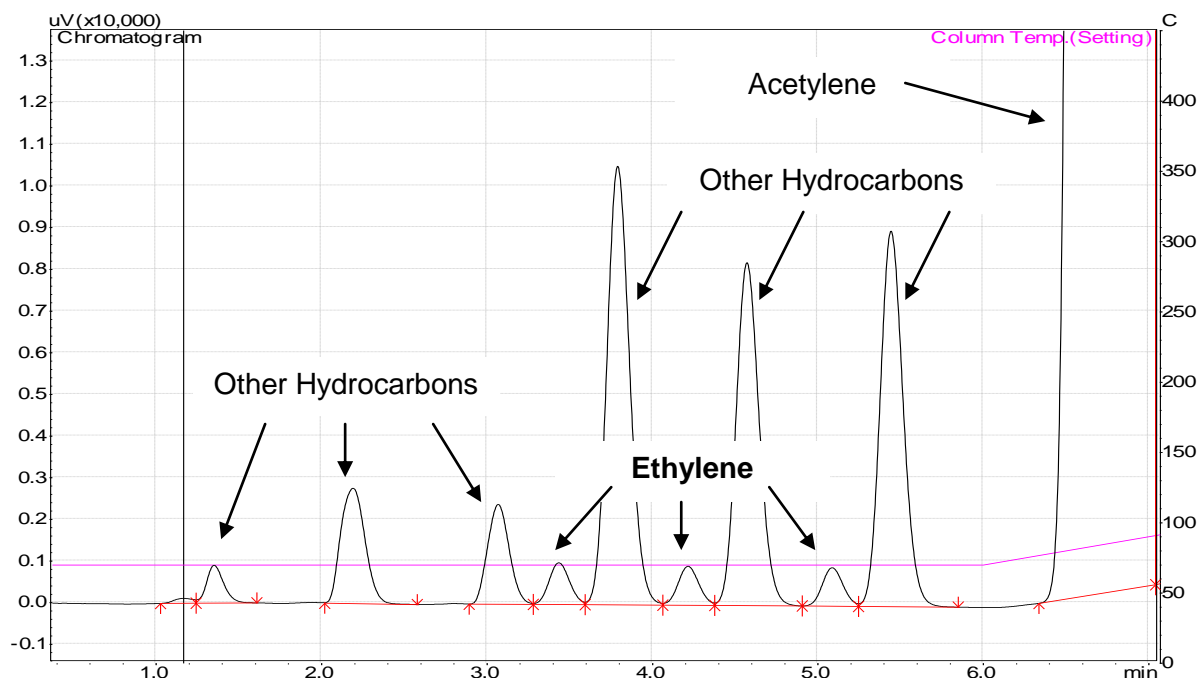


Figure 7 Chromatogram of three headspace samples from oil-treated sediment slurries in one run (GC-2014, Shimadzu Gas Chromatograph, Carrier Gas: Helium, Gas Flow: 22 ml/min, Column Type: HayeSepT, Column Temperature: 70°C, Detector Temperature: 300°C).

The C₂H₄ production was calculated by the following equation (Capone 1993):

$$\text{Eq. 5 } C_2H_4 \text{ [nmol]} = \frac{pk \text{ area, unk}}{pk \text{ area, std}} \times Std \text{ [nmol} \cdot \text{ml}^{-1}] \times GPV \text{ [ml]} \times SC$$

(pk area, unk) = Peak Area for C₂H₄ in 50 µl Sample

(pk area, std) = Peak Area for C₂H₄ in 50 µl Standard

Std = Concentration of C₂H₄ in Standard [nmol · ml⁻¹]

GPV = Gas Phase Volume [ml]

SC = Solubility Correction for C₂H₄

The solubility correction takes into account, that not all C₂H₄ can be found in the headspace but also in the aqueous phase of the sample, which was not sampled. The solubility correction was calculated according to the following equation (Flett et al. 1976):

$$\text{Eq. 6 } SC = 1 + \left(\alpha \cdot \frac{APV}{GPV} \right)$$

α = Bunsen Coefficient for C_2H_4 (depends on temperature and salinity)

APV = Aqueous Phase Volume [ml]

GPV = Gas Phase Volume [ml]

The Bunsen Coefficient was determined to be 0.125 for EBAY (Salinity: 25, Temperature: 13°C) and 0.115 for NAMV (Salinity: 37.5, Temperature: 13°C) according to Breitbarth et al. 2004.

The C_2H_4 concentrations derived from the repeated measure of each replicate were plotted against time. NA was calculated with a linear regression analysis for each replicate. NA was averaged per triplicate. Since no appropriate conversion factors to N_2 fixed were available for the two study sites, NA data is presented as measured in the following, i.e. in terms of C_2H_4 produced (Capone 1993).

2.5.4 Ammonium, Nitrate and Nitrite Measurement (Grasshoff et al. 1983)

Ammonium, nitrate and nitrite concentrations were measured photometrically (SEAL QuAtr Autoanalyzer). The automated procedure for nitrate and nitrite measurements uses the procedure outlined in the following (Grasshoff et al. 1983). For preparation of solutions and standards for ammonium, nitrate and nitrite measurements see Appendix A.3.

Ammonium. 1 cm³ of sample or standard is made up to 5 cm³ with pure water and 0.2 cm³ phenol solution is added. After 2 minutes 0.1 cm³ citrate buffer and 0.2 cm³ DTT reagent are added. After mixing, the samples are kept protected from sunlight at 30-40°C for 1-2 hours, before the absorbance of the indophenol blue is measured at 630 nm.

Nitrate. 1 cm³ of sample or standard are diluted with 9 cm³ buffer and passed through the reductor at 5 cm³ min⁻¹. 100 µl Sulphanilamide and 100 µl N-(1-naphthyl)-ethylenediamine-dihydrochlorid (NED) are added. After 30 minutes, the absorbance of the red azo dye is measured at 540 nm.

Nitrite. To 5.0 cm³ of undiluted sample or standard 0.1 cm³ Sulphanilamide-Solution and 0.1 cm³ NED-Solution are added. After 30 minutes, the absorbance of the red azo dye is measured at 540 nm.

2.5.5 DNA Extraction from Sediment

DNA was extracted from 500 mg sediment per sample, using the FastDNA[®] SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA, USA; see Appendix A.4 for DNA Extraction Protocol).

2.6 Statistical Analysis

The graphical and statistical analysis was done with the software *R*, version 3.0.0 (R Core Team 2013. R Foundation for Statistical Computing, Vienna, Austria). Average values are given by the statistical mean and its standard deviation. The observations from each experiment were analyzed separately (Preliminary Experiment, Experiment 1, Experiment 2). Furthermore, observations obtained from sediment samples originating from different study sites (EBAY, NAMV) were tested separately, if they were present in one experiment, like in Experiment 1. Also the two main dependent variables, TS production and NA, were treated separately, if they were both present in one experiment, like in Experiment 1. The reason for this was that TS and NA were determined from different microcosms (15 ml hungate tubes and 60 ml serum vials respectively).

There was no statistical analysis performed for the TS data collected during the revival process preceding Experiment 1 because measurements were done by different people with different filters (0.22 μm versus 0.45 μm). Also the ammonium, nitrate and nitrite data was not statistically tested because samples were not taken in triplicate for these measurements. One sample per triplicate was enough to get an idea about the ammonium, nitrate and nitrite situation at the beginning and end of Experiment 1 and 2. Regarding Experiment 1, the observations obtained from the controls after day 7, when acetate was added, were excluded from the statistical analysis for both NA and TS production, since these observations did not represent an independent treatment. To get a balanced design for the statistical analysis, only observations from the oil treatment and the controls, collected on day 0, 2, 4 and 6 of Experiment 1 were used in the statistical analysis.

The effect of oil treatment on the two main dependent variables, TS production and NA, was tested by comparing the slopes of linear regressions, calculated for the repeated measure of each replicate over time. The resulting rates (NA in $\text{nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ and TS in $\text{mM cm}^{-3} \text{ d}^{-1}$) were checked for outliers by generating dotcharts and boxplots of the data grouped by treatment. The normality of residuals was checked with the Shapiro-Wilk test and the homogeneity of variances with the Fligner-Killeen test. If the assumptions of statistical tests were fulfilled (no outliers, normality of residuals and homogeneity of variances), the group

means of the rates were compared with either Student's t-test, in the case of two groups (Experiment 1 and 2), or one-factorial ANOVA, in the case of four groups (Preliminary Experiment). If data was not normally distributed, a Mann-Whitney-U-Test was used instead of a Student's t-test. The significance level for all statistical tests applied was $p < 0.05$.

3. RESULTS

The overall goal of this study was to determine whether nitrogen (N_2) fixation is occurring in marine oil-contaminated sediments from Eckernförde Bay (EBAY), Baltic Sea, and North Alex Mud Volcano (NAMV), Mediterranean Sea. If so, is N_2 fixation coupled to sulfate-reducing bacteria (SRB) degrading oil compounds? Are both sulfate reduction and N_2 fixation stimulated by oil addition?

In the Preliminary Experiment, the effect of dilution on the nitrogenase activity (NA) was investigated via the acetylene (C_2H_2) reduction method (see Section 2.2.3 for Experimental Design and Section 2.5.3 for NA Measurement Procedure). Thus the treatment was dilution and four groups were compared: anaerobic undiluted EBAY sediment versus three different sediment slurries with the dilution factor 2, 3 and 4 respectively (see Section 3.1 for Results of the Preliminary Experiment).

In Experiment 1, the effect of oil treatment on total sulfide (TS) production and NA in anaerobic EBAY and NAMV sediment slurries was investigated (see Section 2.3.4 for Experimental Design, Section 2.5.2 for TS Measurement Procedure and Section 2.5.3 for NA Measurement Procedure). Thus the treatment was oil addition and two groups were compared: oil-treated sediment slurries versus control sediment slurries from both study sites (see Section 3.2 for Results of Experiment 1).

In Experiment 2, the effect of oil treatment on NA in aerobic EBAY sediment slurries was investigated (see Section 2.4.3 for Experimental Design and Section 2.5.3 for NA Measurement Procedure). Whereas one set of sediment slurries has been incubated with oil for about three months prior to the start of the experiment (hereafter called pre-adapted sediment slurries), another set of sediment slurries has been incubated with oil only for the duration of the experiment (hereafter called non-adapted sediment slurries). Thus the treatment was oil addition and two groups were compared: oil-treated sediment slurries (pre-adapted and non-adapted) versus the respective control sediment slurries (see Section 3.3 for Results of Experiment 2).

3.1 Preliminary Experiment - Anaerobic Incubation (EBAY)

In the Preliminary Experiment, the effect of dilution on NA was investigated via the C_2H_2 reduction method over the course of 10 days. Thus the treatment was dilution and four groups were compared: anaerobic undiluted EBAY sediment versus three different sediment slurries with the dilution factor 2, 3 and 4 respectively.

The NA in undiluted sediment samples was with $1.37 \pm 0.08 \text{ nmol } C_2H_4 \text{ cm}^{-3} \text{ d}^{-1}$ lower than the three differently diluted sediment slurries. The sediment slurries with the dilution factor 2, 3 and 4 showed a NA of 1.57 ± 0.15 , 1.52 ± 0.05 and $1.61 \pm 0.17 \text{ nmol } C_2H_4 \text{ cm}^{-3} \text{ d}^{-1}$ respectively (Figure 8). The sediment slurries without C_2H_2 and the sediment slurries treated with Formaldehyde showed no NA over the course of ten days, i.e. no natural NA occurred (data not shown here). The sediment slurries without C_2H_2 and the sediment slurries treated with Formaldehyde were excluded from the statistical analysis.

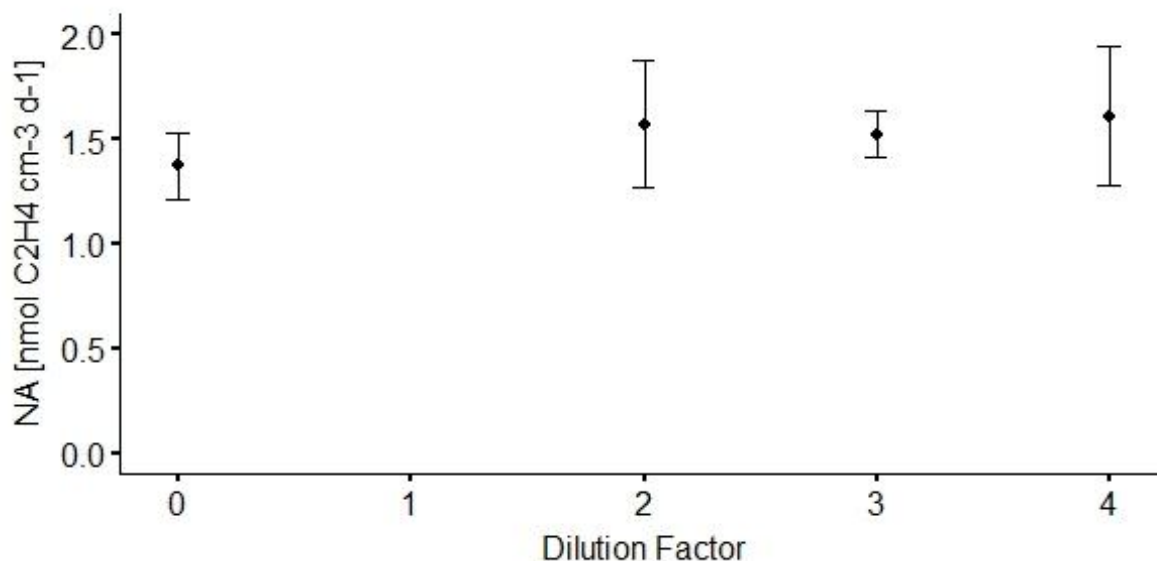


Figure 8 Nitrogenase activity (NA) in undiluted (dilution factor 0) Eckernförde Bay (EBAY) sediment samples and three differently diluted EBAY sediment slurries (dilution factor 2, 3 and 4) during the Preliminary Experiment. Nitrogenase activity is averaged per group ($n = 3$ per group, repeated measure at 5 time points over 10 days). Average values are given in mean \pm SD.

The NA across all groups (undiluted sediment samples and sediment slurries with the dilution factor 2, 3 and 4) was approximately normally distributed according to the insignificance of the Shapiro-Wilk test ($W = 0.95$, $p = 0.62$). The variances were homogenous among groups due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 2.47$, $df = 3$, $p = 0.48$). To test the effect of dilution on NA, a one-way ANOVA was conducted. The ANOVA revealed a significant effect of dilution on the NA ($F(1,10) = 5.63$, $p = 0.039$, $\omega = 0.55$).

The results suggested that it matters, if a sediment sample is diluted or not, when determining NA via the C_2H_2 assay. The undiluted sediment sample showed a significantly lower NA than the three differently diluted sediment slurries. Determining the NA from sediment slurries leads potentially to an overestimation of NA compared to undiluted sediment samples. However, it does not seem to matter how much a sediment sample is diluted with medium, when determining the NA in sediment slurries via the C_2H_2 reduction assay.

3.2 Experiment 1 - Anaerobic Incubation (EBAY and NAMV)

Before the start of Experiment 1, the EBAY and NAMV sediment slurries were revived over nearly 100 days because the sediment slurries had been stored in the fridge at 4°C for a long time (see Section 2.3.1 for History of Sediment Slurries and 2.3.2 for Revival Procedure of Sediment Slurries). During this time, TS concentrations were monitored (see Section 2.5.2 for TS Measurement Procedure). An increase of TS concentrations in oil-treated sediment slurries from both study sites was observed. TS production serves as a measure for sulfate reduction activity by SRB. After the activity of SRB in oil-treated sediment slurries was confirmed by TS production, Experiment 1 was conducted.

In Experiment 1, the effect of oil treatment on TS production and NA in anaerobic EBAY and NAMV sediment slurries was investigated over the course of 14 days. Thus the treatment was oil addition and two groups were compared: oil-treated sediment slurries versus control sediment slurries from both study sites (EBAY and NAMV). Additionally, ammonium, nitrate and nitrite samples were taken at the beginning and end of Experiment 1 (see Section 2.5.4 for Ammonium, Nitrate and Nitrite Measurement Procedure).

3.2.1 Revival of Sediment Slurries

The whole revival process confirmed that SRB were still active in the oil-treated sediment slurries from both study sites, despite their long storage period. Higher TS concentrations could be found in the oil-treated sediment slurries compared to the control sediment slurries from both study sites (Figure 9 and 10). Oil-treated EBAY sediment slurries produced about 1 to 4 mM TS over the course of 100 days. Oil-treated NAMV sediment slurries produced about 4 to 16 mM TS over the course of 100 days. Note that TS data was corrected for the dilution factor of the sediment slurries and the volume of sediment used to prepare the sediment slurries to allow comparisons between study sites.

The revival process consisted of three revival steps. Between each revival step, as much old medium as possible was removed, without losing a substantial amount of sample material, and the same amount of new medium was added shortly afterwards (see Section 2.5.1 for Preparation of Artificial Seawater Medium). These medium exchanges caused the reduction of TS concentrations in the beginning of each following revival step through the removal of TS contained in the old medium (Figure 9 and 10).

A linear positive trend could be observed over time for TS concentrations from oil-treated sediment slurries from both study sites. Linear regressions were applied to each group (oil-treated and control sediment slurries) and revival step (1, 2 and 3). The slope of these linear regressions is a measure of the TS production rate by SRB. The TS production activity of SRB increased during the revival process. This increase can be seen by comparing the slopes of the linear regressions for each group and each revival step (Figure 9 and 10). During the first revival step, which lasted for about 20 days, a slight increase in TS concentrations could be already observed in the oil-treated sediment slurries from both study sites. TS production rates approximately quadrupled in oil-treated EBAY sediment slurries during the second revival step, which lasted for about 30 days (Figure 9 and 10). TS production rates in oil-treated NAMV sediment slurries approximately doubled during the second revival step (Figure 9 and 10). This increase in TS production rates in oil-treated sediment slurries indicates a lag phase of about three to four weeks of an previously oil adapted microbial community towards a new oil input. However, the TS production rates in oil-treated sediment slurries in the third revival step, which lasted for about 30 days, were lower than the TS production rates in oil-treated sediment slurries in the second revival step (Figure 9 and 10).

Although there was some TS present in the control sediment slurries from both study sites, the TS concentrations were generally close to zero and did not change over time (Figure 9 and 10). After the whole revival process, it therefore cannot be said for sure that the microbial communities in the control sediment slurries from both study sites were still alive and active. However, it should be noted that in contrast to the oil-treated sediment slurries, the control sediment slurries did not receive any new carbon source during the revival process. To see, if control sediment slurries were limited by the lack of a carbon source, acetate solution was added at day 7 of Experiment 1.

EBAY

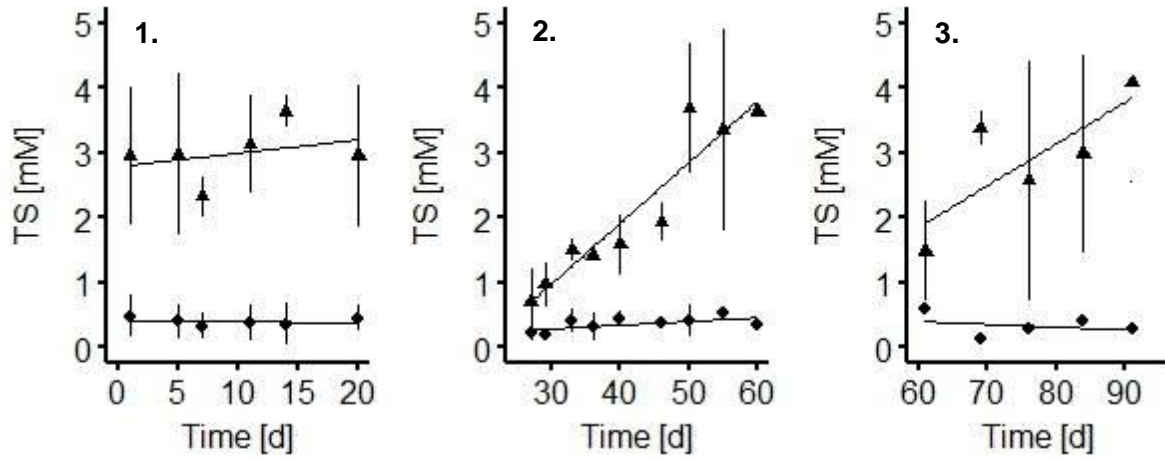


Figure 9 Development of total sulfide (TS) concentrations in (◆) control and (▲) oil-treated Eckernförde Bay (EBAY) sediment slurries during the revival process. TS concentrations are averaged per triplicate at each time point ($n = 3$ per group, repeated measure at 20 time points over nearly 100 days). Average values are given in mean \pm SD. Linear regressions were applied to each group and revival step (1, 2 and 3).

NAMV

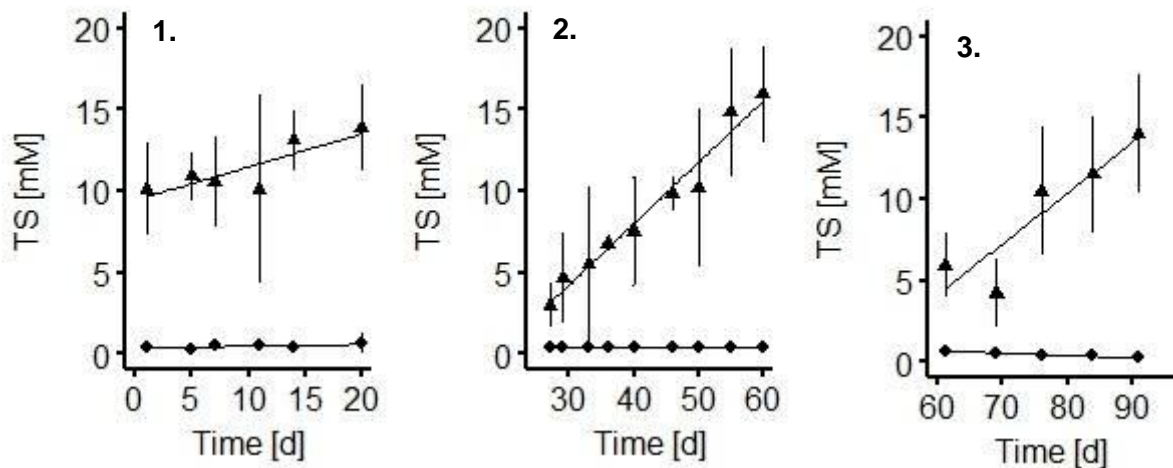


Figure 10 Development of total sulfide (TS) concentrations in control (◆) and oil-treated (▲) North Alex Mud Volcano (NAMV) sediment slurries during the revival process. TS concentrations are averaged per group at each time point ($n = 3$ per group, repeated measure at 20 time points over nearly 100 days). Average values are given in mean \pm SD. Linear regressions were applied to each group and revival step (1, 2 and 3).

The effect of oil treatment on TS production rates from EBAY and NAMV sediment slurries was tested by comparing the slopes of linear regressions, calculated for the repeated measure of each replicate over time. TS production rates were averaged per group (control and oil-treated sediment slurries from each study site) over the whole revival process of nearly 100 days ($n = 3$ per group).

The oil-treated NAMV sediment slurries had a TS production rate about five times as high as the oil-treated EBAY sediment slurries (0.30 ± 0.09 and 0.06 ± 0.04 mM TS $\text{cm}^{-3} \text{d}^{-1}$ respectively, Figure 11). However, the standard deviation was also higher for the oil-treated NAMV sediment slurries than for the oil-treated EBAY sediment slurries (Figure 11). The TS production rates of the control sediment slurries for both EBAY and NAMV (0.005 ± 0.002 and 0.002 ± 0.015 mM TS $\text{cm}^{-3} \text{d}^{-1}$ respectively) were much lower than the TS production rates of the oil-treated sediment slurries (Figure 11).

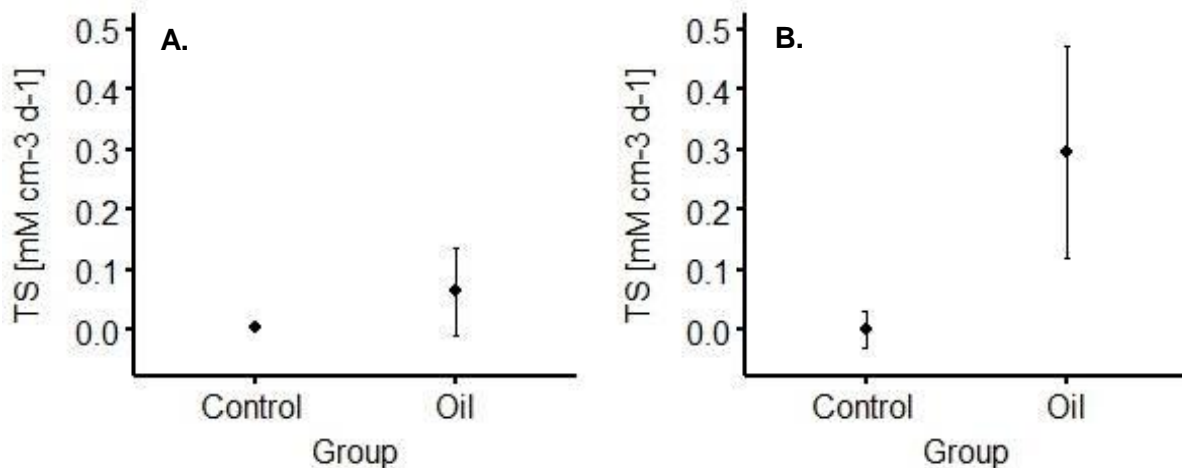


Figure 11 Total sulfide (TS) production rates in control and oil-treated (A.) Eckernförde Bay (EBAY) and (B.) North Alex Mud Volcano (NAMV) sediment slurries during the revival process. TS production rates are averaged per group and the whole revival process ($n = 3$ per group, repeated measure at 20 time points over nearly 100 days). Average values are given in mean \pm SD.

Due to the relatively huge standard deviations among triplicates of oil-treated sediment slurries observed during the revival process, each triplicate was combined and homogenized before the start of Experiment 1 to achieve equal initial conditions.

3.2.2 Eckernförde Bay (EBAY)

In Experiment 1, the effect of oil treatment on TS production and NA in anaerobic EBAY sediment slurries was investigated over the course of 14 days. Additionally, ammonium, nitrate and nitrite samples were taken at the beginning and end of Experiment 1.

In general, oil-treated EBAY sediment slurries produced more TS than control sediment slurries during Experiment 1 (Figure 12). Acetate solution was added to control sediment slurries on day 7 of Experiment 1, to see if the activity of SRB was limited by the lack of a carbon source. After the addition of acetate, TS concentrations in control sediment slurries slightly increased (hereafter called acetate treated control sediment slurries). However, the TS concentrations in acetate treated control sediment slurries were much lower than the TS concentrations in oil-treated sediment slurries (Figure 12). In the oil-treated sediment slurries, TS concentrations ranged from about 8 to 18 mM over the course of 14 days (Figure 12). Note that these TS concentrations were not corrected for the dilution factor of the sediment slurries and the volume of sediment used to prepare the sediment slurries.

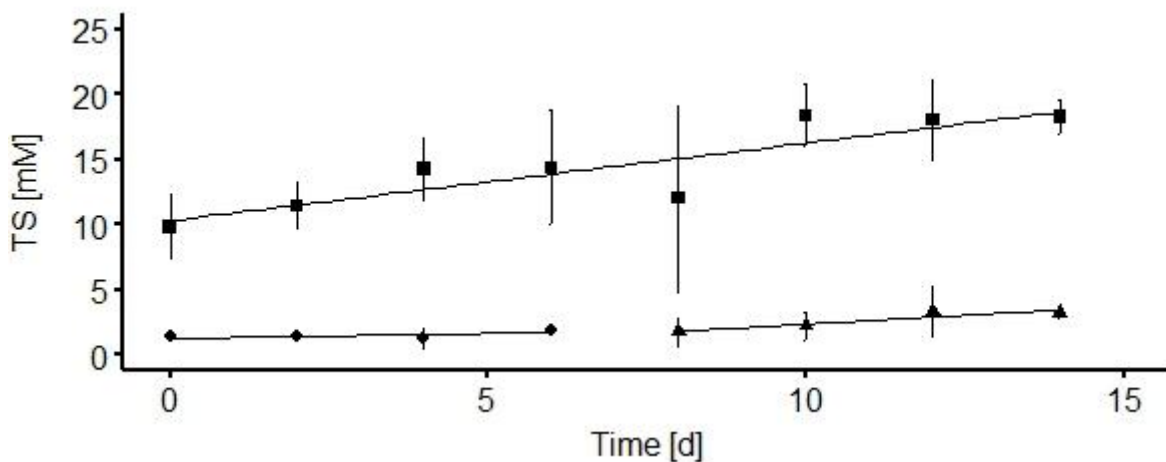


Figure 12 Development of total sulfide (TS) concentrations in (◆) control, (▲) acetate treated control and (■) oil-treated Eckernförde Bay (EBAY) sediment slurries during Experiment 1. TS concentrations are averaged per group at each time point ($n = 3$ per group, repeated measure at 8 time points over 14 days). Average values are given in mean \pm SD. Linear regressions were applied per group.

The effect of oil treatment on TS production rates and NA from EBAY sediment slurries was tested by comparing the slopes of linear regressions, calculated for the repeated measure of each replicate over time. TS production rates and NA were averaged per group (control and oil-treated sediment slurries) over the course of Experiment 1 ($n = 3$ per group).

When comparing TS production rates, the effect of oil treatment becomes even more apparent (Figure 13A). On average, control sediment slurries produced about 0.12 ± 0.008

mM TS cm⁻³ d⁻¹. Acetate treated control sediment slurries produced about 0.37 ± 0.07 mM TS cm⁻³ d⁻¹. Oil-treated sediment slurries produced about 1.10 ± 0.59 mM TS cm⁻³ d⁻¹. Oil-treated sediment slurries produced about ten times more TS cm⁻³ d⁻¹ than control sediment slurries.

Interestingly, NA in control sediment slurries was more than double than in oil-treated sediment slurries (Figure 13B). On average, control sediment slurries produced about 0.91 ± 0.22 nmol C₂H₄ cm⁻³ d⁻¹. Oil-treated sediment slurries produced about 0.37 ± 0.05 nmol C₂H₄ cm⁻³ d⁻¹. Acetate treated control sediment slurries produced about 1.24 ± 0.83 nmol C₂H₄ cm⁻³ d⁻¹. The sediment slurries without C₂H₂ and the sediment slurries treated with Formaldehyde showed no NA over the course of 14 days, i.e. no natural NA occurred (data not shown here). The sediment slurries without C₂H₂ and the sediment slurries treated with Formaldehyde were excluded from the statistical analysis.

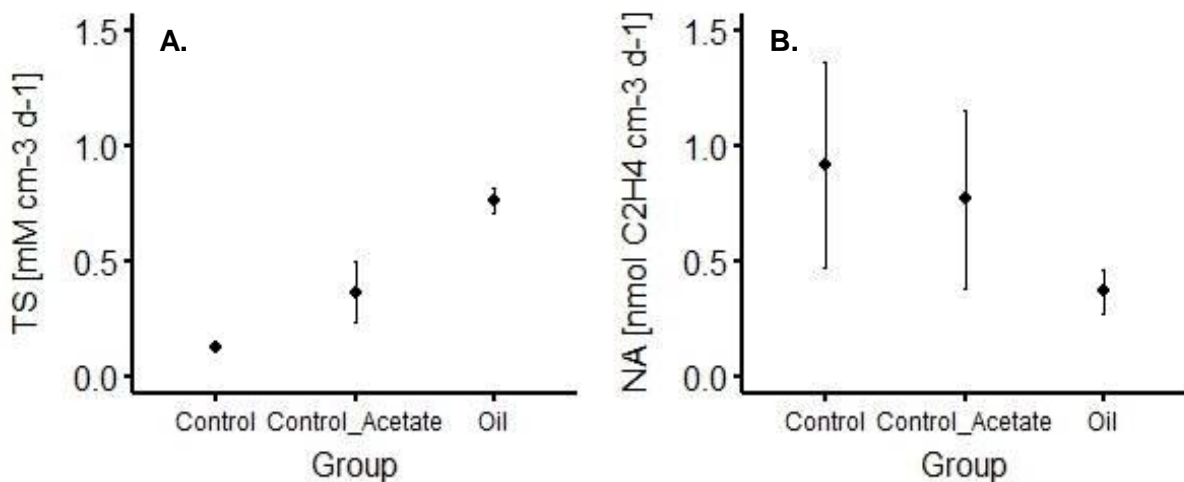


Figure 13 (A.) Total sulfide (TS) production rates and (B.) nitrogenase activity (NA) in control, acetate treated control and oil-treated Eckernförde Bay (EBAY) sediment slurries during Experiment 1. TS production rates and NA are averaged per group ($n = 3$ per group). Average values are given in mean ± SD.

However, TS production rates and NA for control sediment slurries were derived from repeated measures at four time points over seven days. In contrast to TS production rates and NA for oil-treated sediment slurries, which were derived from repeated measures at eight time points over 14 days. To achieve a balanced design for the statistical analysis, TS production rates and NA derived only from the first four time points for both control and oil-treated sediment slurries were used. TS production rates and NA from acetate treated control sediment slurries were not included in the statistical analysis because they did not

represent an independent treatment. Acetate treated sediment slurries (second half of Experiment 1) were based on control sediment slurries (first half of Experiment 1).

The statistical analysis assumed that the TS production rates within each group (control and oil-treated sediment slurries) were approximately normally distributed according to the insignificance of the Shapiro-Wilk test (control: $W = 0.80$, $p = 0.12$, oil: $W = 0.78$, $p = 0.06$). The variances were homogenous among groups due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 1.04$, $df = 1$, $p = 0.31$). Group means of TS production rates were compared with a two-tailed independent Student's t-test. The t-test revealed a significant effect of oil treatment on TS production rates ($t(4) = -2.89$, $p = 0.04$, $r = 0.82$).

The statistical analysis assumed that the NA within each group (control and oil-treated sediment slurries) were approximately normally distributed according to the insignificance of the Shapiro-Wilk test (control: $W = 0.91$, $p = 0.40$, oil: $W = 0.89$, $p = 0.34$). The variances were homogenous for the control and oil-treated sediment slurries due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 1.04$, $df = 1$, $p = 0.31$). Group means of NA were compared with a two-tailed independent Student's t-test. The t-test revealed a significant effect of oil treatment on NA ($t(4) = 4.12$, $p = 0.01$, $r = 0.9$).

Nutrient samples for the analysis of N-species (ammonium, nitrate and nitrite) were taken at the beginning and end of Experiment 1. Surprisingly, the initial nutrient conditions were not similar among groups (control and oil-treated sediment slurries). Therefore the differences in nutrient changes over the course of the Experiment 1 were examined instead of the absolute values. The nutrient changes were calculated by subtracting the nutrient value measured at the beginning of the experiment from the nutrient value measured at the end. Consequently, negative differences stand for a decrease of the certain nutrient over time, i.e. indicating consumption, whereas a positive difference stands for an increase of the respective nutrient species, i.e. indicating production over the course of the experiment. However, these measurements are single observations derived from a combined sample per triplicate/group.

Ammonium concentrations decreased in both groups (control and oil-treated sediment slurries), indicating ammonium consumption over the course of Experiment 1 (Figure 14A). However, ammonium concentrations decreased more in oil-treated sediment slurries ($-96.24 \mu\text{mol/l}$) than in control sediment slurries ($-53.55 \mu\text{mol/l}$). In contrast, nitrate concentrations increased in both groups (Figure 14B). However, nitrate concentrations increased more in control sediment slurries ($2.42 \mu\text{mol/l}$) than in oil-treated sediment slurries ($0.56 \mu\text{mol/l}$). Nitrite concentrations decreased in both groups (Figure 14C). Nitrite concentrations decreased $-1.72 \mu\text{mol/l}$ in control sediment slurries and $-0.18 \mu\text{mol/l}$ in oil-treated sediment slurries.

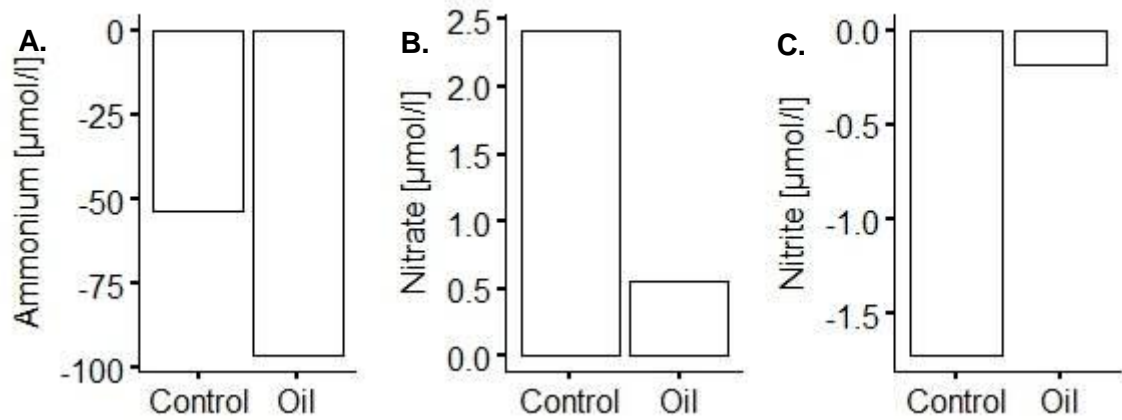


Figure 14 Production and consumption of (A.) ammonium, (B.) nitrate and (C.) nitrite in control and oil-treated Eckernförde Bay (EBAY) sediment slurries during Experiment 1. Negative values indicate consumption, whereas positive values indicate production of the respective N-species ($n = 1$ per group, repeated measure at two time points over 14 days).

3.2.3 North Alex Mud Volcano (NAMV)

In Experiment 1, the effect of oil treatment on TS production and NA in anaerobic NAMV sediment slurries was investigated over the course of 14 days. Additionally, ammonium, nitrate and nitrite samples were taken at the beginning and end of Experiment 1.

In general, oil-treated NAMV sediment slurries produced more TS than control sediment slurries during Experiment 1 (Figure 15). Acetate solution was added to control sediment slurries on day 7 of Experiment 1, to see if the activity of SRB was limited by the lack of a carbon source. After the addition of acetate, TS concentrations in control sediment slurries slightly increased (hereafter called acetate treated control sediment slurries). However, the TS concentrations in acetate treated control sediment slurries were much lower than the TS concentrations in oil-treated sediment slurries (Figure 15). In the oil-treated sediment slurries, TS concentrations ranged from about 11 to 24 mM over the course of 14 days (Figure 15). Note that these TS concentrations were not corrected for the dilution factor of the sediment slurries and the volume of sediment used to prepare the sediment slurries. TS concentrations measured from day 8 to 14 in oil-treated sediment slurries were excluded from further calculations because the values were beyond the calibration range.

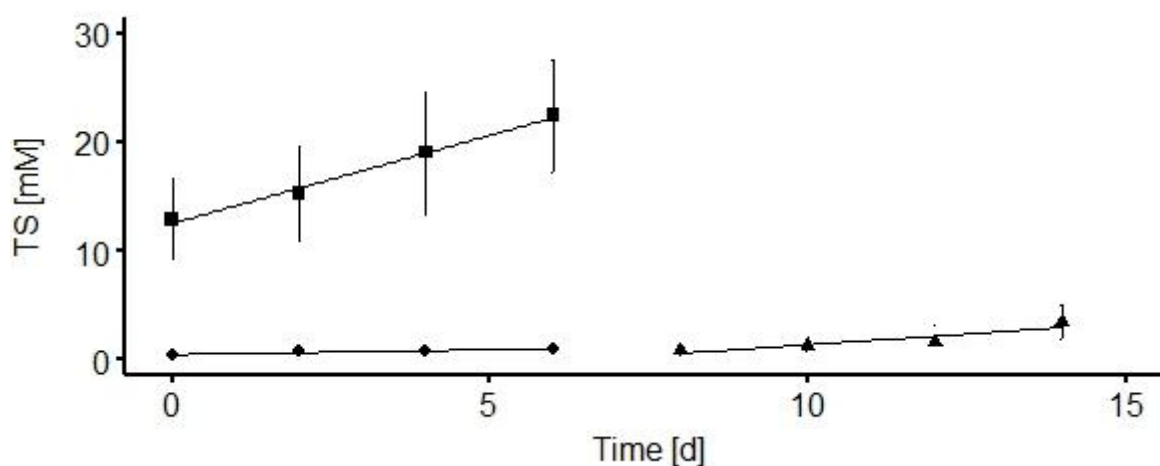


Figure 15 Development of total sulfide (TS) concentrations in (◆) control, (▲) acetate treated control and (■) oil-treated North Alex Mud Volcano (NAMV) sediment slurries during Experiment 1. TS concentrations are averaged per group at each time point ($n = 3$ per group, repeated measure at 8 time points over 14 days for control sediment slurries and repeated measure at 4 time points over 7 days for oil-treated sediment slurries). Average values are given in mean \pm SD. Linear regressions were applied per group.

The effect of oil treatment on TS production rates and NA from NAMV sediment slurries was tested by comparing the slopes of linear regressions, calculated for the repeated measure of each replicate over time. TS production rates and NA were averaged per group (control and oil-treated sediment slurries) over the course of Experiment 1 ($n = 3$ per group).

When comparing TS production rates, the effect of oil treatment becomes even more apparent (Figure 16A). On average, control sediment slurries produced about 0.24 ± 0.05 mM TS $\text{cm}^{-3} \text{d}^{-1}$. Acetate treated control sediment slurries produced about 1.05 ± 0.30 mM TS $\text{cm}^{-3} \text{d}^{-1}$. Oil-treated sediment slurries produced about 4.34 ± 0.32 mM TS $\text{cm}^{-3} \text{d}^{-1}$. Oil-treated sediment slurries produced more than fifteen times more TS $\text{cm}^{-3} \text{d}^{-1}$ than control sediment slurries.

NA in both control and oil-treated sediment slurries was around zero (Figure 16B). On average, control sediment slurries produced about -0.05 ± 0.06 nmol C_2H_4 $\text{cm}^{-3} \text{d}^{-1}$, indicating a loss of C_2H_4 . Possible reasons for this loss are mentioned in the discussion. Acetate treated control sediment slurries produced about 0.04 ± 0.06 nmol C_2H_4 $\text{cm}^{-3} \text{d}^{-1}$. Oil-treated sediment slurries produced about 0.01 ± 0.02 nmol C_2H_4 $\text{cm}^{-3} \text{d}^{-1}$. The sediment slurries without C_2H_2 and the sediment slurries treated with Formaldehyde showed no NA over the course of 14 days, i.e. no natural NA occurred (data not shown here). The sediment slurries without C_2H_2 and the sediment slurries treated with Formaldehyde were excluded from the statistical analysis.

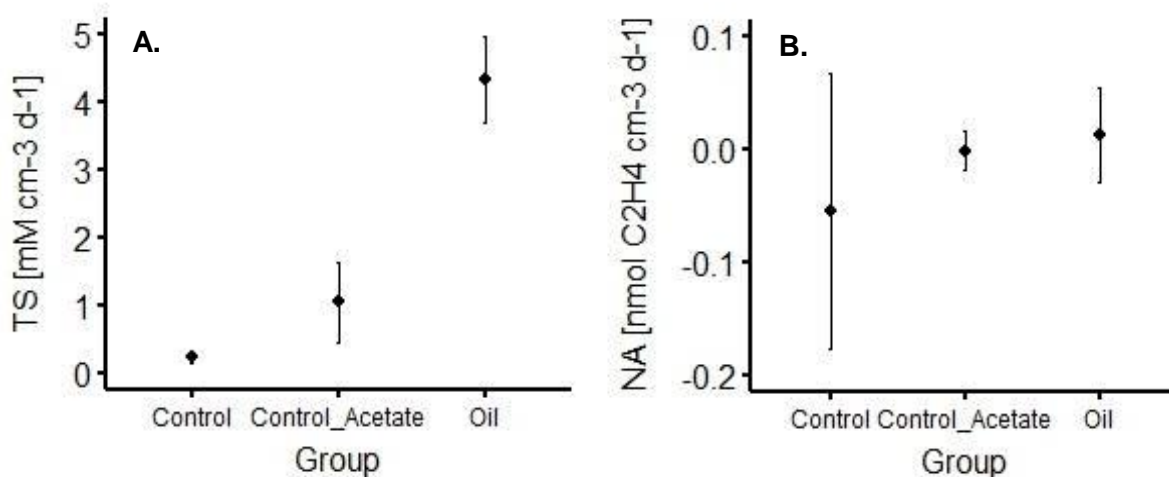


Figure 16 (A.) Total sulfide (TS) production rates and (B.) nitrogenase activity (NA) in control, acetate treated control and oil-treated North Alex Mud Volcano (NAMV) sediment slurries during Experiment 1. TS production rates and NA are averaged per group ($n = 3$ per group). Average values are given in mean \pm SD.

TS production rates and NA from acetate treated control sediment slurries were not included in the statistical analysis because they did not represent an independent treatment. Acetate treated sediment slurries (second half of Experiment 1) were based on control sediment slurries (first half of Experiment 1).

The statistical analysis assumed that the TS production rates within each group (control and oil-treated sediment slurries) were approximately normally distributed according to the insignificance of the Shapiro-Wilk test (control: $W = 0.96$, $p = 0.59$; oil: $W = 0.93$, $p = 0.50$). The variances were homogenous among groups due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 1.04$, $df = 1$, $p = 0.31$). Group means of TS production rates were compared with a two-tailed independent Student's t-test. The t-test revealed a significant effect of oil treatment on TS production rates ($t(4) = -21.94$, $p = 2.55 \cdot 10^{-5}$, $r = 0.99$).

The statistical analysis revealed that NA within each group (control and oil-treated sediment slurries) was not normally distributed due to the significance of the Shapiro-Wilk test ($W = 0.75$, $p = 2.2 \cdot 10^{-16}$) in the control group. However, for the oil-treated group a normal distribution can be assumed ($W = 0.95$, $p = 0.55$). The variances were homogenous for the control and oil-treated sediment slurries due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 6 \cdot 10^{-4}$, $df = 1$, $p = 0.98$). However, due to violation of the normality assumption, the group means of NA were compared with a Mann-Whitney-U test instead of Student's t-test. The Mann-Whitney-U test revealed no significant effect of oil treatment on NA ($W = 0$, $p = 0.07$).

Nutrient samples for the analysis of N-species (ammonium, nitrate and nitrite) were taken at the beginning and end of Experiment 1. Surprisingly, the initial nutrient conditions were not similar among groups (control and oil-treated sediment slurries). Therefore the differences in nutrient changes over the course of the Experiment 1 were examined instead of the absolute values. The nutrient changes were calculated by subtracting the nutrient value measured at the beginning of the experiment from the nutrient value measured at the end. Consequently, negative differences stand for a decrease of the certain nutrient over time, i.e. indicating consumption, whereas a positive difference stands for an increase of the respective nutrient species, i.e. indicating production over the course of the experiment. However, these measurements are single observations derived from a combined sample per triplicate/group.

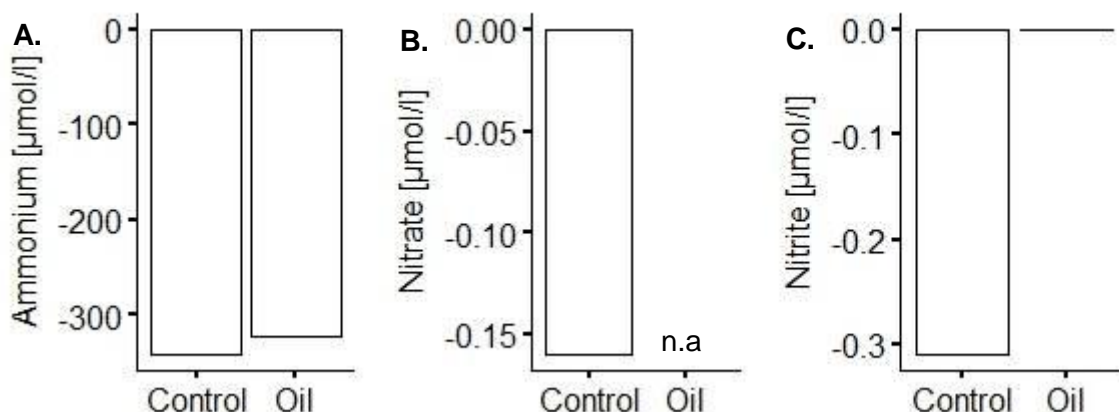


Figure 17 Production and consumption of (A.) ammonium, (B.) nitrate and (C.) nitrite in control and oil-treated North Alex Mud Volcano (NAMV) sediment slurries during Experiment 1. Negative values indicate consumption, whereas positive values indicate production of the respective N-species ($n = 1$ per group, repeated measure at two time points over 14 days).

Ammonium concentrations decreased in both groups (control and oil-treated sediment slurries), indicating ammonium consumption over the course of Experiment 1 (Figure 17A). However, ammonium concentrations decreased more in control sediment slurries ($-342 \mu\text{mol/l}$) than in oil-treated sediment slurries ($-323 \mu\text{mol/l}$). Nitrate concentrations also decreased in control sediment slurries ($-0.16 \mu\text{mol/l}$, Figure 17B). However, nitrate concentrations in oil-treated sediment slurries are unfortunately not available. Nitrite concentrations decreased in control sediment slurries ($-0.31 \mu\text{mol/l}$), but nitrite concentrations did not change in oil-treated sediment slurries (Figure 17C).

3.2.4 Summary

The results of Experiment 1 revealed a significant effect of oil treatment on TS production rates and NA in EBAY and NAMV sediment slurries compared to the respective control sediment slurries (Figure 18). However, there was no significant effect of oil treatment on NA in NAMV sediment slurries (Figure 18 B.2). If a significant effect was observed, oil treatment had a positive effect on TS production rates and NA in EBAY and NAMV sediment slurries compared to the respective control sediment slurries (Figure 18). However, oil treatment had a negative effect on NA in EBAY sediment slurries (Figure 18 A.2). Consequently, TS production and NA did not seem to be correlated in EBAY sediment slurries. However, TS production and NA could be correlated in NAMV sediment slurries because oil treatment had a positive effect on both processes (Figure 18 B). But the effect of oil treatment on NA in

NAMV sediment slurries was not significant. Thus it is uncertain, if TS production and NA in NAMV sediment slurries are actually correlated.

Oil-treated NAMV sediment slurries showed a TS production rate about four times higher than oil-treated EBAY sediment slurries (4.34 ± 0.32 and 1.10 ± 0.59 mM TS $\text{cm}^{-3} \text{d}^{-1}$ respectively). On average, the TS production rate in control NAMV sediment slurries was about twice as high as in the control EBAY sediment slurries (0.24 ± 0.05 and 0.12 ± 0.01 mM TS $\text{cm}^{-3} \text{d}^{-1}$ respectively, Figure 18 A.1 and B.1). A reverse pattern was observed for NA (Figure 18 A.2 and B.2). Control and oil-treated NAMV sediment slurries showed almost no NA over the course of Experiment 1. However, NA in control and oil-treated EBAY sediment slurries was relatively high (0.91 ± 0.22 and 0.37 ± 0.05 nmol C_2H_4 $\text{cm}^{-3} \text{d}^{-1}$ respectively).

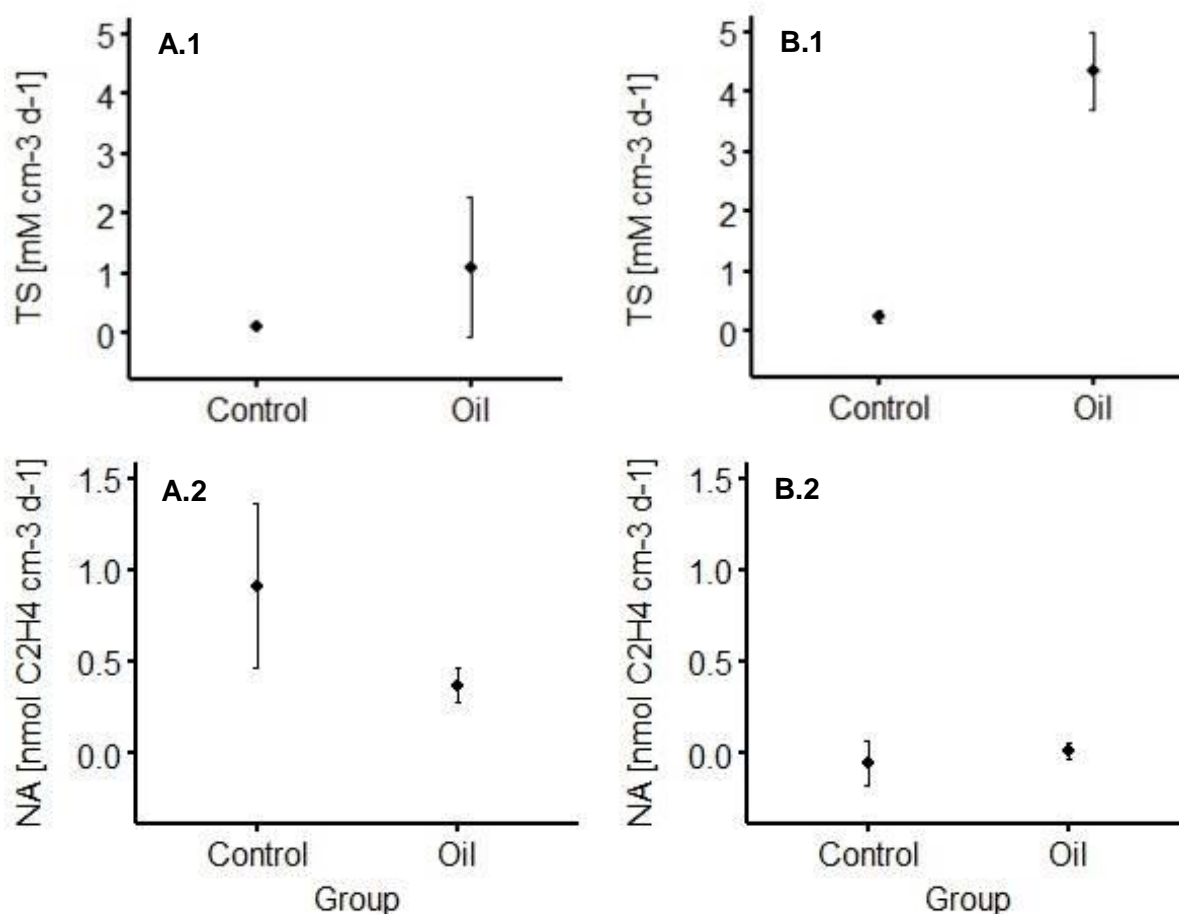


Figure 18 Total sulfide (TS) production rates (A.1 and B.1) and nitrogenase activity (NA, A.2 and B.2) in control, acetate treated control and oil-treated (A.) Eckernförde Bay (EBAY) and (B.) North Alex Mud Volcano (NAMV) sediment slurries during Experiment 1. TS production rates and NA are averaged per group ($n = 3$ per group). Average values are given in mean \pm SD.

3.3 Experiment 2 - Aerobic Incubation (EBAY)

In Experiment 2, the effect of oil treatment on NA in aerobic EBAY sediment slurries was investigated. One set of sediment slurries has been incubated with oil for about three months prior to the start of Experiment 2 (hereafter called pre-adapted sediment slurries), whereas another set of sediment slurries has been incubated with oil only for the duration of the Experiment 2 (hereafter called non-adapted sediment slurries). Experiment 2 lasted for 10 days. The effect of oil treatment on NA in aerobic EBAY sediment slurries was tested by comparing the slopes of linear regressions, calculated for the repeated measure of each replicate over time. NA was averaged per group (control and oil-treated sediment slurries) over the course of Experiment 2 ($n = 3$ per group).

On average, pre-adapted oil-treated sediment slurries produced about 0.22 ± 0.09 nmol C₂H₄ cm⁻³ d⁻¹ over the course of Experiment 2. "Pre-adapted" control sediment slurries produced 0.02 ± 0.02 nmol C₂H₄ cm⁻³ d⁻¹ (Figure 19A). NA in non-adapted sediment slurries was higher than the NA in pre-adapted sediment slurries (Figure 19). Non-adapted oil-treated sediment slurries produced about 0.72 ± 0.32 nmol C₂H₄ cm⁻³ d⁻¹ and "non-adapted" control sediment slurries produced about 0.78 ± 0.65 nmol C₂H₄ cm⁻³ d⁻¹ (Figure 19B). The sediment slurries without C₂H₂ and the sediment slurries treated with Formaldehyde showed no NA over the course of 14 days, i.e. no natural NA occurred (data not shown here). The sediment slurries without C₂H₂ and the sediment slurries treated with Formaldehyde were excluded from the statistical analysis.

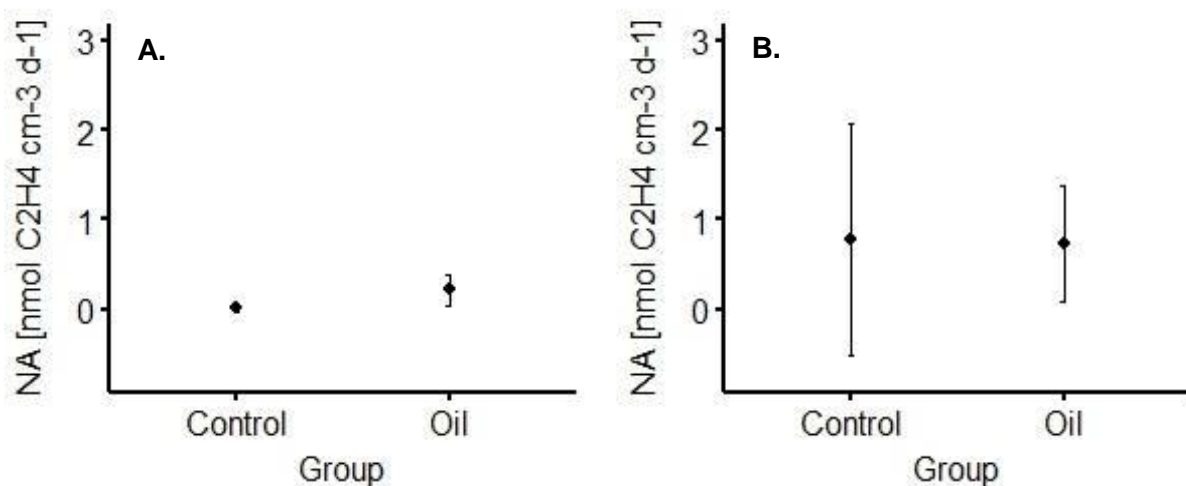


Figure 19 Nitrogenase activity (NA) in (A.) pre-adapted and (B.) non-adapted control and oil-treated Eckernförde Bay (EBAY) sediment slurries. NA is averaged per group ($n = 3$ per group, repeated measure at 5 time points over 10 days). Average values are given in mean \pm SD.

The statistical analysis of NA in pre-adapted EBAY sediment slurries assumed that NA within each group (control and oil-treated sediment slurries) was approximately normally distributed according to the insignificance of the Shapiro-Wilk test (control: $W = 0.96$, $p = 0.64$, oil: $W = 0.99$, $p = 0.78$). The variances were homogenous among groups due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 1.04$, $df = 1$, $p = 0.31$). Group means of NA were compared with a two-tailed independent Student's t-test. The t-test revealed a significant effect of oil treatment on NA ($t(4) = -4.03$, $p = 0.02$, $r = 0.9$).

The statistical analysis of NA in non-adapted EBAY sediment slurries assumed that NA within each group (control and oil-treated sediment slurries) was approximately normally distributed according to the insignificance of the Shapiro-Wilk test (control: $W = 0.99$, $p = 0.92$, oil: $W = 0.99$, $p = 0.94$). The variances were homogenous among groups due to the insignificance of the Fligner-Killeen test (med $\chi^2 = 1.04$, $df = 1$, $p = 0.31$). Group means of NA were compared with a two-tailed independent Student's t-test. The t-test revealed no significant effect of oil treatment on NA ($t(4) = 0.13$, $p = 0.90$, $r = 0.07$).

Ammonium, nitrate and nitrite samples were taken at the end of Experiment 2. However, these measurements are single observations derived from a combined sample per triplicate/group. Interestingly, huge differences in ammonium and nitrate concentrations at the end of Experiment 2 were observed in pre-adapted control and oil-treated sediment slurries (Figure 20 A.1 and A.2). About 140.5 $\mu\text{mol/l}$ ammonium were present in the "pre-adapted" control sediment slurries and about 5.95 $\mu\text{mol/l}$ ammonium were present in the pre-adapted oil-treated sediment slurries (Figure 20 A.1). There was even a higher difference between the nitrate concentrations in the pre-adapted control and oil-treated sediment slurries. About 2450 $\mu\text{mol/l}$ nitrate were present in the "pre-adapted" sediment slurries and about 1.43 $\mu\text{mol/l}$ nitrate were present in the pre-adapted oil-treated sediment slurries (Figure 20 A.2).

In the non-adapted sediment slurries, ammonium concentrations were higher in the control sediment slurries (35.93 $\mu\text{mol/l}$) than in the oil-treated sediment slurries (7.11 $\mu\text{mol/l}$, Figure 20 B.1). Nitrate concentrations did not differ much between control (1.61 $\mu\text{mol/l}$) and oil-treated sediment slurries (1.34 $\mu\text{mol/l}$, Figure 20 B.2).

Nitrite concentrations were below the detection limit of 0.1 $\mu\text{mol/l}$ for all samples in Experiment 2 (data not shown here).

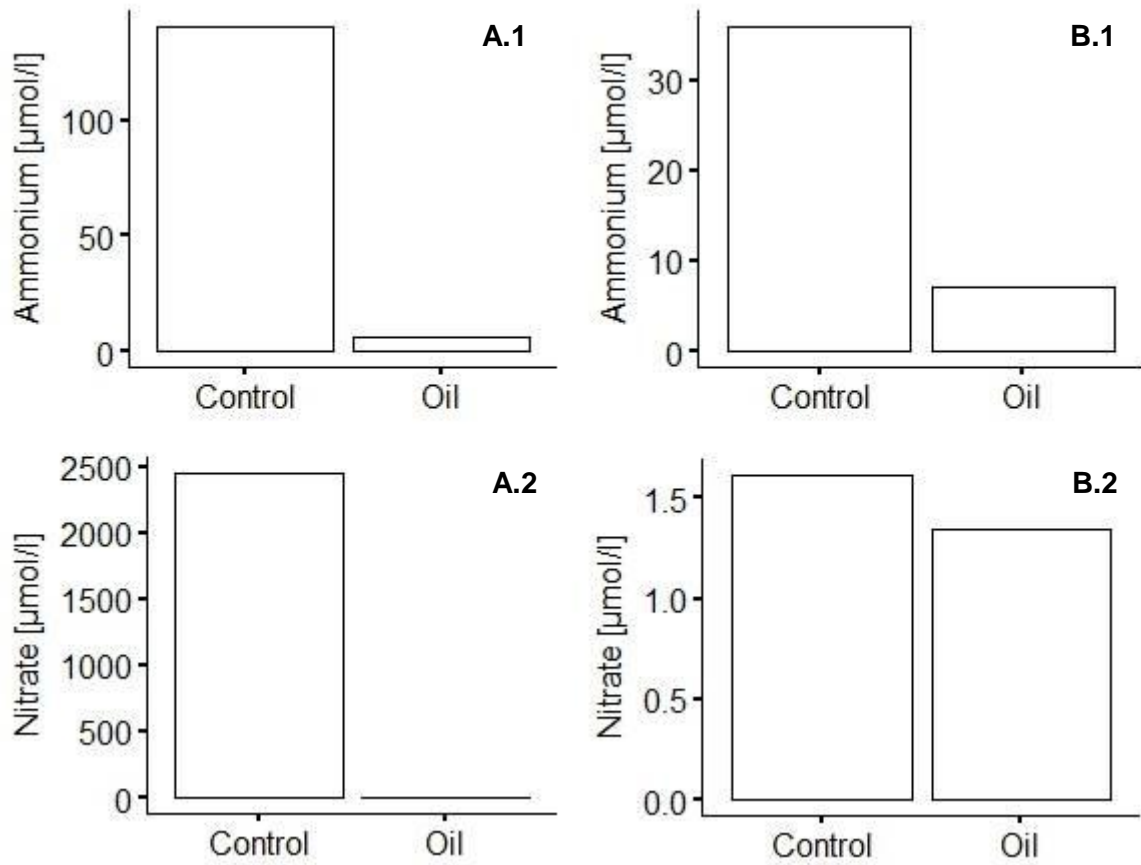


Figure 20 Ammonium (A.1 and B.1) and nitrate (A.2 and B.2) concentrations in aerobic (A.) pre-adapted and (B.) non-adapted control and oil-treated Eckernförde Bay (EBAY) sediment slurries at the end of Experiment 2.

4. DISCUSSION

About 1.3 million tonnes of oil enter the marine environment each year through natural and anthropogenic sources (McGenity et al. 2012). Oil released into the sea is degraded either biologically or abiotically (Harayama et al. 1999). However, biodegradation is thought to be the most important process in the removal of oil from the environment (Head et al. 2006), particularly in the long-term. The major constituents of oil are hydrocarbons. In general, hydrocarbons and their derivatives are ubiquitous in the environment and many microbes have evolved the ability to utilize hydrocarbons (Yakimov et al. 2007). However, the availability of nitrogen and phosphorous can limit the growth and activity of hydrocarbon-degrading microbes, and thus limit oil biodegradation (Harayama et al. 1999). The ability to fix molecular nitrogen (N_2) is highly advantageous since it liberates microbes from the dependence of available fixed nitrogen, such as ammonium, nitrate and nitrite, in their habitat (Madigan et al. 2011).

Microbes can use hydrocarbons either in the presence of oxygen (aerobic) or in the absence of oxygen (anaerobic). However, anaerobic microbes utilizing hydrocarbons always exhibit much slower growth than their aerobic counterparts (Widdel et al. 2010). The aerobic degradation of oil has already been intensely studied (Roger C Prince et al. 2003; Head et al. 2006, and references therein). However, the anaerobic degradation of oil has only been known since about two decades and despite the advances in anaerobic oil degradation research, there are still gaps of knowledge (Widdel et al. 2010). Hydrocarbons can be utilized anaerobically with nitrate, iron(III), or sulfate as electron acceptor. Among anaerobic hydrocarbon-degrading microbes, sulfate-reducing bacteria (SRB) are the main players (Wolicka & Borkowski 2007). Interestingly, SRB are not only involved in the anaerobic degradation of hydrocarbons but many SRB have also the genetic potential to fix N_2 (Zehr et al. 1995). It is therefore possible that SRB could facilitate N_2 fixation in oil-contaminated sediments.

The overall goal of this study was to test whether N_2 fixation is occurring in marine oil-contaminated sediments from Eckernförde Bay (EBAY), Baltic Sea, and North Alex Mud Volcano (NAMV), Mediterranean Sea. It was hypothesized that N_2 fixation is coupled to SRB degrading oil compounds. Furthermore, it was suggested that both sulfate reduction and N_2 fixation are stimulated by oil addition.

As a measure of sulfate reduction, total sulfide (TS) production was determined and as a measure of N₂ fixation, nitrogenase activity (NA) was determined (see Section 2.5.2 for TS Measurement Procedure and Section 2.5.3 for NA Measurement Procedure). The effect of oil treatment on TS production and NA in *anaerobic* EBAY and NAMV sediment slurries was investigated in Experiment 1 (see Section 2.3.4 for Experimental Design). The results of Experiment 1 are discussed in Section 4.2 (see Section 3.2 for Results). The results from the revival process that preceded Experiment 1 are discussed in Section 4.1 (see Section 2.3.2 for Revival Process). The effect of oil treatment on NA in *aerobic* EBAY sediment slurries was investigated in Experiment 2 (see Section 2.4.2 for Experimental Design). The results of Experiment 2 are discussed in Section 4.3 (see Section 3.3 for Results).

The materials and methods used throughout the present study are discussed in Section 4.4. For an outlook regarding future research in the field of microbial oil degradation in marine sediments, especially with regard to the two processes sulfate reduction and N₂ fixation, see Section 4.5.

4.1 Sulfate Reduction in Anaerobic Oil-Contaminated Marine Sediments

The results of the present study suggest that SRB were still alive in the EBAY and NAMV sediment slurries, despite a refrigerated storage period of up to 1.5 years without any carbon source addition. After the addition of new oil to already previously oil-treated sediment slurries from both study sites at the beginning of the revival process, TS concentrations, as a measure of sulfate reduction, increased continuously without any noticeable lag-phase. The absence of a lag-phase after the addition of oil, confirms the previous assumption that microbial communities in EBAY and NAMV sediment slurries were already adapted to oil (see Section 2.3.1 for History of Sediment Slurries). In the previous study, which used the same EBAY sediment slurries as in the present study, a lag-phase about 125 days indicated by increasing TS concentrations was observed after the addition of oil (Laufer 2012). However, the absence of a lag-phase in the present study instead of a shorter lag phase compared to the previous studies, is still striking. According to Widdel et al. 2010, anaerobic hydrocarbon degraders usually grow significantly slower than aerobic hydrocarbon degraders with doubling times ranging from about six hours (in the case of the fastest toluene-degrading denitrifiers) to some months (anaerobic methane-oxidizing consortia reducing sulfate). In the present study, TS concentrations in oil-treated sediment slurries from both

study sites were clearly increasing already during the first 20 days of the revival process (see Section 3.2.1 for Results of Revival Process).

The activity of SRB in oil-treated sediment slurries from both study sites seemed not to be limited throughout the whole present study. TS concentrations, as a measure of sulfate reduction activity, increased continuously within each revival step and during Experiment 1 that followed afterwards. Furthermore, TS production rates in oil-treated sediment slurries from both study sites even increased over the course of the revival process, which lasted about three months. The TS production rates in oil-treated sediment slurries from both study sites increased tremendously during the second revival step compared to the first one. However, in contrast to the second revival step, the TS production rates from oil-treated sediment slurries in the third revival step seemed to be lowered (see Section 3.2.1 for Results of Revival Process). One possible explanation for the lowering might be that the TS measurements in the third revival step were conducted with a 0.22 μm filter, whereas the TS measurements in the first and second revival step were conducted with a 0.45 μm filter (see Section 2.5.2 for TS Measurement Procedure). The use of a filter with a smaller pore size might have caused the impression of a lower TS production rate in the oil-treated sediment slurries during the third revival step compared to the second revival step. However, the impact of different filter sizes on TS measurements according to Cord-Ruwisch should be tested to confirm this hypothesis. Nevertheless, the whole revival process confirmed that SRB in oil-treated sediment slurries from both study sites were alive and active.

On average, TS production rates in oil-treated NAMV sediment slurries were about five times as high as in oil-treated EBAY sediment slurries over the whole revival process. One possible explanation might be that the sediment slurries were prepared with sediment from different sediment depth ranges. EBAY sediment slurries were prepared with sediment ranging from 1 to 10 cm sediment depth, whereas NAMV sediment slurries were prepared with sediment ranging from 5 to 10 cm sediment depth (see Section 2.3.1 for Preparation of Sediment Slurries). Another possible explanation might be that the microbial community in NAMV sediment had already been exposed to hydrocarbons in their natural setting (see Section 2.1.2 for Study Site Description). However, hydrocarbon-degrading microbes are ubiquitous in the environment (Yakimov et al. 2007). For EBAY sediment slurries, Laufer 2012 showed that hydrocarbon-degrading microbes were also present at this study site, although the study site was assumed to be pristine in terms of oil-contamination. Nevertheless, the original diversity of hydrocarbon-degrading microbes may have been different between the two study sites. A decrease in diversity was found to have a strong impact on pollutant degradation in polycyclic aromatic hydrocarbon contaminated sludge (Cravo-Laureau et al. 2011). For the present study, it could be argued that the diversity of

hydrocarbon-degrading microbes in natural NAMV sediment was higher than in natural EBAY sediment. However, this hypothesis remains to be tested.

In contrast to the oil-treated sediment slurries in the present study, the control sediment slurries from both study sites had not received any new carbon source since the sediment has been sampled (see Section 2.3.1 for Sediment Sampling). It was therefore uncertain, if the microbial communities in the control sediment slurries from both study sites were still alive after up to 1.5 years of refrigerated storage without the addition of any new carbon source. It was hypothesized that SRB in control sediment slurries were still alive but that their activity was limited by the lack of a suitable carbon source or a carbon source at all. This hypothesis was confirmed during Experiment 1. After the addition of acetate solution at day 7 of Experiment 1, an increase in TS concentrations was observed in the control sediment slurries from both study sites (hereafter called acetate treated sediment slurries). Consequently, also SRB in control sediment slurries from both study sites were still alive after up to 1.5 years of refrigerated storage without the addition of any new carbon source. However, this should not come as a surprise since theoretical mean generation times calculated for deeply buried microbes in the seabed range from years to thousands of years, challenging the current understanding of the minimum energy requirements for life (Jørgensen & Boetius 2007 and references therein).

4.2 Sulfate reduction and Nitrogen Fixation in Anaerobic Oil-Contaminated Marine Sediments

Eckernförde Bay (EBAY), Baltic Sea

As mentioned before, the EBAY sediment slurries used for Experiment 1 in the present study have already been used previously in another study (see Section 2.3.1 for History of Sediment Slurries). In the previous study (Laufer 2012), one triplicate of EBAY sediment slurries was treated with 0.57 % oil, whereas another triplicate of sediment slurries was left untreated, serving as a control. Both triplicates were incubated at 13°C, in the dark, as in the present study. TS concentrations were measured during incubation in the same manner as in the present study (see Section 2.5.2 for TS Measurement Procedure). The incubation lasted for about 180 days. TS concentrations in oil-treated sediment slurries remained constant over the first 126 days of incubation (0.34 ± 0.15 mM). Afterwards, TS concentrations in oil-treated sediment slurries started to rise, reaching 8.63 ± 1.91 mM after 180 days. TS

concentrations in control sediment slurries remained constant during the whole incubation (0.25 ± 0.15 mM). The author concluded that increasing TS concentrations in oil-treated sediment slurries were due to oil degradation by SRB since no significant increase in TS concentrations in the absence of oil was measured in the control sediment slurries. Furthermore, the author concluded that the relatively long lag-phase in oil-treated sediment slurries indicated that the microbial community in EBAY sediments has not been originally adapted to the degradation of oil compounds before the experimental oil contamination in the study. That EBAY sediment slurries used in the present study were adapted to oil was already confirmed during the revival process preceding Experiment 1 (see Section 4.1).

TS concentrations in oil-treated EBAY sediment slurries measured in the present study (see Section 3.2.2 for Results of EBAY Sediment Slurries in Experiment 1) were much higher than TS concentrations measured in oil-treated sediment slurries by Laufer 2012. Even when taking the different dilution factors and the different amount of sediment used for the preparation of sediment slurries in both studies into account, TS concentrations in oil-treated sediment slurries measured in the present study were still higher. It could be argued that either the number or the activity of SRB in oil-treated sediment slurries differed between the two studies.

The NA measured in control EBAY sediment slurries in the present study (0.91 ± 0.22 nmol $\text{C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$), falls in the range of NA measured by Bertics et al. 2013. The authors determined NA ranging from about 0.5 to 3 nmol $\text{C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ for the top 10 cm of sediment throughout the year of the study (Figure 21). In the present study, also the top 10 cm of sediment were used for the preparation of sediment slurries (see Section 2.3.1). The similarity of NA in EBAY sediment slurries from both studies suggests that the NA in control sediment slurries was not dramatically altered by the refrigerated storage period, which lasted for about 1.5 years prior to the present study.

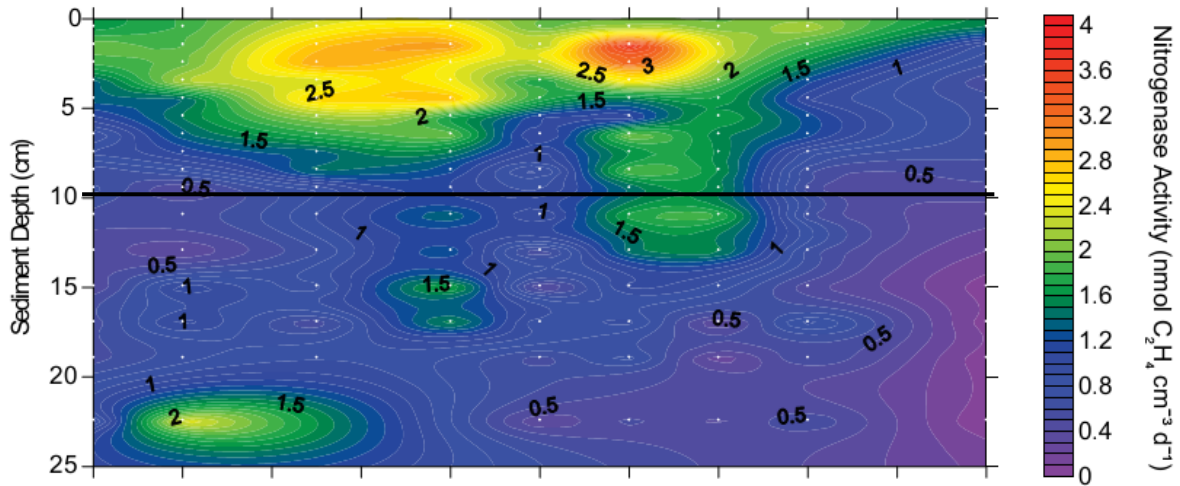


Figure 21 Contour plot of nitrogenase activity (NA) in Eckernförde Bay (EBAY) sediment over the course of the study, from April 2010 (left) to February 2011 (right). The white dots represent data collection points (Bertics et al. 2013).

The results of the present study revealed a significant effect of oil treatment on TS production rates, as a measure of sulfate reduction, as well as on NA, as a measure of nitrogen fixation, in EBAY sediment slurries. Oil treatment had a *positive* effect on TS production rates, as hypothesized. However, oil treatment had a *negative* effect on NA, which was not expected. Consequently, sulfate reduction and nitrogen fixation seem not to be correlated in EBAY sediment slurries, i.e. the two processes may not be carried out by the same group of microbes.

The observation that sulfate reduction and nitrogen fixation seem not to be correlated in EBAY sediment slurries contradicts the findings of another study, which also focused on sulfate reduction and nitrogen fixation in EBAY sediment (Bertics et al. 2013). In this study, monthly samplings were done over the course of one year to assess N_2 fixation, its seasonal variability and its potential coupling to sulfate reduction in EBAY sediment. Similarly to the present study, NA was determined as a measure of N_2 fixation (see Section 2.5.3 for NA Measurement Procedure). The authors found that NA closely mirrored sulfate reduction rates monthly and with sediment depth. Both processes were highest in the top 10 cm of the sediment throughout the year. The authors therefore suggested a coupling of N_2 fixation and sulfate reduction in EBAY sediment. However, N_2 fixation and sulfate reduction in EBAY sediment slurries did not seem to be coupled in the present study, when incubated with oil. It could be argued that SRB with the capability to fix N_2 present in EBAY sediment are not significantly involved in oil degradation.

Bertics et al. further demonstrated via molecular analysis that the presence of *nifH* gene sequences (encoding for one subunit of the enzyme nitrogenase catalyzing N_2 fixation), is related to two known N_2 fixing SRB, namely *Desulfovibrio vulgaris* and *Desulfonema limicola*. These findings support the hypothesis that some of the NA may be attributed to SRB in EBAY sediment. Interestingly, several members of the *Desulfovibrio spp.* group have also been isolated from oil-contaminated habitats (Magot 2005; Birkeland 2005). Thus members of the *Desulfovibrio spp.* may be able to couple sulfate reduction to hydrocarbon degradation, while being capable of fixing N_2 . Due to time constraints, no molecular analysis of EBAY sediment was conducted during the present study. Thus the presence of members of the *Desulfovibrio spp.* cannot be confirmed in EBAY sediment, when incubated with oil.

However, Musat et al. 2006 were able to amplify sequences related to the *nifH* gene from Deltaproteobacteria from oil-contaminated North Sea sediment. Nevertheless, the authors concluded that the contribution of SRB to N_2 fixation was marginal in oil-contaminated sediments incubated permanently under anoxic conditions since only very low NA was measured. The observation of low NA in oil-contaminated sediment is consistent with the results of the present study.

However, that oil treatment had a significant *positive* effect on TS production rates but a significant *negative* effect on NA in EBAY sediment slurries in the present study, might also be explained by the experimental design and methods used. Firstly, the experimental design is not truly multivariate. TS and NA measurements were determined from different microcosms (see Section 2.3.4 for Experimental Design). While TS measurements were determined from sediment slurries in 15 ml hungate tubes, NA measurements were determined from sediment slurries in 60 ml serum bottles. Due to the limited availability of sample material, the supernatant needed for the photometric measurements of TS could not be obtained from the small amount of sediment slurries in 60 ml serum bottles. Thus a potential correlation between the two dependent variables, TS production rates and NA, cannot be statistically analyzed.

Secondly, there are numerous problems and limitations with the application of the C_2H_2 reduction assay used to determine the NA (see Section 2.5.3 for NA Measurement Procedure). For instance, in replacing the natural substrate, C_2H_2 inhibits N_2 fixation itself and may therefore disrupt the nitrogen metabolism (Capone 1993). Furthermore, C_2H_2 itself can be metabolized anaerobically as a growth substrate (Capone 1993). C_2H_2 might have also interfered with the metabolic activities under investigation, namely sulfate reduction and N_2 fixation. It has been argued that the C_2H_2 reduction assay is invalid for alkane-utilizing, N_2 fixing bacteria (Bont & Mulder 1976). The authors found that the supply of energy and reducing power of the enzyme nitrogenase, needed for the reduction of C_2H_2 , was impeded.

Furthermore, the enzyme system involved in the attack on hydrocarbons, called alkane hydroxylase, was inhibited by C_2H_2 . However, the inhibition of alkane hydroxylase by C_2H_2 seemed to depend on the size of the substrate molecule, since growth upon long-chain hydrocarbons was not inhibited by C_2H_2 . Thus it is suggested not to apply the C_2H_2 reduction assay for measuring the NA of bacteria utilizing short-chain alkanes from methane to butane, when alkanes are the sole energy source.

In the present study, light oil was used, which is usually high in alkanes (Head et al. 2006). However, the provider of the oil, RWE Dea AG, declared the oil as 'dead', i.e. the oil did not contain any volatile compounds, such as gaseous short-chain alkanes. Consequently, gaseous, short-chain alkanes were not available in *dead* oil-treated sediment slurries in the present study. Thus it is unlikely that possible NA of alkane degraders has been overlooked due to their inhibition by C_2H_2 .

Nitrogen often becomes limiting in oil-contaminated environments (Head & Swannell 1999). In the present study, ammonium concentrations decreased over the course of Experiment 1 in control and oil-treated EBAY sediment slurries. However, ammonium concentrations decreased more in oil-treated sediment slurries. One possible explanation for this might be a higher consumption of ammonium in oil-treated sediment slurries compared to control sediment slurries. Another possible explanation might be a lower N_2 fixation in oil-treated sediment slurries compared to control sediment slurries resulting in less ammonium produced to compensate the consumption. Surprisingly, nitrate concentrations increased in both control and oil-treated sediment slurries, although nitrification is usually carried out under aerobic conditions. In nature, ammonium is usually preferred over nitrate because it has already the same oxidation state of nitrogen than in amino acids and other nitrogenous biochemicals in the cell (Kirchman 2012b). Because of this preference, ammonium uptake rates are often faster than nitrate uptake rates, even when nitrate uptake rates are higher. Nitrate uptake exceeds ammonium uptake only when ammonium concentrations are substantially lower than nitrate concentrations.

North Alex Mud Volcano (NAMV), Mediterranean Sea

As mentioned before, the NAMV sediment slurries used in the present study have already been used previously in another study (see Section 2.3.1 for History of Sediment Slurries). In the previous study (Sonakshi Mishra, unpublished data), one triplicate of NAMV sediment slurries was treated with 1 % oil, while another triplicate of sediment slurries was left untreated as a control. The sediment slurries were incubated at 20°C, in the dark for about 110 days. TS concentrations were measured regularly during incubation in the same manner

as in the present study (see Section 2.5.2 for TS Measurement Procedure). TS concentrations in oil-treated sediment slurries reached about 15 mM at the end of incubation (Figure 22).

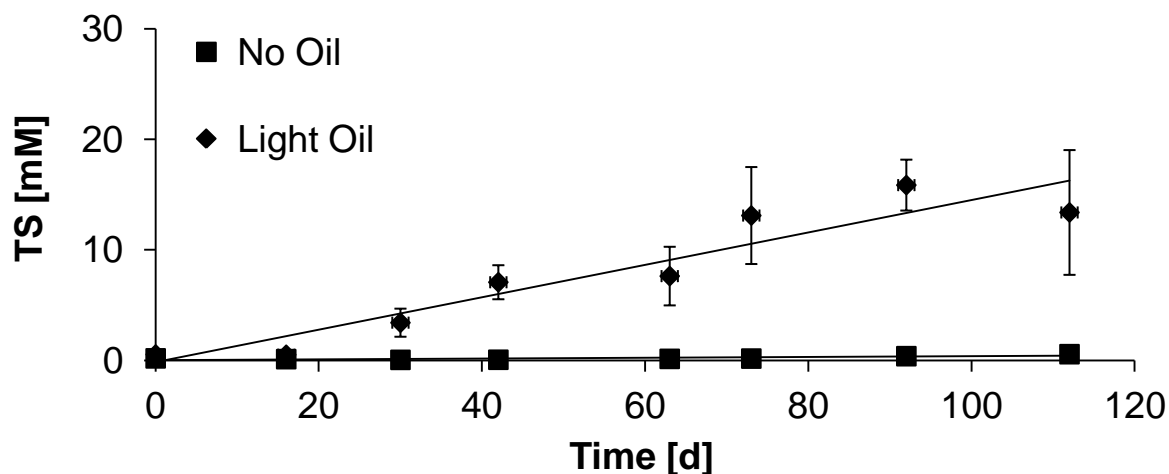


Figure 22 Development of total sulfide (TS) concentrations in control and oil-treated sediment slurries from North Alex Mud Volcano (NAMV) over time (Sonakshi Mishra, unpublished data).

In the present study, TS concentrations up to 24 mM were reached in the oil-treated NAMV sediment slurries at the end of Experiment 1, although the sediment slurries have been incubated at 13°C compared to 20°C in the previous study. It could be argued that either the number or the activity SRB increased between these two studies. Unfortunately, no TS production rates, as a measure of sulfate reduction activity, were calculated in the other study. It would have been interesting to see, if TS production rates had increased in the oil-treated sediment slurries after the one year storage period followed by a new oil input.

A molecular analysis of the *masD* gene, coding for a hydrocarbon-degrading glycy radical enzyme, was performed on control and oil-treated NAMV sediment samples (Marion Stagars, unpublished data). The analysis included nucleic acid extraction from sediment samples, *masD* amplification and clone library construction, followed by sequence analysis. The operational taxonomic units (OTU) were cut-off with a 96 % sequence similarity. The calculated phylogenetic tree shows three groups of NAMV *masD* OTUs_{0.96} (see Appendix A.5). The *masD* OTUs which are of interest for the present study are described in the following.

Interestingly, one *masD* cluster contained OTUs_{0.96} from both control and (light) oil-treated NAMV sediment samples (OTU282, OTU430, OTU327 and OTU314). The OTUs_{0.96} were most similar to each other with the deltaproteobacterial *Desulfatibacillum alkenivorans* strain AK-01 as closest cultured relative. Another *masD* cluster comprised only one OTUs_{0.96}

retrieved from (light) oil-treated NAMV sediment slurries (OTU431), which was closest affiliated with a clone sequence derived from Gowanus canal, a petroleum contaminated aquifer (GU453648).

The molecular analysis of *masD* gene sequences revealed that hydrocarbon-degrading microbes are present in both control and oil-treated NAMV sediment slurries. The presence of hydrocarbon-degrading microbes, particularly in control sediment slurries, confirms the ubiquity of hydrocarbon-degrading microbes in the environment (Yakimov et al. 2007). To test, if a higher number of hydrocarbon-degrading microbes is present in oil-treated sediment slurries compared to control sediment slurries, a quantitative polymerase chain reaction could be done.

Desulfatibacillum alkenivorans strain AK-01 (hereafter referred to as AK-01), the closest cultured relative of OTUs_{0.96} in both control and oil-treated NAMV sediment slurries, was originally isolated from petroleum contaminated sediment collected from the Arthur Kill, an intertidal waterway between Staten Island and New Jersey (So & Young 1999). Its most significant trait is its ability to grow on *n*-alkanes (C₁₃ to C₁₈) and alkenes (1-pentadecene and 1-hexadecene) under sulfate-reducing conditions. Isotope studies using ¹⁴C-labelled hexadecane have demonstrated that AK-01 completely oxidizes *n*-alkanes to CO₂. Another striking feature that makes AK-01 one of the most versatile SRB sequenced to date is its ability to grow chemolithoautotrophically with H₂, CO₂ and sulfate. While growing heterotrophically, AK-01 can utilize sulfate, sulfite and thiosulfate, but not sulfur, nitrate or nitrite, as electron acceptors. AK-01 is known as a model organism for anaerobic alkane biodegradation, coupling the oxidation of alkanes to sulfate reduction (Callaghan et al. 2012). The detection of AK-01 in NAMV sediment slurries in the present study supports the assumption that the increase in TS concentrations in oil-treated sediment slurries is due to the degradation of oil compounds, such as long-chain alkanes.

The results of the present study revealed a significant *positive* effect of oil treatment on TS production rates, as a measure of sulfate reduction, in NAMV sediment slurries, as hypothesized. However, oil treatment had no significant effect on NA, as a measure of N₂ fixation, in NAMV sediment slurries, which was not expected. Control, acetate treated control and oil-treated NAMV sediment slurries showed no or only very low NA (see Section 3.2.3 for Results). One possible explanation for the low NA might be the sediment depth range used for the preparation of sediment slurries (see Section 2.3.1). NAMV sediment slurries were prepared with sediment from 5 to 10 cm depth. However, NA is usually highest in the top few centimeters of the sediment (Bertics et al. 2013), which were excluded in the present study. Another possible explanation might be that although N₂ fixing microbes might have been present in NAMV sediment slurries, N₂ fixation was just not performed during the 14 days of

Experiment 1 in the present study. Since the process of N₂ fixation is highly energy demanding, the activity of the enzyme nitrogenase is highly regulated (Madigan et al. 2011). N₂ fixation can be inhibited by fixed forms of nitrogen, such as ammonia and nitrate, which were present in the artificial seawater medium used to prepare the sediment slurries in the present study (see Section 2.5.1 for Preparation of Medium). It is therefore uncertain if sulfate reduction and N₂ fixation in oil-contaminated NAMV sediment slurries are correlated or not.

In control NAMV sediment slurries, even a slightly negative NA was measured in the present study (-0.05 ± 0.06 nmol C₂H₄ cm⁻³ d⁻¹). Possible reasons for this negative NA might be that C₂H₄ was lost from microcosms or that C₂H₄ itself was consumed within the microcosms over the course of the Experiment 1 (Capone 1993).

Ammonium concentrations decreased dramatically in control and oil-treated sediment slurries over the course of Experiment 2 in the present study. Nitrate and nitrite concentrations decreased as well in the control sediment slurries. The consumption of fixed nitrogen species (ammonium, nitrate and nitrite) is in accordance with the observation that no or only very low NA in control, acetate treated control and oil-treated NAMV sediment slurries could be observed, while relatively high TS production rates were measured in oil-treated NAMV sediment slurries.

However, that oil treatment had no significant effect on NA is in accordance with findings by Musat et al. 2006. The authors found no indication of a significant stimulation by oil of NA in North Sea sediment, when incubated in the dark. Interestingly, N₂ fixation and *nifH* gene sequences have been observed in deep methane seep sediments, resembling the study site NAMV (Dang et al. 2009; Dekas et al. 2013). In the study by Dekas et al., it was observed that N₂ fixation was methane-dependent and that N₂ fixation rates peak in a narrow sediment depth horizon corresponding to increased abundance of aggregates of anaerobic methanotrophic archaea (ANME-2) and SRB. Furthermore, the authors suggested that ANME-2, and possibly physically associated SRB, mediate the majority of new nitrogen production within the seep ecosystem. ANME-2 N₂ fixation was observed while in association with members of two distinct orders of SRB: *Desulfobacteraceae* and *Desulfobulbaceae*. However, the present study focused only on SRB. Sediment slurries were prepared with an artificial seawater medium, especially designed for the needs of SRB (see Section 2.5.1 for Preparation of Medium). Furthermore, light *dead* oil was used in the present study, that did not contain methane according to the provider RWE Dea AG. Consequently, methanotrophic archaea were excluded from the microcosms in the present study. However, laboratory studies have shown that archaea do not play an important role in hydrocarbon degradation on contaminated beaches (Röling et al. 2004).

Summary

Oil treatment had a significant *positive* effect on TS production rates in anaerobic EBAY and NAMV sediment slurries, confirming the hypothesis that SRB are involved in oil degradation in sediment from both study sites. However, oil treatment had a significant *negative* effect on NA in anaerobic EBAY sediment slurries, which was not expected. Consequently, sulfate reduction and N₂ fixation seem not to be correlated in oil-treated EBAY sediment slurries, i.e. the two processes may not be carried out by the same group of microbes. Furthermore, oil treatment had no significant effect on NA in anaerobic NAMV sediment slurries, which was not expected. Control, acetate treated control and oil-treated NAMV sediment slurries showed no or only very low NA. It is therefore uncertain if sulfate reduction and N₂ fixation in oil-contaminated NAMV sediment slurries are correlated or not.

The molecular analysis of *masD* gene sequences revealed that hydrocarbon-degrading microbes are present in both control and oil-treated NAMV sediment slurries. *Desulfatibacillum alkenivorans* strain AK-01, the closest cultured relative of OTU_{S0.96} in both control and oil-treated NAMV sediment slurries is known as a model organism for anaerobic alkane biodegradation coupled to sulfate reduction. The presence of *Desulfatibacillum alkenivorans* in NAMV sediment slurries supports the assumption that the increase in TS concentrations in oil-treated sediment slurries is due to the degradation of oil compounds, such as long-chain alkanes.

As Musat et al. 2006 put it, one may hypothesize that the co-occurrence of hydrocarbon oxidation and nitrogen fixation is rare or physiologically delicate. Further research is needed to understand whether there is a physiological incompatibility between active hydrocarbon degradation and nitrogen fixation.

4.3 Nitrogen Fixation in Aerobic Oil-Contaminated Marine Sediment

Eckernförde Bay (EBAY), Baltic Sea

In Experiment 2, the effect of oil treatment on NA in aerobic EBAY sediment slurries was investigated. One set of sediment slurries have been incubated with oil for about three months prior to the start of Experiment 2 (hereafter called pre-adapted sediment slurries), whereas another set of sediment slurries has been incubated with oil only for the duration of

the Experiment 2 (hereafter called non-adapted sediment slurries). Experiment 2 lasted for 10 days (see Section 2.4.2 for Experimental Design).

The results revealed that oil treatment had a significant *positive* effect on NA in pre-adapted sediment slurries, suggesting that oil degradation and N₂ fixation may be coupled or at least that N₂ fixation is not impeded by oil addition in aerobic EBAY sediment slurries. However, oil treatment had no significant effect on NA in non-adapted sediment slurries. This indicates a lag-phase of at least 10 days after an oil input to aerobic EBAY sediment slurries, which had not been previously adapted to oil.

By far, the highest ammonium and nitrate concentrations at the end of Experiment 2 were observed in the "pre-adapted" control sediment slurries, which showed the lowest NA. One possible explanation for this observation might be the inhibition of N₂ fixation in the presence of high concentrations of fixed nitrogen, such as ammonium and nitrate (Knapp 2012). In contrast, low ammonium and nitrate concentrations were found in pre-adapted oil-treated sediment slurries at the end of Experiment 2, suggesting an almost complete consumption of available fixed nitrogen over the course of 10 days. Accordingly, NA did not seem to be inhibited in oil-treated pre-adapted sediment slurries and a higher NA was observed. Since low ammonium and nitrate concentrations were measured at the end of Experiment 2, it is assumed that fixed nitrogen was quickly consumed again.

Interestingly, Musat et al. 2006 found that NA in oil-contaminated North Sea sediment was lower than NA in pristine sediment during the initial three weeks of incubation, suggesting that the development of certain organisms, such as N₂ fixing microbes, was initially impeded by oil. The authors indicated no significant stimulation of NA by oil in permanently dark microcosms, namely NA associated with aerobic heterotrophs (Musat et al. 2006). Even though sequences related to *nifH* from Betaproteobacteria and Gammaproteobacteria (possible hydrocarbon-degrading bacteria) were directly amplified from oil-contaminated sediment, such sequences were not retrieved via Reverse Transcription Polymerase Chain Reaction, indicating that the corresponding organisms were not or not significantly involved in N₂ fixation. The authors concluded that cyanobacteria contributed most of the fixed nitrogen to oil-contaminated sediment, when nitrogen was a limiting factor and when light was available (Musat et al. 2006). However, in the present study also dark, heterotrophic N₂ fixation could be observed in oil-contaminated EBAY sediment slurries, suggesting that oil degradation and N₂ fixation may be coupled or at least that N₂ fixation is not impeded by oil addition in aerobic EBAY sediment slurries.

4.4 Materials and Methods

Although revealing, microcosm studies are always reductionist and artificial, as they lack the complexity, diversity and dynamics of natural environments (Yakimov et al. 2007). Several issues should be considered, when extrapolating results from the present study to *in situ* conditions. These issues include the experimental design as well as the methods used for the two main dependent variables, TS production and NA, which have already been discussed above. Furthermore, the materials used (sediment, medium, oil) in the present study and the lack of environmental fluctuations as well as community dynamics should be considered. These issues are outlined in the following.

Sediment Slurries. The results of the present study revealed a significant effect of dilution on NA measurements via the C_2H_2 reduction assay (see Section 3.1 for Results of the Preliminary Experiment). Thus it matters, if a sediment sample is diluted or not. Undiluted sediment samples showed a significantly lower NA than three differently diluted sediment slurries (dilution factor 2, 3 and 4). For the preparation of sediment slurries, artificial seawater medium was used, which contained an optimal amount of nutrients, vitamins and trace elements (see Section for Preparation of Medium), which might have caused an increase in NA. Furthermore, in slurried sediment samples the flux of C_2H_2 to sites of N_2 fixation and the flux of C_2H_4 from sites of N_2 fixation is increased, which might have also caused an increase in NA (Capone 1993). Determining NA from sediment slurries thus leads potentially to an overestimation of NA compared to undiluted sediment samples. However, in Experiment 1 and 2 in the present study sediment slurries were used. NA in undiluted sediments from both study sites are likely to be lower than measured.

Sediment. The sediment samples used throughout the present study were of natural origin (see Sections 2.2.1 and 2.3.1 for Sampling of Sediment). However, the sediment samples used in Experiment 2 had a certain lab history due to their use in previous studies prior to the present study (see Section 2.3.1 for History of Sediment Samples). Furthermore, control and oil-treated sediment slurries had been separated quite a long time ago.

Artificial Seawater Medium. For the preparation of sediment slurries in Experiment 1, a selective artificial seawater medium for SRB was used. The medium contained all minerals, trace elements and vitamins that may be required by SRB (Widdel & Bak 1992). Thus SRB were favored. No medium is well suited for all species.

Choice of Carbon Source. In the present study, oil was the only carbon source provided, except for the acetate treated control sediment slurries. However, in nature typically a wide range of organic material is available. Bacteria usually possess global regulation systems to assure a hierarchical assimilation of the individual carbon sources, thus favoring the use of some compounds over other non-preferred substrates (Rojo 2009). Hydrocarbons are usually non-preferred growth substrates.

Oil Type. If oil treatment was applied, light oil was used throughout the whole present study. Light oils are usually high in saturated and aromatic hydrocarbons, with a smaller proportion of resins and asphaltenes (Head et al. 2006). Whereas heavy oils have a much lower content of saturated and aromatic hydrocarbons and a higher proportion of resins and asphaltenes (Head et al. 2006). Heavy oils typically result from the biodegradation of crude oil under anoxic conditions *in situ* in petroleum reservoirs (Head et al. 2006). In another study (Laufer 2012), sulfate reduction rates in sediment slurries treated with light oil were about twice as high as in sediment slurries treated with heavy oil. However, sulfate reduction rates became similar after about 200 days of incubation.

Oil Concentration. If oil treatment was applied, a concentration of 1 % light oil was used throughout the present study. In another study (Laufer 2012), the concentration of crude oil added, had no influence on the duration of the lag-phase, if light oil was added to EBAY sediment. However, the author found a concentration of crude oil at which sulfate reduction rates were highest. Among the tested crude oil concentrations, highest sulfate reduction rates in oil-treated EBAY sediment slurries were observed at a concentration of 0.6 %.

Weathering of Oil. Weathering includes the combined effects of physical, chemical and biological modifications (see Section 1.2 for Fate of Crude Oil in Ocean including Weathering). Weathering not only makes oil more available for microbial degradation, it also alters the composition of oil by affecting some oil compounds more than others. In the present study, 'dead' oil was used, which did not contain any volatile compounds, so that the composition of the oil used might be similar to weathered oil, that has lost its volatile compounds through evaporation. In the case of an oil spill at sea, oil is weathered at the sea surface and within the water column before it reaches the seafloor sediments. Besides the physical and chemical modifications that go along with weathering, this means that oil is firstly degraded aerobically and then secondly anaerobically. However, in the case of an oil seep at the seafloor, oil is firstly degraded anaerobically and then secondly aerobically. Furthermore, oil seeps usually come along with high amounts of gases such as methane, which was not present in the present study. However, another study found that aerobic pre-

digestion of crude oil does not increase potential sulfate reduction rates in sediment slurries (Laufer 2012).

Environmental Fluctuations. The experiments in the present study were restricted to strictly anoxic (Preliminary Experiment, Experiment 1) or oxic (Experiment 2) conditions. However, oxic-anoxic oscillations are widespread in nature, for instance at sediment-water interfaces (Cravo-Laureau et al. 2011). Little is known about microbial oil degradation within the transitional zone from oxic to anoxic conditions. Cravo-Laureau et al. 2011 investigated the impact of environmental fluctuations on pollutant degradation and microbial community structure. The authors demonstrated that redox oscillations stimulated bacterial metabolism by the reworking of available nutrients in sediments, leading to enhanced degradation of organic matter and a decrease in its storage. Thus extrapolations from the present study to *in situ* conditions at sediment-water interfaces should be considered carefully.

Community Dynamics. Individual populations of microbes do not function alone in nature (McGenity et al. 2012). Furthermore, in sediments cells are much more concentrated, resulting in a greater likelihood of interactions. In Experiment 1 in the present study, SRB were favored through the use of a selective medium and the addition of oil as only carbon source. The potential exclusion of other community members might have altered the overall microbial degradation of oil. For instance, the addition of *Alcanivorax borkumensis* to seawater microcosms containing crude oil increased PAH-degradation rates despite the fact that *A. borkumensis* does not mineralise PAHs (McKew et al. 2007). However, *A. borkumensis* is known to produce biosurfactants (biological surface-active agents that have both hydrophilic and hydrophobic moieties), which enhance the uptake of alkanes.

Time. Although the sediment slurries in Experiment 1 in the present study, have been incubated with oil for some time prior to the start of the experiment, TS production and NA were only monitored over 14 days. Long-term studies should be considered, particularly when dealing with microbial oil degradation under anaerobic conditions.

4.5 Outlook

Future studies investigating sulfate reduction and N₂ fixation in marine oil-contaminated sediments, should chose a truly multivariate experimental design to allow the statistical analysis of a potential correlation between sulfate reduction and N₂ fixation, in the presence of oil. Also direct measures for sulfate reduction and N₂ fixation should be considered to avoid the problems and limitations of the indirect measures used in the present study. Radiotracer incubations with ³⁵SO₄²⁻ and ¹⁵N₂ respectively could be used instead (Jørgensen 1978; Kallmeyer et al. 2004). Furthermore, molecular analysis of *masD* and *nifH* gene sequences (coding for key enzymes involved in alkane degradation and N₂ fixation respectively) should be conducted from oil-contaminated sediment samples to see, if the genetic basis for both processes are present in one group of microbes, despite the oil-contamination. When EBAY sediment samples are used, *Desulfovibrio spp.* is a potential candidate to combine those characteristics. The presence of *Desulfovibrio spp.* in oil-contaminated sediment could be also confirmed by Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (Amann et al. 1990; Pernthaler et al. 2002; Ishii et al. 2004).

Furthermore, it would be interesting to see what oil compounds are actually used by SRB changing the composition of oil over time. Hydrocarbon analysis could be done by gas-chromatography coupled to a mass spectrometer to observe changes in oil composition during biodegradation. If possible, fresh sediment samples and natural seawater should be used for preparation of sediment slurries to avoid the potential exclusion of other members of the microbial degradation network, besides the primary oil degraders, such as SRB. Particularly, when studying microbial oil degradation at sediment-water interfaces, oxic-anoxic oscillations should be taken into account. If time allows, long-term experiments should be conducted, when investigating anaerobic microbial oil degradation due to the typically relatively slow growth rates of anaerobic microbes.

5. CONCLUSIONS

The overall goal of this study was to determine whether dark, heterotrophic nitrogen (N_2) fixation is occurring in marine oil-contaminated sediments from Eckernförde Bay (EBAY), Baltic Sea, and North Alex Mud Volcano (NAMV), Mediterranean Sea. It was hypothesized that N_2 fixation is coupled to sulfate-reducing bacteria (SRB) that degrade oil compounds. Furthermore, it was suggested that both sulfate reduction and N_2 fixation are stimulated by oil addition. As a measure of sulfate reduction, total sulfide (TS) production was determined, and as a measure of N_2 fixation, nitrogenase activity (NA) was determined. The results of the present study allow the following conclusions:

Dilution of anaerobic EBAY sediment samples had a significant *positive* effect on NA, when measured via the acetylene reduction assay (Preliminary Experiment). Determining NA from sediment slurries thus leads potentially to an overestimation of NA compared to undiluted sediment samples.

SRB were still active in anaerobic EBAY and NAMV sediment slurries despite a refrigerated storage period of up to 1.5 years without any carbon source addition (Revival Process prior to Experiment 1). SRB in anaerobic EBAY and NAMV control sediment slurries were limited by the lack of a suitable carbon source prior to the addition of acetate at day 7 of Experiment 1. SRB in anaerobic oil-treated EBAY and NAMV sediment slurries were able to degrade light crude oil after being experimentally adapted to oil.

Oil treatment had a significant *positive* effect on TS production rates in anaerobic EBAY and NAMV sediment slurries (Experiment 1), confirming the hypothesis that SRB are involved in oil degradation. However, oil treatment had a significant *negative* effect on NA in anaerobic EBAY sediment slurries. Oil treatment had no significant effect on NA in anaerobic NAMV sediment slurries. Consequently, sulfate reduction and N_2 fixation seem not to be correlated in oil-treated EBAY sediment slurries. Furthermore, oil treatment had no significant effect on NA in anaerobic NAMV sediment slurries, which was not expected. Control, acetate treated control and oil-treated NAMV sediment slurries showed no or only very low NA. It is therefore uncertain, if sulfate reduction and N_2 fixation in oil-contaminated NAMV sediment slurries are correlated or not.

Oil treatment had no significant effect on NA in aerobic EBAY sediment slurries, when incubated with oil for 10 days (Experiment 2). However, oil treatment had a *positive* significant effect on NA in aerobic EBAY sediment slurries, after an incubation with oil for about three months prior to the start of the experiment, suggesting that oil degradation and

N₂ fixation may be coupled or at least that N₂ fixation is not impeded by oil addition in aerobic EBAY sediment slurries.

It could be hypothesized that the co-occurrence of hydrocarbon oxidation and N₂ fixation is rare or physiologically delicate. Further research is needed to understand, whether there is an incompatibility between hydrocarbon degradation and N₂ fixation.

Nevertheless, the present study is one of few focusing on anaerobic oil degradation by marine benthic SRB and dark, heterotrophic N₂ fixation in the presence of oil. More than 90 % of the seafloor lies in water depths with light intensities that do not allow photosynthetic activity. Furthermore, oxygen is usually completely consumed within the upper millimeters, centimeters or the upper meter of marine sediments. Anoxic sediments, where sulfate is one of the main electron acceptors, are typically found in upwelling areas and oxygen-minimum zones that are present at continental margins, as well as at hydrothermal vents and cold seeps. Massive amounts of oil enter the marine environment each year from natural and anthropogenic sources. Thus the findings of the present study could also be relevant for other environments, beside the two study sites investigated.

APPENDIX

A.1 Preparation of Solutions for Artificial Seawater Medium (Widdel & Bak 1992)

1. 6-Vitamine-Solution (sterile filtered)

In 20 mM NaP-buffer (pH 7). For 100 ml use 0.356 g Na_2HPO_4 and 0.270 g NaH_2PO_4 . Control pH. Dissolve, filter sterile and put into autoclaved bottle:

4-aminobenzoate	4 mg
d-biotin	1 mg
nicotinic acid	10 mg
calcium-d-pantothenate	5 mg
pyridoxamine dihydrochloride	15 mg
lipoic acid	1.5 mg
folic acid (non-essential)	4 mg

2. Thiamine (B_1) (sterile filtered)

In 25 mM NaP-buffer (pH 3.4 to 3.7). For 100 ml use 0.345 g NaH_2PO_4 and control pH.

Dissolve 10 mg thiamine, filter sterile and put into autoclaved bottle.

3. Cyanocobalamine (B_{12}) (sterile filtered)

Dissolve 5 mg in 100 ml Milli Q, filter sterile and put into autoclaved bottle.

4. Riboflavin (B_2) (sterile filtered)

Dissolve 5 mg in 100 ml acetic acid (20 mM), filter sterile and put into autoclaved bottle.

5. Selenite/Tungstate (autoclaved)

Dissolve in 1000 ml Milli Q, fill into bottles and autoclave:

NaOH	400 mg
Na_2WO_2	8 mg
Na_2SeO_3	6 mg

6. Trace Elements (autoclaved)

Dissolve in 1000 ml Milli Q and 12.5 ml HCl (37%, fuming), fill into bottles and autoclave:

FeSO ₄ · 7 H ₂ O	2100 mg
H ₃ BO ₃	30 mg
I ₂ · 4 H ₂ O	100 mg
CoCl ₂ · 6 H ₂ O	190 mg
NiCl ₂ · 6 H ₂ O	24 mg
CuCl ₂ · 2 H ₂ O	2 mg
ZnSO ₄ · 7 H ₂ O	144 mg
Na ₂ MoO ₄ · 2 H ₂ O	36 mg
MnCl ₂ · 4 H ₂ O	100 mg

7. Bicarbonate-Solution (autoclaved)

Dissolve 84 g NaHCO₃ in 1000 ml Milli Q. Fill 30 ml aliquots (needed for 1 l medium) in bottles. Close them with a rubber stopper and a crimp cap. Flush them with N₂ and shake bottles well for CO₂ saturation. Repeat this procedure several times.

8. NH₄Cl-/KH₂PO₄-Solution (autoclaved)

Dissolve 4 g KH₂PO₄ and 5 g NH₄Cl in 1000 ml Milli Q. Fill 50 ml aliquots (needed for 1 l medium) in bottles. Close them with a rubber stopper and a crimp cap. Flush them with N₂ for about 5 minutes.

9. 6.5% HCl-Solution (autoclaved)

Add 26 ml 25% HCl (or 17.5 ml 37% HCl) to H₂O, fill up until 100 ml with Milli Q. Fill into two 100 ml Duran bottles, close and autoclave with headspace.

10. 1-M-Na₂CO₃-Solution (autoclaved)

Dissolve 10.6 g Na₂CO₃ in 100 ml Milli Q. Fill into two 100 ml Duran bottles, close and autoclave with headspace.

11. 1-M-Sulfide-Solution (autoclaved)

Dissolve 24 g Na₂S · 9 H₂O (only use clear crystals, no old yellowish ones) in 100 ml anoxic Milli Q to prevent oxidation. Fill into bottle, close with rubber stopper and crimp cap. Flush with N₂ for about 20 seconds (development of toxic H₂S with CO₂ und acids!). Use a syringe and needle to take aliquots out.

A.2 Preparation of Standards for Total Sulfide Measurements (Cord-Ruwisch 1985)

Prepare anoxic Milli Q by flushing Milli Q with N₂ for about 15 min, while shaking the bottle in between. Prepare 20-mM-sulfide-solution by dissolving 0.488 g Na₂S · 9 H₂O in 100 ml anoxic Milli Q. Flush vials with rubber stoppers and crimp caps with N₂ for about 5 minutes. Transfer anoxic Milli Q and 20-mM-sulfide-solution with N₂ flushed syringe into vials (Table 6). Mix well and flush vials again with N₂ for about 5 minutes. Store standards in the fridge at 4°C, if needed. Measure the TS photometrically as described in Section 2.5.2, plot the TS concentration of the standards against the absorbance and do a linear regression analysis for a calibration line (Figure 23).

Table 6 Preparation of standards for the calibration of total sulfide measurements.

Sulfide Conc. [mM]	Vol. 20 mM Sulfide Solution [ml]	Vol. Anoxic Milli Q [ml]
0	0	40
0.5	1	39
1	2	38
2	4	36
5	10	30
10	20	20
20	40	0

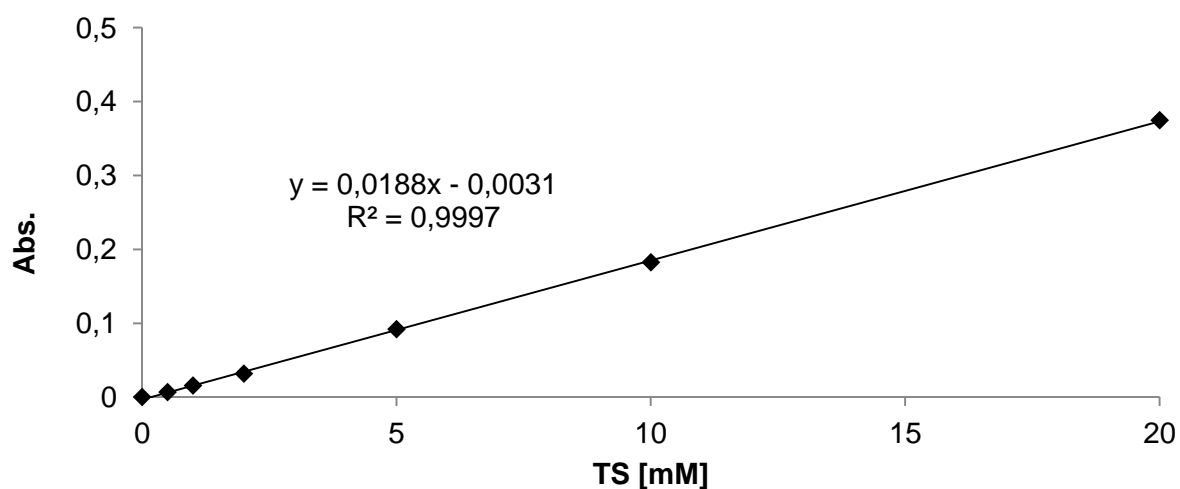


Figure 23 Seven-point calibration line used for the calculation of total sulfide (TS) concentrations.

A.3 Preparation of Solutions and Standards for Ammonia, Nitrate and Nitrite Measurements (Grasshoff et al. 1983)

Ammonia

1. Phenol Solution Dissolve 8.0 g phenol in 30 cm³ ethanol and 60 cm³ pure water. Dissolve 60 mg disodium nitroprusside in 10 cm³ pure water and add to phenol solution. Store refrigerated and tightly closed in glass bottle.

2. Sodium Hydroxide Solution Dissolve 20 g NaOH in 1 dm³ pure water. Store in PE-bottle.

3. Citrate Buffer Dissolve 24 g trisodium citrate in 50 cm³ pure water to which 2 cm³ NaOH-solution is added. Store in PE-bottle at room temperature.

4. DTT Reagent Dissolve 50 mg dichloro isocyanuric acid in 20 cm³ NaOH-solution. Unstable. Prepare immediately before use.

5. Standards Stock standard: NH₄⁺-Standard (MERCK) = 55.56 mmol/l (1 g NH₄Cl per liter)

Work standard: Dilute the stock standard with salt water (36 g/l NaCl in pure water) in different relations. To obtain standards with different concentrations for a calibration (5-6 calibration points).

Nitrate

1. Sulphanilamide Solution Dissolve 1.0 g of sulphanilamide in 10 cm³ conc. HCl and 60 cm³ pure water. After cooling make up to 100 cm³. Store in brown glass bottle.

2. NED Solution Dissolve 100 mg N-(1-naphthyl)-ethylenediamine-dihydrochlorid (NED) in 100 cm³ pure water and stored refrigerated in a glass bottle.

3. Ammoniumchloride Buffer Solution (pH=8) A solution of 10 g NH₄Cl in 1 dm³ water is adjusted to pH 8 by adding conc. NH₃.

4. Preparation of the Reductor Cadmium granules are sieved and the 0.5 - 0.8 mm fraction is washed with 2 N HCl. After thorough rinsing (with water), the cadmium is allowed to react with 1 % CuSO₄ for 5-10 minutes. Then it has to be rinsed again. A funnel is used to pour the copperized granules carefully into the reductor column which is filled with buffer to prevent air inclusion. After both outlets are sealed with glass wool, 20-30 cm³ of buffer solution,

containing about 100-200 $\mu\text{mol/l}$ NaNO_3 , are passed through the column at about 5 cm^3 per min^{-1} to activate the reductor. Once more rinsed with buffer solution, the reductor is ready for use. Between the analysis the reductor should always be kept in buffer/filled with buffer.

5. Standards Stock Standard: NO_3 -Standard (MERCK) = 16.13 mmol/l (1 g NO_3 per liter)

Work Standard: Dilute the Stock standard with salt water (36 g/l NaCl in pure water) in different relations to obtain standards with different concentrations for calibration (5-6 calibration points).

Nitrite

1. Sulphanilamide Solution Dissolve 1.0 g of sulphanilamide in 10 cm^3 conc. HCl and 60 cm^3 pure water. After cooling make up to 100 cm^3 . Store in brown glass bottle.

2. NED Solution Dissolve 100 mg N-(1-naphthyl)-ethylendiamine-dihydrochlorid (NED) in 100 cm^3 pure water and stored refrigerated in a glass bottle.

3. Standards Stock Standard: NO_2 -Standard (MERCK) = 21.734 mmol/l (1 g NO_2 per liter)

Work Standard: Dilute the stock-standard with salt water (36 g/l NaCl in pure water) in different relations to obtain standards with different concentrations for a calibration (5-6 calibration points).

A.4 DNA Extraction Protocol

Modified from FastDNA[®] SPIN Kit for Soil:

1. Add up to 500 mg of soil sample to a Lysing Matrix E Tube.
2. Add 978 μ l Sodium Phosphate Buffer to the sample in Lysing Matrix E Tube.
3. Add 122 μ l MT Buffer.
4. Homogenize in Bead Beater (Mini-BeadBeater 8, 230V MBB8, Biospec Product) for 15 seconds.
5. Centrifuge at 14,000 x g for 5 minutes to pellet debris.

NOTE: Extending centrifugation to 15 minutes can enhance elimination of excessive debris from large samples or from cells with complex cell walls.

6. Transfer supernatant to a clean 2.0 ml microcentrifuge tube. Add 250 μ l PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.
7. Centrifuge at 14,000 x g for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 ml tube.

NOTE: While a 2.0 ml microcentrifuge tube may be used at this step, better mixing and DNA binding will occur in a larger tube.

8. Resuspend Binding Matrix suspension and add 1.0 ml to supernatant in 15 ml tube.
9. Invert tube gently by hand for 2 minutes to allow binding of DNA (do not shake). Place tube in a rack for 3 minutes to allow settling of silica matrix.
10. Remove and discard 500 μ l of supernatant being careful to avoid settled Binding Matrix.
11. Resuspend Binding Matrix in the remaining amount of supernatant using the force of the liquid from the pipet tip. Transfer about 600 - 700 μ l of the mixture to a SPIN[™] Filter and centrifuge at 14,000 x g for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN[™] Filter and centrifuge as before. Empty the catch tube again and repeat Step 11 once again (in total 3 times).
12. Add 500 μ l prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.

NOTE: Ensure that ethanol has been added to the Concentrated SEWS-M.

13. Centrifuge at 14,000 x g for 1 minute. Empty the catch tube.

14. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minutes to 'dry' the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.

15. Air dry the SPIN™ Filter for 5 minutes at room temperature.

16. Gently resuspend Binding Matrix (above the SPIN™ Filter) in 100 µl of DES (DNase/Pyrogen-Free Water) using the force of the liquid from the pipet tip.

NOTE: To avoid over-dilution of the purified DNA, use the smallest amount of DES required to resuspend Binding Matrix pellet.

NOTE: Yields may be increased by incubation for 5 minutes at 55°C in a heat block or water bath.

17. Vortex for 3 seconds (level 4).

18. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN™ Filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or at 4°C until use.

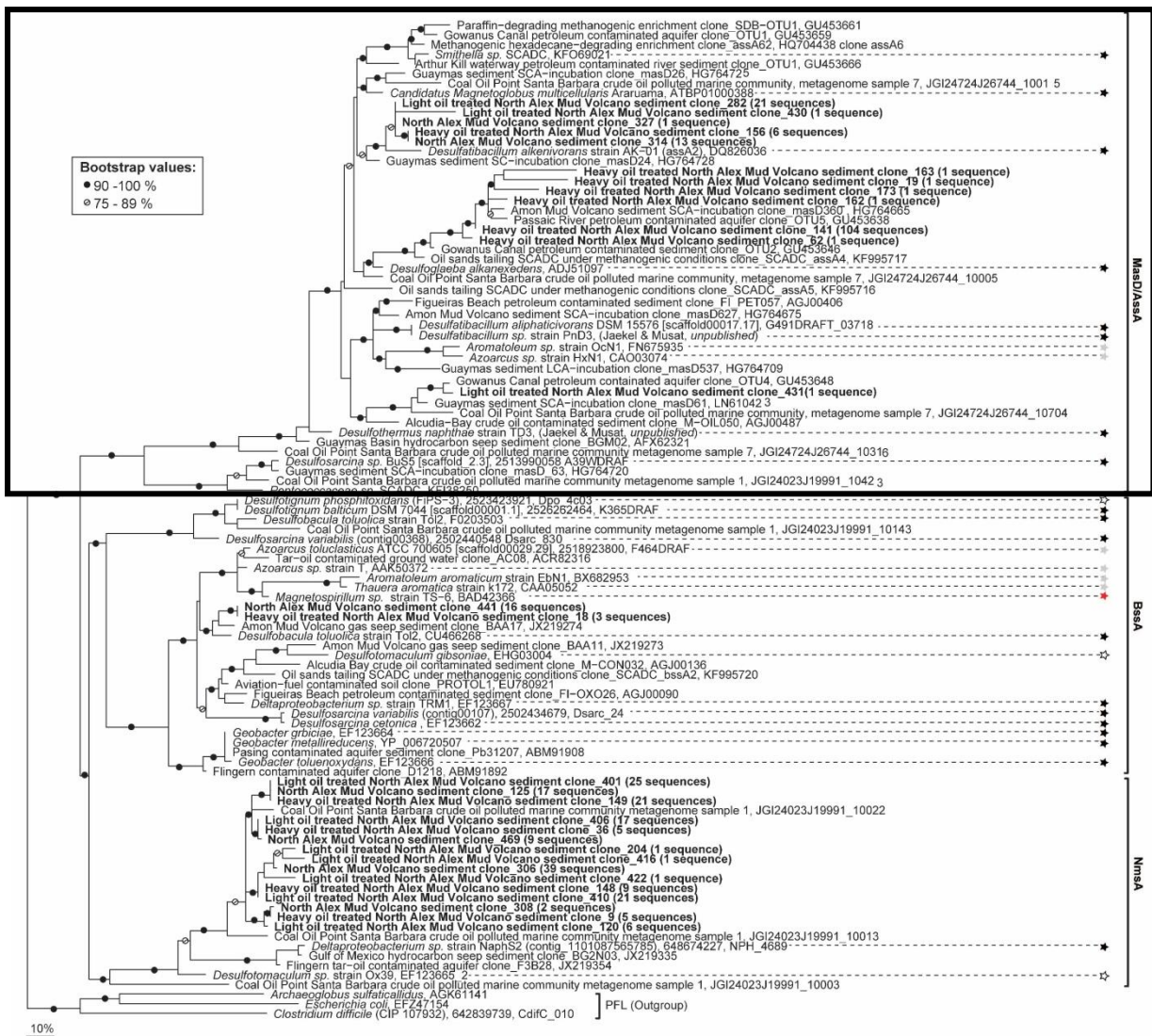
A.5 Phylogenetic Tree of *masD* Sequence Affiliations

Figure 24 Phylogenetic tree showing the affiliation of amino acid deduced *masD*, *bssA* and *nmsA* sequences to selected representative sequences available in the public databases and in deposited metagenomes. The tree was calculated using the maximum likelihood algorithm considering 136 amino acid positions for the analysis. 100 bootstrap replicates and blosum62 correction were used. The tree was rooted with pyruvate formate lyase as outgroup. The 16S rRNA-based phylogenetic affiliation of organisms available in pure culture is indicated on the right side.

Red asterisks, *Alphaproteobacteria*; gray asterisks, *Betaproteobacteria*; black asterisks, *Deltaproteobacteria*; white asterisks, *Firmicutes*. Sequences from this study are printed in boldface type. Sequence abundance for each OTU is indicated in brackets. The scale bar gives 10% estimated sequence divergence. Abbreviations: Ass = alkylsuccinate synthase, Mas = 1-methyl alkyl succinate synthase, Bss = benzylsuccinate synthase, Nms = naphthyl-methylsuccinate synthase (Marion Stagars, unpublished data).

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DECLARATION OF AUTHORSHIP

Herewith I certify that the present thesis,

**"MICROBIAL SULFATE REDUCTION AND NITROGEN FIXATION
IN OIL-CONTAMINATED MARINE SEDIMENTS"**

apart from the consultation of my supervisors, was independently prepared by me. No other than the indicated resources and references were used. This thesis was presented to no other place within the scope of an examination procedure. The written thesis is identical with the electronic one.

Date

Signature