Microbial Response to the Addition of Light and Rich Oil to Natural Marine Sediments

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List of Abbreviations and Formulas

A	Adenine
AOM	Anaerobic oxidation of methane
°API	Degrees Americal Petroleum Institute
BTEX	Benzene, toluene, ethylbenzene, xylene
C	Cytosine
CARD-FISH	Catalyzed reporter deposition FISH
CH_2O	Schematic formula for organic matter
CO_2	Chemical formula for carbon dioxide
$CuSO_4$	Chemical formula for copper sulfate
DAPI	4´, 6-Diamidino-2-phenylindole-polychloride
DMF	Dimenthylformamide
DNA	Desoxyribonucleic acid
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellinien in Braunschweig
EtOH	Ethanol
FID	Flame ionization detector
FISH	Fluorescence in-situ hybridization
G	Guanine
GC	Gas chromatograph
HCO_3^-	Bicarbonate

H_2O	Chemcial formula of water
H_2O_{UP}	Ultrapure water
H_2O_2	Chemical formula of hydrogen peroxide
H_2S	Chemical formula of hydrogen sulfide
HCl	Chemical forlula of hydrochloric acid
HRP	Horseradish peroxidase
IAPSO	International Association for the Physical sciences of the Oceans
IC	Ion chromatograph
MeOH	Methanol
Mini-Muc	Minituarized-multicorer
N_2	Chemical formula of nitrogen gas
PBS	Phosphate buffered saline
PVC	Polyvinyl chloride
rRNA	Ribosomal ribonucleic acid
rpm	Rotations per minute
RWE	Rheinisch-Westfälische Elektrizitätswerke AG
SD	Standard deviation
SSC	Salinesodiumcitrate
SR	Sulfate-reduction
${SO_4}^{2-}$	Chemical formula of sulfate
${}^{35}{SO_4}^{2-}$	Sulfate with radioactive ${}^{35}S$ isotope
T	Thymine
TRIS	Tris(hdydroxymethyl)aminomethane
ZnAc	Zinc acetate

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Abstract

Crude oil, which is the liquid fraction of petroleum, is a complex mixture of hydrocarbons and other more polar organic substances. Main characteristics of crude oil components are low reactivity, low polarity, and high stability. Furthermore, many components crude oil, especially aromatic ones, are toxic. Therefore, if they enter marine ecosystems, crude oil compounds can have harmful effects for marine organisms and even whole ecosystems. However, microorganisms are able to degrade these harmful substances. While much research has already been done for aerobic microbial degradation processes, there are still large gaps of knowledge for the anaerobic degradation processes of petroleum hydrocarbons.

In this study different experiments with batch-incubations of crude-oil treated anoxic sediment slurries and sediment mini-cores were done. Two different types of crude oil, a light and a medium-heavy one, were used in the experiments in different concentrations. Furthermore, the effect of aerobic pre-digestion of the crude oil on subsequent anaerobic degradation was tested. In batch-incubations with sediment slurries also the biodegradability of benzene, a frequent compound of crude oil, under anaerobic conditions was investigated. Sediments used for the experiments were sampled at Boknis Eck Station in the Eckernförde Bay (coastal sediment, south western Baltic Sea). Parameters, which were measured in the batch-incubations with the sediment slurries were: sulfide concentration, total alkalinity, methane content, the ratio of living to death cells, and benzene concentrations. In the incubations with the sediment mini-cores sulfate-reduction rates were measured with a radiotracer technique and bacterial abundances were determined by CARD-FISH.

The results, which were obtained in this study, indicate that the microbial community within the sediment from Boknis Eck was previously not adapted to crude oil, as a long-lag phase of 99-140 days was observed in the batch incubations with sediment slurries. After this lag-phase sulfide concentrations started to increase, indicating that crude oil became degraded. It was

found that initially suflate-reduction rates are higher if light oil is added to the sediment slurries, then when medium-heavy oil is added. The highest sulfate-reduction rates were measured at a concentration of 0.6% (v/v) crude oil, for both tested crude oil types. Furthermore, it was found that if the crude oil was pre-digested under aerobic conditions before addition to the anaerobic sediment slurries, the lag-phase was reduced to half of the time compared to not pre-digested crude oil. For the degradation of benzene, due to insensitivity of the applied methods, no clear results were obtained.

Potential sulfate-reduction rates calculated for the batch-incubations are similar or even higher than rates reported from enrichment- and pure cultures of crude oil degrading bacteria. Also sulfate-reduction rates measured *in-situ* in sediments from natural oil seeps e.g. in the Gulf of Mexico, are not higher than the highest rates found in the present study. CARD-FISH analysis showed, that in the incubation experiments with sediment mini-cores, abundances of bacteria belonging to the *Desulfovibrio spp.* group were slightly elevated after 73 days of incubation. Together with the slightly increased sulfate-reduction rates, which were also measured in the mini-cores, this increase in abundances could indicate a potential involvement of these bacteria in crude oil degradation.

Zusammenfassung

Rohöl, die flüssige Fraktion des Petroleums, ist ein sehr komplexes Gemisch von Kohlenwasserstoffen und anderen polareren organischen Substanzen. Die Bestandteile des Rohöls sind durch eine geringe Reaktivität und Polarität sowie eine hohe Stabilität gekennzeichnet. Da viele Bestandteile des Rohöls, vor allem die aromatischen, giftig sind, kann es schädliche Auswirkungen auf Organismen und sogar ganze Ökosysteme haben, wenn Rohöl in Ökosysteme gelangt. Mikroorganismen besitzen jedoch die Fähigkeit diese schädlichen Substanzen abzubauen. Während die aeroben mikrobiellen Abbauprozesse von Petroleum schon gut untersucht wurden, ist über die anaeroben mikrobiellen Abbauprozesse noch wenig bekannt.

In dieser Studie wurden verschiedene Versuche mit Öl-behandelten anaeroben Batch-Inkubationen von Sediment-Medium-Gemischen und kleineren Sedimentkernen durchgeführt. Zwei unterschiedliche Arten von Rohöl, ein leichtes und ein mittelschweres, wurden in unterschiedlichen Konzentrationen in den Versuchen verwendet. Außerdem wurde der Effekt des aeroben Vorabbaus von Rohöl auf den darauf folgenden anaeroben Abbau getestet. In Batch-Inkubationen mit Sediment-Medium-Gemischen wurde des Weiteren die Abbaubarkeit von Benzol, einem häufigen Bestandteil des Rohöls, unter anaeroben Bedingungen untersucht. Die Sedimente, die für die Versuche verwendet wurden, stammten von der Station Boknis Eck in der Eckernförder Bucht (küstennahes Sediment, SW Ostsee). In den Batch-Inkubationen der Sediment-Medium-Gemische wurden folgende Parameter gemessen: Sulfidkonzentration, Gesamtalkalinität, Methangehalt, die Rate von lebenden zu toten Zellen und die Benzolkonzentration. In den kleineren Sedimentkernen wurden Sulfatreduktionsraten mit radioaktiven Tracern und die bakteriellen Abundanzen mittels CARD-FISH gemessen.

Anhand der langen Lag-Phasen von 100-140 Tage in den Batch-Inkubationen mit den Sediment-Medium-Gemischen wurde deutlich, dass vor den Experimenten die mikrobielle Gemeinschaft in den Sedimenten nicht an den Abbau von Rohöl angepasst war. Nach diesen Lag-Phasen stieg die Sulfid-Konzentration in den Inkubationen an, was darauf hindeutet, dass das Rohöl abgebaut wurde. Anfänglich waren die Anstiegsraten des Sulfides in den Inkubationen zu denen Leichtöl zugegeben wurde höher als in denen mit zugegebenen Mittelschweröl. Nach einiger Zeit waren die Raten aber gleich hoch. Der höchste Anstieg an Sulfid wurde für beide Rohöltypen in Inkubationen mit 0,6% (v/v) Rohöl gemessen. Außerdem zeigte sich, dass der aerobe Vorabbau von Rohöl die Lag-Phase des aeroben Abbaus um etwa die Hälfte verkürzte. Aufgrund mangelnder Sensibilität der verwendeten Methoden wurden für den Benzolabbau keine eindeutigen Ergebnisse gefunden.

Die berechneten potentiellen Sulfatreduktionsraten der Batch-Inkubationen sind gleich hoch bzw. teilweise sogar höher als die in anderen Studien mit Anreicherungs- und Reinkulturen beschriebenen Raten. Auch Raten, die *in-situ* von Sedimenten an natürlichen Ölquellen wie z. B. im Golf von Mexiko gemessen wurden, sind nicht höher als die in der vorliegenden Studie höchsten gemessenen Raten. Eine CARD-FISH Analyse zeigte, dass in den Inkubations-Experimenten mit den kleineren Sedimentkernen, nach 73 Tagen inkubation, leicht erhöhte Abundanzen von Bakterien der *Desulfovibrio spp.*-Gruppe zu finden waren. Zusammen mit den leicht erhöhten Sulfatreduktionsraten, die in den Experimenten mit den kleineren Sedimentkernen gefunden wurde, könnte das auf eine mögliche Beteiligung dieser bakteriellen Gruppe am Rohölabbau hindeuten.

1 Introduction

1.1 Crude oil - formation and characteristics

Crude oil, the liquid fraction of petroleum, consists of a complex mixture of hydrocarbons and other, more polar, organic substances. Petroleum, what literally translated from Latin means "rock oil" (URL 1), consists besides the liquid fraction also of a gaseous fraction. This natural gas consists mainly of methane, but also larger hydrocarbons (up to C4) and smaller amounts of carbon dioxide or sulfide (Tissot & Welte, 1984).

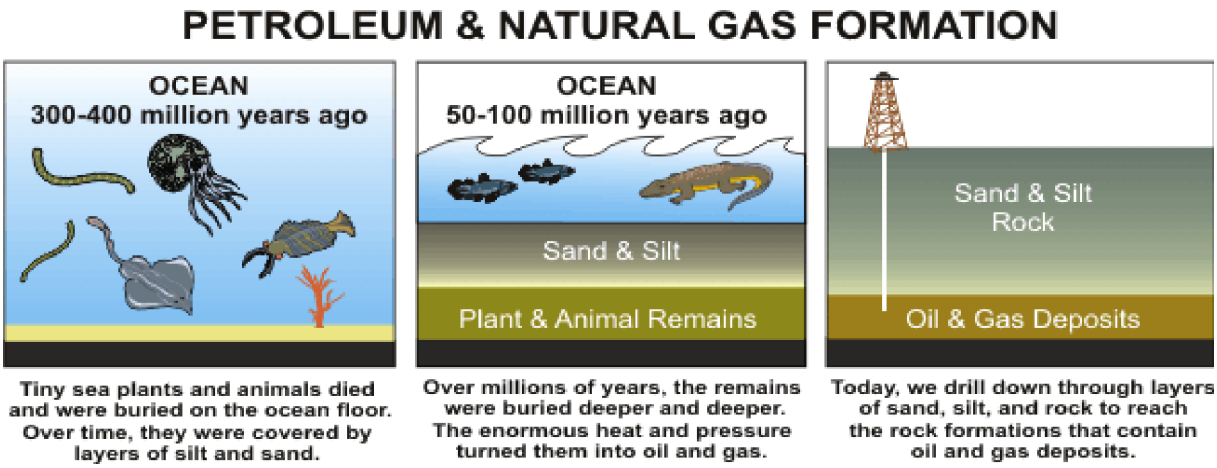

Figure 1.1: *Overview of the history of petroleum formation* (URL 2).

Petroleum generation started up to several 100 million years ago, when huge amounts of organic matter sunk to the ground of lakes or the sea where it then became incompletely degraded and covered by sediment. With time, when the organic matter got buried deeper in the sediment, heat and pressure rose and eventually, within geological timescales, petroleum was formed (Tissot & Welte, 1984; Killops & Killops, 1997; Fig. 1.1).

Petroleum is one of the most complex natural mixtures of substances. It consists of more than 17.000 distinct compounds (Marshall & Rodgers, 2003). But not all of these compounds are present in similar amounts. An average crude oil consists of dozens of major compounds and thousands of minor ones (Committee on Oil in the Sea, 2003). Various types of crude oil can be distinguished based on their different compositions. The composition of a crude oil depends not only on the type and composition of the organic matter, from which it is formed, but also on the temperature and pressure present during its formation. The latter two factors influence the geochemical processes, by which petroleum is generated out of the organic matter (Tissot & Welte, 1984; Killops & Killops, 1997).

Besides the multitude of compounds, of which crude oil can be made up, there are four defined chemical groups, into which all compounds can be arranged (Tissot & Welte, 1984; Killops & Killops, 1997; Prince, 2002).

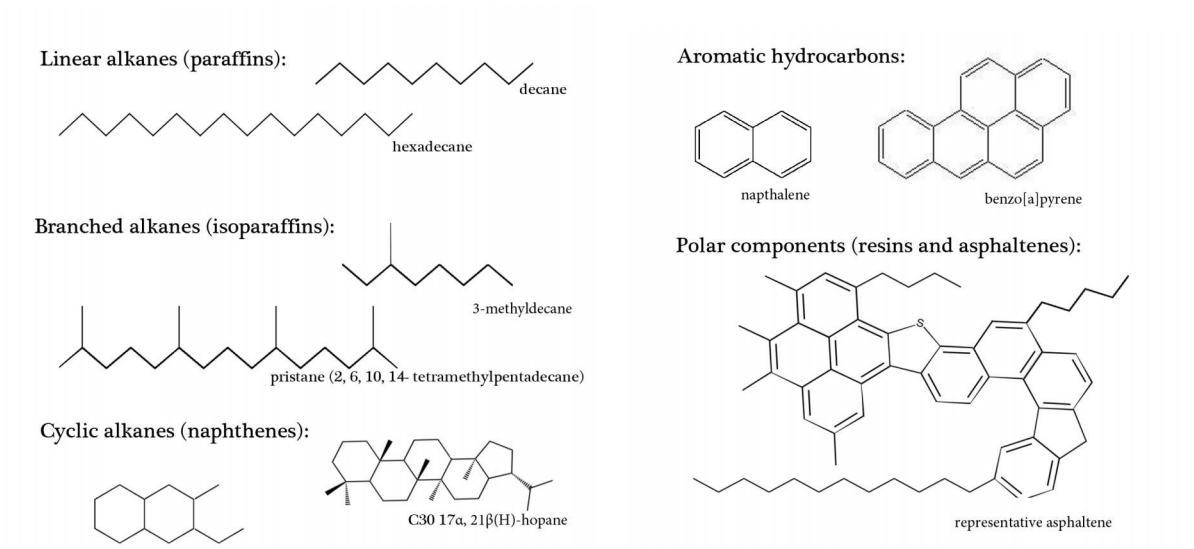

Figure 1.2: *Representative crude oil compounds.* Left: compounds of the alkane group. Right: compounds belonging to the group of the aromatic hydrocarbons and the group of the asphaltenes (after Killops & Killops, 1997; Prince, 2002).

The group of the **alkanes** makes up 58% of an average crude oil (Tissot & Welte, 1984). This group contains linear (paraffins), branched (isoparaffins) as well as cyclic (naphthenes) alkanes (Prince, 2002).

The the group of the **aromatic hydrocarbons** constitute 29% of an average crude oil (Tissot & Welte, 1984) and includes mono- or polycyclic, as well as substituted and unsubstituted aromatic hydrocarbons (Killops & Killops, 1997).

The remaining two groups are the **resins** and the **asphaltenes**. Compounds of these groups are more polar than the compounds found in the groups of the alkanes and the aromatics. Resins and asphaltenes contain multiple aromatic rings and, in addition to carbon and hydrogen, they also contain the hetero-atoms nitrogen, oxygen, and sulfur, as well as small amounts of metals like iron, copper, or vanadium (Mullins, 2007). They account for the residual 14% of an average crude oil (Tissot & Welte, 1984). Their chemical structure is very complex, and for most resins and asphaltenes no exact chemical structure is known (Harayama *et al.*, 1999).

Factors, which can be used to categorize different types of crude oil are: density or gravity, viscosity, sulfur content, and origin. While the most commonly used factor is the specific gravity or density (in degrees API, American Petroleum Institute). Denser fluids have lower °API gravities. For example, water has an API gravity of 10°. Most crude oils have a lower density than water, hence their API gravities are higher than 10°. Per definition, crude oils with an API gravity above 40° are called light oils, crude oils with a API gravity lower than 16° are called heavy oils (Killops & Killops, 1997; Prince, 2002). The differences in density are caused by different compositions. Light oil in general consist of more short-chained hydrocarbon compounds than heavy oil, which contains more higher molecular weight hydrocarbons and also more resins and asphaltenes (Tissot & Welte, 1984; Killops & Killops, 1997).

Crude oil has several characteristics, which all together lead to the fact that crude oil is not only dangerous for organisms and ecosystems but also that biodegradation is slow and the residence time of crude oil in an ecosystem can be really long (several years; Committee on Oil in the Sea, 2003; Head *et al.*, 2006).

Firstly, most of the compounds are quite stable and exhibit a low reactivity. Reasons for that are mainly the lack of functional groups and the high dissociation energies of the carbon–carbon and carbon–hydrogen bonds (Widdel, 2004; Boll & Heider, 2010).

Another factor, which makes crude oil dangerous for many organisms and whole ecosystems, is the high toxicity of many crude oil compounds (Carls *et al.*, 1999; Peterson *et al.*, 2003).

Toxicity of some compounds to microorganisms influences biodegradability (Sikkema *et al.*, 1995; Heipieper & Martinez, 2010). Reasons for the toxicity of crude oil compounds to microorganisms, especially mono-aromatics (e.g. BTEX and phenols), n-alkanols and terpeniols, are that these compounds can enter and disrupt cellular membranes. This disruption of membranes leads to disturbance of the integrity of cell membranes, what means that the functions of membranes: as a barrier, as a matrix for enzymes, and as energy transducer, are interrupted. This effects will lead to growth inhibition and eventually to the death of the microbial cell (Heipieper & Martinez, 2010).

Effects of crude oil contamination in anaerobic habitats are probably even more severe than in aerobic habitats. Biodegradation in anaerobic habitats is in general slower than in aerobic ones (Widdel & Rabus, 2001; Widdel *et al.*, 2010). Additionally, anaerobic microorganisms seem to be more susceptible against the toxicity of hydrocarbons than their aerobic counterparts (Duldhardt, 2007).

The combination of the stability of the crude oil compounds, which leads to long persistence in the environment, and their toxicity makes biodegradation of crude oil compounds an important topic to be studied.

1.2 Sources of crude oil input into the ocean

In the ocean, there are four major sources for crude oil input (Fig. 1.3): (1) by natural seeps (Fig. 1.4), (2) during extraction of petroleum, (3) during transportation of petroleum, and (4) by handling of petroleum during consumption (Committee on Oil in the Sea, 2003; Prince *et al.*, 2003). In total each year about 1.3 million tons of crude oil enter the ocean. The largest source are natural seeps, accounting for one half (600.000 tons per year) of the input (Committee on Oil in the Sea, 2003). However, current estimations about the amount of crude oil input from natural seeps range from 200.000 to 2.000.000 tons per year and are highly debated (Committee on Oil in the Sea, 2003).

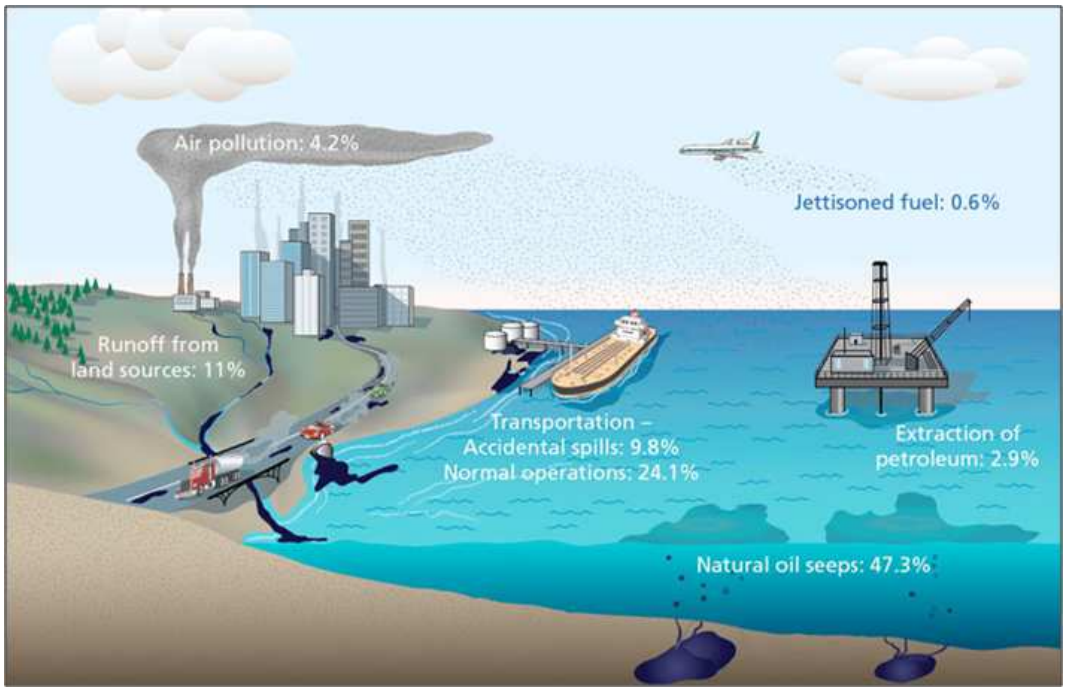

Figure 1.3: *Sources of crude oil input into the ocean* (Illustration by Jack Cook, Woods Hole Oceanographic Institution; URL 3).

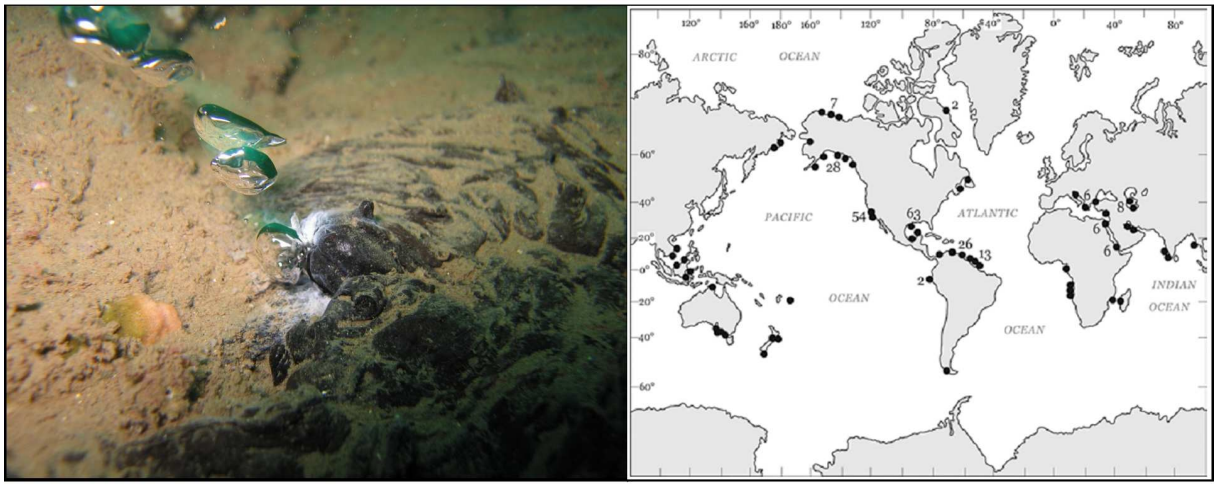

Figure 1.4: *Natural crude oil seeps in the ocean.* Natural oil seep in sediments of the Santa Barbara Channel (left). Crude oil is seeping through the seafloor and methane bubbles are rising (URL 4). Map of known locations of natural oil seeps in the world's oceans (right). Black dots indicate the location of oil seeps. The numbers indicate how many seeps are found in a certain region (Kvenvolden & Cooper, 2003).

1.3 Fates of crude oil in the ocean

Despite the large and constant input of crude oil into the ocean (section 1.2), the ocean is not totally polluted by crude oil. Obviously, there must be mechanisms, by which the crude oil gets removed from the system. There are in fact different biotic and abiotic mechanisms, by which crude oil can be altered or even removed from seawater and sediments (Fig. 1.5). These processes are collectively called "weathering" (Committee on Oil in the Sea, 2003). The natural processes involved in weathering are:

- Emulsification (Fingas *et al.*, 1996; Sjöblom *et al.*, 1999)
- Dissolution and diffusion (Shakir & de Hemptinne, 2007; De Hemptinne *et al.*, 2001)
- Evaporation or volatilization (Fingas, 1999; Stiver & Mackay, 1984)
- Dispersion and spreading (Committee on Oil in the Sea, 2003)
- Photo-oxidation (Hamilton *et al.*, 2003; Garrett *et al.*, 1998)
- Microbial degradation (Leahy & Colwell, 1990; Head *et al.*, 2006)
- Combustion (Buist *et al.*, 1999)

Except for microbial oxidation and combustion, these weathering processes can not completely degrade crude oil. They only can influence composition and distribution and break down larger compounds to smaller ones. The final conversion of the crude oil compounds, to CO_2 and H_2O, can only happen through combustion or biodegradation (Prince, 2010; Committee on Oil in the Sea, 2003). While, for biodegradation, besides CO_2 and H_2O also biomass is produced out of the crude-oil components (Prince, 2002).

Combustion only has a small chance to succeed at sea (Buist, 2003). Consequently, biodegradation of crude oil by microorganisms is probably the major sink for crude oil in the ocean.

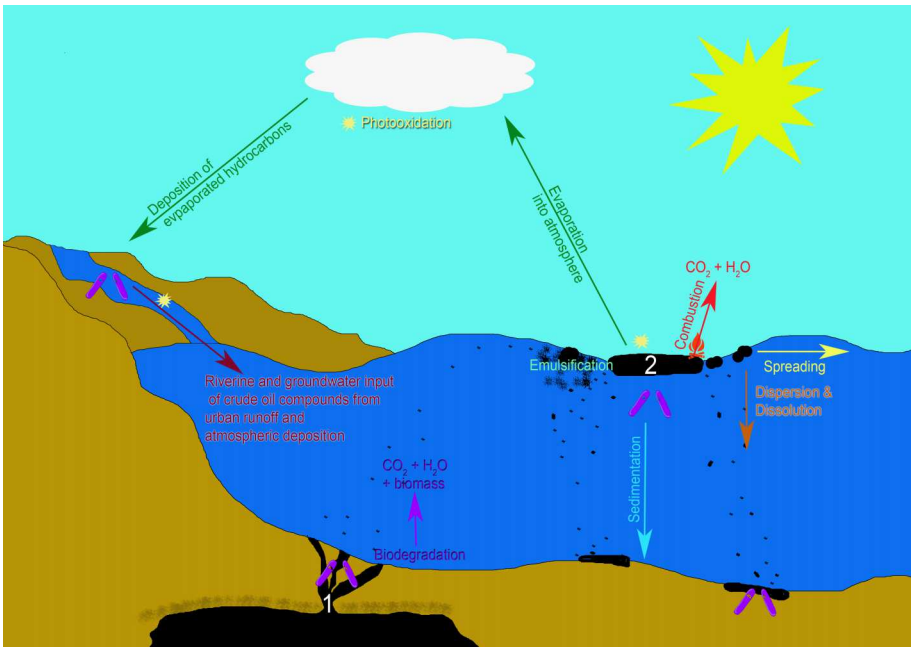

Figure 1.5: *Fates of crude oil in the ocean.* Two major input sources are illustrated: 1.) crude oil seeping through the seafloor from subsurface reservoirs, 2.) input at sea surface e.g by oil spills. Combustion or biodegradation are the only two processes, by which crude oil can be decomposed. While combustion only is possible for oil floating at the surface, biodegradation is possible almost everywhere.

1.3.1 Biodegradation of crude oil

Since the beginning of the twentieth century there is evidence for petroleum hydrocarbon degrading microorganisms. Today, several bacteria as well as algae and some fungi, which have this ability, are known (Prince, 2002). Oil-degrading microorganisms are ubiquitous in the world's oceans (Prince, 2002). This ubiquity is not surprising, as crude oil, seeping naturally through the seafloor, provided an energy source already for millions of years (Prince *et al.*, 2003; Widdel, 2004). In the following, the terms "microbial" and "microorganism" only refer to prokaryotic microorganisms.

Nowadays, it is well established that microbial degradation is the most important process for the removal of harmful crude oil components from the environment (Head *et al.*, 2006). However, the rate of microbial degradation of crude oil compounds is strongly dependent on environmental factors, like: the availability of terminal electron acceptors and nutrients, temperature, salinity, and pressure (Leahy & Colwell, 1990; Committee on Oil in the Sea, 2003).

Within the natural crude oil reservoirs in the subsurface, due to high temperature and pressure, and the deficiency of nutrients and terminal electron acceptors, microbial degradation processes are normally rare and slow or even absent (Head *et al.*, 2003; Foght, 2010). But, once the petroleum is migrating out of its natural reservoirs, it becomes available as food source for many microorganisms (Joye *et al.*, 2004; Orcutt *et al.*, 2010). The microbes then can oxidize the crude oil compounds to gain energy or use it for the production of cell mass (Widdel & Rabus, 2001; Prince, 2002; Boll & Heider, 2010). Smaller alkanes and simple cyclic hydrocarbons can be completely degraded by single microorganisms. Branched alkanes, multicyclic, or other more complex hydrocarbons are often not degraded by a single organism but require multiple metabolic pathways. It is even thought that consortia of several bacterial strains might be involved in the degradation of single compounds (Schink, 1997; Sugiura *et al.*, 1997; Taubert *et al.*, 2012). One organism usually degrades only a narrow range of compounds (Shigeaki *et al.*, 2004; Yakimov *et al.*, 2007; Widdel *et al.*, 2010), and for the degradation of some compounds even several bacterial species are needed. Hence, in the degradation of such a complex mixture like crude oil, probably many different microbes are involved.

Biodegradation does not occur at similar rates for all different hydrocarbons present in crude oil (Prince, 1993; Prince, 2002), and not all constituents of crude oil are readily degradable. It is estimated that the biodegradability of different crude oils ranges from 70 to 97% (Prince, 1993; Prince, 2002; Committee on Oil in the Sea, 2003). What remains undegraded are principally the biologically inert members of the resin and asphaltene groups (Oudot, 1984; Kennicutt, 1988; Prince *et al.*, 2003).

Because of the different biodegradabilities of the different crude-oil compounds, biodegradation happens in a characteristic sequence and produces a characteristic change in crude oil composition. At first, the low-molecular weight aliphatic and aromatic hydrocarbons are degraded. While the higher-molecular weight hydrocarbons are more robust against degradation and therefore become enriched with time (Head *et al.*, 2006). Hence, the fraction of hydrocarbons in general becomes lower and the fraction of the resins and asphaltenes increases (Head *et al.*, 2003; Fig. 1.6). As a result, biodegradation in general increases the density of a crude oil, what means that biodegradation leads to production of heavy- from light oil. This change of crude oil composition is similar to that found in natural petroleum reservoirs where biodegradation is occurring (Head *et al.*, 2003; Röling *et al.*, 2003; Huang & Larter, 2005).

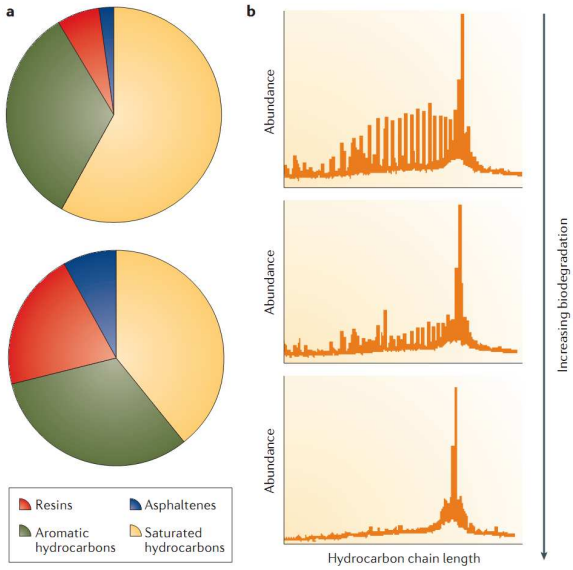

Figure 1.6: *Change of the fractions of the four compound groups of crude oil with increasing biodegradation.* A) in form of a pie-chart and B) as measured with a gas chromatograph. Compounds with a lower molecular weight are degraded first (the high peaks, which remain, are internal standards) (Head *et al.*, 2006).

For biodegradation, the activation of the crude-oil hydrocarbons is an essential step. Activation means that functional groups have to be inserted into the molecules before they can enter the cellular metabolism and can be further processed by conventional dissimilatory (to gain energy) or assimilatory (for the production of cell mass) metabolic pathways (Widdel & Rabus, 2001). Because of the inertness of the bonds within the hydrocarbon molecules this activation is a very energy demanding step (Widdel, 2004; Boll & Heider, 2010). Anyway, this activation can be managed by microorganisms by different biochemical mechanisms. Some of them will be described in the following.

1.3.1.1 Aerobic biodegradation of crude oil

Many of the metabolic capacities and pathways of aerobic crude oil degradation have been extensively studied and are well known. There is much knowledge on organisms, which are capable of aerobic degradation of petroleum hydrocarbons, and there is also increasing evidence, which of these organisms could be important in nature (Prince, 2002; Head *et al.*, 2006, and references therein; Fig. 1.7).

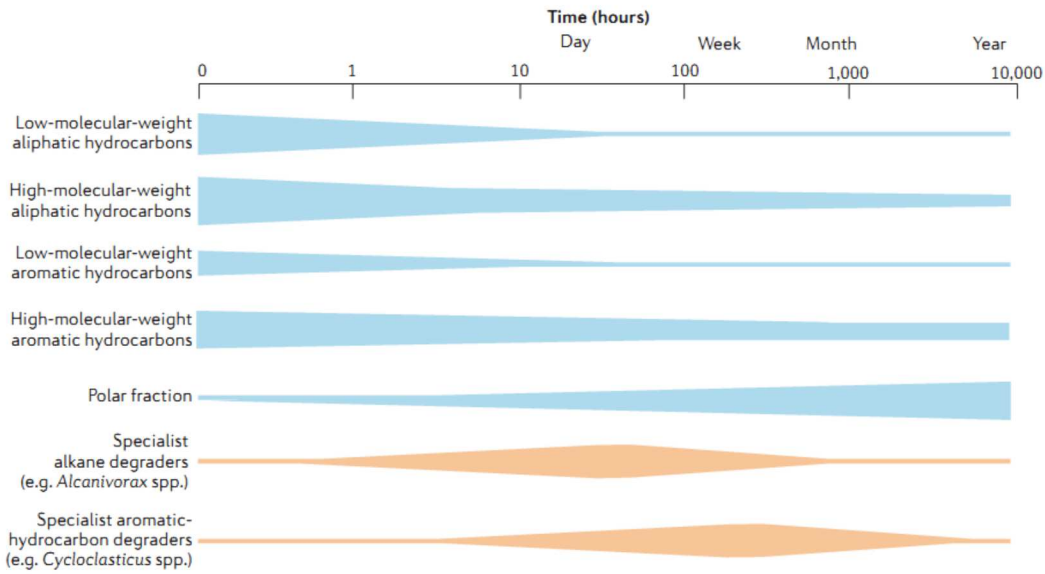

Figure 1.7: *Characteristic changes of composition of spilled crude oil in marine environments and corresponding changes of abundances of known crude-oil degrading bacteria over time* (Head *et al.,* 2006). It can be seen that low-molecular weight aliphatic and aromatic hydrocarbons are degraded fast, while the fraction of the resins and asphaltenes increases over time.

In aerobic organisms activation of the crude-oil hydrocarbons is managed by mono- or dioxygenases. These enzymes incorporate one or two hydroxyl groups into the hydrocarbon. In this reaction, oxygen is used as powerful oxidizing agent (Prince, 2002). What means that, in these organisms, oxygen is needed for two important processes of their metabolism. They use it for the activation of their growth substrate, and as final electron acceptor (Widdel & Rabus, 2001; Fig. 1.8).

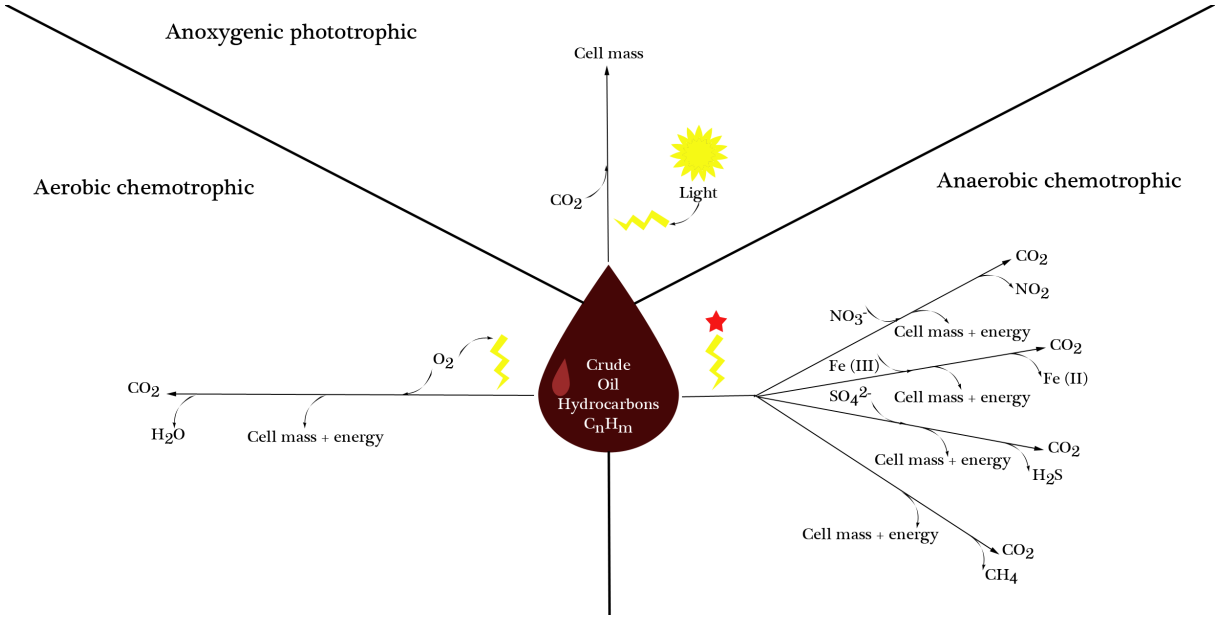

Figure 1.8: *Pathways of petroleum hydrocarbon degradation.* The initial energy-demanding degradation step is indicated by the yellow flash. The red asterisk stands for the manifold activation mechanisms found in anaerobic hydrocarbon degrading organisms. Both chemotrophic reactions can be used for energy production and biomass production. The anoxygenic phototrophic reaction does not yield energy and can only be used for biomass production. In the aerobic degradation oxygen is not only the final electron acceptor but it is also used for the initial activation step of the hydrocarbon (modified according to: Widdel & Rabus, 2001).

1.3.1.2 Anaerobic biodegradation of crude oil

While the aerobic degradation of crude oil hydrocarbons has been extensively studied, for the anaerobic degradation there are still large gaps of knowledge (Widdel *et al.*, 2010). It was only discovered in the late 1980's that crude-oil hydrocarbons can be degraded anaerobically (Vogel & Grbic-Galic, 1986; Grbic-Galic & Vogel, 1987). Prior to that, it was thought that the anaerobic degradation is not possible or at least could not be significant in nature (Atlas, 1981).

Nowadays, it is known that different compounds, like diverse alkanes, for example > C6 alkanes (Caldwell *et al.*, 1998; Anderson & Lovley, 2000) and branched alkanes (Bregnard *et al.*, 1997); or aromatics, like toluene (Langenhoff *et al.*, 1997; Elshahed & McInerney, 2001), naphthalene, and phenanthrene (Coates *et al.*, 1996; Bedessem *et al.*, 1997; Zhang & Young, 1997; Meckenstock *et al.*, 2000) can be degraded anaerobically by microorganisms. Even the aromatic hydrocarbon benzene, which is a frequent constituent of crude oil and the most basic aromatic compound (Tissot & Welte, 1984), is known to be oxidized anaerobically by bacteria (Grbic-Galic & Vogel, 1987; Lovley *et al.*, 1995; Coates *et al.*, 2001). Due to its aromatic π-electron system and the

lack of side chains, benzene is one of the most stable hydrocarbons (Musat & Widdel, 2008). Therefore, it is also one of the hardest to be degraded by microorganisms (Phelps *et al.*, 1996). Furthermore, due to its aromatic ring structure benzene has several characteristics, like good water solubility (Musat & Widdel, 2008), which leads to fast dispersion, and toxicity (Badham & Winn, 2007). This combination makes the biodegradability of this compound interesting to be studied (Lovley, 2000).

Microbes use different terminal electron acceptors, like nitrate (Burland & Edwards, 1999; Coates *et al.*, 2001), sulfate (Edwards *et al.*, 1992; Coates *et al.*, 1996), or ferric iron (Lovley *et al.*, 1994; Anderson *et al.*, 1998) to degrade crude oil compounds under anaerobic conditions. Furthermore, it is shown that crude oil compounds can be degraded by microorganisms under methanogenic conditions (Grbic-Galic & Vogel, 1987; Kazumi *et al.*, 1997), or by anoxygenic photosynthesis (Zengler *et al.*, 1999). An overview about possible pathways of crude oil hydrocarbon degradation with different terminal electron acceptors is shown in Fig. 1.8.

That in former times it was thought that anaerobic degradation of petroleum-hydrocarbons is not possible was mainly because it could not be imagined how the energy demanding initial degradation step, which is the activation of the hydrocarbons by introduction of a functional group, could look like in anaerobic organisms (Chakraborty & Coates, 2004). The discovery of processes, by which anaerobic microorganisms can manage this initial step, was really astonishing as they represented unequaled new metabolic pathways, which were totally different to ones found in aerobic microbes (Widdel & Rabus, 2001). Today, there are five major ways known how anaerobic microorganisms can manage the activation of the of crude-oil hydrocarbons.

Firstly, there is the mechanism of the **carbon-carbon addition to fumarate** by glycly-radical enzymes to yield substituted succinates (Mbadinga *et al.*, 2011). This activation mechanism has been found for toluene, ethylbenzene, p-cresol, methylnaphthalene, cyclohexane, n-hexane, and short chain alkankes (Biegert *et al.*, 1996; Boll & Heider, 2010, and references therein).

The second possibility is the **hydroxylation with water** by molybdenum co-factor containing enzymes (Boll & Heider, 2010). This mechanism has been found for the activation of substituted aromatic hydrocarbons like p-cresol or ethylbenzene (Kniemeyer *et al.*, 2007; Peters *et al.*, 2007).

The third possibility is the **reverse methanogenesis**. This mechanism is found for the degradation of the most simple hydrocarbon, which is methane, in a process called anoxigenic oxidation of methane (AOM) (Boll & Heider, 2010, and references therein). In this activation pathway variants of methyl- co-enzyme M reductase, which usually used by methanogenic organisms for the production of methane, are involved (Boll & Heider, 2010).

The fourth and fifth possibilities are **methylation** and **carboxylation**. Methylation is shown to occur for example in naphthalene degradation (Annweiler *et al.*, 2002). While the initial methylation is usually followed by an further addition to fumarate (Safinowski & Meckenstock, 2004). For naphthalene it is shown that there can be activation by carboxylation with a CO_2-derived carboxyl group (Zhang & Young, 1997).

The ability of anaerobic degradation of crude-oil hydrocarbons is widespread and scattering within bacterial phylogeny, what indicates that this substrate has been used already early in the microbial evolution (Widdel *et al.*, 2010). Most of the anaerobic hydrocarbon degraders can be found within the groups of the α-, β-, γ-, and δ Proteobacteria, the Firmicutes, and the Euryarchaeota. It is remarkable that in particular many are found within the groups of sulfate-reducing bacteria of the δ Proteobacteria (Widdel *et al.*, 2010).

1.4 Crude oil degradation in marine sediments

General characteristics of degradation of organic matter in marine sediments

For the degradation of organic matter (including crude-oil components), bacteria oxidize these reduced organic compounds by the use of an oxidant (terminal electron acceptor), which itself gets reduced during this process. 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” (Jorgensen, 2006; Fig. 1.9). This vertical succession of microbial degradation processes is caused by the decreasing energy yield of the processes and the redox potentials of the oxidants (Jorgensen, 2006).

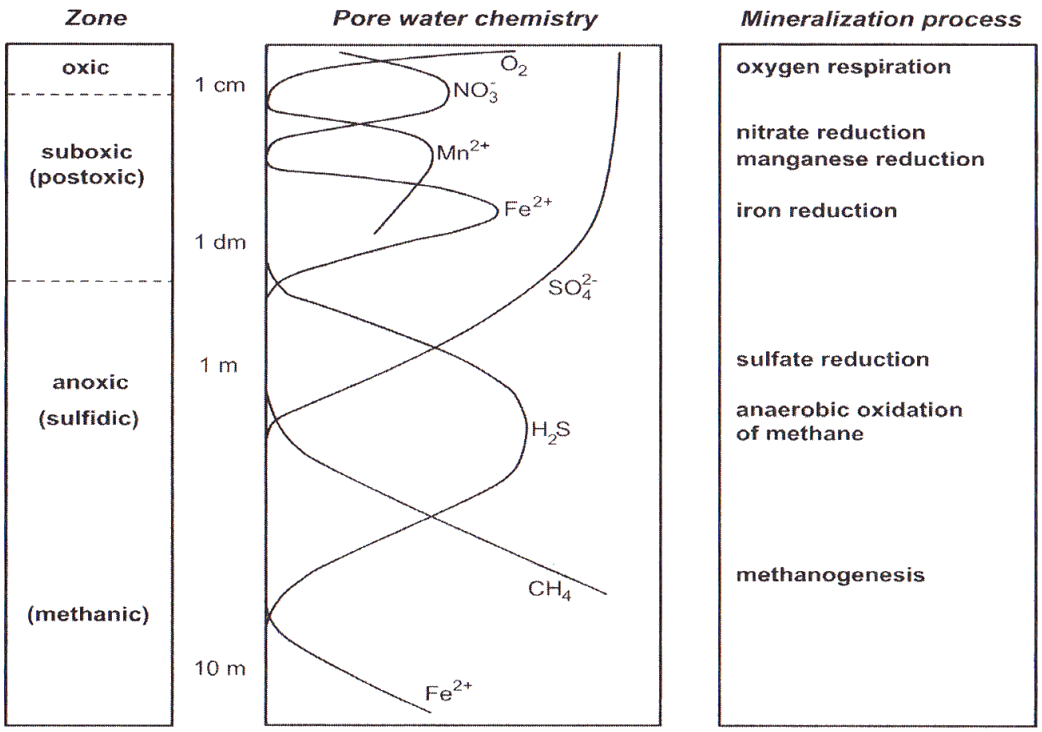

Figure 1.9: *Schematic of the biogeochemical zonation in natural marine sediments.* Left: main zones, proposed by Froelich *et al.* (1979) and Berner (1981) (in brackets). Middle: typical trend of porewater characteristics of the major terminal electron acceptors (and some of their reduced forms, e.g. H_2S for $SO_4{}^{2-}$). Right: dominant processes for mineralization of organic matter in the respective sediment depth (Jorgensen & Kasten, 2006).

In the uppermost sediment layer, degradation of organic matter is done by aerobic microorganisms, using oxygen for oxidation of the organic compounds. As oxygen usually penetrates only few mm to cm into the sediment (Jorgensen, 2006; max. up to dm in deep sea sediments; Reimers, 1987; Wenzhöfer & Glud, 2002), aerobic degradation is limited to this upper layer of the sediment. Underneath this layer, where oxygen is used up, the suboxic- and the anoxic zone are found (Froelich *et al.*, 1979). The anaerobic degradation process which occurs first in a typical vertical profile is nitrate reduction. This process yields the most energy of all anaerobic processes. When most nitrate is used up, manganese reduction, followed by iron reduction, are found. When also most manganese and iron is used up, deeper down in the sediment, sulfate-reduction is the dominating process. Finally, in sediment layers where then most of the sulfate is used up, the process yielding least energy is found, namely methanogenesis (Jorgensen, 1983; Jorgensen, 2006).

Although it does not yield the most energy, sulfate-reduction is the most important anaerobic process for organic matter degradation in marine sediments (Jorgensen, 1982). This importance of sulfate-reduction in marine systems is mainly caused by the fact that sulfate, with a mean concentration of 29 mM in seawater (Vairavamurthy *et al.*, 1995), is the most abundant electron acceptor (Henrichs & Reeburgh, 1987).

Crude oil degradation in marine sediments

Crude oil degradation in marine sediments occurs at natural crude oil seeps. At these places, crude oil is seeping through the seafloor and is already subject to anaerobic microbial degradation during its way through the sediment. During this migration, crude-oil probably runs through the different zones of the redox cascade and is subject to different anaerobic microbial metabolisms. Once it reaches the sediment surface or the free seawater, it is probably also subject to aerobic degradation.

But also when oil spills happen at the sea surface, e.g. when there are tanker accidents, especially in coastal regions, a certain part of the crude oil (about 20-30%) will reach the seafloor (Payne *et al.*, 2003). This crude oil is then probably even worked deeper down into the sediment by bioturbation (Gilbert *et al.*, 1997). In this case, of an oil spill at the sea surface, the crude oil will be at first degraded aerobically in the water column and at the sediment surface. But when then during degradation at or in the sediment, oxygen is used up, what happens fast when large amounts of crude oil compounds reach sediments (Anderson & Lovley, 1997; Greer, 2010), this crude oil will have to be degraded by anaerobic microorganisms.

Therefore, it is seen that anaerobic degradation plays a crucial part for crude oil degradation in marine sediments. As sulfate-reduction is the most important pathway of anaerobic organic matter degradation in marine sediments and many anaerobic crude oil degrading bacteria were found to be sulfate-reducers (Widdel *et al.*, 2010), in this study the main focus was on sulfate-reducing bacteria.

1.4.1 Sulfate-reducing bacteria and their role in crude oil degradation in marine sediments

The term sulfate-reducing bacteria stands for a large and heterogeneous group of obligate anaerobic bacteria (Birkeland, 2005). These organisms use sulfate or other sulfurous compounds, such as sulfite, thiosulfate, or elemental sulfur as terminal electron acceptor (Birkeland, 2005). They reduce these compounds either while oxidizing organic compounds to conserve energy (dissimilatory sulfate reduction) or to incorporate sulfur into organic compounds, which they use for production of biomass (assimilatory sulfate reduction; Jorgensen & Kasten 2006). In this study, the term sulfate reduction in the following only refers to the dissimilatory pathway. The generalized overall equation of dissimilatory sulfate reduction is shown in Equation 1.1 (Jorgensen & Kasten, 2006). In this formula, CH_2O represents organic material.

$$2\,CH_2O\ +\ SO_4{}^{2-}\ \rightarrow 2\,HCO_3{}^{-}\ +\ H_2S \tag{1.1}$$

The idea that sulfate-reducing bacteria could be important for anaerobic crude oil degradation is not only reasonable because of their general importance in the marine sedimentary carbon cycle (Jorgensen, 1982), but also because of their possible role in geochemistry of petroleum reservoirs (Thode *et al.*, 1954; Nazina *et al.*, 1985; Cord-Ruwisch *et al.*, 1987). Already in 1926 sulfate-reducing bacteria were isolated from oil field waters and it was suggested that the hydrogen-sulfide, found in petroleum reservoirs, is produced by their metabolic activity during degradation of crude oil compounds (Bastin *et al.*, 1926). However, it took several decades to confirm this suggestion (Rosnes *et al.*, 1991a; Rosnes *et al.*, 1991b).

Indeed, today hydrocarbon degradation by sulfate-reducing bacteria, is considered to be the most relevant process among the different anaerobic processes (Rothermich *et al.*, 2002). Nowadays, it is well established that, despite the low redox potential of the $SO_4{}^{2-}/H_2S$ couple, sulfate reducing bacteria are able to degrade petroleum hydrocarbons (Edwards *et al.*, 1992; Coates *et al.*, 1996). Furthermore, many sulfate-reducing bacteria have been isolated from oil fields and oil field production waters (Magot *et al.*, 1992; Ben Dhia Thabet *et al.*, 2007). Additionally, oil pollution is known to stimulate the sulfur cycle significantly (Lovley, 1997; Kleikemper *et al.*,

2002). And results of several studies, e.g. by Paisse *et al.* (2008), Coates *et al.* (1997), or Townsend *et al.* (2003) strengthen the idea about the importance of sulfate-reducing bacteria in crude oil degradation in sediments.

Sulfate-reducing bacteria are either able to degrade petroleum hydrocarbons singular or in consortia together with other microorganisms (Mbadinga *et al.*, 2011). Many sulfate-reducing bacteria found in oil contaminated environments belong to the δ Proteobacteria (Mbadinga *et al.*, 2011). Also several members of the *Desulfovibrio spp.* group (δ Proteobacteria) have been isolated from crude oil contaminated habitats (Magot, 2005; Birkeland, 2005). Thus, in this study the main focus will be on the members of the *Desulfovibrio spp.* group.

1.5 Aim of this study

This study served as a pre-study for further, more detailed investigations of crude-oil degradation in marine sediments. Crude-oil degradation is a very complex topic and there are still large gaps of knowledge, especially for the anaerobic part. Most knowledge about crude-oil degradation was obtained through studies where pure cultures or only single crude-oil components were used. It is important to increase the knowledge about microbial degradation of the complex mixture crude oil in natural marine sediments by natural microbial communities. This knowledge about natural processes is important in order to advance the understanding of the potentials and limitations of crude-oil biodegradation in the ocean.

The results of this study could show if there is a response of the natural microbial community, especially of sulfate-reducing bacteria, to the addition of crude oil. Furthermore, the results could show if there are different responses if different kinds of oils are added in different concentrations.

Additionally, in this study it was tested if the methods used for not oil-contaminated sediments also work for oil-contaminated sediments.

The tested hypotheses were:

1. As response to the addition of crude-oil to natural marine sediments sulfate reduction rates will increase.

2. There will be a faster and also a higher response of sulfate-reducing bacteria in the treatment with light oil, compared to the treatment with medium-heavy oil.

3. Intensity of the reaction of sulfate-reducing bacteria and the time until a reaction can be measured (lag-phase) are dependent on crude oil concentration.

4. The addition of crude-oil to natural marine sediments will lead to changes in overall microbial abundance.

5. The addition of crude-oil to natural marine sediments will result in a change of the microbial community species composition. The *Desulfovibrio spp.* group will become more abundant.

6. Crude-oil, which was already subject to aerobic degradation is more readily degradable by anaerobic sediment microorganisms. The response of sulfate-reducing bacteria will be faster compared to sediments, to which crude-oil was added, that has not been subject to aerobic degradation in beforehand.

7. Sulfate-reducing bacteria in natural marine sediments are able to degrade benzene.

8. The response of methanogenic archaea to the addition of crude oil will be higher in deeper sediment layers.

2 Materials and Methods

2.1 Study site

Sediments, used for the experiments, which were conducted in this study, were sampled at Boknis Eck station (54° 31‘ N; 10° 02‘ E). This site is located in the Eckernförde Bay, which is a semi-enclosed bay that lays at the southwestern edge of the Kiel Bight in the western German Baltic Sea (Fig. 2.1). According to Whiticar (2002), Boknis Eck is one of the best studied and most frequently sampled coastal marine environments. Even a time series over nearly half a century, where parameters in the water column were measured, exists (Bange *et al.*, 2011).

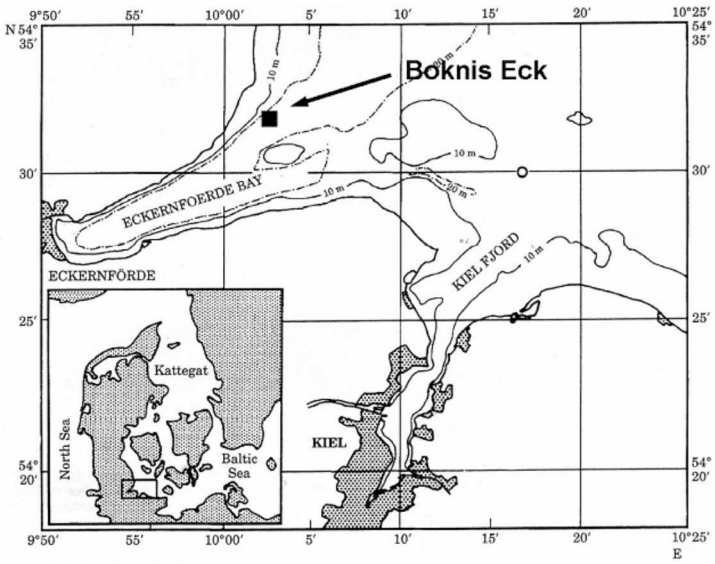

Figure 2.1: *Location of the Boknis Eck station in the Eckernförde Bay* (Bange *et al.*, 2010).

The maximum water depth of the bay is 28 m and sediments below a water depth of 20 m are mostly muddy and organic rich (4-5 wt% organic carbon in uppermost 200 cm; Whiticar, 2002). Sediments at Boknis Eck are largely anoxic already few cm beneath the seafloor (Whiticar, 2002). What is caused by the high content of organic matter, that leads to zones with high rates of sulfate reduction within the upper meter of sediment, followed by methanogenesis deeper down within the sediment (Whiticar, 2002). In Eckernförde Bay a strong seasonality of different parameters is found. Temperature, primary production, and oxygen concentrations in the water

column, as well as organic matter degradation in by microorganisms within the sediment are fluctuating over the year (Whiticar, 2002; Bange *et al.*, 2011; Smetacek, 1985; Meyer-Reil, 1986). The annual cycle in the water column within the bay is characterized by a seasonally developing pycnocline, which develops from March to September in a water depth of ca. 15 m (Bange *et al.*, 2011). During that time low-oxygen zones in the bottom water frequently develop (= hypoxia; $[O_2] < 90$ µM; Diaz & Rosenberg, 2008). In late summer sometimes even anoxia ($[O_2] = 0$ µM; Naqvi *et al.,* 2009) is found. During the last 25 years the frequency of the anoxic events was found to increase (Bange *et al.*, 2011).

2.2 Overview

In total seven different experiments, in which the effect of crude oil addition on the natural bacterial community in sediments from Boknis Eck was tested, were performed in this study. A summary of the experimental setup, the parameters measured, and the research questions of each experiment can be seen in Fig. 2.2. In six of the experiments sediment slurries (sediment diluted with artificial seawater medium) were used. These were: experiment No. 1, 2, 3a and b, 4, and 5. In experiment No. 6 sediment mini-cores were used.

In the following, at first the experimental setup and the experimental procedures of each experiment are described in detail (section 2.3). After that, sediment sampling, preparation of the sediment slurries, and sampling of the sediment mini-cores is described (section 2.4 and 2.5). Next, materials used in the experiments are described (section 2.6). Finally, protocols for analyses and formulas for calculations are given (section 2.7).

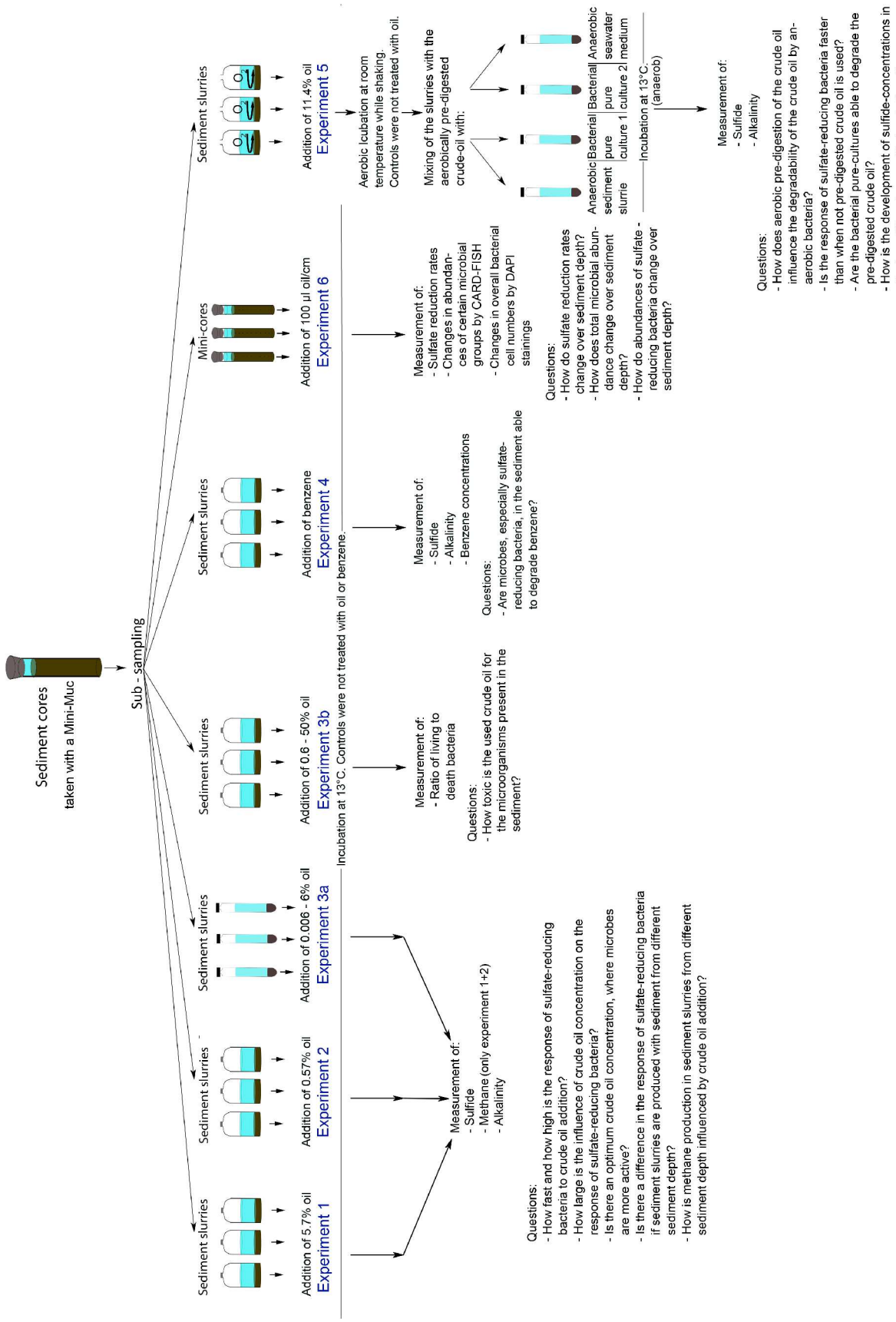

Figure 2.2: *Overview of the setup of the experiments, which were conducted in this study.* Indicated are the setup, the measured parameters, and the main research questions of each experiment.

2.3 Experimental setup

2.3.1 Experiment No. 1: Batch incubations of sediment slurries treated with 5.7% crude oil

The aim of Experiment No. 1 was to investigate how long it can last until sulfate-reducing bacteria in natural sediments, which probably previously were not adapted to the degradation of to crude oil, start to degrade crude oil and how high the rates of sulfate-reduction can become. Additionally, methane production was monitored, to see if there was activity of methanogenic archaea.

Slurries, made of sediment and anoxic artificial seawater medium (mixture 1:7), were incubated with 5.7% crude oil (volume crude oil/volume slurry = 8 ml/140 ml; 0.4 ml crude oil cm^{-3} undiluted sediment). Slurries were prepared in 200 ml serum vials with sediment from 0-10 cm sediment depth from sediments from Eckernförde Bay, which were taken with a miniaturized-multicorer (Mini-Muc; Fig. 2.3; Table 2.1).

For testing the effect of crude oil on sulfate-reducing bacteria and methanogenic archaea, slurries were either treated with light oil or heavy oil (three replicates each). Three slurries remained untreated and served as control to monitor naturally present sulfate-reduction or methanogenic activity.

Vials containing the slurries were incubated horizontally at 13°C for 255 days in the dark. At the beginning only sulfide concentrations in the supernatant of the samples were measured in regular intervals (during the first 0-119 days of incubation weekly; from 123-227 days of incubation in intervals of 3-4 days; finally, one additional measurement was done after 255 days of incubation). After 36 days regular measurements of total alkalinity in the supernatant started (from day 36 on measured simultaneously with sulfide concentrations). After 13, 29, 34, 46, 71, 83, 102, 127, and 211 days methane content of the gas headspace was measured. For analyses see section 2.7.

2.3.2 Experiment No. 2: Batch incubations of sediment slurries treated with 0.57% crude oil

The aim of Experiment No. 2 was, similar to Experiment No. 1, to investigate the effect of crude oil addition on the activity of sulfate-reducing bacteria and methanogenic archaea. As in Experiment No. 1 much crude oil was used compared to other studies, in which comparable experiments were done (Sugiura *et al.*, 1997; Miralles *et al.*, 2007b), in Experiment No. 2, less crude oil was used. Additionally two different sediment depths were used for the production of sediment slurries. In Experiment No. 1 there was an increase in methane content in the gas headspace observed within one month after start of the incubation (after 20-40 days, sediment slurries with sediment from 0-10 cm sediment depth). This increase in methane is why a deeper sediment layer (20-30 cm sediment depth), where a higher abundance of methanogenic archaea was expected, was used additionally to the sediment layer from 0-10 cm, for preparation of sediment slurries. Slurries (1:7; volume sediment:volume anoxic artificial seawater medium) were prepared in 200 ml serum vials. The two depth horizons were sliced from the same Mini-Muc core (Table 2.1).

Heavy- and light oil were each added to three of the slurries from each sediment depth (0 -10 and 20-30 cm). The crude oil concentration chosen for this experiment was 0.57 % (volume crude oil/volume slurry = 800 µl/ 140 ml; 0.04 ml crude oil cm^{-3} undiluted sediment). Three slurries from each sediment depth remained untreated and served as control to monitor naturally present sulfate-reduction or methanogenic activity.

Vials containing the slurries were incubated horizontally at 13°C in the dark for 181 days. The parameters measured were sulfide and total alkalinity, which were measured in the supernatant of the slurries simultaneously in regular intervals (from 0-146 days of incubation measured in weekly intervals; one additional measurement was done after 180 days of incubation). Additionally, methane content of the gas headspace was measured after 4-7, 24-25, 52-54, and 127-130 days of incubation (measurements took too long to measure all samples at one day; triplicates were measured at the same day). For analyses see section 2.7.

2.3.3 Experiment No. 3: Experiments on the influence of crude oil concentration on sulfate-reducing bacteria

The aim of Experiment No. 3 was to study the effect of crude oil concentration on the metabolic activity of sulfate-reducing bacteria and toxic effects of crude oil on sediment bacteria. In Experiment No. 1 and 2 already two different crude oil concentrations were chosen. In Experiment No. 3 more different crude oil concentrations were tested in two different sub-experiments. Used crude oil concentrations ranged from 0 to 50 % (v/v).

Experiment No. 3a: Concentration series for the determination of an optimal crude oil concentration

The concentrations chosen for this experiment ranged from 6 to 0.006 % (v/v) crude oil. The amount of crude oil added to the sediment slurries is shown in the appendix (Table A.3). The highest concentration of crude oil was chosen because in Experiment No. 1, where incubations were done with 5.7% crude oil, there was no response of sulfate-reducing bacteria within the first 80 days of incubation. Therefore, it was not sure if bacterial communities, which are not adapted to crude oil contamination, could cope with such high crude oil concentrations. Thus, the concentration of crude oil used in Experiment No. 1 was set as the highest level of crude oil concentration in Experiment No. 3a. The lower limit of crude oil concentrations used in Experiment No. 3a was 0.006%. This limit was chosen due to technical issues. Lower volumes of crude oil could not be pipetted.

Slurries (1:7; sediment:anoxic artificial seawater medium) for this experiment were prepared with sediment from 0-10 cm sediment depth of a Mini-Muc core (Table 2.1). Experiments were conducted in Hungate tubes (15 ml). Slurries were treated with either light- or heavy oil in the concentrations, which are listed in Table A.3 in the appendix (no replication). One slurry remained untreated and served as control to monitor naturally present sulfate-reduction.

Samples were incubated horizontally at 13°C for 189 days in the dark. Measured parameters were sulfide and total alkalinity, which were measured simultaneously in the supernatant of the samples in regular intervals (from 0-155 days of incubation measured in weekly intervals; one additional measurement was done after 189 days). For analyses see section 2.7.

Experiment No. 3b: Concentration series for the determination of the lethal crude oil concentration

In Experiment No. 3b the ratio of living to death cells in slurries, which were incubated with crude oil in different concentrations, was determinated. Many compounds of crude oil are toxic for microorganisms (Heipieper & Martinez, 2010; Sikkema *et al.*, 1995). Therefore, it was tested, at which concentration the toxic effects of the light- and heavy crude oil, which were used for the experiments in this study become lethal (> 50% death cells). The lowest concentration was 0.6% (v/v) crude oil. It was assumed that this concentration should not be lethal for the sediment bacteria as this concentration was also used in an other experiment on crude oil degradation by sediment bacteria (Miralles *et al.*, 2007b). The upper limit was chosen to be 50% (v/v) crude oil.

Sediment slurries (1:7; sediment:anoxic artificial seawater medium) used for this experiment were prepared with sediment from 0-10 cm sediment depth of a Mini-Muc core (Table 2.1). Slurries were treated with either light- or heavy oil in different concentrations, which are listed in Table A.4 in the appendix. Two slurries remained untreated and served as control for normal ratios of living to death cells. Incubations were done in small serum vials (30 ml).

The ratio of living to death cells was determined by live dead staining after one week of incubation with the crude oil at 13°C in the dark. For analyses see section 2.7.

2.3.4 Experiment No. 4: Anaerobic degradation of benzene

The aim of Experiment No. 4 was to test whether bacteria, which are naturally present in the sediment from Eckernförde Bay, are able to degrade benzene. Benzene is a frequent component of crude oil (Tissot & Welte, 1984). Due to its aromatic π-electron system and the lack of side chains it is one of the most stable and therefore also one of the hardest biodegradable hydrocarbons (Musat & Widdel, 2008; Phelps *et al.*, 1996).

Sediment slurries (1:7; sediment:anoxic artificial seawater medium) used for this experiment were prepared with sediment from 0-10 cm sediment depth of a Mini-Muc core (Table 2.1). One hundred and forty µl of a 0.005 M benzene solution was added to three sediment slurries (140 ml slurry; final concentration of benzene: 5 µM). Two types of controls (three replicates each)

were used for this experiment; One type contained anoxic artificial seawater medium and sediment but no benzene (K1). This first control served as control to monitor naturally occurring sulfate-reduction. The other type of control contained anoxic artificial seawater medium and benzene (140 µl benzene added to 140 ml medium; final concentration 5 µM), but no sediment (K2). This second control should test for loss of benzene processes other than bacterial degradation (e.g. through the butyl rubber stoppers).

Vials containing the samples were incubated horizontally for 105 days at 13°C in the dark. The parameters measured were sulfide and total alkalinity, which were measured simultaneously in the supernatant of the samples in regular intervals (from 0-78 days of incubation measured in weekly intervals; one additional measurement was done after 105 days of incubation). Benzene concentrations in the slurry where benzene was added and K2 were determined at three time-points. After 2, 49, and 104 days of incubation. For analyses see section 2.7.

2.3.5 Experiment No. 5: Experiment on the effect of aerobic pre-digestion of crude oil

The aim of Experiment No. 5 was to test if crude oil, which was subject to degradation by aerobic sediment bacteria, can be more easily degraded by anaerobic microorganisms. Therefore, it was investigated if in incubations with aerobically pre-digested crude oil shorter lag-phases and higher potential sulfate-reduction rates, compared to samples, which were incubated with not pre-digested crude oil, can be observed.

In Experiment No. 5 the first step was to let the crude oil be partially degraded aerobically (aerobic pre-digestion). For this step, aerobic sediment slurries (1:7, sediment:filtered seawater), which were prepared with sediment from 0-2 cm sediment depth, were used. To one of these aerobic slurries 9.12 ml of light oil and to one 9.12 ml of heavy oil was added (per slurry 11.4 % (v/v) crude oil; 9.12 ml crude oil/ 80 ml slurry; 0.8 ml cm^{-3}undiluted sediment). One aerobic slurry remained untreated. The bottles (500 ml glass bottles) containing the samples were sealed with parafilm to allow oxygen diffusion into the bottles. Aerobic slurries were incubated for 14 days while shaking with 60 rpm in the dark at room temperature.

After 14 days of incubation under aerobic conditions, the aerobic slurries were made anoxic. Therefore, at first the parafilm was removed, and the bottles were closed with butyl rubber stoppers and screw-caps. Then, 80 µl of a resazurin solution (1 mg/ml) was added to each slurry. Next, the slurries were flushed for 5 minutes with N_2 and then for 30 minutes with N_2/CO_2 (80:20, v/v), while shaking. During flushing with N_2/CO_2, 80 µl of a Na_2S solution (0.5 M) was added.

After the pink-blue color of the resazurin had disappeared, for each pre-digestion (with heavy-, light-, or without oil) 12 anoxic (N_2-filled), sterile 15 ml Hungate tubes were filled with 5.5 ml of the respective pre-digestion. After that, anoxic slurries (1:7, sediment:anoxic artificial seawater medium), a pure-culture of sulfate-reducing bacteria, or only anoxic artificial seawater medium was added:

- To three of the Hungate tubes from each of pre-digestion (with light oil, heavy oil, and without oil) 5.5 ml of an anoxic slurry (prepared in 200 ml serum vial with sediment from 2-10 cm sediment depth) was added (+ Slurry).

- To three of the Hungate tubes from each pre-digestion (with light oil, heavy oil, and without oil) 560 µl of a *D. mediterraneus* culture and 4.4 ml anoxic artificial seawater medium were added (+ *D. mediterraneus*).

- To three of the Hungate tubes from each pre-digestion (with light oil, heavy oil, and without oil) 70 µl of a *D. marinus* culture and 4.9 ml anoxic artificial seawater medium were added (+ *D. marinus*).

- And to three of the Hungate tubes from each pre-digestion (with light oil, heavy oil, and without oil) 5.5 ml of anoxic artificial seawater medium was added (+ Medium).

After that, all Hungate tubes were flushed with N_2/CO_2 (80:20, v/v).
How much of the bacterial cultures was added to the aerobic pre-digestion was calculated based on cell numbers for *Desulfovibrio spp.* in Eckernförde Bay sediments determined by Gier (2012). This group of sulfate-reducing bacteria was found to be abundant in sediments of Eckernförde

Bay (Bertics *et al.*, 2012). It was estimated that in the amount of sediment added to the + Slurry Hungate tubes, there must have been about 3.2 x 10^7 *Desulfovibrio spp.* cells. After determination of the cell density (cells ml^{-1}) in the bacterial pure-cultures the volume of each culture containing 3.2 x 10^7 cells was added to the Hungate tubes containing the aerobic pre-digestion. In order to achieve a 1:2 dilution of the aerobic pre-digestion, like in the + Slurry Hungate tubes, the remaining volume was added in form of anoxic artificial seawater medium.

Hungate tubes containing the samples were incubated horizontally at 13°C in the dark for 92 days. The measured parameters were sulfide concentrations and alkalinity, which were measured simultaneously in the supernatant of the samples in regular intervals (from 0-62 days of incubation measured in weekly intervals; one additional measurement was done after 92 days of incubation). For analyses see section 2.7.

2.3.6 Experiment No. 6: Incubation Experiment with sediment mini-cores

The aim of this experiment was to investigate the development of sulfate-reduction rates, the abundances of a certain group of sulfate-reducing bacteria and total bacterial abundances over sediment depth after some time of incubation with crude oil. Sulfate-reduction rates and bacterial abundances (total abundances and abundances of the group of sulfate-reducing bacteria) were compared to sediment cores, which were incubated without addition of crude oil. The bacterial group chosen for investigation of changing abundances of sulfate-reducing bacteria, was *Desulfovibrio spp.*. The reasons for choosing this group were that this group was found to be abundant in sediments of Eckernförde Bay (Bertics *et al.*, 2012). Furthermore, several members of the *Desulfovibrio spp.* group have been isolated from crude oil contaminated habitats (Magot, 2005; Birkeland, 2005).

Ten sediment mini-cores were sampled from Mini-Muc cores as described in section 1.3.1. Injection of crude-oil into the sediment mini-cores was done in 1 cm-steps through silicon-sealed holes with a micro syringe. Into four of the mini-cores light oil was injected, and into four cores heavy oil was injected. Injection volume was 100 µl of the respective crude oil for each cm-section (0.025 ml crude oil cm^{-3} sediment). Into two of the mini-cores no crude oil was injected. The

cores where no oil was added served as controls for "naturally" (without treatment with oil) occurring sulfate-reduction rates in the sediment and also for "naturally" occurring bacterial abundances. The mini-cores were incubated for 73 days in an incubator at 13° C in the dark before analyses were done. For analyses see section 2.7.

2.4 Sediment sampling

Sampling of the sediment at Boknis Eck was performed with a miniaturized-multicorer (Mini-Muc; Barnett *et al.* 1986; Fig. 2.3) during one day cruises with the GEOMAR research vessel "Littorina". Each sampled sediment core had a diameter of 10 cm and a length between 30-40 cm (Fig. 2.3).

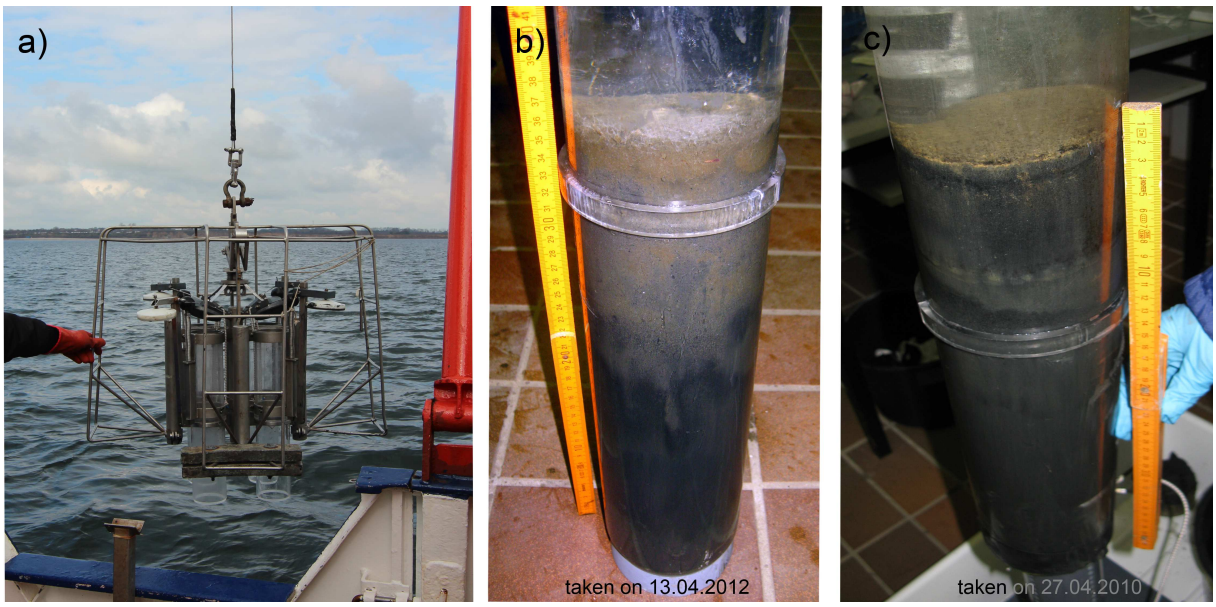

Figure 2.3: *Sampling of sediment cores with a Mini-Muc.* A) Mini-Muc during deployment at sea. B) and C) Sediment cores, which were taken on the 13th of April 2012 and the 27th of April 2010, respectively. These cores were used for the production of some of the sediment slurries for experiments conducted in this study. It can be seen that the layer of oxidized sediment (brownish color) is much thicker in the left sediment core (picture B). The sediment core in picture C) is nearly completely reduced (back color) only the uppermost few mm are still oxidized.

2.5 Sub-sampling of Mini-Muc sediment cores and preparation of sediment slurries

Sub-sampling of Mini-Muc cores for preparation of sediment slurries

Sediment cores from the Mini-Muc (see above), were sub-sampled in a cold room (10°C). Sub-sampling was done as fast as possible and anoxic conditions were re-established immediately to avoid oxidation of the sediment, i.e. sediment was transferred fast into N_2 flushed bottles.

For Experiment No. 1, 3a + b, and 4 only one sediment depth horizon (0-10 cm) was sampled. For Experiment No. 2 and 5 two different sediment depth horizons were sampled. If different sediment horizons were sampled for one experiment, they were sliced from one sediment core. Which sediment horizons were used for which experiments is shown in Table 2.1.

Sub-sampling of the Mini-Muc cores was done as follows: The respective sediment depth was sliced from the sediment core, homogenized, and immediately transferred into sterile, N_2 flushed bottles. Bottles used for the different experiments and amount of sediment per bottle were: 200 ml glass serum vials for Experiment No.1 (20 ml sediment/vial); 1 l glass bottles for the remaining experiments (ca. 400 ml sediment/bottle). After sampling bottles were closed with butyl rubber stoppers and flushed with N_2 for 3 minutes to ensure anoxic conditions.

One exception was Experiment No. 5. For the first sediment horizon (0-2 cm) sampled from the Mini-Muc core, anoxic conditions were not re-established, i.e. bottles were not flushed with N_2 before and after sampling. For the second sediment horizon, sampled from the same core like the first horizon, for Experiment No. 5 (2-10 cm), bottles were flushed with N_2 before and after sampling.

Preparation of sediment slurries

After sampling of the sediment from the Mini-Muc cores, sediment slurries were prepared by diluting the sampled sediment with sterile anoxic artificial seawater medium for sulfate-reducing bacteria (anoxic artificial seawater medium; Widdel & Bak, 1992). The recipe of the medium was slightly altered with respect to ingredients and preparation (see appendix section A.1.1). The salinity of the medium was 23, as this salinity is in the range of salinities usually found for bottom water at the sampling site of the used sediment (Bange *et al.*, 2011; Treude *et al.*, 2005). Sulfate concentration in the medium was 18 mM.

Slurries for Experiment No. 1 were directly prepared in the 200 ml serum vials, in which later the incubation with the oil was done. One hundred and twenty ml anoxic artificial seawater medium was added to each vial containing 20 ml sediment (final dilution; sediment:anoxic artificial seawater medium = 1:7). Afterwards, serum vials were flushed with N_2/CO_2 (80:20, v/v) for 3 minutes.

For preparation of the slurries for the remaining experiments at first 1:2 (sediment:anoxic artificial seawater medium) stock slurries were prepared in the 1 l glass bottles, in which the sediment was sampled from the Mini-Muc core. These stock slurries were then transferred into N_2 filled, sterile vials with a syringe and a thick needle, in which later the incubations were done, by applying the Hungate technique.

Vials used for the single experiments and amounts of 1:2 slurry added were: sterile glass serum vials for Experiment No. 2 (200 ml vials, addition of 40 ml 1:2 slurry, for both depth horizons), Experiment No. 3b (30 ml vials, addition of 5.7 ml 1:2 slurry), Experiment No. 4 (200 ml vials, addition of 40 ml 1:2 slurry), and Experiment No. 5 (first depth horizon (0-2 cm): 500 ml glass bottles, addition of 22.8ml 1:2 slurry; second depth horizon (2-10 cm): 200 ml vials, addition of 40 ml 1:2 slurry), or Hungate tubes for Experiment No. 3a (15 ml Hungate tubes, addition of 3.2 ml 1:2 slurry). Serum vials were closed with butyl rubber stoppers and aluminum crimp-caps. Hungate tubes were closed by butyl rubber stoppers and screw-caps.

Then, the final dilution of 1:7 (sediment:anoxic artificial seawater medium) was prepared in the serum vials and Hungate tubes by adding anoxic artificial seawater medium with a N_2-flushed syringe. Finally, serum vials and Hungate tubes containing sediment slurries were flushed with N_2/CO_2 (80:20, v/v) for 3 minutes.

One exception was again Experiment No. 5. The sediment horizon form 0-2 cm was not diluted with artificial seawater medium but with filtered, oxygenated seawater from the supernatant of the sediment core (final dilution: 1:7, sediment:filtered seawater). The glass bottles containing this aerobic slurry were closed with parafilm.

Details about the used sediment cores, sampled sediment depths, the used vials, and the amounts of sediment and artificial seawater medium or filtered seawater, which were used for the preparation of the sediment slurries for the different experiments, are shown in Table 2.1.

Sub-sampling of Mini-Muc cores with mini-cores

Two of the sediment-cores, which were sampled on October 4th 2011 at Boknis Eck with the Mini-Muc were used for the sub-sampling with mini-cores. The sediment in the used Mini-Muc cores were black, what means that the sediment was reduced. There was just an a few mm thick oxidized brown layer at the surface (Fig. 2.4).

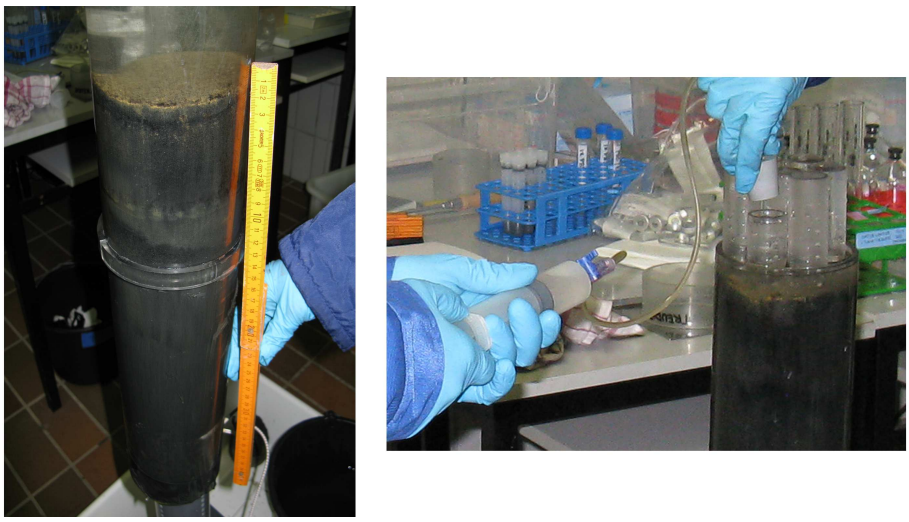

Figure 2.4: *Sub-sampling of the Mini-Muc sediment-cores* with mini-cores. One of the Mini-Muc sediment-cores, used for the sub-sampling with mini-cores (left). Sub-sampling of a Mini-Muc sediment-core with mini-cores (right).

Table 2.1: *Data of the sediment slurries used for the different experiments.* The sediment-cores sampled on the cruises in 2010 and 2011 were stored at 0°C until they were sub-sampled. The sediment-cores sampled on the cruise in 2012 were stored at 10°C until they were sub-sampled. The approximate thickness of the oxidized sediment layer at the top of the core is indicated in brackets underneath the date of the sub-sampling.

Experiment No.	Date of sampling of sediment core	Date of sub - sampling (oxidized layer)	Sediment depth sampled	Media for production of slurry	Vials for set up of slurries	ml medium - ml sediment
1	27th April, 2010	13th December, 2011 (uppermost mm)	0-10 cm	Anoxic artificial seawater medium	200 ml serum vials	120 ml - 20 ml
2	13th April, 2012	16th April, 2012 (uppermost 10 cm)	0-10 cm	Anoxic artificial seawater medium	200 ml serum vials	120 ml - 20 ml
			20-30 cm	Anoxic artificial seawater medium	200 ml serum vials	120 ml - 20 ml
3a	13th April, 2012	16th April, 2012 (uppermost 10 cm)	0-10 cm	Anoxic artificial seawater medium	15 ml Hungate tubes	1.6 ml - 9.4 ml
3b	13th April, 2012	16th April, 2012 (uppermost 10 cm)	0-10 cm	Anoxic artificial seawater medium	30 ml serum vials	2.8 ml - 17.2 ml
4	13th April, 2012	13th June, 2012 (uppermost mm)	0-10 cm	Anoxic artificial seawater medium	200 ml serum vials	120 ml - 20 ml
5	13th April, 2012	13th June, 2012 (uppermost mm)	0-2 cm	Filtrated, oxygen enriched seawater from supernatant of sediment core	500 ml bottle	68.6 ml - 11.4 ml
			2-10 cm	Anoxic artificial seawater medium	200 ml serum vials	120 ml - 20 ml

The mini-cores were 30 cm long PVC cores with 2.5 cm inner diameter. They had silicon-sealed holes in 1 cm intervals, through which later the oil and the radioactive tracers could be injected. In total, ten mini-cores were sampled. Sampling was done by pushing the mini-cores into the upper undisturbed surface of the Mini-Muc sediment cores. While pushing the mini-cores into the sediment, the overlaying water in the mini-core was sucked out with a pipe, which was connected to a syringe in order to not disturb the sediment surface of the core during sampling (see Fig.2.4). After sampling, the PVC cores were closed with rubber stoppers at both sides. Of the ten mini-cores, seven were later used for measurement of sulfate-reduction rates by a radiotracer technique. Three of the mini-cores were used for the determination of bacterial abundances with a biomolecular technique. Sampling was done in a 10°C climate chamber, where the Mini-Muc sediment cores were also stored before the sub-sampling. Sub-sampling was done fast to avoid the sediments from becoming oxidized.

2.6 Materials

2.6.1 Crude oil

Two types of crude oil were used for the experiments. One was a light oil and one a medium heavy oil (in the experiments referred to as light- and heavy oil, respectively). The heavy oil had a much higher viscosity than the light oil. The crude oils were provided by the RWE Dea AG. No exact composition or source area of the crude oil was known. The oils were “dead” oils, i.e. they should not contain any volatile (hydrocarbon) compounds.

2.6.2 Bacterial pure-cultures

Bacterial pure cultures, which were used in Experiment No. 5, were obtained from the German Collection of Microorganisms and Cell Cultures in Braunschweig (DSMZ, URL 5). They were obtained either lyophilized (*Desulfovibrio marinus*) or as actively growing culture (*Desulfobulbus mediterraneus*) and were cultivated according to the recommendations of the DSMZ (URL 6; media used for cultivation see appendix section A.1.2). Growth of the bacterial cultures was checked by regularly measurements of sulfide concentrations in the media.
Both used strains *(Desulfovibrio marinus* (DSM 18311) and *Desulfobulbus mediterraneus* (DSM 13871)) are gram negative, strictly anaerobic, sulfate-reducing bacteria, which belong to the

group of the δ-Proteobacteria. Microscopic pictures of DAPI-stained cells of the cultivated strains can be seen in Fig. 2.5. DAPI staining was performed as described in the CARD-FISH protocol (2.7.9 on page 54).

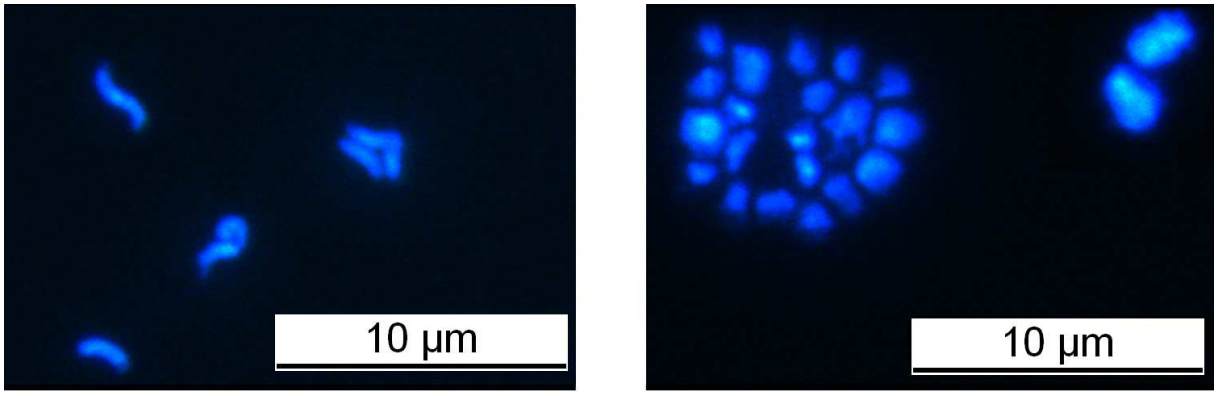

Figure 2.5: *DAPI staining of the pure cultures used in Experiment No 6.*
Left picture: *Desulfovibrio marinus*; Right picture: *Desulfobulbus mediterraneus* (dilution: 1:1000 for *D. marinus,* 1:200 for *D. mediterraneus*; magnification 1000x, filter cube A; see Table 2.2).

D. marinus was first isolated from marine sediments which were influenced by industrial activities and from oilfield production fluids. The cells are elongated, vibrio or s-shaped, and usually 0.5 µm wide and 1.5-2.5 µm long. The cells posses a single polar flagellum and can grow singular or in chains (Ben Dhia Thabet *et al.*, 2007). The second isolation location makes this organism especially interesting for experiments of this study.

D. mediterraneus was first isolated from a deep sea sediment originating from the Mediterranean Sea in ca. 1200 m water depth. The cells are typically oval-shaped with rounded ends and 1.2-1.7 µm wide and 1.4-3.2 µm long. Cells are motile by 1-3 terminal or sub-terminal flagella (Sass *et al.*, 2002). In the cultures *D. mediterraneus* was frequently found to grow in accumulations of 5-25 cells (Fig. 2.5).

2.7 Protocols for analyses and formulas for calculations

2.7.1 Measurement of sulfide concentrations

For the determination of sulfide concentrations in the supernatant of sediment slurries and the bacterial pure cultures the photometric method after Cord-Ruwisch (1985) was applied.

One day before measurements, the vials containing the slurries were shaken and placed upright. Shaking was necessary to dissolve sulfide, precipitated in iron sulfides within the sediment, in the supernatant. As for sulfide measurements the supernatant needed to be clear, it was necessary to let the sediment settle down after shaking for ca. 16 h before the measurements could be done. With a N_2 flushed micro-syringe and a long needle (0.9 x 120 mm), 0.2 ml of the clear supernatant of the sediment slurries were sampled. Zero point one ml of the sample were then added into test-tubes containing 4 ml $CuSO_4$ solution (5 mM in 50 mM HCl, recipe see appendix section A.1.4). This solution was then immediately transferred into a quartz cuvette (4.5 ml, 1 cm optical path length) and the absorption at a wavelength of 480 nm was measured with a photometer (UV- mini-1240 UV-VIS Spectrophotometer, Shimadzu). For the determination of sulfide concentrations at particular absorption values, a calibration with standards ranging from of 0-20 mM sulfide was done. With the sulfide concentrations and corresponding absorption values of the standards, a calibration line was made and a factor for conversion of absorbance to sulfide concentration was calculated (absorption values and calibration line see appendix, section A.1.5). Sulfide concentrations were calculated according to Equation 2.1. The remaining volume of the sample in the syringe was stored in reaction vials and used for subsequent measurement of total alkalinity.

$$Sulfide\,[mM] = Absorption\,(480\,nm)\,/0.021 \tag{2.1}$$

0.021 = Factor calculated with the standard measurements

2.7.2 Calculation of potential sulfate-reduction rates

Potential rates of sulfate-reduction in nmol cm^{-3} undiluted sediment d^{-1} were calculated for the sediment slurries from Experiment No. 1, 2, 3a, and 5, by applying linear regression to time-intervals of the incubation, where a linear relation between sulfide concentration and time of incubation was found. Potential rates of sulfate-reduction were calculated for three time-intervals, if possible, i.e. if a linear relation over at least three successive measurement time-points was found during that phase and the phase was present in the sample.

(1) Time-interval without relevant increase in sulfide concentrations (lag-phase).

(2) Time-interval with increasing sulfide concentrations.

(3) Time-interval where faster increase in sulfide concentrations (compared to the second interval) was measured.

2.7.3 Measurement of total alkalinity

Total alkalinity was determined by titration with HCl (0.01 M) according to a protocol of the Helmholtz Centre for Ocean Research in Kiel (URL 7). Fifty µl of a sample from the supernatant of a slurry (section 2.7.1) was added into a titration vessel after G. Pavlova containing 1 ml of H_2O_{UP} water. Then, 20 µl of an indicator solution (recipe see appendix IV) was added. Next, 0.01 M HCl was added to the sample in 20 µl steps until a stable pink coloration developed (the indicator showed a color change from yellow to pink when the buffer capacity of the sample was exhausted). The titration was done under continuous N_2 bubbling. Calibration of the measurement was done by titration with IAPSO (standard seawater, OSIL; alkalinity 2.325 mM). For calibration, 500 µl IAPSO, 1 ml H_2O_{UP}, and 20 µl indicator solution were used. With the average volume of HCl from three titrations with IAPSO and the actual alkalinity of IAPSO, a factor was calculated, with which the alkalinity of the sample could be calculated (Equations 2.2, 2.3, and 2.4).

$$Total\ Alkalinity = 1000 * F * volume\ HCl\,(ml) * (concentration\ of\ HCl\,(M)\,/volume\ of\ sample\,(ml)) \tag{2.2}$$

$$F = specified\ value\ of\ total\ alkalinity\,(spec.\ Alk.)/ actual\ value\ of\ total\ alkalinity \tag{2.3}$$

$$Spec.\ Alk. = 1000 * 1 * average\ volume\ HCl\ of\ 3\ titrations\,(ml) * (concentration\ of\ HCl\,(M)\,/volume\ of\ sample\,(ml)) \tag{2.4}$$

2.7.4 Measurement of methane content in the gas headspace by gas chromatography

Methane content in the headspace of the sediment slurries from Experiment No. 1 and 2 was determined by measurement of the gas-headspace with a gas chromatograph (GC; Hewlett Packard 6890 Series II). The gas chromatograph was equipped with a packed column (Haye SepT, 6 ft, 3.1 mm ID, Resteck) and a flame ionization detector (FID). The carrier gas was helium, combustion gases were synthetic air and hydrogen. Temperatures were: Column 75°C, injector and detector 160°C. Retention time of methane was 0.9 minutes.

The gas chromatograph was exchanged during the time of the incubations. Until the 22.06.2012 measurements were performed with the HP GC. Afterwards, a Shimadzu GC (Shimadzu GC -2014), equipped with a packed column (Haye SepD, 6 ft, 3.1 mm ID, Resteck) and a FID was used. The carrier gas used for the Shimadzu GC was helium, combustion gases were synthetic air and hydrogen. Temperatures during measurement of methane were: Column 45°C , injector 160°C, and detector 300°C. Retention time of methane was 2.6 minutes. In the Results sections, measurements with the Shimadzu GC are indicated by an asterisk.

For measurement, 100 µl of the headspace of each sample was sampled with a 250 µl SGE syringe and a 0.5 x 40 mm needle and was injected into the GC. Calibration of the measurement was done by measurements with a 100 ppm methane standard (Scotty analyzed gases).

2.7.5 Measurement of benzene concentrations by gas chromatography

Benzene concentrations in the incubations of Experiment No. 4 were determined by measurement of benzene content of the gas headspace with a gas chromatograph (GC, Hewlett Packard 6890 Series Plus). For measurements, sub-samples of 5 ml of the supernatant of the incubations were taken with a N_2-flushed syringe and a needle and transferred into GC-vials (15 ml vials) containing one pellet of NaOH (Merck). GC-vials were closed with aluminum crimp caps equipped with a butyl/ PTFE septum (Bördelkappen, VWR International). For measurement of benzene, GC-vials were placed in an auto-sampler (HP 7694, S/SL), which heated the sample to 80°C (boiling point of benzene = 80°C) before sampling the headspace and injecting the headspace sample into the gas chromatograph. The GC was equipped with a capillary column (DB VRY, 60 m, 250 µm ID, 1.4 µm FD). Carrier gas was helium. Combustion gases were synthetic air and hydrogen. Nitrogen was used as a makeup gas. During measurements, the injector and the detector (FID) were constantly heated to 230°C, while the temperature of the column was step-wise increased from 40°C to 180°C. Retention time of benzene was 10.2 minutes.

2.7.6 Live-dead staining

In order to investigate the viability of the bacterial cells in Experiment No. 3b, live-dead staining with the BacLight cell viability kit (Invitrogen) was done. The working solution mixture of compound A (SYTO® 9) and compound B (propidium iodide) was prepared according to the manual provided by the manufacturer. This working solution was mixed 1:2 with a 1:300 dilution (slurry : 1xPBs) of a sub-sample of a sediment slurry from Experiment No. 3b and incubated for 10 minutes in the dark. This mixture was them filtered onto a black polycarbonate filter (pore size: 0.2 µM, diameter: 25 mm, Nucleopore Track-Etch Membrane Filters, Whatman) and washed 2 times with 1xPBS (phosphate buffered saline, appendix A.1.7). Finally, the proportion of living (green fluorescent, SYTO® 9) to dead (red fluorescent, propidium iodide) cells was determined by counting 500 cells in random microscopic views, under the fluorescence microscope (microscopic filter cube I3, Table 2.2).

2.7.7 Determination of cell density in the bacterial cultures

In Experiment No. 5 cell densities (cells ml^{-1}) of the bacterial pure cultures had to be determined. To count bacterial cells, diluted samples of the cultures were filtered on polycarbonate filters. After filtration, the filters were embedded in agarose to avoid loss of cells. Then, the cells were stained with DAPI. Embedding of filters in agarose and DAPI staining was performed according to the counter-staining in the CARD-FISH protocol provided in section 2.7.9. Counting was performed with a fluorescence microscope (Leitz Aristoplan, 1000x magnification). In random view fields bacterial cells located in the counting grid were counted until a number of 500 cells was exceeded. The cell density in the bacterial cultures was calculated according to equation 2.5.

$$Cells\, ml^{-1} = [(CC\ - BC)\, x\, MF\, x\, DF] \tag{2.5}$$

CC = Average cell number/ counting grid

BC = Blank filter

MF = Microscope factor; MF = FF/GF

FF = Filter area [mm^2]

GF = Size of counting grid [mm^2]

DF = Dilution factor

2.7.8 Determination of sulfate-reduction rates with a radiotracer technique

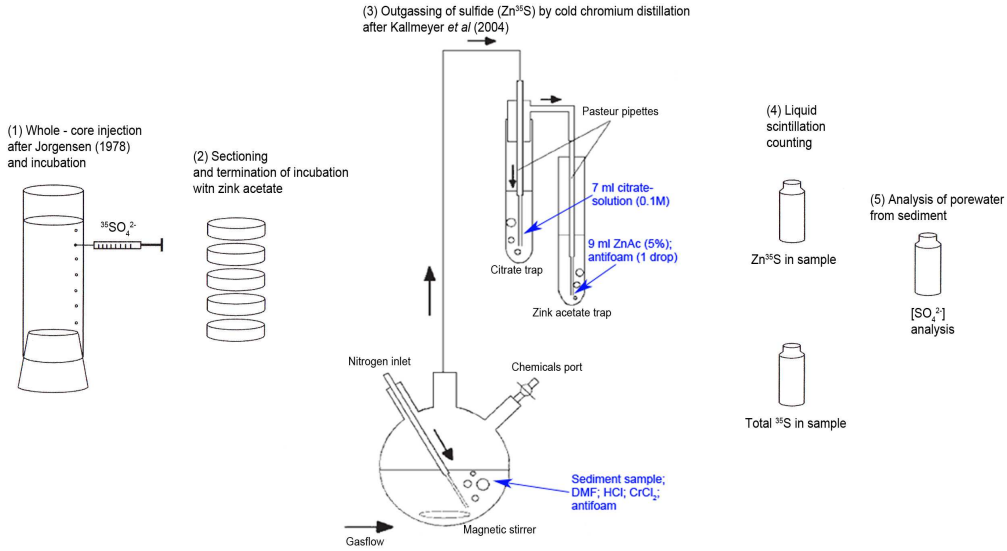

Figure 2.6: *Schematic of the sulfate-reduction rate measurement with radioactive* $^{35}SO_4{}^{2-}$ *tracers.* Procedures and amounts of chemicals are specified in the text, if not specified in the graph. Modified according to Kallmeyer *et al.* (2004) and Jorgensen (2006).

In Experiment No. 6, the activity of sulfate-reducing bacteria over sediment depth was determined by measuring sulfate-reduction rates over sediment depth in the differently treated sediment cores. Therefore, after the mini-cores were incubated for 73 days at 13°C, a radioactive $^{35}SO_4{}^{2-}$ tracer was injected into the mini-cores, according to the whole-core injection method by Jorgensen (1978, Fig. 2.6), which is commonly used for the determination of sulfate reduction rates in marine sediments. After injection of the radioactive tracer and further incubation for about 19 h, sulfate-reduction rates were determined and calculated according the cold chromium distillation method by Kallmeyer *et al.* (2004, Fig. 2.6)

Whole core injection, incubation, and slicing of the mini-cores (Fig. 2.6, 1+2)

Into each of the sediment mini-cores 6 µl of carrier-free $^{35}SO_4{}^{2-}$ tracer (123 kBq/µl) was evenly injected with a micro-syringe through the silicon-sealed holes in 1 cm intervals. After injection, the sediment cores were incubated for 18.5 hours at 13°C in the dark, before the cores were

sliced in 1 cm intervals and the incubation was terminated by transferring each cm section of sediment into a reaction vial (50 ml) containing 20 ml 20% zinc acetate (w/v). The ZnAc stops the microbial activity and prevents the loss of sulfide (ZnS forms out of sulfide).

Cold chromium distillation and measurement of radioactivity by liquid scintillation counting (Fig. 2.6, 3+4)

The cold chromium distillation method was performed according to the protocol by Kallmeyer *et al.* 2004. This method was used for the separation of sulfide, or other reduced sulfur species (including ^{35}S sulfide and other reduced ^{35}S sulfur species), from the remaining sulfate species in the sample (including the remaining $^{35}SO_4{}^{2-}$ tracer). Later, the radioactivity of the reduced ^{35}S species in the sample, and the total ^{35}S radioactivity in the sample were measured with a liquid scintillation counter (Tri-Carb 2100 TR Liquid Scintillation Counter, Packard). So that, with the ratio of $^{35}SO_4{}^{2-}$reduced during the time of incubation, sulfate-reduction rates in the samples could be calculated (Equation 2.6).

Before the cold chromium distillation, vials containing the 1 cm sediment sections and 20 ml ZnAc were weighted and centrifuged for 5 minutes (4500 rpm, 1100 g, room temperature). For the measurement of the total ^{35}S radioactivity of the sample, 100 µl of the supernatant of each sample were sampled and diluted with 1 ml of H_2O in a small scintillation vial (6 ml). Then, the rest of the supernatant was discharged and the vial containing only the sediment was weighted again. Next, the sediment samples were solubilized mechanically in 20 ml DMF (Dimethylformamide, undiluted) and transferred into the round glasses for distillation (see Fig. 2.6). Then, the empty vials were weighted again, so that finally the weight of the sediment-sample could be determined. Into the round glasses, before the addition of the sample, one drop of Antifoam (silicone emulsion, J.T. Baker) was added to avoid foaming when later HCl was added.

Now, the whole distillation apparatus shown in Fig. 2.6 was build up. Next, stirring (magnetic stirrer) and N_2-bubbling was started before 8 ml of HCl (6 M) and 16 ml of chromium-(III)-chloride (1 M) were added to the sample. Afterwards, the distillation was run for 2 h under

constant stirring and N_2 bubbling (2-5 bubbles/second). During the last 15 minutest of the distillation N_2 flow was increased (10-15 bubbles/second). After distillation, all reduced sulfur species should be be trapped in the ZnAc (5%; w/v) trap (Fig. 2.6). The ZnAc trap was then vortexed and the ZnAc containing the reduced sulfur species was transferred into a large scintillation vial (20 ml).

Finally, the liquid scintillation cocktail (Ultima Gold XR, Perkin Elmer) was added into the scintillation vials (3 ml into the small vials, and 12 ml into the large vials), and the ^{35}S radioactivity was determined by scintillation counting. Simultaneously with the samples, also blanks containing 1 ml of H_2O, and 3 ml of the scintillation cocktail (in small scintillation vials), and blanks containing 9 ml ZnAc (5%, w/v), 12 ml scintillation cocktail, and one drop of Antifoam (in large scintillation vials), were measured in the scintillation counter.

Measurement of sulfate concentrations (Fig. 2.6, 5)

Sulfate concentrations in the pore water were measured with a ion chromatograph (Metrohm ion-chromatograph) equipped with an anion-exchange column. Carbonate-bicarbonate solution was used as an eluent. Measurements were calibrated with IAPSO standard seawater.

The porewater for the measurement was sampled from the left-over sediment of the mini-cores, which were used for the CARD-FISH analysis (before slicing these mini-cores were treated in the same way like the mini-cores used for determination of sulfate-reduction rates; mini-cores for CARD-FISH were also sliced in 1 cm sections). Approximately 2 ml of the sediment from each sediment depth of each core was sampled and transferred into reaction vials (2 ml). The reaction vials containing the sediment samples were then centrifuged for 15 minutes at maximum speed in a ultra centrifuge. Afterwards, 1 ml of the supernatant was transferred into new reaction vials (1.5 ml). This supernatant was the porewater sample used for determination of sulfate concentration in the different sediment depths by ion chromatography.

Calculation of sulfate-reduction rates

Sulfate-reduction rates were calculated according to Equation 2.6 (Kallmeyer *et al.*, 2004). The porosity of the sediment, what is a parameter that is needed for calculation of sulfate-reduction rates, was not determined in this study, but already existing data from a recent study by Bertics et al. (2012) were used (appendix section A.2.4.1).

$$SRR = [SO_4^{2-}]\, x\, P_{SED}\, x\, \frac{a_{TRIS}}{a_{TOT}}\, x\, \frac{1}{t}\, x\, 1.06\, x\, 1000 \tag{2.6}$$

SRR	= Sulfate-reduction rate [nmol cm^{-3}sediment d^{-1}]
$[SO_4{}^{2-}]$	= Concentration of sulfate in the porewater of the sample [mM]
P_{SED}	= Porosity of the sediment [ml porewater/ ml sediment]
a_{TRIS}	= Radioactivity of TRIS (total reduced inorganic sulfur) [cpm]
a_{TOT}	= Total radioactivity of ^{35}S [cpm]
t	= Incubation time [days]
1.06	= Correction factor for isotopic fractionation
1000	= Conversion factor for change of units from mmol L^{-1} to nmol cm^{-3}

2.7.9 CARD-FISH analysis of the microbial community

Catalyzed reporter deposition-fluorescence in-situ hybridization (CARD-FISH) is a method, which besides many other possible applications, can be used for the determination of the abundance of specific microbial groups within samples of natural sediments (Ishii *et al.*, 2004).

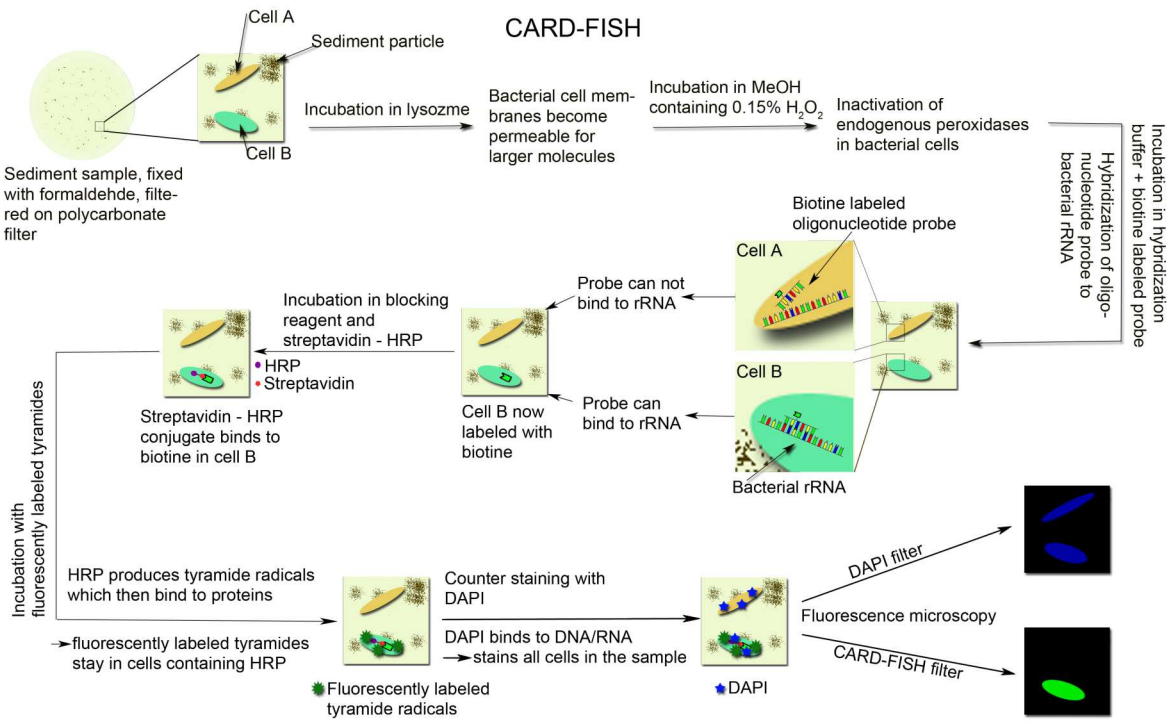

Figure 2.7: *Flow chart of the CARD-FISH method.* Important steps are indicated in this graph. For more detailed description of the method applied, see text.

The principle of the method is, that an oligonucleotide probe, which is specific for the rRNA of a certain microbial group, is hybridized with the rRNA of the microbes present in the sample. The oligonucleotide probe is labeled with the Horseradish peroxidase (HRP). When then fluorescently labeled tyramide is added, this tyramide is converted to radicals by the HRP, which is only present in the cells where the oligonucleotide probe could bind to the rRNA. The radicals of the fluorescently labeled tyramide are likely to bind to electron-rich side chains of amino acids of proteins within the cells. Therefore, the radicals become deposited in cells containing the HRP. One single HRP molecule can produce several tyramide radicals. What leads to the effect, that

many fluorescent molecules become deposited within the oligonucleotide-marked cells, what leads to an amplification of the signal. This amplification increases the sensitivity of the CARD-FISH method compared to the FISH method, where singularly fluorescently labeled oligonucleotides are used. Thus, CARD-FISH makes it possible to detect cells with low rRNA content. The principle of the method, as it was applied here, is illustrated in Fig. 2.7.

In the following, the exact CARD-FISH procedure applied in this study is described. The procedure represented modifications of the protocols by Pernthaler *et al.* (2002), Ishii *et al.* (2004), and a protocol, which is described in detail by Gier (2012). All recipes for the solutions used, are listed in the appendix (section A.1.7).

General remarks for the procedure

(1) Filters could be stored at -20°C after each step that ended with air drying of filters.

(2) If no temperature is specified, steps were done at room temperature (ca. 19°C).

(3) If not specified in the text, all incubation- and washing steps were done in small Petri dishes (55 mm diameter).

(4) If no time for washing steps or air-drying steps are not specified in the text, duration was only ca. 1 minute.

Slicing of the mini-cores and preparation of the sediment samples for CARD-FISH
Sampling of the mini-cores was done after 73 days of incubation at 13 °C. The mini-cores were sliced into 1 cm sections. Each of these sections was mixed well and 0.5 ml of each section was sampled with a cut-off syringe. The 0.5 ml were transferred into a Greiner tube (12 ml) containing 1.5 ml of 4% formalin in 1xPBS. Therein the samples were incubated for 2-4 hours at 4°C. The remaining sediment of each slice was transferred into whirlpack bags and stored at -20°C. After incubation, the formalin had to be removed from the samples. Therefore, three washing steps with 1xPBS, each followed by centrifugation (10 minutes at 4500 rpm, 1400 g, at 4°C), were applied. After the last washing, the sample was re-suspended in 1.5 ml 1xPBS/EtOH (1:2). Finally, the sample was transferred into cryotubes and stored at -20°C.

Preparation of filters

The next step was to filter the samples on white polycarbonate filters (pore size: 0.2 µM, diameter: 25 mm, Nucleopore Track-Etch Membrane Filters, Whatman). Therefore, at first a 1:40 dilution of each of the sediment samples, which were dissolved in 1.5 ml 1xPBS/EtOH (sample already diluted 1:4), was produced. The 1:40 dilution was prepared with 675 µl of 1xPBS/EtOH and 75 µl of the 1:4 diluted sediment sample in a 15 ml reaction vial. The size of the reaction vial was that large in order to avoid loss of sample during the subsequent sonication step. The sonication step is necessary as it separates bacterial cells from sediment particles and helps to achieve a more equal distribution of bacteria on the filter. Sonication was performed with a Bandelin Sonoplus GM 200 for 20 seconds with an intensity of 40 W and a cycle of 20. The tip of the sonicator pointed directly into the sample. During sonication the reaction vial containing the sample was placed in ice-water to avoid heating of the sample. This sonication step was done twice.

Afterwards, the sample was diluted to the working dilution of 1:3000 with 1xPBS (80 µl 1:40 dilution of the sediment sample and 5920 µl 1xPBS). This working solution was then filtered onto the white polycarbonate filters with a filtration device. Therefore, underneath the white polycarbonate filter, which had to be placed shiny side up onto the filtration device, a cellulose nitrate filter (pore size : 0.45 µm, diameter: 25 mm, Satorius) had to be placed. The cellulose nitrate filter served as a carrier filter and could be used for multiple filtrations.

For filtration, at first 5 ml of 1xPBS were added into the filtration tower. Then, 1 ml of the working solution of the sample was added. Next, this mixture of 1xPBS and the working solution was filtered through the filter with -500 mbar pressure. Afterwards, the filtration tower was rinsed with 2 ml 1xPBS, which was then also filtered through the white polycarbonate filter with -500 mbar. After filtration, the white polycarbonate filters were dried on a chromatography paper (Whatman).

Next, the white polycarbonate filters were embedded in agarose to avoid cell-loss during the CARD-FISH procedure. Embedding was done with 0.2% low melting agarose in H_2O_{UP}. The agarose was boiled in beforehand and let cool down to approximately 40°C before usage. Then, 1 drop of agarose (approximately 100 µl) was placed in a Petri dish and the filter was pulled upside down through this drop. Then, the filter was put cell side up into a Petri dish, let dry for

30 minutes at 46°C, and finally dehydrated by washing it shortly in 96 % EtOH. Next, the filter was air dried on a chromatography paper.

After this step, only a 1/8 section of the each filter from each sample was further processed. The remaining 7/8s of each filter were stored at -20°C.

Permeabilization of the microbial cells

In the next step the cell membrane of the microbial cells were made permeable, so that the oligonucleotide probes and other substances used for CARD-FISH can enter the interior of the cells. Therefore, the filters were incubated for 60 minutes at 37°C in a lysozyme solution (10 mg/ml). Afterwards, the filters were washed in H_2O_{UP}. It was important to not let the filters run dry after this step.

Inactivation of endogenous peroxidases

After the cell membrane was made permeable, the next step was to inactivate the endogenous peroxidases. This step was crucial as the endogenous peroxidases are also produce tyramide radicals, what would lead to false positive CARD-FISH stainings. For this inactivation step, the filters were taken out of the H_2O_{UP} and directly transferred into MeOH containing 0.15% H_2O_2. In this solution they were incubated for 30 minutes at room temperature. Afterwards, the filters were washed first in H_2O_{UP} and then in 96% EtOH before they were air dried on a chromatography paper.

Hybridization of the oligonucleotide probe

The next step was the hybridization of the bacterial rRNA with the biotine labeled oligonucleotide probe. Therefore, up to 4 filter sections were placed in a 1.5 ml reaction tube containing 300 µl hybridization buffer (35% formalin) and 1 µl of the biotine marked probe (50 ng/µl). The oligonucleotide probe used was the DSV 698 probe (Probe sequence: GTT CCT CCCA GAT ATC TAC GG; Manz *et al.*, 1998), which is specific for bacteria belonging to the *Desulfovibrio spp.* group. The filters were incubated in this solution for 2.5 h at 46 °C. Afterwards, the filters were first washed for 10 minutes in 48°C warm washing buffer (in a 50 ml reaction tube), for 15 minutes in 1xPBS, and lastly shortly in 2x salinesodiumcitrate (SSC). After washing, the filters should not be let run try.

Coupling of the Horseradish peroxidase to the oligonucleotide probe

Now, as the biotine labeled probes were hybridized with the bacterial rRNA, the probes had to be labeled with the horseradish peroxidase (HRP). This coupling was done with the Tyramide Signal Amplification Kit (Molecular Probes, Invitrogen). Directly after the washing step with 2x SSC, each filter section was placed upside down on a 100 µl drop blocking reagent in 1xPBS and incubated for 30 minutes at room temperature. Then, the filter sections were incubated in a streptavidin-HRP:Blocking Reagent solution (1:100) for 30 minutes at room temperature in a small reaction tube (1.5 ml). Next, the filter sections were washed for 10 minutes in 1xPBS. After this step it was again important to not let the filters run dry, as this would reduce the activity of the HRP.

Catalyzed Reporter Deposition - CARD

In this step, the cells, whose rRNA has bound the now HRP-streptavidin-biotine labeled oligonucleotide probe, were made fluorescent. Therefore, a fluorescently labeled tyramide was used (Alexa 488). As the fluorescent dye is sensitive to light, it was important that all of the following steps were done under low light conditions. For the CARD reaction, up to four filter sections were placed in a reaction vial (1.5 ml) containing 250 ml amplification buffer, 2.5 µl PBS/ H_2O_2 (0.15%), and 0.5 µl of the fluorescently labeled tyramide (Alexa 488, 1 µg/ml) per filter section. After incubation in this mixture for 20 minutes at 46°C, the filter sections were washed for 10 minutes in 1xPBS.

Inactivation of the Horse Radish Peroxidase and Washing

Finally, the HRP was inactivated by washing the filter sections in 0.01 M Tris HCl for 10 minutes. Afterwards, the filter sections were washed in 1xPBS for 5 minutes, shortly in H_2O_{UP}, shortly in 96% EtOH, and were then, finally, air dried on a chromatography paper.

Counter Staining with 4',6-diamidino-2-phenylindole (DAPI)

In order to determine the total microbial abundance, counter-staining with DAPI was done. DAPI is a unspecific fluorescent dye, which binds to DNA and RNA, so that all microbial cells were stained. For the staining 50 µl drops of DAPI (1 µg/ml) were placed on a petri dish. Then, each filter section was dipped upside down onto a drop of DAPI and placed in the petri dish with the cell side up again. After incubation with DAPI for 10 minutes in the dark, the filter sections were washed first in H_2O_{UP} and then in 96% EtOH. Next, the filter sections were let air dry.

Fluorescence microscopy and determination of total microbial abundance and abundance of CARD-FISH stained cells

Finally, for counting of the DAPI and CARD-FISH stained cells under the fluorescence microscope (Leitz Aristoplan, 1000x magnification), filter sections were embedded in Citifluor (Glycerol/PBS solution, Citifluor Ltd.) and placed on glass slides.

For determination of the number of DAPI and CARD-FISH stained cells, for both stains, numbers of bacterial cells stained with the respective dye were determined in random microscopic views. Counting was done until either a cell number of 500 was exceeded or more than 70 random views were counted. Cell numbers were then calculated according to Equation 2.5. The filter cubes used for fluorescence microscopy for the different fluorochromes (DAPI or Alexa 488) are listed in Table 2.2.

Table 2.2: *Data of the filter cubes used for fluorescence microscopy.*

Filter cube	Excitation range [nm], spectrum	Emission range [nm]	Dichromatic mirror [nm]	Fluorochrome
A	340-380, UV	450-490	400	DAPI
L5	460-500, blue	512-542	505	Alexa 488
I3	450 - 490, blue	500-635	510	SYTO 9, propidium iodide

2.8 Statistics

In order to reveal the significance of the results, statistics were applied to results of selected experiments. For which experiments there was statistical analysis done was decided depending on two factors: (1) statistics were only done if the requirements for statistical analyses were met, e.g. if at least3 replicates were present. (2) Statistics were only done if statistical analysis made sense, i.e. if information on significance of observed differences was any helpful or needed for interpretation of the results.

Statistical analyses were done with the program “R” (version 2.15.0., The R Foundation for Statistical Computing). The first step of each statistical analysis was to test for normal distribution of the data. The Shapiro-Wilk test for normal distribution was used to test for that. If normal-

distribution was given, parametric tests were used (T-test for comparison of two samples; one-way ANOVA, followed by a TukeyHSD post-hoc test for comparison of more than two samples). If the parametric tests were significant, a Fligner-Killeen test was done to test for homogenous variances. If normal distribution of data or homogeneity of variances was not given, a non-parametric test, e.g. Mann-Whitney U-Test for two samples, was applied.

3 Results

Five different experiments, to investigate how the addition of crude oil affects the metabolic activity and abundance of sulfate-reducing bacteria in marine sediments, were conducted in this study (Experiment No. 1, 2, 3 a+b, 5, and 6). Additionally, methane production in oil-treated sediment slurries was investigated in two experiments (Experiment No. 1 and 2). In another experiment the degradation of benzene by marine sediment bacteria was studied (Experiment No. 4).

Two different types of crude oil were added to sediment slurries and mini-cores in different concentrations, to observe, if the amount or type of crude oil has an influence on the response of sulfate-reducing bacteria within natural marine sediments. If in the following values are given with ± SD, n was always three.

3.1 General observations and remarks

After addition of the crude oil to the sediment slurries, the crude oil was floating on the surface of the slurries. After 2-3 days of incubation, flocks of oil started to settle down but the majority of the oil was still floating on the surface. At the same time, the overall appearance of the floating oil changed. The volume of the floating oil increased and formation of a "mousse", which is a stable water-in-oil emulsion, was observed (see Fig. 3.1, Committee on Oil in the Sea, 2003). Further, few days after addition of the crude oil, gas bubbles rose from the sediment. These bubbles only appeared in the slurries, which were treated with crude oil, but not in the controls.

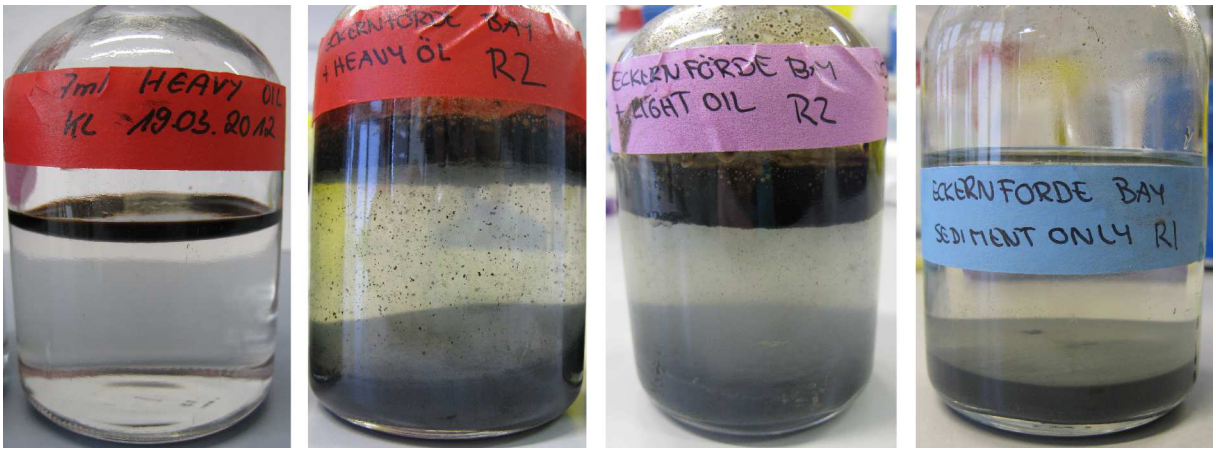

Figure 3.1: *Slurries and medium with crude oil.* From left to right: medium + heavy oil, slurry + heavy oil, slurry + light oil, slurry without oil (all after 3 days of incubation). In the slurries where crude oil was added, the formation of the "mousse" and the flocks of crude oil can be seen. It can also be seen that flocks in the slurry with heavy oil are larger than the flocks in the slurry treated with light oil. In the vial containing just medium and oil, no formation of "mousse" or flocks was observed.

3.2 Experiment No. 1: Batch incubations of sediment slurries treated with 5.7% crude oil

3.2.1 Development of sulfide concentrations and potential rates of sulfate-reduction

In the oil-treated slurries, sulfide concentrations stayed constant (at 0.44 ± 0.31 and 0.46 ± 0.29 mM for the heavy- and light oil treated slurries, respectively) within the first 95 days of the incubation period (Fig. 3.2). After this lag-phase, sulfide concentrations in the oil-treated slurries started to rise, until maximum sulfide concentrations of 16.54 ± 2.9 mM and 20.37 ± 0.4 mM were reached at the end of the incubation time, after 255 days, in the heavy- and light oil treated slurries, respectively.

In the controls, the sulfide concentrations stayed constant (at 0.25 ± 0.15 mM) over the whole time of incubation (Fig. 3.2).

Potential sulfate-reduction rates (in nmol cm^{-3} undiluted sediment d^{-1}) during the first time-interval in the heavy oil samples (lag-phase from 0-95 d) were 18.6 ± 8.08 nmol cm^{-3} d^{-1}

(Fig. 3.3). In the light oil treated samples, potential sulfate-reduction rates during the first time-interval (lag-phase from 0-130 d) were with 30.3 ± 4.04 nmol cm^{-3} d^{-1} about two times higher than in the heavy oil treated samples. The potential rates found for the oil-treated slurries in this phase were about one order of magnitude higher than in the control slurries, for which rates of 3.96 ± 2.14 nmol cm^{-3} d^{-1} were calculated. An ANOVA, followed by a TukeyHSD post-hoc test revealed significant differences between the potential sulfate-reduction rates in this first phase between all three treatments (p-values < 0.05).

In the second time-interval, potential sulfate-reduction rates for the heavy oil treated samples (103-207 d) were 406.00 ± 116.86 nmol cm^{-3} d^{-1} (Fig. 3.3). For the light oil treated slurries potential sulfate-reduction rates during the second interval (134-207 d) were with 845.13 ± 121.64 nmol cm^{-3} d^{-1}, again about twice as high as in the heavy oil treated slurries. A T-test revealed significant differences between the potential sulfate-reduction rates during the second time-interval in the slurries treated with heavy- or light oil (p-value = 0.01).

In the third time-interval (211-227 d), potential rates of sulfate-reduction were almost similar in the slurries treated with the two different crude oil types (3103.10 ± 188.82, heavy oil; and 2946.07 ± 188.82 nmol cm^{-3} d^{-1}, light oil; Fig. 3.3). A Mann-Whitney-U Test revealed no significant difference in potential sulfate-reduction rates between the two treatments during the third time-interval (p-value = 1). For the duration of the three time-intervals for which potential rates of sulfate-reduction were calculated for the different samples see Table 3.1.

Summarized, sulfide concentration started to increase earlier in the heavy oil treated slurries. However, initial rates were lower compared to the light oil treated slurries. Finally, (after 211 d) the turnover rates of sulfate in sediment slurries treated with the two different crude oil types were almost the same (see Fig. 3.2 and 3.3).

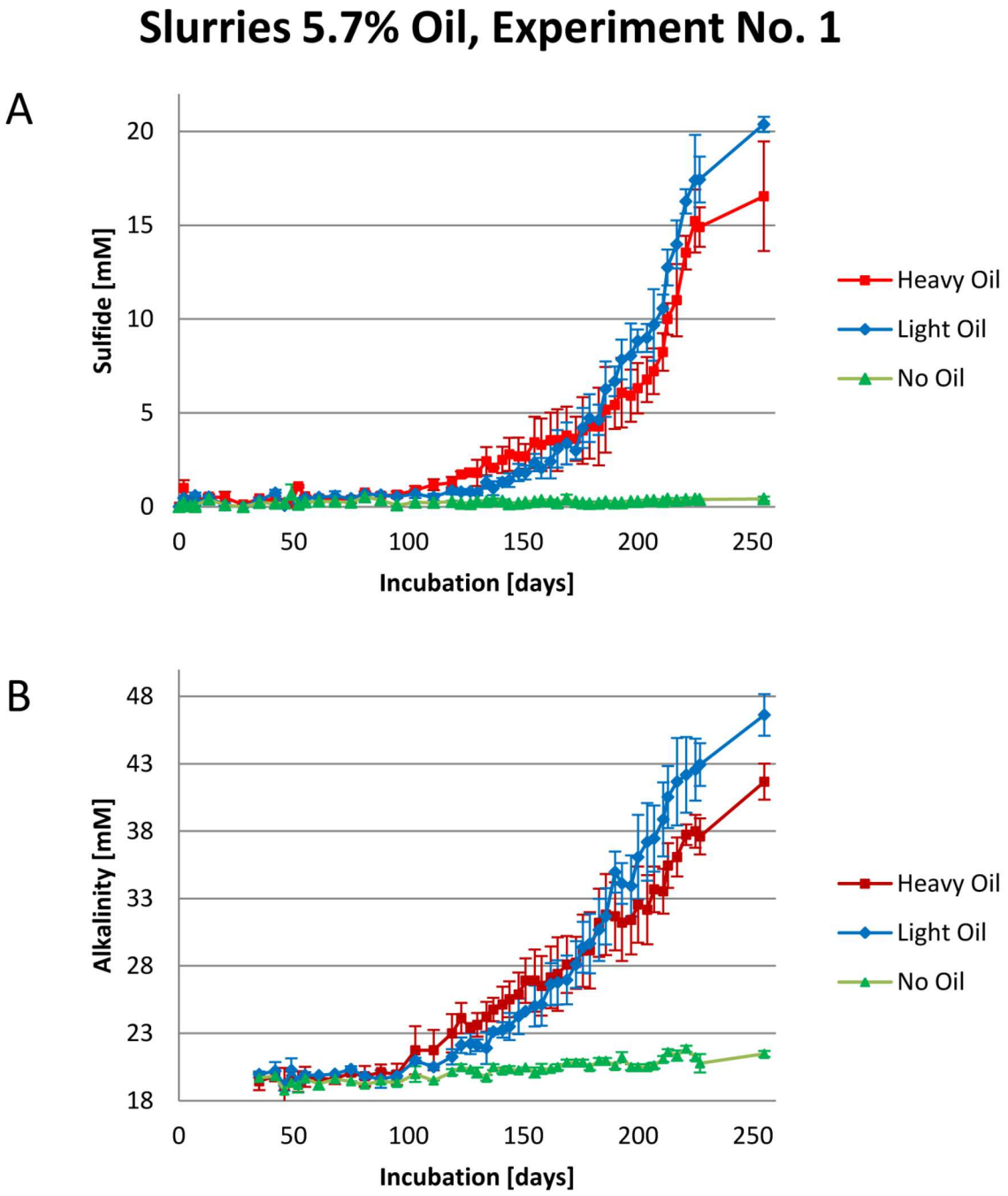

Figure 3.2: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in sediment slurries from Experiment No. 1.* Values are means (± SD, n = 3).

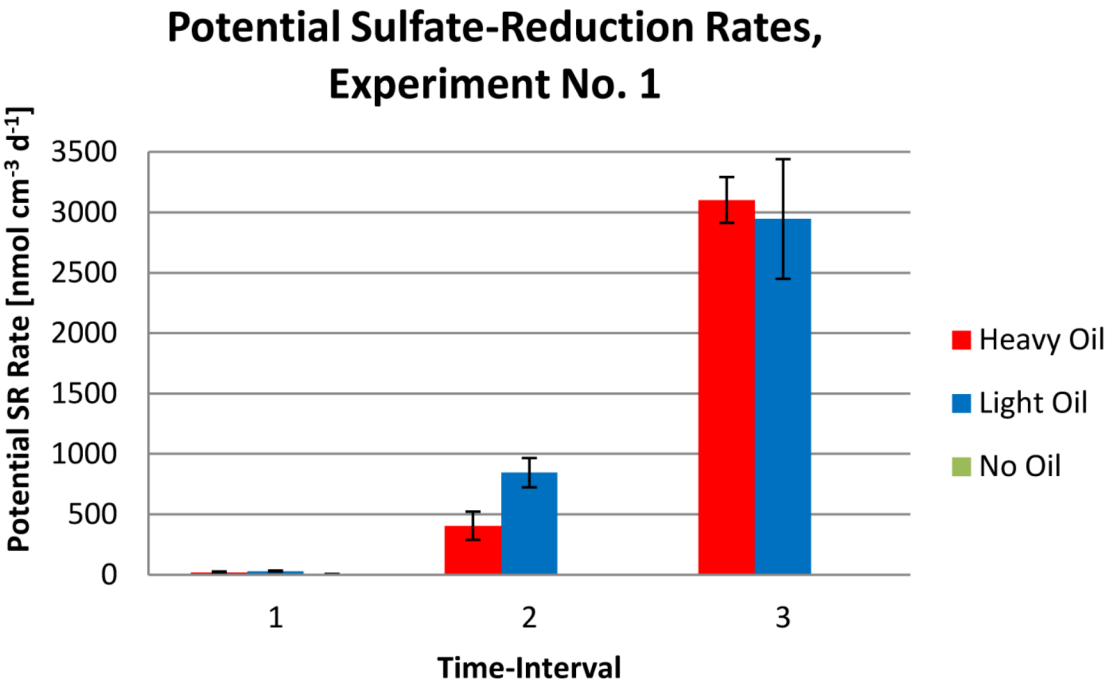

Figure 3.3: *Potential rates of sulfate-reduction in slurries from Experiment No.1 during different time-intervals of incubation.* The duration of the different time intervals for the different samples are shown in Table 3.1 below this graph. Rates were calculated in nmol cm^{-3} undiluted sediment d^{-1}. Values are means (± SD, n = 3).

Table 3.1: *Time-intervals used for calculation of the potential sulfate-reduction rates, which are shown in Fig. 3.3.*

Sample	Time-Interval 1 [days]	Time-Interval 2 [days]	Time-Interval 3 [days]
Heavy Oil	0-95	103-207	211-227
Light Oil	0-130	134-207	211-227
No Oil	0-227	—*	—*

* No rate calculated as no increase in sulfide was observed.

3.2.2 Development of total Alkalinity

Measurement of total alkalinity started after 35 days of incubation. The measurement intervals were the same like for the sulfide measurements.
The development of total alkalinity over time was similar to the one found for sulfide concentrations. Also a long lag-phase of about 100-130 days, without considerable change of total alkalinity, was observed in the oil-treated slurries (Fig. 3.2).

In the heavy oil treated slurries total alkalinity remained constant (at 19.69 ± 0.63 mM) over the first 95 days of incubation (Fig. 3.2). Values of total alkalinity started to rise after 103 days and reached a maximum of 41.67 ± 1.34 mM after 225 days. The high increase after 211 days, like it was found for sulfide, was not found for alkalinity.

In the light oil treated slurries, total alkalinity stayed constant (at 19.98 ± 0.56 mM) over the first 111 days of incubation (Fig. 3.2). A slight increase in total alkalinity was observed after 119 days, five days before sulfide concentrations started to increase in the light oil treated slurries. But this increase was very low. Steeper increase of total alkalinity in the light oil treated slurries started after 141 days of incubation, at the same time when also the rise of sulfate concentrations was observed. Maximum values of total alkalinity in the light oil treated slurries were reached at the end of the incubation time, after 255 days, with 46.62 ± 1.54 mM.

In the control slurries no relevant increase in total alkalinity was observed. Total alkalinity remained constant at 20.27 ± 0.74 mM during the whole incubation time (Fig. 3.2).

3.2.3 Methane content in the gas headspace

Until 100 days of incubation, there was a general increase of methane content measured in all samples, regardless if they were treated with crude oil or not. Afterwards, methane content was decreasing again (Fig. 3.4). This change of methane content was more pronounced in oil-treated slurries. Maximum values of methane content and the change of methane content over time of incubation were highest in heavy oil-treated slurries (maximum content 553.93 ± 164.55 ppm after 102), followed by the light-oil treated slurries (maximum content 389.04 ± 25.18 ppm after 102 d), and lowest in the control slurries (186.57 ± 68.26 ppm after 127 d).

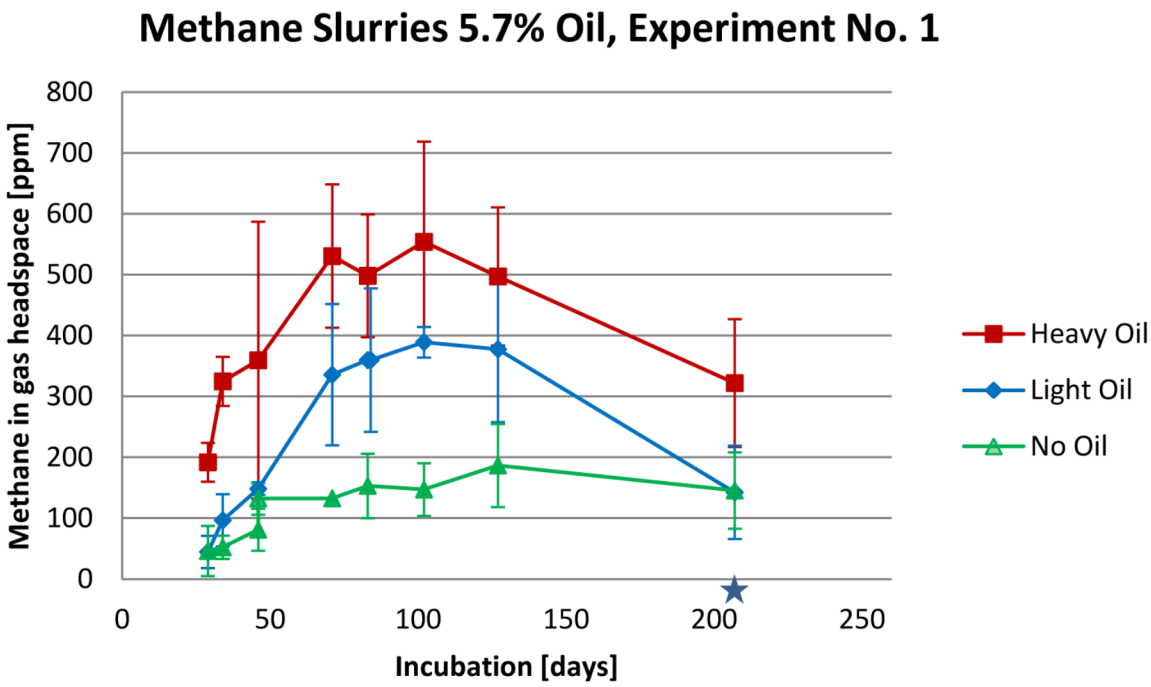

Figure 3.4: *Development of methane content in the gas headspace of the sediment slurries from Experiment No. 1 over time of incubation.* Asterisk marks time-point of measurements, which were done with the Shimadzu GC, others were measured with the HP.
Values are means (± SD, n = 3).

3.3 Experiment No. 2: Batch incubations of sediment slurries treated with 0.57% crude oil

In Experiment No. 2, two different types of slurries (prepared with sediment from 0-10 or 20-30 cm sediment depth) were treated with 0.57% (v/v) of either heavy- or light crude oil and incubated for 181 days. The slurries from the second sediment depth (20-30 cm) were included in this experiment, because of the observed increase in methane content in Experiment No. 1. In Experiment No. 1 only slurries produced with sediment from 0-10 cm sediment depth were used. As in deeper sediment layers usually there should be more methanogens, the effect of crude oil on the activity of methanogens in deeper sediment layers was tested. Furthermore, a different crude oil concentration than in Experiment No. 1 was used to test if crude oil concentration influences activity of sulfate-reducing bacteria. During incubation, sulfide concentration and total alkalinity in the supernatant of the slurries, and methane content in the headspace were determined in regular intervals.

3.3.1 Development of sulfide concentrations and potential rates of sulfate-reduction

In the sediment slurries from 0-10 cm sediment depth, which were treated with crude oil, sulfide concentrations remained constant (0.31 ± 0.13mM, heavy oil; 0.34 ± 0.15 mM, light oil) over the first 126 days of incubation (Fig. 3.5). Afterwards, sulfide concentrations started to rise (max. concentration at end of incubation time, after 180 days: 4.79 ± 0.46 mM for heavy oil; 8.63 ± 1.91 mM for light oil).

In the sediment slurries from 20-30 cm sediment depth, which were treated with crude oil, sulfide concentrations remained constant (at: 0.45 ± 0.28 mM, heavy oil; 0.36 ± 0.16 mM, light oil) during the whole time of incubation (Fig. 3.6).

Sulfide concentrations in the controls from both sediment depths remained constant (0.25 ± 0.15 mM, 0-10 cm; 0.26 ± 0.16 mM; 20-30 cm) during the whole time of incubation (Fig. 3.5 and 3.6).

Potential rates of sulfate-reduction (always calculated in nmol cm^{-3} undiluted sediment d^{-1}) during the first time-interval were negative for all samples (appendix, Table A.5), indicating that there must have been loss of sulfide. What could have caused this loss will be discussed later in the discussion.

In the second time-interval highest potential rates of sulfate-reduction were found in the light oil treated slurries from 0-10 cm sediment depth (1770.30 ± 164.11 nmol cm^{-3} d^{-1}, from 133-146 d; Fig. 3.7), followed by the heavy-oil treated slurries from 0-10 cm sediment depth (826.00 ± 513.50 nmol cm^{-3} d^{-1}, from 133-146 d). Again, similar to Experiment No. 1, potential sulfate-reduction rates in the light-oil treated slurries in the second time-interval were significantly higher (about two times higher; T-test, p-value = 0.02) than in the heavy-oil treated slurries. For the duration of the two time-intervals for which potential rates of sulfate-reduction were calculated for the different samples see Table 3.2.

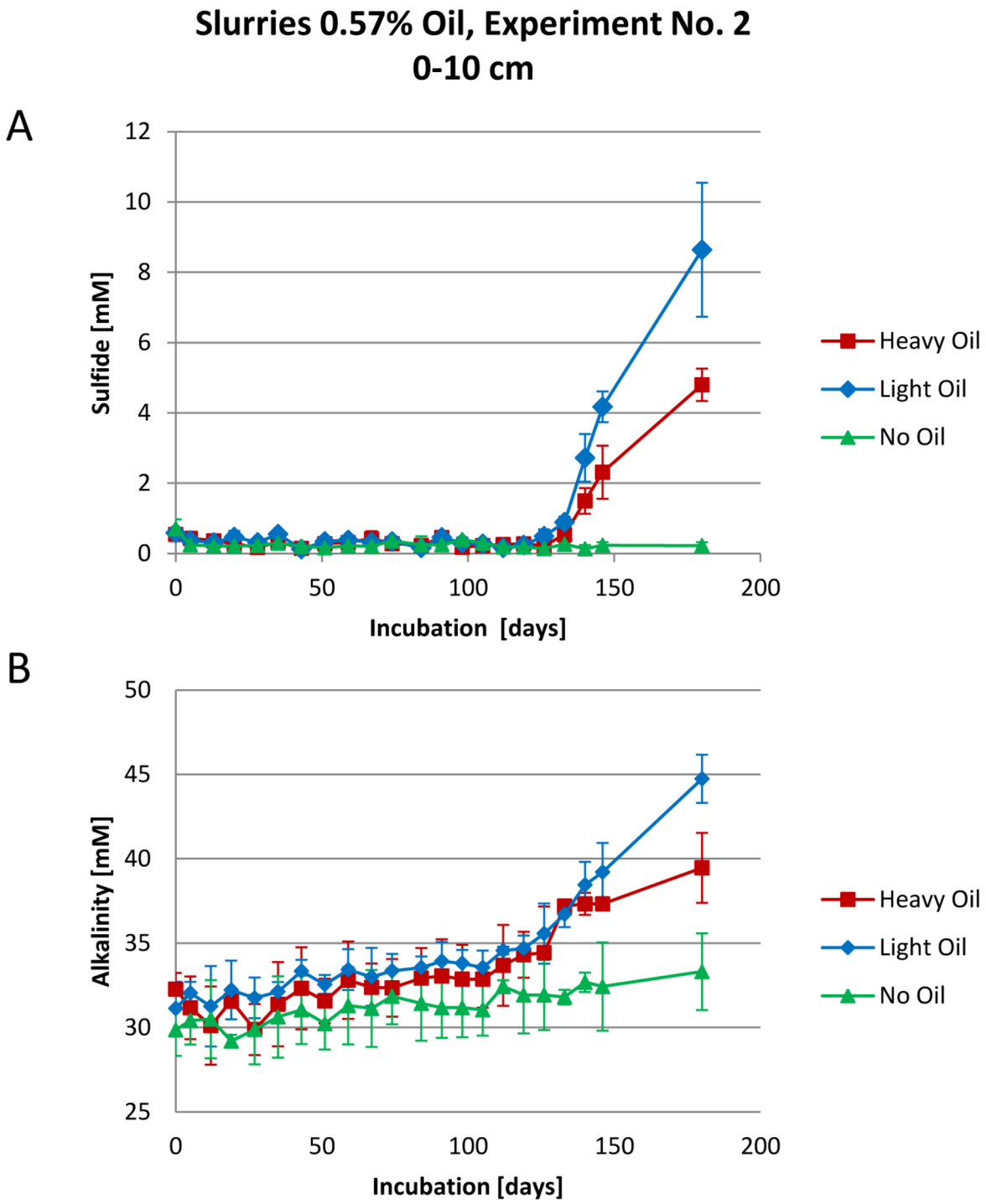

Figure 3.5: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the slurries from 0-10 cm sediment depth from Experiment No. 2.*
Values are means (± SD, n = 3).

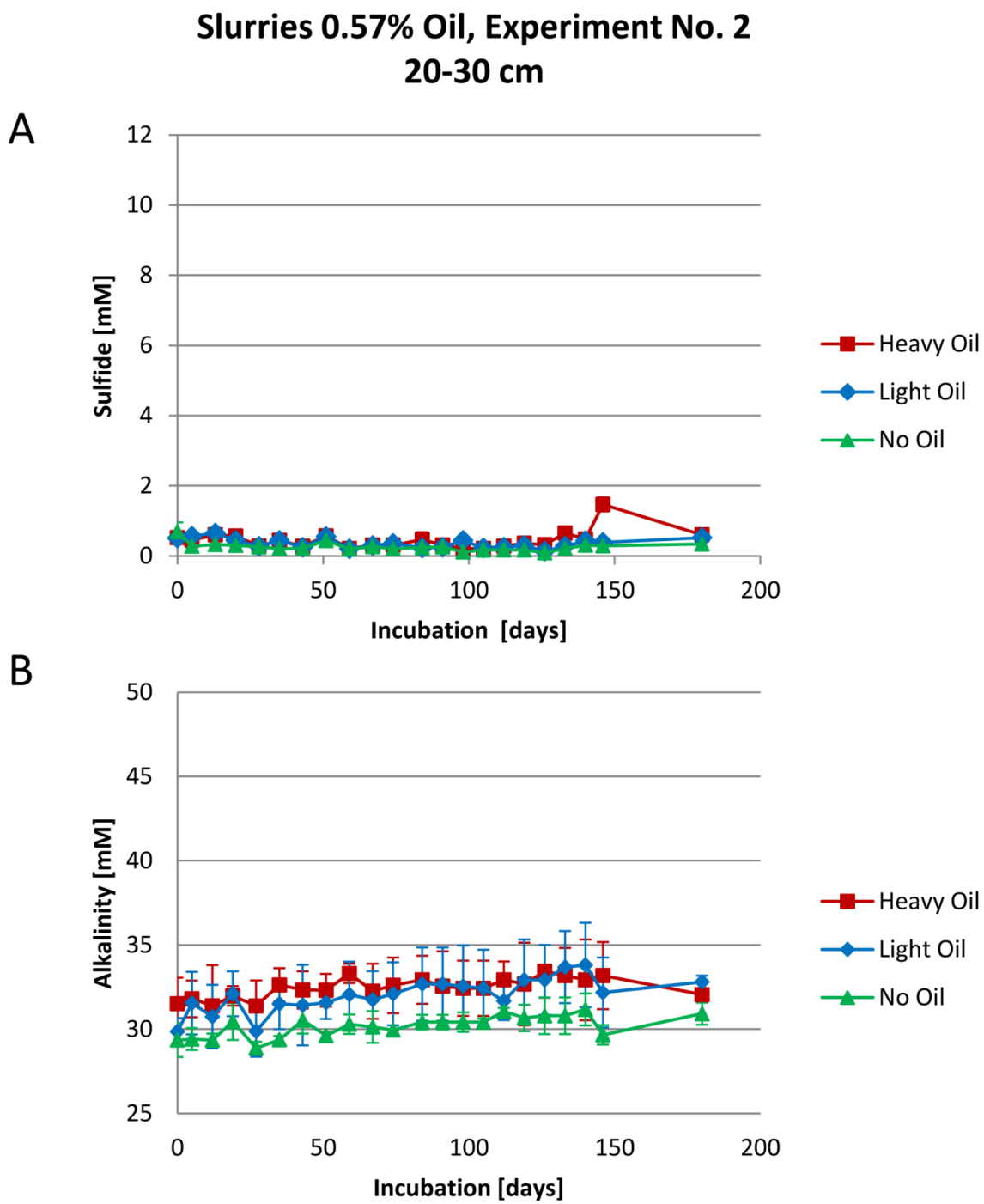

Figure 3.6: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the slurries from 20-30 cm sediment depth from Experiment No. 2.*
Values are means (± SD, n = 3).

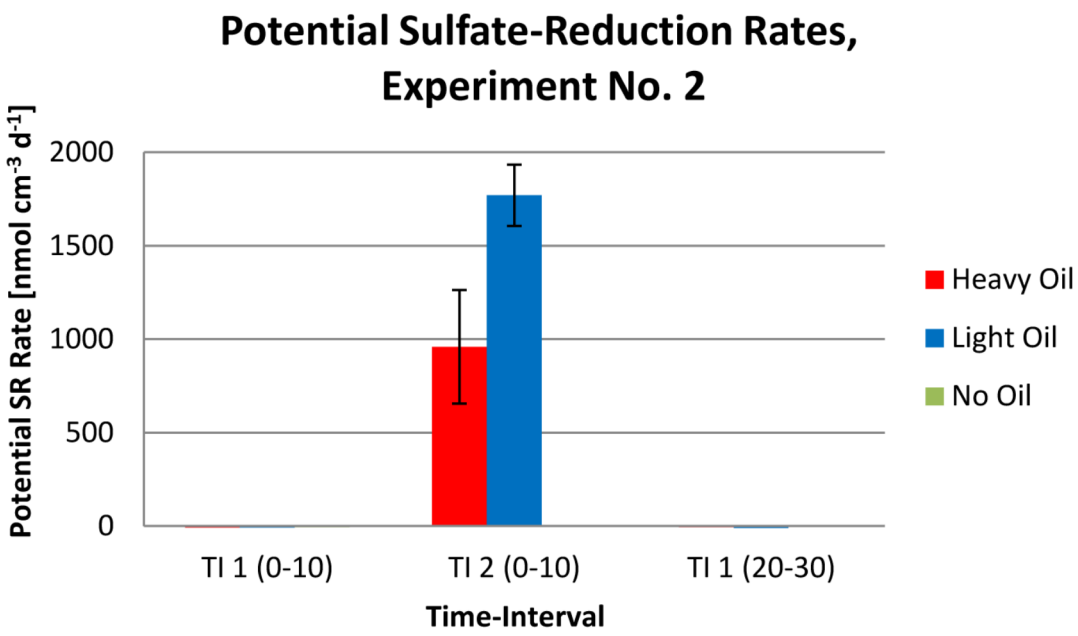

Figure 3.7: *Potential rates of sulfate-reduction in slurries from Experiment No. 2 during different time-intervals of incubation.* The duration of the different time intervals for the different samples are shown in Table 3.2 below this graph. TI 1 (0-10) and TI 1 (20-30)= time-interval with no relevant increase in sulfide concentrations. TI 2 (0-10) = time-interval with relevant increase in sulfide concentrations. Numbers 0-10 and 20-30 stand for sediment slurries prepared with sediment from 0-10 and 20-30 cm sediment depth, respectively. Rates were calculated in nmol cm^{-3} undiluted sediment d^{-1}. Values are means (± SD, n = 3).

Table 3.2: *Time-intervals used for calculation of the potential sulfate-reduction rates, which are shown in Fig. 3.7.* TI 1 (0-10) and TI 1 (20-30)= time-interval with no relevant increase in sulfide concentrations. TI 2 (0-10) = time-interval with relevant increase in sulfide concentrations. Numbers 0-10 and 20-30 stand for sediment slurries prepared with sediment from 0-10 and 20-30 cm sediment depth, respectively.

Sample	Time-Interval 1 (0-10) [days]	Time-Interval 2 (0-10) [days]	Time-Interval 1 (20-30)
Heavy Oil	0-126	133-146	0-146
Light Oil	0-126	133-146	0-140
No Oil	0-146	—*	0-146

* No rate calculated as no increase in sulfide was observed.

3.3.2 Development of total Alkalinity

In the crude oil-treated slurries from 0-10 cm sediment depth total alkalinity stayed constant over the first 105 days of incubation (31.97 ± 1.81 mM, heavy oil; 32.74 ± 1.37 mM, light oil; Fig. 3.5). Afterwards, total alkalinity started to rise in the oil treated slurries from 0-10 cm sediment depth. Maximum values of 39.46 ± 2.07 mM were reached at the end of the incubation time, after 180 days, in the heavy oil treated slurries from 0-10 cm sediment depth. In the light oil treated slurries from 0-10 cm sediment depth, maximum values of 44.74 ± 1.27 mM were reached after 180 days.

In the crude oil-treated slurries from 20-30 cm sediment depth no change of total alkalinity over the time of incubation was observed (remained constant at: 32.46 ± 1.43 mM, heavy oil; 32.04 ± 1.85 mM, light oil; Fig. 3.6). Also in the control slurries, no change in total alkalinity was observed over the time of incubation (remained constant at: 31.14 ± 1.76 mM, 0-10 cm; 30.15 ± 0.84 mM, 20-30 cm; Fig. 3.5 and 3.6).

3.3.3 Methane content in the gas headspace

In the heavy oil treated sediment slurries from both sediment depths, no relevant increase of methane content in the headspace was observed (Fig. 3.8). It stayed constant for the first 52 days of incubation. A decrease of methane content occurred between 52 and 129 days of incubation. Maximum values of methane content in the gas headspace of the heavy oil treated slurries were: 61.55 ± 28.95 ppm in the slurries from 0-10 cm sediment depth (after 25 days of incubation) and 185.20 ± 25.60 ppm in the slurries from 20-30 cm sediment depth (after 52 days of incubation).

In the light oil treated slurries an increase in methane content in the gas headspace until 54 days of incubation was found for the slurries from 0-10 cm sediment depth (Fig. 3.8). Afterwards, methane content started to decrease again. Maximum values of methane content in the gas headspace in the light oil treated slurries from 0-10 cm sediment depth were 135.76 ± 76.03 ppm after 54 days of incubation. In the light oil treated slurries from 20-30 cm sediment depth there was a steady decrease of methane content in the gas headspace over the whole incubation time. Maximum values of methane content of 179.40 ± 8.65 ppm were measured at the first measurement time-point, which was 7 days after start of the incubation.

In the control slurries without oil treatment from 0-10 and 20-30 cm sediment depth there was an increase of methane content in the gas headspace observed during the first 52 days of incubation (Fig. 3.8). Afterwards, between 52 and 129 days of incubation, methane content in all control slurries was decreasing. Maximum values of methane content in the gas headspace of the control slurries were: 95.80 ± 36.83 ppm in the slurries from 0-10 cm sediment depth (after 52 days of incubation) and 208.5 ± 7.93 ppm in the slurries from 20-30 cm sediment depth (after 52 days of incubation).

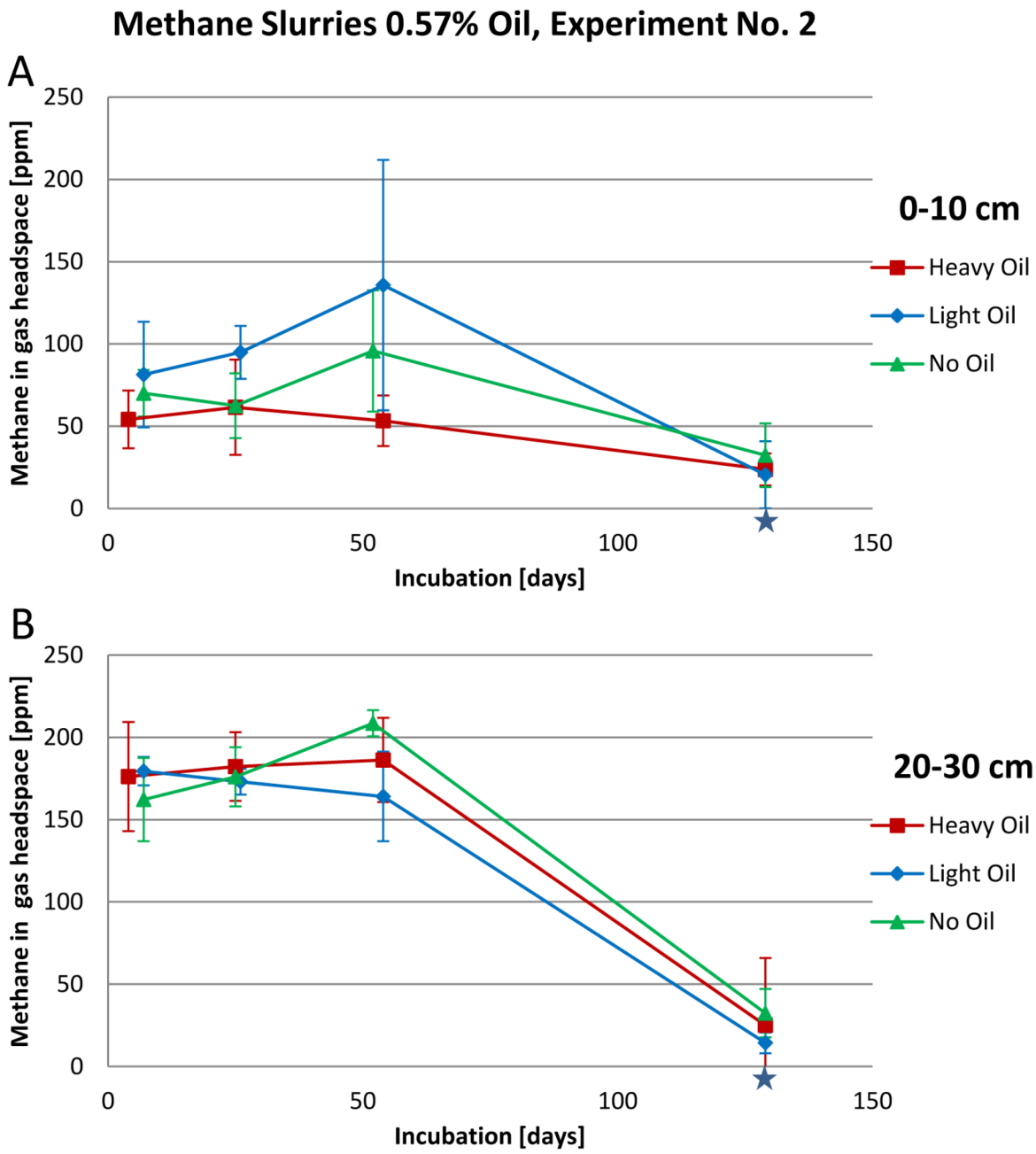

Figure 3.8: *Development of methane content in the gas headspace of the sediment slurries from Experiment No. 2 over time of incubation.* A: Slurries from 0-10 cm sediment depth. B: Slurries from 20-30 cm sediment depth. Asterisk marks time-point of measurements, which were done with the Shimadzu GC, others were measured with the HP).
Values are means (± SD, n = 3).

3.4 Experiment No. 3: Experiments on the influence of crude oil concentration on sulfate-reducing bacteria

Experiment No. 3 was subdivided into two experiments. In Experiment No. 3a the effect of different crude oil concentrations on the metabolic activity of sulfate-reducing sediment bacteria was tested. In Experiment No. 3b it was tested, at which concentrations toxic effects of the two used crude oil types become lethal for marine sediment bacteria.

3.4.1 Experiment No. 3a: Concentration series for the determination of an optimal crude oil concentration

3.4.1.1 Development of sulfide concentrations and potential rates of sulfate-reduction

Similar to the previous experiments (Experiment No. 1 and 2), a long lag-phase of 99 to 141 days with constant sulfide concentrations was observed (Fig. 3.9 and 3.10). A negative correlation was found between the duration of the lag-phase and crude oil concentration in the heavy oil treatment (Fig. 3.9). The lag-phase of the light oil treatment was similar for all tested light-oil concentrations (Fig. 3.10). Durations of the lag-phases and sulfide concentrations measured during the lag-phase are listed in Table A.6 in the appendix. Highest concentrations of sulfide at the end of the incubation were measured in the slurries treated with 0.6% (v/v) crude oil (5.04 mM and 7.36 mM in the heavy- and light oil treated slurry, respectively, after 189 days of incubation). In the control slurries sulfide concentrations remained constant (at 0.29 $\pm$ 0.09 mM) over the whole time of incubation (Fig. 3.9 and 3.10).

Highest potential rates of sulfate-reduction (calculated in nmol cm^{-3} undiluted sediment d^{-1}) were, for both types of crude oil, found in sediment slurries treated with a crude oil concentration of 0.6% (v/v) in the second time-interval (442.4 and 534.8 nmol cm^{-3} d^{-1} in the heavy- and the light oil treated slurry, respectively). Above and below this concentration, rates were always lower (Fig. 3.11, Table A.7). For the duration of the two time-intervals for which potential rates of sulfate-reduction were calculated for the different samples see Table 3.3.

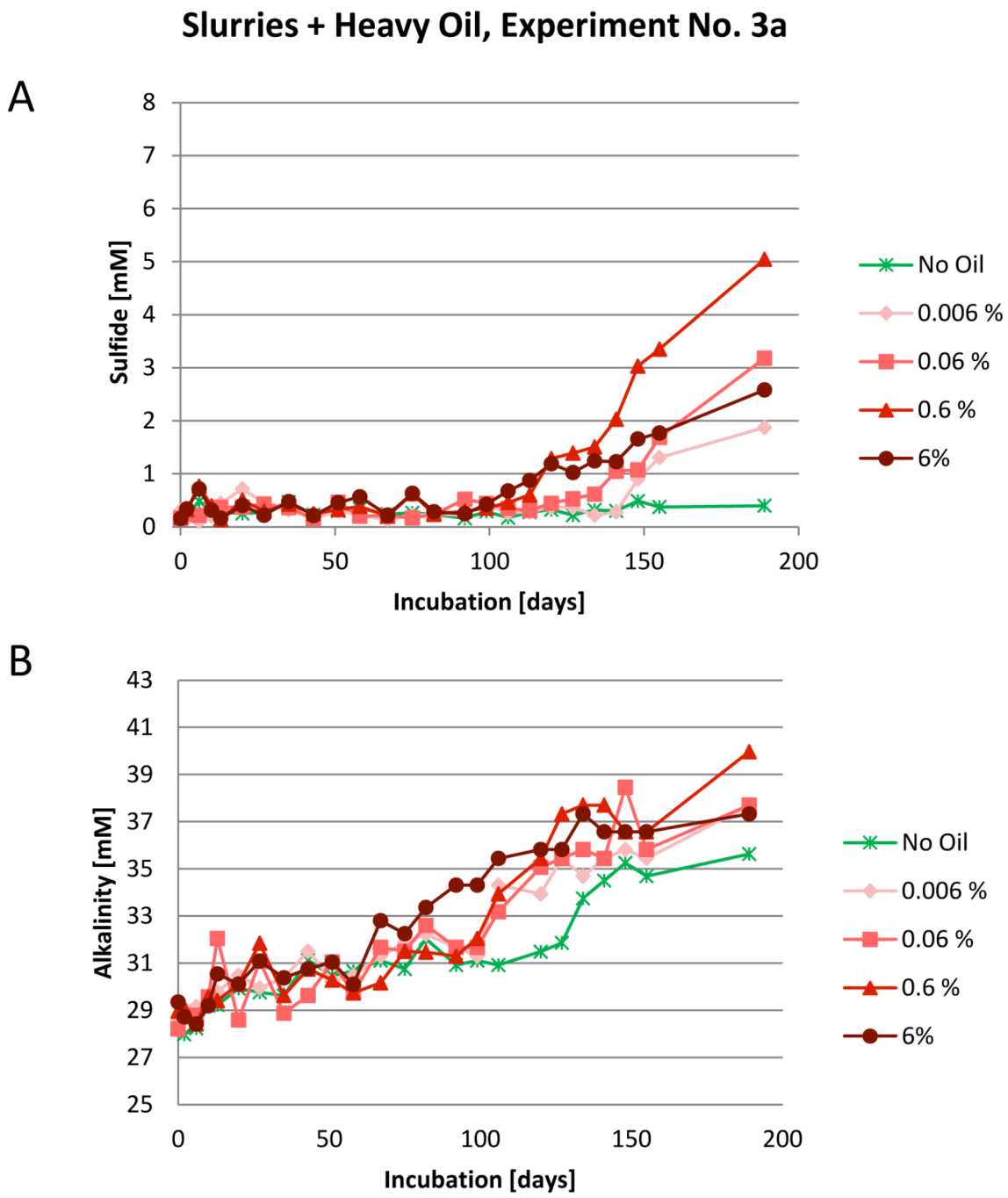

Figure 3.9: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the sediment slurries treated with heavy oil from Experiment No. 3a.*

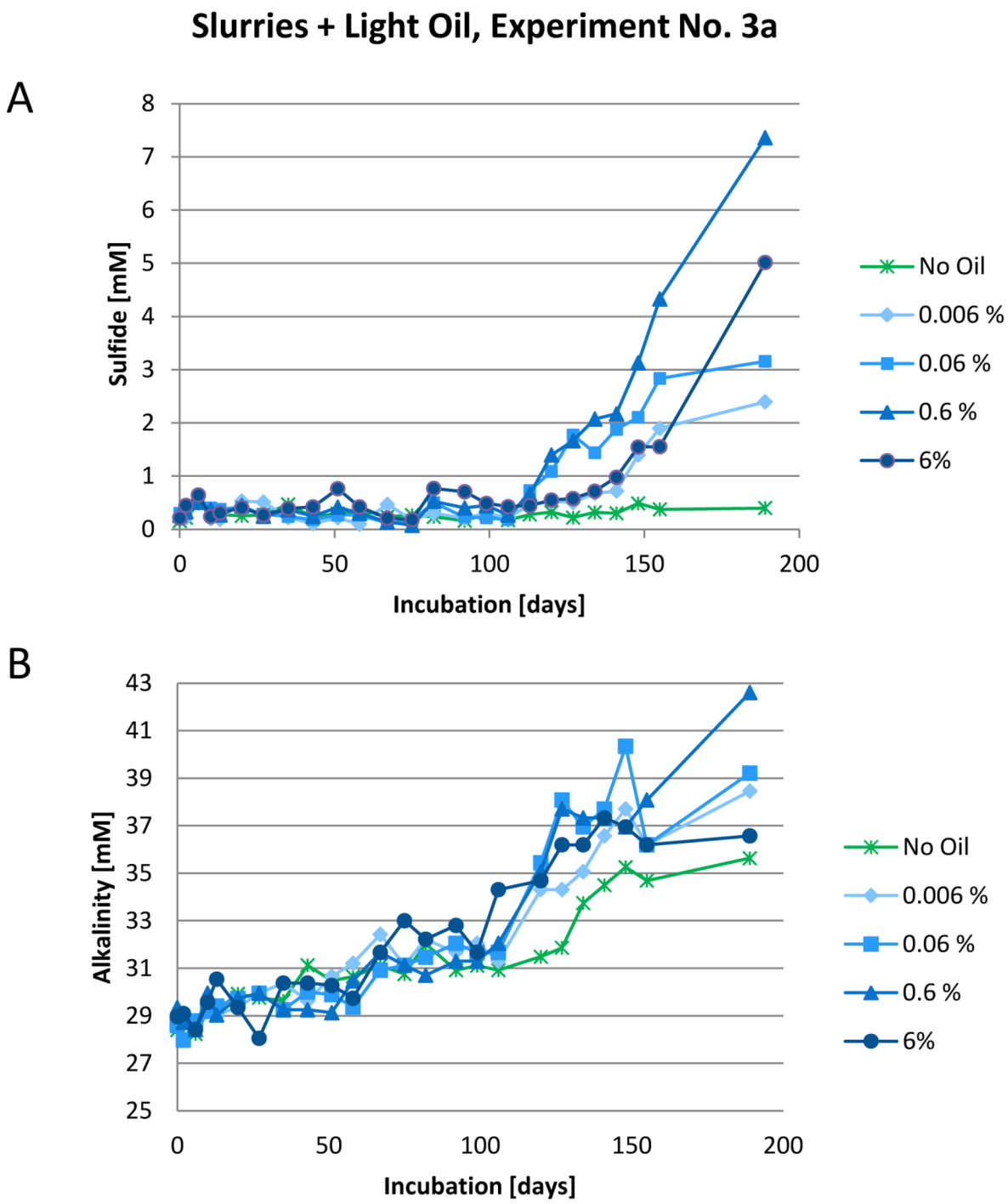

Figure 3.10: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the sediment slurries treated with light oil from Experiment No. 3a.*

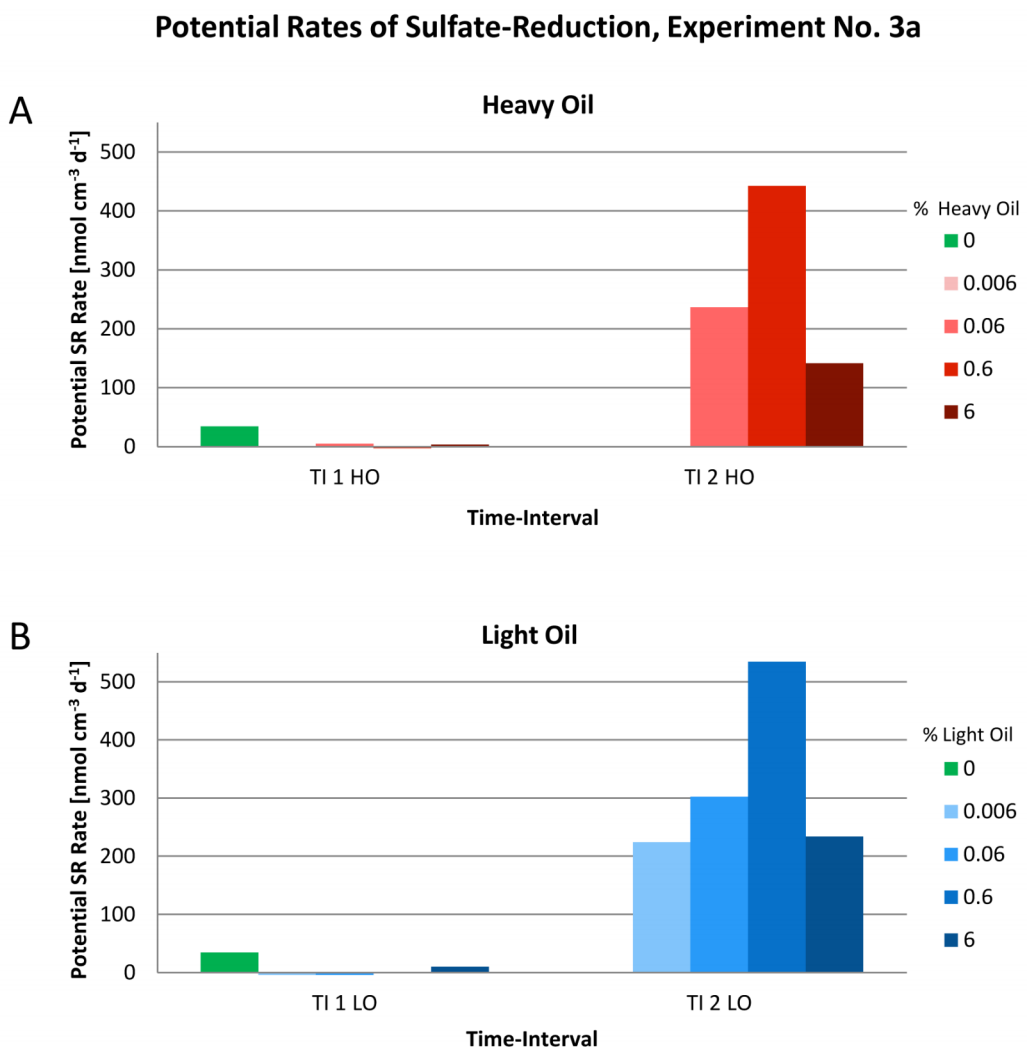

Figure 3.11: *Potential rates of sulfate-reduction in slurries from Experiment No. 3a during different time-intervals of incubation.* A: heavy oil (HO) treated slurries; B: light oil (LO) treated slurries. Time-intervals: TI 1 HO and TI 1 LO = first time-interval with no relevant increase of sulfide concentrations. TI 2 HO and TI 2 LO = second time interval with relevant increase of sulfide concentrations. The duration of the different time-intervals for the different samples are shown in Table 3.3 below this graph.
Rates were calculated in nmol cm^{-3} undiluted sediment d^{-1}.

Table 3.3: *Time-intervals used for calculation of the potential sulfate-reduction rates, which are shown in Fig. 3.11.* Time-interval 1 = first time-interval with no relevant increase in sulfide concentrations. Time-interval 2 = second time-interval with relevant increase in sulfide concentrations.

Sample	Time-Interval 1 [days]	Time-Interval 2 [days]
Heavy Oil 0.006%	0-144	—*
Heavy Oil 0.06%	0-113	120-155
Heavy Oil 0.6%	0-106	113-155
Heavy Oil 6%	0-99	106-155
Light Oil 0.006%	0-106	113-155
Light Oil 0.06%	0-106	113-115
Light Oil 0.6%	0-106	113-155
Light Oil 6%	0-113	120-155
No Oil	0-155	—**

* No rate could be calculated as only two successive measurement points with increasing sulfide concentrations were available.

** No rate calculated as no increase in sulfide was observed.

3.4.1.2 Development of total Alkalinity

Values of total alkalinity were increasing during the whole incubation time, even when there was no simultaneous increase in sulfide concentrations observed (Fig. 3.9 and 3.10). From day 100 on, where also sulfide started to increase in the respective sediment slurries, increase in total alkalinity became steeper. At the beginning, in all samples values of total alkalinity were around 29 mM (28.71 ± 0.40 mM). At the end of the incubation, after 189 days, values for total alkalinity ranged between 35.62 and 42.60 mM in the control slurry and the slurry treated with 0.6% light oil, respectively.

3.4.2 Experiment No. 3b: Concentration series for the determination of the lethal crude oil concentration

3.4.2.1 Live-dead staining for the determination of the lethal crude oil concentration

Determination of the ratio of living to death cells at different crude oil concentrations (0-50%, v/v) revealed an increasing ratio of death cells with increasing crude oil concentration (Fig. 3.12). But only above 18% crude oil concentration for the heavy oil treated slurries, and above 36% of crude oil concentration for the light oil treated slurries, a clear lethal effect of the crude oil could be seen. Below these crude oil concentrations ratios of death cells are even lower than in the controls, in which ratios of death cells of up to 40% were found.

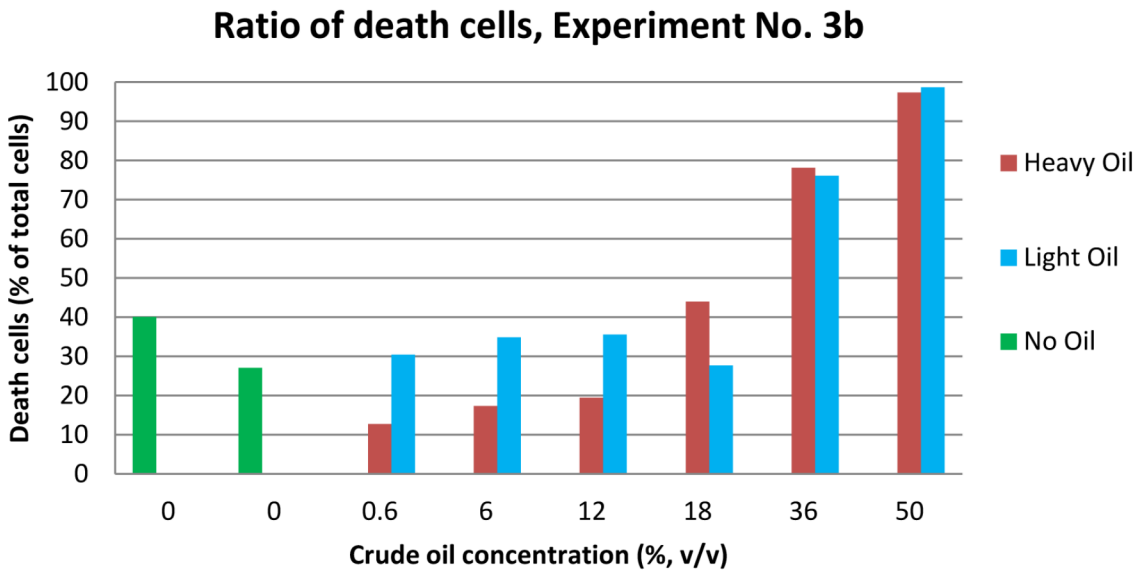

Figure 3.12: *Ratio of living to death cells in sediment slurries treated with different crude oil concentrations.*

3.5 Experiment No. 4: Anaerobic degradation of benzene

Aim of Experiment No. 4 was to investigate the ability of naturally occurring sulfate-reducing bacteria, in sediments from Eckernförde Bay, to degrade benzene. Therefore, sulfide concentrations and total alkalinity in the supernatant were monitored during time of the incubation (104 d) in the benzene treated slurries (benzene + medium + sediment), the control K1 (sediment + medium), and control K2 (benzene + medium). Additionally, benzene concentrations were measured in the benzene treated slurries, and control K2 at three time points (2, 49, and 104 d) during incubation.

3.5.1 Development of benzene concentrations

Two days after preparation of the samples, the initial benzene concentrations in the benzene treated slurries and K2 were determined to be 2.6 ± 0.18 µM (Fig. 3.13). This was lower than the calculated benzene concentration (5 µM, see section 2.3.4). Thus, some benzene was already lost during preparation of the samples. After 49 and 104 days of incubation, benzene concentrations in the benzene treated slurries were decreased by 24.23 ± 14.45 and 60.33 ± 3.75%, respectively (remaining concentration: 1.92 ± 0.46 mM after 49 days and 0.77 ± 0.22 mM after 104 days). Compared to the benzene treated slurries, the loss of benzene in control K2 was smaller after 49 (loss of 7.79 ± 2.63%; remaining concentration: 2.52 ± 0.07 mM) and 104 days of incubation (loss of 44.03 ± 1.67%; remaining concentration: 1.41 ± 0.0 mM). However, there were no significant differences in benzene concentrations in the benzene treated slurries and K2 after two (initial concentration) and 49 days of incubation (p-values: 0.19 and 0.15, respectively; T-test). After 104 days of incubation a T-test revealed a significant difference between the benzene concentrations in K2 and the benzene treated slurries (p-value = 0.038).

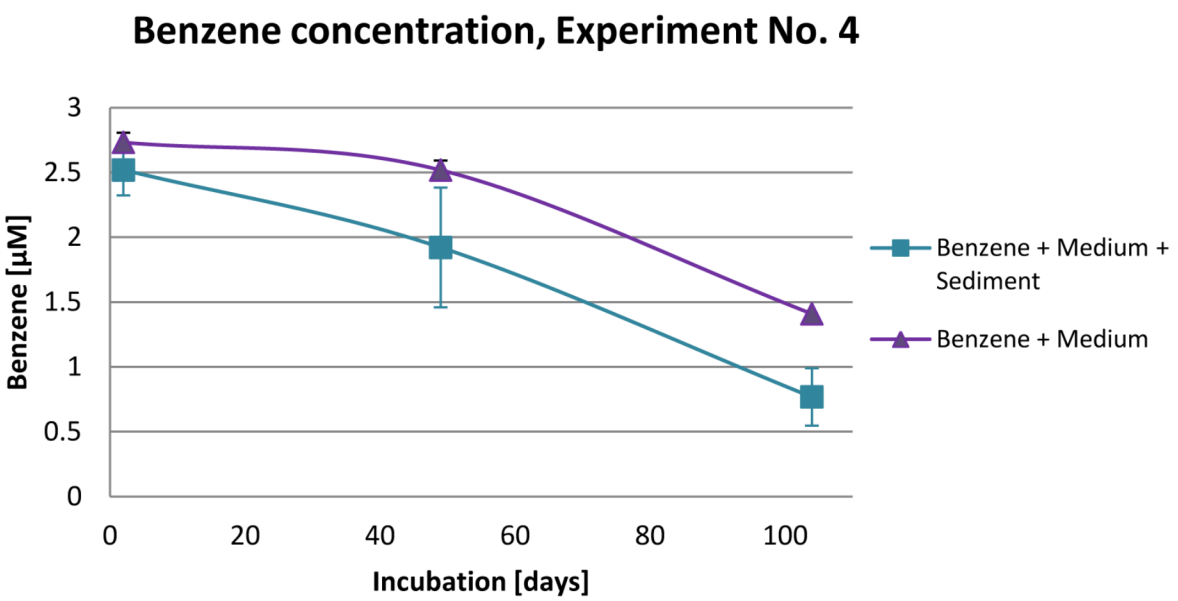

Figure 3.13: *Development of benzene concentrations over time of incubation in Experiment No. 4.* Values are means (± SD, n = 3).

3.5.2 Development of sulfide concentrations and total alkalinity

No relevant change of sulfide concentrations and total alkalinity in the supernatant of the slurries and the controls was observed during time of incubation (Fig. 3.14). Values for sulfide remained constant in slurries treated with benzene, K1, and K2 (0.33 ± 0.11, 0.27 ± 0.10, and 0.13 ± 0.07 mM, respectively). Also, values for total alkalinity remained constant in slurries treated with benzene, K1, and K2 (29.62 ± 1.19, 29.55 ± 0.89, and 29.65 ± 0.98 mM, respectively) over the whole time of incubation.

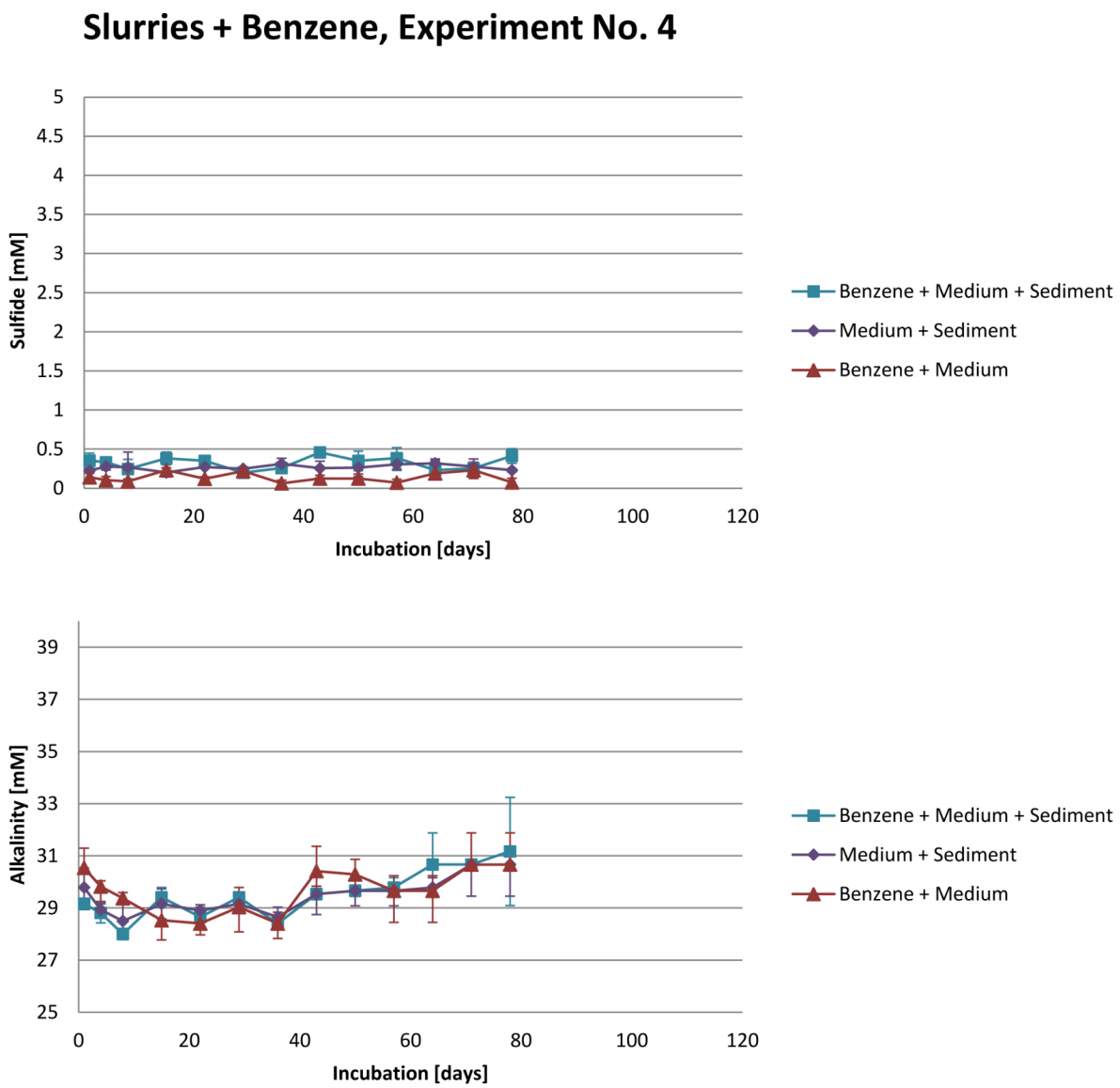

Figure 3.14: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in incubations from Experiment No. 4.* Values are means (± SD, n = 3).

3.6 Experiment No. 5: Experiment on the effect of aerobic pre-digestion of crude oil

The aim of Experiment No. 5 was to test if aerobic pre-digestion of crude oil has an influence on the rapidity and intensity of the reaction of sulfate-reducing bacteria. For that purpose sediment slurries were incubated with two kinds of crude oil (heavy- and light oil) under aerobic conditions, beside a control sediment slurry without crude oil. Subsequently, these three treatments were shifted to anoxic conditions and further incubated after addition of either an anoxic sediment slurry (+ Slurry), a pure-culture of *D. mediterraneus* (+ *D. mediterraneus*), a pure-culture of *D. marinus* (+ *D. marinus*), or anoxic artificial seawater (+ Medium) as a control. Total alkalinity and sulfide concentrations in the supernatant of the slurries were measured over the whole time of incubation (92 d).

3.6.1 General observations and remarks

After 2 weeks of aerobic incubation, resazurin was added to the slurries. It was observed, that the color of the resazurin turned from blue to pink in the oil-treated slurries (added as oxygen indicator for the later anaerobic incubation). No such color transformation was observed in the control slurries. Reasons for this effect will be discussed in the discussion.

3.6.2 Development of sulfide concentrations and potential rates of sulfate-reduction

In the + Slurry samples, an increase in sulfide concentration was found after a lag-phase of 42 and 49 days in the incubations with the pre-digested heavy- and light oil, respectively, but not in the control without oil (Fig. 3.15). Sulfide concentrations in the control stayed constant at 0.20 ± 0.09 mM. Maximum values of 3.71 ± 0.39 and 6.01 ± 0.76 mM sulfide were reached in the heavy- and light oil treated samples, respectively, after 92 days.

Also in the + *D. mediterraneus* samples, an increase of sulfide concentration was found after a lag-phase of 21 and 49 days in the incubations with pre-digested heavy and light oil, respectively, but not in the control without oil (Fig. 3.16). Sulfide concentrations in the control stayed constant at 0.16 ± 0.12 mM. After 92 days of incubation values of 1.38 ± 0.25 and 1.93 ± 0.50 mM sulfide were reached in the heavy- and light oil treated samples, respectively.

For the crude-oil treated samples of the + *D. marinus* and the + Medium incubations, no increase of sulfide concentration was observed during the first 62 days of incubation (stayed constant at 0.13 ± 0.10 and 0.19 ± 0.12 mM, respectively, Fig. 3.17 and 3.18). Between 62 and 92 days of incubation sulfide concentrations in the crude oil treated samples of the + *D.marinus* incubations started to rise and reached maximum values of 3.85 ± 0.13 and 5.07 ± 1.61 mM in the heavy- and light oil treated samples, respectively, at the end of the incubation (Fig. 3.17). Also in the + Medium incubations, after 62 days of incubation an increase of sulfide was measured (Fig. 3.18). Maximum values of 3.74 ± 0.48 and 4.03 ± 0.45 mM in the heavy- and light oil treated samples, respectively, were measured at the end of the incubation time (after 92 days) in the + Medium samples. In the control slurries of the + *D.marinus* and the + Medium incubations sulfide concentrations stayed constant over the whole time of incubation (at: 0.16 ± 0.12 and 0.19 ± 0.12 mM, respectively).

Potential rates of sulfate-reduction (calculated in nmol cm^{-3} undiluted sediment d^{-1}), which were calculated for the different samples for the first two time-intervals are shown in Table 3.4. Highest potential rates of sulfate-reduction in the second time-interval were found in the + Slurry samples incubated with the pre-digested light oil (529.67 ± 23.80 nmol cm^{-3} d^{-1}, 49-62 d). Potential rates in the + Slurry incubation with pre-digested light oil were about twice as high as in the in the + Slurry samples, which were incubated with the pre-digested heavy oil (267.63 ± 29.77 nmol cm^{-3} d^{-1}, 42-62 d). Also for the + *D. mediterraneus* samples, values for the incubation with pre-digested light oil (329.23 ± 50.16 nmol cm^{-3} d^{-1}, 49-62 d) were about twice as high as in the incubations with pre-digested heavy oil (124.83 ± 21.62 nmol cm^{-3} d^{-1}, 21-62 d).

Statistical analysis for all potential rates calculated for the second time-interval in Experiment No. 5, applying ANOVA and post-hoc comparison by a TukeyHSD test, revealed that there are highly significant differences in all potential rates from the different treatments (p-values $<< 0.05$; Table A.8 in the appendix), with one exception. No significant difference was found for the incubation with pre-digested light oil where a *D. mediterraneus* culture was added and the incubation with pre-digested heavy oil where anaerobic slurry has been added (p-value = 0.1856).

Table 3.4: *Potential rates of sulfate-reduction in slurries from Experiment No. 5 during different time-intervals of incubation.* T1 = time-interval 1 (lag-phase) ; T2 = time-interval 2. Rates were calculated in nmol cm^{-3} undiluted sediment d^{-1}.

Pre-digestion with:	+ In anaerobic incubation	T1 - potential SR rate [nmol $cm^{-3}d^{-1}$]	T2 - potential SR rate [nmol $cm^{-3}d^{-1}$]
Heavy Oil	Slurry	-0.7 ± 24.77 (0-35 d)	267.63 ± 29.77 (42-62 d)
	D. mediterraneus	-59.50 ± 52.15 (0-14 d)	124.83 ± 21.62 (21-62 d)
	D. marinus	-14.70 ± 4.26 (0-62 d)	—*
	Medium	5.83 ± 9.93 (0-62 d)	—*
Light Oil	Slurry	-2.33 ± 8.20 (0-42 d)	529.67 ± 23.80 (49-62 d)
	D. mediterraneus	2.1 ± 10.59 (0-42 d)	329.23 ± 50.16 (49-62 d)
	D. marinus	-10.50 ± 17.51 (0-62 d)	—*
	Medium	-2.1 ± 10.57 (0-62 d)	—*
No Oil	Slurry	19.37 ± 7.67 (0-62 d)	—*
	D. mediterraneus	2.33 ± 7.04 (0-62 d)	—*
	D. marinus	-2.05 ± 9.19 (0-62 d)	—*
	Medium	-2.56 ± 4.91 (0-62 d)	—*

* No rate calculated for T2 as no linear increase in sulfide concentrations over more than two measurement time-points was observed.

3.6.3 Development of total Alkalinity

In the incubations where no increase in sulfide was measured during the first 62 days of incubation (+ *D. marinus* and + Medium), also no, or only a slight increase in total alkalinity was measured (Fig. 3.17 and 3.18). Then, in between the last two measurement time-points (between 62 and 92 days of incubation) in the + *D. marinus* incubations values of total alkalinity rose to 29.09 ± 2.54 and 33.29 ± 1.59 mM in the slurries treated with pre-digested heavy- and light oil, respectively (Fig. 3.17). Also in the + Medium incubations values of total alkalinity rose to 29.15 ± 1.06 and 30.74 ± 0.58 mM in the slurries treated with the pre-digested heavy- and light oil, respectively, in between the last two measurement time-points (Fig.3.18)

In the + Slurry incubations with pre-digested heavy- and light oil, during the whole time of incubation there was a slight increase in total alkalinity (Fig. 3.15). This increase in total alkalinity was getting steeper after 42 days of incubation, when also sulfide concentrations started to increase. Maximum values of 29.98 ± 0.96 and 34.05 ± 2.20 mM in the heavy- and light oil treated slurries, respectively, were reached at the end of the incubation time (after 92 days).

In control slurries of the + Medium, the + *D. marinus*, and the + Slurry samples, values of total alkalinity stayed constant over the whole time of incubation (at 20.64 ± 1.03, 19.24 ± 1.15, and 19.01 ± 0.73 mM, respectively, Fig. 3.18, 3.17, and 3.15).

In the + *D. mediterraneus* incubation with pre-digested crude oil total alkalinity continuously rose over the whole time of incubation (Fig. 3.16). Also in the + *D. mediterraneus* control incubation without crude oil, there was a rise of total alkalinity observed (but lower as in the incubations with oil). Maximum values of total alkalinity measured in the samples were 28.20 ± 0.38 and 28,97 ± 1.37 mM, at the end of the incubation time (after 92 days) in the heavy- and light oil treated slurries, respectively. In the control slurries, maximal values of total alkalinity of 25.66 ± 0.22 mM were reached after 49 days of incubation.

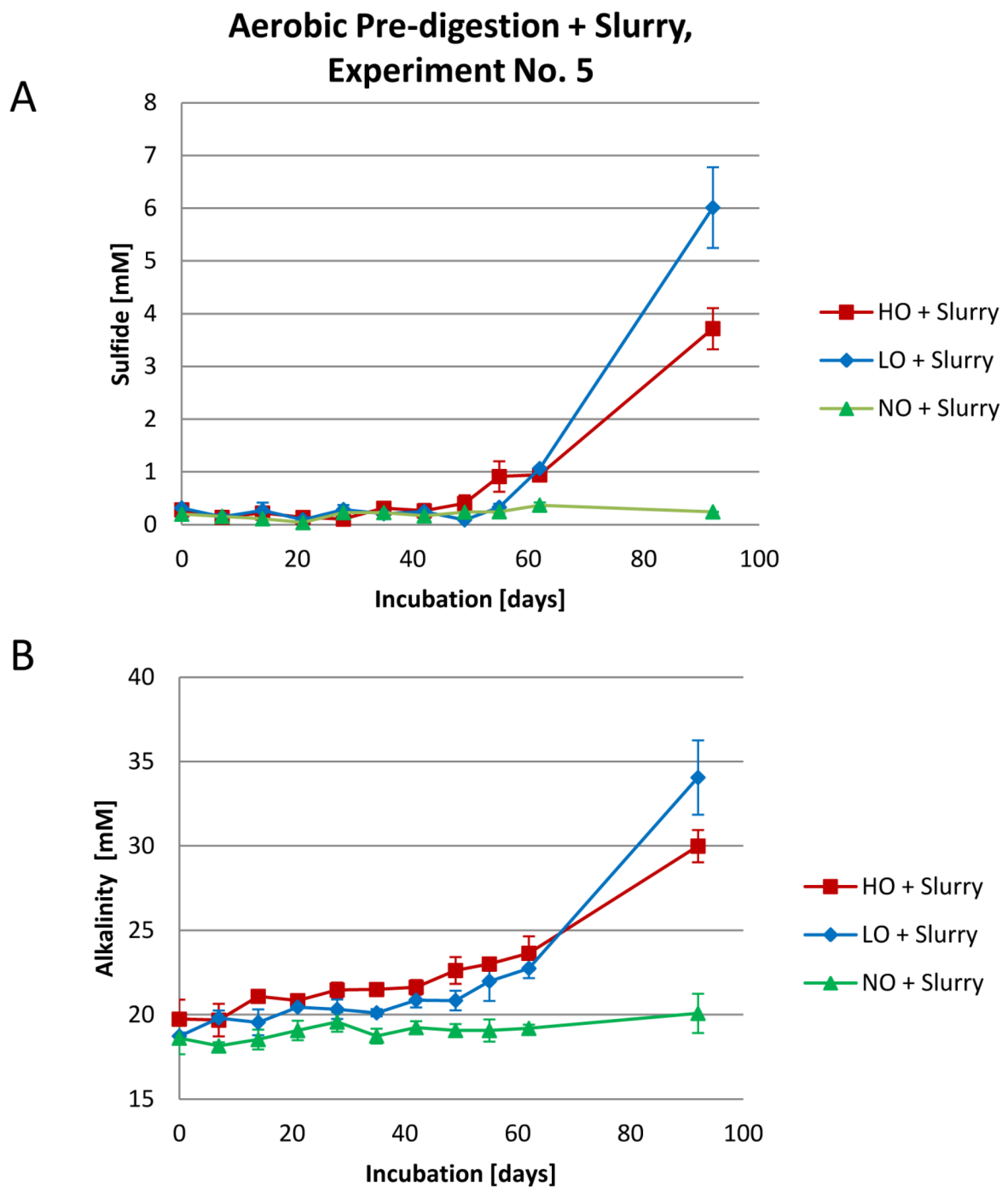

Figure 3.15: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the + Slurry incubations from Experiment No. 5.*
Values are means (± SD, n = 3).

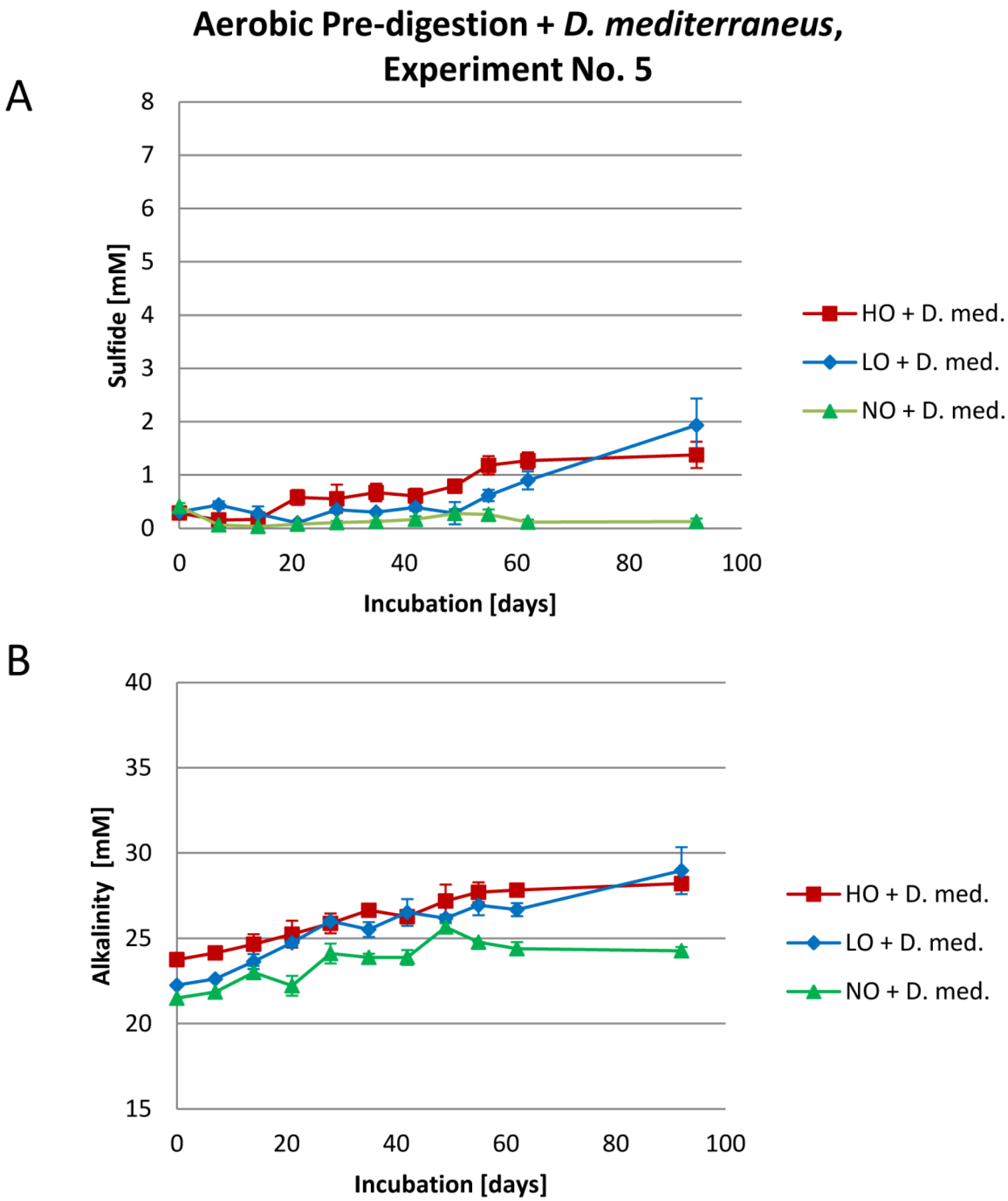

Figure 3.16: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the + D. mediterraneus incubations from Experiment No. 5.*
Values are means (± SD, n = 3).

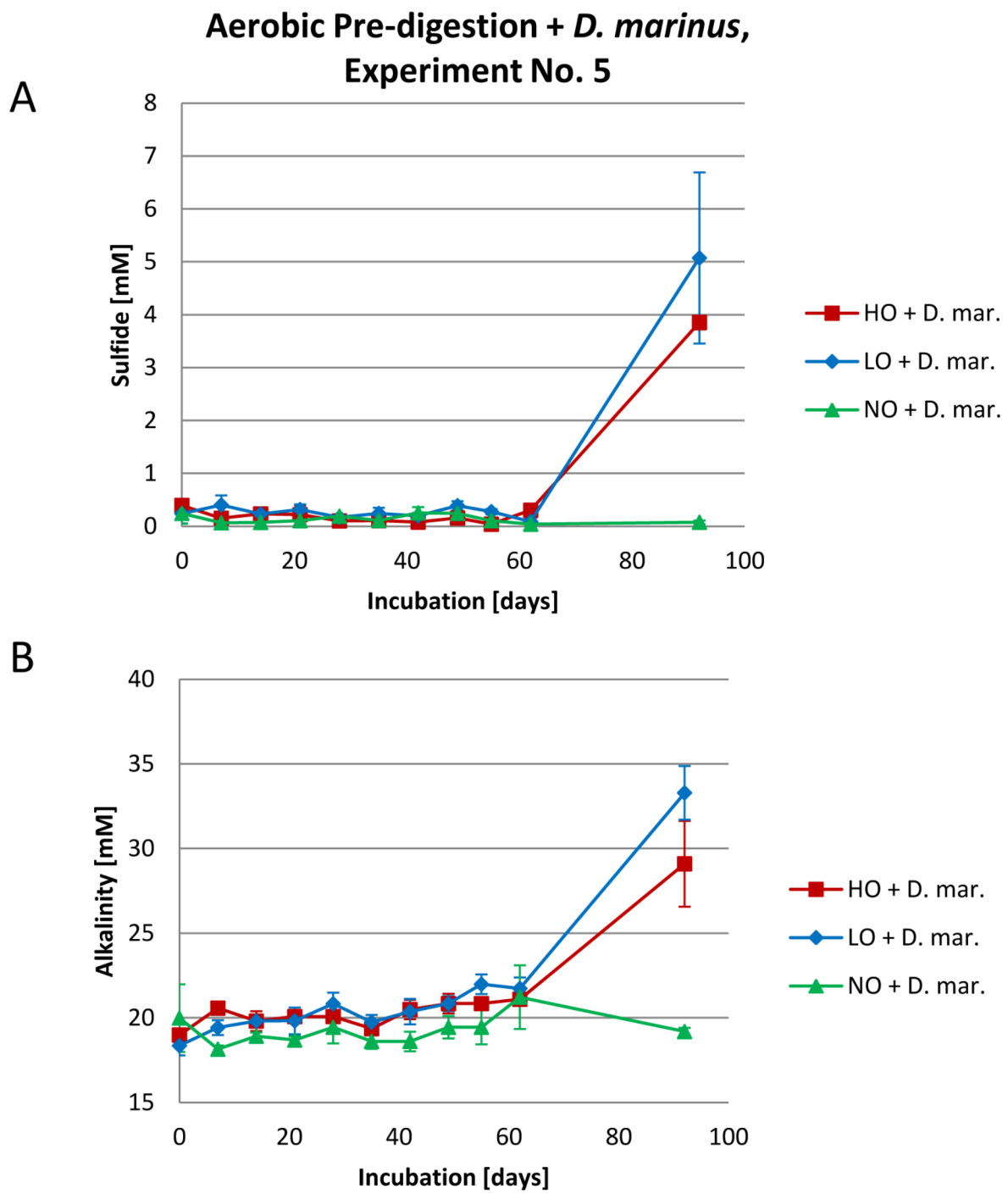

Figure 3.17: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the + D. marinus incubations from Experiment No. 5.*
Values are means (± SD, n = 3).

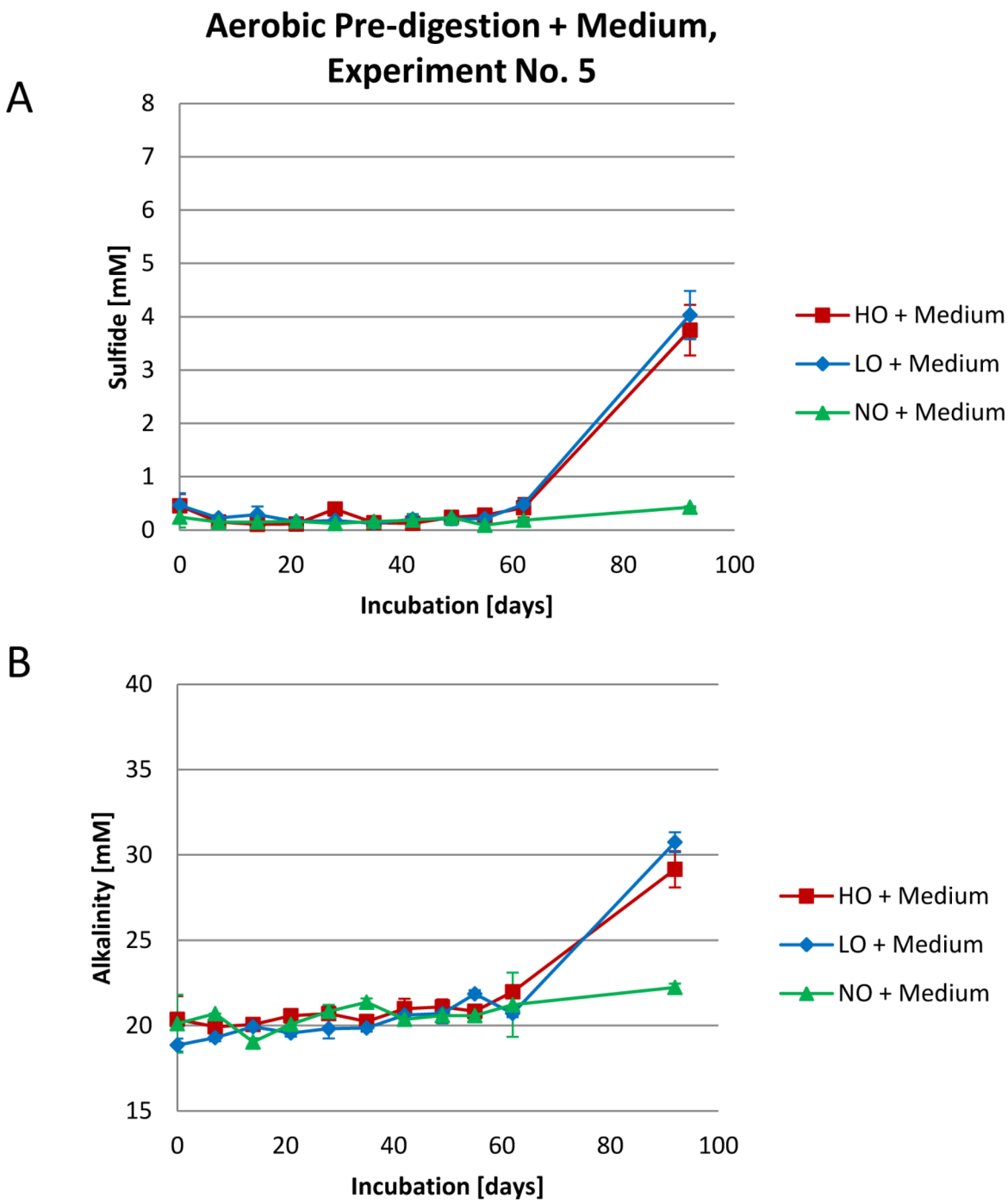

Figure 3.18: *Development of sulfide concentrations (A) and total alkalinity (B) over time of incubation in the + Medium incubations from Experiment No. 5.*
Values are means (± SD, n = 3).

3.7 Experiment No. 6: Incubation Experiment with sediment mini-cores

3.7.1 General observations and remarks

Before incubation, 100 µl of crude oil was injected into the sediment mini-cores at each cm interval. After 73 days of incubation, sediment cores were sliced in 1 cm sections. During slicing of the mini-cores, it was observed that the crude oil, instead of spreading or moving upwards, stayed in line like it was injected (Fig. 3.19).

Figure 3.19: *Sliced sediment mini-core.* The injected crude-oil (heavy oil) is visible (after 73 days of incubation).

3.7.2 Sulfate-reduction rates

Sulfate-reduction rates (in nmol cm^{-3} sediment d^{-1}) were measured after 73 days of incubation (Fig. 3.20). The samples treated with crude oil were present in triplicates, but there was only one control sediment mini-core.

Rates of sulfate-reduction ranged from 0.00 (2-3 cm sediment depth, replicate B) to 134.46 (5-6 cm sediment depth, replicate A) nmol cm^{-3} d^{-1} in the heavy oil treated mini-cores and 29.25 (10-11 cm sediment depth, replicate A) to 134.28 (4-5 cm sediment depth replicate B) nmol cm^{-3} d^{-1} in the light oil treated mini-cores. In the control mini-core rates of sulfate-reduction ranged from 23.79 (2-3 cm sediment depth) to 106 (13-14 cm sediment depth) nmol cm^{-3} d^{-1}.

Integrated areal rates of sulfate-reduction over the uppermost 12 cm were, with 9307.6 ± 3031.5 mmol m^{-2} d^{-1} for the core treated with heavy oil, and 9579.2 ± 786.4 mmol m^{-2} d^{-1} for the core treated with light oil, higher than in the core which was not treated with crude oil (6104.3 mmol m^{-2} d^{-1}). As the control was not present as triplicate, no statistical analysis could be performed. Standard deviations in the heavy oil treated mini-cores were very high compared to the light oil treated mini-cores (Fig. 3.20, and Fig. A.2 for more details about single replicates).

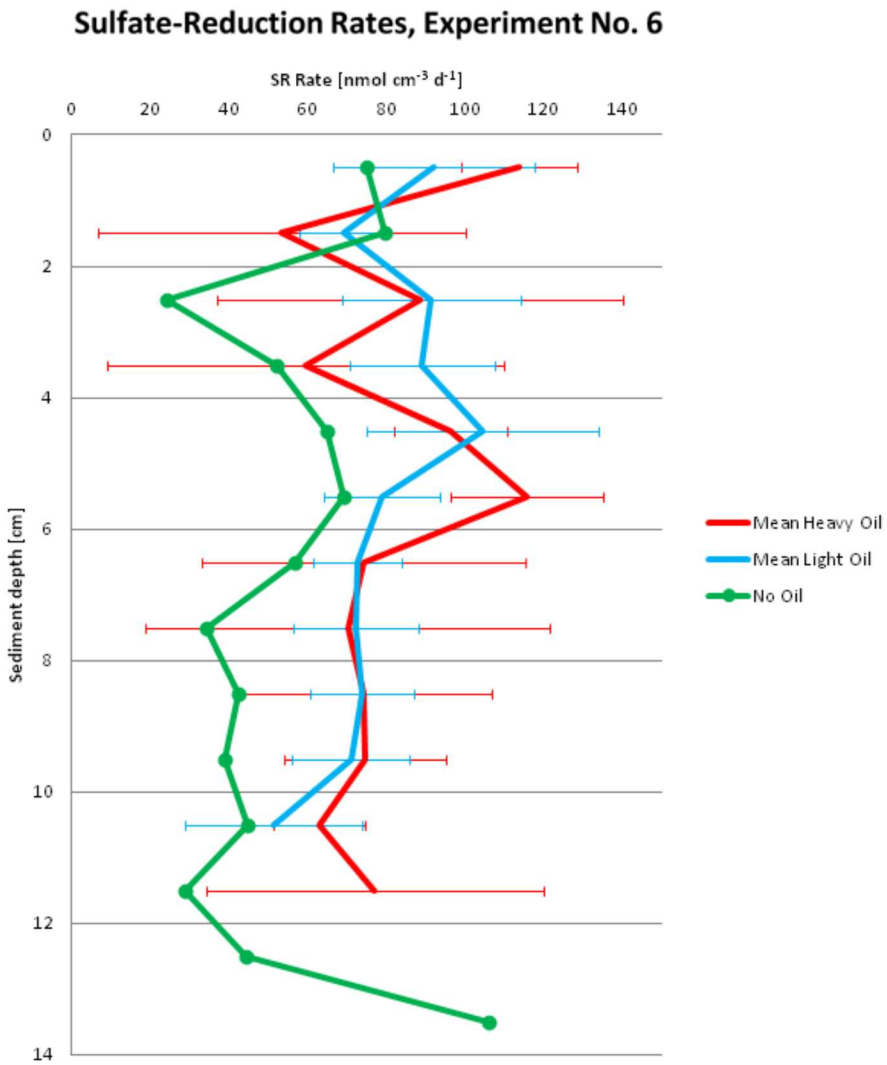

Figure 3.20: *Vertical profile of sulfate-reduction rates in the sediment mini-cores from Experiment No. 6 after 73 days of incubation.*
Values for heavy- and light oil are means (± SD, n = 3).
Rates were calculated in nmol cm^{-3} sediment d^{-1}.

3.7.3 CARD–FISH

After 73 days of incubation with crude oil, total bacterial abundances (counts of DAPI-stained cells) and the abundance of bacteria belonging to the *Desulfovibrio spp.* group (Alexa 488-stained cells), were determined in the sediment mini-cores (examples for stained cells see Fig. 3.21).

Counts were done over a sediment depth of 13 cm. Initially, the cores were sectioned in 1 cm intervals. For CARD-FISH analysis and DAPI counts, only every second cm section was used (started with 0-1 cm). Cell numbers were calculated in: cells ml^{-1}sediment.

In general, total cell numbers were decreasing over sediment depth (Fig. 3.22). The maximum total cell number was found in the uppermost sediment layer for all three treatments (7.48 x 10^9 cells ml^{-1}, heavy oil; 7.1 x 10^9 cells ml^{-1}, light oil; 6.96 x 10^9 cells ml^{-1}, no oil). The minimum total cell number was found in the lowermost sediment section for all three treatments (12-13 cm sediment depth; 3.66 x 10^9 cells ml^{-1}, heavy oil; 4.02 x 10^9 cells ml^{-1}, light oil; 3.66 x 10^9 cells ml^{-1}, no oil).

In the control mini-core, counts of *Desulfovibrio spp.* cells were decreasing over sediment depth in the mini-core not treated with crude oil, similar to the total cell counts (Fig. 3.22). In the top layer (0-1 cm sediment depth), cell numbers of *Desulfovibrio spp.* were 2.94 x 10^8 cells ml^{-1} and decreased to 1.9 x 10^8 cells ml^{-1} in the lowermost layer (12-13 cm sediment depth). The relative contribution of *Desulfovibrio spp.* cells to the total microbial cell numbers stayed relatively constant (compared to the other treatments) at ~ 5% over all investigated sediment depths (Fig. 3.23; mean: 4.92 ± 0.53%).

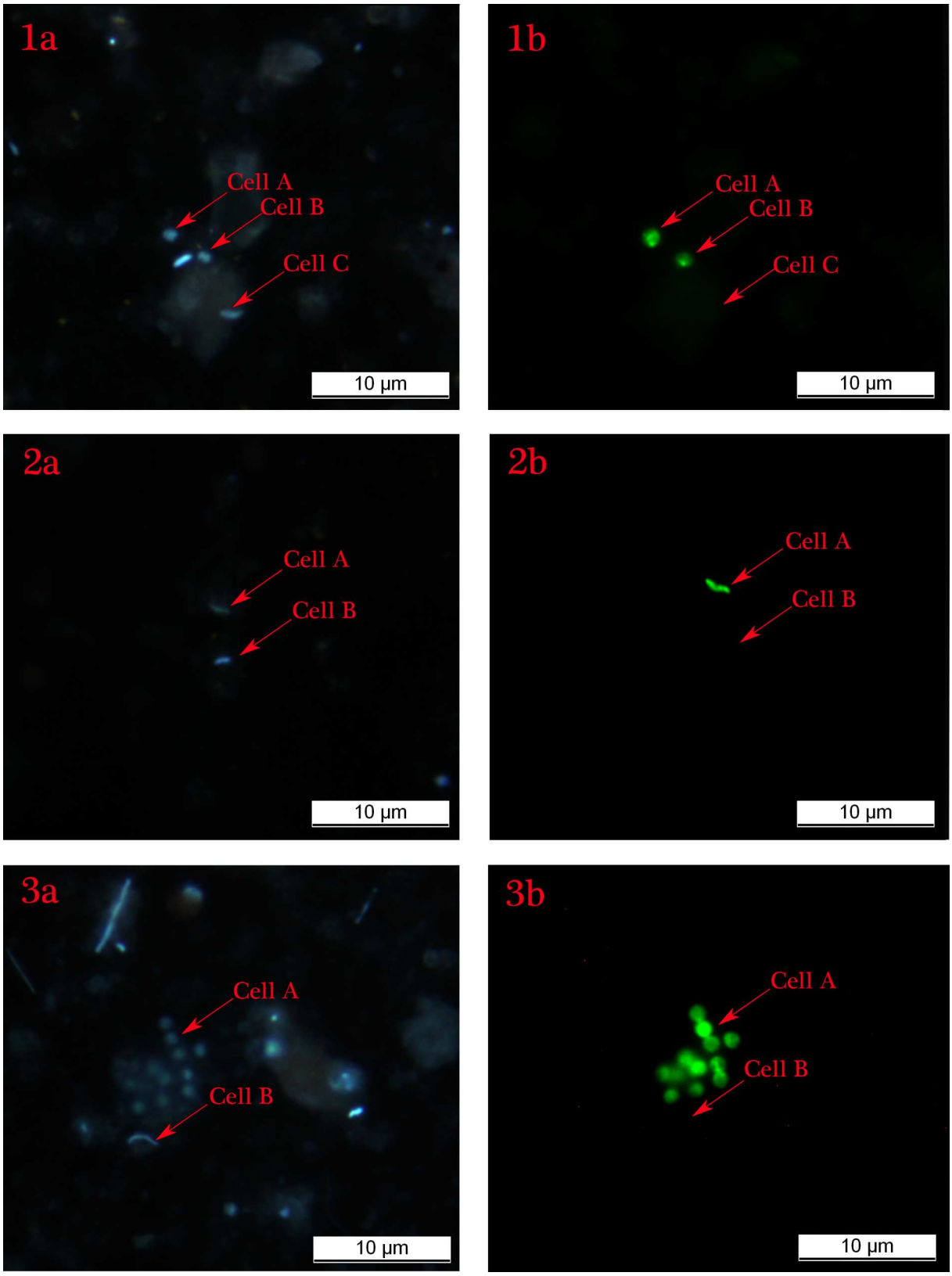

Figure 3.21: *Representative examples for DAPI (left) and CARD-FISH (right) stainings.* DAPI stainings, microscopic filter A; CARD-FISH (fluorochrome = Alexa 488; probe = DSV 698, Manz *et al.*, 1998) microscopic filter L5 (Table 2.2). Some representative cells are marked with arrows. All cells can be seen in the DAPI pictures (left) but only cells belonging to the *Desulfovibrio spp.* group can be seen on the CARD-FISH pictures (right). Magnification 1000x. 1 and 2: No Oil 2-5 cm sediment depth; 3: Heavy Oil 0.5 cm sediment depth.

A similar trend of decreasing cell numbers *of Desulfovibrio spp.* cells with sediment depth was observed in the oil treated mini-cores (Fig. 3.22). Maximum cell numbers of *Desulfovibrio spp.* cells in the oil treated sediment mini-cores were: 4.21 x 10^8 cells ml^{-1} in the heavy oil treated mini-core (2-3 cm sediment depth) and 3.82 x 10^8 cells ml^{-1} in the light oil treated mini-core (0-1 cm sediment depth). Lowest cell numbers of *Desulfovibrio spp.* found in the oil treated mini-cores were: 2.67 x 10^8 cells ml^{-1} in the heavy oil core (12-13 cm sediment depth) and 3.03 x 10^8 cells ml^{-1} in the light oil core (12-13 cm sediment depth). However, relative contribution of *Desulfovibrio spp.* cells to the total microbial cell numbers was increased, compared to the untreated sediment mini-core. Mean percentages of *Desulfovibrio spp.* cells were 6.07 $\pm$ 1.18% for the heavy oil, and 6.69 $\pm$ 1.52% light oil treated sediment mini-cores (Fig. 3.23).

In the oil treated mini-cores, the highest percentages of *Desulfovibrio spp.* cells were found in 8-9 cm sediment depth for the mini-cores treated with heavy- and light oil (7.5 and 8.7%, respectively). The lowest percentages of *Desulfovibrio spp.* cells were found in sediment depths of 2-3 cm for the heavy oil treated mini-core (4.8%) and in a depth of 0-1 cm for the light oil treated mini-core (4.5%).

Summarized, abundances of bacteria belonging to the *Desulfovibrio spp.* group are higher in the oil treated sediment mini-cores, compared to the control core. Also total cell numbers show a weak tendency of increase in the oil treated sediment mini-cores.

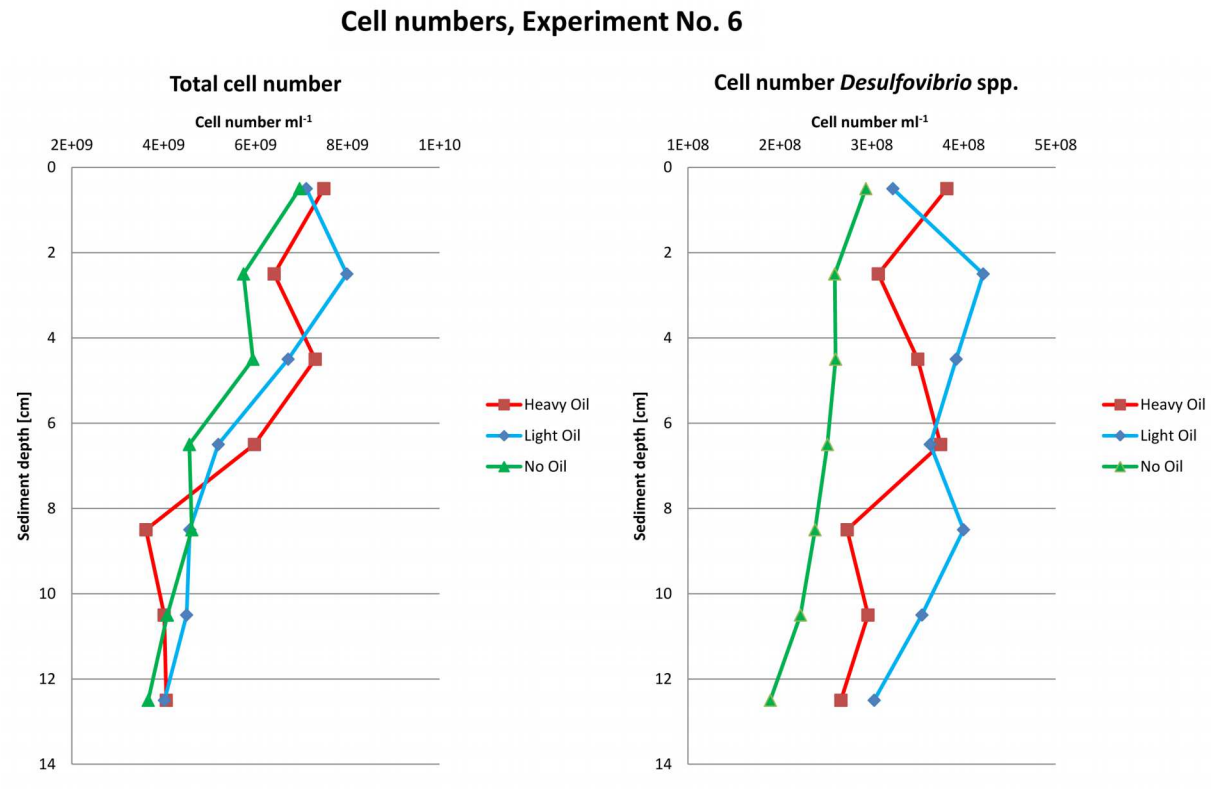

Figure 3.22: *Vertical profile of cell numbers determined by DAPI- and CARD-FISH staining in the sediment mini-cores from Experiment No. 6 after 73 days of incubation.* A: total cell numbers determined by counting of DAPI stained cells. B: Cell numbers of *Desulfovibrio spp.*.

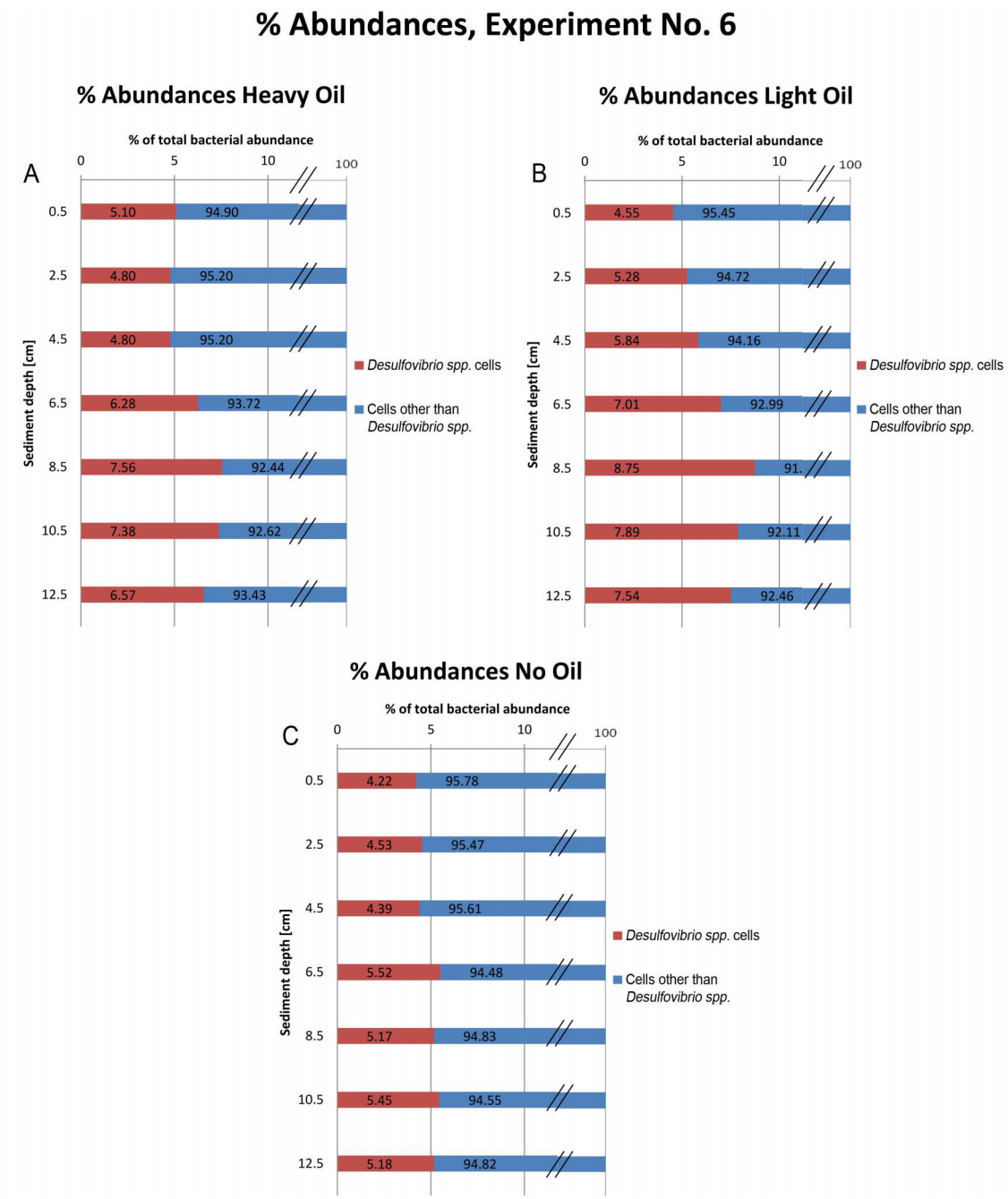

Figure 3.23: *Vertical profile of percentaged abundances of Desulfovibrio spp. in the sediment mini-cores from Experiment No. 6 after 73 days of incubation.* A: mini-core incubated with heavy oil. B: mini-core incubated with light oil. C: control mini-core, incubated without oil addition.

4 Discussion

Degradation by microorganisms is the most important process for the removal of crude oil compounds from marine environments. As many crude oil compounds are toxic and can have harmful effects on organisms and whole ecosystems, microbial degradation of crude oil compounds is crucial in contaminated areas. Aerobic degradation of crude oil components has been intensely studied (Prince, 2002; Head *et al.*, 2006, and references therein). But for crude oil degradation in anaerobic habitats, like marine sediments, there are still large gaps of knowledge (Suárez-Suárez *et al.*, 2011).

In this study it was investigated how fast and how high the response of sulfate-reducing bacteria and methanogenic archaea in natural marine sediments is, when crude oil is added, i.e. the duration of the lag-phase after addition of crude oil, the change of sulfate-reduction rates and methane content, as well as changes in bacterial abundances over time of incubation were measured. The main focus was on sulfate-reducing bacteria, as they are usually the most important organic matter-degraders in marine environments under anoxic conditions (Jorgensen, 1982).

Eight hypotheses, concerning degradation of different types of crude oil and also single crude oil components in natural marine sediments, were tested (section 1.5). In total, seven different experiments were performed (for overview see: Fig. 2.2 on page 26). The sediment, which was used for the experiments, was sampled at Boknis Eck station in the Eckernförde Bay (south-western German Baltic Sea). In the following, it will be discussed if the hypotheses could be confirmed by the results of the experiments. Next, it will be discussed if there were methodical problems. Finally the conclusions, which can be drawn based on the results, followed by an outlook, on which questions should be answered next, are given.

4.1 Adaptation of microbial communities in sediments from Eckernförde Bay to crude oil degradation and the influence of crude-oil addition on sulfate-reduction rates

The **first hypothesis** was that, as a response to crude oil addition to marine sediments, sulfate-reduction rates will increase. This hypothesis was confirmed in several of the conducted experiments. In Experiment No. 1, 2, and 3a, where sediment slurries were treated with different concentrations of two types of crude oil, significant increases of sulfide concentrations in the supernatant of the crude oil treated slurries were measured. But only after long lag-phases of 100-140 days. A similar effect of crude oil addition was also found in Experiment No. 5, where aerobically pre-digested crude oil was used. But in this experiment the lag-phases were only about 60 days long.

If crude oil degradation was happening in the incubations and which compounds were degraded could not be directly measured. Instead, the increase in sulfide-concentration was used as indicator if crude oil-degradation was happening. In the controls, where no crude oil was added, no or no relevant increase in sulfate-reduction was measured. Hence, it can be assumed that increasing sulfide concentrations in the slurries that were treated with crude oil were caused by crude oil degradation by sulfate-reducing bacteria.

In Experiment No. 6, where crude oil was directly injected into sediment mini-cores, the first hypothesis could not be confirmed. Only a small increase in sulfate-reduction rates could be measured in the crude-oil treated mini-cores after 73 days of incubation (Fig. 3.20). From the results of the other experiments it can be estimated that probably a longer incubation time, of at least 140 days, would have been necessary to see significant increases in sulfate-reduction rates. Remarkably were the large standard-deviations in the crude-oil treated mini-cores, especially in the heavy oil treated mini-cores. Similarly to Experiment No. 6, Suàrez-Suàrez *et al.* (2011) incubated sediment cores from a pristine environment with crude oil. After 92 days they also found a slight increase in sulfate-reduction rates and high standard-deviations. Possibly, the high standard-deviations indicate an ongoing change in sulfate-reduction rates. The same was also

found in Experiment No. 1 and 2, where error bars also became larger when sulfide concentrations started to rise.

The long lag-phases, which were found in the incubations with sediment slurries, indicate that the microbial community within sediments from Eckernförde Bay was not adapted to the degradation of crude oil compounds before the experiments of this study. That degradation of crude oil compounds is possible in in pristine environments, harbouring microbial communities that are not adapted to the degradation of crude oil, but starts after a long lag-phase is an already known phenomenon (Committee on Oil in the Sea, 2003). A reason for this lag-phase could be that crude oil degrading bacteria, which are present in small amounts within the sediment, have to become more abundant before substantial crude oil degradation in the sediment can be measured. Several authors report about the "ubiquity" of petroleum-hydrocarbon degrading microorganisms (Atlas, 1981; Leahy & Colwell, 1990; Atlas, 1995; Delille & Delille, 2000; Prince, 2002). Atlas (1981, 1985) state that in pristine petroleum hydrocarbon degrading bacteria usually represent less than 1% of total bacterial community, while in petroleum-contaminated sediments bacteria capable of hydrocarbon degradation can contribute up to 10% of the microbial community. Thus, adapted sediments contain a higher fraction of crude oil utilizing microbes and probably also more microbes, which can survive toxic hydrocarbons (Coates *et al.*, 1997; Paissé *et al.*, 2008). Therefore, no long lag-phase before crude oil degradation starts should be observed when crude oil is added to well adapted sediments. An other cause for the observed lag-phase could be that already abundant facultative hydrocarbon degrading bacteria, which can also grow on substrates other than crude oil compounds, have to adapt to this new substrate and produce the enzymes needed for degradation before crude oil degradation activity can be measured (Committee on Oil in the Sea, 2003).

Due to the close proximity of the sampling site of the sediment, used in the experiments of the present study, to the Schwedeneck oil field (distance ca. 5 km; Fig. 4.1) it is questionable if sediments from Eckernförde Bay can be termed "pristine" in terms of crude oil contamination. The oil field, together with the existing pockmarks (craters in the seabed) and acoustic turbidity (indicating shallow gas in the sediment), found in Eckernförde Bay (see Fig. 4.1), could indicate

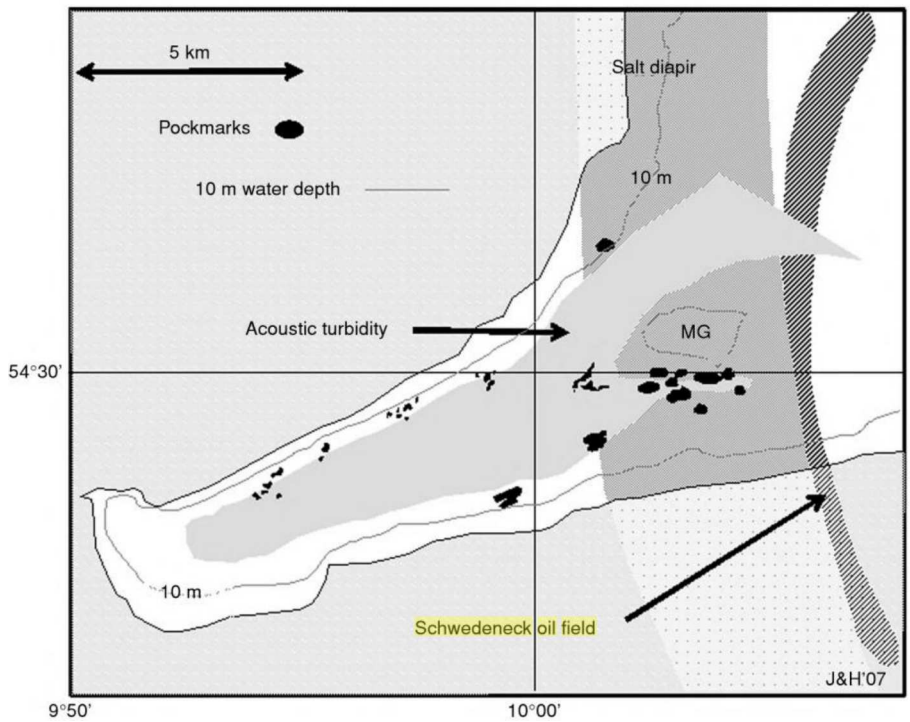

Figure 4.1: *Location of the Schwedeneck oil field within Eckernförde Bay.* Location of the oil field is indicated by the sickle-shaped dark-grey shadow. Also indicated in this map are the locations where acoustic turbidity and pockmarks are found within Eckernförde Bay (Judd & Hovland, 2009). The area where the sediment for the experiment was sampled lies approximately where the arrow from acoustic turbidity points to.

that sediments in the bay could be influenced by hydrocarbon input from the nearby petroleum reservoir. Furthermore, at the Schwedeneck oil field, there has been oil mining activity from 1984 until 2000 (URL 8). Thus, sediments in Eckernförde Bay may be contaminated with crude oil either by natural or anthropogenic sources. But several facts count against that: (1) Whiticar (2002) states that freshwater discharge is the most reasonable source of the pockmarks in Eckernförde Bay. (2) Acoustic turbidity is most likely caused by decomposition of recent organic matter (Dando *et al.*, 2000), so not by degradation of petroleum hydrocarbons. (3) When the mining at Schwedeneck oil field was finished in 2000, the operator of the two offshore oil rigs, the RWE-DEA AG, published a press release, in which it was stated that oil mining occurred without disturbances (URL 8). (4) The long lag-phases found in the experiments of the present study, which indicate that the microbial community within the sediment was not adapted to crude oil degradation. So, based on these statements, one can assume that despite the close proximity to the Schwedeneck oil field, sediments from Boknis Eck were not polluted by crude oil and therefore also not adapted to crude oil degradation before the experiments of this study.

There are a couple of studies, in which *in-situ* sulfate reduction-rates at oil or oil-gas seeps, where the microbial communities are well adapted to the degradation of crude oil- and gas components, were measured (Aharon & Fu, 2000; Joye *et al.*, 2004; Orcutt *et al.*, 2005; Orcutt *et al.*, 2010). Many of them were done in the Gulf of Mexico, where several natural crude oil and gas seeps are found (Behrens, 1988; Kennicutt *et al.*, 1988; Aharon, 1994). Most sulfate-reduction rates reported in these studies are in the same order of magnitude or even lower than the highest rates found in the present study (Table 4.1). But at natural oil seeps mostly not only crude oil but also gas is seeping through the seafloor. Therefore, some part of sulfate-reduction at natural oil seeps is associated to AOM. But AOM associated sulfate-reduction is only a minor part of total sulfate-reduction at sites of active oil seepage (Joye *et al.*, 2004; Orcutt *et al.*, 2005). For example in a study by Joye *et al.* (2004), where sulfate-reduction rates of 359 and 2949 nmol cm^{-3} d^{-1} measured, only AOM rates of 11.7 and 3.4 nmol cm^{-3} d^{-1}, respectively, were found. What means that according to the stoichiometry of AOM (Barnes & Goldberg, 1976) only 11.7 and 3.4 nmol cm^{-3} $d^{-1}$of sulfate reduction were related to AOM. Thus, the majority of measured sulfate-reduction rates in the study by Joye *et al.* (2004) was related to degradation of hydrocarbon compounds other than methane.

There are also studies, which report sulfate-reduction rates measured during petroleum degradation in sediment incubations or in enrichment- and pure-cultures under laboratory conditions (Rueter *et al.*, 1994; Caldwell *et al.*, 1998; Abed *et al.*, 2011). Even in crude-oil degrading pure-cultures of sulfate-reducing bacteria sulfate-reduction rates were not higher then the rates measured in the incubations of the present study. Rather, the highest potential sulfate-reduction rates observed in the present study were about 3-10 times higher than the rates reported in other studies (Table 4.1). In the studies where sediments from contaminated sites or enrichment and pure-cultures were used, no long lag-phases after addition of crude oil were reported.

Comparison of the above mentioned studies and the present study strengthens the theory that prior exposure to petroleum hydrocarbons shortens the lag-phase, as these sediments already contain a higher number of microbes that can utilize or at least survive toxic hydrocarbons (Coates *et al.*, 1997; Paissé *et al.*, 2008), but can not increase the rates of degradation (Committee on Oil in the Sea, 2003). Of course, comparison of the sulfate-reduction rates reported in the above mentioned studies (Table 4.1), to the values measured in the present study, can

Table 4.1: *Comparison of sulfate-reduction rates reported in different studies on crude oil degradation.* Rates were measured either *in-situ* in sediments at oil or oil-gas seeps or *ex-situ* in incubations of sediment slurries or in pure- or enrichment cultures of sulfate-reducing bacteria.

Rates measured *in-situ* or *ex-situ*	Study	Sulfate-reduction rates (min-max) in nmol cm^{-3}sediment d^{-1}	Natural sediment community, pure- or enrichment culture; study area or isolation site
In-situ	Aharon and Fu (2000)	1 - 220	Natural sediment community; natural oil seeps, Gulf of Mexico
	Aharon and Fu (2000)	270 - 2510	Natural sediment community; natural oil seeps, Gulf of Mexico
	Joye *et al.* (2004)	359 - 2949	Natural sediment community; natural oil seeps, Gulf of Mexico
	Orcutt *et al. (2010)*	0.9-800	Natural sediment community; natural oil seeps, Gulf of Mexico
Ex-situ in batch-incubations with sediment or pure- and enrichment cultures	Rueter *et al.* (1994)	900	Thermophilic pure-culture; oil production tank near Hamburg, Germany
	Rueter *et al.* (1994)	550	Enrichment culture; North Sea oil tank, Germany
	Abed *et al.* (2011),	220	Natural community; batch-incubation of microbial mat, Palestine
	Abed *et al.* (2011),	320	Enrichment culture; microbial mat, Palestine
	Present study	140-3100	Natural community; batch-incubation of natural sediment, Eckernförde Bay, Germany *

* Not previously adapted to crude oil degradation. In all other studies well adapted communities or enrichment- or pure cultures of crude oil compound degrading bacteria were investigated.

not be done without any uncertainties. Sulfate-reduction rates are measured under different conditions, with different crude oil types, different incubation conditions and also with different bacterial abundances and species compositions. Especially when comparing the rates of the present studies to rates, which were measured *in-situ*, one has to keep in mind that in the present study incubations were done under ideal conditions for the sulfate reducing bacteria, with media containing many vitamins and trace elements. Thus, the values reported in the present study are potential rates, which were only found under laboratory-conditions. But nevertheless, comparison of the different studies gives a hint that bacteria within sediments from Eckernförde Bay can reach, under ideal conditions, comparable rates of sulfate-reduction during degradation of crude oil compounds like well adapted sediment communities or even pure cultures of crude-oil degrading bacteria.

4.2 The influence of crude oil type on the response of sulfate-reducing bacteria

The **second hypothesis** was that the response of sulfate-reducing bacteria in the samples, which were treated with light oil will be higher and faster than in the samples treated with heavy oil. What means that shorter lag-phases and higher sulfate-reduction rates were expected in the samples treated with the light oil, compared with the samples treated with heavy oil. The hypothesis that the duration of the lag-phase is shorter in light-oil treated samples, could not be confirmed. Rather, it was the other way around. At highest crude oil concentrations used in the experiments, the duration of the lag-phase was shorter in the heavy-oil treated samples. The effect of crude oil concentration on the lag-phase will be discussed in the next section (section 4.3).

For the effect of crude oil type on sulfate-reduction rates in Experiment No. 2, 3a and 5, where sediment slurries were treated with different concentrations of two types of crude oil, it was fund that indeed, potential rates of sulfate-reduction were significantly higher in the light-oil treated slurries compared to the heavy oil treated slurries. Due to missing replication in Experiment

No. 3a no statistics could be done, but potential sulfate-reduction rates in the light oil treated slurries were also higher than in the heavy oil treated samples.

The hypothesis, that addition of lighter oil results in higher sulfate-reduction rates, was based on the assumption that light oil should be more easily degradable by microorganisms because it contains more short-chained hydrocarbons and not as many high-molecular weight hydrocarbons like heavy oil (Tissot & Welte, 1984). It was assumed that short-chained hydrocarbons can be more easily degraded by bacteria because they are usually degraded first (Prince, 2002).

However, the hypothesis that rates of sulfate-reduction become higher in light oil treated samples could not be confirmed in all experiments. In Experiment No. 1 sediment slurries were incubated with crude oil, like in Experiment No. 2, 3 and 5, but incubation time in Experiment No. 1 was longer than in the other experiments. In time-interval two, potential rates of sulfate-reduction in Experiment No. 1 were, similar to Experiment No. 2, 3a, and 5, significantly higher in the light oil treated slurries than in the heavy oil treated slurries. But afterwards, in the third time-interval, potential sulfate-reduction rates in Experiment No.1 became similarly high in slurries treated with both crude oil types. In incubations with both crude-oil types there was an increase of sulfate reduction rates between time-interval two and three. Since the increase in the heavy oil treated slurries was higher, potential sulfate-reduction rates reached the same values in incubations with both crude oil types. The reason therefore could be that heavy oil in general can not be that easily degraded like light oil, what causes the initially lower sulfate-reduction rates in the heavy oil treated slurries. But, after some degradation, the breakdown products of initial degradation of heavy oil compounds are similarly easy degradable as light oil, and therefore the rates became similar. Breakdown products for example could be smaller hydrocarbons, that are produced during the breakdown of large molecules, or organic acids, like acetate, which then could then fuel part of the measured sulfate-reduction (Orcutt *et al.*, 2010).

One question that remains is if similar sulfate-reduction rates, like they were found in the third time-interval in Experiment No. 1, mean that the two used crude oil types were degraded at similar rates. Heavy oil contains more long-chained and more complex molecules than like oil. It is very likely that more complex molecules can not be that easily degraded by microorganisms

and probably require several degradation steps. What could mean that for the same amount of crude oil degraded, in the case of heavy oil there would probably have to be more sulfate reduced than when light oil is degraded. To answer this question direct measurement of rates of crude oil degradation would be necessary. Results of a study by McMillen *et al.* (1995), confirm the assumption that light and heavy oil is usually not degraded at same rates. In the study by McMillen *et al.* (1995) the short-term biodegradability of 17 crude oils in soil microcosms was investigated. They found that $> 61\%$ of the most degradable oil (API = 46°, light oil) is degraded within 4 weeks, while only 10% of the least degradable oil (API = 15°, heavy oil) is degraded after the same time under the same conditions (aerobic conditions). Even though this study was done under aerobic conditions, it is very likely that under anaerobic conditions, like in the experiments of the present study, a similar picture will be found.

A new hypothesis could be that initially sulfate-reduction rates in light oil treated slurries will be higher than in heavy oil treated slurries. But after some time of incubation sulfate-reduction rates in slurries treated with the two different crude oil types will become the same. Nevertheless, within the same time less heavy- than light oil will be degraded.

4.3 The influence of crude oil concentration on the response of sulfate-reducing bacteria

The **third hypothesis** was that the height of sulfate-reduction rates and the duration of the lag-phase are affected by crude oil concentration. The duration of lag-phase seemed to dependent on crude oil concentration only in the heavy oil treated slurries. Lag-phases in heavy oil treated slurries always were shorter in incubations where a higher crude oil concentration was used (Experiment No. 3a, 2 and 1). But there is no simple explanation for that. Heavy oil contains less easily degradable low-molecular weight hydrocarbons than light oil. Thus, maybe, if heavy oil is added at a higher concentration, more low-molecular weight hydrocarbons are available, and therefore degradation starts earlier. But it is questionable why this effect of shortened lag-phases

was not found for light oil. To answer this question it would be necessary to know the composition of the tested crude oils and the characteristics and biodegradability of the compounds.

In Experiment No. 3a, 2 and 1, where sediment slurries were treated with different concentrations of two types of crude oil, it was confirmed that crude oil concentration affects potential rates of sulfate-reduction for both tested types of crude-oil. But highest sulfate-reduction rates were not found at highest crude oil concentrations. In Experiment No. 3a, highest rates of potential sulfate-reduction were found at a concentration of 0.6% (v/v) crude oil. At higher or lower concentrations sulfate-reduction rates were always lower. It is interesting that the same concentration revealed maximum sulfate-reduction rates for both tested crude oils. Also the comparison of potential sulfate-reduction rates from Experiment No. 1 and 2 confirmed this result. In Experiment No. 2, a crude oil concentration of 0.57% (v/v) was used. The sulfate-reduction rates found in Experiment No. 2 were significantly higher than in Experiment No. 1, where a 10 fold higher crude oil concentration was used (ANOVA, TukeyHSD p-values < 0.05).

But why were the highest sulfate-reduction rates in the incubation experiments (Experiment No. 1, 2, 3a) not found in the samples with the highest crude oil concentration? Although, in Experiment 3b, where the ratio of living to death cells was determined at different crude oil concentrations, no toxic effect was found at the crude oil concentrations used in the incubation experiments? One possibility could be that the toxic effects at the highest crude oil concentration used in the incubation experiments were toxic but not lethal. Thus, these toxic effects were not found in Experiment No. 3b. Nevertheless, they could have reduced microbial activity (Heipieper & Martinez, 2010) and this is why measured rates of sulfate-reduction were lower than at the second-highest crude oil concentration.

In studies, where in-situ rates of sulfate-reduction were measured at natural oil seeps, statements on influence of crude oil concentration on sulfate-reduction rates vary. Montagna *et al.* (1986) found higher sulfate-reduction rates at sites of moderate seepage than at sites with low or high seepage, what fits good to the results of the present study. Orcutt *et al.* (2010) investigated five sites with different degrees of crude oil contamination. They conclude that sulfate-reduction rates are higher in sites with higher seepage of crude oil. But only if enough sulfate is present. This

conclusion was refuted by the present study, at least under laboratory conditions. In Experiment No. 3a, 1 and 2 highest potential rates of sulfate-reduction were not found at highest crude oil concentrations, even though there was enough sulfate present.

Also in laboratory studies it was found that degradation rates of crude oil and sulfate-reduction rates are dependent on crude oil concentration. In studies of Rambeloariosa *et al.* (1984) or Rueter *et al.* (1994) it is shown that highest rate of crude oil degradation (aerobic conditions) or sulfate-reduction (anaerobic conditions), respectively, were found at the highest used crude oil concentrations. The present study, however, shows that there is a "threshold concentration", at which rates of sulfate-reduction and consequently probably also rates of crude oil degradation start to decrease again. Thus, crude oil concentrations used in the studies by Rambeloariosa *et al.* (1984) and Rueter *et al.* (1994) were probably too low to find this threshold concentration. How high this threshold concentration is, in every particular case, is probably determined by an interplay of several biotic and abiotic factors, of which the previous adaptation of the microbial community probably is really important.

4.4 The influence of crude oil addition on bacterial abundances

4.4.1 The influence of crude oil addition on total bacterial abundance

The **fourth hypothesis** was that the overall bacterial abundance in the sediment will be affected by the addition of crude oil. This hypothesis could not be confirmed. In Experiment No. 6 total bacterial abundances were determined in sediment mini-cores after 73 days of incubation. No relevant difference of bacterial abundances in mini-cores treated with light- or heavy oil and the cores that were not treated with crude oil were found (Fig. 3.22). That no change in total bacterial abundance was found could be caused by the fact that the incubation time was too short to find increased bacterial abundances. In Experiment No. 1, 2, and 3a it was found that degradation of crude oil probably started after 100-140 days of incubation. Consequently, after 73 days, it is likely that not much microbes were using the crude oil as energy- or carbon source.

Thus, crude-oil degrading microbes could, after 73 days of incubation, presumably not have increased that much in abundance that this could influence overall bacterial abundances.

Also no decrease in total bacterial abundance after addition of crude oil to the sediment mini-cores was observed. Due to the toxicity of many crude oil compounds, it could be expected that there would be a decrease in bacterial abundances after addition of the oil. There are two possibilities why no decrease was detected after 73 days of incubation:

(1) It is possible that there was an initial decrease of bacterial abundance, but that bacterial abundances, after 73 days, were already increasing again. Therefore, no drop in cell numbers could be detected.

(2) The crude oil, in the concentration used in the experiment, had no lethal effect on bacteria. Therefore, bacterial abundances did not decrease. But abundances were also not increased after 73 days, as not much microbes were using crude oil compounds as growth substrate.

The second explanation is more likely to be true. From the results of Experiment No. 3b it can be seen that the crude oil concentration used in Experiment No. 6 probably had no relevant lethal effects on bacteria within the sediment. If assumption No. 1 should be right, for an increase in bacterial abundances, also higher sulfate-reduction rates than the ones measured in the mini-cores should have been measured.

In literature there are many different statements on the of effect of petroleum hydrocarbons on total abundances of sedimentary bacteria. It is reported that either addition of petroleum hydrocarbons enhances (Bunch, 1987), reduces (Griffiths *et al.*, 1981; Suárez-Suárez *et al.*, 2011) or has no effect (Bauer & Capone, 1985; Wyndham, 1985; Carman *et al.*, 1996; Delille & Delille, 2000) on total bacterial abundances within sediments. Thus a statement on the effect of crude oil addition on microbial abundances in sediments is hard to make and probably the direction of the effect can not be generalized. If there is an effect, and which effect is found could be caused by an interplay of many different factors, like the kind of crude oil and previous adaptation of sediments to crude oil.

4.4.2 The influence of crude oil addition on the abundance of certain microbial groups

The **fifth hypothesis** was that the abundance of microbial species within the sediment will be affected by crude oil addition and that the *Desulfovibrio spp.* group will become more abundant. This hypothesis could not be confirmed. Probably due to a too short incubation time of 73 days, only a slight tendency of increased cell numbers of *Desulfovibrio spp.* in the crude oil treated sediment mini-cores from Experiment No. 6 was found (Fig. 3.22). This result is in good agreement with the above mentioned trend of slightly increased sulfate-reduction rates in the sediment mini-cores from Experiment No. 6 after 73 days of incubation (Fig. 3.20).

Although the hypothesis of the changed bacterial abundances was not confirmed, it can be assumed that with longer incubation time one should find an effect on abundances of bacterial groups. In several studies it was clearly shown that oil addition exerts a strong selection pressure on the microbial community and therefore causes fast and drastic changes of species composition (Delille & Delille, 2000; Röling *et al.*, 2002; LaMontagne *et al.*, 2004; Paissé *et al.*, 2008; Orcutt *et al.*, 2010; Rosano-Hernández *et al.*, 2012). In most studies it was found tat community diversity is decreased at sites with crude oil contamination and community diversity is negatively related to crude oil content. What means that for example along a gradient of crude oil contamination, lowest diversity is found at the site with highest contamination (LaMontagne *et al.*, 2004; Paissé *et al.*, 2008).

Most of the above cited studies were done under aerobic conditions. But it is known that also under anaerobic conditions, like in Experiment No. 6 of the present study, the bacterial species diversity in anaerobic environments is directly correlated to the duration and extent of hydrocarbon contamination. For example Miralles *et al.* (2007a) found that bacterial species richness was decreasing after crude oil addition to natural sediments from the Gulf of Fos (France) in *in-situ* incubations.

The tendency of increased abundances for the *Desulfovibrio spp.* group found in the present study and the slight increase in sulfate-reduction rates in sediment mini-cores treated with crude oil, together with isolation sites and potential involvement in crude oil degradation of members

of this group reported in other studies (different contaminated habitats: Magot, 2005; Birkeland, 2005; Miralles *et al.*, 2007a), indicate that it would be interesting to do further experiments targeting this group of sulfate-reducing bacteria and their role in crude oil degradation.

4.5 The effect of aerobic pre-digestion of crude oil on the response of sulfate-reducing bacteria

The **sixth hypothesis** was that crude oil, which has been subject to aerobic pre-digestion can be more easily degraded by sulfate-reducing bacteria. What means that it was expected that in incubations with aerobically pre-digested crude oil the lag-phases are shorter and sulfate-reduction rates become higher, compared to incubations with not pre-digested crude oil. Part of this hypothesis could be confirmed in this study. Indeed, in Experiment No. 5, where incubations with aerobically pre-digested crude oil were done, it was observed that the duration of the lag-phase was approximately only half as long as in incubations with non pre-digested crude oil (compare e.g. Fig. 3.2 and Fig. 3.15). However, the calculated rates of potential sulfate-reduction in incubations from Experiment No. 5 were not higher than those in incubations from the other experiments. What could be due to the fact that only the initial increase of sulfate-reduction rates was measured in the incubations with the pre-digested crude oil (Experiment No. 5) and that the rates would become higher after some more time of incubation.

The lowest increase in sulfide concentrations was found in the incubation where a pure culture of *D. mediterraneus* was incubated with the aerobic pre-digestion. Sulfide concentrations at the end of the incubation time in this sample were even lower than in the + Medium incubations, where just anoxic artificial seawater medium was added to the aerobic pre-digestion. Hence, *D. mediterraneus* must have even hindered the indigenous sulfate-reducing bacteria in the slurry from the aerobic pre-digestion from degrading the crude oil.

During aerobic incubation, it could not be directly measured if degradation of crude oil compounds was happening (e.g. by measurement of CO_2 concentrations or the change of crude oil composition). Nevertheless, the observed color change of the resazurin from blue to pink

in the oil treated incubations is a hint for microbial activity in the aerobic incubation. This change in color could be caused either by activity of aerobic bacteria (resazurin also used for cell viability tests, URL 9) or by pH differences (resazurin is also pH dependent: it turns pink at a pH between 5 and 6, URL 10). As pH changes can also be caused by microbial activity, in both cases the observed color change indicates microbial activity. This color change was not found in the control. Thus, this microbial activity was probably related to crude oil degradation. A further hint that the crude oil was really pre-digested by aerobic microbes are the shortened lag-phases, which were found in the subsequent anaerobic incubations.

The effect of aerobic pre-digestion of crude oil on subsequent degradation by anaerobic microorganisms was already observed about 30 years ago. Jobson *et al.* (1979) did a study on the "interaction of aerobic and anaerobic bacteria in petroleum biodegradation". They found that pure cultures and enrichment cultures of sulfate-reducing bacteria could degrade crude oil, within an incubation period of 55 days, only when this crude oil was subject to aerobic degradation in beforehand. Therefore, they concluded that sulfate-reducing bacteria were not able to degrade petroleum hydrocarbons, what was the common doctrine at that time, but did grow on residues of aerobic degradation of crude oil, like organic acids. The long lag-phases of the experiments, which were conducted in the present study, indicate that the reason why Jobson *et al.* (1979) found no degradation of not pre-digested crude oil was that the incubation time in their study was too short.

For organic matter in general it is known that pre-digestion in the water column (Karl *et al.*, 1988), and also on the seafloor (Lee, 1992; Middelburg *et al.*, 1993) enhances its biodegradability by anaerobic sediment bacteria. In Experiment No. 5, it was found that aerobic pre-digestion probably also enhances the biodegradability of crude oil under anaerobic conditions. The question why aerobic pre-digestion of crude oil shortens the lag-phase could only be answered if the change in crude oil composition and breakdown products during aerobic incubation and later also during anaerobic incubation could be monitored. Probably, like Jobson *et al.* (1979) concluded, a major part of sulfate reduction in the anaerobic incubation of sediment slurries with aerobically pre-digested crude oil was driven by breakdown products of aerobic degradation, e.g. organic acids or smaller hydrocarbon compounds. At least in the beginning, and therefore the lag-phase was shortened.

4.6 Degradation of benzene by sulfate-reducing bacteria

The **seventh hypothesis** was that sulfate-reducing bacteria, which are present in natural marine sediments from Eckernförde Bay, are able to degrade benzene. This hypothesis could not be confirmed. During the time of incubation a decrease in benzene concentrations was observed, but no relevant increase in sulfide concentrations.

Of the three measurement time-points where benzene concentrations were determined only after 104 days there was significant less benzene present in the benzene treated slurries than in the controls. But also in the control a decrease in benzene was observed. Nevertheless, the loss in the benzene treated slurries was significantly higher then in the controls. As the benzene treated slurries and the controls were treated the same, one can expect that the extra-loss was probably caused by microbial activity.

The reason for the absence of an increase in sulfide concentration were probably the low benzene concentrations in the incubations. According to the stoichiometry of benzene degradation by sulfate-reducing bacteria of Lovley *et al.* (1995) the complete degradation of the ca. 2.6 µM benzene, which is the concentration that was initially present in the incubations, would lead to the production of 9.8 µM of sulfide. This increase is far below the detection limit of the method used for measurement of sulfide concentrations (detection limit = 0.1 mM; Cord-Ruwisch, 1985).

Summing up, as the used medium was suitable for sulfate-reducing bacteria, and a decrease in the benzene was observed, it can be assumed that this decrease was probably caused by activity of sulfate-reducing bacteria. But further investigations with refined methods and more sensitive measurements will be needed to confirm this hypothesis.

4.7 Reaction of sulfate-reducing bacteria and development of methane concentrations in batch-incubations of sediment slurries from different sediment depths

The **eighth hypothesis** was that the response of methanogenic archaea after addition of crude oil, is higher in deeper sediment layers. The first hints, which led to the formulation of this hypothesis were the observed gas bubbles and increased methane concentrations after few days of incubation in slurries from Experiment No. 1. If methane production by microorganisms was responsible for the observed increase in methane concentration and the formation of the gas bubbles, a stronger effect should be found in deeper sediment layers. Thus, in Experiment No. 2 not only sediment from 0-10 cm, like in Experiment No. 1, but also sediment from 20-30 cm sediment depth was used for the preparation of sediment slurries. In this deeper sediment layers there should be more methanogenic archaea present, as in methane profiles from Eckernförde Bay an increase of methane concentrations can be already seen in this sediment depth (Treude *et al.*, 2005). But besides higher initial methane content in the slurries from the deeper sediment depth, no substantial difference between the two sediment depths in the extend of changes of methane concentrations during incubation was observed. Thus, the eighth hypothesis could not be confirmed. Nevertheless, the development of methane content in the headspace of the sediment slurries during incubation is interesting. At first there must have been methane production. As methanogenic archaea are the only organisms capable of methanogenesis (Madigan *et al.*, 2011) they could be responsible for the increase in methane content. Then, later methane content was decreasing. As AOM is found in Eckernförde Bay sediments (Treude *et al.*, 2005) this could be a possible explanation for the decrease in methane concentrations.

But, even if there was methane production by methanogenic archaea in the beginning of the incubation, it is questionable if this activity was related to crude oil degradation. Townsend *et al.* (2003) found that methanogenic archaea need even longer to adapt to the degradation of petroleum hydrocarbons than sulfate reducing bacteria. Thus, it can be expected that in sediments from Eckernförde Bay it would probably also take longer to see a response of methanogenic archaea

than of sulfate-reducing bacteria (lag-phase for sulfate-reducing bacteria was 100-140 days). Consequently, it is likely that, if the observed increase in methane concentrations was caused by activity of methanogenic archaea, they probably must have degraded organic matter other than crude oil compounds.

For the observed gas bubbles in the incubations with the crude-oil treated sediment slurries it is very likely, that the gas bubbles were not caused by biotic processes, because: (1) The measured increase in methane concentration was too low to explain the many observed gas bubbles. (2) No relevant development of overpressure was observed in the incubations. Hence, there was probably no production of "new gas" in the incubations. It is very likely that the gas bubbles in the sediment were just caused by shaking of the sediment slurries before measurement. That these bubbles could be only observed in the slurries treated with crude oil could maybe be caused by an increased "stickiness" of oil-treated sediment.

What was furthermore interesting in Experiment No. 2 was that in the two different sediment depths a difference in the response of sulfate-reducing bacteria was found. In the deeper sediment layer no increase in activity of sulfate-reducing bacteria was measured during time of incubation. Also Miralles *et al.* (2007b) report, that in *in-situ* incubation experiments of oil-treated sediment cores in the Gulf of Fos (Mediterranean, France) the reaction of sulfate-reducing bacteria to oil contamination in the uppermost cm of a sediment, is faster than in deeper sediment layers. They only compared 0-1, 2-4 and 8-10 cm sediment depth, but from these results one could expect that in even deeper sediment layers the response of sulfate-reducing bacteria then would be even slower. Causes for the slower response of bacteria in deeper sediment layers could be:

(1) That either initial abundances of sulfate-reducing bacteria ,which are able to degrade crude oil, are lower in deeper sediment layers.

(2) Or that conditions (e.g. abiotic conditions like sulfate concentration) are more favorable for crude oil degrading bacteria in shallower sediment depths.

But as in the incubations in the present study, with the anoxic artificial seawater medium added, conditions in the incubations should have been similar, the first explanation is more reasonable.

4.8 Discussion of methods and experimental procedures

This study served as a pre-study for a further, more detailed investigation on crude oil degradation in marine sediments under natural conditions. Therefore, one aim was to investigate if the methods, used for not oil-contaminated sediments, also work for oil-contaminated sediments.

Most of the methods worked well, despite the oil contamination. For CARD-FISH, in the initial protocol several steps had to be changed. But that CARD-FISH did not work with the initial protocol was probably not caused by the crude oil. In the control sediments, that were not treated with crude oil, the initial protocol did also not work. The adjustments that were made are described in more detail in the appendix (section A.2.4.5).

In the batch-incubations with the sediment slurries, sulfide concentrations in the supernatant were measured with the photometric method after Cord-Ruwisch (1085). Because photometric measurements could be influenced by turbidity in the supernatant, which in the case of the performed experiments could be caused by the crude oil, also total alkalinity was measured. In all experiments, an increase in absorbance in the sulfide measurement was accompanied by an increase in total alkalinity, like it can be conveyed from the formula for sulfate reduction (Equation 1.1 on page 20). Thus, it was confirmed that absorption changes were caused by changes in sulfide concentrations and for the next experiments the photometric method after Cord-Ruwisch (1985) can be used for sulfide measurements in sediment slurries treated with crude oil. Nevertheless, there were three points that are worth being discussed about the sulfide measurements:

(1) In some control slurries and during the lag-phase in the oil-treated slurries (Experiment No. 2 and 3a) negative potential rates of sulfate-reduction were observed. What means that sulfide concentration in the supernatant of the slurries was decreasing. There could be several reasons for that.

> (1a) There is the possibility that some of the sulfide precipitated as iron sulfide within the sediment and was therefore not measured in the supernatant anymore.

(1b) It could be that this trend of decreasing sulfide concentrations is caused by measurement inaccuracy. Cord-Ruwisch (1985) state that the lower detection limit of the method is 0.1 mM. Together with the inaccuracy of measurement of the 0.1 ml sample volume (sampled with a 1 ml syringe) the observed negative rates could maybe be explained.

(1c) It is also possible, that there really was loss of sulfide. Loss of sulfide could for example happen through the rubber stoppers. The rubber stoppers can, especially when they were in usage for some time and several samplings with needles have been done, become untight.

(2) In Experiment No. 3a there was an increase in total alkalinity measured, without increase of sulfide concentration, directly from the beginning of the incubations. This steady increase in total alkalinity, which was also found in the control, indicates that probably organic matter other than crude oil was present in those incubations and became degraded. In Experiment No. 3a, after ca. 100 days, when finally sulfide concentrations started to rise in the oil treated slurries, the increase in total alkalinity in the crude oil treated samples also became steeper. So that the cause for the increased steepness in increase of total alkalinity was probably caused by crude oil degradation, which then started.

That an increase in alkalinity was measured, but no increase in sulfide, can have the same reasons like the ones discussed above for the negative sulfate-reduction rates. It is possible that little sulfate-reduction was happening and therefore total alkalinity increased. But, as there was simultaneously loss of sulfide, no increase of sulfide concentration was measured. Total alkalinity could also be influenced by anaerobic microbial degradation processes other than sulfate-reduction (Kuivila & Murray, 1984; García-Ruiz *et al.*, 1998; Brasse *et al.*, 1999). But, that such a large change in total alkalinity, like it was found in Experiment No. 3a, was caused by other processes than sulfate-reduction is unlikely. For the preparation of the slurries, a selective medium for sulfate-reducing bacteria was used. Therefore, other electron acceptors than sulfate were only present in minor amounts in the incubations.

(3) What that did not work was the measurement of sulfide concentration in the experiment on benzene degradation (Experiment No. 4). The increase of sulfide concentrations during benzene

degradation could probably not be measured, as the increase in sulfide concentration, which could have been expected in the incubations (9.2 µM), was much smaller than the detection limit of the method used for determination of sulfide concentrations. Therefore, for the next experiments, after adaptation of the sediment to small amounts of benzene, higher concentrations of benzene could be used for the actual experiment, as described by Lovley *et al.* (1995). Or, if benzene concentrations have to be low, another method for determination of potential sulfate-reduction rates should be chosen. Either sulfide could be measured by the methylene blue reaction method (Cline, 1969), the detection limit of this method is with 0.01 mM much lower than of the Cord-Ruwisch method. Or one could measure decrease in sulfate by ion chromatography, what also is much more sensitive (Weiss, 2004).

4.9 Conclusions

Based on the results of this study, the following conclusions could be drawn:

1. Sulfate-reducing bacteria, which are naturally present in sediments from Eckernförde Bay, are able to degrade crude oil. But after a long lag-phase of 100-140 days.

2. Potential sulfate-reduction rates in natural sediments from Eckernförde Bay can reach values that are in the range of those found in sediments harbouring communities, which are well adapted to crude oil degradation. For example in sediments at oil seeps in the Golf of Mexico. Also rates of sulfate-reduction found in enrichment or pure-cultures of crude oil degrading bacteria, are not higher than the rates found in the incubations with sediment from Eckernförde Bay.

3. In sediment slurries treated with light oil, initial rates of sulfate-reduction are twice as high as in sediment slurries treated with heavy oil. But rates of sulfate-reduction will become equal after some more time of incubation (after ca. 200 days of incubation in Experiment No. 1).

4. The amount of crude oil added, has no influence on the duration of the lag-phase if light oil is added to the sediment. If heavy oil is added, the lag-phase gets shorter the more crude oil is added, at least within the tested range of crude oil concentrations.

5. There is a "threshold concentration" of crude oil, at which highest sulfate-reduction rates are found. Of the tested crude oil concentrations, 0.6% (v/v) is probably the concentration, at which both types of crude oil used in this study are degraded at highest rates by microorganisms naturally present in sediment from Eckernförde Bay.

6. The addition of crude oil to sediment from Eckernförde Bay has no influence on overall bacterial abundances.

7. A slight increase in relative abundance of bacteria of the *Desulfovibrio spp.* group in oil treated mini-cores indicates a potential involvement of these bacteria in crude-oil degradation.

8. Aerobic pre-digestion of crude oil shortens the lag-phase but does not increase potential sulfate-reduction rates.

9. Sulfate-reducing bacteria in sediments from Eckernförde Bay are able to survive oxic conditions (at least over a time period of two weeks).

10. The observed fluctuations in methane content during the batch incubations were probably not related to crude-oil degradation.

4.10 Open questions and future prospectives

Many of the research questions of this study could be answered. Nevertheless, some questions were left unanswered and also new questions arose from the results of the experiments.

The most prevailing questions are:

(1) How much of the crude oil was degraded and which compounds were degraded? Because of the measured sulfate-reduction rates in the controls and the treatments, it can be assumed with quite high certainty that there must have been crude oil degradation. But how much of the crude oil, and which compounds were degraded could not be directly measured in this study. An ASE (accelerated solvent extraction system) and a GC-MS (gas-chromatograph coupled to a mass spectrometer) would allow to detect changes in crude oil composition. There is still some of the original crude oils, which were added to the sediment slurries, present. So that this crude oil could be compared to the oil in the sediment slurries from the incubations to see if there are differences in composition.

(2) How did the microbial community structure in the crude oil treated slurries, where sulfide concentrations did increase, change compared to the control slurries? Also for this question, the incubated sediment slurries from this study could be further investigated. A detailed analysis of the microbial communities in the different treatments could be done. An idea of diversity changes could be gained by amplifying bacterial 16S rDNA by PCR and analyzing community structure by denaturing gradient gel electrophoresis (DGGE). A more detailed investigation of the species composition of the bacterial communities of the different treatments could be done by restriction fragment length polymorphism (RFLP) analysis, or sequencing of 16S rDNAs and phylogenetic analysis. Also, further investigation e.g. by qPCR or CARD-FISH, of selected bacteria present in the sediment community, could give hints on changed abundances and potential involvements in crude oil degradation of these bacterial species or groups.

(3) Which microbial processes other than sulfate-reduction play a significant role in crude oil degradation in sediments from Eckernförde Bay? As the medium used for preparation of the sediment slurries in the present study was a selective medium for sulfate-reducing bacteria, other

processes than sulfate-reduction probably only played a minor role in the incubations. But in natural sediments, with steep gradients of terminal electron acceptors, this should be different. Therefore, it is important to study also other microbial processes. Even though sulfate reduction is the most important anaerobic process, under natural conditions other anaerobic microbial degradation processes also could contribute a significant part to crude oil degradation.

(4) How can the fluctuations in methane content in the headspace of the sediment slurries be explained? The methane could be either of biotic or abiotic origin. An analysis of the δ13C signature of the methane could give hints on the origin of the methane (Blair, 1998; Judd & Hovland, 2009). To check if the observed subsequent loss of methane was caused by AOM, rates of AOM in the different incubations could be measured.

(5) Why were potential sulfate-reduction rates increasing during the time of incubation? Did rates increase because oil-degrading microorganisms became more abundant? Widdel *et al.* (2010) state that anaerobic petroleum-degrading microbes have long doubling times (up to months), so would this increase be reasonable? Or did just the already present microorganisms "switch" their metabolism? It could also be possible that the steep increase in sulfate-reduction rates after the lag-phase was caused by activity of non-oil degrading sulfate-reducing bacteria, which were degrading breakdown products of crude oil degradation. To answer these questions one could follow degradation of crude oil compounds by ASE and GC-MS and relate them to sulfate-reduction rates. Furthermore, one could measure the turnover of breakdown products, like organic acids or similar compounds. Also analysis of development of bacterial diversity and abundance of certain microbial groups could be helpful to answer this question.

(6) Do similar sulfate-reduction rates in incubations with heavy- and light oil mean that these different types of crude oil were degraded at similar rates? To answer this question one could follow degradation of crude oil compounds and relate them to sulfate-reduction rates.

(7) Why did aerobic pre-digestion decrease the duration of the lag-phase? For answering this question it would be necessary to measure the change of crude oil compound composition during the aerobic and the following anaerobic degradation. Also again, measurement of breakdown products or organic acids should be done. To see if the shortened lag-phases are caused by degradation of them.

Furthermore, it would be interesting to see if communities, when they are adapted to pre-digested crude oil are different to communities, which are adapted to not-aerobically pre-digested crude oil. If there are differences, these differences could also be found in natural situations. What means for example between communities at natural crude oil seeps (where crude oil is also not aerobically pre-digested) and at places where oil spills happened (where oil, if the oil is coming from the water column, should be aerobically pre-digested).

(8) Do sulfate-reduction rates reach similar values if adapted and non-adapted sediments are incubated under the same conditions? In the present study, it was found that potential rates of sulfate-reduction in non-adapted sediments can become similar high like in well adapted sediments or even pure cultures of crude oil degrading bacteria. But conditions, under which sulfate-reduction rates were measured, in the different studies are very different. Thus, this comparison contains many uncertainties. For a better comparability, it would be very interesting to study adapted and not-adapted sediments in parallel, under the same controlled conditions. In such an experiment, it would be good to use crude oil from the source area of the adapted sediment, so that it can be really assumed that the community is perfectly adapted.

It would be interesting to see if, after adaptation of the not-adapted microbial community, the rates of sulfate-reduction during crude-oil degradation would really become the same like in the previously adapted sediment. It would be also interesting to see how similar or different the microbial communities in the previously adapted and non-adapted sediments become and if the same organisms are important for crude oil degradation in the different sediments.

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A Appendix

A.1 Recipes and solutions:

A.1.1 Anoxic sulfate reducer medium (modified according to Widdel & Bak, 1992):

Table A.1: *Concentrations and amounts of salts used for the production of the anoxic sulfate-reducer seawater medium.*

Salts (molecular weight):	Concentrations [mM]; g for 1 l medium:
KBr (119.01 g)	0.4968 mM; 0.05912 g
KCl (74.56 g)	5.29 mM; 0.3944 g
$CaCl_2$*H_2O (147.02 g)	6.5714 mM; 0.9661 g
$MgCl_2$*$6H_2O$ (203.3 g)	18.3277 mM; 3.7260 g
$MgSO_2$*$7H_2O$ (246.4 g)	18.1371 mM; 4.4689 g
NaCl (58.44)	296.3714 mM; 17.3199 g

In Table A.2 the solutions and in Table A.1 salts needed for the production of the anoxic sulfate-reducer medium are shown. At first the salts were dissoluted in 920 ml of ultra-pure water, to which then 1 ml of resazurin was added. Afterwards, this solution was autoclaved for 35 minutes. When the solution was still hot, it was aerated for 5 minutes with N2 while stirring with a magnetic stirrer. Then the gas was changed to N_2/CO_2 (80:20, v/v) and the solution was aerated for other 15 minutes while stirring. After that, still under aeration by N_2/CO_2 and stirring, when the solution has cooled down, 30 ml of the the bicarbonate solution and 50 ml of the the nitrogen / phosphate solution were added and everything was mixed well. Then also 1 ml of each of the vitamins (No. 1-5 in Table A.2) and the trace elements were added. Finally, a sulfide solution (1 M) was added to get rid of the remaining oxygen. The solution was then

further aerated with N_2/CO_2 and stirred until the pink color of the resazurin had disappeared. What meant that there was no oxygen left. Then the pH was adjusted to 7.5 (with help of the HCl – and the NaOH solution). The addition of all solutions after the autoclaving happened through a membrane filter with 0.2 nm pore-size to avoid bacterial contamination.

Table A.2: *Solutions used for the production of the anoxic sulfate reducer medium.*

Solution No.	Name	Ingredients
1.	6 – vitamin – solution (filtered sterile):	20 mM NaP-buffer (0.356 g Na_2HPO_4, 0.27 g NaH_2PO_4; pH 7, 100 ml); 4-aminobenzoate (4 mg); D-(+) biotine (1 mg); nicotinic acid (10 mg); calcium-d-(+)-panthotheanate (5 mg); pyridoxaminedihydro-chloride (15 mg); lipoic acid (1.5 mg)
2.	Thiamine (B1) (filtered sterile):	10 mg of thiamine in 100 ml of 25 mM NaP-buffer pH 3.4 – 3.7, 0.345 g $NaH_2PO_4$4)
3.	Cyanocobalamine (B12) (filtered sterile):	5 mg of cyanocobalamine dissoluted in 100 ml of ultra-clean water
4.	Riboflavine (B2) (filtered sterile):	5 mg of ribovlavine dissoluted in 100 ml of acetic acid (20 mM)
5.	Selenite / wolframate (autoclaved):	In 1000 ml of ultra clean water: 400 mg NaOH, 8 mg Na_2WO_2, 6 mg Na_2SeO_3
6.	Trace elements (autoclaved):	Add 12.5 ml HCl (37 %, fuming), 2100 mg $FeSO_4$ *7 $H_2$2O, 30 mg H_3BO_3, 100 mg I_2*4H_2O, 190 mg $CoCl_2$ * 6 H_2O, 24 mg $NiCl_2$*6H_2O, 2 mg $CuCl_2$*2H_2O, 144 mg $ZnSO_4$, and 36 mg Na_2MoO_2* 2 H_2O into a large cylinder and fill it up to 1000 ml with ultra-pure water
7.	Bicarbonate solution (autoclaved):	Solution with a concentration of 30 mM of $NaHCO_3$
8.	Nitrogen / phosphate – solution (autoclaved):	Solution with a concentration of 4.67 mM of NH_4Cl and 1.47 mM of KH_2PO_4
9.	6.5 % HCl solution (autoclaved)	
10.	1 M NaOH – solution (autoclaved)	
11.	1 M sulfide solution (autoclaved)	
12.	Resazurin (autoclaved)	

A.1.2 Media for cultivation of bacterial pure-cultures:

For cultivation of the pure cultures of *D. mediterraneus* and *D. marinus*, the media recommended by the DSMZ were used (URL 11). For *D. mediterraneus* this was medium 196, where propionate was replaced by 2.5g/l Na-lactate. For. *D. marinus* the medium recommended was medium 1040. For cultivation of the strains, the media were partitioned in 50 ml portions in sterile serum vials, which were closed with butyl rubber stoppers and crimp vials. The gas headspace in the vials was N_2/CO_2(80:20; v/v).

A.1.3 Concentrations of oil added to sediment slurries in experiment No 3:

Table A.3: *Amount of crude oil added to the different samples in Experiment No. 3a.*

Name of samples	% crude oil (volume crude oil/volume slurry)	ml crude oil / cm^3undiluted sediment
Light Oil 6 / Heavy Oil 6	6	0.375
Light oil 0.6 / Heavy Oil 0.6	0.6	0.0375
Light Oil 0.06 / Heavy Oil 0.06	0.06	0.00375
Light Oil 0.006 / Heavy Oil 0.006	0.006	0.000375
No Oil 1 / No Oil 2	0	0.0000375

Table A.4: *Amount of crude oil added to the different samples in Experiment No. 3b.*

Name of samples	% crude oil (volume crude oil/volume slurry)	ml crude oil / cm^3undiluted sediment
No Oil 1 / No Oil 2	0	0
Light Oil 0.6 / Heavy Oil 0.6	0.6	0.0421
Light Oil 6 / Heavy Oil 6	6	0.412
Light Oil 12 / Heavy Oil 12	12	0.842
Light Oil 18 / Heavy Oil 18	18	1.263
Light Oil 36 / Heavy Oil 36	36	2.632
Light Oil 50 / Heavy Oil 50	50	3.509

A.1.4 $CuSO_4$ Solution for the sulfide measurement after Cord-Ruwisch (1985):

$CuSO_4$ solution	
$CuSO_4$x 5 H_2O (MV: 249.69 g mol^{-1})	1.25 g
HCl (25%)	6.51 ml
Adjust to 1 l with H_2O_{UP} water	
Store at room temperature	

A.1.5 Measurement of sulfide standard and straight calibration line:

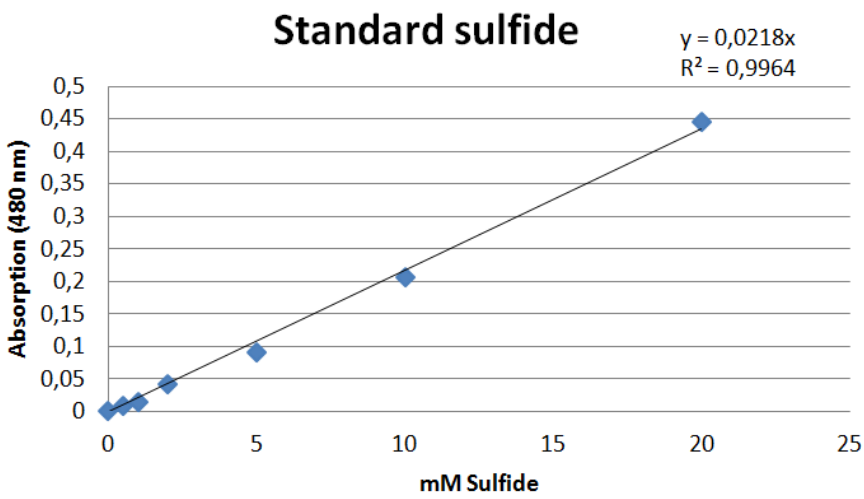

Figure A.1: *Straight calibration line of sulfide standards.* Seven standard solutions with a Na_2S concentration of 0-20 mM were measured.

A.1.6 Indicator used for the measurement of total alkalinity:

Indicator		
Solution 1	Methyl Red	32 g
	0.1 M NaOH	1.19 ml
	EtOH 96%	80 ml
Solution 2	Methylene Blue	10 mg
	EtOH 96%	10 ml
Mix 4.8 ml of solution 2 and 80 ml of solution 1		

A.1.7 Solutions and buffers for CARD-FISH.

H_2O_{UP}	
Fill H_2O_{UP} into 500 ml bottles	
Autoclave	
Store at room temperature	

10xPBS	
137 mM NaCl (MW 58.44)	80 g
2.7 mM KCl (MW 74.55)	2 g
10 mM Na_2HPO_4 (MW 141.96)	14.1 g
2 mM KH_2PO_4 (MW 136.09)	2.7 g
Solve in 800 ml H_2O_{UP}	
Adjust pH to 7.3	
Adjust to 1 L	
Autoclave	
Split into 50 ml Falcons	
Freeze at -20°C	

1xPBS	
10xPBS	100 ml
H_2O_{UP}	900 ml
Sterilized by filtration	
with 0.2 µm bacteria filter	
Store at 4°C	

1xPBS/EtOH	
1xPBS	25 ml
96% EtOH	25 ml
Store at 4°C	

4% Formalin in 1xPBS	
37% Formalin	108.1 ml
1xPBS	891.9 ml
Store at 4°C	

5 M NaCl	
NaCl (MW 58.44)	146.1 g
Solve in 500 ml H_2O_{UP}	
Autoclave	
Store at room temperature	

H_2O_2
General stock solution is 30% H_2O_2
Examples:
3% H_2O_2 – 30µl 30% H_2O_2 +270µl X
0,5% H_2O_2 – 5µl 30% H_2O_2 + 295 µl X
0,15% H_2O_2 - 1 µl 30% H_2O_2 + 200 µl X
Store at 4°C

10% SDS (sodium dodecyl sulfate)	
SDS (MW 288.38 g)	10 g
Adjust volume to 100 ml with H_2O_{UP}	
Autoclave	
Filtrate with 0.2 µm filter	
Store at room temperature	

1 M Tris–HCl pH 7.3	
1M Tris (MW 121.14 g)	121.14 g
H_2O_{UP}	800 ml
Adjust pH to 7.3	
Adjust to 1 L	
Autoclave	
Store at room temperature	

0,5 M EDTA pH 7,3	
0.5 M EDTA (MW 292.24 g)	73.06 g
H_2O_{UP}	400 ml
Adjust pH to 7.3 with NaOH	
Autoclave	
Store at room temperature	

0.1 M Tris-HCl	
1 M Tris - HCl	1 ml
H_2O_{UP}	9 ml

Lysozyme (10 mg/ml)	
Lysozyme	50 mg
0.5 M EDTA	500 µl
0.1 M Tris - HCl	500 µl
H_2O_{UP}	4 ml
Prepare fresh	

Blocking Reagent in 1xPBS	
Blocking Reagent (Invitrogen)	100 mg
1xPBS	10 ml
Prepare fresh	
Or store in aliquots at -20°C	

Hybridization buffer (35 %)	
5 M NaCl	3.6 ml
1M Tris - HCl	0.4 ml
Blocking Reagent	2.0 ml
10 % SDS	20 µl
Formamide (37 %)	7 ml
H_2O_{UP}	7 ml
Dextran sulfate disodium	2 g
Add dextran sulfate at last!	
Dissolve dextran sulfate at 40-50°C	
Store at -20°C	

2 x saline-sodiumcitrate (SSC)	
300 mM NaCl (MW 58.44 g)	17.5 g
34 mM Na-Citrat x $2H_2O$ (MW 294.1 g)	10.03 g
H_2O_{UP}	800 ml
Adjust pH to 7.0	
Fill to 1 L	

Streptavidin-HRP in 1xPBS (stock solution)	
Streptavidin - HRP (Invitrogen TSA Kit)	
Dissolve in 1xPBS	200 µl

Tyramide-Solution	
Amplification buffer (Invitrogen TSA Kit)	1000 µl
0.15 % H_2O_2 in PBS	10 µl
1 µl Tyramide Alexa 488 (Invitrogen TSA Kit)	1 µl
Prepare fresh	

Washing buffer	
1M Tris-HCl	1 ml
0.5 M EDTA	0.5 ml
10% SDS	50 µl
5 M NaCl	700 µl
H_2O_{UP}	47.75 µl
Prepare in 50 ml Falcon tube	
Add SDS at the end	
Prepare fresh	

0.2% Agarose	
Low gelling point Agarose	0.4 g
H_2O_{UP}	200 ml

MeOH with 0.15% H_2O_2	
MeOH	10 ml
30% H_2O_2	50 µl

Biotine labeled oligonucleotide probe
Dissolve probe to a concentration of 50 ng/µl
with nuclease free water (Roth)

A.2 Results

A.2.1 Experiment No. 2 Potential rates of sulfate-reduction

Table A.5: *Potential rates of sulfate-reduction during different time points of incubation in Experiment No. 2.*

Sample	Sulfate-reduction rate [nmol $cm^{-3}d^{-1}$] Time-interval 1	Sulfate-reduction rate [nmol $cm^{-3}d^{-1}$] Time-interval 2
Heavy Oil 0-10 cm	-7.70 ± 2.52 (0-126 d)	826.00 ± 513.50 (133-146 d)
Heavy Oil 20-30 cm	-5.44 ± 4.54 (0-140 d)	—*
Light Oil 0-10 cm	-8.71 ± 4.10 (0-126 d)	1770.30 ± 164.11 (133-146 d)
Light Oil 20-30 cm	-10.73 ± 3.45 (0-146 d)	—*
No Oil 0-10	-6.53 ± 4.55 (0-146 d)	—*
No Oil 20-30	-9.80 ± 1.21 (0-146 d)	—*

* No rate calculated for T2 as no long enough increase in sulfide was observed.

A.2.2 Experiment No. 3a

A.2.2.1 Concentrations during lag-phase

Table A.6: *Concentrations of sulfide during the lag-phase in Experiment No. 3a.*

Sample	Sulfide concentrations [mM] (duration of lag-phase)
Heavy Oil 6%	0.36 ± 0.17 (0-99 d)
Heavy Oil 0.6%	0.36 ± 0.16 (0-106 d)
Heavy Oil 0.06%	0.31 ± 0.11 (0-113 d)
Heavy Oil 0.006%	0.29 ± 0.14 (0-141 d)
Light Oil 6%	0.33 ± 0.12 (0-106 d)
Light Oil 0.6%	0.29 ± 0.11 (0-106 d)
Light Oil 0.06%	0.28 ± 0.15 (0-106 d)
Light Oil 0.006%	0.43 ± 0.19 (0-113 d)
No Oil	0.29 ± 0.09 (0-155 d)

A.2.2.2 Potential rates of sulfate-reduction

Table A.7: *Potential rates of sulfate-reduction during different time-intervals of incubation in Experiment No. 3a.*

Sample	Sulfate-reduction rate [nmol $cm^{-3}d^{-1}$] Time-interval 1	Sulfate-reduction rate [nmol $cm^{-3}d^{-1}$] Time-interval 2
Heavy Oil 6%	3.5 (0-99 d)	141.4 (106-155)
Heavy Oil 0.6%	-2.8 (0-106 d)	442.4 (113-155)
Heavy Oil 0.06%	4.9 (0-113 d)	236.6 (120-155)
Heavy Oil 0.006%	-0.7 (0-141 d)	—*
Light Oil 6%	9.8 (0-106 d)	233.8 (113-155)
Light Oil 0.6%	-1.4 (0-106 d)	534.8 (113-115)
Light Oil 0.06%	-10.5 (0-106 d)	302.4 (113-155)
Light Oil 0.006%	-6.3 (0-113 d)	224.0 (120-155)
No Oil	34.3 (0-155 d)	—*

* No rate calculated for T2 as no long enough increase in sulfide was observed.

A.2.3 Experiment No. 5

Table A.8: *Results of the statistical analysis of potential sulfate-reduction rates in time-interval two of Experiment No. 5.* ANOVA analysis revealed a significant difference between the samples ($p = 0.0000031$, $F = 76.3$). In the table the results of a post-hoc comparison by TukeyHSD test are shown. (Three replicates of each sample were used).

Samples compared	p-value
Heavy Oil + Slurry - Heavy Oil + D. med.	0.0034
Light Oil + D. med. - Heavy Oil + D. med.	0.00031
Light Oil + Slurry - Heavy Oil + D. med.	0.000002
Light Oil + D. med. - Heavy Oil + Slurry	0.1856
Light Oil + Slurry - Heavy Oil + Slurry	0.000052
Light Oil + Slurry - Light Oil + D. med.	0.00036

A.2.4 Experiment No. 6

A.2.4.1 Porosity data

Table A.9: *Data for porosity of sediments from Boknis Eck (Bertics et al., 2012).*

Sediment depth [cm]	Porosity [ml porewater/ml sediment]	Sediment depth [cm]	Porosity [ml porewater/ml sediment]
0-1	0.93	7-8	0.86
1-2	0.90	8-9	0.85
2-3	0.88	9-10	0.85
3-4	0.87	10-11	0.85
4-5	0.87	11-12	0.85
5-6	0.87	12-13	0.85
6-7	0.86	13-14	0.85

A.2.4.2 Results of porewater measurements from Experiment No. 6

Table A.10: *Results of the IC measurement of the porewater samples from Experiment No. 6.*

Sample	Sulfate [mM]	Chloride [mM]	Sample	Sulfate [mM]	Chloride [mM]	Sample	Sulfate [mM]	Chloride [mM]
N 0-1	14.23	384.16	L 0-1	14.122	384.75	H 0-1	15.063	384.47
N 1-2	14.15	378.83	L 1-2	14.183	386.95	H 1-2	15.304	388.52
N 2-3	14.072	376.74	L 2-3	14.401	388.24	H 2-3	15.084	389.2
N 3-4	14.349	384.05	L 3-4	14.174	381.89	H 3-4	14.885	382.65
N 4-5	14.284	384.39	L 4-5	14.252	384.26	H 4-5	15.013	388.34
N 5-6	14.397	385.3	L 5-6	13.829	372.48	H 5-6	15.036	383.74
N 6-7	14.209	382.19	L 6-7	14.224	380.33	H 6-7	15.037	385.53
N 7-8	14.118	381.17	L 7-8	14.233	384.31	H 7-8	14.992	384.69
N 8-9	14.19	383.02	L 8-9	14.082	384.53	H 8-9	14.484	384.75
N 9-10	13.955	380.48	L 9-10	14.246	394.32	H 9-10	14.244	379.69
N 10-11	13.273	373.53	L 10-11	13.875	393.84	H 10-11	13.946	383.8
N 11-12	12.888	379.33	L 11-12	12.775	398.71	H 11-12	13.077	377.76
N 12-13	11.952	375.51	L 12-13	11.304	388.72	H 12-13	12.321	383.05
N 13-14	11.1179	375.69	L 13-14	13.219	390.05			
N = No Oil			L = Light Oil			H = Light Oil		
Number behind N = sediment depth [cm]			Number behind L = sediment depth [cm]			Number behind H = sediment depth [cm]		

A.2.4.3 Results of Sulfate-Reduction Rates Measurements

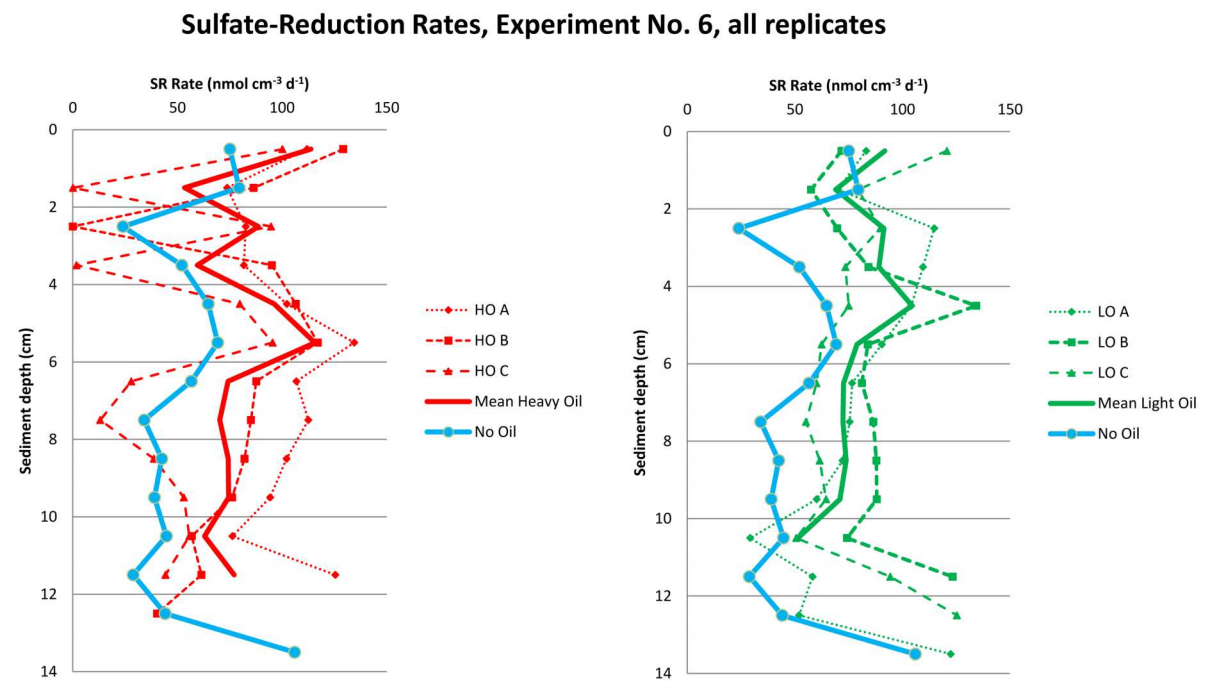

Figure A.2: *Vertical profile of sulfate-reduction rates in the sediment mini-cores from Experiment No. 6 after 73 days of incubation.* For the heavy- and light oil treated mini-cores three replicates (A, B, and C) are shown. For the control core there was no replication.

A.2.4.4 Results of CARD-FISH and DAPI counts

Table A.11: *Results of the CARD-FISH and DAPI counts from the sediment mini-cores of Experiment No. 6.*

Sample	Average cell number / view DAPI	Average cell number / view CARD-FISH	Total cell number DAPI in cm^{-3} sediment	Total cell number CARD-FISH in cm^{-3} sediment
HO 0-1	94.67	4.83	7.48E+09	3.82E+08
HO 2-3	81.00	3.89	6.40E+09	3.07E+08
HO 4-5	92.29	4.43	7.30E+09	3.50E+08
HO 6-7	75.57	4.74	5.97E+09	3.75E+08
HO 8-9	45.73	3.46	3.62E+09	2.73E+08
HO 10-11	50.73	3.74	4.01E+09	2.96E+08
HO 12-13	51.30	3.37	4.06E+09	2.67E+08
LO 0-1	89.83	4.09	7.10E+09	3.23E+08
LO 2-3	101.00	5.33	7.99E+09	4.21E+08
LO 4-5	84.83	4.96	6.71E+09	3.92E+08
LO 6-7	65.63	4.60	5.19E+09	3.64E+08
LO 8-9	57.78	5.06	4.57E+09	4.00E+08
LO 10-11	56.89	4.49	4.50E+09	3.55E+08
LO 12-13	50.80	3.83	4.02E+09	3.03E+08
NO 0-1	88.00	3.71	6.96E+09	2.94E+08
NO 2-3	72.57	3.29	5.74E+09	2.60E+08
NO 4-5	75.14	3.30	5.94E+09	2.61E+08
NO 6-7	57.67	3.19	4.56E+09	2.52E+08
NO 8-9	58.33	3.01	4.61E+09	2.38E+08
NO 10-11	51.60	2.81	4.08E+09	2.23E+08
NO 12-13	46.33	2.40	3.66E+09	1.90E+08

HO = Heavy Oil treated, LO = Light Oil treated, NO = not treated with oil;
Numbers behind HO, LO, and NO indicate sediment depth in cm.

A.2.4.5 Adjustment of the CARD-FISH protocol

In the beginning, it was expected that the CARD-FISH method would be problematic to apply to the oil-treated sediments. For example Orcutt *et al.* (2005) state that they had problems with CARD-FISH in sediments from oil-contaminated sites caused by auto-fluorescence of the crude oil. Surprisingly, the oil did not cause any problems, nevertheless the existing CARD-FISH protocol needed some modifications in order to achieve good signals.

Things that were changed compared to the previously used protocol, which is described in detail by Gier (2012):

- Sonication : The tip of sonicator pointed directly into the 1:40 diluted sample
- Filters were embedded in agarose
- Permeabilization was done before inactivation of endogenous peroxidases
- Inactivation of endogenous peroxidases with H_2O_2 and MeOH
- No pre-hybridization was done
- Concentration of hybridization buffer was changed, less probe was used
- Hybridization was done only for 2.5 hours (over-night did also work, but was too time-consuming)
- A washing buffer was used for washing of filter sections after hybridization
- Lower tyramide concentration was used for CARD

After these modifications, results of the CARD-FISH method, for the samples of this study, were much better. The most important changes were: (1) Change of sonication procedure. Sediments from Eckernförde Bay are organic rich and bacteria used to accumulate in clumps probably attached to organic material, what was hardly countable with the old sonication procedure. (2) The embedding of the filters in agarose is probably also a good improvement. Pernthaler et al. (2002) state that without embedding of the filters in agarose up to 2/3 of cells got lost during the CARD-FISH procedure. Nevertheless, this cell loss should not change relative abundances. So that it is not that problematic for some purposes where only relative abundances are important. (3) Inactivation of endogenous peroxidases with H_2O_2 very likely reduced unspecific staining

and background fluorescence. (4) Usage of lower amounts of tyramide probably also reduced background fluorescence.

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Declaration of Authorship

Hereby I confirm that I have written this thesis with the title:

"Microbial response to the addition of light and rich oil to natural marine sediments"

independently and by my own. Furthermore, I confirm that no other sources have been used than those specified in the thesis itself. This thesis, in same or similar form, has not been submitted as final work at any other place. I also confirm that the printed version is equivalent to the submitted electronic one.

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