

# **Production of greenhouse gases in organic-rich sediments**

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## Abstract

Methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) are greenhouse gases, which atmospheric concentrations increased since preindustrial times by ~150 and ~20%, respectively, mainly due to the increase in anthropogenic emissions. The atmospheric increase of greenhouse gases (incl. carbon dioxide (CO<sub>2</sub>), CH<sub>4</sub> and N<sub>2</sub>O) led to various effects on the Earth's surface and atmosphere, summarized as global climate change. In the marine environment, temperature rise, sea level rise, ocean acidification, and decreased oxygen concentrations are the most significant effects of climate change. To predict possible changes through climate change in the future, natural and anthropogenic sources and production/consumption pathways of greenhouse gases need to be determined carefully. To date, research is sparse on the sources for oceanic CH<sub>4</sub> and N<sub>2</sub>O, thus leading to uncertainties in global ocean emission estimates. Marine, organic-rich sediments (e.g. in coastal areas) are known to be major benthic sources for CH<sub>4</sub> and N<sub>2</sub>O, which eventually could end up in the atmosphere. Both greenhouse gases are produced by microbial processes during the degradation of organic matter in marine sediments, namely methanogenesis and denitrification (next to nitrification), respectively. However, knowledge about magnitude and environmental controls of these microbial processes is still limited. In the present study, benthic CH<sub>4</sub> and N<sub>2</sub>O production was investigated in three different marine areas with the focus on the surface sediment (0-30 cmbsf=centimeter below surface): the upwelling region off Peru, the Eckernförde Bay in the southwestern Baltic Sea and the Gulf of Mexico. In sediments from Peru and Eckernförde Bay, the focus was set on surface methanogenesis within the sulfate-reducing zone, which has been thought to be negligible due to the successful competition of sulfate reducers for the mutual substrates hydrogen (H<sub>2</sub>) and acetate. In oil-influenced sediments from the Gulf of Mexico, the focus was set on benthic denitrification and sulfate reduction, with benthic N<sub>2</sub>O production as a side effect. The investigations showed the following:

- 1) In sediments off Peru, methanogenesis and sulfate reduction co-occurred within the upper 0-30 cmbsf, explained by usage of non-competitive substrates (such as methanol or methylated compounds) by methanogens. In the deeper sediment horizons (>30 cmbsf), usage of the competitive substrates H<sub>2</sub> and acetate was confirmed, probably due to a relief of the competitive situation by sulfate depletion. Surface methanogenesis activity varied spatially along the Peruvian margin (70-1024 m water depth), with the major driving factor being the availability and variety of organic matter, followed by oxygen. Thus, highest surface methanogenesis activity was observed on the shelf, where organic carbon load was highest together with hypoxic or even anoxic conditions in the bottom water. At the same time, dissolved CH<sub>4</sub> concentrations were also highest on the shelf, indicating a previously

underestimated contribution of surface methanogenesis to benthic methane emissions. If not escaping to the water column, the methane produced by surface methanogenesis could act as important methane supplier for anaerobic oxidation of methane in surface sediments.

- 2) Surface methanogenesis was detected on a seasonal basis within the sulfate-reducing zone in sediments (0-30 cmbsf) from the Time Series Station Boknis Eck in Eckernförde Bay (SW Baltic Sea). Methanogenesis activity changed seasonally with highest rates in September/November after the summer/autumn phytoplankton blooms, and lowest rates in March after the period of low production during winter. The main controlling factor for surface methanogenesis was suggested to be the organic matter quantity and quality, followed by temperature and oxygen. The major part of surface methanogenesis was probably facilitated from usage of non-competitive substrates (e.g. methanol and methylated amines) to avoid competition with sulfate reducers, indicated by increased activity after methanol addition and concomitant detection of the family *Methanosarcinaceae*, whose members are known for usage of non-competitive substrates. Accordingly, usage of competitive substrates such as H<sub>2</sub> increased in deeper sediment horizons (>30 cmbsf) when sulfate was depleted. The results revealed that surface methanogenesis could potentially fuel surface anaerobic oxidation of methane with up to 13%. In addition, not only surface methanogenesis activity but also benthic methane emissions showed seasonal variation, indicated by dissolved methane concentrations in the bottom water.
- 3) Denitrification was significantly elevated in oil-influenced (oiled=sedimented oil layer on top) sediments compared to control sediments with no oil influence, identifying this process as a major degrading process. Sulfate reduction was not elevated, hinting towards the restricted availability of sedimented oil to only specific microbial groups. Sulfide-induced N<sub>2</sub>O production, resulting from elevated sulfate reduction, was not observed. However, all investigated sediment samples revealed the potential for being a N<sub>2</sub>O source, probably resulting from elevated denitrification rates.

In summary, the successful detection of surface methanogenesis in two, organic-rich, coastal systems shows its previously underestimated role in benthic methane budgeting, e.g. as a methane supplier for anaerobic oxidation of methane or as a contributor to benthic methane emissions to the water column. In addition to spatial variation, also seasonal variation was identified to play an important role in benthic methane production and emission, which should be included in ocean emission estimates. Production of methane in surface sediments was found to be mainly dependent on organic matter input, temperature and oxygen, and thus could be affected by predicted climate change (e.g. temperature rise).

The findings on benthic N<sub>2</sub>O production indicate the crucial importance for studying environmental controls on denitrification in organic-rich sediments as a source of N<sub>2</sub>O to the water column, including coastal areas and cold seeps. Both systems experience high sulfide concentrations, which would also increase the potential for sulfide-induced N<sub>2</sub>O production in these environments.



## Zusammenfassung

Methan ( $\text{CH}_4$ ) und Distickstoffmonoxid ( $\text{N}_2\text{O}$ ) sind Treibhausgase, deren atmosphärische Konzentration seit Beginn der Industrialisierung jeweils um 150% bzw. 20% angestiegen ist, hauptsächlich durch den Anstieg von anthropogenen Emissionen. Der Anstieg von Treibhausgasen (unter anderem Kohlenstoffdioxid ( $\text{CO}_2$ ),  $\text{CH}_4$  und  $\text{N}_2\text{O}$ ) in der Atmosphäre hat zu unterschiedlichen Auswirkungen in der Erdatmosphäre sowie an der Erdoberfläche geführt, die zusammenfassend als globaler Klimawandel bezeichnet werden. Im marinen Lebensraum gehören Temperaturanstieg, Anstieg des Meeresspiegels, Ozeanversauerung und die abnehmenden Sauerstoffkonzentrationen im Ozean zu den signifikantesten Auswirkungen des Klimawandels. Um zukünftige Veränderungen durch den Klimawandel vorherzusagen, ist es wichtig, die natürlichen und anthropogenen Quellen von Treibhausgasen sowie deren Produktions- und Abbauege zu bestimmen. Die Forschung bzgl. der Quellen für gelöstes  $\text{CH}_4$  und  $\text{N}_2\text{O}$  im Ozean ist immer noch gering, was wiederum zu Unsicherheiten in der Berechnung von globalen Ozean-Emissionen führt.

Marine, organik-reiche Sedimente (z.B. in Küstengebieten) gelten als Hauptquelle für im Ozean gelöstes  $\text{CH}_4$  und  $\text{N}_2\text{O}$ , welches schlussendlich auch in die Atmosphäre gelangen kann. Sowohl  $\text{CH}_4$  als auch  $\text{N}_2\text{O}$  werden in marinen Sedimenten von Mikroben hauptsächlich während der Methanogenese bzw. Denitrifikation (neben der mikrobiellen Nitrifikation) produziert. Beide Prozesse finden während der mikrobiellen Remineralisation von organischem Material statt. Das Wissen in Bezug auf die Größenordnung dieser Prozesse, sowie deren Umwelt-Kontrollen, ist noch sehr lückenhaft.

In der vorliegenden Studie wurde die Produktion von  $\text{CH}_4$  und  $\text{N}_2\text{O}$  in Sedimenten von drei verschiedenen, marinen Gebieten untersucht: dem Auftriebsgebiet vor Peru, der Eckernförder Bucht in der südwestlichen Ostsee und dem Golf von Mexiko. Hierbei lag der Fokus auf der Sediment-Oberfläche (0- 30 cm). In den Gebieten Peru und Eckernförder Bucht wurde die Oberflächen-Methanogenese in der Sulfatreduktionszone untersucht. Diese galt bis dato als vernachlässigbar aufgrund der erfolgreichen Konkurrenz der Sulfatreduzierer für die gemeinsamen Substrate Wasserstoff ( $\text{H}_2$ ) und Acetat. In den Öl-beeinflussten Sedimenten aus dem Golf von Mexiko wurden die benthischen Prozesse Denitrifikation und Sulfatreduktion untersucht, einschließlich der benthischen  $\text{N}_2\text{O}$  Produktion als Nebeneffekt. Die Untersuchungen ergaben folgende Ergebnisse:

- 1) In den Sedimenten vom peruanischen Auftriebsgebiet kamen Methanogenese und Sulfatreduktion gleichzeitig in den oberen 0-30 cm auf vor. Dies war möglich durch Nutzung von nicht-kompetitiven Substraten von den methanogenen Mikroben (Methanogenen), wie z.B. Methanol oder methylierte Verbindungen. In den tieferen Sedimentschichten (> 30 cm) konnte eine verstärkte Nutzung der kompetitiven Substrate  $\text{H}_2$  und Acetat beobachtet

werden. Möglicherweise wurde durch die starke Abnahme an Sulfat die Konkurrenz zwischen Methanogenen und Sulfatreduzierern in diesen Tiefen abgeschwächt. Oberflächen-Methanogenese zeigte räumliche Schwankungen entlang des Kontinentalhanges (70 bis 1024 m Wassertiefe), welche hauptsächlich durch die Verfügbarkeit und die Variabilität des organischen Materials, sowie der Sauerstoffkonzentrationen erklärt wurde. Folglich wurden die höchsten Raten von Oberflächen-Methanogenese auf dem Schelf gemessen, wo der Organik-Gehalt am höchsten und die Sauerstoffkonzentrationen im Tiefenwasser am geringsten waren (hypoxische bis zu anoxische Bedingungen). Gleichzeitig wurden auch die höchsten Methankonzentration im Tiefenwasser auf dem Schelf gemessen, was darauf hindeutet, dass Oberflächen-Methanogenese eine größere Rolle in benthischen Methan-Emissionen spielen kann, als vorher beschrieben. Das an der Oberfläche produzierte Methan kann, wenn es nicht direkt in die Wassersäule diffundiert, auch als wichtige Methanquelle für anaerobe Methanoxidation an der Oberfläche fungieren.

- 2) Oberflächen-Methanogenese wurde saisonal in der Sulfatreduktionszone in Sedimenten der Zeitserienstation Boknis Eck in der Eckernförder Bucht (südwestliche Ostsee) nachgewiesen. Die Methanogenese-Aktivität zeigte saisonale Schwankungen mit den höchsten Raten im September/November nach den Sommer-/Herbst-Phytoplanktonblüten und den niedrigsten Raten im März nach dem weniger produktiven Winter. Die Hauptfaktoren, die die Oberflächen-Methanogenese kontrollierten, waren die Quantität und Qualität des organischen Materials, gefolgt von Temperatur und Sauerstoffgehalt. Der Hauptteil der Oberflächen-Methanogenese wurde wahrscheinlich durch die Nutzung von nicht-kompetitiven Substraten (z.B. Methanol oder methylierte Verbindungen) bestritten. Dies geht auch aus den erhöhten Methanogeneseraten nach Methanolzugabe und der gleichzeitigen Detektion der Familie *Methanosarcinaceae* hervor, deren Angehörige für die Nutzung von nicht-kompetitiven Substraten bekannt sind. Dementsprechend wurden kompetitive Substrate wie z.B.  $H_2$  erst in den tieferen Sedimentschichten (> 30 cm) verwendet, als Sulfat fast aufgebraucht war. Anhand der Ergebnisse konnte errechnet werden, dass Oberflächen-Methanogenese die anaerobe Methanoxidation an der Oberfläche mit bis zu 13% antreiben kann. Außerdem konnte festgestellt werden, dass nicht nur die Oberflächen-Methanogenese saisonale Schwankungen aufwies, sondern auch die benthischen Methan-Emissionen (erkennbar anhand der gelösten Methankonzentrationen im Tiefenwasser).
- 3) Denitrifikationsraten waren signifikant höher in Öl-beeinflussten (ölig=eine sedimentierte Ölschicht an der Oberfläche) Sedimenten im Vergleich zu Kontroll-Sedimenten ohne Öl-Einfluss. Dieses Ergebnis deutet darauf hin, dass Denitrifikation ein Haupt-Abbau-Prozess in

öiligen Sedimenten ist. Sulfatreduktionsraten waren dagegen nicht erhöht in den öiligen Sedimenten, was dadurch zu erklären ist, dass die sedimentierte Ölschicht wahrscheinlich nur für spezielle mikrobielle Gruppen verfügbar war. Eine Sulfid-induzierte  $N_2O$  Produktion, resultierend aus erhöhter Sulfatreduktion, konnte demnach nicht beobachtet werden. Allerdings zeigten alle untersuchten Sedimente erhöhte Sediment- $N_2O$ -Konzentrationen gegenüber dem Tiefenwasser, was sie als potentielle Quelle für  $N_2O$  identifiziert.

Zusammenfassend hat die erfolgreiche Detektion von Oberflächen-Methanogenese in zwei, organikreichen Küstensystemen gezeigt, dass dessen Rolle im benthischen Methan-Zyklus (z.B. als Methan-Lieferant für anaerobe Methanoxidation) sowie dessen Beitrag zu benthischen Methan-Emission in die Wassersäule in der Vergangenheit unterschätzt wurde. Zusätzlich konnte gezeigt werden, dass nicht nur räumliche Schwankungen, sondern auch saisonale Schwankungen eine wichtige Rolle bei der benthischen Methan-Produktion und -Emission spielen, was mit in die Schätzungen von Ozeanemissionen einfließen sollte. Die Methanproduktion in Oberflächen-Sedimenten war hauptsächlich abhängig vom Eintrag des organischen Materials, von der Temperatur und von dem Sauerstoffgehalt. Dies macht deutlich, dass die vorausgesagten Änderungen durch den Klimawandel (wie z.B. der Temperaturanstieg) auch Auswirkungen auf Oberflächen-Methanogenese haben können.

Die Resultate der benthischen  $N_2O$ -Produktion weisen darauf hin, dass weitere Studien benötigt werden, die sich mit den Umwelt-Kontrollen von Denitrifikation in organikreichen Sedimenten als eine Quelle für gelöstes  $N_2O$  in der Wassersäule beschäftigen. Hierbei sollte der Fokus auf Küstengebieten und Kalten Quellen liegen, da beide Systeme hohe Sulfidkonzentrationen im Sediment aufweisen und somit auch eine Sulfid-induzierte  $N_2O$ -Produktion in diesen Sedimenten wahrscheinlich ist.

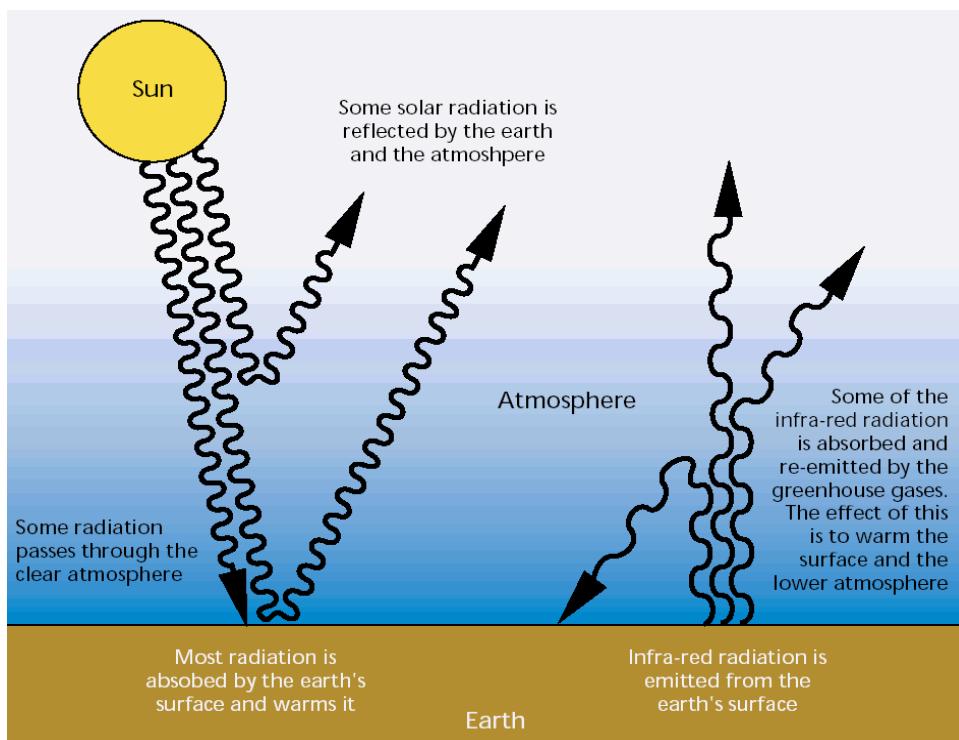
## Chapter 1

# **General Introduction**



## 1. Introduction

The Earth's climate is dependent upon the radiative balance of the atmosphere, which in turn is dependent on solar radiation and the abundances of radiative-active trace gases (greenhouse gases), clouds and aerosols in the atmosphere (IPCC, 1990). Greenhouse gases have the characteristic of adsorbing the long-wave radiation, which is reflected from the Earth's surface. The adsorbed radiation is emitted back to the Earth's surface, leading to warming of the lower atmosphere and surface (IPCC, 1995). This natural greenhouse effect (Fig. 1) has operated in the Earth atmosphere for billions of years. It maintains the Earth's surface temperature at an average of 15°C, 33°C warmer than it would be otherwise (NOAA, 2007).



**Figure 1:** The greenhouse effect. Simplified scheme from IPCC, (1990)

Currently, ocean, atmosphere and earth scientists are facing a challenge with increased public awareness: global climate change. The main cause of climate change is seen in the increase in greenhouse gas concentrations in the atmosphere through anthropogenic emissions (Crowley, 2000). This enhanced greenhouse effect reduces the cooling efficiency of the Earth, thus the temperature of the Earth atmosphere and surface is rising (Harley et al., 2006). In the marine environment, ocean warming, sea-level rise, and ocean acidification are the most significant observations of climate change (Harley et al., 2006; Doney et al., 2009). For example, the temperature of the upper ocean (0-700 m) increased 0.1°C per decade between 1971 and 2010, while the pH decreased 0.1 since the

beginning of the industrial era (mid-18<sup>th</sup> century) (IPCC, 2014). The sea-level rose by ~0.2 m over the time period 1901-2010 (IPCC, 2014). Other observations in the ocean system include decreased oxygen concentrations in coastal waters which led to extension of oxygen minimum zones in recent decades (Stramma et al., 2010; Keeling et al., 2010) and salinity changes due to shifts in evaporation and precipitation (Durack & Wijffels, 2010).

The most important, long-lived greenhouse gases, which are affected by anthropogenic emissions, are carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) (Denman et al., 2007). Their long atmospheric life times of decades (CH<sub>4</sub>) to centuries (N<sub>2</sub>O and CO<sub>2</sub>) lead to well-mixing throughout the atmosphere much faster than their removal, thus leading to long-term influences on climate (Hartmann et al., 2013; Sonnemann & Grygalashvyly, 2013; Bakker et al., 2014). Since the beginning of the industrial era, the atmospheric concentrations of CO<sub>2</sub>, CH<sub>4</sub> and N<sub>2</sub>O increased over 40%, 150% and 20%, respectively (Hartmann et al., 2013). Natural sources of these greenhouse gases need to be determined carefully, to validate the possible changes through climate change and to better distinguish the effect of anthropogenic emissions.

## 1.1 Methane (CH<sub>4</sub>)

CH<sub>4</sub> is the simplest of all hydrocarbons (alkanes), with one carbon atom surrounded by four hydrogen atoms via covalent bonding. It is a colorless, flammable gas, which shows only slight solubility in water due to its non-polarity (Yamamoto et al., 1976). CH<sub>4</sub> is the most abundant organic species in the atmosphere and the most abundant greenhouse gas after water vapor (H<sub>2</sub>O) and CO<sub>2</sub> (Denman et al., 2007). Compared to one molecule of CO<sub>2</sub>, CH<sub>4</sub> has a 25 times stronger global warming potential averaged over 100 years (Denman et al., 2007), explaining why research on CH<sub>4</sub> sources and sinks is crucial. Since preindustrial times (since 1750), atmospheric CH<sub>4</sub> concentrations increased from ~ 722 ppb to currently ~ 1800 ppb, which is mainly caused by increased human activities such as fossil fuel use or waste disposal (Hartmann et al., 2013; Wuebbles & Hayhoe, 2002).

The main part of atmospheric CH<sub>4</sub> (>70%) is produced biologically, i.e. through microbial methanogenesis (Wuebbles & Hayhoe, 2002), which is facilitated by methanogenic archaea during the anaerobic degradation and reduction of organic matter (Jørgensen, 2006). The process of methanogenesis is described in more detail in section 1.1.2 of this introduction.

The sources for biogenic CH<sub>4</sub> can be divided into natural and anthropogenic ones (Table 1). The most important natural sources for biogenic CH<sub>4</sub> within natural and anthropogenic sources are wetlands, followed by ruminants, rice cultivation and waste treatment. Termites, lakes and the ocean (incl. estuaries and rivers) are modest natural sources for biogenic CH<sub>4</sub>. Gas hydrates emissions are in the lower range. The CH<sub>4</sub> source for gas hydrates is predominantly biogenic, deriving from microbial

methanogenesis (Kvenvolden, 1993), however, also non-biogenic sources are possible (e.g. volcanic, hydrothermal or thermogenic) (Bakker et al., 2014). CH<sub>4</sub> hydrates represent an enormous CH<sub>4</sub> reservoir with the potential to be a large source, but research on hydrate contributions is sparse, thus estimations of CH<sub>4</sub> emissions from hydrates involve large uncertainties (Reeburgh, 2007; Bakker et al., 2014).

Non biogenic sources for atmospheric CH<sub>4</sub> are mostly found in combination with anthropogenic activities such as fossil fuel mining and combustion, and biomass burning (Wuebbles & Hayhoe, 2002). In the natural systems, non-biogenic CH<sub>4</sub> is emitted from geological sources and wild fires, in which CH<sub>4</sub> is produced through thermogenic processes or chemical processes like serpentinization (Judd, 2000) and through incomplete combustion, respectively (Wuebbles & Hayhoe, 2002; EPA, 2010). The global production of atmospheric CH<sub>4</sub> is estimated to be in the order of 500-600 Tg CH<sub>4</sub> year<sup>-1</sup> (Conrad, 2009; Denman et al., 2007; Wuebbles & Hayhoe, 2002) with over 60 % being anthropogenic.

In comparison with the numerous sources for CH<sub>4</sub>, only three major sinks are known for atmospheric CH<sub>4</sub>.

1) The reaction with the hydroxyl radical (OH) in the troposphere is the major sink (86 % of total sinks), responsible for removing ~445 Tg CH<sub>4</sub> year<sup>-1</sup> (Table 1). Although CH<sub>4</sub> is destroyed during this reaction, climate is still affected as other greenhouse gases like CO<sub>2</sub>, H<sub>2</sub>O and ozone (O<sub>3</sub>) can be formed during this CH<sub>4</sub> oxidation process (Cicerone & Oremland, 1988; Bakker et al., 2014).

2) The second highest sink (8% of total sinks) is the loss of CH<sub>4</sub> to the stratosphere, where it is removed by reactions with stratospheric OH, excited oxygen atoms O(<sup>1</sup>D) and chlorine (Cl) radicals (Khalil et al., 2000).

3) Lastly, microbial CH<sub>4</sub> oxidation in soils adds up to CH<sub>4</sub> consumption. Here, CH<sub>4</sub>-oxidizing (=methanotrophic) bacteria in the upper soil horizon oxidize CH<sub>4</sub> to CO<sub>2</sub> in the presence of O<sub>2</sub> (see section 1.1.2 for more details on CH<sub>4</sub> oxidation) (Wuebbles & Hayhoe, 2002).

The slightly lower sink strength compared to total source strength leads to a steady increase in atmospheric CH<sub>4</sub> concentration since preindustrial times.

**Table 1.** Estimated natural and anthropogenic sources and sinks of atmospheric CH<sub>4</sub>

<b>SOURCES</b>	<b>Emissions<sup>a</sup></b> <b>(Tg CH<sub>4</sub> year<sup>-1</sup>)</b>
<b>Natural sources</b>	
Natural Wetlands	100
Termites	20
Lakes	20 <sup>b</sup>
Geological sources	14
Oceans (incl. estuaries and rivers)	9
→ <i>Open ocean</i>	1.8 <sup>c</sup>
→ <i>Coastal ocean (incl. estuaries)</i>	6.9 <sup>c</sup>
→ <i>Rivers</i>	0.3 <sup>c</sup>
Wild animals	8 <sup>3</sup>
Gas Hydrates	5
Wildfires	2
<b>Total Natural</b>	<b>178</b>
<b>Anthropogenic sources</b>	
Fossil fuel mining and combustion	106
Ruminants	81
Waste treatment	61
Rice cultivation	60
Biomass burning	50
<b>Total anthropogenic</b>	<b>358</b>
<b>Total sources</b>	<b>536</b>
<b>SINKS</b>	
	<b>Uptake</b> <b>(TgCH<sub>4</sub> year<sup>-1</sup>)</b>
Tropospheric OH	445
Removal to stratosphere	40
Soil uptake	30
<b>Total sinks</b>	<b>515</b>

<sup>a</sup> source: (Wuebbles & Hayhoe, 2002)

<sup>b</sup> source: (Bastviken et al., 2004)

<sup>c</sup> source: (EPA, 2010)

### 1.1.1 The role of the ocean as a CH<sub>4</sub> source

The ocean (together with estuaries and rivers) is considered to play only a modest role in the global CH<sub>4</sub> budget with ocean emissions accounting for ~2% of all natural and anthropogenic emissions (Cicerone & Oremland, 1988; EPA, 2010; Bange et al., 1994). The marine environment in this compilation is estimated to account for ~80% of ocean emissions (EPA, 2010). However, the role of the ocean in the global CH<sub>4</sub> budget might change with ongoing climate change (e.g. ocean deoxygenation), thus research on current oceanic CH<sub>4</sub> sources and sinks is crucial to determine future effects (Keeling et al., 2010). In the following, the focus is set on the ocean including the marine systems open ocean and coastal ocean (i.e. continental shelves and estuaries).

The comparatively low contribution of the ocean emissions to the global CH<sub>4</sub> budget can be explained by a nearly equal balance between CH<sub>4</sub> production and CH<sub>4</sub> consumption=oxidation (Reeburgh, 2007). However, CH<sub>4</sub> production is slightly higher, leading to slightly supersaturated ocean surface waters with respect to the atmosphere, thus CH<sub>4</sub> emissions occur (Reeburgh, 2007; Bange et al., 1994). Generally, emissions from the open ocean are lower compared to the coastal ocean (Lambert & Schmidt, 1993; Bange et al., 1994) due to several factors such as higher organic matter and nutrient input, shallower water depth, and higher mixing dynamics near the coast (EPA, 2010). Accordingly, estuaries and the continental shelves are sites of high biogeochemical turnover and are estimated to contribute with 75% to the oceanic CH<sub>4</sub> emissions even though these areas represent only 7% of the total ocean surface (Bange et al., 1994; Thamdrup & Canfield, 2000; Canfield et al., 1993).

The sources of CH<sub>4</sub> to the ocean water column are poorly quantified (Reeburgh, 2007). They include microbial-mediated methanogenesis during the diagenesis of organic matter, thermogenic production, abiotic production through serpentinization reaction, rock/water reactions occurring in hydrothermal systems, leaks from near-surface petroleum deposits, and the decomposition of CH<sub>4</sub> hydrates (Reeburgh, 2007). Microbial methanogenesis is mainly occurring in marine sediments, but is thought to also occur directly within the water column, which would explain the observed CH<sub>4</sub> maxima in the oxygenated surface mixed layer in most world's oceans (also known as "the ocean methane paradox") (Reeburgh, 2007). The origin of this oceanic CH<sub>4</sub>, which has been thought to be exclusively produced in anaerobic environments, is not known. Hypotheses range from CH<sub>4</sub> production in zoo plankton and fish guts (Oremland, 1979), over CH<sub>4</sub> production in anaerobic microenvironments in sinking particles, e.g. fecal pellets (Holmes et al., 2000), to aerobic CH<sub>4</sub> production using phytoplankton derived dimethylsulfoniopropionate (DMSP) or during methylphosphonate decomposition (Damm et al., 2009; Karl et al., 2008).

The CH<sub>4</sub> source for surface waters in coastal areas may be predominantly explained by microbial methanogenesis in organic-rich sediments, but also by riverine inputs and shallow seeps of ancient microbial, thermogenic or abiogenic CH<sub>4</sub> (Hovland et al., 1993; Bange et al., 1994, 1998; Judd & Hovland, 2007). However, data on seasonality and environmental controls of dissolved CH<sub>4</sub> concentrations and benthic CH<sub>4</sub> production are largely missing, which would increase the accuracy of global CH<sub>4</sub> emission estimates (Bange, 2006b).

Microbial methanogenesis in the sediments of coastal areas is enhanced due to the high load of organic carbon to the seafloor explained by high primary productivity in the water column fueled by high nutrient loads from e.g. riverine inputs or upwelling (Falkowski, 1998; Jørgensen, 2006).

In general, 25-50 % of the biomass originating from primary production reaches the sea floor in coastal regions, whereas only ~1% reaches the sea floor in the deep sea (Suess, 1980; Jørgensen, 2006).

In some circumstances the amount of (buried) organic matter is so high that microbial methanogenesis is intense, resulting in dissolved CH<sub>4</sub> concentrations in the porewater higher than the hydrostatic pressure in the sediment, resulting in gas bubble formation (Wever et al., 1998). Thus CH<sub>4</sub> ebullition (the rapid release of CH<sub>4</sub> bubbles to the water column) is also postulated to be an important contributor to oceanic and atmospheric CH<sub>4</sub> in coastal areas, even though data on sediment CH<sub>4</sub> ebullition is sparse and still difficult to quantify (Bakker et al., 2014; Dimitrov, 2002a).

Most regions with high productivity are associated with coastal upwelling of nutrient-rich deep water, the latter inhabiting ~0.6% of the total ocean area (Bakker et al., 2014; Laruelle et al., 2010). These regions are known “hot-spots” for CH<sub>4</sub> emissions to the atmosphere (Naqvi et al., 2005; Kock et al., 2008), and can be found usually along the eastern margins of ocean basins, e.g. coastal Peru, Chile, the Arabian Sea, western South Africa (also known as the Benguela system), New Zealand, and the Californian coast (EPA, 2010). In these regions, CH<sub>4</sub> is mainly produced in the sediment but could also originate from production directly in the hypoxic or anoxic water column (Naqvi et al., 2005). Upwelling regions are often accompanied with hypoxia or anoxia, explained by the high oxygen consumption during organic matter degradation together with sluggish or restricted water circulation (Wyrski, 1962; Levin, 2003; Kamykowski & Zentara, 1990). These hypoxic or anoxic conditions can also be found in coastal regions without upwelling if water column stratification occurs together with high organic carbon degradation (Bertics et al., 2013; Bange et al., 2010).

### 1.1.2 Microbial CH<sub>4</sub> production and consumption in marine sediments

#### METHANE PRODUCTION=METHANOGENESIS

In marine sediments, biogenic CH<sub>4</sub> is produced via the anaerobic process of **methanogenesis**, which is performed by methanogenic archaea (=methanogens) (Jørgensen, 2006). Within the domain of archaea, the members of the following orders are known for methanogenesis: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* (Boone et al., 1993). The morphology of methanogens is manifold and ranges from rod-shaped, spiral-shaped, plate-shaped, to coccoid cells, which can also form cluster, filaments, or aggregates (Spratt & Beveridge, 1993). Methanogens are strict anaerobes and require highly reducing conditions (redoxpotential < -300 mV) (Hungate, 1950), which explains their occurrence in anoxic environments ranging from aquatic sediments, flooded soils, animal gastrointestinal tracts and sewage (Cicerone & Oremland, 1988). Methanogens reveal a broad level of adaptation, thus they can occur in freshwater and hypersaline environments, at cold temperatures (2°C) up to hydrothermal conditions (100°C), and at acidic pH (around 3) to alkaline habitats (around 9) (Zinder, 1993). Although most methanogens will not produce CH<sub>4</sub> in the presence of O<sub>2</sub>, some species can tolerate oxygen exposure for several hours (Zinder, 1993). Recently, low production of CH<sub>4</sub> despite the presence of O<sub>2</sub> was described in soil methanogenesis (Angel et al., 2011).

Methanogens have a narrow spectrum of simple, small-sized substrates, mostly containing one or two carbon molecules e.g. carbon dioxide or acetate (Zinder, 1993). Due to their substrate specialization, they are reliant upon other microbial groups (e.g. fermenters) which break down macromolecules (e.g. sugar, amino acids), ultimately resulting in the formation of smaller methanogenic substrates (Cicerone & Oremland, 1988).

The catabolic pathways of methanogenesis can be divided into three groups: hydrogenotrophic, acetoclastic and methylotrophic pathways (Zinder, 1993). The hydrogenotrophic pathway is the most widespread catabolic reaction among the methanogens and is characterized by the reduction of CO<sub>2</sub> with H<sub>2</sub> (Zinder, 1993; Stouthamer, 1988). However, some hydrogenotrophic methanogens are also able to use other compounds like formate or secondary alcohols to reduce CO<sub>2</sub> (Zinder, 1993). The acetoclastic pathway is characterized by usage of acetate. During this process, the carboxyl group is oxidized to CO<sub>2</sub> and the methyl group is reduced to CH<sub>4</sub> (Zinder, 1993). During the methylotrophic methanogenesis, compounds containing methyl groups are used for the production of CH<sub>4</sub>, e.g. methanol, methyl amines or dimethyl sulfides (Zinder, 1993). Growth on methylated substrates takes place by oxidation of some part of the substrate to CO<sub>2</sub>, coupled with the reduction of the remaining substrate to CH<sub>4</sub> (Cicerone & Oremland, 1988). While most methylotrophic methanogens are able to

use both methanol and methylated amines, growth on methyl sulfide appears to be restricted to only a few methylotrophic species (Oremland et al., 1989). The reaction equations of the most common methanogenic pathways including the yielded free energy ( $\Delta G^\circ$ ) are summarized in Table 2.

Even though there are multiple different enzymes involved during each methanogenesis pathway, the last step - the formation of  $\text{CH}_4$  - is the same in all methanogens: Methyl coenzyme M is the last intermediary metabolite of all methanogenesis pathways and  $\text{CH}_4$  is formed during its reduction by the enzyme methylreductase (Balch et al., 1979; Ellermann et al., 1988).

**Table 2.** Methanogenic reactions, modified from Zinder, (1993)

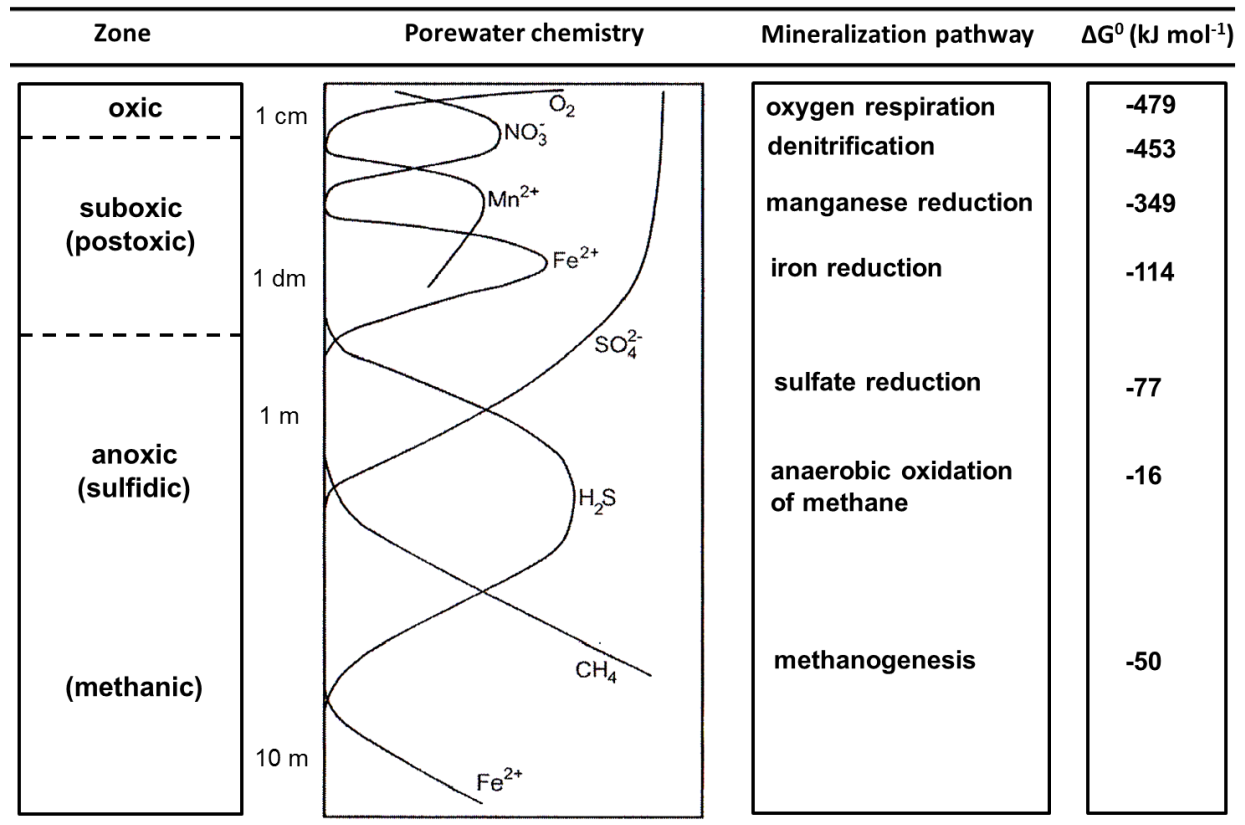
Reaction pathway and equation	Free energy ( $\Delta G^\circ$ )
<u>Hydrogenotrophic pathway:</u>	<u><math>\text{kJ mol}^{-1} \text{CH}_4</math></u>
$4 \text{H}_2 + \text{CO}_2 \longrightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$	-131
<u>Acetoclastic pathway:</u>	
$\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \longrightarrow \text{CH}_4 + \text{HCO}_3^-$	-31
<u>Methylotrophic pathway:</u>	
$\text{CH}_3\text{OH} + \text{H}_2 \longrightarrow \text{CH}_4 + \text{H}_2\text{O}$	-113
$2(\text{CH}_3)_3\text{-NH}^+ + 3 \text{H}_2\text{O} \longrightarrow \text{CH}_4 + \text{HCO}_3^- + 4 \text{NH}_4^+ + 4 \text{H}^+$	-76
$2(\text{CH}_3)_2\text{-S} + 3 \text{H}_2\text{O} \longrightarrow 3 \text{CH}_4 + \text{HCO}_3^- + 2 \text{H}_2\text{S} + 9 \text{H}^+$	-49

Delta G values from Thauer et al., (1977)

In marine sediments, methanogenesis is the terminal pathway of organic matter degradation (Fig. 2) (Jørgensen, 2006). The majority of methanogenesis is restricted to the zone where sulfate is depleted, as sulfate reducing bacteria compete successfully with methanogens for the mutual substrates  $\text{H}_2$  and acetate (Oremland & Polcin, 1982), which are the two most important  $\text{CH}_4$  precursors in many anaerobic environments e.g. marine and freshwater sediments (Crill & Martens, 1986; Lovley & Goodwin, 1988). Thus, methanogenesis is thought to be only of little significance within the sulfate-reducing zone in sulfate-rich environments like marine sediments (Jørgensen, 2006; Zinder, 1993; Whiticar, 2002; Burdige, 2006). The competitive situation can be relieved, however, due to usage of non-competitive substrates by the methanogens (e.g. methanol, methylated amines or sulfides), which are not suitable for sulfate reducers, thus both groups can coexist (Cicerone & Oremland, 1988; Oremland & Polcin, 1982). Non-competitive substrates can be derived from pectin (in plants), choline or from osmoprotectants such as betaine and dimethylpropiothetin needed in marine environments (Zinder, 1993). Coexistence of sulfate reduction and methanogenesis within the sulfate zone has so far been only shown in some organic-



rich sediments, e.g. salt-marsh sediments, coastal sediments or sediments below upwelling regions (Oremland et al., 1982b; Holmer & Kristensen, 1994; Ferdelman et al., 1997; Jørgensen & Parkes, 2010), indicating the importance of these areas for potentially underestimated surface methanogenesis. However, research about magnitude and environmental controls of this surface methanogenesis is still missing.



**Figure 2.** Schematic representation of the biogeochemical zonation in marine sediments, modified after Jørgensen & Kasten, (2006). Left: main zones proposed by Froelich et al., (1979) and Berner, (1981, in parenthesis). Depth scale is quasi-logarithmic, the exact depths can vary strongly. Middle: porewater chemistry of relevant dissolved species (peak heights and scales are arbitrary) and the respective mineralization pathway of organic matter. Right: standard free energy yields for each mineralization process (after Burdige, 2006).

#### METHANE OXIDATION=METHANOTROPHY

CH<sub>4</sub>, which is produced in the anoxic parts of marine sediments, can be oxidized anaerobically and aerobically during its diffusive way upwards to the sediment water interface (Cicerone & Oremland, 1988). Much of the CH<sub>4</sub> produced in sediments never reaches the atmosphere, since it is consumed by methane-oxidizing microbes (methanotrophs). However, a considerable part can reach the atmosphere before oxidation, or is transported directly to the atmosphere in form of bubbles (Zinder, 1993; Whiticar, 1978), thus contributing to the global CH<sub>4</sub> emissions.

While aerobic CH<sub>4</sub> oxidation is restricted to the oxic layer at the sediment surface (Cicerone & Oremland, 1988), which can be limited to only mm-thickness when organic carbon load is high (Jørgensen, 2006), **anaerobic oxidation of methane (AOM)** is happening in the anoxic parts of the sediment, mostly in the area where sulfate and CH<sub>4</sub> are both consumed to depletion (sulfate-methane-transition-zone=SMTZ) (Knittel & Boetius, 2009). Hence, the upwards diffusing CH<sub>4</sub> first gets in contact with the AOM-filter within the SMTZ. AOM is conducted by anaerobic, methanotrophic (methane-oxidizing) archaea (ANME), which form special lines of descent within the euryarchaeota (Knittel & Boetius, 2009). ANME form three phylogenetically distinct clusters: ANME-1, ANME-2 and ANME-3, which are closely related to the methanogenic orders *Methanosarcinales* and *Methanomicrobiales* (Knittel & Boetius, 2009). ANME archaea contain gene homologues of the methyl-coenzyme M reductase, which is the characteristic enzyme in methanogenic archaea, suggesting similar enzymes in both microbial groups (Knittel & Boetius, 2009).

The probably most common and most intensively studied pathway of AOM is the oxidation with sulfate as the terminal electron acceptor (Hoehler et al., 1994; Knittel & Boetius, 2009). Here, the ANMEs are living in a syntrophic relationship with sulfate-reducing bacteria, where the ANME activate the methane and the sulfate-reducing bacteria provide an electron sink (Hoehler et al., 1994; Boetius et al., 2000; Knittel & Boetius, 2009). Recent studies have shown that AOM can also be conducted by ANMEs alone (Thauer & Shima, 2008; Treude et al., 2005a; Milucka et al., 2012), or with other electron acceptors besides sulfate, e.g. nitrate (Raghoebarsing et al., 2006), indicating the diversity of AOM. About 90 % of the CH<sub>4</sub> produced in the ocean is oxidized by AOM, thus AOM is the most efficient control to atmospheric CH<sub>4</sub> efflux from the ocean or specifically the seabed (Knittel & Boetius, 2009).

**Aerobic oxidation of methane** occurs in the oxidized parts of the sediments and water column, thus attenuating the CH<sub>4</sub> flux into the water column and atmosphere, respectively. In a global perspective, aerobic methanotrophs can be found basically in every habitat containing CH<sub>4</sub>, including soils, sea water, and aquatic sediments (Bourne et al., 2000; Eller & Frenzel, 2001; Holmes et al., 1996).

It is conducted by bacteria, which oxidize CH<sub>4</sub> with O<sub>2</sub> to CO<sub>2</sub>. Besides CH<sub>4</sub>, most aerobic methanotrophs are also able to use methanol as a substrate, and only a few strains can use methylamine or other C1-compounds (Bowman, 2006). Aerobic methanotrophs are not able to grow on organic compounds possessing carbon-carbon bonds (Bowman, 2006).

Aerobic methanotrophs are found within a diverse group of specialized alpha and gamma proteobacteria (Lidstrom, 2006). They are separated into two families based on their arrangements of characteristic cell membranes and mode of carbon assimilation (Cicerone & Oremland, 1988; Bowman, 2006).

All aerobic methanotrophs possess an enzyme called methane monooxygenase (MMO), which catalyzes the first step during the oxidation of CH<sub>4</sub> (Bowman, 2006). Besides CH<sub>4</sub>, MMO has been also found to be able to oxidize a wide range of other carbon compounds (Bowman, 2006).

Even though their metabolism is strictly aerobic, methanotrophs can survive anoxia for several months, presumably due to resting cell formation, with a rapid response when CH<sub>4</sub> and O<sub>2</sub> become available (Takeda, 1988; Roslev & King, 1994). In addition, they are able to form cysts, which can survive desiccation or other deprived conditions (e.g. lack of methane availability) for several weeks (Bowman, 2006).

## 1.2 Nitrous oxide (N<sub>2</sub>O)

N<sub>2</sub>O (commonly known as laughing gas) is a colorless, non-flammable gas with two nitrogen atoms connected to one oxygen molecule via covalent bonding. It is fairly soluble in water, alcohols and even fats/oils (Weiss & Price, 1980; Saha et al., 1993; Yokozeki & Shiflett, 2011). It is mostly known for its usage as an inhalation anesthetic and analgetic (Jevtovic-Todorovic et al., 1998). It is the third most important long-lived (~114 years) greenhouse gas after CO<sub>2</sub> and CH<sub>4</sub> contributing to climate change (Hartmann et al., 2013; Bakker et al., 2014). Compared to one molecule of CO<sub>2</sub>, N<sub>2</sub>O has a ~298 times stronger global warming potential if considered over a time period of 100 years (Denman et al., 2007). Its concentration in the atmosphere steadily increased over the last three decades with 0.73 +/- 0.03 ppb year<sup>-1</sup>, reaching unprecedented high concentrations of 326 ppb in 2014 (IPCC, 2014).

Global N<sub>2</sub>O production is mostly attributed to microbial processes in both natural and anthropogenic sources (Denman et al., 2007; EPA, 2010; Bouwman et al., 1995). This biogenic N<sub>2</sub>O originates mainly from microbial nitrification and denitrification, which dominate in the oxic or anoxic environment, respectively (Seitzinger, 1988; Hensen et al., 2006; Burdige, 2006). Further details on microbial N<sub>2</sub>O production are given in section 1.2.2.

The two major natural sources for biogenic N<sub>2</sub>O are soils, followed by the ocean (incl. estuaries and rivers) (Table 3). No data is available for N<sub>2</sub>O emission from lakes. However, this source is hypothesized to contribute to less than 1% towards global N<sub>2</sub>O emission from natural systems (EPA, 2010). Anthropogenic sources for biogenic N<sub>2</sub>O include agriculture and waste disposal (Denman et al., 2007). Non-biogenic sources are mainly found in connection with human activities, namely fossil fuel combustion and industrial processes, biomass and biofuel burning, and the atmospheric deposition of ammonia (NH<sub>3</sub>), which forms N<sub>2</sub>O after oxidation with OH (Dentener & Crutzen, 1994; Mosier et al., 1998). The atmospheric deposition of NH<sub>3</sub> can be separated between anthropogenic and natural NH<sub>3</sub> sources, the latter revealing the same dimension as the anthropogenic source (0.6 Tg N year<sup>-1</sup>) (Dentener & Crutzen, 1994).

In contrast to CH<sub>4</sub>, global N<sub>2</sub>O emissions are larger in natural systems compared to anthropogenic systems (Table 3). It has to be mentioned, however, that even though ocean and rivers are accounted here as natural systems, some studies list rivers and estuaries as anthropogenic sources due to the impact of human activities such as fertilization (Denman et al., 2007). This might be an oversimplification, as it is difficult to determine the origin of the exported, reactive nitrogen (e.g. inorganic nitrogen compounds such as ammonium or nitrate, or organic nitrogen compounds such as proteins, EPA, 2010; Dumont et al., 2005). The dominant sink for atmospheric N<sub>2</sub>O is photolysis via the reaction with O(<sup>1</sup>D) in the stratosphere, which removes about 90 % of atmospheric N<sub>2</sub>O (Mcelroy et al., 1976; Cicerone, 1989; Mosier et al., 1998). During the reaction with O(<sup>1</sup>D), N<sub>2</sub>O is oxidized to nitric oxide (NO), which in turn is a major sink for O<sub>3</sub> (Ravishankara et al., 2009). These reactions identify N<sub>2</sub>O not only as a greenhouse gas but also a dominant ozone-depleting substance (Ravishankara et al., 2009).

The second sink for N<sub>2</sub>O are the soils, which can act as a sink due to the presence of denitrifying microbes, which reduce atmospheric N<sub>2</sub>O to nitrogen gas (N<sub>2</sub>) (Chapuis-lardy et al., 2007; Bouwman et al., 1995). For example, forest and grassland soils have been identified to act as a N<sub>2</sub>O sink (Cicerone, 1989). Again, the slightly higher source strength compared to sink strength leads to steady increase in atmospheric N<sub>2</sub>O.

### **1.2.1 The role of the ocean as a N<sub>2</sub>O source**

The ocean (incl. estuaries and rivers) is one of the major natural sources, contributing with over 30% to global N<sub>2</sub>O emissions (Denman et al., 2007; EPA, 2010; Nevison et al., 2004). In this compilation, rivers are only contributing with ~2% (EPA, 2010). In general, the surface waters of the ocean are slightly supersaturated with respect to atmospheric N<sub>2</sub>O (Bange, 2008), which indicates the global ocean as a net source for N<sub>2</sub>O to the atmosphere. Still, research on oceanic N<sub>2</sub>O and its sources to the water column is sparse, causing large uncertainties in emission estimations (Bange, 2006b; Seitzinger et al., 2000; Galloway et al., 2004; Bakker et al., 2014).

Microbial nitrification (oxidation of ammonium (NH<sub>4</sub><sup>+</sup>), to nitrate (NO<sub>3</sub><sup>-</sup>)) and denitrification (NO<sub>3</sub><sup>-</sup> reduction to N<sub>2</sub>) in the water column and sediment are thought to be the main processes producing N<sub>2</sub>O (Seitzinger, 1988; Nevison et al., 2003; Codispoti et al., 2005), but their distribution and controlling factors are still under research (Naqvi et al., 2010).

**Table 3.** Estimated natural and anthropogenic sources and sinks of atmospheric N<sub>2</sub>O

<b>SOURCES</b>	<b>Emissions<sup>a</sup></b> <b>(Tg N year<sup>-1</sup>)</b>
<b>Natural sources</b>	
Soils	6.6
Oceans (incl estuaries and rivers)	5.5
→ Open ocean	3.2 <sup>b</sup>
→ Coastal ocean (incl. estuaries)	2.2 <sup>b</sup>
→ Rivers	0.1 <sup>b</sup>
Atmospheric chemistry	0.6
<b>Total Natural</b>	<b>12.7</b>
<b>Anthropogenic sources</b>	
Agriculture	2.8
Fossil fuel combustion and industrial processes	0.7
Biomass and biofuel burning	0.7
Atmospheric deposition	0.6
Waste treatment	0.2
<b>Total anthropogenic</b>	<b>5.0</b>
<b>Total sources</b>	<b>17.7</b>
<b>SINKS</b>	
	<b>Uptake</b> <b>(Tg N year<sup>-1</sup>)</b>
Photolysis in stratosphere	12.3 <sup>c</sup>
Soil uptake	2.2 <sup>d</sup>
<b>Total sinks</b>	<b>15</b>

<sup>a</sup> source: (Denman et al., 2007)

<sup>b</sup> source: (EPA, 2010)

<sup>c</sup> source: (Mosier et al., 1998)

<sup>d</sup> source: (Bouwman et al., 1995)

The most productive marine systems are continental shelves (including estuaries) and upwelling regions, contributing to oceanic N<sub>2</sub>O emissions with up to ~60% despite their low area compared to the open ocean (continental shelves: 7%, upwelling regions: 0.2%) (Bange, 2006b; Bange et al., 1996b; Bange, 2006a; Nevison et al., 2004; Arévalo-Martínez et al., 2015). Both systems are characterized by high productivity through nutrient input (mainly reactive N compounds like NO<sub>3</sub><sup>-</sup>, nitrite, (NO<sub>2</sub><sup>-</sup>), or NH<sub>4</sub><sup>+</sup>) from rivers, atmosphere, adjacent continental slope waters or upwelling of nutrient-rich waters, respectively (EPA, 2010). Especially the anthropogenic changes in the N cycle (e.g. due to the use of fertilizers) have increased since pre-industrial times, leading to increased amounts of riverine runoff

of reactive N (Galloway et al., 2004; Green et al., 2004), which has a significant effect on the coastal ocean N<sub>2</sub>O emissions (Bange et al., 1996b), as reactive N directly affects microbial nitrification and denitrification.

In contrast to CH<sub>4</sub> (see 1.1.1), water column N<sub>2</sub>O production is a major source for oceanic N<sub>2</sub>O (Bakker et al., 2014). For example, highest N<sub>2</sub>O-supersaturation in the water column has been observed in areas which experience coastal upwelling together with subsurface O<sub>2</sub>-deficiency, revealing a negative correlation between dissolved O<sub>2</sub> and N<sub>2</sub>O concentrations (Nevison et al., 2004; Bange et al., 1996a; Suntharalingam et al., 2000). Hypotheses range from nitrification as the predominant N<sub>2</sub>O-producing process, to the occurrence of coupled nitrification-denitrification (Goreau et al., 1980; Naqvi et al., 1998; Suntharalingam et al., 2000; Arévalo-Martínez et al., 2015; Freing et al., 2012). If waters are suboxic or anoxic, denitrification is active and can consume N<sub>2</sub>O, thus these waters are often depleted in N<sub>2</sub>O (Naqvi et al., 2010; Bakker et al., 2014).

Also the sediments of the coastal areas such as estuaries, upwelling regions, and continental shelves play an important role in the production of N<sub>2</sub>O. Due to the high organic carbon load and reactive N input, coastal organic-rich sediments reveal high microbial activity (Rullkötter, 2006; Jørgensen, 2006). In organic-rich sediments, the anaerobic degradation of organic matter dominates due to a fast depletion of O<sub>2</sub>, thus the anaerobic microbial processes denitrification or dissimilatory nitrate reduction to ammonium (DNRA) play a more dominant role in N<sub>2</sub>O production than nitrification (Senga et al., 2006; Jørgensen, 2006; Wollast, 1993; Jørgensen & Sørensen, 1985). However, benthic denitrification can also act as a sink for benthic produced N<sub>2</sub>O (Senga et al., 2002; Usui et al., 1998). Research on the environmental controls on N<sub>2</sub>O accumulation in marine sediments is largely missing, but a strong dependence on O<sub>2</sub> concentrations or sulfide concentrations is known (Senga et al., 2006; Bange, 2006b, see section 1.2.2 for more details on environmental controls).

For example, the interference of sulfide with increased N<sub>2</sub>O signals has been observed in organic-rich sediments and could be an important precursor for benthic N<sub>2</sub>O production (Senga et al., 2006, 2001; Okumura et al., 2005), due to the general dominance of sulfate reduction in such organic-rich systems (Jørgensen, 2006). This sulfide effect could also play a dominant role near cold seeps, which are areas of natural gas and/or oil seepage (Joye et al., 2004). Those systems are also characterized by high organic carbon contents in the surrounding sediments originating from e.g. oil seepage. In addition, high sulfide accumulations are observed (Orcutt et al., 2010; Joye et al., 2004). However, research on N<sub>2</sub>O production at cold seeps is missing.

### 1.2.2 Microbial N<sub>2</sub>O production and consumption in marine sediments

In marine sediments, biogenic N<sub>2</sub>O is produced mainly by microbial nitrification and denitrification, processes which are active under oxic and anoxic conditions, respectively (Seitzinger, 1988; Hensen et al., 2006). In addition, DNRA is another process that can produce N<sub>2</sub>O, but research on the mechanism and magnitude of N<sub>2</sub>O production are sparse (Seitzinger, 1988; Knowles, 1982; Senga et al., 2006; Smith & Zimmerman, 1981). DNRA is a strictly anaerobic process, thus occurring in the anoxic sediment layers (Knowles, 1982). The role of anaerobic ammonium oxidation (anammox) in N<sub>2</sub>O production has been postulated but not yet been proven (van der Star, 2008; Naqvi et al., 2010), which is why we focus on nitrification, denitrification and DNRA.

All three processes are important parts of the nitrogen cycling in the marine environment (Fig. 3).

During **nitrification**, NH<sub>4</sub><sup>+</sup> is oxidized to NO<sub>3</sub><sup>-</sup> through a two-step process generally facilitated by two different microbial groups. The first step is done by ammonium-oxidizers (genus names generally start with the prefix *Nitroso*-) and produces NO<sub>2</sub><sup>-</sup>, which in turn is oxidized to NO<sub>3</sub><sup>-</sup> by nitrite-oxidizers (generally starting with *Nitro*-) (Hensen et al., 2006; Herbert, 1999; Burdige, 2006).

Another pathway for nitrification is the so called nitrifier-denitrification (Remde & Conrad, 1990; Ostrom et al., 2000; Wrage et al., 2001). During this reaction, NH<sub>4</sub><sup>+</sup> is oxidized to NO<sub>2</sub><sup>-</sup>, which is then reduced to nitric oxide (NO), N<sub>2</sub>O and N<sub>2</sub> (Wrage et al., 2001). It is facilitated by autotrophic nitrifiers and has been shown to be a predominant pathway in oceans and soils (Remde & Conrad, 1990). However, it cannot be equated with "coupled nitrification-denitrification", as different enzymes are involved (see also section "denitrification"; Wrage et al., 2001; Bakker et al., 2014). Recently, the general belief that nitrification is only carried out by bacteria (Jørgensen, 2006; Santoro et al., 2011) has been rebutted with the finding of ammonia-oxidizing archaea in sea water, which were found to be key organisms in oceanic nitrification (Bakker et al., 2014) and also major contributors for oceanic N<sub>2</sub>O production (Löscher et al., 2012; Santoro et al., 2011).

The exact pathway of N<sub>2</sub>O generation during nitrification (or nitrifier-denitrification) is not clear. Hypotheses range from N<sub>2</sub>O production during the oxidation of NH<sub>4</sub><sup>+</sup> via the intermediate hydroxylamine, to N<sub>2</sub>O production during oxidation of NH<sub>4</sub><sup>+</sup> via the intermediate NO in both pathways nitrification and nitrifier-denitrification (Ritchie & Nicholas, 1972; Naqvi & Noronha, 1991; Yoshida & Alexander, 1970; Bakker et al., 2014).

Even though research on the environmental controls for N<sub>2</sub>O formation is still sparse, O<sub>2</sub> has been clearly identified as an important factor, as studies revealed increased N<sub>2</sub>O production with decreasing O<sub>2</sub> concentrations (Goreau et al., 1980; Ostrom et al., 2000; Santoro et al., 2011). Thus N<sub>2</sub>O production from nitrification might have higher importance in the water column of coastal areas with occurrence

of O<sub>2</sub> deficiency (Suntharalingam et al., 2000; Löscher et al., 2012), while in the underlying organic-rich sediments denitrification might be more dominant.

**Denitrification** is the four-step process during which NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> is reduced to N<sub>2</sub> as the predominant end-product by both bacteria and archaea (Herbert, 1999; Cabello et al., 2004; Philippot, 2002).

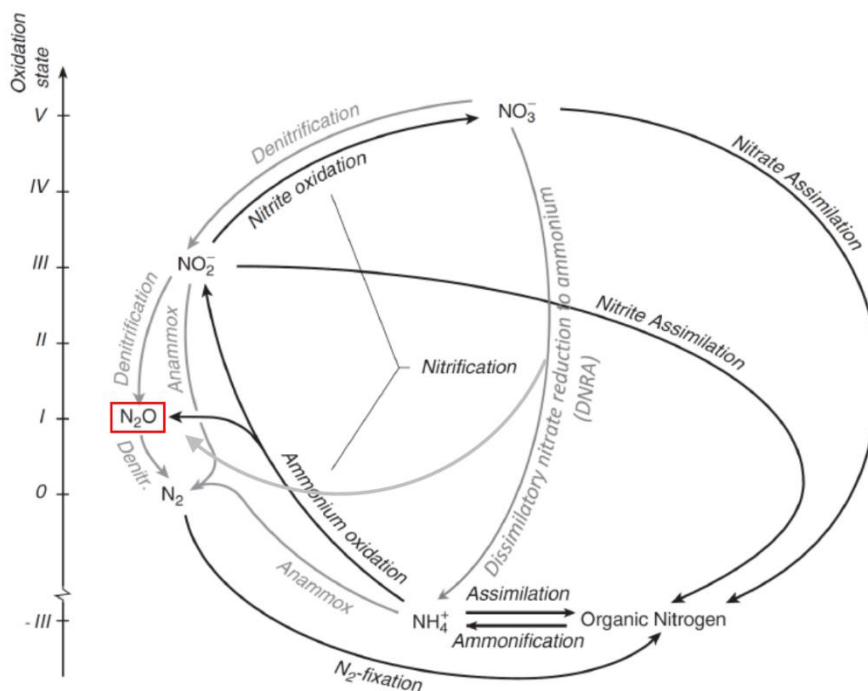
Denitrification is a key process in nitrogen cycling as it removes fixed nitrogen compounds from the environment, thus it is also an important process in removing excess nitrogen in areas which suffer eutrophication, e.g. coastal sediments (Hensen et al., 2006). The reduction of NO<sub>3</sub><sup>-</sup> occurs via multiple steps, each of them catalyzed by a different enzyme: first, NO<sub>3</sub><sup>-</sup> is reduced to NO<sub>2</sub><sup>-</sup> by nitrate reductase, which is then reduced to NO by nitrite reductase, which is reduced to N<sub>2</sub>O by nitric oxide reductase, which is finally reduced to N<sub>2</sub> by nitrous oxide reductase (Knowles, 1982). As N<sub>2</sub>O is an obligatory intermediate of denitrification, some of it can ultimately escape, explaining why denitrification is an important N<sub>2</sub>O source (Canfield et al., 2010). However, not all denitrifying organisms possess all enzymes, thus incomplete denitrification due to the lack of nitrous oxide reductase occurs, resulting in the production of N<sub>2</sub>O as the final product (Knowles, 1982; Canfield et al., 2010).

The major prerequisite for denitrification is the availability of nitrate, which is either provided by nitrification or from the bottom water through bioturbation (the spatial rearrangement of the sediment's solid phase by benthic organisms), bioirrigation (the active transport of bottom water through their habitats by living organisms in the sediment) or diffusion (Hensen et al., 2006). Nitrification is the most important nitrate source for denitrification, except for organic rich sediments underlying O<sub>2</sub>-poor but nitrate-rich waters, e.g. upwelling regions (Middelburg et al., 1996). Hence, a tight coupling between nitrification and denitrification occurs in most sediments, which is called coupled nitrification-denitrification (Burdige, 2006; Jenkins & Kemp, 1984). Additionally, denitrification is strongly dependent on temperature, O<sub>2</sub> concentration, availability of organic matter, and sulfide (Hensen et al., 2006; Middelburg et al., 1996; Senga et al., 2006; Dalsgaard et al., 2014). For example, the O<sub>2</sub> sensitivity of the enzymes involved in denitrification increases step by step along the reduction chain (Codispoti et al., 2005), resulting in slightly increased N<sub>2</sub>O production under O<sub>2</sub> conditions above 200 nM (Dalsgaard et al., 2012). Sulfide can inhibit or fuel denitrification. In general, sulfide is known to inhibit nitrification completely at low concentrations, thus inhibiting the nitrate source for denitrification (Seitzinger, 1988). In addition, sulfide inhibits the last enzymatic step during denitrification, resulting in enhanced formation of N<sub>2</sub>O (Sorensen et al., 1980; Porubsky et al., 2009; Dalsgaard et al., 2014). Recently, a new pathway of autotrophic denitrification coupled to sulfide oxidation was observed, indicating that sulfide interference with denitrification can be diverse (Jensen et al., 2009; Chen et al., 2013; Wang et al., 2005). Some studies also revealed increased N<sub>2</sub>O production during denitrification at acidic pH (Jørgensen et al., 1984; Knowles, 1982).



Besides probably being the most dominant marine benthic  $N_2O$  source, denitrification can also act as a significant  $N_2O$  sink in anaerobic environments such as anaerobic sediments or oxygen-minimum zones (Knowles, 1982; Usui et al., 1998; Naqvi et al., 2010). Hereby,  $N_2O$  is reduced to  $N_2$  by denitrifying bacteria when nitrous oxide reductase is active (Knowles, 1982).

The **dissimilarity nitrate reduction to ammonia (DNRA)** is another  $NO_3^-$  or  $NO_2^-$  reducing process, but in contrast to denitrification, nitrogen is not lost in form of  $N_2O$  or  $N_2$  but conserved as available nitrogen ( $NH_4^+$ ). It is an anaerobic microbial pathway of the nitrogen cycle that reduces  $NO_3^-$  first to  $NO_2^-$  and then to  $NH_4^+$  (Fig. 3; Ussiri & Lal, 2013). DNRA is a widespread physiological ability and has been found in a number of fermentative and strictly anaerobic bacteria (Hensen et al., 2006; Herbert, 1999). While denitrification is often the dominant nitrate-reducing process in most shallow marine sediments, DNRA can be more important in highly reduced sediments when nitrate concentrations are low (Koike & Hattori, 1978; Jørgensen, 1989; Bohlen et al., 2011; Ussiri & Lal, 2013). When nitrate concentrations increase, denitrification becomes the more dominant process (Herbert & Nedwell, 1990; King & Nedwell, 1985).



**Figure 3:** Nitrogen cycling in the marine environment. The various forms of nitrogen are plotted against their oxidation state. Processes shown in grey occur under anoxic conditions. Modified after Gruber, (2008).

There are a few data showing that  $N_2O$  is produced during DNRA (Smith & Zimmerman, 1981; Fazzolari et al., 1990), but the enzymatic pathway and the significance of this pathway for oceanic  $N_2O$  has not been described yet. DNRA is also a known sulfide-oxidizing process in large sulfur bacteria (e.g.

*Beggiatoa*, *Thioploca*, or *Thiomargarita*), which use  $\text{NO}_3^-$  (or  $\text{O}_2$ ) as an electron acceptor for the oxidation of sulfide (Preisler et al., 2007; Fossing et al., 1995). These sulfur oxidizing bacteria occur preferentially in upwelling areas. While *Beggiatoa* are more ubiquitous and occur in areas with enhanced sulfide flux like upwelling regions, hydrothermal vents and seeps or coastal sediments (Jääntti & Hietanen, 2012; Nelson et al., 1989; Ferdelman et al., 1997), *Thioploca* are found in mats in sediments of the upwelling region off Chile and Peru (Fossing et al., 1995). *Thiomargarita* is restricted to the Benguela upwelling region (Schulz et al., 1999).

## 2. Objectives

This study has two objectives:

- 1) In the first, central part of this study, the magnitude and environmental controls of benthic surface methanogenesis, i.e. within the sulfate-reducing zone of marine sediments, are investigated. Thereby the focus was set on coastal, organic-rich sediments, which are estimated to contribute with ~75% to global ocean emissions (Bange et al., 1994).
- 2) The second objective investigates the effect of sedimented oil (and thus the increase of organic material) on benthic microbial activity, including the potential of increased N<sub>2</sub>O-emission as a side effect.

For the first objective, two different coastal areas were examined, which experience either spatial or seasonal variations of environmental conditions such as temperature, O<sub>2</sub> or organic matter input: the upwelling region off Peru and the coastal sediments from Eckernförde Bay in the southwestern Baltic Sea, respectively. Laboratory experiments were conducted to answer the following research questions:

- Is surface methanogenesis present in organic-rich sediments?
- Does microbial activity of surface methanogenesis show spatial (off Peru) or seasonal (in Eckernförde Bay) variation?
- What are potential environmental controls of surface methanogenesis?
- Which substrates are used and which organisms are responsible for surface methanogenesis?
- What are the magnitudes compared to deep methanogenesis and how high is the potential for CH<sub>4</sub> emissions from surface methanogenesis?

For the second objective, sediments from the Gulf of Mexico were examined, which is known for the abundant occurrence for natural gas and oil seeps. Laboratory experiments focused on the detection of microbial denitrification and sulfate reduction to examine the following aspects:

- Is denitrification elevated in sediments with a sedimented oil layer on top (oiled sediments), i.e. with a higher organic carbon content, compared to oil-free (control) sediments?
- Is sulfate reduction elevated in oiled sediments compared to control sediments?
- Are sediment N<sub>2</sub>O concentrations higher in oiled, organic-rich sediments compared to control sediments?
- Is there a potential of increased N<sub>2</sub>O emissions due to sulfide interferences from elevated sulfate reduction?

### 3. Publication outline

The following chapters 2-4 present the results achieved during the PhD-thesis “Production of greenhouse gases in organic-rich sediments”. Each chapter is written in the form of a scientific manuscript, of which chapter 2 and 4 are already submitted to scientific journals, while chapter 3 is in preparation for submission. My contributions to each manuscript are described in the following.

#### **Chapter 2: Microbial methanogenesis in the sulfate-reducing zone of surface sediments traversing the Peruvian margin**

Johanna Maltby, Stefan Sommer, Andrew W. Dale and Tina Treude

*Submitted to: Biogeosciences*

This study was initiated by Tina Treude. Johanna Maltby designed the experiments with input from Tina Treude. Johanna Maltby carried out the sediment sampling, water column sampling, and rate measurements via gas chromatography and usage of radioactive isotopes. Porewater measurements were coordinated by Andy Dale and Stefan Sommer. The manuscript was written by Johanna Maltby with input from all co-authors.

#### **Chapter 3: Methanogenesis within the sulfate-reduction zone in seasonally hypoxic sediments from Eckernförde Bay, SW Baltic Sea**

Johanna Maltby, Lea Steinle, Hermann W. Bange, Carolin R. Löscher, Martin A. Fischer, Mark Schmidt, Ralf Conrad, Tina Treude

*In preparation*

This study was initiated by Tina Treude. Johanna Maltby designed the experiments with input from Tina Treude. The cruises were carried out by Johanna Maltby and Lea Steinle. Sediment sampling and water column sampling were carried out by Johanna Maltby with assistance of Lea Steinle. Johanna Maltby carried out microbial rate measurements via gas chromatography and usage of radioactive isotopes, and porewater measurements. Measurements of  $^{13}\text{C}$ -Methane were carried out by Mark Schmidt. Measurements of  $^{13}\text{C}$ -Methanol were carried out by Ralf Conrad. DNA extraction and qPCR measurements were supervised by Carolin Löscher with assistance of Martin Fischer. The manuscript (in its current form) was written by Johanna Maltby with input of Tina Treude and Hermann Bange.

## Chapter 4: Denitrification and sulfate reduction in oiled and oil-free sediments from the Northern Gulf of Mexico

Johanna Maltby, Tina Treude, Samantha B. Joye

*Submitted to: Deep Sea Research II*

This study was initiated by Samantha (Mandy) Joye. Experiments were designed by Mandy Joye. Sediment sampling and denitrification experiments were carried out by Johanna Maltby. Porewater measurements, sediment parameters, and sulfate reduction rates were carried out by Mandy Joye and Johanna Maltby. The manuscript was written by Johanna Maltby with input from all co-authors.

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## Chapter 2

# **Microbial methanogenesis in the sulfate-reducing zone of surface sediments traversing the Peruvian margin**

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## Abstract

We studied the concurrence of methanogenesis and sulfate reduction in surface sediments (0-25 cm below seafloor) at six stations (70, 145, 253, 407, 770 and 1024 m) along the Peruvian margin (12°S). This oceanographic region is characterized by high carbon export to the seafloor creating an extensive oxygen minimum zone (OMZ) on the shelf, both factors that could favor surface methanogenesis. Sediments sampled along the depth transect traversed areas of anoxic and oxic conditions in the bottom-near water. Net methane production (batch incubations) and sulfate reduction (<sup>35</sup>S-sulfate radiotracer incubation) were determined in the upper 0-25 cmbsf of multicorer cores from all stations, while deep hydrogenotrophic methanogenesis (> 30 cmbsf, <sup>14</sup>C-bicarbonate radiotracer incubation) was determined in two gravity cores at selected sites (78 and 407 m). Furthermore, stimulation (methanol addition) and inhibition (molybdate addition) experiments were carried out to investigate the relationship between sulfate reduction and methanogenesis.

Highest rates of methanogenesis and sulfate reduction in the surface sediments, integrated over 0-25 cmbsf, were observed on the shelf (70-253 m, 0.06-0.1 mmol m<sup>-2</sup> d<sup>-1</sup> and 0.5-4.7 mmol m<sup>-2</sup> d<sup>-1</sup>, respectively), while lowest rates were discovered at the deepest site (1024 m, 0.03 and 0.2 mmol m<sup>-2</sup> d<sup>-1</sup>, respectively). The addition of methanol resulted in significantly higher surface methanogenesis activity, suggesting that the process was mostly based on non-competitive substrates, i.e., substrates not used by sulfate reducers. In the deeper sediment horizons, where competition was probably relieved due to the decline of sulfate, the usage of competitive substrates was confirmed by the detection of hydrogenotrophic activity in the sulfate-depleted zone at the shallow shelf station (70 m).

Surface methanogenesis appeared to be correlated to the availability of labile organic matter (C/N ratio) and organic carbon degradation (DIC production), both of which support the supply of methanogenic substrates. A negative correlation of methanogenesis rates with dissolved oxygen in the bottom-near water was not obvious; however, anoxic conditions within the OMZ might be advantageous for methanogenic organisms at the sediment-water interface.

Our results revealed a high relevance of surface methanogenesis on the shelf, where the ratio between surface to deep (below sulfate penetration) methanogenic activity ranged between 0.13 and 10<sup>5</sup>. In addition, methane concentration profiles indicate a partial release of surface methane into the water column as well as a partial consumption of methane by anaerobic methane oxidation (AOM) in the surface sediment. The present study suggests that surface methanogenesis might play a greater role in benthic methane budgeting than previously thought, especially for fueling AOM above the sulfate-methane transition zone.

## Introduction

Microbial methanogenesis represents the terminal step of organic matter degradation in marine sediments (Jørgensen, 2006). The process is entirely restricted to a small group of prokaryotes within the domain of the Archaea (Thauer, 1998). Methanogens produce methane from a narrow spectrum of substrates, primarily carbon dioxide (CO<sub>2</sub>) and hydrogen (H<sub>2</sub>) (hydrogenotrophic pathway), as well as acetate (acetoclastic pathway, Zinder, 1993). In addition, methanol or methylated compounds such as methylamine are utilized (methylotrophic pathway) (Oremland & Polcin, 1982; Buckley et al., 2008; Zinder, 1993; King et al., 1983b). Substrates for methanogenesis are produced during depolymerization and fermentation of organic macromolecules (e.g., sugars, vitamins, amino acids) to smaller monomeric products (Jørgensen, 2006; Schink & Zeikus, 1982; Neill et al., 1978; Donnelly & Dagley, 1980).

Acetoclastic and hydrogenotrophic methanogenesis are predominantly found in deeper sediment zones below sulfate penetration, owing to the competition with sulfate reducers that outcompete methanogens for H<sub>2</sub> and acetate due to their higher substrate affinity (Oremland & Polcin 1982; Jørgensen 2006). Furthermore, CO<sub>2</sub>/H<sub>2</sub> and acetate are the more abundant substrates in deeper sediments as degradability of organic matter, and with it the substrate variety and availability, decreases with increasing sediment depth (Jørgensen, 2006).

Methanogens avoid competition with sulfate reducers by the utilization of non-competitive substrates, such as methanol or methylamines (Oremland & Polcin, 1982; King et al., 1983b). Facilitated by the usage of such non-competitive substrates, sulfate reduction and methanogenesis were found to co-occur in sulfate-containing salt marsh sediments (Oremland et al., 1982a; Buckley et al., 2008; Senior et al., 1982). Concurrent activity of sulfate reduction and methanogenesis in the marine environment has mostly been postulated for organic-rich sediments (Mitterer, 2010; Jørgensen & Parkes, 2010; Treude et al., 2009, 2005a; Hines & Buck, 1982; Crill & Martens, 1986); however, research on magnitude and environmental controls of surface methanogenesis is still sparse (Holmer & Kristensen, 1994; Ferdelman et al., 1997).

In a study from Eckernförde Bay, southwestern Baltic Sea, considerable *in vitro* methanogenic activity was observed in samples taken from 5 to 40 cm sediment depth (Treude et al. 2005). Although *in vitro* activity was measured in sulfate-free setups, methanogenic activity coincided with zones of *in-situ* sulfate reduction. The authors concluded a coexistence of the two types of organisms, which could be enabled through either the usage of non-competitive substrates, dormancy of methanogens until phases of sulfate depletion, and/or temporal or spatial heterogeneity in the sediments. Eckernförde Bay sediments feature a high input of organic matter due to a shallow water depth (~30 m) and pronounced phytoplankton blooms in spring, summer, and fall (Smetacek, 1985). Furthermore,

seasonal hypoxia ( $O_2 < 90 \mu\text{M}$ ) or even anoxia ( $O_2=0 \mu\text{M}$ ) occur in the deep layers of the water column caused by stratification and degradation of organic matter ( Bange et al. 2011). Oxygen-depleted conditions in the bottom water together with frequent input of fresh organic matter possibly favors methanogenesis in surface sediment by offering reduced conditions and non-competitive substrates. In accordance, methanogenesis activity was observed within the sulfate-reducing zone of organic-rich and seasonally hypoxic sediments from the Limfjorden sound, Northern Denmark (Jørgensen & Parkes, 2010; Jørgensen, 1977b).

The environmental relevance of surface methanogenesis is hitherto unknown. Its closeness to the sediment-water interface makes it a potential source for methane emissions into the water column, unless the methane is microbially consumed before escaping the sediment (Knittel & Boetius, 2009). Methane escapes the sediment either by diffusion or, when methane saturation is exceeded, in the form of gas bubbles (Whiticar, 1978; Wever & Fiedler, 1995; Judd et al., 1997; Dimitrov, 2002b). How much of the released methane reaches the atmosphere mainly depends on water depth, as methane is consumed within the water column through microbial oxidation (Reeburgh, 2007; Valentine et al., 2001). Thus, coastal areas have higher methane emission potentials than the open ocean (Bange et al., 1994). Once in the atmosphere, methane acts as a very potent greenhouse gas (IPCC, 2014).

In the present study we focused on the upwelling region off the Peruvian coast, which is another excellent example of an environment, where both factors that potentially favor surface methanogenesis convene, i.e., a high export of organic carbon and low dissolved oxygen concentrations in the bottom water. This upwelling region represents one of the most productive systems in the world oceans, creating one of the most intense oxygen minimum zones (OMZ, Kamykowski & Zentara 1990; Pennington et al. 2006). Oxygen concentrations in waters impinging on the seafloor are below  $20 \mu\text{M}$  or even reach anoxia. Research on surface methanogenesis in upwelling regions is rare and its potential role in the carbon cycling of the Peruvian OMZ is completely unknown. In a study from the central Chilean upwelling area (87 m water depth, 0.5-6 cm sediment depth), small production of methane was detected despite high sulfate reduction activity, when offering the non-competitive substrate trimethylamine (Ferdelman et al., 1997). The authors concluded that the prevailing methanogens were competing with sulfate reducers for  $H_2$  and with acetogens for methylamines, explaining the overall low methanogenesis activity observed (Ferdelman et al., 1997). Even though the Chilean and Peruvian OMZs are connected, commonly known as OMZ in the eastern South Pacific Ocean (ESP) (Fuenzalida et al., 2009), the core of the ESP-OMZ is centered off Peru with an upper boundary at  $< 100 \text{ m}$  and a vertical distribution to  $> 600 \text{ m}$  versus a thinner OMZ band off Chile constrained between 100-400 m water depth (Fuenzalida et al., 2009). The anoxic conditions in the water column of the OMZ core together with the high export rates of labile organic carbon to the

seafloor (Reimers & Suess, 1983; Dale et al., 2015) provide favorable conditions for methanogenesis activity in surface sediments, thus increasing the potential for benthic methane emissions.

Here, we provide first insights into surface methanogenesis in sediment cores (< 30 cmbsf = centimeters before seafloor) taken along the Peruvian shelf and margin. We hypothesize that methanogenesis coexists with sulfate reduction through the utilization of non-competitive substrates. In addition, we postulate that surface methanogenesis depends on the quantity and quality (= freshness) of organic carbon, and the concentrations of dissolved oxygen in the bottom water. We therefore expect spatial variability of surface methanogenesis along the continental shelf and margin. The observed methanogenic activity will be compared to methane concentrations in the bottom-near water to discuss the potential relevance of surface methanogenesis for methane emissions into the pelagic zone.

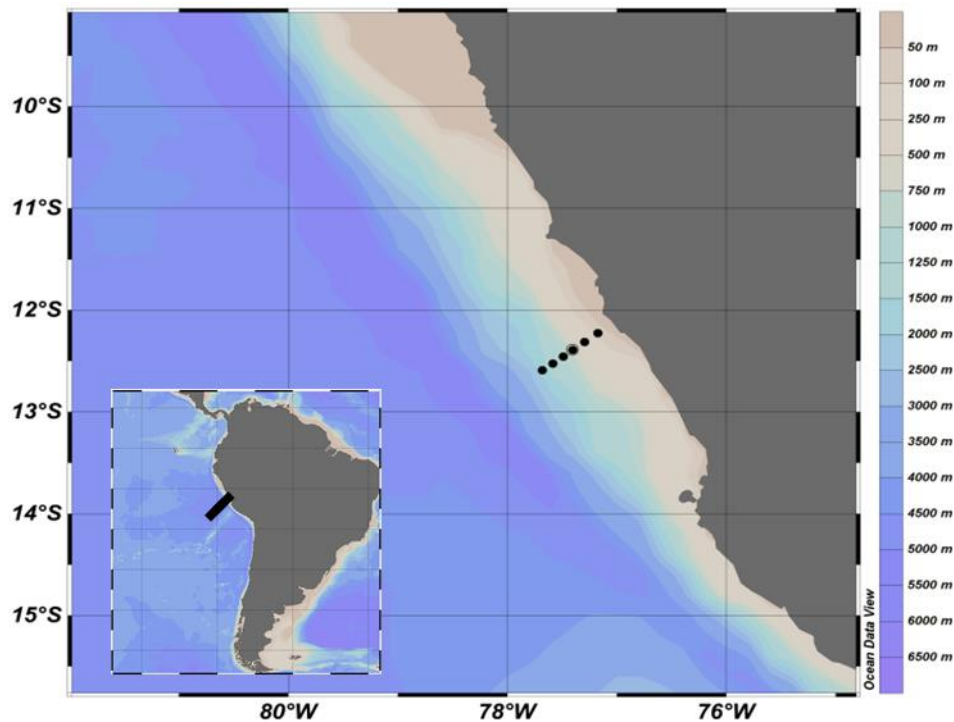
## **Material and Methods**

### **Study site and sediment sampling**

Samples were taken during the R.V. Meteor cruise M92 between 5. Jan and 3. Feb 2013 along a depth transect off the Peruvian coast from the shelf (~70 m) to the continental slope (~1000 m). The transect was located in the central part of the ESP-OMZ (Fuenzalida et al., 2009) at 12°S. Further hydrographic details on the study area can be found elsewhere (Dale et al., 2015).

Sediment cores for the determination of near-surface methanogenesis were collected at six stations along the depth transect at 70, 145, 253, 407, 770 and 1024 m water depth (Fig.1), using a multiple corer with a mounted camera (TV-MUC). The MUC held seven cores (length: 60 cm, inner diameter: 10 cm) and covered an area of ~1 m<sup>2</sup>. If necessary, a second MUC was deployed at the same station, thus sediment cores could originate from different MUC casts. Station numbers were assigned in accordance with Dale et al., (2015). After retrieval, sediment cores were transferred to a ~ 9°C cold room and processed at the same day.

In addition to the MUC, a gravity corer was deployed at two stations (78 and 407 m) for determining deep methanogenesis. The total core length was 400 cm and 206 cm, respectively. The gravity corer was equipped with a 260 kg weight and a 5 m steel barrel (diameter: 14 cm). The replaceable core liner (PVC, diameter: 12.5 cm) was housed within the barrel and fixed with a core catcher. After retrieval, sediment cores from the gravity corer were sliced into 1–m sections, capped on both sides, and brought to the cold room (4°C) for further processing. Relevant station details for MUC and gravity cores are summarized in Table 1.



**Figure 1:** Location of sampling sites off Peru along the depth transect at 12° S. Source: Schlitzer, R., Ocean Data View, <http://odv.awi.de>, 2014

### Water column sampling

CTD/Rosette water column casts were conducted at the same station as sediment coring (for details see Table 1). Temperature and oxygen data are taken from Dale et al. 2015.

For the analysis of methane concentrations in the bottom-near water, water was sampled ca. 1.5 m above the seafloor from 10 L Niskin bottles mounted on a rosette water sampler. The collected water was filled bubble-free into 60 ml vials (triplicates), each vial containing 3 pellets of sodium hydroxide (NaOH, ~ 0.3 M per vial) to stop microbial activity and force dissolved gas into the headspace. After closing the vials with a butyl rubber stopper and a crimp seal, 10 ml of water was removed with a N<sub>2</sub>-flushed 10 ml syringe and replaced with N<sub>2</sub> gas from a second syringe to create a headspace in the sampling vials. Samples were stored and transported at room temperature until further processing. In the home laboratory, 100 µl of the headspace volume was injected into a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector and a HaySep-T 100/120 column (Length 3 m, diameter: 2 mm). Gases were separated isothermally at 75°C with helium carrier gas. Methane concentrations were calibrated against methane standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2%.

**Table 1:** Stations, instruments, chemical/physical parameters in the bottom-near water, and analyses applied to samples along the depth transect on the Peruvian margin (12°S). For abbreviations see footnote.

Station No	Instrument	Latitude (S)	Longitude (W)	Water depth (m)	O <sub>2</sub> (μM)	Temp. (°C)	CH <sub>4</sub> (nM)	Type of analysis
1	MUC 13	12°13.492	77°10.511	70				All
	MUC 38	12°13.517	77°10.084	70				SE
	GC 8	12°14.500	77°9.611	78				GC-All
	CTD 9	12°13.535	77°10.522	73	bdl	14	38.6	WC
4	MUC 10	12°18.704	77°17.790	145				All
	CTD 14	12°18.697	77°18.004	145	bdl	13.4	24.4	WC
6	MUC 5	12°23.321	77°24.176	253				Gas+PW
	MUC 6	12°23.322	77°24.181	253				nMG
	CTD 6	12°24.904	77°26.314	305	bdl	12	79.6	WC
8	MUC 23	12°27.198	77°29.497	407				Gas+ PW
	MUC 24	12°27.197	77°29.497	407				nMG
	GC 3	12°27.192	77°29.491	407				GC-All
	CTD 37	12°29.502	77°29.502	407	bdl	10.6	7.3	WC
9	MUC 17	12°31.374	77°35.183	770				Gas+ PW
	MUC 18	12°31.373	77°35.184	770				nMG
	CTD 27	12°31.327	77°35.265	770	19	5.5	8.4	WC
10	MUC 28	12°35.377	77°40.975	1024				Gas+ PW
	MUC 29	12°35.377	77°40.976	1024				nMG
	CTD 11	12°34.863	77°38.954	1010	53	4.4	3.9	WC

MUC = multicorer, GC= gravity corer, CTD = CTD/Rosette, bdl= below detection limit (5μM), All = methane gas analysis, porewater analysis, net methanogenesis analysis, SE = slurry experiment, GC-All= analysis for gravity cores including methane gas analysis, porewater analysis, hydrogenotrophic methanogenesis analysis, WC= Water column analyses, Gas = methane gas analysis, PW= porewater analysis, nMG= net methanogenesis analysis

### Porewater geochemistry

Porewater sampling for MUC cores has been previously described by Dale et al., (2015). In short, one MUC core per station was subsampled in an argon-filled glove bag, to preserve redox sensitive constituents.

The gravity cores at St. 1 (78 m) and St. 8 (407 m) were subsampled at 10-12 different sediment depths (depending on core length) resulting in depth intervals of 20-33 cm. Before sampling, the plastic core liner was cut open with an electric saw at the specific depths. Porewater was extracted by using anoxic (flushed with argon), wetted rhizons (Rhizosphere Research Products, Seeberg-Elverfeldt et al., 2005).

Sulfate concentrations were determined by ion chromatography (Methrom 761) as described previously by Dale et al., (2015).

For DIC analysis, 1.8 ml of porewater was transferred into a 2 ml glass vial, fixed with 10  $\mu$ l saturated mercury chloride solution and crimp sealed. Samples were stored at 4°C until further processing in the home laboratory. DIC concentration was determined as CO<sub>2</sub> with a multi N/C 2100 analyzer (Analytik Jena) following the manufacturer's instructions. Therefore the sample was acidified with phosphoric acid and the outgassing CO<sub>2</sub> was measured. The detection limit was 20  $\mu$ M with a precision of 2-3%.

### **Sediment porosity and particulate organic carbon/nitrogen**

Methodology and data for porosity, particulate organic carbon (POC) and particulate organic nitrogen (PON) have been previously described by Dale et al., (2015).

In short, wet sediment samples were taken from the porewater MUC core and the gravity cores for determination of porosity from the weight difference of wet and freeze-dried sediment. POC and PON were analyzed with a Carlo-Erba element analyzer (NA 1500). Ratios of POC:PON were calculated by division.

### **Sediment methane**

For sediment methane concentration, one MUC core per station was sliced in 2 cm intervals until 20 cm depth, followed by 5 cm intervals until the end of the core (maximum depth = 48 cm). Gravity cores were subsampled according to the above scheme (see 2.3). From each sampled sediment layer, 2 cm<sup>3</sup> sediment were transferred into a 15 ml serum glass vial containing 5 ml of NaOH (2.5% w/w). The vial was closed with a butyl stopper, crimp sealed and shaken thoroughly to stop microbial activity and to force all methane into the headspace. Vials were stored upside down at room temperature until measurement in the home laboratory.

Sediment methane concentration was determined by injecting 0.1 ml of headspace volume into a Shimadzu GC-2014 gas chromatograph as described under section "Water column sampling".

### **Net methanogenesis activity in MUC cores**

Sediment from MUC cores was used to determine net methanogenesis, which is defined as the sum of total methane production and consumption, including all available methanogenic substrates in the sediment. Net methanogenesis was determined by measuring the linear increase of methane concentration in the headspace of closed incubation vials over time. Therefore, one MUC core per station was sliced in 5 cm intervals, transferring 10 cm<sup>3</sup> of sediment in triplicates into a N<sub>2</sub>-flushed 60 ml serum glass vial. The sediment core lengths ranged between 25-48 cm, resulting in maximum 10 depth intervals. Ten ml of anoxic deep water overlying each MUC core was added to the vial and the



slurry was mixed under a constant N<sub>2</sub> stream (Hungate, 1950) before sealed with a butyl rubber stopper and crimped. The sediment slurry was repeatedly flushed with N<sub>2</sub> through the stopper to guarantee fully anoxic conditions. The vials were incubated in the dark and at 9°C, which reflected the average in situ temperature along the depth transect (see Table 1). The first gas chromatographic measurement was done directly after preparation of the vials, by injecting 100 µl of headspace sample into the gas chromatograph. The on-board Hewlett Packard-5890 gas chromatograph was equipped with a flame ionization detector and a HaySep-T 100/120 column (Length 3 m, diameter: 2 mm). Gases were separated isothermally at 75°C with helium carrier gas. Methane concentrations were calibrated against methane standards. The detection limit was 1 ppm with a precision of < 5%. Measurements were done in 2-4 day-intervals over a total incubation time of ~2 weeks.

### **Potential non-competitive and competitive methanogenesis in sediment slurries from MUC cores**

Sediment slurry experiments were conducted with sediment from St. 1 (70 m) to examine the interaction between sulfate reduction and methanogenesis, as this station revealed highest microbial activity of sulfate reduction and methanogenesis. On board, the sediment core was sliced in 5 cm intervals. Sediment from the 0-5 cm interval and the 20-25 cm interval was transferred completely into 250 ml glass bottles, which were then closed without headspace with a butyl rubber stopper and screw cap. Until further treatment, sediment was stored at 4°C on board and later in a 1°C cold room on shore.

Approximately 6 months after the cruise, sediment slurries from both depth intervals were prepared by mixing 5 ml sediment in a 1:1 ratio with artificial, fully marine seawater (Widdel & Bak, 1992) before further manipulations.

In total, three different treatments, each in triplicates, were prepared per depth: 1) sulfate-rich (28 mM), serving as a control 2) sulfate-rich plus molybdate (22 mM) from now on referred to as molybdate-treatment, and 3) sulfate-rich plus methanol (10 mM) from now on referred to as methanol-treatment.

Molybdate was used as an enzymatic inhibitor for sulfate reduction (Oremland & Capone, 1988). Methanol is a known non-competitive substrate used by methanogens, but not by sulfate reducers (Oremland & Polcin, 1982), which makes it suitable to examine non-competitive methanogenesis.

The sediment slurries were incubated at 9°C in the dark for 23 days and headspace concentration of methane was measured repeatedly over time on a gas chromatograph. Therefore, 100 µl of headspace was removed from the gas vials and injected into a Shimadzu gas chromatograph (GC-2014) equipped with a methanizer (inactive), a packed Haysep-D column and a flame ionization detector. The column temperature was 80°C and the helium flow was set to 12 ml min<sup>-1</sup>. Methane concentrations were

measured against methane standards. The detection limit was 0.1 ppm with a precision of <5%. Rates were determined from the linear increase of methane concentration over time. Due to differences in the linear increase between the three treatments, rates were determined at two different time points: the first period of incubation includes the starting point (day 0) until day 5, the second period includes day 8 to day 23 (Supplementary Material, Fig. S1).

Student's t-test (independent, two-tailed,  $\alpha = 0.05$ ) was applied to detect significant differences between the three different treatments.

### Gross hydrogenotrophic methanogenesis activity in gravity cores

For the determination of surface to deep methanogenesis activity in gravity cores the radiotracer technique using  $^{14}\text{C}$ -bicarbonate was applied (Jørgensen, 1978). With this method only hydrogenotrophic methanogenesis from  $\text{CO}_2/\text{H}_2$  can be determined, which is the expected main pathway in deeper sediment layers.

Sampled sediment depths were according to the above scheme (see 2.3). Circa  $5\text{ cm}^{-3}$  of sediment was sampled in triplicates into glass syringes and then sealed headspace-free with butyl rubber stoppers. Then,  $^{14}\text{C}$ -bicarbonate-tracer (dissolved in water, pH = 8-9, injection volume  $6\ \mu\text{l}$ , activity 222 kBq, specific activity 1.85-2.22 GBq/mmol) was injected through the stopper. The vials were incubated for 48 hours at  $9^\circ\text{C}$  before the reaction was stopped by transferring the sediment into 50 ml glass vials filled with 20 ml NaOH (2.5%), closed with butyl rubber stoppers and shaken thoroughly. Five controls were produced from various sediment depths by injecting the radiotracer directly into the NaOH with sediment.

In the home laboratory,  $^{14}\text{C}$ - methane production was determined with the slightly modified method by Treude et al., (2005a) used for the determination of anaerobic oxidation of methane. The method was identical, except no unlabeled methane was determined by gas chromatography. Instead, DIC values were used to calculate hydrogenotrophic methane production (=  $\text{CO}_2$  reduction):

$$MG\ rate = \frac{{}^{14}\text{CH}_4 * [\text{DIC}]}{({}^{14}\text{CH}_4 + {}^{14}\text{C-DIC}) * t}$$

The methanogenesis rate (*MG rate*) is expressed in  $\text{nmol CH}_4\ \text{cm}^{-3}\ \text{sediment}\ \text{d}^{-1}$ ,  ${}^{14}\text{CH}_4$  is the activity of produced  ${}^{14}\text{CH}_4$ ,  ${}^{14}\text{C-DIC}$  is the activity of residual radioactive dissolved organic carbon ( $\text{DIC} = \text{CO}_2 + \text{HCO}_3^- + \text{CO}_3^{2-}$ ),  $[\text{DIC}]$  is the concentration of dissolved organic carbon in  $\text{nmol cm}^{-3}$  sediment, and  $t$  is the incubation time in days.

### **Sulfate reduction in MUC cores**

One MUC core per station was used for the determination of sulfate reduction. First, two replicate push cores (length 30 cm, inner diameter 2.6 cm) were subsampled from one MUC core. The actual core length varied from 23-25 cmbsf total length. Then, 6  $\mu\text{l}$  ( $\sim 150$  kBq) of carrier-free  $^{35}\text{SO}_4^{2-}$  radiotracer (dissolved in water, specific activity 37 TBq  $\text{mmol}^{-1}$ ) was injected into the replicate pushcores in 1-cm intervals according to the whole-core injection method Jørgensen, (1978). Push cores were incubated for ca. 12 h at 9°C. After incubation, bacterial activity was stopped by slicing the push core into 1-cm intervals and transferring each sediment layer into 50 ml plastic centrifuge tubes filled with 20 ml zinc acetate (20% w/w). Controls were done in triplicates from different depths. Here, the sediment was first fixed with zinc acetate before adding the tracer. Rates for sulfate reduction were determined using the cold chromium distillation procedure according to Kallmeyer et al., (2004). The yielded sulfate reduction rates have to be treated with caution, due to long (up to 3 half-life times of  $^{35}\text{S}$ ) and unfrozen storage. Storage of sulfate reduction samples without freezing has recently been shown to result in the re-oxidation of  $^{35}\text{S}$ -sulfides, which results in an underestimation of sulfate reduction rates (Røy et al., 2014). During this reaction, zinc sulfide ( $\text{Zn}^{35}\text{S}$ ) and iron sulfide ( $\text{Fe}^{35}\text{S}$ ) are re-oxidized to sulfate by reactive Fe(III), which originates from the reaction of  $\text{Fe}^{2+}$  with oxygen.  $\text{Fe}^{2+}$  is released during the gradual conversion of FeS to ZnS, which has the lower solubility product. Still, we do trust the relative distribution of activity along depth profiles and consider a potential underestimation of absolute rates.

## **Results**

### **Water column oxygen and methane concentration**

Dissolved oxygen in the bottom water was below detection limit from St.1 (70 m) to St. 8 (407 m), subsequently increasing with water depth to 53  $\mu\text{M}$  at the deepest site (see Table 1 and Dale et al., 2015). At the shallowest St. 1 (70 m) the water was turbid and smelled of sulfide.

Dissolved methane concentrations in the bottom water were high on the shelf (St.1-6, 70-253 m) and 10 fold lower at the deeper sites (St. 8-10, 407-1024 m; Table 1). The highest measured methane concentration was detected at St. 6 (253 m,  $\sim 80$  nM) and lowest concentrations were detected at St. 10 (1024 m,  $\sim 4$  nM).

### **Sediment core description**

A detailed sediment description for the porewater geochemistry cores has been already published in detail by Dale et al., (2015). In short, sediments revealed a grey color with a black surface layer at St. 1 (70 m), a dark olive green color at St. 4-8 (145-407 m), and a green-brown color at St. 9 and 10 (770

-1024 m). Sediment texture was soft and fluffy at St. 1-6 (70-253 m), and was less soft at the deeper sites. St. 8 (407 m) revealed a fluffy surface layer followed by a dense clay layer > 2 cmbsf sediment depth. In addition, phosphorite nodules were found at the sediment surface (0-2 cmbsf) of St. 8 (407 m).

Mats of the sulfur-oxidizing bacteria *Thioploca spp.* (Gallardo, 1977) were visible at the sediment surface at St.1-6 (70-253 m), with the densest mat at St. 1 (70 m) continuously decreasing with increasing water depth. Sheaths of *Thioploca* were visible until 20-30 cmbsf at St. 1, 4 and 6 (70-253 m).

Foraminifera could be observed at the sediment surface of St. 8 (407 m), St. 9 (770 m) and St. 10 (1024 m). St. 8 (407 m) showed a thick layer of foraminifera ooze on the sediment surface (0-3 cmbsf) while St. 9 (770 m) and St. 10 (1024 m) showed only scattered foraminifera at the sediment surface (0-5 cmbsf).

Macrofauna (large polychaetes, oligochaetes, ophiuroids) were restricted to the sites below the OMZ at St. 9 (770 m) and St. 10 (1024 m), where deep waters were oxygenated. However, small snails (~ 1 cm) were observed at St. 8 (407 m).

### **Geochemical parameters in MUC cores**

Porewater and solid phase geochemistry of sediments retrieved by the MUC cores are shown in Fig. 2. Surface sediment (0-0.5 cmbsf) POC content increased along the continental shelf from 1.6 wt % at the shallow St. 1 (70 m) to a maximum of 15 wt % at St. 8 (253 m). Surface POC content decreased again with increasing water depth showing the lowest POC content at St. 10 (1024 m, 2 wt %). While POC content showed more or less stable profiles throughout the sediment core at St. 1 (70 m, around 3 wt %), St. 9 (770 m, around 4 wt %), and St. 10 (1024 m, around 3 wt %), POC content was stable only in the upper ~ 10 cmbsf at St. 4 (150 m, around 10 wt %) and St. 6 (253 m, around 15 wt %), followed by a decrease until the deepest sampled depth (2 wt % and 9 wt %, respectively). At St. 8 (407 m), POC content increased with sediment depth below 3 cmbsf (from 4 wt % to 9 wt %), which consisted of dense clay (see above). In the upper 3 cmbsf, POC decreased from ~ 7 wt % to ~ 4 wt %, which was the sediment layer with a more fluffy appearance.

The sediment surface C/N ratio was lowest at St. 1 (70 m, 6.2) and increased along the continental shelf showing the highest surface C/N ratio at St. 10 (1024 m, 11). St. 8 (407 m) was exceptional, as it showed slightly lower surface C/N ratio (8) as at St. 6 (253 m, 9). St. 8 (407 m) was also the only site showing an increase of 4 units in the upper 0-5 cmbsf, followed by stable ratios around 12 throughout the rest of the core. St. 1 and 4 (70 and 145 m) showed shallower increases in C/N ratio in the upper ~ 2 cmbsf and upper 1 cmbsf, respectively, followed by stable ratios around 10 until the bottom of the core. At St. 9 and 10 (770 and 1024 m), C/N ratios ranged around 11 and 12, respectively.

The highest increase in methane concentration was observed at St. 1 (70 m). Here, methane increased linearly from the surface (1  $\mu\text{M}$ ) to the bottom of the core (100  $\mu\text{M}$ ). All other stations showed either no clear trend (St. 4= 145 m) or only slight methane increases with depth. At St. 9 (770 m), even a decrease in methane concentration was observed from the surface to the bottom of core.

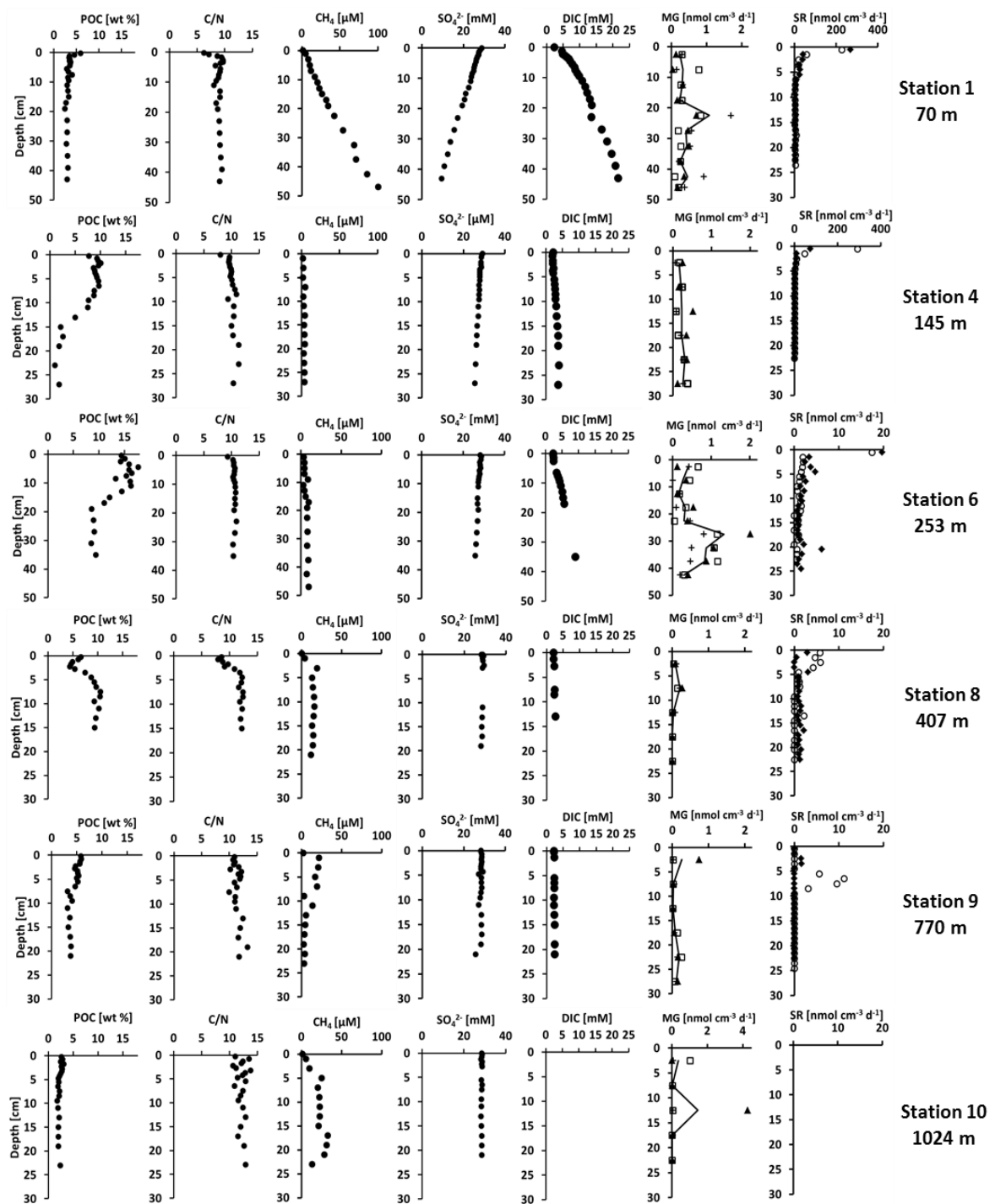
Besides St. 1 (70 m), which showed a strong decrease in sulfate ( $\text{SO}_4^{2-}$ ) concentration with depth from about 28 mM at the top to about 9 mM at the bottom of the core (43 cmbsf), all other stations showed  $\text{SO}_4^{2-}$  concentrations > 25 mM throughout the cores. At St. 4, 6 and 9 (145, 253, 770 m),  $\text{SO}_4^{2-}$  showed very slight decrease with depth from about 28 mM at the top to about 25 mM at the bottom of the core. Porewater  $\text{SO}_4^{2-}$  concentrations were stable around 28 mM throughout the core at St. 8 and 10 (407 and 1024 m).

Dissolved inorganic carbon (DIC) concentration increased with depth at St. 1- 6 (70 -253 m). St. 1 (70 m) showed the steepest increase with depth, showing the lowest DIC concentration at the top (2.3 mM) and the highest at the deepest sampled depth (21.6 mM). At St. 4 (153 m), maximum concentration was reached at  $\sim$  23 cmbsf with 4 mM. St. 6 (253 m) showed maximum concentration at the deepest sampled depth with 9 mM. St. 8 and 9 (407 and 770 m) showed stable DIC concentrations around 2.3 mM throughout the core. No DIC data was available for St. 10 (1024 m).

### **Net methanogenesis and gross sulfate reduction in MUC cores**

Maximum net methanogenesis rates were detected at St. 1 (70 m,  $1.1 \pm 0.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 20-25 cmbsf) and St. 6 (253 m,  $1.3 \pm 0.65 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 25-30 cmbsf). At all other stations, methanogenesis was mostly below  $0.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$  throughout the cores. St. 8 (407 m) showed methanogenesis activity only in the top 10 cmbsf with the maximum at 5-10 cmbsf ( $0.2 \pm 0.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). At St. 9 and 10 (770 and 1024 m), maximum methanogenesis activity was found in the surface layer (0-5 cmbsf) with  $0.3 \pm 0.4 \text{ nmol cm}^{-3} \text{ d}^{-1}$  and  $0.4 \pm 0.6 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , respectively. St. 10 (1024 m) also showed high average methanogenesis at 10-15 cmbsf ( $1.5 \pm 2.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ), which was caused by a single high replicate ( $4.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). In the following, e.g., integration of rates, we will exclude this single high replicate, which will be further elaborated in the discussion.

At all stations beside St. 9 (770 m), sulfate reduction activity was highest in the 0-1 cmbsf horizon, followed by a sharp decrease in activity of 20-90% in the subsequent 1-2 cmbsf horizon. Highest measured rates at 0-1 cmbsf were observed at St. 4 (145 m,  $290 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ), followed by St. 1 (70 m,  $270 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). Surface (0-1 cmbsf) sulfate reduction activity decreased from St. 4 (145 m) to St. 8 (407 m) with concomitant increase in water depth. St. 9 (770 m) was the only site without a surface sulfate reduction maximum.

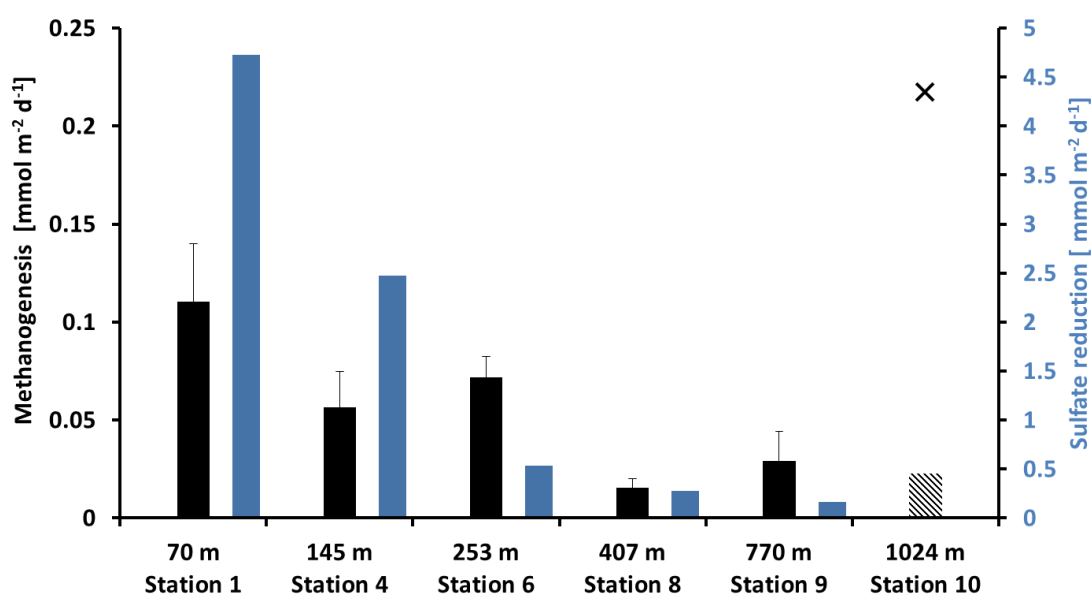


**Figure 2.** Profiles of particulate organic carbon (POC), C/N ratio, methane (CH<sub>4</sub>), sulfate (SO<sub>4</sub><sup>2-</sup>), DIC (dissolved inorganic carbon), net methanogenesis (MG) rates and sulfate reduction (SR) rates in the MUC cores along the depth transect. For MG, triplicates (symbols) and mean (solid line) are shown. For SR, duplicates are shown. Data points from the overlying water in the MUC core (OLW) are set to 0 cm. Note deviant scale dimension for MG at St. 6 and for SR at St. 1 and 2.

Here, highest rates were found at 7 cmbsf ( $11.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). St. 6, 8 and 9 (253, 407, and 770 m) showed a second but smaller maximum of sulfate reduction activity. At St. 6 (253 m), this second maximum was situated at 20.5 cmbsf ( $6.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). St. 8 and 9 (407 and 770 m) showed additional maxima at 4.5 cmbsf ( $3.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) and 2.5 cmbsf ( $1.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ), respectively. At St. 9 (770 m), sulfate reduction activity was not detectable at most depth  $> 10$  cmbsf. At St.10 (1024 m), no sulfate reduction activity was detectable throughout the entire core. At St. 9 and 10 (770 and 1024 m) we cannot exclude that sulfate reduction was present but undetectable due to long, unfrozen storage of the samples (see 2.7).

Fig. 3 shows an overview of integrated methanogenesis and sulfate reduction rates (over the upper 0-25 cm) along the depth transect on the Peruvian margin. Highest integrated surface methanogenesis activity was detected on the shelf (70, 145 and 253 m) with  $0.1 \pm 0.03 \text{ mmol m}^{-2} \text{ d}^{-1}$ ,  $0.06 \pm 0.02 \text{ mmol m}^{-2} \text{ d}^{-1}$ , and  $0.07 \pm 0.01 \text{ mmol m}^{-2} \text{ d}^{-1}$ , respectively. St. 8 (407 m) revealed the lowest integrated methanogenesis rate of all sites ( $0.02 \pm 0.00 \text{ mmol m}^{-2} \text{ d}^{-1}$ ). St. 9 (770 m) and St. 10 (1024 m) showed integrated methanogenesis activity around  $0.03 \pm 0.02 \text{ mmol m}^{-2} \text{ d}^{-1}$ , respectively.

Integrated sulfate reduction activity decreased along the continental margin with increasing water depth, revealing the highest activity at the St. 1 (70 m,  $4.7 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) and the lowest activity at St. 9 (770 m,  $0.2 \text{ mmol m}^{-2} \text{ d}^{-1}$ ). Please note again, that integrated sulfate reduction rates are probably underestimated due to long, unfrozen storage of the samples (see 2.7).

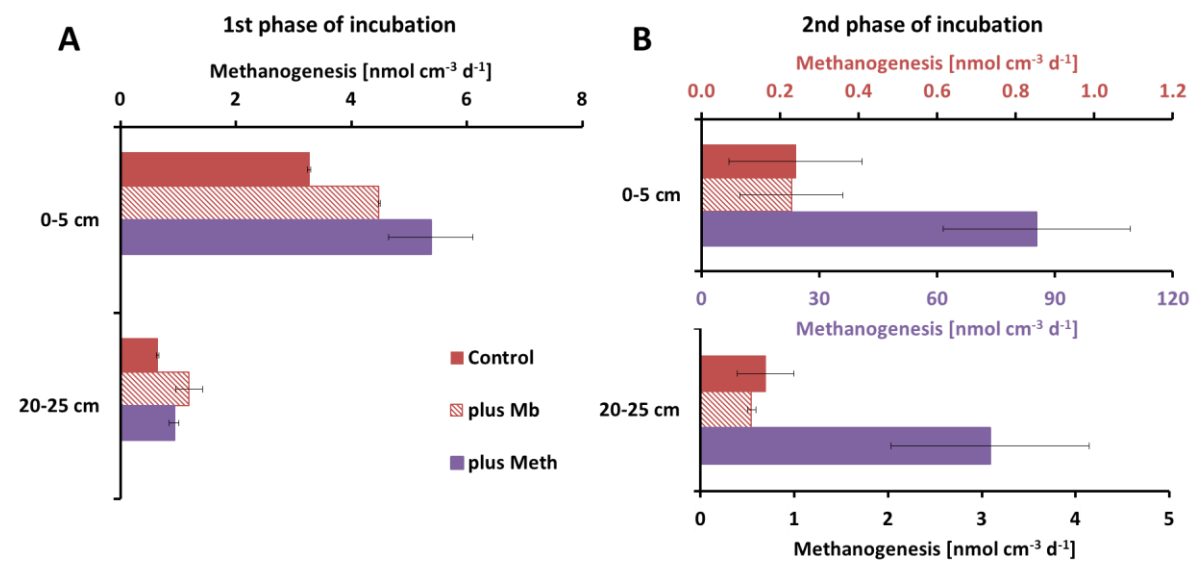


**Figure 3.** Integrated methanogenesis and sulfate reduction rates (0-25 cm) along the depth transect. For methanogenesis rates (black bars), average values are shown with standard deviation. Note for St. 10 a mean from two replicates is shown without standard deviation (pattern-filled bar) and the outlier is shown separately (cross). For sulfate reduction rates (blue bars), means from two replicates are shown without standard deviation.

### Potential competitive and non-competitive methanogenesis in sediment slurries from MUC cores

Results from the sediment slurry experiments, in which we added either the sulfate reduction inhibitor molybdate, the non-competitive substrate methanol, or no additives (control), are shown in Fig. 4. During the first phase of incubation, all three treatments showed rates within the same order of magnitude. Nevertheless, potential methanogenesis rates were significantly higher ( $p < 0.05$ ) in all treatments in the shallow sediment horizon (0-5 cmbsf) compared to the deep horizon (20-25 cmbsf). In addition, potential methanogenesis was always significantly higher in the molybdate and methanol treatment compared to the control.

During the second phase of the incubation (day 8-23), potential methanogenesis showed a different pattern. Rates in the methanol treatment were 350 and 4 times higher compared to the control and molybdate treatment in the 0-5 cm horizon and the 20-25 cm horizon, respectively ( $p < 0.05$ ). Control and molybdate treatments showed no significant difference ( $p > 0.05$ ) in the shallow and deep horizon.



**Figure 4:** Potential methanogenesis rates in sediment slurry experiments from the two sediment intervals (0-5 cm and 20-25 cm) at St. 1 (70 m). The first phase of the incubation shows rates calculated from day 0 to 5 (A), while the second phase of the incubation summarizes the rates from day 8-23 (B). "Control" is the treatment with sulfate-rich (28 mM) artificial seawater medium, "plus Mb" is the treatment with sulfate-rich artificial seawater medium plus molbydate (Mb, 22mM), and "plus Meth" is defined as the treatment with sulfate-rich artificial seawater medium plus methanol (Meth, 10 mM). Per treatment, average values are shown with standard deviation. Please note the split-up in the diagram in part B and the different x-axis for methanogenesis.



### **Geochemical parameters and gross hydrogenotrophic methanogenesis activity in gravity cores**

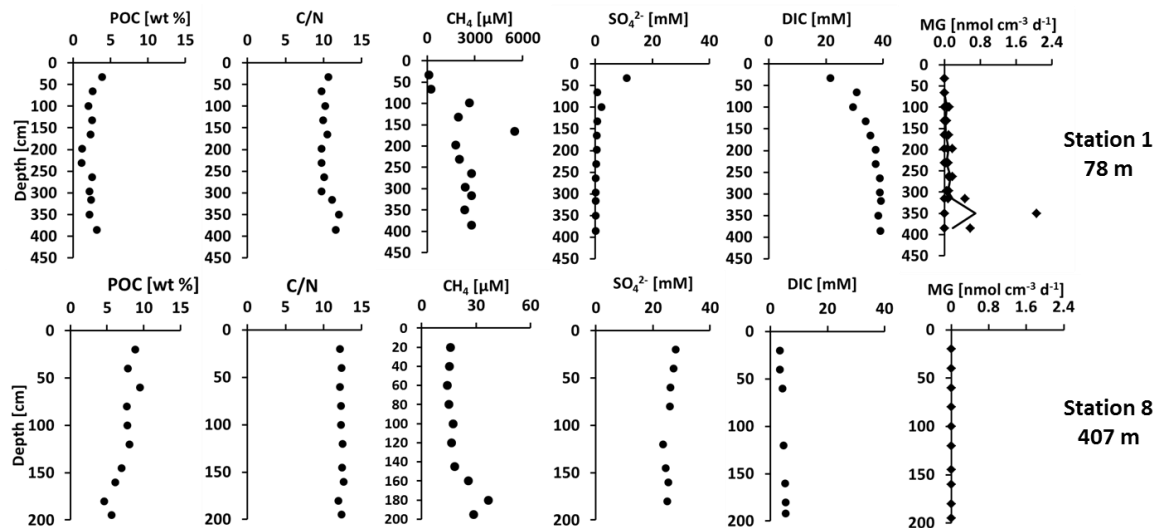
At the shallow St. 1 (78 m), POC concentration slightly decreased with depth, from ~4 wt % at the surface to about 2-3 wt % at the bottom of the core (385 cmbsf, Fig. 5). At St. 8 (407 m), POC concentrations were slightly higher with values ranging around 8-9 wt % in the upper 120 cmbsf, and then decreasing with depth. The C/N ratio at St. 1 (78 m) remained around 10 throughout the core, while it showed slightly higher values around 12 throughout the core at St. 8 (407 m).

At St. 1 (78 m), the methane concentration increased with depth from 0.1 mM at the surface to the highest measured concentration at 165 cmbsf (~5 mM), followed by a decrease to ~ 2 mM at 198 cmbsf. Methane concentration stayed around 2 mM until the deepest measured depth (385 cmbsf). Methane concentrations at St. 8 (407 m) ranged from 14 to 17  $\mu\text{M}$  in the upper 120 cmbsf, then increased to a maximum of 36  $\mu\text{M}$  at 180 cmbsf, followed by a decrease to 28  $\mu\text{M}$  at the deepest sampled depth (195 cmbsf).

$\text{SO}_4^{2-}$  concentration at St. 1 (78 m) decreased with depth with the highest concentration (10 mM) at the shallowest measured sediment depth (33 cmbsf) and the lowest concentration at 350 cmbsf (0.16 mM). At St. 8 (407 m),  $\text{SO}_4^{2-}$  concentration decreased slightly from ~28 mM at the shallowest measured sediment depth (20 cmbsf) to ~24 mM at 145 cmbsf, followed by stable concentrations around 25 mM until the bottom of the core.

DIC concentrations were 5-8 times higher at St. 1 (78 m) compared to St. 8 (407 m) and increased with sediment depth from ~21 mM at 33 cmbsf to ~39 mM at 385 cmbsf. DIC concentrations at St. 8 (407 m) could only be measured at distinct sediment depths due to limited amounts of porewater but still revealed a slight increase with sediment depth (from ~3 mM to ~5 mM).

Hydrogenotrophic methanogenesis at St. 1 (78 m) was present but low below 66 cmbsf until it reached a peak between 300 and 400 cmbsf ( $0.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). In contrast, no hydrogenotrophic methanogenesis activity was detected at St. 8 (407 m).



**Figure 5:** Profiles of particulate organic carbon (POC), C/N ratio, methane (CH<sub>4</sub>), sulfate (SO<sub>4</sub><sup>2-</sup>), dissolved inorganic carbon (DIC), and hydrogenotrophic methanogenesis (MG) rates in the gravity cores at two stations within the depth transect. For MG, triplicates (symbols) and mean (solid line) are shown.

## Discussion

### Concurrent activity of methanogenesis and sulfate reduction in surface sediments

Before we discuss the distribution of methanogenesis in the collected sediment cores, it has to be pointed out that the top soft sediment layer (ca. 0-20 cm) of gravity cores is often disturbed or even lost during the coring procedure. Hence, surface parameters in the gravity cores should not be directly compared to the respective depth layers in MUC cores. According to this likely offset, we will use the term "surface methanogenesis/sediments" when referring to MUC cores and "deep methanogenesis/sediments" when referring to gravity cores.

In the present study, methanogenesis and sulfate reduction concurred in surface sediments along the entire depth transect (70-1024 m) on the Peruvian margin (12°S). Methanogenesis activity was detected in sediment layers that revealed high porewater sulfate concentrations and sulfate reduction activity (besides St. 10, where sulfate reduction was undetectable). Even though absolute sulfate reduction rates were most likely underestimated, we trust relative distribution pattern in the sediment and along the continental margin.

As the competition between methanogens and sulfate reducers for H<sub>2</sub> and acetate was probably never relieved, the detected surface methanogenesis was most likely based on non-competitive substrates such as methanol or methylated compounds including methylated amines or methylated sulfides (Oremland & Polcin, 1982; Oremland & Taylor, 1978; Kiene et al., 1986). Likewise, in a study off Chile (0-6 cm sediment depth, 87 m water depth), surface methanogenesis was found to be coupled to the

non-competitive substrate trimethylamine, and not to  $\text{CO}_2/\text{H}_2$  or acetate, in sediments where sulfate and sulfate reduction was abundant (Ferdelman et al., 1997).

Non-competitive substrate utilization by methanogens in the present study was further confirmed by a significant increase of potential methanogenesis after the addition of methanol to sediment slurries from St. 1 (70 m) (Fig. 4 B). The delayed response of methanogenesis after methanol addition (Supplementary Material, Fig. S1), however, suggests that the present microbial methanogenic community was not primarily feeding on methanol. Potentially other non-competitive substrates like dimethyl sulfides were utilized predominantly. While most methylotrophic methanogens are able to use both methanol and methylated amines, growth on dimethyl sulfide appears to be restricted to only a few methylotrophic species (Oremland et al., 1989). Dimethyl sulfides could have built up during the long storage time (~ 6 months) before experimentation. Even though methylated sulfur compounds (e.g., dimethyl sulfide or methanthiol) are mainly produced by organisms in the marine photic zone (e.g., Andreae & Raemdonck 1983), it was recently postulated that these compounds may also be generated through nucleophilic attack by sulfide on methyl groups in the sedimentary organic matter (Mitterer, 2010). As sulfate reduction was a predominant process in the sediment, it could have delivered sufficient sulfide to produce methylated sulfur compounds. Consequently, results from the sediment slurry experiments might not reflect the activity of the in situ methanogenic community as we cannot exclude community shifts as a response to the availability of alternative substrates that were produced during the long storage.

The utilization of the competitive substrates  $\text{H}_2$  and acetate by the methanogens occurs probably only when sulfate reducers are inhibited. Accordingly, potential methanogenesis rates in the molybdate treatment of the sediment slurry experiment were significantly higher in the two studied horizons (0-5 and 20-25 cmbsf) compared to the controls during the first phase of the incubation (day 0-5), indicating the usage of competitive substrate facilitated by the inhibition of sulfate reduction. However, in the second phase (day 8-23) of the incubation, rates were much lower in both the control and molybdate treatment and did not show significant differences in both horizons ( $p > 0.05$ ). In this second phase, methane production might have slowed down due to depletion of electron donors.

Hydrogenotrophic methanogenesis in the gravity core from St. 1 (78 m) showed no activity at depths where porewater sulfate concentrations were  $> 0.7$  mM. Instead activity peaked where porewater sulfate was lowest (0.16 mM at 350 cmbsf), supporting the above conclusions regarding competition within the sulfate zone. The observation that sulfate was never completely depleted in the porewater until the bottom of the gravity core, in combination with an increase of iron (II) in the porewater at depths  $> 200$  cmbsf (data not shown), hint to the presence of a cryptic sulfur cycle that is responsible for deep formation of sulfate (Holmkvist et al., 2011; Treude et al., 2014).

In comparison, surface net methanogenesis activity along the Peruvian margin was similar to activity found off Chile at 87 m water depth ( $0-0.6 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) (Ferdelman et al., 1997). The slightly higher rates determined in our study (St.1= 70 m;  $0.4-1.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) could be related to different approaches, as our rates represent the sum of net methanogenesis from all available substrates in the sediment, while rates off Chile were based only on  $\text{CO}_2$ , acetate, and trimethylamine utilization. Hence, total methanogenesis could have been easily underestimated, if methanogenesis was supplied by other substrates, which is not unlikely, as methylotrophic methanogens, which are able to use methanol or methylated amines, were the dominant type of methanogens in these sediments (Ferdelman et al., 1997). Interestingly, the authors detected a high number of acetogens, implicating that acetogenesis competed for methylamines or other methylated compounds (Ferdelman et al., 1997). A competition with acetogens for methylated substrates is conceivable for our study, but would require further studies.

### **Surface vs deep methanogenesis**

Maximum single net surface methanogenesis activities detected in our study ( $0.3-4.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) were found to be at the very low end of or even one order of magnitude lower than organic-rich, sulfate-depleted sediments ( $9.8-37 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 0-40 cmbsf, Treude et al., 2005a,  $10-17 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 0-30 cmbsf, Schmaljohann 1996,  $100-300 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 0-30 cmbsf, Crill & Martens, 1983, 1986,  $100-400 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , 0-3 cmbsf, Alperin et al. 1992). To estimate the overall relevance of surface methanogenesis within the sulfate zone compared to deep methane production, we estimated the deep methane production in our study and compiled an overview of published deep methane production data from the sulfate-free zone of organic-rich sediments (Table 2). For this comparison, the deep methane production was assumed to equal the flux of methane into the sulfate-methane-transition zone (SMTZ), where it is consumed by anaerobic oxidation of methane (AOM). Within the SMTZ, both sulfate and methane are depleted steeply as a result of AOM, thus dividing the sulfate-reducing zone above from the methanogenic zone below. The SMTZ is the main niche for AOM in marine sediments, acting as an important filter for upwards migrating methane (Knittel & Boetius, 2009). The SMTZ can be found at decimeters to tens of meters below the seafloor, depending on the burial rate of reactive organic matter, the depth of the methane production zone, and the transport velocity of methane and sulfate as well as their consumption rates (Knittel & Boetius, 2009).

In the present study, a SMTZ was only detected in the gravity core taken at St. 1 (78 m; Fig. 5), where it was located between 66 and 99 cmbsf, i.e., below the penetration depth of the MUC cores. We estimated a methane flux (= deep methane production) into the SMTZ (from 99 to 66 cmbsf) according to Iversen & Jørgensen, (1993) using a seawater methane-diffusion coefficient from Schulz, (2006) which was corrected for porosity resulting in a sediment-diffusion coefficient for methane of  $D_s=$

$1.325 \cdot 10^{-5} \text{ cm}^{-2} \text{ s}^{-1}$  at  $15 \text{ }^\circ\text{C}$ . The resulting deep methane production ( $0.8 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) was slightly higher (ratio of 0.13, surface vs. deep) but still in the same magnitude as the integrated surface methanogenesis at St. 1 (70 m;  $0.1 \text{ mmol m}^{-2} \text{ d}^{-1}$ ). Compared to a different study from the Peruvian OMZ, the ratio between shallow ( $0.07$  to  $0.1 \text{ mmol m}^{-2} \text{ d}^{-1}$ , this study) vs. deep ( $8.9 \cdot 10^{-8}$  to  $2.2 \cdot 10^{-7} \text{ mmol m}^{-2} \text{ d}^{-1}$ ; Arning et al., 2012) methanogenesis on the shelf (150-250 m) was  $3.2 \cdot 10^5$  to  $1.1 \cdot 10^6$ . Both examples highlight the significance of surface methanogenesis, especially on the Peruvian shelf. On the lower Peruvian slope ( $\sim 3800 \text{ m}$  water depth), deep methanogenesis increased (up to  $0.017 \text{ mmol m}^{-2} \text{ d}^{-1}$ ; Arning et al., 2012). In contrast, surface methanogenesis at the deeper St. 10 (1024 m) was lower ( $0.02 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) compared to the shelf indicating a decreasing relevance of surface methanogenesis along the margin with increasing relevance of deep methanogenesis. The decrease of surface methanogenesis with increasing water depth might be correlated to the decreasing organic carbon content and freshness in the sediment (Fig. 6), which will be further discussed in section “Factors controlling methanogenesis along the Peruvian margin”.

**Table 2:** Comparison of deep methanogenesis in organic-rich sediments from different regions with surface methanogenesis ( $0.02$ - $0.1 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) determined in the present study. The ratio range was achieved by dividing the lowest surface by the highest deep and the highest surface by the lowest deep methanogenic activity, respectively.

	Water Depth (m)	Depth of SMTZ (mbsf)	Methane flux into the SMTZ = integrated deep methanogenesis ( $\text{mmol m}^{-2} \text{ d}^{-1}$ )	Ratio between surface methanogenesis (present study) and deep methanogenesis	Reference
Namibia (SE Atlantic)	1312- 2060	3-10	0.07-0.15	0.13-1.43	Niewöhner et al., (1998)
Eckernförde Bay (SW Baltic Sea)	25-28	0.5-1.5	0.66-1.88	0.01-0.15	Treude et al., (2005a)
Chile (SE Pacific)	797-2746	3-4	0.068-0.13	0.15-1.47	Treude et al., (2005b)
Limfjorden (North Sea)	7-10	1-1.5	0.076	0.03-1.32	Jørgensen & Parkes, (2010)
Peru (SE Pacific)	150-3819	2-50	$2.2 \cdot 10^{-7}$ - $0.017$	$1.18$ - $4.55 \cdot 10^5$	Arning et al., (2012)
Peru (SE Pacific)	70-1024	0.7-1	0.8	0.03-0.13	present study

In comparison with other organic-rich sediments (Table 2), surface methanogenesis off Peru was in the same order of magnitude as most reported deep methanogenesis (e.g., off Namibia, off Chile, Limfjorden). The only exemption was Eckernförde Bay (Baltic Sea), where surface methanogenesis off Peru was less than 15% of deep methanogenesis. Eckernförde Bay has a water depth of only ~30 m with high carbon export, featuring extremely high methanogenesis activity below the SMTZ, causing supersaturation and methane gas ebullition (Whiticar, 2002; Treude et al., 2005a).

### **Potential consumption and emission of surface methane**

Due to its closeness to the sediment-water interface, surface methanogenesis along the Peruvian margin could lead to methane emissions from the sediment into the water column. A short diffusion distance, especially in the top most sediment layers, might facilitate a partial escape of methane from consumption by microbes. As surface methanogenesis decreased with water depth (Fig. 3), the methane emission potential appears to be highest on the shelf. Sediment methane concentrations in the 0-2 sediment horizon of all sites along the margin were always higher than bottom-near water methane concentrations (~1.5 m above seafloor; Table 1, Fig. 2), hinting towards an efflux of methane from the sediment. However, more precise profiling of methane at the sediment-water interface would be necessary to confirm this hypothesis. Still, most of the sediment methane profiles suggest methane consumption close to the seafloor to some extent, which would reduce the amount of emitted methane (Fig. 2). AOM might act as an important methane filter at the sediment surface of the shelf stations, where anoxic conditions dominated, while aerobic oxidation might prevail at the deeper stations below the OMZ (St. 9 and 10). The presence of methane oxidation above the SMTZ of organic-rich sediments has been reported earlier (Treude et al., 2005a, 2005b), and could indeed be fueled by surface methanogenesis. An immediate oxidation of the produced methane would explain why sediment methane profiles did not necessarily correlate with peaks in surface methanogenesis (see, e.g., St 6, 253 m). The importance of AOM for the reduction of methane emissions from surface methanogenesis remains speculative, as explicit data is missing. On the basis of our findings, however, we suggest to consider surface methanogenesis as a possible driver for AOM above the SMTZ in earlier and future studies.

### **Factors controlling methanogenesis along the Peruvian margin**

For this discussion we excluded the high integrated methane production observed in one of the replicates at station 10 (1024 m), as we do not think that the detected activity ( $0.23 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) is representative for this deep site, especially as sediment POC content was lowest at station 10 compared to the other stations (<4%, Fig. 2). The outlier might have been caused by additional carbon sources in the sediment, e.g., from fecal pellets or organic carbon released from dead infauna, thus

stimulating below-surface microbial activities during our incubations (Ziervogel et al., 2014; Bertics et al., 2013).

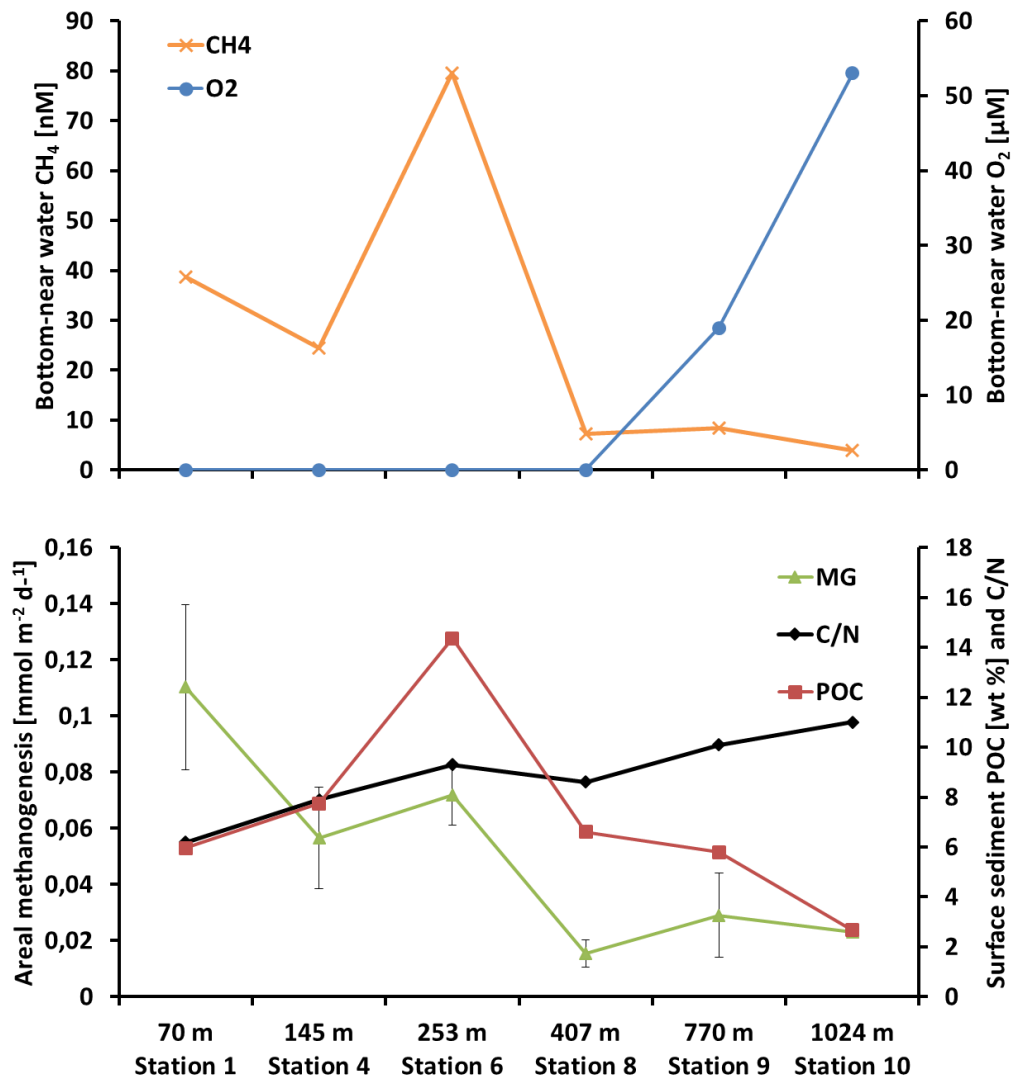
### **Oxygen**

Oxygen is an important controlling factor, as methanogenesis is an oxygen- and redox-sensitive process (Oremland, 1988). Some methanogens can tolerate oxygen exposure for several hours before they die, however, no methane will be produced in the presence of oxygen (Zinder, 1993).

Comparing integrated surface methanogenesis (over 0-25 cmbsf) from the shallowest to the deepest station (Fig. 3), highest rates ( $> 0.05 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) were detected on the shelf (St. 1, 4 and 6=70, 145, 253 m), where oxygen concentrations were below detection (Fig.6), providing advantageous conditions for methanogenesis, particularly at the very sediment surface, where normally aerobic respiration dominates (Jørgensen, 2006). Below the OMZ, integrated methanogenesis decreased. Bioturbating macrofauna and megafauna (e.g., mussels, polychaetes, oligochaetes) were observed at these sites (St. 9 and 10, 770 and 1024 m) (Mosch et al. 2012), which could have transported oxygen into deeper sediment layer (Orsi et al., 1996), thus leading to less reduced conditions ( $> -200 \text{ mV}$ ) unsuitable for methanogens (Oremland, 1988). However, integrated methanogenesis was lowest at St. 8 (407 m), which still revealed anoxic bottom water. Thus, oxygen might just be advantageous but not the driving factor for surface methanogenesis.

### **Organic matter**

The probably most important factor controlling benthic methanogenesis activity is the POC content of the sediment, as it determines the substrate availability and variety, and can thus relieve the competitive situation between methanogens and sulfate reducers (Holmer & Kristensen, 1994; Treude et al., 2009). Hence, we would expect high methanogenesis at sites with high organic carbon load along the Peruvian margin. Conversely, integrated methanogenesis rates are not correlating with sediment POC content (Fig. 6). While POC content was increasing from St. 1 (70 m) to St. 6 (253 m), followed by a decrease until St. 10 (1024 m), integrated methanogenesis showed rather a decreasing trend with increasing water depth. This deviation might be caused by another factor, as not only the quantity of organic matter is important for microbial degradation but also its quality, i.e., freshness. Numerous studies have shown that the quality of the organic matter is important for the rate and magnitude of microbial organic matter degradation (Westrich & Berner, 1984; Canfield, 1994; Amon et al., 2001; Middelburg, 1989).



**Figure 6:** Bottom-near water methane (CH<sub>4</sub>) and oxygen (O<sub>2</sub>) concentrations along the depth transect (above). Surface sediment particulate organic carbon (POC) content and C/N ratio together with integrated methanogenesis (MG) rates (0-25 cmbsf) along the depth transect (below). For MG rates, averages are shown with standard deviation beside St. 10, where a mean from two replicates is shown (see text). Please note the secondary y-axis.

Integrated methanogenesis and C/N ratios (indicating the freshness of organic matter) were negatively correlated along the Peruvian margin (Fig. 6), suggesting that fresh, labile organic matter is advantageous for surface methanogenesis. As methanogens consume mostly short, monomeric substrates, they depend on other microbial groups to break down large organic macromolecules (Zinder, 1993). Hence, labile organic matter offers an important supply of methanogenic substrates. In agreement with this hypothesis, highest integrated methanogenesis rates were observed at St. 1 (70 m), which revealed the freshest organic matter (lowest C/N, Fig. 6) and the highest POC remineralization rates along the Peruvian margin (Dale et al., 2015). The degradation of organic matter



within the water column was probably limited at St. 1 (70 m) due to anoxic conditions and high sedimentation rates (Dale et al., 2015); hence, labile organic matter accumulated at the seafloor, thereby increasing the benthic POC degradation and resulting in high substrate availability and variety for the methanogenic community.

Nevertheless, lowest methanogenesis was measured at St. 8 (407 m), which was neither the site of the highest C/N ratio, lowest POC content (Fig. 6), or the lowest POC mineralization (Dale et al., 2015). In this particular case, methanogenesis was most likely controlled by the sediment properties. Methanogenesis activity was undetectable below 10 cmbsf, which coincided with a very dense and sticky clay layer. The POC profile at St. 8 (407 m) revealed lower concentrations in the upper 5 cmbsf, followed by an increase with depth, suggesting that either the organic matter at this station was resistant to microbial attack (indicated by the increase in C/N) or that microbes were not as frequent/active in the dense clay layer as at the surface. Similarly, sulfate reduction and microbial nitrogen fixation (Gier et al., submitted) showed very low activity at this site (Fig. 2).

## Conclusion

The present study demonstrated that methanogenesis coincides with sulfate reduction in surface sediments (< 30 cmbsf) along the Peruvian margin. The competition with sulfate reducers was partially relieved due to the high load of organic carbon allowing both groups to show concurrent activity through the utilization of non-competitive substrates by the methanogens.

The significance of surface methanogenesis was high on the shelf, where ratios between surface and deep methanogenesis was around 0.13 (this study) or even as high as  $\sim 10^5$  (compared to Arning et al. 2012), and decreased with increasing water depth. Accordingly, we assume that potential methane emissions into the water column, indicated by a higher methane concentration at the sediment surface compared to the bottom water, should be highest on the shelf, where surface methane production rates were highest. Our results further hint towards a partial consumption of methane before reaching the sediment-water interface, probably by anaerobic oxidation of methane (AOM). In this case, surface methanogenesis might act as important supplier of methane for AOM above the SMTZ, which has been largely overseen before.

We postulate that the dominant factor controlling surface methanogenesis is the availability of (primarily labile) organic matter. The high load of organic carbon and resulting high organic carbon mineralization rates secure the supply for methanogenic substrates, especially on the shelf, which mitigates the competition between sulfate reducers and methanogens. Anoxic conditions in the overlying water might be advantageous for the oxygen-sensitive process of methanogenesis, but does not appear to primarily control benthic rates, as they change within the anoxic zones.

Interestingly, organic matter made available by bioturbating infauna (e.g., fecal pellets or dead organisms) could be an important additional factor facilitating methanogenesis in surface sediments. As shown in this study, methanogenesis rates vary strongly in bioturbated sediments below the OMZ, sometimes exceeding all other observed methanogenic rates.

Future studies should seek to (1) identify methanogens and their metabolic capabilities in surface sediments, (2) determine the direct interaction between surface methanogenesis and AOM, (3) evaluate the effect of organic matter hot spots on total benthic surface methanogenesis in organic-rich sediments.

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## Chapter 3

# **Microbial methanogenesis in the sulfate-reducing zone in sediments from Eckernförde Bay, SW Baltic Sea**

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## Abstract

The presence of methanogenesis within the sulfate-reducing zone (0-30 cmbsf=centimeter below surface) was investigated in seasonally hypoxic sediments from Eckernförde Bay, SW Baltic Sea. Water column parameters like oxygen, temperature and salinity together with porewater geochemistry and benthic methanogenesis rates were determined on a seasonal basis from March 2013 to September 2014, to investigate the effect of seasonal environmental changes on the rate and distribution of surface methanogenesis and to estimate its potential contribution to benthic methane emissions. Different experiments were conducted to determine which methanogenic pathway was mainly facilitated in the upper 0-30 cmbsf: 1) batch incubations for the determination of net methanogenesis, 2) radiotracer incubations with  $^{14}\text{C}$ -bicarbonate for the determination of hydrogenotrophic methanogenesis, 3) methanol incubations (with and without  $^{13}\text{C}$ -enriched methanol) for potential methylotrophic methanogenesis and 4) inhibition experiments (molybdate and 2-bromoethane-sulfonate) for potential methanogenesis under different competitive situations. In addition, molecular analysis were carried out after methanol addition to identify responsible microbial groups. Deep methanogenesis below 30 cmbsf was determined by  $^{14}\text{C}$ -bicarbonate radiotracer incubation in gravity cores from two selected time points (September 2013 and September 2014).

Surface methanogenesis changed seasonally in the upper 30 cmbsf with increasing rates from March ( $0.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) to November ( $1.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) 2013 and March ( $0.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) to September ( $0.4 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) 2014, respectively. Its magnitude and distribution is suggested to be mainly controlled by organic matter availability, temperature and oxygen, revealing higher rates and shallower zonation in September/November (high organic carbon load, warm temperatures and low oxygen concentrations) compared to March/June. To avoid competition with sulfate reducers, the major part of surface methanogenesis probably resulted from usage of non-competitive substrates (e.g. methanol and methylated compounds), indicated by highly enhanced methanogenesis rates after methanol addition. Accordingly, hydrogenotrophic methanogenesis increased when sulfate was depleted (sediment layers  $> 30 \text{ cmbsf}$ ). Members of the family *Methanosarcinaceae*, detected by qualitative PCR and known for methylotrophic methanogenesis, are suggested to be responsible for at least some part of the observed surface methanogenesis.

The results of the present time series study reveal a strong seasonality of surface methanogenesis and benthic methane emissions (indicated by dissolved methane concentrations), which should be included in ocean methane emission estimates. Future studies should seek into identifying the interactions between surface methane production and consumption, including the effects of predicted climate change (e.g. temperature rise) on benthic methane budgeting.

## Introduction

After water vapor and carbon dioxide, methane is the most abundant greenhouse gas in the atmosphere (Denman et al., 2007). Its atmospheric concentration increased since preindustrial times over 150 %, mainly through increased human activities such as fossil fuel usage and livestock breeding (Hartmann et al., 2013; Wuebbles & Hayhoe, 2002; Denman et al., 2007). Determining the natural and anthropogenic sources is one of the major goals for ocean, soil and atmosphere scientists to be able to predict further impacts on the world's climate. The ocean is considered to be a modest natural source for atmospheric methane (Wuebbles & Hayhoe, 2002; Reeburgh, 2007; EPA, 2010). However, research is still sparse on the origin of the observed oceanic methane, which automatically leads to uncertainties in current ocean flux estimations (Bange et al., 1994; Naqvi et al., 2010; Bakker et al., 2014).

Within the marine environment, the coastal areas are considered the major source for atmospheric methane, contributing up to 75 % to the global ocean methane production (Bange et al., 1994). The major part of the coastal methane is produced during microbial methanogenesis in the sediments, with only a minor part probably originating from methane production within the water column (Bakker et al., 2014). However, the knowledge on magnitude, seasonality and environmental controls of benthic methanogenesis is still limited.

The muddy sediments in Eckernförde Bay (SW Baltic Sea) are characterized by high organic loading and high sedimentation rates, which lead to anoxic conditions within the uppermost 0.1-0.2 centimeter below seafloor (cmbsf) (Preisler et al., 2007). In addition, seasonal hypoxic (dissolved oxygen < 63  $\mu\text{M}$ ) and even anoxic (dissolved oxygen = 0  $\mu\text{M}$ ) events occur in the bottom water (Middelburg & Levin, 2009; Hansen et al., 1999) featuring ideal conditions for anaerobic microbial processes at the sediment surface.

Sulfate reduction is the dominant pathway of organic carbon degradation in the sediment followed by methanogenesis, which is mostly restricted to the deeper sediment layers beneath the sulfate reduction zone where sulfate is depleted (> 30 cmbsf) (Whiticar 2002; Treude et al. 2005; Martens et al. 1998). The mostly deep location of methanogenesis is explained by the successful competition of sulfate reducers with methanogens for the mutual substrates hydrogen ( $\text{H}_2$ ) and acetate (Burdige, 2006; Crill & Martens, 1986; Hoehler et al., 1998; Jørgensen, 2006). This deep methanogenesis is intense and leads to methane saturation in the porewater below 50 cm sediment depth, resulting in gas bubble formation (Whiticar, 2002; Abegg & Anderson, 1997). Thus, the two processes of molecular diffusion of methane plus the advective transport of methane gas bubbles from the methanogenic zone (> 30 cmbsf) to the surface sediment are prevailing in Eckernförde Bay sediments. Although larger parts of the upward diffusing methane is retained by anaerobic oxidation of methane (AOM)

(Treude et al. 2005), a major part -most likely in form of gas bubbles- is reaching the sediment-water interface (Treude et al. 2005; Jackson et al. 1998), resulting in a supersaturation of the water column with respect to atmospheric concentrations (Bange et al., 2010). The Time Series Station "Boknis Eck" in the Eckernförde Bay is a known site of methane emissions into the atmosphere throughout the year due to this supersaturation of the water column (Bange et al., 2010).

Surface methanogenesis within the sulfate-reducing zone (< 30 cmbsf) is thought to be negligible in Eckernförde Bay (Whiticar, 2002), even though coexistence of sulfate reduction and methanogenesis is possible through the usage of non-competitive substrates (e.g. methanol or methylated compounds) by the methanogens and has been shown in organic-rich sediments like salt marshes (Oremland et al., 1982a; Buckley et al., 2008), coastal sediments (Holmer & Kristensen, 1994; Jørgensen & Parkes, 2010), and sediments in upwelling regions (Ferdelman et al., 1997; Pimenov et al., 1993; Maltby et al., submitted).

Methanogens are restricted to a number of small-size substrates (mostly containing only one C-C bond) and produce methane via three different pathways: hydrogenotrophic (based on H<sub>2</sub> and carbon dioxide (CO<sub>2</sub>)), acetoclastic (based on acetate) and methylotrophic (based on methanol or methylated compounds) methanogenesis (Zinder, 1993). The substrates used in the latter pathway (e.g. methanol, methylamines, dimethyl sulfides) cannot be metabolized by sulfate reducers (Oremland et al., 1982b; King et al., 1983), which is why they are termed non-competitive. Coexistence of sulfate reduction and methanogenesis has been postulated to occur in Eckernförde Bay sediments (Treude et al., 2005a). In this study, either the usage of non-competitive substrates, the temporal and spatial heterogeneity caused by bioturbation and gas ebullition and /or the presence of inactive methanogens, which are activated when sulfate gets depleted, is stated to relieve the competitive situation (Treude et al., 2005a).

Still, the magnitude and environmental controls of this surface methanogenesis is poorly understood in Eckernförde Bay, although it is very likely to contribute to the benthic methane emissions into the water column due to the short diffusive distance to the sediment-water interface and thus the decreased potential of being consumed microbially before escaping the sediment (Knittel & Boetius, 2009). In addition, surface methanogenesis could supply AOM in Eckernförde Bay surface sediments, which was previously attributed to methane flux from deep methanogenesis or rising gas bubbles (Treude et al., 2005a).

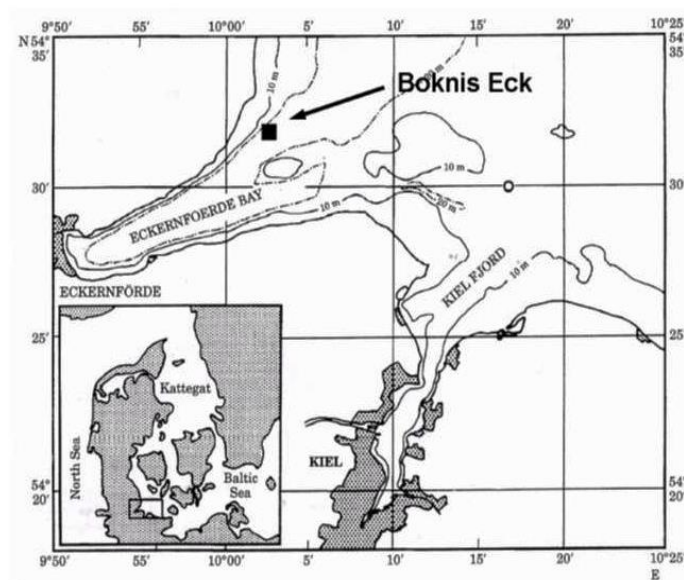
In the present study, we took sediment samples on a seasonal basis at the time series station Boknis Eck in Eckernförde Bay, to validate the existence of surface methanogenesis and to investigate its potential contribution to benthic methane emissions. Water column parameters like oxygen, temperature, salinity and density together with porewater geochemistry and benthic methanogenesis

were measured over a course of 2 years. In addition to seasonal rate measurements, inhibition and stimulation experiments, stable isotope probing, and molecular analysis were carried out to find out if surface methanogenesis 1) shows seasonal variability, 2) is controlled by environmental parameters, 3) is based on non-competitive substrates, and 4) is based on methanogenic groups that are known to consume non-competitive methanogenesis.

## Material and Methods

### Study site

Samples were taken at the Time Series Station "Boknis Eck" (BE, 54°31.15 N, 10°02.18 E; [www.bokniseck.de](http://www.bokniseck.de)) located at the entrance of Eckernförde Bay in the southwestern Baltic Sea with a water depth of about 28 m (Fig. 1). From mid of March until mid of September the water column is strongly stratified due to the inflow of saltier North Sea water and a warmer and fresher surface water (Bange et al., 2011). Organic matter degradation in the deep layers causes pronounced hypoxia (March-September) or even anoxia (August/September) (Smetacek, 1985; Smetacek et al., 1984). The source for the organic material is phytoplankton blooms, which occur regularly in spring (February-March) and fall (September-November) and are followed by pronounced sedimentation of organic matter (Bange et al., 2011). To a lesser extent, phytoplankton blooms and sedimentation are also seen during the summer months (July/August) (Smetacek et al., 1984). For more detailed descriptions of the environmental settings of Eckernförde Bay see Orsi et al., (1996), Treude et al., (2005) and Lennartz et al., (2014).



**Figure 1:** Location of Time Series Station Boknis Eck in Eckernförde Bay (Map H.P. Hansen, GEOMAR)

### Water column and sediment sampling

Sampling was done on a seasonal basis during the years of 2013 and 2014. One-day cruises with either F.S. Alkor, F.K. Littorina or F.B. Polarfuchs were conducted in March, June, and September of each year. In 2013, additional sampling was conducted in November. At each sampling time (=time point), water profiles of temperature, salinity, pressure and oxygen concentration were taken with a CTD (Hydro-Bios, Kiel, Germany) with a 6 Niskin bottle rosette attached. In addition, samples for water column methane concentration were taken at 25 m water depth (Table 1). Complementary samples for water column chlorophyll were taken within the same months during standardized monthly sampling cruises to Boknis Eck organized by GEOMAR.

Sediment cores were taken with a miniature multicorer (MUC, K.U.M. Kiel), holding 4 core liners (length= 60 cm, diameter= 10 cm) at once. The cores had an average length of ~ 30 cm and were stored at 10°C in the home laboratory until further processing (normally within few days after sampling). In September 2013 and 2014, a gravity core was taken in addition to the MUC cores with a length of 330 and 230 cm, respectively. The gravity core was equipped with a plastic (polyethylene, PE) bag (diameter: 13 cm). After retrieval, the PE bag was cut open at 12 different sampling depths resulting in intervals of 20 and 30 cm (in 2014 and 2013, respectively) and sampled directly on board for sediment porewater geochemistry (see section “Sediment porewater geochemistry”), sediment methane (see section “Sediment methane”), sediment solid phase geochemistry (see section “Sediment solid phase geochemistry”), and microbial rate measurements for hydrogenotrophic methanogenesis as described in section “Hydrogenotrophic methanogenesis in gravity cores”.

**Table 1:** Sampling time points with bottom-near water (~ 2 m above seafloor) temperature (Temp.), dissolved oxygen (O<sub>2</sub>) and dissolved methane (CH<sub>4</sub>) concentration

Time Point	Date	Temp. (°C)	O <sub>2</sub> (µM)	CH <sub>4</sub> (nM)
March 2013	13.03.2013	3	340	30
June 2013	27.06.2013	6	94	125
September 2013	25.09.2013	10	0	262*
November 2013	08.11.2013	12	163	13
March 2014	13.03.2014	4	209	41*
June 2014	08.06.2014	7	47	61
September 2014	17.09.2014	13	0	234

\*Concentrations from the standardized monthly Boknis Eck sampling cruises on 24.09.2013 and 05.03.2014

### **Water column parameters**

Concentrations of dissolved methane (CH<sub>4</sub>) were determined as described in Bange et al. (2010). In short, 25 ml glass vials were filled bubble-free and immediately poisoned with saturated mercury chloride solution (HgCl<sub>2</sub>) solution. Per depth, triplicates were sampled. After replacing 10 ml of water with helium and 2 hours equilibration time, headspace methane concentration was determined via gas chromatography-flame ionization detector. Calibration was done by a two-point calibration before the measurement of samples, resulting in an error of <5%.

Dissolved chlorophyll a concentrations were determined using the fluorometric method by Welschmeyer (1994) with an error <10%.

### **Sediment porewater geochemistry**

Porewater was extracted with nitrogen (N<sub>2</sub>) pre-flushed rhizons (0.2 µm, Rhizosphere Research Products, Seeberg-Elverfeldt et al., 2005). In MUC cores, rhizons were inserted into the sediment in 2 cm intervals through pre-drilled holes in the core liner.

Extracted porewater from MUC and gravity cores was immediately analyzed for sulfide using standardized photometric methods (Grasshoff et al., 1999).

Sulfate concentrations were determined using ion chromatography (Methrom 761). Analytical precision was < 1% based on repeated analysis of IAPSO seawater standards (dilution series) with an absolute detection limit of 1 µM corresponding to a detection limit of 30 µM for the undiluted sample. For analysis of dissolved inorganic carbon (DIC), 1.8 ml of porewater was transferred into a 2 ml glass vial, fixed with 10 µl saturated HgCl<sub>2</sub> solution and crimp sealed. DIC concentration was determined as CO<sub>2</sub> with a multi N/C 2100 analyzer (Analytik Jena) following the manufacturer's instructions. Therefore the sample was acidified with phosphoric acid and the outgassing CO<sub>2</sub> was measured. The detection limit was 20 µM with a precision of 2-3%.

### **Sediment methane**

In March 2013, June 2013 and March 2014, one MUC core was sliced in 1 cm intervals until 6 cmbsf, followed by 2 cm intervals until the end of the core. At the other time points, the MUC core was sliced in 1 cm intervals until 6 cmbsf, followed by 2 cm intervals until 10 cmbsf and 5 cm intervals until the end of the core.

Per sediment depth (in MUC and gravity cores), 2 cm<sup>-3</sup> of sediment were transferred into a 10 ml-glass vial containing 5 ml NaOH (2.5%) for determination of sediment methane concentration. The vial was quickly closed with a butyl septum, crimp-sealed and shaken thoroughly. The vials were stored upside down at room temperature until measurement via gas chromatography. Therefore, 100 µl of headspace was removed from the gas vials and injected into a Shimadzu gas chromatograph (GC-2014)

equipped with a packed Haysep-D column and a flame ionization detector. The column temperature was 80°C and the helium flow was set to 12 ml min<sup>-1</sup>. Methane concentrations were calibrated against methane standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2 %.

### **Sediment solid phase geochemistry**

Following the sampling for methane, the same cores described under section “Sediment Methane” were used for the determination for the sediment solid phase geochemistry, i.e. porosity, particulate organic carbon (POC) and particulate organic nitrogen (PON).

Sediment porosity of each sediment horizon was determined by the weight difference of 5 cc wet sediment after freeze drying for 24 hours. Dried sediment samples were then used for analysis of particulate organic carbon (POC) and particulate organic nitrogen (PON) with a Carlo-Erba element analyzer (NA 1500). The detection limit for C and N analysis was < 0.1 dry weight percent (%) with a precision of < 2 %.

### **Methanogenesis in MUC cores**

At each time point, three MUC cores were sliced in 1 cm intervals until 6 cmbsf, in 2 cm intervals until 10 cmbsf, and in 5 cm intervals until the bottom of the core. Every sediment layer was combined in a beaker and quickly homogenized before sampling. The exposure time with air, i.e. oxygen, was kept to a minimum. Sediment layers were then sampled for determination of net methanogenesis (defined as the sum of total methane production and consumption, including all available methanogenic substrates in the sediment), hydrogenotrophic methanogenesis (methanogenesis based on the substrates CO<sub>2</sub>/H<sub>2</sub>), and potential methanogenesis (methanogenesis at ideal conditions, i.e. no lack of nutrients) as described in the following sections.

#### ***Net methanogenesis***

Net methanogenesis was determined with sediment slurry experiments by measuring the headspace methane concentration over time. Per sediment layer, triplicates of 5 cc of sediment were transferred into N<sub>2</sub>-flushed sterile glass vials (30 ml) and mixed with 5 ml filtered bottom water. The slurry was repeatedly flushed with N<sub>2</sub> to remove residual methane and to ensure complete anoxia. Slurries were incubated at in-situ temperature, which varied at each sampling date (Table 1). Headspace samples (0.1 ml) were taken out every 3-4 days over a time period of 4 weeks and analyzed on a Shimadzu GC-2104 gas chromatograph (see section “Sediment methane”). Net methanogenesis rates were determined by the linear increase of the methane concentration over time (minimum of 6 time points).

### ***Hydrogenotrophic methanogenesis***

To determine hydrogenotrophic methanogenesis, radioactive sodium bicarbonate ( $\text{NaH}^{14}\text{CO}_3$ ) was added to the sediment.

Per sediment layer, sediment was sampled in triplicates with glass tubes (5 ml) which were closed with butyl rubber stoppers on both ends according to (Treude et al. 2005). Six controls were taken from various sampling depths. Through the stopper,  $\text{NaH}^{14}\text{CO}_3$  (dissolved in water, injection volume 6  $\mu\text{l}$ , activity 222 kBq, specific activity = 1.85-2.22 GBq/mmol) was injected into each sample and incubated for three days in the dark at in-situ temperature (Table 1). To stop bacterial activity, sediment was transferred into 50 ml glass-vials filled with 20 ml sodium hydroxide (2.5% w/w), closed quickly with rubber stoppers and shaken thoroughly. Five controls were produced from various sediment depths by injecting the radiotracer directly into the NaOH with sediment.

The production of  $^{14}\text{C}$ -methane was determined with the slightly modified method by Treude et al., (2005) used for the determination of anaerobic oxidation of methane. The method was identical, except no unlabeled methane was determined by gas chromatography. Instead, DIC values were used to calculate hydrogenotrophic methane production.

### ***Potential methanogenesis in manipulated experiments***

To examine the interaction between sulfate reduction and methanogenesis, inhibition and stimulation experiments were carried out. Therefore, every other sediment layer was sampled, resulting in the following examined six sediment layers: 0-1cm, 2-3 cm, 4-5 cm, 6-8 cm, 10-15 cm and 20-25 cm. From each layer, sediment slurries were prepared by mixing 5 ml sediment in a 1:1 ratio with adapted artificial seawater medium (salinity 24, Widdel & Bak, 1992) in  $\text{N}_2$ -flushed, sterile glass vials before further manipulations.

In total, four different treatments, each in triplicates, were prepared per depth: 1) sulfate (17 mM), 2) sulfate plus molybdate (22 mM), 3) sulfate plus 2-bromoethane-sulfonate (BES, 60 mM) and 4) sulfate plus methanol (10 mM). From here on, the following names are used to describe the different treatments: 1) control treatment, 2) molybdate treatment, 3) BES treatment, and 4) methanol treatment. The control treatment features the naturally occurring sulfate concentrations. Molybdate was used as an enzymatic inhibitor for sulfate reduction (Oremland & Capone, 1988) and BES was used as an inhibitor for methanogenic archaea (Hoehler et al., 1994). Methanol is a known non-competitive substrate, which is used by methanogens but not by sulfate reducers (Oremland & Polcin, 1982), thus it is suitable to examine non-competitive methanogenesis.



***Potential methanogenesis from methanol using stable isotope probing***

One additional experiment was conducted with sediments from September 2014 by adding  $^{13}\text{C}$ -labelled methanol to investigate the production of  $^{13}\text{C}$ -labelled methane. Three cores were stored at  $1^\circ\text{C}$  after the September 2014 cruise until further processing. Approximately 3.5 months after sampling, the cores were sliced in 2 cm intervals and the upper 0-2 cmbsf sediment layer of all three cores was combined in a beaker and homogenized. Then, sediment slurries were prepared by mixing  $5\text{ cm}^{-3}$  of sediment with 5 ml of artificial seawater medium in  $\text{N}_2$ -flushed, sterile glass vials (30 ml). Then, methanol was added to the slurry with a final concentration of 10 mM (see 2.7.3), but this time the methanol was enriched with  $^{13}\text{C}$ -labelled methanol in a ratio of  $^{13}\text{C}$ -labelled methanol (99.9%  $^{13}\text{C}$ ):non-labelled methanol mostly consisting of  $^{12}\text{C}$  (manufacturer:Roth) = 1:1000. In total, 54 vials were prepared for nine different sampling time points during a total incubation time of two months. At each sampling point, six vials were stopped: one set of triplicates were used for headspace methane and carbon dioxide determination and a second set of triplicates were used for porewater analysis. Headspace methane and carbon dioxide concentrations (volume 100  $\mu\text{l}$ ) were determined on a Shimadzu gas chromatograph (GC-2014) equipped with a packed Haysep-D column a flame ionization detector and a methanizer. The methanizer (reduced nickel) reduces carbon dioxide with  $\text{H}_2$  to methane at a temperature of  $400^\circ\text{C}$ . The column temperature was  $80^\circ\text{C}$  and the helium flow was set to  $12\text{ ml min}^{-1}$ . Methane concentrations were calibrated against methane standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2 %.

Analyses of  $^{13}\text{C}/^{12}\text{C}$ -ratios of methane and carbon dioxide were conducted after headspace concentration measurements by using a continuous flow combustion gas chromatograph (Trace Ultra, Thermo Scientific), which was coupled to an isotope ratio mass spectrometer (MAT253, Thermo Scientific). The isotope ratios of methane and carbon dioxide given in the common delta-notation ( $\delta^{13}\text{C}$  in ‰) are reported relative to Vienna Pee Dee Belemnite (VPDB) standard. Isotope precision was  $\pm 0.5$  ‰, when measuring near the detection limit of 10 ppm.

For porewater analysis of methanol concentration and isotope composition, each sediment slurry of the triplicates was transferred into argon-flushed 15 ml centrifuge tubes and centrifuged for 6 minutes at 4500 rpm. Then 1-ml filtered (0.2  $\mu\text{m}$ ) porewater was transferred into  $\text{N}_2$ -flushed 2 ml glass vials for methanol analysis, crimp sealed and immediately frozen at  $-20^\circ\text{C}$ . Methanol concentrations and isotope composition were determined via liquid chromatography-ion ratio mass spectrometry (LC-IRMS) at the MPI Marburg. The detection limit was 50  $\mu\text{M}$  with a precision of 0.3‰.

**Hydrogenotrophic methanogenesis in gravity cores**

In gravity cores, only hydrogenotrophic methanogenesis using  $^{14}\text{C}$ -bicarbonate was determined at every sampled sediment depth, as it is expected the main pathway in deeper sediment layers.

Therefore, the gravity cores were sampled at sediment intervals as described in 2.2 following the methods described in 2.7.2.

### **Molecular analysis**

In September 2014, additional samples were prepared for the methanol treatment of the 0-1 cmbsf horizon during the stimulation experiment described in 2.7.3 to detect and quantify the presence of methanogens in the sediment. Therefore, additional 15 vials were prepared for five different time points (day 1, day 8, day 16, day 22, and day 36) and stopped at each time point by transferring sediment from the triplicate slurries into whirl-packs (Nasco), which then were immediately frozen at -20°C. DNA was extracted from ~500 mg of sediment using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) technique with a TaqMan probe was used for the detection of methanogens. In total, the sediment was screened for 16S DNA of three orders (*Methanobacteriales*, *Methanosarcinales* and *Methanomicrobiales*) along with two families (*Methanosarcinaceae* and *Methanosaetaceae*) within the order *Methanosarcinales*. In addition, a primer set for detection of the domain *Archaea* was used. Primer and probe sets together with the qPCR conditions were used according to (Yu et al., 2005).

## **Results**

### **Water column parameters**

From March 2013 to September 2014, the water column had a pronounced temporal and spatial variability of temperature, salinity, and oxygen (Fig. 2 and 3). In 2013, temperature of the upper water column increased from March (2°C) to September (16°C), but decreased again in November (11°C). In 2014, lowest temperatures of the upper water column were reached in March (4°C), and warmer temperatures around 17°C were observed in June and September. Salinity also increased over time during 2013, showing the highest salinity of the upper and lower water column in November (18 and 23 PSU, respectively). In 2014, salinity of the upper water column was highest in March and September (both 17 PSU), and lowest in June (13 PSU). The salinity of the lower water column increased from March 2014 (21 PSU) to September 2014 (25 PSU). In both years, June and September showed the most pronounced change of temperature and salinity with depth, supporting the fact of summer stratification with a pycnocline formed at around ~14 m water depth. Summer stratification was also seen in the oxygen profiles, which showed oxygen depleted conditions ( $O_2 = < 200 \mu\text{M}$ ) in the lower water column from June to September in both years, reaching anoxia ( $O_2 = 0 \mu\text{M}$ ) in September of both years (Fig. 2+3). The water column was completely ventilated, i.e. homogenized, in March of both years with  $O_2$  concentrations of 300-400  $\mu\text{M}$  down to the sea floor.

### **Sediment geochemistry in MUC cores**

Sediment porewater and solid phase geochemistry results for the years 2013 and 2014 are shown in Fig. 2 and 3, respectively.

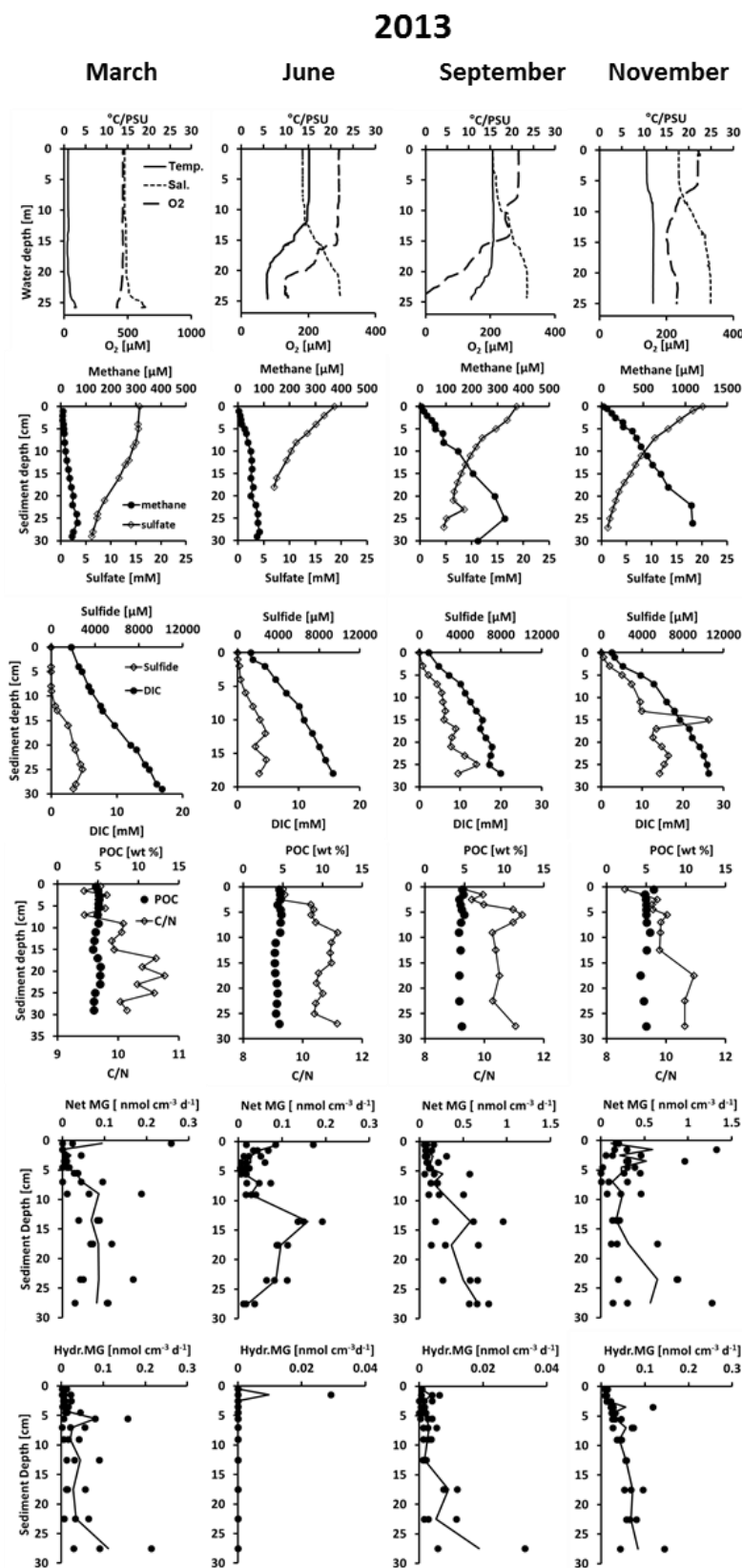
Sulfate concentration decreased with depth at all time points but was never fully depleted until the bottom of the core (between 2 and 7 mM). Sulfate concentrations at the sediment surface ranged between 15-20 mM. November 2013 showed the strongest decrease of sulfate with depth, from ~20 mM at the top to ~2 mM at the bottom of the core.

Opposite to sulfate, methane concentration increased with sediment depth throughout 2013 and 2014 (Fig. 2+3). Over the course of a year (that is: March to November in 2013, and March to September in 2014), maximum methane concentrations increased, reaching the highest measured concentrations in November 2013 (~ 1 mM) and September 2014 (0.2 mM), respectively. In addition, methane profiles became steeper over the course of a year, revealing higher methane concentrations at shallower sediment depth. Magnitudes of methane concentrations were similar in the respective months of 2013 and 2014.

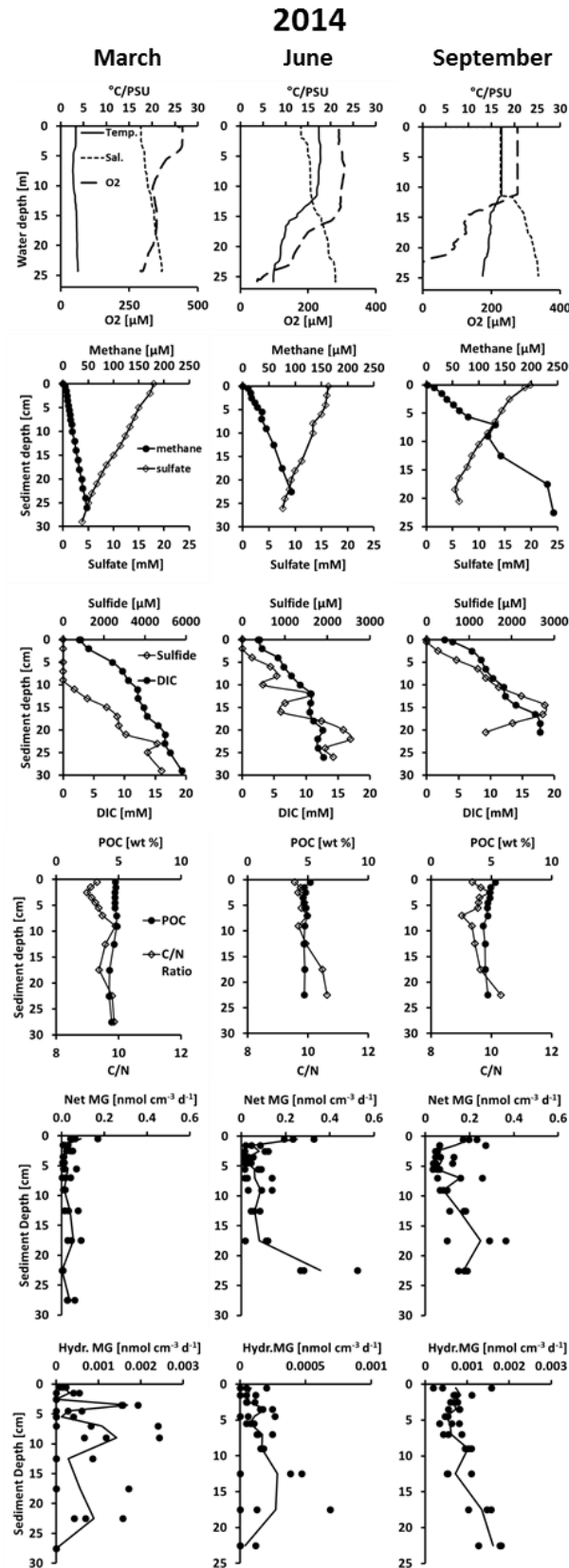
At all time points, sulfide concentration increased with increasing sediment depth. Similar to methane, sulfide profiles revealed higher sulfide concentrations at shallower sediment depth together with increasing maximum sulfide concentrations over the course of a year. Accordingly, November 2013 (~12 mM at 15 cmbsf) and in September 2014 (2.8 mM at 15 cmbsf) revealed the highest measured sulfide concentrations, respectively. September 2014 was the only time point showing a pronounced decrease in sulfide concentration from 15 cmbsf to 21 cmbsf of over 50%.

DIC concentrations increased with increasing sediment depth at all time points. Concomitant with highest sulfide concentrations, highest DIC concentration were measured in November 2013 (26 mM at 27 cmbsf). At the surface, DIC concentrations ranged between 2-3 mM at all time points. In June of both years, DIC concentrations were lowest at the deepest sampled depth compared to the other time points (16 mM in 2013, 13 mM in 2014).

At all time points, POC profiles were around 5 wt % with depth. Only in November 2013, June 2014 and September 2014, POC content exceeded 5 wt % in the upper 0-1 cmbsf (5.9, 5.2 and 5.3 wt %, respectively) with the highest POC content in November 2013. Also in November 2013, surface C/N ratio was lowest of all time points (8.6). In general, C/N ratio increased with depth in both years with values around 9 at the surface and values around 10-11 at the deepest sampled sediment depths.



**Figure 2:** Parameters measured in the water column and sediment at each time point in the year 2013. Rates for net methanogenesis (MG) and hydrogenotrophic (hydr.) MG are shown in triplicate values with the mean (solid line).



**Figure 3:** Parameters measured in the water column and sediment at each time point in the year 2014. Rates for net methanogenesis (MG) and hydrogenotrophic (hydr.) MG are shown in triplicate values with the mean (solid line).

### **Sediment geochemistry in gravity cores**

Results from sediment porewater and solid phase geochemistry in gravity cores are shown in Fig. 4. Please note that usually the upper 20-30 cmbsf of the gravity core get lost during the coring procedure, thus no direct comparison of surface parameters in the gravity cores to the respective depth layers in MUC cores should be done. This off-set in the gravity cores can be clearly seen when comparing the sulfate concentrations and respective sediment depths of MUC (Fig. 2+3) and gravity cores (Fig. 4).

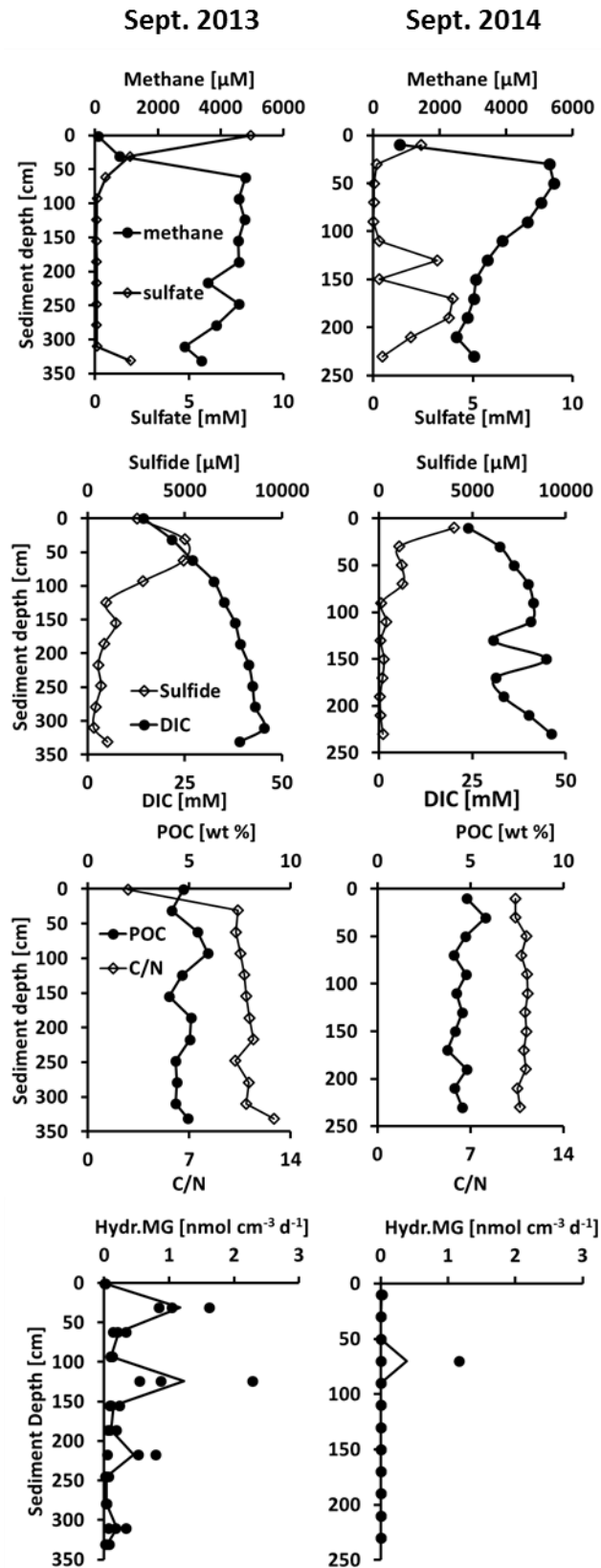
Sulfate concentration decreased with depth in both 2013 and 2014. In September 2013, sulfate concentration decreased below 0.1 mM at 93 cmbsf and stayed below 0.1 mM until 310 cmbsf. At the deepest depth, sulfate increased slightly (1.9 mM), which could result from sulfate formation from sulfide under oxic conditions (Cline, 1969) due to the slowly diffusion of oxygen into the core during sampling on board. In September 2014, sulfate concentrations were highest at the surface (2.4 mM at 10 cmbsf) and decreased to below 0.1 mM at 50 cmbsf. The re-appearance of considerable sulfate concentrations at sediment depths > 110 cmbsf is not regarded as a natural feature but was most likely caused by wave-overwashing during sampling on deck. As wave-overwashing only affected the deeper sediment parts of the core, porewater results in September 2014 at depths > 110 cmbsf have to be treated with caution.

Methane concentration increased steeply with depth reaching a maximum of 4.8 mM at 62 cmbsf. Concentration stayed around 4.7 mM until 248 cmbsf, followed by a slight decrease until the bottom of the core (3.4 mM). In September 2014, methane concentration increased steeply from 10 to 50 cmbsf (from 0.8 to 5.4 mM). Below 50 cmbsf, methane concentration decreased with increasing sediment depth to ~3 mM at the bottom of the core.

In September 2013, sulfide concentrations increased from the surface to a maximum at 31 cmbsf (~5 mM), followed by a sulfide decrease until 330 cmbsf (~1 mM). In September 2014, sulfide decreased with depth, showing the highest concentration at the surface (~4 mM at 10 cmbsf) and concentrations of 0.1-0.2 mM at the bottom of the core.

DIC concentrations increased with depth in both years, revealing a maximum at 310 cmbsf (45 mM) in 2013 and at 230 cmbsf (46 mM) in 2014.

POC concentrations varied around 5 wt % throughout the cores in both years. While C/N ratio slightly increased with depth in 2013, revealing the lowest ratio at the surface (~3) and the highest ratio at the bottom of the core (~13), the C/N ratio in 2014 showed a stable profile around 10 throughout the core.



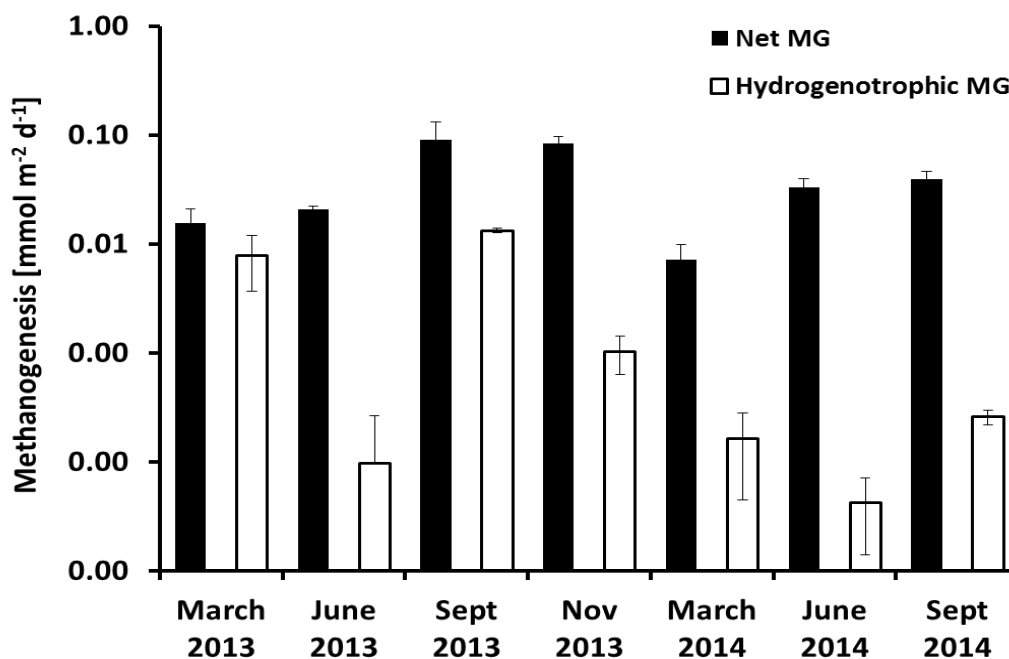
**Figure 4:** Parameters measured in the sediment in the gravity cores in September 2013 and September 2014. Rates for hydrogenotrophic methanogenesis (Hydr.MG) are shown in triplicates per depth with the mean as a solid line.

## Methanogenesis activity in MUC cores

### *Net methanogenesis*

Net methanogenesis activity was detected throughout the cores at all time points (Fig. 2+3). The activity increased over the course of the year in 2013 and 2014 (that is: March to November in 2013 and March to September in 2014) with lower rates mostly  $< 0.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$  in March and higher rates  $> 0.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$  in November 2013 and September 2014, respectively. In general, November 2013 revealed highest measured net methanogenesis rates ( $1.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$  at 1-2 cmbsf). Peak rates were detected at the sediment surface (0-1 cmbsf) at all time points except for September 2013 where the maximum rates were situated between 10-15 cmbsf. In addition to the surface peaks, net methanogenesis showed also subsurface (=below 1 cmbsf until 30 cmbsf) maxima at all time points, but with altering sediment depth. In March 2013, the subsurface maximum was found at  $\sim 9$  cmbsf, while in June and September 2013 it was situated between 10 -15 cmbsf. In November 2013 and June 2014, the subsurface maximum was even found at 20-25 cmbsf. In March and September 2014, subsurface maximum rates were found between 15-20 cmbsf.

Comparison of net integrated methanogenesis rates (0-25 cmbsf) over the course of the whole sampling period revealed highest rates in September and November 2013 and lowest rates in March 2014 (Fig. 5). Excluding November, both years showed a trend of increasing areal net methanogenesis rates from March to September.



**Figure 5:** Integrated net methanogenesis (MG) rates and hydrogenotrophic MG rates (0-25 cmbsf) for each time point. Please note the logarithmic scale of the y-axis.



**Hydrogenotrophic methanogenesis**

Hydrogenotrophic methanogenesis rates from  $^{14}\text{CO}_2$  plus  $\text{H}_2$  are shown in Fig. 2+3. In 2013, maximum hydrogenotrophic methanogenesis ranged between 0.03-0.2  $\text{nmol cm}^{-3} \text{d}^{-1}$ , while in 2014, maximum hydrogenotrophic methanogenesis was even lower ranging from  $7 \cdot 10^{-4}$  to  $2 \cdot 10^{-3} \text{nmol cm}^{-3} \text{d}^{-1}$ . In comparison, maximum net methanogenesis ranged between 0.2-1.3 in 2013 and between 0.2-0.5 in 2014, showing that maximum hydrogenotrophic methanogenesis was up to 700 times lower than net methanogenesis. Only in March 2013 magnitudes of hydrogenotrophic methanogenesis and net methanogenesis were similar.

Overall, hydrogenotrophic methanogenesis rates increased with depth in March, September, and November 2013 and in June and September 2014. In June 2013 and March 2014, hydrogenotrophic methanogenesis rates decreased with sediment depth, showing the highest rates in the upper 0-5 cmbsf and the lowest at the deepest sampled depth.

Depths of maximum hydrogenotrophic methanogenesis rates were different to net methanogenesis and changed between time points. Maximum hydrogenotrophic methanogenesis rates appeared at 5-6 cmbsf and 25-30 cmbsf in March 2013, while in June 2013 maximum rates were found at 1-2 cmbsf. In September and November 2013 as well as September 2014, maximum rates were found at the deepest sampled depth (25-30 cmbsf and 20-25 cmbsf, respectively). The depth profile in March 2014 showed highest rates < 10 cmbsf (3-4 cmbsf and 9 cmbsf). In June 2014, peak rates were measured between 10 and 20 cmbsf.

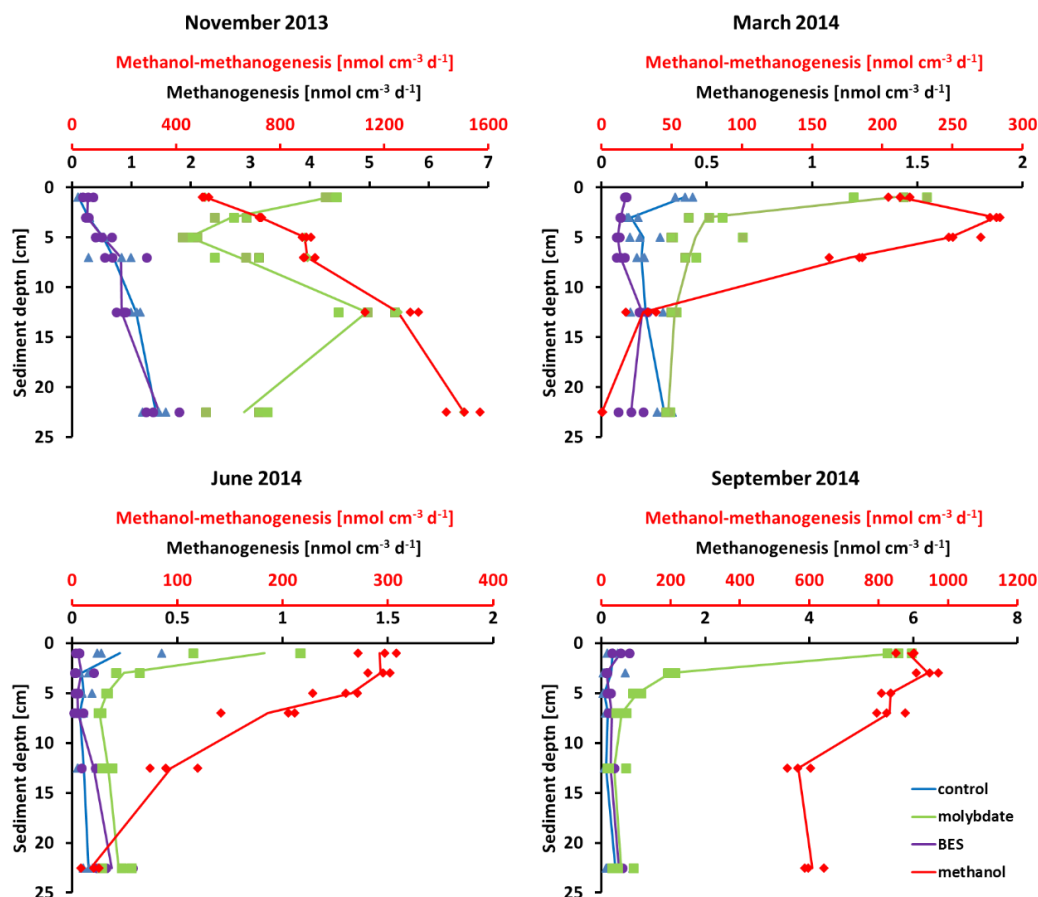
Concomitant with integrated net methanogenesis, integrated hydrogenotrophic methanogenesis rates (0-25 cmbsf) showed highest rates in September 2013, followed by March 2013 (Fig. 5). Lowest areal rates of hydrogenotrophic methanogenesis were seen in June 2014.

**Potential methanogenesis**

Potential methanogenesis rates in the control treatment were below  $0.5 \text{nmol cm}^{-3} \text{d}^{-1}$  from March 2014 to September 2014 (Fig. 6). Only in November 2013, control rates exceeded  $0.5 \text{nmol cm}^{-3} \text{d}^{-1}$  below 6 cmbsf. While rates increased with depth in November 2013 and June 2014, rates decreased with depth at the other two time points.

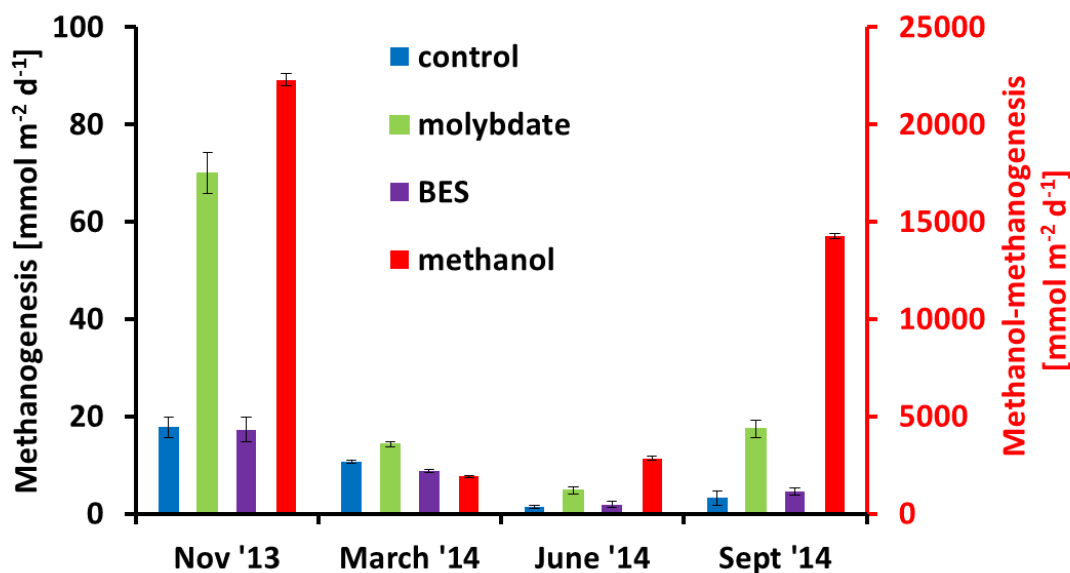
The profiles of potential methanogenesis rates in the BES treatment were similar to the control mostly in the lower range  $< 0.5 \text{nmol cm}^{-3} \text{d}^{-1}$ , showing rates  $> 0.5 \text{nmol cm}^{-3} \text{d}^{-1}$  only in November 2013. Rates increased with depth at all time points, except for September 2014, where highest rates were found at the sediment surface (0-1 cmbsf). Peak potential methanogenesis rates in the molybdate treatment were found in the uppermost sediment interval (0-1 cmbsf) at almost every time point with rates being 3-30 times higher compared to the control treatment. Only in November 2013 potential methanogenesis showed two maxima, one at 0-1 cmbsf and the highest one at 10-15 cmbsf. Highest

measured rates were found in September 2014 ( $\sim 6 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ), followed by November 2013 ( $\sim 5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ). At all time points, potential rates in the methanol treatment were 1000-3000 times higher compared to the control treatment. Except for November 2013, potential methanogenesis rates in the methanol treatments were highest in the upper 0-5 cmbsf and decreased with depth. In November 2013, highest rates were detected at the deepest sampled depth (20-25 cmbsf).



**Figure 6:** Potential methanogenesis rates of the four different treatments in November 2013, March 2014, June 2014 and September 2014. Control is describing the treatment with sediment plus artificial seawater containing natural salinity (24 PSU) and sulfate concentrations (17 mM), molybdate is the treatment with addition of molybdate (22 mM), BES is the treatment with 60 mM BES addition, and methanol is the treatment with addition of 10 mM methanol. Shown are triplicate values per depth interval and the mean as a solid line. Please note the different x-axis for the methanol treatment (red).

Over the course of the entire sampling period (November 2013 to September 2014) highest integrated rates (0-25 cmbsf, rates for every other non-sampled sediment horizon were gained by interpolation) of all four treatments were detected in November 2013 (Fig. 7). Lowest rates were detected in March and June 2014. In general, magnitudes of the integrated rates of the control treatment and the BES treatment were similar, while the rates of the molybdate treatment and the methanol treatment were up to 1 and 4 magnitudes higher compared to the control treatment.



**Figure 7:** Integrated potential methanogenesis rates (0-25 cmbsf, rates for not sampled sediment horizons were gained via interpolation) from the four different treatments. Control is describing the treatment with sediment plus artificial seawater (salinity: 24, sulfate: 17 mM), molybdate is the treatment with addition of molybdate (22 mM), BES is the treatment with 60 mM BES addition, and methanol is the treatment with addition of 10 mM methanol. Shown are mean values with standard deviation. Please note the different y-axis for the methanol treatment (red).

#### **Potential methanogenesis from <sup>13</sup>C-labelled methanol**

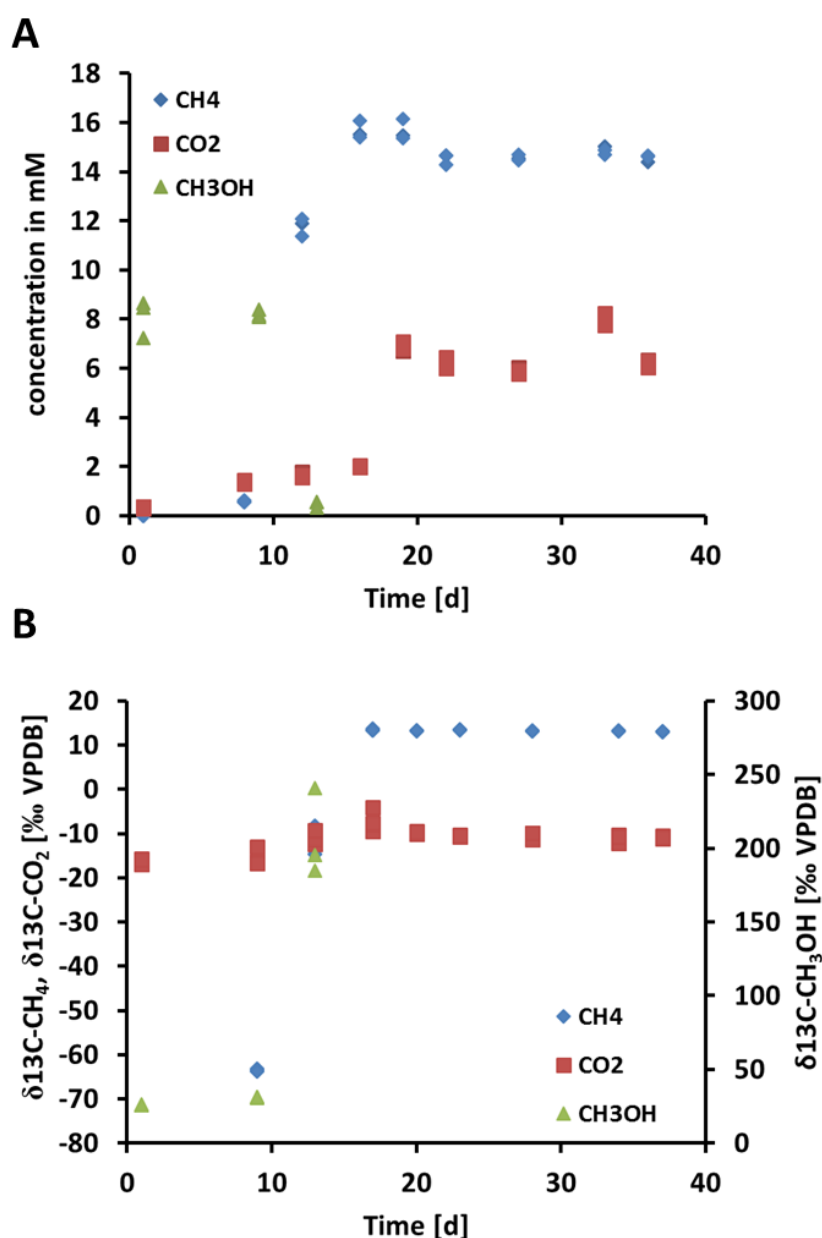
The concentration of methanol in the sediment decreased sharply in the first 2 weeks from ~8 mM at day 1 to 0.5 mM at day 13 (Fig. 8). At day 17, methanol was below the detection limit. Concomitant with the decrease in the methanol concentration, the residual methanol got enriched with <sup>13</sup>C in the first two weeks, reaching ~200 ‰ at day 13.

In contrast to methanol, the concentration of methane increased from 0 mM at day 1 to ~15 mM at day 17 and stayed around that value until the end of the incubation (Fig. 8). Concomitant with the increase in methane concentration, the carbon isotopic signature of methane ( $\delta^{13}\text{C}_{\text{CH}_4}$ ) showed a clear enrichment of the heavier isotope <sup>13</sup>C from day 9 to 17 (no methane was detectable at day 1). After day 17,  $\delta^{13}\text{C}_{\text{CH}_4}$  stayed around 13 ‰ until the end of the incubation. The concentration of CO<sub>2</sub> also increased from 0.3 mM at day 1 to ~7 mM at day 20 and stayed between 6-8 mM until the end of the incubation (Fig 8). CO<sub>2</sub> was also enriched with <sup>13</sup>C during the first 2 weeks (from -16.2 to -7.3 ‰) but then stayed around 11 ‰ until the end of the incubation.

#### **Methanogenesis activity in gravity cores**

Hydrogenotrophic methanogenesis activity in gravity cores varied strongly between the September samplings in 2013 and 2014 (Fig. 4). In 2013, methanogenesis activity revealed several peaks throughout the core at 31, 124, 217 and 310 cmbsf (1.0, 1.2, 0.5, and 0.1 nmol cm<sup>-3</sup> d<sup>-1</sup>, respectively).

In 2014, hydrogenotrophic methanogenesis was only detected at 70 cmbsf (mean:  $0.4 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) and 10 cmbsf (mean  $0.02 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ).



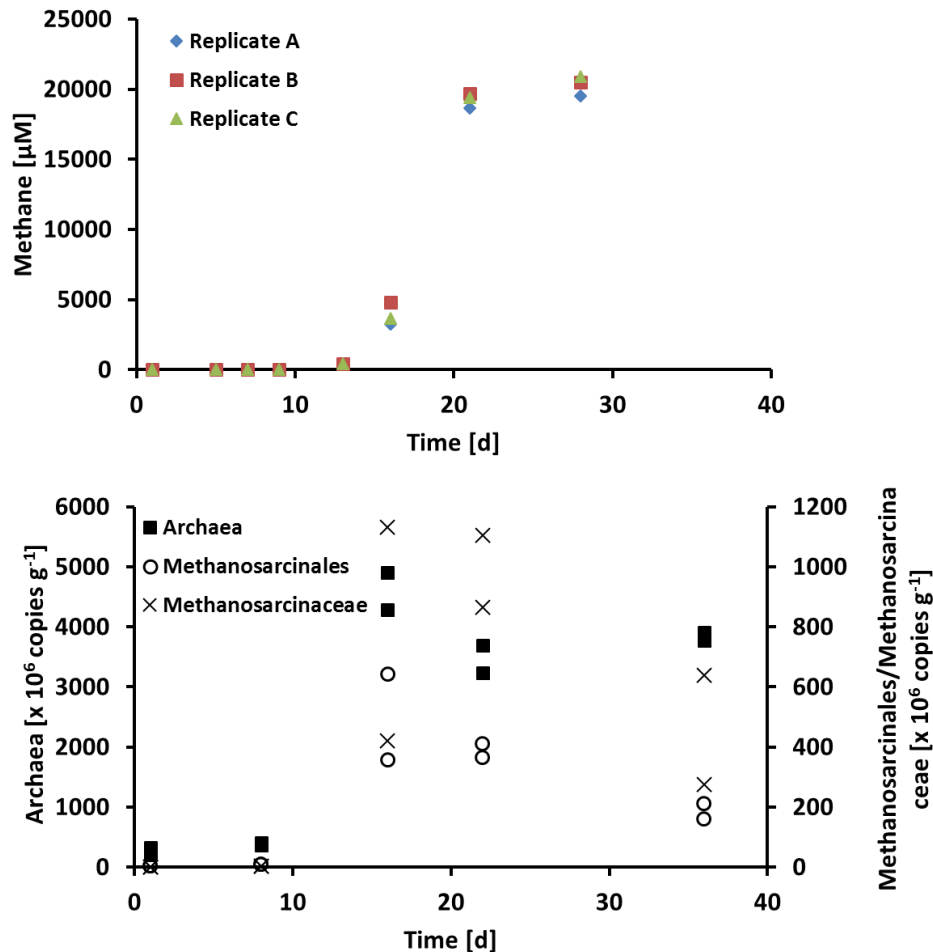
**Figure 8:** Concentrations (A) and isotope composition (B) of methanol (CH<sub>3</sub>OH), methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) during the sediment-slurry experiment with addition of <sup>13</sup>C-enriched methanol (<sup>13</sup>C:<sup>12</sup>C= 1:1000).

### Molecular analysis of benthic methanogens

In September 2014, additional samples were run during the methanol treatment (see 2.7.3) for the detection of benthic methanogens via qPCR. The qPCR results are shown in Fig.9. For a better comparison, the qPCR results are plotted together with the sediment methane concentrations from

the methanol treatment, from which the rate calculation for the methanol-methanogenesis at 0-1 cmbsf was done (shown in Fig. 6).

Methane concentrations increased over time revealing a slow increase in the first ~10 days, followed by a steep increase between day 13 and day 20 and ending in a stationary phase.



**Figure 9:** Sediment methane concentrations over time in the treatment with addition of methanol (10 mM) are shown above. Shown are triplicate values per measurement. DNA copies of *Archaea*, *Methanosarcinales* and *Methanosarcinaceae* are shown below in duplicates per measurement. Please note the different y-axis for *Archaea*.

A similar increase was seen in the numbers of total and methanogenic archaea. In general, the domain *Archaea*, the order *Methanosarcinales* and, within the order *Methanosarcinales*, the family *Methanosarcinaceae* were successfully detected by qPCR in the methanol-treatment in September 2014, with 10 fold higher DNA counts for *Archaea* compared to DNA counts for *Methanosarcinales*. The DNA counts for *Methanosarcinaceae* were 500 fold lower than for *Methanosarcinales*. All detected groups increased sharply in the second week of the incubation and showed a maximum after 16 days (~5000\*10<sup>6</sup> copies g<sup>-1</sup> for *Archaea*, ~500\*10<sup>6</sup> copies g<sup>-1</sup> for *Methanosarcinales*, 1\*10<sup>6</sup> copies g<sup>-1</sup>

<sup>1</sup> for *Methanosarcinaceae*). While DNA counts for *Methanosarcinales* and *Methanosarcinaceae* decreased to about a third of the maximum ( $\sim 200 \cdot 10^6$  copies  $g^{-1}$  and  $\sim 0.4 \cdot 10^6$  copies  $g^{-1}$ ) until the end of the incubation, DNA counts for Archaea stayed high around  $3000 \cdot 10^6$ - $4000 \cdot 10^6$  copies  $g^{-1}$ .

## Discussion

### Methanogenesis in the sulfate-reducing zone

Even though sulfate reduction rates were not directly measured, sulfate concentrations decreasing with sediment depth and a concomitant increase in sulfide suggest that sulfate reduction was present within the upper 30 cmbsf (Fig. 2+3). Earlier studies in Eckernförde Bay sediments confirmed the dominance of sulfate reduction in the surface sediment, which revealed an activity of 100-10000  $nmol\ cm^{-3}\ d^{-1}$  in the upper 25 cmbsf (Treude et al. 2005; Bertics et al. 2013).

On the basis of the results presented above, it appears obvious that methanogenesis and sulfate reduction coexisted in the surface sediments of Eckernförde Bay. Furthermore, we propose that surface methanogenesis in Eckernförde Bay sediments was mostly based on non-competitive substrates like methanol or methylated compounds (methylotrophic methanogenesis). Eckernförde Bay sediments revealed high POC contents of around 5 wt% (Fig. 2+3) in comparison to typical sediments of continental slopes (0.3-1 wt %) (Rullkötter, 2000). Due to this high organic carbon content, microbial fermentation of organic matter was probably high, increasing the substrate availability and variety and thus the presence of non-competitive substrates.

To support the hypothesis of mainly methylotrophic methanogenesis in surface sediments, we offer the following arguments:

1) in most cases, hydrogenotrophic methanogenesis only accounted for a seventh down to a seven hundredth part of the net methanogenesis (Fig. 2+3), 2) competitive substrates could only be used when sulfate reduction was inhibited (Fig. 7), 3) addition of BES did not result in inhibited methanogenesis (Fig. 7), 4) addition of methanol increased potential methanogenesis rates up to 1000-3000 times compared to the control treatment (Fig. 7), 5) we successfully detected methylotrophic methanogens of the order *Methanosarcinales* in the methanol-treatment (Fig. 9), and 6) stable isotope probing revealed highly  $^{13}C$ -enriched methane produced from  $^{13}C$ -labelled methanol (Fig. 8). In the following, the arguments will be discussed in more detail.

1) As results from MUC cores revealed,  $H_2/CO_2$  was not the main substrate of the observed ex-situ net methanogenesis at most time points, because hydrogenotrophic methanogenesis was much smaller than net methanogenesis. March 2013 was the only time point, where rates of hydrogenotrophic methanogenesis exceeded ex-situ net methanogenesis in discrete depths (5-6 cmbsf and 25-30

cmbsf). It is probable that additional carbon sources from dying organisms, burrow constructions or release of fecal pellets could have resulted in enhanced microbial activity at these depths (Ziervogel et al., 2014; Aller & Aller, 1986; Jørgensen, 1977; Bertics et al., 2013). These additional carbon sources could have led to increased local fermentation processes, which produced high amounts of  $H_2$ , thus fueling hydrogenotrophic methanogenesis at these sites. Eckernförde Bay sediments are known for bioturbation especially during early spring by mollusks and polychaetes (D'Andrea et al., 1996; Orsi et al., 1996; Bertics et al., 2013; Dale et al., 2013), and mollusk shells were observed even at depth of ~20 cmbsf during sampling in the present study (personal observation). Accordingly, it has been shown that the increase in organic carbon associated with bioturbation leads to the formation of reduced microniches with increased rates of  $N_2$  fixation coupled to sulfate reduction rates in Eckernförde Bay sediments (Bertics et al., 2013).

Hydrogenotrophic methanogenesis was also detected in gravity cores. Please note that gravity core results are only used for deep methanogenesis > 30 cmbsf, as surface sediments in gravity cores are strongly disturbed and usually the upper 20-30 cmbsf get lost. In September 2013 and 2014, maximum hydrogenotrophic rates were found at 132 cmbsf and 70 cmbsf, respectively, indicating a higher usage of  $CO_2$  and  $H_2$  at depths > 50 cmbsf, where sulfate was depleted and thus the competitive situation between sulfate reducers and methanogens was relieved.

2) We are confident that methylotrophic methanogenesis was the main pathway within the sulfate reduction zone, as the highly competitive situation between methanogens and sulfate reducers within the upper 30 cmbsf prevented methanogens from using competitive substrates. Competitive substrates could only be used when sulfate reducers were inhibited as seen in the results from the manipulating experiment with addition of molybdate (Fig. 6+7). The successful inhibition of sulfate reduction by molybdate led to enhanced (up to 30 times) potential methanogenesis rates compared to the control treatment. During the inhibition, the substrate spectrum of the present methanogenic community was broadened by enabling the utilization of the competitive substrates  $H_2/CO_2$  and acetate, which are preferably consumed by sulfate reducers (Oremland & Polcin, 1982; King et al., 1983). Interestingly, highest rates in the molybdate treatment were measured at the shallowest sediment depth at most time points (except November 2013), pointing towards the strongest competition between sulfate reducers and methanogens directly at the top 0-1 cmbsf, which is also confirmed by highest measured sulfate reduction rates at 0-1 cmbsf in earlier studies (Bertics et al. 2013; Treude et al. 2005).

3) Addition of BES did not result in an inhibition of potential methanogenesis but rates were in the same range as the control treatment (Fig. 7). Either the inhibition of BES was incomplete, or present

methanogens were insensitive to BES (Hoehler et al., 1994; Smith & Mah, 1981; Santoro & Konisky, 1987). The latter was more likely in the presented sediments, as rates in the BES treatment were never lower compared to rates in the control treatment. Insensitivity to BES would also support our hypothesis of methylotrophic methanogenesis, as BES resistance was shown in *Methanosarcina* mutants in earlier studies (Smith & Mah, 1981; Santoro & Konisky, 1987), a genus which we successfully detected in our samples (for more details see point 5) and which is known for facilitating the methylotrophic pathway (Keltjens & Vogels, 1993).

4) High potential methanogenesis rates observed after the addition of the non-competitive substrate methanol leads to the assumption that non-competitive substrates relieve the competition between methanogens and sulfate reducers in surface sediments of Eckernförde Bay. Except for November 2013, highest rates in the methanol-treatment were detected in the upper 0-5 cmbsf and decreased with depth (Fig. 6). As mentioned above, sulfate reduction was most dominant in the 0-5 cmbsf (Treude et al., 2005a; Bertics et al., 2013). Thus prevalent methanogens at the sediment surface were probably more adapted to usage of non-competitive substrates due to the highly competitive situation. Please note, that rates were calculated from the linear increase in methane concentration over the entire incubation period to easier compare between treatments. However, a delayed increase in methane concentration was observed in the methanol treatments at each time point (Supplementary Material, Fig. S1). This delayed response of methanogenesis after methanol addition has been observed in an earlier study from organic-rich sediments off Peru and was explained by the predominant use of other non-competitive substrates such as methylated sulfides (e.g. dimethyl sulfides or methanthiol; Maltby et al, submitted). In the marine environment, dimethyl sulfides mainly originate from the algae osmoregulatory compound dimethylsulfoniopropionate (DMSP) (Van Der Maarel & Hansen, 1997), which could have accumulated in Eckernförde Bay sediments, due to intense sedimentation of algae blooms (Bange et al., 2011). Certain *Methanosarcina* species have been shown to use DMSP as a substrate (Sieburth et al., 1993; Van Der Maarel & Hansen, 1997), a genus, which also has been detected in our samples (see more details under point 5).

Additionally, there are hints that methylated sulfur compounds may be generated through nucleophilic attack by sulfide on the methyl groups in the sedimentary organic matter (Mitterer, 2010). As shown in the present study, sulfide was an abundant species (up to mM levels) due to high sulfate reduction activity (Fig. 2+3).

5) Simultaneously with the increase in methane concentration in the headspace after methanol addition, the DNA counts for the order *Methanosarcinales* and the family *Methanosarcinaceae* within the order *Methanosarcinales* increased 100 to 500 000 times, respectively, in the surface layer (0-1



cmbsf) of September 2014 (Fig. 9). The successful enrichment of *Methanosarcinaceae* indicates that this family is present in the natural environment and thus could be responsible for the observed surface methanogenesis. As the members of the family *Methanosarcinaceae* are known to be exclusive to utilization of methylated substrates (Boone et al. 1993), our hypothesis for the predominant usage of non-competitive substrates is supported. The delay in growth of *Methanosarcinales* and *Methanosarcinaceae*, however, also hints towards the predominant usage of other non-competitive substrates besides methanol (see also point 4).

6) In September 2014, we were able to show that methanol ( $^{13}\text{C}$ -labelled methanol: non-labelled methanol mostly existing of  $^{12}\text{C}= 1:1000$ ) was completely consumed (after 17 days) and converted to methane, which revealed concomitant enrichment in  $^{13}\text{C}$ . Consumption of  $^{12}\text{C}$ -methanol was preferred by the microbes, leading to an enrichment of  $^{13}\text{C}$  in the residual methanol in the first 13 days. We postulate that members of the family *Methanosarcinaceae* were responsible for the observed methane production, which is mainly known for facilitating the methylotrophic pathway (Keltjens & Vogels, 1993). Please note, however, that the storage of the cores (3.5 months) could have led to shifts in the microbial community and thus might not reflect in-situ conditions.

### **Environmental control of surface methanogenesis**

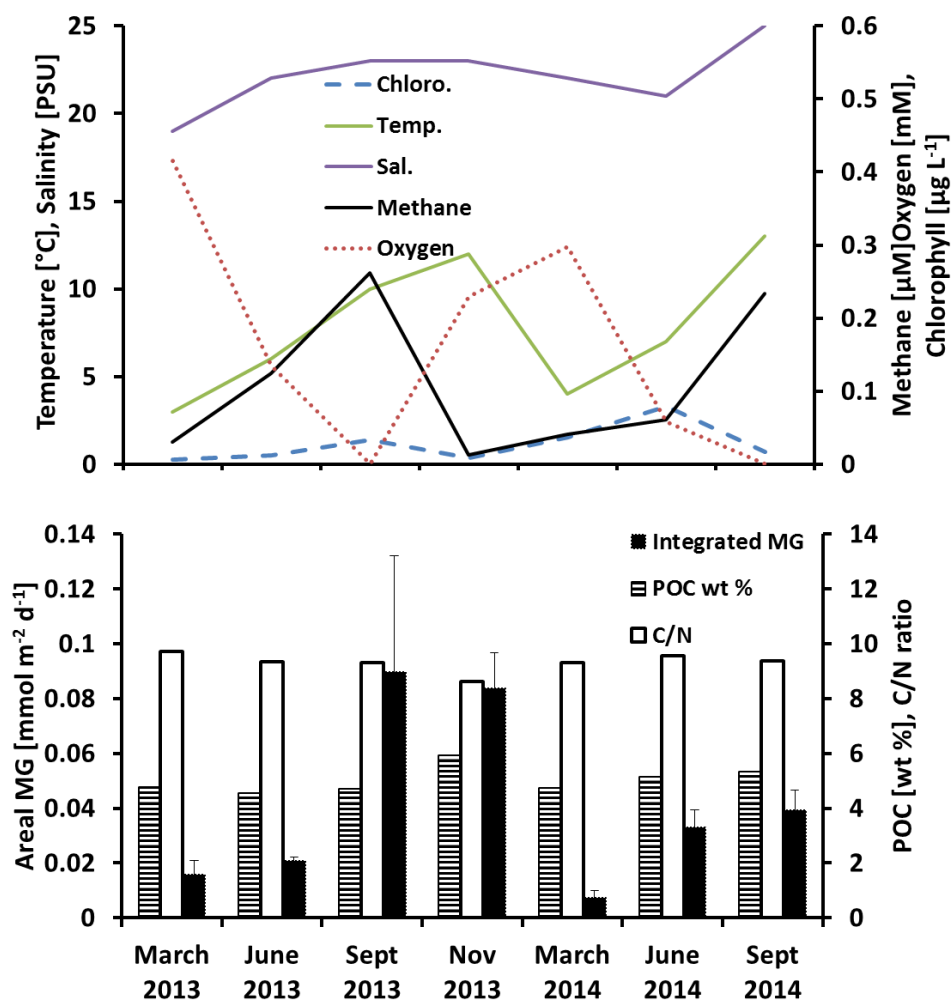
Surface methanogenesis in Eckernförde Bay sediments showed variations throughout the sampling period, which could be explained by various environmental factors namely salinity, temperature, oxygen, and organic matter content. In the following, we will discuss the potential impact of those factors on magnitude and distribution of surface methanogenesis.

#### **Salinity**

An increase of salinity was concurrent with an increase in sulfate concentration, as sulfate is one of the major ions in seawater (Fig. 2+3). The availability of sulfate and resulting sulfate reduction are major factors controlling the presence and magnitude of surface methanogenesis (Burdige, 2006), because sulfate reducers outcompete methanogens for the mutual substrates  $\text{H}_2$  and acetate (Oremland & Polcin, 1982).

From March 2013 to November 2013, and from March 2014 to September 2014, salinity increased in the bottom-near water from 19 PSU to 23 PSU and from 22 PSU to 25 PSU, respectively, due to the inflow of saline North Sea water (Bange et al., 2011). Thus, an increase in surface sulfate concentrations and its penetration depth occurred over the course of a year, potentially increasing sulfate reduction, which in turn could have led to a stronger inhibition effect on surface methanogenesis in November 2013 and September 2014, respectively. However, highest integrated surface methanogenesis occurred during the time points when salinity was highest and porewater

sulfate decrease and sulfide increase were strongest, indicating increased sulfate reduction activity (Fig. 2+3). Hence, we postulate that surface methanogenesis was not affected directly by variation in salinity (and thus the variation in surface sulfate concentration) but was rather controlled by substrate availability.



**Figure 10:** Development of water column parameters temperature (Temp.), salinity (Sal.), oxygen, methane, and chlorophyll (Chloro.) over the total sampling period (above). Development of sediment integrated net methanogenesis (0-25 cmbsf), sediment surface particulate organic carbon (POC) content and sediment surface C/N ratio over the total sampling period (below). MG rates are shown in means with standard deviation.

### Temperature

Depending on the temperature optimum of the methanogens, the in-situ temperature could have an effect on microbial activity. During the sampling period, bottom water temperatures increased from spring (March, 3-4 °C) to winter (November 12°C, Fig. 2+3). Due to stratification from June to September, bottom water temperatures did not exceed 10°C at most time points (Fig. 2+3).

November 2013 and September 2014 revealed the warmest bottom water (12 and 13°C, respectively), which coincided with high methanogenesis activity (Fig. 10). Temperature methanogenesis experiments with deep sediment from ~75 cmbsf in September 2014 revealed a temperature optimum at 20°C (Supplementary Material, Fig. S2), thus deep methanogenic archaea seem to be mesophilic. Whether surface methanogenic archaea in Eckernförde Bay have the same temperature optimum remains speculative. At least, AOM organisms, which are related to methanogenic organisms, revealed a mesophilic metabolism at this site (Treude et al. 2005).

### **Oxygen**

In the years 2013 and 2014, Eckernförde Bay sediments experienced hypoxic conditions in the bottom water from June to September, with even anoxic conditions occurring in September of each year (Supplementary Material, Fig. S3). The development of hypoxia/anoxia in Eckernförde Bay is explained by high organic matter (from phytoplankton blooms) degradation within the water column in addition to water column stratification (Lennartz et al., 2015; Orsi et al., 1996). In October, autumn storms break up the summer stratification bringing oxygenated waters to the bottom (Fig. S3; Lennartz et al., 2015; Orsi et al., 1996).

Oxygen is an important factor for methanogenesis, as methane is normally only produced under strictly anoxic, highly reduced (<-200 mV) conditions (Oremland, 1988; Zinder, 1993). Interestingly, high integrated methanogenesis rates were detected in September of both years, when the bottom water was anoxic (Fig. 10), suggesting that anoxic conditions in the bottom water could be advantageous for surface methanogenesis. The anoxic conditions in the water column probably led to decreased organic matter degradation, thus less-degraded organic matter reached the seafloor in September 2013 and 2014, enhancing benthic mineralization processes including methanogenesis. Additionally, an establishment of a methanogenic community up to the sediment-water interface was favored under anoxic bottom water conditions. However, integrated surface methanogenesis showed similar high magnitudes in November 2013, where bottom water oxygen concentrations were already above 200 µM. Thus, rather substrate availability and variety was controlling surface methanogenesis with anoxic bottom waters as a potential supporting factor as seen in other organic-rich sediments (Maltby et al, submitted). Similar conclusions were made in an earlier study from the Time Series Station Boknis Eck in Eckernförde Bay, where hypoxic conditions were found to be advantageous for sedimentary methanogenesis (based on water column methane concentrations), but the main controlling factor was postulated to be the input of organic material (Bange et al., 2010).

### ***Particulate organic carbon (POC)***

The POC content is probably the most important factor controlling methanogenesis in surface sediments, as it determines substrate availability and variety and thus can relieve the competitive situation between sulfate reducers and methanogens in sulfate-containing, organic-rich marine sediments (Oremland, et al. 1982; Holmer & Kristensen 1994; Treude et al. 2009; Maltby et al., submitted). Due to their substrate speciation, which mainly includes small molecules with only one C-C bonding, methanogens are dependent on other microbial groups (e.g. fermenters) to degrade large organic compounds (e.g. amino acids) for them (Zinder, 1993). A high POC content results in high microbial degradation processes within the sediments, which in turn secures the substrate availability of methanogens.

In Eckernförde Bay, the organic material reaching the sediment floor, originates mainly from phytoplankton blooms in spring, summer and autumn (Bange et al., 2011). It has been estimated that > 50% in spring, > 25% in summer and > 75% in autumn of these blooms is reaching the sediment floor (Smetacek et al., 1984), resulting in high organic carbon content (5 wt %) and high sedimentation rates ( $\sim 1.4 \text{ mm yr}^{-1}$ ) in Eckernförde Bay sediments (Whiticar, 2002; Treude et al., 2005a).

In fact, a positive correlation could be seen in the present study with increasing methanogenesis rates and increasing surface POC content in 2013 and 2014 (Fig. 10). In addition, highest rates (Nov. 2013) coincided with lowest C/N ratios indicating less degraded (=fresher) organic material. In support of this observation, highest bottom water chlorophyll concentrations coincided with highest bottom water methane concentrations and highest benthic areal methanogenesis in September 2013, probably as a result of the summer phytoplankton bloom (Fig. 10). Thus, methane production and emission was high when carbon load was high. However, no correlation with chlorophyll was seen in 2014. While highest chlorophyll concentrations occurred in June 2014, highest areal methanogenesis rates and dissolved methane concentrations were detected in September 2014 (Fig. 10). This off-set could be explained by the time lag until the chlorophyll finally reaches the seafloor, which could even take longer when a strong stratification occurs. Earlier studies also revealed enhanced sedimentary methane formation (from enhanced bottom water methane concentration) with a time lag of  $\sim$ one month after the chlorophyll maximum (Bange et al., 2010).

The fact that the subsurface maximum of net methanogenesis moved downwards over the course of a year (Fig. 2+3) could as well be correlated with the availability of POC. First, the high sedimentation rates lead to increased burial of organic matter, as it limits the initial degradation at the sediment surface (Zabel & Hensen, 2003). This buried organic material could then fuel microbial processes deeper in the sediment. As mentioned earlier, dissolved organic material produced from dying organisms or the organic matter used in burrow constructions could lead to “hot spots” of microbial

activity below surface (Ziervogel et al., 2014; Bertics et al., 2013). Bioturbation and bioirrigation in Eckernförde Bay sediments were found to be highest from spring to summer, with no activity in late summer due to the low oxygen concentrations in the water column (Dale et al., 2013; Bertics et al., 2013). The hypoxic/anoxic events in September 2013 of the present study could have led to an enhanced dying of bioturbating organisms, which then served as an organic source for benthic processes in November 2013, including methanogenesis.

### **Relevance of surface methanogenesis in Eckernförde Bay sediments**

The time series station Boknis Eck in Eckernförde Bay is known for being a methane source to the atmosphere throughout the year due to supersaturated waters, which result from significant benthic methanogenesis and emission (Bange et al., 2010). The benthic methane formation is thought to take place mainly in the deeper, sulfate-depleted sediment layers (Treude et al., 2005a; Whiticar, 2002).

In the present study, we show that surface methanogenesis within the sulfate zone is present despite sulfate concentrations  $> 1$  mM, above which methanogenesis has been thought to be negligible (Alperin et al., 1994; Hoehler et al., 1994; Burdige, 2006), and thus could also contribute to benthic methane emissions. In support of this hypothesis, high dissolved methane concentration in the water column occurred with concomitant high surface methanogenesis activity (Fig. 10).

Maximum net methanogenesis rates ranged from 0.2 to 1.3  $\text{nmol cm}^{-3} \text{d}^{-1}$  and were in the same magnitude as other surface (within the upper 25 cmbsf), sulfate-containing, organic-rich sediments like the upwelling region off Chile and Peru (0-0.6  $\text{nmol cm}^{-3} \text{d}^{-1}$ , Ferdelman et al., 1997; 0-1.5  $\text{nmol cm}^{-3} \text{d}^{-1}$ , Maltby et al., submitted). However, our rates were only on the very low end or even two orders of magnitude lower in comparison with sulfate-depleted, organic-rich sediments, (9.8-37  $\text{nmol cm}^{-3} \text{d}^{-1}$ , 0-40 cmbsf, Treude et al., 2005a, 10-17  $\text{nmol cm}^{-3} \text{d}^{-1}$ , 0-30 cmbsf, Schmaljohann 1996, 100-300  $\text{nmol cm}^{-3} \text{d}^{-1}$ , 0-30 cmbsf, Crill & Martens, 1983, 1986), which is explained by the highly competitive situation between sulfate reducers and methanogens in the presented sediments.

To identify the relevance of surface methanogenesis in the sulfate-zone of Eckernförde Bay sediments, we also performed a comparison with deep methanogenesis in the sulfate-depleted zone.

As a spatial border for surface and deep methanogenesis we identified the layer where both sulfate and methane are depleted, generally known as sulfate-methane-transition zone (SMTZ, Knittel & Boetius, 2009). The SMTZ is known as the main niche for anaerobic oxidation of methane (AOM), which acts as an important methane filter retaining up to 90 % of deeply produced methane in marine sediments (Knittel & Boetius, 2009).

In the gravity core of September 2013, the SMTZ was situated between 31 and 62 cmbsf, while in September 2014 the SMTZ was situated between 10-30 cmbsf (Fig.4). Again please note that these depths layers have to be treated with caution, as during the gravity coring procedure, the top soft

sediment layer (ca. 0-20 cm) often gets disturbed or even lost. Hence, the depth layer of the SMTZ might have been deeper at in situ conditions especially in September 2014.

The methane flux was estimated according to Iversen & Jørgensen, (1993) using a sediment methane diffusion coefficient of  $D_s = 1.64 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  for September 2013 and  $D_s = 1.79 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  for September 2014, respectively. Sediment diffusion coefficients were derived from the seawater methane-diffusion coefficient at 10°C (Schulz, 2006), which was corrected by porosity according to Iversen & Jørgensen, (1993).

The resulting deep methane production in September 2013 and 2014 (1.5 and 3.0  $\text{mmol m}^{-2} \text{ d}^{-1}$ , respectively) was similar to earlier calculated deep methanogenesis in Eckernförde Bay (0.66 – 1.88  $\text{mmol m}^{-2} \text{ d}^{-1}$ ; Treude et al., 2005a). The relatively high deep methane production in September 2014 could be a result of the overall warmer temperatures in September 2014 resulting in increased microbial activity. Deep methanogenesis in September 2013 was probably dominated by the hydrogenotrophic pathway, as integrated hydrogenotrophic methanogenesis below 31 cmbsf, i.e., below the SMTZ, was similar to the flux of methane into the SMTZ ( $1.1 \pm 0.4 \text{ mmol m}^{-2} \text{ d}^{-1}$ ). This finding is also supported by an earlier study in Eckernförde Bay, where the authors identified hydrogenotrophic methanogenesis as the main pathway on the basis of  $\delta^{13}\text{C}$ -methane concentrations (Whiticar, 2002). In September 2014, however, integrated hydrogenotrophic methanogenesis ( $0.13 \pm 0.17 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) below 30 cmbsf was 10-100 times lower compared to the calculated deep methane production. An explanation for this deviation could be the wave-overwashing during gravity core sampling, thus hydrogenotrophic rates in September 2014 have to be treated with caution.

Comparing surface ( $0.09 \pm 0.04 \text{ mmol m}^{-2} \text{ d}^{-1}$  in September 2013 and  $0.04 \pm 0.007 \text{ mmol m}^{-2} \text{ d}^{-1}$  in September 2014) and deep methanogenesis of the respective months, surface methanogenesis represented 3-9 % and 1-2 % of deep methanogenesis in 2013 and 2014, respectively. While this seems low, surface methanogenesis in Eckernförde Bay sediments is in the same magnitude as deep methane production in other organic-rich sediments from the North Sea (0.076  $\text{mmol m}^{-2} \text{ d}^{-1}$ , Jørgensen & Parkes, 2010), or from the upwelling region off Chile/Peru (0.068-0.13  $\text{mmol m}^{-2} \text{ d}^{-1}$ , Treude et al., 2005b,  $2.2 \cdot 10^{-7}$ -0.017  $\text{mmol m}^{-2} \text{ d}^{-1}$ , Arning et al., 2012), indicating the general importance of this process. The low magnitude of surface methanogenesis in comparison with deep methanogenesis in the present study can be explained by the high carbon export in Eckernförde Bay, which leads to extremely high deep methanogenesis activity below the SMTZ, resulting in even gas bubble formation due to methane supersaturation in the porewater (Whiticar, 2002; Treude et al., 2005a).

How much of the surface methane is emitted into the water column, depends on the rate of methane consumption, i.e. aerobic and anaerobic oxidation of methane (Knittel & Boetius, 2009). In organic-

rich sediments such as in the presented study, the oxic sediment layer is often only mm-thick, due to the high rates of microbial organic matter degradation, which rapidly consumes oxygen (Revsbech et al., 1980; Emerson et al., 1985; Jørgensen, 2006). Thus the anaerobic oxidation of methane (AOM) might play a more dominant role in the present study. In an earlier study from Eckernförde Bay, AOM rates were measured above the SMTZ (0-25 cmbsf), but the authors concluded that it was only fueled by deep methanogenesis (Treude et al., 2005a), as surface integrated AOM rates ( $0.8\text{-}1.5\text{ mmol m}^{-2}\text{ d}^{-1}$ ) were in the same magnitude as deep methane flux ( $0.66\text{-}1.88\text{ mmol m}^{-2}\text{ d}^{-1}$ ) from below the SMTZ (Treude et al., 2005a).

With the presented data set we postulate that surface AOM is not only fueled by deeply produced methane, which diffuses upwards, but also by surface methanogenesis with up to 13% (percentage of  $0.1\text{ mmol m}^{-2}\text{ d}^{-1}$  integrated surface methanogenesis from  $0.8\text{ mmol m}^{-2}\text{ d}^{-1}$  integrated surface AOM). More detailed measurements of deep methanogenesis and AOM within the SMTZ together with methane flux measurements from the sediment to the water column are needed, to better determine the potential of benthic methane emissions from surface methanogenesis.

## Conclusion

The present study revealed that methanogenesis and sulfate reduction coexisted within the sulfate-reducing zone in Eckernförde Bay sediments. Observed methanogenesis was probably based on non-competitive substrates due to the competition with sulfate reducers for the substrates  $\text{H}_2$  and acetate. Accordingly, members of the family *Methanosarcinaceae*, which are known for methylotrophic methanogenesis, are likely to be responsible for the observed surface methanogenesis using the substrates methanol, methylamines or methylated sulfides.

The most important controlling factor for surface methanogenesis was the availability and quality of organic material, resulting in highest methanogenesis activity after summer and autumn phytoplankton blooms. The increasing bottom water temperatures during the course of a year also revealed a positive correlation with surface methanogenesis, leading to the hypothesis that prevalent methanogens were mesophilic. In addition to organic matter and temperature, also the hypoxic/anoxic events during later summer were advantageous for surface methanogenesis, probably due to less organic carbon degradation within the water column and an easier establishment of a methanogenic community directly at the sediment surface.

Even though surface methanogenesis was low compared to deep methanogenesis, it is likely to contribute to methane emissions, indicated by concomitant high dissolved methane concentrations in the water column when surface methanogenesis activity was high. The benthic methane emissions might even increase in the future, as an increase in temperature and oxygen depletion are predicted

for Eckernförde Bay (Lennartz et al., 2014), both factors, which have an enhancing effect on surface methanogenesis. The predicted decrease in nutrients and chlorophyll, however, might compensate increased rates by increased temperature and hypoxic events, leading to overall constant emissions.

Our results reveal a previously underestimated role of surface methanogenesis as a 1) methane supplier for AOM above the SMTZ and 2) contributor to benthic methane emissions.

Future studies should seek into identifying the role of surface methanogenesis in benthic methane emissions, including the interactions between surface methanogenesis and aerobic and anaerobic methane oxidation, and the effects of predicted climate change on surface methanogenesis.

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## Chapter 4

# **Denitrification and sulfate reduction in oiled and oil-free sediments from the Northern Gulf of Mexico**

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## Abstract

Sulfate reduction activity and potential denitrification activity was determined in oil-impacted sediments (oiled sediments) from the Gulf of Mexico and compared to sediments devoid of oil-deposition (control sediments). Oiled sediments were characterized by a surface layer of sedimented, weathered oil, which originated from either the Deepwater Horizon (DWH) oil spill or from natural seepage. Porewater DOC and sediment TPC and POC data revealed differences between sites affected by the DWH oil spill and sites affected by natural seepage, suggesting that the oil from the DWH oil spill was more weathered and thus had a lower organic carbon content. This was also apparent in the higher rates of microbial activity at the site affected by natural seepage due to greater lability of less-degraded oil. Sulfate reduction rates were low within the oiled layer, but were significantly higher in the 5-10 cmbsf horizon below the oiled layer, suggesting that either the sulfate reducing community was not established yet within the oiled layer or that the organic matter within the oiled layer was not available to the native sulfate reducers. Potential denitrification rates were significantly higher within the oiled layer compared to deeper sediment layers in oiled cores and to surface sediments in control cores. These results suggest that denitrification might be a dominant process in degrading weathered, sedimented oil, and that sulfate reduction remains important in deeper layers. Sulfide produced by sulfate reduction was probably consumed quickly by sulfide-removing processes (e.g. dissimilatory nitrate reduction to ammonium (DNRA) by sulphur-oxidizing bacteria, microcable bacteria, chemical oxidation through metal oxides) before it could reach the sediment surface layer, limiting sulfide accumulation in these sediments and thus no sulfide-driven N<sub>2</sub>O production was observed in oiled sediments. However, both types of sediment (oiled and control) showed high N<sub>2</sub>O concentrations at the sediment surface, illustrating the potential for these sediments to be a source for N<sub>2</sub>O. Our results indicate that weathered sedimented oil persists on the sea floor for long time periods (>2 years) probably due the fact that it is more resistant to microbial attack, thus being available only to very specific microbial communities.

## Introduction

The Gulf of Mexico (Gulf) is a prolific hydrocarbon basin and natural hydrocarbon seeps abound, particularly in the Northern Gulf (MacDonald et al., 1996). The Gulf is located between North America and Central/South America, and it supports widespread gas and oil reservoirs housed beneath and within thick layers of Jurassic salt. This salt is ductile and moves as it is compressed, generating salt tectonics and faults that serve as conduits for gas and oil migration to the seabed from deep reservoirs (Kennicutt & Brooks, 1990). Gulf seeps are commonly characterized by a high diversity of specialized macrofauna and free-living sulfur-oxidizing bacteria like *Beggiatoa* (Kennicutt et al., 1985; MacDonald

et al., 1989, 1990, 1996). Naturally-occurring seafloor microbial processes such as sulfate reduction (Orcutt et al., 2005; Bowles et al., 2011) methanogenesis (Orcutt et al., 2005), anaerobic oxidation of methane (AOM; (Joye et al., 2004, 2010; Bowles et al., 2011) and denitrification (Bowles & Joye, 2010) have been evaluated at natural seeps in the Gulf, revealing high rates of microbial activity coupled to oil degradation (Joye et al., 2004; Orcutt et al., 2010).

Sedimentation of oil in surface slicks can also return to the seafloor following weathering processes (NRC, 2003). The processes that transport oil to the sea floor may include oil-associated marine snow formation (Passow, this issue), sinking oil-mineral aggregates (Khelifa et al., 2005) or weathered oil/tar-like residues (Patton et al., 1981; Joye et al., submitted). The size of the oil discharge - either through natural seepage or from an anthropogenic discharge - and the resulting oil slick and the physical transport processes in the area (dispersion, oxidation, overwashing etc., NRC, 2003) determine the potential area of oil contamination through sedimentation, which often exceeds the area around the discharge source (NRC, 2003).

Recently, the Gulf received international attention during to the Deepwater Horizon (DWH) oil spill, which occurred between April and July 2010, and is at present the largest offshore marine oil spill in US history. The DWH disaster discharged roughly 5 million barrels of oil into offshore Gulf waters (Joye et al., 2011). In the aftermath of the discharge, a large sedimentation event occurred, blanketing a substantial area around the wellhead (up to 20 to 40 km away) with an often thick (2-10 cm) layer of weathered oil (Montagna et al., 2013; Joye et al., 2014; Valentine et al., 2014; Passow, this issue; Joye et al., submitted). In contrast to natural seep sediments, the impact of this weathered oil on seafloor microbial activity has been sparsely reported (Ziervogel et al., this issue). If weathered, sedimented oil fuels benthic microbial activity in similar magnitudes to that of natural seep sediments, oxygen would be rapidly consumed (Atlas, 1981) and anaerobic processes such as denitrification or sulfate reduction would dominate. The latter two processes are often elevated in sediments from Gulf oil seeps (Bowles & Joye, 2010; Joye et al., 2004, 2011) due to the ability of sulfate reducers (Kniemeyer et al., 2007; Orcutt et al., 2005) and denitrifiers (Alain et al., 2012; Rabus & Widdel, 1995) to biodegrade complex petroleum. Thus, these two processes in particular, could reveal the effect of oil on microbial activity in sediments.

Hydrocarbon seep sediments typically exhibit high concentrations of hydrogen sulfide due to high sulfate reduction rates coupled to methane and oil degradation (up to mM ranges, Joye et al., 2010). The coupling between AOM and sulfate reduction is often loose at Gulf oil seeps, with higher sulfate reduction rates exceeding AOM rates, suggesting that the majority of sulfate reduction is fueled by the oxidation of other alkanes and oil rather than by AOM (Joye et al., 2004, 2010).



If sulfate reduction is elevated in sediments impacted by oil sedimentation, a build-up of pore water sulfide is expected. High sulfide concentrations block the terminal enzyme of denitrification, nitrous oxide reductase, which catalyzes the reduction of nitrous oxide ( $\text{N}_2\text{O}$ ) to dinitrogen gas ( $\text{N}_2$ ). As a result, the inhibition causes the accumulation of  $\text{N}_2\text{O}$  (Bowles et al., 2012; Porubsky et al., 2009; Brunet & Garcia-Gil, 1996; Joye, 2002). Because  $\text{N}_2\text{O}$  is a very potent greenhouse gas (approximately 300 times more effective than  $\text{CO}_2$  in trapping heat, (Jain et al., 2000), research on its global sinks and sources is critical (IPCC, 2014).

Some marine areas act as  $\text{N}_2\text{O}$  sources and emit  $\text{N}_2\text{O}$  into the atmosphere due to microbial  $\text{N}_2\text{O}$  production in the water column (Bange et al., 1998; Bange, 2006b; Farías et al., 2009), thereby exacerbating global warming through ozone depletion (Denman et al., 2007). Benthic  $\text{N}_2\text{O}$  production could contribute to ocean  $\text{N}_2\text{O}$  emissions; however studies of  $\text{N}_2\text{O}$  dynamics in the ocean are rare and have focused on near-shore and offshore oxygen minimum zones (Middelburg et al., 1995; Usui et al., 1998; Meyer et al., 2008).

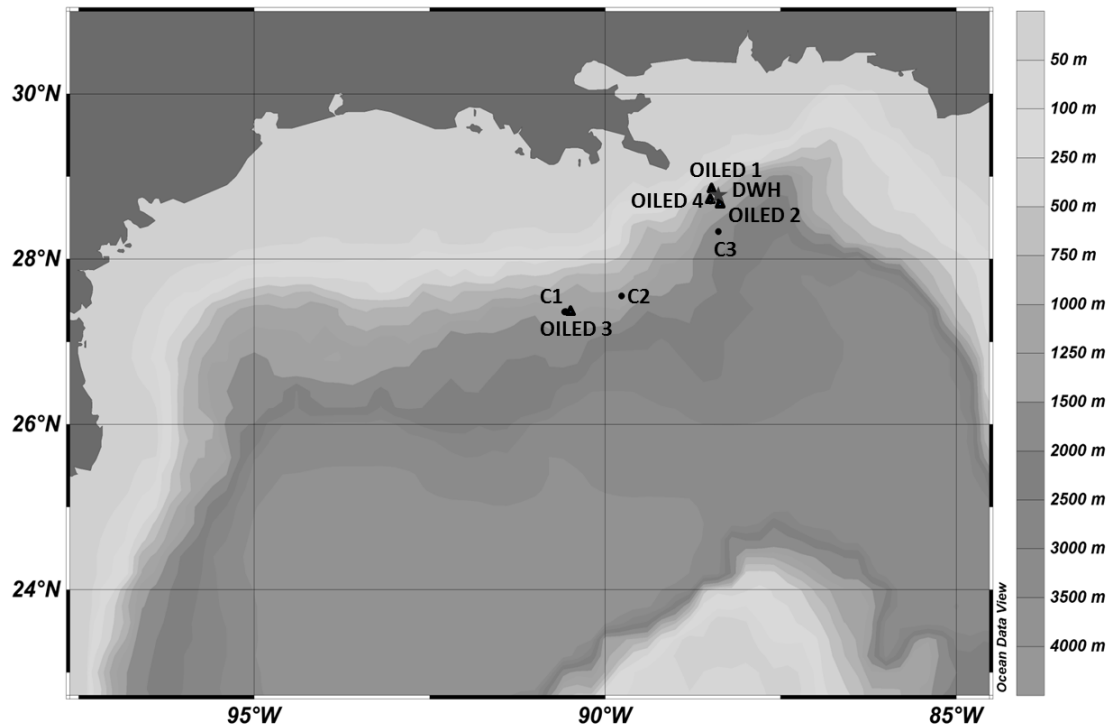
With the present data set, we offer insight into denitrification and sulfate reduction processes (including the coupling to AOM) in sediments overlain by weathered oil (hereafter, oiled sediments), which originated either from natural seepage or from the DWH discharge and subsequent rapid sedimentation event. We compare processes measured in oiled sediments to those in oil-free control sediments (hereafter, control sediments) to reveal the potential impact of the weathered oil on sediment biogeochemical dynamics. We hypothesized that weathered oil deposition stimulates sulfate reduction, leading to the accumulation of sulfide and giving rise to sulfide-inhibited denitrification, which ultimately leads to an increase in  $\text{N}_2\text{O}$  production.

## Material and Methods

### Study sites and core descriptions

Sediments were sampled from 7 different locations in the northern Gulf of Mexico (Gulf) during two research cruises (EN509 and EN510) aboard the *R/V Endeavor* in May - June 2012 (Fig. 1). Sediments from four stations (St. 2.02, 4.07, 2.18 and 12.05) showed a visible layer of weathered surface oil (Fig. 2), while the remaining three stations (St. 2.18, 3.08, and 6.06) were visually oil-free. The presence or absence of the weathered oil layer served as criteria for the separation between the following two sediment types: cores with oiled layer are termed "Oiled Sites 1, 2, 3 and 4" (hereafter OILED1, OILED2, OILED3, OILED4), while cores without visible oiled layer served as controls and are termed "Control Sites 1, 2, and 3" (hereafter C1, C2, C3). Station OILED1 was in the vicinity (< 1 km) of a known gas and oil seep, site MC118 (Mississippi Canyon lease block 118, 28°51,132'N, 88°29,568'W, 900 meters below sea level (mbsl), Sassen et al., 2013) and ~16 km from the Macando wellhead, site of the

Deepwater Horizon (DWH) well blowout. Thus, the oiled layer at OILED1 is most likely a mixture of oil from both sources. Station OILED2 and OILED4 were located  $\sim 7$  km and  $\sim 12$  km, respectively from the Macondo wellhead, and this oiled layer most likely originated from the DWH discharge. Station OILED3 was located near ( $< 1$  km) a prolific oil seep, GC600 (Green Canyon block 600,  $27^{\circ}22,176'N$ ,  $90^{\circ}34,158'W$ , 1250 mbsl, Joye et al., 2010) and thus represents a site influenced by natural oil seepage (Joye et al. submitted).

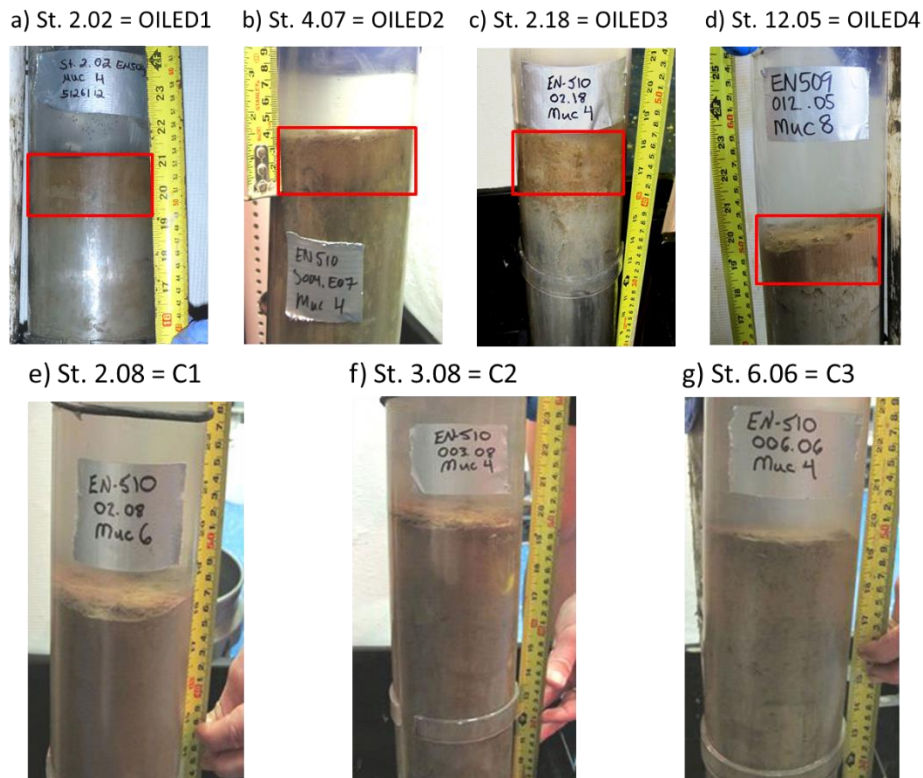


**Figure 1:** Location of sampling sites in the Northern Gulf of Mexico. Oiled sites are marked with black triangles, the Deepwater Horizon oil spill site (DWH) is marked with a dark grey star, and control sites are marked with black circles. Source: Schlitzer, R., Ocean Data View, <http://odv.awi.de>, 2014

Sediment cores from control sites were collected from areas away from known sites of natural seepage, lacked visible evidence of seepage, were not impacted by the DWH blowout, and lacked a weathered oiled surface layer. Site C1 was situated  $\sim 1$  km northwest of OILED3, C2 was located  $\sim 80$  km northeast of OILED3, and C3 was located  $\sim 40$  km south of OILED2. Station details are presented in Table 1. The oiled layer of the cores OILED1 through 4 had a thickness of about 4-5 cm, a characteristic reddish-brown color, and a greasy consistency (Joye et al., 2014; Ziervogel et al., this issue). For later calculations an average oil layer thickness of 5 cm was assumed. Due to absence of oil veins or stains in deeper layers of the sediment, these oiled layers clearly originated from sedimentation, rather than through upward advection of oil. No sulfur-oxidizing bacteria or other seep-related macrofauna were visible on the sediment surface of any of the oiled cores.

**Table 1:** Overview of Stations

Station	Cruise	Station No	Date	Latitude	Longitude	Water Depth (m)
OILED1	EN509	2.02	26 May 2012	28°51.093	88°29.279	890
OILED2	EN510	4.07	03 July 2012	28°40.483	88°22.289	1652
OILED3	EN510	2.18	29 June 2012	27°21.772	90°33.878	1300
OILED4	EN509	12.05	11 June 2012	28°42.133	88°30.000	1386
C1	EN510	2.08	27 June 2012	27°21.741	90°34.713	1168
C2	EN510	3.08	01 July 2012	27°33.327	89°45.969	1131
C4	EN510	6.06	04 July 2012	28°19.955	88°23.206	1789

**Figure 2:** Photographs of sediment cores from oiled stations with visible oiled layer (a-d, top row) and from control stations (e-g, bottom row). Oiled layers are marked by a red frame.

### **Sediment sampling, processing and porewater /solid phase analysis**

Eight replicate cores (average core length: 50 cm) were recovered with a multicorer at each station from an area of 1-2 m<sup>2</sup>. One replicate core was used for sediment porewater and solid phase analyses, one core was used for sulfate reduction and AOM rate determinations, and one core was used for denitrification experiments. The porewater and rate cores were sliced in the same manner: in case of the oiled cores, the oiled layer was sliced as a separate layer (~5 cm layer), followed by slicing the sediment in 3 cm (until 14 cmbsf), and 5-10 cm intervals (below 14 cmbsf). In case of the control cores, 3 cm sediment intervals were sliced until 9 cmbsf, followed by 5-15 cmbsf intervals below 9 cmbsf. The denitrification core was sliced in the following intervals: 0-5, 5-10, and 20-25 cm (oiled sediments), and 0-5 and 20-25 cm (control sediments).

First, a 3-ml subsample was taken from each depth of the porewater core for dissolved gas quantification (CH<sub>4</sub> and N<sub>2</sub>O) using a cut-off syringe. The subsample was transferred immediately into a 12-ml helium purged serum vial, containing 3 ml of 2M NaOH, and quickly sealed with a rubber stopper and a crimp. For determination of porosity, a 2-ml sediment subsample was transferred into a pre-weighed and pre-combusted glass vial. The remaining sediment was used for porewater extraction using a mechanical pore water press (Joye et al., 2010). Separate subsamples for nitrite (NO<sub>2</sub><sup>-</sup>) nitrate (NO<sub>3</sub><sup>-</sup> = NO<sub>x</sub> minus nitrite), ammonium (NH<sub>4</sub><sup>+</sup>), dissolved organic carbon (DOC), sulfate (SO<sub>4</sub><sup>2-</sup>), and sulfide (HS<sup>-</sup>) were obtained from the extracted porewater. Sample fixation and analysis of these parameters followed previously described methods (Joye et al., 2004). Porosity was determined by the change in weight after drying 2 ml sediment at 80°C for 48 hours. The dried sample was used to determine total particulate carbon (TPC) and organic carbon content following standard methods (Joye et al., 2010).

### **Microbial activity**

#### ***Sulfate reduction***

Rates of sulfate reduction were determined using standard radioisotope techniques. Triplicate modified Hungate tubes were filled with sediment and closed with a butyl rubber stopper at one end and with septa and cap on the other end (Bowles et al., 2011). Samples were amended with ultra-high purity methane gas to achieve a concentration of dissolved gas of 5 mM when samples were put under pressure. This methane amendment was done to check for the potential coupling between sulfate reduction and AOM under quasi *in situ* conditions (Bowles et al., 2011). Methane additions were made to all cores for both SR and AOM assays. During core recovery, methane is known to degass and since we do not know the methane concentration upon initiation of the rate assays, we amended all samples with the same methane concentration (5 mM, which is in the mid-range of *in situ*

measurements of methane concentration) making rates between different cores directly comparable. In parallel, AOM rates were determined by using  $^{14}\text{C-CH}_4$  radiotracer (see 2.3.2). After methane amendment, 100  $\mu\text{l}$  of slightly alkaline ultrapure water containing about 33 kBq of  $\text{Na}^{35}\text{SO}_4$  (specific activity 5 mCi/mmol) was injected through the septa into two sample tubes. A third tube was used as a control by injecting 3 ml of 20% (w/v) zinc acetate solution before radioisotope addition to halt microbial activity. All samples were incubated for 24 hours at *in situ* temperature (8 °C) and pressure (varied according to depth) using hydrostatic pressure vessels (Bowles et al., 2011). Termination of incubation and the first processing step was done according to Bowles et al. (2011). Samples were distilled via one-step chromous-acid reduction and the evolved total reduced inorganic sulfur (TRIS) was trapped in 5% zinc acetate (Canfield et al., 1986). The rate of sulfate reduction was calculated according to Eq. 1:

$$\text{SR Rate} = [\text{SO}_4^{2-}] \times \alpha_{\text{SO}_4} / t \times (\text{DPM TRIS} / (\text{DPM } ^{35}\text{SO}_4 + \text{DPM TRIS})) \quad \text{Eq. 1}$$

The sulfate reduction (SR) rate is expressed as  $\text{nmol cm}^{-3} \text{d}^{-1}$ .  $[\text{SO}_4^{2-}]$  is the sediment sulfate concentration in  $\text{nmol cm}^{-3}$ ,  $\alpha_{\text{SO}_4}$  is the isotope fractionation factor for sulfate reduction (1,06; Jørgensen, 1978),  $t$  is the incubation time in days (d), DPM TRIS is the activity of  $^{35}\text{S}$ -sulfide generated (minus activity in killed controls) and  $\text{DPM } ^{35}\text{SO}_4 + \text{DPM TRIS}$  is the total  $^{35}\text{S}$ -tracer activity injected.

#### **Anaerobic oxidation of methane (AOM)**

AOM rates were determined using standard radioisotope techniques. Triplicate modified Hungate tubes were filled with sediment and closed with a butyl rubber stopper at one end and with septa and cap on the other end (Bowles et al., 2011). For AOM, 100  $\mu\text{l}$  of dissolved  $^{14}\text{CH}_4$  tracer (ca. 3.3 kBq of tracer with a specific activity 25 mCi/mmol) in slightly alkaline milliQ water) was injected through the septa into two sample tubes. The activity of the tracer ( $^{14}\text{CH}_4$ ) added was determined beforehand by injecting 100  $\mu\text{l}$  directly into scintillation cocktail followed by liquid scintillation counting (Joye et al., 1999). A third tube was used as a control by injecting 3 ml of 2 M NaOH solution before radioisotope addition to halt microbial activity. Incubation conditions were the same as for sulfate reduction (see chapter 2.3.1). After incubation, samples were terminated by injecting 3 ml of NaOH (2M) into each tube, followed by transferring the sediment slurry to a 20-ml vial containing 10 ml of 2 M NaOH. After mixing the samples with the base the vials were frozen until further processing. The processing of the samples was done by acid digestion following the method of Joye et al., (2004). The AOM rate was calculated according to Eq. 2:

$$\text{AOM rate} = [\text{CH}_4] * \alpha_{\text{CH}_4} / t * (\text{DPM-}^{14}\text{CO}_2 / \text{DPM-}^{14}\text{CH}_4) \quad \text{Eq. 2}$$

The AOM rate is expressed as  $\text{nmol cm}^{-3} \text{ d}^{-1}$ .  $[\text{CH}_4]$  is the methane concentration ( $\text{nmol cm}^{-3}$ ),  $\alpha_{\text{CH}_4}$  is the isotope fractionation factor for AOM (1.06; (Alperin et al., 1988),  $t$  is the incubation time in days,  $\text{DPM-}^{14}\text{CO}_2$  is the activity of the product pool, and  $\text{DPM-}^{14}\text{CH}_4$  is the activity of the substrate pool.

### **Potential DNF rates**

For determination of denitrification rates, sediment samples were stored in argon-purged 50-ml centrifuge tubes at 4°C and used for laboratory experiments within 2-3 weeks of collection. Potential DNF rates were measured in the University of Georgia laboratory using the acetylene ( $\text{C}_2\text{H}_2$ ) inhibition method (Joye & Paerl, 1993).  $\text{C}_2\text{H}_2$  inhibits the nitrous oxide reductase enzyme, which catalyzes the transformation of  $\text{N}_2\text{O}$  to  $\text{N}_2$  in the denitrification pathway (Sørensen, 1978b). This inhibition causes the accumulation of  $\text{N}_2\text{O}$  in the sample in the presence of DNF activity.  $\text{N}_2\text{O}$  accumulation was quantified via electron capture gas chromatography (Joye & Paerl, 1994). Nitrate ( $150 \mu\text{M}$ ) was added to the incubations (Joye et al., 1996), to avoid nitrate limitation (Hynes & Knowles, 1978; Cornwell et al., 1999). Consequently, the reported DNF activity must be viewed as a potential rate.

In total, three treatments, each in triplicate, were prepared per depth: 1)  $T_0$  sediment slurry +  $150 \mu\text{M}$  nitrate; 2)  $T_{5\text{h}}$  sediment slurry +  $150 \mu\text{M}$  nitrate + acetylene; 3)  $T_{5\text{h}}$  sediment slurry +  $150 \mu\text{M}$  nitrate without acetylene. Sediment slurries were prepared under an Argon stream as follows: 3 g of sediment was weighed into a 36 ml serum glass vial and 10 ml of filtered ( $0.2 \mu\text{m}$ ), argon-purged bottom water was added and the phases were mixed using a spatula. After mixing, the vial was closed with a butyl rubber stopper and crimp-sealed. Then,  $100 \mu\text{l}$  of a  $19.6 \text{ mM}$  potassium-nitrate solution was added through the stopper to reach a final concentration of  $\sim 150 \mu\text{M}$  in the sediment slurry and the headspace was purged with Argon. One set of samples was amended with 10% acetylene per volume ( $3.6 \text{ ml}$ ) injected through the stopper (Joye & Paerl, 1993). After amendment, each bottle was shaken gently to mix the slurries.

$T_0$  slurries were terminated immediately after nitrate addition. One set of samples with acetylene and one set without acetylene were incubated for five hours at 4°C on a shaking table before stopping the incubation. Afterwards, vials were vigorously shaken to quantitatively force  $\text{N}_2\text{O}$  into the headspace. Then, 10 ml of headspace was removed by displacement with 10 ml anoxic  $2 \text{ mM}$  NaCl brine through the stopper. The gas sample was injected into a 10 ml vacutainer and stored until  $\text{N}_2\text{O}$  quantification (see below).

### **Analytical procedures**

For pore water  $\text{CH}_4$  gas analysis, 0.5 ml headspace sample was injected into a Shimadzu gas chromatograph equipped with a HayeSep D column (40°C) and a flame ionization detector. Helium

was used as a carrier gas. Certified standard gas mixtures (Scott Specialty Gases) served for calibration of the methane signals, thereby achieving analytical precision of <1%.

After CH<sub>4</sub> analysis the same vials were used for N<sub>2</sub>O gas analysis. For this, a Shimadzu gas chromatograph (Shimadzu-GC-8A) with an electron capture detector (300°C) was used. A gas volume of ~ 5 ml was removed from the vacutainer by displacement with anoxic brine and then flushed through into a 3 ml sample loop. N<sub>2</sub>O was separated on a HayeSep DB column (80°C) and Ultrapure CH<sub>4</sub> (5%) in Argon was used as carrier gas. N<sub>2</sub>O concentration was quantified in comparison to purified gas standards (Scott Specialty Gases). The analytical precision (<1%) was based on repeated analysis of gas standards.

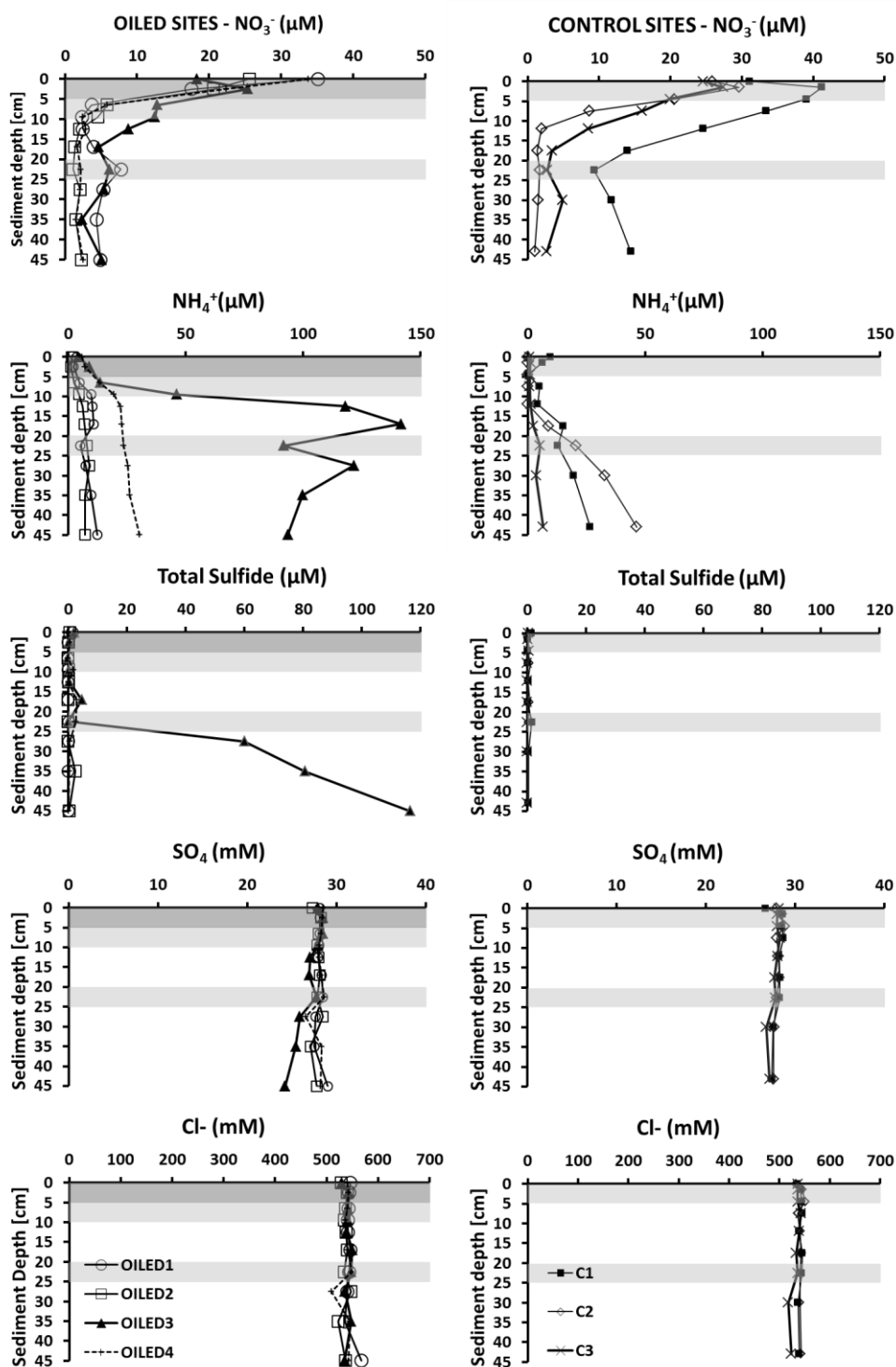
### **Statistical analysis**

For statistical analysis of the microbial activity the free statistic program R (source: R-project) was used. For comparison of rates from oily sites and control sites either the parametric Welch two sample t-test or the non-parametric Wilcoxon rank sum test (if normal distribution was not fulfilled) was used. Please note that for comparison of sulfate reduction OILED1, and for the comparison of denitrification OILED4 were not included due to missing data.

## **Results**

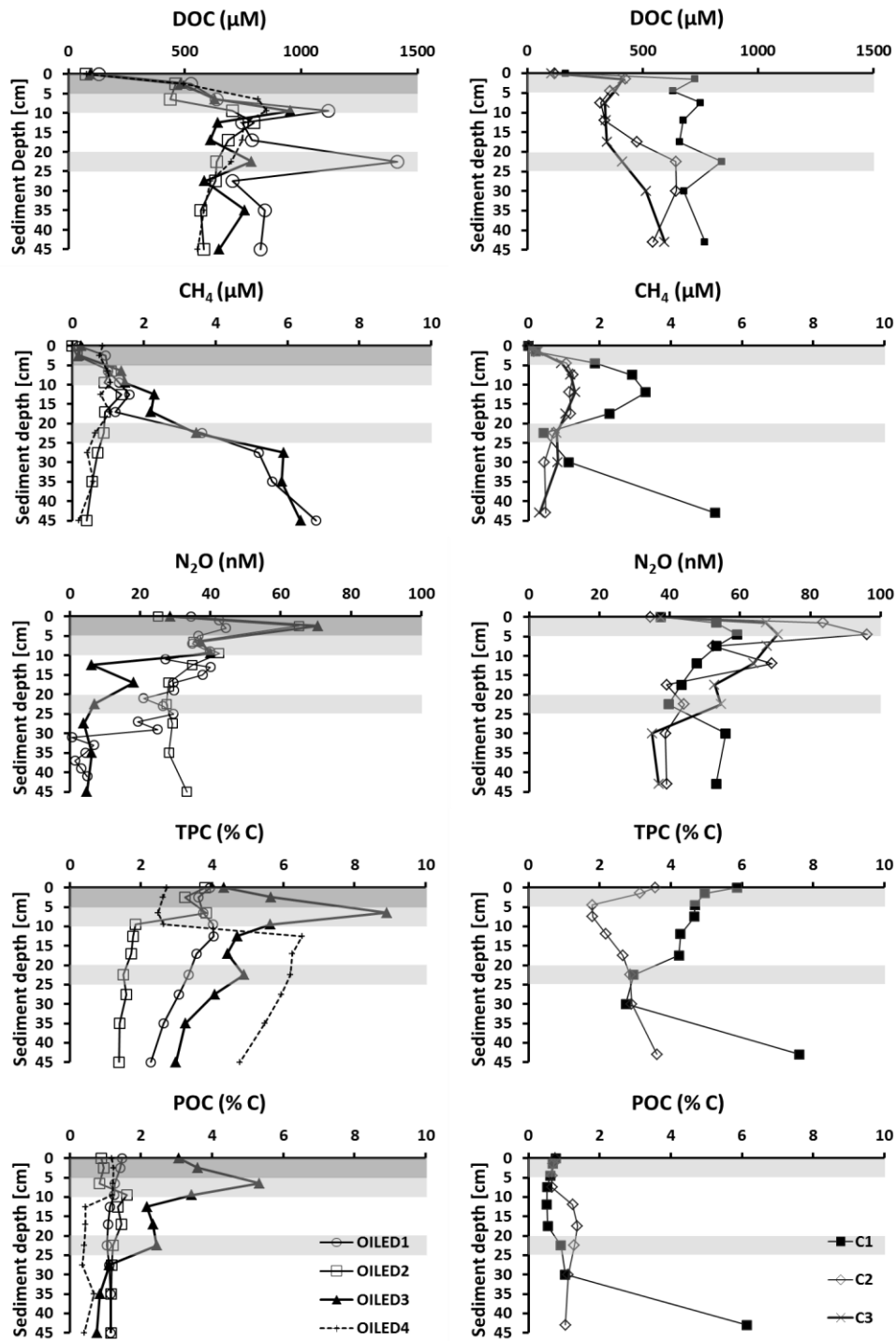
### **Geochemical parameters**

Porewater profiles of oiled and control stations are shown in Fig. 3. Because porewater nitrite concentrations were below detection limit (<1 µM), data are not shown. Porewater nitrate (NO<sub>3</sub><sup>-</sup>) concentrations data at OILED1, OILED2 and OILED4 showed a strong decrease (~80 %) in NO<sub>3</sub><sup>-</sup> concentration from 0 to 10 cmbsf (Fig. 3), afterwards values remained around 2 µM throughout the core at OILED2 and OILED4, while OILED1 showed a sub-surface peak of 8 µM at about 23 cmbsf. At OILED3, NO<sub>3</sub><sup>-</sup> concentration increased in the upper 3 cmbsf from 18 to 25 µM, followed by a decrease to a minimum of 3 µM at 35 cmbsf. At the control sites C1, C2 and C3, nitrate increased between the overlying water and 2 cmbsf and then decreased. The decrease of NO<sub>3</sub><sup>-</sup> concentration was only about 5-30% in the first 5 cmbsf of the control cores.



**Figure 3:** Porewater profiles of nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), total sulfide, sulfate ( $\text{SO}_4$ ), and chloride ( $\text{Cl}^-$ ) at oiled sites (left) and control sites (right). Grey bars mark the sediment layers investigated for denitrification activity; dark grey bars mark the oiled layer at the oily stations (0-5 cm). Data points from the overlying water of the core (OLW) are set to 0 cm.





**Figure 3 continued:** Porewater profiles of dissolved organic carbon (DOC), dissolved methane ( $\text{CH}_4$ ), nitrous oxide ( $\text{N}_2\text{O}$ ), total particulate carbon (TPC) and particulate organic carbon (POC) at oiled sites (left) and control sites (right). Grey bars mark the sediment layers investigated for denitrification activity, dark grey bars mark the oiled layer at the oily stations. Data points from the overlying water of the core (OLW) are set to 0 cm.

Ammonium ( $\text{NH}_4^+$ ) concentration in the oiled cores was low at the surface (1- 5  $\mu\text{M}$ ) but increased slightly with depth at OILED1 and OILED2 (maximum concentration 13 and 9  $\mu\text{M}$ , respectively; Fig. 3). At OILED3, a sharp  $\text{NH}_4^+$  increase from 1 $\mu\text{M}$  to about 150  $\mu\text{M}$  was observed between 0 and 17 cmbsf. At OILED4,  $\text{NH}_4^+$  concentrations increased until 10 cmbsf (22  $\mu\text{M}$ ), followed by a less sharp increase until the end of the core (30  $\mu\text{M}$ ). At the control sites,  $\text{NH}_4^+$  increased with depth in all 3 cores. The highest concentration at the control sites was seen at C2 with 46  $\mu\text{M}$  at 43 cmbsf.

In the oiled cores sulfide was elevated only at OILED3 (45 cmbsf, 120  $\mu\text{M}$ ); the other three cores showed much lower sulfide concentrations in the range of 0-2  $\mu\text{M}$  throughout the core. In the control cores, sulfide concentration was largely undetectable (C3), or ranged between 0-1  $\mu\text{M}$  (C1, C2).

At the oiled sites, OILED3 was the only sediment that showed a slight decrease in sulfate (from 28 to 24 mM) over depth. OILED1, OILED2 and OILED4 revealed stable sulfate profiles with values around 28 mM. C1, C2, and C3 revealed stable sulfate profiles with values around 28 mM throughout the core. Chloride ( $\text{Cl}^-$ ) profiles ranged around 540 mM throughout the core at all oiled and control stations.

Dissolved organic carbon (DOC) was low in the over lying water ( $\sim 100 \mu\text{M}$ ) and increased with sediment depth at OILED1 and OILED3 (1100 and 950  $\mu\text{M}$ , respectively, at 10 cmbsf) followed by a decrease and a second peak at 23 cmbsf at both sites (1450 and 790  $\mu\text{M}$ , respectively). At OILED2, DOC concentration increased until 13 cmbsf (800  $\mu\text{M}$ ), and decreased thereafter (570  $\mu\text{M}$ ). At OILED4, DOC concentration increased until 10 cmbsf (850  $\mu\text{M}$ ) but then decreased until the end of the core (550  $\mu\text{M}$ ). At all control stations, DOC concentration increased with depth. DOC concentrations ranged between 400 and 700  $\mu\text{M}$  in the first 5 cmbsf. At C1 and C2 the highest concentration was reached at 23 cmbsf (840  $\mu\text{M}$  and 640  $\mu\text{M}$ , respectively), at C3 at 45 cmbsf (595  $\mu\text{M}$ ).

Methane profiles at the oiled sites were similar in OILED1 and OILED3 showing increasing concentrations with depth, i.e., from non-detectable at the surface to 6-7  $\mu\text{M}$  at 45 cmbsf. At OILED2 and OILED4, methane was undetectable at the surface and reached only 1  $\mu\text{M}$  at  $\sim 7$  cmbsf and  $\sim 10$  cmbsf, respectively. At control sites, methane concentration was undetectable at the sediment surface, but increased until 13 cmbsf in all cores reaching  $\sim 1\mu\text{M}$  in C2 and C3 and  $\sim 3 \mu\text{M}$  in C1. Then methane concentration decreased in C2 and C3 until the end of the core. In C1, methane concentration decreased until 23 cmbsf (0.4  $\mu\text{M}$ ), followed by an increase with the maximum  $\text{CH}_4$  concentration at the bottom of the core ( $\sim 5 \mu\text{M}$ , Fig. 3 continued).

At the oiled sites, the maximum  $\text{N}_2\text{O}$  concentration occurred in the top 0-5 cmbsf and ranged between 60 - 80 nM at OILED2 and OILED3 and was  $\sim 44$  nM at OILED1. No  $\text{N}_2\text{O}$  data are available for OILED4. At OILED1 and OILED3, the  $\text{N}_2\text{O}$  concentration decreased with depth to less than 10 nM. OILED2 showed a second peak at 10 cmbsf (43 nM) followed by a decrease to about 30 nM at 18 cmbsf. The

N<sub>2</sub>O concentration remained around 35 nM until the deepest depth of the core. At the control sites, N<sub>2</sub>O concentrations in the surface layer (0-5 cmbsf) ranged between 56 and 96 nM with C2 showing the highest concentration at 5 cmbsf (96 nM). At C2 and C3, N<sub>2</sub>O concentration decreased with depth to a minimum of about ~38 nM at 45 cmbsf. At C1, N<sub>2</sub>O concentration decreased below 5 cmbsf until 23 cmbsf, showing a second peak at 30 cmbsf (56 nM).

The total particulate carbon (TPC) concentration increased with depth at OILED3 and OILED4 with peak TPC concentration at 7 cmbsf (9% C) and at 13 cmbsf (6.5 %C), respectively, after which concentration decreased until the bottom of the core. At OILED2, TPC concentration varied between 3-4 %C in the upper 6 cmbsf, then decreased sharply at 10 cmbsf to 2 %C, and then further decreased to ~1 %C until the bottom of the core. At OILED1, TPC concentration slightly decreased in the upper 3 cmbsf, then increased to the maximum concentration at 13 cmbsf (4 % C), after which TPC concentration decreased again until the end of the core. Surface TPC concentration at oiled stations 1-3 was similar (~ 4 %C). Only OILED4 showed a slightly lower TPC concentration at 0-5 cmbsf (~ 3 %C). At the control sites, TPC data are only available for C1 and C2. At both control sites, TPC concentration decreased in the upper 0- 5 cmbsf (to ~5%C in C1 and to ~2 %C in C2). At C2, TPC concentration increased with depth below 5 cmbsf reaching the highest measured TPC concentration at the end of the core (3.6 %C). C1 showed TPC concentration higher than 5 %C at the surface (6 %C). Here, concentrations of TPC decreased until a depth of 30 cmbsf (3 %C), followed by an increase until 43 cmbsf (8 %C).

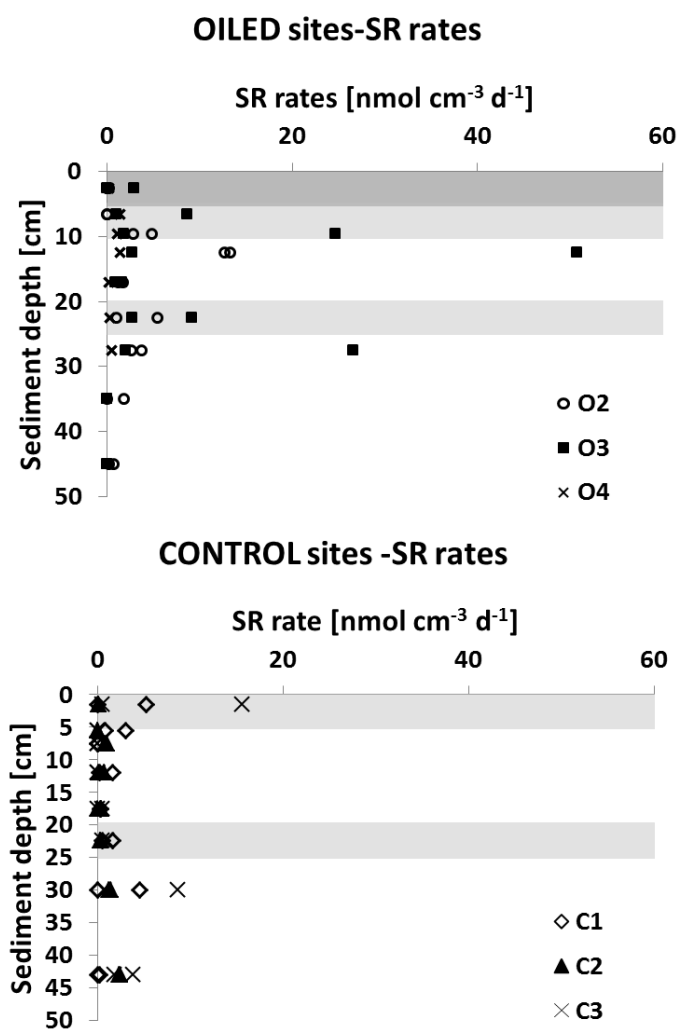
POC (particulate organic carbon) represents the organic proportion of TPC. At OILED1, POC content was 1%C (= 30-40% of TPC) in the top 5 cmbsf and stayed around 1 %C throughout the core. At the deepest depth (45 cmbsf), the highest organic content in TPC was detected (50%). At OILED2, POC increased until a depth of 10 cmbsf (2 %C = 87% of TPC), followed by slight decrease until the end of the core (~1 %C= 84% of TPC). At OILED3, the POC profile followed TPC, with two peaks in the 5-10 cmbsf horizon and in the 20-25 cmbsf horizon (5 and 2 %C, respectively). The organic percentage of TPC decreased with depth, showing the highest organic content at the surface (71%) and the lowest at the deepest depth (26%). At OILED4, POC concentration stayed around ~1 %C (43-49% of TPC) in the upper 10 cmbsf, followed by a decrease to ~ 0.4 %C until ~28 cmbsf (~ 7% of TPC). A second POC peak of 0.7 %C (=12% of TPC) was detected at 35 cmbsf. At the control sites, POC increased with depth in C1 and C2. Again, no POC data are available for C3. C1 showed the highest measured POC concentration of all control sites at the deepest depth (43 cmbsf) with 6 %C (=81% of TPC). POC concentration in C2 increased to 1.4 %C (52% of TPC) at a depth of 18 cmbsf, followed by a decrease to 1 %C (29% of TPC) at the end of the core.

### **Sediment sulfate reduction rates**

Benthic sulfate reduction rates are shown in Fig. 4 and were determined at regular intervals between 0 and the deepest depth, 50 cmbsf. No data are available for OILED1 core. Maximum sulfate reduction rates were on average 2-3 times higher in oiled (13-50  $\text{nmol cm}^{-3} \text{d}^{-1}$ ) compared to control sediments (5-15  $\text{nmol cm}^{-3} \text{d}^{-1}$ ). At oiled sites, sulfate reduction increased with depth to a maximum at about 13 cmbsf in OILED2 and OILED3 (13.3 and 50.7  $\text{nmol cm}^{-3} \text{d}^{-1}$ , respectively). While duplicates in OILED2 showed similar sulfate reduction activity at 13 cmbsf (13.3 and 12.7  $\text{nmol cm}^{-3} \text{d}^{-1}$ ), duplicates in OILED3 showed a high variation in activity from 2.7  $\text{nmol cm}^{-3} \text{d}^{-1}$  to 50.7  $\text{nmol cm}^{-3} \text{d}^{-1}$  at this depth. Below 13 cmbsf, activity decreased with depth in both oiled sites. OILED3 showed a second peak in sulfate reduction at ~28 cmbsf reaching 26.6  $\text{nmol cm}^{-3} \text{d}^{-1}$  in one replicate. Again, high variation between replicates was observed at that depth (26.6 and 2.0  $\text{nmol cm}^{-3} \text{d}^{-1}$ ). OILED4 showed highest rates below the oiled layer in the 5-8 cmbsf horizon (2.1  $\text{nmol cm}^{-3} \text{d}^{-1}$ ), which then decreased with depth until the end of core.

The control sites showed a different pattern in the sulfate reduction profile. C1 revealed a peak at the surface layer (replicate 1: 2 cmbsf, 5.2  $\text{nmol cm}^{-3} \text{d}^{-1}$ , replicate 2: 0  $\text{nmol cm}^{-3} \text{d}^{-1}$ ) and at 30 cmbsf (replicate 1: 4.6  $\text{nmol cm}^{-3} \text{d}^{-1}$ , replicate 2: 0  $\text{nmol cm}^{-3} \text{d}^{-1}$ ). C2 revealed a peak at 8 cmbsf (replicate 1: 1.0  $\text{nmol cm}^{-3} \text{d}^{-1}$ , replicate 2: 0.84  $\text{nmol cm}^{-3} \text{d}^{-1}$ ) followed first by a decrease until 18 cmbsf and second by an increase with depth to a maximum rate of 2.4  $\text{nmol cm}^{-3} \text{d}^{-1}$  and 2.3  $\text{nmol cm}^{-3} \text{d}^{-1}$  at the end of the core (43 cmbsf). Replicate values were similar at all depths at C2. C3 revealed the highest activity at the surface (replicate 1: 15.6  $\text{nmol cm}^{-3} \text{d}^{-1}$ , replicate 2: 0.5  $\text{nmol cm}^{-3} \text{d}^{-1}$ ), followed by a second peak at 30 cmbsf (replicate 1: 8.7  $\text{nmol cm}^{-3} \text{d}^{-1}$ , replicate 2: 0.7  $\text{nmol cm}^{-2} \text{d}^{-1}$ ).

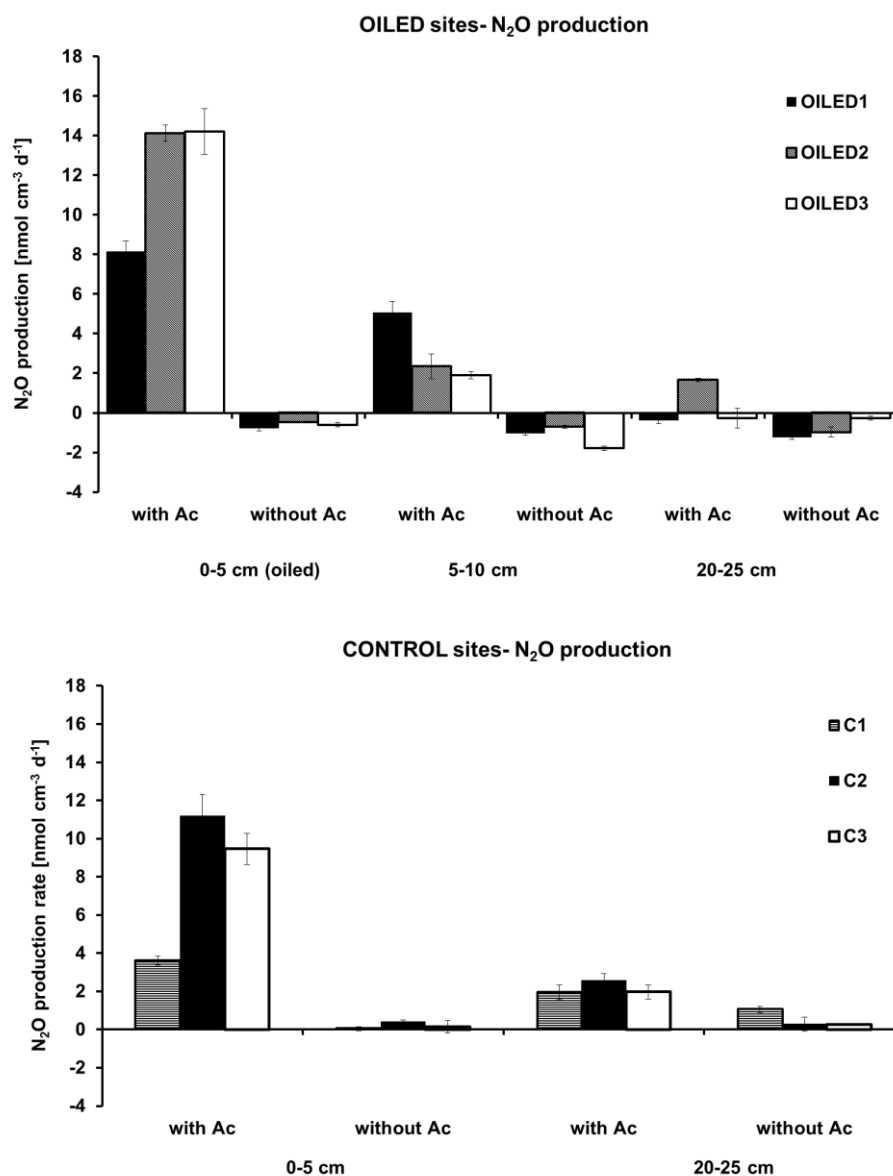
The top layers 0-5 and 5-10 cm were of specific interest in this study with regard to the potential influence of sedimented weathered oil. Statistical analysis revealed no significant difference between areal sulfate reduction rates in the surface layer (0-5 cmbsf) of oiled versus control sites which is likely caused by the high variability between replicates. Interestingly, areal sulfate reduction rates integrated over 5-10 cmbsf were significantly higher at oiled sites (0.19 +/- 0.16  $\text{mmol m}^{-2} \text{d}^{-1}$ ,  $p < 0.05$ ) compared to control sites (0.02 +/- 0.01  $\text{mmol m}^{-2} \text{d}^{-1}$ ). In addition, areal sulfate reduction rates integrated over 5-10 cmbsf at the oiled sites were significantly higher (0.19 +/- 0.16  $\text{mmol m}^{-2} \text{d}^{-1}$ ,  $p < 0.05$ ) compared to the surface rates at the oiled sites integrated over 0-5 cmbsf (0.03 +/- 0.05  $\text{mmol m}^{-2} \text{d}^{-1}$ ) but rates were not significantly different compared to the surface rates integrated over 0-5 cmbsf at control sites (0.12 +/- 0.17  $\text{mmol m}^{-2} \text{d}^{-1}$ ,  $p > 0.05$ ). At control sites, areal sulfate reduction rates between 0-5 cmbsf and 5-10 cmbsf showed no significant difference ( $p > 0.05$ ).



**Figure 4:** Sulfate reduction (SR) rates at the oiled sites (A) and control sites (B), duplicates are shown per depth. Sediment horizon investigated for denitrification are marked with grey bars, the oiled layer is marked with a dark grey bar.

### Potential denitrification rates

$\text{N}_2\text{O}$  production rates for the treatments with and without acetylene addition were calculated from the change in  $\text{N}_2\text{O}$  concentration measured in the headspace (Fig. 5). No data are available for OILED4. It should be noted that the  $\text{N}_2\text{O}$  production in the treatments with acetylene addition is equivalent to the potential denitrification rate, while in treatments without acetylene addition the production is equivalent to background  $\text{N}_2\text{O}$  production such as that resulting from exposure to an excess of substrate (here: nitrate, (Hutchinson & Davidson, 1993) or from sulfide-inhibition (Joye, 2002)).

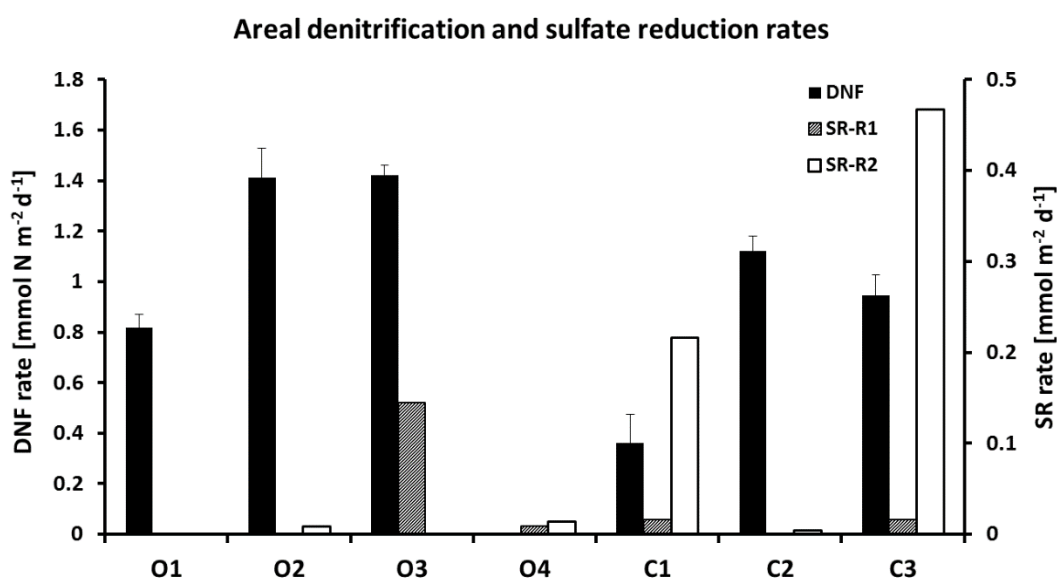


**Figure 5:** N<sub>2</sub>O production rates with acetylene (Ac) (equals potential denitrification rate) and without addition of acetylene from oiled sites (A) and control sites (B). Shown are means from triplicates with standard deviation.

At all three oiled sites, potential denitrification rates in the acetylene treatments decreased from shallow to deep sediment layers. Rates were 4-40 times higher in the oiled layer (0-5 cm) compared to the layer beneath it (5-10 cmbsf), or to the deepest measured horizon (20-25 cmbsf) (12.2, 3.1 and 0.3 nmol cm<sup>-3</sup> d<sup>-1</sup>, respectively). OILED3 showed the lowest potential denitrification rate at 0-5 cmbsf of all three oiled sites (8.2 nmol cm<sup>-3</sup> d<sup>-1</sup>). At 20-25 cmbsf, OILED2 (and in part OILED3, see standard deviation) showed potential denitrification, while at OILED1 and in part at OILED3 N<sub>2</sub>O was consumed in the acetylene treatments. In the treatments without acetylene, N<sub>2</sub>O was consumed at all oiled sites at rates between 0.3 and 1.8 nmol cm<sup>-3</sup> d<sup>-1</sup>. Except for OILED3, the consumption was higher at 5-10

cmbsf and 20-25 cmbsf compared to the oiled layer (0-5 cmbsf). At OILED3, the  $\text{N}_2\text{O}$  consumption was lowest at the 20-25 cmbsf sediment horizon. At all control sites, potential denitrification rates in the acetylene treatments were higher in the 0-5 cmbsf horizon compared to the 20-25 cmbsf horizon (Fig. 5). The highest potential denitrification rate was observed at C2 at 0-5 cmbsf with  $11.2 \text{ nmol cm}^{-3} \text{ d}^{-1}$ . Potential denitrification rates were in a similar range around  $2.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$  ( $1.9\text{-}2.6 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) in the 20-25 cmbsf horizon at all control sites. Interestingly, low  $\text{N}_2\text{O}$  production (ranging from  $0.1$  to  $1.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) was detected in all control treatments (=without acetylene addition) in both sediment horizons (0-5 cmbsf, 20-25 cmbsf). In the 0-5 cmbsf horizon without acetylene addition, C2 showed a  $\text{N}_2\text{O}$  production rate of  $0.4 \text{ nmol cm}^{-3} \text{ d}^{-1}$  while C1 and C3 revealed  $\text{N}_2\text{O}$  production rates of only  $0.04$  and  $0.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , respectively. In the 20-25 cmbsf horizon without acetylene addition, C1 showed the highest the  $\text{N}_2\text{O}$  production rate ( $1.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$ ) while C2 and C3 showed  $\text{N}_2\text{O}$  production rates 2-3 times lower ( $0.3$  and  $0.5 \text{ nmol cm}^{-3} \text{ d}^{-1}$ , respectively).

Areal potential denitrification rates integrated from 0-5 cmbsf are shown in Fig. 6. All seven sites revealed denitrification activity in the upper sediment layer. While potential denitrification rates ranged between  $0.8$  and  $1.4 \text{ mmol N m}^{-2} \text{ d}^{-1}$  at the oiled sites, potential denitrification rates at control sites ranged between  $0.4$  and  $1.1 \text{ mmol N m}^{-2} \text{ d}^{-1}$ . Statistical analysis revealed significantly higher areal potential denitrification rates in the surface oiled layer compared to the deeper layers (5-10 cmbsf and 20-25 cmbsf,  $p\text{-value} < 0.05$ ) and compared to surface control sediments ( $p\text{-value} < 0.05$ ).



**Figure 6:** Areal rates of potential denitrification (DNF) and sulfate reduction (SR) integrated over 0- 5 cm sediment depth. Oiled sites are marked with O1, O2, O3 and O4. Shown are mean DNF rates (triplicates) with standard deviation. Duplicates of SR-rates are shown in separate bars (SR-R1 and SR-R2).

## Discussion

To our best knowledge, this is the first documentation of the response of sediment microbial activity, namely sulfate reduction and denitrification, to weathered oil sedimentation. We examined sediments covered with weathered oil from the Deepwater Horizon (DWH) oil spill and from the natural seepage site GC600 and compared them to control sediments without oil influence. Our study revealed: 1) low to non-detectable sulfate reduction activity within the oiled layer, 2) significantly higher sulfate reduction activity in the layer beneath the oiled layer (5-10 cmsbf) compared to the oiled layer, 3) significantly higher sulfate reduction activity in the layer beneath the oiled layer (5-10 cmsbf) compared to 5-10 cmsbf of the control sites, 4) significantly higher potential denitrification rates in the oiled surface layer compared to control sites, 5) low porewater sulfide concentrations at most sites and, 6) highest porewater  $N_2O$  concentrations at control sites. The different aspects of the porewater biogeochemistry, the microbial activity patterns observed, and the potential of elevated  $N_2O$  production in those types of sediments are discussed below.

### Porewater biogeochemistry in oiled sediments

The porewater components and their concentrations at the control sites were similar to other deep-sea sites without oil influence in the northern Gulf (Joye et al., 2004, 2010). We are therefore confident that these sediments reflect sites lacking active advection of hydrocarbon. The concentrations of typical seep porewater constituents like  $CH_4$  and sulfide were not high at the oiled sites, as would be expected for natural seep sediments (Joye et al., 2004, 2010). Thus, the presence of a surface oil-containing layer can only be explained by the fact that the oil did not seep from below but instead sedimented onto the seafloor from above (Joye et al., 2014; Valentine et al., 2014; Passow, this issue; Joye et al., submitted). Despite the visible confirmation of oil presence in our cores, biogeochemical analyses showed distinct differences between oiled cores.

Sediment from the OILED3 site featured elevated concentrations of ammonium ( $NH_4^+$ ), sulfide (the sum of  $H_2S$ ,  $HS^-$  and  $S_2^-$ ), dissolved organic carbon (DOC), methane ( $CH_4$ ), total particulate carbon (TPC), and particulate organic carbon (POC). Although this site featured no hydrocarbon seepage, it did lie near (ca. 1 km) a very active hydrocarbon seep (GC600, Joye et al., 2014), known for oil-, gas- and brine-flows (Brooks et al., 2008), but far west of the area impacted by the DWH oil spill (> 200 km away from DWH wellhead). Porewater chloride data confirm that OILED3 did not have contact with the actual seep but showed only surface oil contamination. Interestingly, enormous surface oil slicks are spotted frequently in the area of GC600 (<http://bit.ly/1xIS4DK>; Joye et al., submitted). Formation of marine snow after an accidental discharge (e.g. DWH oil incident) has been described and is probably a main cause for the transport of the spilled oil to the seafloor (Joye et al., 2014; Passow et



al., 2012; Passow, this issue). The same mechanism could consequently also transport oil from smaller-scale slicks formed at GC600 to the seafloor at OILED3. Oil composition and weathered status differ at OILED3 compared to the other three oiled sites (Joye et al., submitted). Due to the natural discharge of oil at GC600 and the frequent surface oil slicks observed, the oiled layer at OILED3 is most likely less weathered and thus more bioavailable for microbial degradation. Enhanced microbial activity at OILED3 would explain elevated porewater  $\text{NH}_4^+$  and sulfide concentrations, which could result from dissimilatory nitrate reduction to ammonium (DNRA) and sulfate reduction activity (as confirmed in the present study, see section "Sulfate reduction activity in oiled vs. control sediments"), respectively. In comparison, porewater constituents (e.g.  $\text{HS}^-$ ,  $\text{NH}_4^+$  etc.) were lower at OILED1, OILED2, and OILED4, but still showed high DOC and POC concentrations in the oiled surface layer. In general, it has been observed that seep sediment POC content is substantially higher than at non-seep sites in the northern Gulf (~1%, (Kennicutt & Brooks, 1990; Goñi et al., 1998). We observed 1-5% POC concentrations at oiled sites showing the difference between control sites and oiled sites. As highlighted above, differences in porewater geochemistry between OILED3 and the other three oiled sites (OILED1, 2, and 4) might be due to the proximity of OILED3 to an active natural oil source. In OILED2 and OILED4 the oiled layer most likely originated from the DWH oil spill (~7 km and ~12 km away, respectively), because sediments even in a good distance (up to 40 km) from the DWH wellhead showed oil contamination (Liu et al., 2012; Montagna et al., 2013; Joye et al., 2014; Valentine et al., 2014). At OILED1 the oiled layer was probably a mixture of oil sedimented from the DWH oil spill (~16 km distance) and the hydrocarbon seep MC118 (~1 km distance). The partial influence of a natural seep in OILED1 is indicated by the elevated  $\text{CH}_4$  concentrations compared to OILED 2 and OILED4. The oiled layer at OILED1 (in part), OILED2 and OILED4 was probably up to ~2 years old according to the timing of the DWH oil spill (between April and July 2010). We therefore conclude that the sedimented oil was in a highly weathered condition and was therefore less bioavailable to the microbial community.

### **Sulfate reduction activity in oiled vs. control sediments**

Sulfate reduction samples in the present study were spiked with 5 mM methane, to account for the fact that degassing of methane occurred during core retrieval. This methane concentration was higher than the natural  $\text{CH}_4$  concentrations (Fig. 3), but it was chosen to check for the full potential of coupling between sulfate reduction and anaerobic methane oxidation (AOM) in those types of sediments. AOM rates were undetectable or 10-1000 times lower compared to sulfate reduction (Supplementary Material, Fig. S1), thus coupling of sulfate reduction to AOM is of minor importance in these sediments. In the following discussion, we therefore neglect AOM as a source for the observed sulfate reduction activity.

Sulfate reduction rates of the control stations (integrated over a depth of 12 cm for comparison, 0.02-0.47 mmol m<sup>-2</sup> d<sup>-1</sup>) were in the range of other Gulf sediments without seepage (integrated over similar depths, (Aharon & Fu, 2000; Joye et al., 2004, 2010). Sulfate reduction rates in the oiled sediments (integrated over a depth of 12 cm, 0.11-0.94 mmol m<sup>-2</sup> d<sup>-1</sup>) were up to four orders of magnitude lower compared to oil-containing (oil stains throughout the core), hydrocarbon-rich sediments from active Gulf seeps (integrated over similar depths, 5.6 -244.3 mmol m<sup>-2</sup> d<sup>-1</sup>; (Joye et al., 2004, 2010; Orcutt et al., 2010). It is worth noting that these rates were obtained from areas of active hydrocarbon discharge; none of the oiled cores in the present study were from areas of active discharge. Microbial activity in the investigated sediments was thus not fueled by a steady seepage of substrates, which explains lower sulfate reduction rates.

In the following, we will focus on the areal sulfate reduction rates of the surface layer (0-5 cmbsf) and the layer beneath it (5-10 cmbsf) in oiled and control cores to actually determine if the sedimented oiled material had an effect on sulfate reduction activity. Interestingly, not the oiled layer but the visually oil-free layer beneath it (5-10 cmbsf) showed higher sulfate reduction rates in the oiled cores. The rates were (1) significantly higher than in the surface oiled layer ( $p < 0.05$ ) and (2) significantly higher compared to the equivalent depth interval at the control sites ( $p < 0.05$ ). Because there was no significant difference observed in the control cores between 0-5 cmbsf and 5-10 cmbsf ( $p > 0.05$ ), we are confident that the sulfate reduction activity at 5-10 cmbsf in the oiled cores was influenced by the oiled layer.

The fact that the sulfate reducing activity was not different in the actual oiled layer compared to the respective surface layer of the control cores could arise from different explanations. First, the sulfate reducing community had not yet established within the oiled layer as a result of the fast sedimentation pulse of the “oil snow” after the DWH oil spill. However, increased proportions of sulfate-reducing Deltaproteobacteria were reported in sediments near the well head (0.5 km and 2.7 km) in sediment depths of 1.5 -3 cmbsf in September and October 2010 (Kimes et al., 2013). This might not account for the samples in the presented study, as sampling was conducted further away from the well head (> 7 km). Another reason for the lower sulfate reduction rates within the oiled layer could be the weathered status of the sedimented oil material. During the weathering process the most biodegradable substrates (e.g. n-alkanes) are consumed first, leaving those fractions, which are more refractory to microbial attack (aromatic or long chained hydrocarbons; Colwell & Walker, 1977). The DWH Macando oil contained up to 10% polar components, which are naturally highly resistant to biodegradation (Reddy et al., 2012). Sulfate reducers mostly use short-chain fatty acids (acetate, formate etc.) and other small molecules (like H<sub>2</sub>) that are produced from the degradation and fermentation of organic matter (Jørgensen, 2006). It has been shown that sulfate reducers can oxidize

more complex molecules like n-alkanes, toluene and benzene (Heider et al., 1998; Rueter et al., 1994), however, the energy gain of toluene oxidation is low and relatively low growth rates are observed (Heider et al., 1998), thus using smaller molecules is more feasible for them. Hence, instead of sulfate reducing bacteria rather fermenting bacteria might dominate within the oiled layer, providing substrates for the sulfate reducers below. A third possibility for elevated sulfate reduction below the oiled layer could be decaying macrobenthic organisms. In a recent study, sedimented DWH oil stimulated benthic microbial hydrolysis at 0-2 cm sediment depth at sites proximate to the DWH wellhead (<10 km), when compared to deeper sediment layers (Ziervogel et al., this issue). But compared to other, oil-free deep-sea environments, only moderate stimulation of microbial metabolic rates was observed (Ziervogel et al., this issue). The authors concluded that the sedimented oil-snow material was unsuited to promote greatly elevated levels of microbial activity and growth (Ziervogel et al., this issue).

The enhanced microbial activity in the water column during the initial phase of the DWH oil spill (Hazen et al., 2010; Valentine et al., 2010) probably lead to less bioavailable material which then sedimented down. Only some cores showed higher microbial metabolic rates at sediment depths > 2 cm. The reason for that was seen in the harmful effect of the oil sedimentation on macrobenthic organism (e.g. dead polychaetes), whose decaying biomass would then stimulate below-surface bacterial activities by releasing organic carbon (Ziervogel et al., this issue). However, as we did not observe any dead (or living) macrobenthic organisms during sampling in our study, we exclude this organic source and conclude that rather the quality of the weathered oil material and/or the delayed enrichment of sulfate reducers slowed sulfate reduction activity. Slow oil degradation was also observed in sediments adjacent to the DWH wellhead (within 2-6 km) one year after the oil spill documented by the slight to moderate degradation of petroleum hydrocarbons (Liu et al., 2012). This observation agrees with low sulfate reduction activity observed within the oiled layer.

The oiled site OILED3 showed by far the highest sulfate reduction rates in the 0-5 cmbsf horizon (~10 times higher) as well as in the 5-10 cmbsf horizon (3-10 times higher) compared to the other 2 oiled sites (OILED2 and OILED4). This higher sulfate reduction activity could be a result of less weathered oil, which sedimented in the area of GC600 (Joye et al., submitted; see also discussion above); hence, more bioavailable substrates for microbes were present in the oil (Colwell & Walker, 1977). OILED3 was also the only site of elevated sulfide concentration starting at a depth of 23 cmbsf, which can be explained by the higher sulfate reduction activity. OILED3 showed peak of sulfate reduction at 13 cmbsf and 27 cmbsf (Fig. 4), which correlated with the sulfide increase and sulfate decrease in the porewater profiles (Fig. 3). Interestingly, low to no sulfide could be detected in the surface sediments (< 13 cmbsf) of OILED3. An explanation could be that sulfide was consumed before it reached the

sediment surface. DNRA by sulphur-oxidizing bacteria like *Beggiatoa* is a known sulfide-consuming process, in which nitrate or oxygen is used to oxidize sulfide (Preisler et al., 2007). We did not observe a visible mat of sulphur-oxidizing bacteria at the sediment surface, but *Beggiatoa* can occur in upper sediment layers without forming obvious mats (Jørgensen & Nelson, 2004). Recently it has been discovered that sulfide oxidizers of the family Desulfobulbaceae are able to form filaments (up to 1.5 cm length), which can act as electron transporters to overcome the spatial separation between the oxygenated surface layer and the anoxic sediment part where sulfide is produced (Pfeffer et al., 2012). Besides bacterial sulfide consumption also chemical processes could remove sulfide, e.g. through the precipitation with  $\text{Fe}^{2+}$ -ions or oxidation by Fe (III) (Jørgensen & Nelson, 2004; Preisler et al., 2007).

### **Potential denitrification activity in oiled vs. control sediments**

The applied acetylene block technique is a widespread method to determine denitrification rates (Sørensen, 1978b; Yoshinari et al., 1977). This method provides a reliable proxy of potential denitrification when nitrate is not limiting and sulfide concentrations are low. The appeal of this method is that it is applicable to a large number of samples and is not as cost- or labor- intensive as stable isotope tracer methods. Here, samples were spiked with 150  $\mu\text{M}$  nitrate to prevent nitrate limitation and have thus to be regarded as potential denitrification rates. Sulfide was low or undetectable in most of the stations (except for OILED3, Fig. 3), so an alleviation of the acetylene inhibition by sulfide was unlikely.

In all control sites the 0-5 cmbsf horizon showed higher potential denitrification rates compared to the 20-25 cmbsf horizon, which can be explained by the higher DOC and TPC concentrations at the surface.

At all oiled sites, potential denitrification rates were highest in the oiled layer compared to the deeper sediment layers (5-10 cmbsf and 20-25 cmbsf), probably due to high availability of electron donors. Even though the presented sediments were not affected by direct oil seepage, the sedimented oil material provided additional organic matter as indicated by the DOC, TPC and POC contents (Fig. 3). Nitrate-reducing bacteria have been demonstrated to degrade petroleum hydrocarbons including alkanes and aromatic hydrocarbons (Alain et al., 2012; Grishchenkov et al., 2000). Areal potential denitrification rates integrated over 0-10 cmbsf at the oily sites ( $1.3\text{-}1.8 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) were similar to integrated rates from Gulf cold-seep sediment covered with *Beggiatoa* mats ( $0.6\text{-}1.9 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the upper 12 cmbsf, Bowles & Joye, 2010). It should be noted that sediments in the present study, which were visually devoid of sulfur-oxidizing bacterial mats, were spiked with 150  $\mu\text{M}$  nitrate and therefore concentrations in the incubations exceeded the *in situ* porewater nitrate concentrations by

about 4-15 times. However, porewater nitrate concentrations in Bowles & Joye, (2010) were in a similar range (150-270  $\mu\text{M}$ ) of the spiked concentrations (150  $\mu\text{M}$ ).

The significantly higher areal potential denitrification rates in the oiled layer compared to control sites correlated with higher TPC and POC concentrations at oiled sites compared to control sites. Interestingly, this result contrasts the sulfate reduction activity, which did not show a significant difference between 0-5 cmbsf oiled and 0-5 cmbsf control ( $p > 0.05$ ). One explanation for this difference in activity might be that denitrifiers are more versatile in exploiting substrates compared to sulfate reducers and do not depend on fermentation (Jørgensen, 2006). Thus, we hypothesize that denitrifying bacteria can better degrade the weathered oil than sulfate-reducing bacteria. Metagenomic sequence data revealed increased denitrification genes in surface sediments (0-1 cmbsf) within 3 km of the well head (Mason et al., 2014), which supports our hypothesis that denitrification could be the prevalent process in degrading the weathered oil.

### **Inhibition of denitrification by sulfide with resulting $\text{N}_2\text{O}$ production**

In the investigated sediments, peak porewater  $\text{N}_2\text{O}$  concentrations (oiled sites: 42-70 nM, control sites: 55-96 nM) were found in the shallowest (0-5 cmbsf) horizon in both oiled and control sediments (Fig. 3), where potential denitrification rates were maximal. Little is known about  $\text{N}_2\text{O}$  concentration in marine sediments, but the measured  $\text{N}_2\text{O}$  concentrations agreed with coastal (10-130 m) and deeper sediments (300- 5000 m) (5-250 nM, (Koike & Terauchi, 1996; Usui et al., 1998).

High potential denitrification rates in the 0-5 cmbsf sediment horizon at oiled and control sites suggest that denitrification could be a major  $\text{N}_2\text{O}$ -producing process in the natural environment. In support of this assumption of existing denitrification, high activity of natural denitrification was indicative from the porewater  $\text{NO}_3^-$  profiles, which showed a decrease of  $\text{NO}_3^-$  concentration at the sediment surface of oiled and control sites.

A correlation of elevated  $\text{N}_2\text{O}$  with elevated sulfide concentration was, however, not observed, because sulfate reduction rates were low within the weathered oil layer, and thus sulfide did not accumulate in the sediment porewater. It is possible that sulfide, which was produced in deeper sediment layers, was consumed by sulfide-removing processes (e.g. DNRA by sulfur-oxidizing bacteria, chemical oxidation with iron) before it reached the sediment surface.

Still, the presented study shows that Gulf sediments may act as an  $\text{N}_2\text{O}$  source, as benthic  $\text{N}_2\text{O}$  concentration in the surface sediment exceeded the mean  $\text{N}_2\text{O}$  concentration in the bottom water in the Northern Gulf (~20 nM, Kim et al., 2013). Aside from denitrification, other microbial processes like DNRA (Smith & Zimmerman, 1981) and nitrification (Middelburg et al., 1995) could act as an  $\text{N}_2\text{O}$  source in the presented sediments as  $\text{NH}_4^+$  and  $\text{NO}_3^-$  porewater profiles indicate an  $\text{NH}_4^+$  - and  $\text{NO}_3^-$  -

consuming process, respectively,. Future studies should seek to decipher the microbial processes producing N<sub>2</sub>O as well as to determine the N<sub>2</sub>O fluxes from the sediment into the water column to better understand N-cycling in Gulf sediments.

### **Summary and conclusion**

Gulf sediments covered by weathered, sedimented oil revealed significantly lower sulfate reduction activity within the oiled layer (0-5 cmbsf) in comparison to the sediments directly beneath the oiled layer (5-10 cmbsf). As no similar pattern was seen in the control sediments, the oiled layer probably had a fueling effect on the sulfate reducers directly below the oiled layer. The fact that sulfate reduction was low within the oiled layer could have two explanations: 1) the rapidly sedimented oiled layer was not yet enriched with sulfate reducers, or 2) the quality of the sedimented oil material was not suitable for the indigenous sulfate reducers, and hence rather denitrifying and fermenting bacteria were active in the oiled layer breaking down larger molecules that ultimately fed degradable substrates to sulfate reduction beneath it. We propose that the oiled layer in these sediments consisted mostly of weathered oil, in which the labile substrates were already consumed, leaving those molecules behind that were more resistant to microbial degradation (e.g. long-chained hydrocarbons, polycyclic aromatic hydrocarbons).

In contrast, potential denitrification activity was significantly higher in the 0-5 cmbsf- horizon at oiled sites compared to control sites, suggesting that denitrifying bacteria were able to further break down weathered oil, as they are more versatile in using substrates. We therefore conclude that denitrification is an important process in the degradation of sedimented, weathered oil in the sediments around the DWH well head as well as around the natural hydrocarbon seep GC600, and could be the source for the measured N<sub>2</sub>O concentrations in the surface sediments. Even though no correlation between sulfide concentrations and N<sub>2</sub>O concentrations at the oiled sites was observed, we were able to show that Gulf sediments have the potential of being an N<sub>2</sub>O source. Sulfate reduction dominated below the oiled layer, showing that the indigenous sulfate reduction community was probably not well adapted to the degradation of oil in a weathered status. Our study indicates that microbial degradation of sedimented, weathered oil could take long time-periods of >2 years as it might not be instantaneously available to some microbial groups including sulfate reducers.

More research is needed in this area to determine the persistence of sedimented oil on the sea floor and their long-term effects on microbial communities.

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## Chapter 5

### **Final Summary and conclusions**

## Preface

The presented thesis gives new insights into the occurrence and environmental controls of benthic surface production of the greenhouse gases methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) in marine, organic-rich sediments. Following environments have been investigated:

### CH<sub>4</sub>-production:

- Sediments from the upwelling region off Peru (eastern-south Pacific) traversing the continental margin including the oxygen minimum zone (OMZ) on the shelf
- Sediments from the Time Series Station Boknis Eck situated in the seasonally hypoxic Eckernförde Bay, SW Baltic Sea

### N<sub>2</sub>O-production:

- Oil-affected sediments in the Gulf of Mexico

In the following sections I present a summary of the major findings and conclusions, which is followed by a critical appraisal of the used methods and a short account on future research questions.

## **1. Benthic CH<sub>4</sub> and N<sub>2</sub>O production in different marine habitats**

### **1.1 Production of CH<sub>4</sub> in surface, organic-rich sediments**

The results presented in chapter 1 show that benthic surface methanogenesis (0-30 cmbsf) was detectable within the sulfate-reducing zone at all stations along the Peruvian margin (70-1024 m), but with decreasing magnitude from the shelf to the deeper stations. Due to the highly competitive situation with sulfate reducers in the upper, sulfate-penetrated sediment layers (0-30 cmbsf), surface methanogenesis was mostly based on non-competitive substrates. Our results revealed the importance of availability and variety of organic material for surface methanogenesis, with anoxic conditions in the water column being advantageous for the establishment of a surface methanogenic community.

In chapter 2, investigations of surface methanogenesis in the seasonal hypoxic Eckernförde Bay are described. Measurements were done on a seasonal basis covering seven time points from March 2013 to September 2014. Surface methanogenesis activity (0-30 cmbsf) was detected throughout the whole sampling period. The results showed a strong seasonality of surface methanogenesis rates, mainly controlled by the availability of organic matter, temperature and oxygen. The presence of surface

methanogenesis was mainly based on usage of non-competitive substrates by methylotrophic methanogens such as the family *Methanosarcinaceae*. Seasonal variations could also be observed in the benthic methane emissions, indicated by dissolved methane concentrations in the bottom water.

### 1.1.1 Environmental controls of methanogenesis within the sulfate-reducing zone

Surface methanogenesis within the sulfate-reducing zone has been thought to be only of minor importance, due to the successful competition of sulfate reducers for the mutual substrates H<sub>2</sub> and acetate (Capone & Kiene, 1988; Hoehler et al., 1994; Burdige, 2006; Jørgensen, 2006).

In the presented studies, we could not only detect methanogenesis in surface sediments where sulfate reduction was dominating, but were also able to identify some controlling factors on magnitude and depth distribution of this microbial process. Both systems - the Peru upwelling region and the coastal Eckernförde Bay - revealed spatial or seasonal variations, respectively, of biological and physical environmental parameters such as organic carbon load, dissolved oxygen and temperature distribution.

**Table 1:** Environmental parameters in the two investigated systems. In Peru, conditions changed spatially along the continental margin, while in Eckernförde the conditions changed seasonally.

	Temperature range (°C)	Oxygen range ( $\mu\text{mol L}^{-1}$ )	Sedimentatio n rate ( $\text{mm year}^{-1}$ )	POC content (wt %)	Surface methanogenesis ( $\text{mmol m}^{-2} \text{d}^{-1}$ )
<b>Peruvian margin</b>	4-14	bdl-53	0.1-4.5 <sup>a</sup>	3-15	0.02-0.1
<b>Boknis Eck in Eckernförde Bay</b>	3-13	bdl-340	1.4 <sup>b</sup>	4-5	0.01-0.09

bdl= below detection limit, POC= particulate organic carbon, <sup>a</sup>= Dale et al., (2015), <sup>b</sup>=Whiticar, (2002)

#### **Organic matter**

Our studies revealed that the quantity and quality (=freshness) of organic matter were the main factors controlling methanogenesis within the sulfate-reducing zone, as it determines the substrate availability and variety and thus can relieve the competitive situation between sulfate reducers and methanogens (Holmer & Kristensen, 1994; Treude et al., 2009). Both systems, the Peruvian margin and the time series station Boknis Eck in Eckernförde Bay, revealed similar magnitudes of surface methanogenesis, which is explained by the high productivity in both areas, resulting in high sedimentation of organic matter onto the seafloor and high organic carbon content in the sediments (Table 1). Accordingly, highest methanogenesis rates in Eckernförde Bay (seasonal variability) were

found in September/November after the summer/autumn phytoplankton blooms, while on the Peruvian margin (spatial variability) highest methanogenesis rates were found on the shelf, where both sedimentation rates and organic matter content were highest.

While surface methanogenesis in Eckernförde Bay was positively correlated with both particulate organic matter content (POC) and freshness of the organic matter (C/N ratio), no direct correlation with POC was observed in sediments off Peru. Here, a negative correlation was observed between surface methanogenesis and the C/N ratio. These results reveal the importance of the freshness of the sedimenting organic matter for surface methanogenesis. As methanogens are only able to use short monomeric substrates, they are dependent on other microbial groups (e.g. fermenters) to break down large organic macromolecules (Zinder, 1993). Fresh (=less degraded) organic matter leads to increased magnitudes of microbial organic matter degradation (Westrich & Berner, 1984; Canfield, 1994; Amon et al., 2001; Middelburg, 1989), which in turn leads to increased availability of methanogenic substrates. The results presented here from the Peruvian margin and Eckernförde Bay suggest that surface methanogenesis within the upper 30 cmbsf was fueled by either sedimented organic material originating from the primary production in the upper water column, or by additional carbon sources associated with bioturbation. The dissolved organic material from dead infauna, fecal pellets or burrow constructions can lead to the formation of microniches with elevated microbial activity due to the local abundance of methanogenic substrates (Ziervogel et al., 2014; Bertics et al., 2013).

In both systems, the observed surface methanogenesis (0-30 cmbsf) was mainly based on non-competitive substrates such as methanol or methylated compounds, thus avoiding competition with sulfate reducers (Oremland & Polcin, 1982; King, 1984). Stimulation experiments with addition of methanol were successful in both systems, identifying the adaptation of prevalent methanogens (including the genus *Methanosarcinales*) to the methylotrophic pathway. In addition, methylated amines and methylated sulfides probably served as additional substrates. In the deeper sediment horizons (> 30 cmbsf), usage of competitive substrates such as H<sub>2</sub> increased in both systems, probably as competition was relieved due to the decline of sulfate.

### ***Abiotic controls***

To a minor extent, temperature and dissolved oxygen concentration of the bottom-near water showed some controlling effect on benthic surface methanogenesis in both systems (Peru and Eckernförde Bay).



In Eckernförde Bay, highest surface methanogenesis was observed at highest bottom water temperatures ( $\sim 12^{\circ}\text{C}$ ). The same accounts for the Peruvian margin, but with highest temperatures of  $\sim 14^{\circ}\text{C}$ . Thus, we postulate the presence of a mesophilic (Madigan et al., 2006) methanogenic community facilitating surface methanogenesis in the upper 0-30 cmbsf in both systems.

The depleted dissolved oxygen concentrations in the water column in both systems, either due to the establishment of an OMZ on the Peruvian shelf or the development of seasonal hypoxia/anoxia in Eckernförde Bay, also had positive effects on surface methanogenesis. Firstly, sedimenting organic material was not degraded extensively in the water column due to depleted oxygen conditions. Hence, less-degraded organic material reached the sea floor, which led to enhanced microbial organic matter degradation within the sediment. Secondly, anoxic waters impinging on the sea floor prevent benthic aerobic degradation at the sediment surface, thus making room for an establishment of an anaerobic microbial community up to the sediment-water interface including methanogens, which are known to be sensitive to oxygen (Oremland, 1988; Zinder, 1993).

### **1.1.2 Relevance of surface methanogenesis and its potential for benthic emissions**

In the presented studies from Peru and Eckernförde Bay, methanogenesis was always present within the sulfate-reducing zone (0-30 cmbsf), and was mainly based on usage of non-competitive substrates. Rates of surface methanogenesis were similar in both systems and also in comparison with other organic-rich, sulfate-containing sediments off Chile (Ferdelman et al., 1997). Due to highly competitive situation between methanogens and sulfate reducers in Peru and Eckernförde Bay, those surface methanogenesis rates were up to two magnitudes lower compared to surface sediments with similar organic content but with depleted sulfate concentrations (Kuivila et al., 1990; Schmaljohann, 1996; Treude et al., 2005a).

The major part of benthic methanogenesis is proposed to occur in deeper sediment layers, when sulfate is depleted to  $<1$  mM (Burdige, 2006; Jørgensen, 2006). The results from Peru and Eckernförde Bay revealed, however, that surface methanogenesis can reach similar (Niewöhner et al., 1998; Treude et al., 2005b) and sometimes even higher magnitudes (Jørgensen & Parkes, 2010; Arning et al., 2012) compared to deep methanogenesis from similar organic-rich systems (see Table 2 in chapter 2). Accordingly, benthic methane emissions are not only explained by deep methanogenesis but can be partly or -in some systems- primarily be explained by surface methanogenesis. This contribution to benthic methane emissions could be as high as 100 % in upwelling regions as seen on the Peruvian shelf, or could be in the lower range in coastal inlets such as Eckernförde Bay (up to 9%). In addition, the observation of spatial and seasonal variation in surface methane production indicates also spatial

and seasonal variation in benthic methane emissions, which has been largely overlooked in current global methane emission estimations, due to the lack of data (Bange et al., 1994; Naqvi et al., 2010). How much of this surface produced methane can escape the sediment and reaches the water column (and finally the atmosphere) is dependent on the microbial consumption of methane, namely aerobic and anaerobic oxidation of methane (AOM), with the latter probably being the more dominant process in organic-rich sediments, due to the lack of oxygen (Jørgensen, 2006; Revsbech et al., 1980). The presented results indicate a previously underestimated role of surface methanogenesis in the benthic methane budgeting, as it could act as an important methane supplier for surface AOM in organic-rich sediments.

## **1.2 Production of N<sub>2</sub>O in surface, organic-rich sediments**

Chapter 3 reports the results from experiments with oil-impacted (oiled) sediments compared to non-oiled (control) sediments from the Gulf of Mexico. The sedimented oil originated either from the Deepwater Horizon (DWH) oil spill or natural seepage sites. We investigated the microbial processes of sulfate reduction and denitrification, the latter being known to be a source for N<sub>2</sub>O. The results showed that microbial denitrification was elevated in oiled sediments compared to control sediments, indicating the importance of this process for oil degradation. In contrast, sulfate reduction was not elevated in oiled sediments, illustrating that sedimented oil is available only to specific microbial communities. Accordingly, no sulfide-induced N<sub>2</sub>O production was observed, however, all sediments revealed the potential for being a N<sub>2</sub>O source.

### **1.2.1 Impact of increased microbial activity in oil-contaminated sediments on benthic N<sub>2</sub>O production and its potential for N<sub>2</sub>O emissions**

In the presented results from sediments in the Gulf of Mexico, denitrification was a major degrading process in oiled sediments, i.e. sediments with increased organic matter content, compared to control sediments. Accordingly, the observed sediment N<sub>2</sub>O could originate from denitrification. To what extent other microbial processes such as dissimilatory nitrate reduction to ammonium (DNRA) or nitrification might have played a role in the benthic N<sub>2</sub>O production was not investigated; however, hints towards DNRA and nitrification were seen in the porewater profiles of ammonium and nitrate in oiled and control sediments. As organic-rich sediments generally have only low oxygen penetration depths, due to high oxygen consumption from enhanced aerobic degradation (Jørgensen, 2006; Revsbech et al., 1980), N<sub>2</sub>O production from nitrification could be a possible feature within the oxic sediment horizon, as previous studies showed increased N<sub>2</sub>O production during nitrification when oxygen concentration decreased (Goreau et al., 1980; Ostrom et al., 2000).

Interestingly, all oiled and control sediments investigated in the presented study revealed the potential of being a N<sub>2</sub>O source, due to elevated N<sub>2</sub>O concentrations at the sediment surface, compared with dissolved N<sub>2</sub>O concentrations in the bottom-near water. Sulfide-induced N<sub>2</sub>O production was not observed in either oiled or control sediments, thus the sediment N<sub>2</sub>O concentrations are rather explained by increased denitrification (and probably DNRA and nitrification) due to increased organic matter input. The potential of sulfide-induced N<sub>2</sub>O production might be of more importance in other marine sediments with high sulfate reduction activity such as coastal sediments or cold seep sediments, where sulfide concentrations can reach up to mM levels.

The general observation of enhanced denitrification in sediments with a higher organic carbon content reveals the crucial importance for further research on denitrification and its potential for benthic N<sub>2</sub>O emissions in other organic-rich sediments such as coastal areas or upwelling regions, which are thought to be the dominant source for atmospheric N<sub>2</sub>O (Bange, 2006a; Bakker et al., 2014).

## **2. Critical review on used methodology**

### **2.1 Acetylene inhibition method**

The acetylene inhibition method for the detection of denitrification is a widely used method, but also subject of controversial discussion (Groffmann et al., 2006). The method uses the ability of acetylene to block the last enzymatic step during denitrification, i.e. the reduction of N<sub>2</sub>O to N<sub>2</sub> by the enzyme nitrous oxide reductase (Yoshinari et al., 1977; Sørensen, 1978). Thereby denitrification is measured as N<sub>2</sub>O production, a gas which is much easier to measure than N<sub>2</sub>. The appeal of this method is that it is applicable to a large number of samples and is not as cost- or labor- intensive as stable isotope tracer methods (Groffmann et al., 2006). However, the underestimation of denitrification rates is a known phenomenon when using this method. This underestimation can occur in systems with low or dynamic nitrate pools, as acetylene also inhibits nitrification, which is a main nitrate supplier for denitrification (Groffmann et al., 2006). Other concerns are the alleviation of the acetylene blockage in presence of sulfide or the slow diffusion of acetylene into fine-grained sediments (Groffmann et al., 2006). Still, this method provides a reliable proxy of potential denitrification when nitrate is not limiting and sulfide concentrations are low, which is why it was used in the present study. However, methods including the usage of stable isotopes such as <sup>15</sup>N (Nielsen, 1992) followed by mass spectrometric measurements of substrates and products are more sensitive and thus should be used in the future, especially when working in natural systems.

## 2.2 Radiotracer experiments

Radioactive tracers are used to determine and identify microbial processes in marine sediments (Jørgensen, 2006). Due to the high sensitivity of this method, only small injection quantities of radioactive tracer and shorter incubation times are needed (compared to the solely observation of chemical change in e.g. sediment slurry experiments), which reduces disturbances of the natural sample. In the presented thesis, the radioactive tracer  $^{35}\text{S}$ -sulfate was used for detection of sulfate reduction and the tracer  $^{14}\text{C}$ -bicarbonate was used for the detection for hydrogenotrophic methanogenesis.

While sulfate reduction was detected via the whole-core injection method (Jørgensen, 1978), small glass tubes were used for the detection of hydrogenotrophic methanogenesis. The advantage of the whole-core injection is minimized disturbances of the sediment structure and minimized diffusion of e.g. methane or oxygen due to usage of thick rubber stoppers, thus this method should be favored.

The method using glass tubes could lead to artifacts, as sampling is accompanied by more disturbance, because a smaller sediment volume has to be transferred into the glass tubes without headspace. In addition, leakages of methane or oxygen diffusion could occur through the less thick rubber stoppers, which seal the tube on both sides. However, rather an underestimation of activity would occur than an overestimation. Also, this technique is a good alternative for sampling hard or dry sediments.

Other problems address the radiotracer themselves. For example, applications with  $^{35}\text{S}$ -sulfate revealed the re-oxidation of  $^{35}\text{S}$ -sulfides during unfrozen storage, which results in an underestimation of sulfate reduction rates (Røy et al., 2014). During this reaction, zink sulfide ( $\text{Zn}^{35}\text{S}$ ) and iron sulfide ( $\text{Fe}^{35}\text{S}$ ) are re-oxidized to sulfate by reactive  $\text{Fe(III)}$ , which originates from the reaction of  $\text{Fe}^{2+}$  with oxygen.  $\text{Fe}^{2+}$  is released during the gradual conversion of  $\text{FeS}$  to  $\text{ZnS}$ , which has the lower solubility product.

Even if samples have not been frozen during storage, results can still be used to detect the distribution of sulfate reduction. However, to also compare the magnitude of sulfate reduction, the frozen storage is an unavoidable procedure in future studies.

### 3. Future research questions

In case of benthic surface methanogenesis, future studies should seek into identifying responsible microbes, including spatial or seasonal changes in the methanogenic community. It would also be advisable to measure surface methanogenesis in a variety of marine environments (coastal and deep-sea sediments) to get a better global estimation about the importance of this process. To clarify the role of surface methanogenesis in the benthic methane cycling, the following aspects should be addressed:

- Can surface methanogenesis act as an important methane supplier for surface AOM?
- How much of the methane produced in surface sediments is consumed before it reaches the water column and atmosphere, respectively?

In addition, the effects of on-going global and regional environmental changes (warming, deoxygenation, acidification, eutrophication etc.) on surface methanogenesis should be investigated to also evaluate its potential role as a methane source in a changing environment. Surface methanogenesis could experience the consequences of environmental changes much faster than deep methanogenesis due its closeness to the sediment-water interface. For example, the predicted increase in temperature or the increase in hypoxic areas as a result of climate change and eutrophication (IPCC, 2014; Hoegh-Guldberg & Bruno, 2010; Keeling et al., 2010) could be advantageous for surface methanogenesis and thus could lead to enhanced activity.

For surface N<sub>2</sub>O production, more research is needed to determine the potential of N<sub>2</sub>O production and emission in organic-rich sediments, especially as the presented results revealed increased denitrification rates in sediments with increased organic matter content. However, as continental margins are the most productive areas in terms of N<sub>2</sub>O emissions (Bange, 2006b; Bange & Andreae, 1996), future studies should concentrate on these environments. In general, the following aspects should be investigated:

- What are the environmental controls of benthic N<sub>2</sub>O production in organic-rich sediments?
- Which processes are responsible for benthic N<sub>2</sub>O production?
- Which microbial groups can be identified as major N<sub>2</sub>O producers?
- How much N<sub>2</sub>O is consumed before escaping the sediment?
- How much N<sub>2</sub>O is emitted into the water column?

By answering the above stated research questions, the global importance of benthic surface N<sub>2</sub>O production could be validated and uncertainties in coastal N<sub>2</sub>O emissions minimized.

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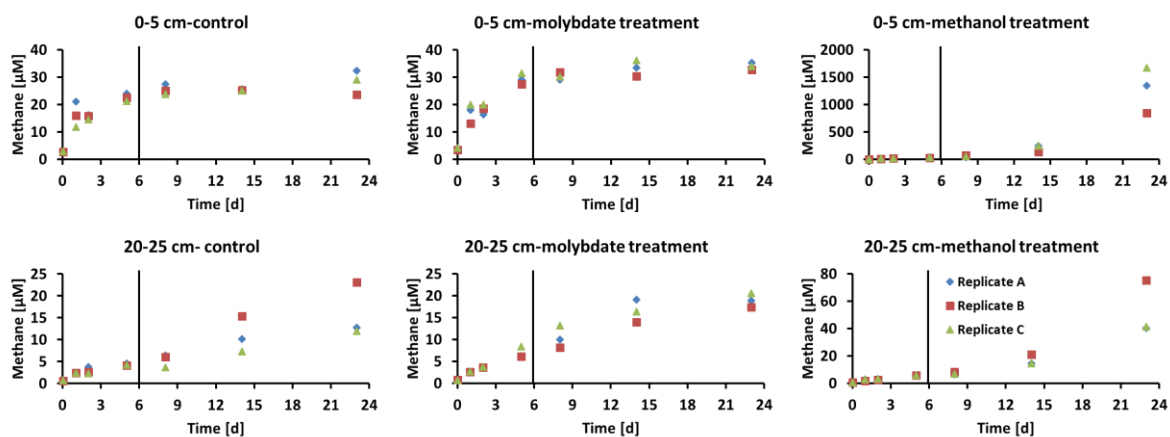
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## **Supplementary Material**

## Supplementary Material to Chapter 2:



**Figure S1:** Methane concentration over time in the three different treatments of the sediment slurry experiment in the 0-5 cm and 20-25 cm horizon of St. 1 (70 m). "Control" is defined as the treatment with sulfate-rich (28 mM) artificial seawater medium, "molybdate treatment" is defined as the treatment with sulfate-rich artificial seawater medium plus molybdate (22 mM), and "methanol treatment" is defined as the treatment with sulfate-rich artificial seawater medium plus methanol (10 mM). Shown are triplicate measurements per time point. The vertical black line marks the separation of two different phases that were used for methane production rate calculations. Please note the different scale at the x-axis of the methanol treatments.

Supplementary Material to Chapter 3

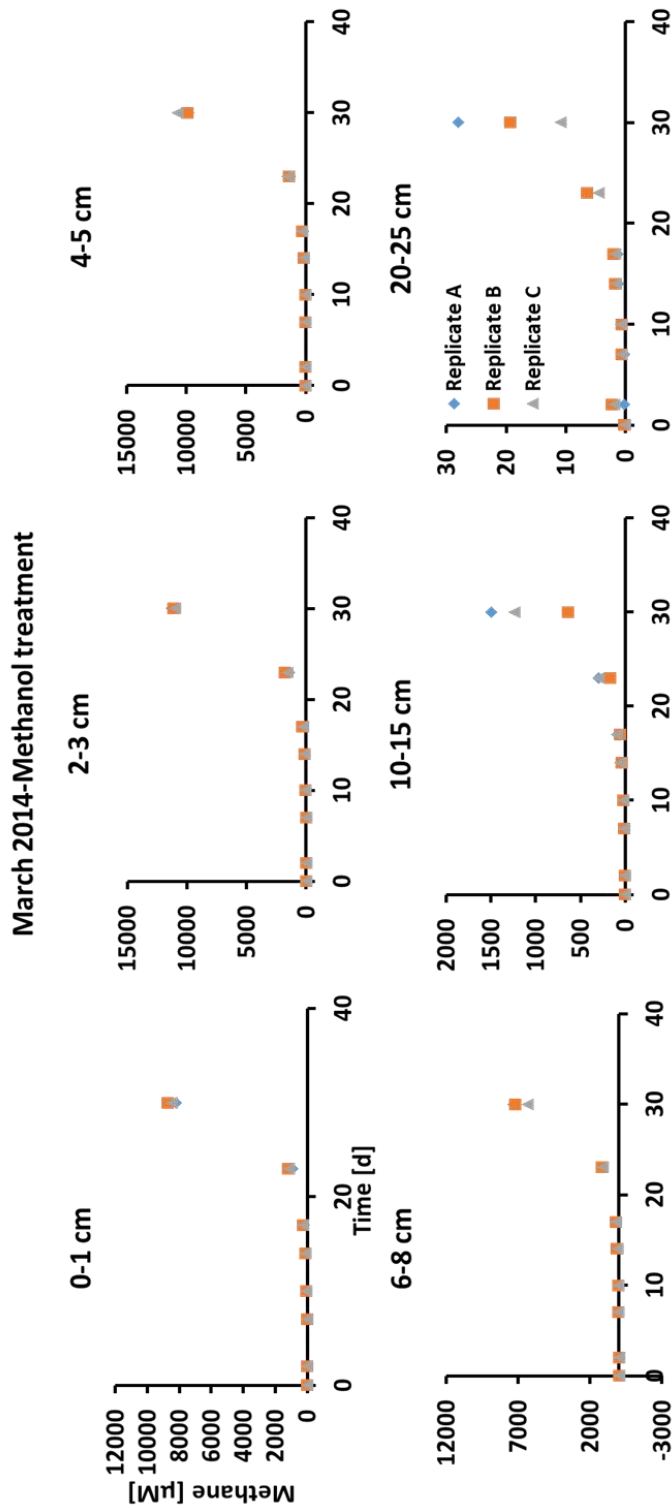
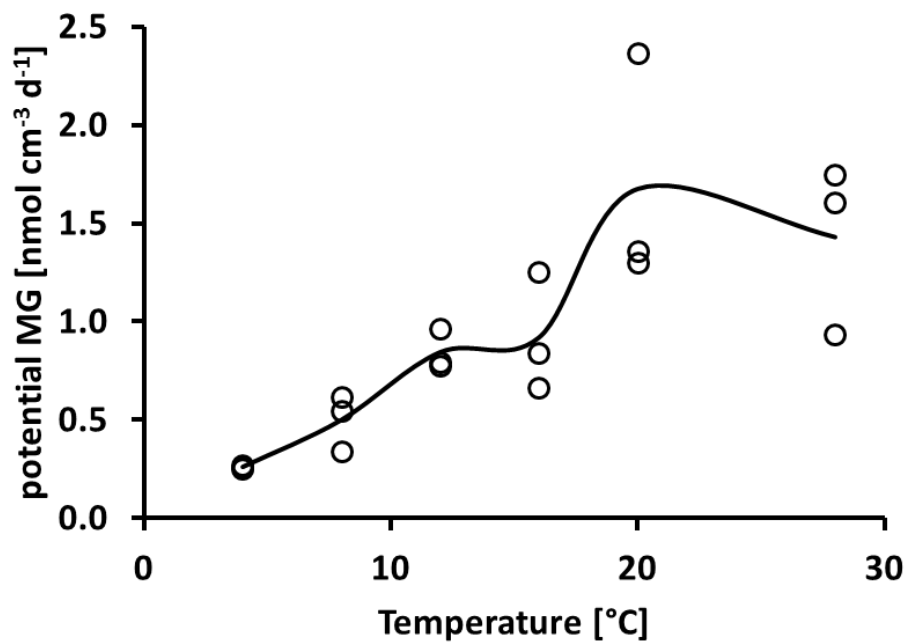
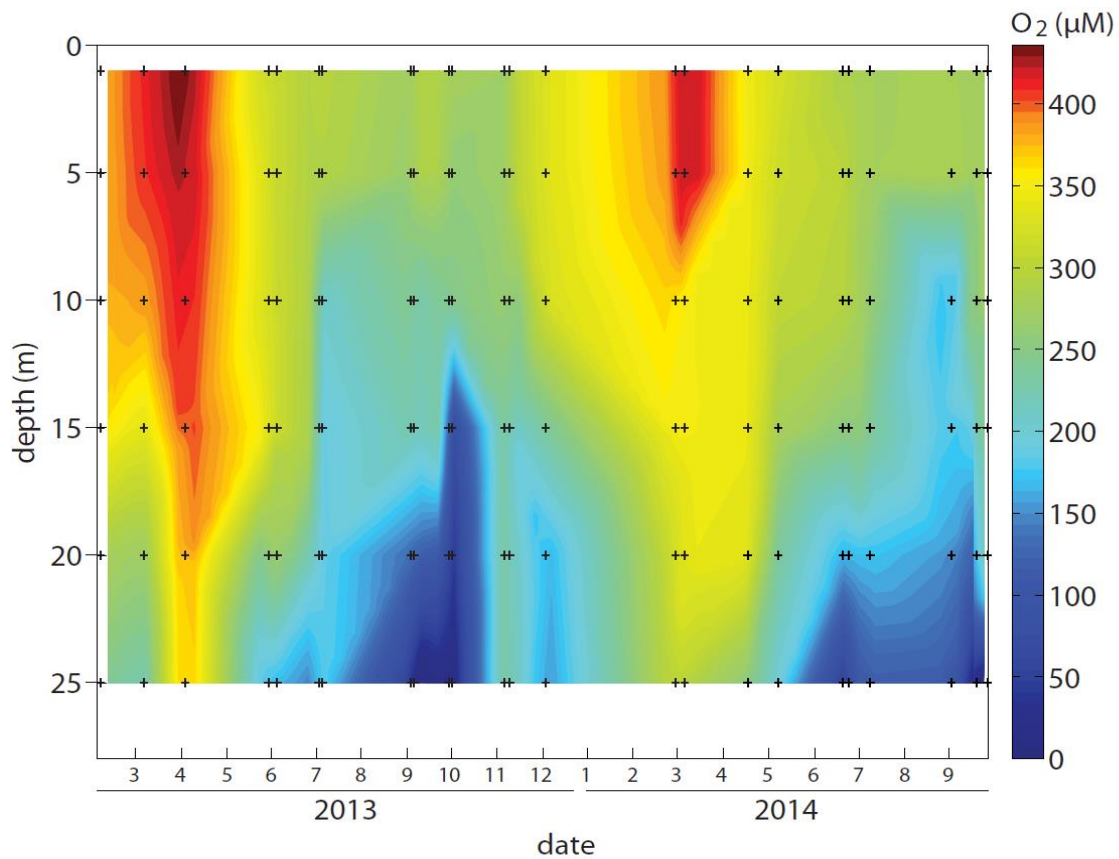


Figure S1: Example for the sediment methane increase after addition of methanol (=methanol treatment) in March 2014. Shown are methane concentrations of triplicate values. Methane showed a similar increase at the other three time points November 2013, June 2014 and September 2014.



**Figure S2:** Potential methanogenesis rates from 75 cmbsf sediment depth from the gravity core in September 2014 at different temperatures. 1:1 sediment slurry was prepared with 5 cm<sup>-3</sup> sediment plus 5 ml sulfate-free artificial medium (Widdel & Bak, 1992, salinity 24). Headspace methane samples (100  $\mu$ l) were taken out every 4-7 days and injected into a Shimadzu gas chromatograph equipped with a packed Haysep-D column (80°C) and a flame ionization detector. Methane concentrations were calibrated against methane standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2 %. Methanogenesis rates were determined from the linear methane increase over time (including a total of nine time points). Shown are values of triplicates (circles) and the average (solid line).



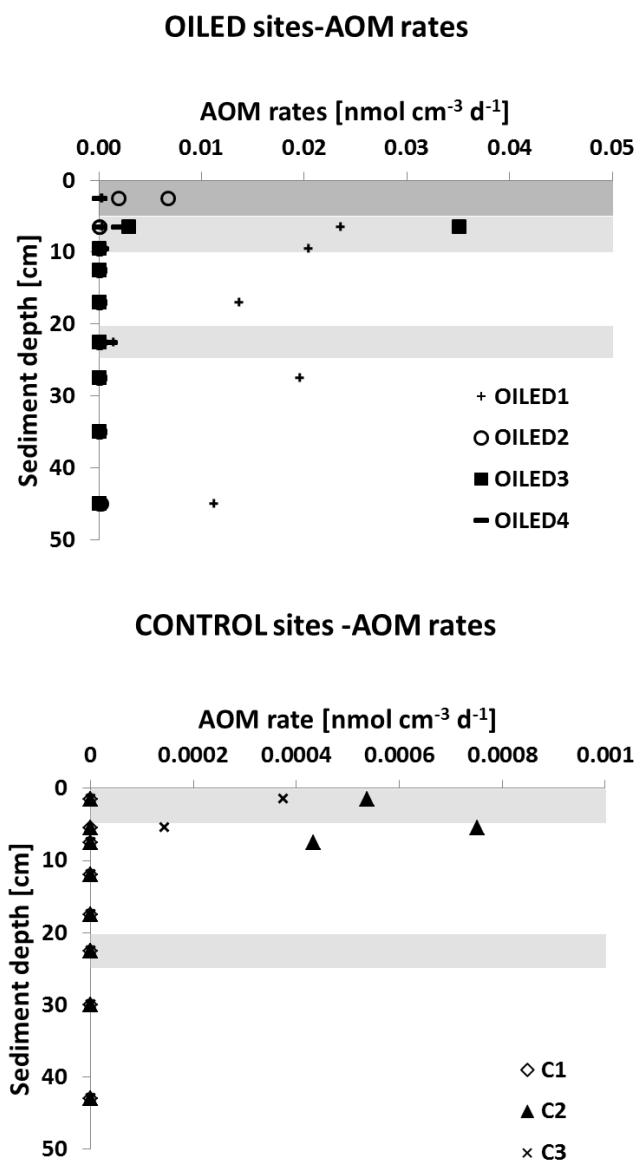


**Figure S3:** Dissolved oxygen ( $O_2$ ) concentrations in the water column from February 2013 to September 2014. Data extracted from the Boknis Eck database: [www.bokniseck.de/database](http://www.bokniseck.de/database).

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## Supplementary Material to Chapter 4



**Figure S1:** Potential anaerobic oxidation of methane (AOM) rates at oiled sites (above) and control sites (below). Samples were amended with 5 mM methane before addition of tracer. Duplicates per depth are shown. Sediment horizons investigated for denitrification are marked with grey bars, the oiled layer is marked with a dark grey bar. Please note the different scale at the x-axis.